Structure-based design of GNE-495, a potent and selective MAP4K4 inhibitor with efficacy in retinal angiogenesis

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Supporting Information

MAP4K4 Inhibition Biochemical Assay Protocol

The kinase activity of purified human MAP4K4 kinase domain on a peptide substrate (5-FAM-LGRDKYKTLRQIRQ-COOH) was monitored using Z′-LYTE™ technology according to manufacturer's protocol as previously described.[1] In a related assay, the activity was measured by monitoring the phosphorylation of a peptide substrate derived from moesin protein (Leu-Gly-Arg-Asp-Lys-Tyr-Lys-Thr-Leu-Arg-Gln-Ile-Arg-Gln) fluorescently labeled on the N-terminus with 5-carboxyfluorescein using the Caliper LabChip technology (Caliper Life Sciences, Hopkinton, MA). To determine inhibition constants (IC50), compounds were serially diluted in DMSO and added to 10 uL kinase reactions containing 1 nM purified MAP4K4 enzyme, 1 uM peptide substrate, 10 uM ATP, 10 mM $MgCl_2$, 1 mM EGTA, 50 mM Hepes pH 7.2, 1 mM DTT, 0.01% Triton X-100, and 2% DMSO. Reactions were incubated at room temperature in Perkin Elmer Proxiplates for 45 minutes and stopped by the addition of 10 uL of an EDTA-containing solution (50 mM Hepes pH 7.2, 40 mM EDTA, 0.02% Triton X-100). The fraction of phosphorylated peptide was determined as a fraction

of total peptide substrate using the Caliper Lab Chip 3000 (LC3K) according to the manufacturer's instructions. IC_{50} values were determined using the four-parameter non-linear fit model.

HUVEC Incucyte angiogenesis assay

Cellular potency of Map4K4 inhibitors was determined utilizing the Essen 96 Well Angiogenesis Cryokit assay previously described[2] (Essen Biosciences Cat# 4452). GFP expressing human umbilical vein endothelial cells (HUVEC) are co-cultured with normal human dermal fibroblasts. All cell growth and assay procedures are carried out in a cell culture incubator at 37 °C with 5% CO_2 and 80% relative humidity. Following two days of growth, cells are treated with VEGF to stimulate tube formation by the HUVECs. Cells are concurrently treated with controls or Map4K4 inhibitors (10 point dose- response, final [DMSO] = 0.5% for all wells). Following compound treatment, formation of tubes by HUVEC cells is monitored on the Essen Incucyte FLR system (Essen Biosciences, Ann Arbor, MI) for 5 days. Potency of Map4K4 inhibitors is determined by the ability of a compound to inhibit uniform tube formation relative to untreated controls.

Kinase selectivity

Evaluation of the 3 compounds in the Invitrogen® KCS assay at 1 μM concentration against either 76, 244 or 246 representative kinases indicated that we maintained exquisite selectivity for MAP4K4 and it's closely related kinases Mink1 and TNIK, which all have a nearly identical (>90%) sequence homology in their ATP-binding pocket. These compounds also showed lesser % inhibition against other off-targets such as MLK1, GCK and NIK.

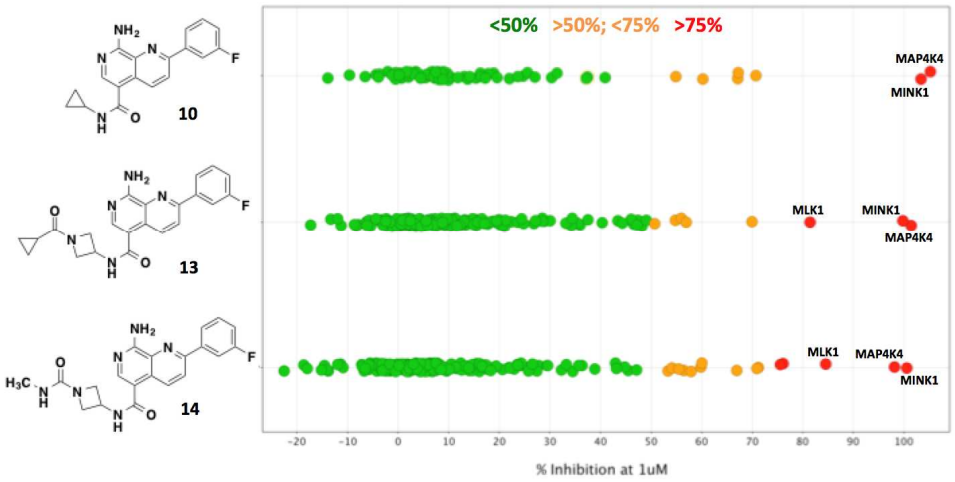


Figure S1. Graphical depiction of kinase selectivity for **10**, **13** and **14** assayed at 1 μM concentration. Each dot represents a single kinase in the panel.

Table S1. Invitrogen® KCS % inhibition for **10**, **13** and **14** at 1 μM test concentration:

Kinases	**10** (# kinases=76)	**13** (# kinases=246)	**14** (# kinases=244)
ACVR1B		2.9	-0.7
AKT1	0.7	0.8	4.0
AKT2		-0.1	-1.0
AKT3		4.4	-14.5
Abl	67.1	47.8	66.3
Arg		19.6	13.3
Aurora_A	70.7	47.6	53.5
Aurora_B		36.3	34.7
Aurora_C		17.7	22.5
Axl		27.3	27.3
B-Raf	1.0	13.9	20.8
BTK		-0.9	9.5
Blk		-0.2	-1.9
Bmx		7.3	10.9
BrSK1		36.7	46.1
Brk	1.2	0.9	1.7
CDK1/cyclinB	3.6	3.2	7.7
CDK2/cyclinA		-1.9	3.2
CDK5/p25		-3.4	-2.5
CDK5/p35		-1.9	-4.6

CDK7/cyclinH		11.2	-6.2
CDK8/cyclinC	-2.6	5.1	-1.0
CDK9/cyclinT1		-5.8	19.1
CHK1	13.2	13.8	9.0
CHK2	6.3	0.2	1.4
CK1_alpha1	7.3	11.1	24.9
CK1_delta		22.1	46.8
CK1_epsilon1		1.0	75.7
CK1_gamma1		3.3	0.5
CK1_gamma2		6.6	3.6
CK1_gamma3		1.7	-6.2
CK2_alpha1	6.5	6.0	4.2
CK2_alpha2		7.0	2.5
CLK1		8.9	0.0
CLK2		-1.5	16.1
CLK3		1.3	2.6
CSF1R	19.1	22.9	24.4
CSK	8.6	9.3	-1.3
CaMKI		8.7	4.2
CaMKII_beta		10.7	-0.4
CaMKI_delta	-3.9	-2.6	9.6
CamKII_alpha		3.4	0.7
CamKII_delta		-0.1	3.2
CamKIV		7.9	3.3
Cot	17.2	14.8	9.1
DAPK1		-0.7	-5.1
DCAMKL2		1.1	3.5
DNA-PK		11.1	4.4
DYRK1A		25.3	27.0
DYRK1B		7.9	5.5
DYRK3		4.3	8.3
DYRK4		0.4	-1.1
EGFR	10.3	2.4	8.3
EGFR(T790M)	3.6	-0.1	-5.0
ERK1		1.8	3.7
ERK2	6.8	-6.4	2.5
EphA1	5.1	5.1	4.1
EphA2	10.8	3.5	-0.9
EphA4		7.7	7.4
EphA5	9.8	0.9	-1.0
EphA8		9.0	10.8
EphB1	8.2	3.3	6.5

