Discovery of a Potent and Selective Covalent Inhibitor and Activity-Based Probe for the Deubiquitylating Enzyme UCHL1, with Anti-Fibrotic Activity

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Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) is a deubiquitylating enzyme which is proposed as a potential therapeutic target in neurodegeneration, cancer, and liver and lung fibrosis. Herein we report the discovery of the most potent and selective UCHL1 probe (IMP-1710) to date based on a covalent inhibitor scaffold and apply this probe to identify and quantify target proteins in intact human cells. IMP-1710 stereoselectively labels the catalytic cysteine of UCHL1 at low nanomolar concentration in cells, and we show that a previously claimed UCHL1 inhibitor (LDN-57444) fails to engage UCHL1 in cells. We further demonstrate that potent UCHL1 inhibitors block pro-fibrotic responses in a cellular model of idiopathic pulmonary fibrosis, supporting a potential therapeutic role for UCHL1 inhibition and providing a basis for future therapeutic development of selective UCHL1 inhibitors.

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Discovery of a potent and selective covalent inhibitor and activity-based probe for the deubiquitylating enzyme UCHL1, with anti-fibrotic activity

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**ABSTRACT:** Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) is a deubiquitylating enzyme which is proposed as a potential therapeutic target in neurodegeneration, cancer, and liver and lung fibrosis. Herein we report the discovery of the most potent and selective UCHL1 probe (IMP-1710) to date based on a covalent inhibitor scaffold and apply this probe to identify and quantify target proteins in intact human cells. IMP-1710 stereoselectively labels the catalytic cysteine of UCHL1 at low nanomolar concentration in cells, and we show that a previously claimed UCHL1 inhibitor (LDN-57444) fails to engage UCHL1 in cells. We further demonstrate that potent UCHL1 inhibitors block pro-fibrotic responses in a cellular model of idiopathic pulmonary fibrosis, supporting a potential therapeutic role for UCHL1 inhibition and providing a basis for future therapeutic development of selective UCHL1 inhibitors.

Targeting of the ubiquitin (Ub) proteasome system has emerged as a highly promising therapeutic strategy, and deubiquitylating enzymes (DUBs) have attracted increasing interest as drug targets.¹ To study DUB biology and inhibition, Ub-derived activity-based probes (ABPs) have been developed that covalently bind to DUB active sites allowing isolation or profiling in cell lysates.² However, Ub-based probes are non-selective between DUBs and membrane-impermeable, limiting their applications to lysates and highlighting the need for complementary small molecule ABPs to profile DUB activity in intact cells. We recently characterized a cell-permeable small molecule probe for ubiquitin-specific proteases (USPs), alongside an alkyne-permeable ABP analogue that enabled activity-based protein profiling (ABPP) of USP activity for the first time in intact cells.³

Ubiquitin Carboxy-Terminal Hydrolase L1 (UCHL1) belongs to the UCH protease family, which feature a characteristic Cys-His-Asp catalytic triad. Although UCHL1 has well-characterized DUB activity, some studies suggest UCHL1 may also function as a Ub ligase or mono-Ub stabilizer, and its biological functions are not yet fully understood.⁴ UCHL1 is abundantly expressed in the brain where it is involved in apoptosis regulation, learning, and memory, whilst UCHL1 dysregulation is linked to diseases including neurodegeneration,⁴ cancers⁵⁻⁶ and fibrosis.⁷

A screening and hit optimization campaign by Mission Therapeutics identified a series of novel cyanamide-containing UCHL1 inhibitors.⁸ Recognizing the potential of these inhibitors as covalent probes for UCHL1, we selected a potent inhibitor (1; example 27 in reference 8) and designed analogue IMP-1710 (2) bearing an alkyne tag strategically placed in line with established structure-activity relationships (Figure 1a, Figure S1).⁸ The alkyne enables functionalization post-inhibition with ‘capture’ reagents via copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) for analysis.⁹⁻¹⁰ We first examined biochemical UCHL1 inhibition in a fluorescence polarization (FP) assay using Ub-Lys-TAMRA.¹¹ Compound 2 showed improved
UCHL1 inhibition (IC\textsubscript{50} 38 nM, 95% CI 32-45 nM) over parent compound 1, (IC\textsubscript{50} 90 nM, 95% CI 79-100 nM), following 30 min pre-incubation. However, the (R)-enantiomer of 1 (IMP-1711, 3) was >1000-fold less active than 1, demonstrating a highly stereoselective interaction with UCHL1 and providing a useful negative control (Figure S1). We further characterized inhibition kinetics by 1 and 2 through determination of \( k_{obs}/1 \) (7400 (95% CI 5200-9700) 11000 (95% CI 7700-13000) M\textsuperscript{-1}s\textsuperscript{-1}, respectively, Figure. S2), and slow recovery of activity following dilution demonstrated these inhibitors are slowly reversible, in line with previous reports of this warhead class (Figure. S2).\textsuperscript{12} Cross-screening against a panel of 20 DUBs demonstrated that compounds 1 and 2 exhibit exquisite selectivity for UCHL1 (Figure 1b), whereas control compound 3 was inactive against all tested DUBs (Figure S1). Cellular UCHL1 activity was demonstrated in breast cancer cells (Cal51) stably expressing FLAG-UCHL1 using a Ub-vinyl methyl ester probe (HA-Ub-VME) in a homogeneous time resolved fluorescence (HTRF) assay.\textsuperscript{13} Compounds 1 and 2 effectively engaged UCHL1, with in-cell IC\textsubscript{50} values of 820 and 110 nM, respectively (Figure S3). Concentration-dependent competition by 1 and 2 for selective UCHL1 HA-Ub-VME labeling was confirmed through immunoblot analysis (Figure 1c, S4). Potencies were in line with biochemical data, whilst compound 3 did not inhibit UCHL1 in cells, consistent with previous results (Figure S1).

We next compared our probes to the most potent literature UCHL1 inhibitor, isatin O-acyl oxime LDN-57444 (Figure 1a). LDN-57444 has been reported as a reversible, Ub-competitive UCHL1 inhibitor with IC\textsubscript{50} 880 nM against recombinant UCHL1,\textsuperscript{14} and has been widely used as a tool inhibitor in UCHL1 studies in disease models.\textsuperscript{6-7, 15-16} However, engagement of UCHL1 in intact cells has not previously been demonstrated. We profiled LDN-57444 in a range of assays, including biochemical activity and capacity to bind UCHL1 in intact cells (Figure S1, S3, S4), and were surprised to discover that LDN-57444 failed to engage UCHL1 in any assay (IC\textsubscript{50} >300 \mu M) or inhibit any DUBs tested (Table S1, Figure S1), effectively invalidating this compound as a DUB inhibitor.

Direct target engagement using 2 was examined in HEK293 cells treated with compound for 60 min, followed by lysis and ligation to azide-TAMRA-biotin capture reagent (AzTB, Figure S5) through CuAAC (Figure 2a).\textsuperscript{9} In-gel fluorescence revealed a single major target at \~25 kDa, labeled in a concentration-dependent manner. Labeling appeared complete at 125 nM 2, whilst concentrations >500 nM suggested some labeling of other proteins (Figure 2b). Labeling was confirmed by biotin pulldown and immunoblotting, with UCHL1 significantly enriched at 30 nM 2, and maximal at 125 nM, with minimal or no detectable labeling of UCHL3, UCHL5 and DUBs from other families (Figure 2c, Figure S6). Time course experiments demonstrated maximal labeling within 60 min (Figure S7), and 2 remained stable in media for >72 h without loss of activity (Figure S8). Incubation of recombinant UCHL1
(5 μM) with 1 or 2 (12.5 μM) for 60 min led to single modification of UCHL1 by LC-ESI-MS (Figure 3a). Modification specifically at the UCHL1 catalytic cysteine (Cys90) was confirmed by tryptic digest and nanoLC-MS/MS (Figure S9, Supplementary Data S1). To confirm the catalytic Cys requirement for modification in intact cells, we overexpressed FLAG-tagged wild type (WT) UCHL1, or C90A or C90S mutants in HeLa cells, which lack endogenous UCHL1, and treated with 2. In-gel fluorescence and immunoblot analyses demonstrated that only WT UCHL1 is labeled by 2, confirming dependence on UCHL1 catalytic activity for cellular target engagement (Figure 3b, Figure S10). Taken together, these data demonstrate 2 is a bona fide UCHL1 ABP that rapidly targets endogenous UCHL1 in cells in a strictly activity-dependent manner.

To determine selectivity of 2 across the proteome, unbiased quantitative chemical proteomic profiling (Figure 2a) was employed in HEK293 cells treated with 2 (2, 20 or 200 nM) or vehicle (DMSO) control, for 10, 60 or 180 min. Cell lysates were ligated by CuAAC to azide-arginine-biotin (AzRB, Figure S5) capture reagent, and labeled proteins enriched on dimethylated NeutrAvidin-agarose beads. Proteins were digested on-resin with LysC followed by trypsinization, labeled with tandem mass tags (TMT) to enable relative quantification between conditions, and samples analyzed by nanoLC-MS/MS. UCHL1 was significantly enriched by 2 in a concentration-dependent manner, with marginal enrichment of UCHL3 at higher concentrations and no significant enrichment of any other DUB (Figure 2d, Figure S11); UCHL1 enrichment was similar at all time points, consistent with previous results (Figure S7). Only two proteins, UCHL1 and fibroblast growth factor receptor 2 (FGFR2), were significantly enriched at 20 nM ABP 2 treatment, with UCHL1 by far the major target (Figure S12, Supplementary Data 2). No significant change protein abundance was detected in whole proteome analysis following ABP 2 treatment (Figure S13, Supplementary data 3), suggesting that 2 does not substantially alter global protein homeostasis. In-gel fluorescence and immunoblot analysis further confirmed ABP 2 can profile activity of endogenous UCHL1 across different cell types, including endothelial cells (EA.hy926) and adenocarcinoma human alveolar basal epithelial cells (A549) with excellent selectivity (Figure S14).

To identify selective targets of parent compound 1 and differentiate between selective labeling and non-specific pulldown, competitive ABPP was performed. HEK293 cells were treated with 1, 3 or LDN-57444 for 1 h, followed by 2 (20 nM) for 10 min. Immunoblot analysis demonstrated dose-dependent UCHL1 labeling reduction with parent compound 1 at nanomolar concentrations, whereas no competition was observed with control compound 3 or LDN-57444, confirming 3 as an effective negative control and LDN-57444 as inactive against UCHL1 in cells (Figure 4a-b). In-cell proteome-wide competitive ABPP was performed by quantitative chemical proteomics, showing that across the whole proteome UCHL1
Responds strongly to 1 in a concentration-dependent manner, but does not respond with inactive control 3 or LDN-57444 (Figure 4c, Figure S15, Supplementary Data 4). UCHL3 shows a small response to 1, whilst outside the DUB family FGFR2 responds (Figure S15), suggesting these proteins are possible minor off-targets of 1.

UCHL1 has recently been reported as an emerging therapeutic target in development of fibrosis.7, 21 Pharmacological UCHL1 inhibition was therefore investigated in primary human lung cells derived from idiopathic pulmonary fibrosis (IPF) patients. Fibroblast-to-myofibroblast transition (FMT) was stimulated by transforming growth factor beta 1 (TGF-β1) using alpha-smooth muscle actin (αSMA) as a disease-relevant marker for transition (Figure 5a).22-23 αSMA production was validated by high content imaging analysis (HCA) of three donor cell lines in response to TGF-β1 stimulation, alongside response to 1 h pre-treatment with TGF-β1 receptor kinase inhibitor SB525334 (1 µM).24 FMT was consistently induced by TGF-β1 and inhibited by SB525334 in all donor fibroblasts, with strong Spearman correlation between biological replicates (Figure S16). Donor cells were treated with compounds 1, 2, 3, LDN-57444 or FDA-approved IPF drug nintedanib,25 and response to TGF-β1 measured after 3 days by staining and HCA quantification of αSMA and nuclei (DAPI). Compounds 1 and 2 (1 µM) demonstrated >50% FMT inhibition (IC_{50} 15 nM and 410 nM, respectively), with comparable potency to nintedanib (Figure 5b). Nuclear count was stable <5 µM compound, providing a therapeutic window between inhibition and cytotoxicity (Figure S17). Compound 3 showed reduced αSMA inhibition compared to 1 and 2, with similar cytotoxicity to 2. Although LDN-57444 showed evidence for weak αSMA inhibition, this was concurrent with increased cytotoxicity over the same concentration range, suggesting that LDN-57444 toxicity drives decreased αSMA (Figure S18).

