Structure-based Design of 2-Aminopyridine Oxazolidinones as Potent and Selective Tankyrase Inhibitors

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Experimental Section

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Anhydrous solvents were obtained from Aldrich and used directly. All reactions involving air- or moisture–sensitive reagents were performed under a nitrogen or argon atmosphere. All final compounds were purified to ≥95% purity as determined by high-performance liquid chromatography (HPLC). Purity was measured using Agilent 1100 Series high performance liquid chromatography (HPLC) systems with UV detection at 254 nm (System A: Agilent Zorbax Eclipse XDB-C8 4.6 x 150 mm, 5 micron, 5 to 100% CH_3CN in H_2O with 0.1% TFA for 15 min at 1.5 mL/min; System B: Zorbax SB-C8, 4.6 x 75 mm, 10 to 90% CH_3CN in H_2O with 0.1% formic acid for 12 min at 1.0 mL/min). Silica gel columns were performed using prepacked silica gel cartridges (Biotage). ^1H NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer or on a Bruker AV-500 (500 MHz) spectrometer at ambient temperature. NMR spectra were processed using ACD SpecManager (version 12; ACD, Toronto, Canada). All observed protons are reported as parts per million (ppm) downfield from tetramethylsilane (TMS) or other internal reference in the appropriate solvent indicated. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants, and number of protons. Low-resolution mass spectral (MS) data were determined on an Agilent 1100 Series LCMS with UV detection at 254 nm and a low resonance electrospray mode (ESI). Exact mass confirmation was performed on an Agilent 1200 series high performance liquid chromatography (HPLC) system (Santa Clara,

CA, U.S.) by flow injection analysis, eluting with a binary solvent system A and B (A, water with 0.1% FA; B, ACN with 0.1% FA) under gradient conditions (5-95% B over 3 min) at 0.3 mL/min with MS detection by an Agilent 6510-Q-TOF mass spectrometer (Santa Clara, CA, U.S.).

Preparation of 2-(2-fluoro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)pyrimidine (10)

Step 1: 2-(5-bromo-2-fluoropyridin-3-yl)pyrimidine

2-bromopyrimidine (5.42 g, 34.1 mmol), sodium carbonate (2M, 22.75 mL, 45.5 mmol), 5-bromo-2-fluoropyridine-3-boronic acid (5 g, 22.75 mmol, Frontier Scientific, Inc.), and dichloro(1,1-bis(diphenylphosphinoferrocene))palladium(II) (1.858 g, 2.275 mmol, Sigma - Aldrich Chemical Company, Inc.) were combined in dioxane (32.5 mL) and stirred at 100 ºC in a heating block overnight. LCMS indicated good conversion to desired product. The crude material was absorbed onto a plug of silica gel and purified by chromatography through a Redi-Sep pre-packed silica gel column (12 g), eluting with a gradient of 0% to 10% MeOH in CH_2Cl_2, to provide 2-(5-bromo-2-fluoropyridin-3-yl)pyrimidine (4.37 g, 17.20 mmol, 76%) as yellow solid. m/z (ESI) 253.8

Step 2: 2-(2-fluoro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)pyrimidine

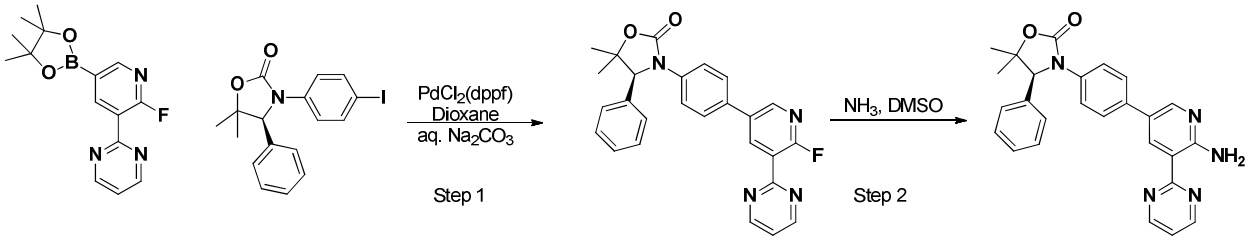
To a solution of 2-(5-bromo-2-fluoropyridin-3-yl)pyrimidine (1.7 g, 6.69 mmol) and dioxane (13.38 mL) were added potassium acetate (3.28 g, 33.5 mmol, Sigma-Aldrich Chemical Company, Inc.), 4,4,4',4',5,5,5',5'-octamethyl-2,2'-bi(1,3,2-dioxaborolane) (1.869 g, 7.36 mmol, Sigma-Aldrich Chemical Company, Inc.) and 1,1'-bis (diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (0.546 g, 0.669 mmol, Sigma-Aldrich Chemical Company, Inc.). Reaction vessel was flushed with argon, sealed and heated at 130 °C for 1h in a microwave oven. LCMS showed complete conversion to desired product. Reaction was cooled to rt and was filtered through Celite, the crude material was absorbed onto a plug of silica gel and purified by chromatography through a Redi-Sep pre-packed silica gel column (40 g), eluting with a gradient of 0% to 50% EtOAc in hexane, to provide 2-(2-fluoro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)pyrimidine (1.6 g, 5.31 mmol, 79%). ^{1}H NMR

(400 MHz, DMSO-d_6) δ 9.00 (d, *J* = 4.89 Hz, 2H), 8.82 (dd, *J* = 1.96, 10.37 Hz, 1H), 8.55 (br. s, 1H), 7.57 (t, *J* = 4.89 Hz, 1H), 1.34 (s, 12H). m/z (ESI) 219.9 (M-C_6H_9).

