Supporting Information

Fragment-based Discovery of a Small-molecule Protein Kinase C-iota Inhibitor Binding Post-kinase Domain Residues

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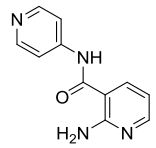
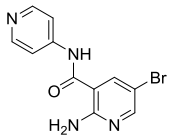
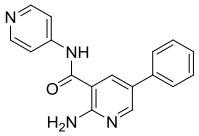
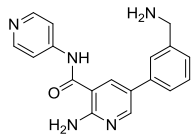
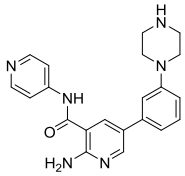
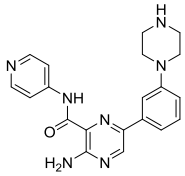
Contents

1. Isoform selectivity

To better understand selectivity profile of the inhibitor series, several compounds were screened against representative PKC isoenzyme from each class: classical (PKC-α), novel (PKC-ε) and atypical (PKC-ζ and ι, **SI Table 1**). Interestingly, compound **15** while inactive against PKC-ι, was inhibiting PKC-α with IC_{50} = 17.9 μM indicating opportunity to pursue classical PKC inhibitors based on compound **15**. Replacement of halogen in **15** with a phenyl ring increased the potency against all PKC isoforms, most notably bringing about activity towards atypical PKCs (compound **16**). Introduction of the basic methylamino- group onto the phenyl ring resulted in a dramatic improvement of inhibitory activity against aPKCs (**17**, 44-fold for PKC-ι and nearly 100-fold for PKC-ζ). It is likely, that the boost in potency is due to the involvement of post-kinase residue Asp-553. On the other hand, this modification did not significantly affect IC_{50}s against other isoenzymes. Taken together, this could indicate an opportunity to increase isoform selectivity of the inhibitors towards aPKCs by designing-in interactions with the post-kinase domain residues. The subsequent modification of compound **17** by replacement of methylamino- group with piperazine, improved potency of the resulting compound **19** towards PKC-α to a larger extent (4-fold) than towards PKC-ι (2-fold), consequently equalizing the respective IC_{50}s. Lastly, modification of the core from aminopyridine to aminopyrazine (**20**) resulted in a slight improvement in activity towards all PKC isoforms, most notably PKC-α by 4 folds. In short, the initial SAR across PKC isoforms indicated opportunities to increase selectivity for aPKC and cPKC isoenzymes.

SI Table 1. Activity of selected compounds against atypical PKC (ι, ζ), classical PKC (α) and novel PKC (ε)

Cmpd / IC_{50} [μM]	PKC-ι	PKC-α	PKC-ε	PKC-ζ
 **2**	22	–	–	–
 **15**	> 384	17.9	153	> 384

 **16**	31.6	2.5	7.1	63
 **17**	0.71	1.5	1.4	0.68
 **19**	0.34	0.34	1.6	0.51
 **20**	0.27	0.073	0.94	0.28

2. Biochemical assays

PKC isonzymes (Carna Biosciences, Chuo-ku, Kobe Japan) were assayed by Microfluidics LabChip® technology (PerkinElmer, Waltham, MA, USA). In brief, the assay was performed in buffer with 100 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM DTT (Dithiothreitol), 0.003% of Brij35, 0.004% of Tween 20. Reactions, in a total volume of 26 µl, contained varying concentrations of PKC isonzymes, Km of ATP and 1.5 µM synthetic peptide (5FAM) RFARKGSLRQKNV (GenScript). The enzyme concentration for PKC-ι, PKC-ζ, PKC-α and PKC-ε assay was 0.15 nM, 0.6 nM, 0.15 nM, 0.15 nM respectively. The ATP concentration for PKC-ι, PKC-ζ, PKC-α and PKC-ε assay was 36 µM, 4 µM, 18 µM, 26 µM respectively. Reactions were incubated at RT for 60 minutes and then stopped by 45 µl termination buffer with 10 mM EDTA. The terminated reaction was then analyzed on a LabChip EZ Reader II (Perkin Elmer, Waltham, MA, USA). The assays were repeated at least 3 times.

Percent inhibition values were calculated in Caliper's EZReviewer Software and plotted vs. the log of inhibitor concentration. GraphPad Prism was used to fit the data with dose-response curves and determine IC_{50} values.

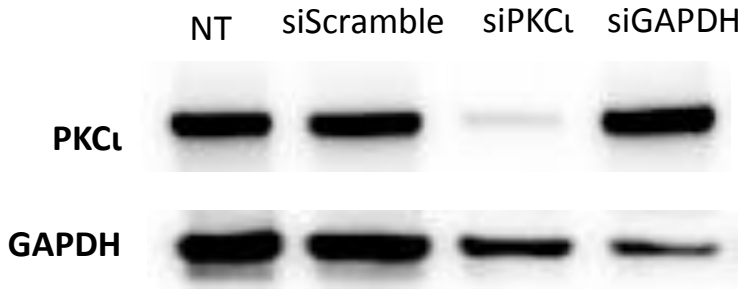
3. Cellular assay

Cell line selection

PKC-ι promotes cell proliferation of Huh7 cells

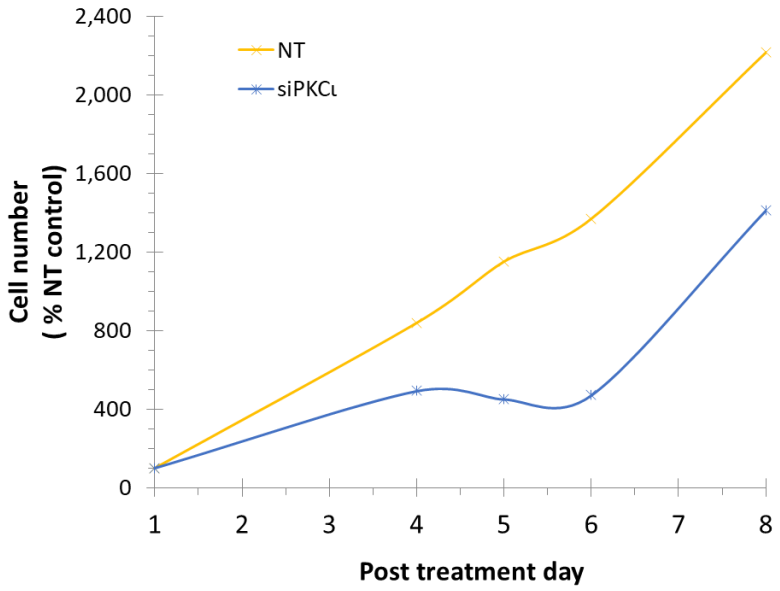
In NSCLC, PKC-ι regulates cell proliferation and growth and it has been shown before in multiple cancer cell lines, that knockdown of PKC–ι results in decrease in ECT2 phosphorylation.[1] To investigate whether PKC-ι also plays a role in proliferation and growth of hepatocellular carcinoma (HCC) cells, the effect of siRNA mediated PKC-ι knockdown (KD) on Huh7 cells proliferation was investigated (Huh7 cells were selected due to relatively high content of PKC-ι as compared to other HCC cell lines). As shown on **SI Figure 1a**, immunoblot analysis of cellular extracts from treated Huh7 cells demonstrated that PKC-ι expression level decreased by over 90% in siPKC-ι cells when compared with non-treated and siScramble control cells. We next assessed the functional role of PKC-ι in Huh7 cells. PKC-ι KD significantly attenuated the growth of Huh7 cells *in vitro*, indicating that PKC-ι promotes Huh7 cell proliferation (**SI Figure 1b**).

