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Article

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Discovery of Covalent Inhibitors Targeting the Transcriptional Enhanced Associate Domain (TEAD) Central Pocket

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ABSTRACT: Transcriptional enhanced associate domain (TEAD) transcription factors together with coactivators and corepressors modulate the expression of genes that regulate fundamental processes, as organogenesis and cell growth, and elevated TEAD activity is associated with tumorigenesis. Hence novel modulators of TEAD and methods for their identification are in high demand. We describe the development of a new "thiol conjugation assay" for identification of novel small molecules that bind to the TEAD central pocket. The assay monitors prevention of covalent binding of a fluorescence turn-on probe to a cysteine in the central pocket by small molecules. Screening of a collection of compounds revealed kojic acid analogs as TEAD inhibitors, which covalently targets the cysteine in the central pocket, blocks the interaction with coactivator Yes-Associated Protein (YAP) with nanomolar apparent IC_{50} values and reduces TEAD target gene expression. This methodology promises to enable new medicinal chemistry programs aimed at the modulation of TEAD activity.

Introduction

Transcriptional enhanced associate domain (TEAD) transcription factors play significant roles during development, tissue regeneration, cell growth and migration, and in human disease progression.[1-4] Elevated TEAD expression has been observed in solid tumors such as prostate,[5] lung,[6] colorectal,[7] gastric,[8] and breast cancers[9]. A number of TEAD target genes including cell surface receptor tyrosine kinase Axl[10], connective tissue growth factor CTGF[11,12], apoptosis inhibitor survivin[13] and tumor marker mesothelin[14] are also frequently associated with tumorigenesis. Therefore, TEAD transcription factors are potential therapeutic targets in cancer therapy.

There are four TEAD homologs (TEAD1–4) in mammalian cells that are expressed in a tissue and development stage specific manner. TEAD1 is involved in cardiogenesis, while TEAD2 is critical for neural development and TEAD4 is necessary for embryo implantation.[15-18] Thus, pharmacological modulation of TEAD activity may enable novel medicinal chemistry approaches both in oncology and regenerative medicine.[19,20] Accordingly, the development of small molecule and peptidic modulators of TEAD function has been approached.[1,20-23]

TEAD homologs 1–4 share two highly conserved N- and C-terminal domains. The N-terminal DNA binding domain forms a homeodomain and the C-terminal transactivation domain adopts a β-sandwich capped with a helix-turn-helix motif (Figure 1A, left panel, cyan).[1] The transactivation domain maintains the interaction with transcriptional co-modulators, i.e. coactivators YAP (Figure 1A, left panel, pale yellow), transcriptional coactivator with PDZ-binding motif (TAZ), Vestigial-like (Vgll) family proteins, as well as corepressor Vgll4 (Figure S1).[24,25]

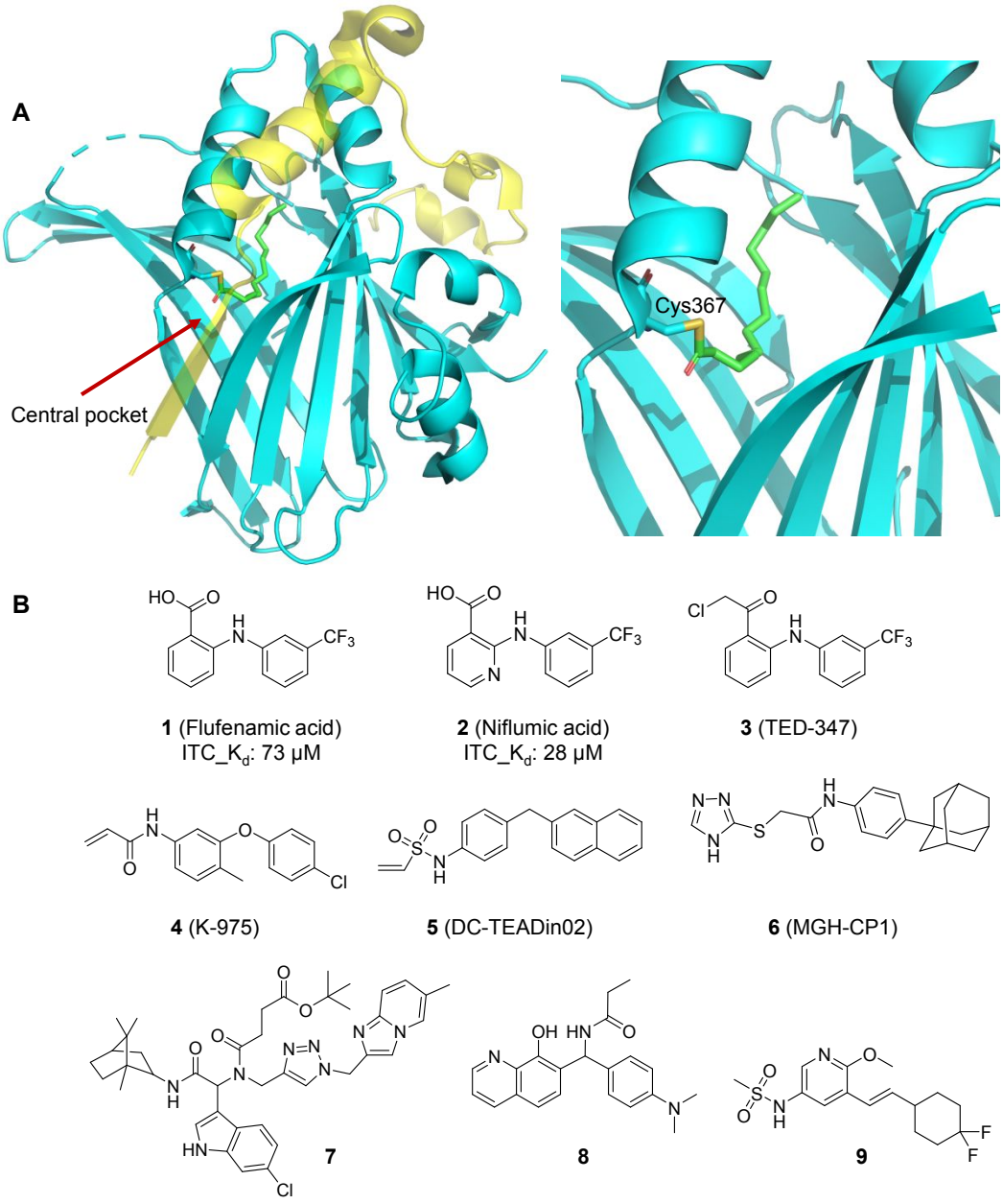


Figure 1. hTEAD4 transactivation domain. (A) Structure of TEAD transactivation domain (cyan) and the acylated cysteine residue in the central pocket (PDB ID: 5OAQ). Cofactor YAP (PDB ID: 3KYS) is shown (pale yellow). Right panel zoomed into the acylated cysteine residue (Cys367). (B) Small molecules that bind the TEAD central pocket.

TEADs contain a large hydrophobic central pocket within the globular β-sandwich to which the cofactors bind. The pocket embodies a conserved cysteine residue that can be acylated with palmitic or myristic acid (Figure 1A).[26-28] Recent studies suggest that TEAD acylation is dynamically regulated by auto-palmitoylation and depalmitoylases[26,29], pointing to a possibility of targeting the TEAD central pocket via the non-acylated form (Figure S2).

Indeed, the non-acylated hydrophobic central pocket can be targeted by small molecules that bind the lipidation site (Figure 1B). Nonsteroidal anti-inflammatory drugs flufenamic acid (**1**, K_d= 73 μM) and niflumic acid (**2**, K_d= 28 μM) bind the TEAD central pocket and inhibit expression of TEAD-YAP target genes and growth of cancer cell lines.[30] Furthermore, a chloromethylketone analog of flufenamic acid (**3**, **TED-347**) was developed as a covalent TEAD inhibitor that can allosterically inhibit the TEAD-YAP interaction (K_i = 10.4 μM).[31] Another covalent TEAD inhibitor (**4**, **K-975**) showed efficacy in human malignant pleural mesothelioma xenograft models.[32] More recently, a vinylsulfonamide derivative (**5** , **DC-TEADin02**) was developed via structure based virtual screening and was shown to covalently bind to TEAD in cells transfected with Flag-TEAD4.[33] Triazole **6** efficiently inhibits TEAD palmitoylation in intestinal epithelium in vivo[34] and compound **7** was identified via screening a DNA-Encoded Library with indole-focused Ugi-peptidomimetics.[35] Furthermore, the hydroxyquinoline analog **8** was reported to bind the central pocket, however, it activates TEAD function.[36] A recently identified TEAD palmitoylation inhibitor (**9**) mimics the palmitate binding mode, activates TEAD to act as a transcriptional repressor independent from YAP and inhibits tumor growth in a xenograft model.[37] Overall, the number and diversity of small molecules that target the TEAD central pocket has been growing, and any methodology that will aid the development of novel TEAD modulators will be invaluable.

We describe the development of a new methodology for the identification of novel small molecule inhibitors binding to the TEAD central pocket. By means of a newly developed "thiol conjugation assay" we identified kojic acid derivatives that covalently target the palmitoylatable cysteine in the TEAD central pocket and inhibit the TEAD-YAP interaction with nanomolar apparent IC_{50} values. By means of a representative analog, we have shown the TEAD engagement in cell lysates and the inhibition of the expression of TEAD target gene ANKRD1. The novel methodology in general promises to enable new medicinal chemistry programs aimed at the discovery of covalent or non-covalent TEAD inhibitor classes.

RESULTS AND DISCUSSION

Development of a "Thiol Conjugation Assay"

In order to develop a robust, homogeneous and rapid binding assay for the TEAD central pocket, we utilized the thiol reactive pro-fluorescent probe, N-(4-(7-diethylamino-4-methylcoumarin-3-yl) phenyl) maleimide (CPM) that has been used for site specific protein labeling, thermal stability analysis of membrane proteins and assaying N-acyltransferase activity.[38-42] The fluorescence in CPM is quenched due to the maleimide substitution on the phenyl group that modulates the resonance between the coumarin carbonyl and 7-amino groups. However, upon reaction with a thiol, CPM fluorescence increases strongly (Figure 2A).[41] We hypothesized that reaction of a free cysteine residue in the TEAD central pocket would yield a fluorescence signal, and that small molecules binding to the TEAD central pocket with appreciable potency would prevent covalent labeling of the cysteine by CPM.

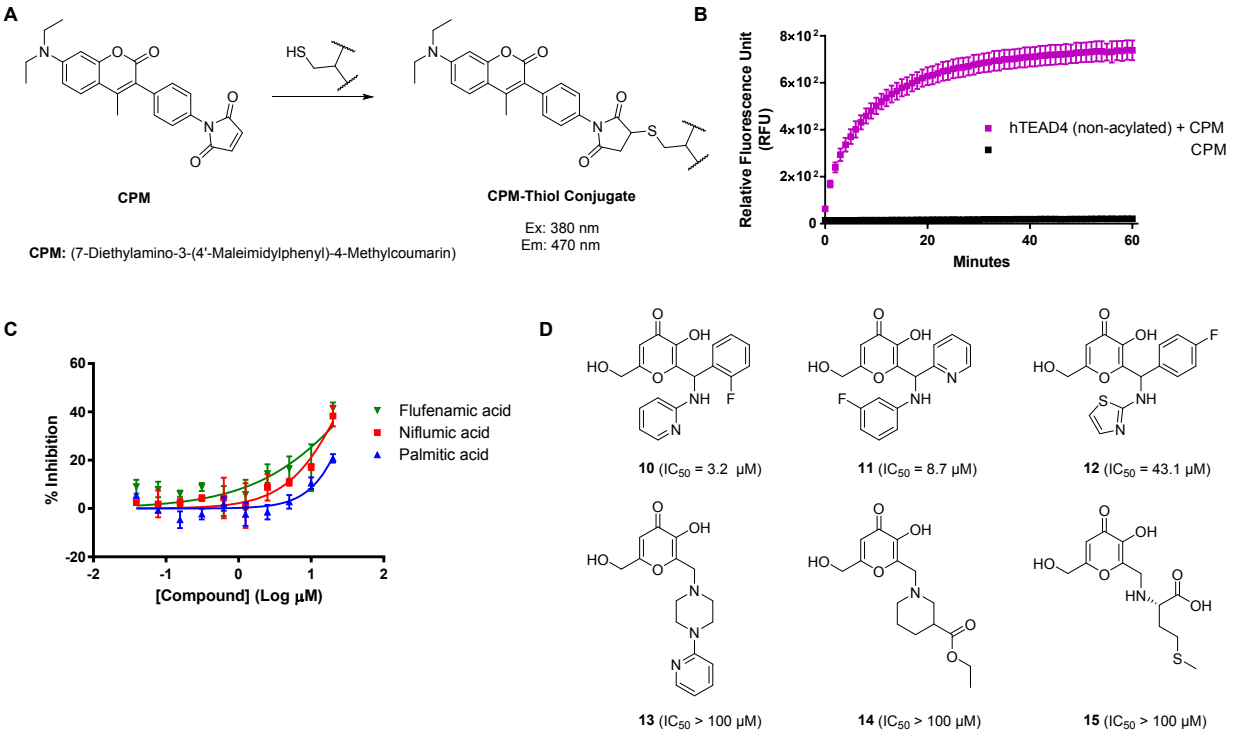


Figure 2: Targeting the central pocket with small molecule inhibitors. (A) Thiol conjugation assay principle. The free cysteine residue in the TEAD central pocket reacts with CPM yielding a fluorescence signal. (B) Kinetic measurement of hTEAD4 (non-acylated) treatment with CPM (purple square) yields a fluorescence signal, while CPM alone (black square) has only a minimal signal. (C) Competitive inhibition of hTEAD4 (non-acylated)-CPM with known non-covalent central pocket binders. (D) Selected screening hits (**10–12**) and inactive analogs (**13–15**) with similar structures.

For assay development, hTEAD4 was delipidated with hydroxylamine to cleave the covalent thioester bond between the fatty acid and the cysteine (Cys367 for hTEAD4, Figure 1A right panel). Delipidation was monitored and confirmed with whole-protein electron spray ionization (ESI)-mass spectrometry (Figure S3). Through expression, a mixture of the three recombinant[28]

non-acylated hTEAD4 (MW: 26,495) and hTEAD4 covalently bound to either myristic acid (C14-fatty acid, MW:26,705) or palmitic acid (C16-fatty acid, MW:26,733) (Figure S3A) was converted to a homogeneous non-acylated hTEAD4 protein (MW: 26,495) (Figure S3B).

