Supporting information

Developing potent anti-inflammatory IRAK4-targeting PROTACs with simplified CRBN ligands

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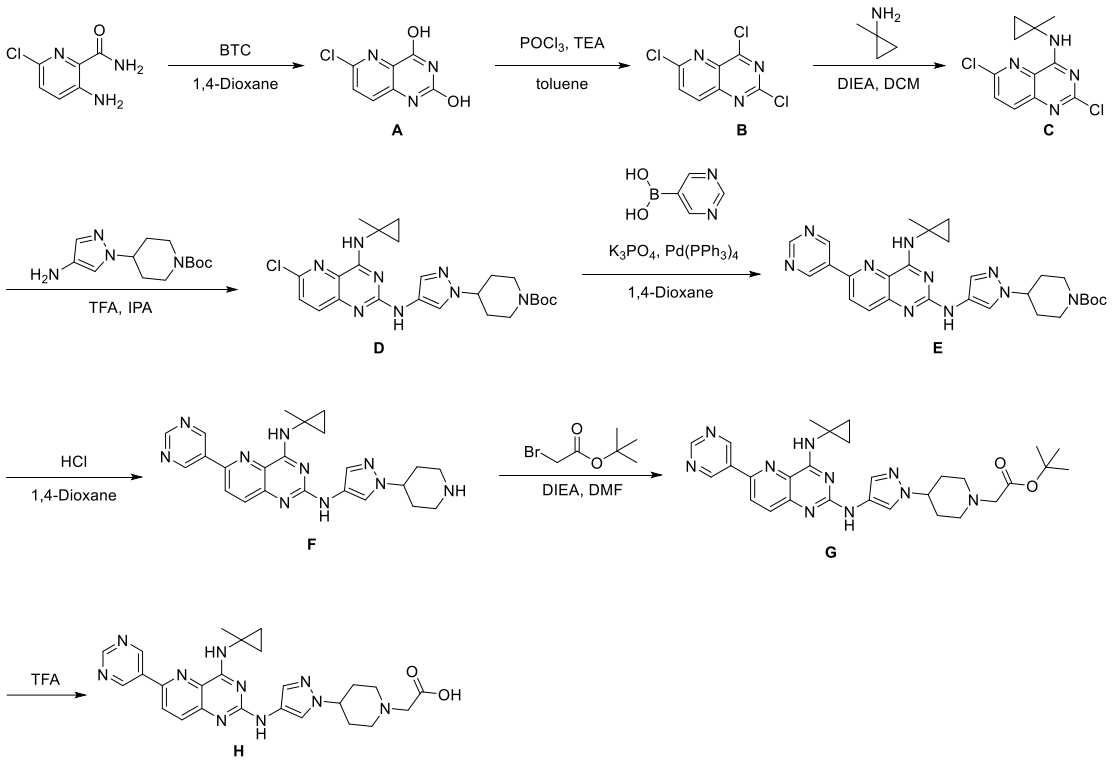
Table of contents

Supplementary information, Data S1

Materials and methods

All commercial materials (Selleck, Alfa Aesar, Aladdin, Bide Pharmatech LTD, J&K Chemical LTD, Energy Chemical.) were utilized without further purification. All solvents were analytical grade. The NMR spectra was generated on a Bruker AVANCE^{III}400 MHz spectrometer in DMSO-d_6, $CDCl_3$ using solvent peak as standard. Low-resolution mass spectral analyses were performed with Waters AQUITY UPLC^{TM}/MS. Flash column chromatography was performed on Qingdao Haiyang Chemical Co. Ltd silica gel 60 (200-300mesh). Analytical TLC was performed on Yantai Chemical Industry Research Institute silica gel 60 F254 plates and the rotavapor was BUCHI's Rotavapor R-3.

Synthesis of IRAK4 binder



Scheme S1: Synthetic route of IRAK4 binders

The preparation of compound **A**:

3-amino-6-chloropyridine-2-carboxamide (3 g, 17.5 mmol) was dissolved in1,4-dioxane (100 mL), and then trichloroisocyanuric acid (2.6 g, 8.75 mmol) was added. The reaction mixture was stirred at 105°C for 3 hours. After the reaction is completed, it was allowed to cool to room temperature, following by addition of 1 mL of water with stirring for 30 minutes. Then the mixture was filtered, and washed with ethyl acetate twice. The cake was dryed by concentrating under vacuum to give a pale yellow product **A** with a yield of 85%.

The preparation of compound **B**:

Compound **A** (1 g, 5 mmol) was dispersed in toluene (10 mL), then phosphorus trichloride (1.7 g, 11.1 mmol) and N, N-diisopropylethylamine (1.94 mL, 11.1 mmol) were added. The reaction mixture was heated to reflux and stirred for 5 hours. After the reaction was completed, it was allowed to cool to room temperature, followed

by dilution with ethyl acetate. The organic layer was washed with saturated sodium bicarbonate and saturated brine, then dried with anhydrous sodium sulfate. The mixture was concentrated under vacuum, and the product was purified by silica gel chromatography using a petroleum ether to ethyl acetate ratio of 50:1 to obtain product **B** with a yield of 65%.