(a)



[1] Justilien, V.; Lee, J.; Der, C. J.; Rossman, K. L.; Fields, A. P. "Oncogenic Activity of Ect2 Is Regulated through Protein Kinase C-ι-Mediated Phosphorylation." *J. Biol. Chem.* 2011, 286, 8149–8157.

(b)



SI Figure 1. PKCι is important for proliferation of Huh7 cells. a) Huh7 cells were transfected with either non-treated (NT), siScramble, siPKC-ι or siGAPDH. Cell lysates were assayed for PKC-ι and GAPDH content by immunoblot; **b**) growth of Huh7 cell transfectants was determined by Celltiter Glo assay.

Materials and methods

Antibody reagents and cell line

Antibodies used were: anti-PKC-ι (BD Transduction Labs, San Jose, CA, USA), anti-GAPDH (ThermoFisher Scientific, Waltham, MA, USA), ECL-anti mouse IgG-HRP and ECL-anti rabbit IgG-HRP (GE Healthcare, Chicago, IL, USA). Huh7 cells were cultured in DMEM medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (ThermoFisher Scientific) and maintained in a 37 °C incubator with 5% CO_2.

siRNA knockdown

Using the standard transfection protocol, cells were seeded in a 6-well plate 24 hrs prior to siRNA (small interfering RNA) treatment. The following ON-TARGETplus SMARTpool siRNA against human PKC-ι was predesigned and purchased from GE Dharmacon (Lafayette, CO, USA). GAPDH siRNA and Negative Control / Non-targetting siRNA were purchased from ThermoFisher Scientific. The cells were transfected with siRNA using Lipofectamine™ RNAiMAX in serum free Opti-MEM Medium (ThermoFisher Scientific). 5 µl of Lipofectamine™ RNAiMAX and siRNA were incubated in 500 µl of Opti-MEM for 20

minutes at room temperature. The transfection mix was added to the cells in a dropwise manner with 2.5 ml of growth media each well to a final siRNA concentration of 20 nM. After 72 hrs, the transfection solution was removed from the cells and replaced with standard medium for assay. The siRNA knockdown cells were harvested for either proliferation or western blot experiment.

Western blot

Cell lysate was prepared in RIPA buffer (ThermoFisher Scientific) supplemented with protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology) and SDS (Sodium dodecyl sulphate). The lysate was quantitated with Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and denatured by boiling with 2 X Laemmli sample buffer supplemented with β-mercaptoethanol (Bio-Rad Laboratories) for 10 minutes. Equal amount of protein was loaded and separated using the NuPAGE Novex 4 -12 % gradient Bis-Tris Protein Gel (ThermoFisher Scientific) and subsequently transferred to a PVDF membrane. Membrane was blocked for 1 hr at RT in 5 % w / v BSA in PBST and incubated at 4 °C overnight with primary antibody (anti-PKC-ι and anti-GAPDH) as described above. Secondary antibody (ECL-anti mouse IgG-HRP or ECL-anti rabbit IgG-HRP) incubation was performed for 1 hr at RT. ECL-Plus (GE Healthcare Life Sciences,Pittsburgh, PA, USA) was used for western blot detection using the FluorChem R Imaging system (ProteinSimple, San Jose, CL, USA) Chemiluminescent channel. Quantification of the immublot was performed with the AlphaView software (ProteinSimple).

Cell Proliferation Assay

Cell proliferation assay was performed using CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI, USA) following manufacturer's instructions. The Huh7 cells were treated with compounds that were serial diluted in respective growth medium. Plates were incubated for 72 hrs at 37 ˚C in 5 % CO_2. After 72 hrs, an equal volume of Cell Titer Glo reagent was added. Plates were rocked on a rotator for 2 hrs. Luminescence emitted was measured with the Tecan Safire II (Perkin Elmer).

4. Molecular modelling & dynamics

The X-ray structure of PKC-ι complexed with ATP was downloaded from the protein data bank (www.rcsb.org, PDB code 3A8W). The structure was prepared using the protein preparation wizard in Maestro release 2017-3 (www.schrodinger.com) with standard settings. This included the addition of hydrogen atoms, bond assignments, removal of all water molecules, protonation state assignment and optimization of the hydrogen bond network. ATP was deleted from the prepared structure and the inhibitors were then manually docked into the ATP-site using Maestro. It was assumed that the amino-pyridine or amino-pyrazine part of our compounds forms the same hydrogen bonds with the kinase hinge residues Val335 and Glu333 as the adenine of ATP. Residues beyond 9 Å of the inhibitor were constrained and the complex was then subjected to 500 steps of TNCG minimization using the OPLS3 force field and

GB/SA solvation model implemented in MacroModel release 2017-3 (www.schrodinger.com). A map of the binding site was calculated for the minimized complex with compound **2** using SiteMap release 2017-3 (www.schrodinger.com).

The in-house X-ray structure of PKC-ι in complex with compound **19** was prepared using the protein preparation wizard. The missing sidechains and residues were added using Prime release 2017-3 (www.schrodinger.com). This structure was then subjected to 250 ns of unconstrained molecular dynamics (MD) simulation using Desmond 4.8 (www.schrodinger.com). The system for MD was build using the System Builder in Desmond. The protein-inhibitor complex was placed in an orthorhombic box with a 10Å buffer in an orientation that minimized total box volume. The SPC explicit solvent model using water as solvent was employed. The system was neutralized by adding Na+ ions and the NaCl salt concentration was set to 0.15 M. The MD simulation was set up with the following settings: NPT ensemble class with T = 300 K, P=1.01325 bar, system relaxation before MD, and 250 ns run time. Analysis of secondary structure, RMSF, and ligand-protein interactions were performed using the Simulation Interactions Diagram tool that is part of Desmond (see additional report as supplementary information). The binding orientation and ligand-protein interactions shown in **Figure 2** (main text) is representative of the interactions observed during the MD simulation

5. X-ray supplementary information

Protein purification and crystallization

PKC-ι kinase domain (residues 240-579) with N-terminal His_6 tag and a TEV site expressed in Sf9 cells was purchased from Genscript. The His6 tagged protein was purified using the published protocol.[2]

The purified protein was concentrated to 10 mg/ml and incubated with the compound in a 1:5 stoichiometry ratio for crystallization. The diffracting crystals were grown in a hanging drop containing 0.1 M BIS-TRIS 6.5, 28 % w/v Polyethylene Glycol monomethyl ether 2,000. The crystals were flash frozen in liquid nitrogen with 20% glycerol. A 3.26Å dataset was collected in the MX1 beamline at the Australian synchrotron, Victoria, Australia.[3]

The structure of PKC-ι (PDB ID: 3A8W) after the removal of water molecules was used as the search model for molecular replacement using the PHENIX suite of programs. COOT Ligand Builder was used to generate the PDB and CIF files necessary for the geometry restraints for the compound of interest during refinement. The final PKC-ι-compound co-crystal structure

[2] Takimura,T.; Kamata, K.; Fukasawa, K.; Ohsawa, H.; Komatani, H.; Yoshizumi, T; Takahashi, I; Kotani, H.; Iwasawa, Y. Structures of the PKC-ι Kinase Domain in its ATP-bound and Apo Forms Reveal Defined Structures of Residues 533-551 in the C-terminal Tail and Their Roles in ATP Binding. Acta Cryst., 2010, D66, 577-583.

