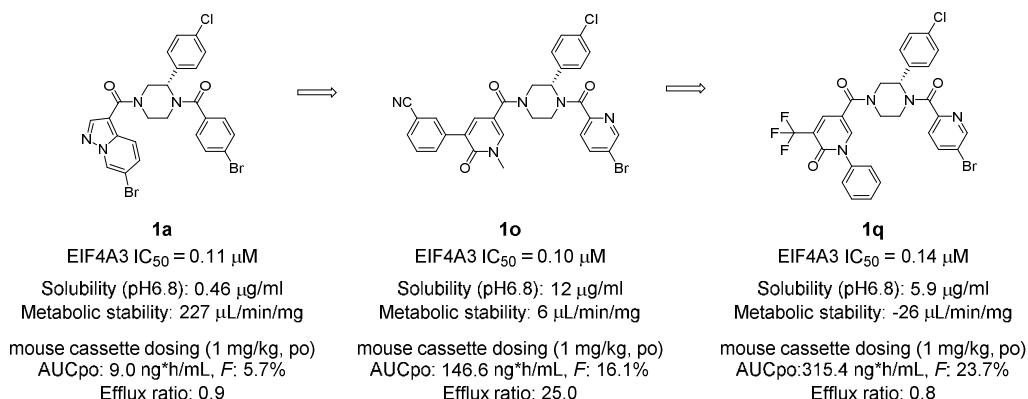


Discovery of Novel 5-(Piperazine-1-carbonyl)pyridin-2(1*H*)-one Derivatives as Orally eIF4A3-selective Inhibitors

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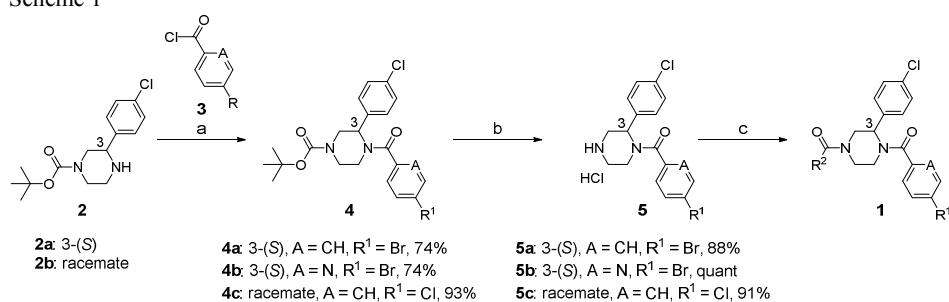
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Chemistry.

The general method for the preparation of compound **1** is shown in Scheme 1. Optically active (S)-3-(4-chlorophenyl)piperazine **2a** was commercially available or synthesized according to the literature method¹ in 91–99% ee. Condensation of **2** with acyl chloride **3** afforded 1-Boc-4-acylpiperazine derivative **4**. After removal of the Boc group under acidic conditions, compound **1** was synthesized by condensation with the appropriate carboxylic acid.

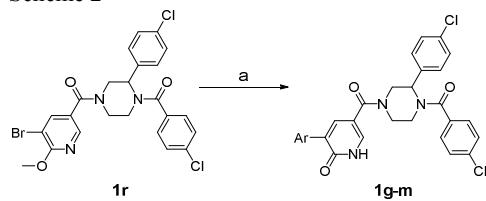
Scheme 1



Reagents and conditions: a) Et₃N, THF, rt, 2 h, 74–93%; b) 2 M HCl MeOH solution, 60 °C, 1 h, 88%–quant; c) R²CO₂H, HATU, Et₃N, DMF, rt, 2 h, 25–97%.

Parallel synthesis of derivatives **1g–m** was carried out as shown in Scheme 2. Suzuki-coupling of 3-bromo-2-methoxypyridine derivative **1r** with the appropriate boronic acid utilizing (A-taPhos)₂PdCl₂ under microwave irradiation afforded the expected coupling product, which was subjected to the cleavage of the methyl group from the methoxypyridine scaffold by treatment with TMSCl and NaI in CH₃CN to provide the desired 3-arylpyridin-2(1*H*)-one derivative **1g–m**.

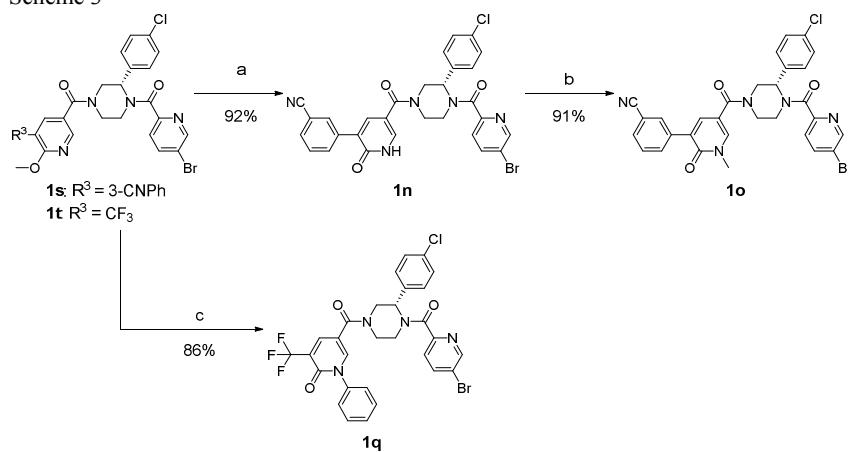
Scheme 2



Reagents and conditions: a) i) boronic acid, (A-taPhos)₂PdCl₂, Cs₂CO₃, DME, 110 °C, 1 h, microwave; ii) TMSCl, NaI, CH₃CN, 60 °C, 2 h, 7–39%.

The preparation of pyridin-2(1*H*)-one derivatives **1n**, **1o** and **1q** was conducted as shown in Scheme 3. Removal of the methyl group from methoxy compound **1s** under TMSCl/NaI conditions in CH₃CN produced pyridin-2(1*H*)-one derivative **1n**. Subsequent *N*-methylation of **1n** afforded *N*-methyl-pyridin-2(1*H*)-one derivative **1o**. Removal of the methyl group of **1t** followed by Cu-mediated coupling reaction with phenylboronic acid yielded *N*-phenyl-pyridin-2(1*H*)-one derivative **1q**.