In summary, we report discovery and characterization of the most potent and selective small-molecule DUB ABP (2) to date, enabling robust detection of UCHL1 activity in living cells at low nanomolar concentrations. The only previously reported UCHL1 ABP is >150-fold less potent than 2 and has multiple significant off-targets,12 with no selectivity over UCHL3. UCHL1 labeling by 2 is strictly activity-dependent, occurring only at the catalytic cysteine, and providing a new chemical tool to examine UCHL1 activity in various intact cell types. ABPP demonstrated that parent compound 1 is a potent UCHL1 inhibitor that targets the active site cysteine residue with impressive selectivity in cells, and that UCHL1 inhibition is highly stereoselective providing an ideal control compound (3) for future studies. Further, evidence from multiple assays (biochemical, cellular, proteomics) demonstrates that, contrary to previous reports, LDN-57444 does not engage UCHL1, which invalidates this compound as a tool inhibitor and suggests that previously reported studies using LDN-57444 may be subject to reinterpretation.14, 26 Finally, we show that 1 and 2, but not the inactive enantiomer 3, can
completely suppress fibrotic phenotypes in IPF cellular models without substantial cytotoxicity, highlighting the potential of UCHL1 inhibitors for treatment of fibrosis. 1 and 2 therefore represent powerful and selective probes to explore UCHL1 activity with potential application to substrate identification, mode of action studies, and cellular target profiling, which can accelerate future development of UCHL1 inhibitors as potential fibrosis therapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXXXXXX.

Additional results and materials and methods for protein production, biochemical assay, cell-based experiment, chemical proteomic sample preparation and data analysis, FMT assay. Detailed chemical synthesis for all synthesized compounds, 1H, 13C NMR spectra and HRMS data (PDF).

Datafiles of protein identification and quantification from the proteomic experiments (XLSX)
Supplementary Data S1: Peptide lists of compound-modified peptide identification
Supplementary Data S2: Protein lists of ABP 2 target engagement
Supplementary Data S3: Protein lists of whole proteome analysis
Supplementary Data S4: Protein lists of competitive ABPP

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository27 with the dataset identifier PXD015825

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Author Contributions

N.P. and A.G. undertook experiments, analyzed data and wrote the initial draft of the manuscript. T.L.-H. contributed to enzyme expression, assay data analysis and supervised experiments. S.L.-O. contributed to enzyme expression and assay data. E.W. and C.S. contributed to synthesis of compounds. K.M. performed biochemical screening assays, S.E. performed cellular activity probe assays, L.M.S. analyzed data and supervised experiments, and J.A.H generated UCHL1 expression constructs and supervised experiments. E.W.T. conceived and supervised the study. All authors contributed to the final version of the manuscript.

Notes

K.M., S.E., L.M.S., and J.A.H. are employees of Mission Therapeutics Ltd; E.W.T. is a Director and shareholder in Myricx Pharma Ltd.

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Figure 1. Specificity of UCHL1 inhibitor (1), alkyne ABP (IMP-1710, 2), control compound (IMP-1711, 3) and LDN-57444. (a) Inhibitor structures and UCHL1 IC₅₀ values (Ub-Lys-TAMRA FP assay). Maximum assay concentration. (b) Selectivity profiling of 1 and 2 against DUBs in FP and FI assays (Ub-Lys-TAMRA and Ub-Rho110, respectively). See Supplementary Information for 3 and LDN-57444 profiling. Data represent mean ± SEM (n=2). (c) Immunoblot analysis of HA-Ub-VME UCHL1 labeling following HEK293T treatment with 1, 2 or 3 for 1 h. Dose-dependent decrease in UCHL1 labeling occurs with increasing active compounds, but not with inactive enantiomer 3.
Figure 2. UCHL1 activity profiling using ABP 2 in HEK293 cells. (a) Chemical proteomics workflow. Cells were incubated with ABP 2, and labeled proteins ligated via CuAAC to AzTB for in-gel fluorescence and/or affinity enrichment for immunoblotting and proteomic profiling. (b) In-gel fluorescence shows dose-dependent labeling by ABP 2. (c) Probe-labeled protein identification by enrichment and immunoblotting. (d) Quantitative proteomic analysis of selected DUBs by ABP 2, demonstrating dose-dependent enrichment of UCHL1. Data represent mean ± SEM (n=3).
Figure 3. UCHL1 labeling with 1 and 2 exclusively at catalytic Cys90 in vitro and in intact cells. (a) LC-ESI demonstrating single UCHL1 covalent modification with 1 or 2. (b) HeLa cells lacking endogenous UCHL1, transfected to express UCHL1 wild type (WT) or catalytic cysteine mutants (C90A and C90S); pulldown following treatment with 2 and AzTB functionalization confirms Cys90 specific labeling.
Figure 4. Compound 1 selectively competes ABP 2, whilst 3 and LDN-57444 do not. Competition for ABP 2 labeling was confirmed by: (a) in-gel fluorescence; (b) pulldown and UCHL1 immunoblotting; (c) pulldown and whole proteome quantitative proteomic analysis. Data represent mean ± SEM (n=3).
Figure 5. Phenotypic effects of selective UCHL1 inhibition in idiopathic pulmonary fibrosis (IPF). (a) Schematic of TGF-β1-mediated fibroblast-to-myofibroblast transition in primary human lung fibroblasts, increasing alpha-smooth muscle actin (αSMA) transdifferentiation marker. (b) Primary fibroblasts from IPF donors were pre-incubated with 1 μM 1, 2, 3, LDN-57444, or IPF approved drug (nintedanib) for 1 h followed by TGF-β1 treatment for 3 days. αSMA expression was analyzed by high content imaging, demonstrating 1, 2, and Nintedanib, but not 3 or LDN-57444 inhibit transdifferentiation (N.S. (non-significant), *P≤0.05, ***P≤0.01). Plots represent median values (center lines) and 25th/75th percentiles (box limits) with Tukey whiskers.
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Supplementary Information

Discovery of a potent and selective covalent inhibitor and activity-based probe for the deubiquitylating enzyme UCHL1, with anti-fibrotic activity

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(a) Biochemical enzyme assay. UCHL1 activity was measured via FP with Ub-Lys-TAMRA. UCHL1 (5 nM) was incubated with 8-point dilutions of test compounds for 30 min, and measurement initiated by adding Ub-Lys-TAMRA (50 nM). Data represents mean ± SEM (n=3). IC$_{50}$ and 95% confidence intervals (95% CI) were calculated from a four-parameter dose-response curve.

(b) Biochemical selectivity profiling of compound 3 and LDN-57444 at 1 µM using FP and FI assays. For each DUB enzyme, the substrate and enzyme concentration were optimized, as described in Materials and Methods. Data represent mean ± SEM of assay in duplicate (n=1-3).

| Compound   | FP IC$_{50}$ (95% CI) |
|------------|-----------------------|
| 1          | 90 (79-100) nM        |
| 2          | 38 (32-45) nM         |
| 3          | >100 µM*              |
| LDN-57444  | >300 µM*              |

*Maximum concentration used in the assay

Figure S1. Biochemical enzyme assay. (a) UCHL1 activity was measured via FP with Ub-Lys-TAMRA. UCHL1 (5 nM) was incubated with 8-point dilutions of test compounds for 30 min, and measurement initiated by adding Ub-Lys-TAMRA (50 nM). Data represents mean ± SEM (n=3). IC$_{50}$ and 95% confidence intervals (95% CI) were calculated from a four-parameter dose-response curve. (b) Biochemical selectivity profiling of compound 3 and LDN-57444 at 1 µM using FP and FI assays. For each DUB enzyme, the substrate and enzyme concentration were optimized, as described in Materials and Methods. Data represent mean ± SEM of assay in duplicate (n=1-3).
Figure S2. UCHL1 kinetics
(a) Kinetic analysis of UCHL1 (3 nM)-catalyzed cleavage of varied concentrations of Ub-AMC. (b) Non-linear fit of Michaelis–Menten equation to initial reaction velocities versus Ub-AMC concentrations, giving $K_m$ of 220 nM (95% CI 140-310 nM). (c) Apparent rate of inhibition ($k_a$) of compound 1 and 2 at varying concentrations. Ub-AMC (130 nM) fluorescence intensity was measured in the presence of UCHL1 (3 nM). The apparent rate constant for inhibition, $k_a/I$ was determined by linear regression, giving the absolute value of the rate constant $k_{obs}/I$ values for compounds 1 and 2 of 7400 (95% CI 5200-9700) M$^-1$s$^-1$ and 11000 (95% CI 7700-13000) M$^-1$s$^-1$, respectively. (d) Reaction progression curves for Ub-AMC cleavage after 100x dilution of UCHL1 (300 nM) treated with DMSO (positive control), compound 1, 2, LDN-57444 and iodoacetamide (IAA, negative control).
Figure S3. Compound characterization in Cal51 cells.
In-cell UCHL1 activity profiling of 1, 2, 3 and LDN-57444 by HTRF. Cal51 cells overexpressing UCHL1 were treated with compounds 1, 2, 3 or LDN-57444 for 1 h at 37°C before lysis. Lysates were combined with HA-Ub-VME (100 nM) and incubated for 30 min at room temperature. Detection antibodies were added to the reaction plate and incubated overnight at 4°C before measurement. HTRF ratios were calculated as described in Materials and Methods. Data represents mean ± SEM (n=4). IC_{50} and 95% CI values were calculated from a four-parameter dose-response curve.

| Compound     | HTRF IC_{50} (95% CI)   |
|--------------|-------------------------|
| 1            | 820 (500-1100) nM       |
| 2            | 110 (86-130) nM         |
| 3            | 23 (18-29) µM*          |
| LDN-57444    | >30 µM*                 |

*Maximum concentration used in the assay
Figure S4. In-cell target engagement of 1, 2, 3 and LDN-57444 using HA-Ub-VME ABP. Immunoblot analysis of HEK293T cells incubated with different concentrations of 1, 2, 3 and LDN-57444 for 1 h. The compound-treated lysates were labeled with HA-Ub-VME ABP for 15 min, separated by SDS-PAGE and probe-labeled proteins visualized by immunoblotting using the indicated antibodies.
**Figure S5. Structures of ‘capture’ reagents.**
Structure of azido-TAMRA-biotin (AzTB) and azido-arginine-biotin (AzRB). Capture reagents contain key functional moieties: an azide (red) for copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) reaction, a TAMRA fluorophore (pink) for in-gel fluorescence visualization, a biotin affinity handle (blue) for enrichment and purification, and an arginine residue (green) for on-bead trypsin digestion.
Figure S6. Immunoblot analysis of ABP 2 labeling in HEK293 cells.
HEK293 cells were incubated with 1.9, 7.8, 31, 130, 500 or 2000 nM of 2 for 1 h then lysed. The lysate was ligated to AzTB capture reagent and labeled proteins were enriched on Streptavidin magnetic beads. The total lysate (TL), pulldown (PD), and supernatant (SN) samples were separated by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies.
a) In-gel fluorescence analysis of ABP 2 labeling.

| Incubation time | 60 min | 180 min |
|-----------------|--------|---------|
| [2] (nM)        |        |         |
| 0               | 1.9    | 0       |
| 1.9             | 7.8    | 1.9     |
| 7.8             | 31.3   | 7.8     |
| 31.3            | 125    | 31.3    |
| 125             | 500    | 125     |
| 500             |        | 500     |

Figure S7. In-gel fluorescence analysis of ABP 2 labeling.
(a) HEK293 cells were incubated with 1.9, 7.8, 31, 130 or 500 nM of ABP 2 for 1 or 3 h. The lysate was ligated to AzTB capture reagent, separated by SDS-PAGE and visualized by in-gel fluorescence. (b) The labeling intensity on the gel was quantified using ImageJ and plotted against 2 concentration, demonstrating little further labeling after 1 h. Values were background corrected to DMSO vehicle control.
Figure S8. In-gel fluorescence analysis of ABP 2 stability.
Probe stability was assessed by measuring incorporation via in-gel fluorescence after incubation in cell culture medium. ABP 2 was incubated in medium for 2, 6, 24, 48 or 72 h. Probe-containing medium was then transferred to HEK293 cells and further incubated for 1 h, flowed by lysis, AzTB functionalization and in-gel fluorescence analysis. Protein loading was assessed by Coomassie Blue staining.
a) 1 modification: 82% peptide sequence coverage

Oxidation (M), +15.99
Carbamidamethylation (C), +57.02
NP_Cys-Compound 1 (C), +357.16
b) 2 modification: 96% peptide sequence coverage

