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

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Table of contents

Table S1. Kinetic study of probe **1** with ERK2 by mass spectrometry.[a]

Time point (min)	Observed mass (Da)[b]	Difference of mass (DA)[c]	Identity	% probe **1** bound
0	42284.8	1.2	Unmodified ERK2	
	42868.3	583.5	Modified ERK2, single copy	20
2	42285.3	1.7	Unmodified ERK2	
	42871.5	586.2	Modified ERK2, single copy	52
4	42285.3	1.7	Unmodified ERK2	
	42871.1	585.8	Modified ERK2, single copy	88
8	42287.3	3.7	Unmodified ERK2	
	42871.5	584.2	Modified ERK2, single copy	96
16	42284.0	0.4	Unmodified ERK2	
	42871.2	587.2	Modified ERK2, single copy	94
32	42287.2	3.6	Unmodified ERK2	
	42871.6	584.4	Modified ERK2, single copy	93
64	42873.0	589.4	Modified ERK2, single copy	100
128	42872.6	589.0	Modified ERK2, single copy	100

[a]ERK2 (20 mM) and probe **1** (200 mM) were mixed in buffer containing 100 mM ammonium acetate pH 7.5 at r.t.; [b]ERK2 (42284 Da) and probe **1** (585.2 Da); [c]Difference between the mass observed and the theoretical mass of ERK2.

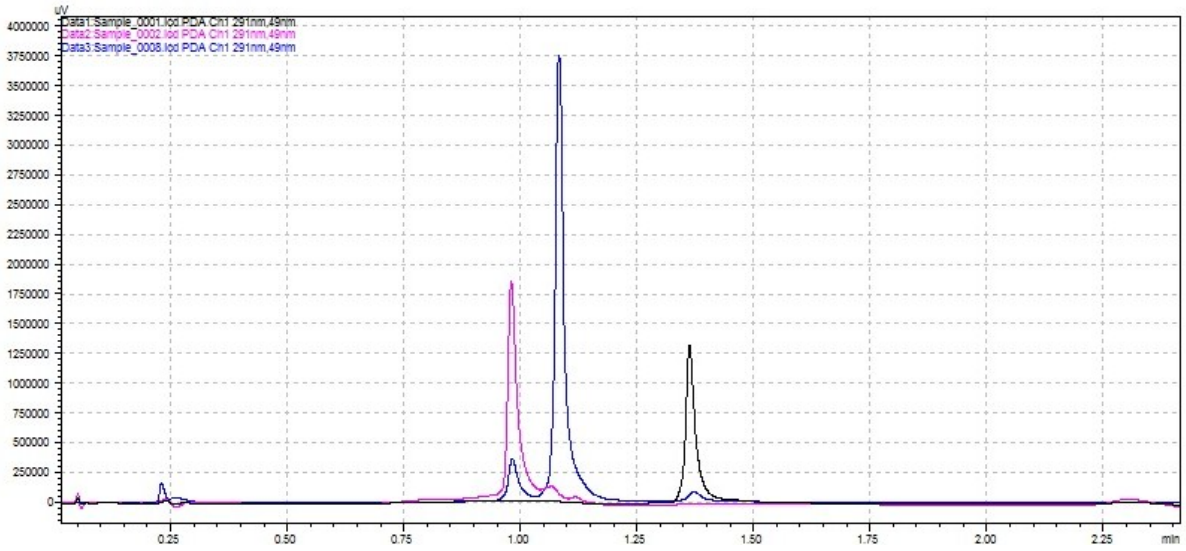

Figure S1. LC-MS analysis of probe **1** (black), Tz-dye **11** (pink) and the reaction mixture after 15 min (blue, probe **1** and Tz dye **11**).

