Discovery and Pharmacological Characterization of Novel Quinazoline-based PI3K delta-selective Inhibitors

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In Vitro Biology

Biochemical assays for PI3K isoforms

KinaseGlo format

The inhibitory kinase activities on PI3K α and PI3K β were determined using phosphatidyl inositol (PI) as substrate in n-Octyl-Glucoside (OG) using a luminescence assays based on ATP consumption (KinaseGlo). Some 50 nl of compound dilutions were dispensed onto black 384-well plates (Greiner Cat. No.784076). L- α -phosphatidylinositol (PI), provided as 10 mg/ml solution in methanol, was transferred into a glass tube and dried under nitrogen beam. It was then resuspended in 3% (v/v) Octylglucoside by vortexing and stored at 4°C. Some 4.5 μ l of a mix of PI/OG with 10 nM PI3K α or 0.75 μ g/ml PI3K β was added. The kinase reactions were started by addition of 4.5 μ l of ATP-mix containing in a final volume of 9 μ l the following: 10 mM TRIS-HCl pH 7.5, 3 mM MgCl₂ or 3 mM MnCl₂, 50 mM NaCl, 0.05% CHAPS, 1m M DTT and 1 μ M ATP. The reactions were carried out at room temperature for either 30 or 60 minutes (PI3K α or PI3K β , respectively) stopped with 9 μ l of Kinase Glo and plates were read 10 minutes later in a Synergy2 reader using an integration time of 0.1 seconds per well. To generate the 100% inhibition of the kinase reaction PI/OG in kinase buffer without addition of recombinant kinases was used while the 0% inhibition was given by the solvent vehicle (90% v/v) DMSO) in water in the presence of recombinant kinases.

ADAPTA format

The kinase assay format used for measuring the activity on PI3K γ and PI3K δ is non-radioactive and monitors the formation of ADP TR-FRET (ADAPTA).

Some 50 nl of compound dilutions were dispensed onto white 384-well small volume polystyrene plates. Then, 4.5 μ l of PI3K substrate PI followed by 4.5 μ l of ATP (final assay volume 9 μ l) are incubated at RT. The standard reaction buffer for the Adapta™ TR-FRET assay contained 10 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 50 mM NaCl, 1 mM DTT, and 0.05% (v/v) CHAPS. Reactions were stopped with 4.5 μ l of a mixture of EDTA containing the Eu-labeled anti-ADP antibody and the Alexa Fluor® 647-labeled ADP tracer in TR-FRET dilution buffer. Plates are read 30 to 60 minutes (PI3K γ or PI3K δ , respectively) later in a Synergy2 reader using an integration time of 0.4 seconds and a delay of 0.05 seconds. Control for the 100% inhibition of the kinase reaction was performed by replacing the PI3K by the standard reaction buffer. The control for the 0% inhibition was given by the solvent vehicle of the compounds (90% DMSO in H₂O).

IC₅₀ values obtained via KinaseGlo and ADAPTA values have been shown to be equivalent/comparable.

Radiometric format

The radiometric protein kinase assay (33PanQinase® Activity Assay) was performed in 96-well Flash-Plates™ from Perkin Elmer (Boston, MA, USA) in a 50 μ l reaction volume. The reaction cocktail was pipetted in 4 steps in the following order:

- 10 μ l of non-radioactive ATP solution (in H₂O)
- 25 μ l of assay buffer/ [γ -33P]-ATP mixture
- 5 μ l of test sample in 10% DMSO
- 10 μ l of enzyme/substrate mixture

The assay contained 70 mM HEPES-NaOH pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 3 μM Na-orthovanadate, 1.2 mM DTT, 1.0 μM ATP, [γ -³³P]-ATP (approx. 8 x 10⁵ cpm per well), 2.4 nM protein kinase, and 20 μg/ml substrate (casein).

All protein kinases provided by ProQinase were expressed in Sf9 insect cells or in E.coli as recombinant GST-fusion proteins or His-tagged proteins, either as full-length or enzymatically active fragments. All kinases were produced from human cDNAs. Kinases were purified by either GSH-affinity chromatography or immobilized-metal affinity chromatography. Affinity tags were removed from a number of kinases during purification. The purity of the protein kinases was examined by SDS-PAGE/Coomassie staining, the identity was checked by mass spectroscopy.

The reaction cocktails were incubated at 30 °C for 60 minutes. The reaction was stopped with 50 μl of 2 % (v/v) H₃PO₄, plates were aspirated and washed two times with 200 μl 0.9 % (w/v) NaCl. Kinase activity dependent transfer of ³³Pi (counting of "cpm") was determined with a microplate scintillation counter (Microbeta. Wallac).

All assays were performed with a BeckmanCoulter Biomek 2000/SL robotic system.

For each kinase, the median value of the cpm of three wells with complete reaction cocktails, but without kinase, was defined as "low control" (n=3). This value reflects unspecific binding of radioactivity to the plate in the absence of protein kinase but in the presence of the substrate. Additionally, for each kinase the median value of the cpm of three other wells with the complete reaction cocktail, but without any compound, was taken as the "high control", i.e. full activity in the absence of any inhibitor (n=3). The difference between high and low control was taken as 100 % activity for each kinase.

As part of the data evaluation the low control of each row of a particular plate was subtracted from the high control value as well as from their corresponding 10 "compound values". The residual activity (in %) for each well of each row of a particular plate was calculated by using the following formula:

Res. Activity (%) = 100 X [(cpm of compound - low control) / (high control - low control)]

Since 10 distinct concentrations of each test compound were tested against each kinase, the evaluation of the raw data resulted in 10 values for residual activities per kinase. Based on each 10 corresponding residual activities, IC₅₀ values were calculated using Prism 5.04 for Windows (Graphpad, San Diego, California, USA; www.graphpad.com). The mathematical model used was "Sigmoidal response (variable slope)" with parameters "top" fixed at 100% and "bottom" at 0 %.

Cellular assays for PI3K isoforms

Rat-1 fibroblast cells stably overexpressing a myristoylated form of the catalytic subunit of human PI3K α , β or δ were plated in 384-well plates at a density of 7500, 6200, or 4000 cells (PI3K α , β and δ , respectively) in 30 μl complete growth medium (Dulbecco's modified Eagle's medium (DMEM high glucose) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) MEM non-essential amino acids, 10 mM HEPES, 2 mM L-glutamine, 10 μg/ml puromycin and 1% (v/v) Penicillin/Streptomycin) and were incubated at 37°C / 5%CO₂ / 95% humidity for 24 hours. Compounds were diluted in 384-well plates to obtain 8-point serial dilutions in 90% DMSO, as well as 4 reference compounds plus 16 high controls and 16 low (inhibited) controls. Pre-dilution plates were prepared by dispensing pipetting 250 nl of compound solutions into 384-well polypropylen plates using a Hummingwell nanoliter dispenser. Compounds were pre-diluted by the addition of 49.75 μl complete growth medium. 10 μl of pre-diluted compound solution were transferred to the cell plate using a 384-well pipettor, resulting in a final DMSO concentration

of 0.11%. Cells were incubated for 1 h at 37°C / 5%CO₂ / 95% humidity. The supernatant was removed, the cells were lysed in 20 µl of lysis buffer for AlphaScreen® SureFire® detection. For detection of p-AKT(Ser473), the SureFire® p-Akt 1/2 (Ser473) Assay Kit (PerkinElmer, U.S.A) was used. Some 5 µl of cell lysate was transferred to 384-well low volume Proxiplates for detection using a 384-well pipettor. Addition of AlphaScreen® SureFire® reagents was done according to the manufacturer's protocol. First, 5 µl of reaction buffer plus activation buffer mix containing AlphaScreen® acceptor beads was added, the plate was sealed, and incubated on a plate shaker for 2 hours at room temperature. Second, 2 µl of dilution buffer containing AlphaScreen® donor beads was added, and the plate was incubated on plate shaker as above for a further 2 hours. The plate was read on an AlphaScreen® compatible plate reader, using standard AlphaScreen® settings.

Cellular assays for B cell activation in vitro

Mouse B cell activation (CD86 expression) and proliferation after B cell receptor stimulation.

Compound **11** was first dissolved and diluted in DMSO followed by a 1:50 dilution in medium. Splenocytes from Balb/c mice were isolated, resuspended and transferred to 96 well plates (200 µl/well). The diluted compound or solvent were added to the plates (25 µl) and incubated at 37 °C for 1 hour. Then the cultures were stimulated with 25 µl anti-IgM mAb/well (final concentration 30 µg/ml) for 24 hours at 37 °C and stained with anti-mouse CD86-FITC and anti-mouse CD19-PerCP (2 µl of each antibody/well, both Becton Dickinson). CD86 expression on CD19 positive B cells was quantified by flow cytometry. Based on the reduction of CD86 expression IC₅₀ values were determined.