Scheme 3



Reagents and conditions: a) 1s, TMSCl, NaI, CH₃CN, 80 °C, 2 h, 92%; b) MeI, K₂CO₃, DMF, rt, 1 h, 91%; c) i) 1t, TMSCl, NaI, CH₃CN, 80 °C, 2 h; ii) PhB(OH)₂, Cu(OAc)₂, pyridine, 80 °C, 1 h, 86%.

EXPERIMENTAL SECTION

General.

Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on Bruker AVANCE-300 (300 MHz) and Bruker AVANCE-400 (400 MHz) instruments in CDCl₃ or DMSO-*d*₆ solution. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, brs = broad singlet. Coupling constants (*J* values) are given in hertz (Hz). Melting points (mp) were determined on an OptiMelt MPA100 melting point apparatus and were uncorrected. Elemental analyses were carried out by Sumika Chemical Analysis Service, Ltd. or Toray Research Center, Inc. and were within 0.4% of the theoretical values. Low-resolution mass spectra (MS) were acquired using a Shimadzu UFLC/MS (Prominence UFLC high pressure gradient system/LCMS-2020) operating in an electron spray ionization mode (ESI⁺). The column used was an L-column 2 ODS (3.0 x 50 mm I.D., 3 μ m, CERI, Japan) with a temperature of 40 °C and a flow rate of 1.2 or 1.5 mL/min. Condition 1: Mobile phases A and B under an acidic condition were 0.05% TFA in water and 0.05% TFA in CH₃CN, respectively. The ratio of mobile phase B was increased linearly from 5% to 90% over 0.9 min, 90% over the next 1.1 min. The ratio of mobile phase B was increased linearly from 5% to 90% over 0.9 min, 90% over the next 1.1 min. Specific rotation values were carried out by Sumika Chemical Analysis Service, Ltd. and observed in MeOH (1w/v%). The purities of all compounds tested in biological systems were assessed as being > 95% using elemental analysis or analytical HPLC. Purity data were collected by HPLC with NQAD (Nano Quality Analyte Detector) or Corona CAD (Charged Aerosol Detector). The column was an L-column 2 ODS (30 x 2.1 mm I.D., CERI, Japan) or a Capcell Pak C18AQ (50 mm x 3.0 mm I.D., Shiseido, Japan) with a temperature of 50 °C and a flow rate of 0.5 mL/min. Mobile phases A and B under a neutral condition were a mixture of 50 mmol/L ammonium acetate, water and acetonitrile (1/8/1, v/v/v) and a mixture of 50 mmol/L ammonium acetate and CH₃CN (1/9, v/v), respectively. The ratio of mobile phase B was increased linearly from 5% to 95% over 3 min, 95% over the next 1 min. Reagents and solvents were obtained from commercial sources and used without further purification. Reaction progress was determined by thin layer chromatography (TLC) analysis on Merck Kieselgel 60 F254 plates or Fuji SiliSyl NH plates. Chromatographic purification was carried out on silica gel columns ((Inject column and Universal column, YAMAZEN Co.) or on Purif-Pack (Si or NH, Shoko Scientific Co., Ltd.)). All commercially available solvents and reagents were used without further purification. Yields were not optimized.

General procedure for the preparation of compound 4:

tert-Butyl (3*S*)-4-(4-bromobenzoyl)-3-(4-chlorophenyl)piperazine-1-carboxylate (**4a**): A mixture of (*S*)-*tert*-butyl 3-(4-chlorophenyl)piperazine-1-carboxylate (**2a**) (4.2 g, 14.2 mmol), 4-bromobenzoyl chloride (3.5 g, 16 mmol) and Et₃N (5 ml, 35.9 mmol) in THF (50 ml) was stirred at room temperature for 1 h. The mixture was extracted with AcOEt and water. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, eluent: 5/95 to 50/50 AcOEt/hexane) to afford **4a** (5 g, 74 %) as an off-white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 1.33 (9H, s), 3.00 (2H, brs), 3.39 (2H, brs), 3.75 (1H, brs), 4.46 (1H, d, *J* = 14.4 Hz), 5.62 (1H, s), 7.30–7.52 (6H, m), 7.67 (2H, d, *J* = 7.5 Hz). LCMS *m/z* calcd for C₂₂H₂₄BrClN₂O₃: 478.07, found 422.9 [M+1-tBu].

tert-Butyl (3*S*)-4-((5-bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazine-1-carboxylate (**4b**): Product was prepared according to the general procedure from 5.51 g of **2a** and obtained (6.74 g, 74%) as a beige amorphous solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.34 (9H, s), 2.82–3.23 (2H, m), 3.37–4.00 (3H, m), 4.20–4.60 (1H, m), 5.07–5.78 (1H, m), 7.31–7.53 (4H, m), 7.67 (1H, d, *J* = 8.7 Hz), 8.09–8.32 (1H, m), 8.59–8.88 (1H, m). LCMS *m/z* calcd for C₂₁H₂₃BrClN₃O₃: 479.06, found 424.0 [M+1-tBu].

tert-Butyl (4-chlorobenzoyl)-3-(4-chlorophenyl)piperazine-1-carboxylate (**4c**): Product was prepared according to the general procedure from 4 g of **2b** and obtained (5.45 g, 93%) as an off-white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 1.33 (9H, brs), 3.00 (2H, brs), 3.33–3.55 (2H, m), 3.76 (1H, brs), 4.46 (1H, d, *J* = 13.4 Hz), 5.41–5.92 (1H, m), 7.18–7.66 (8H, m). LCMS *m/z* calcd for C₂₂H₂₄Cl₂N₂O₃: 434.12, found 379.0 [M+1-tBu].

General procedure for the preparation of compound **5**:

(*S*)-(4-Bromophenyl)(2-(4-chlorophenyl)piperazin-1-yl)methanone hydrochloride (**5a**): A mixture of **4a** (5 g, 10.4 mmol) and 2 M HCl MeOH solution (30 ml, 60 mmol) was stirred at 60 °C for 1 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. The residue was extracted with AcOEt and saturated NaHCO₃ aqueous solution. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was dissolved in AcOEt (30 ml) and 4 M HCl AcOEt solution (8 ml) was added to the mixture. After stirring at room temperature for 30 min, the precipitated solid was collected by filtration to afford **5a** (3.82 g, 88 %) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 2.51–2.53 (2H, m), 2.99–3.29 (3H, m), 3.51 (1H, dd, *J* = 13.6, 4.5 Hz), 3.90–4.06 (2H, m), 5.46–6.03 (1H, m), 7.39–7.56 (6H, m), 7.69 (2H, d, *J* = 8.4 Hz). LCMS *m/z* calcd for C₁₇H₁₇N₂OBrCl₂: 413.99, found 379.0 [M+1-HCl].

