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Fragment-based covalent ligand screening enables rapid discovery of inhibitors for the RBR E3 ubiquitin ligase HOIP

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ABSTRACT: Modification of proteins with polyubiquitin chains is a key regulatory mechanism to control cellular behavior and alterations in the ubiquitin system are linked to many diseases. Linear (M1-linked) polyubiquitin chains play pivotal roles in several cellular signalling pathways mediating immune and inflammatory responses and apoptotic cell death. These chains are formed by the linear ubiquitin chain assembly complex (LUBAC), a multi-protein E3 ligase that consists of 3 subunits, HOIP, HOIL-1L and SHARPIN. Herein, we describe the discovery of inhibitors targeting the active site cysteine of the catalytic subunit HOIP using fragment-based covalent ligand screening. We report the synthesis of a diverse library of electrophilic fragments and demonstrate an integrated use of protein LC-MS, biochemical ubiquitination assays, chemical synthesis and protein crystallography to enable the first structure-based development of covalent inhibitors for an RBR E3 ligase. Furthermore, using cell-based assays and chemoproteomics we demonstrate that these compounds effectively penetrate mammalian cells to label and inhibit HOIP and NF- κ B activation, making them suitable hits for the development of selective probes to study LUBAC biology. Our results illustrate the power of fragment-based covalent ligand screening to discover lead compounds for challenging targets, which holds promise to be a general approach for the development of cell-permeable inhibitors of thioester-forming E3 ubiquitin ligases.

Introduction

Ubiquitination represents one of the most diverse post-translational modifications of proteins and constitutes an essential route for the regulation of protein signalling and degradation. The process is carried out through a complex interplay of enzymes (E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases and deubiquitinating (DUB) enzymes), which provides an intricate network of regulation and substrate specificity (Figure 1A).¹⁻² E3 ubiquitin ligases are the key determinants for substrate specificity in this cascade and as such represent attractive yet notoriously challenging pharmacological targets. There are over 600 known human E3 ligases that fall into three main classes (RING, HECT and RBR) based on their structure and mechanism of ubiquitin transfer, which in the case of HECT and RBR E3 ligases involves the formation of a covalent thioester intermediate with ubiquitin.³⁻⁴ However, a deeper understanding of their underlying biology has long been hampered by the lack of selective pharmacological tools. The linear ubiquitin chain assembly complex (LUBAC) is a multi-protein E3 ubiquitin ligase of the RBR family that catalyzes the formation of polyubiquitin chains linked between the C-terminal carboxylate of ubiquitin and the N-terminal α -amino group

of methionine 1, called linear or M1-linked chains.⁵⁻⁶ Linear polyubiquitin chains play crucial roles in the regulation of multiple cellular functions including immune and inflammatory signaling via the NF- κ B pathway, cell death and cancer.⁷⁻¹⁰ LUBAC consists of three core components, the RBR-domain containing subunits HOIP and HOIL-1L plus SHARPIN,¹¹⁻¹³ with HOIP constituting the catalytic machinery required for linear chain formation (Figure 1B).¹⁴⁻¹⁶ Recent crystal structures of truncated HOIP have provided valuable insight into the mechanism of linear polyubiquitin chain formation.^{15, 17} However, in order to interrogate the physiological function of LUBAC in a cellular context, chemical tools that selectively target the E3 ligase activity of HOIP would be of great value. To this end, a handful of HOIP modulators have been reported, including small molecule inhibitors BAY 11-7082 (**1**, Figure 1C)¹⁸ and gliotoxin (**2**),¹⁹ as well as stapled peptides that target protein-protein interactions at the HOIP/HOIL-1L and HOIL-1L/SHARPIN interfaces.²⁰⁻²² Although these modulators all have demonstrated effects on LUBAC activity, the molecules are also associated with drawbacks that may limit their utility as tools. Compound (**1**), which was originally identified as an inhibitor of TNF α -induced phosphorylation of I κ B α and NF- κ B signaling, has been

shown to covalently inhibit HOIP activity yet exhibits broad reactivity across multiple proteins.¹⁸

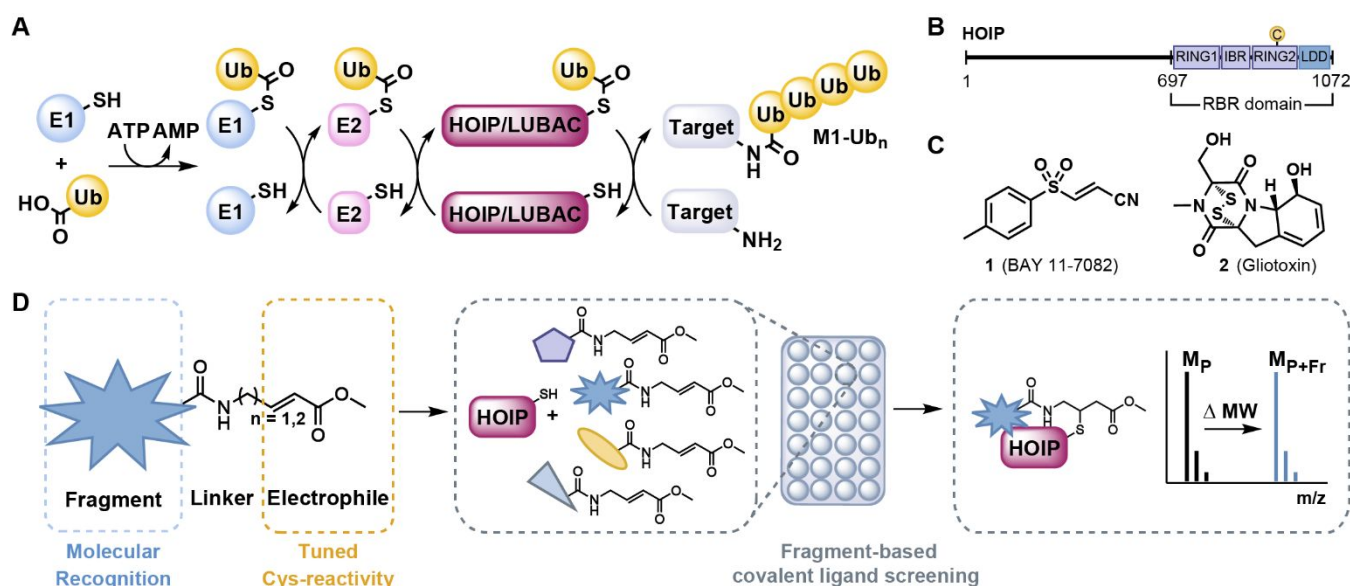


Figure 1 – Targeting HOIP using fragment-based covalent ligand screening. A) Schematic of the ubiquitination cascade highlighting the formation of linear (M1) polyubiquitin chains (Ub_n) by LUBAC, a reaction that proceeds via a covalent thioester intermediate between the HOIP subunit and ubiquitin. B) Schematic representation of the HOIP E3 ligase, highlighting the RBR which contains the active-site cysteine residue C885 (yellow circle) and the linear chain-determining domain, LDD (amino acids 697- 1072). C) Small molecule LUBAC inhibitors (**1**) BAY 11-7082 and (**2**) gliotoxin. D) Overview of our approach to develop covalent probes targeting the active site cysteine residue of HOIP using fragment-based covalent ligand screening by protein LC-MS.

Compound (**2**) is a well-characterized fungal metabolite and was recently identified in a high-throughput screen as an inhibitor of LUBAC.¹⁹ However, (**2**) interacts with multiple specific targets in mammalian cells,²³⁻²⁴ and as a complex natural product it does not easily lend itself to structure-based optimization. Stapled peptides suffer from similar disadvantages as staple type and position, and changes in peptide sequence can have profound effects on cell permeability.²⁵⁻²⁷ Furthermore, a recent report describing a novel MALDI-TOF MS-based E2/E3 ligase screening assay identified the chemotherapeutic bendamustine as an inhibitor of HOIP RBR *in vitro*, albeit additional studies are warranted to verify its utility as a tool in a cellular setting.²⁸

Inspired by recent reports describing the targeting of reactive cysteine residues in proteins using fragment-based covalent ligand screening of α,β -unsaturated methyl esters,²⁹⁻³⁰ we decided to take a rational approach to target the active-site cysteine residue in HOIP as a means to covalently block its catalytic activity (Figure 1D). Herein, we describe the first inhibitors targeting the active-site cysteine of an RBR E3 ligase discovered by fragment-based covalent ligand screening and go on to show that such fragments serve as a useful starting point for structure-based development of selective, cell-permeable probes to interrogate the biology of LUBAC and other thioester-forming E3 ubiquitin ligases.