To assess effects on proliferation, murine B cells were stimulated via the B cell receptor by anti-IgM antibody in the presence of titrated amounts of compound and proliferation was assessed by incorporation of radioactive ³H-Thymidine over the last 16 hours of a 88 hour incubation period as described (Julius et al 1984).

Rat B cell activation in diluted whole blood (CD86).

For assessing effects on B cell activation upon surface B cell receptor stimulation, heparinized blood was pre-diluted to 50% with medium. Then, 180.5 µl pre-diluted blood was spiked with 9.5 µl of pre-diluted compound **11** in 96 well U-bottomed microtiter plates (Nunc) resulting in a 2-fold serial dilution with a concentration range from 50 to 0.008 µM. Control wells were pretreated with DMSO to obtain a final concentration of 0.5 % DMSO. Cultures were set up in duplicates, mixed well by agitation on a plate shaker, pipetting up and down and agitated on the plate shaker again. Cultures were incubated at 37 °C, 5% CO₂ for 1 hr. Then 10 µl of anti-rat IgM/rIL-4 at a final concentration of anti-rat IgM of 50 µg/ml and rIL-4 of 10 ng/ml was added. Control samples were left unstimulated. Samples were mixed thoroughly and incubated for 24 hours at 37 °C, 5% CO₂. After incubation, 15 µl of a 25 mM EDTA solution was added per well and shaken for 15 min to detach adherent cells.

Samples were then stained with PE-Cy5-labeled anti-ratCD45RA (Becton Dickinson) to allow gating on B cells in FACS analysis. In addition, samples were stained with PE-labeled anti-rat CD86 (Becton Dickinson).

All staining procedures were performed at room temperature for 30 min in the dark. After incubation, samples were transferred to 96-deep well V-bottomed microtiter plates (Corning) containing 2 ml/well of 1x BD Lysing Solution (Becton Dickinson). After lysis of erythrocytes samples were washed with 2 ml of CellWASH (Becton Dickinson). Data was acquired on an LSRII or FACScalibur flow cytometer (BD Biosciences) using Cellquest Plus or DIVA (version 6.1.1) software, respectively. Lymphocytes were gated in the FSC/SSC dot blot according to size and granularity and further analyzed for expression of CD45RA and activation markers. Data were calculated from dot blots or histograms as percentage of cells positively stained for activation markers within the CD45RA+ population.

Rat B cell activation in diluted whole blood (pAkt). For assessment of the in vitro effects of compound **11** on intracellular activation marker pAkt, heparinized blood was prediluted to 50% with medium. Then, 180 µl prediluted blood was spiked with 10 µl of pre-diluted compound **11** in 5ml U-bottom tubes (Becton Dickinson) resulting in a dilution with a concentration range from 16666 nM to 0.8 nM. Control wells were pretreated with DMSO to obtain a final concentration of 0.17 % DMSO. Samples were set up in duplicates, mixed well by agitation on a vortexer (3 times 1 sec, speed 1800). Samples were incubated at 37 °C in the water bath for 1.5 hours. 10 µl of diluted goat anti-rat IgM antibody (Serotec) and 10 µl of diluted rIL-4 (Becton Dickinson) was added, mixed (3 times 1 sec, speed 1800) and incubated for 10 minutes at 37 °C in the water bath. After incubation, 2 ml of pre-warmed (37 °C in water bath) BD Phosflow Lyse/Fix buffer (Becton Dickinson) was added per tube and shaken for 3 seconds on a vortexer and incubated for 20 minutes at 37°C in the water bath. Samples were centrifuged at 400 g for 5 minutes. After centrifugation 2 ml of BD Phosflow Perm/wash buffer I (Becton Dickinson) was added per tube and incubated at room temperature in the dark for 10 minutes. After centrifugation at 400 g for 5 minutes the pellets were washed with 2 ml of BD Phosflow Perm/wash buffer I and again centrifuged at 400 g for 5 minutes.

Samples were stained with PE-labeled anti-rat IgM, (Becton Dickinson) to allow gating on B cells in FACS analysis. In addition, samples were stained with Alexa647 conjugated anti-pAkt (Ser473; Becton Dickinson).

Staining procedures were performed in BD Phosflow Perm/wash buffer I at RT for 30 min in the dark. After incubation, samples were washed with 2 ml of 1xBD Phosflow Perm/wash buffer I and centrifuged at 400 g for 5 minutes and the pellets were resuspended in 300 µl BD Stain Buffer (Becton Dickinson). Samples were kept on ice until data were acquired on an LSRII flow cytometer (BD Biosciences) using DIVA (version 6.1.2) software. Lymphocytes were gated in the FSC/SSC dot blot according to size and granularity and further analyzed for expression of IgM and phosphorylation of Akt. Data were calculated from dot blots or histograms as percentage of cells positively stained for Akt-phosphorylation within the IgM+ population

Human B cell activation in diluted whole blood (pAkt).

For assessment of the in vitro effects of compound **11** on intracellular pAkt, 180 µl heparinized blood was spiked with 10 µl of pre-diluted compound **11** in 5 ml U-bottom tubes (Becton Dickinson) resulting in a dilution with a concentration range from 16666 nM to 0.8 nM. Control samples were pretreated with DMSO to obtain a final concentration of 0.17 % DMSO. Samples were set up in duplicates, mixed well by agitation on a vortex (3 times 1 sec, speed 1800). Samples were incubated at 37 °C in the water bath for 1.5 hr. Then, goat anti-human IgM antibody (Southern Biotech) in a volume of 10 µl was added, mixed (3 times 1 sec, speed 1800) and incubated for 20 min at 37 °C in the water bath. After incubation, 2 ml of

pre-warmed (37 °C in water bath) BD Phosflow Lyse/Fix buffer (Becton Dickinson) was added per tube and shaken for 3 seconds on a vortexer and incubated for 20 min at 37°C in the water bath. Samples were centrifuged at 400g for 5 min. After centrifugation 2 ml of BD Phosflow Perm/wash buffer I (Becton Dickinson) was added per tube and incubated at room temperature in the dark for 10 min. After centrifugation at 400 g for 5 min the pellets were washed with 2 ml of BD Phosflow Perm/wash buffer I and again centrifuged at 400 g for 5 min. Supernatants were discarded and the samples were stained with anti-hu CD20 (Alexa488-labeled anti-huCD20, Becton Dickinson) to allow gating on B cells in the cytometric analysis. In addition, samples were stained with Alexa647 conjugated anti-human pAkt (Ser473; Becton Dickinson).

Staining procedures were performed in BD Phosflow Perm/wash buffer I at RT for 30 min in the dark. After incubation, samples were washed with 2 ml of BD Phosflow Perm/wash buffer I and centrifuged at 400 g for 5 minutes and the pellets were resuspended in 300 µl BD Stain Buffer (Becton Dickinson). Samples were kept on ice until data were acquired on an LSRII flow cytometer (BD Biosciences) using DIVA (version 6.1.2) software. Lymphocytes were gated in the FSC/SSC dot blot according to size and granularity and further analyzed for expression of CD20 and phosphorylation of Akt. Data were calculated from dot blots or histograms as percentage of cells positively stained for Akt-phosphorylation within the CD20+ population.

Human B cell activation in diluted whole blood (CD69, CD86).

For assessment of the in vitro effects of compound **11** on B cell surface activation marker, 180.5 µl heparinised whole blood was spiked with 9.5 µl of pre-diluted compound **11** in 96 well U-bottomed microtiter plates (Nunc) resulting in a 2-fold serial dilution with a concentration range from 50 to 0.008 µM. Control wells were pretreated with DMSO to obtain a final concentration of 0.5 % DMSO. Cultures were set up in duplicates, mixed well by agitation on a plate shaker, pipetting up and down and agitated on the plate shaker again. Cultures were incubated at 37 °C, 5% CO₂ for 1 hr. Then 10 µl stimulus solution as described and mixed as above and cultures were incubated at 37 °C, 5% CO₂ for a further 24 h. Following incubation, cell aggregates were broken up by addition of 15 µl/well of a 25 mM EDTA solution, pH 7.4, to the cultures. Samples were mixed thoroughly by agitation on a plate shaker for 15 min. Cells were stained by addition of 25 µl of a mixture of fluorescent labelled antibodies, mixing on a plate shaker and incubation for 30 min in the dark at room temperature. Samples were stained with anti-huCD19-APC (Becton Dickinson) to allow gating on B cells in FACS analysis. In addition, samples were stained with anti-huCD69-PE-Cy7 and anti-huCD86-PE-Cy5 (both Becton Dickinson).