The preparation of compound **C**:

Compound **B** (1 g, 4.27 mmol) was added to a mixture containing 1-methylcyclopropane amine hydrochloride (0.48 g, 4.5 mmol) and N, N-diisopropylethylamine (2.4 mL, 13.5 mmol) in dichloromethane (20 mL), and the reaction mixture was stirred at 0°C for 30 minutes. After the reaction was completed, it was washed once with saturated brine, dried with anhydrous sodium sulfate, and concentrated under vacuum to obtain a pale yellow product **C** with a yield of 94%.

The preparation of compound **D**:

Compound **C** (1 g, 3.9 mmol) and *tert*-butyl 4-(3-amino-1H-pyrazol-1-yl)piperidine-1-carboxylate (1 g, 3.9 mmol) were dissolved in tert-butanol (20 mL), and a catalytic amount of trifluoroacetic acid (20 μL) was added. The mixture was heated to reflux and stirred for 8 hours. After the reaction was complete, the solvent tert-butanol was removed under reduced pressure. Then, 10% sodium bicarbonate solution (10 mL) was added, and the mixture was stirred for 30 minutes. The reaction mixture was filtered, and the cake was washed with a small amount of water three times. The cake was dried to obtain a crude product **D** with a yield of 82%.

The preparation of compound **E**:

Compound **D** (1 g, 2 mmol), 5-pyrimidinylbenzene boronic acid (330 mg, 2.6 mmol), and tetrakis(triphenylphosphine)palladium (120 mg, 0.1 mmol) were dispersed in 10 mL of 1,4-dioxane, and an aqueous solution of potassium phosphate (850 mg, 4 mmol) in water (2 mL) was added to the suspension. The suspension was deoxygenated under vacuum and purged with argon gas three times. It was then heated to 80°C and stirred under argon for 4 hours. After the reaction was complete, the reaction mixture was diluted ethyl acetate. The organic layer was washed with water and saturated brine, dried with anhydrous sodium sulfate, and concentrated under vacuum. The product was purified by silica gel chromatography using a dichloromethane to methanol ratio of 30:1 to obtain a yellow product **E** with a yield of 55%.

The preparation of compound **F**:

Compound **E** (1 g, 1.84 mmol) was dissolved in a 1 M solution of hydrochloric acid in 1,4-dioxane, and the reaction mixture was stirred at 25°C for 4 hours. After the reaction was complete, the reaction mixture was concentrated under vacuum to obtain a pale yellow product **F** with a yield of 95%.

The preparation of compound **G**:

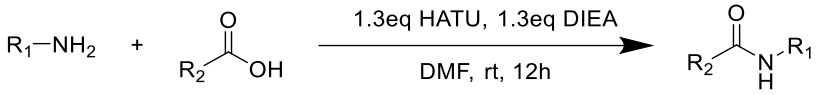
Compound **F** (530 mg, 1.2 mmol), tert-butyl bromoacetate (254 mg, 1.3 mmol), and potassium carbonate (330 mg, 2.4 mmol) were dissolved in DMF, and the reaction mixture was stirred at 60°C for 1 hour. After the reaction was complete, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and saturated brine, dried with anhydrous sodium sulfate, and concentrated under vacuum. The product was purified by silica gel chromatography using a dichloromethane to methanol ratio of 50:1 to obtain a yellow product **G** with a yield of 82%.

The preparation of compound **H**:

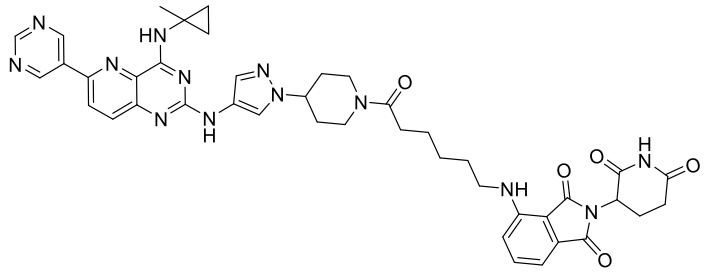
Compound **G** (300 mg, 0.54 mmol) was dissolved in TFA (1.5 mL) and stirred at room temperature overnight. After the reaction was complete, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and saturated brine, dried with anhydrous sodium sulfate, and concentrated under vacuum. The product was purified by silica gel chromatography using a dichloromethane to methanol ratio of 30:1 to obtain a yellow product **H** with a yield of 74%.