Results and Discussion

Design and synthesis of a structurally diverse electrophilic fragment library: We synthesized a small library of fragments linked to α,β -unsaturated methyl ester electrophiles by amide bond formation between carboxylic acid fragments, and amines (**3**) and (**4**) (Figure 2, Supplementary Schemes S1, S2). Compound collections based on the α,β -unsaturated methyl ester scaffold (**3**) have been shown to exhibit a narrower reactivity profile compared to other commonly used electrophiles for covalent approaches, such as acrylamides.^{29, 31-32} This advantageous reactivity profile promotes hit identification governed by molecular recognition rather than variations in the reactivity of the electrophile.²⁹ We chose to synthesize a small number of amides using the extended amine (**4**), based on the hypothesis that a longer linker between the fragment and electrophile may allow a greater sampling of potential protein interactions in the vicinity of the reactive cysteine residue. Fragments were selected from the GSK compound collection and commercial sources, based on physicochemical properties such as molecular weight, clogP, hydrogen bond acceptors and donors (Supplementary Figure S1, Supplementary Table S1), and clustered to ensure scaffold and functional group diversity. In total 106 amides were synthesized in parallel to give a physicochemically favorable and topologically diverse screening set of electrophilic fragments (Figure 2). A major concern of working with electrophilic compounds is their potential for poor stability. We monitored the DMSO stock solutions of the synthesized compounds over time to

domains of the E3 ligases HOIL-1L, RNF144A, HHARI and TRIAD3A, the HECT E3 ligases NEDD4L and E6AP, which all form covalent thioester intermediates with ubiquitin, and the two DUBs known to cleave linear polyubiquitin chains OTULIN and CYLD-1 (Figure 3F). Compound (5) was screened at a 10-fold stoichiometric excess, and for comparison BAY 11-7082 (1) was used as a positive control

at just 2-fold excess. Unsurprisingly, compound (1) caused rapid and extensive labelling for most proteins while compound (5) displayed encouraging selectivity for HOIP, resulting in <40% labelling after 24 h incubation for all other proteins screened (Figure 3F, Supplementary Figure S6), indicating that compound (5) is a useful starting point for further ligand optimization.

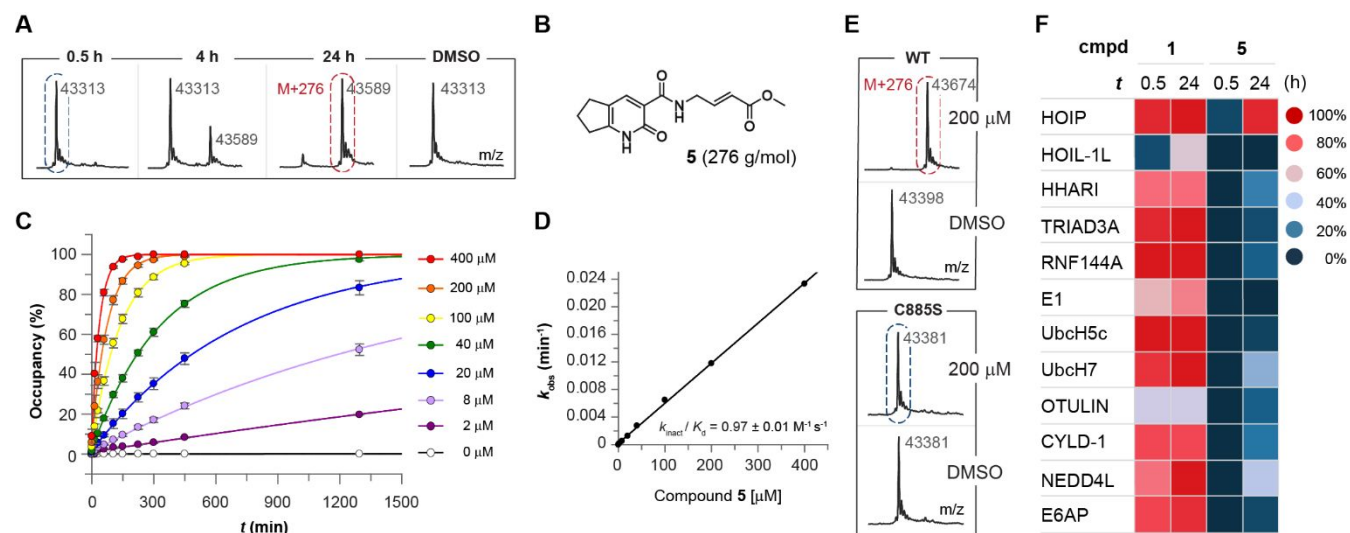


Figure 3 – Compound (5) labels HOIP RBR at the active site cysteine residue C885 in a time- and concentration-dependent manner. A) Compound (5) (20 μ M) labelled HOIP RBR R1032A (2 μ M) over time in the library screen. B) The chemical structure of compound (5). C) Labelling of wild type HOIP RBR (2 μ M) was validated by screening different concentrations of (5). Each time course was normalized to percentage occupancy, fitted to a single exponential equation and plotted in GraFit (v.7) as an average of three independent experiments to determine the observed first-order rate constant (k_{obs}). D) k_{obs} measurements were plotted against the concentration of (5) to give the ratio of k_{inact}/K_d as the slope of the curve.³⁴ E) The active-site C885S mutant of HOIP RBR (2 μ M) was not labelled by compound (5) at 200 μ M after 4 h, suggesting that (5) targets the catalytic cysteine. F) LC-MS based counter screen of compounds (1) (2-fold excess) and (5) (10-fold excess) against a panel of RBR E3 ligases (HOIL-1L, HHARI, TRIAD3A, RNF144A RBR domains), HECT E3 ligases (NEDD4L, E6AP), E2 enzymes (UbcH5c, UbcH7), E1 enzyme and DUBs (OTULIN, CYLD-1). Data shown as percentage labelling after 0.5 and 24 h incubation.

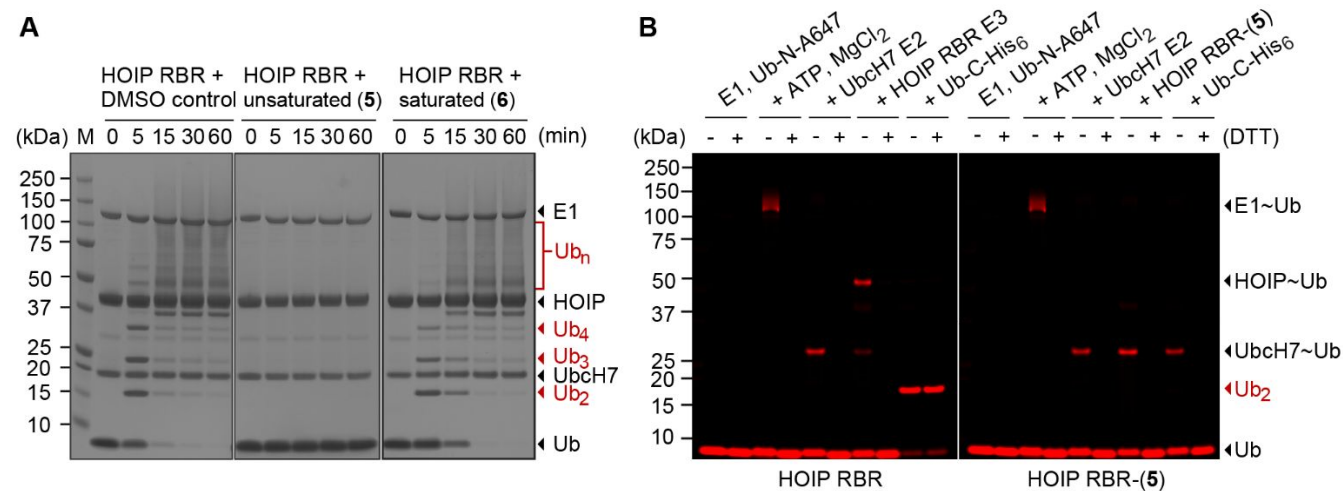


Figure 4 – Compound (5) inhibits the formation of polyubiquitin chains by preventing loading of ubiquitin onto the HOIP RBR domain. A) *In vitro* polyubiquitination assay monitoring the formation of unanchored polyubiquitin chains. Pre-labelling of the HOIP RBR domain with compound (5) (middle) led to complete inhibition of polyubiquitin chain formation, whereas treatment with the saturated analogue (6) had no effect on catalytic activity. B) *In vitro* ubiquitin transfer assay