Preparation of (S)-3-(4-iodophenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (12)

A resealable tube was charged with (S)-5,5-dimethyl-4-phenyloxazolidin-2-one (1.01 g, 5.28 mmol), 1,4-diiodobenzene (3.48 g, 10.56 mmol), potassium phosphate, tribasic (5.61 g, 26.4 mmol) and dioxane (20.0 mL). The mixture was purged with argon and then copper(I) iodide (1.00 g, 5.28 mmol) and N,N'-dimethylethylenediamine (1.14 mL, 10.56 mmol) were added. The system was purged with argon, the tube was sealed, and the reaction mixture was heated at 100 °C for 12 h. LCMS inidcated complete conversion. The reaction mixture was filtered through Celite and concentrated to afford a purple solid. This material was purified via column chromatography on silica gel (RediSep 80 g column, gradient elution with 0-25% ethyl acetate-hexane) to afford (S)-3-(4-iodophenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (1.27 g, 3.23 mmol, 61.2%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ7.64 - 7.59 (m, 2 H), 7.39 - 7.33 (m, 2 H), 7.33 - 7.27 (m, 3 H), 7.26 - 7.20 (m, 2 H), 5.43 (s, 1 H), 1.61 (s, 3 H), 0.89 (s, 3 H).

Preparation of (S)-3-(4-(6-amino-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (3)



Step 1 : (S)-3-(4-(6-fluoro-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one

(S)-3-(4-iodophenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (2.61 g, 6.64 mmol), sodium carbonate (2M, 6.64 mL, 13.28 mmol), 2-(2-fluoro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)pyrimidine (2 g, 6.64 mmol), and dichloro(1,1-bis(diphenylphosphinoferrocene))palladium(II) (0.54 g, 0.66 mmol, Sigma-Aldrich Chemical Company, Inc.) were combined in dioxane (16.6 mL) and stirred at 110° C overngight. LCMS indicated complete conversion to desired product. The crude material was absorbed onto a plug

of silica gel and purified by chromatography through a Redi-Sep pre-packed silica gel column (12 g), eluting with a gradient of 0% to 50% EtOAc in hexane, to provide (S)-3-(4-(6-fluoro-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (1.82g, 62%) as yellow solid. m/z (ESI) 441.3$(M+H)^+$

Step 2 : (S)-3-(4-(6-amino-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one

A 2-mL glass microwave reaction vessel was charged with ammonia, 2M solution in 2-propanol (123 µL, 5.68 mmol, Sigma-Aldrich Chemical Company, Inc.) and (S)-3-(4-(6-fluoro-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (25 mg, 0.057 mmol) in DMSO (568 µL). The reaction mixture was stirred and heated at 100°C in a heating block overnight. LCMS indicated clean and complete conversion to desired prodcut. The crude material was purified by reverse-phase preparative HPLC using 0.1% TFA in CH_3CN/H_2O, gradient 15% to 90% over 20 min to provide (S)-3-(4-(6-amino-5-(pyrimidin-2-yl)pyridin-3-yl)phenyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (21 mg, 83% yield) as a yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.93 (d, *J* = 5.02 Hz, 2H), 8.84 (d, *J* = 2.46 Hz, 1H), 8.42 (d, *J* = 2.56 Hz, 1H), 7.56 (s, 4H), 7.43 (t, *J* = 4.81 Hz, 1H), 7.34 - 7.41 (m, 2H), 7.25 - 7.34 (m, 3H), 5.48 (s, 1H), 1.65 (s, 3H), 0.91 (s, 3H). HRMS (ESI^+) $[M+H]^+$, calcd for $C_{26}H_{24}N_5O_2$: 438.1930 Found 438.1930.

Preparation of (S)-5,5-dimethyl-3-(4-oxocyclohexyl)-4-phenyloxazolidin-2-one (15)

Step 1: (S)-1-(1,4-dioxaspiro[4.5]decan-8-ylamino)-2-methyl-1-phenylpropan-2-ol

To a flask charged with 1,4-cyclohexanedione monoethylene acetal (41.6 g, 266 mmol) was added 1,2-dichloroethane (484 mL), acetic acid (18.02 mL, 315 mmol), (S)-1-amino-2-methyl-1-phenylpropan-2-ol (40g, 242 mmol) and sodium triacetoxyborohydride (71.8 g, 339 mmol) respectively. The resulting orange suspension was stirred at room temperature, during the process heat was build up in the reaction. The mixture was cooled in an ice bath. The ice bath was removed after 15 min. The reaction mixture was stirred at rt for 3h, LCMS indicated good conversion to desired product. The reaction mixture was cooled in an ice bath, and was added 6N NaOH (80mL), the reaction mixture was diluted with water (200 mL). The mixture was transferred to a separation funnel and extracted with DCM (2 x 200 mL). The combined organic

layers were dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was used directly without purification. m/z (ESI) 306.2 $(M+H)^+$