Synthesis of IRAK4 degraders based on IMiDs-like CRBN ligands

General procedure for IRAK4 degraders based on IMiDs-like CRBN ligands

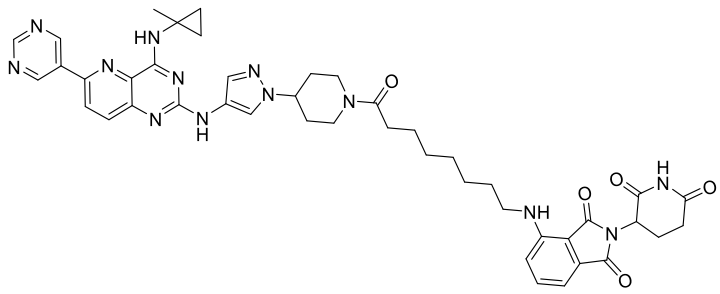


A mixture of amine derivative (1 eq.), carboxylic acid derivative (1 eq.), HATU (1.2 eq.), DIEA (2 eq.), in DMF (10 mL) was stirred in a round bottom flask for 2 h at room temperature. The reaction was quenched by saturated aqueous NaCl and the mixture was extracted by ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (DCM: MeOH = 30: 1) to give the corresponding amide derivatives.



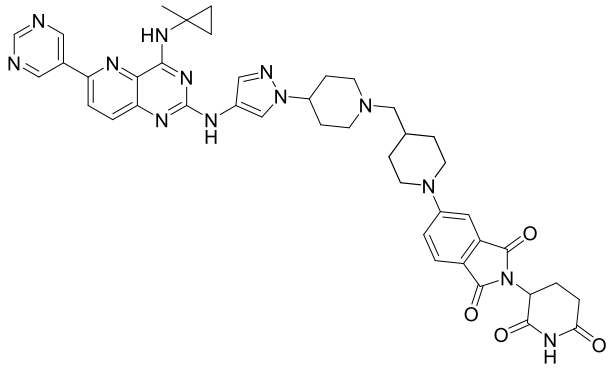
2-(2,6-dioxopiperidin-3-yl)-4-((6-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)-6-oxohexyl)amino)isoindoline-1,3-dione (PL-14)

^{1}H NMR (400 MHz, Chloroform-d) δ 9.44 (s, 2H), 9.21 (s, 1H), 8.10 (s, 1H), 8.05 (d, *J* = 8.8 Hz, 1H), 7.88 (d, *J* = 8.8 Hz, 1H), 7.79 (s, 1H), 7.56-7.49 (m, 1H), 7.08 (d, *J* = 7.1 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 1H), 5.00-4.93 (m, 1H), 4.75-4.71 (m, 1H), 4.08-4.05 (m, 1H), 3.36-3.26 (m, 3H), 2.87-2.75 (m, 4H), 2.46 (t, *J* = 7.5 Hz, 2H), 2.30-2.21 (m, 2H), 2.16-2.11 (m, 1H), 1.98-1.92 (m, 2H), 1.78-1.69 (m, 4H), 1.63 (s, 3H), 1.56-1.48 (m, 2H), 1.06 (s, 2H), 0.89-0.86 (m, 2H). LC-MS(ESI$^+$) m/z 812.1 (M+ H) $^+$



2-(2,6-dioxopiperidin-3-yl)-4-((8-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)-8-oxooctyl)amino)isoindoline-1,3-dione (PL-15)

^{1}H NMR (400 MHz, Chloroform-d) δ 9.60 (s, 2H), 9.39 (s, 1H), 8.27 (s, 1H), 8.21 (d, *J* = 8.7 Hz, 1H), 8.06-7.96 (m, 2H), 7.69 (dd, *J* = 8.5, 7.1 Hz, 1H), 7.25 (d, *J* = 7.0 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 1H), 5.14-5.09 (m, 1H), 4.92-4.89 (m, 1H), 4.58-4.52 (m, 1H), 4.25-4.21 (m, 1H), 3.50-3.42 (m, 3H), 3.05-2.93 (m, 4H), 2.59 (t, *J* = 7.7 Hz, 2H), 2.48-2.38 (m, 2H), 2.33-2.29 (m, 1H), 2.16-2.10 (m, 2H), 1.90-1.82 (m, 4H), 1.80 (s, 3H), 1.65-1.57 (m, 6H), 1.24 (s, 2H), 1.07-1.04 (m, 2H). LC-MS(ESI$^+$) m/z 840.1 (M+ H) $^+$

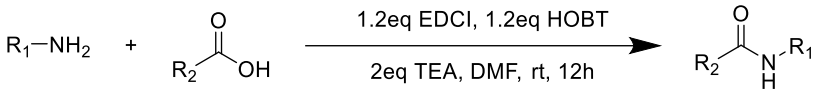


2-(2,6-dioxopiperidin-3-yl)-5-(4-((4-(4-((4-(((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)methyl)piperidin-1-yl)isoindoline-1,3-dione (PL-18)

