Discovery of a Chemical Probe Bisamide (CCT251236): An Orally Bioavailable Efficacious Pirin Ligand from a Heat Shock Transcription Factor 1 (HSF1) Phenotypic Screen

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Supporting Information

ABSTRACT: Phenotypic screens, which focus on measuring and quantifying discrete cellular changes rather than affinity for individual recombinant proteins, have recently attracted renewed interest as an efficient strategy for drug discovery. In this article, we describe the discovery of a new chemical probe, bisamide (CCT251236), identified using an unbiased phenotypic screen to detect inhibitors of the HSF1 stress pathway. The chemical probe is orally bioavailable and displays efficacy in a human ovarian carcinoma xenograft model. By developing cell-based SAR and using chemical proteomics, we identified pirin as a high affinity molecular target, which was confirmed by SPR and crystallography.

INTRODUCTION

Despite the recent extraordinary progress seen in cancer therapy using molecularly targeted drugs, the disease commonly remains resistant to effective long-term treatment. Even when excellent responses to drugs are initially observed, resistance is almost inevitable and patients are then left with few treatment options as the discovery of targeted therapies in oncology has focused on relatively few protein families. To break this cycle and expand the treatment options for cancer patients, new approaches are needed to discover novel druggable protein targets.

In phenotypic screens, small molecules undergo high-throughput screening against intact cells, rather than recombinant proteins, and discrete phenotypic changes in the cell are measured and quantified. Interest in phenotypic screens has increased significantly in recent years due to their potential to effectively discover new drugs. Phenotypic screens have several advantages over screens using recombinant proteins. Hits from a phenotypic screen will, by definition, be cell permeable and have cellular activity, potentially reducing optimization cycles and timelines. Also, because the screening approach is unbiased, established knowledge of the biology of molecular targets is not required. Finally, polypharmacology is often observed with small molecules and structurally related protein families; this can be crucial for efficacy and is perfectly compatible with a phenotypic screening approach.

In contrast, progressing hits from a phenotypic screen can generate a number of unique challenges. Discovering pharmacodynamic (PD) biomarkers in vivo for use in animals can be difficult when developing hits from phenotypic screens, as the pathways commonly need to be activated with an external stimulus. Cell-based screens are typically more expensive and time-consuming and so may require a greater commitment prior to the screening campaign. Furthermore, molecular target identification, deconvolution, and validation are crucial steps if new chemical probes and drugs are to be discovered. These are often a bottleneck in phenotypic screening. Even with successful target deconvolution, the target discovered may not be of interest; for example, the target may already be drugged or be a known antitarget. Finally, polypharmacology may be a serious impediment to compound progression because the interaction with multiple structurally related protein targets may prove impossible to deconvolute. These challenges alter the balance between prioritizing druglike properties of compounds and an efficient target identification strategy.

To execute a successful phenotypic screening campaign, it is critical to select an appropriate phenotype for small molecule intervention. HSF1 is a transcription factor and the master
regulator of the ancient, canonical heat shock response. A large body of work has verified the importance of HSF1 to tumorigenesis and cancer progression. HSF1 has been proposed to be activated by various elements of the cancer state, potentially reprogramming the transcriptome in a way that is overlapping with, but distinct from, the heat shock response. Also, a strong correlation has been reported between the expression of activated HSF1 in tumors and adverse clinical outcomes. This evidence indicates that the inhibition of HSF1-mediated transcription could be a viable strategy in cancer treatment. Moreover, inhibiting the HSF1 stress pathway would represent an attempt at targeting non-oncogene addiction and proteotoxic stress, which has been proposed to be advantageous. However, HSF1 is a ligandless transcription factor and so is unlikely to be amenable to standard drug discovery strategies and direct inhibition with small molecules. Therefore, we proposed that an inhibitor of HSF1-mediated transcription, which antagonized the HSF1 pathway but without necessarily binding directly to HSF1, could be discovered and developed via a cell-based phenotypic screen.

■ RESULTS

HSF1 Phenotypic Assay. To observe HSF1-mediated transcription in an in vitro setting, the HSF1 pathway is activated by a validated heat shock protein 90 (HSP90) inhibitor, or another form of external stress, which initiates the heat shock response. Commonly, the output of the heat-shock response is quantified by measuring the induction of heat shock 70 kDa protein 1 (HSP72) expression, the stress-inducible HSP70 isoform. HSF1 pathway inhibitors are then defined by their ability to block the induction of HSP72. Several HSF1-mediated HSP72 induction inhibitors have been discovered via this method with different proposed molecular mechanisms of action (Figure 1).

With the aim of discovering novel hit-matter that inhibits HSF1-mediated transcription, we previously carried out a cell-based high-throughput phenotypic screen in U2OS human osteosarcoma cells of ~200000 compounds, including ~35000 compounds from a kinase-focused library. The screen quantified the inhibition of HSP72 induction using the Arrayscan assay following treatment with the HSP90 inhibitor tanespimycin (17-AAG).

The Bisamide Series. Using this screen, we identified a potent hit from the kinase-focused deck, bisamide 1 (CCT245232) (Figure 2). Following resynthesis, the screening hit was confirmed and displayed a pIC_{50} = 8.55 ± 0.09 (IC_{50} = 2.8 nM, n = 49) in our HSP72 cell-based enzyme-linked immunosorbent assay (ELISA) in U2OS cells. The HSP72 cell-based ELISA assay is an alternative assay format to the Arrayscan assay for quantifying the induced expression of HSP72 and was used as our primary phenotypic pathway assay throughout the study. The IC_{50} was defined as the concentration that inhibited the signal to 50% of the 17-AAG (250 nM) induced HSP72 expression, relative to the control 17-AAG alone (see Supporting Information for details). The clear structural feature defining this chemotype was the N,N′-4-methyl-1,3-phenylenediamide core (Figure 2).

We had previously demonstrated that pan cyclin-dependent kinase (CDK) inhibitors, and potent CDK9 inhibitors in particular that act as transcription antagonists, can inhibit the HSF1-mediated HSP72 induction phenotype; we initially suspected a similar mechanism for this chemotype. However, upon biochemical screening of bisamide 1 against CDK2 and CDK9, no inhibition was observed (<10% inhibition at 1 μM, see Supporting Information). Therefore, we hypothesized that bisamide 1 was acting through a different mechanism of action and consequently 1 was submitted for further characterization.

Hit Characterization: Kinase Activity. HSF1 is regulated by multiple post-translational phosphorylations, so we hypothesized that kinase inhibition, other than CDK2 and CDK9, was causing the observed HSF1-mediated HSP72 induction inhibition. Bisamide 1 was screened against a broad kinase set using the KINOMEscan biochemical screening platform, a binding assay which gave single-point percentage inhibition values at 1 μM (DiscoverX, http://www.discoverx.

Figure 1. Inhibitors of HSF1-mediated HSP72 induction.

Figure 2. HSF1 pathway inhibitor, bisamide 1.
inhibit cancer cell growth. Because our biochemical kinase screening had failed to reveal two primary aims to our medicinal chemistry strategy. First, for the cellular activity of the bisamide chemotype. There were matched pair changes to establish which features were crucial pathway with an HSP90 inhibitor was not required for ranging anticancer activity and that activation of the HSF1

\[ \text{pGI}_{50} = 7.74 \pm 0.08 \text{ (GI}_{50} = 18 \text{ nM, n = 13), when compared to vehicle.} \]

To assess the broader single agent cellular activity of I, the compound was assayed for growth inhibition against a large, genetically diverse panel of human cancer cell lines (Genomics of Drug Sensitivity in Cancer, www.cancerrxgene.org). Of the 635 cell lines assayed, 628 (99%) displayed a pGI50 > 6 (GI50 < 10 nM), indicating that BRAF inhibition was not an important contributor to the observed HSF1 transcription inhibition phenotype (see Supporting Information).

**Hit Characterization: Cellular.** To establish whether bisamide I displays cellular activity without activation of the heat shock response using the HSP90 inhibitor 17-AAG, the growth inhibitory effects were assayed in the U2OS cell line using the CellTiter Blue (CTB) assay (Promega). Following 4 days of treatment, bisamide I displayed a highly potent pGI50 = 7.74 ± 0.08 (GI50 = 18 nM, n = 13), when compared to vehicle.

**Cell-Based SAR.** Given the complexity of factors underlying cell-based structure-activity relationships (SAR), we focused on matched pair changes to establish which features were crucial for the cellular activity of the bisamide chemotype. There were two primary aims to our medicinal chemistry strategy. First, because our biochemical kinase screening had failed to reveal any potential kinase targets, a broader target identification strategy would need to be developed. Second, although bisamide I had excellent cellular potency, the compound had poor aqueous solubility (kinetic solubility <2 μM), which would need to be addressed to validate HSF1 pathway inhibition as an anticancer strategy in an in vivo human tumor xenograft model. Both goals required us to improve our understanding of the cellular SAR, particularly to identify a vector-to-solvent through which either a chemical probe linker or a solubilizing group could be attached, without disrupting the ability of the compound to bind to the primary pharmacological target or targets.

To develop the cell-based SAR, we switched to the human ovarian carcinoma cell line SK-OV-3, which was relatively sensitive to growth inhibition and is commonly used in translational drug discovery both in vitro and in vivo. When the activity of bisamide I in the SK-OV-3 cell line was assayed, it gave a pIC50 = 7.17 ± 0.07 (IC50 = 68 nM, n = 4) using the HSP72 cell-based ELISA assay induced with 250 nM 17-AAG. Bisamide I also inhibited the proliferation of SK-OV-3 cells in the CTB assay with a pGI50 = 8.08 ± 0.12 (GI50 = 8.4 nM, n = 12).

We first focused the exploration on the benzodioxane motif (Scheme 1). The left-hand ring system analogues (Table 1, entries 1–6) were synthesized via a three-step procedure from the commercially available 2-methyl-5-nitroaniline 2. Following amide bond formation, through the reaction of 2-methyl-5-nitroaniline 2 with 2-methylquinoline-6-carboxylic acid 3 and subsequent reduction of the nitro-group using iron, the second amide bond was generated using the corresponding carboxylic acid in a HATU-mediated coupling to afford the various benzodioxide analogues in good to moderate yields.