Figure S9. Identification of modification sites by nanoLC-MS/MS. Recombinant UCHL1 (1 equiv.) was incubated with compound 1 or 2 (2.5 equiv.) for 1 h at room temperature. The labeled protein was digested with trypsin and analyzed by nanoLC-MS/MS. The data was processed in PEAKS 8.0. 82% and 96% of UCHL1 peptide sequence coverage for (a) 1, or (b) 2 modification, respectively. The high confidence modification of compound 1 or 2 was observed on catalytic cysteine 90(*). Statistical data and peptide lists from PEAKS are detailed in Supplementary Data S1.
Figure S10. In cell UCHL1 modification site identification.
HeLa cells overexpressing FLAG-UCHL1 (WT, C90A or C90S) were incubated with 20 or 200 nM of 2 for 1 h. (a) Lysates were functionalized with AzTB, labeled proteins separated by SDS-PAGE and visualized by in-gel fluorescence. (b) The labeled proteins were enriched using Streptavidin magnetic beads. The total lysate (TL) and pulldown (PD) were separated by SDS-PAGE and analyzed by immumoblotting using α-FLAG antibody.
Figure S11. ABP 2 shows concentration- and time-dependent DUB incorporation. HEK293 cells were treated with ABP 2 (20, 200 nM) or vehicle (DMSO) for 10, 60 or 180 min. Following CuAAC ligation to the AzRB cleavable capture reagent, probe-labeled proteins were enriched on dimethylated NeutrAvidin agarose beads. Enriched proteins were digested on-bead with LysC followed by in-solution tryptic digestion. Peptides were labeled with 10-plex tandem mass tag (TMT) reagents, desalted and analyzed by nanoLC-MS/MS. Data was processed in MaxQuant and analyzed in Perseus as described in Materials and Methods. Data represent mean ± SEM (n=3). Selected DUBs were normalised to DMSO controls and compared side-by-side. (a-c) Concentration-dependent incorporation of 2; (d-e) Time-dependent incorporation of 2; (f) DUBs identified in this experiment in HEK293 cells. The protein lists from MaxQuant and Perseus analysis are detailed in Supplementary Data S2.
Figure S12. Target profiling of ABP 2 in HEK293 cells.
Volcano plots showing the log₂ difference (fold change) and significance (-log₁₀ p-value) between protein enrichment at different concentrations of ABP 2 ((a) 2 nM, (b) 20 nM and (c) 200 nM) compared to DMSO control (two sample t-test, n=3, Permutation-based FDR=0.01, S0=1). Data indicates concentration-dependent UCHL1 incorporation with minimal off-targets. Protein lists from MaxQuant and Perseus analysis are detailed in Supplementary Data S2.
Figure S13. ABP 2 does not affect global protein levels.
HEK293 cells were treated with ABP 2 ((a) 20 nM, (b) 200 nM or (c) 2000 nM) or vehicle (DMSO) for 60 min. Following cell lysis, reduction and alkylation, the samples were tryptic digested and labeled with 10-plex tandem mass tag (TMT) reagents, desalted and analyzed by nanoLC-MS/MS. Data was processed in MaxQuant and analyzed in Perseus as described in Materials and Methods. Volcano plots showing the \( \log_2 \) difference (fold change) and significance (-\( \log_{10} \) p-value) between protein enrichment at different concentration of ABP 2 (20, 200 or 2000 nM) and DMSO control (two sample t-test, n=3, Permutation-based FDR=0.01, S0=1). ABP 2 treatment does not affect protein abundance across the whole proteome. The protein lists from MaxQuant and Perseus analysis are detailed in Supplementary Data S3.
**Figure S14. ABP 2 target engagement in A549 and EA.hy926 cells.**

EA.hy926 and A549 cells were incubated with 1.9, 7.8, 31, 130 or 500 nM ABP 2 for 3 h, and labeled proteins ligated to AzTB capture reagent. (a) Labeled proteins were separated by SDS-PAGE and visualised by in-gel fluorescence. (b) Labeled proteins were enriched by NeutrAvidin magnetic beads. Total lysate (TL), supernatant (SN) and pulldown (PD) samples were separated by SDS-PAGE and analyzed by immunoblotting using anti-UCHL1 antibody.
Figure S15. Competitive activity-based proteome profiling.
Competitive activity-based proteome profiling of ABP 2 with UCHL1 inhibitor 1, control compound 3 or LDN-57444 competition using quantitative proteomics. HEK293 were incubated with (a) vehicle (DMSO), compound (b, c) 1 (1, 4 µM), (d, e) 3 (1, 4 µM) or (f, g) LDN-57444 (5, 25 µM) for 60 min and further incubated with ABP 2 (20 nM) for 10 min. Following CuAAC ligation to AzRB cleavable capture reagent, probe-labeled proteins were enriched on dimethylated NeutrAvidin agarose beads and on-bead digested with LysC followed by in solution trypptic digestion. Peptides were labeled with 10-plex TMT reagents and
analyzed by nanoLC-MS/MS. Data was processed in MaxQuant and analyzed in Perseus as described in Materials and Methods n. Volcano plots show log₂ difference (fold change) and significance (-log₁₀ p-value) of inhibitor-treated samples versus non-treated samples (two sample t-test, n=3, permutation-based FDR=0.01, S0=1). The protein lists from MaxQuant and Perseus analysis are detailed in Supplementary Data S4.
Figure S16. Quality control of fibroblast-to-myofibroblast transition (FMT) assay.
Quality control of FMT assay in human lung cells derived from idiopathic pulmonary fibrosis (IPF) patients. Patient-derived primary human bronchial fibroblasts from three donors were FMT triggered by 1.25 ng/mL TGF-β1 in the presence or absence of positive control inhibitor (1 µM SB525334). After 3 days incubation, cells were fixed and stained using αSMA for imaging using high content analysis (HCA). αSMA immunoreactivity was quantified by an HCA algorithm with density x area output. A Kruskal–Wallis test with Dunn’s post-test was performed (***, P ≤ 0.01). Plots represent median values (center lines) and 25th/75th percentiles (box limits) with Tukey whiskers.
Figure S17. Effect of UCHL1 inhibitors on fibrotic activity and cell viability.
Patient-derived primary human bronchial fibroblasts from three donors were FMT trigged by 1.25 ng/mL TGF-β1 in combination with 8-point serial dilution of (a) reference compound nintedanib, compound (b) 1, (c) 2, (d) 3 or (e) LDN-57444. After 3 days incubation, the cells were fixed and stained using αSMA and DAPI staining and imaged using HCA. Data represent mean ± SEM (n=6) of percentage αSMA inhibition (blue) and percentage remaining cells (red). αSMA IC₅₀ and remaining cells EC₅₀ values were calculated from a four-parameter dose-response curve.
Figure S18. Uncropped gels/blots

Figure 1
\( \alpha \)-UCHL1

| Compound 1 treatment | Compound 2 treatment |
|----------------------|----------------------|
| ![Image](image1.png)  | ![Image](image2.png)  |
| ![Image](image3.png)  | ![Image](image4.png)  |

Figure 2
In-Gel Florencecence

| ABP 2 labelling | ABP 2 labelling |
|-----------------|-----------------|
| ![Image](image5.png)  | ![Image](image6.png)  |

Coomassie

| ![Image](image7.png)  |

Figure 3
\( \alpha \)-UCHL1 (PD)

| \( \alpha \)-UCHL1 (PD) | \( \alpha \)-UCHL1 (TL) | \( \alpha \)-HSP90 |
|------------------------|------------------------|-----------------|
| ![Image](image8.png)  | ![Image](image9.png)  | ![Image](image10.png)  |
Figure 4
In-Gel Fluorescence
Coomassie

α-UCHL1 (PD)

α-UCHL1 (TL)

Figure S4
Compound 1 treatment

α-UCHL3

α-UCHL5

α-BAP1

α-HSP90

Compound 2 treatment

α-UCHL3

α-UCHL5

α-BAP1

α-HSP90
Figure S4 (cont.)

Compound 3 treatment

α-UCHL3

α-UCHL5

LDN-57444 treatment

α-UCHL1

α-HSP90

Figure S6

α-BAP1

α-USP7

α-USP14

α-HSP90

α-β-ACTIN
Figure S14

EA.hy926 In-Gel Florecence

Coomassie

A549 In-Gel Florecence

Coomassie

α-UCHL1

EA.hy926

A549
Supplementary Tables

Ranges:
- **A** < 0.1 µM
- 0.1 < **B** < 1 µM
- 1 < **C** < 10 µM
- **D** > 10 µM

| DUBs   | Biochemical IC$_{50}$ range |
|--------|----------------------------|
|        | 1  | 2  | 3  | LDN-57444 |
| USP1   | D  | D  | D  | D          |
| USP4   | D  | D  | D  | D          |
| USP8   | D  | D  | D  | D          |
| USP10  | D  | D  | D  | D          |
| USP16  | D  | D  | NA | D          |
| USP19  | D  | D  | D  | D          |
| USP21  | D  | D  | D  | D          |
| USP22  | D  | D  | D  | D          |
| USP25  | D  | D  | D  | D          |
| USP28  | D  | D  | NA | D          |
| USP30  | C  | C  | D  | D          |
| USP34  | C  | C  | D  | D          |
| USP35  | D  | D  | D  | D          |
| BAP1   | C  | C  | D  | D          |
| **UCHL1** | **A** | **A** | **D** | **D** |
| UCHL3  | C  | C  | D  | D          |
| UCHL5  | D  | D  | D  | D          |
| Cezanne1 | D  | D  | D  | D          |
| OTUB2  | D  | D  | D  | D          |
| TRABID | D  | D  | D  | D          |

Table S1. Activity profiling of 1, 2, 3 and LDN-57444 against 20 DUBs. Biochemical IC$_{50}$ values were measured by fluorescence polarization (FP) and fluorescence intensity (FI) assays using Ub-Lys-TAMRA, Ub-FANCD2-TAMRA, and Ub-Rho110 substrates (NA=Not assessed).
| Target          | Species | Dilution | Catalogue no. | Supplier                  |
|-----------------|---------|----------|---------------|---------------------------|
| BAP1            | Mouse   | 1:500    | sc-28383      | Santa cruz               |
| FLAG, HRP       | Mouse   | 1:1000   | A8592         | Sigma-Aldrich            |
| HA-tag (C29F4)  | Rabbit  | 1:1000   | 3724          | Cell Signaling Technology|
| HSP90           | Mouse   | 1:1000   | sc-69703      | Santa Cruz Biotechnology |
| UCHL1/PGP9.5    | Rabbit  | 1:2000   | 14730-1-AP    | ProteinTech              |
| UCHL3           | Mouse   | 1:1000   | sc-100340     | Santa Cruz               |
| UCHL5/UCH37     | Rabbit  | 1:1000   | ab124931      | Abcam                     |
| USP14           | Rabbit  | 1:2000   | 11931S        | Cell Signalling          |
| USP7/HAUSP      | Rabbit  | 1:1000   | ab4080        | Abcam                     |
| β-ACTIN         | Rabbit  | 1:5000   | Ab8227        | Abcam                     |
| Mouse IgG, HRP  | Mouse   | 1:10000  | R-05071-500   | Advansta                  |
| Rabbit IgG, HRP | Rabbit  | 1:10000  | R-05072-500   | Advansta                  |

**Table S2. Primary and secondary antibodies used.**

Primary and secondary antibodies were used at indicated concentrations for immunoblotting as described in Materials and Methods.
| DUB     | Assay Buffer | Substrate          | Substrate Concentration (nM) |
|---------|--------------|--------------------|------------------------------|
| BAP1    | A            | Ub-Lys-TAMRA       | 50                           |
| Cezanne1| B            | Ub-Lys-TAMRA       | 50                           |
| OTUB2   | B            | Ub-Lys-TAMRA       | 50                           |
| TRABID  | B            | Ub-Rho110          | 400                          |
| UCHL1   | A            | Ub-Lys-TAMRA       | 50                           |
| UCHL3   | A            | Ub-Lys-TAMRA       | 50                           |
| UCHL5   | A            | Ub-Lys-TAMRA       | 50                           |
| USP1    | A            | Ub-FANCD2-TAMRA    | 50                           |
| USP4    | A            | Ub-Lys-TAMRA       | 50                           |
| USP8    | B            | Ub-Lys-TAMRA       | 50                           |
| USP10   | B            | Ub-Rho110          | 400                          |
| USP16   | B            | Ub-Lys-TAMRA       | 50                           |
| USP19   | A            | Ub-Lys-TAMRA       | 50                           |
| USP21   | A            | Ub-Lys-TAMRA       | 50                           |
| USP22   | B            | Ub-Rho110          | 400                          |
| USP25   | B            | Ub-Lys-TAMRA       | 50                           |
| USP28   | B            | Ub-Lys-TAMRA       | 50                           |
| USP30   | B            | Ub-Lys-TAMRA       | 50                           |
| USP34   | B            | Ub-Rho110          | 400                          |
| USP35   | B            | Ub-Lys-TAMRA       | 50                           |

*Table S3. Conditions of DUB selectivity profiling assays.*
The selectivity profile of compounds 1, 2, 3 and LDN-57444 for inhibition of the DUB panel was assessed by FP (TAMRA) or FI (Rho110) using the indicated substrates and concentrations in buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween-20, 0.5 mg/mL BSA, 5 mM BME) or buffer B (40 mM Tris, pH 7.5, 0.005% Tween-20, 0.5 mg/mL BSA, 5 mM BME).
Materials and Methods

Abbreviations

AzRB (azido-arginine-biotin); AzTB (azido-TAMRA-biotin); BSA (bovine serum albumin); CuAAC (copper(I)-catalyzed azide-alkyne cycloaddition); DCM (dichloromethane); DMF (dimethylformamide); DMSO (dimethyl sulfoxide); DIPEA (N,N-disopropylethylamine); EDTA (ethylenediaminetetraacetic acid); FI (fluorescence intensity); FP (fluorescence polarization) GST (glutathione-S-transferase); HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate); HA-Ahx-Ahx-Ub-VME (HA-tagged ubiquitin vinyl methylester); HCD (higher-energy collisional dissociation); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); IPTG (isopropyl-β-D-thiogalactoside); LB (lysogeny broth); PBS (phosphate buffered saline); SDS (sodium dodecyl sulfate); SDS-PAGE (SDS-Polyacrylamide gel electrophoresis); TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine); TCEP (tris(carboxyethyl)phosphine hydrochloride); TFA (trifluoroacetic acid); THF (tetrahydrofuran); TLC (thin-layer chromatography); TMT (tandem mass tag); TRIS (tris(hydroxymethyl)aminomethane); Ub-AMC (ubiquitin-7-amino-4-methylcoumarin); Ub-FANCID2-TAMRA (ubiquitin-Fanconi anemia group D2-TAMRA); Ub-Lys-TAMRA (ubiquitin-ε-Lys-TAMRA); Ub-Rho110 (ubiquitin-Rhodamine 110); XPhos Pd G2 (2nd Generation XPhos Precatalyst, chloro(2-dicyclohexylphosphino-2',4',6'-trisopropyl-1,1'-biphenyl)[2-(2-amino-1,1'-biphenyl)]palladium(II)).