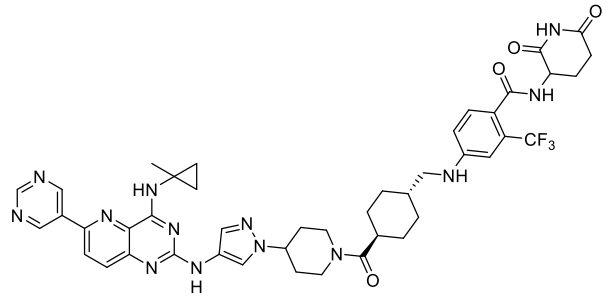
^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.34 (s, 2H), 9.24 (s, 1H), 7.98 (d, *J* = 8.8 Hz, 1H), 7.91 (d, *J* = 8.7 Hz, 1H), 7.68 (d, *J* = 8.5 Hz, 2H), 7.46 (s, 1H), 7.30 (d, *J* = 2.3 Hz, 2H), 7.05 (dd, *J* = 8.6, 2.3 Hz, 1H), 4.98 (dd, *J* = 12.1, 5.2 Hz, 1H), 4.14-4.09 (m, 1H), 4.00-3.97 (m, 2H), 3.03-2.93 (m, 4H), 2.89-2.72 (m, 3H), 2.29-2.28 (m, 2H), 2.23-2.12 (m, 5H), 2.02 (s, 2H), 1.82 (s, 4H), 1.59 (s, 3H), 1.39-1.30 (m, 2H), 1.00 (s, 2H), 0.86-0.80 (m, 2H). LC-MS(ESI$^+$) m/z 796.3 (M+ H) $^+$

Synthesis of IRAK4 degraders based on simplified CRBN ligands

General procedure for IRAK4 degraders based on simplified CRBN ligands

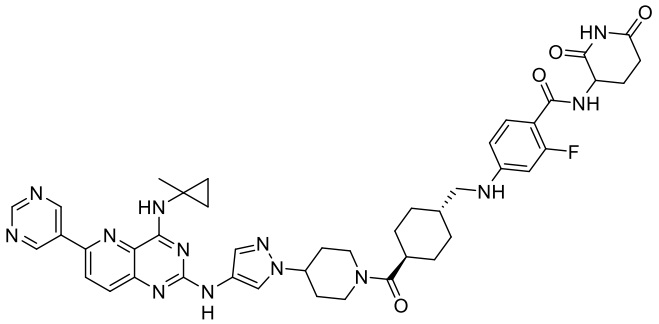


A mixture of amine derivative (1 eq.), EDCI (1.2 eq.), HOBT (1.2 eq.), TEA (2 eq.), in DMF (10 mL) was stirred in a round bottom flask for 20 min at room temperature, then carboxylic acid derivative (1 eq.) was added and stirred for another 12 h, The reaction was quenched by saturated aqueous NaCl and the mixture was extracted by ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (DCM: MeOH = 30: 1) to give the corresponding amide derivatives.



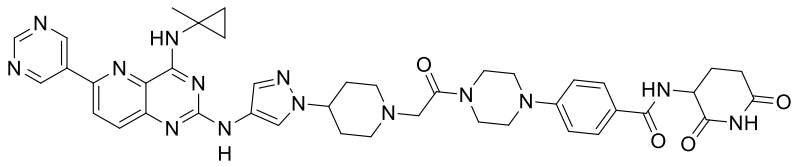
N-(2,6-dioxopiperidin-3-yl)-4-((((1r,4r)-4-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido [3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidine-1-carbonyl)cyclohexyl)methyl)amino)-2-(trifluoro methyl)benzamide (LZ-02)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.81 (s, 1H), 9.78 (s, 2H), 9.40 (s, 0.53H), 9.23 (s, 1H), 9.16 (s, 0.31H), 8.84 (s, 0.53H), 8.58 (s, 0.37H), 8.44 (d, *J* = 8.4 Hz, 1H), 8.35 (d, *J* = 8.8 Hz, 1H), 8.17 (s, 1H), 7.93 (s, 0.39H), 7.75 (s, 0.53H), 7.64 (s, 1H), 7.35 (d, *J* = 8.4 Hz, 1H), 6.92 (d, *J* = 2.3 Hz, 1H), 6.79 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.47 (t, *J* = 5.6 Hz, 1H), 4.70-4.64 (m, 1H), 4.52-4.39 (m, 2H), 4.10-4.03 (m, 1H), 3.26-3.17 (m, 1H), 2.97-2.94 (m, 2H), 2.82-2.70 (m, 2H), 2.67-2.60 (m, 1H), 2.56-2.54 (m, 1H), 2.10-1.95 (m, 4H), 1.88-1.85 (m, 3H), 1.75-1.71 (m, 3H), 1.56 (s, 4H), 1.40 (s, 2H), 1.14-1.08 (m, 2H), 1.01-0.92 (m, 2H), 0.88-0.81 (m, 2H). LC-MS (ESI$^+$) m/z 880.50 (M+ H) $^+$



N-(2,6-dioxopiperidin-3-yl)-2-fluoro-4-((((1r,4r)-4-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl) pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidine-1-carbonyl)cyclohexyl)methyl)amino)benza mide (LZ-03)