After staining, samples were transferred to 96-deep well V-bottomed microtiter plates, (Corning) containing 2 ml/well of 1x BD Lysing Solution (Becton Dickinson). Plates were mixed by pipetting up and down and incubated for 10 min in the dark at room temperature. Plates were centrifuged at 450 x g for 5 min and after removal of the supernatant, 2 ml of CellWASH (Becton Dickinson) was added to each well. Plates were centrifuged at 450 x g for 5 min again, the supernatant removed and the cell pellet resuspended in 0.5 ml CellWASH.

Data was acquired on a Becton Dickinson LSR II flow cytometer using BD FACSDiva software (version 4.1.2). Lymphocytes were gated in the FSC/SSC dot plot according to size and granularity and further analyzed for expression of CD19 and activation markers. Data were calculated from dot blots as percentage of cells positively stained for activation markers within the CD19+ population.

High-throughput equilibrium solubility

Sample preparation for high-throughput equilibrium solubility determination was performed in microtiter plate format. Starting from 10 mM DMSO stock solution, each sample was dispensed in triplicates. The DMSO was evaporated in an evaporator (Combidancer, Hettich) under vacuum and moderate heat of 35 °C. Afterwards the buffer media was added, the plate sealed and shaken overnight at 1000 rpm (Tritrimax, Heidolph) at ambient temperature. After phase separation of supernatant and undissolved solid by centrifugation, an adequate volume of the supernatant was diluted for analysis. Quantitation was done using a four point external calibration curve, performed by mass spectrometry using LC-MS/MS (TSQ Discovery, Thermo). Data evaluation was performed by Excel (Microsoft) and results were stored in a proprietary laboratory information system.

Kinase selectivity and safety panel assays for compound 11

List of lipid kinase assays:

PIK3C3, KGlo	IC ₅₀ = 4.1 uM
PI4Kβ, KGlo	IC ₅₀ > 9 uM
mTOR, FRET	IC ₅₀ > 9 uM

List of protein kinase assays, all IC₅₀ > 10 uM:

ALK, ABL1, AKT1, AURKA, BTK, CDK2A, CDK4D1, GSK3B, JAK1, JAK2, JAK3, MAP3K8, MAPK1, MAPK14, MAPKAPK2, MAPKAPK5, MKNK2, PDPK1, PKN1, PKN2, PRKACA, PRKCA, PRKCQ, ROCK2, TYK2, EphA4, EphB4, FGFR4, FGFR3K, HER1, HER2, IGF1R, INS1R, KDR, LCK, PDGFRα, RET, SYK, cKIT, cMET

List of safety panel assays:

Adenosine 1 receptor binding assay, human recombinant.....	IC ₅₀ > 30 uM
Adenosine 2A receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Adenosine 3 receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Adenosine Transporter binding assay, human non-recombinant	IC ₅₀ > 30 uM
Adrenergic Alpha 2C receptor assay, human recombinant	IC ₅₀ > 30 uM
Alpha1A receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Androgen receptor agonist/antagonist FRET assay, rat recombinant.....	IC ₅₀ > 30 uM
Angiotensin II AT1 receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Beta 1 adrenergic receptor assay, human recombinant	IC ₅₀ > 30 uM
Beta 2 adrenergic receptor assay, human recombinant	IC ₅₀ > 30 uM
Bradykinin 2 receptor binding assay, human recombinant	IC ₅₀ > 30 uM
COX-1 assay, ovine recombinant	IC ₅₀ > 30 uM
COX-2 assay, human recombinant	IC ₅₀ > 30 uM
Cholecystokinin A receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Cholecystokinin B receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Dopamine D2 receptor assay, human recombinant	IC ₅₀ > 30 uM

Estrogen alpha receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Ghrelin receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Glucocorticoid receptor binding assay, human recombinant	IC ₅₀ > 30 uM
HERG dofetilide binding assay, human recombinant	IC ₅₀ > 30 uM
Histamine H1 receptor assay, human recombinant	IC ₅₀ > 30 uM
Human histamine H3 receptor binding assay	IC ₅₀ > 30 uM
Melanocortin MC3 receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Melanocortin MC4 receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Monoamine Oxidase A, human recombinant baculovirus	IC ₅₀ > 30 uM
Motilin receptor binding assay, human recombinant	IC ₅₀ > 30 uM
Muscarinic M1 receptor assay, human recombinant	IC ₅₀ > 30 uM
Muscarinic M2 Calcium flux assay, agonist and antagonist mode	IC ₅₀ > 10 uM
Nicotinic (CNS) Receptor binding in human IMR32 cells	IC ₅₀ > 30 uM
Norepinephrine Transporter assay, human recombinant	IC ₅₀ > 30 uM
Opiate Mu OpM receptor assay, human recombinant	IC ₅₀ > 30 uM
Phosphodiesterase 3 from human platelets	IC ₅₀ > 30 uM
Phosphodiesterase 4D, human recombinant	IC ₅₀ = 7 uM
Progesteron receptor agonist/antagonist FRET assay, human recombinant ..	IC ₅₀ > 30 uM
Serotonin Transporter assay, human recombinant	IC ₅₀ > 30 uM
Serotonin 5HT2A Calcium flux assay, agonist and antagonist mode.....	IC ₅₀ > 10 uM

Animal studies – general

All aspects of animal care, use, and welfare for research animals used for in vivo studies were performed in compliance with local governmental regulations. Furthermore, all animals were handled in accordance with Novartis Animal Care and Use Committee approved protocols and regulations.

PK/PD study

Animals

PK/PD experiments were performed with adult male Lewis rats (LEW/HanHsd) weighing 220-280 g. SRBC experiments were performed with female albino OFA rats weighing 150 to 160g. Studies were performed according to the Swiss federal law for animal protection and approved by the Veterinary Office Basel (BS No. 425).

Reagents

KZI/Gluc.5%; 1 part ‘Konzentrat zur Infusion’ (Ethanol (Merck)/Cremophor EL (Fluka) 1+1 V/V) and 2 parts of D-Glucose (Fluka) Solution 5% was used as the vehicle for oral compound administration. High molecular weight sodium heparin B.Braun (B.Braun, Melsungen, Germany; 5000 I.U./ml) was used as anticoagulant.

Compound dosing, PK/PD blood collection

Compound 11 was administered p.o. at a volume of 4 ml/kg body weight. For blood collection, animals were anaesthetized with isofluran using a Fluvac airflow system. Whole blood was collected from the abdominal aorta using a 10 ml syringe with hypodermic needle pre-coated with sodium heparin. Blood was transferred into 50 ml Falcon tubes and the anticoagulant concentration was adjusted to 100 U/ml final concentration.

Pharmacokinetic analysis of PK/PD experiment

For pharmacokinetic analysis, 150 µl of each whole blood sample was stored at -80 °C prior analysis. After addition of 20 µl internal standard (conc = 400 ng/ml) to an 80 µl aliquot of each whole blood sample, cells and proteins were removed by centrifugation following precipitation with 400 µl acetonitrile. The organic upper layer was then evaporated to dryness. The residues were dissolved in 20 % acetonitrile containing 0.2 % formic acid, centrifuged and then stored at 10 °C prior to analysis. For calibration, 8 blank whole blood samples were spiked with amounts of compound from 1 to 1000 ng/ml. Quality and recovery control samples were set to 100 ng/ml.

For analysis, a 10 µl aliquot of each sample extract was injected into a LC-MS system. Compounds were resolved on a Uptisphere 3 HDO C18 0.5 x 50 mm reversed phase column applying a linear gradient from 0.02 % heptafluoro-butyric acid in water to acetonitrile, containing 0.02 % trifluoroacetic acid, within 5 minutes. For detection, mass spectrometry in MRM (multiple reaction monitoring) was used. After ionization of the column effluent in an AP electrospray source, compound and internal standard were detected as their [MH]⁺ product ions.

Compound concentrations were calculated by Thermo Xcalibur® and Excel®, based on the extracted peak area ratio, obtained from the relative intensity of the MS signals.

SRBC

Five female OFA rats per group were immunized on day 0 with 2x10⁸/ml SRBCs (SRBC from Laboratory animal services LAS, Novartis) in a volume of 0.5 ml by i.v. injection. Animals were treated for 4 consecutive days starting on the day of immunization.