EphB2	2.6	6.9	5.0
EphB3	6.0	2.4	-0.4
EphB4	5.6	1.0	-4.8
ErbB2		-3.2	-3.0
ErbB4		-1.4	-3.3
FAK	-3.1	-0.2	9.7
FAK2		2.6	-4.5
FGFR1	-6.8	-10.4	9.8
FGFR2		5.5	-6.7
FGFR3		1.0	-14.1
FGFR4		2.0	-1.0
Fer		1.1	0.3
Fes		2.4	-0.4
Fgr		5.5	17.4
Flt1		3.7	-3.9
Flt3	11.9	21.6	23.7
Flt4		17.4	17.9
Frk		-0.2	7.2
Fyn	7.5	2.8	-0.9
GCK		54.9	**76.2**
GRK2		-3.0	5.0
GRK3		-2.8	0.4
GRK4		-12.6	-12.1
GRK5		0.0	-4.0
GRK6		-0.9	-4.7
GRK7		3.3	4.7
GSK3_alpha		15.2	7.1
GSK3_beta	14.7	14.1	14.7
HIPK1		0.3	-0.9
HIPK2		7.9	8.4
HIPK4		6.6	6.2
Haspin		28.2	13.7
Hck		4.2	10.8
Hyl		0.7	10.8
IGF1R		-7.2	9.9
IKK_alpha		-2.3	0.3
IKK_beta	5.2	5.5	2.2
IKK_epsilon		3.5	1.4
IRAK4		-2.0	8.9
IRR		0.2	3.5
InsR		0.7	-1.4
Itk	0.1	9.1	-3.5

JAK1	-0.8	-4.0	-22.4
JAK2	-8.8	5.0	-18.4
JAK3	2.4	2.1	0.4
JNK1_alpha1		-0.1	4.8
JNK2		12.1	2.0
JNK3		4.9	0.3
KDR	24.1	16.4	16.6
KHS1	37.9	38.2	29.2
Kit		4.9	2.7
LRRK2		18.1	30.1
LTK		8.3	3.5
Lck	31.1	19.9	15.3
Lyn		2.4	8.2
LynB		7.0	-2.4
MAP4K4	**104.7**	**102.0**	**97.4**
MAPKAPK2	0.5	6.0	-2.5
MAPKAPK3		3.3	17.4
MARK1	54.2	35.8	36.0
MARK2		46.9	45.3
MARK3		39.9	54.2
MARK4		49.5	60.3
MEK1	15.9	2.6	21.5
MEK2		14.0	16.8
MELK		12.6	25.9
MKK6		0.0	2.2
MKNK1	31.8	25.0	18.9
MLK1		**81.9**	**84.2**
MLK2		46.0	
MRCK_alpha		-6.4	-0.9
MRCK_beta		-16.8	0.9
MSK1	6.9	10.0	3.9
MSK2		5.3	11.9
MSSK1		-0.5	6.0
MST1		10.9	14.7
MST2		6.9	17.4
MST3		10.8	14.8
MST4		5.6	6.8
MYLK2(skMLCK)	67.7	33.7	56.8
Mer		56.1	59.3
Met	0.9	6.7	7.6
Mink1	**103.3**	**100.6**	**101.2**
MuSK		-0.3	-4.5

NEK1		15.7	7.5
NEK2	1.4	6.5	-14.6
NEK4		4.3	2.4
NEK6		10.0	3.0
NEK7		7.5	2.8
NEK9		2.6	-3.5
NIK	60.7	43.7	55.6
PAK1		0.4	5.2
PAK2		-5.5	6.2
PAK3		7.6	7.7
PAK4	22.6	50.9	43.6
PAK6		14.2	6.9
PAK7		30.6	32.4
PASK		-0.1	5.1
PDGFR_alpha	14.5	12.8	18.3
PDGFR_beta		0.9	2.5
PDK1	7.8	2.2	12.8
PDK1(direct)		-11.8	4.1
PI3K-A		1.6	2.6
PI3K-D		0.9	3.6
PI3K-G		0.5	-2.5
PI3KC2a	-14.3	-8.3	-3.0
PI3KC2b	4.7	9.7	-10.6
PI3KC3_hVPS34	9.1	1.3	2.9
PI4Ka	-0.6	-0.2	-6.8
PI4Kb	16.7	20.6	26.0
PIM1	-3.2	2.2	57.5
PIM2		5.2	6.0
PKA	1.4	2.1	5.5
PKC_alpha	26.1	47.0	26.2
PKC_beta1		20.4	14.3
PKC_beta2		33.9	24.3
PKC_delta		-5.2	-3.0
PKC_epsilon		16.3	-2.7
PKC_eta		9.5	7.9
PKC_gamma		34.5	6.7
PKC_iota		2.8	-3.0
PKC_theta		9.7	3.3
PKC_zeta		3.3	2.3
PKD1		2.0	6.6
PKD2		-1.9	-1.6
PKD3		14.2	8.1

PKG1_alpha		0.6	3.8
PKG2		-2.4	6.2
PLK1	4.7	2.0	1.4
PLK2		1.1	0.7
PLK3		-6.3	17.1
PRAK		4.0	5.5
PRK1		-0.7	0.0
PRKAA1		5.9	12.3
PRKAA2		29.4	38.6
PhK_gamma1	2.7	3.2	3.3
PhK_gamma2		3.4	0.4
PrKX		4.0	4.0
RAF1(Y340D,Y341D)		19.5	14.4
ROCK1	-0.9	0.5	-0.6
ROCK2		-8.0	2.4
Ret		-1.9	14.3
Ron		9.6	15.5
Ros		10.6	4.3
Rse		11.4	7.9
Rsk1	36.8	28.1	32.5
Rsk2		27.8	35.7
Rsk3		57.6	71.5
Rsk4		69.4	71.3
SGK1	41.2	13.3	16.6
SGK2		9.8	5.1
SGK3		0.9	11.8
SIK2		15.3	12.9
SLK		9.6	
SPHK1		-3.7	-14.8
SPHK2		4.4	-5.8
SRPK1		1.9	6.5
SRPK2		8.8	2.0
Src	19.5	11.0	-5.3
Src_N1		7.7	8.8
Srm		-4.8	-12.1
Syk	9.2	4.9	1.3
TAK1-TAB1	26.3	44.4	42.5
TAO1	1.1	3.5	2.6
TBK1		9.3	18.1
TSSK1	14.1	12.1	9.3
TSSK2		3.9	5.1
TYK2	4.0	0.6	4.4

Tie2	9.1	-1.9	11.1
TrkA	23.5	11.6	9.2
TrkB		-1.2	-5.8
TrkC		18.1	9.4
YSK1		3.3	4.0
Yes	16.4	9.0	13.7
ZAP-70	0.6	3.1	3.4
ZIPK		-1.4	0.4
eEF-2K		4.5	4.0
mTOR		-2.7	1.5
p38_alpha	8.1	16.4	3.9
p38_alpha(direct)		1.0	2.2
p38_beta		3.2	8.2
p38_delta		5.8	-17.9
p38_gamma		6.1	9.0
p70S6K		20.3	23.1