monitoring the sequential loading of N-terminally AlexaFluor647-labelled ubiquitin (Ub-N-A647) onto E1, UbCH7, HOIP RBR domain and a C-terminally His-tagged ubiquitin substrate (Ub-C-His₆). Pre-labelling of the HOIP RBR domain with compound (**5**) (right) effectively prevented the loading of ubiquitin onto the E3, thereby inhibiting the formation of di-ubiquitin. For full gels see Supplementary Figures S9 and S11.

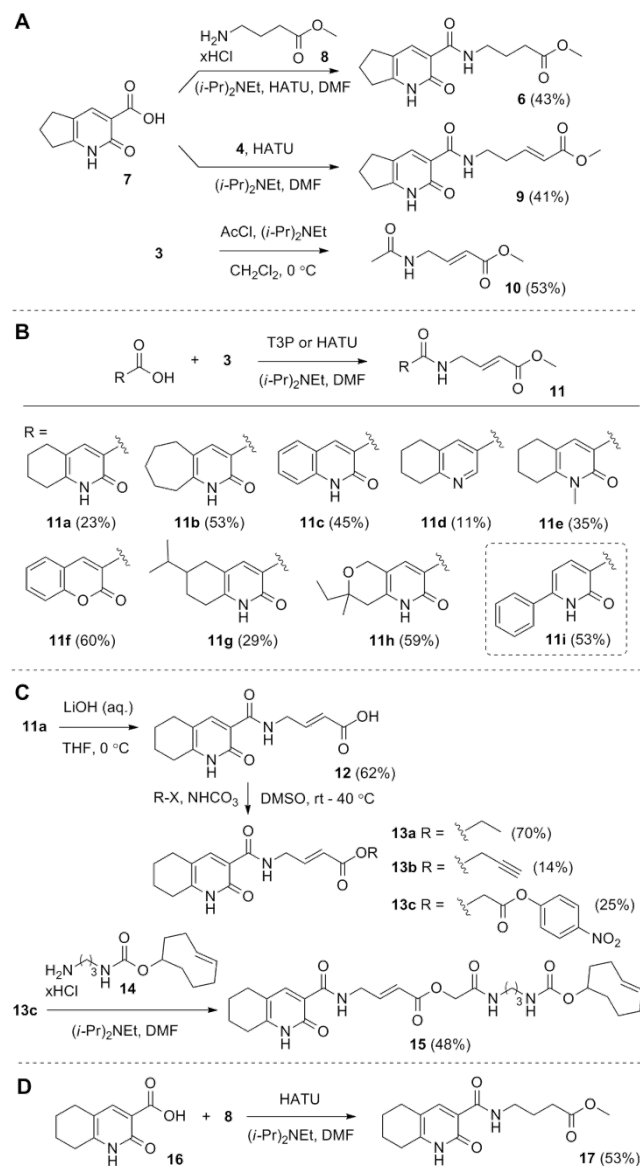
The HOIP-reactive hit compound inhibits enzyme function: The functional effects of HOIP labelling were investigated in an *in vitro* polyubiquitination assay. The HOIP RBR domain was preincubated with compound (**5**) to ensure full labelling and mixed with E1, E2 (UbCH7 or Supplementary Figure S9). In contrast, treatment of HOIP with the saturated analogue (**6**) (Scheme 1A) which is not able to form a covalent adduct had no inhibitory effect on polyubiquitin chain formation, demonstrating that the labelling occurs through the α,β -unsaturated ester electrophile. Furthermore, a fluorescence-based ubiquitin transfer assay that monitors the sequential formation of E1, E2 and E3-ubiquitin thioesters,¹⁴ showed that labelling of the HOIP RBR domain with compound (**5**) inhibits formation of the E3~Ub thioester intermediate, but not any of the previous steps, effectively blocking only ubiquitin transfer onto the protein substrate (Figure 4B).

Exploration of SAR through targeted compound modifications: To investigate the structural determinants for compound recognition and labelling of the HOIP RBR domain we synthesized a set of closely related analogs, including saturated compounds (**6**) and (**17**), linker-extended analog (**9**) and fragment control (**10**) (Scheme 1A, D). Moreover, the pyridone scaffold was systematically altered to identify key protein-binding elements, including ring expansion (**11a** and **b**), aromatic rings (**11c**), changes to the hydrogen bond donor and acceptor abilities (**11d-f**), and fragment growth in different vectors (**11g-i**; Scheme 1B). Compound (**11i**) was part of the original screening library. Lastly, we established a synthetic route based on sequential alkaline hydrolysis and alkylation to enable structural variation of the ester (**13a** and **b**), and the synthesis of a clickable *trans*-cyclooctene (TCO) probe (**15**) by amide bond formation between activated ester (**13c**), and amine (**14**; Scheme 1C). The analogs were screened by protein LC-MS against the HOIP RBR domain at a 10-fold stoichiometric excess, and the percentage labelling was measured after 0.5, 4, and 24 h incubation (Figure 5A, Supplementary Figure S7). The data confirmed that saturation of the α,β -unsaturated ester (**6** and **17**) abolished activity. Furthermore, it showed that extension of the linker between fragment and electrophile (**9**), or removal of the fragment portion of the molecule (**10**) resulted in significantly lower activity, suggesting that the fragment is essential for establishing key molecular interactions with the protein. The loss of reactivity observed when the hydrogen bonding ability of the pyridone was modified (*e.g.* analogues **11d-f**) highlighted the importance of this group for binding and labelling. Alternative substitution patterns on the pyridone were more tolerated. Ring-expanded, and aromatic analogs (**11a-c**) gave a comparable or increased rate of labelling, while fragment growth from different vectors of the pyridone (*e.g.* **11g** vs **11h** and **11i**), or growing the ester substituent (**13a**, **13b** and **15**) had varying effects on activity, suggesting

UbCH5c), ubiquitin and ATP to initiate unanchored polyubiquitin chain synthesis. Labelling of HOIP fully abolished chain formation when compared to DMSO control (Figure 4A,

possible binding modes in the active site of the HOIP RBR domain for this chemical series.

Structural analysis of the HOIP-inhibitor complex: To rationalize the observed reactivity of the analogs tested and to provide a basis for structure-based ligand optimization, we solved the crystal structure of the RING2-LDD domain of HOIP in covalent complex with inhibitor (**5**), at 2.15 Å resolution (Figure 5B, Supplementary Table S5). Compound (**5**) is bound within the active site of HOIP with clear contiguous electron density between C885 and the beta carbon of ester (**5**).



Scheme 1 – Analog synthesis. A) Control compounds (**6**), (**9**), and (**10**) were synthesized by amide bond formation using amines (**8**), (**4**), and (**3**) respectively. B) Analogs (**11a-i**) were synthesized by amide bond formation between the corresponding carboxylic acid and amine (**3**). Compound (**11i**) was synthesized as part of the original screening library. C) Ester analogs (**13a-c**) were synthesized in two steps from ester (**11a**) by alkaline hydrolysis and alkylation reactions using the appropriate alkyl halide (ethyl iodide, propargyl bromide and 4-nitrophenyl iodoacetate respectively), and (**13c**) was further converted into TCO

probe (**15**). D) Saturated control (**17**) was synthesized from carboxylic acid (**16**) and amine (**8**).

