Supporting Information

Privileged Structures and Polypharmacology within and between Protein Families

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Pocket Analysis

The SiteHopper PatchScore represents a summation of four Color and Shape Tanimoto coefficients weighted 3:1 in favor of Color similarity. Five pharmacophoric pseudocenter types make up the color forcefield: hydrogen bond donor, hydrogen bond acceptor, anion, cation and hydrophobe. Binding sites were identified with fpocket (version 2.0) which was implemented for ligand-independent cavity detection using default settings with two parameter alterations; the \(-r\) flag was set to 3.0 (default 4.5) and the \(-n\) flag was set to 3 (default 2). For each protein structure, chains were treated independently. For each chain, the cavity with the greatest fpocket Score was considered as the binding site – this was confirmed visually for binding sites pertinent to this analysis.

Table S1. CDK9 Pocket Analysis Dataset

| Protein       | N (homology models) | PDB IDs                                                                 |
|---------------|---------------------|------------------------------------------------------------------------|
| CDK9          | 21 (1)              | 3BLH, 3BLQ, 3BLR, 3LQS, 3MB, 3MLA, 3MY1, 3TN8, 3TNH, 3TNI, 4BCF, 4BCG, 4BCH, 4BCI, 4BCJ, 4ECS, 4EC9, 4IMY, 4OR5, HM(template:4IMY, identity:100%, source:SWISSMODEL) |
| ERK5          | 4                   | 2Q8Y, 4B99, 4IC7, 4IC8                                                 |
| FPPK5         | 4                   | 1IOB, 1IV0, 1V0O, 1V0P                                                 |
| CDKL5         | 1                   | 4BCQ                                                                  |
| CDC2L5        | 1                   | 5EFQ                                                                  |
| CDK16         | 1                   | 3M7L                                                                  |
| CDK7          | 1                   | 1UA2                                                                  |
| CDK5          | 6                   | 1H4L, 1UNG, 1UNH, 1UNL, 300Q, 4AU8                                    |
| CDK2 – structures taken from the sc-PDB database (2013) | 180  |
| CDK4- cyclinD3 | 1                   | 3G33                                                                  |
| CDK4- cyclinD1 | 5(1)                | 2W96, 2W99, 2W9F, 2W9Z                                               |
| PCTK3         | 3(3)                | HM(template:2W9Z, identity:99%, source:MODBASE)                        |
| CDK14         | 3(3)                | HM(template:1GZ8, identity:53%, source:MODBASE), HM(template:3PR, identity:52%, source:MODBASE), HM(template:3COL, identity:36%, source:NYSGXR) |
| TLK1          | 3(3)                | HM(template:3M7, identity:60%, source:MODBASE), HM(template:300G, identity:54%, source:SWISSMODEL), HM(template:3M7, identity:56%, source:SWISSMODEL) |
| TLK2          | 3(3)                | HM(template:2RAJ, identity:41%, source:MODBASE), HM(template:3O8, identity:33%, source:SWISSMODEL), HM(template:2RAJ, identity:37%, source:SWISSMODEL) |
| ICK           | 3(3)                | HM(template:3GBZ, identity:43%, source:MODBASE), HM(template:3G2Z, identity:31%, source:MODBASE), HM(template:3HY, identity:42%, source:SWISSMODEL) |
| ERK8          | 3(3)                | HM(template:3L5, identity:32%, source:MODBASE), HM(template:3Q26, identity:50%, source:MODBASE), HM(template:3Q26, identity:49%, source:SWISSMODEL) |
| GSK3A         | 2(2)                | HM(template:1JJ, identity:86%, source:MODBASE), HM(template:4ACQ, identity:86%, source:SWISSMODEL) |
| TAO1K         | 2(2)                | HM(template:1US, identity:89%, source:MODBASE), HM(template:1US, identity:89%, source:SWISSMODEL) |
| HIPK2         | 3(3)                | HM(template:3AN, identity:42%, source:MODBASE), HM(template:3KV, identity:35%, source:SWISSMODEL), HM(template:4MQ2, identity:40%, source:SWISSMODEL) |
| HIPK3         | 3(3)                | HM(template:3AN, identity:42%, source:MODBASE), HM(template:3O7, identity:38%, source:MODBASE), HM(template:4MQ2, identity:40%, source:SWISSMODEL) |