Addition of the non-acylated hTEAD4 to 1 equivalent CPM yielded a significant increase in the fluorescence signal, which reached a plateau in 30-40 min (Figure 2B). Analysis of the reaction mixture with ESI-mass spectrometry indicated that two cysteine residues might have been labeled with CPM (Figure S4A). Subsequent tandem mass spectrometry (MS-MS) analysis showed that Cys367 and Cys330, both of which are located in the hTEAD4 central pocket, were covalently modified with CPM (Figures S4B and S5). These findings suggested that monitoring of CPM binding to the TEAD central pocket and its prevention by small molecules could be used to identify novel TEAD central pocket targeting inhibitors.

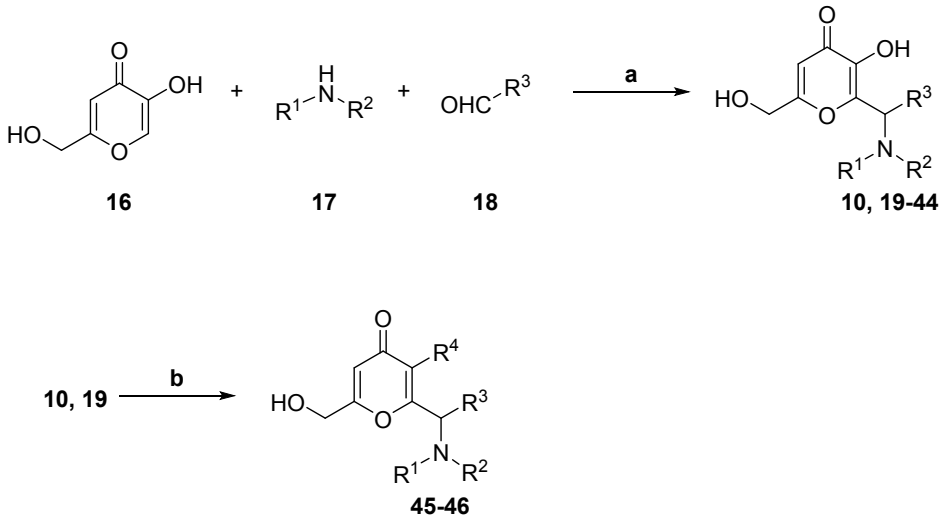
For validation, we investigated inhibition of CPM binding and fluorescence decrease by the known TEAD central pocket binders, flufenamic acid (**1**), niflumic acid (**2**), palmitic acid and the covalent inhibitor **3** (TED-347). As shown in Figure 2C for non-covalent binders and in Figure S6 for TED-347, these compounds inhibited the signal increase in a dose dependent manner. To our best knowledge, this is the first example that CPM is used for a binding assay. Since a cysteine thiol is necessary in the reaction with CPM, we termed this novel assay as "Thiol Conjugation Assay".

Discovery of Small Molecule Inhibitors of the TEAD Central Pocket

Investigation of a collection of 14,000 compounds using the newly established thiol conjugation assay identified 329 compounds, which showed > 50% inhibition at a concentration of 12.5 μM (2.3 % primary hit rate). Kojic acid analogs **10**–**12** (Figure 2D) were among the most potent hits. Analogues **13**–**15** lacking aryl substituents on the methyl amine, however, were

inactive (Figure 2D) such that for initial structure-activity relationship (SAR) investigations a phenyl group at both R^1 and R^3 positions was retained (Scheme 1). Compounds **19**–**44** were synthesized using a one pot Betti reaction[43] starting from kojic acid (**16**), amines **17** and aldehydes **18** (Scheme 1). In addition, the 2-hydroxyl group in compounds **19** and **10** was methylated to yield compounds **45** and **46**, respectively.

Scheme 1. Synthesis of kojic acid analogs.



Reagents and conditions: (a) EtOH, rt, 2-20 days. (b) MeI, K_2CO_3, DMF, 24h, rt.

Direct binding of compounds **19**–**46** to the TEAD central pocket was monitored by means of a fluorescence polarization (FP)-based competitive binding assay employing a FITC-labelled palmitate tracer (Scheme S1), which bound with K_d = 28 nM to non-acylated hTEAD4 (Figure S7A). The FP assay was validated employing the previously reported competitive inhibitor niflumic acid (**2**) as a positive control (Figure S7B).

IC_{50} values obtained from the thiol conjugation assay and the FP-based competitive binding assay are shown in Table 1. Not surprisingly, due to the different assay principles (i.e. competition with the covalent cysteine binder CPM, *versus* competition with non-covalent binder FITC-palmitate) and protein concentrations, the IC_{50} values obtained from the FP and thiol conjugation assays differ for all kojic acid analogues, consistently 5-10 fold.

Initial SAR investigations focused on modifications of amine substituents R^1 and R^2 (Table 1). Replacement of the phenyl group in **19** by an isopropyl group (**20**) led to a 7-fold increase in IC_{50} values for both assays. Although, a benzyl group (**21**) was slightly (2-fold) less favorable than the phenyl group (**19**), the introduction of the constrained *bis*-benzyl moiety in **22** lowered the affinity nearly 5-fold. A fluorine group at the *meta*-position of R^1 (**23**) did not influence TEAD binding, and introduction of an electron deficient 2-pyridine ring (**24**) gave a similar IC_{50} value as for a phenyl group (**19**). Substitution of the 2-pyridine ring (**25**) with a methyl group on the 5-position also did not alter the binding. However, the introduction of 2-pyrimidine- (**26**), 2-thiazole- (**27**) and 2-benzimidazole (**28**) groups resulted in more than 20-fold increase in the IC_{50} values. Equipment of the phenyl ring at R^1 with an electron withdrawing 4-carboxamide (**29**) did not influence the affinity.

Replacement of the benzylic phenyl group at the R^3 position with an aliphatic cyclohexyl- (**30**) or isopropyl group (**31**) or with a hydrogen (**32**) dramatically (100-fold) reduced TEAD binding in the thiol conjugation assay showing the importance of an aromatic residue at the R^3 position. Introduction of an electron withdrawing carboxamide substituent at the *para*-position (**33**) slightly (2-fold) improved binding. Hydrophobic substituents at the *para*-position (**34**–**38**) did not change binding significantly, and similarly introduction of a fluorine group (**39**) at the

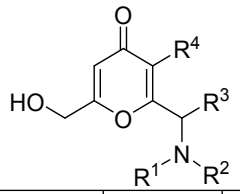
ortho-position was tolerated. Replacement of the phenyl ring with an electron deficient 2-pyridine group (**40**), however, lowered the inhibitory activities by 2-3-fold in both assays.

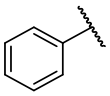
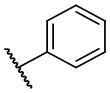
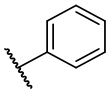
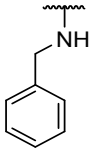
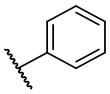
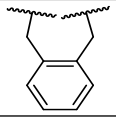
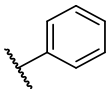
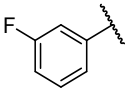
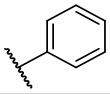
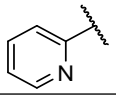
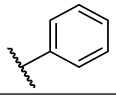
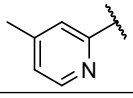
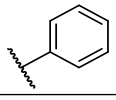
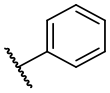
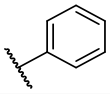
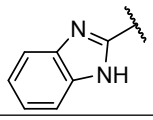
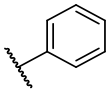
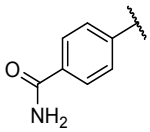
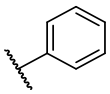
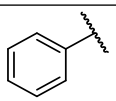
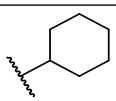
Additional analogs with 2-pyridine residues at R^1 and R^3 (**41**–**44**), in particular the combination of two 2-pyridines at both R^1 and R^3 positions (**43**) lowered the affinity significantly.

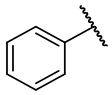
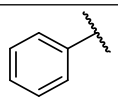
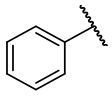
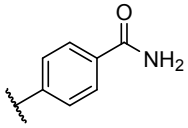
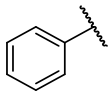
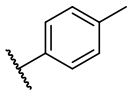
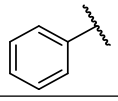
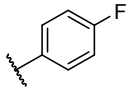
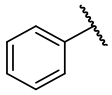
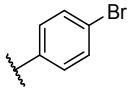
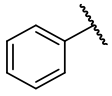
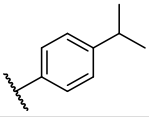
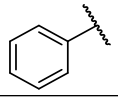
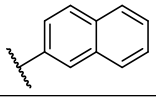
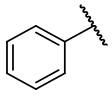
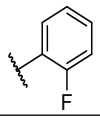
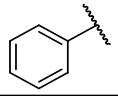
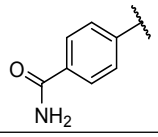
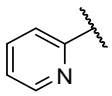
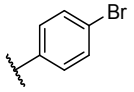
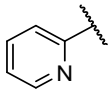
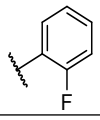
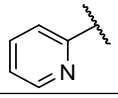
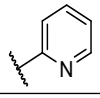
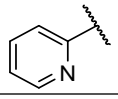
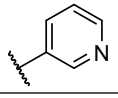
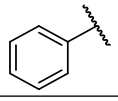
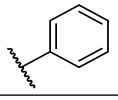
The importance of a 2-OH group at the R^4 position was proven by investigation of compounds **45** and **46**, i.e. the 2-OMe analogs of compounds **19** and **10**, respectively. Both compounds were inactive in the thiol conjugation assay and had > 200-fold lower affinity in the FP assay, showing the critical influence of the 2-OH group for TEAD binding (Figure 3A-3C).

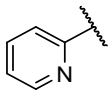
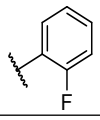
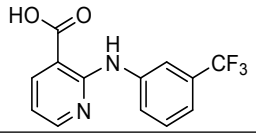
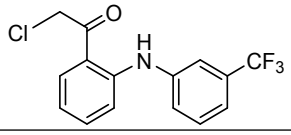
We additionally confirmed the TEAD binding of selected analogs (**19, 27, 42, 45**) using differential scanning fluorimetry (Figure 3D). Compound **19** lowered the melting point (Tm) of non-acylated hTEAD4 by 5 °C, and its 2-pyrido (R^1), 4-bromo (R^3) analog (**42**) induced a smaller Tm shift (-2 °C) aligning with the binding experiments (Table 1). Consistent with the competitive binding experiments, compounds **27** and **45** did not induce any change in the Tm of non-acylated hTEAD4.

Table 1. SAR studies of the kojic acid analogs.



Compound ID	R^1	R^2	R^3	R^4	Apparent IC_{50} (μM)	
					Thiol Conjugation	Fluorescence Polarization
19		-H		-OH	2.3 ± 0.3	0.2 ± 0.04
20		-H		-OH	16.8 ± 0.9	1.5 ± 0.2
21		-H		-OH	4.0 ± 0.6	0.4 ± 0.03
22				-OH	13.2 ± 2.7	0.9 ± 0.3
23		-H		-OH	1.5 ± 0.4	0.2 ± 0.05
24		-H		-OH	3.1 ± 0.6	0.3 ± 0.03
25		-H		-OH	2.4 ± 0.3	0.3 ± 0.08
26		-H		-OH	> 100	13.4 ± 3.3
27		-H		-OH	53.4 ± 11.5	5.6 ± 0.9
28		-H		-OH	> 100	6.4 ± 0.9
29		-H		-OH	1.7 ± 0.2	0.3 ± 0.09
30		-H		-OH	> 100	22.6 ± 2.8

31		-H		-OH	> 100	38.1 ± 8.4
32		-H		-OH	> 100	36.4 ± 11.9
33		-H		-OH	1.5 ± 0.2	0.3 ± 0.1
34		-H		-OH	1.3 ± 0.3	0.2 ± 0.03
35		-H		-OH	1.9 ± 0.2	0.3 ± 0.01
36		-H		-OH	1.2 ± 0.4	0.2 ± 0.06
37		-H		-OH	1.0 ± 0.3	0.2 ± 0.04
38		-H		-OH	1.1 ± 0.3	0.2 ± 0.05
39		-H		-OH	0.7 ± 0.1	0.1 ± 0.03
40		-H		-OH	18.9 ± 6.2	0.5 ± 0.1
41		-H		-OH	9.2 ± 2.1	1.1 ± 0.2
42		-H		-OH	3.7 ± 0.1	0.4 ± 0.06
10		-H		-OH	3.2 ± 0.6	0.3 ± 0.03
43		-H		-OH	> 100	16.0 ± 2.6
44		-H		-OH	> 100	14.7 ± 3.9
45		-H		$-OCH_3$	> 100	41.6 ± 7.3

46		-H		$-OCH_3$	> 100	49.9 ± 4.8
2					41.9 ± 10.5	5.9 ± 1.1
3 (TED-347)					0.8 ± 0.4	14.1 ± 4.0

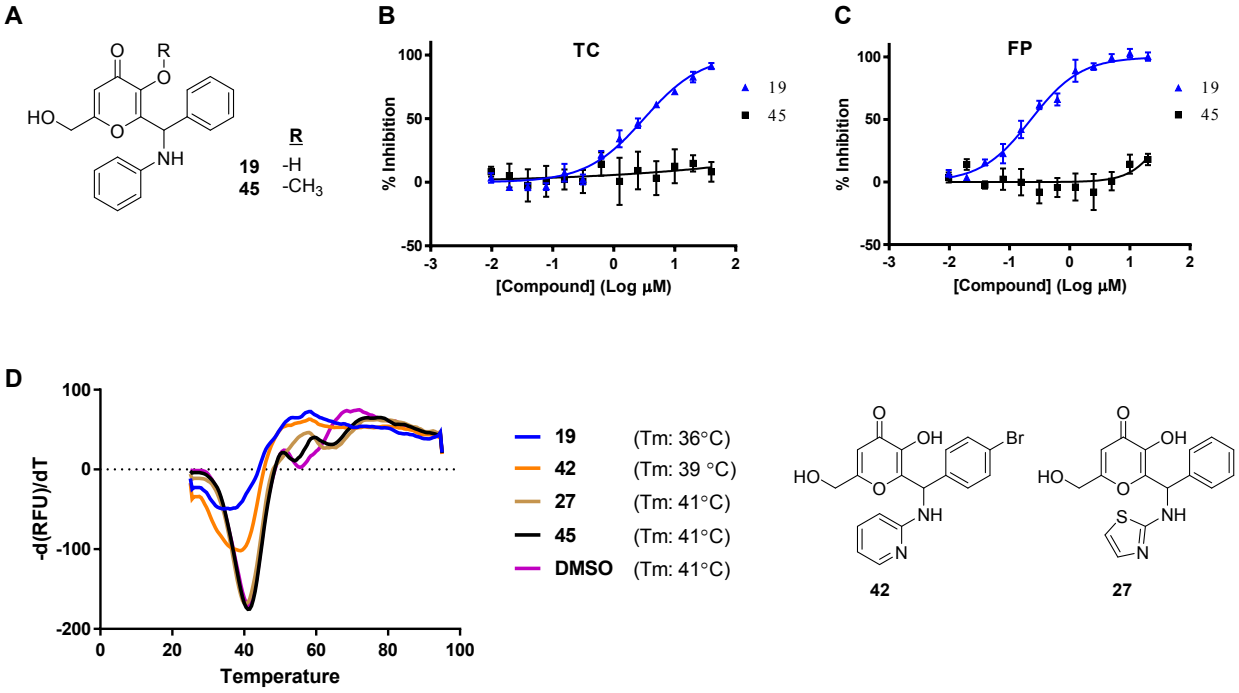


Figure 3: Target engagement studies with compound **19** and its inactive analog **45** in cell-free systems. (A) Structures of the compounds **19** and **45**. Representative inhibition curves in the (B) thiol conjugation assay, and (C) FP-based competitive binding assay. (D) Differential scanning fluorimetry measurements of hTEAD4 (non-acylated) with selected derivatives.

