In-Gel Activity-Based Protein Profiling of a Clickable Covalent ERK1/2 Inhibitor

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INTRODUCTION

- The selectivity of a tool inhibitor is a key parameter in order to interpret biological data with accuracy and to predict potential for off-target toxicities, particularly for covalent inhibitors. In-gel Activity-Based Protein Profiling (ABPP) is a rapid and cost effective method to screen an inhibitor against the entire proteome and provide an overall assessment of its selectivity, which can be potentially followed up by identification of off-targets by proteomics. This technique requires a clickable and covalent chemical probe designed from the inhibitor of interest.
- Here we describe the structure-based design of a TCO-tagged ERK1/2 covalent probe based on a series of inhibitors which were recently shown to bind covalently to Cys166 of ERK2.[1, 2] The tagged probe was evaluated in bioassay, MS and cellular activity screens, and used to determine target engagement and selectivity in A375 and HCT116 cancer cell lines, whose proliferation is driven by upregulated ERK1/2 pathway signalling as a result of activating mutations in BRAF or KRAS respectively.

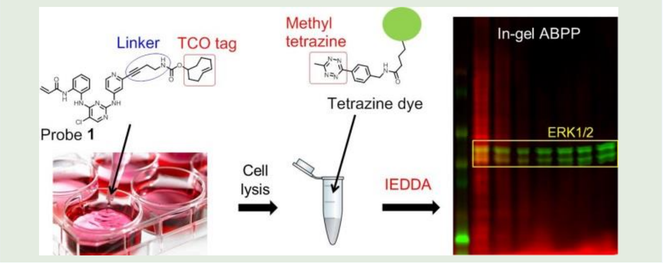


Figure 1: Representative scheme of the method.

DESIGN AND SYNTHESIS OF PROBE 1

- The TCO ERK1/2 covalent probe **1** was designed using the co-crystal structures of ERK2 with the published acrylamide covalent inhibitors, **2** and **3** (Figure 2A).[1, 2]
- Replacing the tetrahydropyran (THP) group with a pyridine ring would provide a vector pointing into the solvent (red arrows) and allow facile introduction of a linker to attach the TCO tag while maintaining affinity for the enzyme (Figure 2B). From the crystal structure, the available space appeared limited, so a rigid alkyne group was chosen as part of a four-carbon linker long enough to place the TCO tag in the solvent and avoid steric clashes.

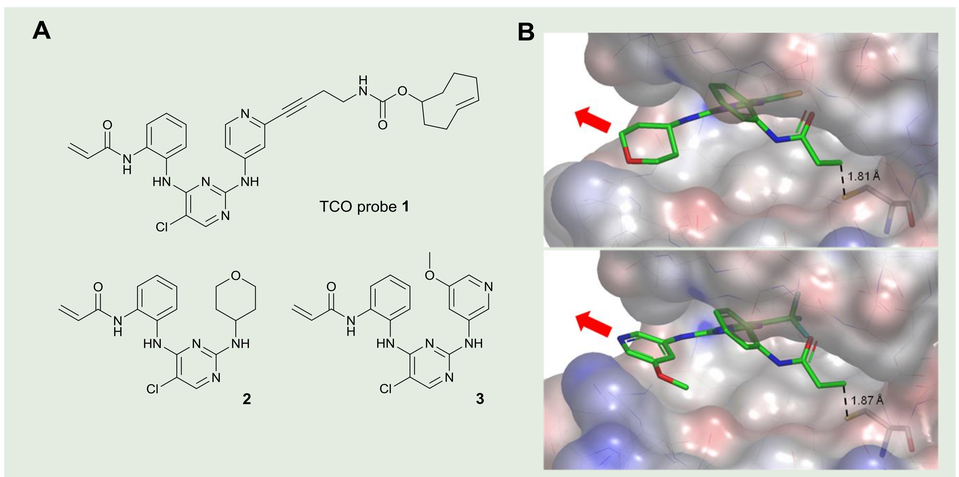
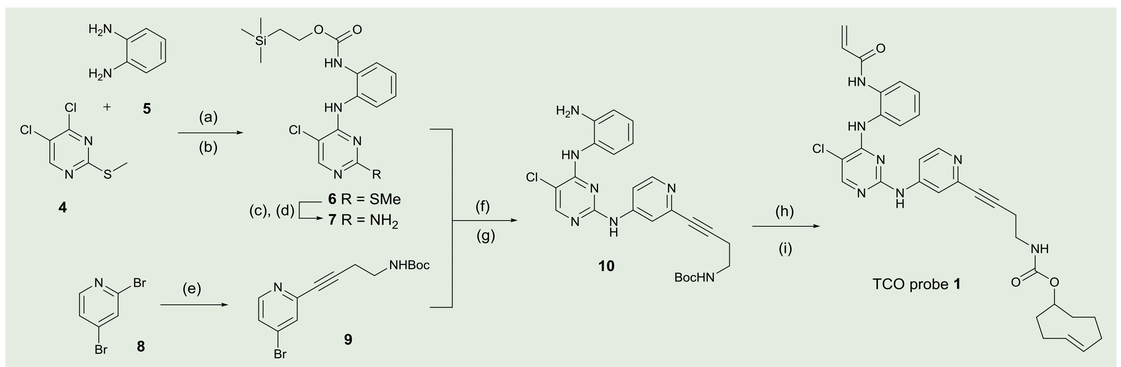