Pharmacokinetics

For the brain cassette study, three male Sprague-Dawley (SD) rats were dosed with intravenous (IV) bolus of six test compounds (0.5 mg/kg in 10%/60%/30% DMSO/PEG400/sterile water). For the mouse PK study, female CD-1® mice from Taconic were administered IV bolus doses of GNE-495 (**13**) (1 mg/kg in 40% PEG400/60% 50 mM Citrate pH 5 buffer or 10% DMSO/35% PEG400/55% water, respectively). In addition, female CD-1® mice from Taconic were administered **13** (5 mg/kg in 0.5% (w/v) methylcellulose/0.2% (w/v) Tween 80 in sterile water (MCT)) via oral (PO) gavage. A dosing volume of 2 ml/kg was used for the rat brain cassette PK and 5 mL/kg was used for all other dosing. Animals were not fasted prior to dose administration, and water and food were available ad libitum. Following administration of the compound of interest, three blood samples (~60 μL) were collected at each time point from individual mice up to either 9 or 24 hours post-dose using a serial sampling approach. Immediately upon collection, the blood was mixed with K_2EDTA and stored on ice or in a chilled Kryorack prior to centrifugation to obtain plasma. Within 1 hr of collection, blood samples were centrifuged at

approximately 1000-2000 × g for 10–15 min at 4 °C, and plasma was harvested. The plasma samples were stored at –70 to –80°C until analysis. For neonate PK, 3-day old CD1 pups were injected with 25 mg/kg and 50 mg/kg GNE-495 intraperitoneally, blood samples were collected at the time points indicated in Figure S2A, retinas were collected one hour post-dose and snap frozen in liquid nitrogen and stored at –80°C until analysis. Plasma and retinal lysate concentrations were determined by LC/MS/MS.

Sample Analysis

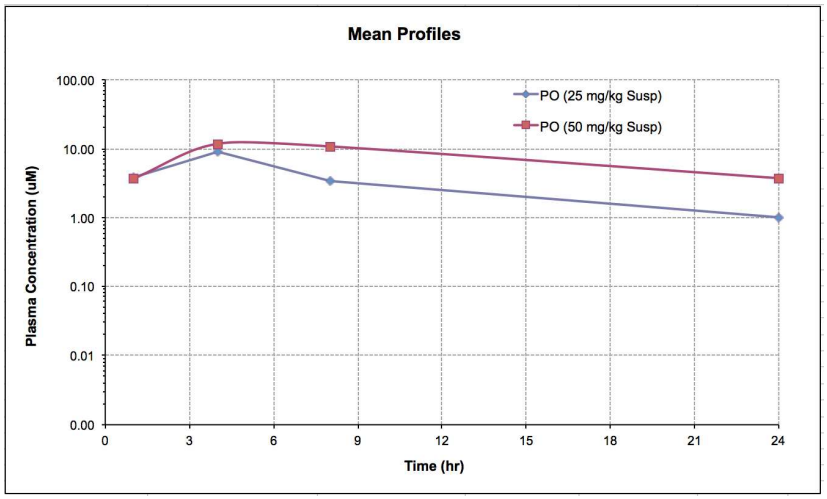
Concentrations of all test compounds in plasma, brain, cerebral spinal fluid (CSF) or retinal lysates were determined using a LC/MS/MS assay. For all samples, 10 µL of internal standard (IS) in 50% DMSO/50% water was added to a 25 µL aliquot of plasma from each sample placed in a 96-well plate. Following this, 200 µL of acetonitrile was added to precipitate plasma or retinal proteins and then the entire plate containing sample aliquots was centrifuged and supernatant was harvested for analysis. A 2.5 to 10 µL aliquot of the supernatant was injected onto a HPLC column. Compounds were run on an ACE 5 phenyl column (100 × 2.1 mm) using a gradient pump program beginning at 20% acetonitrile containing 0.1% formic acid (FA) and 80% aqueous containing 0.1% FA with a flow rate of 0.75 mL/min for 5 minutes. The acetonitrile with FA increased linearly from 20 to 80% from 0.5 to 2 minutes and then was maintained at 80% from 2 to 3.5 minutes. The system returned to the initial conditions in a single step and was allowed to equilibrate for 1.4 minutes. The lower limit of quantitation for plasma was 0.005 µM.

Pharmacokinetic Analysis

PK parameters were determined by non-compartmental methods using WinNonlin, Version 5.2 or 6.3 (Mountain View, CA). The area under the plasma concentration-time curve from t=0 extrapolated to infinity ($AUC_{0-\infty}$) or from t=0 to the last

measurable time point (AUC_{last}) was assessed by log linear trapezoidal approximation. The highest plasma concentration achieved (C_{max}) and T_{max}, time when C_{max} is observed was directly derived from the experimental data. CL, volume of distribution at steady state (V_{ss}) and terminal or elimination half-life ($t_{½}$) were determined as appropriate.

A



B

GNE-495 concentration in Retina (µM)					
GNE-495 dose	Animal1	Animal2	Animal3	Mean	SD
25 mg/kg	0.480	0.350	0.690	0.507	0.172
50 mg/kg	0.600	0.530	0.410	0.513	0.0961

Figure S2. A. Plasma concentration–time curve for **13** (GNE-495) administered intraperitoneally (IP) into P3 neonatal mice at either 25 mg/kg and 50 mg/kg dose. B. Retinal lysate concentration for GNE-495 administered intraperitoneally (IP) into P3 neotnates at either 25 mg/kg and 50 mg/kg.

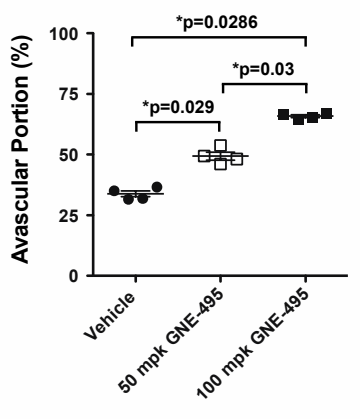


Figure S3. Dose-dependent effect of GNE-495 on neonatal retinal development. Neonatal pups were IP-injected with 50 or 100 mg/kg (mpk) GNE-495 respetively from P1-P5 and their retinas were harvested and analyzed at P6. Quantification of avascular portion is desribed in Figure 4.

Neonatal retinal angiogenesis model

To determine the doses for *in vivo* function analyses, we compared the retinal lysate concentrations (Figure S2B) with the *in vitro* endothelial cell functional assays reported in the Extended Data Figure 8e-g in Vitorino et al[2]. Since the retinal lysate concentration at 50 mg/kg (~ 0.5 μM) is below the concentration required to achieve maximal *in vitro* activity (~ 1 μM), we decided to evaluate two dose levels: 50 mg/kg and 100 mg/kg via daily IP injection.

