Discovery of AG-120 (Ivosidenib): A First-in-Class Mutant IDH1 Inhibitor for the Treatment of IDH1-Mutant Cancers

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Supporting Information

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Inhibitory Behavior of AG-120

In vitro, AG-120 showed no time-dependence in IC_{50} values between 1 and 16 hours of incubation for the mIDH1-R132 homodimer. AG-120 showed non-competitive inhibitory behavior with respect to the α-KG substrate (Figure S1) and appears to be non-competitive with respect to the NADPH cofactor by standard equilibrium kinetic analysis (Figure S2). These observations are consistent with AG-120 being a rapid equilibrium inhibitor of the mIDH1-R132H homodimer in the presence or absence of substrate and cofactor. However, we cannot confirm this mode of action because NADPH is found to be tightly pre-bound in recombinant enzyme preparations.[1] Against the IDH1-WT homodimer, AG-120 showed un-competitive inhibition with respect to the NADP cofactor (Figure S3). In the presence of increasing concentrations of AG-120, the observed rate constant (k_{obs}) was a hyperbolic function of inhibitor concentration. Along with the observed time-dependence in IC_{50} values between 1 and 16 hours, these data are consistent with AG-120 being a slow-binding inhibitor of the IDH1-WT homodimer (Figure S4).

With respect to the inhibition of the IDH1-WT enzyme, it is worth noting that mice with complete knock-out of Idh1 showed completely normal development and fertility, although they were more susceptible to lethal doses of lipopolysaccharide than WT litter-mates,[2] suggesting that inhibition of IDH1 under homeostasis should be tolerable. Overall, preclinical profiling of AG-120 fully supports further clinical studies in the context of diseases associated with mutation of IDH1.

Supplementary Figures and Tables

Table S1. *In vitro* ADME and *in vivo* PK properties of AGI-14100

In vitro ADME	Liver microsomes E_h Human/Mouse/Rat/Dog/Cynomolgus	0.23/0.63/0.17/0.36/0.43
	Caco2 A→B (10^{-6} cm/s) /Efflux ratio	14.2/2.3
	hPXR % rifampicin @ 1/10 μM	69/70
In vivo PK	CLp (L/hr/kg), from 1 mg/kg IV dose Rat/Dog/Cynomolgus	0.45/0.12/0.22
	t½ (hr) Rat/Dog/Cynomolgus	9.9/32.5/12.5
	F (%), from 10 mg/kg PO dose Rat/Dog/Cynomolgus	44/18/43

ADME, absorption, distribution, metabolism, and excretion; CLp, clearance; CYP, cytochrome P450; E_h, microsome stability recorded as hepatic extraction ratio in liver microsomes; F, oral bioavailability; hPXR, human pregnane X receptor; IV, intravenous; PK, pharmacokinetic; PO, oral; t½, half-life.

Table S2. Biochemical profiling of AG-120 against IDH2-wild type (WT) and mutant IDH2 (mIDH2)

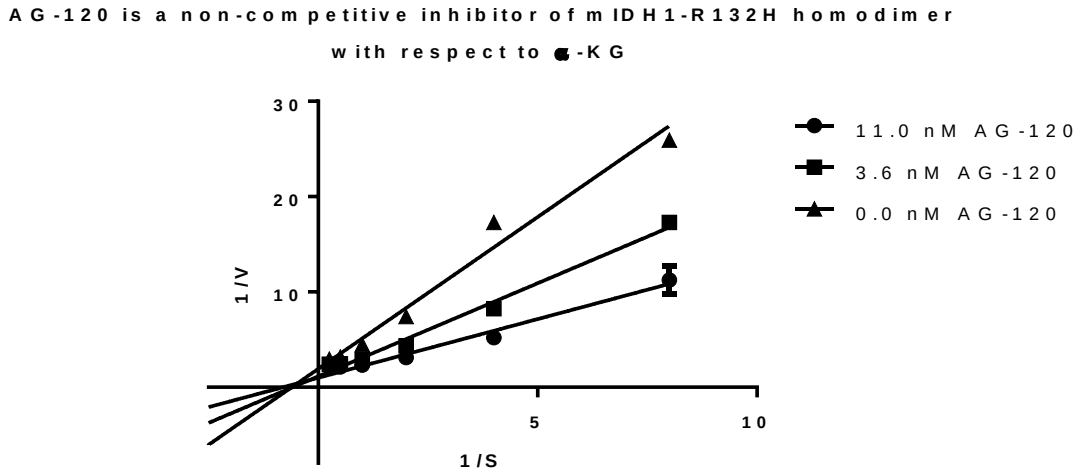
Enzyme	Reverse (NADPH consumption), IC_{50} (μM)
mIDH2-R140Q/IDH2-WT (16 hr)	No fit
mIDH2-R140Q/mIDH2-R140Q (16 hr)	No fit
mIDH2-R172K/IDH2-WT (16 hr)	72 (88%)
mIDH2-R172K/mIDH2-R172K (1 hr)	No fit

Table S3. Profiling of AG-120 against dehydrogenases

Phosphoglycerate dehydrogenase	No inhibition @ 100 μM
Lactate dehydrogenase A[a]	No inhibition @ 100 μM
Glutamate dehydrogenase	No inhibition @ 100 μM
Glucose-6-phosphate dehydrogenase	No inhibition @ 100 μM

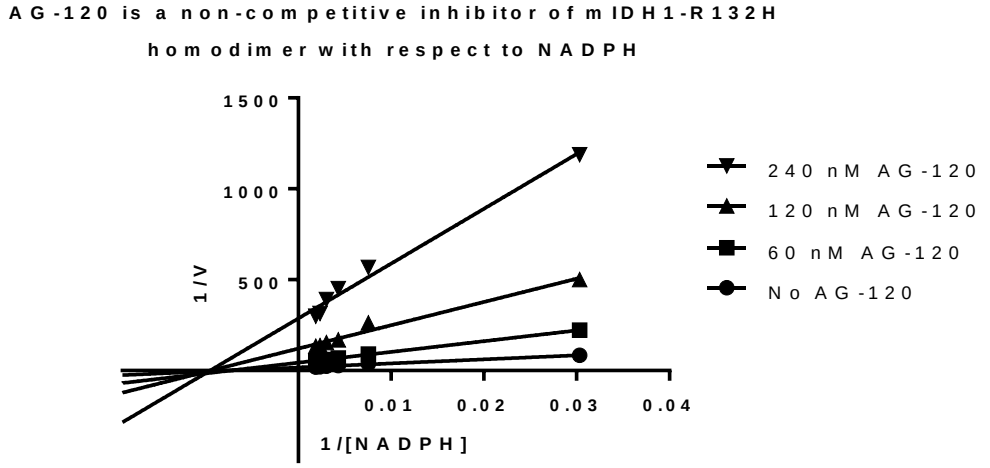
[a]Includes both forward and reverse reactions.

Figure S1. AG-120 showed non-competitive inhibition with respect to alpha-ketoglutarate (α-KG)



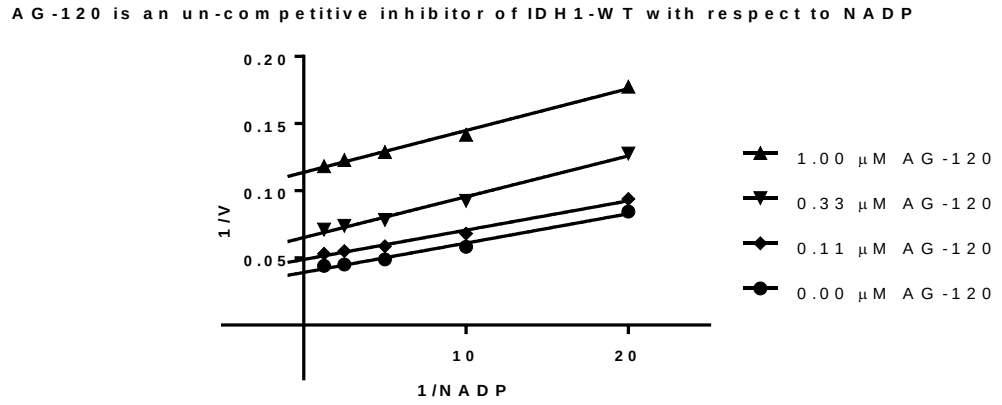
Linear regression of the reciprocal plots for each titration series of varying substrate versus varied fixed concentration of inhibitor intersects at a negative value on the x-axis, indicative of a non-competitive mode of inhibition.

Figure S2. AG-120 showed non-competitive inhibition with respect to the NADPH cofactor



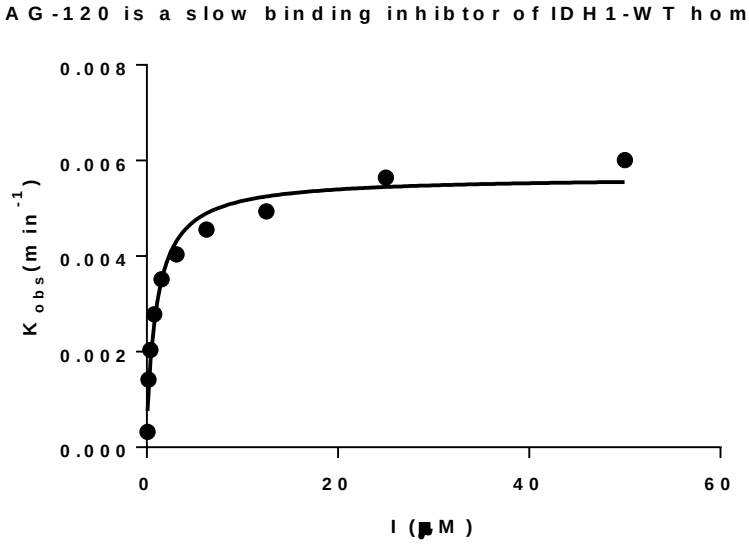
AG-120 is a non-competitive inhibitor of the mIDH1-R132H homodimer with respect to NADPH. Linear regression of the reciprocal plots for each titration series of varying cofactor versus fixed concentration of inhibitor intersects at a negative value on the x-axis, indicative of a non-competitive mode of inhibition. However, it is not conclusive that AG-120 can bind to the apo-form of the enzyme given that recombinantly expressed mIDH1-R132 enzymes exhibit low nM K_m values for NADPH and already contain pre-bound co-factor (observed in crystallography studies, data not shown).

Figure S3. AG-120 is an uncompetitive inhibitor of the IDH1-WT homodimer with respect to NADP



Linear regression of the reciprocal plots for each titration series of varying cofactor versus fixed concentration of inhibitor yields parallel lines, indicative of an uncompetitive mode of inhibition.

Figure S4. AG-120 is a slow-binding inhibitor of the IDH1-WT homodimer



In the presence of increasing concentrations of AG-120, the observed rate constant (k_{obs}) is a hyperbolic function of inhibitor concentration, indicative of AG-120 being a slow-binding inhibitor of the IDH1-WT homodimer.