Figure 2: A. Structures of Probe 1 and compounds 2 and 3. B. ERK2 co-crystal structures of compound 2 (PDB: 4ZZO) and 3 (this work).

- The TCO probe **1** was synthesised in 8 steps as outlined in Scheme 1.



Scheme 1: Synthesis of Probe 1.[a]

[a]Reagents and conditions: (a) DIPEA, *n*-BuOH, 110 °C, 3 h, 67%; (b) Et_3N, 1-[2-(trimethylsilyl) ethoxycarbonyloxy]pyrrolidin-2,5-dione, MeCN/DMF (1: 1), 70 °C, 5 h, 80%; (c) *m*-CPBA, DCM, 0 °C, 2 h, 95%; (d) NH_4OH, dioxane, 80 °C, 20 h, 58%; (e) *Tert*-Butyl but-3-ynylcarbamate, DIPEA, $Pd(PPh_3)_2Cl_2$, Cu(I)I, THF, r.t., 24 h, 48%; (f) K_2CO_3, XPhos, $Pd(dba)_2$, MeCN, 80 °C, 18 h, 43%; (g) TBAF, THF, 40 °C, 2 h, 83%; (h) 1. HCl in dioxane (4M), DCM/MeOH (5: 1), r.t., 18 h. 2. DIPEA, TCO-NHS ester, DMF, r.t., 30 min, 91% over two steps; (i) acryloyl chloride, DIPEA, THF, 0 °C, 30 min, 44%.

CLICK FORMATION OF THE FLUORESCENT-TAGGED INHIBITOR

- We selected the inverse electron demand Diels Alder (IEDDA) cycloaddition with tetrazine (Tz) and *trans*-cyclooctene (TCO)[3] due to its high reaction rate ($k_2 \sim 10^2$ -10^5 $M^{-1}.s^{-1}$)[4] and yield. The reaction requires only a small excess of dye, allowing increased signal-to-noise ratios. The tetrazine reacts with a strained alkene without the need for catalyst, which simplifies the experimental procedure, and nitrogen is the only by-product.[5]
- Consistent with reported rates, the reaction between TCO probe **1** and Tz-dye **11** (Figure 4) in DMSO was shown to be 95% complete after 15 min by LC-MS.

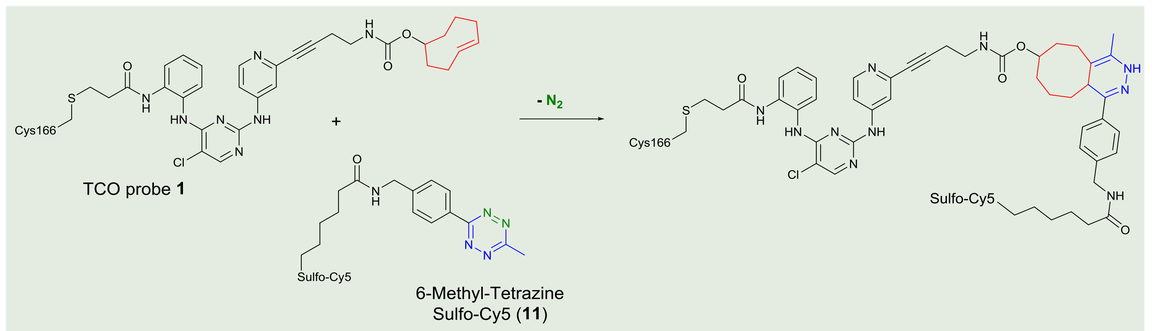


Figure 4: 'Click' reaction between Probe 1 and tetrazine dye 11.