For vascular coverage analysis, newly born C57BL/6 mice were injected peritoneally with vehicle (MCT), 50 mg/kg, or 100 mg/kg GNE-495 everyday from postnatal day 1 to day 5, and euthanized at day 6. For long membrane protrusion analysis, newly born C57BL/6 mice were injected peritoneally with vehicle (MCT) or 100 mg/kg GNE-495 everyday from postnatal day1 to day 6 and euthanized at day 7. Analysis and quantification of the retinal vasculature were performed as previously described.[3]

Chemistry: General Methods. All solvents and reagents were used as obtained. Reactions involving air or moisture sensitive reagents were carried out under nitrogen atmosphere. Microwave reactions were performed using CEM Discover and Biotage

Initiator reactors. NMR spectra were recorded in a deuterated solvent with a Bruker Avance 300- or 400-MHz NMR spectrometer, and referenced to trimethylsilane (TMS). Chemical shifts are expressed as δ units using TMS as the external standard (in NMR description, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad peak). All coupling constants (*J*) are reported in Hertz. Mass spectra were measured with a Finnigan SSQ710C spectrometer using an ESI source coupled to a Waters 600MS HPLC system operating in reverse mode with an X-bridge Phenyl column of dimensions 150 mm by 2.6 mm, with a 5 μm sized particles. Analytical purity was >95% unless stated otherwise. The following analytical method was used to determine chemical purity of final compounds unless otherwise stated: HPLC-Agilent 1200, water with 0.05% TFA, acetonitrile with 0.05% TFA, Agilent Zorbax SD-C18, 1.8 μM, 2.1x30 mm, 40 ºC, 3-95% B in 8.5 min, 95% in 2.5 min, 400 μL/min, 220 nm and 254 nm, equipped with Agilent quadrupole 6140, ESI positive, 110-800 amu. Preparative HPLC (prep-HPLC) were performed on a Varian Prostar instrument, using a Phenomenex Gemini-NX C-18 (0.3 cm × 5 cm; 5 μm particle size) stationary phase, with 0.1% aqueous formic acid/acetonitrile (for acidic or neutral compounds) or 0.1% aqueous ammonium hydroxide/acetonitrile (for basic compounds) gradients as the mobile phase (typically 5–85% acetonitrile over 10 min) with a flow rate of 60 mL/min.

Table 1 compounds:

7-Bromo-1-chloroisoquinoline. A mixture of 7-bromoisoquinolin-1-ol (**15**) (20 g, 89.3 mmol) in $POCl_3$ (400 g, 2.61 mol) was stirred at 100 °C for 3 h. $POCl_3$ was removed under reduced pressure. The residue was quenched with ice-water, neutralized with a Na_2CO_3 solution and extracted with DCM. The combined organic layers was dried over Na_2SO_4 and concentrated in vacuum to give the crude product (17.3 g, 80.1%).

LCMS (0-60AB, 2 min.): RT = 1.253 min, $M+H^+$ = 243.8

7-Bromoisoquinolin-1-amine (16). A solution of 7-Bromo-1-chloroisoquinoline (17.3 g, 71.3 mmol) in a mixture of aq. conc. ammonia/NMP (250 mL/250 mL) was stirred in a sealed container at 148 ˚C for 20 h. The mixture was suspended in brine and extracted with EtOAc (100 mL × 3). The combined organic layers was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography to give the title product (14.1 g, 88.7%).

LCMS (0-60AB, 2 min): RT = 0.912 min, $M+H^+$ = 224.6

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.48 (s, 1H), 7.83 (d, J = 5.6 Hz, 1H), 7.74-7.72 (m, 1H), 7.66 (d, J = 8.8 Hz, 1H), 6.92-6.89 (m, 3H).

7-(3-Fluorophenyl)isoquinolin-1-amine. To a solution of compound **16** (8.0 g, 35.9 mmol) in dioxane/H_2O (125 mL/25 mL) was added Pd(dppf)Cl_2 (4.0 g, 5.5 mmol) and $NaCO_3$ (7.6 g, 71.7 mmol). 3-Fluorophenylboronic acid (10.0 g, 71.5 mmol) was added and the mixture was stirred at reflux for 15 h under N_2. The mixture was partitioned between DCM and H_2O, and the combined organic layers was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography to give the title compound (8.5 g, 98.8%).

LCMS (0-60AB, 2 min): RT = 1.080 min, $M+H^+$ = 238.9.

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.56 (s, 1H), 7.99 (m, 1H), 7.81-7.71 (m, 4H), 7.57-7.51 (m, 1H), 7.24-7.19 (m, 1H), 6.95-6.91 (m, 3H).

4-Bromo-7-(3-fluorophenyl)isoquinolin-1-amine (17). To a solution of 7-(3-fluorophenyl)isoquinolin-1-amine (4.0 g, 16.8 mmol) in anhydrous DMF (60 mL) was added NBS (3.6 g, 20.2 mmol) in anhydrous DMF (40 mL) portion-wise at 0 ˚C. After the mixture was stirred at 0˚C for 3 h, it was quenched with saturated Na_2SO_3 solution and partitioned between EtOAc and H_2O. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by column chromatography to give the title compound (2.6 g, 49.0%).

LCMS (0-60AB, 2 min): RT = 1.152 min, $M+H^+$ = 318.9.

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.96 (s, 1H), 8.45 (m, 1H), 8.08 (t, J = 8.8 Hz, 2H), 7.77-7.74 (m, 2H), 7.58 (dd, J = 14.4 Hz, 8.0, 1H), 7.29-7.25 (m, 1H).

Methyl 1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxylate. To a mixture of compound **17** (5.4 g, 17.0 mmol) in MeOH (60 mL) was added Pd(dppf)Cl_2 (1.3 g, 1.8 mmol) and DMAP (4.1 g, 33.6 mmol). After the mixture was stirred under CO (50 psi) at 70 ˚C overnight, it was filtrated, concentrated in vacuum. The residue was purified by column chromatography to give the title compound (2.2 g, 43.7%).
LCMS (0-60AB, 2 min): RT = 1.152 min, M+H^+ = 296.9.

Methyl 1-(*tert*-butoxycarbonylamino)-7-(3-fluorophenyl)isoquinoline-4-carboxylate. To a solution of methyl 1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxylate (2.4 g, 8.1 mmol) in anhydrous DCM (60 mL) was added Et_3N (2.3 mL, 16.5 mmol), DMAP (0.1 g, 0.8 mmol), and $(Boc)_2O$ (3.5 mL, 16.4 mmol) (slowly). After the mixture was stirred at room temperature for 2 days, it was concentrated in vacuum and purified by column chromatography to give the title compound (2.0 g, 62.5%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 (s, 2H), 8.17 (s, 1H), 8.07 (d, J = 8.8 Hz, 1H), 7.49-7.45 (m, 2H), 7.39-7.35 (m, 1H), 7.16-7.12 (m, 1H), 4.07 (s, 3H), 1.32 (s, 9H).

1-(*tert*-Butoxycarbonylamino)-7-(3-fluorophenyl)isoquinoline-4-carboxylic acid. To a solution of methyl 1-(*tert*-butoxycarbonylamino)-7-(3-fluorophenyl)isoquinoline-4-carboxylate (1.0 g, 2.5 mmol) in a mixture of MeOH, H_2O, and THF (15 mL, 5 mL, and 20 mL) was added NaOH (0.30 g, 7.5 mmol). After the mixture was stirred at room temperature overnight, it was concentrated in vacuum, and the residue was partitioned between EtOAc and H_2O. The water layer was neutralized with a diluted HCl solution, extracted with EtOAc. The EtOAc layer was dried and concentrated in vacuum to give the title compound (0.50 g, 52.3%).