Changing the 5-substituted dioxane isomer 1 (Table 1, entry 1) to the 6-substituted dioxane isomer 6 (Table 1, entry 2) resulted in a complete loss of cellular activity (pIC50 < 5), indicating that the isomer of the benzodioxane was crucial. The diacetyl dimethoxy analogue 7 (Table 1, entry 3) also displayed a complete loss of cellular activity. Next, we investigated the role of the two oxygen atoms of the dioxane ring. Removing the para-oxygen of the dioxane to give the meta-chroman 8 (Table 1, entry 4) resulted in a modest 5-fold decrease in cellular HSF1-mediated HSP72 induction inhibition and a 7-fold decrease in growth inhibition compared to I; while the para-chroman isomer 9 (Table 1, entry 5) displayed a further 6-fold decrease in HSF1-mediated HSP72 induction inhibition and an 8-fold decrease in growth inhibition compared to 8, which represents a 37- and 51-fold decrease in cellular activity respectively compared to the original hit, bisamide I. Surprisingly, isochroman 10 (Table 1, entry 6) displayed no measurable cellular activity. From these results, it was clear that the benzodioxane moiety was crucial for the cellular activity of the bisamide series. Because the SAR surrounding the benzodioxane was steep and complex, we decided that this region was unlikely to be solvent exposed and so would be incompatible with linker and solubilizing group attachment.

Second, we investigated the role of the central ring and amide moieties in the cellular activity of the bisamide chemotype. We hypothesized that the amide groups could play an important role in hydrogen bonding with a potential target and in controlling the overall shape of the ligand, while substitution at the central ring could lead to the discovery of solvent-exposed vectors. The synthesis of these analogues...
Table 1. Bisamide Analogues

| Entry | Compd | Structures | SK-OV-3 pIC50±SEM (IC50 nM) | SK-OV-3 pGI50±SEM (GI50 nM) | Pirin SPR pIC50±SEM (Kd nM) |
|-------|-------|-----------|-----------------------------|-----------------------------|-----------------------------|
| 1     | 1     | ![Structure](image1) | 7.17±0.07 (68 nM, n=4)     | 8.08±0.12 (8.3 nM, n=12)   | 7.42±0.03 (38 nM, n=3)     |
| 2     | 6     | ![Structure](image2) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | ND                         |
| 3     | 7     | ![Structure](image3) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | <6.00 (>1000 nM, n=3)     |
| 4     | 8     | ![Structure](image4) | 6.35±0.07 (450 nM, n=3)   | 7.26±0.09 (55 nM, n=3)    | 7.57±0.01 (27 nM, n=3)    |
| 5     | 9     | ![Structure](image5) | 5.59±0.09 (2600 nM, n=4)  | 6.37±0.12 (430 nM, n=5)   | 7.74±0.03 (18 nM, n=3)   |
| 6     | 10    | ![Structure](image6) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | 7.57±0.03 (27 nM, n=3)   |
| 7     | 11    | ![Structure](image7) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | ND                         |
| 8     | 12    | ![Structure](image8) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | ND                         |
| 9     | 13    | ![Structure](image9) | <5.00 (>10000 nM, n=2)    | <6.00 (>10000 nM, n=2)   | <6.00 (>10000 nM, n=3)   |
| 10    | 14    | ![Structure](image10) | <5.00 (>10000 nM, n=2)    | <6.00 (>1000 nM, n=2)     | ND                         |
| 11    | 15    | ![Structure](image11) | 7.92±0.05 (12 nM, n=5)    | 8.60±0.11 (8.6 nM, n=4)   | ND                         |
| 12    | 16    | ![Structure](image12) | 7.29±0.23 (51 nM, n=6)    | 8.29±0.05 (5.1 nM, n=3)   | ND                         |
| 13    | 17    | ![Structure](image13) | 6.65±0.09 (220 nM, n=6)   | 8.05±0.08 (8.9 nM, n=7)   | 7.42±0.00 (38 nM, n=3)   |

*All results are quoted as the geometric mean ± SEM, pIC50/pGI50/pKd = -log IC50/GI50/Kd (M). The number of repeats, n, are described in parentheses. ND = not determined. *c* Cell-based ELISA assay for inhibition of HSP72 induction; SK-OV-3 cells were pretreated with compound at the relevant concentration for 1 h before the addition of 250 nM 17-AAG. HSP72 levels were then quantified after 18 h. *d* Growth inhibition was measured after 96 h of treatment and compared to vehicle control. *c* pKd values were measured through analysis of the sensorgram at equilibrium where possible. Values were then fitted to a one-site specific binding model using Graphpad Prism Version 6.

(Table 1, entries 7–10) was similar to that described in Scheme 1. Substitution at both the 6-position (Table 1, entry 7) and the 4-position (Table 1, entry 8) of the central benzene ring resulted in a complete loss of cellular activity, suggesting that neither of these vectors represented a viable route to solvent. Owing to the potential for the toluene methyl group to affect the conformation of the right-hand amide, we decided to leave this group unchanged. N-Methyl substitution of the left-hand amide (Table 1, entry 9) and an attempted sulfonamide bioisosteric replacement (Table 1, entry 10) also resulted in a complete loss of cellular activity. Again, owing to the steep SAR in this region of the chemotype, it was clearly incompatible with linker and solubilizing group attachment.

Finally, we examined the role of the 2-methylquinoline ring in the cellular activity of the bisamide series. All analogues (Table 1, entries 11–13) were synthesized via a method similar to that described in Scheme 1. First, we removed the 2-methyl group, which is both lipophilic and has the potential to be a weak hydrogen bond donor, to give quinoline 15 (Table 1, entry 11). Removal of this group resulted in no significant change in the cellular activity when compared to 1 (Table 1, entry 1). Next we moved the quinoline nitrogen from the 1- to the 3-position to give isoquinoline 16 (Table 1, entry 12). Again, no significant change in cellular activity was observed when compared to bisamide 1. Finally, we partially reduced the quinoline ring to give tetrahydroquinoline 17 (Table 1, entry 13). By changing to tetrahydroquinoline 17, the nitrogen atom now acts as a hydrogen bond donor rather than an acceptor. Despite this reversal in anticipated binding properties, only a 3.5-fold decrease in HSF1-mediated HSP72 induction inhibition and no significant change in antiproliferative activity was observed when compared to bisamide 1. The broad SAR of the quinoline region of the molecule suggested that this moiety is solvent exposed and so could be exploited for solubilizing group and linker attachment.
Exploiting Vectors-to-Solvent: Solubilizing Group Optimization. Owing to the complexities of the cellular SAR, attaching a solubilizing group to the solvent-exposed region of the ligand was considered a more expeditious strategy to generate an in vivo chemical probe suitable for use in animal models rather than attempting to optimize the intrinsic solubility of the bisamide series without insight from structural information. To address this objective, we adopted an iterative strategy to exploit the rapid synthesis of analogues that would exhibit improved physicochemical properties and demonstrate the appropriate mouse pharmacokinetic (PK) parameters for in vivo study (Scheme 2).

The optimal route to the synthesis of these analogues would be to introduce the solubilizing group via nucleophilic aromatic substitution.

**Scheme 2. Synthesis of Solubilizing Group Analogues**

**Table 2. Optimization of the Bisamide Solubilizing Group**

| Entry | Compd | Sol. Group | SK-OV-3 pGI50SEM (GL_{100}, n) | MLM Cl_{in} (μL/min/mg) | KS (μM) | LogD_s, e | Calculated pK_a (MoKa) f |
|-------|-------|------------|-------------------------------|------------------------|--------|----------|-----------------------|
| 1     | 1     |            | 8.08±0.12 (8.3 nM, n=12)      | 35                     | 1      | 2.6      | NA                    |
| 2     | 21    |            | 8.37±0.06 (4.3 nM, n=3)       | 92                     | 5      | 3.3      | NA                    |
| 3     | 22    |            | 8.94±0.10 (1.1 nM, n=4)       | 170                    | 90     | 2.0      | 9.5                   |
| 4     | 23    |            | 8.13±0.18 (7.4 nM, n=4)       | 76                     | 7      | 2.9      | 7.7                   |
| 5     | 24    |            | 8.87±0.09 (1.3 nM, n=8)       | 24                     | 40     | 2.4      | 9.7                   |
| 6     | 25    |            | 8.97±0.03 (1.1 nM, n=6)       | 57                     | 30     | 2.5      | 8.8                   |
| 7     | 26    |            | 8.65±0.10 (2.2 nM, n=25)      | 23                     | 70     | 2.1      | 8.9                   |

aReagents and conditions: (i) oxalyl chloride, DMF, 1,4-benzodioxane-6-carboxylic acid, DCM, then, 4-methyl-3-nitroaniline, pyridine, DCM, RT, then Pd/C (10%), H_2 (1 atm), EtOH; (ii) NaH, RCH_2OH, THF, 0 °C to reflux; (iii) n-BuLi, CO_2(s), THF, −78 °C; (iv) HATU, DIPEA, DMF, RT.

bReagents and conditions: (i) oxalyl chloride, DMF, 1,4-benzodioxane-6-carboxylic acid, DCM, then, 4-methyl-3-nitroaniline, pyridine, DCM, RT, then Pd/C (10%), H_2 (1 atm), EtOH; (ii) NaH, RCH_2OH, THF, 0 °C to reflux; (iii) n-BuLi, CO_2(s), THF, −78 °C; (iv) HATU, DIPEA, DMF, RT.

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Moving to piperidine to balance the physicochemical properties of the compound. 

suggested that the solubilizing group was not sufficient to cause a reduction in lipophilicity required for better balance in physicochemical properties, with the lipophilicity as measured by LogD7.4. To optimize the solubilizing group, we used four in vitro parameters (Table 2).