[3] McPhillips, T. M., McPhillips, S. E., Chiu, H. J., Cohen, A. E., Deacon, A. M., Ellis, P. J., Garman, E., Gonzalez, A., Sauter, N. K., Phizackerley, R. P., Soltis, S. M., Kuhn, P. Blu-Ice and the Distributed Control System: Software for Data Acquisition and Instrument Control at Macromolecular Crystallography Beamlines. J. Synchrotron Rad., 2002, 9, 401-406.

was refined to a final R_{free} value of 34.8% using Phenix.refine with four molecules in the asymmetric unit.

Complete electron density was obtained for all residues shown in the X-ray renders with exception of ASP553, for which electron density was incomplete in one of the four PKC-ι – compound **19** complex instances recorded.

SI Table 2. Data collection and refinement statistics

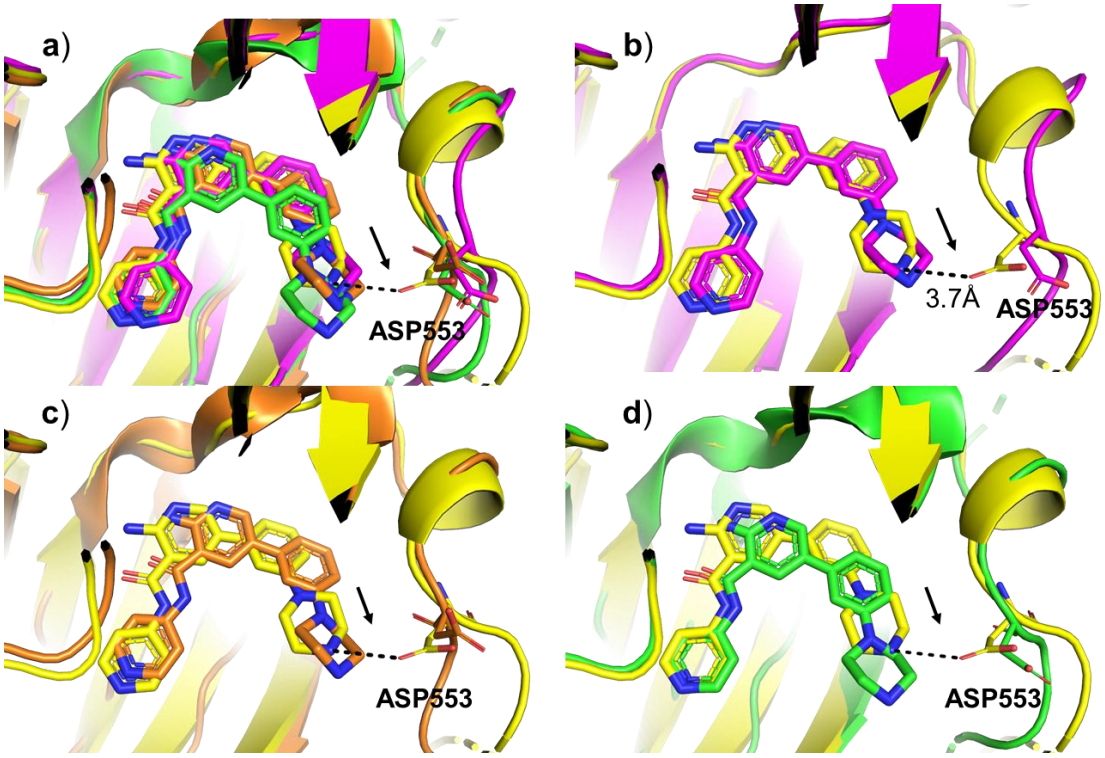
Parameter	**PKC-ι_refine60**
Resolution range	44.45 - 3.26 (3.38 - 3.26)
Space group	P 1 21 1
Unit cell	87.87 87.63 105.18 90 114.69 90
Total reflections	43411 (3895)
Unique reflections	22181 (2066)
Multiplicity	2.0 (1.9)
Completeness (%)	97.23 (91.58)
Mean I/sigma(I)	9.71 (3.08)
R-merge	0.08004 (0.1936)
R-meas	0.1132 (0.2737)
R-pim	0.08004 (0.1936)
CC1/2	0.947 (0.803)
CC*	0.986 (0.944)
Reflections used in refinement	22181 (2066)
Reflections used for R-free	1998 (187)
R-work	0.3005 (0.4046)
R-free	0.3398 (0.3710)
CC(work)	0.631 (0.484)
CC(free)	0.498 (0.566)
Number of non-hydrogen atoms	9676
macromolecules	9564
ligands	112
Protein residues	1267
RMS(bonds)	0.009
RMS(angles)	1.02
Ramachandran favored (%)	95.71
Ramachandran allowed (%)	3.63
Ramachandran outliers (%)	0.66
Rotamer outliers (%)	0.00
Clashscore	22.92
Average B-factor	33.10
macromolecules	33.11
ligands	31.87
Number of TLS groups	1

Statistics for the highest-resolution shell are shown in parentheses.