Table S4. *In vitro* ADME and *in vivo* PK properties of AG-120

In vitro ADME	Liver microsomes E_h Human/Mouse/Rat/Dog/Cynomolgus	0.15/0.12/0.13/stable/0.1
	Caco2 A→B (10^{-6} cm/s) /Efflux	15/2
	hPXR % rifampicin 1/10 μM	2/21
In vivo PK	CLp (L/hr/kg), from 1 mg/kg IV dose Rat/Dog/Cynomolgus	0.2/0.02/0.2
	t½ (hr) Rat/Dog/Cynomolgus	8.9/19/5.3
	F (%), from 10 mg/kg PO dose Rat/Dog/Cynomolgus	42/21/49

ADME, absorption, distribution, metabolism, and excretion; CLp, clearance; CYP, cytochrome P450; E_h, microsome stability recorded as hepatic extraction ratio in liver microsomes; F, oral bioavailability; hPXR, human pregnane X receptor; IV, intravenous; PK, pharmacokinetic; PO, oral; t½, half-life.

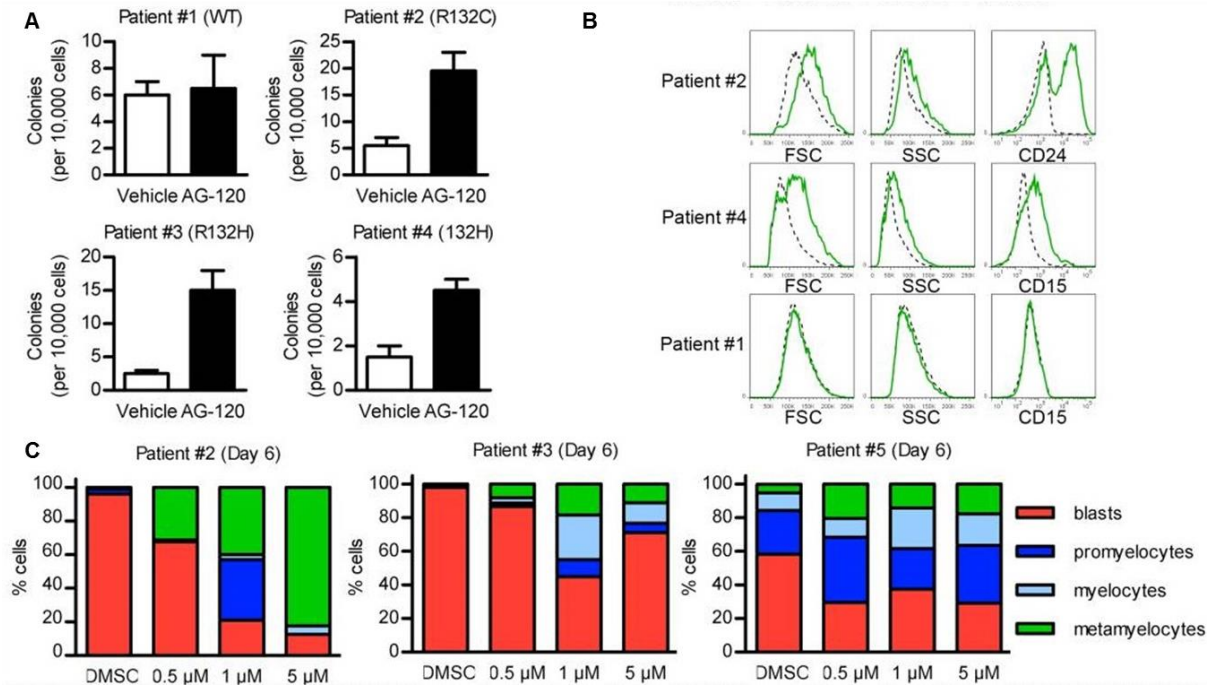
Table S5. AML diagnosis of bone marrow aspirates

Patient code	IDH mutation	Other mutations	FAB	Karyotype	Sample source
1	WT	FLT3-ITD DNMT3A	AML M1	Normal	Peripheral blood
2	R132C	NPM1	AML M1	Normal	Peripheral blood
3	R132H	None	AML M4	t(6:9)(p26;q34)	Bone marrow
4	R132C	FLT3-ITD NPM1	AML M1	Normal	Bone marrow
5	R132H	FLT3-ITD	AML M1	Normal	Bone marrow

Diagnosis was morphologically confirmed according to the French–American–British (FAB) classification. Description of the karyotypes followed the International System for Human Cytogenetic Nomenclature.[3]

Figure S5. AG-120 induces cellular differentiation in preclinical models.

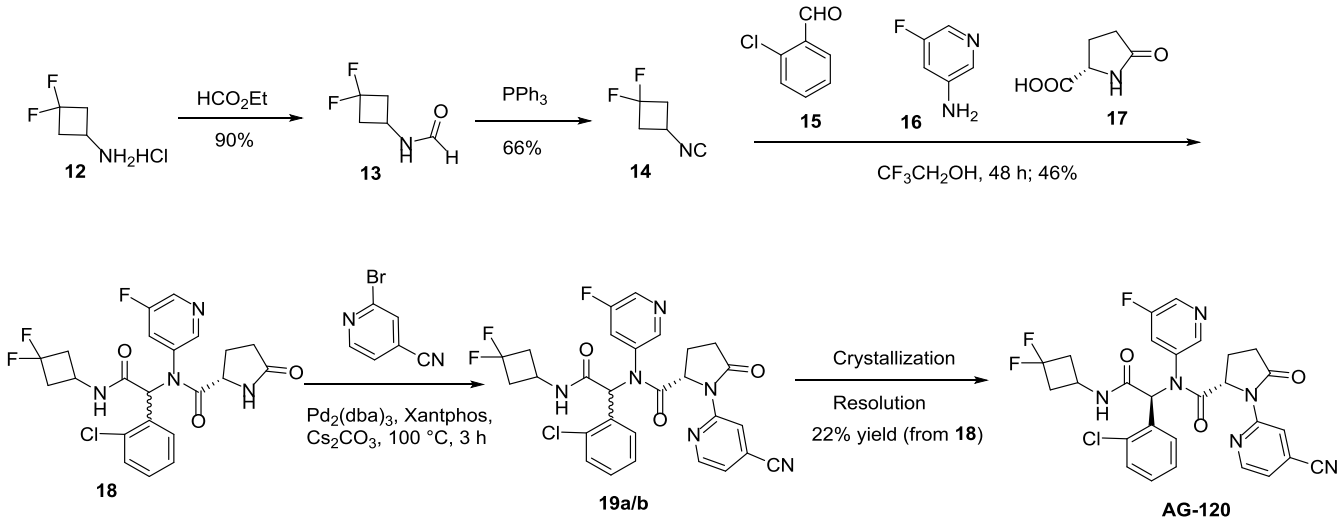
(A) Number of colony-forming units following treatment with AG-120 in primary human patient samples cultured in methylcellulose. (B) Flow cytometry analysis in mIDH1 primary human patient samples treated *in vitro* with AG-120 for 6 days. Solid green lines indicate AG-120 treatment, dashed black lines indicate DMSO control treatment. (C) Percentages of cell types in human acute myeloid leukemia bone marrow samples following 6 days of treatment with control (DMSO) or AG-120 (0.5, 1.0, and 5.0 μM).



Enhanced ability of progenitor cells to form differentiated colonies, consistent with the onset of cellular differentiation, was observed for mIDH1, but not IDH1-WT, patient myeloblasts when grown for 12 days in methylcellulose in the presence of AG-120 (5 μM) compared with vehicle control (DMSO, Figure S5A). Maturation of AML blasts was also evaluated by flow cytometry analysis for changes in cell-surface markers associated with myeloid differentiation (CD15, CD24, CD11b, and CD14). Treatment of mIDH1 AML cells with AG-120 always induced increases in one or more of these differentiation markers, although there was significant heterogeneity in the marker(s) induced, as well as in the kinetics of their appearance, with both features varying from one patient sample to another. This is illustrated in Figure S5B, which shows that treatment of mIDH1 blasts (from Patients #2 and #4) in liquid culture with AG-120 for 6 days induced an increase in the mean fluorescence

intensities for CD15 and CD24 (the most uniform features of the AG-120-induced differentiation effect across AML samples), and in the side scatter (SSC, granularity) and forward scatter (FSC, relative size) parameters. Treatment of IDH1-WT AML cells with AG-120 never induced increases in the expression level of any of these markers (e.g. Patient #1 in Figure 5B), indicating that AG-120 is effective solely on mIDH1 cells. These results were confirmed by cytology as a decrease in the number of blast cells and an increase in the number of more mature myeloid cells (myelocytes and metamyelocytes) in the mIDH1 patient samples following 6 days of treatment with AG-120 (0.5 μM, 1 μM, or 5 μM) compared with DMSO control (Figure S5C). These data show that AG-120 induces myeloid differentiation in mIDH1-R132H and -R132C, but not IDH1-WT, primary human AML BM samples.

Scheme S1. Synthesis of AG-120



Starting from the commercially available 3,3-difluorocyclobutan-1-amine hydrochloride 12, isocyanide 14 was easily obtained following procedures reported in the literature (see Supporting Information references) and used without further purification in the next reaction. Ugi four-component reaction using isocyanide 14, o-Cl benzaldehyde 15, 5-fluoropyridin-3-amine 16, and (S)-5-oxopyrrolidine-2-carboxylic acid 17 gave phenyl glycine key intermediate 18 as a racemate at R2 in 46% isolated yield. Next, Pd-mediated coupling of 18 with 2-bromoisonicotinonitrile provided diastereomeric mixture 19 a/b, which after crystallization and concomitant chiral resolution provided AG-120 in 22% isolated yield from intermediate 18. This route proved to be an effective scalable synthesis that provided a multi-

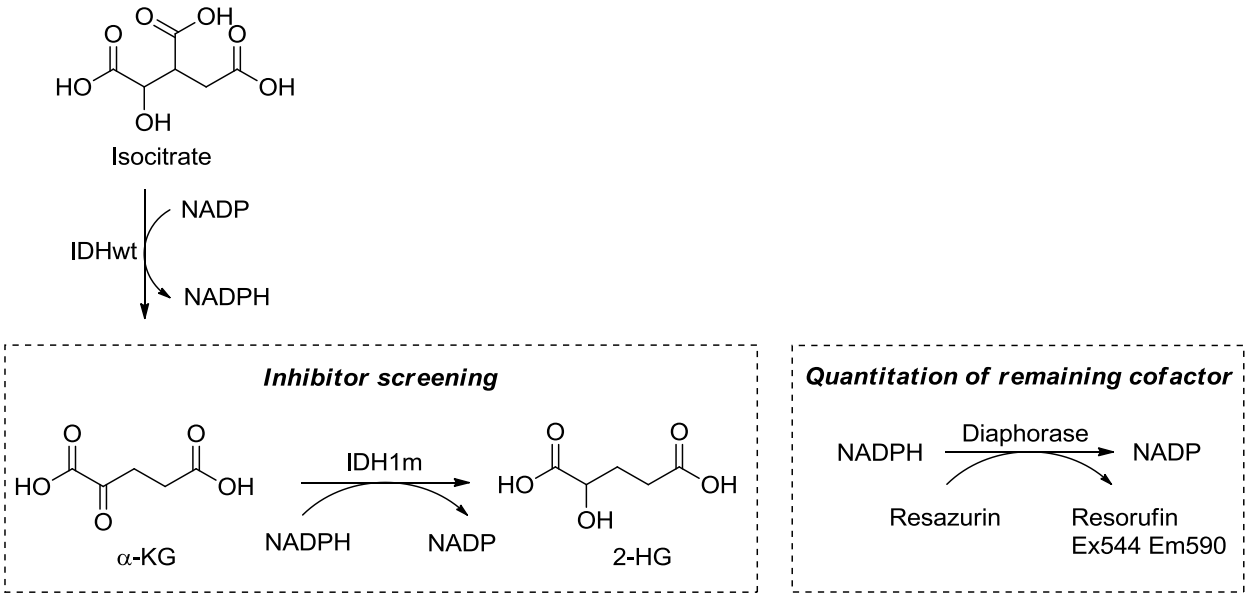
hundred gram quantity of AG-120 to support additional *in vivo* pharmacology and preclinical toxicology studies.