The cyclopentyl pyridone sits on a shallow ledge perfectly placed for its aromatic ring to stack above F888 and be anchored on one side by hydrogen bonds formed between the pyridone and the backbone nitrogen and oxygen of H889. This arrangement helps to explain why an extension of the linker between the pyridone and Michael acceptor in compound (**9**) led to reduced activity. Residues Y878, H889 and

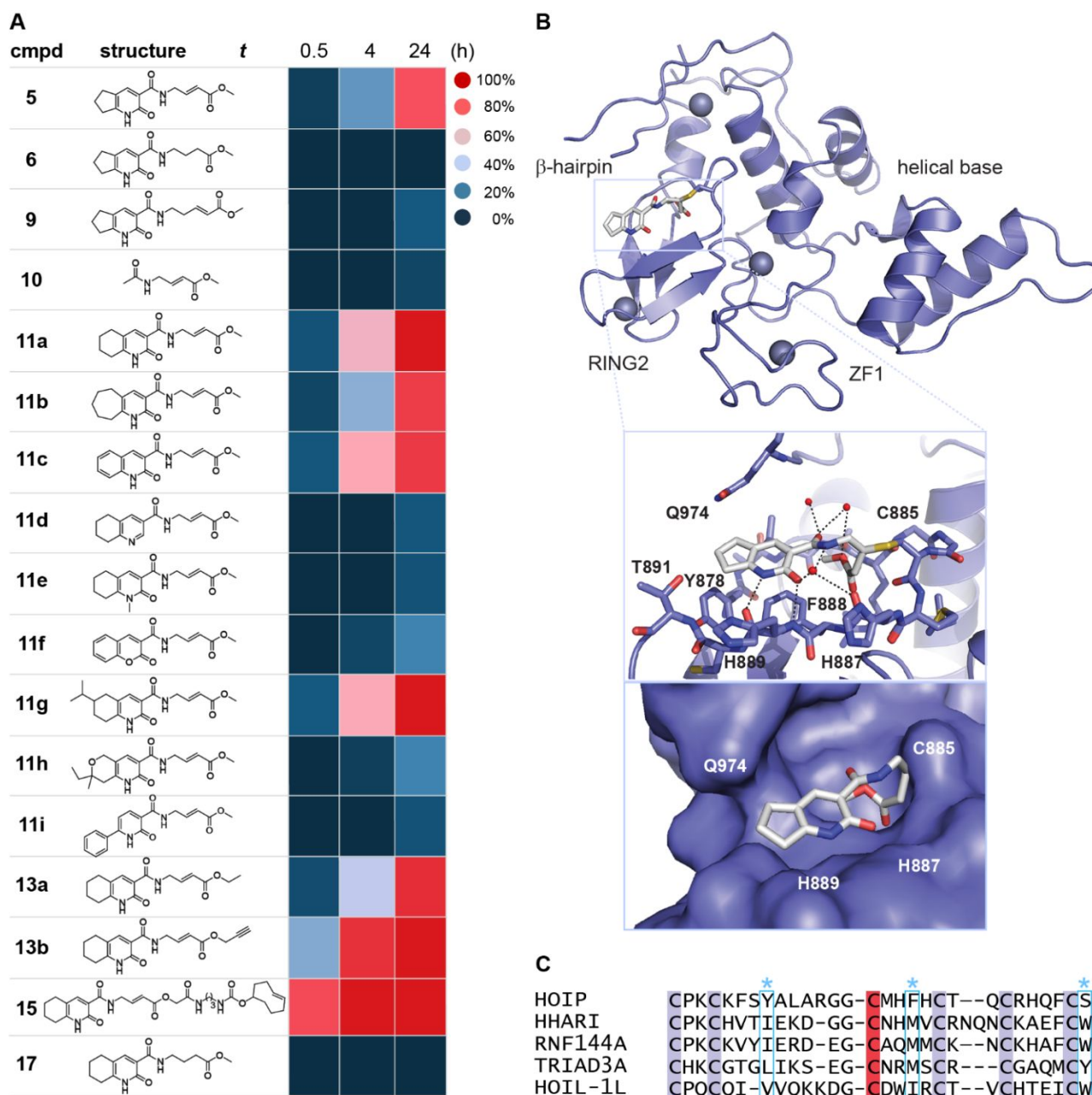


Figure 5 – Molecular determinants for interactions between the HOIP RING2 domain and compound (5). A) LC-MS screening data plotted as percentage labelling of the HOIP RBR domain (2 μ M) by key analogues (**6**), (**9**)-(11), (**13**), (**15**) (20 μ M), and (**17**) (200 μ M) after 0.5, 4, and 24 h incubation. For full screening data see Supplementary Figures S7, S8. B) Ribbon representation of the HOIP RING2-LDD domain structure in complex with compound (**5**) at 2.15 Å resolution (PDB: 6GZY). Zn^{2+} ions are shown as grey spheres and compound (**5**) as ball and stick. The inset shows a zoom into the active site showing hydrogen bonds (dashed lines) made by compound (**5**) with water molecules (red spheres) and HOIP (top), and the pocket

around the active site which accommodates compound (**5**) (bottom). C) Sequence alignment of RING2 domains of those RBR E3 ligases that were screened against compound (**5**). The catalytic cysteine residue is highlighted in red, non-catalytic cysteine residues in purple, and non-conserved residues contributing to the compound-binding pocket in HOIP are highlighted with a blue asterisk.

T891 enclose the other side of the ring system to form a defined, but probably somewhat malleable site, as demonstrated by the ability to accommodate small changes in ring sizes and substitutions in this region of the compound (e.g. **11a-c** and **11g**). Additional water-mediated and direct hydrogen bonds are made by all the other heteroatoms of (**5**) (Figure 5B). The ester chain of (**5**) lies

within a pocket orthogonal to the ledge, with the carbonyl oxygen held in position by the hydroxyl side chain of S899, in a conformation that may facilitate the reaction of the catalytic cysteine and the Michael acceptor. Sequence alignment of the RING2 domains of the RBR E3 ligases HOIP, HOIL-1L, HHARI,

