Demonstrating In-Cell Target Engagement Using a Pirin Protein Degradation Probe (CCT367766)

Nicola E. A. Chessum,† Swee Y. Sharp,† John J. Caldwell,† A. Elisa Pasqua,† Birgit Wilding,† Giampiero Colombano,† Ian Collins,† Bugra Ozer,† Meirion Richards, Martin Rowlands, Mark Stubbs,† Rosemary Burke,† P. Craig McAndrew,† Paul A. Clarke,*† Paul Workman,*† Matthew D. Cheeseman,*† and Keith Jones*†

†Cancer Research UK Cancer Therapeutics Unit at The Institute of Cancer Research, London SW7 3RP, United Kingdom

Supporting Information

ABSTRACT: Demonstrating intracellular protein target engagement is an essential step in the development and progression of new chemical probes and potential small molecule therapeutics. However, this can be particularly challenging for poorly studied and noncatalytic proteins, as robust proximal biomarkers are rarely known. To confirm that our recently discovered chemical probe 1 (CCT251236) binds the putative transcription factor regulator pirin in living cells, we developed a heterobifunctional protein degradation probe. Focusing on linker design and physicochemical properties, we generated a highly active probe 16 (CCT367766) in only three iterations, validating our efficient strategy for degradation probe design against nonvalidated protein targets.

INTRODUCTION

Drug discovery is reliant on recombinant proteins and biochemical screens to develop structure−activity relationships (SAR) and progress compounds. However, the conditions in biochemical assays often display little relevance to the intracellular environment, which can result in a failure to translate high target affinity to activity within a living cell. Target-proximal biomarker modulation is the most important confirmation of intracellular target engagement. Unfortunately, this is often not possible in early stage chemical probe or drug discovery projects, especially against novel biological targets, where poorly understood biology and pharmacology make it difficult to discover and robustly validate biomarkers, a process that is particularly challenging for noncatalytic proteins.

Owing to the importance of confirming intracellular target engagement, several techniques have been developed. The overexpression of fusion proteins allows for a direct readout of target occupancy. However, the engineered cell lines are often difficult to generate and the protein label can impact compound binding. The cellular thermal shift assay (CETSA) is a label-free technique for intracellular target engagement. It exploits compound-induced stabilization of protein melting temperatures, but CETSA cannot be applied to all targets. Activity-based protein profiling (ABPP) methods utilize nonselective irreversible covalent ligands, or intracellular fluorescence polarization probes, combined with intracellular reversible ligand competition, to study target engagement. Proteolysis targeting chimeras (PROTACs) and specific and nongenetic IAP-dependent protein erasers (SNIPERs) are heterobifunctional molecules that induce rapid and selective protein degradation, via the proteasome, within living cells. One portion of the molecule engages the target protein, while the other, attached via a flexible linker, recruits an E3 ligase to ubiquitinate the target, marking it for degradation as part of the cullin−RING finger machinery.

We recently reported the development of a high affinity pirin chemical probe 1 (CCT251236, SPR $K_D = 44$ nM) discovered through a cell-based phenotypic screen for inhibitors of the heat shock transcription factor 1 (HSF1) stress pathway. Pirin is an iron-binding member of the cupin super family of proteins and has been reported as a putative transcription factor regulator. It has no known enzymatic function in mammalian cells, no
endogenous ligands have been reported, and no validated proximal biomarkers have been described. This makes demonstrating intracellular target engagement in living cells very challenging. We hypothesized that we could demonstrate chemical probe binding to pirin within living cells by developing a pirin-targeting protein degradation probe (PDP).

**RESULTS AND DISCUSSION**

**Protein Degradation Probe Design.** PDPs have been described against various proteins, with the bromodomain epigenetic target, BRD4, the most extensively studied. These heterobifunctional PDP molecules have utilized several ligands that bind to the E3 ligases: VHL, the IAP proteins, and CRBN. Despite the rapid expansion in PDP research, there remains no clear methodology to determine which proteins are amenable to PDP-mediated degradation, which E3 ligase ligand should be exploited or the optimal features of probe design. The structure of the linker, its length, and physicochemical properties have all been demonstrated to be important for PDP activity. The linker controls the formation of the essential ternary complex, with evidence that it may stabilize the protein–protein interaction (PPI) between the target and the E3 ligase rather than forming a detached linear ternary complex (Figure 1), although it is unclear whether the ternary complex is part of the multiprotein cullin–RING finger complex.

Although we had discovered a high affinity ligand for pirin, there was no evidence that this protein would be a suitable substrate for ubiquitination by a PDP-recruited E3 ligase, without which extensive linker optimization could be futile. Therefore, we initially designed a synthetically tractable 15-atom linker that we predicted would not affect the affinity of the PDP for the isolated target proteins. Analysis of the crystal structure of the chemical probe bound to pirin (Figure 1, PDB 5JCT) suggested that the solvent-exposed solubilizing group vector should be amenable to linker attachment. We selected a CRBN-targeting thalidomide ligand as the basis of our E3 ligase binding motif due to its low molecular weight. The CRBN-targeting ligand would be attached via the solvent exposed hydroxyl group of the 4-hydroxythalidomide analogue. The linker would then attach the pirin- and CRBN-targeting specific binding groups via two amide moieties to give our first generation pirin-targeting PDP 3 (Scheme 1).

The CRBN-targeting motif 4 of PDP 3 was synthesized from 4-hydroxythalidomide in two steps and 84% yield, in a similar manner to that previously described. The pirin-binding motif of the PDP 3 was synthesized from 6-bromoquinoline via a palladium-mediated carbonylation reaction on the ether derivative. Trapping the carbonylation intermediate with 2-(trimethylsilyl)ethan-1-ol gave the (trimethylsilyl)ethyl ester, which facilitated TBAF-selective hydrolysis. Amide coupling to the previously described bis-aniline derivative was followed by hydrolysis of the aliphatic linker ester to give acid. Final amide coupling to the CRBN-targeting derivative gave the first-generation pirin-targeting PDP 3 in 10 steps and 0.4% overall yield.

*The First Generation PDP.* Analysis of the heterobifunctional PDP 3 revealed that it possessed good affinity for recombinant protein...
pirin (Table 1, entry 1) when measured using SPR, confirming the success of the rationally designed attachment vector. The affinity of PDP 3 was then assessed against the CRBN-DDB1 complex, with DDB1 acting as a scaffolding protein, using an FP-assay similar to that previously described (Supporting Information, Figure S6). PDP 3 displayed moderate affinity for CRBN-DDB1, with $K_i = 230 \text{ nM}$, comparable to the affinities of the parent CRBN ligands, thalidomide and lenalidomide (Supporting Information, Figure S7).

Following confirmation that both binding motifs of the PDP 3 retained high affinity for their respective targets, we then investigated its activity against pirin in human cancer cell lines. Several cell lines were assessed for CRBN expression by quantitative capillary electrophoresis (Supporting Information, Figure S17). The SK-OV-3 ovarian carcinoma cell line displayed good basal CRBN and also pirin expression, and there was no observable depletion of pirin in these cells when treated with chemical probe 1 ($1 \mu M$, data not shown), so this line was selected for further study. Unfortunately, treatment of SK-OV-3 cells with PDP 3, at high concentrations (>1 $\mu M$) and for extended time periods (>48 h), resulted in no measurable effects on the cancer cells (data not shown).