We first attached the oxygen-linked ether chain to give glycol 21 (Table 2, entry 2). No significant decrease in either HSF1-mediated HSP72 induction inhibition (see Supporting Information) or the antiproliferative activity was observed when compared to lead bisamide 1 (Table 2, entry 1). This suggested first that the oxygen linker had no detrimental effect on activity and, second, that this region of the molecule was indeed solvent exposed. To carry out multiparameter optimization of the solubilizing group, we used four in vitro properties to assess each compound’s potential for in vivo mouse PK: microsomal stability, kinetic solubility at pH 7.4, lipophilicity as measured by LogD7.4, and the predicted basicity of the solubilizing group. The glycol 21 displayed an increase in lipophilicity compared to lead bisamide 1; this increase was reflected in the 2.4-fold decrease in microsomal stability with a modest increase in kinetic solubility. However, glycol 21 remained a low solubility compound, which is inconsistent with good oral bioavailability. From these data, we concluded that the compound would need to be charged to deliver the correct balance of properties. To introduce a charged moiety, we first focused on the dimethylamine group with a 3-carbon linker. Dimethylamine 22 (Table 2, entry 3) displayed no significant change in cellular activity despite this analogue being predominantly charged at physiological pH according to the calculated pK_a. Consistent with its cationic character, dimethylamine 22 displayed a 1.4 log unit decrease in lipophilicity when compared to 21, which was accompanied by an 18-fold improvement in kinetic solubility. Unfortunately, the decrease in lipophilicity was not reflected in an improvement in microsomal stability. We hypothesized that this was due to oxidation of the N-methyl groups, so we moved to cyclic amines to reduce the CYP450 mediated degradation. Morpholine 23 (Table 2, entry 4) showed little improvement in microsomal stability or kinetic solubility when compared to 21. Analysis of the LogD7.4 and calculated pK_a for morpholine 23 suggested that the solubilizing group was not sufficiently basic to balance the physicochemical properties of the compound. Moving to piperidine 24 (Table 2, entry 5), we observed a better balance in physicochemical properties, with the reduction in lipophilicity reflected in improvements in both microsomal stability and kinetic solubility. However, piperidine 24 was predicted to be highly basic and we were concerned that this would have a detrimental effect on permeability and therefore on oral bioavailability. To reduce the basicity of the solubilizing group without increasing lipophilicity, we reduced the linker length to give 25 (Table 2, entry 6), thereby exploiting the inductive effect of the oxygen. Despite the 0.9 log unit decrease in predicted basicity, the kinetic solubility and lipophilicity were unchanged. Unfortunately, 25 displayed a decrease in microsomal stability, so we reduced the ring size to give pyrrolidine 26 (CCT251236, Table 2, entry 7), which displayed the desired balance of in vitro properties, while maintaining excellent cellular activity with a pIC50 = 7.73 ± 0.07 (IC50 = 19 nM, n = 15, see Supporting Information, Table S3 for details) for inhibition of HSF1-mediated HSP72 induction. The free GI50 in SK-OV-3 cells was then calculated from the free fraction in the cell assay, which gave a free GI50 = 1.1 nM. Western blotting confirmed that pyrrolidine 26 blocked the HSF1-mediated induction of both HSP72 and HSP27 as representative heat shock proteins, following treatment with the HSP90 inhibitor 17-AAG. Also, qPCR analysis demonstrated that 26 inhibited the induction of HSP72 at the mRNA level, clearly blocking the induction of HSPA1A gene expression with a pIC50 = 7.40 (IC50 = 40 nM, n = 1, see Supporting Information). Taking these results together, pyrrolidine 26 was selected for in vivo study.

**Mouse Pharmacokinetics (PK).** To assess the potential of bisamide 26 as an in vivo chemical tool to study HSF1-mediated transcriptional activity inhibition, the compound was dosed in BALB/c mice at 5 mg/kg as an oral solution and iv bolus. Blood concentrations were then measured over a 24 h period (Table 3).

Analysis of the mouse PK data revealed that bisamide 26 possessed low total blood clearance (10% hepatic blood flow) and moderate oral bioavailability, with a half-life sufficient to allow once-daily dosing. In vitro assessment of the plasma protein binding and the blood to plasma ratio revealed that 26 readily binds to tissues, consistent with the basicity of the compound. Bisamide 26 Displays Efficacy in a Human Ovarian Carcinoma Xenograft Model. Despite the high unbound clearance of bisamide 26 in mouse, the good oral bioavailability, half-life, and excellent in vitro cellular activity (free GI50 = 1.1 nM) encouraged us to test the potential efficacy of 26 in an in vivo human tumor xenograft model. On the basis of the free C_{av}^0−24h = 2.0 mM observed in nontumor bearing immunocompetent BALB/c mice following the 5 mg/kg po qd dose, a 20 mg/kg po qd dose in immunodeprived athymic mice was selected to cover ~10 times the in vitro free GI50 in SK-OV-3 cells.

To our surprise, following this 20 mg/kg po dose, the free exposure was actually a disappointing AUC_{0−24h} = 29 h·nM, equivalent to a free C_{av}^0−24h = 1.2 mM (Table 3, see Supporting Information for details). Despite the lower than expected free exposure of bisamide 26, this dose still represented coverage of the in vitro free GI50 in SK-OV-3
cells and was well tolerated in a mouse multidose tolerability study, therefore, the 20 mg/kg po qd dose was selected for further investigation.

SK-OV-3 cells were injected subcutaneously into athymic mice for tumor formation. Once tumors were established, the mice were randomized into treatment and control groups and were dosed orally once-a-day with either vehicle or 20 mg/kg of bisamide 26. Tumor volumes and mouse body weights were measured throughout and tumor weights were measured at the end of the study, while total tumor concentrations were measured 2 and 6 h post final dose (Figure 3 and Supporting Information).

![Figure 3](image-url)

**Figure 3.** Efficacy of bisamide 26 against SK-OV-3 human ovarian carcinoma xenograft model. Blue, vehicle control, n = 8; red, 26, 20 mg/kg po qd, n = 8 (vehicle = 10% DMSO, 90% of a 25% (2-hydroxypropyl)-β-cyclodextrin in 50 mM citrate buffer pH 5). Error bars: arithmetic mean ± SEM. Dosing breaks were carried out on days 5–12, 14, 16, 18, 20, 22, 24, 26, 29, 31.

The mice were dosed intermittently throughout the study with 26 to maintain their condition, as assessed through monitoring body weights (see Supporting Information). Clear therapeutic efficacy was observed with bisamide 26, with a tumor growth inhibition (%TGI) of 70% based on final tumor volumes. The study was terminated after 33 days, and comparison of the control and treated arms indicated a 64% reduction in mean tumor weights (p = 0.015) with total tumor concentrations of 26 as high as 940 nM, consistent with the compound’s basicity and high volume of distribution (see Supporting Information).

**Target Identification.** Following the successful efficacy study, the potential for bisamide 26 as a chemical probe to study the effects of HSF1 transcription inhibition, both in vitro and in vivo, were clear. However, the variety of HSF1 transcription inhibitors in the literature suggests there are multiple mechanisms through which this phenotype may be observed, each with differing potential for drug discovery.

To decipher the molecular mechanism of the bisamide series, we needed to discover their protein targets. The potency of the bisamide 26 in cell-based assays suggests that only high affinity efficacy and epistatic targets would be of interest. Our biochemical kinase screening had indicated that there were no high affinity kinase targets. We therefore expanded our biochemical screening to include other protein families. Bisamide 26 was submitted to the Cerep Diversity Screen (Cerep, http://www.cerep.fr) comprising 98 molecular targets, including receptors and enzymes, measured at 10 μM. Analysis of these screening data revealed that bisamide 26 displays a good selectivity profile. Only six targets (the receptors: adenosine A2A and A3, histamine H2 and H3, muscarinic, and the enzyme acetylcholine esterase) displayed >80% inhibition, and these generally represented the highly promiscuous receptor protein targets (see Supporting Information for details). No hits displayed sufficient activity to clearly relate the molecular target to the efficacious free concentrations achieved in vitro or in vivo.

Because no clear protein families had been revealed from our biochemical screening, a different approach was necessary. We decided to exploit a chemical proteomics strategy and using our knowledge of the cellular SAR, we designed a protein pull-down chemical probe to identify high affinity molecular targets from a human cancer cell lysate (Figure 4).

![Figure 4](image-url)

**Figure 4.** Tool compounds for target identification. All quoted cellular activities are in the SK-OV-3 cell line, the numbers of repeats are in parentheses.
Table 4. Molecular Targets from the Pull-down Assay Using the Bisamide Probes in SK-OV-3 Cell Lysate

| Protein | EC_{50} (μM) | AC_{50} (μM) | K_{i} (μM) | AC_{50} (μM) | K_{i} (μM) | AC_{50} (μM) |
|---------|--------------|--------------|------------|--------------|------------|--------------|
| GSK3β   | 1.3          | 1.2          | NA         | 10           | NA         | 10           |
| Pirin   | 1.3          | 1.5          | 0.028      | 10           | 0.19       | NA           |
| PDE6D   | 26           | 17           | 28         | NA           | NA         | NA           |

(A) SK-OV-3 cell lysate protein binding curves for immobilized 29. (B) Protein displacement curves for pyrrolidine 26. Curves are an average of two different mixing conditions. EC_{50} and AC_{50} are determined from analysis of the curves without limits. EC_{50} is the apparent immobilized bisamide probe concentration at which 50% of the available protein is bound. The probe bisamide is assumed to be >10-fold molar excess over proteins in the lysate. AC_{50} is the apparent bisamide concentration at which 50% of the protein is displaced from the immobilized bisamide probe.

Supporting Information) had demonstrated that the bisamide series have no measurable affinity for this kinase; we therefore concluded that GSK3β was potentially an indirect target of the bisamide series. The final putative protein target was pirin. The binding curve for pirin to the bead-bound bisamide probe was quite shallow; however, the displacement curves for pirin, which were very similar to the displacement curves for GSK3β with both cell active probe ligands, gave robust data with an apparent K_{i} = 28 nM for 26 and no affinity was observed with the inactive control. Using this method on a SK-OV-3 cell lysate, pirin emerged as the only remaining putative target of the bisamide series.