Reagents

Reagents were purchased from Sigma-Aldrich Chemical Co. Ltd., Acros Organics Ltd., WVR international Ltd. or Fluorochem Ltd. and were used without further purification. Common organic solvents used in chemical reactions (THF, DMF, DCM, 1,4-dioxane, and MeOH) were purchased anhydrous from Acros Organics Ltd. over molecular sieves (where available) in AcroSeal® bottles and handled under nitrogen. Water used for chemical reactions was obtained from a Sartorius Arium® Mini Plus lab water system (ASTM Type 1 ultrapure). For purifications, HPLC-grade solvents (≥99% purity) were used as purchased from Sigma-Aldrich Chemical Co. Ltd. or Fisher Scientific UK. For reaction work-up, technical grade solvents (≥95% purity) were purchased from WVR or Fisher. LDN-57444 was obtained from Tocris Bioscience (Cat. No. 3998). Recombinant DUBs were purchased from Boston Biochem or Ubiquigent or produced in-house as described below. The synthesis of capture reagents AzTB, and AzRB have been described previously. All compounds were dissolved in DMSO and stored at -20°C.

Biological and Biochemical Methods and Proteomics

Recombinant UCHL1 production

UCHL1 expression plasmid (GST-tagged UCHL1) was a generous gift from Dr C. Das (Purdue University, USA). Full-length human UCHL1 DNA (residues 1-223, UniProt P09936) was produced as previously described, with the following modifications. The GST-UCHL1 DNA construct in pGEX-6P-1 vector was transformed into E. coli Rosetta™ 2(DE3) Singles™ competent cells (Novagen). A single transformed colony was inoculated into LB (50 mL) supplemented with chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) and cells grown overnight at 37 °C with shaking (220 rpm). The starter culture (40 mL) was expanded in LB (2 L) supplemented with chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) and incubated at 37 °C with shaking (220 rpm). After reaching an OD600 of 0.6, protein expression was induced by addition of IPTG (0.5 mM) at 18 °C for 18 h with shaking (220 rpm). Cells were harvested by centrifugation (4000 rpm, 4 °C, 30 min). The resulting cell pellet was resuspended in lysis buffer (50 mL PBS, pH 7.4 (Sigma-Aldrich) containing Complet™ EDTA-free protease inhibitor cocktail (Roche) and DNase I (Invitrogen)) and lysed via one passage at 25 kPSI through a Cell Disruptor (Constant Systems). The lysate was centrifuged
Glutathione

Inhibition of

×

- 

- ×

ash frozen, and stored at 20

- × -

were initiated by the addition of diluted compound prepared by plotting a four-parameter dose-response curve (Graphpad Prism 5.03).

Biochemical UCHL1 fluorescence polarisation (FP) assay using Ub-Lys-TAMRA

Reactions were performed in duplicate in black 384 well plates (Greiner #784076) in a final reaction volume of 21 µL. Compound dilutions were prepared at 21× final concentration (2100 µM for a final concentration of 100 µM) in 50% DMSO/water in a 96-well polypropylene V-bottom plate (Greiner #651201). 50% DMSO (1 µL) or diluted compound (1 µL) was added to the plate. UCHL1 was diluted in reaction buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween-20, 0.5 mg/mL BSA, 5 mM BME) and 10 µL of diluted UCHL1 was added to the compound (final concentration 5 nM). Enzyme and compound were incubated for 30 min at room temperature. Reactions were initiated by the addition of 50 nM of TAMRA labeled peptide linked to ubiquitin via an iso-peptide bond (Ub-Lys-TAMRA, UbiQ Bio). Reactions were read immediately after substrate addition and following a 2 h incubation at room temperature using a Pherastar Plus (BMG Labtech; λ<sub>Ex</sub> 540 nm, λ<sub>Em</sub> 590 nm). IC<sub>50</sub> values were determined by plotting percentage UCHL1 activity against compound concentration and fitting a four-parameter dose-response curve (Graphpad Prism 5.03).

Biochemical DUB selectivity profiling assays

DUB selectivity assays were performed as outlined in Table S3. For USP10, USP22, USP34 and TRABID fluorescence intensity assays, enzymes were diluted in reaction buffer B (40 mM Tris, pH 7.5, 0.005% Tween-20, 0.5 mg/mL BSA, 5 mM BME), reactions were initiated by the addition of 400 nM Ub-Rho110 (Boston Biochem, U-555), and read using a Pherastar Plus (λ<sub>Ex</sub> 487 nm, λ<sub>Em</sub> 535 nm). Enzyme concentrations were optimized for each DUB to ensure activity was in the linear range.

UCHL1 kinetic studies

UCHL1 was added to Ub-AMC substrate (UbiQ Bio) in black flat bottom 384-well microplates (Corning #3575) in reaction buffer C (100 mM sodium phosphate, pH 8.5, 0.01% Tween-20) in 40 µL total reaction volume. The fluorescence intensity was read at room temperature using a 2104 EnVision™ Multilabel reader (Perkin Elmer; λ<sub>Ex</sub> 380 nm, λ<sub>Em</sub> 460 nm).

Michaelis-Menten kinetic analysis: Michaelis constant (K<sub>m</sub>) determination was performed by preparing Ub-AMC substrate (20 µL) at 2× final concentration in reaction buffer C. Reactions were initiated by adding UCHL1 (20 µL, 6 nM) and fluorescence intensity recorded immediately over 1 h. K<sub>m</sub> was calculated by plotting initial velocities against Ub-AMC concentration with a non-linear fit of the Michaelis–Menten equation (Graphpad Prism 5.03).

Inhibitor kinetic analysis: Inhibition kinetics for compound 1 and 2 were determined by preparing compound dilution plates at 40× final concentration in DMSO. DMSO (1 µL) or diluted compound (1 µL) was added to reaction buffer C (9 µL) in the reaction plate. Substrate Ub-AMC (500 nM, 10 µL) was prepared in buffer C and added to the reaction plate. Reactions were initiated by the addition of UCHL1 (6 nM, 20 µL), and fluorescence intensity read
immediately for 1 h. The apparent rate of inhibition ($k_a$) at each compound concentration was determined by curve fitting using non-linear regression analysis in GraphPad Prism 5.03. $k_a$ values were plotted against compound concentration to afford the apparent second order rate constant for inhibition, $k_{obs}$/l. The absolute value of the second order rate constant $k_{obs}/l$ was calculated by using $k_{obs}/l = k_a/l \times (1+|S|/K_m)$ where $K_m$ of UCHL1 and Ub-AMC is ~0.22 µM.\(^3\)\(^4\)

**Reversible inhibition assay:** Jump dilution assays were performed by incubating UCHL1 (300 nM) with compound 1, 2 (400 nM), LDN-57444 (1700 nM), or positive (DMSO) and negative controls (iodoaceticamide (IAA) 10 mM) for 30 min at room temperature. The reaction mixtures were diluted 100× with buffer C containing Ub-AMC (125 nM), and the fluorescence intensity read immediately for 1 h.

**UCHL1 covalent adduct determination**

UCHL1 (5 µM, 50 µL) in buffer D (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) was incubated with compound 1, 2 (2.5 equiv.) or DMSO for 1 h at room temperature. The excess ligand was removed by washing with buffer D in an Amicon\textsuperscript{®} Ultra MWCO 10 kDa centrifugation spin filter three times. The protein-adducts were desalted using 3× SDB-XC polystyrene-divinylbenzene copolymer extraction disks, following published methods.\(^5\)

Samples were eluted with 50% (v/v) MeCN 0.5% (v/v) formic acid in in LC-MS grade water and analyzed by LC-ESI (ACQUITY UPLC H-Class PLUS instrument (Waters)) using an ACQUITY UPLC BEH C\(_4\) column (Waters) coupled with Waters LCT Premier MS detector.

**UCHL1 covalent adduct site identification**

Compound-protein adduct samples were prepared as described above and digested with trypsin (Promega, 1:50 trypsin:UCHL1 ratio) at 37 °C, overnight. The resulting peptides were desalted using SDB-XC disks and eluted with 80% (v/v) MeCN in MilliQ water and dried in a Savant SPD1010 SpeedVac\textsuperscript{®} Concentrator (Thermo Fisher Scientific). Dried peptides were dissolved in 2% (v/v) MeCN, 0.5% (v/v) TFA in LC-MS grade water and analyzed on a Quadrupole Orbitrap nanoLC-MS/MS via an EASY-Spray\textsuperscript{™} source (Thermo Fisher Scientific). The MS data was analyzed by PEAKS Studio 8.5 (Bioinformatics Solutions Inc.).

**Cell Culture**

Cal51 and HEK293T cells were culture in Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose (Gibco #41965) supplemented with 10% fetal bovine serum (FBS, Gibco® #10500) and penicillin/streptomycin (P/S, Gibco #15070). HEK293, A549, EA,hy926 and HeLa cells were culture in DMEM low glucose (Sigma-Aldrich #D6046) supplemented with 10% FBS. All cells were maintained in a humidified incubator at 37 °C and 5% CO\(_2\). Cells were seeded on plates at least 24 h before experiment.

**Homogeneous time resolved fluorescence (HTRF) assay**

Cal51 cells stably expressing wild-type or Cys90Ala mutant FLAG-UCHL1 were seeded in a 96-well plate (Nunc #167008) at 45,000 cells/well and incubated overnight at 37 °C, or seeded at 75000 cells/well and incubated for 4 h at 37 °C. Compounds were diluted in medium and incubated with 1 h at 37 °C. Cells were washed twice with PBS, lysed in 20 µL of cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.5% CHAPS, 5 mM MgCl\(_2\), 5 mM BME, Complete™ EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche)) and incubated on ice for 30 min. Lysate (5 µL) was dispensed (in duplicate) in a white 384-well plate (Greiner #784075), combined with Ub-VME probe (100 nM, 5 µL) in HTRF buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.05% Tween-20, 0.5 mg/mL BSA, 5 mM BME) and incubated for 30 min at room temperature. Detection antibodies (10 µL) were diluted in detection buffer (50 mM HEPES pH 7.5, 400 mM KF, 0.5 mg/mL BSA), 0.033 µL of anti-FLAG cryptate antibody (Cisbio #61FG2KLA) and 0.1 µL of anti-HA-XL antibody (Cisbio #610HAXLB), and added to plates. The plates were sealed and incubated overnight at 4 °C then read using a PHERAstar (BMG), and the Delta F calculated:
% Delta F = 100 × \left( \frac{\text{ratio}_{\text{sample}} - \text{ratio}_{\text{negative control}}}{\text{ratio}_{\text{negative control}}} \right)

\text{Ratio} = 10000 \times \left( \frac{\text{fluorescence emission at 665 nm}}{\text{fluorescence emission at 620 nm}} \right)

The IC₅₀ values were determined by plotting the HTRF signal (% Delta F normalised against the vehicle treated sample) against concentration with a four-parameter dose-response curve (Graphpad Prism 5.03).

**In-cell target engagement using HA-Ub-VME**

HEK293T cells were seeded in 6-well plates (VWR #734-1596) at 1,000,000 cells/well and incubated overnight at 37 °C. Compounds 1, 2, 3 and LDN-57444 were diluted in media and incubated with cells for 1 h at 37 °C (final concentration 0.3% (v/v) DMSO). The media was removed and cells were washed with PBS prior to lysing in TRIS-lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 0.1% (v/v) NP-40, 0.5% (w/v) CHAPS, 2 mM MgCl₂, 5 mM BME, 10 U Benzonase, cOmmplete™ EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche)). HA-Ahx-Ahx-Ub-VME (0.5 µg) was added to lysate (20 µg) and incubated for 15 min at room temperature. 5× sample loading buffer (160 mM Tris pH 8, 5% (w/v) SDS, 0.025% (w/v) bromophenol blue, 25% (v/v) glycerol) containing 4% (v/v) BME was added and samples heated for 5 min at 95 °C prior to SDS-PAGE and immunoblotting. Blots were developed with Amersham ECL Prime Western Blotting Reagent and imaged using Amersham Imager 680. Inhibition was observed as a loss of HA-Ub-VME-labeled upper band intensity, and percentage inhibition was calculated using Image Quant (GE Healthcare).