Biochemistry and Cell Biology Assay Methods

Expression and purification of WT and mutant IDH enzymes

WT and mutant IDH1 and IDH2 enzymes were expressed and purified as previously reported.[4]

Determination of compound inhibition potency against the mIDH1-R132H enzyme reaction using a diaphorase/resazurin coupled system



In the primary reaction, the reduction of α-KG acid to D-2-hydroxyglutarate (2-HG) is accompanied by a concomitant oxidation of NADPH to NADP. The amount of NADPH remaining at the end of the reaction time is measured in a secondary diaphorase/resazurin reaction in which the NADPH is consumed in a 1:1 molar ratio with the conversion of resazurin to the highly fluorescent resorufin. Uninhibited reactions exhibit a low fluorescence at the end of the assay, while reactions in which the consumption of NADPH by mIDH1-R132H has been inhibited by a small molecule show a high fluorescence. The primary reaction was performed in a volume of 50 μL 1X Buffer (150 mM NaCl, 20 mM Tris 7.5, 10 mM $MgCl_2$, 0.05% w/v bovine serum albumin [BSA]), contained 2 nM mIDH1-R132H, 1 mM α-KG, and 4 μM NADPH, and was conducted for 60 minutes at 25°C. To perform the secondary reaction, 25 μL of 1X buffer containing 36 μg/mL diaphorase and 30 mM

resazurin was added to the primary reaction and incubated for a further 10 minutes at 25°C. Florescence was read on a Spectramax plate reader at Ex 544 Em 590. Recombinant protein was expressed and purified as previously described.[5] Compounds or compound dilutions were prepared in 100% dimethyl sulfoxide (DMSO) concentration and diluted 1:100 into the final reaction. mIDH1-R132C was assayed under similar conditions, with the exception that the 1X Buffer was 50 mM K_2HPO_4 pH 6.5, 40 mM $NaHCO_3$, 5 mM $MgCl_2$, 10% glycerol, 0.03% w/v BSA.

Assay of the IDH1-WT enzyme reaction for determination of inhibitor potency

IDH1-WT enzyme was assayed in a modified version of the assay used for mIDH1-R132H. Since this enzyme converts NADP to NADPH stoichiometrically with the conversion of isocitrate to α-KG, NADPH product can be continuously assayed by direct coupling to the diaphorase/resazurin system and reading resorufin production at Ex 544 Em 590. Assays were conducted in 50 μL of 1X Buffer (150 mM NaCl, 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.05% (w/v) BSA, 2 mM beta-mercaptoethanol [B-ME]) containing 50 μM NADP, 70 μM DL-isocitrate, and 31.2 ng/mL IDH1-WT enzyme (reaction time 1 or 16 hours). The direct coupling system comprised 20 μg/mL diaphorase and 40 μM resazurin.

Assay of the IDH2-WT enzyme reaction for determination of inhibitor potency

Inhibitory potency of compounds against the IDH2-WT enzyme was determined in a coupled assay to diaphorase. In this assay, production of NADPH by IDH2-WT was linked to a concomitant reduction of resazurin to the highly fluorescent resorufin. Enzyme was diluted to 0.06 μg/mL in 40 μL 1X Assay Buffer (150 mM NaCl, 50 mM potassium phosphate pH 7, 10 mM $MgCl_2$, 10% glycerol, 2 mM B-ME, 0.03% BSA), to which 1 μL of compound was added in DMSO. The mixture was incubated for 16 hours at room temperature (RT). The reaction was started with the addition of 10 μL of Substrate Mix (200 μM isocitrate, 175 μM NADP, 60 μg/mL diaphorase, 200 μM resazurin, in 1X Assay Buffer), and run for 30 minutes at RT. The reaction was halted with the addition of 25 μL of 6% sodium dodecyl sulfate and read on a Spectramax Plate Reader at Ex544/Em590.

Assay of the mIDH2-R140Q and mIDH2-R172K enzyme reaction for determination of inhibitor potency

Inhibitory potency against the mIDH2-R140Q and mIDH2-R172K enzymes was determined in an endpoint assay in which the amount of NADPH remaining at the end of the reaction was measured by the addition of a large excess of diaphorase and resazurin. mIDH2-R140Q

was diluted to 0.25 μg/mL in 40 μL 1X Assay Buffer (150 mM NaCl, 50 mM potassium phosphate pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 2 mM B-ME, 0.03% BSA) and incubated for 16 hours at 25°C in the presence of 1 μL of compound in DMSO. The reaction was started with the addition of 10 μL of Substrate Mix (20 μM NADPH, 8 μM α-KG, in 1X Assay Buffer) and incubated for 1 hour at 25°C. Then, remaining NADPH was measured by the addition of 25 μL of Detection Mix (36 μg/mL diaphorase, 18 μM resazurin in 1X Assay Buffer), incubated for 5 minutes at 25°C, and read as described above. mIDH2-R172K was assayed as for mIDH2-R140Q with the following modifications: 1.25 μg/mL of protein was used, the Substrate Mix contained 50 μM NADPH and 6.4 μM α-KG, and the compound was incubated for 1 hour before starting the reaction.

Cell lines

Human glioblastoma astrocytoma (U87MG) pLVX-IDH1-R132H was generated in-house using U87MG (ATCC, #HTB-14) cells and pLVX-IRESNeo. High titers of recombinant, replication-incompetent virions were generated using a Lenti-X HTX Packing system (Clontech), in which pLVX vector containing the IDH1-R132H gene was co-transfected along with a Lenti-X HTX Packing Mix (Cat# 631249, Clontech) into the Lenti-X 293T cell line (Cat#632180, Clontech). All procedures were performed according to the manufacturer's protocol. Cells were selected and maintained in 500 μg/mL Geneticin in RPMI medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

HT1080 (ATCC, #CCL-121), COR-L 105 (ECACC, # 92031918), and HCCC-9810 (Shanghai Cell Bank, ChemPartner) cells were maintained in RPMI medium with 10% FBS and penicillin/streptomycin.

Neurospheres (603) were derived from glioma patients who underwent tumor resection at the Memorial Sloan-Kettering Cancer Center (MSKCC) by the Ingo Mellinghoff laboratory. All human samples were collected after patients signed informed consent according to MSKCC institutional review board guidelines. Cells were maintained as spheres in Human Neurocult media (Stem Cell Technologies) with Neurocult supplement, 1% Primocin, 1% Normocin, 0.0002% Heparin, 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor.

Cell assays

Cells were seeded in their respective growth media at a density of 5000 (U87MG, HCCC-9810, COR-L 105) or 2500 (HT1080) cells/well into 96-well microtiter plates and incubated overnight at 37°C and 5% CO_2. The next day, AG-120 was prepared in 100% DMSO as a 10 mM stock and then diluted in media for a final concentration of 0.1% DMSO. Highest concentration dose was 3 µM. Medium was removed from the cell plates and 200 µL of the compound dilutions were added to each well. For neurospheres, compounds and cells (40,000/well) were plated together at the same time. After 48 hours of incubation with compound at 37°C, 100 µL of media was removed from each well and analyzed as described below. The cell plates were then allowed to incubate another 24 hours. At 72 hours post compound addition, a 10 mL/plate of Promega Cell Titer Glo reagent was thawed and mixed. The cell plate was removed from the incubator and allowed to equilibrate to RT. Then 100 µL of reagent was added to each well of media. The cell plate was placed on an orbital shaker for 10 minutes and then allowed to sit at RT for 20 minutes. The plate was then read for luminescence with an integration time of 500 ms to determine any compound effects on growth inhibition (half maximal inhibition of cell proliferation, GI_{50}).

Cellular half-maximal inhibitory concentration (IC_{50}), 90% maximal IC (IC_{90}), percent maximum 2-HG inhibition, and growth inhibition were calculated as described below.

The concentrations of 2-HG in media were determined using a non-validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) method.

The following protein precipitation method was used for sample preparation:

- A 30 µL aliquot of 80:20 methanol (MeOH):H_2O extract was mixed with 170 µL of acetonitrile (MeCN) with internal standard (^{13}C5-2-HG, 0.2 µg/mL).
- Samples were vortexed for 5 minutes to mix well and centrifuged at 400 rpm for 10 minutes.
- 100 µL of supernatant was transferred and diluted with 100 µL of H_2O and mixed well.
- 40 µL was injected onto the LC-MS/MS for analysis.

The instrument setup consisted of a Thermo TSQ Vantage mass spectrometer equipped with Accela high-performance liquid chromatography (HPLC) pumps. An isocratic gradient method was used at a flow rate of 1 mL/min. The HPLC column used was Bio-Rad, Aminex

Fast Acid Analysis, 100 mm × 7.8 mm, 9 μm. 2-HG and stable isotope labeled 2-HG (^{13}C-2-HG – used as internal standard) were ionized under negative ion spray mode and detected through the multiple-reaction monitoring of a mass transition pair at m/z = 147.0/129.0 and 152.0/134.0, respectively. Stock solutions of 2-HG and ^{13}C-2-HG were prepared separately in H_2O at a target concentration of 1 mg/mL as free acid. Stock working solutions of 2-HG ranging from 10 ng/mL to 30,000 ng/mL were made in phosphate-buffered saline and were used to prepare calibration standards and quality control samples (QCs). The internal standard solution was prepared in MeCN at a concentration of 200 ng/mL. Samples were run with two standard curves and QCs. Standard curves and QCs were acceptable, ie, two-thirds of the QCs had a precision and accuracy within 15% of theoretical values except in lower limit of quantitation within 20%; a standard curve has a coefficient of determination (R^2) value >0.98 in linear regression. The peak area ratios of the analyte relative to the internal standard were used for 2-HG quantitation. Linearity was achieved in the 2-HG concentration range from 10 to 30,000 ng/mL for cell pellets.

Generation of HT1080 mIDH1-R132C xenografts

All animal studies were approved by the Institutional Animal Care and Use Committee and conducted in compliance with all national and local guidelines and regulations.

HT1080 mIDH1-R132C cells were grown and 3×10^6 cells were inoculated subcutaneously on the flank of female BALB/c mice. When tumors reached approximately 200 mm^3 the mice were randomized into dosing groups according to tumor size and treated with AG-120. Mice were dosed orally by gavage with a single dose of AG-120 at 50 or 150 mg/kg (n = 21 per dose group). Blood and tumor tissue samples were collected at 1, 3, 6, 12, 24, 48, and 72 hours following the dose (n = 3 at each time point) and were analyzed for AG-120 and 2-HG via LC-MS/MS.

Primary patient sample differentiation

Primary AML samples

Peripheral blood (PB) or bone marrow (BM) aspirate samples were obtained at the time of diagnosis at the Gustave Roussy Institut (Villejuif, France). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. AML diagnosis was morphologically proven according to the French–American–British classification. Description of the karyotypes followed the International System for Human Cytogenetic Nomenclature.[3] Immunophenotyping of the blasts was performed using the following

antibodies: phycoerythrin (PE)-CD34 (BD Pharmingen), allophycocyanin (APC)-CD38 (BD Pharmingen), and PECy7-CD45 (clone H130, BD Pharmingen).