Pirin Validation. Very little is known about pirin, and few papers have been published discussing its possible function. Pirin was first identified in 1997 by Wendler et al. from a yeast two-hybrid screen to discover interactors of the transcription factor NFI/CTF1. Pirin was described as an iron-binding, metal-dependent protein, predominately localized within the nucleus, highly conserved across species, and ubiquitously expressed in all tissue types. Following this initial discovery, Scheidereit et al. reported that human pirin interacts with the proto-oncoprotein, BCL3, linking pirin with the NFκB pathway. The role of pirin in the NFκB pathway was further demonstrated by Lui et al.; using SPR, they revealed the metal-dependent formation of a pirin/p65/DNA complex and hypothesized the role of human pirin to be a redox-sensing transcription factor regulator. The redox activity of pirin has previously been discussed, as the expression level of pirin was proposed to be under the control of the transcription factor NRF2 through changes in cellular oxidative stress. Wang et al. previously reported that human pirin interacts with the tumor suppressor protein EAF2/U19 and that exogenous over-expression of pirin increased colony formation in LNCaP human prostate cancer cells. In the human melanoma cell line WM266.4, Alcalay et al. demonstrated that shRNA knockdown of pirin could induce a senescence phenotype and suppress colony formation. Also in the WM266.4 cell-line, Osada et al. demonstrated that siRNA knockdown of pirin could suppress cell migration. These results are consistent with the observation by Simizu et al. that siRNA knockdown of pirin
suppressed migration of human adenocarcinoma HeLa cells and that pirin was important for epithelial–mesenchymal transition (EMT).63 However, little evidence has emerged of an antiproliferative phenotype from genetic inhibition of pirin expression and our in-house data using siRNA in SK-OV-3 cells was consistent with this finding.64 Nonetheless, because our original screening paradigm was designed to discover inhibitors of transcription, the proposed role of pirin as a redox-sensitive transcription factor regulator was certainly intriguing.

To confirm pirin as a high affinity molecular target of the bisamide series, we needed to assess the affinity of the compounds and establish SAR in an orthogonal assay format. Pirin has no known catalytic function and no known endogenous ligand in mammalian cells, consequently we decided to focus on surface plasmon resonance (SPR) to measure the affinity of the bisamide series for pirin. Purified recombinant human pirin was attached to the SPR chip through a standard amide coupling. The affinity molecular target for the bisamide analogues was selected for further study. We focused on the two other analogues used in target binding. This could be due to the coupling of pirin to the SPR chip impairing protein folding or because pirin is a metal-dependent protein, and the metal was leaching out into the running buffer; both effects could negatively impact the apparent binding affinity and stoichiometry.

To establish the SAR surrounding pirin binding, several bisamide analogues were selected for further study. We first focused on the two other analogues used in target identification. The cell active analogue tetrahydroquinoline 17 (Table 1, entry 13) was also found to display tight-binding affinity for pirin; in contrast, the negative control dioxane isomer 28 (pIC_{50} < 5.00, IC_{50} > 10000 nM, n = 2) displayed no measurable affinity. These data suggested that pirin SAR and the cellular SAR were linked. Increasing the steric bulk of the solubilizing group, as exemplified by piperidine 24 (Table 2, entry 4), again returned a high affinity pirin ligand (pK_{D} = 7.52 ± 0.02, K_{D} = 30 nM, n = 3), consistent with the potent cellular activity. However, structural changes to the benzodioxide amide motif proved more complex. The original hit, bisamide 1 (Table 1, entry 1), was a tight-binding pirin ligand; whereas the cell inactive analogues, methylated amide 13 (Table 1, entry 9) and dimethoxy 7 (Table 1, entry 3), displayed no affinity for pirin. However, the two chroman isomers 8 and 9 (Table 1, Entries 4 and 5) were tight-binding ligands for pirin but their target affinity did not reflect their decrease in cellular activity when compared to bisamide 1. Finally, the isochroman analogue 10 (Table 1, entry 6) was still a high affinity ligand.
for pirin, despite displaying no cellular activity (see Supporting Information, Table S11 for details).

Pirin is a member of the cupin super family of proteins, which are so-called because they all possess a conserved β-barrel. However, due to minimal sequence homology between members of the cupin family, crystallography is often needed to identify them. The cupins display a huge array of functionality, including oxidases and isomerases. To better understand pirin SAR, we determined the crystal structure of efficacious bisamide 26 bound to pirin (Figure 6).

Pirin is a metal-binding bicupin characterized by the two β-barrels that are clearly visible in the crystal structure. Pirin binds a single metal-ion in one of the β-barrels, which is where a deep, small molecule binding pocket is located. The metal-ion is clearly visible in the electron density, although it is unclear which metal-ion is actually bound. The metal-ion binding site is formed of three histidine residues (His56, His58, and His101), a glutamic acid (Glu103) and two water molecules, showing the metal-ion possesses octahedral coordination. Bisamide 26 forms no direct interactions with the metal-ion, instead a water-mediated interaction is created by the two amide functional groups, which form a hydrogen bonding pincer around this metal-coordinated water molecule, revealing why the two amides are crucial for binding. The methyl-distal amide also acts as a hydrogen-bond donor with an aspartic acid (Asp43) at the base of the binding site, explaining why methylation of that group is not tolerated. The methylpyridine ring of the quinoline substituent is clearly solvent exposed and the solubilizing group cannot be resolved due to poor stability in the lysate. The dual targeting of proteins is often observed; for example, CDK4 and CDK6, where simultaneous inhibition of both kinases is necessary to observe an antiproliferative phenotype and owing to the similarity of their respective binding sites, small-molecule inhibitors inevitably inhibit both proteins. RNAi knockdown of pirin by several groups has demonstrated very limited effects on cell proliferation, and as pirin has no known enzymatic function, discovering and validating a cellular biomarker to investigate its role in the antiproliferative phenotype is an ongoing challenge.

One phenotype that has previously been associated with a small-molecule binding to pirin is cancer cell migration. Miyazaki et al. demonstrated that their pirin ligand, TPh A, inhibited the migration of the melanoma cell line, WM266.4. To investigate whether our tool compound, bisamide 26, phenocopied this distinct pirin-ligand chemotype, we decided to investigate its antimigratory activity using a scratch-wound assay. The scratch-wound assay is commonly used to assess the effects of small-molecules on cell migration. Bisamide 26 demonstrated excellent antiproliferative activity against the WM266.4 cell line (pGI_{50} = 7.92 ± 0.10, GI_{50} = 12 nM, n = 11). To exclude the effect of inhibiting proliferation on migration in the scratch-wound assay, the cells were plated at
high confluence and the timeframe of the assay was reduced to 30 h. The relative wound-density was then measured at various concentrations and time-points. The negative control isomer 28 displayed no measurable antimigratory activity in stark contrast to the potent pirin ligand bisamide 26, which was able to strongly inhibit the migration of WM266.4 cells at 100 nM (Figure 7).

■ DISCUSSION

Bisamide 26 displays excellent and wide-ranging in vitro cellular potency, inhibiting both HSF1-mediated transcriptional activity (defined by the inhibition of the induced expression of the heat-shock proteins HSP72 and HSP27 and HSPA1A mRNA) and cancer cell proliferation. Also, 26 has good mouse PK and demonstrated its anticancer effects in vivo at low free exposures with clear tumor growth inhibition at tolerable doses. Kinase screening and broader proteome screening, in addition to the chemical proteomics using SILAC quantified pull-down with rigorous controls and follow-up testing, indicated that pirin was the sole specific protein target that could be identified to date. SPR analysis of the binding of the bisamide series to pirin confirms that these compounds are high affinity ligands, with most aspects of the pirin SAR being consistent with the cellular SAR. However, there was an apparent disconnect surrounding the dioxane group of the chemotype. This requires further investigation to determine whether pirin is crucial for the in vitro antiproliferative activity and in vivo efficacy via an allosteric modulation mechanism or whether binding to a second high affinity protein target is needed to fully account for the observed phenotype. Because the SILAC quantified pull-down assay can only identify protein targets that are stable to cell lysis, additional in-cell target-ID methods are currently under investigation to probe for a potential second target. Nevertheless, the tool compound, bisamide 26, demonstrated the previously proposed anticancer phenotype of pirin ligands by inhibiting the migration of WM266.4 melanoma cells in vitro. The role of pirin in the bisamide phenotype and the cellular effects of modulating the HSF1 pathway with bisamide 26 are currently also under investigation and will be reported subsequently.

■ CONCLUSIONS

Using a high-throughput screen to identify inhibitors of the HSF1-mediated stress pathway, we have discovered an extremely potent inhibitor of human cancer cell proliferation in vitro from the bisamide chemotype. By exploring the SAR from the cellular assays, we designed a chemical probe, bisamide 26, which is highly potent and displays an appropriate mouse pharmacokinetic profile to significantly inhibit growth in a human ovarian carcinoma xenograft model. The chemical probe 26 was also used to design a chemical proteomic pull-down experiment, which identified the putative transcription factor regulator, pirin, as a protein target. The high affinity of chemical probe 26 for pirin was confirmed by SPR. Comparison of the biophysical with the cellular data indicated that active molecules bind pirin but that the cellular SAR is more complex, although 26 did display a potent inhibitory effect on the migration of human melanoma cells, consistent with the putative pirin cancer phenotype. Despite this, we propose that bisamide 26, in combination with the physicochemically matched negative control dioxane isomer 28, are promising chemical probes to investigate the role of HSF1 pathway inhibition and pirin binding in vitro and in vivo.