(*S*)-((5-Bromopyridin-2-yl)carbonyl)(2-(4-chlorophenyl)piperazin-1-yl)methanone hydrochloride (**5b**): Product was prepared according to the general procedure from 8.64 g of **4b** and obtained (7.81 g, quant) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.75–4.03 (6H, m), 5.37–6.06 (1H, m), 7.38–7.58 (4H, m), 7.72 (1H, d, *J* = 7.7 Hz), 8.28 (1H, brs), 8.44–9.02 (2H, m), 9.39 (1H, brs). LCMS *m/z* calcd for C₁₆H₁₆N₃OBrCl₂: 414.99, found 380.0 [M+1-HCl].

(*S*)-(4-Chlorophenyl)(2-(4-chlorophenyl)piperazin-1-yl)methanone hydrochloride (**5c**): Product was prepared according to the general procedure from 5.45 g of **4c** and obtained (4.24 g, 91%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.02–3.32 (3H, m), 3.52 (1H, dd, *J* = 13.8, 4.9 Hz), 3.84–4.12 (2H, m), 5.59 (1H, brs), 7.38–7.46 (2H, m), 7.47–7.61 (6H, m), 8.12–10.09 (2H, m). LCMS *m/z* calcd for C₁₇H₁₇N₂OCl₃: 370.04, found 335.0 [M+1-HCl].

General procedure for the preparation of compound **1**:

((3*S*)-4-(4-Bromobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)(6-bromopyrazolo[1,5-a]pyridin-3-yl)methanone (**1a**): A mixture of 6-bromopyrazolo[1,5-a]pyridine-3-carboxylic acid (120 mg, 0.50 mmol), **5a** (200 mg, 0.48 mmol), HATU (250 mg, 0.66 mmol) and Et₃N (0.15 mL, 1.08 mmol) in DMF (2 mL) was stirred at room temperature for 2 h. The mixture was extracted with AcOEt and water. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by column chromatography (NH, silica gel, eluent: 10/90 to 100/0 AcOEt/hexane) and crystallized from AcOEt and hexane to afford **1a** (173 mg, 60 %, 99.1% ee) as a white crystalline solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.46 (2H, brs), 3.64–3.90 (2H, m), 4.06 (1H, brs), 4.68 (1H, d, *J* = 9.9 Hz), 5.69 (1H, brs), 7.32 (4H, brs), 7.45 (2H, brs), 7.55 (1H, d, *J* = 9.4 Hz), 7.68 (3H, d, *J* = 7.4 Hz), 8.21 (1H, s), 9.20 (1H, s). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 37.55, 45.11, 53.39, 57.61, 106.43, 108.01, 116.03, 119.69, 123.70, 128.41, 128.88, 128.91, 129.48, 129.93, 132.04, 132.31, 135.24, 137.89, 139.46, 142.27, 148.89, 163.47, 169.73. LCMS *m/z* calcd for C₂₅H₁₉N₄O₂Br₂Cl: 599.96, found 601.0 [M+1]. Anal. Calcd for C₂₅H₁₉N₄O₂Br₂Cl: C, 49.82; H, 3.18; N, 9.30. Found: C, 49.64; H, 3.09; N, 9.28. Mp. 184–186 °C. [α]_D²⁵ -10.2° (c 1.4515, DMSO).

(6-Bromopyrazolo[1,5-a]pyridin-3-yl)((3*S*)-4-((5-bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)methanone (**1b**): Product was prepared according to the general procedure from 200 mg of **5b** and obtained (72 mg, 25 %, >99.9% ee) as a white crystalline solid after crystallization from AcOEt and hexane.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.30 (1H, s), 3.44–3.88 (3H, m), 3.98–4.32 (1H, m), 4.39–4.79 (1H, m), 5.25–5.80 (1H, m), 7.36 (4H, brs), 7.55 (1H, dd, *J* = 9.4, 1.7 Hz), 7.64–7.81 (2H, m), 8.14–8.30 (2H, m), 8.62–8.86 (1H, m), 9.20 (1H, dd, *J* = 1.7, 0.8 Hz). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 38.41, 43.39, 53.07, 57.05, 106.43, 108.01, 119.73, 121.95, 125.56, 125.85, 128.84, 128.91, 129.94, 132.33, 137.58, 137.91, 139.47, 140.73, 142.33, 149.71, 152.61, 163.50, 167.41. LCMS *m/z* calcd for C₂₄H₁₈N₅O₂Br₂Cl: 600.95, found 602.0 [M+1]. Anal. Calcd for C₂₄H₁₈N₅O₂Br₂Cl: C, 47.75; H, 3.01; N, 11.60. Found: C, 47.81; H, 3.22; N, 11.64. Mp. 160–162 °C. [α]_D²⁵ -7.4° (c 0.9560, DMSO).

((3*S*)-4-(4-bromobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)(isoquinolin-4-yl)methanone (**1c**): Product was prepared according to the general procedure from 40 mg of **5a** and obtained (35 mg, 68 %, 95.9% ee) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 2.54–2.63 (2H, m), 3.19 (3H, brs), 4.03 (1H, d, *J* = 8.0 Hz), 5.26 (1H, s), 7.26–7.88 (12H, m), 8.17 (1H, brs), 9.25–9.53 (1H, m). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 38.72, 42.17, 43.49, 46.72, 123.47, 123.76, 123.99, 127.51, 128.19, 128.56, 128.74, 129.25, 129.33, 129.47, 131.80, 131.92, 132.01, 132.72, 134.94, 135.21, 136.96, 140.16, 153.82, 154.03, 166.87, 169.72. LCMS

m/z calcd for $C_{27}H_{21}N_3O_2BrCl$: 533.05, found 534.1 [M+1]. Anal. Calcd for $C_{27}H_{21}N_3O_2BrCl-0.5H_2O$: C, 59.63; H, 4.08; N, 7.78. Found: C, 59.81; H, 4.23; N, 7.96. $[\alpha]^{25}_D +97.5^\circ$ (c 1.3520, DMSO).

((3*S*)-4-(4-bromobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)(quinolin-4-yl)methanone (1d): Product was prepared according to the general procedure from 200 mg of **5a** and obtained (248 mg, 97 %, 94% ee) as a white amorphous solid.