1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxamide (3). To a solution of 1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxylic acid (5.0 g, 0.017 mol) in DMF (50 mL), was added DIPEA (11 g, 0.085 mol), HATU (8.4 g, 0.022 mol), and NH_4Cl (3.6 g, 0.068 mol). After the mixture was stirred at 40–50 ºC for 4 h, it was poured into water (500 mL) and the layers were partitioned. The aqueous layer was extracted with DCM twice. The combined organic layers were washed with brine and subsequently dried with $MgSO_4$. The mixture was filtered and the filtrate concentrated to give the desired product (4.0 g, yield 80%).
HRMS (ESI) found for $C_{16}H_{13}ON_3F$ ($M+H^+$): 281.10

1-amino-*N*-(cyanomethyl)-7-(3-fluorophenyl)isoquinoline-4-carboxamide (4).
This compound was prepared in a similar fashion to the one above.
LCMS: (10-80, AB, 2 min), 1.065 min, $M+1^+$ =321.1
^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.21 (bs, 1H), 8.82 (s, 1H), 8.66 (bs, 1H), 8.47 (d, J = 8.8 Hz, 1H), 8.31 (d, J = 8.0 Hz, 1H), 8.04 (s, 1H), 7.79-7.75 (m, 2H), 7.61-7.56 (m, 1H), 7.30-7.25 (m, 1H), 4.34 (d, J = 5.6 Hz, 2H).

1-amino-*N*-cyclopropyl-7-(3-fluorophenyl)isoquinoline-4-carboxamide (5). This compound was prepared in a similar fashion to the one above.
LCMS: (10-80, AB, 2 min), 1.164 min, $M+1^+$ =322.1
^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.95 (s, 1H), 8.68 (d, J = 4.4 Hz, 1H), 8.43-8.38 (m, 2H), 7.86 (s, 1H), 7.81-7.77 (m, 2H), 7.63-7.57 (m, 1H), 7.32-7.27 (m, 1H), 2.90-2.84 (m, 1H), 0.75-0.70 (m, 2H), 0.60-0.56 (m, 2H).

***tert*-butyl (7-(3-fluorophenyl)-4-((3-hydroxycyclobutyl)carbamoyl)isoquinolin-1-yl)carbamate.** To a solution of 1-((*tert*-butoxycarbonyl)amino)-7-(3-fluorophenyl)isoquinoline-4-carboxylic acid (200 mg, 0.50 mmol) in THF (10 mL), was added DIPEA (528.9 mg, 2.6 mmol), HATU (290 mg, 0.75 mmol), and N-3-aminocyclobutan-1-one (140 mg, 1.0 mmol). The mixture was stirred at room temperature overnight and poured into water (20 mL). The resulting mixture was

extracted with EtOAc (30 mL×2), and concentrated to give the title compound (250 mg, crude).

***tert*-butyl (7-(3-fluorophenyl)-4-((3-hydroxycyclobutyl)carbamoyl)isoquinolin-1-yl)carbamate.** To a solution of 1-amino-7-(3-fluorophenyl)-*N*-(3-oxocyclobutyl)isoquinoline-4-carboxamide (250 mg, 0.5 mmol) in MeOH (10 mL), was added $NaBH_4$ (77.8 mg, 2.0 mmol). The mixture was stirred at room temperature for 1 h, and poured into water (30 mL). The mixture was extracted with EtOAc (30 mL×2), and washed with NaCl (20 mL), dried over Na_2SO_4, and concentrated to give the crude product (200 mg).

1-amino-7-(3-fluorophenyl)-*N*-(3-hydroxycyclobutyl)isoquinoline-4-carboxamide (6). To a solution of *tert*-butyl (7-(3-fluorophenyl)-4-((3-hydroxycyclobutyl)carbamoyl)isoquinolin-1-yl)carbamate (200 mg, crude) in THF (10 mL), was added conc. HCl (2.0 mL). After it was stirred at room temperature for 5 h, organic solvent was removed and the residue was poured into water (20 mL), and pH was adjusted to 8-9 with sat $NaHCO_3$. It was extracted with EtOAc (20 mL×3). The combined organic layers was washed with sat NaCl (30 mL), dried over Na_2SO_4, concentrated, and purified by prep-HPLC to give the title compound.
LCMS: (0-60, AB, 2 min), 1.059 min, $M+1^+ = 351.9$
^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.64 (s, 1H), 8.51 (d, J = 7.2 Hz, 1H), 8.40 (d, J = 9.2 Hz, 1H), 8.11-8.04 (m, 2H), 7.77-7.72 (m, 4H), 7.57-7.54 (m, 1H), 7.25-7.21 (m, 1H), 5.08 (bs, 1H), 3.93-3.82 (m, 2H), 2.57-2.55 (m, 2H), 1.91-1.84 (m, 2H).

***tert*-Butyl 3-(1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxamido)azetidine-1-carboxylate**. To a solution of 1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxylic acid (1.0 g, 3.5 mmol) in DMF (40 mL), was added HATU (1.6 g, 4.2 mmol), DIPEA (1.4 g, 10.5 mmol), and *tert*-butyl 3-aminoazetidine-1-carboxylate (790 mg, 4.6 mmol). After the mixture was stirred at 40-50^{o}C for 3 h, it was cooled to room temperature and water (100 mL) was added. The mixture was extracted with EtOAc

(100 mL×2) and washed with H_2O (100 mL), sat. NaCl (50 mL), concentrated and purified by column chromatography (PE: EtOAc = 1:3) to give the *tert*-butyl 3-(1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxamido)azetidine-1-carboxylate as a yellow solid (1.0 g, yield 67 %).

1-amino-*N*-(azetidin-3-yl)-7-(3-fluorophenyl)isoquinoline-4-carboxamide (7). To a solution of *tert*-butyl 3-(1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxamido)azetidine-1-carboxylate (1.0 g, 2.3 mmol) in DCM (30 mL), was added EtOAc/HCl (5 mL), and the mixture was stirred at room temperature for 2 h. The mixture was concentrated to give the title compound (800 mg).
LCMS: (0-60, AB, 2 min), 0.959 min, $M+1^+$ = 337.1
^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.92 (d, *J* = 6.8 Hz, 1H), 8.60 (s, 1H), 8.49 (d, *J* = 8.8 Hz, 1H), 8.22-8.17 (m, 3H), 8.07 (dd, *J* = 8.8 1.6 Hz, 1H), 7.76-7.72 (m, 2H), 7.57-7.52 (m, 3H), 7.25-7.20 (m, 1H), 4.83-4.77 (m, 1H), 4.07-4.02 (m, 2H), 43.97-3.93(m, 2H).