EXPERIMENTAL SECTION

Experimental Procedures (Chemistry). All final compounds were screened through our in-house computational PAINS filter and gave no structural alerts as potential assay interference compounds. 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 (particle size 40–63 μm). Column chromatography was also performed on a Biotage SP1 purification system using Biotage Flash silica cartridges (SNAP KP-Sili). Ion exchange chromatography was performed using acidic Isolute Flash SCX-II columns. Semi-preparative HPLC was performed on an Agilent 1210 system, flow 20 μL/min, eluents 0.1% acetic acid in water and 0.1% acetic acid in methanol, gradient of 10–100% organic phase. 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 doubles (dd), doublet of doublet of doubles (ddd), broad (br), or obscured (obs). Coupling constants, J, were 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 0.01 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 fitted with a multimode ESI/APCI source. Analytical separation was carried out according to the methods listed below. The mobile phase was a mixture of methanol (solvent A) and water (solvent B), both containing formic acid at 0.1%, UV detection was at 254 nm. Method I: Agilent 1200 series HPLC, Merck Purospher STAR (RP-18e, 30 mm × 4 mm) column using a flow rate of 1.5 mL/min in a 4 min gradient elution. Gradient elution was as follows: 10:90 (A/B) to 90:10 (A/B) over 2.5 min, 90:10 (A/B) for 1 min, and then reversion back to 10:90 (A/B) over 0.3 min, finally 10:90 (A/B) for 0.2 min. Method II: Agilent 1200 series HPLC, Merck Chromolith flash column (RP-18e, 25 mm × 2 mm) at 30 °C using a flow rate of 0.75 mL/min in a 4 min gradient elution. Gradient elution was as follows: 5:95 (A/B) to 100:0 (A/B) over 2.5 min, 100:0 (A/B) for 1 min, and then reversion back to 5:95 (A/B) over 0.1 min, finally 5:95 (A/B) for 0.4 min. Method III: Waters Acquity UPLC, Phenomenex Kinetex XB-C18 column (30 mm × 2.1 mm, 1.7 μm, 100 A) at 30 °C using flow rate of 0.3 mL/min in a 4 min gradient elution. Gradient elution was as follows: 10:90 (A/B) to 90:10 (A/B) over 3 min, 90:10 (A/B) for 0.5 min, and then reversion back to 10:90 (A/B) over 0.3 min, finally 10:90 (A/B) for 0.2 min; Method IV: Waters Acquity UPLC, Phenomenex Kinetex C18 column (30 mm × 2.1 mm, 2.6 μm, 100 A), flow rate and gradient elution according to Method III. The following reference masses were used for HRMS analysis: Agilent 1200 series, caffeine [M + H]+ 195.087652, hexakis[1H,1H,3H-tetrafluoropentyl]phosphazene [M + H]+ 922.007978, and hexakis[2,2-difluoroethoxy]phosphazene [M + H]+ 622.028966 or reserpine [M + H]+ 569.280657. Waters Acquity UPLC: leucine enkephalin fragment ion [M + H]+ 397.1876. All compounds were >95% purity by LC/MS analysis unless otherwise stated.

General Synthetic Procedures. Method A. Oxalyl chloride (1.2–1.7 equiv) was added dropwise to a solution of the carboxylic acid (1.0–1.5 equiv) and DMF in anhydrous dichloromethane. The reaction mixture was stirred at room temperature for 3–4 h and then...
concentrated. The remaining residue was redissolved in dichloromethane and concentrated again. Then, the aniline (1.0 equiv) was added to the remaining residue and the combined solids were dissolved in anhydrous pyridine or a mixture of anhydrous pyridine and dichloromethane. The resulting reaction mixture was stirred at room temperature overnight.

**Method B.** 1-(3-Dimethylamino)pyridin-1-yl-1H-1,2,3-triazolo[4,5-b]pyrimidine-3-oxide hexafluorophosphate (HATU, 1.2–1.3 equiv) was added to a solution of the carbonylic acid (1.0–1.2 equiv) and N,N-diisopropylethylamine (2.0–5.0 equiv) in anhydrous DME. The reaction mixture was stirred for 10 min before the aniline (1.0 equiv) was added. The reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with water. The resulting precipitate was isolated by filtration and washed with water.

**Method C.** The (3-nitrophenyl)amide (1.0 equiv), iron powder (9–11 equiv), and ammonium chloride (9–11 equiv) in ethanol/water (4/1) were added to a round-bottom flask and heated to reflux overnight. Then the reaction mixture was filtered over a short pad of Celite or silica gel. The filtrate was reduced in vacuo until dryness. The remaining residue was resuspended in dichloromethane and washed with satd NaHCO₃ (aq), water, and brine. The organic layer was dried over sodium sulfate or magnesium sulfate and reduced in vacuo until dryness to afford the corresponding (3-aminophenyl)amide.

**Method D.** Palladium (10% on activated carbon, 42–68 mg/1 mmol) was added to a suspension of the nitro compound in a 1:1 mixture of ethanol/ethyl acetate and stirred under H₂ (1 atm.) at 28 °C overnight. The reaction mixture was then filtered through Celite and the Celite pad washed with further ethyl acetate. The filtrate was concentrated in vacuo to afford the corresponding aniline.

**Method E.** NaH (60% in mineral oil, 1.1–2.2 equiv) was added to a solution of the alcohol (1.2–2.8 equiv) in anhydrous THF at 0 °C. The reaction was allowed to stir at 0 °C for 10 min, then at room temperature for 30 min. 6-Bromo-2-chloroquinoline was added and the resulting suspension heated to reflux for 1–16 h. The reaction was allowed to cool to room temperature, then diluted with water/satd NaHCO₃ (aq) and extracted with dichloromethane (3x). The organic layers were combined, washed with water, dried over magnesium sulfate, and concentrated in vacuo to afford the crude product.

**Preparation of Compounds in Table 1.** 2-Methyl-N-(2-methyl-5-nitrophenyl)quinolinoine-6-carboxamide 5. 2-Methyl-N-(2-methyl-5-nitrophenyl)quinolinoine-6-carboxamide 4 (4.0 g, 12.45 mmol), iron powder (6.95 g, 124.0 mmol), and ammonium chloride (2.12 g, 124.0 mmol) in ethanol (50 mL) and water (12.5 mL) were reacted according to method C to afford the product as a light-yellow amorphous solid (1.95 g, 54%). IR (thin film): νmax 3340, 3252, 1645, 1582, 1487, 1278 cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆) δ 9.86 (s, 1H), 8.61–8.51 (m, 1H), 8.38 (d, J = 8.4 Hz, 1H), 8.21 (dd, J = 8.8, 1.7 Hz, 1H), 8.00 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 8.4 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.65 (d, J = 2.0 Hz, 1H), 6.42 (dd, J = 8.1, 2.2 Hz, 1H), 4.94 (s, 2H), 2.70 (s, 3H), 2.09 (s, 3H).

¹³C NMR (126 MHz, DMSO-d₆) δ 165.18, 161.09, 148.86, 147.31, 137.58, 137.01, 132.25, 130.87, 128.72, 128.49, 128.38, 125.84, 123.38, 120.72, 112.75, 112.61, 25.49, 17.50. HRMS (ESI⁺): calcd for C₂₇H₂₄N₃O₄ [M + H]⁺, 454.1751; found, 454.1765.

5-(2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylquinolinoine-6-carboxamide 6. 2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxylic acid (46 mg, 0.26 mmol), HATU (127 mg, 0.34 mmol), and N-(3-amino-2-methylphenyl)quinolinoine-6-carboxamide 5 (75 mg, 0.26 mmol) in N,N-diisopropylethylamine (0.14 mL, 0.82 mmol) and anhydrous DMF (2.5 mL) were reacted according to method B to afford the product as a white amorphous solid (95 mg, 51%). IR (thin film): νmax 3370, 3256, 1644, 1598, 1532, 1450 743 cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆) δ 10.16 (s, 1H), 10.09 (s, 1H), 8.61 (s, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.27–8.21 (m, 2H), 8.03 (d, J = 8.7 Hz, 1H), 7.82 (br s, 1H), 7.58–7.52 (m, 2H), 7.25 (d, J = 8.3 Hz, 1H), 7.18–7.11 (m, 2H), 7.02–6.98 (m, 1H), 6.92 (t, J = 7.8 Hz, 1H), 4.40–4.27 (m, 4H), 2.71 (s, 3H), 2.24 (s, 3H).

¹³C NMR (126 MHz, DMSO-d₆) δ 165.43, 164.26, 161.23, 148.94, 144.11, 141.65, 137.62, 135.72, 136.89, 131.90, 130.74, 129.44, 128.81, 128.67, 128.38, 128.14, 125.85, 123.45, 121.73, 121.17, 119.51, 118.44, 118.00, 64.93, 64.21, 25.51, 17.97. HRMS (ESI⁺): calcd for C₂₉H₂₅N₃O₄ [M + H]⁺, 454.1761; found, 454.1773.

5-(2,3-Dimethylbenzamido)-2-methylquinolinoine-6-carboxamide 7. 3,4-Dimethylbenzamide (34 mg, 0.189 mmol), HATU (82 mg, 0.215 mmol), and N-(3-amino-2-methylphenyl)quinolinoine-6-carboxamide 5 (50 mg, 0.172 mmol) in N,N-diisopropylethylamine (0.075 mL, 0.429 mmol) and anhydrous DMF (1.2 mL) were reacted according to method B. The crude product was purified by flash column chromatography (1–5% methanol in dichloromethane) to afford compound 7 as a white amorphous solid (52 mg, 67%). IR (thin film): νmax 1648, 1601, 1508, 1418, 1268, 1225 cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆) δ 10.13 (s, 1H), 10.09 (s, 1H), 8.61 (d, J = 2.0 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.24 (dd, J = 8.8, 2.0 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.85 (d, J = 2.2 Hz, 1H), 7.62 (d, J = 8.8, 2.2 Hz, 1H), 7.55 (s, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.26 (d, J = 8.5 Hz, 1H), 7.08 (d, J = 8.5 Hz, 1H), 3.85 (s, 3H), 3.84 (s, 3H), 2.71 (s, 3H), 2.26 (s, 3H).

¹³C NMR (126 MHz, DMSO-d₆) δ 165.01, 164.74, 160.76, 151.58, 148.48, 148.26, 137.32, 137.16, 136.27, 131.51, 130.15, 128.75, 128.37, 128.16, 127.91, 126.92, 125.40, 122.98, 121.03, 118.76, 118.32, 110.98, 110.91, 55.67.
4.23 (d, J = 8.4 Hz, 1H), 6.84 (d, J = 8.6 Hz, 1H), 2.64 (dd, J = 8.8, 1.9 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.69 (dd, J = 8.3, 2.1 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.43 (dd, J = 7.9, 1.7 Hz, 1H), 7.36 (d, J = 1.6 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.19 (d, J = 7.9 Hz, 1H), 4.23−4.14 (m, 2H), 2.80 (t, J = 6.4 Hz, 2H), 2.71 (s, 3H), 2.25 (s, 1H), 1.94 (p, J = 6.3 Hz, 2H). 13C NMR (126 MHz, DMSO-d6) δ 165.44, 165.31, 154.79, 148.93, 137.70, 137.63, 136.73, 134.25, 131.96, 130.61, 130.29, 129.32, 128.81, 128.64, 128.38, 128.70, 125.86, 123.44, 119.61, 119.08, 118.64, 115.94, 66.57, 25.51, 24.73, 22.00, 17.95. HRMS (ESI+): calcld for C28H26N3O3 (M + H)+, 452.1969; found, 452.1964.