IDH mutational status

Genomic DNA was purified from diagnostic PB and/or BM specimens using the Qiagen DNeasy extraction kit following the manufacturer's instructions. IDH1/2 mutational status was analyzed by PCR amplification of IDH genes (exon 4 genomic regions, containing the mutational hotspot codon mIDH1-R132 and codons mIDH2-R140 and -R172) and direct sequencing, as reported.[6-7]

Ex vivo AG-120 treatment

Patient PB or BM samples were sorted according to their immunophenotype using an Influx cell sorter (BD Biosciences). Live cells were cultured in 48-well tissue culture plates (Becton Dickinson) in serum-free medium supplemented with cytokines (human interleukin 3, interleukin 6, stem cell factor, thrombopoietin, erythropoietin, FMS-like tyrosine kinase 3 ligand, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, all from PeproTech) either in the presence of DMSO (0.05% v/v, used as a vehicle control) or in the presence of increasing doses of AG-120 (0.5, 1, and 5 μM) over a 6-day time course.

Cytometry

Cytometric analysis was performed on a BD Biosciences LSRII using the anti-CD24 (clone SN3, eBioscience SAS) and anti-CD15 (clone VIMC6, Miltenyi Biotec SAS) antibodies.

Methylcellulose assays

Primary AML samples (2 × 104 cells from PB or BM) were plated in methylcellulose-based medium (Methocult H4434, StemCell Technologies) in duplicate 35 mm dishes per condition in the presence of AG-120 (5 μM) or vehicle (0.05% v/v). Dishes were incubated in a humidified incubator at 37°C. Colonies containing at least 30 cells were counted at day 12.

General Synthetic Procedures and Experimental Notes

In the following examples, the chemical reagents were purchased from commercial sources (such as Alfa, Acros, Sigma Aldrich, TCI, and Shanghai Chemical Reagent Company) and used without further purification. Flash chromatography was performed on an Isolera One

column (Biotage) with silica gel particles of 200–300 mesh. Analytical and preparative thin-layer chromatography (prep-TLC) plates were HSGF 254 (0.15–0.2 mm thickness, Shanghai Anbang Company). Nuclear magnetic resonance (NMR) spectra were recorded using an AMX-300 or AMX-400 NMR spectrometer (Brucker). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Mass spectra were run with electrospray ionization (ESI) from an LCT TOF mass spectrometer (Waters). High-resolution mass spectra were run with ESI from a Premier UPLC-Q-TOF mass spectrometer (Waters). HPLC chromatographs were recorded on an LC-20AHT (Shimadzu) liquid chromatography system (column: Ultimate 4.6 mm × 50 mm [Welch], 5 μm, mobile phase A:H_2O/MeCN/trifluoroacetic acid (TFA) = 90/10/0.1; mobile phase B: MeCN/H_2O/TFA = 90/10/0.1). Chiral HPLC was recorded on an LC-2010A HT (Shimadzu) system (column: Chiralpak IB 4.6 mm × 50 mm [Daicel], 5 μM, mobile phase: hexane/ethanol (EtOH) = 80/20). Microwave reactions were run on an Initiator 2.5 Microwave Synthesizer (Biotage).

General procedure for the Ugi reaction[8-15]

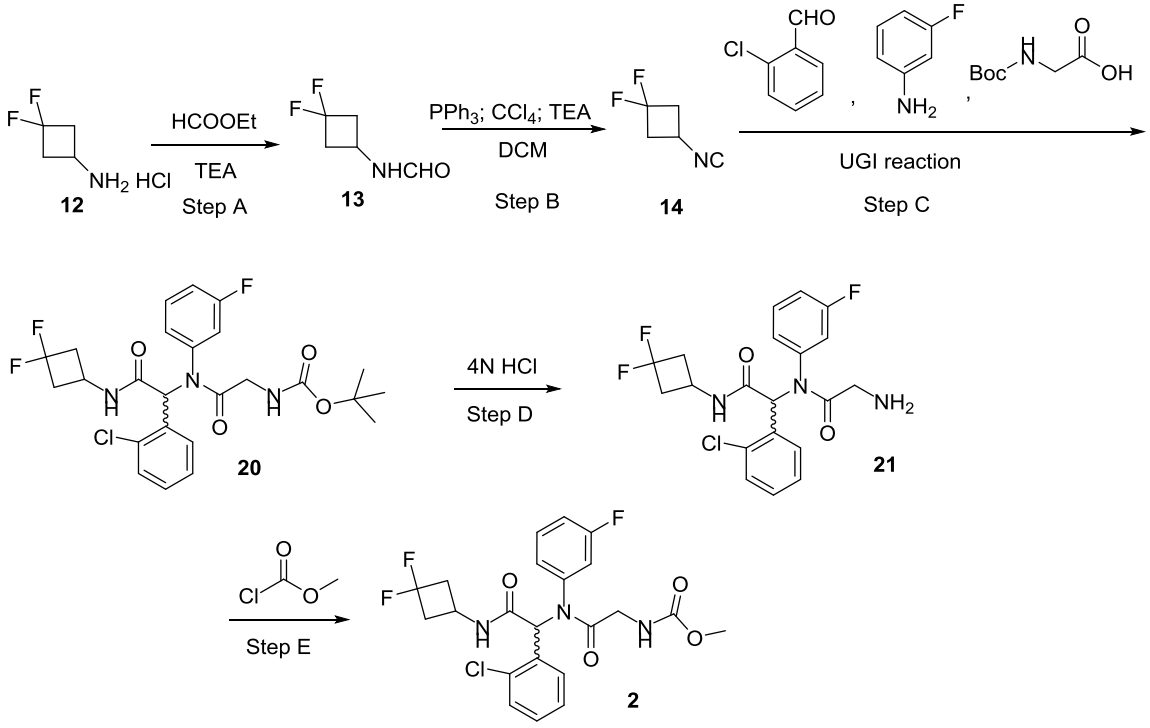
A mixture of aldehyde (3.5 mmol) and aniline (3.5 mmol) in MeOH (8 mL) was stirred at RT for 30 minutes. Then, acid (3.5 mmol) was added and the reaction mixture was stirred for another 30 minutes. Isocyanide (3.5 mmol) was then added. The resulting mixture was then stirred at RT overnight and quenched by addition of H_2O. The resulting mixture was partitioned between ethyl acetate (EtOAc) and H_2O. The organic layer was separated, washed with brine, dried over anhydrous Na_2SO_4, and then concentrated. The residue was purified by silica gel column chromatography using dichloromethane (DCM)/MeOH as eluent to afford the desired product.

General procedure for the Buchwald reaction[16-19]

A mixture of amine (0.30 mmol), aryl bromide (0.30 mmol), Cs_2CO_3 (129 mg, 0.39 mmol), $Pd_2(dba)_3$ (18 mg, 0.02 mmol), and Xantphos (9.4 mg, 0.02 mmol) in 1,4-dioxane (10 mL) was stirred under nitrogen atmosphere at 80°C overnight. After filtration, the filtrate was concentrated in high vacuo and the residue was purified by prep-TLC to give the desired products.

Synthetic Details and Characterization of Compounds

*Compound **2** was prepared according to the following scheme, using the following protocol:*



Step A: N-(3,3-difluorocyclobutyl)formamide (13). A 2-L sealed reactor was charged with 3,3-difluorocyclobutanamine hydrochloride **(12)** (87 g, 0.61 mol), trimethylamine (TEA; 170 mL, 1.22 mol), ethyl formate (HCOOEt; 850 mL, 10.57 mol). The mixture was heated at 85°C overnight. The reaction mixture was cooled and filtered. The cake was washed with EtOAc (3 × 200 mL), and the combined filtrate was concentrated under reduced pressure. The residual solid was dissolved in EtOAc (600 mL) and filtered. The filtrate was concentrated under reduced pressure. The residue was triturated with *n*-hexane (400 mL) and filtered. The solid was collected and dried to afford 74 g desired product **(13)** as off-white solid (86% yield).

Step B: 1,1-Difluoro-3-isocyanocyclobutane (14). To a solution of N-(3,3-difluorocyclobutyl)formamide **(13)** (37 g, 0.274 mol) and triphenylphosphine (PPh_3; 79.1 g, 0.301 mol) in DCM (500 mL) were added CCl_4 (42.1 g, 0.274 mol) and TEA (27.73 g, 0.274 mol). The reaction mixture was stirred overnight at 45°C under the atmosphere of N_2. The resulting mixture was evaporated *in vacuo* at 0°C. The residue was suspended in diethyl ether (Et_2O) (50 mL) at 0°C for 30 minutes and filtered. The precipitate was filtered and the filtrate was evaporated to ~50 mL at 0°C under reduced pressure. The residue was purified by

column chromatography eluting with Et_2O to afford the desired product **14** as light brown oil (36 g [67% purity, based on ^{1}H NMR], 75% crude yield).

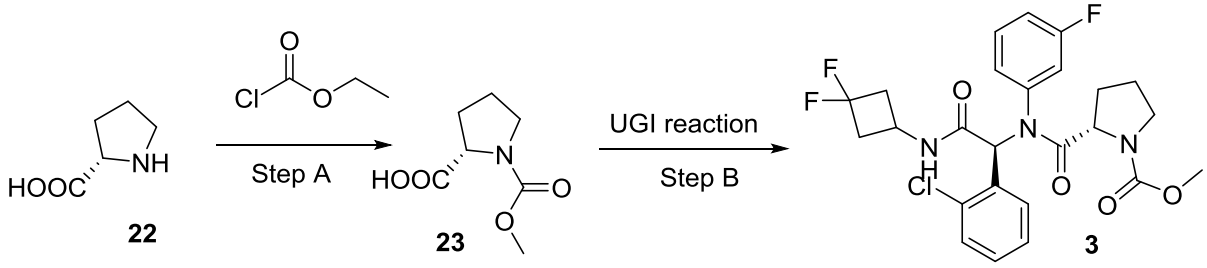
Step C: Tert-butyl 2-((1-(2-chlorophenyl)-2-(3,3-difluorocyclobutylamino)-2-oxoethyl)(3-fluorophenyl)amino)-2-oxoethylcarbamate (20). A mixture of 2-chlorobenzaldehyde (0.31 mL, 2.74 mmol) and 3-fluoroaniline (0.26 mL, 2.74 mmol) in dry MeOH (2 mL) was stirred at RT for 0.5 hour, followed by addition of 2-(*tert*-butoxycarbonylamino)acetic acid (479 mg, 2.74 mmol). The resulting mixture was stirred at RT for 10 minutes followed by addition of 3,3-difluoroisocyanobutane (67% purity, 500 mg, 4.27 mmol). The mixture was stirred at RT overnight. The resulting mixture was filtered to give the desired compound (717.7 mg, 41.9 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.88 (m, 1H), 7.61 (m, 1H), 7.42 (d, *J* = 7.9 Hz, 1H), 7.32–6.42 (m, 7H), 6.33 (s, 1H), 4.12 (m, 1H), 3.72–3.48 (m, 1H), 3.28–3.23 (m, 1H), 3.05–2.75 (m, 2H), 2.55–2.50 (m, 1H), 2.50–2.40 (m, 1H), 1.37 (s, 9H).MS: 526.2 $(M+1)^+$.