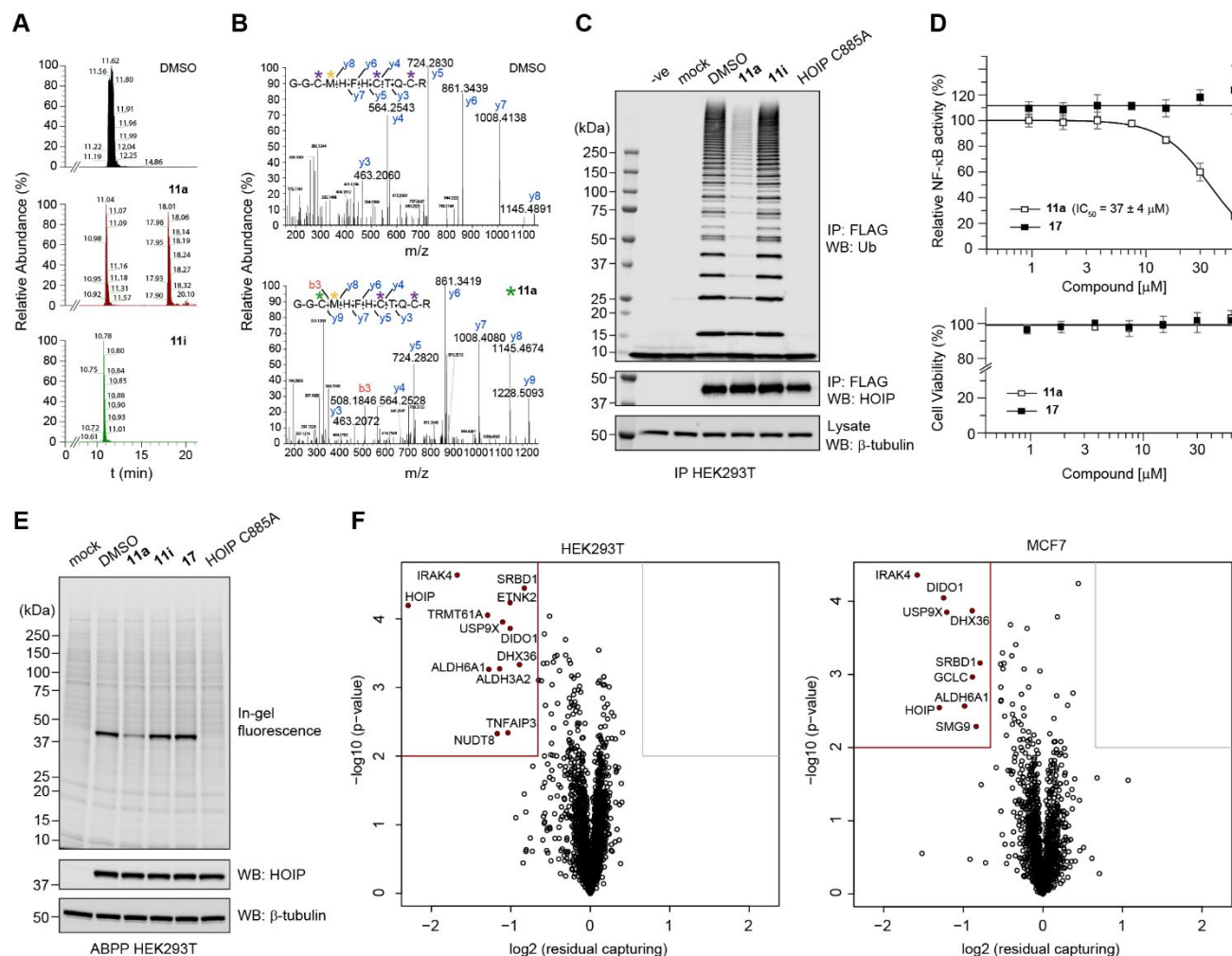


Figure 6 - Compound (11a) labels and inhibits HOIP in HEK293T and MCF7 cells. A) Total ion count traces of the dodecapeptide GGC-MHFHCTQ-C-R containing C885 after FLAG-IP and tryptic digest. Incubating HEK293T cells with compounds (**11a**) and (**11i**) at 30 μ M for 8 h gave significant labelling of overexpressed 3xFLAG-tagged HOIP RBR by compound (**11a**, middle) but not (**11i**, bottom). B) LC-MS/MS analysis of the GGC-MHFHCTQ-C-R peptides from DMSO control (top) and (**11a**)-treated cells (bottom), showing labelling of (**11a**) at C885 (green star). The samples were globally labelled with iodoacetamide (purple asterisk) prior to tryptic digest, and methionine oxidation (yellow asterisk) was taken into account. C) On-bead polyubiquitination assay. In-cell labelling of overexpressed 3xFLAG-tagged HOIP RBR by compound (**11a**) (30 μ M) led to a significant decrease in free polyubiquitin chain formation compared to DMSO and (**11i**) (30 μ M) controls. Non- (-ve), mock or 3xFLAG HOIP RBR C885A transfected HEK293T cells did not show formation of polyubiquitin chains in the polyubiquitin assay. The data is representative of two independent experiments. D) NF- κ B Luciferase reporter and CellTiter-Glo® Luminescent cell viability (Promega) assays. Compound (**11a**) but not saturated analog (**17**) inhibited NF- κ B activity in HEK293T cells overexpressing full-length HOIP, HOIL-1L and SHARPIN in a dose-dependent manner (top), with no effect on cell viability (bottom). The graphs show average data of three independent experiments, each carried out in triplicate. E) Activity-based protein profiling (ABPP) of HEK293T cells overexpressing 3xFLAG-tagged HOIP RBR (wt and C885A mutant). Cells were pre-incubated with DMSO (control and HOIP C885A) or compounds (**11a**), (**11i**) or (**17**) (30 μ M).

for 7 h, then treated with TCO probe (**15**) (3 μ M) for 2 h and lysed. The cell lysates were treated with Cy5-Tz (3 μ M) for 1 h. F) ABPP of HEK293T (overexpressed HOIP RBR) and MCF7 cells (endogenous LUBAC expression), by competition of (**15**) (3 μ M) with (**11a**) (30 μ M), using quantitative MS. Specific labelling (maroon boxes) of HOIP (RNF31, Supplementary datafile) was observed in both cell types. Treatments were carried out in triplicate. For full blots see Supplementary Figures S13, S16.

TRIAD3A and RNF144A (Figure 5C) reveal sequence variation around the active-site cysteine, with Y878, F888 and S899 that contact the compound via their side chains not being conserved. This variation may account for some of the selectivity observed in labelling HOIP over other RBRs. Taken together, these observations suggest that specific molecular interactions between the fragment and protein help to position the electrophile favorably and promote sufficient residence time to support covalent bond formation.

Targeting of HOIP in mammalian cells: To provide further mechanistic characterization of the inhibitors we evaluated HOIP targeting in mammalian cells. For this purpose, we overexpressed the 3 \times FLAG-tagged HOIP RBR domain in HEK293T cells and used the slightly more potent analog (**11a**; Figure 5A). The cells were transiently transfected with 3 \times FLAG HOIP RBR for 48 h, then incubated in media supplemented with inhibitor (**11a**) or inactive analogue (**11i**) at 30 μ M for 8 h before cell lysis. Following immunoprecipitation (IP) of the 3 \times FLAG-HOIP RBR domain with anti-FLAG magnetic beads, the protein was digested on the beads with trypsin. Subsequent LC-MS/MS analysis showed that the HOIP RBR domain was significantly labelled by compound (**11a**) but not (**11i**) at C885, demonstrating that these inhibitors can effectively bind and label HOIP RBR in live cells (Figure 6A, B and Supplementary Figure S12). Furthermore, immunoprecipitated 3 \times FLAG-tagged HOIP RBR (wild type and catalytically inactive C885A mutant) from HEK293T cells was used for an *in vitro* polyubiquitination assay with E1, UbcH7 and ubiquitin, to demonstrate that in-cell labelling of over-expressed HOIP RBR with compound (**11a**) leads to significant loss of the catalytic activity of HOIP, compared to (**11i**) and controls (Figure 6C). Given the central role of LUBAC in canonical NF- κ B signalling,⁸ we then adopted an NF- κ B Luciferase reporter assay to further demonstrate functional effects of HOIP inhibition. Treating HEK293T cells overexpressing full length HOIP, HOIL-1L and SHARPIN with compound (**11a**) overnight led to inhibition of NF- κ B activation in a concentration-dependent manner, with an estimated IC₅₀-value of 37 ± 4 μ M (Figure 6D, top, Supplementary Figure S14). Conversely, saturated analog (**17**) had no effect on NF- κ B activity, and neither compound had any effect on cell viability (Figure 6D, bottom, Supplementary Figure S14). Finally, we assessed the selectivity of compound (**11a**) on a proteome-wide level using activity-based protein profiling (ABPP). ABPP is a powerful technique that utilizes covalent probes as handles for protein visualization or enrichment to evaluate protein engagement in cells,³⁵ an approach that has recently enabled proteome-wide in-cell screening of covalent fragments.³⁶⁻³⁷ We synthesized probe (**15**), based on the structure of (**11a**) and functionalized with a TCO moiety as enrichment handle to enable inverse-electron demand Diels-Alder (IED-DA) click chemistry with tetrazine (Tz); IED-DA reactions typically give superior efficiency and