N-(5-Chroman-7-carboxamido)-2-methylphenyl-2-methylquinoline-6-carboxamide 9. Chroman-4-iodo acid (40 mg, 0.227 mmol), HATU (98 mg, 0.257 mmol), and N-(5-amino-2-methylphenyl)-2-methylquinoline-6-carboxamide (60 mg, 0.206 mmol) in N,N-diisopropylethylamine (0.988 mL, 0.505 mmol) and anhydrous DMF (1.5 mL) were reacted according to method B. The crude product was purified by flash column chromatography (1−3% methyl ethyl ketone in cyclohexane) to afford 2-methylquinoline-6-carboxylic acid (87 mg, 0.30 mmol) in N,N-diisopropylethylamine (0.14 mL, 0.82 mmol) and anhydrous DMF (2.5 mL) were reacted according to method B. The crude product was purified by column chromatography (Biotage, gradient of 0−5% methanol in dichloromethane) to afford a pale-yellow amorphous solid (78 mg, 63%). IR (thin film): νmax 2965, 1650, 1600, 1528, 1499, 1285 cm−1. 1H NMR (500 MHz, DMSO-d6) δ 10.04 (s, 1H), 8.61 (d, J = 2.0 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.24 (dd, J = 8.8, 2.0 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.88 (dd, J = 2.1 Hz, 1H), 7.78 (dd, J = 8.0, 1.8 Hz, 1H), 7.67−7.65 (m, 1H), 7.60 (dd, J = 8.3, 2.2 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.26 (d, J = 8.6 Hz, 1H), 4.76 (2H), 3.91 (t, J = 5.7 Hz, 2H), 2.86 (t, J = 5.7 Hz, 2H), 2.71 (s, 3H), 2.25 (s, 3H). 13C NMR (126 MHz, DMSO-d6) δ 165.58, 165.46, 161.22, 148.91, 137.50, 137.49, 135.49, 132.87, 131.96, 130.65, 129.34, 129.26, 128.80, 126.85, 124.37, 124.36, 119.05, 118.63, 64.79, 64.82, 28.24, 25.50, 17.95. HRMS (ESI+): calcld for C28H26N3O3 (M + H)+, 452.1964; found, 452.1965.

N-(5-Chroman-6-carboxamido)-2-methylphenyl-2-methylquinoline-6-carboxamide 9. Chroman-6-carboxylic acid (40 mg, 0.227 mmol), HATU (98 mg, 0.257 mmol), and N-(5-amino-2-methylphenyl)-2-methylquinoline-6-carboxamide (60 mg, 0.206 mmol) in N,N-diisopropylethylamine (0.988 mL, 0.505 mmol) and anhydrous DMF (1.5 mL) were reacted according to method B. The crude product was purified by flash column chromatography (1−3% methyl ethyl ketone in cyclohexane) to afford 2-methylquinoline-6-carboxylic acid (87 mg, 0.30 mmol) in N,N-diisopropylethylamine (0.14 mL, 0.82 mmol) and anhydrous DMF (2.5 mL) were reacted according to method B. The crude product was purified by column chromatography (Biotage, gradient of 0−5% methanol in dichloromethane) to afford a pale-yellow amorphous solid (78 mg, 63%). IR (thin film): νmax 2965, 1650, 1600, 1528, 1499, 1285 cm−1. 1H NMR (500 MHz, DMSO-d6) δ 10.04 (s, 1H), 8.61 (d, J = 2.0 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.24 (dd, J = 8.8, 2.0 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.88 (dd, J = 2.1 Hz, 1H), 7.78 (dd, J = 8.0, 1.8 Hz, 1H), 7.67−7.65 (m, 1H), 7.60 (dd, J = 8.3, 2.2 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.26 (d, J = 8.6 Hz, 1H), 4.76 (2H), 3.91 (t, J = 5.7 Hz, 2H), 2.86 (t, J = 5.7 Hz, 2H), 2.71 (s, 3H), 2.25 (s, 3H). 13C NMR (126 MHz, DMSO-d6) δ 165.58, 165.46, 161.22, 148.91, 137.50, 137.49, 135.49, 132.87, 131.96, 130.65, 129.34, 129.26, 128.80, 126.85, 124.37, 124.36, 119.05, 118.63, 64.79, 64.82, 28.24, 25.50, 17.95. HRMS (ESI+): calcld for C28H26N3O3 (M + H)+, 452.1964; found, 452.1965.
2.3-Dihydrobenzo[b][1,4]dioxine-6-carboxylic acid (415 mg, 2.30 mmol) and oxalyl chloride (350 mL, 3.92 mmol) were then reacted in anhydrous dichloromethane (10 mL) to afford product N-(2,4-dimethyl-3-nitroaniline as a red amorphous solid (1.1 g), which was used in the next synthetic step without further purification. 2.4. Dimethyl-5-nitroaniline (105 mg, 0.76 mmol) and ammonium chloride (1.36 g, 80 mmol) in ethanol (20 mL) and water (5 mL) were reacted according to method C. The crude product was isolated by filtration and washed with water and diethyl ether. The crude product was isolated as a beige amorphous solid (1.18 g, 52%). IR (solid): ν_{max} 3447, 3354, 2934, 1600, 1579, 1509, 1426, 1282 cm⁻¹. ^1H NMR (500 MHz, DMSO-d₆) δ 6.85–6.82 (m, 1H), 6.60 (d, J = 8.4 Hz, 1H), 6.40–6.32 (m, 1H), 6.23–6.15 (m, 1H), 4.92 (s, 2H), 4.18 (d, J = 8.0 Hz, 4H), 3.42 (m, 4H), 2.70 (s, 3H), 2.25 (s, 3H), 2.20 (s, 3H), 1.97 (s, 3H), 1.79 (s, 3H). ^1C NMR (126 MHz, DMSO-d₆) δ 165.43, 146.55, 144.85, 143.36, 134.81, 131.82, 128.22, 121.45, 121.11, 119.64, 117.23, 117.00, 113.13, 64.81, 64.46, 17.41, 17.29. HRMS (ESI⁺): calcld for C_{12}H_{12}N_{2}O_{2} (M + H)⁺, 226.0918; found, 226.0907.

2.3-Dihydrobenzo[b][1,4]dioxine-6-carboxylic acid (1.63 g, 9.03 mmol), oxalyl chloride (970 mL, 10.86 mmol), and DMF (140 mL, 1.81 mmol) in anhydrous dichloromethane (15 mL) were reacted according to method A. The resulting acid chloride and N-4-dimethyl-3-nitroaniline (1.5 g, 9.03 mmol) were then reacted in anhydrous dichloromethane (15 mL) and the acid chloride was then reacted in anhydrous pyridine (15 mL). The reaction mixture was concentrated in vacuo. The remaining residue was triturated with diethyl ether and dried in vacuo to give product 2.4-Dimethyl-5-nitroaniline (105 mg, 0.76 mmol) and ammonium chloride (1.36 g, 80 mmol) in ethanol (20 mL) and water (5 mL) were reacted according to method C to afford product N-(5-amino-2,4-dimethylphenyl)-2,3-dihydrobenzo[b][1,4]-dioxine-6-carboxamide (298 mg, 0.96 mmol). IR (solid): ν_{max} 3351, 2934, 1600, 1579, 1509, 1426, 1282 cm⁻¹. ^1H NMR (500 MHz, DMSO-d₆) δ 6.32 (m, 1H), 5.37 (d, J = 2.2 Hz, 1H), 4.92 (s, 2H), 4.18 (d, J = 2.2 Hz, 4H), 3.42 (m, 4H), 2.70 (s, 3H), 2.25 (s, 3H), 2.20 (s, 3H), 1.97 (s, 3H), 1.79 (s, 3H). ^1C NMR (126 MHz, DMSO-d₆) δ 164.53, 146.55, 144.85, 143.36, 134.81, 131.82, 128.22, 121.45, 121.11, 119.64, 117.23, 117.00, 113.13, 64.81, 64.46, 17.41, 17.29. HRMS (ESI⁺): calcld for C_{12}H_{12}N_{2}O_{2} (M + H)⁺, 226.0918; found, 226.0907.

2-Methylquinoline-6-carboxylic acid 3 (345 mg, 1.84 mmol), oxalyl chloride (180 μL, 2.06 mmol), and DMF (300 μL, 3.87 mmol) in anhydrous dichloromethane (10 mL) were reacted according to method A. The resulting acid chloride and N-(3-amino-4-methylphenyl)-N-methyl-2,3-dihydrobenzo[b][1,4]-dioxine-6-carboxamide (500 mg, 1.68 mmol) were then reacted in anhydrous dichloromethane (13 mL) and pyridine (1.2 mL, 14.89 mmol). The reaction mixture was diluted with water and brine. The product was isolated by filtration and washed with water and diethyl ether. The crude product was then purified with a cation exchange sorbent (Isolute SCX-II, washing first with methanol, and then with 10% 2 M ammonia in methanol). The product was further purified by semipreparative HPLC according to the method specified in the general procedures to afford a yellow amorphous solid (22.4 mg, 2.9%). IR (solid): ν_{max} 2927, 1624, 1493, 1282 cm⁻¹. ^1H NMR (500 MHz, DMSO-d₆) δ 10.09 (s, 1H), 5.89 (d, J = 1.8 Hz, 1H), 8.39 (d, J = 8.4 Hz, 1H), 8.21 (dd, J = 8.8, 1.9 Hz, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.72 (d, J = 8.4 Hz, 1H), 7.36 (d, J = 8.8 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 6.90 (dd, J = 8.1, 2.2 Hz, 1H), 6.87 (d, J = 2.0 Hz, 1H), 6.76 (d, J = 2.0 Hz, 1H), 6.68 (d, J = 8.4 Hz, 1H), 4.27–4.14 (m, 4H), 3.17 (s, 3H), 2.70 (s, 3H), 2.23 (s, 3H). ^13C NMR (126 MHz, DMSO-d₆) δ 169.06, 161.29, 148.95, 144.99, 143.28, 142.94, 137.62, 137.39, 131.93, 131.75, 131.25, 129.48, 128.80, 128.75, 128.61, 128.40, 128.77, 125.84, 125.00, 123.43, 121.60, 117.31, 117.11, 116.84, 116.48, 25.50, 17.97, 17.89. HRMS (ESI⁺): calcld for C_{19}H_{16}N_{2}O_{4} (M + H)⁺, 486.1923; found, 486.1914.