6. Binding mode of compound 19 – additional comments

Interaction with ASP553

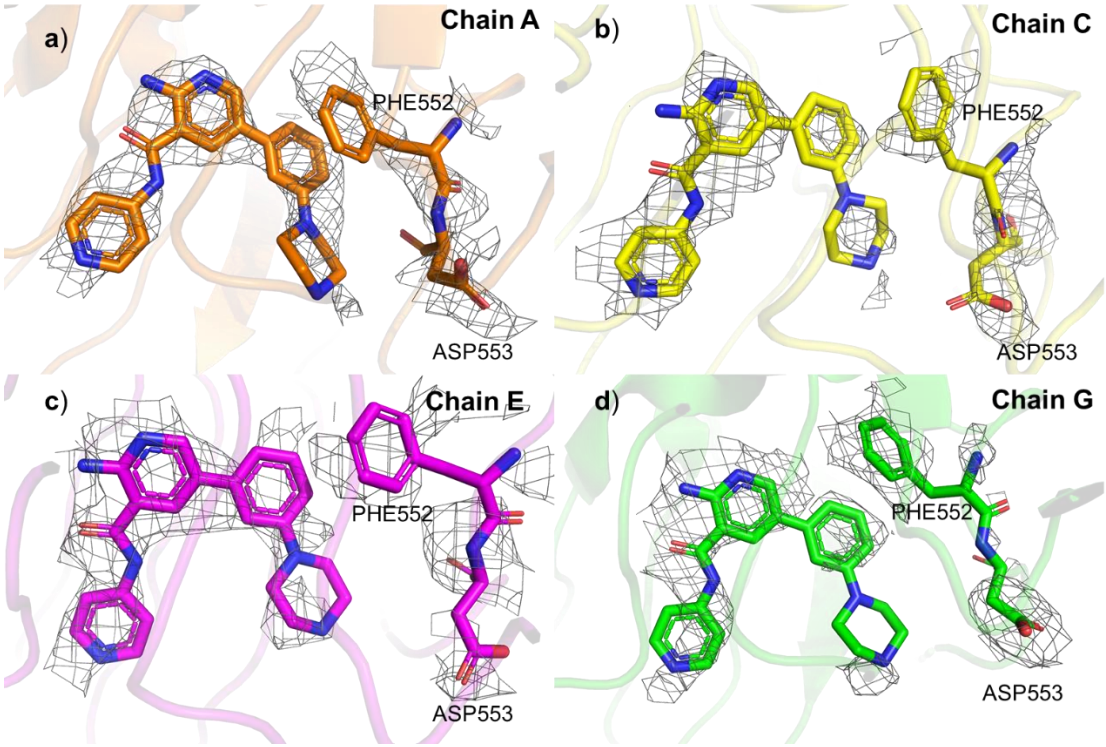
Each asymmetric unit of the X-ray structure contained 4 complexes of protein and compound **19** (numbered: chain A, C, E, G; PDB: 6ILZ). Each complex was analyzed and indicated nearly identical binding mode, except for the interaction of Asp553 and piperazine of **19**. Clear interaction was detected between **19** and ASP553 in chain C (**SI Figure 2a**), while in another instance (chain E, **SI Figure 2b**) piperazine and carbonyl of Asp553 were facing away from one another. In the other two chains (C and G), piperazine appeared shifted, with angles and distances not supporting a clear, direct hydrogen bond to ASP553. Notably, complete electron density was obtained for all key protein residues and ligand, with exception of chain Z, where electron density for Asp553 was partially missing.



SI Figure 2. X-ray crystal structure of compound 19 with PKC-ι: interaction with ASP553 shown in individual complex instances. a) Comparison of chains A, C, E and G (coloured orange, yellow, magenta and green respectively) of PKC-ι-ligand complex in one asymmetric unit. Chain C (coloured yellow) indicates a clear interaction between piperazine and ASP553; **b**) overlay of chains C and E show that Asp553 in chain C interacts with the ligand (3.7Å) whereas in chain E, the entire loop is shifted away from the ligand; **c**) overlay of chains C and A; **d**) overlay of chains C and G. Figures c) & d) show that the ligands bound to chains A and G slide "downwards" compared to the ligand bound to chain C thus disrupting the interaction with Asp 553.

On the other hand, SAR shown in **Table 2** in the main text, suggests a strong interaction *in solution* of the basic residue of our inhibitors as a 45-fold gain in potency was achieved by addition of methylamino- moiety onto the phenyl ring and a 100-fold gain by installation of piperazine. To further increase confidence that the interaction between Asp553 and piperazine

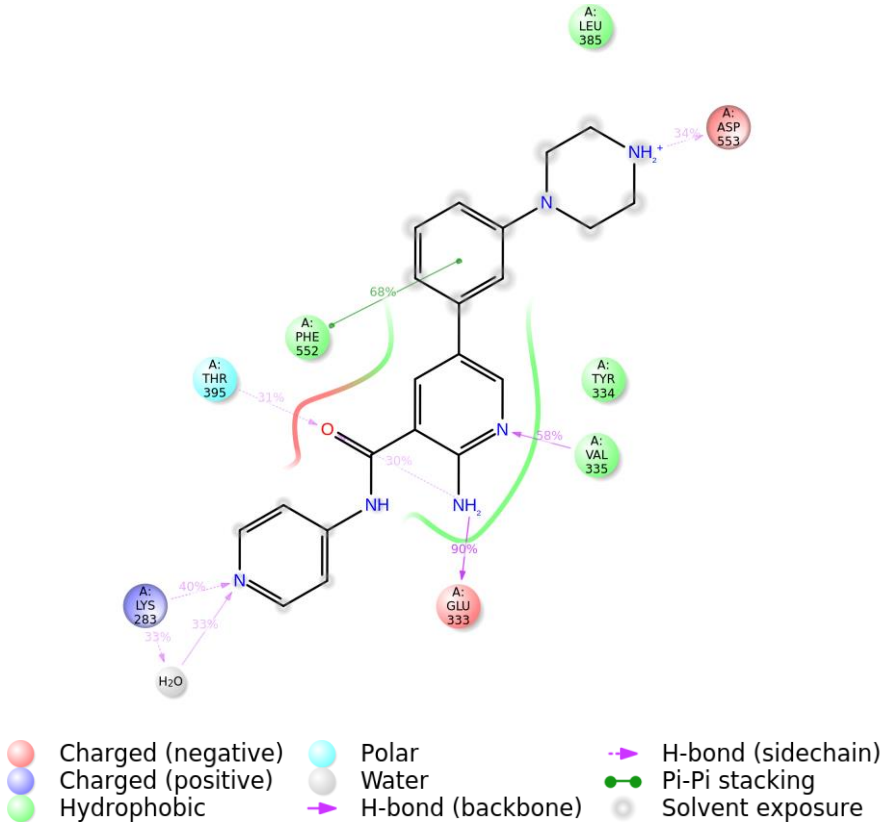
moiety of **19** is not artefactual, composite omit map[4] was generated with Phenix suite of programs (**SI Figure 3 a-d**). The omit map validated our PKC-ι – compound **19** complex interpretation.



SI Figure 3. Composite omit map with annealing generated for each PKC-ι – compound 19 complex: a) chain A; **b**) chain C; **c**) chain E; **d**) chain G.

Furthermore, molecular dynamics simulation (MD) was performed on the PKC-ι – compound **19** complex and the result reinforced our binding hypothesis (**SI Figure 4** and **MD SI**). MD model indicated a water-mediated H-bond between peripheral pyridine and Lys283 as well as a stable interaction of piperazine moiety and Asp553, in addition to π-π contacts of the phenyl ring of **19** with Phe552 and the expected interactions of aminopyridine with hinge-binding protein residues and Thr395 with carbonyl oxygen atom.

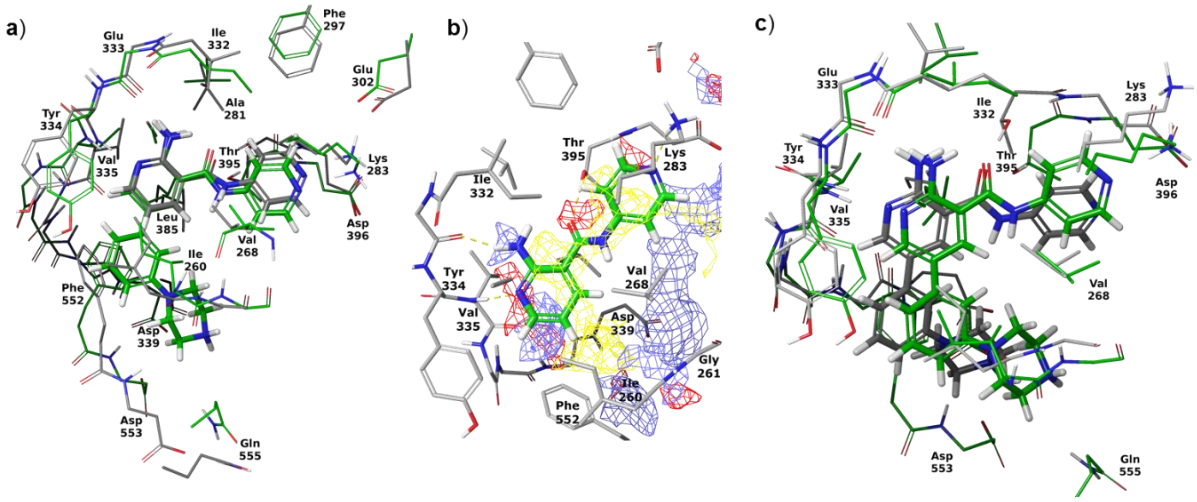
[4] The composite omit map with annealing ignores small portion of the data and re-creates the map, then moves on to remove and re-create another portion and so on, until the composite view of the final map is obtained. Simulated annealing is added to this iteration to remove any bias. This type of electron density is often considered fool-proof to show that electron density is correct and conclusive.