**Preparation of lysates for ABP 2 profiling and competition**

Near-confluent (ca. 90%) plates of HEK293 cells were incubated with media containing 2 (2-2,000 nM, 0.1% (v/v) DMSO) or vehicle for indicated times. For competition experiments, HEK293 cells were pre-incubated with indicated concentrations of compound 1, 3, LDN-57444 or DMSO control for 1 h before incubation with 20 nM of 2 for 10 min. In both cases, treated cells were washed twice with PBS (500 µL) and lysed in PBS-lysis buffer (1% (v/v) Triton X-100, 0.1% (w/v) SDS in PBS supplemented with 1× cOmplete™ EDTA-free protease inhibitor cocktail). The lysate was clarified by centrifugation (17,000 g, 4°C, 10 min) and the supernatant recovered. The protein concentration was determined using the DC™ protein assay (Bio-Rad) following the manufacturer's protocol. After cell lysis and CuAAC ligation, samples were analyzed by in-gel fluorescence analysis, immunoblotting or proteomics (described below).

**Preparation of lysates for ABP 2 stability evaluation**

ABP 2 was pre-incubated in DMEM medium for 2, 6, 24, 48 or 72 h in a humidified incubator (37 °C, 5% CO₂) before being transferred to near-confluent HEK293 cells and incubated for 1 h. After cell lysis and CuAAC ligation, samples were analyzed by in-gel fluorescence analysis and Coomassie blue staining (described below).

**Preparation of UCHL1 DNA constructs**

WT UCHL1 was cloned as a N-terminal FLAG tag fusion protein in the multiple cloning site of pFLAG-CMV-6a plasmid using the HindIII and BglII restriction sites. UCHL1 mutants C90A and C90S were generated by PCR using the QuikChange Lightening Site-Directed Mutagenesis kit (Agilent) and the following primers:

UCHL1 C90A forward: 5' - GCAGACCATTGGGAATTCCGCTGGCACAATCGGACTTATT-3'
UCHL1 C90A reverse: 5' - AATAAGTCGATTGTGCCAGCGGAATTCCCAATGCTTCGCTGC -3'
UCHL1 C90S forward: 5' - GCCATTGGGAATTCCAGTGCCGACAATCGGACT-3'

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UCHL1 C90S reverse: 5’- AGTCCGATTGTGCCACTGGAATTCCCAATGGTC -3

Preparation of UCHL1 overexpression cell lysates
HeLa cells were transfected with plasmid encoding for UCHL1 WT, C90A or C90S using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) and Opti-MEM™ reduced serum medium (Gibco™) according to the manufacturer’s instructions with the following modifications. DNA (0.5 µg) was mixed with lipofectamine (3 µL) and added to cells in a 6-well plate. After 6 h transfection, medium was removed and replaced by fresh culture medium supplemented with 2 (10 and 100 nM) and incubated for 1 h. After cell lysis and CuAAC ligation, samples were analyzed by in-gel fluorescence and immunoblotting (described below).

Click chemistry ligation
Lysates were normalized to 1-2 mg/mL in PBS-lysis buffer and incubated with pre-mixed click reagents, containing AzTB (100 µM) for pulldown and in-gel fluorescence or AzRB (100 µM) for proteomics, CuSO₄ (1 mM), TCEP (1 mM) and TBTA (100 µM), with shaking for 1 h at room temperature. The reaction was quenched with addition of EDTA (5 mM final concentration). Protein was precipitated by adding MeOH/CHCl₃/water at a ratio of 2:0:5:1, relative to the sample volume. The precipitate was washed twice with cold MeOH, and air-dried for 5 min. The protein pellet was first resuspended in 2% (w/v) SDS in PBS to 10 mg/mL and then diluted to 1 mg/mL and 0.2% (w/v) SDS by addition of PBS.

SDS-PAGE and in-gel fluorescence imaging
4× sample loading buffer (250 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, supplemented fresh with 1.25% (v/v) BME) was added to samples (10 µg) which were incubated for 5 min without heating. The protein samples were separated on 12% (w/v) acrylamide Tris-HCl gels using Tris/glycine/SDS running buffer (0.25 M Tris, 0.2 M glycine, 0.1% (w/v) SDS) at 90 V for 30 min followed by 120 V for 1 h. Gel were imaged on a Typhoon imager (GE Healthcare; λ_ex 532 nm, λ_em 575 nm for TAMRA fluorophore; λ_ex 635 nm, λ_em 575 nm for protein MW markers (Precision Plus All Blue Standards, Bio-Rad)). For protein loading quantification, gels were stained with Coomassie blue staining solution overnight, rinsed with water and imaged on an ImageQuant™ LAS 4000 imager (GE Healthcare).

Biotin affinity enrichment for immunoblot analysis
Protein samples (150 µg) were ligated to the capture reagent (AzTB), precipitated and re-suspended as described previously. An aliquot (10 µg) was kept for total lysate (TL) input before enrichment. The samples (100 µg) were incubated with pre-washed Streptavidin Magnetic Beads (New England Biolabs, 30 µL beads per 100 µg protein) for 2 h at room temperature. Beads were washed with 0.2% (w/v) SDS in PBS (0.5 mL, three times) and eluted by boiling in 1× sample loading buffer (95 °C, 10 min). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham™ Protran®, GE Healthcare) by wet-tank transfer (Bio-RAD) in Tris-Glycine transfer buffer supplemented with 20% (v/v) MeOH for 1 h at 100 V. Membranes were blocked in 3% (w/v) BSA in Tris-buffer (50 mM Tris pH 7.4, 150 mM NaCl) containing 0.01% (v/v) Tween-20 (TBST) for 1 h before incubation with primary antibody in 0.3% (w/v) BSA in TBST for 1.5 h at room temperature or overnight at 4°C. The membrane was washed three times with TBST for 5 min and incubated with the corresponding HRP-conjugated secondary antibody (α-mouse-HRP or α-rabbit-HRP) in 0.3% (w/v) BSA in TBST for 1 h at room temperature. After three washes with TBST (5 min each), the membrane was incubated with HRP substrate (Luminata Crescendo, Millipore) and the chemiluminescence signal captured with an ImageQuant™ LAS 4000 imager.

Sample preparation for MS-based proteomic analysis
Proteomics samples were prepared in LoBind microcentrifuge tubes (Eppendorf®). All buffer solutions were prepared fresh and filtered with a 0.22 µm syringe filter prior to use. NeutrAvidin® Agarose Beads (Thermo Fisher Scientific) were dimethylated prior to the
enrichment procedure as described previously and washed twice with 0.2% (w/v) SDS in PBS before use. Protein lysates (400 μg, in biological triplicates) were ligated with AzRB capture reagent via CuAAC click chemistry, followed by protein precipitation and re-suspension as previously described. The protein solution (in 0.2% (w/v) SDS, PBS) was cleared by centrifugation (17,000 g, 10 min, room temperature) and incubated with dimethylated NeutrAvidin Agarose Beads (60 μL beads per starting 1 mg protein) for 2 h at room temperature with gentle shaking. The supernatant was removed by centrifugation (3,000 g, 2 min) and the protein-immobilised beads were sequentially washed three times with 1% (w/v) SDS in PBS (0.5 mL) and three times with 50 mM HEPES pH 8.0 (0.5 mL). After the final wash, the beads were resuspended in 50 mM HEPES pH 8.0 (50 μL) before addition of Lysyl endopeptidase®/ LysC (0.4 μg, FUJIFILM Wako) and incubated for 2 h at 37 °C. Beads were pelleted and the supernatant containing free peptides released by LysC cleavage was transferred to a new LoBind tube. TCEP (5 mM) and chloroacetamide (10 mM) were added to each sample and samples incubated for 10 min at room temperature, before addition of sequence grade trypsin (0.2 μg, Promega) and incubation overnight at 37 °C. Digested peptides were then chemically labeled with 10-plex TMT reagents (Thermo Fisher Scientific). Each peptide sample (40 μL) was labeled with different TMT reagents (0.08 mg, dissolved in 40 μL MeCN) and incubated for 2 h at room temperature with gentle shaking. The reaction was quenched by addition of hydroxylamine (1 μL, 5% (w/v)) and the labeled peptides combined and dried in SpeedVac Concentrator at 45 °C. The dried TMT-labeled peptides were re-suspended in 150 μL 1% (v/v) TFA in MilliQ water and cleared via centrifugation, followed by fractionation using 3× SDB-RPS styrenedivinylbenzene-reverse phase sulfonated disks and separated into three fractions as previously described. The samples were dried in a SpeedVac Concentrator and stored at −80°C. Peptides were re-suspended in 2% (v/v) MeCN and 0.5% (v/v) TFA in LC-MS grade water for analysis by nanoLC-MS/MS on a Q Exactive Orbitrap mass spectrometer.

**Preparation of samples for MS-based proteomic analysis of whole proteomes**

Protein lysates (10 μg) were reduced and alkylated with TCEP (5 mM) and chloroacetamide (10 mM), respectively, for 45 min at room temperature. Lysates were then precipitated using CHCl₃/MeOH/water as described previously. The protein pellets were re-suspended in 50 mM HEPES pH 8.0 to 1 mg/mL and further digested with trypsin (1:100, trypsin:lysate) at 37 °C overnight. After digestion, the peptides were labeled with TMT reagents (0.08 μg TMT reagent per peptide sample), fractionated using SDB-RPS disks and analyzed by nanoLC-MS/MS.

**NanoLC-MS/MS analysis of compound-UCHL1 adduct samples**

Peptides were separated on an EASY-Spray™ Acclaim PepMap C18 column (50 cm × 75 μm inner diameter, Thermo Fisher Scientific) using a 70-min linear gradient separation of 0-100% solvent B (80% (v/v) MeCN supplemented with 0.1% (v/v) formic acid): solvent A (2% (v/v) MeCN supplemented with 0.1% (v/v) formic acid) at a flow rate of 250 nL/min. The liquid chromatography was coupled to a Q Exactive mass spectrometer via an easy-spray source (Thermo Fisher Scientific) which operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at m/z 200. Scans were acquired from 350 to 1650 m/z. Up to 10 of the most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 2.0 m/z and fragmented by HCD with normalized collision energy of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17,500 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10⁶ and for MS/MS to 10⁷, and the intensity threshold was set to 8.3 × 10².

**NanoLC-MS/MS analysis of TMT-labeled samples**

Peptides were separated on an EASY-Spray™ Acclaim PepMap C18 column (50 cm × 75 μm inner diameter, Thermo Fisher Scientific) using a 3-hour linear gradient separation of 0-100% solvent B (80% (v/v) MeCN supplemented with 0.1% (v/v) formic acid): solvent A (2% (v/v)
MeCN supplemented with 0.1% (v/v) formic acid) at a flow rate of 250 nL/min. The liquid chromatography was coupled to a Q Exactive mass spectrometer via an easy-spray source which operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at m/z 200. Scans were acquired from 350 to 1800 m/z. Up to 10 of the most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 1.6 m/z and fragmented by HCD with normalized collision energy of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 35,000 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5, and the intensity threshold was set to 8.3 × 10^2.

**Proteomics data analysis and TMT-labeling quantification**

MS raw files were processed in MaxQuant (version 1.6.4.0) using the built-in Andromeda search engine and searched against the human reference proteome including isoforms (Uniprot, Taxonomy 9606, accessed 17 January 2019). For protein quantification, MS2 based quantification was used, selecting pre/defined TMT 10-plex labeling on lysine side chains and peptide N-termini. Cysteine carbamidomethylation was used as a fixed modification while methionine oxidation and N-terminus acetylation were used variable modifications. Trypsin/P was selected as digestion enzyme, with a maximum of 2 missed cleavages and minimum 7 residues of peptide length allowed. Unique and razor peptides were selected for protein identification and quantification. Other default parameters were used. MaxQuant-processed data was further analyzed using Perseus (version 1.6.2.3) and GraphPad Prism (version 5.03).

**TMT data analysis in Perseus**

Protein groups and the corresponding corrected TMT intensity values were analyzed in Perseus (version 1.6.2.3) and filtered against Only ID by site, Reverse and Potential contaminant. The TMT intensity values were grouped per TMT multiplex set (TMT set - A/B/C) and per condition (TMT channel - 126-131). The values were log2 transformed and filtered to require at least 2 valid values per condition and 2 unique and razor peptides for each protein. The mean values within each row (TMT set) were subtracted followed by subtraction of the median values across TMT channels (conditions). A two-sample t-test was performed across all quantified proteins to compare the TMT intensity values of condition 1 (eg. ABP 2-treated samples) versus condition 2 (eg. DMSO control) and to determine the significantly enriched proteins in each condition (n=3, Permutation-based FDR=0.01, S0=1). Fold-changes for each comparison were computed and plotted against calculated p-values in a Volcano plot. The protein groups were matched with the known DUBs database and further processed in GraphPad Prism 5.03.

**UCHL1 modified site identification analysis in PEAKS**

The MS raw files were processed in PEAKS (version 8.0) as described. Trypsin was selected as the digestion enzyme, allowing for maximum 2 missed cleavages. Cysteine carbamidomethylation, methionine oxidation and cysteine-compound 1 or 2 adducts (compound 1 C21H19N2O exact mass 357.16, compound 2 C20H16N2O exact mass: 381.16) were used as variable modifications. Other default software parameters were used as described in Supplementary Data 3. The peptide -log10P was set as ≥60 for the modified site identification analysis.

Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD015825.

**Fibroblast-to-myofibroblast transition (FMT) assay**

FMT assays in human lung cells derived from Idiopathic Pulmonary Fibrosis (IPF) patients were performed by Charles River (Assay OTS101-FMT-LUNG-IPF). Briefly, patient-derived primary human bronchial fibroblasts were seeded at 750 cells/well in 384-well plates and
subsequently pre-treated with reference compounds SB525334 (ALK-5 inhibitor, αSMA inhibition positive control) and nintedanib (clinically approved IPF drug), test compounds (1, 2, 3 and LDN-57444) or vehicle control (0.1% (v/v) DMSO) for 1 h, followed by triggering of FMT with 1.25 ng/mL TGF-β1. After 3 days, the cells were fixed and stained for αSMA and DAPI and imaged using high content analysis (HCA).

The αSMA immunoreactivity was quantified by an HCA algorithm with density x area (DxA) output. A percentage of αSMA inhibition was calculated:

\[
\% \text{ αSMA inhibition} = 100 - \frac{\mu_p - X_i}{\mu_p - \mu_n} \times 100
\]

Where \( \mu_p \) is an average αSMA value of positive control (TGF-β1 + 1 µM SB525334) \( \mu_n \) is an average αSMA value of vehicle control (TGF-β1 + 0.1% (v/v) DMSO) \( X_i \) are the αSMA values of the compound treatment

The nuclei were stained with DAPI and quantified by an HCA-based quantification of the number of imaged cells. A percentage of remaining cells was calculated:

\[
\% \text{ remaining cells} = \frac{X_i}{\mu_n} \times 100
\]

Where \( \mu_n \) is an average αSMA value of vehicle control (TGF-β1 + 0.1% (v/v) DMSO) \( X_i \) are the nuclei numbers of the compound treatment

The IC\(_{50}\) and EC\(_{50}\) values were determined by plotting the percentages of αSMA inhibition or remaining cells, respectively, against compound concentration with the four-parametric variable slope function (Graphpad Prism 5.03).

**Chemical Methods**

**General synthetic methods**

Air and moisture sensitive reactions were performed under nitrogen atmosphere using standard Schlenk manifold techniques with anhydrous solvent unless otherwise stated. All glassware was oven dried at 200°C and allowed to cool to room temperature under positive pressure of nitrogen. Solids were dried under vacuum and solvents were sparged with nitrogen for 30 min. The reaction containers were kept under positive nitrogen pressure for the whole reaction time. Compound 1, 2 and 3 were synthesized as described in chemical synthesis section. Compound 1 was synthesized based on the original report, following a modified procedure.\(^{13}\)

**Analytical techniques**

Reactions were monitored by TLC on Merck silica gel 60 Å aluminum plates and visualized with UV light, unless otherwise stated. Preparative TLC was performed using Merck PLC silica gel 60 Å glass plates (20 x 20 cm, 1 mm thickness). Flash column chromatography was performed using Merck Geduran® 60 Å silica gel (40-63 μm particle size) or on a Biotage Isolera™ One (SNAP KP-Sil columns, 60 Å pore size, 45-50 μm particle size). NMR spectra were recorded in CDCl₃, CD₂Cl₂, (CD₃)₂SO or CD₃OD (Acros Organics) on a 400 MHz Bruker AV NMR spectrometer at 298 K (400 MHz for ¹H and 101 MHz for ¹³C). Chemical shifts (δ) are reported in parts per million (ppm) and multiplicities reported as: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; br., broad. Coupling constants are reported in hertz (Hz). Spectra were analyzed using MestReNova 12 software. Residual
solvent signals were used as an internal standard and the characteristic solvent peaks were corrected to the data reported in *Organometallics*, **2010**, 29 (9), 2176-2179.\(^{14}\)

All high-resolution mass spectrometry and LC-MS analyses were performed by the Imperial College Mass Spectrometry Service using a Waters Acquity UPLC i-Class system comprised of a Waters BEH Acquity C18 column (2.1 mm × 50 mm, operating at 0.5 mL/min), a Waters LCT Premier ES-ToF mass spectrometer, and a photodiode array (detecting from 210 to 280 nm). Eluents of MeCN and water (each with 0.1% (v/v) formic acid) were used as linear gradients (% (v/v) MeCN in water) of: 5% to 95% (0 to 3.2 min), 95% to 5% (3.2 to 3.5 min), and 5% (3.5 to 4.0 min).

Preparative LC-MS of all final compounds was performed on a Waters RP-HPLC system comprised of a Waters 2767 auto-sampler, a Waters 515 HPLC pump, a XBridge C18 column (5 μm, 4.6 mm × 100 mm, operating at 1.2 mL/min), a Waters 3100 ESI mass spectrometer, and a Waters 2998 photodiode array (detecting from 190 to 700 nm). MeCN and water (each with 0.1% (v/v) formic acid) were used as eluent, with the method described in the table below.

Chiral HPLC to determine % enantiomeric excess (ee) was performed on a PerkinElmer Series 200 HPLC system with a Chiralpak AD column operating at 0.7 mL/min. n-Hexane/i-ProOH (75:25) were used as eluent, with detection at 220 nm.

### Linear gradients used on the Waters 2767 LC-MS system (% (v/v) MeCN in water, with 0.1% (v/v) formic acid)

| Method, 18 min | 0–0.2 min | 0.2–10 min | 10–13 min | 13–14 min | 14–18 min |
|---------------|-----------|------------|-----------|-----------|-----------|
| A (40-98%)    | 40%       | 40–98%     | 98%       | 98–40%    | 40%       |
| B (20–98%)    | 20%       | 20–98%     | 98%       | 98–20%    | 20%       |
| C (2–98%)     | 2%        | 2–98%      | 98%       | 98–2%     | 2%        |

### Chemical synthesis

**Scheme S1. Synthetic route to Compound 1**

**Synthesis of tert-butyl (S)-2-(4-bromoindoline-1-carbonyl)pyrrolidine-1-carboxylate (4)**

To a solution of N-(tert-butoxycarbonyl)-L-proline (260 mg, 1.2 mmol, 1.2 equiv.) in THF (5 mL) was added HATU (570 mg, 1.5 mmol, 1.5 equiv.) and DIPEA (350 µL, 2.0 mmol, 2.0 equiv.). The reaction mixture was stirred at room temperature for 1 h then 4-bromoindoline (130 µL, 1.0 mmol, 1.0 equiv.) was added and the solution stirred at room temperature for 15 h. The resulting reaction mixture was added to saturated aqueous NaHCO₃ (15 mL) and
extracted with EtOAc (3 × 15 mL). The combined organic phases were washed with brine (15 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure to afford a crude material that was purified by automated flash chromatography (Biotage SNAP KP-Sil 50 g cartridge, 6-48% EtOAc in n-Hexane). Collected fractions were combined and concentrated under reduced pressure to yield an off-white solid. The resulting product was triturated in saturated aqueous NaHCO₃, then washed with water (1 mL) and n-Hexane (3 mL) to yield compound 4 as a fine white powder (270 mg, 0.68 mmol, 68% yield). Rf = 0.25 (25% EtOAc in n-Hexane); ¹H NMR (400 MHz, CDCl₃) δ 8.18 (dd, J = 13.3, 8.0 Hz, 1H), 7.21-6.97 (m, 2H), 4.66-4.52 (m, 0.5H), 4.48-4.34 (m, 1H), 4.31-4.17 (m, 0.5H), 4.14-4.02 (m, 1H), 3.75-3.57 (m, 1H), 3.56-3.40 (m, 1H), 3.28-3.08 (m, 2H), 2.32-1.83 (m, 4H), 1.45 and 1.33 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 171.70, 171.44, 154.68, 153.77, 144.31, 144.10, 131.81, 131.54, 129.65, 129.35, 126.75, 126.59, 119.34, 119.21, 116.19, 115.94, 80.00, 79.92, 58.79, 58.56, 47.25, 47.02, 46.83, 30.82, 29.64, 28.61, 28.41, 24.41, 23.85; LC-MS (method A: 7.2 min); HRMS (ES+): m/z calc. for C₂₉H₂₈N₂O₃NaBr [M+CH₃CN+Na]⁺: 548.1055, found: 548.1050.

**Synthesis of tert-butyl (S)-2-(4-bromoindoline-1-carbonyl)pyrrolidine-1-carboxylate (5)**

Synthesis followed literature protocols with the following adaptions.¹⁵ Compound 4 (180 mg, 0.45 mmol, 1.0 equiv.), tert-butyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrrolo[2,3-b]pyridine-1-carboxylate (200 mg, 0.58 mg, 1.3 equiv.), K₂PO₄ (190 mg, 0.89 mmol, 2.0 equiv.) and XPhos-Pd-G2 (5.3 mg, 0.006 mmol, 0.015 equiv.) were dissolved in 4.4 mL of 5:1 degassed 1,4-dioxane: water, and the mixture was stirred at 60 °C overnight. After cooling to room temperature, water (5 mL) was added and the solution extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (10 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The recovered material was purified by automated flash chromatography (Biotage SNAP KP-Sil 50 g cartridge, 13-100% EtOAc in n-Hexane) to yield compound 5 as an off-white solid (180 mg, 0.33 mmol, 75% yield); ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.62-8.55 (m, 1H), 8.38-8.29 (m, 1H), 7.93-7.84 (m, 1H), 7.67 (d, J = 12.8 Hz, 1H), 7.36 (t, J = 7.9 Hz, 0.5H), 7.32 (t, J = 7.9 Hz, 0.5H), 7.26 (ddd, J = 7.6, 4.7, 2.5 Hz, 1H), 7.20 (dd, J = 1.8 Hz, 1H), 7.05-6.97 (m, 1H), 5.68-5.61 (m, 0.5H), 4.55-4.42 (m, 1H), 4.29-4.18 (m, 0.5H), 4.17-4.06 (m, 1H), 3.78-3.63 (m, 1H), 3.62-3.44 (m, 1H), 3.34-3.11 (m, 2H), 2.36-1.85 (m, 4H), 1.71 (s, 5H), 1.49 (s, 4H), 1.39 (s, 4H), 1.26 (s, 5H); ¹³C NMR (101 MHz, CDCl₃) δ 171.34, 171.14, 154.59, 153.71, 148.31, 147.87, 145.61, 145.54, 143.88, 143.70, 129.82, 129.44, 128.50, 128.10, 124.61, 123.86, 122.16, 118.11, 118.72, 117.55, 117.42, 116.76, 116.47, 84.41, 84.33, 79.80, 79.74, 75.01, 58.81, 58.49, 47.75, 46.94, 46.74, 30.50, 29.64, 28.52, 28.34, 28.14, 24.86, 24.33, 23.73; LC-MS (method B: 8.32 min); HRMS (ES-ToF): m/z calc. for C₂₉H₃₇N₂O₅ [M+H]⁺: 533.2764, found: 533.2770.
Synthesis of (S)-3-(1-prolylindolin-4-yl)-1H-pyrrolo[2,3-b]pyridine (6)

To a solution of 5 (100 mg, 0.19 mmol, 1.0 equiv.) in DCM (0.5 mL) was added TFA (0.3 mL, 3.9 mmol, 20 equiv.) and the solution stirred at room temperature for 3 h. The reaction mixture was diluted with DCM (2 mL) and concentrated under reduced pressure to yield a crude oil that was triturated with Et₂O. The recovered solid was dried under high vacuum to yield 6 as an off-white solid which was used without further purification (58 mg, 0.17 mmol, 93% yield).

**¹H NMR** (400 MHz, CD₃OD) δ (ppm): 8.28 (dd, J = 4.8, 1.5 Hz, 1H), 8.16 (dd, J = 5.8, 3.4 Hz, 1H), 8.09 (dd, J = 8.0, 1.5 Hz, 1H), 7.55 (s, 1H), 7.40-7.31 (m, 2H), 7.19 (dd, J = 7.9, 4.8 Hz, 1H), 4.71 (dd, J = 8.8, 6.5 Hz, 1H), 4.24-4.12 (m, 2H), 3.59-3.53 (m, 1H), 3.47-3.39 (m, 1H), 3.31 (t, J = 8.2 Hz, 2H), 2.71-2.58 (m, 1H), 2.24-2.07 (m, 3H); ¹³C NMR (101 MHz, CD₃OD) δ (ppm): 167.34, 149.39, 144.02, 143.80, 133.07, 131.35, 129.65, 128.85, 126.67, 125.49, 120.51, 117.14, 116.37, 114.78, 61.15, 48.95, 47.62, 29.79, 29.40, 25.28; HRMS (ES-ToF): m/z calc. for C₂₀H₁₈N₂O [M+H]⁺: 333.1715, found: 333.1723.