Step 2: (S)-5,5-dimethyl-4-phenyl-3-(1,4-dioxaspiro[4.5]decan-8-yl)oxazolidin-2-one

To a 1L flask charged with (S)-1-(1,4-dioxaspiro[4.5]decan-8-ylamino)-2-methyl-1-phenylpropan-2-ol (40 g, 131 mmol) was added THF (218 mL) followed by diisopropylethylamine (114 mL, 655 mmol). The resulting suspension was cooled in an ice water bath. Triphosgene (38.9 g, 131 mmol) was dissolved in 50 mL of THF and was added dropwise to the reaction mixture through an addition funnel over 2h. After stirring for another 1h, LCMS indicated good conversion to desired product. To the mixture was added sat. aq. NH_4Cl (200 mL). The resulting mixture was transferred to a separation funnel and extracted with EtOAc (2 x 200 mL). The combined organic layers were dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The material was used without purification. m/z (ESI) 332.2 $(M+H)^+$

Step 3: (S)-5,5-dimethyl-3-(4-oxocyclohexyl)-4-phenyloxazolidin-2-one

To a 1L round bottomed flask was added (S)-5,5-dimethyl-4-phenyl-3-(1,4-dioxaspiro[4.5]decan-8-yl)oxazolidin-2-one (43.4 g, 131 mmol) and trifluoroacetic acid (292 mL, 3929 mmol) in THF (175 mL) and Water (87 mL). The reaction mixture was stirred at rt overnight. Another 100 mL of TFA was added to the reaction mixture and was stirred at rt for 24h. LCMS indicated good conversion to desired product. The mixture was concentrated in vacuo, the residue was redisolved in EtOAc. The solution was cooled in a ice bath and was neutralized with 6N NaOH(80mL) and then diluted with sat. aq. $NaHCO_3$. The reaction mixture was extracted with EtOAc (3 x 200mL). The organic extracts were concentrated in vacuo, the crude material was absorbed onto a plug of silica gel and purified by chromatography through a Redi-Sep pre-packed silica gel column (330 g), eluting with a gradient of 0% to 10% MeOH in DCM (DCM/MeOH/NH4OH 90/10/1), to provide (S)-5,5-dimethyl-3-(4-oxocyclohexyl)-4-phenyloxazolidin-2-one (30.5 g, 81%) as light-yellow solid. ^1H NMR (400 MHz, DMSO-d_6) δ 7.09 - 7.59 (m, 5H), 4.66 (s, 1H), 3.87 - 4.02 (m, J = 3.30 Hz, 1H), 2.36 - 2.55 (m, 2H), 1.94 - 2.26 (m, 4H), 1.68 - 1.85 (m, 1H), 1.36 - 1.56 (m, 4H), 0.78 (s, 3H). m/z (ESI) 288.3 $(M+H)^+$

Preparation of 4-((S)-5,5-dimethyl-2-oxo-4-phenyloxazolidin-3-yl)cyclohex-1-en-1-yl trifluoromethanesulfonate (16)

A solution of (S)-5,5-dimethyl-3-(4-oxocyclohexyl)-4-phenyloxazolidin-2-one (24 g, 83.5 mmol) in anhydrous THF (450 mL) was cooled to -78 °C using a dry ice-acetone bath. Lithium bis(trimethylsilyl)amide, 1.0 M in THF (96 mL, 96 mmol) was added over 5 min and the resulting mixture was stirred at -78 °C for 45 min. N-Phenyltrifluoromethanesulfonimide (47.3 g, 0.132 mol) was added and the mixture was allowed to stir at room temperature under nitrogen overnight (16.5 h). Water (250 mL) was added to quench the reaction and the mixture was concentrated. The resulting aqueous phase was extracted with ethyl acetate (250 mL x 3) and the organic extracts were combined, washed with saturated aqueous sodium bicarbonate solution (100 mL x 2), water (100 mL x 2), brine (100 mL), dried, filtered and concentrated. The residue was purified by column chromatography (eluting with hexanes/ethyl acetate = 20: 1 to 5:1) to afford 4-((S)-5,5-dimethyl-2-oxo-4-phenyloxazolidin-3-yl)cyclohex-1-en-1-yl trifluoromethanesulfonate (25 g, 71%). ^{1}H NMR (300 MHz, d_6-DMSO) δ ppm 7.50-7.20 (m, 5H), 5.90-5.75 (m, 1H), 4.68 (d, J = 9 Hz, 1H), 3.70-3.50 (m, 1H), 2.85-2.70 (m, 1H), 2.50-2.10 (m, 3H), 2.05-1.90 (m, 1H), 1.80-1.55 (m, 1H), 1.47 (s, 3H), 0.80 (s, 3H). m/z (ESI) 420 $(M+H)^+$.