Animals were euthanized with CO₂ 4 days after immunization. Agarose dishes were prepared, 0.7% Agarose (SERVA) in Hanks Balanced Salt solution pH 7.3 (Gibco, added with 1 mg Phenol red /100ml). Spleens were removed and a single cell suspension (30x10⁶/ml) of each animal was prepared and plated with fresh antigen (SRBC, 40 x 10⁸/ml) onto the soft agar (50 µl splenocytes + 50 µl SRBCs). After an incubation for 60 minutes at 37 °C, 1.4 ml guinea pig complement 10% (Harlan) was added and incubation continued for another 60 minutes at 37 °C. During this time, B cells released antibodies formed against SRBCs, which, upon incubation bound to the antigen (SRBC) in the vicinity of the secreting B cells. This antigen-antibody complex activated complement and led to lysis of the complex leaving a bright spot (plaque) within the red antigen layer (erythrocytes). Plaques were counted with a microscope.

The following formula for calculation of % Inhibition was used:

$C \times 100 / V - 100$ with V= mean# plaques/spleen for vehicle group; C= mean# plaques/spleen for compound treated group.

For *ex vivo* PD analysis of blood from drug-treated animals, 90 µl heparinized blood was mixed in 5 ml U-bottom tubes (BD, cat# 352063) with 100 µl of RPMI medium (Gibco, cat# 31870) immediately after

blood collection, and activated with 10 µl of anti-rat IgM/rIL-4 at a final concentration of anti-rat IgM of 50 µg/ml and rIL-4 of 10 ng/ml. Control samples were left unstimulated. Samples were then processed, stained for pAkt and CD86 and analyzed as described above.

Full statistics: At a dose of 10 mg/kg bid, plaque formation was inhibited by 79% from a mean of $4.18 \cdot 10^5$ PFC/spleen (95% CI = $3.02 \cdot 10^5$ to $5.33 \cdot 10^5$) to a mean of $8.89 \cdot 10^4$ PFC/spleen (95% CI = $4.47 \cdot 10^4$ to $13.3 \cdot 10^4$, $p < 0.001$). At 30 mg/kg bid, an inhibition of 94% from a mean of $4.18 \cdot 10^5$ PFC/spleen (95% CI = $3.02 \cdot 10^5$ to $5.33 \cdot 10^5$) to a mean of $2.37 \cdot 10^4$ PFC/spleen (95% CI = $1.46 \cdot 10^4$ to $3.28 \cdot 10^4$, $p < 0.001$) was achieved comparable to the inhibition seen with the positive control CsA (Cyclosporin A, mean = $3.00 \cdot 10^4$ PFC/spleen, 95% CI = $1.30 \cdot 10^4$ to $4.70 \cdot 10^4$, $p < 0.001$).

Pharmacokinetic studies

In vivo pharmacokinetic studies in rats

Female wild-type Sprague Dawley rats (Iffa Credo, France) were kept in standard cages and conditions according to Swiss Animal Welfare guidelines (12h light/dark cycles, room temperature at 22-24 °C, humidity at least 45 % but <70 %) with free access to Ringer solution (glucose 5%, NaCl 0.9% and KCl 0.5%) and pelleted rodent chow. 96-120 hours before administration of the test substance the animals were anesthetized with isoflurane and catheters were surgically implanted under aseptic precautions (use of sterile instruments and surgical material in combination with local antibiotic prophylaxis) into the femoral artery and vein. The catheters were exteriorized in the neck region, connected to a Harvard swivel system (Harvard Instruments) and filled with 0.9% saline containing 100 U mL⁻¹ heparin. After recovery from anesthesia the animals were housed individually in special cages with free access to food and tap water until and throughout the experiment. Analgesic treatment with Temgesic® (10 µg/kg s.c., application volume 1 mL/kg) was performed in the evening following surgery and in the next morning. Compound administration was in the morning (6-8 AM). Blood samples were collected at various time points from the femoral artery catheter into Eppendorf tubes coated with sodium EDTA. Blood samples were immediately frozen at -20 °C until final processing (maximum storage was 8 days). Intravenous and oral dosing was performed in the same animals after a 48 h wash-out interval between the single dose applications. The test substance was administered intravenously as a solution in 1-methyl-2-pyrrolidone and polyethylene glycol 200 (30:70, v/v) at a dose of 1 mg/kg and orally as a homogenous aqueous suspension in Tween 80 and carboxy methyl cellulose sodium 0.5/0.5/99 (w/w) at a dose of 3 mg/kg.

In vivo pharmacokinetic studies in dogs

Adult male beagle dogs (originating from Marshall Farms at Montichiari, Italy) from the permanent MAP/DMPK stock were kept under standard conditions in dog pens according to Swiss Animal Welfare guidelines (standard dog chow once daily, free access to tap water throughout the study). The dogs were fasted 12 h before dosing and then fed 2 h post dose. Compound administration was done in a cassette

dosing format of five compounds and in the morning (7-8 AM). Intravenous and oral dosing was performed in different animals. The test substance were administered intravenously as a solution in 1-methyl-2-pyrrolidone and polyethylene glycol 200 (10:90, v/v) at a dose of 0.1 mg/kg and orally as a homogenous aqueous suspension in Tween 80 and carboxy methyl cellulose sodium 0.5/0.5/99 (w/w) at a dose of 0.3 mg/kg.

Bioanalytical method for pharmacokinetics

Chemicals and reagents: CHROMASOLV[®] Plus HPLC grade water (Sigma, Buchs, Switzerland) was further purified by distillation using a Water Still Distinction D4000 (GMB Glasmechanik, Therwil, Switzerland). CHROMASOLV[®] HPLC grade acetonitrile (MeCN) and methanol (MeOH), phosphate buffer (PBS, 1.0 M pH 7.4), trifluoroacetic acid (TFA), nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt hydrate (NADPH), raloxifene, 1-methyl-2-pyrrolidone (NMP) and polyethylene glycol-200 (PEG200), uridine 5-diphosphoglucuronic acid trisodium salt (UDPGA), glutathione, isocitrate dehydrogenase, isocitrate, alamethicin and carboxy methyl cellulose sodium (C9481) were purchased from Sigma (Buchs, Switzerland). Tween 80 (Polysorbate 80, 59924) and ammonium bicarbonate was purchased from Fluka (Switzerland).

Optima LC/MS grade methanol and acetonitrile were purchased from Fisher Scientific (Waltham, Massachusetts USA). CHROMASOLV[®] NMR grade acetonitrile (used for metabolite desorption), ammonium formate and formic acid were purchased from Fluka (Buchs, Switzerland). Nitrogen (purity 99.5 %) and Argon (purity 99.995 %) were purchased from Carbogas (Lausanne, Switzerland). Perdeuterated dimethyl sulfoxide (DMSO-d₆, 100 atom% D) was purchased from Armar Chemicals (Döttingen, Switzerland).

Oasis HLB columns and SPE cartridges were purchased from Waters (Milford, Massachusetts USA). Synergi Polar-RP HPLC columns were purchased from Phenomenex (Brebühler, Switzerland). XBridge BEH130 C18 HPLC columns were purchased from Waters (Switzerland). Maisch Reprosil C18 HPLC columns were purchased from Morvay (Basel, Switzerland).

Blood samples were spiked with a structurally closely related internal standard, then lysed and deproteinized using acetonitrile. After centrifugation the supernatant was evaporated to dryness. The remainder was re-dissolved in methanol (60%) and 1% aqueous formic acid (pH 3.0). The solution was separated on a Synergi Polar-RP HPLC column (particle size: 2.5 µm; column dimensions: 2 × 50 mm) using a linear gradient (0 min, 98% A/2% B; 4.1 min, 30% A/70% B; 4.2 min, 1% A/99% B; 5.4 min, 1% A/99% B at a flow rate of 0.35 or 0.45 mL/min. Mobile phase A consisted of 1% formic acid in HPLC grade water. Mobile phase B consisted of 1% formic acid in HPLC grade acetonitrile. The flow from the HPLC system was directly introduced into the ion source of a Sciex Q-Trap 5500 mass spectrometer (AB Sciex, Massachusetts, USA) and subjected to electrospray ionization (positive ion mode). The test substance was detected via a specific daughter ion of its protonated quasi-molecular ion. Quantitation of blood levels of the test substance was based on a 8-level calibration curve (in triplicate) using blank rat blood samples spiked with stock solutions of external and internal standards.

Supplementary Table 1. PK parameters^a for compounds 6d and 11.