Step D: 2-Amino-N-(1-(2-chlorophenyl)-2-(3,3-difluorocyclobutylamino)-2-oxoethyl)-N-(3-fluorophenyl) acetamide hydrochloride (21). A mixture of *tert*-butyl 2-((1-(2-chlorophenyl)-2-(3,3-difluorocyclobutylamino)-2-oxoethyl)(3-fluorophenyl)amino)-2-oxoethylcarbamate (500 mg) in 4N HCl in EtOAc (20 mL) was stirred at RT overnight. The mixture was filtered to give the desired product (quant. yield). MS: 426.2 $(M+1)^+$.

Step E: Compound 2 To a solution of 2-amino-N-(1-(2-chlorophenyl)-2-(3,3-difluoro cyclobutylamino)-2-oxoethyl)-N-(3-fluorophenyl) acetamide hydrochloride **21** (100 mg) in saturated aqueous $NaHCO_3$ (10 mL) and tetrahydrofuran (THF) (10 mL) was added methyl carbonochloridate (1 mL). The mixture was stirred at RT overnight. Saturated aqueous $NaHCO_3$ was added to the mixture until pH reached 9 and the mixture then extracted by EtOAc. Combined organic layers were dried, filtered, and concentrated. The residue was purified by flash chromatography using DCM/MeOH (V:V, 20:1) as eluent to give the desired product (66.5 mg, 60% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.83 (m, 1H), 7.63 (m, 1H), 7.39 (d, *J* = 7.8 Hz, 1H), 7.27–7.13 (m, 3H), 7.05 (m, 2H), 6.84 (d, *J* = 7.6 Hz, 1H), 6.72 (m, 1H), 6.28 (s, 1H), 4.09 (dt, *J* = 8.0, 6.1 Hz, 1H), 3.58 (m, 1H), 3.49 (s, 3H), 3.31–3.24 (m, 1H), 2.97–2.84 (m, 2H), 2.57 (m, 1H), 2.40 (m, 1H). High-resolution mass spectrometry (HRMS) (ESI) calculated for $C_{22}H_{21}ClF_3N_3O_4$ $[M+H]^+$ 484.1251, found 484.1258.

*Compound **1** was prepared via the procedure described above;* 1H NMR (400 MHz, CDCl3), δ 7.16-6.74 (m, 8H), 6.34 (s, 1H), 5.54 (s, 1H), 5.54-5.26 (m, 1H), 3.88-3.64 (m, 6H), 2.38 (s, 3H), 1.98-1.62 (m, 4H), 1.42-0.98 (m, 6H); HRMS(ESI) calculated for $C_{25}H_{30}FN_3O_4$ [M+ H]$^+$ 456.2220, found 456.2307.

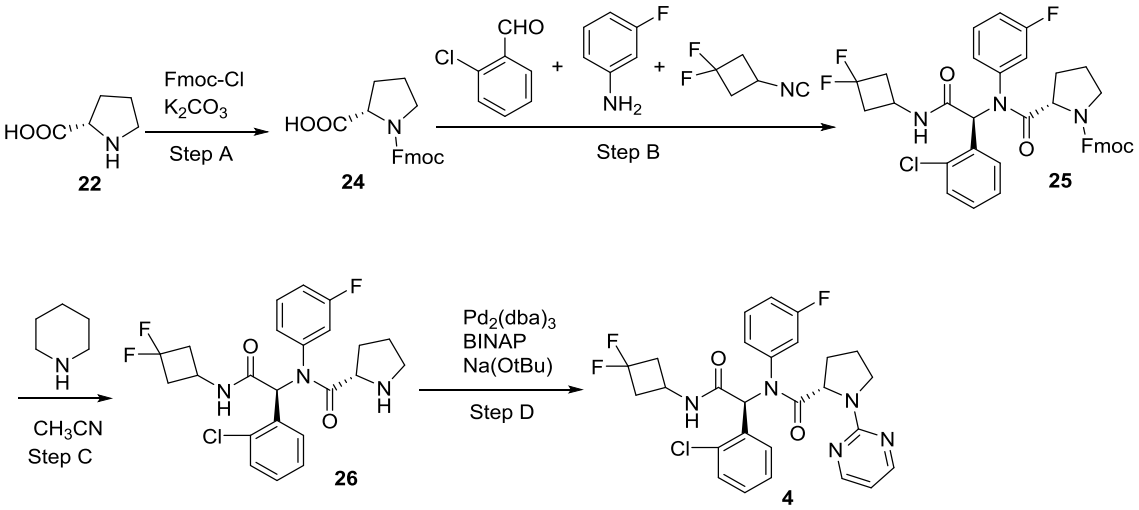
*Compound **3** was prepared according to the following scheme, using the following protocol:*



Step A: (S)-1-(methoxycarbonyl)pyrrolidine-2-carboxylic acid. Methyl carbonochloridate (1.65 g, 17.4 mmol) was added to a solution of (S)-pyrrolidine-2-carboxylic acid **(22)** (1 g, 8.7 mmol) in a solution of THF (10 mL) and saturated $NaHCO_3$ (10 mL) at 0°C and stirred at RT overnight. The mixture was extracted with EtOAc and the organic layer was concentrated to afford the desired product **23** (1.2 g, 80% yield) as colorless oil.

Step B: Compound 3. The product was synthesized via the *general procedure* for the Ugi reaction set forth above. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (s, 0.4H), 7.37-7.31 (m, 2H), 7.27–7.07 (m, 3H), 7.03–6.86 (m, 2H), 6.73–6.39 (m, 1H), 6.13 (s, 0.6H), 4.39 (m, 1H), 4.23–4.08 (m, 1H), 3.72 (m, 3H), 3.58–3.37 (m, 2H), 3.12–2.92 (m, 2H), 2.79–2.31 (m, 2H), 2.09–1.86 (m, 3H), 1.77–1.66 (m, 1H). Integration (24) for $C_{25}H_{25}ClF_3N_3O_4$ due to one active proton (NH) not shown in $CDCl_3$. HRMS (ESI) calculated for $C_{25}H_{25}ClF_3N_3O_4$ [M+ H]$^+$ 524.1564, found 524.1560.

Compound 4 was prepared according to the following scheme, using the following protocol:



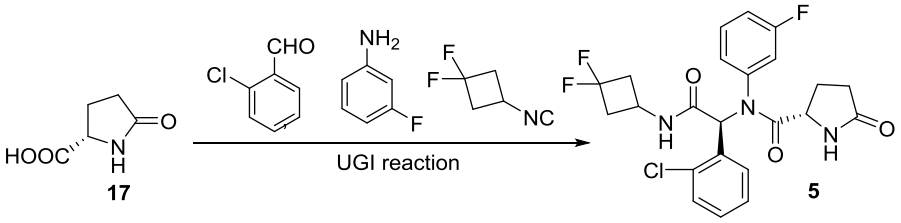
Step A: (S)-1-(((9H-fluoren-9-yl)methoxy)carbonyl)pyrrolidine-2-carboxylic acid (24). (S)-pyrrolidine-2-carboxylic acid **22** (1 g, 8.7 mmol) was dissolved in 1,4-dioxane (4 mL) and H_2O (15 mL), and cooled to 0°C. K_2CO_3 (3.24 g, 23 mmol) was added, and then (9H-fluoren-9-yl)methyl chloroformate (2.3 g, 8.3 mmol) was added in portions. The mixture was stirred at RT overnight and H_2O (10 mL) was added. The mixture was extracted with Et_2O (2 × 20 mL).The aqueous phase was acidified with aqueous HCl (1 M) to pH 2–3, and extracted with DCM (3 × 50 mL). Combined organic layers were dried over Na_2SO_4, filtered, concentrated to dryness to give the desired product **24** as a white solid (2.8 g, 95% yield). MS: 338 $(M+1)^+$.

Step B: (S)-(9H-fluoren-9-yl)methyl 2-(((S)-1-(2-chlorophenyl)-2-(3,3-difluorocyclo -butyl-amino)-2-oxoethyl)(3-fluorophenyl)carbamoyl)pyrrolidine-1-carboxylate (25). A mixture of 2-chlorobenzaldehyde (230 mg, 1.5 mmol) and 3-fluorobenzenamine (180 mg, 1.5 mmol) in MeOH (4 mL) was stirred at RT for 30 minutes. (S)-1-(((9H-fluoren-9-yl)-methoxy)-carbonyl)pyrrolidine-2-carboxylic acid **24** (500 mg, 1.5 mmol) was added and the reaction mixture stirred for 10 minutes. 1,1-Difluoro-3-isocyanocyclobutane (360 mg, 3.0 mmol) was added and the reaction mixture was stirred at RT overnight. The mixture was concentrated to dryness, and purified by chromatography eluting with DCM/acetone (V:V, 150:1 to 30:1), then with DCM/EtOAc (V:V, 1:1) to give the desired two isomers, (S)-(9H-fluoren-9-yl)methyl-2-(((R)-1-(2-chloro-phenyl)-2-(3,3–difluoro-cyclobutylamino)-2-oxo-ethyl)(3-fluorophenyl)carbamoyl)pyrrolidine-1-carboxylate (350 mg, 33% yield) and (S)-(9H-fluoren-9-yl)methyl-2-(((S)-1-(2-chloro-phenyl)-2-(3,3–difluorocyclo-butylamino)-2-oxoethyl)(3-fluorophenyl)carbamoyl)pyrrolidine-1-carboxylate (370 mg, 33% yield). MS: 688$(M+1)^+$.

Step C: (S)-N-((S)-1-(2-chlorophenyl)-2-(3,3-difluorocyclobutylamino)-2-oxoethyl)-N-(3-fluorophenyl)pyrrolidine-2-carboxamide (26). (S)-(9H-fluoren-9-yl)methyl-2-(N-((S)-(3,3-difluoro-cyclobutylcarbamoyl)(2-chlorophenyl)methyl)-N-(3-fluorophenyl) carbamoyl)pyrrolidine-1-carboxylate **25** (370 mg, 0.53 mmol) was dissolved in CH_3CN (5 mL). Piperidine (1 mL) was added dropwise. The mixture was stirred at 40°C overnight, then H_2O (10 mL) was added. The resulting mixture was extracted with EtOAc (3 × 50 mL). Combined organic layers were dried over $MgSO_4$, filtered, concentrated to dryness to give crude product **26** (145 mg, 57% yield). MS: 466 $(M+1)^+$.