Preparation of (R)-3-((1r,4R)-4-(6-aminopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (7)

Step1: 3-(4-(6-aminopyridin-3-yl)cyclohex-3-en-1-yl)-5,5-dimethyl-4-phenyloxazolidin-2-one

A microwave vial was charged with 5,5-dimethyl-2-oxo-4-phenyloxazolidin-3-yl)cyclohex-1-en-1-yl trifluoromethanesulfonate (500 mg, 1.19 mmol), 2-amino-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (262 mg, 1.19 mmol, Sigma-Aldrich), sodium carbonate (1.8 mL, 3.58 mmol, Sigma-Aldrich), tetrakis(triphenylphosphine)palladium(0) (138 mg, 0.12 mmol, Strem) and dioxane (4 mL) was added, the system was purged with argon, and the tube was sealed. The mixture stirred at 100 °C in the microwave for 1 h. LCMS indicated clean and good conversion to desired product. The reaction mixture was filtered through Celite and the filtrate was concentrated to afford a yellow oil. This oil was purified via column chromatography on silica gel (RediSep 40 g column, gradient elution with 0 to 100% ethyl acetate-heptane) to afford

3-(4-(6-aminopyridin-3-yl)cyclohex-3-en-1-yl)-5,5-dimethyl-4-phenyloxazolidin-2-one (419 mg, 93% as a white solid. m/z (ESI) 364.2 $(M+H)^+$.

Step 2: (R)-3-((1r,4R)-4-(6-aminopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one

To a 50 mL high pressure round-bottomed flask was added 3-(4-(6-aminopyridin-3-yl)cyclohex-3-en-1-yl)-5,5-dimethyl-4-phenyloxazolidin-2-one (11g, 30.3 mmol) and palladium, 10% wt. on activated carbon (3.22 g, 3.03 mmol, Sigma-Aldrich) in MeOH. The reaction mixture was stirred at 50°C under 45 psi of hydrogen over night. LCMS inidcated good conversion to desired product. After cooling to rt, the reaction mixture was filtered and concentrated in vaccuo. The reaction mixture was subject to chiral separation (Purified using SFC Column: Chiralpak AD, 5 micron, 5 cm id x 15 cm length; Mobile Phase: 35% methanol w/ 0.2% diethylamine / 65% CO_2, Flowrate: 350 ml/min; Back pressure regulator setting: 100 bar), which provided (R)-3-((1r,4R)-4-(6-aminopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (5.5g, 39%) in good optical purity (>97%ee). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.69 (d, *J* = 2.25 Hz, 1H), 7.14 - 7.51 (m, 5H), 6.33 (d, *J* = 8.41 Hz, 1H), 5.59 (s, 2H), 4.62 (s, 1H), 4.02 - 4.15 (m, 1H), 3.38 - 3.59 (m, 1H), 2.08 - 2.24 (m, 1H), 1.69 - 1.90 (m, 3H), 1.54 - 1.66 (m, 2H), 1.47 (overlap s, 4H), 1.25 - 1.39 (m, 1H), 1.08 - 1.24 (m, 1H), 0.79 (s, 3H). m/z (ESI) 366.2 $(M+H)^+$

Preparation of (R)-3-((1r,4R)-4-(6-amino-5-bromopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (17)

To a solution of (R)-3-((1r,4R)-4-(6-aminopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (250 mg, 0.684 mmol) in DCM (3.4 mL) at 0°C, was added N-bromosuccinimide (134 mg, 0.752 mmol, Sigma-Aldrich). The reaction mixture was stirred for 1h at the same temperature. LCMS indicated clean and complete conversion to desired product. The solution was concentrated and water was added. The solid was filtered and washed with water three times. The solid was air-dried to afford (R)-3-((1r,4R)-4-(6-amino-5-bromopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (310 mg, 0.698 mmol, 100%) as yellow solid. The material was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.77 (d, *J* = 1.86 Hz, 1H), 7.59 (d, *J* = 1.76 Hz, 1H), 7.12 - 7.51 (m, *J* = 7.10, 19.00 Hz, 5H), 5.94 (br. s., 2H), 4.62 (s, 1H), 3.43 - 3.58 (m, 1H), 2.14 - 2.30 (m, 1H), 1.69 - 1.90 (m, 2H), 1.55

- 1.66 (m, 2H), 1.47 (overlap s, 4H), 1.30 - 1.42 (m, 1H), 1.05 - 1.28 (m, 1H), 0.79 (s, 3H). m/z (ESI) 444.0

Preparation of 2-amino-5-((1S,4r)-4-((S)-5,5-dimethyl-2-oxo-4-phenyloxazolidin-3-yl)cyclohexyl)nicotinonitrile (6)