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128.39, 125.81, 125.05, 124.52, 124.37, 122.42, 118.16, 116.71, 64.60, 64.33, 49.06, 38.94, 25.51, 18.04. HRMS (ESI1): calcd for C26H22N3O4 (M + H)+, 440.1605; found, 440.1604.

N-(5-(2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)-1,2,3,4-tetrahydroquinoline-6-carboxamide 17. A suspension of N-(5-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)quinoline-6-carboxamide 15 (0.097 g, 0.22 mmol) and Pd/C (10%, 0.025 g) in methanol (8 mL), ethyl acetate (6 mL), and 10 drops of glacial acetic acid was stirred under 1 atm hydrogen at 40 °C overnight, filtered through Celite with ethyl acetate, and concentrated. The residue was purified by flash column chromatography using a gradient of 10–22% ethyl acetate in dichloromethane to afford the title compound (72 mg, 74%) as a white amorphous solid.

N-(3-Amino-4-methylphenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide 18. 1,4-Benzodioxane-6-carboxylic acid (2.486 g, 13.80 mmol), oxalyl chloride (1.40 mL, 16.66 mmol), and DMF (0.34 mL) in anhydrous dichloromethane (34 mL) were reacted according to method A. The resulting acid chloride was dissolved in anhydrous dichloromethane (165.65 mL), 164.74, 148.79, 146.76, 143.38, 137.56, 137.50, 130.31, 129.43, 127.51, 127.29, 126.74, 119.07, 117.74, 117.30, 117.12, 64.85, 64.48, 17.93. HRMS (ESI2): calcd for C28H26N3O4 (M + H)+, 444.1718; found, 444.1812.

N-(3-Amino-4-methylphenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide 18. 1,4-Benzodioxane-6-carboxylic acid (2.486 g, 13.80 mmol), oxalyl chloride (1.40 mL, 16.66 mmol), and DMF (0.34 mL) in anhydrous dichloromethane (34 mL) were reacted according to method A. The resulting acid chloride was dissolved in anhydrous dichloromethane (12 mL) and added dropwise to a solution of 4-methyl-3-nitroaniline (2.10 g, 13.80 mmol) and pyridine (2.23 mL, 27.6 mmol) in anhydrous dichloromethane (35 mL). The reaction mixture was stirred at room temperature for 2 h and then concentrated. The resulting amorphous solid was suspended in methanol, diluted with water, and then isolated by filtration. The water was collected and concentrated under reduced pressure to afford 1.363 g (73%) of product as a white amorphous solid.

Palladium (10% on activated carbon, 0.567 g) and N-(4-methyl-3-nitrophenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide (4.237 g, 13.48 mmol) in ethanol (90 mL) and ethyl acetate (90 mL) were reacted according to method D to afford the desired product (3.803 g, 99%) as a pale-yellow amorphous solid. IR (thin film): νmax 1614, 1615, 1329, 1172, 1027, 873, 760, 643, 612, 296 cm−1. HRMS (ESI2): calcd for C29H28N3O5 (M + H)+, 477.2156; found, 477.2121.
1611, 1582, 1289, 1064 cm⁻¹. 1H NMR (500 MHz, DMSO-d₆) δ 9.70 (s, 1H), 7.49 (d, J = 2.2 Hz, 1H), 7.46 (dd, J = 8.3, 2.2 Hz, 1H), 7.10 (d, J = 2.0 Hz, 1H), 6.95 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.1 Hz, 1H), 6.79 (dd, J = 8.1, 2.0 Hz, 1H), 4.81 (s, 2H), 4.32–4.26 (m, 4H), 2.01 (s, 3H). 13C NMR (126 MHz, DMSO-d₆) δ 164.48, 146.89, 146.54, 143.30, 138.09, 130.04, 128.60, 125.14, 117.18, 117.05, 109.26, 106.88, 64.82, 64.47, 17.46. (1 carbon missing) HRMS (ESI⁺): calculated for C₁₀H₉N₂O₄ (M + H)⁺, 2851234; found, 2851233.

**Preparation of Compounds in Table 2.** **N-(5-(2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)-2-(2-methoxyethoxy)quinoline-6-carboxamide 21.** NaH (60% in mineral oil, 0.198 g, 4.950 mmol) was added. 2-Methoxethylamine (2.20 mmol, 0.188 mmol), HATU (104 mg, 0.275 mmol), and N-(3-aminomethyl-4-methylphenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide 18 (50 mg, 0.176 mmol) in N,N-diisopropylethylamine (0.154 mL, 0.879 mmol) and anhydrous DMF (1.5 mL) were reacted according to method B. The crude product was purified by flash column chromatography (3.5–6% 2-MeOH in dichloromethane) to afford the product 22 as a light-yellow amorphous solid (0.078 g, 38% over 3 steps). IR (thin film): vmax, 2945, 1647, 1603, 1583, 1528, 1502, 1286, 1262 cm⁻¹. 1H NMR (500 MHz, DMSO-d₆) δ 10.07 (s, 2H), 8.56 (d, J = 2.0 Hz, 1H), 8.37 (d, J = 8.8 Hz, 1H), 8.22 (dd, J = 8.7, 2.0 Hz, 1H), 7.88–7.84 (m, 2H), 7.58 (dd, J = 8.3, 2.2 Hz, 1H), 7.54 (d, J = 2.1 Hz, 1H), 7.51 (dd, J = 8.4, 2.2 Hz, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.10 (d, J = 8.8 Hz, 1H), 6.98 (d, J = 8.4 Hz, 1H), 4.47 (t, J = 6.7 Hz, 2H), 4.34–4.27 (m, 4H), 3.93 (t, J = 7.1 Hz, 2H), 2.24 (s, 3H), 1.27 (s, 6H), 1.93 (p, J = 6.8 Hz, 2H). 13C NMR (126 MHz, DMSO-d₆) δ 164.94, 164.35, 162.88, 147.63, 146.34, 142.93, 140.17, 137.29, 136.33, 130.01, 128.76, 128.38, 214.80, 127.69, 126.98, 126.73, 123.98, 121.70, 118.62, 111.81, 116.84, 116.66, 113.98, 63.48, 64.02, 55.76, 45.21, 26.55, 17.48. HRMS (ESI⁺): calculated for C₁₄H₁₀N₂O₅ (M + H)⁺, 5412446; found, 5412442.

**N-(2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-(3-morpholinopropoxy)quinoline-6-carboxamide 23.** 4-(3-Morphoxypropyl)morpholine (0.171 mL, 1.24 mmol), NaH (60% in mineral oil, 0.47 g, 1.19 mmol), and 6-bromo-2-quinoline-3-carboxylic acid (0.250 g, 1.03 mmol) in anhydrous THF (3.50 mL) were reacted according to method E. The reaction mixture was heated for 16 h. Purification using flash column chromatography (1.5–3.5% methanol in dichloromethane) gave 4-(3-(6-morphoxynquinolin-2-yl)oxy)propyl)morpholine (221 mg) as a white amorphous solid, which was used directly in the next step.

**N-(2,3-Dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-(3-(Dimethylamino)propoxy)quinoline-6-carboxamide 24.** 3-(Dimethylamino)propoxyquinoline (1.56 g, 0.160 mmol) and 6-bromo-2-chloroquinoline (4.0 mL, 50.7 mmol) at 0 °C were reacted according to method E. The reaction mixture was heated for 5 h. Purification using flash column chromatography (1.5–3.5% methanol in dichloromethane) gave 4-(3-(6-morphoxynquinolin-2-yl)oxy)propyl)morpholine (221 mg) as a white amorphous solid, which was used in the next step without further purification.
\( n\)-BuLi (1.56 M in hexanes, 0.732 mL, 1.14 mmol) and 6-bromo-2-(3-(piperidin-1-yl)propoxy)quinoline (0.319 g, 0.913 mmol) in anhydrous THF (3.10 mL) were reacted according to method F to afford the crude product, which was used in the next step without further purification.

2-(3-(Piperidin-1-yl)propoxy)quinoline-6-carboxylic acid hydrochloride (76 mg, 0.188 mmol), HATU (89 mg, 0.234 mmol), and N-(3-amino-4-methylphenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide (18 mg, 0.141 mmol) in \( N,N\)-diisopropylethylamine (0.139 mL, 0.797 mmol) and anhydrous DMF (1.35 mL) were reacted according to method B. The crude product was purified by flash column chromatography (3.5–15% methanol in dichloromethane) to afford compound 24 as a white amorphous solid (53 mg, 49% over 3 steps).