selectivity for most protein labelling applications in cell lysates and in live cells compared to other types of click chemistries.³⁸⁻³⁹ In brief, HEK293T cells overexpressing 3 \times FLAG-tagged HOIP RBR domain were pre-treated with (**11a**) followed by (**15**) before cell lysis. The impact of pretreatment time and concentration of (**11a**) and (**15**) was evaluated by in-gel fluorescence using Cy5-Tz (Supplementary Figure S15). Pretreating the HEK293T cells with 30 μ M (**11a**) for 7 h, then 3 μ M (**15**) for 2 h, followed by lysis and TCO-Tz click reaction of the lysates gave significant reduction of the in-gel fluorescence of HOIP RBR compared to (**11i**), (**17**), DMSO and transfection controls (Figure 6E). Encouraged by these results, we then used quantitative MS to identify specifically labelled proteins. HEK293T cells overexpressing 3 \times FLAG-tagged HOIP RBR, as well as MCF7 cells endogenously expressing LUBAC were treated with DMSO or (**11a**), then (**15**) as described. Proteins labelled by (**15**) were enriched using Tz-beads,³⁸ treated with trypsin for on-bead digest, and the resulting peptides were analyzed by quantitative MS. To ensure specificity, the residual capturing of every protein was calculated as log₂-fold change competition versus DMSO control (mean ratio), p-values were calculated using student's t-test, and the results plotted so that specifically bound proteins with high p-value and strong fold-change populate the upper left quadrant (maroon boxes, Figure 6F). In addition to HOIP, 11 other proteins in HEK293T cells, and 8 other proteins in MCF7 cells were identified as being specifically covalently labelled by (**11a**). Among these proteins, IRAK4 is a kinase upstream of NF- κ B and therefore labelling by (**11a**) could impact physiological NF- κ B signalling. However, in an IRAK4 substrate phosphorylation assay, compound (**11a**) showed no inhibition at concentrations up to 200 μ M (Supplementary Figure S17), indicating that it is unlikely to affect NF- κ B activation. Considering that no other RBR or HECT E3 ligases were identified as off-targets in the proteomics experiment, and that (**11a**) is an unoptimized fragment hit, the observed cellular selectivity profile is striking. Furthermore, specific labelling of HOIP in MCF7 cells provides the first evidence of targeting endogenous LUBAC in cells, showing that (**11a**) already constitutes a valuable tool compound and an excellent starting point for further optimization.

Conclusion

Despite the crucial biological roles of E3 ubiquitin ligases the development of selective tools that target their activity has been limited to date. Here, we describe how fragment-based covalent ligand screening allowed the rapid identification of inhibitors targeting the active site cysteine of the catalytic HOIP subunit of LUBAC, a member of the RBR family of E3s. A diverse library of fragments linked to α,β -unsaturated ester electrophiles was synthesized and rapidly screened by protein LC-MS to identify compound (**5**) as a covalent binder of HOIP. The established screening protocol required minimal sample handling and enabled expedient screening of multiple proteins. Biochemical

characterization of (**5**) and its analog (**11a**) demonstrated that these compounds label HOIP with promising proteome-wide selectivity and effectively inhibit linear polyubiquitin chain formation *in vitro* and in a cellular environment. The combination of analog synthesis and a high-resolution crystal structure of the HOIP RING2-LDD domain in covalent complex with (**5**) provides the first insight into the molecular requirements for fragment recognition by HOIP and will allow the rational design of inhibitors with increased activity and specificity for this RBR family member. Taken together, our results show the first proof-of-concept for screening and rational design of a covalent inhibitor library designed to target the active site of an RBR E3 ligase, an approach which is generally applicable to other thioester-forming E3 ligases and should help in the search for novel inhibitors of this important class of enzymes.

ASSOCIATED CONTENT

Detailed synthetic procedures for all synthesized compounds, ¹H NMR spectra and LC-MS chromatograms for all library compounds, ¹H, ¹³C NMR, LC-MS and HRMS data for all synthesized hit analogs, physicochemical analyses of the screening library, details of recombinant protein expression and purification, experimental procedures for biochemical assays, TOF LC-MS protocol and protein deconvolution data for LC-MS-based compound screening, protein X-ray crystallography, ABPP and cell based experiment, as well as a supplementary datafile for the quantitative MS-based ABPP experiment. The supporting information is available free of charge on the ACS Publications website.

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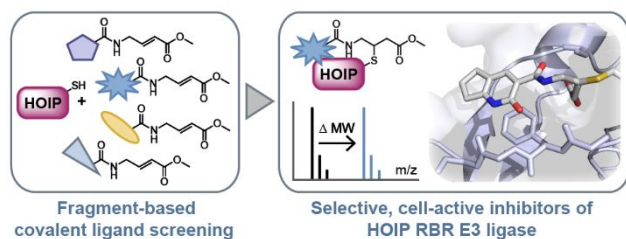
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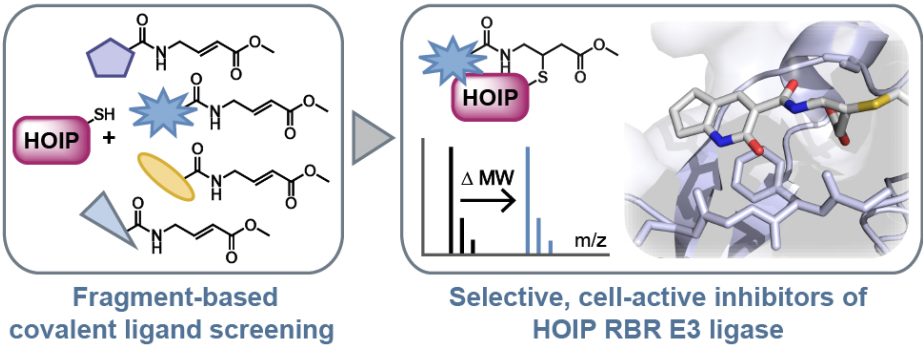
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TOC Graphic





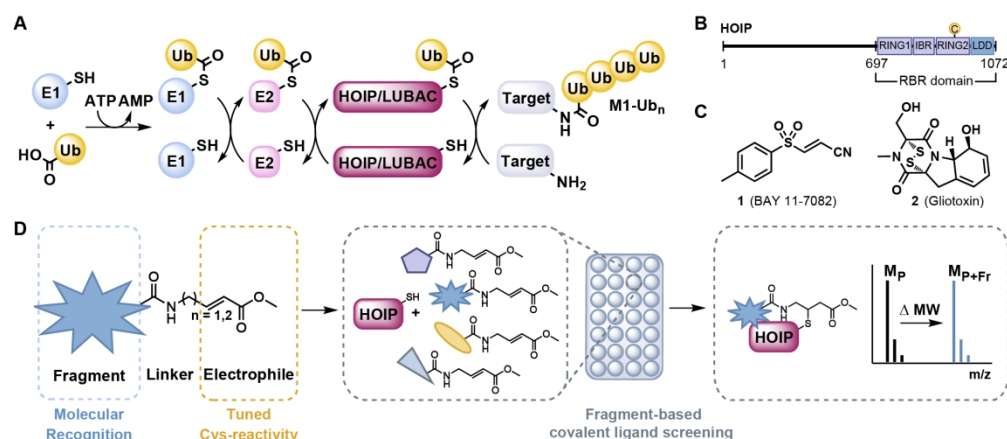


Figure 1 – Targeting HOIP using fragment-based covalent ligand screening. A) Schematic of the ubiquitination cas-cade highlighting the formation of linear (M1) polyubiquitin chains (Ubn) by LUBAC, a reaction that proceeds via a covalent thioester intermediate between the HOIP subunit and ubiquitin. B) Schematic representation of the HOIP E3 ligase, highlighting the RBR which contains the active-site cysteine residue C885 (yellow circle) and the linear chain-determining domain, LDD (amino acids 697- 1072). C) Small molecule LUBAC inhibitors (1) BAY 11-7082 and (2) gliotoxin. D) Overview of our approach to develop covalent probes targeting the active site cysteine residue of HOIP using fragment-based covalent ligand screening by protein LC-MS.