### Table 1. Physicochemical Properties and Affinities for Recombinant Protein Targets of the Three Generations of PDPs

| Entry | Compd | X | R | Linker | HBD | ALogP | LogD2 | iPSA (Å²) | KS (μM) | Pirin SPR/IC50 (pIC50±SEM) | CRBN/DDB1 IC50 (μM±SEM) | CRBN/Ki (nM) |
|-------|-------|---|---|--------|-----|-------|-------|-----------|--------|--------------------------|------------------------|----------|
| 1     | 3     | Me |    |        | 5   | 2.8   | 2.2   | 258       | 1      | 110 nM (6.97±0.02)       | 850 nM (6.07±0.14)     | 220      |
| 2     | 10    | F  |    |        | 4   | 2.0   | 1.9   | 244       | 5      | 230 nM (6.63±0.01)       | 410 nM (6.38±0.07)     | 95       |
| 3     | 16    | Cl |    |        | 3   | 3.9   | 2.7   | 207       | 2      | 55 nM (7.26±0.04)        | 490 nM (6.61±0.10)     | 120      |
| 4     | 21    | Cl |    |        | 3   | 3.9   | 2.9   | 207       | 2      | >1300 nM (<5.90)         | 420 nM (6.38±0.10)     | 98       |

*HBD = hydrogen bond donor count. ALogP was calculated using Biovia Pipeline Pilot, version 9.5, 2 SF. Log $D_{2-4}$ measured using a HPLC-based method, $n = 1, 2$ SF. iPSA was calculated using ChemDraw (16.0.1) based on the O- and N-count, 3 SF. $KS = \text{kinetic solubility in pH 7.4 phosphate buffer at room temperature, } n = 1, 1 \text{ SF.}^1$ $K_{i0}$ values are reported to 2 SF and are calculated by equilibrium analysis using a one site specific binding model from SPR sensorgrams at equilibrium where possible, $K_{i0} = -\log(C_{i0} (\text{M}) \times 10^{-9})$ and represents the geometric mean of $n = 3$ independent biological repeats. $IC_{50}$ values are reported to 2 SF and are calculated from an FP-assay dose–response curve to displace a thalidomide derived fluorescent probe using a log[Inhibitor] vs response – variable slope (four parameters) model, $IC_{50} = -\log(C_{i0} (\text{M}) \times 10^{-9})$ and represents the geometric mean of $n = 3$ independent biological repeats, also see ref 31. $IC_{50}$ values are calculated from the geometric mean CRBN-DDB1 complex $IC_{50}$ and the FP-probe $K_{i0}$ using methods described in ref 32. SF = significant figure. All data was reprocessed using GraphPad Prism 7.01. See Supporting Information, Figures S8–S16. SEM = standard error of the mean.
incompatible with this methodology, and if so, no further improvements could be made.35 Alternatively, CRBN could be the wrong E3 ligase target to deplete pirin,36 or the linker length could be inconsistent with formation of the ternary complex.26 Finally, the physicochemical properties of PDP3 could be limiting its intracellular free concentration.37 Given these variables, designing a PDP against a protein target that has not previously been validated as being susceptible to E3 ligase-directed degradation is challenging, as optimization cycles can appear lengthy and the difficult and low yielding synthesis discourages the generation of multiple analogues. It was also unclear how strict and narrow the requirements for optimal PDP design would be. We hypothesized that the physicochemical properties of the PDP were the primary cause of the failure of the first generation probe, and so began a redesign process that should increase cell membrane flux while maintaining the same linker length and CRBN-targeting ligand (Table 1). By carrying out multiple changes to the PDP, we aimed to minimize the number of iterative design cycles, so we could more rapidly validate this approach.

Reagents and conditions: (a) (i) X = F 12, 2-methylquinoline carboxylic acid, oxalyl chloride, DMF, DCM, RT, 3 h, then pyridine, 18 h quant, (ii) Fe(0), NH4Cl, EtOH/H2O, 90 °C, 1 h, quant; (b) 2,3-dihydrobenzo-[b][1,4]-dioxine-6-carboxylic acid, oxalyl chloride, DMF, DCM, RT, 3 h, then pyridine, RT, 2 h, 85%; (c) (i) SeO2, 1,4-dioxane/DMF, reflux, 1 h, (ii) N-Boc-piperazine, DCM, RT, 12 h then NaBH(OAc)3, DCM, RT, 2 h, 94% (over 2 steps), (iii) TFA, DCM, RT, 2 h, 69%; (d) (i) 2, PPh3, butyl 2-hydroxyacetate, DTBAD, THF, 0 °C → RT, 16 h, 75%, (ii) HCO2H, DCM, 40 °C, 16 h, 54%, (iii) HATU, DIPEA, DMF, 4-butyldi(2-aminoethoxy)propanolate, RT, 16 h, 72%, (iv) HCO2H, DCM, 40 °C, 6 h, 93%; (e) HATU, DIPEA, DMF, RT, 16 h, 52%; (f) (i) X = Cl 17, 2-methylquinoline carboxylic acid, oxalyl chloride, DMF, DCM, RT, 3 h, then pyridine 2 h, 88%, (ii) Fe(0), NH4Cl, EtOH/H2O, 90 °C, 1 h, quant; (g) 2,3-dihydrobenzo-[b][1,4]-dioxine-6-carboxylic acid, oxalyl chloride, DMF, DCM, RT, 3 h, then pyridine 2 h, 61%; (h) (i) SeO2, DMF, 1,4-dioxane, 50 °C, 16 h, (ii) N-Boc-piperazine, NaBH3CN, AcOH, DMF, 0 °C → RT, 16 h, (iii) 4 M HCl in dioxane, MeOH 0 °C → RT, 16 h, 32% over 3 steps; (i) 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-methylbenzenesulfonate, K2CO3, DMF, RT, 16 h, 48%; (j) PPh3, DTBAD, THF, RT, 2 h, 27%; (k) (i) 23, 2,3-dihydro-1,4-benzodioxine-5-carboxylic acid, oxalyl chloride, DMF, DCM, RT, 2 h, then pyridine 48 h, 84%, (ii) Pd/C, EtOH/DCM, H2 (1 atm), 77%, (iii) 2-methylquinoline carboxylic acid, oxalyl chloride, DMF, DCM, RT, 2 h, then pyridine 16 h, 58%, then same procedure as from 18, 6% yield over 5 steps. (l) (i) SeO2, 1,4-dioxane/DMF, 50 °C, 5.5 h, (ii) 1,2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-methylbenzenesulfonate, K2CO3, DMF, RT, 16 h, 58%, then same procedure as from 18, 6% yield over 5 steps. (m) (i) 23, 2,3-dihydro-1,4-benzodioxine-5-carboxylic acid, oxalyl chloride, DMF, DCM, RT, 2 h, then pyridine 16 h, 58%, then same procedure as from 18, 6% yield over 5 steps. (n) (i) SeO2, 1,4-dioxane/DMF, 50 °C, 5.5 h, (ii) N-ethylpiperazine, DCM, RT, 20 h, then NaBH(OAc)3, DCM, RT, 2 h, 35% (over 2 steps).
This poor chemical stability was consistent with the known reduced tPSA (244 Å²). Using selenium dioxide was followed by reductive amination of yield. Amide coupling to give bisamide 11, synthesized in a similar manner to that previously described from 2-fluoro-5-nitroaniline 12 in three steps and 85% yield. Amide coupling to give bisamide 13 and benzylic oxidation using selenium dioxide was followed by reductive amination of the resulting aldehyde with N-Boc-piperazine, and subsequent deprotection, to give 14. The CRBN-targeting thalidomide derivative precursor 15 was prepared in four steps and 27% yield from 4-hydroxylthalidomide 2. Final amide coupling with monosubstituted piperazine 14 gave the second generation pirin-targeting PDP 10 in 11 steps and 8% overall yield (Scheme 2).

PDP 10 displayed a similar affinity for recombinant pirin and CRBN-DDB1 to our first generation probe 3 (Table 1, entry 2), so it was progressed to cellular assessment of pirin degradation. Pleasingly, treatment of SK-OV-3 ovarian cancer cells with so it was progressed to cellular assessment of pirin degradation. The synthesis of PDP 10 began from the fluoroaniline carbamid 11, synthesized in a similar manner to that previously described from 2-fluoro-5-nitroaniline 12 in three steps and 85% yield. Amide coupling to give bisamide 13 and benzylic oxidation using selenium dioxide was followed by reductive amination of the resulting aldehyde with N-Boc-piperazine, and subsequent deprotection, to give 14. The CRBN-targeting thalidomide derivative precursor 15 was prepared in four steps and 27% yield from 4-hydroxylthalidomide 2. Final amide coupling with monosubstituted piperazine 14 gave the second generation pirin-targeting PDP 10 in 11 steps and 8% overall yield (Scheme 2).