***N*-(1-acetylazetidin-3-yl)-1-amino-7-(3-fluorophenyl)isoquinoline-4-carboxamide (8)**. To a solution of 1-amino-*N*-(azetidin-3-yl)-7-(3-fluorophenyl)isoquinoline-4-carboxamide (**7**) (80 mg, 0.24 mmol) in THF (10 mL), was added DIPEA (180 mg, 1.5 mmol), HATU (136.8 mg, 0.36 mmol), and acetic acid (0.36 mmol, 1.5 eq). After the mixture was stirred at r.t for 3 h, it was poured into water (20 mL), extracted with EtOAc (30 mL×2), concentrated and purified by prep HPLC to give the product.
LCMS: (0-60, AB, 2 min), 1.058 min, $M+1^+$ = 378.9
^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.59 (s, 1H), 8.45 (d, *J* = 8.8 Hz, 1H), 8.14 (dd, *J* = 8.8 2.0 Hz, 1H), 8.09 (s, 1H), 7.68-7.52 (m, 3H), 7.20-7.15 (m, 1H), 4.85-4.84 (m, 1H), 4.63 (t, *J* = 8.8 Hz, 1H), 4.40 (t, *J* = 8.8 Hz, 1H), 4.27-4.23 (m, 1H), 4.06-4.02 (m, 1H), 1.93 (s, 3H).

1-amino-7-(3-fluorophenyl)-*N*-(1-(methylcarbamoyl)azetidin-3-yl)isoquinoline-4-carboxamide (9). To a solution of 1-amino-*N*-(azetidin-3-yl)-7-(3-

fluorophenyl)isoquinoline-4-carboxamide (**7**) (150 mg, 0.47 mmol) in THF (10 mL), was added DIPEA (290 mg, 2.2 mmol), CDI (100 mg, 0.61 mmol). After the mixture was stirred at room temperature for 1 h, methylamine (150 mg, 47 mmol) was added, and the mixture was stirred for 2 h, and purified by (basic) prep-HPLC to give the title compound.

LCMS: (0-60, AB, 2 min), 1.041 min, $M+1^+ = 393.9$

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.84 (d, J = 7.2 Hz, 1H), 8.61 (s, 1H), 8.47 (d, J = 8.8 Hz, 1H), 8.18 (s, 2H), 8.08 (dd, J = 8.8 2.0 Hz, 1H), 7.78-7.74 (m, 2H), 7.59-7.53 (m, 1H), 7.47 (bs, 2H), 7.26-7.21 (m, 1H), 6.28-6.27 (m, 1H), 4.66-4.64 (m, 1H), 4.08-4.06 (m, 2H), 3.82-3.79 (m, 2H), 2.55 (d, J = 4.8 Hz, 3H).

Table 2 compounds:

5-Fluoro-2-(3-fluorophenyl)pyridine. After a mixture of 2-bromo-5-fluoropyridine (50 g, 0.284 mol), 3-fluorophenylboronic acid (48 g, 0.343 mol), Pd(dppf)Cl_2 (5.0 g, 6.8 mmol,) and K_2CO_3 (178.5 g, 0.568 mol) in dioxane/H_2O (500 mL /150 mL) was degassed 3 times, the mixture was heated to 80–100 $^{\circ}$C for 3 h under N_2. The mixture was filtered through diatomite, and dioxane was removed under reduced pressure. EtOAc (1.0 L) was added, and the organic phase separated, concentrated, and purified by column chromatography (20:1~10:1 Petroleum: EtOAc) to give desired product (50 g, 92%). ^{1}H NMR (400MHz, $CDCl_3$) δ 8.54 (s, 1H), 7.65-7.72 (m, 3H), 7.39-7.49 (m, 2H), 7.08 (m, 1H)

5-Fluoro-2-(3-fluorophenyl)pyridine 1-oxide. 5-Fluoro-2-(3-fluorophenyl)pyridine (50 g, 0.262 mol), *m*-CPBA (106 g, 0.523 mol, 2 equiv.) in DCM was heated at reflux for 16 h. After most of the starting material was consumed (as indicated by TLC), the mixture was cooled to room temperature and a $Na_2S_2O_3$ solution was added slowly, until there was no peroxide exist (KI starch paper). (*Caution: peroxide may be explosive.*). DCM was removed and the yellow precipitate was collected and washed with saturated $NaHCO_3$ solution, until 3-Chloro-benzoic acid was completely

removed. The crude compound was dried and was used in next step without further purification (45 g, 83%).

^{1}H NMR (400MHz, $CDCl_3$) δ 8.23-8.25 (m, 1H), 7.53 (m, 1H), 7.36-7.49 (m, 3H), 7.09-7.16 (m, 2H).

3-Fluoro-6-(3-fluorophenyl)picolinonitrile. To a solution of 5-Fluoro-2-(3-fluorophenyl)pyridine 1-oxide (38 g, 0.183 mol) in acetonitrile (400 mL) was added TMSCN (73 g, 0.734 mol) and Et_3N (93 g, 0.917 mol). After the mixture was heated at reflux for 12 h under N_2, it was concentrated and purified by column chromatography (20% EtOAc in petroleum ether) to give the title compound (20 g, 50 %).

^{1}H NMR (DMSO-d_6 400MHz) δ 8.44 - 8.47 (m, 1H), 8.19-8.21 (m, 1H), 7.83-7.91 (m, 2H), 7.54-7.56 (m, 1H), 7.29 - 7.34 (m, 1H).

Diethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)malonate. To a stirred solution of NaH (11.15 g, 278.81 mmol, 60%) in THF (100 mL) was added diethyl malonate (44.66 g, 278.81 mmol) in THF (50 mL) dropwise at 0 ^{o}C under N_2. After the reaction mixture was stirred at 0 ^{o}C for 10 min, 3-Fluoro-6-(3-fluorophenyl)picolinonitrile (30 g, 138.8 mmol) in THF (100 mL) was added. After the reaction mixture was heated at reflux for 4 h under N_2, it was extracted with EtOAc (500 mLx2), washed with sat NaCl (300 mL), dried over Na_2SO_4, and concentrated to give the crude product, which was used for the next directly (50 g, 100%).

Ethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)acetate. To a solution of diethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)malonate (50 g, 140.31 mmol) in DMSO (300 mL), was added H_2O (6 mL), LiCl (23.32 g, 550.02 mmol) and the mixture was stirred at 100 ^{o}C overnight. After cooling to room temperature, it was extracted with EtOAc (300 mL×3), washed with sat NaCl (300 mL), dried over Na_2SO_4, concentrated and purified by column (PE:EtOAc = 8:1) to give the product as white solid (20 g, 50% over two steps).

^{1}H NMR (400MHz, DMSO-*d6*) δ 8.33 (d, J = 8.4 Hz, 1H), 8.11 (d, J = 8.4 Hz, 1H), 7.96-7.87 (m, 2 H), 7.59-7.54 (m, 1H), 736-7.31 (m, 1H), 4.16-4.10 (m, 2H), 4.02 (s, 2H), 1.19 (t, J = 7.0 Hz, 3H).

Ethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)-3-(dimethylamino)acrylate. A solution of ethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)acetate (50 g, 0.176 mol) and DMF-DMA (168 g, 1.41 mol) in DMF (200 mL) was heated at 80 °C overnight. The mixture was concentrated and purified by column chromatography to give the product (45 g, 75%).