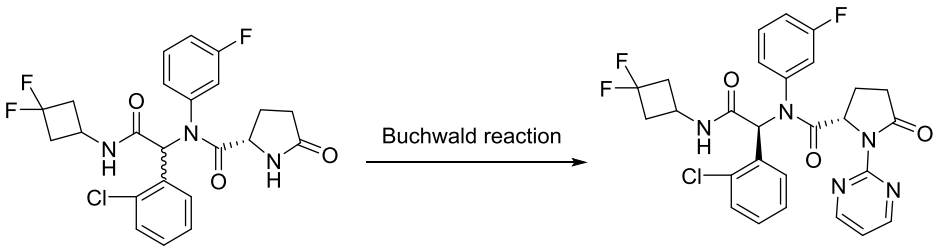
Step D: Compound 4. A mixture of (2S)-N-((S)-(3,3-difluorocyclobutylcarbamoyl)-(2-chlorophenyl)-methyl)-N-(3-fluorophenyl)pyrrolidine-2-carboxamide (40 mg, 0.08 mol), 2-bromo-pyrimidine (100 mg, 0.5 mmol), $Pd_2(dba)_3$ (10 mg, 0.008 mmol), BINAP (10 mg, 0.012 mmol), sodium *tert*-butoxide (NaOtBu; 24 mg, 0.2 mmol) in toluene was stirred at 100°C under the atmosphere of N_2 overnight. The mixture was filtered, and then washed with EtOAc (3 × 20 mL). Combined organic layers were concentrated. The residue was purified by prep-TLC using petroleum ether/EtOAc (V:V, 3:2) then by DCM/acetone (V:V, 30:1) as eluent to give the desired compound **4** (10 mg, 23% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.309–8.298(m, 2H), 7.315–7.296(m, 1H), 7.151–7.108(m,3H), 7.305–6.948(m, 2H), 6.880–6.832(m,1H), 6.505–6.482(t, 1H), 6.430(s, 1H), 6.203–6.187(m, 1H), 4.417–4.385(m, 1H), 4.305–4.302(m, 1H), 3.770–3.645(m, 2H), 3.014–2.937(m, 2H), 2.509–2.351(m, 2H), 2.164–2.073(m, 2H), 1.974–1.835(m, 2H). HRMS (ESI) calculated for $C_{27}H_{25}ClF_3N_5O_2$ $[M+H]^+$ 544.1727, found 544.1727.

Compound 5 was prepared according the general procedure for the Ugi reaction:



^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 7.31 (m, 7H), 6.86 (m, 1H), 6.34 (m, 1H), 4.13 (s, 1H), 4.01–3.72 (m, 1H), 3.31 (s, 1H), 3.05–2.77 (m, 2H), 2.55 (m, 1H), 2.42 (d, *J* = 9.1 Hz, 1H), 2.23–1.65 (m, 4H). HRMS (ESI) calculated for $C_{23}H_{21}ClF_3N_3O_3$ $[M+H]^+$ 480.1302, found 480.1305.

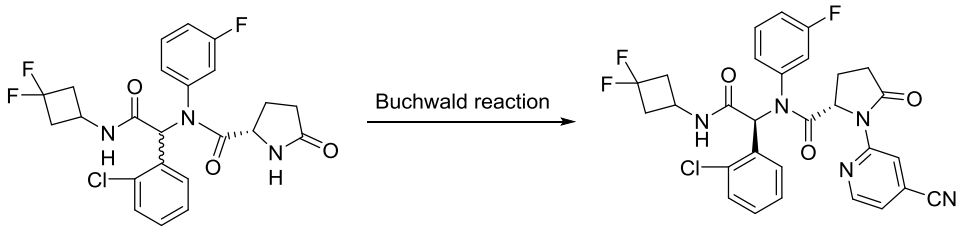
Compound 6 was prepared according the general procedure for the Buchwald reaction:



rac 5 **6**

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.69 (d, *J* = 4.8 Hz, 2H), 7.71 (s, 1H), 7.31 (m, 1H), 7.18 (m, 1H), 7.13–6.77 (m, 6H), 6.46 (s, 1H), 6.22 (s, 1H), 5.00–4.62 (m, 1H), 4.35 (s, 1H), 3.19–2.71 (m, 3H), 2.69–1.83 (m, 5H). HRMS (ESI) calculated for $C_{27}H_{23}ClF_3N_5O_3$ [M+ H]$^+$558.1520, found 558.1525.

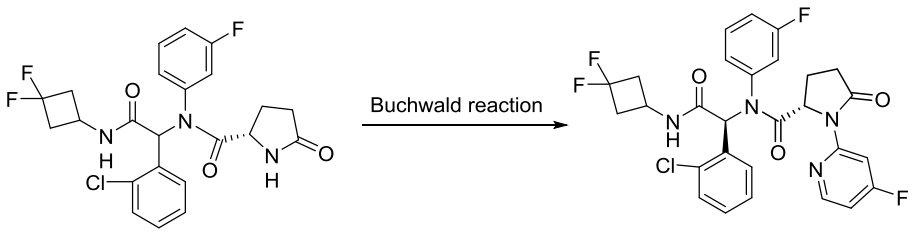
Compound 8 was prepared according the general procedure for the Buchwald reaction:



rac 5 **8**

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.74 (s, 1H), 8.52 (s, 1H), 7.72 (d, *J* = 7.1 Hz, 1H), 7.43–7.33 (m, 1H), 7.25–7.17 (m, 1H), 7.13–6.81 (m, 4H), 6.43 (s, 1H), 6.12 (s, 1H), 4.92 (d, *J* = 6.8 Hz, 1H), 4.37–4.28 (m, 1H), 3.10–2.82 (m, 3H), 2.59–2.49 (m, 2H), 2.42–2.36 (m, 1H), 2.31–2.22 (m, 1H), 2.06–1.88 (m, 2H). HRMS (ESI) calculated for $C_{29}H_{23}ClF_3N_5O_3$[M+ H]$^+$582.1520, found 582.1526.

Compound 7 was prepared according the general procedure for the Buchwald reaction:

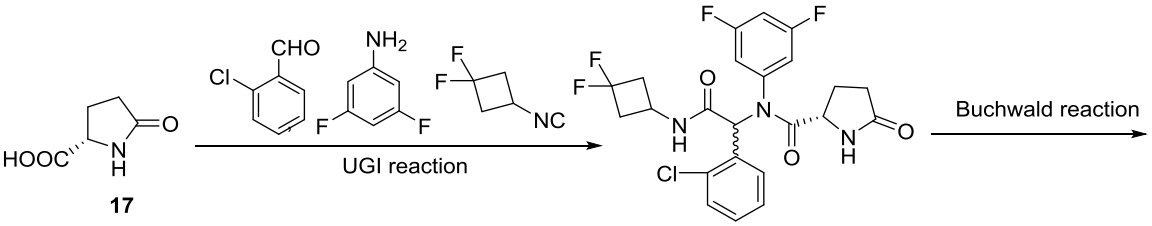


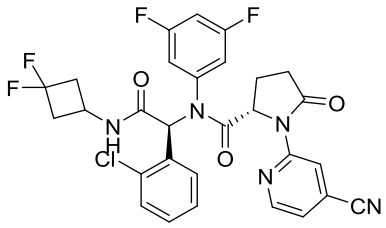
rac 5 **7**

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32 (s, 1H), 8.17 (d, *J* = 11.2 Hz, 1H), 7.06 (dd, *J* = 6, 3.2 Hz, 1H), 7.33–6.82 (m, 7H), 6.42 (s, 1H), 6.34 (s, 2H), 4.93 (d, *J* = 4 Hz, 1H), 4.34 (s, 1H),

3.05–2.80 (m, 3H), 2.55–2.37 (m, 3H), 2.42–2.36 (m, 1H), 2.21–2.16 (m, 1H), 2.03–1.97 (m, 1H). HRMS (ESI) calculated for $C_{28}H_{23}ClF_4N_4O_3$[M+ H]$^+$575.1473, found 575.1469.

Compound AGI-14100 was prepared according to the general procedures for the Ugi reaction and the Buchwald reaction:

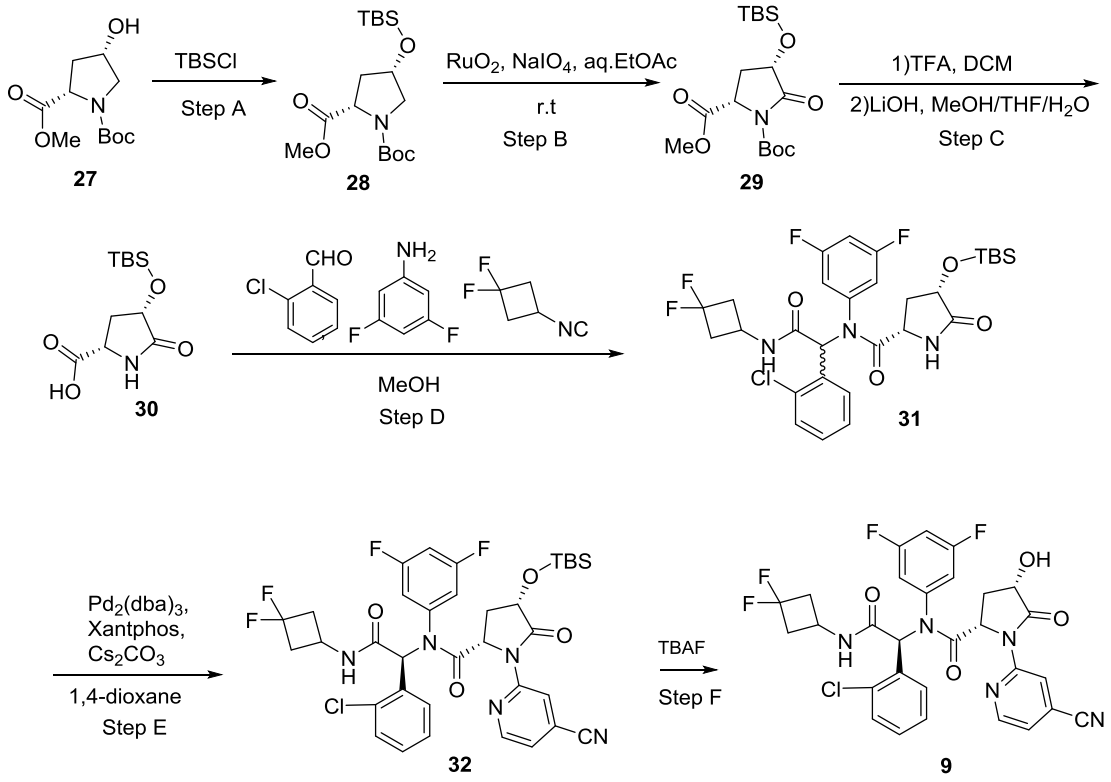




AGI-14100

^{1}H NMR (400 MHz, $CDCl_3$) : δ 8.73 (d, *J* = 7.1 Hz, 1H), 8.60–8.46 (m, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.38–7.32 (m, 1H), 7.31–7.27 (m, 1H), 7.26–7.18 (m, 1H), 7.14–7.00 (m, 1H), 6.96 (m, 1H), 6.85 (s, 1H), 6.69 (m, 1H), 6.40 (s, 1H), 6.02 (d, *J* = 6.6 Hz, 1H), 4.98–4.74 (m,1H), 4.39–4.10 (m, 1H), 3.11–2.67 (m, 3H), 2.64–1.95 (m, 5H). HRMS (ESI) calculated for $C_{29}H_{22}ClF_4N_5O_3$ [M+ H]$^+$ 600.1426, found 600.1419.

Compound 9 was prepared according to the following scheme, using the following protocol:



Step A: (2S,4S)-1-tert-butyl 2-methyl 4-((tert-butyldimethylsilyl)oxy)pyrrolidine-1,2-dicarboxylate (28). Imidazole (2.8 g, 40.8 mmol) was added to a solution of (2S,4S)-1-*tert*-butyl 2-methyl 4-hydroxypyrrolidine-1,2-dicarboxylate **27** (5.0 g, 20.4 mmol) and *tert*-butyldimethylsilyl chloride (TBSCl; 4.6 g, 30.6 mmol) in anhydrous dimethylformamide (DMF; 100 mL). The mixture was stirred at RT overnight. The resulting mixture was partitioned between EtOAc and H_2O. The organic layer was separated, washed in sequence with aqueous LiCl (10%) and brine, dried over anhydrous Na_2SO_4, and then concentrated. The residue was purified by column chromatography (silica gel, 0–25% DCM in petroleum ether) to afford the desired product **28** (6.7 g, 91.5% yield) as colorless oil. MS: 360.2 $(M+1)^+$.