Compound (route of administration)	6d (i.v.)	6d (p.o.)	11 (i.v.)	11 (p.o.)	11 (p.o.)	11 (i.v.)	11 (p.o.)
Species (gender)	Rat (female) ^b	Rat (female) ^b	Rat (female)	Rat (female)	Rat (female)	Dog (male)	Dog (male)
Dose [mg·kg ⁻¹]	1	3	1	3	30	0.1	0.3
CL [mL·min ⁻¹ ·kg ⁻¹]	33 (4)		15 (3)			13 (3)	
V _{ss} [L·kg ⁻¹]	3.9 (0.5)		2.0 (0.3)			1.0 (0.2)	
t _{1/2} term. [h]	2.1 (0.4)		2.0 (0.5)			0.9 (0.1)	
AUC d.n. ^c [nM·h]	1152 (148)	918 (144)	2315 (537)	500 (168)	237 (24)	2798 (715)	1188 (251)
abs. oral BAV [%]		80 (13)		22 (7)	10 (1)		42 (9)
C _{max} d.n. ^c [nM]		230 (92)		48 (14)	20 (4)		440 (112)
T _{max} [h]		0.7 ^d		3.5 (3.1)	5.3 (2.2)		0.8 (0.3)

^amean values (rat: n = 4, dog: n= 3) with standard deviation in brackets, ^bcassette dosing, ^cd.n. = dose normalized to 1 mg·kg⁻¹, ^dhigh variability.

Structural biology

The human protein was used for PI3K α and the mouse protein for PI3K δ . The only isoform divergence within 12 Å of the ligand binding site is amino acid 830 (R830 in the human sequence, H830 for mouse), both residues are solvent exposed (see Supplementary Figure 1). Based on at least partial protonation of H830 at physiological pH, we are confident that the PI3K δ structure of compound 11 with the mouse protein is very close to that of the human protein.

Supplementary Figure 1. Difference between human and mouse PI3K δ around the ligand binding site: R830 (orange) in human versus H830 in mouse.

Supplementary Figure 2. Main interactions of compound 11 in PI3K δ (a) and PI3K α (b).

Expression and purification (PI3K α)

PI3K α BV2244 p85(307-593) + p110 α (M232K/L233K) was expressed in Tn5 insect cells. Cell pellets were resuspended in Buffer A + 5 mM imidazole + Benzonase (1:10K dilution), 1:6 (pellet:lysate volume)

and lysed by Dounce, 20 strokes. The lysate was cleared by centrifugation, 20K rpm in Beckman JA-20 rotor for 30 m. The first step of purification was performed by immobilized metal ion affinity column (IMAC:Nickel SepharoseFF) with one wash with Buffer B, followed by step elution with Buffer C. The pooled IMAC fractions were adjusted with 10mM EDTA. IMAC fractions were diluted with 15x Buffer D and purified over Ion Exchange Column, Sepharose SP-HP, with a gradient from 30 mM to 500 mM over 20 CV using Buffer E and Buffer F. Concentrated IEX fractions with 50K MWCO were loaded onto gel filtration column (Superdex 200) pre-equilibrated with Buffer G. Final sample was concentrated 6-6.5mg/mL.

Buffer A: 20 mM Tris pH 7.5, 500 mM NaCl, 1 mM MgCl₂, 1% Betaine, 1% Ethylene Glycol, 1xComplete Protease Inhibitor Tablet, 5mM bME.

Buffer B: Buffer A + 45 mM Imidazole

Buffer C: Buffer A + 250 mM Imidazole

Buffer D: 50 mM Hepes pH 7.5, 1% Betaine, 1% Ethylene Glycol, 0.02% CHAPS, 5 mM DTT

Buffer E: Buffer D + 30 mM NaCl

Buffer F: BufferD + 1 M NaCl

Buffer G: 20 mM Tris pH 7.2, 200 mM NaCl, 1% Betaine, 1% Ethylene Glycol, 0.02% CHAPS, 5 mM DTT

Co-crystallization of the PI3K α /compound 11 complex and structure refinement

Compound 11, dissolved in DMSO, was added to the protein solution containing PI3K α to a final concentration of 1 μ M. This solution was incubated on ice for 1 h and then passed through a 0.2 μ m filter. Crystals were grown by the hanging-drop vapor diffusion method at 30 °C by mixing 5 μ L protein-inhibitor solution with 3 μ L well solution composed of 120 mM potassium thiocyanate and 12% w/v polyethylene glycol (PEG) 3350. The drops were streak seeded and then sealed with a screw cap. Crystals were transferred to a new drop containing the well solution supplemented with 20% ethylene glycol and then flash cooled in liquid nitrogen. Diffraction data was collected at beamline 5.0.2 of the Advanced Light Source.

The diffraction images were integrated and data scaled and merged with autoPROC. The initial structural solution was solved by Molecular Replacement using Phaser and an in-house model. The structure was improved to convergence through successive rounds of rebuilding and refinement using Coot¹ and auto-Buster,² respectively.

Expression and purification (PI3K δ)

Virus stock was obtained from the laboratory of Roger Williams (Medical Research Council, Laboratory of molecular biology, Cambridge, UK).

5L- bottles with a culture volume of 3.7 L were used for the large-scale expression. Sf9-DE cells were diluted at a density of $1.5 \times 10^6 \text{ ml}^{-1}$ in SF4 medium. Recombinant baculoviruses were added simultaneously at 10 - 20 parts of PI3K δ and 1 part for iSH2p85 α (dependent on virus batch and as determined by functional titration). The culture was incubated at 27 °C and shaken at 120 rpm. At 48 h - 72 h post infection when the viability was less than 80% the culture was harvested by centrifugation at 500x g for 20 min at room temperature. The pellet was stored at -80 °C.

Purification was performed as described³ with minor modifications. Briefly, after thawing of the cell pellet and resuspension in buffer A (20mM Tris pH 8, 100 mM NaCl, 5% (v/v) glycerol and 2 mM TCEP) and addition of Complete EDTA-free proteinase inhibitors (Boehringer), the cells were lysed manually using a glass homogenizer. After spinning down the lysate at 42,000 rpm for 45 minutes, the supernatant was filtered and loaded onto a NiNTA column (GE healthcare). After washing with buffer A, the column was eluted using a gradient from 0-100% buffer B (buffer A + 200 mM imidazole). Fractions of the PI3K δ -iSH2 complex were pooled and loaded onto a heparin column equilibrated with heparin A buffer (20 mM Tris pH 8, 100 mM NaCl, 2 mM TCEP) then the column was washed and eluted with a gradient from 0 - 100% heparin B buffer (heparin A+ 1 M NaCl), to separate the excess His6-iSH2 from the PI3K δ -iSH2 complex. The PI3K δ -iSH2 fractions were pooled and TCEP adjusted to 5 mM. TEV protease was added and the mixture incubated overnight at 4 °C. The resulting solution was adjusted to 30 mM imidazole and passed over a second NiNTA column to remove the cleaved ABD/His6-iSH2 PI3K δ was collected in the flow-through, and NaNO₄ was added to a final concentration of 100 mM. Following a concentration step the protein was subjected to gel filtration on an S200 16/60 HiLoad column (GE healthcare) and eluted in 20 mM Tris pH 7.2, 50 mM ammonium sulphate, 1 % (v/v) ethylene glycol, 1 % (w/v) betaine, 0.2 % CHAPS and 5 mM TCEP. Fractions were then pooled and concentrated to 5 mg/ml, flash frozen in liquid N₂ and stored at -80 °C.

To prepare the complex with inhibitor, 0.2 μL of compound 11 in a 100 mM stock solution of 100% DMSO was mixed with 2.4 μL of 5% (w/v) dodecylmaltoside and 22 μL of PI3K δ at 6 mg/ml, incubated at RT for 30min and then spun for 5 min. The supernatant was used to set up the crystallization drop.

Co-crystallization of the PI3K δ /compound 11 complex

Plate-like crystals were obtained by the sitting drop vapor diffusion method at 17° C (290 K) after mixing 0.25 μL of complex at 6 mg/ml protein concentration with 0.16 μL volume of reservoir solution consisting of 13 mM NaNO₄, 13 mM ammonium sulfate, 13 mM Na₂HPO₄, 16.4% (v/v) glycerol, 13% (w/v) PEG4000 and 0.1M imidazole pH6.8 in 96-well MRC-SD2 crystallisation plates. Crystals were harvested from their mother liquor and directly flash-frozen in liquid nitrogen.

X-ray diffraction data collection and processing (PI3K δ)

Data sets were collected at 1.0 Å wavelength with a PILATUS 6M detector at the Swiss Light Source beamline X10SA (Villigen, Switzerland). Diffraction data were collected by Expose GmbH. Diffraction images recorded at 0.25° oscillation angle wedges were processed and scaled using XDS and XSCALE,⁴

respectively, in the APRV program suite.⁵ Data collection and processing statistics are summarized in supplementary Table 2.