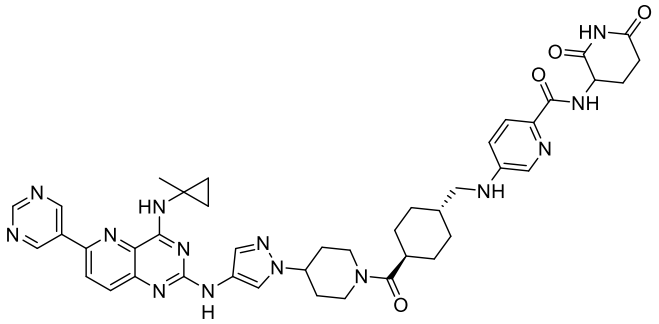
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.78 (s, 2H), 9.42 (s, 0.53H), 9.23 (s, 1H), 9.16 (s, 0.31H), 8.86 (s, 0.53H), 8.59 (s, 0.37H), 8.35 (d, *J* = 8.8 Hz, 1H), 8.17 (s, 1H), 7.94 (s, 0.39H), 7.84 (t, *J* = 7.7 Hz, 1H), 7.75 (s, 0.53H), 7.64 (s, 1H), 7.55 (t, *J* = 8.9 Hz, 1H), 6.61 (t, *J* = 5.6 Hz, 1H), 6.49-6.46 (m, 1H), 6.37-6.32 (m, 1H), 4.75-4.68 (m, 1H), 4.52-4.39 (m, 1H), 4.09-4.06 (m, 1H), 3.25-3.19 (m, 1H), 2.95-2.92 (m, 2H), 2.82-2.69 (m, 2H), 2.66-2.30 (m, 1H), 2.54-2.53 (m, 1H), 2.17-2.10 (m, 4H), 1.88-1.85 (m, 3H), 1.75-1.71 (m, 3H), 1.56 (s, 4H), 1.40 (s, 2H), 1.13-1.07 (m, 2H), 1.00-0.91 (m, 2H), 0.88-0.79 (m, 2H). LC-MS(ESI$^+$) m/z 830.33 (M+ H) $^+$



N-(2,6-dioxopiperidin-3-yl)-4-(4-(2-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d] pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)acetyl)piperazin-1-yl)benzamide (LZ-04)

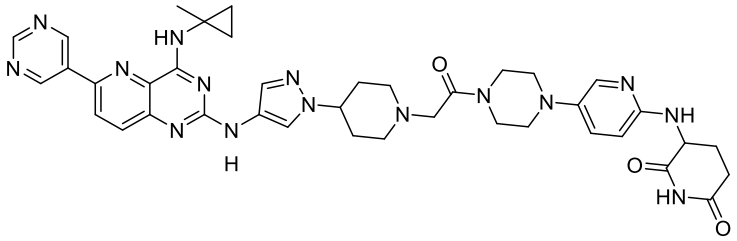
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.78 (s, 2H), 9.40 (s, 0.48H), 9.22 (s, 1H), 9.14 (s, 0.37H), 8.84 (s, 0.52H), 8.58 (s, 0.36H), 8.49 (d, *J* = 8.3 Hz, 1H), 8.34 (d, *J* = 8.8 Hz, 1H), 8.19 (s, 1H), 7.91 (s, 0.48H), 7.79 (d, *J* = 8.4 Hz, 2H), 7.74 (s, 0.50H), 7.67 (s, 0.42H), 7.57 (s, 0.56H), 7.03 (d, *J* = 8.5 Hz, 2H), 4.78-4.72 (m, 1H), 4.14-4.08 (m, 1H), 3.77-3.75 (m, 2H), 3.63-3.61 (m, 2H), 3.40-3.37 (m, 2H), 3.30-3.25 (m, 4H), 2.98-

2.95 (m, 2H), 2.83-2.74 (m, 1H), 2.57-2.55 (m, 1H), 2.27-2.22 (m, 2H), 2.17-2.09 (m, 1H), 2.07-2.02 (m, 2H), 1.97-1.92 (m, 3H), 1.55 (s, 3H), 0.99-0.93 (m, 2H), 0.88-0.80 (m, 2H). LC-MS (ESI^+) m/z 799.07 $(M+H)^+$