Total 252
Table S2. Results of SiteHopper Pocket Analysis:

| Rank | Kinase  | Npockets | Max SiteHopper PatchScore (CDK9) | CDK9 code | Kinase code |
|------|---------|----------|----------------------------------|-----------|-------------|
| 1    | CDK9    | 33       | 2.66 (excluding self)            | 4BCI      | 4BCH        |
| 2    | CDK2    | 180      | 1.86                             | 3LQ5      | 2C5V        |
| 3    | TAO4K1  | 2        | 1.81                             | 3BLR      | HM          |
| 4    | CDC2L5  | 3        | 1.58                             | 3BLQ      | 5EFQ        |
| 5    | CDK14   | 4        | 1.58                             | 3BLR      | HM          |
| 6    | HIPK2   | 4        | 1.48                             | 3MY1      | HM          |
| 7    | CDKL5   | 1        | 1.47                             | 3BLR      | 4BGQ        |
| 8    | HIPK3   | 3        | 1.42                             | 3BLR      | HM          |
| 9    | ICK     | 4        | 1.38                             | 4EC8      | HM          |
| 10   | PFPK5   | 5        | 1.35                             | 3BLR      | 1V0P        |
| 11   | CDK4-cyclinD1 | 6    | 1.31                             | 3BLR      | HM          |

B-Raf Docking Study

An enzyme-inhibitor cocrystal structure of human B-Raf kinase with a bisamide chemotype (PDB 4G9C) [1] was prepared for modeling using Protein Preparation Wizard in Maestro.[2] To propose predicted binding modes of ligands, Glide (Grid-based Ligand Docking with Energetics) [3] was used for the docking experiments. The receptor grid was defined by a grid box of $30 \times 30 \times 30 \text{Å}^3$ with a default inner box $(10 \times 10 \times 10 \text{Å}^3)$ centered on the cocrystallized ligand in PDB 4G9C.

The dual pirin/B-Raf ligand compound 2 was prepared using LigPrep,[4] applying the OPLS_2005 force field with possible tautomeric and ionization states within pH range $5.0–9.0$ generated. Using Glide Extra Precision (XP) settings, flexible docking of the ligand was conducted without any constraints. The docked pose with the lowest RMSD to the bisamide-containing aromatic ring of the crystallized ligand in PDB 4G9C was selected as the predicted binding pose.

[1] Wenglowesky et al. (2012) Bioorg. Med. Chem. Lett., 22, 6237.
[2] Maestro version 11; Schrödinger, LLC: New York, NY, 2017.
[3] Glide, version 7.5; Schrödinger, LLC: New York, NY, 2017.
[4] LigPrep, version 2.5; Schrödinger, LLC: New York, NY, 2011.

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**Chemistry Experimental**

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 pre-coated 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 40-65 µm). Column chromatography was also performed on Biotage SP1 or Isolera 4 purification systems using Biotage Flash silica cartridges (SNAP KP-Sil). Ion exchange chromatography was performed using acidic Biotage Isolute Flash SCX-2 columns.

Semi-preparative HPLC: 500 µL standard injections (with needle wash) of the sample were made on a Phenomenex Gemini C18 column (5µ, 250x21.2 mm, Phenomenex, Torrence, USA). Chromatographic separation at room temperature was carried out using a 1200 Series Preparative HPLC (Agilent, USA) over a 15 minutes gradient elution from 90:10 to 0:100 water:methanol (both modified with 0.1% formic acid) at a flow rate of 20 mL/min. UV-Vis spectra were acquired at 254 nm on a 1200 Series Prep Scale diode array detector (Agilent). Post-UV and pre-MS splitting was achieved using an Active Split (Agilent) before being infused into a 6120 Series Quad mass spectrometer fitted with an ESI/APCI Multimode ionization source (Agilent). Collection was triggered by UV signal and collected
on a 1200 Series Fraction Collector (Agilent). $^1$H-NMR spectra were recorded on Bruker Avance 500 (500 MHz) spectrometers using an internal deuterium lock. Chemical shifts are quoted in parts per million (ppm) using the following internal references: CDCl$_3$ (δH 7.26), MeOD (δH 3.31) and DMSO-d$_6$ (δH 2.50). Signal multiplicities are recorded as singlet (s), doublet (d), triplet (t), quartet (q), quintet (qn), and multiplet (m), doublet of doublets (dd), doublet of doublet of doublets (ddd), broad (br), obscured (obs) or apparent (app). Coupling constants, $J$, are measured to the nearest 0.1 Hz. $^{13}$C-NMR spectra were recorded on Bruker Avance 500 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: CDCl$_3$ (δC 77.0), MeOD (δC 49.0) and DMSO-d$_6$ (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 (Methods I-IV) 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.