Step B: (2S,4S)-1-tert-butyl 2-methyl 4-((tert-butyldimethylsilyl)oxy)-5-oxopyrrolidine- 1,2-dicarboxylate (29). To a solution of sodium periodate ($NaIO_4$; 7.5 g, 35.0 mmol) in H_2O (80 mL) was added ruthenium oxide (RuO_2; 370 mg, 2.8 mmol) under nitrogen. The resulting green–yellow solution was stirred for 5 minutes followed by addition of (2S,4S)-1-*tert*-butyl-2-methyl4-((*tert*-butyldimethylsilyl)oxy)pyrrolidine-1,2-dicarboxylate **28** (5.0 g, 14.0 mmol) in EtOAc (44 mL) in one portion. The mixture was stirred at RT overnight. The resulting mixture was then diluted with EtOAc and filtered through a pad of Celite. The organic layer

was separated and washed with saturated aqueous $NaHSO_3$, which resulted in precipitation of Ru black. The organic layer was then washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave the desired product **29** (4.8 g, 92.3% yield) as colorless oil. MS: 374.2 $(M+1)^+$.

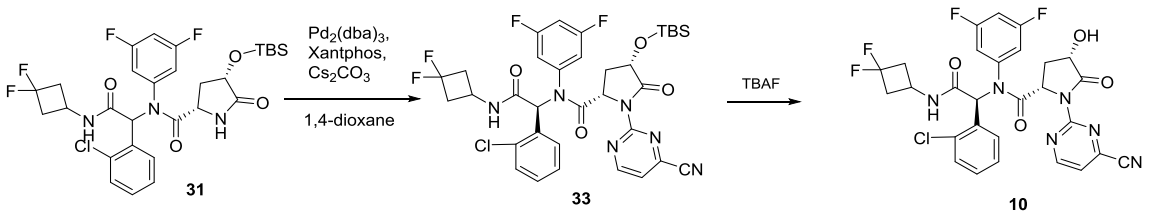
Step C: (2S,4S)-4-((tert-butyldimethylsilyl)oxy)-5-oxopyrrolidine-2-carboxylic acid (14). TFA (6 mL) was added to a solution of (2S,4S)-1-*tert*-butyl 2-methyl 4-((*tert*-butyldimethylsilyl)oxy)-5-oxopyrrolidine-1,2-dicarboxylate **29** (2.5 g, 6.68 mmol) in DCM (18 mL) at 0°C. The mixture was stirred at RT for 1 hour, then concentrated. The residue was dissolved in MeOH/THF (10 mL/10 mL) followed by addition of a solution of LiOH (842 mg, 20.1 mmol) in H_2O (5 mL). The resulting mixture was stirred at RT for 1 hour and then partitioned between EtOAc and H_2O. The aqueous layer was separated and then pH adjusted to 6 by 1N HCl aqueous and extracted with EtOAc (20 mL × 3). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and then concentrated to afford the desired product **30** (1.3 g, 75.1% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.87 (s, 1H), 8.17 (s, 1H), 4.21 (t, *J* = 8.0 Hz, 1H), 4.02 (d, *J* = 8.4 Hz, 1H), 2.39–2.23 (m, 1H), 2.09 (m, 1H), 0.84 (s, 9H), 0.07 (s, 6H). MS: 260.1 $(M+1)^+$.

Step D: The same as general procedure for Ugi reaction set forth above.

Step E: The same as general procedure for Buchwald reaction set forth above.

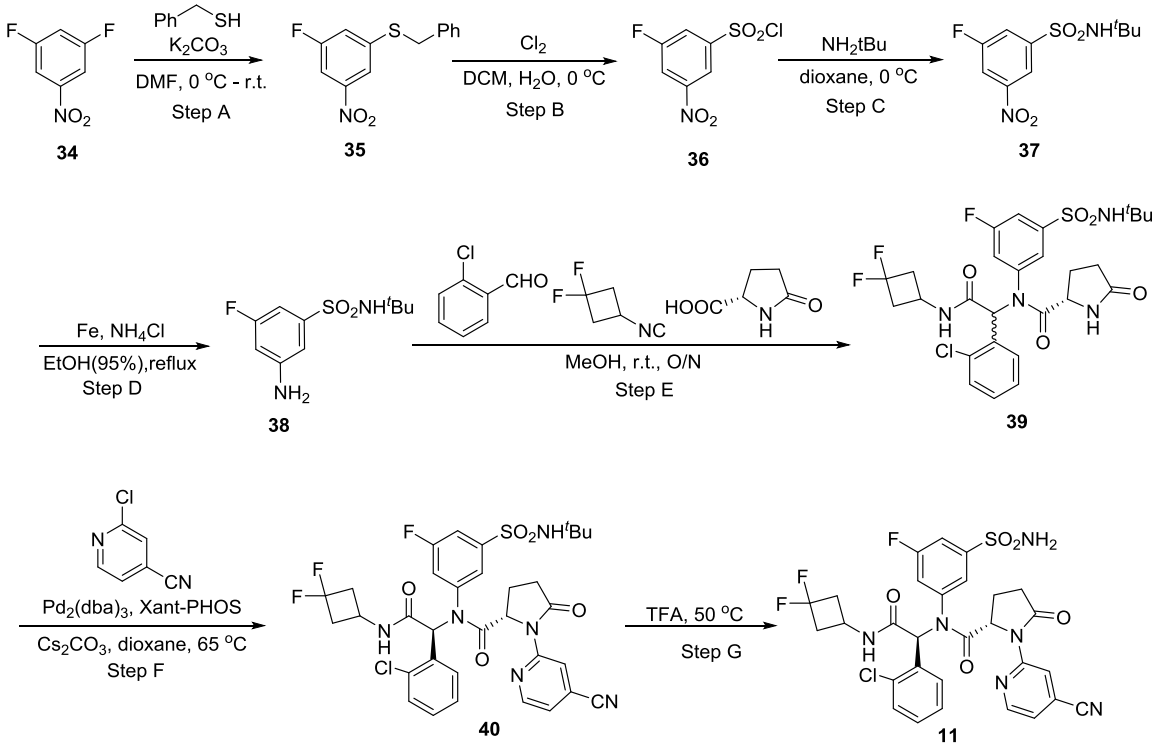
Step F: (2S,4S)-4-((tert-butyldimethylsilyl)oxy)-N-((S)-1-(2-chlorophenyl)-2-((3,3-difluorocyclobutyl)amino)-2-oxoethyl)-1-(4-cyanopyridin-2-yl)-N-(3,5-difluorophenyl)-5-oxopyrrolidine-2-carboxamide (9). Tetrabutylammonium fluoride (TBAF) in THF (1N, 0.3 mL) was added to a solution of (2S,4S)-4-((*tert*-butyldimethylsilyl)oxy)-N-((S)-1-(2-chlorophenyl)-2-((3,3-difluorocyclo-butyl)amino)-2-oxoethyl)-1-(4-cyanopyridin-2-yl)-N-(3,5-difluorophenyl)-5-oxopyrrolidine-2-carboxamide (0.15 mmol) in THF at 0°C and the reaction solution was stirred at this temperature for 20 minutes. The resulting mixture was concentrated and the residue was purified by prep-TLC (6% MeOH in DCM) to afford the desired product **9**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (s, 1H), 8.52 (d, *J* = 5.0 Hz, 1H), 7.60 (d, *J* = 8.2 Hz, 1H), 7.45–7.17 (m, 4H), 7.15–6.91 (m, 2H), 6.84 (d, *J* = 8.7 Hz, 1H), 6.69 (t, *J* = 8.7 Hz, 1H), 6.54–6.36 (m, 2H), 4.87–4.60 (m, 1H), 4.31 (m, 2H), 3.99–3.77 (m, 1H), 3.15–2.78 (m, 2H), 2.62–2.26 (m, 3H), 2.26–2.08 (m, 1H). HRMS (ESI) calculated for $C_{29}H_{22}ClF_4N_5O_4$ $[M+H]^+$ 616.1375, found 616.1377.

Compound 10 was prepared following the procedure for Compound 9:



^{1}H NMR (400 MHz, CD_3OD): δ 8.97 (d, *J* = 4.9 Hz, 1H), 7.68 (d, *J* = 4.8 Hz, 1H), 7.61 (d, *J* = 9.2 Hz, 1H), 7.42 (d, *J* = 7.2 Hz, 1H), 7.25 (td, *J* = 7.8, 1.5 Hz, 1H), 7.11 (t, *J* = 7.2 Hz, 1H), 7.00 (dd, *J* = 13.3, 5.5 Hz, 2H), 6.90 (m, 1H), 6.47 (s, 1H), 4.82 (m, 1H), 4.36 (t, *J* = 9.2 Hz, 1H), 4.21 (dd, *J* = 14.9, 7.2 Hz, 1H), 2.98–2.80 (m, 2H), 2.73–2.33 (m, 3H), 2.04–1.84 (m, 1H). Integration (19) for $C_{28}H_{21}ClF_4N_6O_4$ due to two active protons (NH and OH) not shown in MeOD. HRMS (ESI) calculated for $C_{28}H_{21}ClF_4N_6O_4$ $[M+H]^+$ 617.1327, found 617.1322.

Compound 11 was prepared according to the following scheme:



Step A: Benzyl(3-fluoro-5-nitrophenyl)sulfane (35). To a solution of 1, 3-difluoro-5-nitrobenzene **34** (15.9 g, 100 mmol) in DMF (160 mL) was added K_2CO_3 (15.8 g, 110 mmol)

and phenylmethanethiol (Ph-SH; 12.4 g, 100 mmol) at 0°C. The mixture was stirred at RT for 2 hours. The resulting mixture was concentrated in high vacuo to afford the crude product **35** as yellow oil, which was used for the next step without further purification.

Step B: 3-Fluoro-5-nitrobenzene-1-sulfonyl chloride (36). Benzyl(3-fluoro-5-nitrophenyl)sulfane **35** (3.0 g) was dissolved in DCM (30 mL). To this solution, deionized H_2O (30 mL) was added. Then chlorine was bubbled slowly into the stirred reaction mixture until the complete consumption of the starting material as monitored by TLC. The organic layer was separated, washed with saturated aqueous $Na_2S_2O_3$ solution, dried and concentrated to afford the crude product **36**, which was used for the next step without further purification.

Step C: N-tert-butyl-3-fluoro-5-nitrobenzenesulfonamide (37). 3-Fluoro-5-nitrobenzene-1-sulfonyl chloride **36** was dissolved in dry dioxane (30 mL). The solution was cooled to 0°C, and *tert*-butylamine (NH_2tBu; 10 mL) was slowly added, after which the mixture was warmed to RT and stirred for 2 hours. The mixture was concentrated and the residue was purified by column chromatography (silica gel, MeOH/DCM = 0:100 to 10:90) to afford the desired product **37** (2.2 g, 70% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.43 (s, 1H), 8.40–8.32 (m, 1H), 8.10–8.05 (m, 1H), 7.99 (s, 1H), 1.12 (s, 9H).