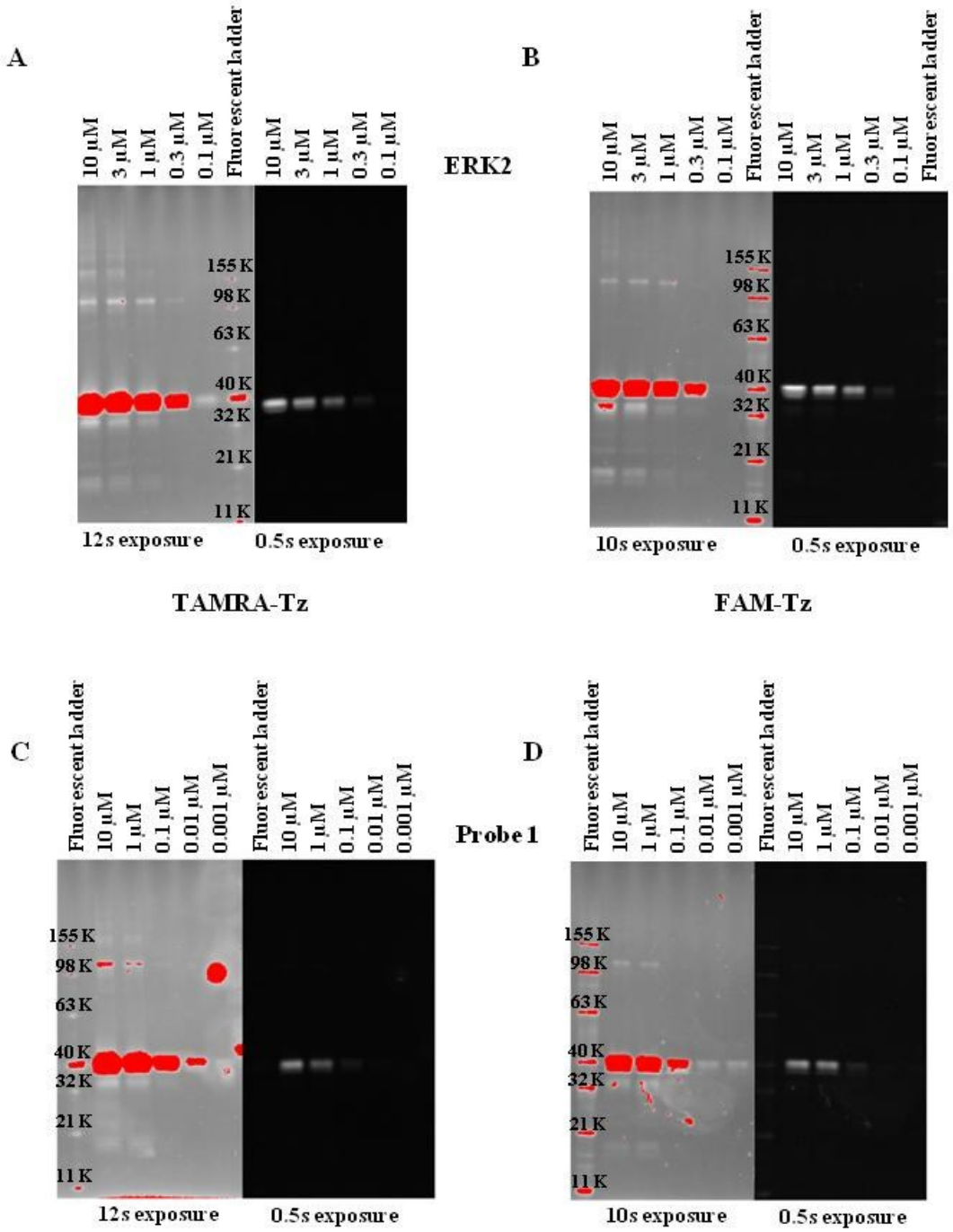

Figure S2. Determination of the detection limits by varying the ERK2 concentration, probe **1** concentration, tetrazine dye and exposure time. (A). Variation of ERK2 concentration with 1 µM probe **1** and 10 µM TAMRA-Tz. (B). Variation of ERK2 concentration with 1 µM probe **1** and 10 µM FAM-Tz. (C). Variation of probe **1** concentration with 1 µM ERK2 and 10 µM TAMRA-Tz. (D). Variation of probe **1** concentration with 1 µM ERK2 and 10 µM FAM-Tz. The experiments were performed on a Bio-rad EZ Gel doc using a UV tray.