For details of Steps (i) to (vi) see: Cheeseman, M. D.; Chessum, N. E.; Rye, C. S.; Pasqua, A. E.; Tucker, M. J.; Wilding, B.; Evans, L. E.; Lepri, S.; Richards, M.; Sharp, S. Y.; Ali, S.; Rowlands, M.; O’Fee, L.; Miah, A.; Hayes, A.; Henley, A. T.; Powers, M.; Te Poel, R.; De Billy, E.; Pellegrino, L.; Raynaud, F.; Burke, R.; van Montfort, R. L.; Eccles, S. A.; Workman, P.; Jones, K. J. Med. Chem. 2017, 60, 180-201.

6-amino-9-[(2-[(2-((2-(5-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)carbamoyl)quinolin-2-yl)oxy)ethyl]amino)-4-oxobutyl](methyl)carbamoyl)phenyl)-3-imino-3H-xanthene-4,5-disulfonic acid 4

To a solution of 2-(2-aminoethoxy)-N-(5-(2,3-dihydrobenzo[b][1,4]dioxine-6-carboxamido)-2-methylphenyl)quinoline-6-carboxamide (1.0 mg, 2.0 μmol) in dry DMF (0.18 mL) in the dark was added 6-amino-3-imino-9-(2-(methyl(4-oxo-4-(pyrrolidin-1-yloxy)butyl)carbamoyl)phenyl)-3H-xanthene-4,5-disulfonic acid (1.5 mg, 2.2 μmol) and iPr$_2$NEt (drop) and the mixture was stirred overnight. After this time, the solvent was removed under reduced pressure and the resulting residue was purified by semi-prep chromatographic separation. Semi-prep HPLC as carried out at room temperature using a Gilson GX-281 Liquid Handler system combined with a Gilson 322 HPLC pump (Gilson, Middleton, USA) over a 15 minute gradient elution (Grad15mins20mls.m) from 10:90 to 100:0 methanol:water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min. UV-Vis spectra were acquired at
254 nm on a Gilson 156 UV-Vis detector (Gilson, Middleton, USA). Collection was triggered by UV signal, and collected using a Gilson GX-281 Liquid Handler system (Gilson, Middleton, USA), to give the title compound 4 as an orange oil in 25% yield HRMS (ESI) \( \text{C}_{35}\text{H}_{45}\text{N}_{7}\text{O}_{4}\text{S}_{2} \) \((\text{M}+\text{H})^+\) requires 1070.2695, found 1070.2748.

![Chemical structure](image)

\( \text{N}(2\text{-chloro}-5\text{-}(2,3\text{-dihydrobenzo}[b][1,4]\text{dioxine}-6\text{-carboxamido})\text{phenyl})\text{-2-formylquinoline-6-carboxamide} \)

For the synthesis of the chlorobisamide aldehyde intermediate see: Chessum, N. E. A.; Sharp, S. Y.; Caldwell, J. J.; Pasqua, A. E.; Wilding, B.; Colombano, G.; Collins, I.; Ozer, B.; Richards, M.; Rowlands, M.; Stubbs, M.; Burke, R.; McAndrew, P. C.; Clarke, P. A.; Workman, P.; Cheeseman, M. D.; Jones, K. *J. Med.Chem.* **2018**, *61*, 918-933.