SELECTIVITY PROFILE OF PROBE 1

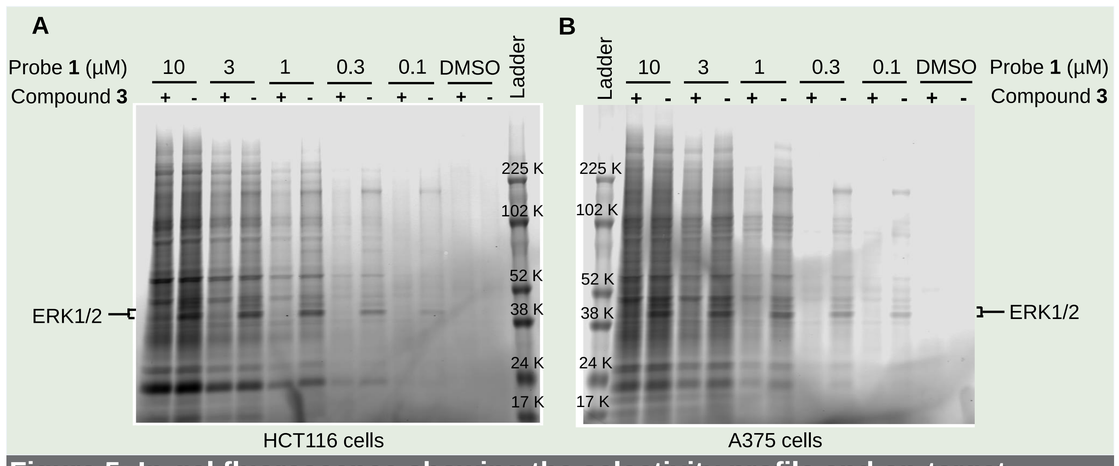


Figure 5: In-gel fluorescence showing the selectivity profile and on-target activity of Probe 1 in HCT116 cells (A) and in A375 cells (B) using Tz-dye 11.

- The selectivity profiles of probe **1** in A375 and HCT116 cells were found to be similar, with no major off-target present preferentially in one cell line (Figures 5A and 5B).
- At low concentrations (<1 μM), probe **1** showed a relatively clean profile with few off-targets, whereas at higher concentrations (>1 μM), probe **1** was found to be more promiscuous.
- No fluorescence signal was detected in the absence of TCO probe **1**, showing that the background fluorescence is minimal and that the fluorescence observed in the presence of TCO probe **1** is due to on- and off-target activities but not from unspecific binding of the dye.
- When cells were pre-treated with the untagged covalent ERK1/2 inhibitor (**3**), no fluorescence was observed around 40 kDa, supporting the on-target activity of probe **1** with ERK1/2.
- On-target activity of probe **1** was also validated by immunostaining using a total ERK1/2 antibody (see Figure 1).

BIOLOGICAL & STRUCTURAL EVALUATION

- TCO probe **1** showed moderate activity against ERK1/2 (IC_{50} = 0.35 ± 0.14 μM, Table 1).
- The co-crystal structure shows that the binding mode of TCO probe **1** is largely identical to that of the untagged inhibitor, and confirms the presence of the covalent bond between Cys166 and the acrylamide group (Figure 3). No electron density was observed for the TCO group, implying significant mobility resulting from its positioning into the solvent.
- The submicromolar growth inhibition values exhibited by TCO probe **1** (Table 1) are within 10-fold of the range of potencies described in the literature for ERK1/2 covalent inhibitors.
- The covalent ERK1/2 inhibitor **3** was evaluated against ERK1/2 and showed good enzymatic and cellular potencies against both cell lines.

Table 1: Biological evaluation of Probe 1 and the untagged analogue 3.

Compounds	IC_{50}[a]	GI_{50}	
		A375 cells	HCT116 cells
1	0.23[b]	0.45[c]	0.41[b]
3	0.008[b]	0.040[d]	0.057[b]

[a]1 h pre-incubation with ERK2; [b]n = 2; [c]n=3; [d]n=4.

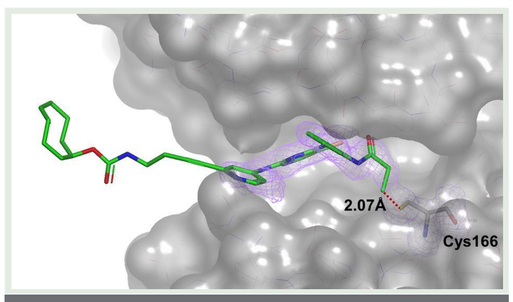


Figure 3: Co-crystal structure of Probe 1 with ERK2.

CONCLUSIONS

- We developed a TCO tagged covalent ERK1/2 probe which was used to study the proteome-wide selectivity of a class of covalent ERK1/2 inhibitors.
- We showed that the IEDDA cycloaddition is an attractive alternative to the traditional copper catalysed alkyne-azide cycloaddition (CuAAC) for in-gel ABPP.
- The selectivity profile of probe **1**, in both A375 and HCT116 cell lines, indicates a narrow range of concentrations at which the target is engaged selectively. This might arise from the reactivity of the covalent warhead.
- Our results support the literature findings on covalent inhibitors bearing this unsubstituted acrylamide motif which should be treated with caution both when relating target engagement to cellular phenotypic data, and when considering potential therapeutic index.

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