Synthesis of (S)-2-(4-(1H-pyrrolo[2,3-b]pyridin-3-yl)indoline-1-carbonyl)pyrrolidine-1-carbonitrile (1)

A solution of 6 (60 mg, 0.18 mmol, 1.0 equiv.) and K₂CO₃ (74 mg, 0.54 mmol, 3.0 equiv.) was prepared in dry THF (1.5 mL) and cooled to 0°C. A solution of BrCN in DCM (3 M, 72 µL, 0.22 mmol, 1.2 equiv.) was added dropwise and the reaction mixture allowed to warm to room temperature and stirred for 1 h. The resulting mixture was added to water (5 mL) and extracted with EtOAc (3 × 5 mL). The combined organic phases were washed with brine (5 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude material was purified by automated flash chromatography (Isolera, SNAP KP Sil cartridge 10 g, gradient 0 to 6% MeOH in DCM). Fractions were combined, concentrated under vacuum and further purified by preparative LCMS using method A to yield compound 1 as a white solid (24 mg, 0.067 mmol, yield 37%). ¹H NMR (400 MHz, (CD₃)₂SO) δ (ppm): 11.95 (s, 1H), 8.28 (d, J = 3.7 Hz, 1H), 8.08 (d, J = 6.4 Hz, 1H), 8.05 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 2.3 Hz, 1H), 7.36-7.23 (m, 2H), 7.13 (dd, J = 7.9, 4.6 Hz, 1H), 4.75 (dd, J = 7.9, 3.6 Hz, 1H), 4.29 (q, J = 9.6 Hz, 1H), 4.05 (q, J = 9.8 Hz, 1H), 3.53 (m, J = 8.3 Hz, 2H), 3.38-3.16 (m, 2H), 2.32 (dq, J = 15.1, 7.7 Hz, 1H), 2.04-1.83 (m, 3H); ¹³C NMR (101 MHz, (CD₃)₂SO) δ (ppm): 168.70, 149.05, 143.87, 143.49, 132.03, 130.00, 128.00, 127.88, 125.18, 124.49, 118.41, 116.76, 116.41, 114.67, 112.90, 62.27, 51.49, 47.72, 29.96, 28.48, 24.31; HRMS (ES-ToF): m/z Calc. for C₂₁H₂⁰N₄O [M+H]⁺: 358.1662, found 358.1658; ee >99%, tᵢ = 27.93.
Scheme S2. Synthetic route to IMP1710 (2)

To a solution of 3-bromo-5-iodo-1H-pyrrolo[2,3-b]pyridine (500 mg, 1.5 mmol, 1.0 equiv.) in degassed, dry DMF (4 mL) was added dichlorobis(triphenylphosphine)palladium (33 mg, 0.046 mmol, 0.03 equiv.), copper iodide (3.0 mg, 0.015 mmol, 0.02 equiv.), triethylamine (1.1 mL, 7.7 mmol, 5.0 equiv.) and ethynyl(trimethyl)silane (230 µL, 1.6 mmol, 1.1 equiv.). The reaction mixture was heated at 65 °C for 2 h then allowed cool to room temperature and quenched by addition of water (4 mL). The aqueous mixture was extracted with EtOAc (3 × 15 mL) and combined organic extracts were washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude material was purified by automated flash chromatography (Biotage SNAP KP-Sil 100 g cartridge, 5-40% EtOAc in n-Hexane) to yield compound 7 as a pale beige solid (330 mg, 1.1 mmol, 73%). \( R_f = 0.30 \) (30% EtOAc in n-Hexane); \(^1\)H NMR (400 MHz, CDCl₃) δ (ppm): 11.38 (s, 1H), 8.47 (d, \( J = 1.8 \) Hz, 1H), 8.04 (d, \( J = 1.8 \) Hz, 1H), 7.40 (d, \( J = 1.8 \) Hz, 1H), 0.29 (s, 9H); \(^{13}\)C NMR (101 MHz, CDCl₃) δ (ppm): 147.07, 146.57, 131.52, 125.50, 119.54, 113.07, 102.81, 95.68, 89.81, 0.14; HRMS (ES-TOF): m/z Calc. for C₁₂H₁₃N₂BrSi [M+H]+: 293.0110, found 293.0101.

Synthesis of 3-bromo-5-((trimethylsilyl)ethynyl)-1H-pyrrolo[2,3-b]pyridine (7)

Synthesis of 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5-((trimethylsilyl)ethynyl)-1H-pyrrolo[2,3-b]pyridine (8)
Synthesis followed literature protocols with the following adaptations.\textsuperscript{16} A solution of 2 (90 mg, 0.30 mmol, 1 equiv.), bis(pinacolato)diboron (230 mg, 0.92 mmol, 3.0 equiv.), KOAc (90 mg, 0.92 mmol, 3.0 equiv.) and XPhos-Pd-G2 (9.7 mg, 0.012 mmol, 0.04 equiv.) in 0.7 mL of degassed, dry 1,4-dioxane was heated to 65 °C and stirred overnight. The reaction mixture was allowed to cool to room temperature, filtered through a silica plug and the silica was washed with EtOAc (3 × 5 mL). The recovered solution was concentrated under reduced pressure and purified using automated flash chromatography (Biotage SNAP KP-Sil 25 g cartridge, 7-40% EtOAc in n-Hexane) to yield compound 3 as a pale-yellow solid (69 mg, 0.20 mmol, yield 68%). \( R_t = 0.25 \) (30% EtOAc in n-Hexane); \( ^{1} \text{H NMR} \) (400 MHz, CDCl\(_3\) \( \delta \) (ppm): 10.93 (s, 1H), 8.44 (d, \( J = 1.8 \) Hz, 1H), 8.40 (d, \( J = 1.8 \) Hz, 1H), 7.81 (d, \( J = 2.4 \) Hz, 1H), 1.37 (s, 12H), 0.29 (s, 9H); \( ^{13} \text{C NMR} \) (101 MHz, CDCl\(_3\) \( \delta \) (ppm): 149.03, 146.56, 135.65, 134.31, 123.55, 112.86, 103.90, 94.68, 83.36, 25.05, 0.23; HRMS (ES-ToF): m/z [M+H]+ Calcd. for C\(_{18}\)H\(_{25}\)BN\(_3\)O\(_3\)Si [M+H]+: 341.1857, found 341.1864.

**Synthesis of tert-butyl (S)-2-(4-(5-ethynyl-1H-pyrrolo[2,3-b]pyridin-3-yl)indoline-1-carbonyl)pyrrolidine-1-carboxylate (9)**

Synthesis followed literature protocols with the following adaptations.\textsuperscript{15} A solution of 8 (43 mg, 0.13 mmol, 1.3 equiv.), 4 (46 mg, 0.12 mmol, 1.0 equiv.), K\(_2\)PO\(_4\) (49 mg, 0.23 mmol, 2.0 equiv.) and XPhos-Pd-G2 (1.4 mg, 0.0017 mmol, 0.015 equiv.) in 6:1 degassed 1,4-dioxane:water (0.7 mL) was stirred at 60 °C overnight. The reaction mixture was allowed to cool to room temperature, filtered through a silica plug, and the silica washed with EtOAc (3 × 5 mL). The filtrate concentrated under reduced pressure to yield a black solid. The crude residue and K\(_2\)CO\(_3\) (32 mg, 0.23 mmol, 2.0 equiv.) were dissolved in 1:1 THF:MeOH (1.3 mL), and stirred at room temperature for 1.5 h. The mixture was added to saturated aqueous NH\(_4\)Cl (3 mL), then extracted with EtOAc (3 × 5 mL). The combined organic phases were dried with Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The residue was dissolved in EtOAc, filtered through a silica plug and the silica washed with EtOAc (3 × 5 mL). The filtrate concentrated under reduced pressure to yield a black solid which was purified by preparative TLC (75% EtOAc in n-Hexane) to yield compound 9 as an off-white amorphous solid (15 mg, 0.043 mmol, yield 37%). \( R_t = 0.15 \) (75% EtOAc in n-Hexane); \( ^{1} \text{H NMR} \) (400 MHz, Chloroform-d) \( \delta \) 10.95 (d, \( J = 16.8 \) Hz, 1H), 8.55 (dd, \( J = 6.4 \), 1.9 Hz, 1H), 8.30 (dd, \( J = 19.9 \), 8.0 Hz, 1H), 8.17 (dd, \( J = 14.2 \), 1.8 Hz, 1H), 7.44 (dd, \( J = 24.5 \), 2.1 Hz, 1H), 7.41 – 7.16 (m, 2H), 4.72 – 4.64 (m, 0.5H), 4.56 – 4.49 (m, 0.5H), 4.48 – 4.39 (m, 0.5H), 4.30 – 4.19 (m, 0.5H), 4.18 – 4.03 (m, 1H), 3.79 – 3.45 (m, 2H), 3.37 – 3.14 (m, 3H), 2.37 – 1.85 (m, 3H), 1.50 (s, 4.5H), 1.40 (s, 4.5H); \( ^{13} \text{C NMR} \) (101 MHz, CDCl\(_3\) \( \delta \) ppm): 171.42, 171.30, 171.16, 154.79, 153.91, 149.03, 146.56, 135.65, 134.31, 123.55, 112.86, 103.90, 94.99, 83.36, 25.05, 0.23; HRMS (ES-ToF): m/z [M+H]+ Calcd. for C\(_{27}\)H\(_{32}\)N\(_4\)O\(_3\) [M+H]+: 457.2234, found 457.2227.
Synthesis of (S)-5-ethynyl-3-(1-prolylindolin-4-yl)-1H-pyrrolo[2,3-b]pyridine (10)

Synthesis followed literature protocols with the following adaptions. \(^{17}\) A suspension of 9 (32 mg, 0.069 mmol, 1 equiv.) and K\(_2\)CO\(_3\) (96 mg, 0.69 mmol, 10 equiv.) in degassed, dry DCM (1.0 mL) was cooled to 0°C. TMSI (19.0 \(\mu\)L, 0.136 mmol, 2.0 equiv.) was slowly supplemented to the reaction mixture using a micro syringe. The solution was then stirred at room temperature for 30 min. The mixture was then added to a saturated solution of NaHCO\(_3\) (5 mL) and extracted with DCM (3 \(\times\) 5 mL). The combined organic layers were washed with brine (5 mL), dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (10% 2 M methanolic ammonia in DCM) to yield compound 10 as a pale-beige solid (17 mg, 0.048 mmol, 69% yield). \(R_f\) = 0.30 (10% 2 M methanolic ammonia in DCM); \(^1\)H NMR (400 MHz, CD\(_2\)OD) \(\delta\) (ppm): 8.34 (d, \(J = 1.8\) Hz, 1H), 8.13 (d, \(J = 7.7\) Hz, 1H), 8.10 (d, \(J = 1.9\) Hz, 1H), 7.54 (s, 1H), 7.28 (t, \(J = 7.8\) Hz, 1H), 7.23 (dd, \(J = 7.7, 1.2\) Hz, 1H), 4.19 (q, \(J = 8.5\) Hz, 1H), 4.09 (q, \(J = 9.9\) Hz, 1H), 4.00-3.93 (m, 1H), 3.53 (s, 1H), 3.26-3.16 (m, 3H), 2.92-2.81 (m, 1H), 2.35-2.23 (m, 1H), 1.94-1.76 (m, 3H); \(^{13}\)C NMR (101 MHz, CD\(_2\)OD) \(\delta\) (ppm): 173.31, 148.65, 147.13, 144.73, 132.69, 132.20, 126.71, 119.70, 116.48, 115.06, 112.66, 82.18, 60.92, 48.13, 31.12; LC-MS (method C: 9.4 min); HRMS (ES-ToF): \(m/z\) calc. for C\(_{22}\)H\(_{21}\)N\(_4\)O [M+H]\(^{+}\): 357.1710, found: 357.1704.

Synthesis of (S)-2-(4-(5-ethynyl-1H-pyrrolo[2,3-b]pyridin-3-yl)indoline-1-carbonyl)pyrroldidine-1-carbonitrile (2)

To a stirred solution of 10 (18 mg, 0.049 mmol, 1.0 equiv.) in THF (1 mL) was added K\(_2\)CO\(_3\) (27 mg, 0.20 mmol, 4.0 equiv.). The reaction mixture was cooled to -20°C and a solution of BrCN in DCM (3 M, 20 \(\mu\)L, 0.059 mmol, 1.2 equiv.) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. The resulting mixture was added to water (5 mL), supplemented with K\(_2\)CO\(_3\) (25 mg) and extracted with DCM (3 \(\times\) 5 mL). The combined organic layers were dried over MgSO\(_4\), filtered and concentrated under reduced pressure. The crude material was purified by automated flash column chromatography (SNAP KP-Sil 10 g, 1 to 6% MeOH in DCM, 36 mL/min) to yield compound 2 as a white solid (18 mg, 0.048 mmol, 97% yield). \(R_f\) = 0.30 (8% MeOH in DCM); \(^1\)H NMR (400 MHz, CD\(_2\)Cl\(_2\)) \(\delta\) (ppm): 9.89 (s, 1H), 8.47 (s, 1H), 8.24 (d, \(J = 8.0\) Hz, 1H), 8.18 (d, \(J = 1.7\) Hz, 1H), 7.47 (d, \(J = 2.1\) Hz, 1H), 7.34 (t, \(J = 7.8\) Hz, 1H), 7.27 (dd, \(J = 7.7, 1.2\) Hz, 1H), 4.48 (dd, \(J = 8.3, 3.6\) Hz, 1H), 4.19 (q, \(J = 9.2\) Hz, 1H), 4.05 (q, \(J = 9.2\) Hz, 1H), 3.70 (q, \(J = 7.6, 7.1\) Hz, 1H), 3.61-3.50 (m, 1H), 3.31-3.21 (m, 2H), 3.18 (s, 1H), 2.40-2.27 (m, 1H), 2.16-1.94 (m, 3H); \(^{13}\)C NMR (101 MHz, CD\(_2\)Cl\(_2\)) \(\delta\) (ppm): 168.41, 147.96, 147.13, 143.92, 132.62, 130.87, 129.88, 128.50, 125.37,
125.25, 124.80, 116.53, 116.20, 114.99, 82.05, 78.24, 62.84, 54.31, 52.05, 48.19, 30.55, 28.89, 24.69; **LC-MS** (method B: 9.4 min); **HRMS** (ES-ToF): \( m/z \) calc. for \( C_{23}H_{26}N_5O \) [M+H]^+: 382.1662, found: 382.1657; ee >94%, \( t_R = 19.77 \).