![Chemical structure](image)

2-((4-(tert-butyl)piperazin-1-yl)methyl)-\( \text{N}(2\text{-chloro}-5\text{-}(2,3\text{-dihydrobenzo}[b][1,4]\text{dioxine}-6\text{-carboxamido})\text{phenyl})\text{-2-formylquinoline-6-carboxamide} \)

To a solution of \( \text{N}(2\text{-chloro}-5\text{-}(2,3\text{-dihydrobenzo}[b][1,4]\text{dioxine}-6\text{-carboxamido})\text{phenyl})\text{-2-formylquinoline-6-carboxamide} \) (300 mg, 0.62 mM) in \( \text{CH}_{2}\text{Cl}_{2}/\text{MeOH} \) (5 mL, 5:1) was added 1-((tert-butyl)piperazine (260 mg, 1.9 mM) and the mixture was stirred at room temperature for 12 hours. After this time, \( \text{NaBH(OAc)}_{3} \) was added and the mixture for 2 h, then quenched with sat. \( \text{NaHCO}_{3} \) solution, extracted with \( \text{CH}_{2}\text{Cl}_{2}/\text{MeOH} \) (9:1), dried and the solvent removed under reduced pressure. The resulting residue was purified by silica gel chromatography, eluting with a 10% \( \text{CH}_{2}\text{Cl}_{2}/\text{MeOH} \) gradient to give the desired product (60 mg, 16%) as a beige solid. 1H NMR (500 MHz, DMSO-d6) \( \delta \) 10.34 (s, 1H), 10.29 (s, 1H), 8.66 (d, \( J=1.89 \) Hz, 1H), 8.49 (d, \( J=8.51 \) Hz, 1H), 8.26 (dd, \( J=2.21,8.83 \) Hz, 1H), 8.14 (d, \( J=2.52 \) Hz, 1H), 8.09 (d, \( J=8.83 \) Hz, 1H), 7.69-7.80 (m, 2H), 7.49-7.61 (m, 3H), 7.00 (d, \( J=8.20 \) Hz, 1H), 4.28-4.36 (m, 4H), 3.79 (s, 2H), 1.03 (br s, 9H). 13C NMR (126 MHz, DMSO-d6) \( \delta \) 165.5, 165.1, 162.2, 148.7, 147.0, 143.4, 139.1, 138.0, 135.4, 131.8, 129.8, 129.3, 128.9, 128.4, 127.8, 126.7, 124.1, 122.3, 121.8, 120.3, 119.7, 117.4, 117.2, 65.4, 64.9, 64.5, 54.2, 45.8, 26.0. HRMS (ESI+): calcd for \( \text{C}_{33}\text{H}_{39}\text{Cl}_{15}\text{N}_{5}\text{O}_{4} \) \((\text{M}+\text{H})^+\), 614.2534; found 614.2530.

**Pirin Fluorescence Polarization Assay**

**General Information:** Unless otherwise stated, the assay buffer contained 50 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (w/w) CHAPS, 2% DMSO and the assay was conducted in 384 Plus F ProxiPlates (PerkinElmer). Plates were centrifuged at 1000 rpm for 1 minute prior to incubation and read using a 2103 Envision Multilabel Plate Reader. Excitation and emission wavelengths used for green probes were 480 nm and 535 nm, respectively. Fluorescence polarization was measured in units of millipolarization (mP) and all experiments were performed in triplicate.
**Determination of optimal probe concentration:** 5 μL of pirin (400nM in assay buffer) or 5 μL assay buffer and increasing concentrations of probe (5 μL, dilution series from 0.2 up to 1000nM) were added. From the plotted data, a probe concentration of 2 nM was selected and gave an assay window of 4.0 with a Z’ of 0.74.

**Fluorescent probe K_D determination:** 5 μL of probe molecule (4 nM in assay buffer) to increasing concentrations of the pirin protein (5 μL, dilution series from 0.2 to 200 nM). Fluorescence polarization values for tracer control wells (2 nM probe in assay buffer only) were subtracted from each data point prior to data analysis. The K_D determination was analyzed using non-linear regression analysis (one site–specific binding model, GraphPad Prism 6) and gave a K_D of 12 nM.

**Compound IC_{50} determination:** Compounds (0.2 μL at 50 x screening concentration in DMSO) were dispensed using an ECHO 550 Liquid Handler (Labcyte Inc.). To the corresponding wells, 5 μL of probe molecule (4 nM in assay buffer) and 5 μL of pirin protein (60 nM) were added. Tracer controls (2 nM probe molecule only) and bound tracer controls (2 nM probe in the presence of appropriate protein concentration) were included on each assay plate. IC_{50} determination was performed using non-linear least squares curve fitting (GraphPad Prism 6, log(inhibitor) vs. response-variable slope (four parameters)).