Covalent Binding to the TEAD Central Pocket

In light of the observation that a non-modified, acidic 2-OH group in kojic acid (pKa = 7.9) [44] is required for activity, we reasoned that the compounds of type **19** might undergo a retro-Mannich reaction to yield a Michael acceptor, which then could covalently react with Cys367 in non-acylated hTEAD4 (Figure 4A). In agreement with this hypothesis, methylation of the 2-OH group (**45**, **46**) led to compounds inactive in the thiol conjugation assay (Figure 3B, Table 1). Indeed,

treatment of non-acylated hTEAD4 with 8-equivalents of compound **19** or **45**, and subsequent analysis of the mixture with whole protein ESI-MS spectrometry revealed that- consistent with our hypothesis- modification of the protein (+230 Da) was observed upon incubation with **19** (Figure 4A-4B), while modification of TEAD was not observed upon co-incubation with **45** (Figure 4C).

The hypothesis that compound **19** specifically targets Cys367 that is lipidated under physiological conditions was further strengthened by the observation that incubation of **19** with acylated (i.e. not treated with hydroxylamine) hTEAD4 did not result in formation of an acylated hTEAD4-**19** adduct (Figures 4D and S3A).

In addition, mutants of mouse TEAD4 (mTEAD4) in which the reactive cysteine was replaced with alanine or serine and that should not react with compound **19**, were investigated in the thiol conjugation assay. As shown in Figure 4E, compound **19** cannot inhibit an increase in the fluorescence signal when the Ser or Ala mutant of mTEAD4 is used. Collectively, these observations show that kojic acid analog **19** covalently and specifically binds non-acylated TEAD4.

To confirm the covalent reaction between compound **19** and non-acylated hTEAD4, thiol conjugation and fluorescence polarization assays were used to determine the time-dependent inhibition (TDI) of hTEAD4. Compound **19** showed a low-micromolar inhibition constant (K_i = 2.6 μM), which describes the potency of reversible binding between the compound and the protein before the covalent bond formation (Figure S8A-S8B). Compound **19** inhibited non-acylated hTEAD4 with k_{inact}/K_i = 8.3 μM in the thiol conjugation assay and with k_{inact}/K_i = 4.0 μM in the FP assay (Figures S8B and S8D).

Investigation of the specificity of compound **19** for binding to TEAD homologs by means of the thiol conjugation assay showed that **19** inhibits hTEAD1–4 with similar low micromolar IC_{50}

values (2.6-6.9 μM, Figure S9) consistent with the high homology (72.0-87.9 %)[1] of the transactivation binding domains of TEAD1–4.

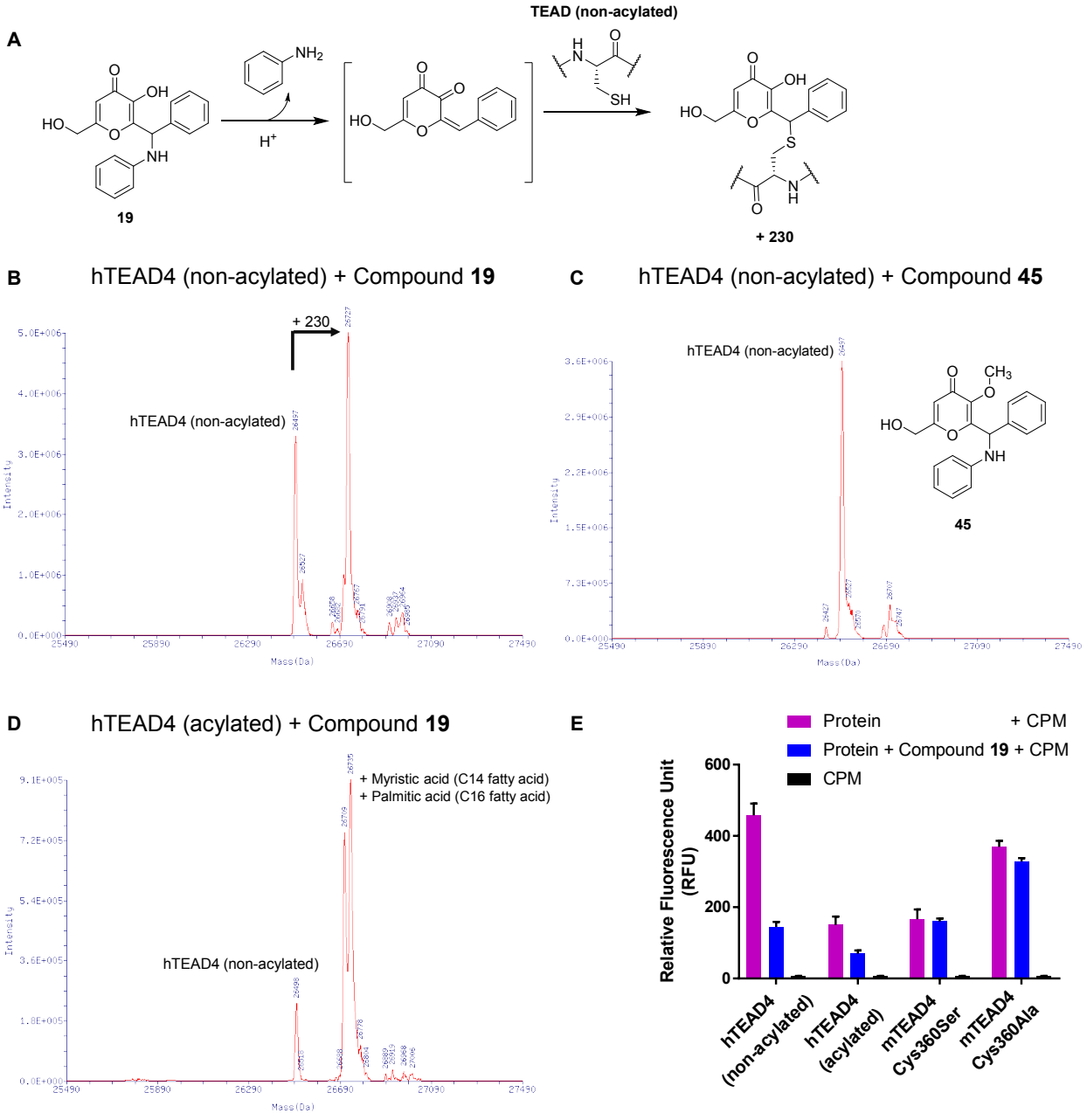


Figure 4. Mechanism of action studies with kojic acid analogs. (A) Hypothesized mechanism of action of compound **19**. Whole protein ESI-MS analysis of hTEAD4 (non-acylated) treatment with kojic acid analog (B) compound **19** and (C) compound **45**. Compound **19** is a covalent binder for

non-acylated hTEAD4 and the -OH group at the 2nd position is required for the predicted reaction with a thiol group. The -OMe analog (**45**) is not a covalent TEAD binder. (D) Whole protein ESI-MS analysis of hTEAD4 (acylated) incubation with **19**. No protein adduct with compound **19** was observed when Cys367 in hTEAD4 is lipidated. (E) Comparison of thiol conjugation inhibition with 10 μM compound **19** in the presence of hTEAD4 (non-acylated), hTEAD4 (acylated) or Cys to Ala/Ser mutants of mTEAD4. When the cysteine residue is replaced with alanine or serine, compound **19** cannot inhibit the thiol conjugation.

Binding Mode Analysis

To get insight into the binding mode and binding site of the new covalent inhibitors, ^{1}H-^{15}N-HSQC spectra of mTEAD4 in the absence and presence of compound **19** or **45** were recorded, and signals were assigned based on the corresponding analysis[45] of the transactivation domain of mTEAD4. Compound **19,** induced line broadening of several residues (Figure S10A), including Phe386 (F386) and Phe408 (F408). These amino acids are in close proximity to the palmitoylation site. Conversely, the negative control **45** did not induce a significant change compared to the DMSO control (Figure S10B). These findings support the biochemical data suggesting that compound **19** binds the central pocket in TEAD.

Covalent TEAD Binders Inhibit the TEAD-YAP Interaction

The chloromethyl ketone flufenamic acid analog (**3**, TED-347) that binds the TEAD central pocket covalently, allosterically inhibits TEAD and YAP interaction and consequently YAP mediated target gene expression.[31] In order to determine whether the new inhibitor class can also disrupt this protein-protein interaction, a fluorescence polarization assay that monitors the binding

of fluorescently-labelled YAP peptide to non-acylated hTEAD4 was used. Pre-incubation with compound **19** for 10 min potently inhibited the interaction of non-acylated hTEAD4 with human YAP^{50-100} with an IC_{50} value of 70 nM. In contrast, and in agreement with the covalent inhibition mechanism proposed above, the OMe analog **45** had > 100-fold lower activity (IC_{50} = 9.5 μM) (Figure 5A), and covalent inhibitor **19** did not inhibit the interaction of YAP with acylated hTEAD4 and mTEAD4 with Cys360Ser or Cys360Ala mutations (Figure 5B-5D) (saturation binding curves of FITC-YAP^{50-100} with the TEAD4 proteins are shown in Figure S11A-D). Overall, these results are in accordance with previous findings[31] for covalent allosteric inhibition of TEAD and show that the covalent and potent TEAD central pocket binding small molecules identified here can block the TEAD-YAP interaction efficiently.

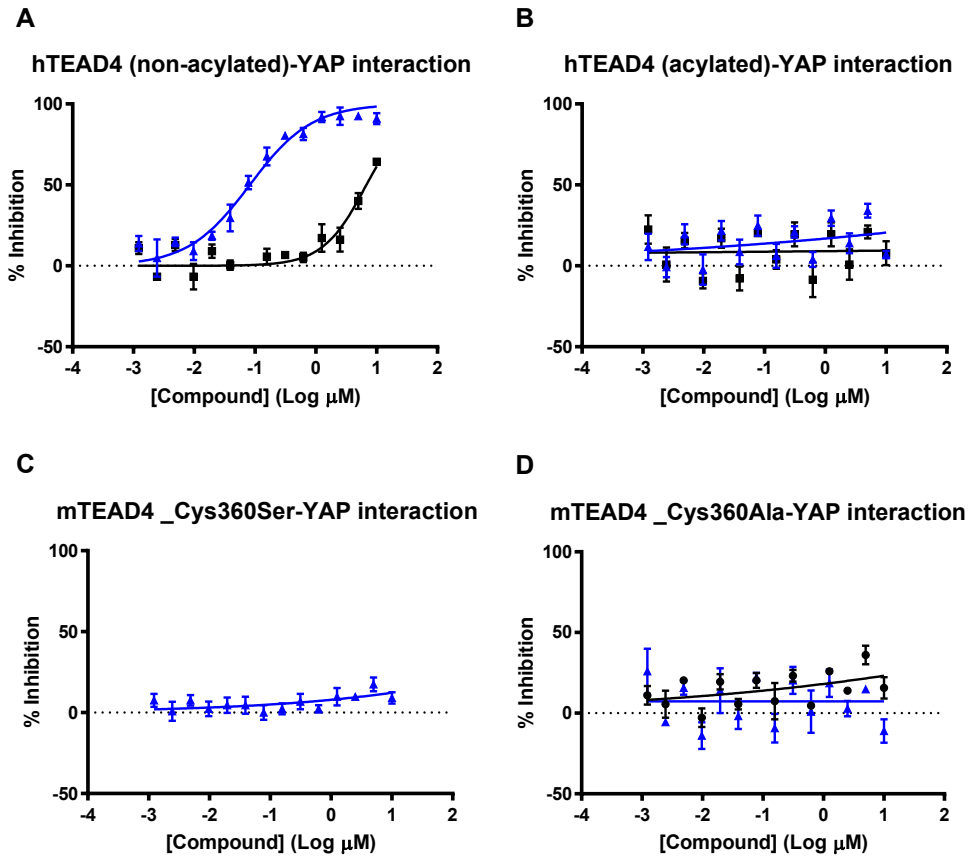


Figure 5. Inhibition of TEAD-YAP interaction as determined with an FP-based competitive binding assay using FITC labeled hYAP$^{50-100}$. Compound **19** (blue triangle) and compound **45** (black square) were tested. (A) Compound **19** can prevent YAP interaction with hTEAD4 (non-acylated), but not with (B) hTEAD4 (acylated), mTEAD4 with (C) Cys360Ser or (D) Cys360Ala mutation.

TEAD Engagement in Cellular Environment

Target engagement in HCT116 cell lysates was proven by means of a Cellular Thermal Shift Assay (CETSA). Treatment of the lysates with compound **19** for 10 mins increased the melting temperature of hTEAD4 from 49.8 °C to 53.1 °C (ΔTm = 3.3 °C), providing evidence for the interaction with hTEAD4 in a biologically relevant environment.