\( \text{H NMR (500 MHz, DMSO-} \delta \text{)} 10.07 (\text{s, 2H}), 8.57 (d, J = 2.1 Hz, 1H), 8.38 (d, J = 8.9 Hz, 1H), 8.23 (dd, J = 8.8, 2.1 Hz, 1H), 7.89–7.83 (m, 2H), 7.58 (dd, J = 8.3, 2.1 Hz, 1H), 7.54 (d, J = 2.1 Hz, 1H), 7.51 (dd, J = 8.8, 2.2 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 8.8 Hz, 1H), 6.98 (d, J = 8.4 Hz, 1H), 4.49 (tt, J = 6.5 Hz, 2H), 4.34–4.26 (m, 4H), 2.70–2.30 (m, 6H), 2.24 (br s, 3H), 2.02 (br s, 2H). 13C NMR (126 MHz, DMSO-d<sub>6</sub>) \( \delta \) 164.99, 164.37, 162.80, 147.59, 146.35, 142.94, 140.25, 140.37, 131.36, 133.12, 130.06, 128.78, 128.84, 128.15, 127.69, 126.72, 124.03, 121.20, 118.60, 118.14, 116.85, 116.67, 113.96, 64.40, 64.21, 64.03, 63.54, 53.67, 24.99, 23.34, 17.48, (3 carbons missing). HRMS (ESI+): calcd for C<sub>31</sub>H<sub>31</sub>N<sub>4</sub>O<sub>5</sub> (M + H)<sup>+</sup>, 553.2253; found, 553.2246; 11C NMR (126 MHz, DMSO-d<sub>6</sub>) \( \delta \) 165.39, 164.81, 163.15, 148.00, 146.80, 143.39, 140.72, 137.76, 136.79, 130.57, 130.52, 129.23, 128.88, 128.15, 127.21, 124.49, 121.65, 119.08, 118.58, 117.30, 117.12, 114.48, 65.03, 64.86, 64.48, 54.92, 54.58, 42.04, 40.32, 10.15, 23.61, 19.75. HRMS (ESI+): calcd for C<sub>31</sub>H<sub>31</sub>N<sub>4</sub>O<sub>5</sub> (M + H)<sup>+</sup>, 553.2246; found, 553.2246.

Preparation of Compounds in Figure 4. N-(4-Methyl-3-(6-(2-propionamidoethoxy)-2-naphthalimido)phenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide 27. Propionyl chloride (2.5 µL, 0.029 mmol) was added to a solution of 2-(2-aminoethoxy)-N-(5-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)-2-(5-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide-2-carboxamido)-2-methylphenyl)ethylenepyrrolidine (1.1H, 0.026 mmol) in anhydrous dichloromethane (0.75 mL) and DMF (0.15 mL). The reaction mixture was stirred at room temperature for 2 h, concentrated to remove dichloromethane, and then diluted with water, extracted with dichloromethane (3×), dried over magnesium sulfate, filtered, and concentrated. Heptane was added and concentrated (2×) to remove residual DMF. The crude material was purified by flash column chromatography using a gradient of 5–12% methanol in dichloromethane to afford the title compound 27 (10 mg, 71%) as a white amorphous solid.

IR (thin film): \( \nu \text{max} \) 2934, 1645, 1529, 1502, 1486, 1462, 1465, 1440, 1382, 1375, 1369, 1357, 1308, 1293, 1289, 1281, 1259, 1256, 1244, 1215, 1196, 1158, 1159, 1170, 1172, 112, 46.53, 64.86, 64.48, 54.48, 54.58, 42.04, 40.32, 10.15, 23.61, 19.75. HRMS (ESI+): calcd for C<sub>31</sub>H<sub>31</sub>N<sub>4</sub>O<sub>5</sub> (M + H)<sup>+</sup>, 553.2246; found, 553.2246.

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Palладium (10% on activated carbon, 0.325 g) and N-(4-methyl-3-nitrophenyl)-2,3-dihydrobenzo[b][1,4]dioxine-5-carboxamide (1.50 g, 4.77 mmol) in ethanol (32 mL) and ethyl acetate (32 mL) were reacted according to method D to afford N-(3-amino-4-methylphenyl)-2,3-dihydrobenzo[b][1,4]dioxine-5-carboxamide (1.30 g, 96%) as a white amorphous solid.1 H NMR (500 MHz, CDCl3) δ 9.35 (s, 1H), 7.80 (dd, J = 7.8, 1.6 Hz, 1H), 7.34 (br s, 1H), 7.03 (dd, J = 8.0, 1.7 Hz, 1H), 7.01−6.92 (m, 2H), 6.76 (dd, J = 8.0, 2.0 Hz, 1H), 4.49−4.44 (m, 3H), 4.36−4.30 (m, 3H), 3.54 (br s, 2H).1 C NMR (126 MHz, CDCl3) δ 162.58, 145.09, 143.61, 141.73, 137.17, 130.51, 124.23, 122.59, 121.51, 121.00, 118.39, 114.07, 107.22, 65.13, 63.59, 16.91. HRMS (ESI): calcd for C18H13NO3 (M + H)+: 315.0975; found, 315.0975.

Trifluoroacetic acid (0.75 mL) was added to a solution of tert-butyl (2-(6-((5-(2,3-dihydrobenzo[b][1,4]dioxine-5-carboxamido)-2-methylphenyl)carbamoyl)quinolin-2-yl)oxy)ethyl)carbamate (0.039 g, 0.065 mmol) in anhydrous dichloromethane (0.75 mL), and the reaction mixture was stirred at room temperature for 75 min, concentrated, dichloromethane added, and concentrated again, diluted with a small amount of methanol, and then a 1:1 mixture of saturated NaHCO3 (aq) was slowly added. The resulting precipitate was isolated by filtration and washed with water. The crude material was purified by flash column chromatography using 10% methanol in dichloromethane then a gradient of 5−9% 2M NH4 methanol/DCM to afford the title compound (22 mg, 68%) as an off-white amorphous solid.1 H NMR (500 MHz, CDCl3) δ 10.08 (s, 2H), 8.57 (d, J = 2.0 Hz, 1H), 8.39 (d, J = 8.9 Hz, 1H), 8.22 (d, J = 8.7, 2.1 Hz, 1H), 7.89−7.84 (m, 2H), 7.58 (dd, J = 8.3, 2.2 Hz, 1H), 7.54 (d, J = 2.1 Hz, 1H), 7.51 (dd, J = 8.5, 2.2 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.12 (d, J = 8.7 Hz, 1H), 6.99 (d, J = 8.4 Hz, 1H), 4.42 (t, J = 5.8 Hz, 2H), 4.33−4.28 (m, 48H). The reaction mixture was dried over sodium sulfate, filtered, and then evaporated to give a white amorphous solid (77 mg, 46%).1 H NMR (500 MHz, CDCl3) δ 13.06 (br s, 1H), 8.56 (d, J = 1.9 Hz, 1H), 8.41 (d, J = 8.9 Hz, 1H), 8.13 (dd, J = 8.7, 2.0 Hz, 1H), 7.80 (d, J = 8.8 Hz, 1H), 7.07 (d, J = 8.9 Hz, 1H), 7.04 (t, J = 5.7 Hz, 1H), 4.43 (t, J = 5.8 Hz, 2H), 3.37 (q, J = 5.6 Hz, 2H), 1.37 (s, 9H). HRMS (ESI): calcd for C19H14N3O3 (M + H)+: 333.1445; found, 333.1447.

2-(2-((tert-Butoxycarbonyl)amino)ethoxy)quinoline-6-carboxylic acid (46 mg, 0.138 mmol), HATU (66 mg, 0.173 mmol), and N-(3-aminophenyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamide (39 mg, 0.138 mmol) in NN-diisopropylprolylamine (0.073 mL, 0.415 mmol) and anhydrous DME (2 mL) were reacted according to method B. The crude material was purified by flash column chromatography using a gradient of 1−3% methanol in dichloromethane to afford the product as an off-white amorphous solid (77 mg, 56%).1 H NMR (500 MHz, DMSO-d6) δ 10.07 (s, 1H), 10.07 (s, 1H), 8.57 (d, J = 2.1 Hz, 1H), 8.39 (d, J = 8.8 Hz, 1H), 8.22 (dd, J = 8.8, 2.1 Hz, 1H), 7.86 (d, J = 8.8 Hz, 1H), 7.86 (d, J = 2.2 Hz, 1H), 7.58 (dd, J = 8.5, 2.2 Hz, 1H), 7.51 (d, J = 8.4, 2.2 Hz, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.09 (d, J = 8.9 Hz, 1H), 7.06 (s, J = 5.6 Hz, 1H), 6.98 (d, J = 8.4 Hz, 1H), 4.45 (t, J = 5.7 Hz, 2H), 4.33−4.28 (m, 2H), 3.39 (ap q, J = 5.7 Hz, 2H), 2.24 (s, 3H), 1.38 (s, 9H).1 C NMR (126 MHz, CDCl3) δ 165.39, 164.81, 163.12, 147.95, 146.80, 143.39, 140.71, 137.76, 137.69, 130.57, 129.23, 128.88, 128.58, 128.15, 127.21, 125.43, 125.16, 119.08, 118.58, 117.30, 117.12, 114.47, 78.21, 65.13, 64.86, 64.48, 39.72, 39.70, 17.95 (2 carbon signals). HRMS (ESI): calcd for C21H15N3O3 (M + H)+: 599.2500; found, 599.2495.

NMR spectra of final compounds, CDK inhibition assay, Cancerogene cell line profiling, DiscoveRx KinomeScan, kinase inhibition assays, BRAF inhibitors, solubilizing group optimization, mouse pharmacokinetics, in vivo mouse efficacy studies, Cerep Diversity Screen, SIILAC target identification, surface plasmon resonance (SPR), X-ray crystallography, full experimental procedures (PDF)

SMILES molecular formula strings (CSV)

1% DMSO WM266.4 migration assay VC (MOV)

Bisamide 26 WM266.4 migration assay 100 nM (MOV)

Bisamide 28 WM266.4 migration assay 100 nM (MOV)
Accession Codes

Atomic coordinates and structure factors for the crystal structures of pirin with compound 26 can be accessed using PDB code 5JCT. Authors will release the atomic coordinates and experimental data upon article publication.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The Institute of Cancer Research has a potential financial interest in inhibitors of the HSF1 pathway and operates a Rewards to Discoverers.

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ABBREVIATIONS USED

CTB, Cell Titer Blue; ELISA, enzyme-linked immunosorbent assay; HSFl, heat shock transcription factor 1; HSP70, 70 kDa heat shock proteins; SAR, structure-activity relationship; HSP72, heat shock protein 70; 90 h, average concentration over a 24 h period; po, per os, oral dose; qd, quae die, once a day dose; Cl, clearance; Clm, unbound clearance; Vss, volume of distribution at steady-state; %F, percentage oral bioavailability; fub, fraction unbound in plasma; fbb, fraction unbound in blood; rol, blood to plasma ratio; CYP450, cytochrome P450; DMF, dimethylformamide; DCM, dichloromethane; DIPEA, diisopropylethylamine; THF, tetrahydrofuran; satd, saturated

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