In a 2 mL sealed tube, added (S)-3-((1r,4S)-4-(6-amino-5-bromopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (50 mg, 0.113 mmol), dicyanozinc (13.21 mg, 0.113 mmol, Sigma-Aldrich) and $Pd(Ph_3P)_4$ (13.00 mg, 0.011 mmol, Strem) in DMF (375 µl), purged solvent with nitrogen for 5 minutes, sealed. The vessel was heated to 85 °C in a microwave oven for 1h. LCMS indicated complete conversion to desired product. The crude mixture was passed through a syringe filter, the crude material was purified by reverse-phase preparative HPLC using 0.1% TFA in CH_3CN/H_2O, gradient 50% to 95% over 20 min to provide 2-amino-5-((1S,4r)-4-((S)-5,5-dimethyl-2-oxo-4-phenyloxazolidin-3-yl)cyclohexyl)nicotinonitrile (26 mg, 60%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.06 (d, *J* = 2.46 Hz, 1H), 7.70 (d, *J* = 2.35 Hz, 1H), 7.39 - 7.47 (m, 2H), 7.35 - 7.38 (m, 1H), 7.26 (br. S, 1H), 6.58 - 6.61 (m, 2H), 4.62 - 4.64 (m, 1H), 3.43 - 3.53 (m, 1H), 2.21 - 2.31 (m, 1H), 1.80 - 1.87 (m, 2H), 1.71 - 1.79 (m, 1H), 1.56 - 1.65 (m, 2H), 1.47 (overlap s, 4H), 1.29 - 1.42 (m, 1H), 1.11 - 1.23 (m, 1H), 0.78 - 0.82 (m, 3H). HRMS (ESI^+) $[M+H]^+$, calcd for $C_{23}H_{27}N_4O_2$: 391.2134 Found 391.2135.

Preparation of (S)-3-((1r,4S)-4-(6-amino-5-(pyrimidin-2-yl)pyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (4)

A microwave vial was charged with (S)-3-((1r,4S)-4-(6-amino-5-bromopyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (900 mg, 2.03 mmol), 2-(tributylstannyl)pyrimidine (736 µL, 2.23 mmol), lithium chloride (172 mg, 4.05 mmol), copper(I) iodide (38.6 mg, 0.20 mmol), and DMF (6.8 mL). Tetrakis(triphenylphosphine)palladium(0) (234 mg, 0.20 mmol) was added, the system was purged with argon, and the tube was sealed. The mixture stirred at 120 °C in the microwave for 1h. LCMS indicated good conversion to desired product. The mixture was cooled to the room temperature and partitioned between water (100 mL) and ethyl acetate (3x150 mL). The organic layers were combined and were washed twice with water then brine. The solution was dried over MgSO4, filtered and concentrated in vacuo to give the crude material as a black oil.The crude

material was absorbed onto a plug of silica gel and purified by chromatography through a Redi-Sep pre-packed silica gel column (40 g), washing with heptane for 15 min. and then eluting with a gradient of 0% to 10% MeOH in CH2CL2 for 60 min, to provide (S)-3-((1r,4S)-4-(6-amino-5-(pyrimidin-2-yl)pyridin-3-yl)cyclohexyl)-5,5-dimethyl-4-phenyloxazolidin-2-one (422 mg, 47% yield) as yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.88 (d, *J* = 4.81 Hz, 2H), 8.45 (d, *J* = 2.35 Hz, 1H), 7.97 (d, *J* = 2.46 Hz, 1H), 7.39 - 7.46 (m, 3H), 7.33 - 7.39 (m, 3H), 7.20 - 7.33 (m, 2H), 4.64 (s, 1H), 3.47 - 3.59 (m, 1H), 2.32 (tt, *J* = 3.21, 12.18 Hz, 1H), 1.81 - 1.92 (m, 3H), 1.58 - 1.73 (m, 2H), 1.48 (overlap s, 4H), 1.34 - 1.44 (m, 1H), 1.18 - 1.29 (m, 1H), 0.80 (s, 3H). HRMS (ESI$^+$) $[M+H]^+$, calcd for $C_{26}H_{30}N_5O_2$: 444.2399 Found 444.2404.

TNKS1/2 Enzymatic Assays.

The tankyrase 1 biochemical activity of the compounds was assayed in the following assay buffer (50 mM MOPS pH7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.01% Tween-20, 0.05% BSA, and 1mM DTT) as follows: 0.25 nM of 6XHIS-tankyrase1 (1091-1325) was incubated in the presence of compound (DMSO 1.85% final) in a Perkin Elmer 384 well Proxiplate PlusTM (cat.no. 6008289) with 400 nM of NAD for 60 minutes at RT. The assay was then stopped with the above assay buffer containing a 0.6 µM inhibitor and the following detection components: 0.05 µg/mL monoclonal anti-PAR antibody (Trevigen cat.no. 4335-MC-01K-AC) prebound for 60 minutes with 0.63 µg/mL protein G AlphaLisa® acceptor bead (Perkin Elmer cat.no. AL102M) and 5 µg/mL AlphaLisa® nickel chelate donor bead (Perkin Elmer cat.no. AS101M). The assay was incubated for 16 hours at RT in the dark and read on a Perkin Elmer Envision® multi label reader using the default program set with laser excitation at 680 nM and emission at 615 nM. Assay standards: 66 runs with standard which yielded a mean of 0.0086 µM with a standard deviation of 0.00038; 42 runs with a different standard which yielded a mean of 0.0013 µM with a standard deviation of 0.00064.

PARP 1/2 Assays.

PARP1 biochemical assay was purchased as a kit from Trevigen (cat.# 4676-096-K) and used per manufacturers recommendations. PARP2 biochemical assay was purchased as a kit from BPS (cat.# 80552) and used per manufacturers recommendations.

Axin2 Accumulation Assay.