TEADs, together with the coactivators (e.g. YAP), regulate the expression of genes involved in critical processes as cardiogenesis, cell growth and migration.[2] Small molecules that bind the TEAD central pocket have been shown to modulate the TEAD transcriptional activity, such as, inhibition of the expression of well-established TEAD target genes (i.e. ANKRD1, CTGF and CYR61).[34,37] To evaluate the influence of kojic acid analogs on TEAD transcriptional activity, HEK293 cells were incubated with compound **19**, negative control **45** or TED-347. Treatment with compound **19** for 6 h resulted in 90 % reduction of the mRNA level of the TEAD target gene ANKRD1 compared to the DMSO control, while compound **45** had only a minimal effect at the same concentration, further supporting the on target activity of the kojic acid analogs.

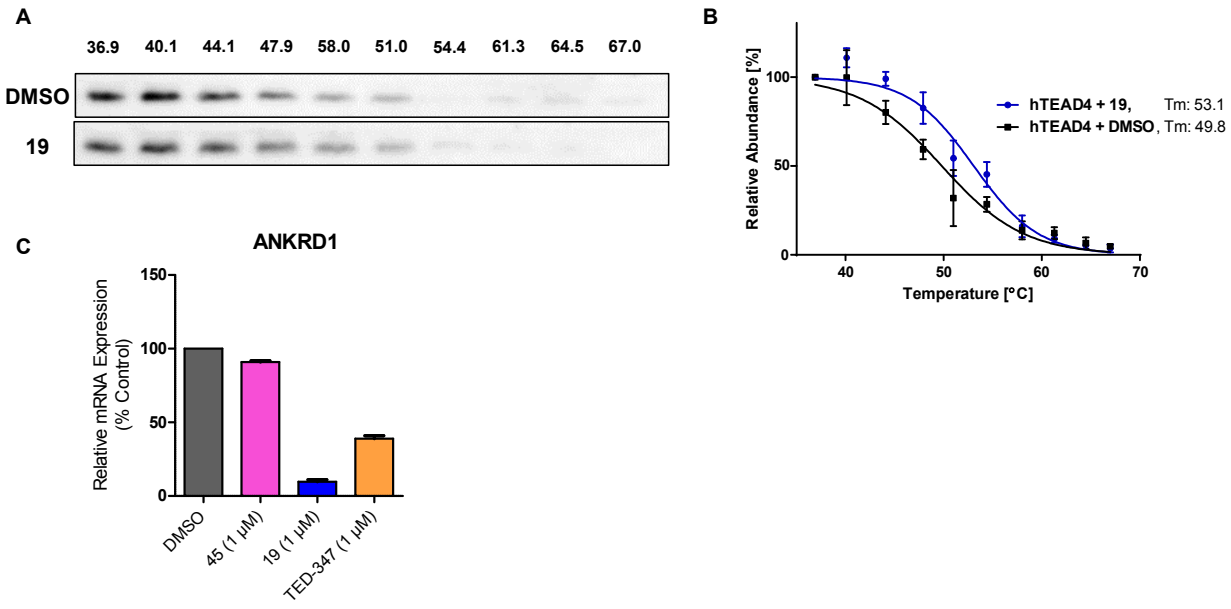


Figure 6. TEAD inhibitory activity of **19** in cellular environment. (A, B) Target engagement in HCT116 cell lysates. (A) Representative immunoblots of hTEAD4 treated with vehicle control (DMSO) or compound **19**. (B) CETSA melting curves of hTEAD4 treated with vehicle control (DMSO) or compound **19**. (C) Inhibition of TEAD target gene ANKRD1 expression by compound **19** in comparison to the control compounds **45** and TED-347.

CONCLUSION

We have developed a new "thiol conjugation assay" that enables the systematic identification of small molecule inhibitors of the TEAD central pocket. By means of this assay, kojic acid analogs were identified as a new class of TEAD inhibitors. Our data show that these inhibitors covalently bind to the Cys residue in the TEAD central pocket that is S-acylated under physiological conditions. The representative kojic acid analog **19** inhibited the interaction of YAP with non-acylated hTEAD4, but not with the corresponding acylated protein and mutants, in which the Cys had been replaced by a serine or an alanine. Furthermore, CETSA in lysate provided evidence for

compound **19** interacting with hTEAD4 in a biologically relevant medium. Consistent with the binding experiments, kojic acid analog **19** inhibited TEAD target gene expression. Taken together we have established a novel methodology that could be a guide for the development of novel classes of TEAD inhibitors interacting with the protein covalently or non-covalently.

EXPERIMENTAL SECTION

Plasmids. hTEAD4 plasmid was isolated from cDNA clone of hTEAD4 (Dharmacon, clone ID: 3913870). Plasmid with mTEAD4 with Cys360Ala mutant was obtained through site-directed mutagenesis using mTEAD4 wild-type template (Dharmacon, clone ID: 40142595). All plasmids were verified by DNA sequencing.

Cloning, expression and purification of recombinant proteins. Sequences of the proteins used in the study are shown in Table S1.

N-His hTEAD4: Plasmid of N-terminal His_6-tagged $hTEAD4^{217-434}$ was subcloned into pOPIN-neo vector, which was used to transform *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent, 230280). The transformed cells were grown in TB medium supplemented with 0.01 % alpha-lactose monohydrate, 2 mM $MgSO_4$, 100 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C for 4 h. Then the temperature was reduced to 25 °C and the culture was incubated for a further 20-24 h. The cells were harvested, and the pellets were taken in lysis buffer containing 50 mM Hepes, 300 mM NaCl, 20 mM imidazole, 1 mM TCEP, pH 8 and lysed by sonication followed by ultracentrifugation. Then the cleared lysate was loaded on to the ÄktaXpress (GE Healthcare) for affinity purification (His trap FF, GE Healthcare) which was followed by size exclusion chromatography (SD75 26/60, GE Healthcare) using 20 mM HEPES, 100 mM NaCl, 1 mM TCEP,

2 mM $MgCl_2$, 5 % glycerol, pH 8. The purified protein was concentrated using Amicon Ultra Centrifugal Filters, 10K (Merck), aliquots were snap frozen and stored at -80 °C.

N-His mTEAD4_Cys360Ser: A method provided earlier was used for the protein expression using 1 mM IPTG.[45] The harvested cell pellets were lysed, and the protein was purified as described above for N-His hTEAD4.

N-His-MBP mTEAD4_Cys360Ala: cDNA of N-terminal His_6- and MBP-tagged mTEAD4$^{209-427}$ with Cys360Ala mutation was used to transform *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent, 230280). The protein was expressed, and the collected pellets were lysed as described for N-His hTEAD4 above. The cleared lysate was loaded on to the ÄktaXpress (GE Healthcare) for affinity purification (His trap FF, GE Healthcare) which was followed by size exclusion chromatography (SD200 16/60, GE Healthcare) using 25 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.2. The purified protein was concentrated using Amicon Ultra Centrifugal Filters, 50K (Merck), aliquots were snap frozen and stored at -80 °C. The storage buffer was exchanged with pH 8 Tris-HCl (20 mM), NaCl (100 mM) $MgCl_2$ (2 mM) using Amicon Ultra Centrifugal Filters (10K) for the binding studies.

N-His-GST hTEAD1: Plasmid of N-terminal His_6-tagged GST-hTEAD1$^{209-426}$ was subcloned into pOPIN-neo vector, which was used to transform *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent, 230280). The transformed cells were grown in TB medium supplemented with 2 mM $MgSO_4$, 100 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37 °C until the optical density reached 0.6. Then the temperature was reduced to 18 °C and the culture was incubated for a further 16 h. The cells were harvested, and the pellets were taken in lysis buffer containing 50 mM Hepes, 300 mM NaCl, 20 mM imidazole, 1 mM TCEP, pH 8 and lysed by sonication followed by ultracentrifugation. Then the cleared lysate was loaded on to the ÄktaXpress (GE Healthcare) for

affinity purification (His trap FF, GE Healthcare) which was followed by size exclusion chromatography (SD75 26/60, GE Healthcare) using 20 mM HEPES, 100 mM NaCl, 1 mM TCEP, 2 mM $MgCl_2$, 5 % glycerol, pH 8. The purified protein was concentrated using Amicon Ultra Centrifugal Filters, 10K (Merck), aliquots were snap frozen and stored at -80 °C.

N-His hTEAD2: Plasmid of N-terminal His_6-tagged $hTEAD4^{217-447}$ was subcloned and the protein was expressed as described for N-His hTEAD4 above.

N-His hTEAD3: N-terminal His_6-tagged $hTEAD4^{216-434}$ was expressed using the protocol described for hTEAD4 earlier.[36]

Deacylation (depalmitoylation) of TEAD. To 500 µl 105 µM (2.8 mg/ml) recombinant hTEAD4 (acylated) in an eppendorf tube, 30 µl of freshly prepared 105 mM hydroxyl amine solution was added slowly and the mixture was gently shaken at room temperature for 3-4 h. The deacylation process was monitored with whole protein ESI-Mass spectrometry. The protein was then purified over two consecutive size exclusion columns (i) Amicon, 0.5 ml 10K (Merck) ii) Zebaspin, 7K (Thermo Fisher) using Tris (20 mM pH 8), NaCl (100 mM), $MgCl_2$ (2 mM) as the final storage buffer. 5 µL aliquots were snap frozen and stored at -80 °C.

4M hydroxylamine solution: 0.46 g (6.67 mmol) hydroxylamine HCl was dissolved in 0.53 ml of H_2O in a glass vial. Then, 0.33 ml of diethylamine was added dropwise, followed by additional 0.2 diethylamine and 0.63 ml H_2O addition. Freshly prepared 4M hydroxylamine solution was further diluted to 105 mM with MilliQ water.

Binding assays. All the experiments were performed using black, low volume, round-bottom, non-binding, 384-well plate plates (Corning 4514). The plates were imaged using Tecan Spark. Spectramax Paradigm (Molecular Devices) was used for imaging the plates during low-throughput

screening. 10 mM stock solutions of the compounds in DMSO were used. The K_d of the tracers and the IC_{50} value of the inhibitors were calculated using GraphPad Prism 7 software.

Binding assays for TEAD central pocket:

a) Thiol conjugation assay: To serially diluted solutions of the tested compounds in 5 µl assay buffer (Hepes (25 mM), NaCl (150 mM) pH 6.5), freshly prepared 5 µl 0.45 µM protein solution was added and incubated at room temperature for 10 mins followed by 5 µl of 0.45 µM CPM solution addition, giving a 15 µl final assay volume, 150 nM protein and 150 nM CPM. The assay plate was shaken at room temperature for 1h before measuring the fluorescence (Ex/Em: 380/470 nm). Compound **19** was used as an internal standard to determine 100 % inhibition.

b) Thiol conjugation assay for low-through-put screening: A library of 14,000 compounds were screened in 1536-well plates with using pH 6.5 MES buffer. To the pre-incubated solution of non-acylated hTEAD4 (0.15 µM final concentration) and compound solution (12.5 µM final concentration), CPM (0.15 µM final concentration) was added, and the assay plates were incubated at room temperature for 50 mins before measuring the fluorescence (Ex/Em: 360/465 nm) using Spectramax Paradigm (Molecular Devices). Z' = 0.6-0.8 (10-plates).

c) Saturation binding experiment: To a serially diluted solution of non-acylated hTEAD4 in 12 µl assay buffer (Hepes (25 mM), NaCl (150 mM) pH 6.5), 8 µl of 25 nM of FITC-Palmitate solution was added, giving 20 µl final assay volume with 10 nM tracer. The assay plate was incubated at room temperature for 1h before measuring fluorescence polarization (Ex/Em: 485/535 nM).

d) FP-based competitive inhibition experiments: To serially diluted solution of the tested compounds in 5 µl assay buffer (Hepes (25 mM), NaCl (150 mM) pH 6.5 buffer), freshly prepared 5 µl 0.15 µM protein solution was added and incubated at room temperature for 10 mins followed by 5 µl of 30 nM FITC-Palmitate solution addition, giving 15 µl final assay volume, 50 nM protein

and 10 nM tracer. The assay plate was shaken at room temperature for one hour before measuring the fluorescence polarization (Ex/Em: 485/535 nm).

Binding assays for TEAD-YAP interaction: All the TEAD-YAP binding experiments for were performed in PBS (pH 7.5).

a) Saturation binding experiments: To a serially diluted solution of the protein in 12 µl PBS, 8 µl of FAM-YAP solution was added giving 20 µl final assay volume. The assay plate was incubated at room temperature for 1h before measuring fluorescence polarization (Ex/Em: 485/530 nM). The final FAM concentrations are as following: hTEAD4 (non-acylated): 2 nM, hTEAD4 (acylated): 2 nM, mTEAD4_Cys360Ser: 10 nM, mTEAD4_Cys360Ala: 10 nM.

b) FP-based competitive inhibition experiments: To serially diluted solution of the tested compounds in 5 µl PBS, freshly prepared 5 µl protein solution was added and incubated at room temperature for 10 mins followed by 5 µl of FAM-YAP$^{50\text{-}100}$ solution addition. The assay plate was shaked at room temperature for one hour before measuring the fluorescence polarization (Ex/Em: 485/535 nm). Final protein and FAM concentrations are as following (protein/FAM): hTEAD4 (non-acylated): 5 nM/2 nM, hTEAD4 (acylated): 3 nM/15 nM, mTEAD4_Cys360Ser: 10 nM/50 nM, mTEAD4_Cys360Ala: 10 nM/50 nM.

Inhibition efficiency experiments: Time-dependent inhibition of hTEAD4 (non-acylated) by compound **19** was monitored in FP (central pocket binding) and thiol conjugation assays at 5-7 different concentration (0.15-40 µM) following 1, 2, 4, and 24 h incubation at 4°C (mean ± SD; n = 2). By fitting the percent of inhibition versus preincubation time at different concertation, k_{obs} was calculated. Next, k_{obs} at was plotted against concentration and was further fitted to a non-linear regression model to determine the kinetic parameters of covalent inhibition of hTEAD4, where plateau is showing k_{inact} (min^{-1}) and $t_{1/2}$ is K_i (µM).

Differential Scanning Fluorometry (DSF). Non-acylated hTEAD4 (20 μM) was incubated in the absence or presence of inhibitors (80 μM) at 25°C for 10 min. Then, SYPRO orange (Sigma Aldrich) at a final concentration of 5x was added and the mixture was heated at a ramp rate of 1°C/min in a qPCR machine (Applied Biosystems). Derivative of the SYPRO orange fluorescent intensity is plotted as a function of temperature.