**Scheme S3. Synthetic route to IMP1711 (3)**

**Synthesis of tert-butyl \((R)-2-(4-bromoindoline-1-carbonyl)pyrrolidine-1-carboxylate (11)**

To a solution of \( N-(\text{tert-butoxycarbonyl})-D\)-proline (260 mg, 1.2 mmol, 1.2 equiv.) in THF (5 mL) was added HATU (570 mg, 1.5 mmol, 1.5 equiv.) and DIPEA (350 µL, 2.0 mmol, 2.0 equiv.). The reaction mixture was stirred at room temperature for 1 h. 4-Bromoindoline (130 µL, 1.0 mmol, 1.0 equiv.) was dissolved in dry THF (0.5 mL) and added to the reaction mixture. The solution was stirred at room temperature overnight. The resulting reaction mixture was added to saturated aqueous NaHCO₃ solution (15 mL), and extracted with EtOAc (3 × 15 mL). The combined organic phases were washed with brine (15 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude material was purified by automated flash chromatography (Biotage SNAP KP--Sil 25 g cartridge, 6 to 48% EtOAc in \( n \)-Hexane). The collected fractions were combined and concentrated under reduced pressure to yield an off-white solid. The resulting product was triturated in saturated aqueous NaHCO₃, and washed with water (1 mL) and \( n \)-Hexane (3 mL) to yield compound 11 as a fine white powder (220 mg, 0.55 mmol, 54% yield). \(^1H\) NMR (400 MHz, CDCl₃) \( \delta \) (ppm): 8.18 (dd, \( J = 13.1, 8.0 \) Hz, 1H), 7.22-6.95 (m, 2H), 4.58 (dd, \( J = 7.7, 3.2 \) Hz, 0.5H), 4.43 (dt, \( J = 11.7, 5.8 \) Hz, 1H), 4.23 (q, \( J = 9.5 \) Hz, 0.5H), 4.16-4.01 (m, 1H), 3.75-3.58 (m, 1H), 3.58-3.41 (m, 1H), 3.29-3.09 (m, 2H), 2.35-2.06 (m, 2H), 2.06-1.81 (m, 2H), 1.46 (s, 4.5H), 1.34 (s, 4.5H); \(^13C\) NMR (101 MHz, CDCl₃) \( \delta \) (ppm): 171.57, 171.31, 154.55, 153.63, 144.20, 143.99, 131.67, 131.40, 129.53, 129.22, 126.61, 126.47, 119.22, 119.08, 116.08, 115.82, 79.86, 79.78, 58.67, 58.44, 47.14, 46.72, 46.32, 30.45, 29.73, 29.58, 28.50, 28.31, 24.30, 23.73. **HRMS** (ES-ToF): \( m/z \) calc. for \( C_{20}H_{36}N_{3}O_{3}NaBr \) [M+CH₃CN+Na]^+: 460.1035, found: 460.1027.
Synthesis of tert-butyl (R)-2-(4-bromoindoline-1-carbonyl)pyrrolidine-1-carboxylate (12)

Synthesis followed literature protocols with the following adaptions.\textsuperscript{15} Compound 11 (180 mg, 0.45 mmol, 1.0 equiv.), tert-butyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1\textit{H}-pyrrolo[2,3-\textit{b}]pyridine-1-carboxylate (200 mg, 0.58 mg, 1.3 equiv.), K\textsubscript{3}PO\textsubscript{4} (190 mg, 0.89 mmol, 2.0 equiv.) and XPhos-Pd-G2 (5.3 mg, 0.006 mmol, 0.015 equiv.) were dissolved in 5:1 degassed 1,4-dioxane: water (2.4 mL), and the mixture stirred at 60 °C overnight. After cooling to room temperature, water (5 mL) was added and the solution was extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (10 mL), dried over MgSO\textsubscript{4}, filtered and concentrated under reduced pressure. The crude material was purified by automated flash chromatography (Biotage SNAP KP-Sil 50 g cartridge, 7 to 100% EtOAc in \textit{n}-Hexane) to yield compound 12 as an off-white solid which was used without further purification (220 mg, 0.42 mmol, 93% yield). \(R_f = 0.26\) (55% EtOAc in \textit{n}-hexane); \textbf{LC-MS} (method B: 8.70 min); \textbf{MS} (Scan ES\textsuperscript{+}): \mbox{m/z} \text{calc. for } C\textsubscript{30}H\textsubscript{37}N\textsubscript{4}O\textsubscript{5} [M+H]\textsuperscript{+}: 533.2764, found: 533.40.

Synthesis of (R)-3-(1-prolylindolin-4-yl)-1\textit{H}-pyrrolo[2,3-\textit{b}]pyridine (13)

To a solution of 12 (200 mg, 0.38 mmol, 1.0 equiv.) in DCM (0.5 mL) was added TFA (0.5 mL, 6.5 mmol, 17 equiv.). The solution was stirred at room temperature for 3 h, then diluted with DCM (2 mL) and concentrated under reduced pressure to yield a yellowish oil. The crude material was triturated with Et\textsubscript{2}O and the recovered solid dried under high vacuum to yield compound 13 as an off-white solid which was used without further purification (100 mg, 0.30 mmol, 80% yield). \textbf{LC-MS} (method B: 2.43 min); \textbf{MS} (Scan ES\textsuperscript{+}): \mbox{m/z} \text{calc. for } C\textsubscript{20}H\textsubscript{21}N\textsubscript{4}O [M+H]\textsuperscript{+}: 333.1715, found: 333.24.
Synthesis of (R)-2-(4-(1H-pyrrolo[2,3-b]pyridin-3-yl)indoline-1-carbonyl)pyrrolidine-1-carbonitrile (3)

A solution of 13 (100.0 mg, 0.30 mmol, 1.0 equiv.) and K₂CO₃ (120 mg, 0.90 mmol, 3.0 equiv.) was prepared in dry THF (2 mL) and cooled to 0°C. A solution of BrCN in DCM (3 M, 120 µL, 0.36 mmol, 1.2 equiv.) was added dropwise and the reaction mixture allowed to warm to room temperature and stirred for 1 h. The resulting reaction mixture was added to water (5 mL) and extracted with EtOAc (3 × 5 mL). The combined organic phases were washed with brine (5 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude material was purified by automated flash chromatography (Biotage SNAP KP Sil cartridge 10 g, gradient 0 to 6% MeOH in DCM). The collected fractions were combined and concentrated under reduced pressure. The recovered material was dissolved in 1:1 MeCN:H₂O (1 mL) supplemented with TFA (0.1% (v/v)). The solution was filtered and lyophilized to yield 3 as white solid (35 mg, 0.098 mmol, yield 33%). ¹H NMR (400 MHz, (CD₃)₂SO) δ (ppm): 11.97 (s, 1H), 8.28 (dd, J = 4.6, 1.4 Hz, 1H), 8.08 (dd, J = 6.6, 2.3 Hz, 1H), 8.05 (dd, J = 8.0, 1.3 Hz, 1H), 7.70 (d, J = 2.5 Hz, 1H), 7.35-7.25 (m, 2H), 7.13 (dd, J = 7.9, 4.6 Hz, 1H), 4.75 (dd, J = 8.3, 3.9 Hz, 1H), 4.35-4.23 (m, 1H), 4.10-3.98 (m, 1H), 3.60-3.45 (m, 2H), 3.32-3.16 (m, 2H), 2.38-2.24 (m, 1H), 2.03-1.83 (m, 3H); ¹³C NMR (101 MHz, (CD₃)₂SO) δ (ppm): 168.70, 149.05, 143.87, 143.01, 132.03, 130.00, 128.00, 127.88, 125.18, 124.49, 118.41, 116.76, 116.41, 114.67, 112.90, 62.27, 51.49, 47.72, 29.96, 28.48, 24.31; HRMS (ES-ToF): m/z Calc. for C₂₁H₁₉N₅O [M+H]⁺: 358.1668, found 358.1673. ee >99%, tₘ = 24.42.

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Appendix 1: $^1$H and $^{13}$C NMR Spectra

$^1$H-NMR of compound 4 in CDCl$_3$ (400 MHz)

$^{13}$C-NMR of compound 4 in CDCl$_3$ (101 MHz)
$^1$H-NMR of compound 5 in CDCl$_3$ (400 MHz)

\[ \text{Diagram of } ^1\text{H-NMR spectrum} \]

$^{13}$C-NMR of compound 5 in CDCl$_3$ (101 MHz)

\[ \text{Diagram of } ^{13}\text{C-NMR spectrum} \]
$^1$H-NMR of compound 6 in CD$_3$OD (400 MHz)

$^{13}$C-NMR of compound 6 in CD$_3$OD (101 MHz)
$^1$H-NMR of compound 1 in (CD$_3$)$_2$SO (400 MHz)

$^{13}$C-NMR of compound 1 in (CD$_3$)$_2$SO (101 MHz)
$^1$H-NMR of compound 7 in CDCl$_3$ (400 MHz)

$^{13}$C-NMR of compound 7 in CDCl$_3$ (101 MHz)
$^1$H-NMR of compound 8 in CDCl$_3$ (400 MHz)

$^{13}$C-NMR of compound 8 in CDCl$_3$ (101 MHz)
$^1$H-NMR of compound 9 in CDCl$_3$ (400 MHz)

$^{13}$C-NMR of compound 9 in CDCl$_3$ (101 MHz)
$^1$H-NMR of compound 10 in CD$_3$OD (400 MHz)

$^{13}$C-NMR of compound 10 in CD$_3$OD (101 MHz)
\(^1\text{H-NMR}\) of compound 2 in \(\text{CD}_2\text{Cl}_2\) (400 MHz)

\[^{13}\text{C-NMR}\) of compound 2 in \(\text{CD}_2\text{Cl}_2\) (101 MHz)
$^1$H-NMR of compound 11 in CDCl$_3$ (400 MHz)

$^{13}$C-NMR of compound 11 in CDCl$_3$ (101 MHz)
$^1$H-NMR of compound 3 in (CD$_3$)$_2$SO (400 MHz)

$^{13}$C-NMR of compound 3 in (CD$_3$)$_2$SO (101 MHz)
### Appendix 2: Chiral HPLC traces

#### Compound 1

| Peak # | Time (min) | Area (μV·s) | Height (μV) | Area (%) | Norm. Area (%) | Area/Height (s) |
|--------|------------|-------------|-------------|----------|----------------|----------------|
| 1      | 5.03       | 27139.7     | 7104.48     | 0.5      | 0.5            | 3.8201         |
| 2      | 27.933     | 5367244     | 68966.5     | 99.5     | 99.5           | 77.824         |

#### Compound 2

| Peak # | Time (min) | Area (μV·s) | Height (μV) | Area (%) | Norm. Area (%) | Area/Height (s) |
|--------|------------|-------------|-------------|----------|----------------|----------------|
| 1      | 4.242      | 329.07      | 39.53       | 0.01     | 0.01           | 8.3254         |
| 2      | 5.102      | 264.56      | 130.15      | 0.01     | 0.01           | 2.0327         |
| 3      | 5.490      | 6283.15     | 1082.68     | 0.21     | 0.21           | 5.8033         |
| 4      | 5.631      | 6514.30     | 1239.12     | 0.22     | 0.22           | 5.2572         |
| 5      | 19.770     | 2809296.75  | 45592.13    | 93.94    | 93.94          | 61.6180        |
| 6      | 29.663     | 167765.04   | 1294.69     | 5.61     | 5.61           | 129.5791       |
| 7      | 39.665     | 23.77       | 56.45       | 0.00     | 0.00           | 0.4211         |

#### Diagrams

- **Compound 1**
- **Compound 2**
| Peak | Time (min) | Area (μV·s) | Height (μV) | Area (%) | Norm. Area (%) | Area/Height (s) |
|------|------------|-------------|-------------|----------|----------------|----------------|
| 1    | 5.059      | 15806.51    | 3989.40     | 0.06     | 0.06           | 3.9621         |
| 2    | 24.423     | 28216910.36 | 381202.79   | 99.94    | 99.94          | 74.0207        |

28232716.87  385192.19  100  100
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