SI Figure 4. Molecular Dynamics Simulation of interactions between PKC-ι and compound 19. The model indicated stable interaction of piperazine moiety with post-kinase residue ASP553.

Comparison of binding mode derived from docking with crystal structure of protein-inhibitor complex

Superimposition of the structure of the binding site and compound **19** derived from X-ray analysis with docking of compound **2** revealed that our docking model was very accurate (**SI Figure 5a**). The aminopyridine core of the two compounds locks the molecules in the same orientation, with slight difference in positioning of the peripheral pyridine moieties pointing towards the flexible Lys283. Interestingly, the model indicated possibility of interaction between post-kinase residue Phe552 and inhibitor substituted with lipophilic group at C-5 (**SI Figure 5b**). This interaction was enabled by phenylpiperazine moiety of compound **19**, where clear π-π interaction was detected between the phenyl and Phe552 with additional possible interaction between piperazine and Asp553 (**Figure 2a**, main text). Consequently, binding mode of **19** remained very similar to that of **2** (**SI Figure 5c)** and different from binding modes observed for similar kinase inhibitors, where post-kinase domain was displaced by substituents of the hinge-binding motif (see e.g. PDB: 3ZH8, 1ZRZ)**.** On retrospect, docking model correctly indicated possible interaction of **19** with post-kinase loop of PKC-ι, as indicated in the superimposition of an X-ray structure with docking (**SI Figure 5c**), which normally would be regarded as unrealistic due to the flexibility of the post-kinase domain.



SI Figure 5. Overlay of the docking model with binding site and ligand shown in X-ray crystal structure of PKC-ι – 19 complex. a) Overlay of modelled binding site and compound **2** (in grey) with X-ray crystal structure of PKC-ι-**19** complex (in green); **b**) the grid superimposed on the binding site shows areas favorable for binding ligand atoms with the following properties: hydrophobic (yellow), acceptor or negatively charged (red), donor or positively charged (blue); **c**) overlay of modelled binding site and compound **19** (in grey) with X-ray crystal structure of PKC-ι-**19** (in green); binding modes derived from docking and X-ray crystal structure are nearly identical.

7. Chemistry: synthesis and characterization of compounds

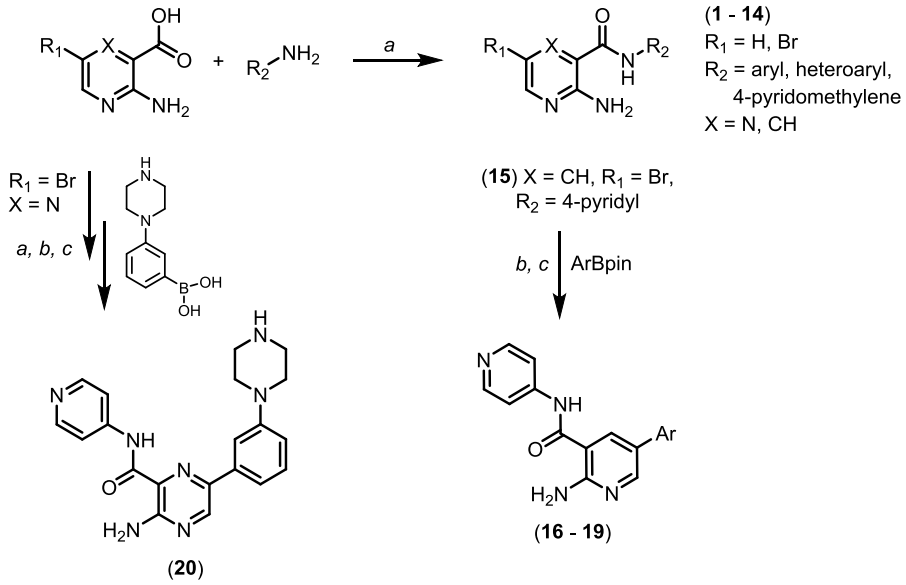
General information

All solvents and reagents were purchased from commercial source and used without further purification. ^{1}H NMR spectra were obtained using a Bruker Ultrashield 400 PLUS/R system, operating at 400 MHz. The compounds' purities were ≥ 95% determined by VARIAN ProStar HPLC instrument with Phenomenex Luna 5u C18(2) 100A, 150x4.60 mm 5 micron, reversed phase column, λ = 254 nm, 5% - 95% CH_3CN/H_2O (with 0.1% formic acid) as eluent, flow rate = 1.0 mL/min, total acquisition time 15 mins. 2-Aminopyrazine-3-carboxylic acid was purchased from Combi Blocks and used as received.

Synthesis

All compounds were synthesized as shown on **SI Scheme 1**.

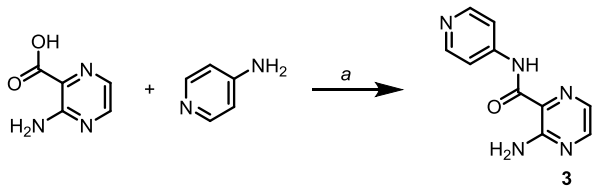
SI Scheme 1. Synthetic route to compounds 1-20.[a]



[a] Regents and conditions: (a) HATU (1.1 equiv.), DIPEA (2 equiv.), CH_2Cl_2/DMF, rt, 4 h; (b) $Pd(DPPF)Cl_2 \bullet CH_2Cl_2$ (0.05 equiv.), K_3PO_4 (3 equiv.), dioxane/water (4/1), 110 °C, 0.5 - 1.5 h; (c) TFA/CH_2Cl_2 (10 vol.%), rt, 1 h.

Compounds **2 – 15** were synthesized through an amide coupling of 2-Aminopyridine-3-carboxylic acid or 2-Aminopyrazine-3-carboxylic acid and an appropriate amine. The representative procedure is described for compound **3** below (**SI Scheme 2**).

SI Scheme 2. Synthesis of compound 3.[a]

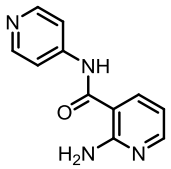


[a] Regents and conditions: HATU (1.1 equiv.), DIPEA (2 equiv.), CH_2Cl_2 / DMF, rt, 4 h.