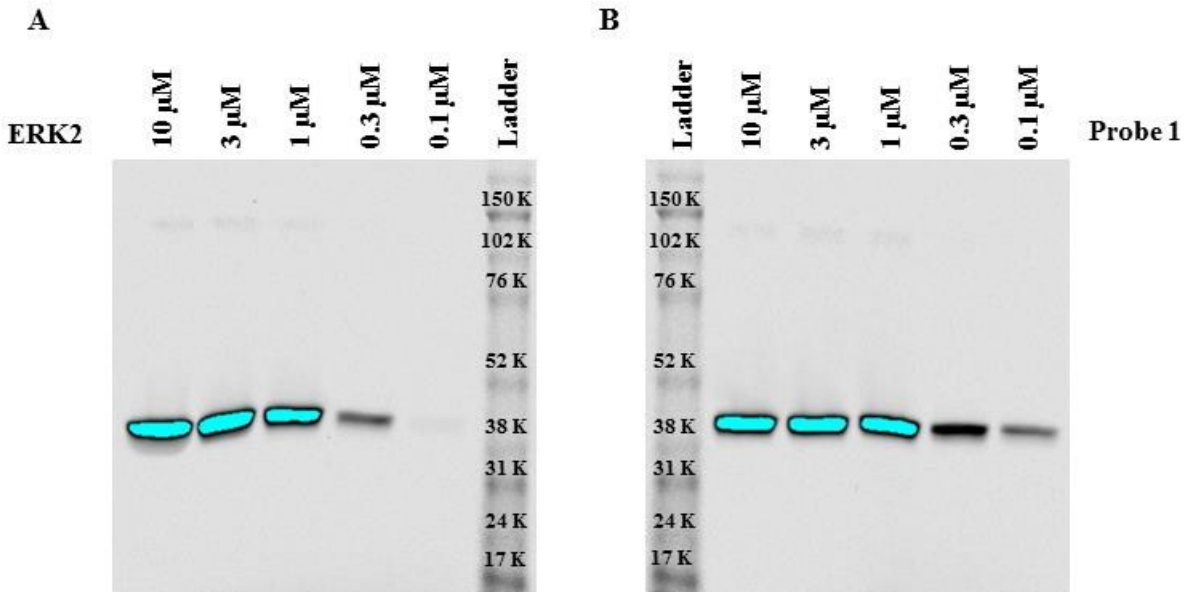

Figure S3. Determination of the detection limits by varying the concentrations of ERK2 and probe **1** using 6-methyl-tetrazine-sulfo-cy5 dye (**11**). (A). Variation of ERK2 concentration with 1 µM probe **1** and 10 µM 6-methyl-tetrazine-sulfo-cy5 dye (**11**). (B). Variation of probe **1** concentration with 1 µM ERK2 and 10 µM 6-methyl-tetrazine-sulfo-cy5 dye (**11**). The experiments were performed on a Li-Cor Biosciences Odyssey system in the 700 nm channel.