1H NMR (300 MHz, DMSO-*d*₆) δ 3.08 (2H, brs), 3.38–3.77 (3H, m), 4.05–4.39 (1H, m), 5.27 (1H, brs), 7.38–7.76 (11H, m), 8.04 (2H, d, *J* = 8.6 Hz), 8.92 (1H, d, *J* = 3.8 Hz). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 38.54, 41.93, 43.68, 46.51, 118.35, 123.73, 123.78, 123.89, 124.59, 125.17, 127.52, 128.25, 128.64, 129.28, 130.03, 130.53, 132.01, 132.76, 134.91, 135.17, 136.81, 141.91, 148.13, 150.84, 166.71, 169.73. LCMS *m/z* calcd for $C_{27}H_{21}N_3O_2BrCl$: 533.05, found 534.1 [M+1]. Anal. Calcd for $C_{27}H_{21}N_3O_2BrCl-0.2H_2O$: C, 60.23; H, 4.01; N, 7.80. Found: C, 60.03; H, 4.10; N, 7.63. $[\alpha]^{25}_D +76.9^\circ$ (c 1.4545, DMSO).

4-(((3*S*)-4-(4-Bromobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)isoquinolin-1(2*H*)-one (1e): Product was prepared according to the general procedure from 310 mg of **5a** and obtained (140 mg, 31 %, 95% ee) as a white crystalline solid after crystallization from AcOEt and hexane.

1H NMR (300 MHz, DMSO-*d*₆) δ 2.30–2.49 (2H, m), 3.30 (2H, brs), 3.48 (1H, d, *J* = 14.0 Hz), 4.05–4.47 (1H, m), 5.08 (1H, brs), 7.07 (3H, brs), 7.28–7.79 (9H, m), 8.08–8.34 (1H, m), 11.46 (1H, brs). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 14.45, 22.55, 31.44, 60.23, 112.09, 123.72, 124.13, 124.24, 126.19, 127.31, 127.55, 128.94, 129.05, 129.43, 132.04, 132.47, 132.55, 132.90, 133.15, 135.10, 137.14, 137.25, 161.61, 161.72, 166.52, 169.74. LCMS *m/z* calcd for $C_{27}H_{21}N_3O_3BrCl$: 549.05, found 550.1 [M+1]. Anal. Calcd for $C_{27}H_{21}N_3O_3BrCl$: C, 58.87; H, 3.84; N, 7.63. Found: C, 58.51; H, 3.80; N, 7.52. Mp. 225–227 °C. $[\alpha]^{25}_D +66.3^\circ$ (c 1.3770, DMSO).

4-((4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)isoquinolin-1(2*H*)-one (1f): Product was prepared according to the general procedure from 50 mg of **5c** and obtained (35 mg, 52%) as a white amorphous solid.

1H NMR (300 MHz, DMSO-*d*₆) δ 2.39–2.50 (4H, m), 3.21–3.32 (2H, m), 3.39–3.66 (1H, m), 6.94–7.63 (12H, m), 8.11–8.28 (1H, m), 11.45 (1H, brs). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 14.44, 22.54, 31.44, 60.23, 112.15, 123.14, 124.16, 124.34, 126.20, 127.32, 127.52, 128.92, 129.13, 129.37, 132.04, 132.48, 132.93, 134.73, 135.00, 135.16, 137.16, 137.25, 161.63, 161.72, 166.53, 169.72. LCMS *m/z* calcd for $C_{27}H_{21}N_3O_3Cl_2$: 505.10, found 506.1 [M+1]. Anal. Calcd for $C_{27}H_{21}N_3O_3Cl_2$: C, 64.04; H, 4.18; N, 8.30. Found: C, 63.71; H, 4.36; N, 8.12.

5-((3*S*)-4-((5-Bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-1-methyl-3-(trifluoromethyl)pyridin-2(1*H*)-one (1p): Product was prepared according to the general procedure from 35 mg of **4b** and obtained (45 mg, 92%, 96% ee) as a white crystalline solid after crystallization from AcOEt and hexane.

1H NMR (300 MHz, DMSO-*d*₆) δ 3.32–3.43 (3H, m), 3.50 (3H, s), 3.80 (2H, brs), 4.33–4.70 (1H, m), 5.21–5.82 (1H, m), 7.22–7.49 (4H, m), 7.52–7.78 (2H, m), 8.12–8.30 (2H, m), 8.61–8.84 (1H, m). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 38.01, 38.03, 43.17, 52.66, 56.89, 111.46, 117.08 (q, *J* = 30.44 Hz), 121.43, 122.00, 125.02, 125.66, 125.91, 129.02, 132.46, 137.49, 138.92, 140.75, 145.70, 149.71, 152.51, 157.86, 166.00, 167.42. LCMS *m/z* calcd for $C_{24}H_{19}N_4O_3BrClF_3$: 581.03, found 583.1 [M+1]. Anal. Calcd for $C_{24}H_{19}N_4O_3BrClF_3$: C, 49.38; H, 3.28; N, 9.60. Found: C, 49.53; H, 3.43; N, 9.55. Mp. 201–203 °C. $[\alpha]^{25}_D -1.5^\circ$ (c 1.3805, DMSO).

(5-Bromo-6-methoxypyridin-3-yl)(4-(4-chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)methanone (1r): Product was prepared according to the general procedure from 2.6 g of **5c** and obtained (3.51 g, 91%) as a white amorphous solid.

1H NMR (300 MHz, DMSO-*d*₆) δ 2.49 (2H, brs), 3.32 (3H, s), 3.96 (3H, s), 4.88 (1H, brs), 5.53 (1H, s), 7.04–7.85 (9H, m), 8.05 (1H, brs). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 33.20, 43.35, 46.29, 55.23, 62.67, 106.32, 126.44, 128.94, 129.08, 129.13, 129.32, 132.44, 134.75, 135.02, 137.63, 137.72, 141.13, 141.20, 144.92, 160.39, 166.46, 169.60, 169.66. LCMS *m/z* calcd for $C_{24}H_{20}N_3O_3BrCl_2$: 547.01, found 548.0 [M+1]. Anal. Calcd for $C_{24}H_{20}N_3O_3BrCl_2$: C, 52.48; H, 3.67; N, 7.65. Found: C, 52.31; H, 3.73; N, 7.68.

3-((3*S*)-4-((5-Bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-2-methoxypyridin-3-yl)benzonitrile (1s): Product was prepared according to the general procedure from 2 g of **5b** and obtained (2.5 g, 85%, 94% ee) as a white amorphous solid.