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**Figure S1. Pirin FP-Probe Binding Data**

**Figure S2. Example Pirin FP-probe Displacement Data**
**Pirin FP-Probe Binding Analysis**

Huang’s equation was used to calculate $K_i$ values from the measured $IC_{50}$s. See equation below:

$$IC_{50} = \left( \frac{f_0 K_d}{(1-f_0)(2-f_0)} + \frac{f_0 L_0}{2} \right) \left( \frac{K_i(2-f_0)}{K_d f_0} + 1 \right)$$

The equation states that the $IC_{50}$ for a ligand that is competitive for binding with the assay probe is related to the binding affinity of the ligand ($K_i$), the bound fraction of the probe ($f_0$), the binding affinity of the probe ($K_d$) and the concentration of the probe ($L_0$). For competition experiments, it is recommended that a protein concentration giving a bound fraction between 0.5 and 0.8 be selected. A bound fraction below 0.7 will often result in an assay that is not statistically robust due to the decreased size of the binding window, however as the bound fraction approaches 1 the relationship between $K_i$ and $IC_{50}$ deviates from linear and the resolvable range of the assay decreases. For these reasons, a bound fraction of 0.72 was used for all assays.

$$R_0 = \frac{K_d f_0}{1-f_0} + f_0 L_0$$

Where $R_0$=[protein]

When $K_i$=11 nM, $F_b$=0.72 and $L_0$=2 nM

$R_0$=30 nM

All $IC_{50}$ values within 2-fold of the protein concentration were considered tight-binding.

For details see Huang, X. *J. Biomol. Screen.* 2003 8, 34-38.

**Pirin and B-Raf Crystallography Analysis**

![Figure S3. Overlay of CDK9 (PDB: 4EC8, turquoise) and homology models of TAOK1 (brown) and HIPK2 (yellow)](image-url)
Figure S4. Key Residues Leading to the Intra-Family Polypharmacology as Detected by the *SiteHopper* Pocket Analysis. CDK9 (PDB: 4EC8, turquoise), CDK2 (PDB: 4BZD, green) and homology models of TAOK1 (brown) and HIPK2 (yellow).

Figure S5. Crystal structures of the bisamide chemotype bound to EphA3 (PDB: 3DZQ).

Figure S6. Crystal structures of the bisamide chemotype bound to p38 (PDB: 3KQ7).
Figure S7. Overlay of the binding site of BRD4 (PDB: 4OGI, salmon) with PLK1 (PDB ID: 2RKU, white), using the bound ligand, BI-2536.

Figure S8. Structure of Bisamide 2 bound to Pirin highlighting key residues.

Figure S9. Docking Pose of Bisamide 2 Bound to B-Raf
Figure S10. Structure of PLX4720 7 bound to Pirin highlighting key residues. NB: the sulfonamide moiety could not be observed in the electron density due to its flexibility but is shown here in one possible conformation for clarity.

Figure S11. Structure of PLX4720 7 bound to B-Raf highlighting key residues.

Figure S12. Overlay of bisamide 2 (purple) and PLX4720 7 bound to pirin.
Figure S13. FP-Assay Probe Design. Surface representation of Pirin with Bisamide 2 (gold) bound. The methylene quinoline group is clearly solvent exposed.

Figure S14. Analysis of key interactions in B-Raf
Kinase Assay Data

Table S3. B-Raf Assay Data

| Activity | Part# / Lot# |
|----------|-------------|
| MAPK1 (ERK2) : ATP [Km app] | CCT250879 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

| Activity | Part# / Lot# |
|----------|-------------|
| MAPK1 (ERK2) : ATP [Km app] | CCT245232 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

| Activity | Part# / Lot# |
|----------|-------------|
| BRAF : ATP [100] | CCT250879 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

| Activity | Part# / Lot# |
|----------|-------------|
| MAP2K1 (MEK1) : ATP [100] | CCT245232 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

| Activity | Part# / Lot# |
|----------|-------------|
| BRAF : ATP [100] | CCT251236 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

| Activity | Part# / Lot# |
|----------|-------------|
| BRAF V599E : ATP [100] | CCT251236 |
| Concentration (nM) | 10 100 1000 10000 |
| Percent Inhibition | -20 0 20 40 60 80 100 120 |