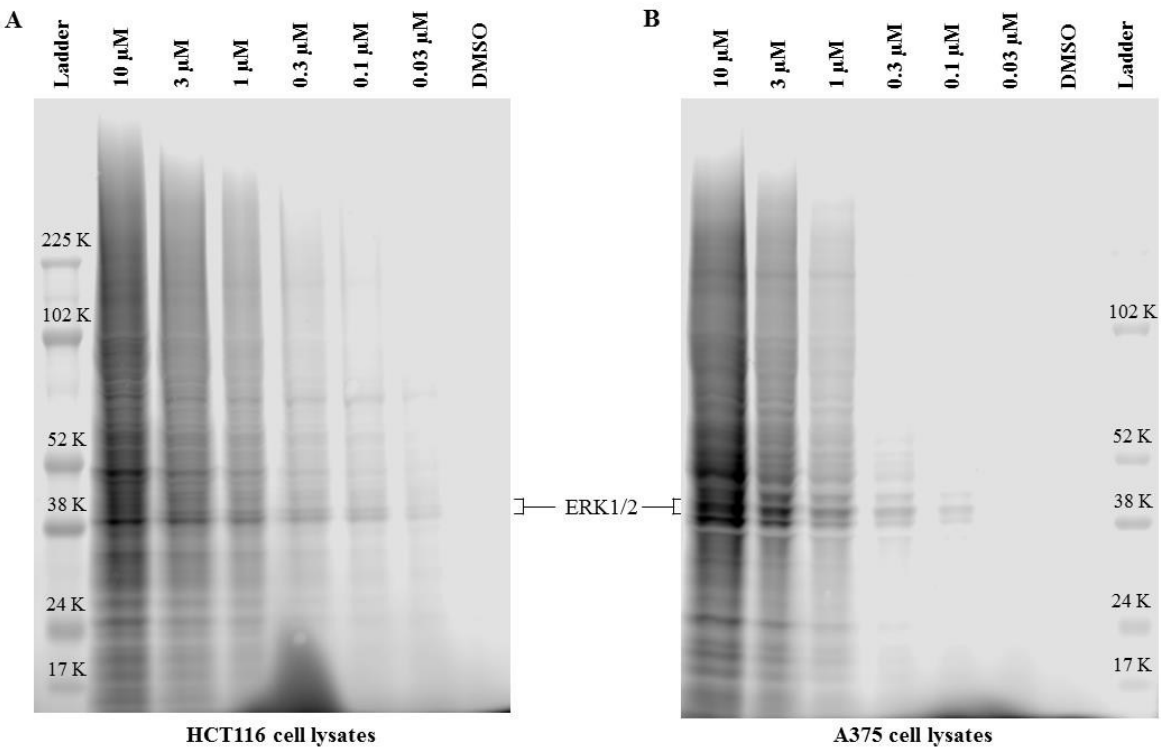

Figure S4. (A). In-gel fluorescence showing the selectivity profile of TCO probe **1** in HCT116 cell lysates using Tz-sulfo-Cy5 dye **11**. (B). In-gel fluorescence showing the selectivity profile of TCO probe **1** in A375 cell lysates using Tz-sulfo-Cy5 dye **11**.