Supplementary Table 2. Summary of data collection and processing statistics*

Data set name	PI3K δ /compound 11 (PDB ID 5IS5)	PI3K α /compound 11 (PDB ID 5ITD)
Space group name	C2	P212121
Unit cell a [Å]	141.8	104.6
Unit cell b [Å]	64.6	107.2
Unit cell c [Å]	116.7	134.6
Unit cell alpha [°]	90.0	90.0
Unit cell beta [°]	103.2	90.0
Unit cell gamma [°]	90.0	90.0
Resolution [Å]	69.9-2.6 (2.68-2.61)	83.86-2.82 (2.82-2.83)
Rsym [%]	10.1 (47.7)	7.7 (91.5.8)
I/sig(I) [%]	10.1 (2.7)	20.4 (2.3)
Completeness [%]	98.0 (99.1)	98.6 (100.0)
Redundancy/Multiplicity [%]	3.3 (3.4)	7.3 (7.4)
Number of observed reflections (overall)	102166	268859
Number of unique reflections (overall)	30985	36718
Wilson Bfactor [Å ²]	49.8	70.5

* Values in parentheses are for highest resolution shell.

Structure determination and refinement (PI3K δ)

Coordinates of the protein component of a published PI3K δ structure (PDB 2wxf) were refined against experimental data using autoBuster in order to obtain a start model and electron density maps. The initial model was subjected to iterative cycles of manual rebuilding and subsequent structure refinement in Coot and autoBuster, respectively. The ligand structure was built into unbiased Fo-Fc difference electron density calculated by autoBuster. Final structure refinement statistics are summarized in Supplementary Table 3. Refined coordinates were deposited to the PDB with entry number [PDB ID 5IS5].

Supplementary Table 3. Summary of refinement statistics

Structure	PI3K δ /compound 11	PI3K α /compound 11
Resolution [Å]	65.95-2.85	83.86-3.02
No. reflections	23616	29827
Rwork/Rfree	0.2294/0.2709	0.1907/0.2545
No. atoms		
Protein	5648	9772

Solvent	232	51
R.m.s deviations		
Bond lengths [Å]	0.008	0.010
Bond angles [°]	0.97	1.10

In silico methods

Supplementary Figure 3. Compound **2d** docked into a homology model of PI3K δ (based on a crystal structure of PI3K γ).

For further force field refinement of the protein-ligand complexes, only the following residues were used: for PI3K α , residues A657 to C862 and N873 to T1052, for PI3K δ , sequences L638 to N840, A848 to N918, and R929 to S1026. All resulting N- and C-termini were capped by acetyl and N-methyl, respectively. Water molecules further away than 8 Å of any ligand heavy atom were discarded.

The structures were prepared with the ff14SB force field⁶ for the protein and the GAFF force field⁷ for the ligand compound **11**. *mbondi2* atom radii were assigned for subsequent Generalized-Born (GB) implicit solvent computations. Partial charges on compound **11** were generated with the AM1/BCC approach.⁸

The energy was refined with *sander* (AmberTools15),⁹ using the GB option *igb*=5, running 500 steps of conjugate gradient, with virtually no cutoff for non-bonded interactions (*cut*=99.0). All C α atoms were constrained with a harmonic force of 1 kcal/mol/Å² to their original positions.

MM/GBSA computations were carried out on the resulting energy-minimized structures after discarding all explicit water molecules, using the GB options *igb* = 2 and 5 (both compatible with the *mbondi2* radius settings chosen during the data preparation).

Experimental part chemistry

General

Unless otherwise stated, all reagents and solvents were purchased from commercial suppliers and used without further purification. ¹H NMR spectra were recorded on a Bruker 400 MHz or a Bruker 600 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to an internal solvent reference. Recorded peaks are listed in the order multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet; m, multiplet; br, broad), coupling constants, and number of protons. Final compounds were purified to ≥95% purity as assessed by analytical liquid chromatography using the methods below. High resolution mass analyses (HRMS) were performed by using electrospray ionization in positive mode after separation by liquid chromatography. The elemental composition was derived from the averaged mass spectra acquired at a high resolution of about 30 000 on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The high mass accuracy below 1 ppm was obtained by using a lock mass.

HPLC Method 1: column XBridge MS C18 30/3.0 2.5m at 40°C; eluent A: water + 0.1% formic acid; eluent B: acetonitrile + 0.1% formic acid; gradient: from 10 to 95% B in 1.7 min; flow 1.2 mL/min.

UPLC Method 2: column Acquity HSS T3 1.8 μ m 2.1 x 50 mm at 60 °C; eluent A: water + 0.05% formic acid + 3.75 mM ammonium acetate; eluent B: acetonitrile + 0.04% formic acid; gradient: from 5 to 98% B in 1.4 min; flow 1.0 mL/min.

General synthetic sequence towards 4,6-diaryl quinazolines

Supplementary Scheme 1: General synthetic sequence towards 4,6-diaryl quinazolines. Reagents and conditions: a) POCl₃, neat, 80%; b) i. Pd(0), K₃PO₄, CH₃CN, 31-72%; ii. LiOH, dioxane, 88-98%; iii. Amine, HBTU, DIPEA, CH₂Cl₂, 81-97%; b') Pd(0), K₃PO₄, CH₃CN, 17-24%; c) Pd(0), Na₂CO₃, DME, H₂O, 24-60%.

Two routes towards 4,6-diaryl quinazolines are outlined in Supplementary Scheme 1. In the first route outlined by the solid arrows, 6-bromoquinazolin-4(3H)-one **A** was converted to 6-bromo-4-chloroquinazoline **B** using neat POCl₃. The aromatic substituent in position 4 was then installed using a Pd(0) catalyzed Suzuki coupling ($\rightarrow$ **C**). Aromatic amides were introduced in one step (b), whereas aromatic esters were converted to the corresponding amides via saponification and amide bond formation following the cross-coupling reaction (b'). Finally, the position 6 aromatic ring was attached using another Pd(0) catalyzed cross-coupling to yield final compounds of type **D**. As indicated by the dashed arrow, this general sequence was also used in reversed order. Overall, this modular approach enabled the chemistry team to modify either aromatic ring and/or the amide as the final synthetic step allowing for maximal diversity in the most efficient manner.

Synthesis and full characterization of 5-(4-(3-(4-acetylpiperazine-1-carbonyl)phenyl)quinazolin-6-yl)-2-methoxynicotinonitrile (11)

6-Bromo-4-chloroquinazoline CAS [38267-96-8]

6-bromo-3H-quinazolin-4-one (20 g, 89 mmol) was suspended in phosphorus oxychloride (140 ml), and the resulting mixture was stirred for 3 h at rt. After that time, the reaction mixture was concentrated under reduce pressure. The residue was dissolved in dichloromethane (500 ml) and neutralized with solid NaHCO_3 (200 g). The mixture was filtered, and the filtrate was concentrated under reduce pressure to yield the title compound (21 g, 86.2 mmol, 95%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.98 (d, 1H) 8.09 (d, 1H) 8.5 (s, 1H) 9.1 (s, 1H). HPLC/MS Method 1: 1.24 min, 243.0 $[\text{M}+\text{H}]^+$.

Ethyl 3-(6-bromoquinazolin-4-yl)benzoate

6-Bromo-4-chloroquinazoline (2.0 g, 8.21 mmol), (3-(ethoxycarbonyl)phenyl)boronic acid (1.673 g, 8.62 mmol), $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (0.288 g, 0.411 mmol) and K_3PO_4 (2.62 g, 12.32 mmol) were dissolved in acetonitrile (16 ml). The reaction mixture was flushed with argon, then H_2O (2 ml) was added, and the tube was capped and heated to 100°C for 15 min in a microwave oven. After cooling, the formed yellow solid was filtered, washed with ether and dried under reduce pressure to yield the title compound (1.54 g, 4.3 mmol) as a yellow solid. EtOAc was added to the filtrate, after phase separation the organic layer was washed with brine, dried (MgSO_4), filtered and concentrated under reduced pressure. The obtained residue was triturated with MeOH to afford the title compound as a yellow solid (580 mg, 1.6 mmol). The two solids were combined to yield the title compound (2.12 g, 5.9 mmol, 72%) as a yellow solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 1.42 (t, 3H) 4.43 (q, 2H) 7.77 (t, 1H) 7.97-8.07 (m, 2H) 8.16 (dd, 1H) 8.22 (d, 1H) 8.29 (d, 1H) 8.41 (s, 1H) 9.34 (s, 1H). HPLC/MS Method 1: 1.52 min, 357.0 $[\text{M}+\text{H}]^+$.