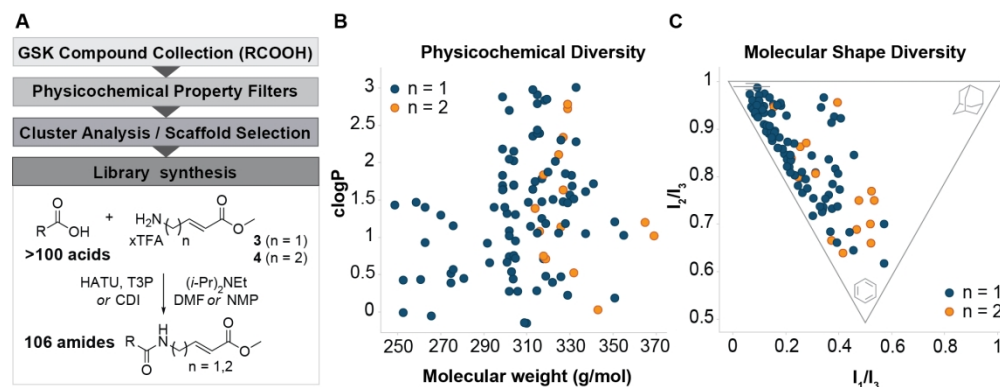


Figure 2 – Design and synthesis of an electrophilic fragment library. A) Parallel synthesis of the electrophilic fragment library from commercial and in-house sourced fragment carboxylic acids and the trifluoro acetic acid (TFA) salts of amines (3) and (4) by amide bond formation, using coupling reagents 1-bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), propylphosphonic anhydride (T3P) or 1,1'-carbonyldiimidazole (CDI). B) The fragment library members clogP plotted against molecular weight illustrates the physicochemical diversity of the library. C) The fragment library members normalized principal moment of inertia (PMI) were calculated in Molecular Operating Environment (MOE)33 based on low energy conformations and plotted as ratios in a triangular graph to illustrate the molecular shape diversity of the library. The vertices of this triangular plot (0,1), (0.5,0.5) and (1,1) represent a perfect rod, disc, and sphere, respectively.

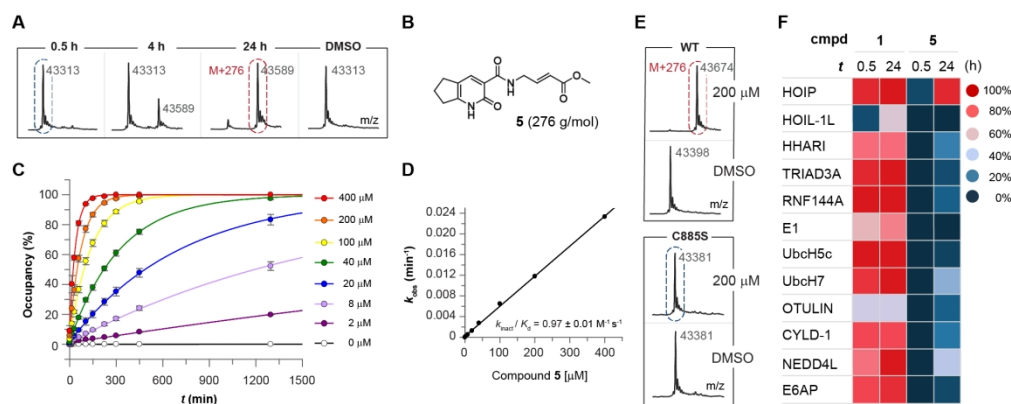


Figure 3 – Compound (5) labels HOIP RBR at the active site cysteine residue C885 in a time- and concentration-dependent manner. A) Compound (5) (20 μ M) labelled HOIP RBR R1032A (2 μ M) over time in the library screen. B) The chemical structure of compound (5). C) Labelling of wild type HOIP RBR (2 μ M) was validated by screening different concentrations of (5). Each time course was normalized to percentage occupancy, fitted to a single exponential equation and plotted in GraFit (v.7) as an average of three independent experiments to determine the observed first-order rate constant (k_{obs}). D) k_{obs} measurements were plotted against the concentration of (5) to give the ratio of k_{obs}/K_d as the slope of the curve.³⁴ E) The active-site C885S mutant of HOIP RBR (2 μ M) was not labelled by compound (5) at 200 μ M after 4 h, suggesting that (5) targets the catalytic cysteine. F) LC-MS based counter screen of compounds (1) (2-fold excess) and (5) (10-fold excess) against a panel of RBR E3 ligases (HOIL-1L, HHARI, TRIAD3A, RNF144A RBR domains), HECT E3 ligases (NEDD4L, E6AP), E2 enzymes (UbcH5c, UbcH7), E1 enzyme and DUBs (OTULIN, CYLD-1). Data shown as percentage labelling after 0.5 and 24 h incubation.

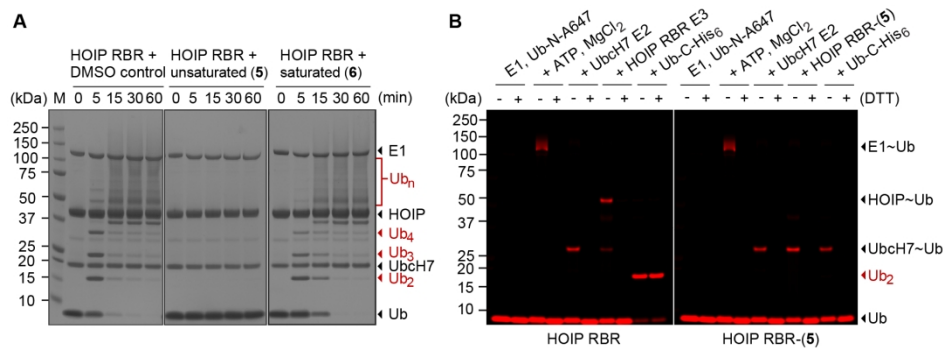


Figure 4 – Compound (5) inhibits the formation of polyubiquitin chains by preventing loading of ubiquitin onto the HOIP RBR domain. A) In vitro polyubiquitination assay monitoring the formation of unanchored polyubiquitin chains. Pre-labelling of the HOIP RBR domain with compound (5) (middle) led to complete inhibition of polyubiquitin chain formation, whereas treatment with the saturated analogue (6) had no effect on catalytic activity. B) In vitro ubiquitin transfer assay monitoring the sequential loading of N-terminally AlexaFluor647-labelled ubiquitin (Ub-N-A647) onto E1, Ubch7, HOIP RBR domain and a C-terminally His-tagged ubiquitin substrate (Ub-C-His6). Pre-labelling of the HOIP RBR domain with compound (5) (right) effectively prevented the loading of ubiquitin onto the E3, thereby inhibiting the formation of di-ubiquitin. For full gels see Supplementary Figures S9 and S11.

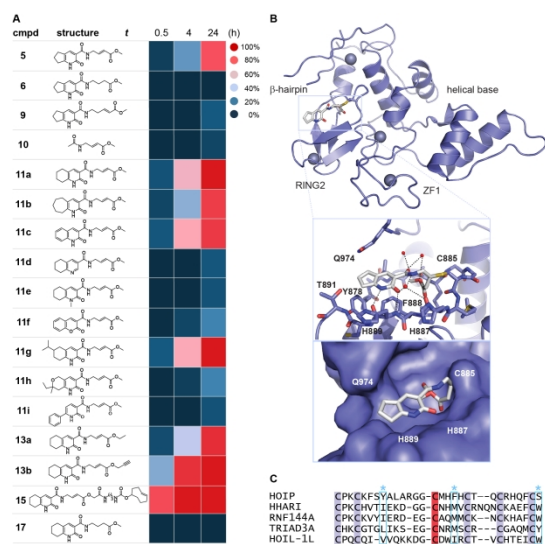


Figure 5 – Molecular determinants for interactions between the HOIP RING2 domain and compound (5). A) LC-MS screening data plotted as percentage labelling of the HOIP RBR domain (2 μ M) by key analogues (6), (9)-(11), (13), (15) (20 μ M), and (17) (200 μ M) after 0.5, 4, and 24 h incubation. For full screening data see Supplementary Figures S7, S8. B) Ribbon representation of the HOIP RING2-LDD domain structure in complex with compound (5) at 2.15 Å resolution (PDB: 6GZY). Zn²⁺ ions are shown as grey spheres and compound (5) as ball and stick. The inset shows a zoom into the active site showing hydrogen bonds (dashed lines) made by compound (5) with water molecules (red spheres) and HOIP (top), and the pocket around the active site which accommodates compound (5) (bottom). C) Sequence alignment of RING2 domains of those RBR E3 ligases that were screened against compound (5). The catalytic cysteine residue is high-lighted in red, non-catalytic cysteine residues in purple, and non-conserved residues contributing to the compound-binding pocket in HOIP are highlighted with a blue asterisk.