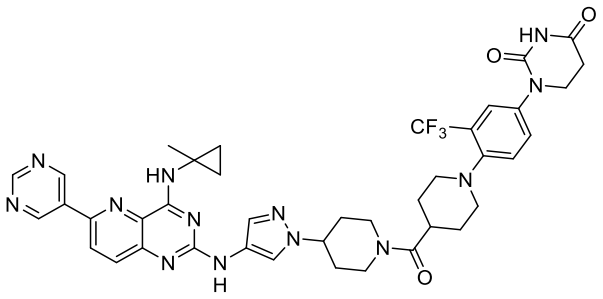
N-(2,6-dioxopiperidin-3-yl)-5-((((1R,4R)-4-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidine-1-carbonyl)cyclohexyl)methyl)amino)picolinamide (LZ-07)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 9.78 (s, 2H), 9.40 (s, 0.55H), 9.23 (s, 1H), 9.16 (s, 0.38H), 8.84 (s, 1H), 8.58 (d, J = 8.2 Hz, 1H), 8.35 (d, J = 8.8 Hz, 1H), 8.17 (s, 1H), 8.00-7.96 (m, 2H), 7.77 (d, J = 8.6 Hz, 1H), 7.63 (s, 1H), 6.99 (d, J = 8.0 Hz, 1H), 6.62 (t, J = 5.7 Hz, 1H), 4.75-4.69 (m, 1H), 4.52-4.42 (m, 2H), 4.10-4.07 (m, 1H), 3.40-3.39 (m, 1H), 3.26-3.17 (m, 2H), 2.98 (t, J = 6.2 Hz, 2H), 2.83-2.76 (m, 1H), 2.67-2.60 (m, 1H), 2.55-2.54 (m, 1H), 2.20-2.10 (m, 2H), 2.03-1.99 (m, 2H), 1.91-1.86 (m, 3H), 1.75-1.72 (m, 3H), 1.56 (s, 3H), 1.40-1.34 (m, 2H), 1.15-1.09 (m, 2H), 1.00 -0.94(m, 2H), 0.86-0.81 (m, 2H). LC-MS (ESI^+) m/z 813.23 $(M+H)^+$



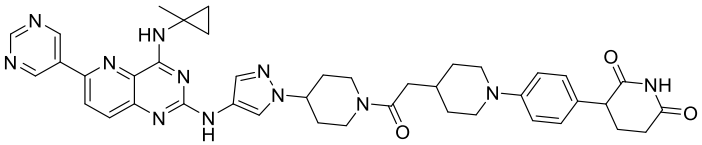
3-((5-(4-(2-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)acetyl)piperazin-1-yl)pyridin-2-yl)amino)piperidine-2,6-dione (LZ-09)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.73 (s, 1H), 9.77 (s, 2H), 9.39 (s, 0.48H), 9.22 (s, 1H), 9.14 (s, 0.35H), 8.83 (s, 0.52H), 8.57 (s, 0.39H), 8.34 (d, J = 8.8 Hz, 1H), 8.19 (s, 1H), 7.92 (s, 0.44H), 7.74 (s, 0.48H), 7.69 (d, J = 2.9 Hz, 1H), 7.58 (s, 1H), 7.28 (dd, J = 8.9, 2.9 Hz, 1H), 6.58 (d, J = 8.9 Hz, 1H), 6.46 (d, J = 7.5 Hz, 1H), 4.70-4.64 (m, 1H), 4.14-4.08 (m, 1H), 3.73-3.71 (m, 2H), 3.61-3.58 (m, 2H), 3.25 (s, 2H), 3.00-2.90 (m, 6H), 2.78-2.69 (m, 1H), 2.55-2.53 (m, 1H), 2.27-2.21 (m, 2H), 2.13-1.92 (m, 6H), 1.55 (s, 3H), 0.99 (s, 2H), 0.88-0.80 (m, 2H). LC-MS (ESI^+) m/z 772.28 $(M+H)^+$



1-(4-(4-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidine-1-carbonyl)piperidin-1-yl)-3-(trifluoromethyl)phenyl)dihydropyrimidine-2,4(1H,3H)-dione (LZ-20)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 1H), 9.78 (s, 2H), 9.41 (s, 0.55H), 9.22 (s, 1H), 9.17 (s, 0.35H), 8.85 (s, 0.54H), 8.58 (s, 0.42H), 8.35 (d, *J* = 8.7 Hz, 1H), 8.18 (s, 1H), 7.93 (s, 0.49H), 7.74 (s, 0.61H), 7.65-7.59 (m, 3H), 7.53 (d, *J* = 8.7 Hz, 1H), 4.56-4.41 (m, 2H), 4.15-4.11 (m, 1H), 3.82 (t, *J* = 6.6 Hz, 2H), 3.27-3.23 (m, 1H), 2.97 (s, 2H), 2.89-2.77 (m, 4H), 2.72 (t, *J* = 6.6 Hz, 2H), 2.15-2.05 (m, 2H), 1.82-1.75 (m, 6H), 1.56 (s, 3H), 1.01 (s, 2H), 0.81 (s, 2H). LC-MS(ESI$^+$) m/z 810.10 (M+ H) $^+$