Step D: 3-Amino-N-tert-butyl-5-fluorobenzenesulfonamide (38). *N-tert*-butyl-3-fluoro-5-nitrobenzenesulfonamide **37** (1.0 g, 3.6 mmol), iron powder (1.0 g, 18 mmol) and NH_4Cl (1.0 g, 18 mmol) were mixed in EtOH (95%, 10 mL). The mixture was refluxed for 16 hours then filtered. The filtrate was concentrated and the residue was purified by column chromatography to afford the desired product **38** (0.62 g, 70% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.45 (s, 1H), 6.88–6.85 (m, 1H), 6.66–6.62 (m, 1H), 6.48–6.42 (m, 1H), 5.89 (s, 2H), 1.11 (s, 9H).

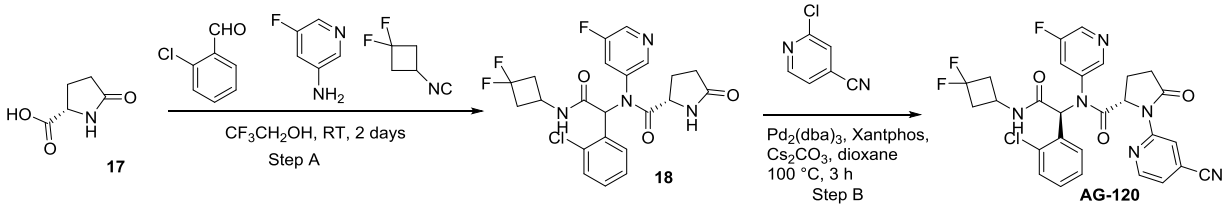
Step E: The same as general procedures for Ugi reaction set forth above.

Step F: The same as general procedures for Buchwald reaction set forth above.

Step G: (S)-N-((S)-1-(2-chlorophenyl)-2-(3,3-difluorocyclobutylamino)-2-oxoethyl)-1-(4-cyanopyridin-2-yl)-N-(3-fluoro-5-sulfamoylphenyl)-5-oxopyrrolidine-2-carboxamide (11). (2S)-N-(3-(N-*tert*-butylsulfamoyl)-5-fluorophenyl)-N-(1-(2-chlorophenyl)-2-(3,3-difluorocyclo-butylamino)-2-oxoethyl)-1-(4-cyanopyridin-2-yl)-5-oxopyrrolidine-2-carboxamide **40** (80 mg, 0.11 mmol) was dissolved in TFA (1 mL). The solution was stirred at RT for 16 hours and neutralized with saturated aqueous $NaHCO_3$. The mixture was then

extracted with EtOAc (10 mL × 3). Combined organic layers were dried and concentrated. The residue was purified by prep-TLC to afford **11** (34 mg, 46% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.90–8.84 (m, 1H), 8.67–8.62 (m, 1H), 8.55 (s, 1H), 8.19 (s, 1H), 7.87–7.76 (m, 1H), 7.65–7.60 (m, 2H), 7.45–7.40 (m, 3H), 7.21 (d, *J* = 7.0 Hz, 2H), 7.11–7.04 (m, 1H), 6.93–6.86 (m, 1H), 6.33–6.26 (m, 1H), 4.83 (m, 1H), 4.13 (s, 1H), 2.94 (m, 2H), 2.63–2.53 (m, 3H), 2.42–2.32 (m, 1H), 1.97 (s, 2H). HRMS (ESI) calculated for $C_{29}H_{24}ClF_3N_6O_5S$ [M+H]$^+$ 661.1248, found 661.1262.

Compound AG-120 was prepared according to the following scheme, using the following protocol:



Step A: 2-(2-chlorophenyl)-N-(3,3-difluorocyclobutyl)-3-(5-fluoropyridin-3-yl)-4-oxo-4-((S)-5-oxopyrrolidin-2-yl)butanamide (18). A mixture of 2-chlorobenzaldehyde (230 g, 1.64 mol) and 5-fluoropyridin-3-amine (184 g, 1.64 mol) in CF_3CH_2OH (1.64 L) was stirred at RT for 30 minutes. (S)-5-oxopyrrolidine-2-carboxylic acid **17** (212 g, 1.64 mol) was added and the reaction mixture stirred for 10 minutes. 1,1-difluoro-4-isocyanocyclohexane (192 g, 1.64 mol) was then added and the reaction mixture was stirred at RT for 2 days. The mixture was concentrated, and then the residue was purified by column chromatography on silica gel eluted with DCM/MeOH (100:1 to 20:1) to afford the desired product **18** (360 g, 45.8% yield).

Step B: Compound AG-120. $Pd_2(dba)_3$ (17 g, 18.6 mmol) was added to a solution of 2-(2-chlorophenyl)-N-(3,3-difluorocyclobutyl)-3-(5-fluoropyridin-3-yl)-4-oxo-4-((S)-5-oxopyrrolidin-2-yl)butanamide **18** (355 g, 0.74 mol), 2-chloroisonicotinonitrile (123 g, 0.89 mol), Cs_2CO_3(290 g, 0.89 mol) and Xantphos (21 g, 36.3 mmol) in 1,4-dioxane (4 L) and stirred at 100°C for 3 hours. The mixture was filtered and the filtrate was concentrated, then the diastereomeric residue was separated by column chromatography on silica gel and eluted with DCM/EtOAc (100:1 to 4:1) to give the desired isomer, which was then further purified by crystallization from EtOAc to get the desired product **AG-120** (95 g, 22% yield, 99.7 %*de*) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.01–8.49 (*m*, 2.5H), 8.36–8.11 (m, 2.2H), 7.33 (d, *J* = 8.0 Hz, 1H), 7.25 (m, 1.3H), 7.21 (t, *J* = 7.8 Hz, 1H), 6.91 (m, 1H),

6.48–6.41 (m, 1H), 6.30–6.21 (m, 1H), 4.82 (m, 1H), 4.38–4.30 (m, 1H), 3.11–2.74 (m, 3H), 2.61–1.91 (m, 5H); integration (21) for $C_{28}H_{22}ClF_3N_6O_3$ due to one active proton (NH) not shown in $CDCl_3$. HRMS (ESI) calculated for $C_{28}H_{22}ClF_3N_6O_3$ $[M+H]^+$ 583.1472, found 583.1477.

Abbreviations

2-HG: D-2-hydroxyglutarate
α-KG: alpha-ketoglutarate
ADME: absorption, distribution, metabolism, and excretion
AML: acute myeloid leukemia
B-ME: beta-mercaptoethanol
BINAP: (2,2'-bis(diphenylphosphino)-1,1'-binaphthyl)
BM: bone marrow
Boc: tert-butyloxycarbonyl
BSA: bovine serum albumin
CLp: clearance
CYP: cytochrome P450
Dba: dibenzylideneacetone
DCM: dichloromethane
DMF: dimethylformamide
DMSO: dimethylsulfoxide
E_h: hepatic extraction ratio
Enz: enzymatic
Et_2O: diethyl ether
EtOAc: ethyl acetate
EtOH: ethanol
ESI: electrospray ionization
F: bioavailability
FAB: French-American-British
FBS: fetal bovine serum
Fmoc: fluorenylmethyloxycarbonyl
FSC: forward scatter
GBM: glioblastoma multiforme
^1H NMR: proton nuclear magnetic resonance
HRMS: high-resolution mass spectrometry
HPLC: high-performance liquid chromatography
hPXR: human pregnane X receptor
IC_{50}: half-maximal (50%) inhibitory concentration
IC_{90}: 90% inhibitory concentration
IDH: isocitrate dehydrogenase

IV: intravenous
k_{obs}: observed rate constant
LC-MS/MS: liquid chromatography–tandem mass spectrometry
MeCN: acetonitrile
MeOH: methanol
mIDH: mutant isocitrate dehydrogenase
MSKCC: Memorial Sloan Kettering Cancer Center
NaOtBu: sodium *tert*-butoxide
ND: not determined
NMR: nuclear magnetic resonance
PB: peripheral blood
$Pd_2(dba)_3$: tris(dibenzylideneacetone)dipalladium(0)
PK: pharmacokinetic
PO: oral
PPh_3: triphenylphosphine
prep-TLC: preparative thin-layer chromatography
QCs: quality control samples
RPMI: Roswell Park Memorial Institute
SSC: side scatter
t½: half-life
TBAF: tetrabutylammonium fluoride
TBSCl: *tert*-Butyldimethylsilyl chloride
TEA: trimethylamine
TFA: trifluoroacetic acid
THF: tetrahydrofuran
TLC: thin-layer chromatography
tPSA: total polar surface area
WHO: World Health Organization
WT: wild type
Xantphos: 4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene

Full Conflict of Interest Statement

Janeta Popovici-Muller: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder, patents. Decibel Therapeutics – employment.

René M. Lemieux: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder, patents. KSQ Therapeutics – employment.

Erin Artin: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. KSQ Therapeutics – employment, stock/shareholder.

Jeffrey Saunders: Agios Pharmaceuticals, Inc. – prior employment, patents. KSQ Therapeutics – advisory board. Praxis Medicines – consultancy. SV Health – consultancy.

Francesco G. Salituro: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. Sage Therapeutics – employment.

Jeremy Travins: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder, patents. Shire – employment.

Giovanni Cianchetta: Agios Pharmaceuticals, Inc. – employment, stock/shareholder, patents.

Zhenwei Cai: PharmaResources – employment, patents (as coinventor).

Ding Zhou: PharmaResources – prior employment. GSK R&D Shanghai – employment.

Dawei Cui: PharmaResources – prior employment.

Ping Chen: PharmaResources – employment.

Kimberly Straley: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. Vertex Pharmaceuticals –employment.

Erica Tobin: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. KSQ Therapeutics – employment.

Fang Wang: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Muriel D. David: INSERM/AVIESAN, French National Cancer League (LNCC), French National Cancer Association (ARC) – funding to institution. Agios Pharmaceuticals, Inc. – travel/accommodation expenses. Celgene – travel/accommodation expenses.

Virginie Penard-Lacronique: INSERM/AVIESAN, French National Cancer League (LNCC), French National Cancer Association (ARC) – funding to institution. Celgene – travel/accommodation expenses.

Cyril Quivoron – no competing financial interest.

Véronique Saada – no competing financial interest.

Stéphane de Botton: Agios Pharmaceuticals, Inc. – advisory board member, research funding, travel/accommodation expenses. Celgene – advisory board member, research funding, travel/accommodation expenses. Pfizer – advisory board member, travel/accommodation expenses. Novartis – advisory board member, travel/accommodation expenses. Servier – advisory board member. Pierre Fabre – advisory board member, travel/accommodation expenses.

Stefan Gross: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Lenny Dang: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Hua Yang: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Luke Utley: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. Alnylam Pharmaceuticals – prior employment. Spero Therapeutics – employment, stock/shareholder.

Yue Chen: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Hyeryun Kim: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Shengfang Jin: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Zhiwei Gu: ChemPartner – employment.

Gui Yao: ChemPartner – employment.

Zhiyong Luo: ChemPartner – employment.

Xiaobing Lv: ChemPartner – employment.

Cheng Fang: ChemPartner – employment.

Liping Yan: ChemPartner – employment.

Andrew Olaharski: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder. Akebia Therapeutics – employment.

Lee Silverman: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

Scott Biller: Agios Pharmaceuticals, Inc. – employment, stock/shareholder. Syros Pharmaceuticals, Inc. – advisory board.

Shin-San M. Su: Agios Pharmaceuticals, Inc. – prior employment, stock/shareholder, consultancy. Decibel Therapeutics – employment.

Katharine Yen: Agios Pharmaceuticals, Inc. – employment, stock/shareholder.

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