3-(6-Bromoquinazolin-4-yl)benzoic acid

An aqueous solution of LiOH.H₂O (1.0 M, 8.22 ml, 8.22 mmol) was added to a solution of ethyl 3-(6-bromoquinazolin-4-yl)benzoate (1.41 g, 4.11 mmol) in dioxane (45 ml), and the reaction mixture was stirred for 3 h at rt. The reaction mixture was quenched with the addition of an aqueous solution of HCl (1.0 M, 5 mL), and the formed precipitate was filtered and dried under vacuum to yield the title compound (880 mg, 2.67 mmol) as a light yellow solid. The filtrate was extracted with EtOAc, the organic layer was washed with brine, dried (MgSO₄) and concentrated under reduced pressure to give the title compound (555 mg, 1.68 mmol) as a light yellow solid. The two isolated solids were combined to yield the title compound (1.34 g, 4.07 mMol, 99%) which was used in the next step without further purification. HPLC/MS Method 1: 1.14 min, 331.0 [M+H]⁺.

1-(4-(3-(6-Bromoquinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one

HBTU (2.53 g, 6.68 mmol) and DIPEA (2.12 mL, 12.2 mmol) were added to a solution of 3-(6-bromoquinazolin-4-yl)benzoic acid (2.0 g, 6.08 mmol) in dichloromethane (60 ml). The reaction mixture was stirred for 10 min at rt, then 1-(piperazin-1-yl)ethan-1-one (0.935 g, 7.29 mmol) was added, and the reaction mixture was stirred at rt for a further 2 h. The reaction mixture was quenched with a saturated aqueous NaHCO₃ solution, and then extracted with dichloromethane. The organic layer was washed with brine, dried (MgSO₄) and concentrated under reduce pressure. The product was purified by flash chromatography using a dichloromethane/methanol gradient to yield the title compound (2.64 g, 6.02 mmol, 99%). ¹H-NMR (400 MHz, DMSO-*d*₆): δ 2.03 (br s, 3H) 3.52 (br s, 8H) 7.69-7.76 (m, 2H) 7.84 (s, 1H) 7.91 (d, 1H) 8.09 (d, 1H) 8.19-8.22 (m, 2H) 9.43 (s, 1H). HPLC/MS Method 1: 1.02 min, 439.6 [M+H]⁺.

5-(4-(3-(4-Acetylpiperazine-1-carbonyl)phenyl)quinazolin-6-yl)-2-methoxynicotinonitrile (11)

2-Methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)nicotinonitrile (89 mg, 0.273 mmol) and Pd(PPh₃)₄ (13 mg, 0.011 mmol) were added to a mixture of 1-(4-(3-(6-bromoquinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one (100 mg, 0.228 mmol) in DME (3ml). The reaction mixture was flushed with argon, an aqueous Na₂CO₃ solution (1.0 M, 0.455 mL, 0.455 mmol) was added, and the vial

was capped. The reaction mixture was heated to 140 °C for 10 min in a microwave oven. After cooling to rt, the mixture was diluted with EtOAc, filtered through a pad of celite and partitioned between H₂O/EtOAc. The organic layer was washed with brine, dried (MgSO₄) and concentrated under reduced pressure. The product was purified by preparative reverse phase HPLC HPLC (Waters Sunfire C18 OBD 30x100 mm, flow 30 ml/min, water + 0.1% TFA and acetonitrile + 0.1% TFA gradient), subsequent neutralization of the combined fractions using PL-HCO₃ MP gave the title compound (47 mg, 0.095mmol, 41% yield) as a white powder. ¹H-NMR (500 MHz, DMSO-*d*₆): δ 9.36 (s, 1H) 8.84 (br s, 1H) 8.73 (br s, 1H) 8.37 (d, *J* = 8.7Hz, 1H) 8.29 (d, *J* = 1.7Hz, 1H) 8.16 (d, *J* = 8.7Hz, 1H) 7.99 (dt, *J* = 7.6Hz, 1H) 7.88 (br s, 1H) 7.73 (t, *J* = 7.6Hz, 1H) 7.68 (dt, *J* = 7.7Hz, 1H) 4.04 (s, 3H) 3.64-3.44 (m, 8H) 1.98 (br s, 3H). ¹³C-NMR (500 MHz, DMSO-*d*₆): δ 168.6 (1C), 167.0 (1C), 163.1(1C), 154.6(1C), 150.2(1C), 150.0(1C), 142.6(1C), 136.6(1C), 136.2(1C), 134.9(1C), 133.2(1C), 131.3(1C), 129.3(1C), 129.1(1C), 128.8(1C), 128.5(1C), 128.4(1C), 124.2(1C), 122.4(1C), 115.1(1C), 95.9(1C), 61.3(1C), 54.8(1C), 47.0(2C), 45.3(2C), 21.2(1C). UPLC/MS Method 2: 0.86 min, 493.3[M+H]⁺. HRMS (ESI⁺) calcd for C₂₈H₂₅N₆O₃ [M+H]⁺ 493.19827, C₂₈H₂₄NaN₆O₃ [M+Na]⁺ 515.18021, found 493.19818, 515.18018.

Synthetic sequence towards compound 6f.

Supplementary Scheme 2: Synthetic sequence towards compound 6f. Reagents and conditions: a) POCl₃, 140 °C; b) triethylamine, 2-propanol, rt; c) 1-methylpiperazine, HBTU, DIPEA, DCM, rt; e) (6-methoxypyridin-3-yl)boronic acid, Pd(PPh₃)₄, 1M aqueous Na₂CO₃, DME, 140 °C.

(S)-1-(6-bromoquinazolin-4-yl)piperidine-3-carboxylic acid

(S)-piperidine-3-carboxylic acid (0.80 g, 6.16 mmol) and trimethylamine (1.72 ml, 12.3 mmol) were added to a solution of 6-bromo-4-chloroquinazoline (1.0 g, 4.11 mmol) in 2-propanol (15 ml), and the resulting mixture was stirred for 30 min at rt. After that time, the reaction mixture was diluted with water and acidified to pH=4 with an aqueous HCl solution (1 M). The precipitated solid was filtered off and dried under reduce pressure to yield the title compound (1.29 g, 3.84 mmol, 93%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.65 (s, 1H), 8.20 (d, 1H), 7.95 (dd, 1H), 7.75 (d, 1H), 4.25-4.20 (m, 1H), 4.00-3.85 (m, 1H), 3.55-3.35 (m, 2H), 2.80-2.65 (m, 1H), 2.05-1.95 (m, 1H), 1.90-1.60 (m, 3H). HPLC/MS Method 1: 0.72min, 336.3[M+H]⁺.

Characterization of compounds 2a-d, 3d, 4d, 5d, 6d, 6e, 6f, 7, 8, 9 and 10

6-(quinoline-3-yl)quinazoline 2a

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.72 (s, 1H), 9.41 (d, J = 35.7 Hz, 2H), 9.05 - 8.52 (m, 3H), 8.16 (dd, J = 34.1, 7.1 Hz, 3H), 7.94 - 7.62 (m, 2H). UPLC/MS Method 2: 0.83 min, 258.1 [M+H]⁺.

4-phenyl-6-(quinoline-3-yl)quinazoline 2b

¹H NMR (400 MHz, CD₃OD) δ 9.27 (d, J = 58.1 Hz, 2H), 8.69 (s, 1H), 8.57 - 8.43 (m, 2H), 8.29 (d, J = 8.6 Hz, 1H), 8.08 (t, J = 9.2 Hz, 2H), 7.97 - 7.60 (m, 7H). UPLC/MS Method 2: 1.10 min, 334.2 [M+H]⁺.

4-(3,4-dimethoxyphenyl)-6-(quinoline-3-yl)quinazoline 2c

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.35 (d, J = 13.3 Hz, 2H), 8.78 (s, 1H), 8.73 - 8.49 (m, 2H), 8.32 - 8.03 (m, 3H), 7.88 - 7.48 (m, 4H), 7.22 (d, J = 8.2 Hz, 1H), 3.89 (d, J = 5.1 Hz, 6H). UPLC/MS Method 2: 1.04 min, 394.2 $[\text{M}+\text{H}]^+$.