Whole protein ESI-mass spectrometry. The protein solution (1 μl 5 μM) was injected to UPLC-MS (ThermoScientific_UltiMate 3000 LC coupled to a Velos Pro MS) through a desalting column (MassPrepTM Online desalting, 2.1×10 mm). For reactions, 1 μM of the protein was incubated at room temperature for one hour with 8 equiv. of the compound in Hepes (25 mM), NaCl (150 mM) pH 6.5 buffer. Then, the mixture was centrifuged before injecting 5 μl. The deconvolution of m/z values was performed using Promass Xcalibur software.

Tandem mass spectrometry experiments

Non-acylated hTEAD4-CPM reaction: To 5 μl assay buffer (Hepes (25 mM), NaCl (150 mM) pH 6.5), 5 μl 0.6 μM non-acylated hTEAD4 and 5 μl 0.6 μM CPM solution was added and incubated at room temperature for 1h in a 384-well plate (Corning 4514). The reaction mixtures were collected from 16-replicates and transferred to a non-binding eppendorf tube followed by co-incubating with 10 μl 60 μM DTT for 30 mins to quench the unreacted CPM. The protein mixture was then purified through Amicon (10K) to exchange the buffer with 50 mM Tris-HCl (pH 7.5).

In solution digestion and purification: To 50 μl of the protein sample in 50 mM Tris-HCl (pH 7.5), 250 μl denaturizing/reducing buffer (10 M urea, 1.2 mM TCEP, 50 mM Tris, pH 7.5) was added and shaken at room temperature (350 rpm, 30 mins). Next, 33.3 μl 50 mM alkylation solution (iodoacetamide prepared in denaturizing/reducing buffer) was added and shaken (350 rpm) at room temperature for 30 mins. Subsequently, 23.5 μl of 1 ng/μl solution of LysC (Wako,

125-05061) was added at 37 °C, and shaken (350 rpm) for 1h, followed by 7.8 µl of 6 ng/ µl Trypsin (Roche, 03708969001) addition. The mixture was shaked (350 rpm) at 37 °C overnight). The digested samples were stage tip purified using C18 (octadecyl) tips (3M High Performance Extraction Discs, Empore) using a protocol described earlier.[46] Bovine serum albumin (BSA) (50 µl, 0.5 µM prepared in 50 mM Tris-HCl, pH 7.5) was used in parallel as a control for the digestion-purification experiments and treated as described above. The peptide solution was injected to nanoLC-MS (ThermoScientific_UltiMate 3000 nanoLC coupled to a QExactive Plus or QExactive HF MS) through a nano column (ThermoScientific PEPMAP C18 2UM 75UMX500MM).

mTEAD4 (non-acylated) HSCQ Experiments. The $^{13}C/^{15}N$-labeled mTEAD4 was prepared following a protocol established earlier,[45] and the protein was deacylated as described above for hTEAD4. ^{1}H-^{15}N-HSQC spectra in the presence of DMSO or the test compound (0.5 mM) were collected at 37 °C on a Bruker 600 MHz magnet equipped with a cryo-probe in 20 mM HEPES, pH 7.3 buffer containing 150 mM NaCl and 1 mM DTT. The spectra were processed and overlaid.

Cellular Thermal Shift Assay (CETSA) Experiments

Lysate preparation: HCT116 cells were grown in DMEM (*Sigma Aldrich*, #P04-03550) containing 10% FBS, 1 x NEAA (*Sigma Aldrich*, # P08-32800) and 1 mM sodium pyruvate (*Sigma Aldrich,* #S8636) until they reached 90% confluency. Cells were detached, transferred to two separate tubes and washed three times with PBS. Then 0.6 mL PBS containing 0.04% NP-40 were added to each tube and cells were lysed by means of freeze and thaw. Lysates were centrifuged (Beckman Optima MAX-TL) at 100,000 *g*, 4°C for 25 min and protein concentration was determined using the DC™ Protein Assay Kit I (*BioRad*, #5000111). 1.5 mL of aliquots containing 1 mg/mL protein were stored at -80°C until further usage.

CETSA: 50 µM of compound **19** (2.75 µL 10 mM stock) or DMSO (2.75 µL) were added to 550 µL of HCT116 lysates (1 mg/mL), samples were mixed and incubated for 10 min at room temperature. Treated and non-treated lysates were divided into ten aliquots, each 50 µL in PCR tubes. The aliquots were individually heated at different temperatures (Eppendorf Mastercycler ep Gradient S). After the heat treatment the cell lysates were directly, completely transferred to polycarbonate tubes and centrifuged (Beckman Optima MAX-TL) at 100,000 *g*, 4°C for 25 min. To 16 µL of each supernatant was added 4 µL of 5x loading buffer and incubated for 5 min at 95°C, before samples were analyzed by Immunoblotting. Proteins were separated by SDS-PAGE, and transferred to PVDF membrane using wet transfer. The membranes were blocked with Odyssey Blocking Buffer (TBS; *Li-Cor*) for 1 h and incubated with the primary antibody (Mouse Anti-TEAD4 antibody corresponding to amino acids 151-261 of Human TEAD4 - ChIP Grade (ab58310)) in Odyssey blocking buffer containing 0.2% Tween-20 at 4°C overnight. After washing with TBS-T (TBS containing 0.1 % Tween-20) the membrane was incubated with the secondary antibody coupled to IRDye® 800CW (goat anti-mouse IgG, *Li-COR*) for 1 h, in Odyssey blocking buffer containing 0.2 % Tween-20 and 0.1 % SDS at room temperature. Membranes were washed with TBS-T, then TBS before images were taken (*Bio-Rad ChemiDoc™ MP Imaging System*).

Analysis: Images were analyzed with ImageJ (FUJI). Normalization and all calculations were done with GraphPad Prism 7 software. (Curves: IC_{50} variable slope → turning points correspond to melting temperature in obtained curves).

RNA purification and RT-qPCR. HEK293 cells were treated with DMSO or the indicated compound for the indicated duration. RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's procedure. DNA was removed on

column by DNase digestion with RNase-free DNase Set (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Following the manufacturer's protocol, cDNA templates were synthesized from 700 ng total RNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany).

The expression levels of the TEAD target gene ANKRD1 was assessed by real-time quantitative PCR. 100 ng cDNA was amplified using 500 nM of gene-specific primers and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Feldkirchen, Germany) in a total volume of 10 µL for 50 cycles. All measurements were performed in both technical and biological triplicates using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Feldkirchen, Germany).

The sequences of primers for ANKRD1 were forward 5'-AGACTCCTTCAGCCAACATGATG-3' and reverse 5'-CTCTCCATCTCTGAAATCCTCAGG-3'. The sequences of primers for GAPDH were forward 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Assay media stability. The compounds were incubated in MES (25 mM), pH 6.5 buffer or Hepes (25 mM), NaCl (150 mM) pH 6.5 buffer for an hour at room temperature before injecting to a reverse phase HPLC (ThermoScientific_Ultimate 3000) equipped with a C18 column (2/50 Nucleodur C18 gravity 1.8 µm von Macherey-Nagel).

CHEMISTRY

All the final compounds were characterized with ^{1}H-NMR, ^{13}C-NMR (500, 700 MHz, Bruker), and HR-MS (ESI+) (LTQ Orbitrap mass spectrometer). The intermediates were characterized with ^{1}H-NMR, ^{13}C-NMR (500, 700 MHz, Bruker), and LR-MS (ESI+) (Agilent 1290 Infinity

UPLC/MS coupled to Agilent 6120 Quadripole or ThermoScientific_UltiMate 3000 LCMS coupled to LQC Fleet). Chemical shifts were reported in ppm relative to TMS. CD_3OD (3.31 ppm), or DMSO-d_6 (2.50 ppm) was used as the internal standard for ^{1}H-NMR spectra. CD_3OD (49.0 ppm) or DMSO-d_6 (39.52 ppm) was used as internal standard for ^{13}C-NMR spectra. All the final compounds have purity ≥ 95% as determined by reverse phase UPLC (Agilent 1260 Infinity or ThermoScientific_Dionex Ultimate 3000 LCMS) equipped with a C18 column (Nucleodur C18 Gravity, 5 μm).

Compounds **1**–**3**, **10-16** were obtained from commercial sources and used without further purification.

2-((2-fluorophenyl)(pyridin-2-ylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (10)

2 mmol of each component (kojic acid, 2-fluorobenzaldehyde and 2-amino pyridine) in DMF were stirred at room temperature for 2 days before concentrated in vacuo. Then the remaining crude was purified over silica column using ethyl acetate:methanol (20:1) as eluent. The collected fractions were concentrated and further washed with methanol yielding compound **10** as a white solid. HRMS (ESI): m/z calculated for $C_{18}H_{15}FN_2O_4 + H^+$ $[M+H]^+$: 343.1089, and found 343.1089. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.20 (s, 1H), 7.94 (dd, *J* = 5.2, 1.8 Hz, 1H), 7.51 (td, *J* = 7.8, 1.9 Hz, 1H), 7.44 – 7.37 (m, 2H), 7.33 (tdd, *J* = 7.4, 5.2, 1.8 Hz, 1H), 7.17 (q, *J* = 9.3, 8.1 Hz, 2H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 6.53 (dd, *J* = 7.0, 5.0 Hz, 1H), 6.30 (s, 1H), 5.64 (t, *J* = 6.2 Hz, 1H), 4.31 – 4.18 (m, 2H).

^{13}C NMR (126 MHz, DMSO) δ 173.8, 167.5, 160.8, 158.8, 157.1, 148.1, 147.4, 141.2, 136.9, 129.5, 129.4, 128.99, 128.96, 126.8, 126.7, 124.5, 124.4, 115.4, 115.3, 112.8, 109.1, 108.9, 59.4, 44.80, 44.77, 40.1, 40.0, 39.94, 39.85, 39.78, 39.7, 39.5, 39.4, 39.2, 39.0.

3-hydroxy-6-(hydroxymethyl)-2-(phenyl(phenylamino)methyl)-4H-pyran-4-one (19)

2 mmol of each component (kojic acid, benzaldehyde and aniline) in DMF were stirred at room temperature for 3 days before concentrated in vacuo. Then the remaining crude was washed with ethanol yielding compound **19** as a white solid (34 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{17}NO_4$ + H^+ $[M+H]^+$: 324.1230, and found 324.1236. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.36 (s, 1H), 7.50 – 7.45 (m, 2H), 7.37 (t, *J* = 7.7 Hz, 2H), 7.32 – 7.27 (m, 1H), 7.09 – 7.04 (m, 2H), 6.73 – 6.68 (m, 2H), 6.60 – 6.55 (m, 1H), 6.41 (d, *J* = 8.2 Hz, 1H), 6.27 (s, 1H), 5.89 (d, *J* = 8.2 Hz, 1H), 5.61 (t, *J* = 6.3 Hz, 1H), 4.25 (qd, *J* = 15.5, 5.8 Hz, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.5, 148.9, 147.2, 141.5, 139.2, 128.9, 128.6, 127.6, 127.0, 116.9, 112.9, 108.9, 59.4, 52.9.

^{1}H-NMR, ^{13}C- NMR, purity and assay media stability spectra of compound **19** is shown in Supporting Information (pages S17, S19, S20-21).

3-hydroxy-6-(hydroxymethyl)-2-((phenylamino)(p-tolyl)methyl)-4H-pyran-4-one (20)

1 mmol of each component (kojic acid, benzaldehyde and isopropylamine) in 15 ml EtOH were stirred at room temperature for 7h before concentrating in vacuo. The remaining crude was silica column purified using ethyl acetate:methanol (25:1) as eluent. The compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 19 mg compound **20** (6.5 % yield). HRMS (ESI): m/z calculated for $C_{16}H_{19}NO_4$ + H^+ $[M+H]^+$: 290.1387, and found 290.1391. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 7.43 – 7.40 (m, 2H), 7.33 (dd, *J* = 8.4, 6.9 Hz, 2H), 7.28 – 7.24 (m, 1H), 6.26 (s, 1H), 5.62 (t, *J* = 6.4 Hz, 1H), 5.16 (s, 1H), 4.31 – 4.22 (m, 2H), 2.64 (h, *J* = 6.2 Hz, 1H), 1.01 (dd, *J* = 10.4, 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 173.5, 167.1, 150.2, 141.9, 140.5, 128.4, 127.3, 127.1, 108.9, 59.5, 55.8, 45.8, 22.9, 22.3.

2-((benzylamino)(phenyl)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (21)

1 mmol of each component (kojic acid, benzaldehyde and benzyl amine) in 15 ml EtOH were stirred at room temperature for 3 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. A second silica column purification step was pursued using CH_2Cl_2:MeOH (10:0.75) as eluent. The purified compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 22 mg compound **21** as a cream color solid (0.065 mmol, % 65 yield). HRMS (ESI): m/z calculated for $C_{20}H_{19}NO_4$ + H^+ $[M+H]^+$: 338.1387, and found 338.1375. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.09 (s, 1H), 7.43 – 7.40 (m, 2H), 7.36 – 7.25 (m, 7H), 7.24 – 7.20 (m, 1H), 6.26 (s, 1H), 5.65 (t, J = 6.4 Hz, 1H), 5.06 (s, 1H), 4.27 (qd, J = 15.5, 6.2 Hz, 2H), 3.66 (s, 2H).

^{13}C NMR (126 MHz, DMSO) δ 173.5, 167.2, 149.8, 141.9, 140.3, 140.1, 128.5, 128.14, 128.11, 127.4, 127.1, 126.8, 108.8, 59.5, 57.4, 50.7.

3-hydroxy-6-(hydroxymethyl)-2-(isoindolin-2-yl(phenyl)methyl)-4H-pyran-4-one (22)

1 mmol of each component (kojic acid, benzaldehyde and isoindoine) in 15 ml EtOH were stirred at room temperature overnight before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 38 mg compound **22** as an off white solid (11 % yield). HRMS (ESI): m/z calculated for $C_{21}H_{19}NO_4$ + H^+ $[M+H]^+$: 350.1387, and found 350.1369. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.23 (s, 1H), 7.57 – 7.54 (m, 2H), 7.38 (t, J = 7.6 Hz, 2H), 7.32 (td, J = 7.1, 1.4 Hz, 1H), 7.22 – 7.16 (m, 4H), 6.30 (s, 1H), 5.65 (t, J = 6.2 Hz, 1H), 5.19 (s, 1H), 4.35 – 4.25 (m, 2H), 3.85 – 3.78 (m, 4H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.7, 148.5, 142.0, 139.4, 139.1, 128.6, 128.1, 127.9, 126.6, 122.3, 108.8, 64.4, 59.5, 57.1.