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.24 (d, J = 8.4 Hz, 2H), 7.96 (d, J = 8.0 Hz, 2H), 7.92 (m, 1H), 7.88 (d, J = 8.4 Hz, 1H), 7.72 (s, 1H), 7.53-7.57 (m, 1H), 7.29-7.34 (m, 1H), 3.97-4.07 (m, 2H), 2.70-2.74 (s, 6H), 1.11 (t, 3H).

Ethyl 8-amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylate. A mixture of ethyl 2-(2-cyano-6-(3-fluorophenyl)pyridin-3-yl)-3-(dimethylamino)acrylate (6.5 g, 19.15 mmol) and NH_4OAc (37 g, 478.84 mmol) in HOAc (60 mL) was heated at 80-100 ℃ overnight. The mixture was cooled to room temperature and poured into ice-water. The precipitate was collected and washed with EtOH (30 mL) to give the desired product, which was used directly in the next step (4.0 g, 67%).

^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.21 (d, J = 8.8 Hz, 1H), 8.63 (s, 1H), 8.44 (d, J = 9.2 Hz, 1H), 8.43 – 8.40 (m, 1H), 8.23 – 8.21 (m, 2H), 8.04 – 8.00 (br, 1H), 7.57 – 7.53 (m, 1H), 7.32 – 7.29 (m, 1H), 4.32 – 4.27 (q, 2H), 1.34 – 1.31 (t, 3H).

8-Amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylic acid. To a solution of ethyl 8-amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylate (6.5 g, 20.88 mmol) in THF/MeOH/H_2O (10:2:1) (300 mL) was added NaOH (3.34 g, 83.5 mmol) at room temperature After the solution was stirred for 2 h at 65 °C, organic solvent was removed under reduced pressure and H_2O (50 mL) was added. The pH was

adjusted to 8.0 and the precipitate was collected by filtration and dried to give the desired product (5.9 g, 100%).

8-amino-N-cyclopropyl-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide (10). To an 8 mL screw-cap vial was added 8-amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylic acid (230 mg, 0.81 mmol), followed by dimethylformamide (3 mL), HATU (1.5 equiv., 1.22 mmol, 468 mg), triethylamine (4 equiv., 3.25 mmol, 0.46 mL), and cyclopropanamine (2 equiv., 1.6 mmol, 93 mg). The reaction was capped and shaken at room temperature for 3 h. The reaction was then diluted with 5 mL ethyl acetate, and 3 mL water. The resulting precipitate was collected by filtration, and washed with ethyl acetate, yielding 150 mg of 8-amino-N-cyclopropyl-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide. ^{1}H NMR (400 MHz, DMSO) δ 9.00 – 8.92 (d, J = 9.0 Hz, 1H), 8.42 – 8.36 (d, J = 9.1 Hz, 2H), 8.36 – 8.32 (d, J = 3.9 Hz, 1H), 8.25 – 8.21 (d, J = 7.7 Hz, 1H), 8.19 – 8.16 (s, 1H), 7.71 – 7.53 (m, 3H), 7.36 – 7.29 (t, J = 8.4 Hz, 1H), 2.92 – 2.83 (m, 1H), 0.74 – 0.67 (m, 2H), 0.61 – 0.56 (m, 2H). LCMS m/z (M+H) = 323.

8-amino-2-(3-fluorophenyl)-*N*-((1*s*,3*s*)-3-hydroxycyclobutyl)-1,7-naphthyridine-5-carboxamide (11). This compound was prepared in a similar vein to **10** but instead replacing cyclopropanamine with 3-amino-cyclobutanol hydrochloride. The *cis*/*trans* isomers were separated by prep-HPLC.
^{1}H NMR (400 MHz, DMSO) δ 8.95 – 8.90 (d, J = 9.0 Hz, 1H), 8.50 – 8.46 (d, J = 7.3 Hz, 1H), 8.42 – 8.36 (m, 2H), 8.25 – 8.21 (m, 2H), 7.73 – 7.53 (m, 3H), 7.35 – 7.29 (dd, J = 9.7, 7.3 Hz, 1H), 5.09 – 5.05 (d, J = 5.6 Hz, 1H), 3.98 – 3.80 (m, 2H), 2.64 – 2.54 (m, 2H), 1.97 – 1.84 (m, 2H). LCMS m/z (M+H) = 353.

8-amino-*N*-(azetidin-3-yl)-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide. To a solution of 8-amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylic acid (300 mg, 1.06 mmol) and *tert*-butyl 3-aminoazetidine-1-carboxylate (218 mg, 1.27 mmol) in DMF (20 mL) was added HATU (600 mg, 1.6 mmol) followed by DIPEA (270 mg, 2.1 mmol). After the mixture was stirred for 3 h at room temperature, water

(30 mL) was added, and the precipitate was collected to give the crude product (378 mg 85%). To a solution of this compound (378 mg, 0.86 mmol) in DCM (12 mL) was added TFA (3 mL). After the mixture was stirred overnight, it was concentrated to give the crude product, which was used for the next step without purification (400 mg). LC/MS (ESI+): m/z 338.4 (M+H).

8-amino-2-(3-fluorophenyl)-*N*-(1-propionylazetidin-3-yl)-1,7-naphthyridine-5-carboxamide (12). Propionic acid (0.014 mL, 0.18mmol) in DMF (1 mL) was treated with HATU (70 mg, 0.18 mmol) and DIPEA (0.13 mL, 0.74 mmol). The mixture was stirred at room temperature for 15 minutes. 8-amino-N-(azetidin-3-yl)-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide (50 mg, 0.15 mmol) was added, continue stirred for 30 minutes until the reaction went to completion. The reaction mixture was diluted with EtOAc, washed with 10% citric acid followed by brine. The organic layer was dried and concentrated. The crude was purified by prep-HPLC to give the title compound (**12**).

LC/MS (ESI+): m/z 394.4 (M+H).

^{1}H NMR (400 MHz, DMSO) δ 9.03 – 8.85 (m, 2H), 8.47 – 8.36 (m, 2H), 8.36 – 8.29 (s, 1H), 8.28 – 8.18 (d, *J* = 7.8 Hz, 1H), 8.00 – 7.64 (s, 2H), 7.62 – 7.51 (q, *J* = 7.4 Hz, 1H), 7.43 – 7.24 (dd, *J* = 9.5, 7.2 Hz, 1H), 4.79 – 4.62 (q, *J* = 6.8 Hz, 1H), 4.49 – 4.35 (t, *J* = 8.3 Hz, 1H), 4.23 – 4.12 (t, *J* = 8.9 Hz, 1H), 4.12 – 4.03 (dd, *J* = 8.6, 5.3 Hz, 1H), 3.94 – 3.79 (dd, *J* = 9.8, 5.4 Hz, 1H), 2.19 – 1.96 (q, *J* = 7.5 Hz, 2H), 1.07 – 0.88 (t, *J* = 7.5 Hz, 3H).

8-Amino-*N*-(1-(cyclopropanecarbonyl)azetidin-3-yl)-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide (**13**, GNE-495).