Procedure. To the solution of 2-Aminopyrazine-3-carboxylic acid (0.5 mmol, 70 mg) in CH_2Cl_2 (4 ml) and DMF (1 ml) DIPEA (2 equiv., 127 mg) was added, followed by HATU portion-wise (1.1 equiv., 209 mg). After 5 minutes of stirring, the amine (1.2 equiv., 56 mg) was added and stirring was continued at room temperature for 4 h. Upon completion as judged by LC-Mass, the reaction mixture was diluted with CH_2Cl_2 (15 ml) and water was added (15 ml). The white precipitate formed was collected and lyophilised to afford the pure product 3-amino-N-(pyridin-4-yl)pyrazine-2-carboxamide (**3**, 94 mg).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (br. s., 1H), 8.47 (d, *J* = 5.62 Hz, 2H), 8.33 (d, *J* = 1.71 Hz, 1H), 7.95 (d, *J* = 1.71 Hz, 1H), 7.88 (d, *J* = 5.75 Hz, 2H), 7.61 (br. s., 2H); ^{13}C NMR (101 MHz, DMSO-d6) δ 165.0, 154.9, 149.6, 147.4, 144.4, 130.5, 123.9, 113.6; LCMS (ESI) m/z calcd for $C_{10}H_{10}N_5O$ $[M+H]^+$ = 216.2, found = 216.1; purity 96.8%, t_R = 4.33 min.

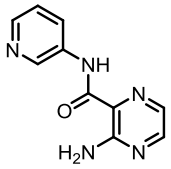
2-Amino-N-(pyridin-4-yl)nicotinamide (**2**)



The title compound was synthesized as described for compound **3** with one modification: 2-Aminopyridine-3-carboxylic acid was used in place of 2-Aminopyrazine-3-carboxylic acid.

65 mg, brown solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.43 (bs, 1H), 8.46 (d, *J* = 6.4 Hz, 2H), 8.16 (dd, *J* = 4.4 Hz, 1.2 Hz, 1H), 8.06 (dd, *J* = 8.0 Hz, 1.2 Hz, 1H), 7.70 (dd, *J* = 4.8 Hz, 1.2 Hz, 2H), 7.00 (bs, 2H), 6.68 (dd, *J* = 8.0 Hz, 4.8 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d6) δ 166.8, 158.2, 151.7, 149.6, 145.2, 137, 113.5, 110.8, 108.7; LCMS (ESI) m/z calcd for $C_{11}H_{10}N_4O$ $[M+H]^+$ = 215.2, found = 215.1.

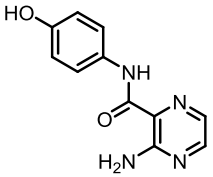
3-Amino-N-(pyridin-3-yl)pyrazine-2-carboxamide (**4**)



The title compound was synthesized as described for compound **3** with one modification: 3-Aminopyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

79 mg, off-white solid ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H), 9.00 (d, *J* = 2.20 Hz, 1H), 8.29 - 8.37 (m, 2H), 8.24 (d, *J* = 8.31 Hz, 1H), 7.94 (d, *J* = 2.08 Hz, 1H), 7.60 (br. s., 2H), 7.39 (dd, *J* = 4.71, 8.25 Hz, 1H); LCMS (ESI) m/z calcd for $C_{10}H_{10}N_5O$ $[M+H]^+$ = 216.2, found = 216.1

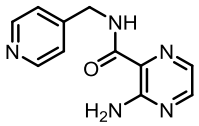
3-Amino-N-(4-hydroxyphenyl)pyrazine-2-carboxamide (**5**)



The title compound was synthesized as described for compound **3** with one modification: 4-Aminophenol was used in place of 4-Aminopyridine.

75 mg, off-white solid ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.26 (br. s., 1H), 9.27 (s, 1H), 8.25 (s, 1H), 7.89 (br. s., 1H), 7.58 (d, *J* = 8.56 Hz, 4H), 6.73 (d, *J* = 8.44 Hz, 2H); LCMS (ESI) m/z calcd for $C_{11}H_{11}N_4O_2$ $[M+H]^+$ = 231.2, found = 231.1

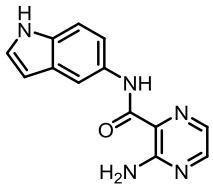
3-Amino-N-(pyridin-4-ylmethyl)pyrazine-2-carboxamide (**6**)



The title compound was synthesized as described for compound **3** with one modification: 4-Aminomethylpyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

92 mg, white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.40 (t, *J* = 6.17 Hz, 1H), 8.45 - 8.53 (m, 2H), 8.24 (d, *J* = 2.32 Hz, 1H), 7.86 (d, *J* = 2.32 Hz, 1H), 7.51 (br. s., 2H), 7.29 (d, *J* = 5.87 Hz, 2H), 4.47 (d, *J* = 6.36 Hz, 2H); LCMS (ESI) m/z calcd for $C_{11}H_{11}N_5O$ $[M+H]^+$ = 230.2, found = 230.1

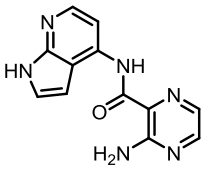
3-Amino-N-(1H-indol-5-yl)pyrazine-2-carboxamide (**7**)



The title compound was synthesized as described for compound **3** with one modification: 5-Aminoindole was used in place of 4-Aminopyridine.

81 mg, light-brown solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.97 - 11.11 (m, 1H), 10.29 (s, 1H), 8.26 (d, *J* = 2.08 Hz, 1H), 8.03 (s, 1H), 7.91 (d, *J* = 2.08 Hz, 1H), 7.48 - 7.73 (m, 2H), 7.42 (d, *J* = 1.47 Hz, 1H), 7.26 - 7.38 (m, 2H), 6.41 (br. s., 1H); LCMS (ESI) m/z calcd for $C_{13}H_{12}N_5O$ $[M+H]^+$ = 254.3, found = 254.2

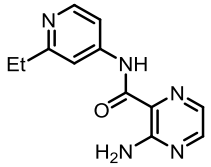
3-Amino-N-(1H-pyrrolo[2,3-b]pyridin-4-yl)pyrazine-2-carboxamide (**8**)



The title compound was synthesized as described for compound **3** with one modification: 4-Amino-7-azaindole was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

66 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.64 - 11.80 (m, 1H), 10.40 - 10.55 (m, 1H), 8.35 (d, *J* = 1.96 Hz, 1H), 8.15 - 8.24 (m, 1H), 7.98 (d, *J* = 2.08 Hz, 1H), 7.76 - 7.87 (m, 1H), 7.55 - 7.71 (m, 2H), 7.39 - 7.49 (m, 1H), 6.53 - 6.64 (m, 1H); LCMS (ESI) m/z calcd for $C_{12}H_{11}N_6O$ $[M+H]^+$ = 255.3, found = 255.2

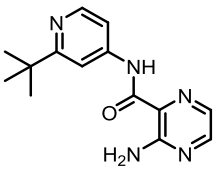
3-Amino-N-(2-ethylpyridin-4-yl)pyrazine-2-carboxamide (**9**)



The title compound was synthesized as described for compound **3** with one modification: 4-Amino-2-ethylpyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