TNKS inhibition results in Axin2 accumulation into distinct cytoplasmic foci which were visualized and quantified using a high-content imaging system (Cellomics ArrayScan). SW480 cells were grown under normal culture conditions (RPMI 1640, 10% HI FBS and 1x Sodium Pyruvate). On the day of the assay, cells were plated at 2,500 cells per well in 60 µL of assay media in Perkin Elmer Black 384 ViewPlates (Fisher, 509052489). TNKS compounds were diluted to generate a 22-point dose titration in media and incubated with the cells for 24 hours at 37 degrees, 5% CO_2. The next day the cells were fixed for 15 min in 4% paraformaldehyde and 0.1% Triton, washed in PBS and blocked in PBS with 0.1% Tween-20 and1% Normal Goat Serum. The cells were stained with an Axin2 primary antibody(Sigma, SAB1100677-200UL) at 1:10000 overnight at 4 ºC and an Alexa 488-labeled secondary antibody (Invitrogen, A11008). The nuclei were visualized with Hoechst dye. The Axin2 foci were quantified on the Cellomics ArrayScan (a variation of the compartmental analysis protocol was optimized and data was analyzed using MEAN_RingSpotAvgIntenCh2) and EC_{50} values were calculated. Assay standards: 18 runs with a standard which yielded a mean of 0.311µM with a standard deviation of 0.173. 18 runs with another standed which yielded a mean of 0.114 µM with a standard deviation of 0.065.

Quantitative Total β-Catenin Assay.

TNKS inhibition results in degradation of the total pool of β-catenin in SW-480 cells colorectal cells. SW480 cells do not express E-cadherin and thus do not have membrane associated ("non-signaling") β-catenin which interferes with Wnt pathway activity analysis. Cells were seeded at 10,000/well in CellBIND 96-well in 60 µL of normal growth medium (MEM alpha supplemented with 10% heat inactivated FBS, GlutaMAX, pyruvate, and 10mM HEPES). A 10-point, 3-fold dilution series for each TNKS inhibitor was constructed and 20 ul of each diluted compound was transferred to the plated SW-480 cells (resulting in a in final vehicle (DMSO) concentration of 0.1%). The plates were incubated at 37°C for 40 to 48 hours after which the media was removed and the cells were lysed with 75 µL/well MSD lysis buffer. A goat anti-rabbit MSD plate (catalog no. L41RA-1) was coated with 25 µL of 5 µg/mL of Cell Signaling anti-total β-catenin polyclonal (catalog no. 9562, lyophilized, carrier-free special order) and incubated overnight in a cold room with gentle shaking. The plate was then blocked with 150 µL of Blocker "A" per well and washed 4 times with 150 µL/well TBS-T wash buffer (150 mM

NaCl, 50 mM Tris, pH 7.5, 0.02% Tween-20). Cell lysates (75 µL) were transferred to prepared MSD plates and incubated at 4 °C overnight with gentle shaking. The next day MSD plates were washed with TBS-T wash buffer and the anti-total β-catenin mAb (catalog no. 610153) detection antibody conjugated to SULFO-TAG was added. The detection antibody was incubated for 1 hour at room temperature with vigorous shaking after which plates were washed and processed for analysis by the addition of 150 µL/well MSD Read 4X Buffer T with surfactant. The plates were read and the data was captured on the SECTOR Imager 6000. Assay standards: 54 runs with a standard which yielded a mean of 0.157µM with a standard deviation of 0.116; 38 runs with another standard which yielded a mean of 0.063 µM with a standard deviation of 0.041.

DLD-1 STF assay.

Constitutively activated Wnt pathway reporter assay (*APC*-mutant cancer cell context): DLD-1 colorectal cells engineered with an 8X TCF promoter-driven Firefly (FF) luciferase gene (*Wnt reporter*) along with an EF1a promoter-driven Renilla (RN) luciferase gene (*control reporter*) were used to measure the potency of tankyrase compounds in the context of the constitutively activated Wnt pathway due to mutated APC in colorectal cancer cells. The engineered DLD-1 cells were plated at a density of 10,000 cells/well in black, clear-bottom, 96-well View plates (PerkinElmer) in normal growth medium (RPMI with10%FBS with no antibiotics). Tankyrase inhibitors were transferred to cells from a three-fold serially diluted compound plate. A 10-point dilution series was tested starting at a concentration of 10 mM. The plates were incubated at 37 ºC for 40 to 48 hr. The Dual-Glo Reagents (Promega) were added as directed by manufacturer to assess the Firefly (FF) and Renilla (RN) luciferase activity. Luciferase activity was measures using the EnVision multilabel plate reader (PerkinElmer).

Pharmacodynamic Assay.

Mice were housed in sterile caging with water and food supplied ad libitum. The laboratory housing the cages met all AAALAC specifications. Clinical observations of all mice on study occurred at least once daily. Athymic nude mice were injected subcutaneously with 5x10e6 DLD1 tumor cells that had been engineered to express firefly luciferase under the control of the SuperTopFlash promoter and Renilla luciferase under a constitutive promoter. Once tumors reached approximately 250mm^3, tumor bearing animals were randomized into groups of 4 and

dosed for 3 days with either vehicle or 50 mg/kg Compound 7 b.i.d. PO formulated in 30%HPBCD 10% Pluronic F68 pH 2.0. Tumors were harvested after 3 days, snap-frozen, disaggregated and lysed. Quantitation of Axin 2 protein was done using an MSD assay (MesoScale Discovery) using an Axin 2 antibody from Genetex for capture and a sulfotagged antibody from R&D Systems for detection. STF transcription assay was done using the Dual-Glo luciferase assay from Promega and firefly luciferase values were normalized using renilla luciferase values.