1H NMR (300 MHz, DMSO-*d*₆) δ 2.51–2.56 (2H, m), 3.40–3.88 (3H, m), 3.94 (3H, s), 4.42 (1H, brs), 5.25–5.92 (1H, m), 7.38 (4H, brs), 7.60–7.77 (3H, m), 7.88 (2H, t, *J* = 7.4 Hz), 8.02 (1H, s), 8.10–8.30 (2H, m), 8.61–8.85 (1H, m). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 38.22, 43.24, 52.62, 54.51, 56.75, 112.03, 119.13, 121.69, 121.98, 125.61, 125.73, 125.89, 128.97, 129.98, 132.01, 132.45, 132.52, 133.07, 134.43, 136.99, 137.61, 138.42, 140.73, 146.16, 149.70, 152.52, 161.10, 167.40, 167.62. LCMS *m/z* calcd for $C_{30}H_{23}N_5O_3BrCl$: 615.07, found 616.1 [M+1]. Anal. Calcd for $C_{30}H_{23}N_5O_3BrCl$: C, 58.41; H, 3.76; N, 11.35. Found: C, 58.06; H, 4.00; N, 11.22. $[\alpha]^{25}_D +7.8^\circ$ (c 1.4535, DMSO).

(5-Bromopyridin-2-yl)((2*S*)-2-(4-chlorophenyl)-4-((6-methoxy-5-(trifluoromethyl)pyridin-3-yl)carbonyl)piperazin-1-yl)methanone (1t): Product was prepared according to the general procedure from 2 g of **5b** and obtained (2.52 g, 90%, 94% ee) as a white amorphous solid.

1H NMR (300 MHz, DMSO-*d*₆) δ 3.32–3.35 (3H, m), 3.95–4.06 (5H, m), 4.40 (1H, s), 4.90–5.40 (1H, m), 7.40 (5H, brs), 7.68 (1H, d, *J* = 8.7 Hz), 8.25 (1H, d, *J* = 7.3 Hz), 8.36 (1H, brs), 8.79 (1H, brs). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ 38.11, 43.13, 52.04, 55.07, 56.86, 111.72 (q, *J* = 32.83 Hz), 121.27, 121.99, 124.83, 125.70, 125.92, 128.48, 128.98, 132.50, 136.77, 137.60, 140.72, 149.68, 150.22, 152.48, 160.94, 166.65, 167.40. LCMS *m/z* calcd for $C_{24}H_{19}N_4O_3BrClF_3$: 582.03, found 583.0 [M+1]. Anal. Calcd for $C_{24}H_{19}N_4O_3BrClF_3$: C, 49.38; H, 3.28; N, 9.60. Found: C, 49.32; H, 3.52; N, 9.63. $[\alpha]^{25}_D +10.9^\circ$ (c 1.6155, DMSO).

General method for high-throughput chemistry: A mixture of **1r** (55 mg, 100 μ mol), boronic acid (130 μ mol), (A-taPhos)₂PdCl₂ (7.08 mg, 10.00 μ mol), Cs₂CO₃ (98 mg, 300 μ mol) and DME (1 mL) was heated at 110 °C for 1 h under microwave irradiation. The reaction mixture was poured into AcOEt (3 mL) and water (1 mL), and stirred for 5 min. The organic layer was filtered, and the filtrate was evaporated by blowing away with the air at 60 °C. The residue was dissolved in CH₃CN (1 mL). TMSCl (0.064 mL, 500 μ mol) and sodium iodide (0.03 g, 200 μ mol) were added to the solution. The mixture was stirred at 60 °C for 2 h. The reaction mixture was diluted with AcOEt (3 mL) and quenched with water (1 mL), and stirred for 2 min. The organic layer was separated and then the reaction mixture was extracted

with AcOEt (2 mL) from an aqueous layer. The combined organic layers were evaporated by blowing away with the air at 60 °C. The residue was purified by preparative HPLC (Actus Triart C18, eluted with CH₃CN / 10 mM NH₄HCO₃ aqueous solution 5:95→100:0). Colored impurity was removed by filtration through StratoSpheres SPE (PL-HCO₃ MP-Resin). Pure fractions were combined and concentrated by evaporation under air at 60 °C to afford product (**1g–1m**).

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-phenylpyridin-2(1*H*)-one (1g**):** Product was prepared according to the general procedure and obtained (4.9 mg, 9%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.33 (3H, brs), 3.69 (1H, d, *J* = 10.8 Hz), 3.83–4.02 (1H, m), 4.57 (1H, d, *J* = 12.6 Hz), 5.64 (1H, brs), 7.17–7.43 (8H, m), 7.45 (1H, brs), 7.52 (4H, brs), 7.65 (2H, dd, *J* = 8.2, 1.4 Hz), 12.09 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.45, 22.55, 31.44, 45.58, 113.47, 127.28, 128.14, 128.44, 128.67, 129.01, 129.04, 129.14, 129.32, 129.48, 129.88, 132.40, 134.78, 135.01, 136.02, 136.13, 136.40, 137.64, 161.28, 167.19, 169.61, 169.67. LCMS *m/z* calcd for C₂₉H₂₃N₃O₃Cl₂: 531.11, found 532.2 [M+1]. Anal. Calcd for C₂₉H₂₃N₃O₃Cl₂: C, 65.42; H, 4.35; N, 7.89. Found: C, 65.65; H, 4.65; N, 7.66.

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-(2-chlorophenyl)pyridin-2(1*H*)-one (1h**):** Product was prepared according to the general procedure and obtained (8.4 mg, 15%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.16–3.48 (3H, m), 3.67 (1H, d, *J* = 9.1 Hz), 3.92 (1H, brs), 4.35–4.68 (1H, m), 5.63 (1H, brs), 7.21 (1H, brs), 7.27–7.43 (7H, m), 7.46–7.61 (6H, m), 12.07 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.57, 21.25, 34.84, 60.23, 112.70, 127.39, 128.93, 129.01, 129.14, 129.29, 129.56, 129.68, 129.91, 132.28, 132.36, 133.17, 134.78, 135.00, 135.78, 137.31, 137.62, 137.69, 139.56, 160.60, 166.99, 169.69, 170.80. LCMS *m/z* calcd for C₂₉H₂₂N₃O₃Cl₃: 565.07, found 566.1 [M+1]. Anal. Calcd for C₂₉H₂₂N₃O₃Cl₃–0.3H₂O: C, 60.87; H, 3.98; N, 7.34. Found: C, 60.67; H, 3.91; N, 7.47.