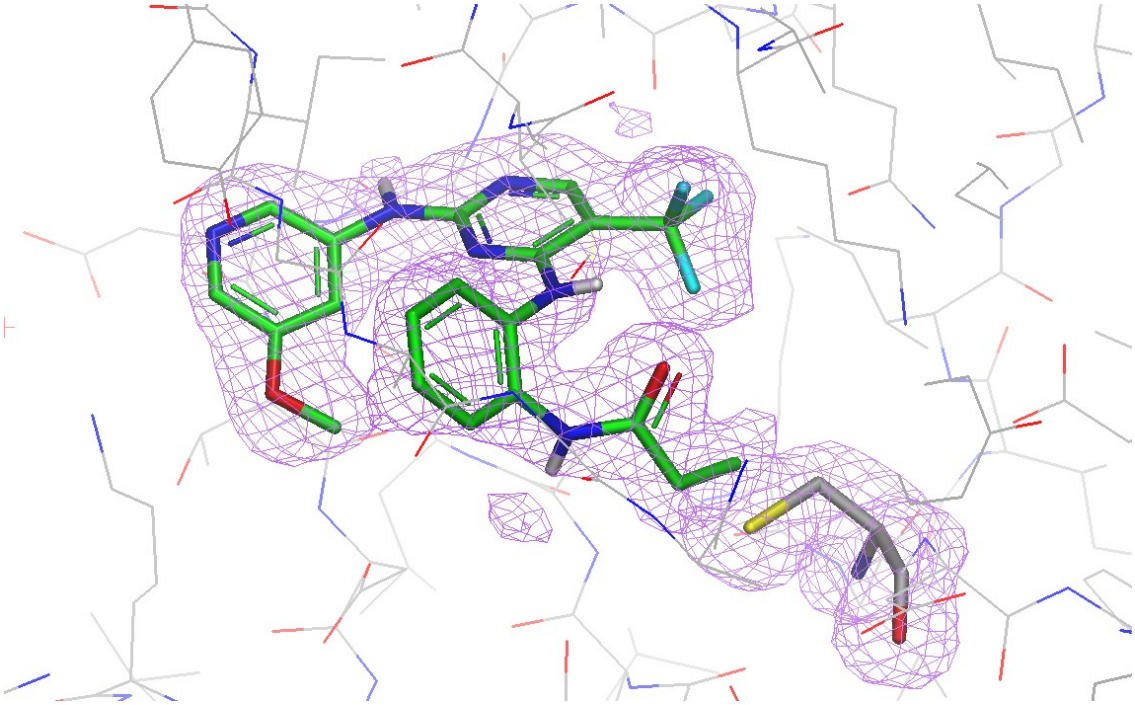

Figure S5. Electron density map for compound 3 in complex with ERK2.

Supporting experimental procedures

Co-crystal structures of probe 1 and compound 3 with ERK2

Human ERK2 (full length ERK2 with N-ter MAHHHHHH tag) was crystallised using 15mg/mL ERK2, hanging drop, 1µL reservoir + 1µL protein, 18C. 0.2M ammonium sulphate, (31-36)% 2KMPEG, 20mM beta-mercaptoethanol (BME), 0.1M Hepes/NaOH pH7.2. After soaking, crystals were dipped in a cryo protectant containing all buffer components and 35% 2KMPEG and flash frozen in liquid N_2. Crystals were soaked in BME free crystallisation buffer containing either (96hrs) 5mM (nominal) AT34170, 15% DMSO, 10mM DTT or (18hrs) 40mM AT31777 (nominal) 10% DMSO 7.5mM TCEP.

Table S2. X-ray collection statistics for ERK2 co-structures.

	ERK2 – (**1**)	ERK2 – (**3**)
PDB Code	5LCJ	5LCK
Data Collection		
Space group	$P2_1$	$P2_1$
Cell Dimensions		
a,b,c (Å)	48.73, 70.81, 60.21	48.86, 70.91, 60.42
α,β,g (^{0})	90, 109.18, 90	90, 109.43, 90
Resolution (Å)	30.1 – 1.78	30.1 – 1.89
Measured reflections	102572	105730
Unique reflections	32145	28915
R_{merge}	3.6	8.5
I/sI	1.9	2.0
Completeness (outer)	87.1 (75.8)	96.4 (73.7)
Redundancy	3	3.4
Refinement		
Resolution (Å)	26.7 – 1.78	28.5 – 1.89
No. reflections (test set)	32060 (1678)	30041 (1550)
R_{work}/R_{free} (%)	0.163/0.212	0.164/0.212
No. atoms		
Protein	2817	2822
water	535	484
B-factors (Å²)		
Protein	33.0	33.0
Ligand	58.3	42.0
water	48.3	45.5
RMSD		
Bond lengths (Å)	0.008	0.006
Bond angles (^{0})	1.7	1.3
Ramachandran plot % residues		
Favoured	89.0	87.5
Additional allowed	10.8	12.2
Generously allowed	0.3	0
Disallowed	0	0.3

Quantitation of covalent modification of ERK2 protein with Probe 1 by LC-MS

ERK2 (20 mM) and probe **1** (200 mM in DMSO) were mixed in buffer containing 100 mM ammonium acetate pH 7.5 at r.t. Reactions were terminated at the specified time points (see Table S1) by precipitation of the protein in neat ethanol at -80 ºC. The supernatant was pipetted away and the protein resolubilised in 75% acetonitrile/ 2% formic acid.

Reacted protein samples were analysed by LC-MS with an Agilent 1200 HPLC connected to a Bruker MicroTOF MS using a Waters Xbridge Xbridge BEH300 C4 column (100 x 2.1mm, 3.5 µm).