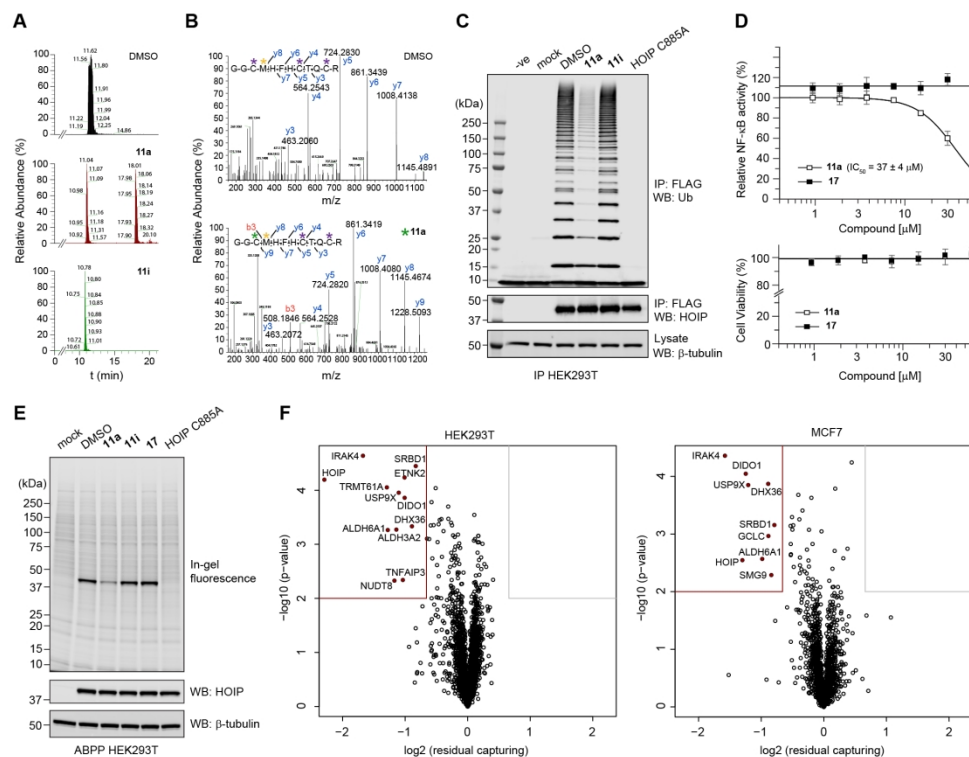
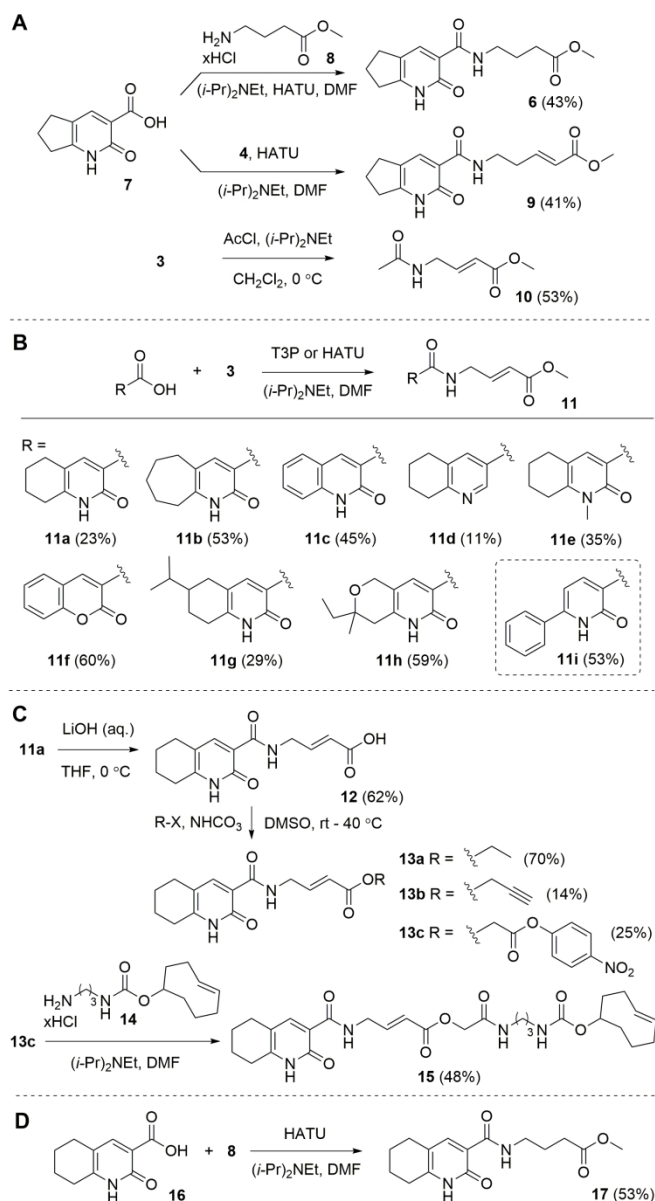


Figure 6 – Compound (11a) labels and inhibits HOIP in HEK293T and MCF7 cells. A) Total ion count traces of the dodecapeptide GGCMMHFHCTQCR containing C885 after FLAG-IP and tryptic digest. Incubating HEK293T cells with compounds (11a) and (11i) at 30 μM for 8 h gave significant labelling of overexpressed 3×FLAG-tagged HOIP RBR by compound (11a, middle) but not (11i, bottom). B) LC-MS/MS analysis of the GGCMMHFHCTQCR peptides from DMSO control (top) and (11a)-treated cells (bottom), showing labelling of (11a) at C885 (green star). The samples were globally Ia-labelled with iodoacetamide (purple asterisk) prior to tryptic digest, and methionine oxidation (yellow asterisk) was taken into account. C) On-bead polyubiquitination assay. In-cell labelling of overexpressed 3×FLAG-tagged HOIP RBR by compound (11a) (30 μM) led to a significant decrease in free polyubiquitin chain formation compared to DMSO and (11i) (30 μM) controls. Non- (-ve), mock or 3×FLAG HOIP RBR C885A transfected HEK293T cells did not show formation of polyubiquitin chains in the polyubiquitin assay. The data is representative of two independent experiments. D) NF-κB Luciferase reporter and CellTiter-Glo Luminescent cell viability (Promega) assays. Compound (11a) but not saturated analog (17) inhibited NF-κB activity in HEK293T cells overexpressing full-length HOIP, HOIL-1L and SHARPIN in a dose-dependent manner (top), with no effect on cell viability (bottom). The graphs show average data of three independent experiments, each done in triplicate. E) Activity-based protein profiling (ABPP) of HEK293T cells overexpressing 3×FLAG-tagged HOIP RBR (wt and C885A mutant). Cells were pre-incubated with DMSO (control and HOIP C885A) or compounds (11a), (11i) or (17) (30 μM) for 7 h, then treated with TCO probe (15) (3 μM) for 2 h and lysed. The cell lysates were treated with Cy5-Tz (3 μM) for 1 h. F) ABPP of HEK293T (overexpressed HOIP RBR) and MCF7 cells (endogenous LUBAC expression), by competition of (15) (3 μM) with (11a) (30 μM), using quantitative MS. Specific labelling (maroon boxes) of HOIP (RNF31, Supplementary datafile) was observed in both cell types. Treatments were done in triplicate. For full blots see Supplementary Figures S13, S16.



Scheme 1 – Analog synthesis. A) Control compounds (6), (9), and (10) were synthesized by amide bond formation using amines (8), (4), and (3) respectively. B) Analogs (11a-i) were synthesized by amide bond formation between the corresponding carboxylic acid and amine (3). Compound (11i) was synthesized as part of the original screening library. C) Ester analogs (13a-c) were synthesized in two steps from ester (11a) by alkali-line hydrolysis and alkylation reactions using the appropriate alkyl halide (ethyl iodide, propargyl bromide and 4-nitrophenyl iodoacetate respectively), and (13c) was further converted into TCO probe (15). D) Saturated control (17) was synthesized from carboxylic acid (16) and amine (8).

149x271mm (300 x 300 DPI)