For the screening protocol and assay conditions see: https://www.thermofisher.com/uk/en/home/life-science/drug-discovery/target-and-lead-identification-and-validation/kinasebiology/kinase-activity-assays/z-lyte.html

**BRAF: [ATP]=100 μM Cascade**

The 2X BRAF/inactive MAP2K1 (MEK1)/inactive MAPK1 (ERK2)/Ser/Thr 03 mixture is prepared in 50 mM HEPES pH 7.5, 0.01 % BRIJ-35, 10 mM MgCl₂, 1 mM EGTA. The final 10 μL kinase reaction consists of 0.03-0.1 ng B RAF, 1X inactive MAP2K1 (MEK1)/inactive MAPK1 (ERK2), and 2 μM Ser/Thr 03 in 50 mM HEPES pH 7.5, 0.01 % BRIJ-35, 10 mM MgCl₂, 1 mM EGTA. After the 1 hour kinase reaction incubation, 5 μL of a 1:1024 dilution of development reagent A is added.

**Kinase Panel Data**

http://www.kinase-screen.mrc.ac.uk/services/premier-screen, accessed April 2017.

The principal method utilized is a radioactive filter binding assay using 32P ATP (Hastie, et al 2006. Nat Protoc. 2006;1(2):968-71; Bain, et al 2007. Biochem J. 2007 Dec 15;408(3):297-315). This method is sensitive, accurate and provides a direct measure of activity.

**Assay step-by-step process:**

1. Upon receipt of your small molecule, staff at the ICKP will dilute each to the appropriate concentration (if required)

2. This compound is added to a ‘mother plate’ consisting of customer samples, controls and blanks
   - These serve as the source for ‘daughter plates’ which are stored at -20° until assay initiation
   - Note: All compounds are screened in duplicate

3. Next there will be 3 additions to the assay:
   - Enzyme-substrate mixture
- Incubation Time: 5 minutes at Room Temperature (RT)
- $^{33}$P ATP - Assay begins with this addition
  - Incubation time varies based upon optimal designated incubation time (for each enzyme @ RT)
- Orthophosphoric acid - Assay is halted with this addition

4. Assay components are harvested onto P81 filter plate
5. Filter plates are air-dried
6. Scintillation fluid is added to plates
7. Counts are read on a Topcount NXT

Data Analysis:
1. Bar codes assigned to each file ensure that data corresponding to the correct compound is being analysed
2. After completion of each assay, ICKP staff ensure that the run has passed standard quality control measures by examining reference compounds on the QC plate
3. Upon determination that the run has met QC standards, a Z-Prime ($Z'$) value is calculated utilizing data from the controls/blanks on each individual plate
   - This QC measure is in place to ensure that each individual plate in the run has passed QC
4. Finally, a mean percentage activity is calculated for each customer.
   - A standard deviation for all the duplicates is also calculated
### Table S4. Aminopyrimidine 1 TAOK1 Screening Data

| Conc.(µM) | 0.0003 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 10 |
|-----------|--------|-------|------|------|-----|----|--|---|
| CCT250006 | 0.0003 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 10 |
| Control   | 18454.5| 2268  | 18343.5| 18454.5| 2268 | 18343.5| 18454.5| 2268 |
| AVR       | 0.025  | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 |

### Table S5. Aminopyrimidine 1 HIPK2 Screening Data

| Conc.(µM) | 0.0003 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 10 |
|-----------|--------|-------|------|------|-----|----|--|---|
| CCT250006 | 0.0003 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 10 |
| Control   | 15668.75| 15625.88| 15613| 15613| 15613| 15613| 15613| 15613|
| AVR       | 0.025  | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 | 0.025 |

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![Graph](image-url)
Crystallography Experimental

Protein production, purification and crystallogenesis;

Full-length 6His-tagged Pirin was produced in E.coli and purified as previously described.(1) Purified Pirin was crystallized in the apo form at 4 °C using the hanging-drop vapor-diffusion method. The crystallization drops were composed of 1 μL of protein (50 mg/mL) and 1 μL of reservoir solution containing 0.1 M HEPES (pH 7.5), 8 % ethylene glycol, 20 % (w/v) PEG 8,000, placed over 200 μL of reservoir solution. Needle-shaped crystals typically grew in two weeks. Pirin ligand complexes were formed by soaking apo crystals for 48 h in 25 mM of ligand in matching reservoir condition, and 20 % (v/v) DMSO. Soaked protein crystals were then briefly transferred to cryoprotectant solution of 0.1 M HEPES (pH 7.5), 8 % ethylene glycol, 22 % (w/v) PEG 8,000, 20 % Glycerol and cryocooled to 100 K in liquid nitrogen prior to data collection.