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-(3-chlorophenyl)pyridin-2(1*H*)-one (1i**):** Product was prepared according to the general procedure and obtained (9.1 mg, 16%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.19–3.35 (3H, m), 3.72 (1H, brs), 3.85–4.00 (1H, m), 4.54 (1H, brs), 5.61 (1H, brs), 7.22–7.44 (6H, m), 7.45–7.57 (6H, m), 7.60 (1H, dt, *J* = 6.6, 1.9 Hz), 7.81 (1H, d, *J* = 1.8 Hz), 12.21 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.57, 21.24, 31.44, 60.23, 113.56, 127.17, 127.50, 127.96, 128.06, 128.38, 129.03, 129.14, 129.28, 130.34, 132.39, 133.14, 134.32, 134.77, 135.02, 136.80, 137.56, 137.65, 138.27, 138.41, 161.06, 166.99, 169.67, 170.77. LCMS *m/z* calcd for C₂₉H₂₂N₃O₃Cl₃: 565.07, found 566.1 [M+1]. Anal. Calcd for C₂₉H₂₂N₃O₃Cl₃: C, 61.45; H, 3.91; N, 7.41. Found: C, 61.33; H, 4.28; N, 7.03.

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-(4-chlorophenyl)pyridin-2(1*H*)-one (1j**):** Product was prepared according to the general procedure and obtained (8.8 mg, 16%) as a white crystalline solid after crystallization from AcOEt and hexane.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.14–3.36 (3H, m), 3.69 (1H, d, *J* = 13.4 Hz), 3.90 (1H, brs), 4.53 (1H, brs), 5.65 (1H, brs), 7.17–7.40 (4H, m), 7.41–7.64 (8H, m), 7.67–7.76 (2H, m), 12.16 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.63, 21.13, 34.59, 60.54, 113.53, 128.44, 129.01, 129.04, 129.14, 129.26, 129.29, 130.41, 132.39, 132.78, 134.77, 135.01, 135.16, 136.48, 137.68, 137.85, 161.11, 167.07, 169.65, 170.83. LCMS *m/z* calcd for C₂₉H₂₂N₃O₃Cl₃: 565.07, found 566.1 [M+1]. Anal. Calcd for C₂₉H₂₂N₃O₃Cl₃: C, 61.45; H, 3.91; N, 7.41. Found: C, 61.24; H, 4.05; N, 7.33. Mp. 184–186 °C.

3-(5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-2-oxo-1,2-dihydropyridin-3-yl)benzonitrile (1k**):** Product was prepared according to the general procedure and obtained (11.3 mg, 20%) as a white crystalline solid after crystallization from AcOEt and hexane.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.33 (3H, brs), 3.72 (1H, brs), 3.96 (1H, d, *J* = 18.7 Hz), 4.54 (1H, brs), 5.63 (1H, brs), 7.25–7.42 (4H, m), 7.46–7.59 (6H, m), 7.60–7.65 (1H, m), 7.81 (1H, dt, *J* = 7.8, 1.3 Hz), 8.02 (1H, dt, *J* = 8.0, 1.4 Hz), 8.16 (1H, t, *J* = 1.4 Hz), 12.27 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.57, 21.24, 31.07, 60.23, 111.64, 113.64, 119.31, 127.38, 129.03, 129.14, 129.29, 129.72, 131.63, 132.15, 132.38, 132.68, 133.38, 134.77, 135.02, 137.28, 137.51, 137.67, 138.59, 161.10, 162.35, 166.93, 167.19, 169.67, 170.80. LCMS *m/z* calcd for C₃₀H₂₂N₄O₃Cl₂: 556.11, found 557.1 [M+1]. Anal. Calcd for C₃₀H₂₂N₄O₃Cl₂–0.2H₂O: C, 64.22; H, 4.02; N, 9.99. Found: C, 64.02; H, 4.22; N, 9.88. Mp. 214–216 °C.

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-(3-(trifluoromethyl)phenyl)pyridin-2(1*H*)-one (1l**):** Product was prepared according to the general procedure and obtained (23.6 mg, 39%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.24–3.32 (3H, m), 3.71 (1H, brs), 3.86–4.02 (1H, m), 4.55 (1H, brs), 5.47–5.74 (1H, m), 7.36 (4H, brs), 7.52 (5H, brs), 7.60–7.77 (3H, m), 7.93 (1H, d, *J* = 7.6 Hz), 8.11 (1H, s), 12.24 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.55, 21.23, 31.75, 60.22, 113.64, 122.95, 124.71 (q, *J* = 4.77 Hz), 125.16 (q, *J* = 3.85 Hz), 126.55, 128.00, 128.60, 129.02, 129.13, 129.29, 129.43, 129.63, 129.86, 130.16, 132.38, 132.50, 134.77, 135.01, 136.91, 137.31, 137.61, 138.54, 161.11, 166.98, 169.66, 170.80. LCMS *m/z* calcd for C₃₀H₂₂N₃O₃Cl₂F₃: 599.10, found 600.1 [M+1]. Anal. Calcd for C₃₀H₂₂N₃O₃Cl₂F₃–0.7H₂O: C, 58.78; H, 3.85; N, 6.85. Found: C, 59.01; H, 3.98; N, 6.94.

5-((4-(4-Chlorobenzoyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-3-(3-(methylsulfonyl)phenyl)pyridin-2(1*H*)-one (1m**):** Product was prepared according to the general procedure and obtained (4.2 mg, 7%) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.24 (3H, s), 3.32 (3H, brs), 3.71 (1H, brs), 3.89–4.03 (1H, m), 4.57 (1H, d, *J* = 8.4 Hz), 5.49–5.78 (1H, m), 7.27–7.44 (4H, m), 7.52 (5H, brs), 7.61 (1H, brs), 7.68 (1H, t, *J* = 7.8 Hz), 7.89 (1H, d, *J* = 8.4 Hz), 8.02 (1H, d, *J* = 7.9 Hz), 8.28 (1H, s), 12.27 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 14.57, 21.25, 31.31, 44.07, 60.24, 113.59, 126.46, 126.92, 127.90, 128.35, 128.99, 129.05, 129.14, 129.27, 129.63, 132.40, 133.54, 134.76, 135.02, 136.68, 137.15, 137.53, 137.66, 138.70, 141.13, 161.20, 167.01, 169.66, 170.60. LCMS *m/z* calcd for C₃₀H₂₅N₃O₃Cl₂S: 609.09, found 610.1 [M+1]. Anal. Calcd for C₃₀H₂₅N₃O₃Cl₂S–0.5H₂O: C, 58.16; H, 4.23; N, 6.78. Found: C, 58.34; H, 4.40; N, 6.69.