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Although demonstrating pirin depletion with the second generation probe 10 was a very encouraging result, we found the effects were poorly reproducible. The concentrations of PDP 10 needed to observe pirin degradation were high and close to its kinetic solubility (KS). Furthermore, at least 24 h of compound exposure was needed before pirin degradation was observed (Supporting Information, Figure S18). To explore the cause of the variable results obtained with PDP 10, we assessed its chemical stability. At room temperature, PDP 10 was stable as a solid and in DMSO stock solution (>1 month, data not shown), but at 37 °C in pH 7.4 phosphate buffer, consistent with the cell assay conditions, it underwent rapid decomposition (Supporting Information, Table S1), displaying a half-life of only ~4 h. This poor chemical stability was consistent with the known decomposition of the parent CRBN-targeting thalidomide ligand under these conditions, where multiple hydrolysis products of the imide and glutaramide moieties are observed. The chemical probe 1 displayed no instability under these conditions; therefore, we concluded that the facile hydrolysis of the CRBN-targeting motif limited the reproducibility of our slow-acting second generation pirin-targeting PDP 10.

The Third Generation PDP. In the design of a third generation probe, we aimed to increase permeability further. This should result in higher intracellular free concentrations more quickly, mitigating the poor stability of the CRBN-targeting motif. We decided to carry out a bioisosteric replacement of the central ring fluorine for the larger and more sterically hindering chlorine substitute. However, we were concerned that the increase in lipophilicity from this exchange would negatively impact both the solubility and permeability of the probe. Large lipophilic heterobifunctional molecules are particularly susceptible to aggregation, and this decreases the free concentration that drives cell membrane flux. To balance the lipophilicity, we removed the tertiary amide bond to the piperazine, introducing a cationic amine, which would be substantially charged at pH 7.4 (MoKa, version 2.5.2, pKₐ = 8.0). The second amide in the linker was also removed, reducing the HBD count further and increasing the overall flexibility of the ligand, consistent with the formation of the crucial PDP ternary protein complex. The linker length was reduced by one atom to accommodate these changes and resulted in the design of the third generation pirin-targeting PDP 16 (CCT367766), which now displayed a notably reduced, but still high, tPSA (207 Å²).

The synthesis of the pirin-targeting motif of the third generation PDP 16 was carried out in a similar manner to that previously described starting from 2-chloro-5-nitroaniline 17 to give chlorobisamide 18 in three steps and 54% yield (Scheme 2). Oxidation of the methylquinoline moiety with SeO₂ and reductive amination of the resulting aldehyde with N-Boc-piperazine and N-Boc deprotection gave 19 in 32% yield. Following S₉₂ alkylation with the ether linker to give 20, selective Mitsunobu alkylation with 4-hydroxylthalidomide 2 gave the third generation PDP 16 in eight steps and 2% overall yield. A nonpirin-binding negative control matched pair compound, PDP 21, based on our negative control pirin chemical probe, was synthesized from regiosomer 23 in a similar manner in 2% overall yield. A non-CRBN binding control 22 was also synthesized from 18, utilizing reductive amination with N-ethylpiperazine in 35% yield (Scheme 2).

Analysis of the third generation PDP 16 confirmed that it retained acceptable lipophilicity (Table 1, entry 3) but also displayed a 4.2-fold increase in affinity for recombinant pirin compared to the second generation PDP 10 (Figure 2A) and comparable affinity for CRBN (Figure 2B). SK-OV-3 cells were
then treated with PDP 16 at concentrations from 50 to 1500 nM for up to 4 h (Figure 3).

In contrast to our earlier generation PDPs, near complete pirin degradation was now observed with just 50 nM treatment and only 2 h exposure (Figure 3A). Increasing the total initial concentration of pirin-targeting PDP 16 resulted in a clear hook effect. This bell-shaped concentration–response is consistent with the formation of a ternary complex.22 Interestingly the hook effect was seen to decrease over time (24 h), possibly either due to the slower degradation at high concentration or from the depletion of PDP 16 due to thalidomide hydrolysis (half-life = ~3 h, Supporting Information, Figure S1), which would reduce the effective free concentration below the negative cooperativity threshold of the ternary complex.23 Pirin degradation was subsequently shown to be concentration-responsive with activity at concentrations as low as 0.5 nM (Figure 3B), which was confirmed with a quantitative capillary electrophoresis-based immunoassay (Figure 3C). The negative control benzodioxane regioisomer PDP 21 (Table 1, entry 4) displayed no pirin depletion at equimolar concentrations (Supporting Information, Figure S19),20 and degradation was also confirmed to be proteasome-dependent by rescue following preincubation with the proteasome inhibitor, MG132 (500 nM, Supporting Information, Figure S19).21 We then carried out whole proteome mass spectrometry to estimate the cellular selectivity of the pirin-targeting PDP 16 in an unbiased manner, quantified using tandem mass tagging (TMT) (Figure 3D, www.proteomics.com). After treating SK-OV-3 cells with 50 nM PDP 16 for 4 h, and comparing to vehicle treated cells using Benjamini–Hochberg corrected \( p \) values, we found that from 8547 quantifiable proteins identified, only pirin (2.3-fold reduction, \( p(\text{adj}) = 1.4 \times 10^{-4} \)) displayed a statistically significant (\( p(\text{adj}) < 0.05 \))22 difference in protein expression.

To confirm that our chemical probe 1 also bound pirin within SK-OV-3 cancer cells, we carried out competition experiments designed to rescue pirin depletion by PDP 16. The concentrations required for a mutually exclusive binding ligand to displace a probe molecule are dependent on the affinity of the ligand relative to the ratio of the free concentration of the probe and its affinity for the protein target complex.53 Because the depletion of pirin is a nonequilibrium event that does not necessarily require complete target occupancy, it is more difficult to observe competition at later time points owing to continued protein turnover.22 Pretreating SK-OV-3 cells with 10 \( \mu \)M thalidomide, as a CRBN-binding competitive ligand, demonstrated that after 2 h treatment with PDP 16 (5 nM), we successfully rescued pirin depletion (Supporting Information, Figure S19). The non-CRBN-binding control chlorobisamide 22 displayed high affinity for recombinant pirin (SPR, \( K_D = 21 \text{ nM}, \ pK_D = 7.68 \pm 0.03, \ n = 3 \)). Pretreatment of SK-OV-3 cells with chlorobisamide 22 displayed concentration-dependent rescue of pirin expression following treatment with the PDP 16 (Figure 4, top), with complete rescue observed at 1 \( \mu \)M. Finally, pretreatment with the chemical probe 1 demonstrated clear rescue of pirin depletion, confirming intracellular target engagement (Figure 4, bottom).

**CONCLUSION**

Exploiting cell-based phenotypic screens to identify new disease-associated therapeutic targets is an increasingly frequent strategy in drug discovery. While this approach can identify novel targets with unique mechanisms of action, these proteins are often poorly characterized and can lack identifiable enzymatic activity, ligands, and biomarkers of target engagement. The main focus of research into PDPs has been as potential therapeutics with novel mechanisms of action. We designed a PDP as an intracellular...
probe against the poorly understood and noncatalytic molecular target, pirin. Developing PDPs to confirm intracellular target engagement, and potentially develop intracellular SAR, against challenging proteins, is an important addition to the current methods for compound profiling.

For PDPs to be used as target engagement probes, their rapid development and validation is crucial. The ideal strategies for efficient and successful PDP design are still under investigation and will clearly improve as more protein targets are modulated and additional crystallographic evidence of the target protein/E3 ligase/PDP ternary complexes are discovered. The number of variables involved in PDP design against nonvalidated target proteins can make the process daunting. By focusing on the physicochemical properties of our probe molecules, in only three iterations, we developed a selective degradation probe that eliminates pirin at low concentration and in a short time period. This confirmed our chemical probe 1 does bind pirin in an intracellular environment, and PDP 16 provides another chemical tool to study a largely unexplored protein.