2-(((3-fluorophenyl)amino)(phenyl)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (23)

1 mmol of each component (kojic acid, benzaldehyde and 3-fluoroaniline) in 15 ml EtOH were stirred at room temperature for 5 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated and the remaining crude was silica column purified using ethyl acetate as eluent. Next, the collected fractions were concentrated and washed with CH_2Cl_2. The remaining white powder was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 46 mg compound **23** as a cream color solid (0.135 mmol, 13.5 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{16}FNO_4$ + H^+ $[M+H]^+$: 342.1136, and found 342.1139. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.48 (s, 1H), 7.50 – 7.43 (m, 2H), 7.42 – 7.35 (m, 2H), 7.34 – 7.25 (m, 1H), 7.08 (td, *J* = 8.2, 7.0 Hz, 1H), 6.81 (d, *J* = 7.9 Hz, 1H), 6.55 – 6.51 (m, 1H), 6.46 (dt, *J* = 12.3, 2.3 Hz, 1H), 6.35 (td, *J* = 8.5, 2.5 Hz, 1H), 6.29 (s, 1H), 5.88 (d, *J* = 8.0 Hz, 1H), 5.63 (t, *J* = 6.2 Hz, 1H), 4.30 – 4.20 (m, 2H).
^{13}C NMR (126 MHz, DMSO) δ 173.6, 167.6, 164.2, 162.3, 149.37, 149.28, 148.3, 141.7, 138.7, 130.45, 130.37, 128.7, 127.8, 127.1, 108.99, 108.98, 108.95, 103.1, 102.9, 99.4, 99.2, 59.4, 52.9.

3-hydroxy-6-(hydroxymethyl)-2-(phenyl(pyridin-2-ylamino)methyl)-4H-pyran-4-one (24)

2 mmol of each component (kojic acid, benzaldehyde and 2-aminopyridine) in DMF were stirred at room temperature for 3 days before concentrated in vacuo. Then the remaining crude was washed with ethanol yielding compound **24** as a white solid. HRMS (ESI): m/z calculated for

$C_{18}H_{16}N_2O_4$ + H^+ $[M+H]^+$: 325.1183, and found 325.1169. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.25 (s, 1H), 7.95 (dd, *J* = 5.2, 1.8 Hz, 1H), 7.44 – 7.31 (m, 6H), 7.28 – 7.23 (m, 1H), 6.72 (d, *J* = 8.4 Hz, 1H), 6.67 (d, *J* = 8.6 Hz, 1H), 6.53 (dd, *J* = 7.0, 5.1 Hz, 1H), 6.29 (s, 1H), 5.64 (t, *J* = 6.3 Hz, 1H), 4.28 (qd, *J* = 15.6, 6.2 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ 173.8, 167.4, 157.4, 149.5, 147.4, 141.0, 140.2, 137.0, 128.5, 127.4, 126.8, 112.7, 109.1, 108.9, 59.5, 50.1.

3-hydroxy-6-(hydroxymethyl)-2-(((4-methylpyridin-2-yl)amino)(phenyl)methyl)-4H-pyran-4-one (25)

1 mmol of each component (kojic acid, benzaldehyde and 2-Amino-4-methylpyridine) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was taken in a mixture of ethyl acetate: MeOH (10:0.5) and sonicated for 10 mins. The solution was discarded and the wash step was repeated. Next, the was step was repeated using EtOH. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 156 mg compound **25** as a white solid (0.46 mmol, 46 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{18}N_2O_4$ + H^+ $[M+H]^+$: 339.1339, and found 339.1325. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.25 (s, 1H), 7.82 (d, *J* = 5.2 Hz, 1H), 7.41 – 7.36 (m, 2H), 7.36 – 7.30 (m, 2H), 7.29 – 7.22 (m, 2H), 6.67 (d, *J* = 8.7 Hz, 1H), 6.54 (d, *J* = 1.5 Hz, 1H), 6.39 (dd, *J* = 5.3, 1.4 Hz, 1H), 6.29 (s, 1H), 5.64 (t, *J* = 6.3 Hz, 1H), 4.28 (qdd, *J* = 15.6, 6.3, 0.9 Hz, 2H), 2.15 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 173.8, 167.4, 157.6, 149.6, 147.23, 147.07, 140.9, 140.3, 128.5, 127.3, 126.8, 114.4, 109.0, 108.9, 59.5, 50.1, 20.6.

3-hydroxy-6-(hydroxymethyl)-2-(phenyl(pyrimidin-2-ylamino)methyl)-4H-pyran-4-one (26)

1 mmol of each component (kojic acid, benzaldehyde and 2-aminopyrimidine) in 15 ml EtOH were stirred at room temperature for 6 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers together were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using CH_2Cl_2:MeOH (10:0.5) as eluent. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 8 mg compound **26** as a white solid (0.025 mmol, 2.5 % yield). HRMS (ESI): m/z calculated for $C_{17}H_{15}N_3O_4$ + H^+ $[M+H]^+$: 326.1135, and found 326.1128. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.29 (s, 1H), 8.33 (d, *J* = 4.8 Hz, 2H), 8.05 (d, *J* = 9.1 Hz, 1H), 7.41 (dd, *J* = 8.0, 1.4 Hz, 2H), 7.34 (t, *J* = 7.7 Hz, 2H), 7.30 – 7.22 (m, 1H), 6.69 (d, *J* = 9.1 Hz, 1H), 6.66 (t, *J* = 4.8 Hz, 1H), 6.29 (d, *J* = 0.9 Hz, 1H), 5.65 (t, *J* = 6.5 Hz, 1H), 4.29 (dddd, *J* = 56.1, 15.4, 6.5, 0.9 Hz, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.8, 167.3, 158.14, 157.99, 148.6, 140.8, 139.8, 128.5, 127.4, 126.6, 111.2, 108.9, 59.5, 50.4.

3-hydroxy-6-(hydroxymethyl)-2-(phenyl(thiazol-2-ylamino)methyl)-4H-pyran-4-one (27)

1 mmol of each component (kojic acid, benzaldehyde and thiazol-2-amine) in 15 ml EtOH were stirred at room temperature for 14 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using CH_2Cl_2 : MeOH (10:1) as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 34 mg compound **27** as an off-white solid (10 % yield). HRMS (ESI): m/z calculated for

$C_{16}H_{14}N_2O_4S$ + H^+ $[M+H]^+$: 331.0747, and found 331.0735. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 9.34 (s, 1H), 8.48 (d, *J* = 8.3 Hz, 1H), 7.42 – 7.38 (m, 2H), 7.36 (t, *J* = 7.7 Hz, 2H), 7.31 – 7.26 (m, 1H), 7.01 (d, *J* = 3.6 Hz, 1H), 6.67 (d, *J* = 3.6 Hz, 1H), 6.39 (d, *J* = 7.9 Hz, 1H), 6.30 (s, 1H), 5.63 (t, *J* = 6.3 Hz, 1H), 4.34 – 4.21 (m, 2H).

^{13}C NMR (151 MHz, DMSO) δ 173.7, 167.54, 167.41, 148.3, 141.1, 139.1, 138.4, 128.6, 127.7, 126.8, 109.0, 107.6, 59.4, 53.8.

2-(((1H-benzo[d]imidazol-2-yl)amino)(phenyl)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (28)

1 mmol of each component (kojic acid, benzaldehyde and 1*H*-benzimidazol-2-amine in 15 ml EtOH were stirred at room temperature for 20 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was taken in a mixture of ethyl acetate: MeOH (10:0.5) and sonicated for 5 mins. The solution was discarded, and the wash step was repeated twice. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 22 mg compound **28** as an off-white solid (0.06 mmol, 6 % yield). HRMS (ESI): m/z calculated for $C_{20}H_{17}N_3O_4$ + H^+ $[M+H]^+$: 364.1292, and found 364.1268. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 10.69 (bs, 1H), 9.62 (bs, 1H), 7.60 (d, *J* = 9.1 Hz, 1H), 7.47 – 7.43 (m, 2H), 7.37 (t, *J* = 7.6 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 1H), 7.17 (d, *J* = 8.7 Hz, 2H), 6.89 (s, 2H), 6.49 (d, *J* = 8.5 Hz, 1H), 6.31 (s, 1H), 5.65 (bs, 1H), 4.29 (q, *J* = 15.4 Hz, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.9, 167.2, 154.1, 149.0, 141.1, 139.5, 128.6, 127.6, 126.7, 109.2, 59.5, 52.6.

4-(((3-hydroxy-6-(hydroxymethyl)-4-oxo-4H-pyran-2-yl)(phenyl)methyl)amino)benzamide (29)

1 mmol of each component (kojic acid, benzaldehyde and 4-aminobenzamide) in 15 ml EtOH were stirred at room temperature for 3 days before concentrating in vacuo. The remaining crude was partitioned between ethyl acetate and water. The observed white precipitate from both layers were collected, washed with ethyl acetate and water. The compound was then taken in acetonitrile-H_2O mixture and lyophilized yielding 95 mg compound **29** as an off-white solid (26 % yield). HRMS (ESI): m/z calculated for $C_{20}H_{18}N_2O_5 + H^+$ $[M+H]^+$: 367.1288, and found 367.1289. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.46 (s, 1H), 7.65 – 7.61 (m, 2H), 7.55 (bs, 1H), 7.50 – 7.45 (m, 2H), 7.38 (t, J = 7.7 Hz, 2H), 7.34 – 7.30 (m, 1H), 6.94 (d, J = 7.9 Hz, 1H), 6.90 (bs, 1H), 6.71 – 6.67 (m, 2H), 6.29 (d, J = 0.9 Hz, 1H), 5.96 (d, J = 7.9 Hz, 1H), 5.62 (t, J = 6.3 Hz, 1H), 4.30 – 4.21 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.8, 167.6, 149.7, 148.3, 141.7, 138.7, 128.96, 128.67, 127.8, 127.1, 122.4, 111.7, 108.9, 59.4, 52.7.

2-(cyclohexyl(phenylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (30)

1 mmol of each component (kojic acid, cyclohexanecarboxaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 5-days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and brine. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate:CH_2Cl_2 (1:1) as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 80 mg compound **30** as an off-white solid (0.24 mmol, 24 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{23}NO_4 + H^+$ $[M+H]^+$: 330.1700, and found 330.1702. ^{1}H NMR (600 MHz, DMSO-d_6): δ

= 9.03 (s, 1H), 7.04 – 6.95 (m, 2H), 6.64 – 6.56 (m, 2H), 6.52 – 6.44 (m, 1H), 6.24 (d, J = 0.9 Hz, 1H), 5.88 (d, J = 8.7 Hz, 1H), 5.59 (t, J = 6.3 Hz, 1H), 4.37 (t, J = 9.0 Hz, 1H), 4.25 (qdd, J = 15.4, 6.3, 0.9 Hz, 2H), 2.12 – 2.05 (m, 1H), 1.84 – 1.76 (m, 1H), 1.73 – 1.56 (m, 3H), 1.43 (d, J = 12.8 Hz, 1H), 1.27 – 1.11 (m, 3H), 1.08 – 0.95 (m, 2H).

^{13}C NMR (151 MHz, DMSO) δ 173.3, 167.4, 149.6, 148.0, 142.7, 128.9, 116.0, 112.2, 108.7, 59.5, 54.3, 30.0, 29.0, 25.9, 25.4.

3-hydroxy-6-(hydroxymethyl)-2-(2-methyl-1-(phenylamino)propyl)-4H-pyran-4-one (31)

1 mmol of each component (kojic acid, isobutyraldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 5-days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and brine. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate:CH_2Cl_2 (1:1) as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 75 mg compound **31** as an off-white solid (0.26 mmol, 26 % yield). HRMS (ESI): m/z calculated for $C_{16}H_{19}NO_4$ + H^+ $[M+H]^+$: 290.1387, and found 290.1389. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 9.04 (s, 1H), 7.04 – 6.98 (m, 2H), 6.65 – 6.59 (m, 2H), 6.50 (tt, J = 7.3, 1.1 Hz, 1H), 6.24 (d, J = 0.9 Hz, 1H), 5.90 (d, J = 8.8 Hz, 1H), 5.59 (t, J = 6.2 Hz, 1H), 4.32 – 4.18 (m, 3H), 2.09 (dp, J = 9.0, 6.6 Hz, 1H), 1.08 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.7 Hz, 3H).

^{13}C NMR (151 MHz, DMSO) δ 173.3, 167.3, 149.8, 147.9, 142.5, 128.9, 116.1, 112.2, 108.7, 59.5, 55.5, 30.8, 20.1, 19.1.

3-hydroxy-6-(hydroxymethyl)-2-((phenylamino)methyl)-4H-pyran-4-one (32)

1 mmol of each component (kojic acid, formaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 5 days before concentrating in vacuo. The remaining crude was silica column purified using CH_2Cl_2: MeOH (10:1) as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 100 mg compound **32** as a light brown-orange solid (40 % yield). HRMS (ESI): m/z calculated for $C_{13}H_{13}NO_4 + H^+$ $[M+H]^+$: 248.0917, and found 248.0918. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.19 (s, 1H), 7.09 – 7.01 (m, 2H), 6.67 – 6.61 (m, 2H), 6.57 – 6.50 (m, 1H), 6.29 (s, 1H), 6.16 (t, *J* = 6.1 Hz, 1H), 5.65 (t, *J* = 6.1 Hz, 1H), 4.26 (dd, *J* = 6.2, 0.9 Hz, 2H), 4.20 (d, *J* = 6.0 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ 173.6, 167.6, 148.0, 147.8, 142.3, 128.9, 116.3, 112.1, 108.9, 59.5.