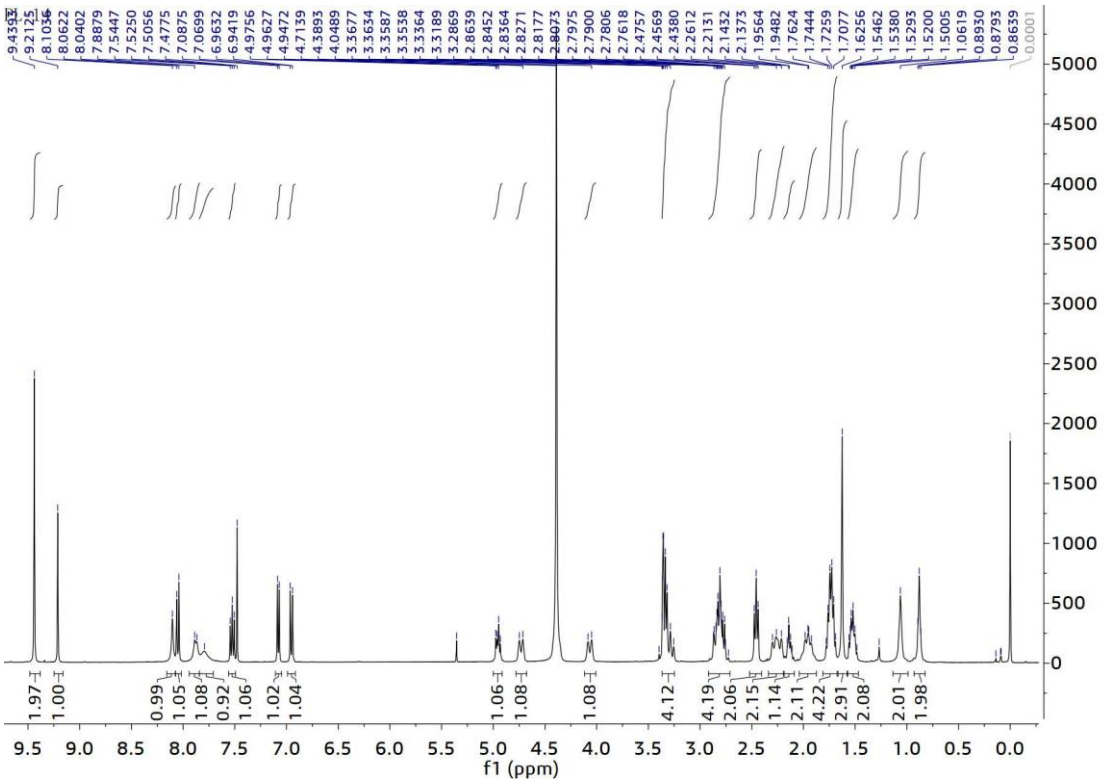
3-(4-(4-(2-(4-(4-((4-((1-methylcyclopropyl)amino)-6-(pyrimidin-5-yl)pyrido[3,2-d]pyrimidin-2-yl)amino)-1H-pyrazol-1-yl)piperidin-1-yl)-2-oxoethyl)piperidin-1-yl)phenyl)piperidine-2,6-dione (LZ-22)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.76 (s, 1H), 9.77 (s, 2H), 9.40 (s, 0.53H), 9.23 (s, 1H), 9.16 (s, 0.36H), 8.85 (s, 0.57H), 8.58 (s, 0.45H), 8.35 (d, *J* = 8.8 Hz, 1H), 8.16 (s, 1H), 7.93 (s, 0.45H), 7.75 (s, 0.49H), 7.65 (s, 1H), 7.03 (d, *J* = 8.3 Hz, 2H), 6.89 (d, *J* = 8.4 Hz, 2H), 4.54-4.51 (m, 1H), 4.45-4.38 (m, 1H), 4.07-4.03 (m, 1H), 3.74-3.65 (m, 3H), 3.24-3.18 (m, 1H), 2.79-2.73 (m, 1H), 2.70-2.59 (m, 3H), 2.48-2.43 (m, 1H), 2.35 (d, *J* = 6.8 Hz, 2H), 2.13-1.97 (m, 4H), 1.87-1.76 (m, 5H), 1.56 (s, 3H), 1.36-1.23 (m, 2H), 1.00 (s, 2H), 0.86-0.81 (m, 2H). LC-MS(ESI$^+$) m/z 755.46 (M+ H) $^+$