**EXPERIMENTAL SECTION**

**General Experimental.** Unless otherwise stated, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen or argon using anhydrous solvents. All commercially obtained reagents and solvents were used as received. Thin layer chromatography (TLC) was performed on precoated aluminum sheets of silica (60 F254 nm, Merck) and visualized using short-wave UV light. Flash column chromatography was carried out on Merck silica gel 60 (partial size 40–65 μm). Column chromatography was also performed on a Biotage SP1 or Biotage Isolera Four purification system using Biotage Flash silica cartridges (SNAP KP-Sil) or for reverse phase purifications SNAP Ultra C18 cartridges. Ion exchange chromatography was performed using acidic Isolute Flash SCX-II columns. Semipreparative HPLC was performed on an Agilent 1100 system with 20 mL/min, eluents 0.1% acetic acid in water and 0.1% acetic acid in methanol, gradient of 10–100% organic phase. Lipophilic method: Chromatographic separation at room temperature was carried out using a 1200 series preparative HPLC (Agilent, Santa Clara, USA) over a 15 min gradient elution (gradient 15 min, 20 mL) from 60:40 to 100:100 water:methanol (both modified with 0.1% formic acid) at a flow rate of 20 mL/min. 1H NMR spectra were recorded on Bruker AMX500 (500 MHz) spectrometers using an internal deuterium lock. Chemical shifts are quoted in parts per million (ppm) using the following internal references: CDCl3 (δH 7.26), MeOD (δH 3.31), and DMSO-d6 (δH 2.50). Signal multiplicities are recorded as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), doublet of doublet of doublets (ddd), broad (br), or obscured (obs). Coupling constants, J, are measured to the nearest 0.1 Hz. 13C NMR spectra were recorded on Bruker AMX500 spectrometers at 126 MHz using an internal deuterium lock. Chemical shifts are quoted to 0.1 ppm, unless greater accuracy was required, using the following internal references: CDCl3 (δC 77.0), MeOD (δC 49.0), and DMSO-d6 (δC 39.5). High resolution mass spectra were recorded on an Agilent 1200 series HPLC and diode array detector coupled to a 6210 time-of-flight mass spectrometer with dual multimode APCI/ESI source or on a Waters Acquity UPLC and diode array detector coupled to a Waters G2 QToF mass spectrometer equipped with a multimode ESI/APCI source. All compounds were >95% purity by LCMS analysis unless otherwise stated.

Thalidomide ([http://www.sigmaaldrich.com/catalog/product/sigma/t144?lang=en&region=GB, accessed August 29, 2017](http://www.sigmaaldrich.com/catalog/product/sigma/t144?lang=en&region=GB, accessed August 29, 2017)), Lenalidomide ([http://www.sigmaaldrich.com/catalog/product/aldrich/cds022536?lang=en&region=GB, accessed August 29, 2017](http://www.sigmaaldrich.com/catalog/product/aldrich/cds022536?lang=en&region=GB, accessed August 29, 2017)), and MG132 ([http://www.sigmaaldrich.com/catalog/product/sigma/m8699?lang=en&region=GB, accessed August 29, 2017](http://www.sigmaaldrich.com/catalog/product/sigma/m8699?lang=en&region=GB, accessed August 29, 2017)) were purchased from Sigma-Aldrich and used without further purification. The pirin chemical probe 1 CECT251236 was synthesized using the previously described procedure. 1

2-(2,6-Dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione 2. Aminopiperidine-2,6-dione hydrochloride (300 mg, 1.82 mmol) was added to a solution of 4-hydroxybenzofuran-1,3-dione (299 mg, 1.82 mmol) and triethylamine (0.38 mL, 2.73 mmol) in anhydrous THF (36.5 mL) and the reaction heated to reflux at 43 °C for 2 h under inert atmosphere. The reaction mixture was allowed to cool to room temperature, then 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (384 mg, 2.00 mmol) and DMAP (21 mg, 0.18 mmol) were added. The reaction was heated to reflux for 24 h. Then the reaction mixture was allowed to cool, and the solvents were removed under reduced pressure. The resulting residue was taken up in methanol and passed sequentially through two ion exchange columns (Isolute SCX-II), eluting with methanol to afford the title compound as a white solid (422 mg, 84%). 1H NMR (500 MHz, DMSO-d6) δ 11.18 (s, 1H), 11.09 (s, 1H), 7.65 (dd, J = 8.4, 7.2 Hz, 1H), 7.32 (dd, J = 7.1, 0.7 Hz, 1H), 7.25 (dd, J = 8.4, 0.7 Hz, 1H), 5.07 (dd, J = 12.8, 5.5 Hz, 1H). 13C NMR (126 MHz, DMSO-d6) δ 172.78, 169.89, 167.14, 166.71, 165.12, 155.03, 145.84, 130.71, 92.46, 61.97, 19.69. HRMS (ESI+): calcd for C15H13N2O7 (M + H, m/z) 297.28. This compound is also commercially available from several suppliers.

tert-Butyl 2-((2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxyacetate. Triphenylphosphine (0.261 g, 0.996 mmol) and tert-butyl 2-hydroxyacetate (101 mg, 0.77 mmol) were dissolved in anhydrous THF (3 mL) while stirring at 0 °C. Then a solution of DTBAD (229 mg, 0.10 mmol) in anhydrous THF (2 mL) was added dropwise. Finally, a solution of (2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetate (1.30 g, 3.35 mmol) was dissolved in anhydrous THF (2 mL) and the reaction was stirred at 4 °C for 16 h. The mixture was stirred for 1 h at 0 °C then allowed to warm to room temperature and stirred overnight. Then the solvent was removed under reduced pressure. The crude product was purified by Biotage chromatography using a gradient of 0–50% EtOAc in cyclohexane to afford the title compound as an amorphous white solid (224 mg, 0.577 mmol, 75% yield). 1H NMR (500 MHz, DMSO-d6) δ 11.11 (s, 1H), 7.80 (dd, J = 8.5, 7.3 Hz, 1H), 7.48 (dd, J = 7.2 Hz, 1H), 7.38 (dd, J = 8.5 Hz, 1H), 5.10 (dd, J = 12.8, 5.5 Hz, 1H), 4.97 (s, 2H), 2.89 (dd, J = 17.0, 13.9, 5.4 Hz, 1H), 2.64–2.52 (m, 2H), 2.07–2.00 (m, 1H), 1.43 (s, 9H). 13C NMR (126 MHz, DMSO-d6) δ 172.78, 169.89, 167.14, 166.71, 165.12, 155.03, 136.76, 133.25, 119.92, 116.44, 116.74, 81.92, 81.15, 65.50, 48.80, 30.95, 27.68, 21.97. HRMS (ESI+): calcd for C22H18N2O7 (M + H, [M + H]–Bu) 333.0717, found 333.0722.

2-((2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)(oxy)acetic Acid. tert-Butyl 2-((2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetate (1.30 g, 3.35 mmol) was dissolved in anhydrous DCM, then formic acid (12.8 mL, 335 mmol) was added while stirring at room temperature. The reaction was stirred overnight at 40 °C. The solvents were removed under reduced pressure and the resulting residue purified by reverse phase Biotage chromatography using a gradient of 0–50% MeOH in water + 0.1% formic acid to afford the title...
that was dissolved in anhydrous DMF (3.3 mL) at room temperature under inert atmosphere. HATU (0.35 mL, 1.96 mmol) was added and the reaction stirred for 16 h. The reaction mixture was filtered and the filtrate was concentrated by vacuum. The resulting crude was purified by reverse phase Biotage chromatography using a gradient of 0–100% MeOH in water + 0.1% formic acid to afford the title compound as an amorphous white solid (236 mg, 72%).