4-((3-hydroxy-6-(hydroxymethyl)-4-oxo-4H-pyran-2-yl)(phenylamino)methyl)benzamide (33)

1 mmol of each component (kojic acid, 4-formylbenzamide and aniline) in 25 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using ethyl acetate:MeOH (10:1) as eluent. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 40 mg compound **33** as a bright yellow solid (11 % yield). HRMS (ESI): m/z calculated for $C_{20}H_{18}N_2O_5 + H^+$ $[M+H]^+$: 367.1288, and found 367.1289. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.43 (s, 1H), 7.94 (bs, 1H), 7.87 – 7.83 (m, 2H), 7.56 – 7.52 (m, 2H), 7.34 (bs, 1H), 7.10 – 7.05 (m, 2H), 6.73 – 6.68 (m, 2H), 6.59 (tt, *J* = 7.3, 1.1 Hz, 1H), 6.48 (d, *J* = 8.2 Hz, 1H), 6.28 (d, *J* = 0.9 Hz, 1H), 5.94 (d, *J* = 8.2 Hz, 1H), 5.62 (t, *J* = 6.3 Hz, 1H), 4.30 – 4.20 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.57, 167.55, 148.4, 147.1, 142.4, 141.6, 133.7, 128.9, 127.8, 126.8, 117.0, 113.0, 108.9, 59.4, 52.8.

3-hydroxy-6-(hydroxymethyl)-2-((phenylamino)(p-tolyl)methyl)-4H-pyran-4-one (34)

2 mmol of each component (kojic acid, 4-methylbenzaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was silica column purified using ethyl acetate as eluent. The purified compound was further washed with diethyl ether. Then the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 0.16 g compound **34** as a light brown solid (24 % yield). HRMS (ESI): m/z calculated for $C_{20}H_{19}NO_4$ + H^+ $[M+H]^+$: 338.1387, and found 338.1390. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.32 (s, 1H), 7.36 (d, *J* = 8.0 Hz, 2H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.09 – 7.02 (m, 2H), 6.69 (d, *J* = 8.0 Hz, 2H), 6.57 (t, *J* = 7.3 Hz, 1H), 6.35 (d, *J* = 8.2 Hz, 1H), 6.27 (s, 1H), 5.84 (d, *J* = 8.1 Hz, 1H), 5.61 (t, *J* = 6.3 Hz, 1H), 4.25 (qd, *J* = 15.5, 6.2 Hz, 2H), 2.27 (s, 3H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.5, 149.1, 147.3, 141.4, 136.8, 136.2, 129.13, 128.89, 126.9, 116.8, 112.9, 108.8, 59.4, 52.6, 20.6.

2-((4-fluorophenyl)(phenylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (35)

1 mmol of each component (kojic acid, 4-Fluorobenzaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using CH_2Cl_2 : MeOH (10:1) as eluent, and the dried solid was further washed with ether. Then, the compound was dissolved in

acetonitrile-H_2O mixture and lyophilized yielding 20 mg compound **35**as a brown-orange solid (6 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{16}FNO_4$ + H^+ $[M+H]^+$: 342.1136, and found 342.1139. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.44 (s, 1H), 7.55 – 7.48 (m, 2H), 7.20 (t, *J* = 8.9 Hz, 2H), 7.11 – 7.04 (m, 2H), 6.72 – 6.65 (m, 2H), 6.58 (td, *J* = 7.3, 1.2 Hz, 1H), 6.45 (d, *J* = 8.2 Hz, 1H), 6.28 (s, 1H), 5.89 (d, *J* = 8.2 Hz, 1H), 5.63 (t, *J* = 6.3 Hz, 1H), 4.32 – 4.18 (m, 2H).

^{13}C NMR (126 MHz, DMSO) δ 173.6, 167.6, 162.5, 160.6, 148.6, 147.1, 141.5, 135.43, 135.41, 129.14, 129.07, 128.97, 117.0, 115.51, 115.34, 113.0, 108.9, 59.4, 52.3.

2-((4-bromophenyl)(phenylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (36)

1 mmol of each component (kojic acid, 4-bromobenzaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 21 mg compound **36** as a light brown solid (5 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{16}BrNO_4$ + H^+ $[M+H]^+$: 402.0335, and found 402.0334. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.44 (s, 1H), 7.59 – 7.53 (m, 2H), 7.45 – 7.39 (m, 2H), 7.10 – 7.04 (m, 2H), 6.70 – 6.67 (m, 2H), 6.59 (dd, *J* = 7.9, 6.6 Hz, 1H), 6.46 (d, *J* = 8.1 Hz, 1H), 6.28 (s, 1H), 5.87 (d, *J* = 8.1 Hz, 1H), 5.61 (t, *J* = 6.3 Hz, 1H), 4.29 – 4.18 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.6, 148.3, 147.0, 141.5, 138.7, 131.5, 129.2, 128.9, 120.7, 117.1, 113.0, 108.9, 59.4, 52.4.

3-hydroxy-6-(hydroxymethyl)-2-((4-isopropylphenyl)(phenylamino)methyl)-4H-pyran-4-one (37)

2 mmol of each component (kojic acid, 4-isopropylbenzaldehyde and aniline) in 10 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was silica column purified using ethyl acetate as eluent. The purified compound was further washed with diethyl ether, dried. Then the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 0.3 g compound **37** as a white-bright yellow solid (41 % yield). HRMS (ESI): m/z calculated for $C_{22}H_{23}NO_4 + H^+$ $[M+H]^+$: 366.1700, and found 366.1682. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.32 (s, 1H), 7.41 – 7.38 (m, 2H), 7.25 – 7.23 (m, 2H), 7.08 – 7.04 (m, 2H), 6.71 – 6.68 (m, 2H), 6.57 (tt, *J* = 7.3, 1.2 Hz, 1H), 6.35 (d, *J* = 8.2 Hz, 1H), 6.27 (s, 1H), 5.84 (d, *J* = 8.1 Hz, 1H), 5.62 (t, *J* = 6.3 Hz, 1H), 4.30 – 4.22 (m, 2H), 2.86 (hept, *J* = 6.9 Hz, 1H), 1.18 (d, *J* = 7.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 173.5, 167.4, 149.0, 147.8, 147.3, 141.5, 136.6, 128.9, 127.1, 126.5, 116.8, 112.8, 108.9, 59.4, 52.7, 33.1, 23.85, 23.80.

3-hydroxy-6-(hydroxymethyl)-2-(naphthalen-2-yl(phenylamino)methyl)-4H-pyran-4-one (38)

1 mmol of each component (kojic acid, 2-naphthaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 10 mg compound **38** as a cream color solid (3 % yield). HRMS (ESI): m/z calculated for $C_{23}H_{19}NO_4 + H^+$ $[M+H]^+$: 374.1387, and

found 374.1388. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.43 (s, 1H), 8.00 (d, J = 1.8 Hz, 1H), 7.94 – 7.88 (m, 3H), 7.62 (dd, J = 8.5, 1.8 Hz, 1H), 7.51 (qd, J = 7.2, 3.4 Hz, 2H), 7.08 (dd, J = 8.4, 7.1 Hz, 2H), 6.75 (d, J = 8.0 Hz, 2H), 6.58 (t, J = 7.3 Hz, 1H), 6.55 (d, J = 8.0 Hz, 1H), 6.28 (s, 1H), 6.06 (d, J = 8.0 Hz, 1H), 5.61 (t, J = 6.3 Hz, 1H), 4.26 (qd, J = 15.6, 6.2 Hz, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.6, 148.8, 147.3, 141.6, 136.8, 132.8, 132.4, 128.9, 128.3, 127.84, 127.50, 126.34, 126.11, 125.5, 125.2, 116.9, 113.0, 108.9, 59.4, 53.1.

2-((2-fluorophenyl)(phenylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (39)

1 mmol of each component (kojic acid, 2-Fluorobenzaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 5-days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and brine. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 135 mg compound **39** as an off-white solid (40 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{16}FNO_4$ + H^+ $[M+H]^+$: 342.1136, and found 342.1139. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.29 (s, 1H), 7.55 (td, J = 7.8, 1.8 Hz, 1H), 7.29 (tdd, J = 7.5, 5.3, 1.8 Hz, 1H), 7.17 – 7.12 (m, 2H), 7.03 – 6.98 (m, 2H), 6.59 (d, J = 8.0 Hz, 2H), 6.52 (t, J = 7.3 Hz, 1H), 6.39 (d, J = 8.0 Hz, 1H), 6.22 (s, 1H), 6.07 (d, J = 8.0 Hz, 1H), 5.56 (t, J = 6.2 Hz, 1H), 4.24 – 4.15 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.7, 167.6, 160.4, 159.0, 147.4, 146.9, 141.5, 129.78, 129.73, 129.13, 129.11, 129.0, 125.85, 125.77, 124.63, 124.61, 117.1, 115.45, 115.33, 112.7, 108.9, 59.4, 46.56, 46.54.

3-hydroxy-6-(hydroxymethyl)-2-((phenylamino)(pyridin-2-yl)methyl)-4H-pyran-4-one (40)

1 mmol of each component (kojic acid, 2-Pyridinecarboxaldehyde and aniline) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated. The remaining crude was silica column purified using ethyl acetate as eluent. Then, the compound was dissolved in acetonitrile-H_2O mixture and lyophilized yielding 43 mg compound **40** as a bright yellow solid (13 % yield). HRMS (ESI): m/z calculated for $C_{18}H_{16}N_2O_4$ + H^+ $[M+H]^+$: 325.1183, and found 325.1188. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.37 (s, 1H), 8.56 – 8.53 (m, 1H), 7.82 (td, *J* = 7.7, 1.8 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.36 – 7.31 (m, 1H), 7.11 – 7.06 (m, 2H), 6.72 (d, *J* = 8.0 Hz, 2H), 6.59 (t, *J* = 7.3 Hz, 1H), 6.49 (d, *J* = 8.0 Hz, 1H), 6.26 (s, 1H), 5.99 (d, *J* = 8.0 Hz, 1H), 5.59 (t, *J* = 6.3 Hz, 1H), 4.23 – 4.13 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.5, 157.8, 148.9, 148.4, 147.0, 142.3, 137.2, 129.0, 123.0, 121.9, 117.0, 112.9, 108.8, 59.4, 54.7.

4-(((3-hydroxy-6-(hydroxymethyl)-4-oxo-4H-pyran-2-yl)(pyridin-2-yl)methyl)amino)benzamide (41)

1 mmol of each component (kojic acid, 2-pyridinecarboxaldehyde and 4-aminobenzamide) in 15 ml EtOH were stirred at room temperature for 3 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous MgSO4, concentrated, dried. The crude was then taken in a mixture of ethyl acetate: MeOH (10:1) and sonicated for 5 mins. The solution was discarded and the wash step was repeated with ethyl acetate. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 235 mg compound **41** as a cream color solid (64 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{17}N_3O_5$

+ H^+ $[M+H]^+$: 368.1241, and found 368.1242. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.46 (s, 1H), 8.56 (ddd, *J* = 4.8, 1.8, 0.9 Hz, 1H), 7.83 (td, *J* = 7.7, 1.8 Hz, 1H), 7.66 – 7.63 (m, 2H), 7.57 (bs, 1H), 7.54 (dd, *J* = 8.0, 1.2 Hz, 1H), 7.35 (ddd, *J* = 7.5, 4.8, 1.1 Hz, 1H), 7.01 (d, *J* = 7.8 Hz, 1H), 6.91 (bs, 1H), 6.73 – 6.69 (m, 2H), 6.28 (d, *J* = 1.0 Hz, 1H), 6.06 (d, *J* = 7.7 Hz, 1H), 5.59 (t, *J* = 6.2 Hz, 1H), 4.25 – 4.11 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.6, 167.78, 167.64, 157.2, 149.5, 149.0, 147.9, 142.4, 137.3, 129.0, 123.1, 122.5, 122.0, 111.7, 108.9, 59.4, 54.5.

2-((4-bromophenyl)(pyridin-2-ylamino)methyl)-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (42)

2 mmol of each component (kojic acid, 4-bromobenzaldehyde and 2-aminopyridine) in DMF were stirred at room temperature for 3 days before concentrated in vacuo. Then the remaining crude was washed with ethanol yielding compound **42** as a white solid. HRMS (ESI): m/z calculated for $C_{18}H_{15}BrN_2O_4$ + H^+ $[M+H]^+$: 403.0288 , and found 403.0259. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.31 (s, 1H), 7.95 (ddd, *J* = 5.1, 1.9, 0.9 Hz, 1H), 7.55 – 7.52 (m, 2H), 7.43 (ddd, *J* = 8.8, 7.1, 1.9 Hz, 1H), 7.39 (d, *J* = 8.5 Hz, 1H), 7.35 – 7.32 (m, 2H), 6.71 (dt, *J* = 8.4, 1.0 Hz, 1H), 6.64 (d, *J* = 8.5 Hz, 1H), 6.55 (ddd, *J* = 7.0, 5.1, 1.0 Hz, 1H), 6.30 – 6.29 (m, 1H), 5.62 (t, *J* = 6.3 Hz, 1H), 4.32 – 4.22 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.8, 167.5, 157.2, 148.8, 147.3, 141.0, 139.6, 137.0, 131.4, 128.9, 120.4, 112.9, 109.1, 108.9, 59.4, 49.7.

3-hydroxy-6-(hydroxymethyl)-2-(pyridin-2-yl(pyridin-2-ylamino)methyl)-4H-pyran-4-one (43)

1 mmol of each component (kojic acid, 2-pyridinecarboxaldehyde and 2-aminopyridine) in 15 ml EtOH were stirred at room temperature for 1 day before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using ethyl acetate:MeOH (1:2) as eluent. Then, the compound was taken in acetonitrile-H_2O mixture and lyophilized yielding 75 mg compound **43** as an off-white solid (23 % yield). HRMS (ESI): m/z calculated for $C_{17}H_{15}N_3O_4$ + H^+ $[M+H]^+$: 326.1135, and found 326.1122. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.21 (s, 1H), 8.51 (ddd, *J* = 4.9, 1.8, 0.9 Hz, 1H), 7.95 (ddd, *J* = 5.1, 1.9, 0.9 Hz, 1H), 7.78 (td, *J* = 7.7, 1.8 Hz, 1H), 7.46 (dt, *J* = 8.0, 1.0 Hz, 1H), 7.43 (ddd, *J* = 8.7, 7.0, 1.9 Hz, 1H), 7.37 (d, *J* = 8.3 Hz, 1H), 7.29 (ddd, *J* = 7.5, 4.8, 1.1 Hz, 1H), 6.76 (dt, *J* = 8.4, 1.0 Hz, 1H), 6.69 (d, *J* = 8.3 Hz, 1H), 6.55 (ddd, *J* = 7.0, 5.0, 1.0 Hz, 1H), 6.28 (d, *J* = 0.9 Hz, 1H), 5.60 (t, *J* = 6.3 Hz, 1H), 4.28 – 4.14 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.8, 167.4, 158.6, 157.3, 149.11, 148.87, 147.3, 141.8, 136.99, 136.97, 122.6, 121.6, 112.8, 109.1, 108.9, 59.4, 52.3.