3-(5-((3S)-4-((5-Bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-2-oxo-1,2-dihydropyridin-3-yl)benzonitrile (1n**):** A mixture of **1s** (2.5 g, 4.1 mmol), TMSCl (1 mL, 7.8 mmol) and sodium iodide (1.2 g, 8 mmol) in CH₃CN (15 mL) was stirred at

80 °C for 1 h. After cooling to room temperature, the mixture was extracted with AcOEt and water. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, eluent: 0/100 to 20/80 MeOH/AcOEt) and crystallized from AcOEt and hexane to afford **1n** (2.24 g, 92 %, 93% ee) as a white crystalline solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.33–3.42 (2H, m), 3.64–4.02 (3H, m), 4.34–4.68 (1H, m), 5.23–5.79 (1H, m), 7.39 (4H, brs), 7.51–7.71 (4H, m), 7.81 (1H, dt, *J* = 7.8, 1.3 Hz), 7.99–8.07 (1H, m), 8.17 (1H, t, *J* = 1.5 Hz), 8.25 (1H, d, *J* = 9.0 Hz), 8.63–8.85 (1H, m), 12.27 (1H, brs). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 38.22, 43.23, 52.63, 56.83, 111.63, 113.67, 119.31, 121.88, 121.98, 125.66, 125.89, 127.43, 128.99, 129.73, 131.64, 132.16, 132.40, 133.38, 137.23, 137.47, 137.60, 138.65, 140.75, 149.54, 149.71, 152.53, 161.04, 166.93, 167.39. LCMS *m/z* calcd for C₂₉H₂₁N₅O₃BrCl: 601.05, found 602.1 [M+1]. Anal. Calcd for C₂₉H₂₁N₅O₃BrCl-0.5H₂O: C, 56.93; H, 3.62; N, 11.45. Found: C, 57.12; H, 3.83; N, 11.34. Mp. 231–233 °C. [α]²⁵_D -0.3° (c 1.5220, DMSO).

3-((3S)-4-((5-Bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-1-methyl-2-oxo-1,2-dihydropyridin-3-ylbenzonitrile (**1o**): A mixture of **1n** (2.24 g, 3.7 mmol), MeI (0.4 mL, 6.4 mmol) and K₂CO₃ (1 g, 7.2 mmol) in DMF (15 mL) was stirred at room temperature for 1 h. The mixture was extracted with AcOEt and water. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by column chromatography (NH silica gel, eluent: 10/90 to 100/0 AcOEt/hexane) to afford **1o** (2.08 g, 91 %, 94% ee) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.32–3.45 (2H, m), 3.54 (3H, s), 3.68–4.03 (3H, m), 4.34–4.70 (1H, m), 5.24–5.82 (1H, m), 7.39 (4H, brs), 7.62 (2H, t, *J* = 7.8 Hz), 7.69 (1H, d, *J* = 8.1 Hz), 7.81 (1H, dt, *J* = 7.8, 1.3 Hz), 7.93–8.04 (2H, m), 8.14 (1H, t, *J* = 1.5 Hz), 8.23–8.33 (1H, m), 8.62–8.83 (1H, m). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 38.19, 38.36, 43.30, 52.67, 56.83, 111.63, 113.26, 119.29, 121.98, 125.65, 125.88, 126.35, 129.00, 129.70, 131.66, 132.34, 132.41, 133.60, 137.35, 137.59, 137.66, 137.72, 140.75, 141.80, 149.71, 152.53, 160.66, 166.81, 167.42. LCMS *m/z* calcd for C₃₀H₂₃N₅O₃BrCl: 615.07, found 616.1 [M+1]. Anal. Calcd for C₃₀H₂₃N₅O₃BrCl-0.4H₂O: C, 57.73; H, 3.84; N, 11.22. Found: C, 57.98; H, 4.04; N, 10.94. [α]²⁵_D +3.5° (c 0.4380, DMSO).

5-((3S)-4-((5-Bromopyridin-2-yl)carbonyl)-3-(4-chlorophenyl)piperazin-1-yl)carbonyl)-1-phenyl-3-(trifluoromethyl)pyridin-2(1H)-one (**1q**): A mixture of **1t** (2.52 g, 4.32 mmol), sodium iodide (1.3 g, 8.67 mmol) and TMSCl (1.2 mL, 9.39 mmol) in CH₃CN (15 mL) was stirred at 80 °C for 30 min. After cooling to room temperature, the mixture was extracted with AcOEt and water. The organic layer was washed with brine, dried over MgSO₄ and concentrated under reduced pressure. To the residue were added pyridine (15 mL), phenylboronic acid (0.9 g, 7.38 mmol) and Cu(OAc)₂ (0.9 g, 4.96 mmol). The mixture was stirred at 80 °C for 1 h. After cooling to room temperature, the residue was purified by column chromatography (NH silica gel, eluent: 10/90 to 100/0 AcOEt/hexane) to afford **1q** (2.4 g, 86 %, 94% ee) as a white amorphous solid.

¹H NMR (300 MHz, DMSO-*d*₆) δ 3.35 (2H, brs), 3.69–4.02 (3H, m), 4.32–4.68 (1H, m), 5.23–5.80 (1H, m), 7.30–7.43 (4H, m), 7.48–7.59 (5H, m), 7.67 (1H, d, *J* = 8.4 Hz), 7.82 (1H, brs), 8.07 (1H, brs), 8.13–8.30 (1H, m), 8.59–8.84 (1H, m). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 38.05, 43.09, 52.64, 56.51, 112.11, 118.87 (q, *J* = 30.44 Hz), 121.26, 122.01, 124.86, 125.69, 125.91, 127.49, 129.00, 129.55, 129.65, 132.47, 137.31, 137.52, 139.77, 140.05, 140.09, 140.75, 144.17, 149.71, 152.48, 157.35, 165.77, 167.38. LCMS *m/z* calcd for C₂₉H₂₁N₄O₃BrClF₃: 644.04, found 645.0 [M+1]. Anal. Calcd for C₂₉H₂₁N₄O₃BrClF₃-0.5H₂O: C, 53.19; H, 3.39; N, 8.56. Found: C, 53.48; H, 3.66; N, 8.53. [α]²⁵_D +5.5° (c 1.4995, DMSO).