**1H NMR** (500 MHz, MeOD) δ 7.82 (dd, J = 8.4, 7.4 Hz, 1H), 7.55 (dd, J = 7.3, 0.5 Hz, 1H), 7.46–7.43 (m, 1H), 5.16 (dd, J = 16.2, 5.5 Hz, 1H), 4.78 (s, 2H), 3.71 (t, J = 6.2 Hz, 2H), 3.62–3.56 (m, 2H), 3.52–3.48 (m, 2H), 3.15 (dd, J = 18.0, 4.4, 5.2 Hz, 1H), 2.82–2.70 (m, 2H), 2.50 (d, J = 6.2, 3.8 Hz, 2H), 2.16 (dddd, J = 10.8, 7.9, 5.1, 2.4 Hz, 1H), 1.42 (s, 9H).

**13C NMR** (126 MHz, MeOD) δ 174.55, 172.81, 171.33, 170.00, 168.31, 167.59, 156.21, 153.18, 154.96, 121.60, 119.30, 117.89, 118.75, 79.01, 69.26, 67.75, 50.56, 40.15, 37.22, 32.20, 28.34, 23.66. HRMS (ESI-): calcd for C24H30N4O9Na (M + Na)+ 541.1911, found 541.1911.

**2-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (3) (217 mg, 0.65 mmol), and DIPEA (0.34 mL, 1.96 mmol) were dissolved in anhydrous DMF (3.27 mL) at room temperature under inert atmosphere.** HATU (230 mg, 0.98 mmol) was added and the reaction stirred overnight. The solvents were removed under reduced pressure, and the resulting residue was purified by reverse phase Biotage chromatography using a gradient of 20–100% MeOH in water + 0.1% formic acid to afford the title compound as a beige solid (32 mg, 74%).

**1H NMR** (500 MHz, DMSO-d6) δ 13.29 (s, 1H), 11.11 (s, 1H), 7.79 (dd, J = 8.5, 7.3 Hz, 1H), 7.47 (d, J = 7.2 Hz, 1H), 7.39 (d, J = 8.6 Hz, 1H), 5.10 (dd, J = 12.8, 5.4 Hz, 1H), 4.98 (s, 2H), 2.89 (ddd, J = 16.9, 13.9, 5.4 Hz, 2H), 2.64–2.52 (m, 2H), 2.04 (d, J = 13.0, 5.4, 2.3 Hz, 1H). LCMS (ESI+): RT = 0.71 min, 100%, M + Na+ 355.

**tert-Butyl (2-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (2) (217 mg, 0.65 mmol), and DIPEA (0.34 mL, 1.96 mmol) were dissolved in anhydrous DMF (3.27 mL) at room temperature under inert atmosphere.** HATU (230 mg, 0.98 mmol) was added and the reaction stirred overnight. The solvents were removed under reduced pressure, and the resulting residue was purified by reverse phase Biotage chromatography using a gradient of 20–100% MeOH in water + 0.1% formic acid to afford the title compound as a beige solid (32 mg, 74%).

**1H NMR** (500 MHz, MeOD) δ 7.82 (dd, J = 8.4, 7.4 Hz, 1H), 7.55 (dd, J = 7.3, 0.5 Hz, 1H), 7.46–7.43 (m, 1H), 5.16 (dd, J = 16.2, 5.5 Hz, 1H), 4.78 (s, 2H), 3.71 (t, J = 6.2 Hz, 2H), 3.62–3.56 (m, 2H), 3.52–3.48 (m, 2H), 3.15 (dd, J = 18.0, 4.4, 5.2 Hz, 1H), 2.82–2.70 (m, 2H), 2.50 (d, J = 6.2, 3.8 Hz, 2H), 2.16 (dddd, J = 10.8, 7.9, 5.1, 2.4 Hz, 1H), 1.42 (s, 9H).

**13C NMR** (126 MHz, MeOD) δ 174.55, 172.81, 171.33, 170.00, 168.31, 167.59, 156.21, 153.18, 154.96, 121.60, 119.30, 117.89, 118.75, 79.01, 69.26, 67.75, 50.56, 40.15, 37.22, 32.20, 28.34, 23.66. HRMS (ESI-): calcd for C24H30N4O9Na (M + Na)+ 541.1911, found 541.1911.
(3 × 15 mL). The combined organic layer was washed with brine (20 mL) and dried (Na$_2$SO$_4$) to afford the crude product as a yellow oil. This material was used directly in the next step without further purification. LCMS (ESI): RT = 1.57 min, 84%, (M + H)$^+$ 304. 2-(4-Ethoxy-4-oxobutyl)quino line-6-carboxylic acid (74 mg, 0.24 mmol) was dissolved in anhydrous DMF (2 mL), and DIPEA (0.12 mL, 0.70 mmol) was added, followed by HATU (105 mg, 0.28 mmol). The reaction was allowed to stir for 5 min, and then N-(3-amino-4-methylphenyl)-2,3-dihydrobenzof[1,4]dioxine-6-carboxamide (63 mg, 0.22 mmol) was added. The reaction was stirred at room temperature overnight. The reaction mixture was poured into water and the resulting precipitate collected by filtration. The precipitate was dissolved in DCM/MeOH and preabsorbed onto silica. The crude material was purified by Biotech chromatography using a gradient of 0–5% MeOH in DCM and then by preparative HPLC (20 mL, lipophilic method) to afford the title compound as an amorphous beige solid (48 mg, 38% over two steps). H NMR (500 MHz, CDCl$_3$) δ 8.30 (d, $J = 1.9$ Hz, 1H), 8.13–8.05 (m, 3H), 7.93–7.87 (m, 3H), 7.68 (d, $J = 8.3$, 2.1 Hz, 1H), 7.44 (d, $J = 2.1$ Hz, 1H), 7.38 (dd, $J = 8.4, 2.2$ Hz, 1H), 7.23 (d, $J = 8.4$ Hz, 1H), 6.97 (d, $J = 8.8$ Hz, 1H), 6.94 (d, $J = 8.4$ Hz, 1H), 4.57 (t, $J = 6.3$ Hz, 2H), 4.31 (dd, $J = 11.1, 3.7$, 1.8 Hz, 4H), 4.18 (q, $J = 7.1$ Hz, 2H), 2.56 (t, $J = 7.4$ Hz, 2H), 2.35 (s, 3H), 2.21 (p, $J = 6.7$ Hz, 2H), 1.28 (t, $J = 7.1$ Hz, 3H). $^1$C NMR (126 MHz, CDCl$_3$) δ 173.3, 165.49, 165.09, 148.69, 146.89, 143.67, 139.54, 136.92, 136.11, 131.17, 130.22, 128.35, 128.05, 127.62, 127.30, 125.17, 124.61, 120.59, 117.63, 117.52, 116.89, 114.86, 114.55, 65.40, 65.71, 64.34, 60.62, 31.26, 24.58, 17.52, 14.39. HRMS (ESI): calculated for C$_{30}$H$_{28}$N$_3$O$_7$ (M + H)$^+$ 542.1922, found 542.1905. To a suspension of ethyl 4-((6-(5-(2,3-dihydrobenzo[1,4]dioxine-6-carboxamido)-2-fluorophenoxy)propanoyl)pirezan-1-ylmethyl)quino line-6-carboxamide 9. 3-(2-(2-(2,6-Dioxopiperdin-3-yl)-1,3-dioxoisindolin-4-yl)acetamido)ethoxypropanoyl)pirezan-1-ylmethyl)quino line-6-carboxamide 10. 3-(2-(2-(2,6-Dioxopiperdin-3-yl)-1,3-dioxoisindolin-4-yl)acetamido)ethoxypropanoyl acid 11. To a solution of N-(2-fluoro-5-nitrophenyl)-2-methylquinoline-6-carboxamide (104 g, 32.0 mmol) in ethanol (120 mL) and water (40 mL), ammonium chloride (120 g, 224 mmol) and iron powder (125.2 g, 224 mmol) were added in one portion, and the resulting suspension was allowed to stir at 90 °C for 1 h. The reaction mixture was filtered through a pad of Celite. The resulting filtrate was concentrated under vacuum to afford a light-brown solid which was redissolved in a mixture of DCM:MeOH (9:1, 150 mL) and washed with saturated aqueous NaHCO$_3$ (150 mL). The organic phase was dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to afford a yellow solid as crude product, which was taken directly onto the next step without further purification (9.46 g, quant.). H NMR (500 MHz, DMSO-d$_6$) δ 10.50 (s, 1H), 8.57 (d, $J = 1.8$ Hz, 1H), 8.39 (d, $J = 8.5$ Hz, 1H), 8.19 (dd, $J = 8.8, 2.0$ Hz, 1H), 8.01 (d, $J = 8.8$ Hz, 1H), 7.52 (d, $J = 8.4$ Hz, 1H), 6.94 (dd, $J = 10.3, 8.8$ Hz, 1H), 6.89 (dd, $J = 6.6$, 2.7 Hz, 1H), 6.64–6.39 (m, 1H), 5.05 (brs 2H), 2.70 (s, 3H). $^1$C NMR (126 MHz, DMSO-d$_6$) δ 164.93, 160.84, 148.49, 147.72 (d, $J = 23.9$ Hz), 143.05 (d, $J = 1.9$ Hz), 137.19, 131.19, 128.44, 128.33, 127.95, 125.50 (d, $J = 13.1$ Hz), 125.34, 122.99, 115.54 (d, $J = 20.6$ Hz), 112.4, 111.39, 11.31 (d, $J = 6.6$ Hz), 25.05. $^1$F NMR (470 MHz, DMSO-d$_6$) δ −138.12. HRMS (ESI): calculated for C$_{30}$H$_{28}$N$_3$O$_7$ (M + H)$^+$ 597.3170, found 597.3134. N-(5-Amino-2-fluorophenyl)-2-methylquinoline-6-carboxamide 12. To a solution of N-(2-fluoro-5-nitrophenyl)-2-methylquinoline-6-carboxamide (104 g, 32.0 mmol) in ethanol (120 mL) and water (40 mL), ammonium chloride (120 g, 224 mmol) and iron powder (125.2 g, 224 mmol) were added in one portion, and the resulting suspension was allowed to stir at 90 °C for 1 h. The reaction mixture was filtered through a pad of Celite. The resulting filtrate was concentrated under vacuum to afford a light-brown solid which was redissolved in a mixture of DCM:MeOH (9:1, 150 mL) and washed with saturated aqueous NaHCO$_3$ (150 mL). The organic phase was dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to afford a yellow solid as crude product, which was taken directly onto the next step without further purification (9.46 g, quant.). H NMR (500 MHz, DMSO-d$_6$) δ 10.50 (s, 1H), 8.57 (d, $J = 1.8$ Hz, 1H), 8.39 (d, $J = 8.5$ Hz, 1H), 8.19 (dd, $J = 8.8, 2.0$ Hz, 1H), 8.01 (d, $J = 8.8$ Hz, 1H), 7.52 (d, $J = 8.4$ Hz, 1H), 6.94 (dd, $J = 10.3, 8.8$ Hz, 1H), 6.89 (dd, $J = 6.6$, 2.7 Hz, 1H), 6.64–6.39 (m, 1H), 5.05 (brs 2H), 2.70 (s, 3H). $^1$C NMR (126 MHz, DMSO-d$_6$) δ 164.93, 160.84, 148.49, 147.72 (d, $J = 23.9$ Hz), 143.05 (d, $J = 1.9$ Hz), 137.19, 131.19, 128.44, 128.33, 127.95, 125.50 (d, $J = 13.1$ Hz), 125.34, 122.99, 115.54 (d, $J = 20.6$ Hz), 112.4, 111.39, 11.31 (d, $J = 6.6$ Hz), 25.05. $^1$F NMR (470 MHz, DMSO-d$_6$) δ −138.12. HRMS (ESI): calculated for C$_{30}$H$_{28}$N$_3$O$_7$ (M + H)$^+$ 597.3170, found 597.3134.
concentrated in vacuo to afford a dry pale-green solid. The solid was dissolved in pyridine (100 mL), and NaHCO₃ saturated aqueous solution (5 mL) and extracted with a 0.541 mmol) and tert-difluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