(4-methylpiperazin-1-yl)(3-(6-(quinoline-3-yl)quinazolin-4-yl)phenyl)methanone **2d**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.44 (s, 1H), 9.33 (d, J = 2.2 Hz, 1H), 8.81 (d, J = 1.9 Hz, 1H), 8.60 (d, J = 8.9 Hz, 1H), 8.49 (s, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.07 (dd, J = 15.0, 8.3 Hz, 3H), 7.88 (s, 1H), 7.82 (t, J = 7.7 Hz, 1H), 7.76 (t, J = 7.7 Hz, 1H), 7.73 - 7.63 (m, 2H), 3.51 (m, 4H), 2.20 (m, 4H), 1.99 (s, 3H). UPLC/MS Method 2: 0.69 min, 460.3 $[\text{M}+\text{H}]^+$.

(3-(6-(3,4-dimethoxyphenyl)quinazolin-4-yl)phenyl)(4-methylpiperazin-1-yl)methanone **3d**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.36 (s, 1H), 8.40 (dd, J = 8.8, 2.0 Hz, 1H), 8.21 - 8.16 (m, 2H), 8.01 (dt, J = 7.7, 1.3 Hz, 1H), 7.86 (s, 1H), 7.74 (t, J = 7.7 Hz, 1H), 7.66 (dt, J = 7.6, 1.3 Hz, 1H), 7.32 (d, J = 2.1 Hz, 1H), 7.27 (dd, J = 8.3, 2.1 Hz, 1H), 7.08 (d, J = 8.4 Hz, 1H), 3.86 (s, 3H), 3.81 (s, 3H), 3.69 - 3.39 (m, 4H), 2.45 - 2.09 (m, 7H). UPLC/MS Method 2: 0.70 min, 469.3 $[\text{M}+\text{H}]^+$.

2-methoxy-5-(4-(3-(4-methylpiperazine-1-carbonyl)phenyl)quinazolin-6-yl)nicotinonitrile **4d**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.41 (s, 1H), 8.90 (d, 1H), 8.79 (d, 1H), 8.43 (dd, 1 H), 8.34 (d, 1H), 8.23 (d, 1H), 8.01 (dt, 1H), 7.83 (br s, 1H), 7.74 (t, 1 H) , 7.65 (dt, 1H), 4.07 (s, 3H), 3.62 (br s, 2H), 3.46 (br s, 2H), 2.35 (br s, 2H), 2.22 (br s, 2H), 2.15(s, 3H). HPLC/MS Method 1: 0.94 min, 465.1 $[\text{M}+\text{H}]^+$.

(3-(6-(6-methoxy-5-(trifluoromethyl)pyridin-3-yl)quinazolin-4-yl)phenyl)(4-methylpiperazin-1-yl)methanone **5d**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.41 (s, 1H), 8.87 (d, $J = 2.1$ Hz, 1H), 8.52 - 8.19 (m, 4H), 8.03 (d, $J = 7.6$ Hz, 1H), 7.86 (s, 1H), 7.77 - 7.59 (m, 2H), 4.06 (s, 3H), 3.71 - 3.35 (m, 4H), 2.40-2.10 (m, 7H). UPLC/MS Method 2: 0.82 min, 508.3 $[\text{M}+\text{H}]^+$.

(3-(6-(6-methoxypyridin-3-yl)quinazolin-4-yl)phenyl)(4-methylpiperazin-1-yl)methanone **6d**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.38 (s, 1H), 8.59 (d, $J = 2.3$ Hz, 1H), 8.39 (dd, $J = 8.8, 2.1$ Hz, 1H), 8.25 - 8.18 (m, 2H), 8.12 (dd, $J = 8.6, 2.6$ Hz, 1H), 8.00 (dt, $J = 7.7, 1.3$ Hz, 1H), 7.84 (t, $J = 1.4$ Hz, 1H), 7.74 (t, $J = 7.7$ Hz, 1H), 7.65 (dt, $J = 7.7, 1.3$ Hz, 1H), 6.96 (d, $J = 8.6$ Hz, 1H), 3.92 (s, 3H), 3.75 - 3.33 (m, 4H), 2.41 - 2.17 (m, 4H), 2.15 (s, 3H). UPLC/MS Method 2: 0.69 min, 440.2 $[\text{M}+\text{H}]^+$.

(5-(6-(6-methoxypyridin-3-yl)quinazolin-4-yl)pyridin-3-yl)(4-methylpiperazin-1-yl)methanone **6e**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.43 (s, 1H), 9.14 (d, J = 2.1 Hz, 1H), 8.84 (d, J = 2.0 Hz, 1H), 8.67 – 8.61 (m, 1H), 8.43 (dd, J = 8.8, 2.1 Hz, 1H), 8.32 (t, J = 2.1 Hz, 1H), 8.28 – 8.21 (m, 2H), 8.17 (dd, J = 8.7, 2.6 Hz, 1H), 7.07 – 6.88 (m, 1H), 3.92 (s, 3H), 3.73 – 3.37 (m, 4H), 2.42 – 2.20 (m, 4H), 2.17 (s, 3H). UPLC/MS Method 2: 0.61 min, 441.2 $[\text{M}+\text{H}]^+$.

(s)-(1-(6-(6-methoxypyridin-3-yl)quinazolin-4-yl)piperidin-3-yl)(4-methylpiperazin-1-yl)methanone **6f**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.69 – 8.52 (m, 2H), 8.18 – 8.07 (m, 3H), 7.88 (d, J = 9.3 Hz, 1H), 6.97 (d, J = 8.6 Hz, 1H), 4.43 – 4.23 (m, 2H), 3.93 (s, 3H), 3.72 – 3.61 (m, 1H), 3.60 – 3.49 (m, 2H), 3.36–3.29 (m, 3H), 3.29 – 3.20 (m, 1H), 3.20 – 3.07 (m, 1H), 2.39 – 2.07 (m, 7H), 1.96 – 1.84 (m, 1H), 1.82 – 1.61 (m, 3H). UPLC/MS Method 2: 0.53 min, 447.3 $[\text{M}+\text{H}]^+$.

1-(4-(3-(6-(quinolin-3-yl)quinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one **7**

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.44 (s, 1H), 9.35 (d, J = 1.9 Hz, 1H), 8.83 (s, 1H), 8.62 (dd, J = 8.8, 2.0 Hz, 1H), 8.51 (d, J = 1.7 Hz, 1H), 8.31 (d, J = 8.8 Hz, 1H), 8.13 – 8.03 (m, 3H), 7.97 (s, 1H), 7.86 – 7.65 (m, 4H), 3.77 – 3.39 (m, 8H), 1.91 (d, J = 90.5 Hz, 3H). UPLC/MS Method 2: 0.84 min, 488.3 $[\text{M}+\text{H}]^+$.

1-(4-(3-(6-(3,4-dimethoxyphenyl)quinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one 8

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.37 (s, 1H), 8.42 (dd, J = 8.8, 2.0 Hz, 1H), 8.27 – 8.13 (m, 2H), 8.01 (d, J = 7.6 Hz, 1H), 7.93 (s, 1H), 7.83 – 7.64 (m, 2H), 7.34 (d, J = 2.1 Hz, 1H), 7.30 (dd, J = 8.3, 2.1 Hz, 1H), 7.09 (d, J = 8.4 Hz, 1H), 3.86 (s, 3H), 3.82 (s, 3H), 3.76 – 3.36 (m, 8H), 2.00 (s, 3H). UPLC/MS Method 2: 0.87 min, 497.2 $[\text{M}+\text{H}]^+$.

1-(4-(3-(6-(6-methoxypyridin-3-yl)quinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one 9

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 9.39 (s, 1H), 8.61 (d, J = 2.3 Hz, 1H), 8.40 (dd, J = 8.8, 2.0 Hz, 1H), 8.27 – 8.18 (m, 2H), 8.13 (dd, J = 8.6, 2.6 Hz, 1H), 8.01 (dt, J = 7.6, 1.4 Hz, 1H), 7.90 (s, 1H), 7.79 – 7.64 (m, 2H), 6.97 (d, J = 8.6 Hz, 1H), 3.92 (s, 3H), 3.73 – 3.36 (m, 8H), 2.00 (s, 3H). UPLC/MS Method 2: 0.82 min, 468.2 $[\text{M}+\text{H}]^+$.

1-(4-(3-(6-(6-methoxy-5-(trifluoromethyl)pyridin-3-yl)quinazolin-4-yl)benzoyl)piperazin-1-yl)ethan-1-one 10

^1H NMR (400 MHz, $\text{Methanol-}d_4$) δ 9.36 (s, 1H), 8.74 (s, 1H), 8.43 – 8.21 (m, 4H), 8.10–7.90 (m, 2H), 7.80–7.71 (m, 2H), 4.11 (s, 3H), 4.00–3.50 (m, 8H), 2.14 (s, 3H). UPLC/MS Method 2: 0.98 min, 536.3 $[\text{M}+\text{H}]^+$.

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