Protein Crystallography.

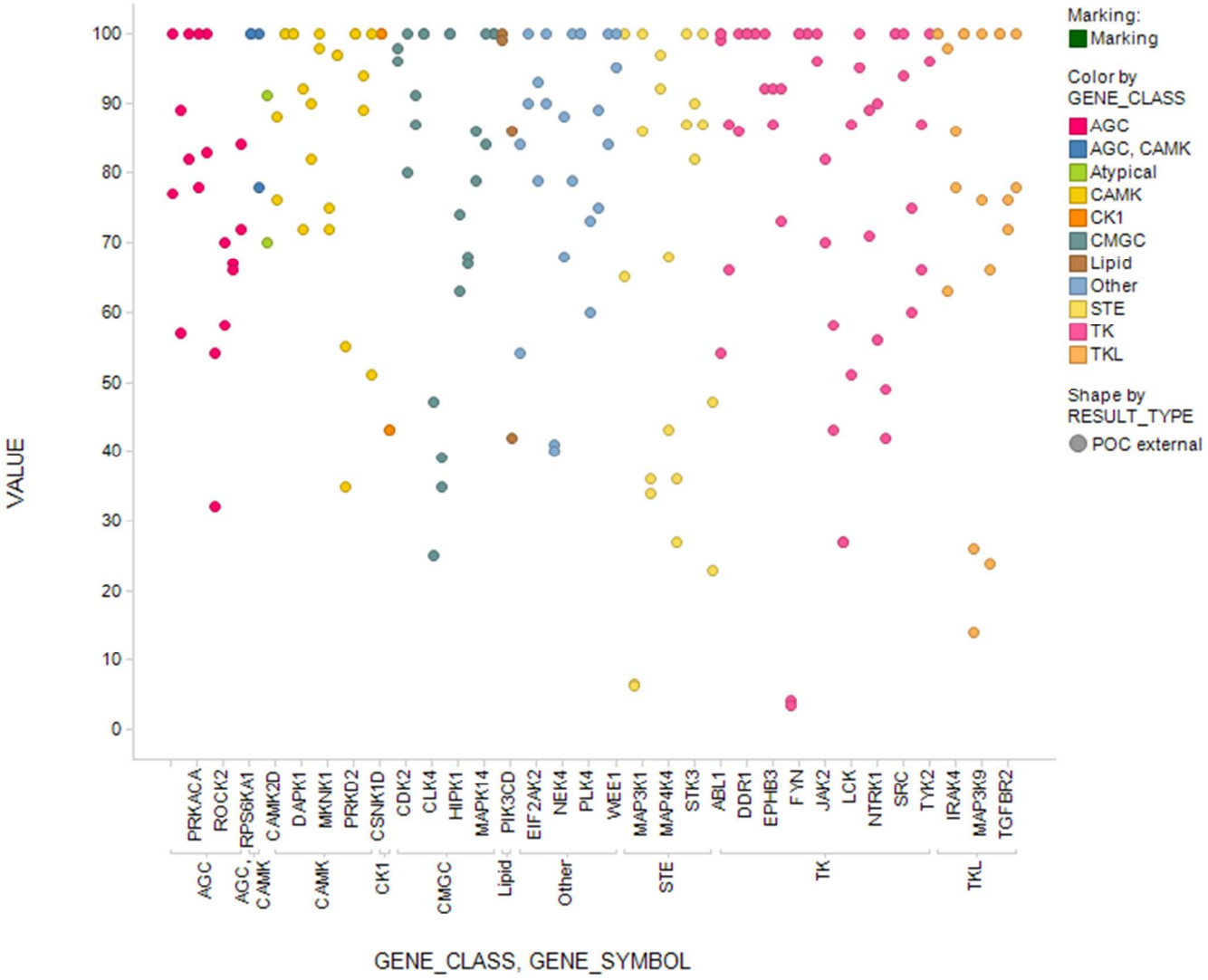
Human TNKS1 (1104-1314) with a C-terminal His_6 tag was expressed and purified as previously described.[1] The TNKS1/inhibitor complexes were obtained by incubating TNKS1(1104-1314)His_6 at 10 mg/ml with inhibitors in 2-fold molar excess for 30 minutes at 4 °C. Crystals of TNKS1/inhibitor were obtained at 4 °C in hanging drops by mixing 0.5 µL of TNKS1/4 complex with 0.5 µL of well solution containing 100 mM citrate pH 5.0, 0.2 M or 0.4 M diammonium tartrate, 12.5-25% PEG3350. Crystals appeared overnight and grew to maximum size in a few days. These crystals belong to the spacegroup $P2_12_12_1$ with unit cell parameters of a=42.5, b=73.4, c=148.2 Å. Paratone-N mineral oil was used as cryo protectant and diffraction data were collected on beamline 5.0.1 at the Advanced Light Source (ALS), Berkeley, CA and processed with HKL2000. The TNKS1/inhibitor complexes structures were solved by molecular replacement with AMoRe. Model building was carried out with QUANTA and refinement was done using CNX.