Preparation of enzymes. The human recombinant proteins, full-length eIF4A3, MLN51(residues 137-283), full-length eIF4A1, full-length eIF4B, and eIF4G (residues 712-1451) were expressed in *Escherichia coli* BL21(DE3) as fusion proteins with 6 × histidine (His)-small ubiquitin-like modifier (SUMO) or His tags followed by a tobacco etch virus (TEV) protease cleavage site at the N terminus and purification by Ni-NTA superflow affinity column (QIAGEN) and Superdex 200 gel-filtration column (GE Healthcare). The His-SUMO or His tags were cleaved with SUMO protease or TEV protease. Human recombinant full-length DHX29 and full-length Brr2 were expressed in Sf-9 insect cells as fusion proteins with FLAG-His or His tags at the N terminus, respectively, using the BaculoDirect™ C-term Baculovirus expression system (Thermo Fisher Scientific Inc.). FLAG-His-DHX29 and His-Brr2 were purified by Ni-NTA superflow affinity column and Superdex 200 gel-filtration column. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) with bovine serum albumin as a standard. Recombinant human His-eIF4A2 protein was purchased from GeneCopoeia Inc..

RNA-dependent ATPase assay. The RNA-dependent ATPase assay was performed using the ADP-Glo™ assay system (Promega Corp.). Single-stranded RNA poly(U) was purchased from MP Biomedicals, LLC. The assay buffer comprised 20 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol (DTT), and 0.01% (v/v) Tween20. To enhance ATPase activity for eIF4A, the equivalent molar concentration of MLN51 for 150 nM eIF4A3 or eIF4B and eIF4G for 100 nM eIF4A1 or eIF4A2 were added. Regarding the ATPase assays for DHX29 or Brr2, the optimal concentrations were 6.4 nM and 6.25 nM, respectively. Concentrations of ATP or RNA were set at the *Km* value of each substrate for each enzyme as follows: 35 μM ATP and 1.5 μg mL⁻¹ poly(U) for eIF4A1 and eIF4A3; 20 μM ATP and 3.0 μg mL⁻¹ poly(U) for eIF4A2; 30 μM ATP and 1.8 μg mL⁻¹ poly(U) for DHX29; and 20 μM ATP and 2.5 μg mL⁻¹ poly(U) for Brr2. After the addition of the substrates and test compounds, the ATPase reactions were started by the addition of the enzymes. They were incubated at room temperature for 30 min for eIF4A3, DHX29, and Brr2 or 40 min for eIF4A1 and eIF4A2. The enzymatic reactions were terminated by ADP-Glo™ reagent, and then ADP-Glo™ detection reagent was added to detect adenosine diphosphate. Luminescent signals were measured using an EnVision™ 2102 Multilabel Plate Reader (PerkinElmer). We defined the luminescent signals of the reaction without enzyme as 100% inhibitory activity and those of the complete reaction mixture as 0% inhibitory activity. Curve fittings and calculations of IC₅₀ values were performed using the program XLfit version 5 (ID Business Solutions Ltd.) with the maximum and minimum of the curve constrained to 100 and 0, respectively.

In vitro PD assay. HCT 116 cells (American Type Culture Collection) were seeded in 96-well plate at 5,000 cells/well in McCoy's 5A (Modified) medium (Thermo Fisher Scientific) containing 10% fetal bovine serum. Two days after plating, cells were treated with test compounds dissolved in DMSO. Six hours after the treatment, cells were lysed, reverse transcribed, and subjected to quantitative polymerase chain reaction (qPCR) using TaqMan Gene Expression Cells-to-Ct Kit (Thermo Fisher Scientific). The expression levels of SC35 1.6 kb and GAPDH were measured using the following oligonucleotides (forward primer: 5'-GAAAATGATGTATCGGCAAGCA-3', TaqMan MGB probe: 5'-FAM-TGTAAACGGAGGACTTG-MGB-3', reverse primer: 5'-TACACAACTGCGCTTTCAA-3') and TaqMan Gene Expression Assays for GAPDH (4326317E, Thermo Fisher Scientific), respectively. The SC35 1.6 kb mRNA expression levels were normalized using GAPDH mRNA expression for each sample, and relative expression levels to control groups were calculated.

In vitro cell growth assay. HCT 116 cells were seeded in 96-well plate at 1,500 cells/well. Twenty-four hours after plating, cells were treated with test compounds at 0.3 to 30 μ M. After 3 days, cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). GI₅₀ values were calculated using GraphPad Prism 6 software by setting cell viability in control groups as 100%.

In vivo PD study. Female BALB/cA Jcl-*nu/nu* (Nude) mice (CLEA Japan) were maintained under specific pathogen-free conditions and used in compliance with the guidelines of the Takeda Institutional Animal Care and Use Committee in the facility accredited by the American Association for Accreditation of Laboratory Animal Care. HCT 116 cells (1×10^6 cells) were inoculated with Matrigel (Corning) into the flank of 6-week old nude mice. Thirteen days after the inoculation, the mice were orally administered with test compounds dissolved in 10% DMSO / 10% Cremophor EL / 30% PEG400 / 20% propylene glycol / 30% 0.4 mol/L Citrate solution (n = 3 at each time point). The mice were euthanized 4, 8, and 24 hours after the administration, and tumor samples were collected in RNAlater Stabilization Solution (Thermo Fisher Scientific). Total RNA was prepared using an RNeasy Mini Kit (Qiagen) and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Expression levels of SC35 1.6 kb and GAPDH mRNA in aliquots of the cDNA were measured by qPCR as described in "in vitro PD assay" section.

In vivo efficacy study. HCT 116 cells (1×10^6 cells) were inoculated with Matrigel into the flank of female nude mice. Eleven days after the inoculation, tumor size was measured and tumor volume was calculated using following equation: tumor volume (mm^3) = A \times B² \times 0.5, where A is the largest diameter of the tumor (mm) and B is the smallest diameter of the tumor (mm). The mice were randomized into three groups (n = 6) based on their tumor volumes. Test compounds were dissolved in the solution described in "in vivo PD study" section, and orally administered twice-daily for 14 days. Tumor size and body weight were measured twice a week. Percentage treated/control ratio (T/C (%)) was calculated by dividing the change in volume of the tumors in the treated mice by the change in volume in the mice treated by vehicle.

Statistical analysis. Data were represented as mean \pm standard deviation (SD). Statistical differences between the control and the treated groups were analyzed by Dunnett's test, and a value of P < 0.05 was considered significant.

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