Step1: Cyclopropanecarboxylic acid (0.64 mL, 8 mmol) in DMF (1 mL) was treated with HATU (2.4 g, 6.4 mmol) and DIPEA (2.8 mL, 16 mmol). The mixture was stirred at room temperature 10 minutes, and *t*-butyl N-(azetidin-3-yl)carbamate (0.92 g, 5.3 mmol) was added, and the reaction mixture was stirred overnight. TLC showed the reaction completed (stained by ninhydrin spray solution). The reaction mixture

was diluted with EtOAc, washed with 5% citric acid, followed by 10% $NaHCO_3$ and sat. brine. The organic layers were combined and dried, and concentrated to dryness. The crude was used in the deprotection without further purification.

^{1}H NMR (400 MHz, DMSO) δ 4.48 – 4.37 (t, *J* = 8.2 Hz, 1H), 4.37 – 4.22 (t, *J* = 6.7 Hz, 1H), 4.09 – 3.95 (dd, *J* = 8.4, 5.9 Hz, 2H), 3.75 – 3.57 (dd, *J* = 9.7, 5.6 Hz, 1H), 1.54 – 1.45 (ddd, *J* = 7.4, 4.8, 2.7 Hz, 1H), 1.44 – 1.33 (s, 8H), 0.75 – 0.58 (m, 4H).

Step 2: *tert*-butyl N-[1-(cyclopropanecarbonyl)azetidin-3-yl]carbamate (5.1 g, 21 mmol) was treated with trifluoroacetic acid (15 mL, 194 mmol) and DCM (15 mL). The reaction was stirred at room temperature overnight. Concentrated to dryness and purified by flash column chromatography with 10% MeOH/DCM to give (3-aminoazetidin-1-yl)-cyclopropyl-methanone as a white solid (2.7 g, 91%). The product was monitored with TLC using ninhydrin stain.

^{1}H NMR (400 MHz, DMSO) δ 4.58 – 4.36 (t, *J* = 8.2 Hz, 1H), 4.24 – 4.13 (dd, *J* = 9.5, 3.7 Hz, 1H), 4.13 – 3.96 (m, 2H), 3.90 – 3.71 (dd, *J* = 9.4, 3.3 Hz, 1H), 1.63 – 1.42 (m, 1H), 0.80 – 0.56 (m, 4H).

Step 3: 8-amino-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxylic acid (0.8 g, 3 mmol) in DMF (5 mL) was treated with HATU (1 g, 3 mmol) and DIPEA (1.2 mL, 8 mmol). The mixture was stirred at room temperature for 15 minutes, and (3-aminoazetidin-1-yl)-cyclopropyl-methanone (1.5 equiv., 4 mmol) was added. The reaction mixture was stirred at room temperature 1 hour until the reaction was completed. The reaction mixture was diluted with EtOAc, washed with 5% citric acid, followed by 10% $NaHCO_3$ and sat. brine solution. The organic layers were combined and dried, and concentrated to dryness. The crude was purified with FCC using 10% MeOH/EtOAc, and followed by trituration with EtOAc to obtain pure product as pale yellow solid.

LC/MS (ESI+): m/z 406.4 (M+H).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.04 – 8.86 (m, 2H), 7.63 – 7.50 (m, 1H), 8.43 (d, *J* = 9.2 Hz, 2H), 8.34 (s, 1H), 8.25 (d, *J* = 8.1 Hz, 1H), 7.90 – 7.48 (m, 3H), 7.28 (td, *J*

= 8.5, 2.6 Hz, 1H), 4.86 – 4.68 (m, 1H), 4.55 (t, *J* = 8.2 Hz, 1H), 4.24 (dd, *J* = 8.6, 5.3 Hz, 1H), 4.16 (t, *J* = 8.9 Hz, 1H), 3.89 (dd, *J* = 9.7, 5.5 Hz, 1H), 1.67 – 1.45 (m, 1H), 0.71 (dd, *J* = 7.9, 4.7, 4H).

8-amino-2-(3-fluorophenyl)-*N*-(1-(methylcarbamoyl)azetidin-3-yl)-1,7-naphthyridine-5-carboxamide (14). To a solution of 8-amino-N-(azetidin-3-yl)-2-(3-fluorophenyl)-1,7-naphthyridine-5-carboxamide in DMF (1 mL), was added DIPEA (0.26 mL, 1.48 mmol) , *N*,*N*'-carbonyldiimidazole (74 mg, 0.44 mmol) , and the mixture was stirred at room temperature for 1 hour. 0.16 mL of methylamine (2 mol/L) in THF (2 mol/L) was added, and the mixture was stirred overnight. The reaction mixture was concentrated to dry and purified with prep HPLC to afford the product.

LC/MS (ESI+): m/z 395.4 (M+H).

^{1}H NMR (400 MHz, DMSO) δ 9.04 – 8.95 (d, *J* = 9.0 Hz, 1H), 8.95 – 8.85 (d, *J* = 6.8 Hz, 1H), 8.46 – 8.37 (m, 2H), 8.37 – 8.31 (s, 1H), 8.31 – 8.18 (m, 1H), 7.87 – 7.64 (s, 2H), 7.63 – 7.52 (td, *J* = 8.0, 6.1 Hz, 1H), 7.38 – 7.26 (td, *J* = 8.5, 2.6 Hz, 1H), 4.82 – 4.61 (m, 1H), 4.53 – 4.35 (t, *J* = 8.3 Hz, 1H), 4.21 – 4.12 (t, *J* = 8.9 Hz, 1H), 4.12 – 4.03 (dd, *J* = 8.7, 5.3 Hz, 1H), 3.94 – 3.82 (dd, *J* = 9.8, 5.5 Hz, 1H), 2.24 – 1.97 (q, *J* = 7.5 Hz, 2H), 1.08 – 0.85 (t, *J* = 7.5 Hz, 3H).

Protein Crystallography

The MAP4K4 kinase domain (residues 2-328) was purified and crystallized as previously described.[1] Compound 13 was soaked into apo protein crystals for several hours, serially transitioned to MES pH 6.5 with 35% PEG 3350, and flash frozen in liquid nitrogen. Data were collected at the Stanford Synchrotron Radiation Lightsource beamline 11-1. The structure was determined by fourier synthesis from our prior models and refined by iterative rounds of manual rebuilding (PyMOL,[4] COOT)[5] and automated refinement (BUSTER,[6] Phenix).[7] Structure statistics are

reported in Table S1. Coordinates and structure factors are deposited in the Protein Data Bank (4ZK5).

Table S2. Crystallographic data collection and refinement statistics

MAP4K4 : GNE-495 (4ZK5)	
Wavelength (Å)	0.97945
Resolution range (Å)	91.01 - 2.89 (2.992 - 2.889)
Space group	P 2_1 2_1 2_1
Unit cell	79.35 89.34 91.01 90 90 90
Total reflections	98672 (9937)
Unique reflections	15003 (1481)
Multiplicity	6.6 (6.7)
Completeness (%)	99.83 (99.80)
Mean I/sigma(I)	17.57 (3.27)
Wilson B-factor	64.75
R-merge	0.0892 (0.6231)
R-meas	0.0971
R-work	0.2186 (0.3038)
R-free	0.2672 (0.4047)
Number of atoms	4817
macromolecules	4678
ligands	43
water	96
Protein residues	580
RMS(bonds)	0.012
RMS(angles)	1.7
Ramachandran favored (%)	94
Ramachandran outliers (%)	0.17
Clashscore	2.33
Average B-factor	71.9
macromolecules	72.3
ligands	69.4
solvent	56.5

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