To a suspension of tert-butyl 4-((S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was added to anhydrous DCM (5 mL) to afford the crude product as a pale-yellow solid which was taken directly onto the next step without further purification (5.15 g).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).

A solution of N-(S)-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-fluorophenyl)-2-formylquinoline-6-carboxamide (255 mg, 0.541 mmol) and tert-butyl piperazine-1-carboxylate (302 mg, 1.62 mmol) in anhydrous DCM (5 mL) was allowed to stir at 20 °C for 12 h, after which time sodium triacetoxysilylhydride (344 mg, 1.62 mmol) was added in one portion and the resulting mixture was allowed to stir at 20 °C for 2 h. The reaction was quenched with NaHCO₃ saturated aqueous solution (5 mL) and extracted with DCM:MeOH, 9:1 mixture (3 × 5 mL). The crude product (pale-yellow solid) was purified by column chromatography on silica gel using a gradient of 0–6% MeOH in DCM, followed by trituration in diethyl ether to afford the desired product as a pale-beige solid (325 mg, 94%).
(2.00 g, 6.42 mmol) was added in one portion. The resulting dark-yellow suspension was stirred for 2 h, then it was poured onto water and the yellow precipitate was filtered and washed several times with water, EtOAc, and finally with a minimum amount of DCM to afford the crude product as a pale-yellow amorphous solid, which was carried onto the next step without purification (1.86 g, 61%). H NMR (500 MHz, DMSO-d6) δ 10.13 (s, 1H), 10.27 (s, 1H), 8.65 (d, J = 1.9 Hz, 1H), 8.43 (d, J = 8.8 Hz, 1H), 8.25 (dd, J = 8.8, 2.2 Hz, 1H), 8.14 (d, J = 2.2 Hz, 1H), 8.04 (d, J = 8.8 Hz, 1H), 7.75 (dd, J = 8.8, 2.9 Hz, 1H), 7.58–7.49 (m, 4H), 7.00 (d, J = 8.8 Hz, 1H), 4.37–4.26 (m, 4H), 2.71 (s, 3H). HRMS (ESI+): found [M + H]+ 474.1210, C38H35Cl3N4O4 requires 474.1215.

N-(2-Chloro-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-carboxamido)phenyl)-2-piperazin-1-ylmethyliquinoline-6-carboxamide 19. N-(2-Chloro-5-(2,3-dihydro-1,4-benzodioxin-6-carboxamido)phenyl)-2-methylquinoline-6-carboxamide 18 (4.00 g, 8.44 mmol) was taken up in anhydrous DMF (20.00 mL) and anhydrous 1,4-dioxane (20 mL). Selenium dioxide (1.03 g, 9.28 mmol) was added, and the reaction was degassed via 3× vacuum/nitrogen cycles. The reaction mixture was heated to 50 °C and stirred overnight. A bleach bubbler was used to vent the reaction. The reaction was quenched by slow addition of an aqueous solution of 1 M NaOH (50 mL), and the reaction mixture was stirred at room temperature under nitrogen for 2 h. The reaction mixture became darker red/brown in color. The reaction was allowed to warm to room temperature overnight. Further 1H NMR (500 MHz, CDCl3) showed almost complete disappearance of the starting material, and a new compound formed as a pale-yellow amorphous solid, which was carried onto the next step, assuming 100% yield, without further purification. HRMS (ESI+): calcd for C30H29Cl3N3O4 requires 474.1215.