LC methodology

Eluant A: 0.1% TFA/water

Eluant B: 0.1%TFA 95% acetonitrile/water

Linear HPLC gradient: 5 - 95% B over 25.3 minutes after 20 minutes pre-injection column - equilibration in 100% solvent A

Flow rate: 0.2 mL/min

Column temperature: 60 ºC

ERK2 Bioassay

Activity of ERK2 enzyme (Life Technologies) was determined using a time-resolved fluorescence format measuring the phosphorylation of a truncated version of Activating transcription factor 2 labelled with green fluorescent protein (ATF2-GFP) (Life Technologies). Compound, ERK2 (0.25 nM) and ATF2-GFP (400 nM) were pre-incubated for 1 hour at room temperature in buffer containing 50 mM Tris pH 7.5, 10mM $MgCl_2$, 1 mM EGTA, 0.01% Triton X-100, 1mM DTT and 2.5% DMSO. ATP (20 µM) was added and the reactions were allowed to proceed for 30 min at room temperature. Reactions were then stopped using TR-FRET dilution buffer (Life Technologies), 25mM EDTA and 2 nM Tb-Anti-pATF2 (Thr71) (Life Technologies). After a further incubation period of at least 30 minutes, fluorescence was read on a Pherastar reader (Lanthascreen optic module; excitation 340 nm, emission 520 nm (channel A), 495 nm (channel B)). The ratio between A and B counts was used to calculate signal. IC_{50} values were calculated using a sigmoidal dose response equation (Prism GraphPad software, La Jolla, CA, USA).

A375/HCT116 Cell proliferation assay

The anti-proliferative activities of compounds of the invention were determined by measuring the ability of the compounds to inhibit growth in the Human melanoma cell line A375.

Cell proliferation was determined by measuring the conversion of rezasurin (Alamar Blue) to resorufin in reponse to mitochondrial activity (Nociari, M. M, Shalev, A., Benias, P., Russo, C. *Journal of Immunological Methods* 1998, 213, 157-167). A375 cells (American Type Culture Collection, Teddington, UK) or HCT116 cells (American Type Culture Collection,

Teddington, UK) were grown in Dulbecco's Modified Eagle Medium + 10 % FBS. Each well of a black 96-well flat-bottomed plate was seeded with $2x10^3$ cells in 200 µL of complete culture medium one day before the compound treatment. Cells were incubated with compound in 0.1% (v/v) dimethyl sulfoxide (DMSO) for 4 days before addition of 20 µL Alamar blue. After a further 6 h incubation at 37°C the plate was read on a Spectramax Gemini reader (Molecular Devices; excitation 535 nm, emission 590 nm). GI_{50} values were calculated using a sigmoidal dose response equation (Prism GraphPad software, La Jolla, CA, USA).

Study of IEDDA cycloaddition between probe 1 and Tz-dye 11 by LC-MS

6-Methyl-tetrazine Sulfo-Cy5 (**11**) was bought from Jena Bioscience and used as received. The samples were analysed by LC-MS (liquid chromatography mass spectrometry) on a Shimadzu Nexera UPLC coupled with a Shimadzu LCMS-2020 single-quadrupole MS using a YMC-Triart C18 column (50 x 2.0 mm, 1.9 µm).

LC-MS methodology

Eluent A: 10 mmoL ammonium bicarbonate pH 9.4

Eluent B: acetonitrile

Gradient: 3 – 99% B over 0.7 min

Flow: 0.7 mL/min

Column T: 45 ºC

A 10mM sample of probe **1** in DMSO-d_6 was analysed by LC-MS (black spectrum). A 10 mM sample of 6-methyl-tetrazine Sulfo-Cy5 (**11**) in DMSO- d_6 was analysed by LC-MS (pink spectrum). Probe **1** and dye **11** were mixed in DMSO- d_6 in a 1:9 ratio with a final concentration of 0.5 mM for probe **1** and 5 mM for dye **11**. The mixture was analysed by LC-MS after 15 min (blue spectrum). The reaction was more than 95% complete after 15 min with less than 5% of probe **1** remaining in the reaction mixture.

Cell culture and cell lysates

A375 and HCT116 cells were cultured in DMEM supplemented with 10% FBS (Gibco, Life Technologies). Cells have been lysed using TG lysis buffer supplemented with protease inhibitor cocktail (Mini EDTA free protease inhibitor cocktail, Roche) on ice for 20 min. The lysates were centrifuged at 14000 rpm for 10 min at 4 °C and protein concentration was determined by a Pierce™ BCA Protein Assay Kit.