71 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 8.37 (d, *J* = 5.62 Hz, 1H), 8.32 (d, *J* = 2.20 Hz, 1H), 7.94 (d, *J* = 2.20 Hz, 1H), 7.79 (d, *J* = 1.59 Hz, 1H), 7.69 (dd, *J* = 1.96, 5.62 Hz, 1H), 7.60 (br. s., 2H), 2.71 (q, *J* = 7.58 Hz, 2H), 1.23 (t, *J* = 7.58 Hz, 3H); LCMS (ESI) m/z calcd for $C_{12}H_{14}N_5O$ $[M+H]^+$ = 244.3, found = 244.1

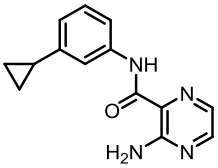
3-Amino-N-(2-(tert-butyl)pyridin-4-yl)pyrazine-2-carboxamide (**10**)



The title compound was synthesized as described for compound **3** with one modification: 4-Amino-2-*tert*-butylpyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

65 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.41 (d, *J* = 5.50 Hz, 1H), 8.32 (d, *J* = 1.96 Hz, 1H), 7.87 - 7.98 (m, 2H), 7.75 (dd, *J* = 1.53, 5.44 Hz, 1H), 7.60 (br. s., 2H), 1.31 (s, 9H); LCMS (ESI) m/z calcd for $C_{14}H_{17}N_5O$ $[M]^+$ = 271.3, found = 271.2

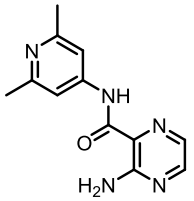
3-Amino-N-(3-cyclopropylphenyl)pyrazine-2-carboxamide (**11**)



The title compound was synthesized as described for compound **3** with one modification: 3-Cyclopropylaniline was used in place of 4-Aminopyridine.

87 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H), 8.28 (d, *J* = 2.20 Hz, 1H), 7.91 (d, *J* = 2.32 Hz, 1H), 7.61 - 7.67 (m, 1H), 7.58 (br. s., 1H), 7.53 (t, *J* = 1.83 Hz, 1H), 7.21 (t, *J* = 7.89 Hz, 1H), 6.85 (d, *J* = 7.70 Hz, 1H), 1.90 (ddd, *J* = 3.36, 4.98, 8.41 Hz, 1H), 0.91 - 1.00 (m, 2H), 0.62 - 0.71 (m, 2H); LCMS (ESI) m/z calcd for $C_{14}H_{14}N_4O$ $[M]^+$ = 254.3, found = 254.1

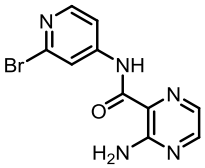
3-Amino-N-(2,6-dimethylpyridin-4-yl)pyrazine-2-carboxamide (**12**)



The title compound was synthesized as described for compound **3** with one modification: 2,6-Dimethyl-4-aminopyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

58 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, *J* = 2.20 Hz, 2H), 7.91 (d, *J* = 2.20 Hz, 2H), 7.29 (br. s., 2H), 3.85 (s, 6H); LCMS (ESI) m/z calcd for $C_{12}H_{14}N_5O$ $[M+H]^+$ = 244.3, found = 244.4.

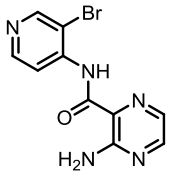
3-Amino-N-(3-bromopyridin-4-yl)pyrazine-2-carboxamide (**13**)



The title compound was synthesized as described for compound **3** with one modification: 3-Bromo-4-aminopyridine was used in place of 4-Aminopyridine.

79 mg, yellow solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.58 (s, 1H), 8.76 (s, 1H), 8.52 (d, *J* = 5.50 Hz, 1H), 8.36 - 8.43 (m, 2H), 7.96 (d, *J* = 2.08 Hz, 1H), 7.66 (br. s., 2H); LCMS (ESI) m/z calcd for $C_{10}H_9BrN_5O$ $[M+H]^+$ = 295.1, found = 295.4

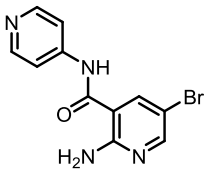
3-Amino-N-(2-bromopyridin-4-yl)pyrazine-2-carboxamide (**14**)



The title compound was synthesized as described for compound **3** with one modification: 2-Bromo-4-aminopyridine was used in place of 4-Aminopyridine. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane.

100 mg, yellow solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.05 (s, 1H), 8.34 (d, *J* = 1.83 Hz, 1H), 8.18 - 8.31 (m, 2H), 7.95 (d, *J* = 1.83 Hz, 1H), 7.91 (d, *J* = 4.52 Hz, 1H), 7.62 (br. s., 2H); LCMS (ESI) m/z calcd for $C_{10}H_9BrN_5O$ $[M+H]^+$ = 295.1, found = 295.4

3-Amino-N-(3-bromopyridin-4-yl)pyrazine-2-carboxamide (**15**)

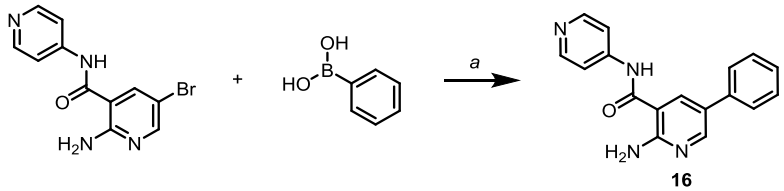


The title compound was synthesized as described for compound **3** with one modification: 2-Amino-5-bromopyridine-3-carboxylic acid was used (2 mmol) instead of 2-Aminopyrazine-3-carboxylic acid. The pure product was obtained after column chromatography on silica gel column eluting with a gradient of ethyl acetate / hexane. Compound **15** was subsequently used as a substrate for preparation of compounds **16** – **19**.

490 mg, yellow solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.52 (s, 1H), 8.44 - 8.54 (m, 2H), 8.25 (dd, *J* = 2.32, 10.88 Hz, 2H), 7.67 - 7.73 (m, 2H), 7.20 (s, 2H); LCMS (ESI) m/z calcd for $C_{11}H_9BrN_4O$ $[M]^+$ = 293.1, found = 293.2

Compounds **16** – **19** were synthesized through a Suzuki coupling of compound **15** with the appropriate boronic acid / pinacol ester (purchased from commercial vendors; majority from Combi Blocks). Representative procedure is described below for compound **16** (**SI Scheme 3**).

SI Scheme 3. Synthesis of compound 16.[a]



[a] Regents and conditions: Pd(DPPF)Cl_2•CH_2Cl_2 (0.05 equiv.), K_3PO_4 (3 equiv.), dioxane/water (4/1), 110 ^{o}C, 0.5 h.

Procedure

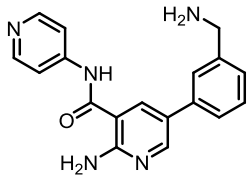
To the solution of compound **15** (3-Amino-N-(3-bromopyridin-4-yl)pyrazine-2-carboxamide, 0.3 mmol, 88 mg) in a mixture of dioxane and water (4/1, total 5 ml), phenylboronic acid was added (1.1 equiv., 40 mg) followed by potassium phosphate (3 equiv., 191 mg). The reaction mixture was degassed by bubbling nitrogen through over 10 mins. Subsequently, palladium catalyst Pd[DPPF]Cl_2•CH_2Cl_2 (5 mol%, 12 mg) was added and the reaction mixture was heated to 110 ^{o}C under nitrogen atmosphere. The reaction was complete in 30 mins (LC-Mass analysis).