To a solution of tert-butyl 4-((6-(2-Chloro-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-carboxamido)phenyl)quinoxalin-2-yl)methyl)piperazin-1-carboxylate (4.57 g, 6.94 mmol) in anhydrous MeOH [1,4]dioxine-6-carboxamido)phenyl)-2-piperazin-1-ylmethyliquinoline-6-carboxamide (4.12 g, 8.44 mmol) and 1-Boc-piperazine (3.15 g, 16.89 mmol) in anhydrous DMF (60 mL) at 0 °C under nitrogen. Then acetic acid (0.53 mL, 9.29 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. A bleach bubbler was used to vent the reaction. The reaction was quenched by slow addition of an aqueous solution of 1 M NaOH (50 mL), and the reaction mixture concentrated in vacuo by using heptane for azeotropic removal of DMF. The reaction mixture was heated to 50 °C and stirred overnight. A bleach bubbler was used to vent the reaction. The reaction was quenched by slow addition of an aqueous solution of 1 M NaOH (50 mL), and the reaction mixture was stirred at room temperature under nitrogen for 2 h. The reaction mixture had become fully dissolved. Then the solvents were removed in vacuo. Anhydrous DCM (10 mL) was added and the reaction concentrated again. The residue was taken up in anhydrous DCM (5 mL, then 1 mL to rinse the vial) and added dropwise (effervescence observed) and the reaction stirred at room temperature under nitrogen for 2 h. After this time, the effervescence had ceased and the reaction mixture had become fully dissolved. Then the solvents were removed in vacuo. Anhydrous DCM (10 mL) was added and the reaction concentrated again. The residue was taken up in anhydrous DCM (5 mL, then 1 mL to rinse flask) and added slowly to a solution of 4-chloro-3-nitro-aniline (1.11 g, 6.41 mmol) and pyridine (1.55 mL, 19.22 mmol) in anhydrous DCM (45 mL). The reaction was stirred at room temperature under nitrogen for 2 h. The solvent was removed in vacuo and the resulting residue partitioned between saturated aqueous NaHCO3 solution and 10% MeOH in DCM (30 mL). The aqueous layer was extracted with two further portions of 10% MeOH in DCM (30 mL), the combined organic layer was washed with water, brine, dried (Na2SO4) and concentrated in vacuo, and then dried under high vacuum to afford the crude product as a pale-brown solid (2.24 g).

However, this material was contaminated with 2,3-dihydro-1,4-benzodioxin-6-carboxylic acid, therefore the solid was suspended in a small amount of MeOH and dried under high vacuum to afford the crude product as a pale-brown solid (2.42 g). Purification by Biotage chromatography using a gradient of 0–10% MeOH in DCM + 1% 7N NH3 in MeOH afforded the title compound as a yellow amorphous solid (297 mg, 48%). H NMR (500 MHz, CDCl3) δ 8.67 (s, 1H), 8.56 (s, 1H), 8.52 (s, 1H), 8.27, 8.25 (dd, J = 8.8, 2.8 Hz, 1H), 7.76–7.71 (m, 4H), 7.58–7.47 (m, 3H), 7.00 (d, J = 8.4 Hz, 1H), 4.40–4.33 (m, 4H), 3.76 (s, 2H), 2.74 (t, J = 4.8 Hz, 2H), 1.98–1.83 (br, m, 4H). 13C NMR (126 MHz, DMSO-d6) δ 165.00, 164.64, 161.79, 148.29, 146.57, 142.97, 138.58, 137.45, 134.89, 131.32, 132.90, 132.88, 128.39, 127.85, 127.31, 125.61, 123.54, 121.85, 121.32, 119.81, 119.23, 116.92, 116.74, 65.11, 64.42, 64.03, 54.26, 45.54. HRMS (ESI+): found [C15H12Cl3N3O4]+ 358.0552, C15H12Cl3N3O4 requires 358.0555.

2-(2-(2-(4-(6-(2-Chloro-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-carboxamido)phenyl)carbamoyl)quinolin-2-yl)ethoxy)ethoxy)ethyl 4-Methylbenzenesulfonate 20. N-(2-Chloro-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-carboxamido)phenyl)-2-piperazin-1-ylmethyliquinoline-6-carboxamide 19 (500 mg, 0.90 mmol) was dissolved in anhydrous DCM (8 mL) under nitrogen at room temperature. K2CO3 (372 mg, 2.69 mmol) was added, followed by a solution of 2-(2-hydroxyethoxy)ethoxyethyl 4-methylbenzenesulfonate (573 mg, 1.88 mmol) in anhydrous DCM (2 mL), and the reaction stirred at room temperature overnight. Further 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (286 mg, 0.94 mmol) in anhydrous DCM (1 mL) was added and the reaction stirred overnight. The reaction mixture was poured into water and the aqueous layer extracted three times with 10% MeOH in DCM. The combined organic layers were washed with brine, dried (Na2SO4) and concentrated in vacuo. EtOAc/heptane was added to remove remaining traces of DMF by azetroping. The crude oil was purified by Biotage chromatography using a gradient of 0–10% MeOH in DCM + 1% 7N NH3 in MeOH to afford the title compound as a yellow amorphous solid (297 mg, 48%).
added and the reaction mixture stirred for 3 days under 1 atm H₂. The reaction mixture was filtered through Celite, eluting with 10% MeOH in DCM. The filtrate was concentrated in vacuo and dried under high vacuum to afford the crude product as a pale-brown solid (1.13 g, 93% purity, 77%), which was used directly in the next reaction. A portion of this material (320 mg) was purified by Biotage chromatography using a gradient of 0–5% EtOAc in DCM to afford the title compound as a pale-yellow amorphous solid (198 mg, 52% based on starting material).

\(^1\)H NMR (500 MHz, DMSO-\(d_6\), J = 5.0, 2.7 Hz, 2H), 2.71 (s, 3H). 13C NMR (126 MHz, DMSO-\(d_6\), J = 8.5 Hz, 1H), 6.93 (t, J = 8.0, 1.5 Hz, 1H), 6.91 (t, J = 7.8 Hz, 1H), 6.81 (d, J = 8.6, 2.3 Hz, 1H), 5.37 (s, 2H), 4.39–4.32 (m, 2H), 4.31–4.27 (m, 2H). 13C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 136.77, 144.72, 143.63, 141.13, 138.48, 128.82, 125.80, 121.21, 120.70, 118.98, 111.77, 108.78, 106.29, 64.43, 63.74. HRMS (ESI\(^+\)): calcd for C\(_{15}\)H\(_{14}\)Cl\(_2\)N\(_2\)O\(_3\) \(\text{M} + \text{H}\)\(^+\) 305.0687, found 305.0688.

\(N\)-(2-Chloro-5-(2,3-dihydrobenz[b][1,4]dioxine-5-carboxamido)phenyl)-2-methylquinoline-6-carboxamide. 2-Methylquinoline-6-carboxylic acid (685 mg, 3.66 mmol) was dissolved in anhydrous DCM (24 mL) at room temperature under inert atmosphere and 1 drop anhydrous DMF added. Then oxalyl chloride (0.31 mL, 3.66 mmol) was added dropwise (ervescence observed) and the reaction stirred at room temperature for 2 h, over which time ervescence ceased and the solution became dark green. The solvent was removed in vacuo. The resulting residue was redissolved in anhydrous DCM (2 mL) and concentrated in vacuo (\(\times\)2). The solid was taken up in anhydrous DCM (1 mL, then 1 mL to rinse and concentrated in vacuo (\(\times\)3)). The resulting residue was redissolved in anhydrous DCM (2 mL) at room temperature under inert atmosphere. The reaction was stirred overnight. A precipitate formed which was isolated by filtration, washed with water (\(\times\)2) and ether, and then dried under vacuum to afford the product as a beige amorphous solid (672 mg, 58%).

\(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.35 (s, 1H), 10.32 (s, 1H), 8.71–8.54 (m, 1H), 8.49 (d, J = 8.5 Hz, 1H), 8.26 (dd, J = 8.8, 1.8 Hz, 1H), 8.09 (app. d, J = 8.6, 2.9 Hz), 7.74 (d, J = 8.5 Hz, 1H), 7.68 (dd, J = 8.8, 2.3 Hz, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.14 (dd, J = 7.6, 1.3 Hz, 1H), 7.02 (dd, J = 8.0, 1.5 Hz, 1H), 6.93 (t, J = 7.8 Hz, 1H), 4.42–4.34 (m, 2H), 4.33–4.29 (m, 2H), 3.77 (s, 2H), 2.76 (t, J = 4.5 Hz, 4H), 2.42 (br s, 4H) (1 proton missing). 13C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 165.00, 164.24, 162.71, 143.80, 141.67, 141.20, 138.27, 132.23, 135.32, 130.87, 129.53, 128.46, 128.44, 127.85, 125.51, 125.39, 123.79, 123.07, 121.19, 120.76, 119.31, 119.19, 118.67, 64.43, 63.77, 20.53. HRMS (ESI\(^+\)): calcd for C\(_{26}\)H\(_{21}\)Cl\(_2\)N\(_2\)O\(_3\) \(\text{M} + \text{H}\)\(^+\) 558.1903, found 558.1890.