In-gel ABPP

Cell lysate (20 µL) was added to Tris-glycine SDS PAGE sample buffer (20 µL) 2 x (Novex). The resulting mix (15 µL) was then analysed by SDS-PAGE using 4-20% Tris-

glycine gels (Novex) and was run with Tris-glycine running buffer (Novex). The gels were imaged on a Li-Cor Biosciences Odyssey system in the 700 nm channel.

Immunoblotting

Once the gel has been imaged, it was transferred onto a nitrocellulose membrane (Novex). The membrane was blocked in blocking buffer (Odyssey) at r.t. for 1 hour and subjected to immunodetection using a total ERK1/2 primary antibody (p44/42 MAPK ERK1/2, Cell Signaling Technologies®, 1:1000) in blocking buffer. After washing 3x with a Tris-buffered saline (TBS) with 0.1% Tween-20 solution, the membrane was incubated with fluorescently labelled secondary antibody (IRDye800CW Donkey Anti-Rabbit, 1:10 000) for 1 hour at r.t. in the dark. After washing 2x with a TBS solution, the membrane was imaged on a Li-Cor Biosciences Odyssey system in the 800 nm channel.

This membrane was also imaged at 700 nm to see superposition of the probe and ERK1/2.

Labelling of pure ERK2 with probe 1

Probe **1** was added from a 100x stock in DMSO to samples of ERK2 in 15 mM Tris pH 7, 150 mM NaCl, 4 mM DTT, 25% glycerol. ERK2 was incubated with probe **1** for 2 hours at r.t. FAM-Tz, TAMRA-Tz or 6-methyl-tetrazine-sulfo-cy5 dye (**11**) (Jena Bioscience) was added from a 100x stock in DMSO for a 10 µM final concentration. The samples were incubated for 15 min at r.t. and analysed by SDS-PAGE as described above. The gels were imaged on a Bio-rad EZ Gel doc using a UV tray (for FAM-Tz and TAMRA-Tz) or an a Li-Cor Biosciences Odyssey system in the 700 nm channel (for 6-methyl-tetrazine-sulfo-cy5 dye (**11**)).

Labelling of cell lysates with probe 1

A375 and HCT116 were lysed as described above. Probe **1** was added from a 100x stock in DMSO (2 µL) to cell lysates (200 µL, $C_{A375\ cell\ lysates}$ = 4.30 mg/mL and $C_{HCT116\ cell\ lysates}$ = 3.71 mg/mL). The cell lysates were shaken at r.t. for 2 hours. 6-Methyl-tetrazine-Sulfo-Cy5 **11** (Jena Bioscience) was added from a 100x stock in DMSO for a 10 µM final concentration. The samples were shaken at r.t. for 15 min and the samples were analysed as described above for in-gel ABPP.

Labelling of cells with probe 1

6-Well plates were seeded with 1.10^6 cells in 2 mL of medium. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and air for 18 hours. Probe **1** was added from a 1000x stock in DMSO (2 µL) to the plates. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and air for 2 hours. The medium was removed and the cells were washed with PBS (2x). TG Lysis buffer (150 µL) was added to each well and the plates were kept on ice for 20 min. The cell lysates were centrifuged at 14000 rpm for 10 min at 4 °C and the protein concentration was determined by a Pierce™ BCA Protein Assay Kit. Samples were

normalised to 2.2 mg/mL for A375 cell lysates and to 1.5 mg/mL for HCT116 cell lysates. 6-Methyl-tetrazine SulfoCy5 **11** was added from a 100x stock in DMSO and the cell lysates were shaken for 15 min at r.t. The samples were analysed as described above for in-gel ABPP.

When the cells were pre-incubated with the untagged inhibitor (**3**), the latter was added to the cells from a 1000x stock in DMSO. The cells were incubated at 37 °C in an atmosphere of 5% CO_2 and air for 2 hours and washed with fresh medium (2x) before addition of probe **1**.