Work up: reaction mixture was filtered through paper filter to a separating funnel, diluted with ethyl acetate and washed with water. The aqueous phase was back-extracted with ethyl acetate once. Combined ethyl acetate layers were dried over sodium sulfate and evaporated. The crude product was purified by flash silica gel chromatography eluting with a gradient of ethyl acetate

/ hexane to afford 2-Amino-5-phenyl-N-(pyridin-4-yl)nicotinamide (**16**) as a white solid, 53 mg.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (br. s, 1H), 8.49 (s, 3H), 8.35 (d, *J* = 2.32 Hz, 1H), 7.72 (d, *J* = 6.72 Hz, 4H), 7.47 (t, *J* = 7.70 Hz, 2H), 7.34 (t, *J* = 7.46 Hz, 1H), 7.07 - 7.21 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d6) δ 167.8, 158.6, 150.7, 150.6, 146.4, 137.6, 136.2, 129.4, 127.3, 126.3, 124.2, 114.8, 109.7; LCMS (ESI) m/z calcd for $C_{17}H_{15}N_4O$ $[M+H]^+$ = 291.3, found = 291.5; purity > 99 %, t_R = 4.87 min.

2-Amino-5-(3-(aminomethyl)phenyl)-N-(pyridin-4-yl)nicotinamide (**17**)



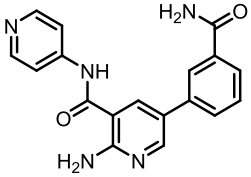
The title compound was synthesized analogically to compound **16** using [3-(Tert-butoxycarbonylaminomethyl)phenyl]boronic acid. Following the Suzuki coupling an additional step of deprotection of the amine was necessary. The deprotection of the amine was performed on the crude product of Suzuki coupling after workup.

Procedure – **deprotection of the amine**:

The crude Suzuki coupling product was dissolved in CH_2Cl_2 (5 ml) and TFA was added (0.5 ml). Upon completion as judged by LC-Mass, the reaction mixture was evaporated. The residue was re-dissolved in methanol, filtered and purified on preparative HPLC eluting with a gradient of acetonitrile/water (containing 0.1 % formic acid). Free base form of the product was obtained upon filtration through Agilent ion exchange column (Stratospheres) to yield 2-Amino-5-(3-(aminomethyl)phenyl)-N-(pyridin-4-yl)nicotinamide (**17**) as an off-white solid, 47 mg.

^{1}H NMR (400 MHz, METHANOL-d_4) δ 8.41 - 8.46 (m, 3H), 8.38 (d, *J* = 2.32 Hz, 1H), 7.79 - 7.84 (m, 2H), 7.64 (s, 1H), 7.55 (d, *J* = 7.70 Hz, 1H), 7.42 (t, *J* = 7.64 Hz, 1H), 7.29 - 7.34 (m, *J* = 7.60 Hz, 1H), 3.88 (s, 2H); LCMS (ESI) m/z calcd for $C_{18}H_{18}N_5O$ $[M+H]^+$ = 320.4, found = 320.1; purity > 99 %, t_R = 2.71 min.

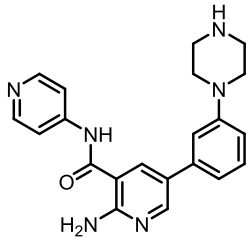
2-Amino-5-(3-carbamoylphenyl)-N-(pyridin-4-yl)nicotinamide (**18**)



The title compound was synthesized analogically to compound **16** using (3-carbamoylphenyl)boronic acid.

54 mg, off-white solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 8.60 (d, *J* = 2.08 Hz, 1H), 8.50 (d, *J* = 5.26 Hz, 2H), 8.42 (d, *J* = 2.08 Hz, 1H), 8.21 (s, 1H), 8.07 (br. s., 1H), 7.89 (d, *J* = 7.82 Hz, 1H), 7.84 (d, *J* = 7.70 Hz, 1H), 7.74 (d, *J* = 6.11 Hz, 2H), 7.56 (t, *J* = 7.70 Hz, 1H), 7.44 (br. s., 1H), 7.17 (s, 2H); LCMS (ESI) m/z calcd for $C_{18}H_{15}N_5O_2$ $[M]^+$ = 333.3, found = 333.5; purity = 95.0 %, t_R = 4.91 min.

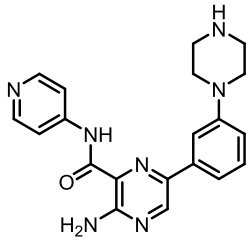
2-Amino-5-(3-(piperazin-1-yl)phenyl)-N-(pyridin-4-yl)nicotinamide (**19**)



The title compound was synthesized analogically to compound **17**, using 3-{4-[(Tert-butoxy)carbonyl]piperazin-1-yl}phenyl)boronic acid.

65 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (br. s., 1H), 8.43 - 8.53 (m, 3H), 8.30 (d, *J* = 2.32 Hz, 1H), 7.69 - 7.76 (m, 2H), 7.29 (t, *J* = 7.95 Hz, 1H), 7.18 (s, 1H), 7.05 - 7.14 (m, 3H), 6.89 (dd, *J* = 1.96, 8.19 Hz, 1H), 3.17 (s, 1H), 3.08 - 3.15 (m, 4H), 2.81 - 2.89 (m, 4H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.9, 158.5, 152.8, 150.7, 146.3, 138.4, 136.3, 129.9, 125.0, 117.0, 114.7, 114.4, 113.4, 109.6, 49.9, 46.1, 25.4; LCMS (ESI) m/z calcd for $C_{21}H_{23}N_6O$ $[M+H]^+$ = 375.4, found = 375.3.

3-Amino-6-(3-(piperazin-1-yl)phenyl)-N-(pyridin-4-yl)pyrazine-2-carboxamide (**20**)



The title compound was synthesized in a three-step sequence: amide coupling between 4-Aminopyridine and 2-Amino-5-bromopyrazine-3-carboxylic acid (limiting substrate, 0.5 mmol), followed by Suzuki coupling of the resulting amide with 3-{4-[(Tert-butoxy)carbonyl]piperazin-1-yl}phenyl)boronic acid and deprotection of the piperazine. All steps were performed following the procedures described above (see compounds **3**, **16** and **17**).

64 mg, off-white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.66 (s, 1H), 8.93 (s, 1H), 8.47 - 8.54 (m, 2H), 7.82 - 7.90 (m, 2H), 7.64 (br. s., 3H), 7.53 - 7.61 (m, 1H), 7.33 (s, 1H), 6.92 - 7.00 (m, 1H), 3.14 - 3.21 (m, 4H), 2.82 - 2.91 (m, 4H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.0, 153.7, 151.4, 149.7, 144.9, 144.3, 138.7, 135.7, 128.7, 122.4, 115.9, 114.7, 113.8, 112.0, 48.4, 44.3; LCMS (ESI) m/z calcd for $C_{20}H_{21}N_7O$ $[M+H]^+$ = 376.4, found = 376.6.