Ambit KINOMEscan Assays.[2]

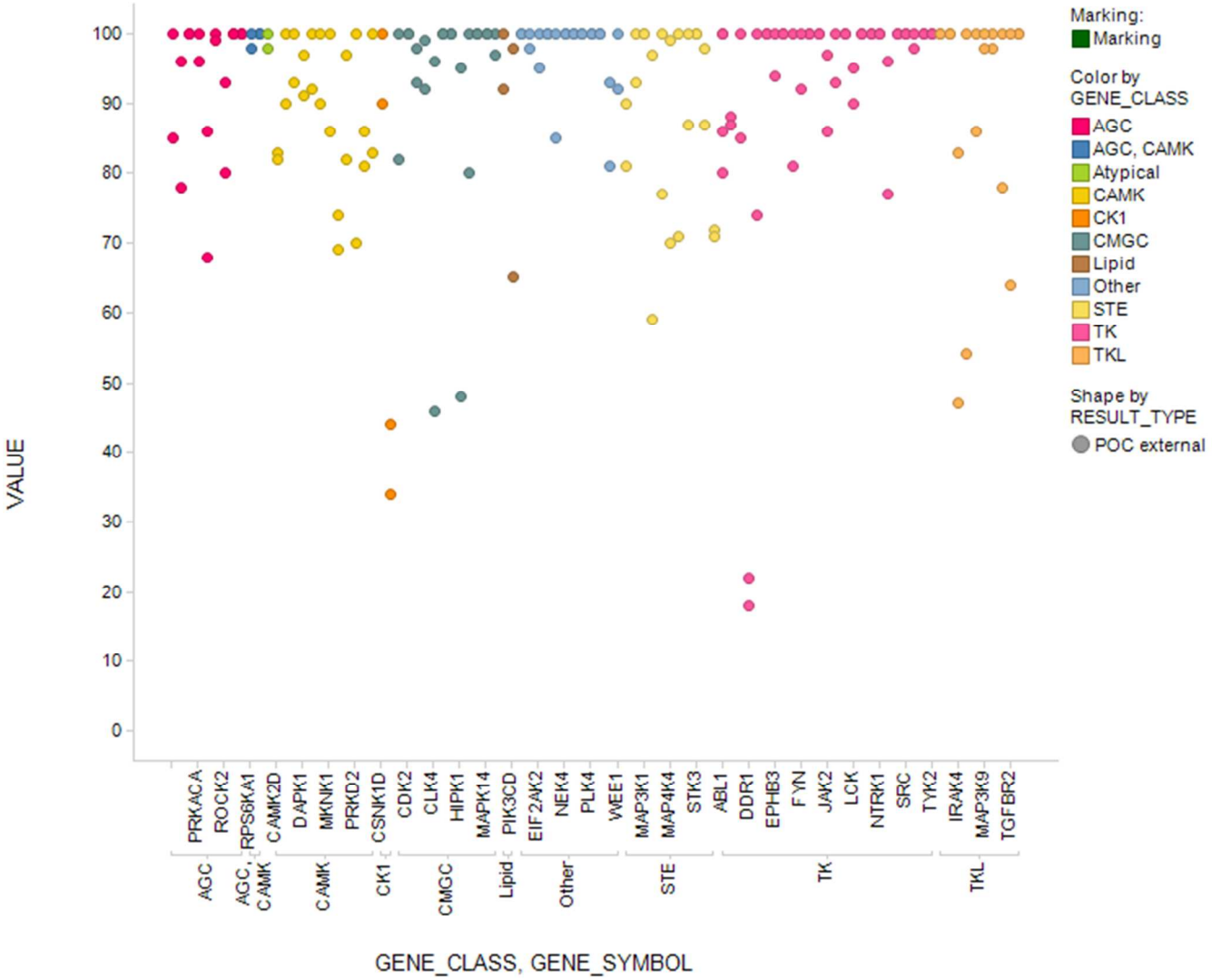
Kinase-tagged T7 phage strains were grown in parallel in 24-well blocks in an *E. coli* host derived from the BL21 strain. *E. coli* were grown to log-phase and infected with T7 phage from a frozen stock (multiplicity of infection = 0.4) and incubated with shaking at 32°C until lysis (90-150 minutes). The lysates were centrifuged (6,000 x g) and filtered (0.2µm) to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1 % BSA, 0.05 % Tween 20, 1 mM DTT) to remove unbound ligand and to reduce

nonspecific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20 % SeaBlock, 0.17x PBS, 0.05 % Tween 20, 6 mM DTT). Test compounds were prepared as 40x stocks in 100% DMSO and directly diluted into the assay. All reactions were performed in polypropylene 384-well plates in a final volume of 0.04 ml. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05 % Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5 μM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

Kinase selectivity of **3** in Ambit KINOMEscan @1 μM:



Kinase selectivity of **4** in Ambit KINOMEscan @1 μM:



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