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Data collection, processing and refinement:

X-ray data were collected at the European Synchrotron Radiation Facility (ESRF) on beam-lines ID30A-1/MASSIF-1 and ID23-2. Crystals belonged to the space group P2_1_2_1_2_1 and diffracted to a resolution between 1.54 and 1.69 Å. Datasets were integrated with XDS (1) and scaled and merged with AIMLESS (2). Structures were solved by molecular replacement using PHASER (3, 4) with a publicly available Pirin structure (PDB codes 5JCT) with ligand and water molecules removed used as molecular replacement model. The protein–ligand structure was manually corrected and rebuilt in COOT (5) and refined with BUSTER (6) in iterative cycles. Ligand restraints were generated with GRADE (7) and MOGUL (8). The quality of the structures was assessed with MOLPROBITY (9, 10).

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**Table S6: Crystallographic data collection and refinement statistics:**

| Protein construct       | Pirin 1-290 | Pirin 1-290 |
|-------------------------|------------|------------|
| Ligand                  | 2          | 7          |
| PDB code                | 6H1I       | 6H1H       |

| Crystal                 |            |            |
|-------------------------|------------|------------|
| Space group             | P 2 2 1    | P 2 2 1    |
| Unit cell dimensions    | 42.05/67.15/107.24 | 42.30/67.41/107.68 |
| Unit cell angles        | 90/90/90   | 90/90/90   |

| Data collection and processing |            |            |
|-------------------------------|------------|------------|
| Beamline                      | ESRF ID30A-1 | ESRF ID23-2 |
| Wavelength (Å)                | 0.9650     | 0.8726     |
| Integration program           | XDS        | XDS        |
| Reduction program             | AIMLESS    | AIMLESS    |
| Resolution range              | 41.90 – 1.69 | 42.30 – 1.54 |
| Number of unique reflections  | 33746 (1702) | 46420 (2277) |
| Completeness Æ                | 97.3 (98.4) | 99.8 (99.6) |
| Redundancy Æ                  | 4.3 (4.5)  | 6.5 (6.4)  |
| Rmerge (%)                    | 12 (144.6) | 10.5 (136.7) |
| I/σ(I) Æ                      | 7.7 (1.2)  | 10.1 (1.3) |
| CC1/2 Æ                       | 0.986 (0.375) | 0.998 (0.344) |

| Refinement                 |            |            |
|----------------------------|------------|------------|
| Program                    | BUSTER     | BUSTER     |
| Rwork (%)                  | 17.87      | 15.86      |
| Rfree (%)                  | 22.42      | 18.29      |
| Number of residues         | 287        | 288        |
| Number of water molecules  | 272        | 349        |
| Average B-factor (Å)       | 25.73      | 24.76      |
| Ramachandran favoured (%)  | 97.54      | 97.55      |
| Ramachandran outliers (%)  | 0          | 0          |
| RMSD bonds (Å)             | 0.014      | 0.014      |
| RMSD angles (°)            | 1.75       | 1.66       |

Æ Values in parentheses are for the highest resolution shell.

Half-dataset correlation coefficient, see: Karplus, P. A.; Diederichs, K. Linking crystallographic model and data quality. *Science* **2012**, *336*, 1030–1033.
Figure S15. Structure of Bisamide 2 bound to Pirin, with final 2Fo-Fc map contoured at 1 σ shown as blue mesh, and omit Fo-Fc map contoured at 3 σ shown as green mesh.

Figure S16. Structure of PLX4720 7 bound to Pirin, with final 2Fo-Fc map contoured at 1 σ shown as blue mesh, and omit Fo-Fc map contoured at 3 σ shown as green mesh.