\(N\)-(2-Chloro-5-(2,3-dihydrobenz[b][1,4]dioxine-5-carboxamido)phenyl)-2-(4-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)piperazin-1-yl)methylquinoline-6-carboxamide. \(N\)-(2-Chloro-5-(2,3-dihydrobenz[b][1,4]dioxine-5-carboxamido)phenyl)-2-piperazin-1-yl)methylquinoline-6-carboxamide (248 mg, 0.440 mmol) was dissolved in anhydrous DCM (3 mL) at room temperature under inert atmosphere, and KCO\(_3\) (371 mg, 2.69 mmol) was added. Then a portion of the crude product as a pale-brown solid (1.13 g, 93% purity, 77%), which was used directly in the next reaction. A solution of 2-(2-hydroxyethoxy)ethoxyethyl 4-methylbenzenesulfonate (573 mg, 1.88 mmol) in anhydrous DCM (1 mL) was added and reaction stirred overnight. The reaction was diluted with water and the aqueous layer extracted (\(\times\)3) with 10% MeOH in DCM. The combined organic layer was washed with brine and dried (Na\(_2\)SO\(_4\)). Purification by Biotage chromatography using a gradient of 0–10% MeOH in DCM + 1% 7N NH\(_3\) in MeOH gave the title compound as a pale-yellow amorphous solid (253 mg, 43% over 3 steps).

1H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.35 (s, 1H), 10.32 (s, 1H), 8.71–8.54 (m, 1H), 8.49 (d, J = 8.5 Hz, 1H), 8.26 (dd, J = 8.8, 1.8 Hz, 1H), 8.09 (app. d, J = 8.6, 2.9 Hz), 7.74 (d, J = 8.5 Hz, 1H), 7.68 (dd, J = 8.8, 2.3 Hz, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.14 (dd, J = 7.6, 1.3 Hz, 1H), 7.02 (dd, J = 8.0, 1.5 Hz, 1H), 6.93 (t, J = 7.8 Hz, 1H), 4.42–4.34 (m, 2H), 4.33–4.29 (m, 2H), 3.77 (s, 2H), 2.76 (t, J = 4.5 Hz, 4H), 2.42 (br s, 4H) (1 proton missing). 13C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 165.00, 164.24, 162.71, 143.80, 141.67, 141.20, 138.27, 132.23, 135.32, 130.87, 129.48, 128.12, 128.44, 127.85, 125.50, 123.78, 121.86, 121.19, 120.76, 119.32, 119.19, 118.70, 65.02, 64.47, 63.77, 53.94, 48.60, 45.37 (1 missing/overlapping signal). HRMS (ESI\(^+\)): calcd for C\(_{30}\)H\(_{29}\)Cl\(_2\)N\(_2\)O\(_3\) \(\text{M} + \text{H}\)\(^+\) 558.1903, found 558.1890.
The reaction was allowed to stir overnight, and then NaBH(OAc)$_3$ (1.34 g, suspended in anhydrous DCM (20 mL) at room temperature under phenyl)-2-formylquinoline-6-carboxamide (1.03 g, 2.11 mmol) was added, and the reaction was degassed via 3 vacuum/nitrogen cycles and stirred at 50 °C under nitrogen for 3.5 h. Further selenium dioxide (47 mg, 0.42 mmol) was added and the reaction stirred at 50 °C for an additional 2 h. The reaction mixture was cooled, filtered through Celite (eluting with DCM/EtOAc), and concentrated in vacuo, using EtOAc/ pentane azetropes to remove DMF. This material was used without further purification, assuming 100% yield.

N-(2-Chloro-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-carboxamido)phenyl)-2-(4-ethylpirazin-1-yl)methylquinoline-6-carboxamide ($\text{C}_{32}\text{H}_{33}\text{N}_{3}\text{O}_{4}$) was prepared by semipreparative HPLC to give the desired product as an amorphous yellow solid (0.60 mg, 0.0013 mmol) dissolved in 20 μL of triethylamine and 300 μL of DMF, and the deep-blue solution was left to stand for 16 h. The crude product was then purified by semipreparative HPLC to give a desired product. LCMs (ESI$^+$) RT = 2.59 min, 77%, M + H$^+$ 1043.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01406.

Full chemistry experimental, NMR spectra of final compounds, physicochemical properties and PDP stability experimental, PDP design pictures, biochemical experimental, biological experiment, proteomics experimental, additional information, proteomics data (PDF)

**SMILES molecular formula strings (CSV)**

**AUTHOR INFORMATION**

**Corresponding Authors**

*For M.D.C.: phone, (+44) 208 722 4168; E-mail, matthew.cheeseman@icr.ac.uk.*

*For P.A.C.: E-mail, paul.clarke@icr.ac.uk.*

*For P.W.: E-mail, paul.workman@icr.ac.uk.*

*For K.J.: E-mail, keith.jones@icr.ac.uk.*

**ORCID**

Nicola E. A. Chessum: 0000-0003-4125-320X

A. Elisa Pasqua: 0000-0002-7966-4672

Birgit Wilding: 0000-0002-1896-3708

Ian Collins: 0000-0002-8143-8498

Bugra Ozer: 0000-0002-1441-4162

Mark Stubbs: 0000-0001-7855-9435

Rosemary Burke: 0000-0002-0111-9701

Paul A. Clarke: 0000-0001-9342-1290

Paul Workman: 0000-0003-1659-3034

Matthew D. Chesseman: 0000-0003-1123-6985

Keith Jones: 0000-0002-9440-4094

**Author Contributions**

N.E.A.C. and S.Y.S. contributed equally. S.Y.S. carried out in vitro cellular biology experiments. M.R. and designed the physicochemical and stability analysis of compounds. P.C.M. expressed and purified the recombinant proteins. B.O. analyzed proteomics data. M.R., M.S., and R.B. designed and carried out biochemical experiments. N.E.A.C., S.Y.S., P.C., P.W., M.D.C., and K.J. contributed to the design of compounds. M.R. ran and designed the physicochemical and stability analysis of compounds. P.C.M. expressed and purified the recombinant proteins. B.O. analyzed proteomics data. M.R., M.S., and R.B. designed and carried out biochemical experiments. N.E.A.C., S.Y.S., P.C., P.W., M.D.C., and K.J. designed studies and interpreted results. N.E.A.C. and M.D.C. wrote the manuscript. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare the following competing financial interest(s): The Institute of Cancer Research has a potential financial interest in ligands of pinir and operates a Rewards to Discoverers scheme.

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**ABBREVIATIONS USED**

SAR, structure–activity relationships; CETSA, cellular thermal shift assay; ABPP, activity-based protein profiling; PROTAC, proteolysis targeting chimera; SNIPER, specific and nongenetic IAP-dependent protein eraser; FDP, protein degradation probe; SPR, surface plasmon resonance spectroscopy; SEM, standard error of the mean; HPLC, high-performance liquid chromatography; KS, kinetic solubility; IFSA, topological polar surface area; DMSO, dimethyl sulfoxide; HSF1, heat shock transcription factor 1; BRD4, bromodomain-containing protein 4; VHL, Von Hippel–Lindau disease tumor suppressor; IAP, inhibitors of apoptosis proteins; CRBN, protein cereblon; PPI, protein–protein interaction; TBAF, tetra-n-butylammonium fluoride; HDD, hydrogen bond donor; TMT, tandem mass tagging MS2, MS/MS mass spectrometry; DTBAD, di-tetra-butylazocarboxylate; THF, tetrahydrofuran; DCM, dichloromethane; RT, room temperature; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA, diisopropylethylamine; DMP, dimethylformamide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; SF, significant figures.

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