3-hydroxy-6-(hydroxymethyl)-2-((pyridin-2-ylamino)(pyridin-3-yl)methyl)-4H-pyran-4-one (44)

1 mmol of each component (kojic acid, 3-pyridinecarboxaldehyde and 2-aminopyridine) in 15 ml EtOH were stirred at room temperature for 2 days before concentrating in vacuo. The remaining crude was extracted with ethyl acetate and water. The organic layers were dried over anhydrous $MgSO_4$, concentrated, dried. The remaining crude was silica column purified using ethyl CH_2Cl_2 : MeOH (20:1) as eluent. The crude then was washed with ethyl acetate and sonicated. The wash step was repeated two more times. Then, the compound was taken in acetonitrile-H_2O mixture and

lyophilized yielding 110 mg compound **44** as a cream color solid (34 % yield). HRMS (ESI): m/z calculated for $C_{17}H_{15}N_3O_4$ + H^+ $[M+H]^+$: 326.1135, and found 326.1122. ^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.21 (s, 1H), 8.51 (ddd, *J* = 4.9, 1.9, 0.9 Hz, 1H), 7.95 (ddd, *J* = 5.1, 1.9, 0.8 Hz, 1H), 7.78 (td, *J* = 7.7, 1.8 Hz, 1H), 7.46 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.43 (ddd, *J* = 8.6, 7.0, 1.9 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 1H), 7.29 (ddd, *J* = 7.5, 4.8, 1.2 Hz, 1H), 6.76 (dt, *J* = 8.4, 1.0 Hz, 1H), 6.69 (d, *J* = 8.3 Hz, 1H), 6.55 (ddd, *J* = 7.1, 5.0, 1.0 Hz, 1H), 6.28 (d, *J* = 1.0 Hz, 1H), 5.60 (t, *J* = 6.3 Hz, 1H), 4.26 – 4.14 (m, 2H).

^{13}C NMR (176 MHz, DMSO) δ 173.8, 167.4, 158.6, 157.3, 149.1, 148.9, 147.3, 141.8, 137.00, 136.97, 122.6, 121.6, 112.8, 109.10, 108.88, 59.4, 52.3.

6-(hydroxymethyl)-3-methoxy-2-(phenyl(phenylamino)methyl)-4H-pyran-4-one (45)

To a stirring solution of 33 mg (0.1 mmol) compound **19** and 13.8 mg K_2CO_3 (1 equiv.) in 1 ml DMF, 14.1 mg (1 equiv.) iodomethane in 1 ml DMF was added, stirred for 1 day in ambient temperature. The reaction mixture was then filtered and concentrated. The remaining crude was silica column purified using CH_2Cl_2:MeOH (10:0.5) as eluent yielding 29 mg white powder (86 % yield). HRMS (ESI): m/z calculated for $C_{20}H_{19}NO_4$ + H^+ $[M+H]^+$: 338.1387, and found 338.1379. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.54 – 7.49 (m, 2H), 7.42 – 7.37 (m, 2H), 7.34 – 7.29 (m, 1H), 7.09 (dd, *J* = 8.6, 7.2 Hz, 2H), 6.75 – 6.71 (m, 2H), 6.60 (tt, *J* = 7.3, 1.1 Hz, 1H), 6.46 (d, *J* = 8.5 Hz, 1H), 6.27 (d, *J* = 1.0 Hz, 1H), 5.94 (d, *J* = 8.5 Hz, 1H), 5.67 (t, *J* = 6.3 Hz, 1H), 4.32 – 4.20 (m, 2H), 3.79 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 174.5, 167.8, 158.0, 147.1, 143.3, 138.8, 129.0, 128.7, 127.9, 127.2, 117.2, 113.2, 111.7, 59.9, 59.2, 53.4.

^{1}H-NMR, ^{13}C- NMR, purity and assay media stability spectra of compound **45** is shown in Supporting Information (pages S18–S21).

2-((2-fluorophenyl)(pyridin-2-ylamino)methyl)-6-(hydroxymethyl)-3-methoxy-4H-pyran-4-one (46)

To a stirring solution of 25 mg (0.067 mmol) compound **10** and 9.3 mg K_2CO_3 (0.063 mmol) in 1 ml DMF, 4.5 mg (0.5 equiv.) iodomethane in 1 ml DMF was added, stirred for 30 mins. Then another portion of 4.5 mg (0.5 equiv.) iodomethane in 0.5 ml DMF was added and stirred for 30 mins. The reaction mixture was then concentrated. The remaining crude was silica column purified using ethyl acetate as eluent yielding 10 mg (0.028 mmol) compound **46** as a white powder (42 % yield). HRMS (ESI): m/z calculated for $C_{19}H_{17}FN_2O_4$ + H^+ $[M+H]^+$: 357.1245, and found 357.1222. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.97 (dd, *J* = 5.2, 1.8 Hz, 1H), 7.56 (td, *J* = 7.8, 1.9 Hz, 1H), 7.47 (d, *J* = 8.3 Hz, 1H), 7.43 (ddd, *J* = 8.7, 7.0, 2.0 Hz, 1H), 7.39 – 7.33 (m, 1H), 7.24 – 7.18 (m, 2H), 6.90 (d, *J* = 8.3 Hz, 1H), 6.71 (d, *J* = 8.3 Hz, 1H), 6.58 – 6.53 (m, 1H), 6.29 (s, 1H), 5.69 (t, *J* = 6.2 Hz, 1H), 4.30 – 4.20 (m, 2H), 3.69 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 174.7, 167.8, 160.7, 158.7, 157.7, 157.0, 147.4, 143.0, 137.0, 129.86, 129.80, 129.08, 129.06, 126.27, 126.16, 124.67, 124.64, 115.55, 115.38, 113.0, 111.8, 109.3, 59.34, 59.23, 44.82, 44.78.

tert-butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (S2)

Synthesis of **S2** was achieved using the procedure as described earlier.[47] To a solution of 1.01 g (4.6 mmol) 1,13-Diamino-4,7,10-trioxatridecane (**S1**) in 20 ml of dry CH_2Cl_2, 0.5 g (2.3 mmol) Di-tert-butyl dicarbonate in 10 ml dry CH_2Cl_2 was added dropwise over 1 hour and stirred at room

temperature for overnight. Then, the reaction mixture was washed with H_2O three times, dried over anhydrous $MgSO_4$, concentrated in vacuo. The remaining colorless oil was used without further purification.

tert-butyl (15-oxo-4,7,10-trioxa-14-azatriacontyl)carbamate (S3)

Intermediate **S2** (30 mg, 0.1 mmol) was added to a solution of palmitic acid (38.5 mg, 0.15 mmol), EDCI (29 mg, 0.15 mmol), HOAt (20 mg, 0.15 mmol) and DIPEA (26 µl, 0.15 mmol) in 10 ml CH_2Cl_2, and the reaction mixture was stirred at room temperature for 4 hours. Then, the reaction was quenched with H_2O, extracted to CH_2Cl_2. The organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. The remaining crude was purified over silica column using n-Hex-EtAc (1:1, then 1:4, then Et Ac only) as eluent. **S3** was obtained as a white solid (60 mg, 0.11 mmol, 98 % yield). LRMS (ESI): m/z calculated for $C_{31}H_{62}N_2O_6 + H^+$ $[M+H]^+$: 559.47, and found 559.06. ^{1}H NMR (500 MHz, Methanol-d_4): δ = 3.64 (dp, *J* = 5.1, 2.4 Hz, 4H), 3.59 (dq, *J* = 7.0, 3.7, 3.0 Hz, 4H), 3.51 (td, *J* = 6.1, 4.2 Hz, 4H), 3.25 (t, *J* = 6.8 Hz, 2H), 3.12 (t, *J* = 6.8 Hz, 2H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.74 (dp, *J* = 17.2, 6.5 Hz, 4H), 1.60 (q, *J* = 7.2 Hz, 2H), 1.43 (s, 9H), 1.36-1.25 (m, 24H), 0.90 (t, *J* = 6.9 Hz, 3H).

^{13}C NMR (126 MHz, MeOD) δ 176.3, 158.5, 79.8, 71.6, 71.26, 71.25, 69.89, 69.86, 38.7, 37.8, 37.2, 33.1, 30.91, 30.83, 30.81, 30.79, 30.75, 30.67, 30.51, 30.47, 30.43, 30.32, 28.8, 27.1, 23.8, 14.5.

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(3-(15-oxo-4,7,10-trioxa-14-azatriacontyl)thioureido)benzoic acid (S4)

2 ml 4M HCl solution in 1,4-dioxane was added to a solution of **S3** (10 mg, 0.017 mmol) in 10 ml MeOH and was stirred at room temperature overnight. Then, the reaction mixture was

concentrated, the remaining crude (7.4 mg, 0.015 mmol) was taken in 2 ml DMF, DIPEA (7.8 µl, 0.045 mmol, 3 equiv.) was added followed by FITC addition (8.2 mg, 0.021 mmol, 1.4 equiv.) in 1 ml DMF. The reaction was stirred at room temperature overnight. Then, the solvent was evaporated and the remaining crude was purified over reverse phase HPLC-MS (Agilent 1260 Infinity coupled to Agilent 6120 Quadripole), with a C18 reverse phase column (VP125/21 Nucleodur C18 Gravity, 5 µm). **S4** was taken in a mixture of acetonitrile-H_2O and lyophilized yielding 5 mg orange powder (43 % yield). HRMS (ESI): m/z calculated for $C_{47}H_{65}N_3O_9S + H^+$ $[M+H]^+$: 848.4514, and found 848.4467. ^{1}H NMR (500 MHz, Methanol-d_4): δ = 8.14 (d, *J* = 2.0 Hz, 1H), 7.79 (d, *J* = 8.3 Hz, 1H), 7.17 (d, *J* = 8.2 Hz, 1H), 6.82 – 6.66 (m, 4H), 6.65 – 6.48 (m, 2H), 3.74 (s, 2H), 3.63 (qq, *J* = 5.0, 3.3, 2.5 Hz, 8H), 3.56 (dd, *J* = 5.8, 3.3 Hz, 2H), 3.49 (t, *J* = 6.2 Hz, 2H), 3.23 (t, *J* = 6.9 Hz, 2H), 2.15 (t, *J* = 7.5 Hz, 2H), 1.93 (p, *J* = 6.2 Hz, 2H), 1.73 (p, *J* = 6.5 Hz, 2H), 1.57 (t, *J* = 7.3 Hz, 3H), 1.28 (d, *J* = 9.6 Hz, 23H), 0.89 (t, *J* = 6.9 Hz, 3H).^{13}C NMR (176 MHz, MeOD): δ = 182.6, 176.3, 171.2, 154.2, 142.4, 130.3, 113.6, 111.5, 103.5, 71.52, 71.44, 71.23, 71.14, 70.4, 69.9, 37.8, 37.2, 33.1, 30.80, 30.79, 30.76, 30.72, 30.63, 30.48, 30.45, 30.44, 30.30, 29.9, 27.1, 23.7, 14.4.

Synthesis of FITC-YAP$^{50-100}$. The YAP peptide (Fmoc-AGHQIVHVRGDSETDLEALFNAVMNPKTANVPQTVPMRLRKLPDSFFKPPE) was synthesized on a Syro I peptide synthesizer (Multisyntech) following standard Fmoc-protocols for solid-phase peptide synthesis using Rink amide ChemMatrix resin (Aldrich, 0.28 mmol/g, 0.05 mmol). N-terminal Fmoc group in the resulting peptide on the resin was deprotected using 25 % piperidine and reacted with Fmoc-O_2Oc-OH (0.2 mmol, 4 eq.), HATU (0.2 mmol, 4 eq.) and DIPEA (0.4 mmol, 8 eq.) in DMF for 2 hours. The N-terminal Fmoc group was then deprotected followed by reaction with 5-isothiocyanatefluorescein (0.250 mmol, 5 eq.) and DIPEA (0.5 mmol,

10 eq) in DMF at room temperature for 3 h in the dark. The resin was washed and the coupling procedure was repeated for an additional time and this time shaken in the dark at rt for 16 h. The peptidyl resin (0.05 mmol, 1 eq.) was cleaved with 1 ml of cleavage solution (triisopropylsilane 2.5%, water 2.5% in TFA) for 3 hours at room temperature. The peptide was precipitated in 40 ml of cold diethyl ether/petroleum ether (1:1) upon 10 minutes of centrifugation. The pellet was washed twice with 40 ml of diethyl ether. The crude was dried over vacuum, dissolved in water and lyophilized. Then, the crude was dissolved in 1 ml of DMSO and the peptide was purified by reverse phase HPLC. HRMS (ESI): m/z calculated for $C_{280}H_{425}N_{75}O_{81}S_3$ + $[M+5H]^{5+}$: 1247.0194, and found 1247.0158 calculated for + $[M+6H]^{6+}$: 1039.3507, and found 1039.3481; calculated for + $[M+7H]^{7+}$: 891.0159, and found 891.0145.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS publications website.

Full protein ESI-mass spectrometry analysis of recombinant hTEAD4 (acylated), hTEAD4 (non-acylated) and hTEAD4 (non-acylated) reaction with CPM; tandem-MS analysis of hTEAD4 (non-acylated) reaction with CPM; competitive inhibition of thiol conjugation signal with TED-347; saturation binding curve of FITC-palmitate to hTEAD4 (non-acylated) and competitive inhibition curve of niflumic acid (**2**) in the FP assay; time and concentration dependent inhibition of hTEAD4; specificity of **19** for TEAD homologs; ^{1}H-^{15}N-HSQC spectrum of mTEAD4 (non-acylated) treated with **19** or **45**; saturation binding curves of FITC labeled YAP^{50-100} with TEAD4 proteins; sequences of the TEAD proteins used in the study; scheme for the synthesis of FITC-palmitate; ^{1}H-NMR, ^{13}C-NMR and

UPLC spectra of compounds **19** and **45**; Molecular Formula Strings (CSV) with biological data.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Ala, alanine; brs, broad signal; CPM, N-(4-(7-diethylamino-4-methylcoumarin-3-yl) phenyl) maleimide; Cys, cysteine; ESI-MS, electron spray ionization-mass spectrometry; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; mTEAD4, mouse TEAD4; Phe, phenylalanine; Ser, serine; TEAD, Transcriptional enhanced associate domain; Tm, melting temperature; YAP, yes-associated protein.

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