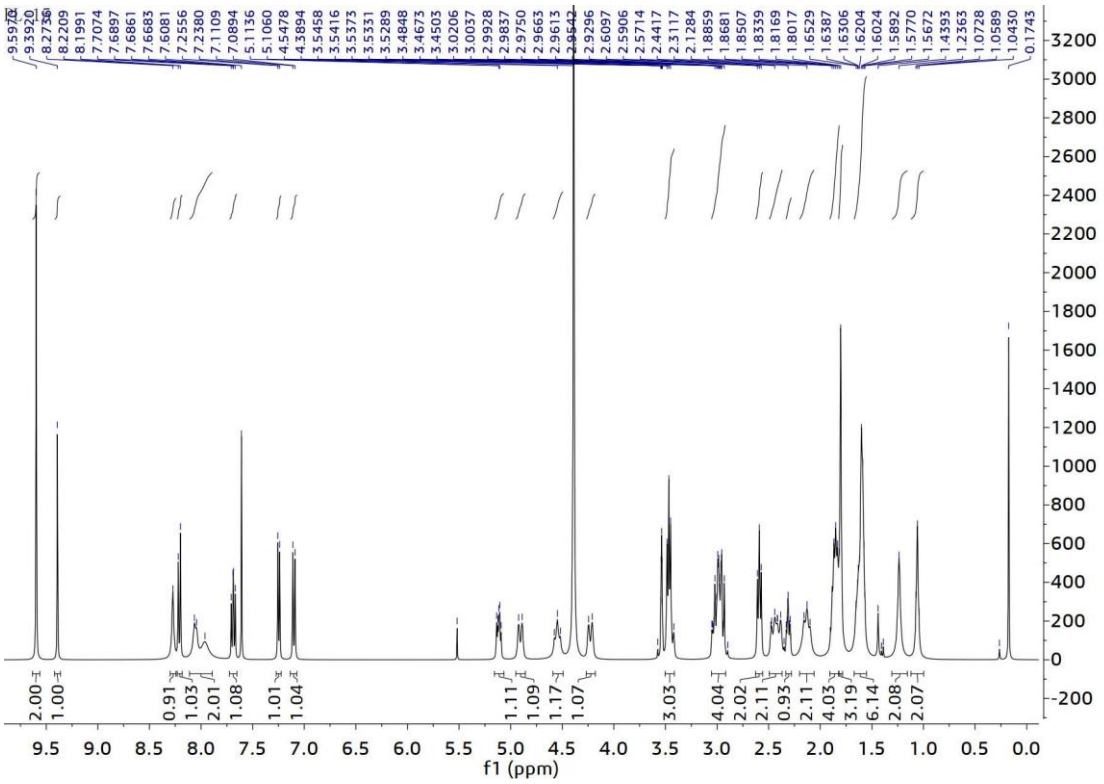
NMR data of a subset of compounds

PL series

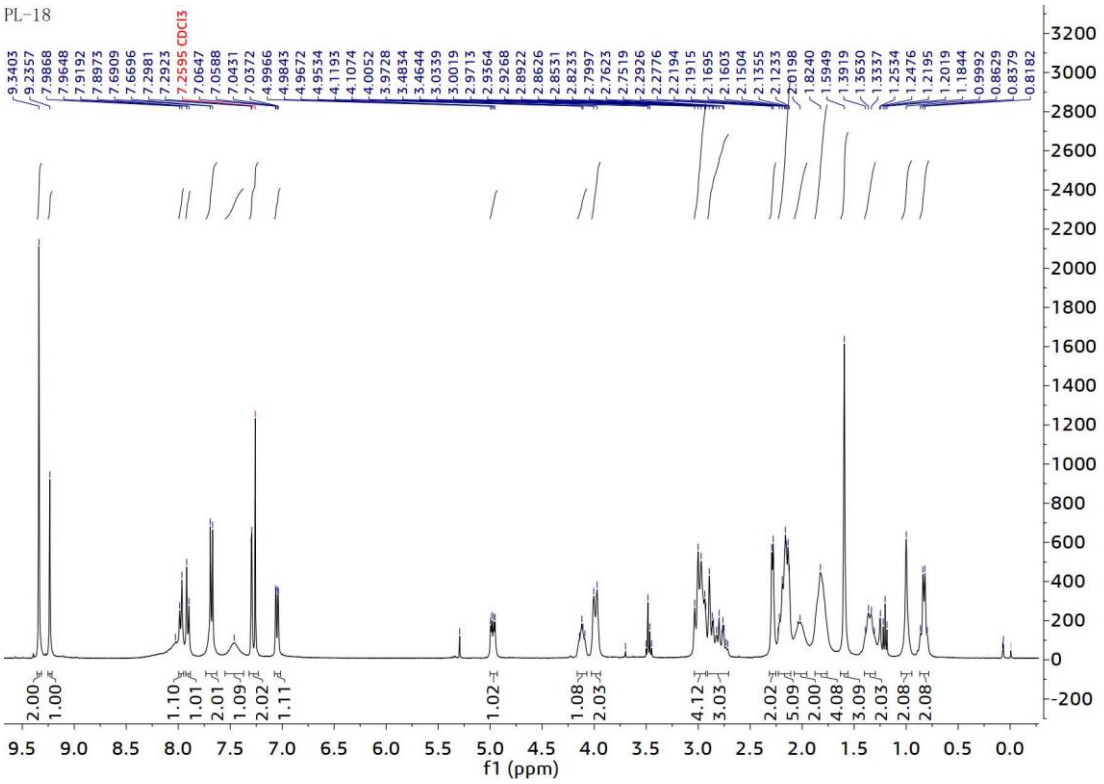
^{1}H-NMR of compound PL-14



^{1}H-NMR of compound PL-15



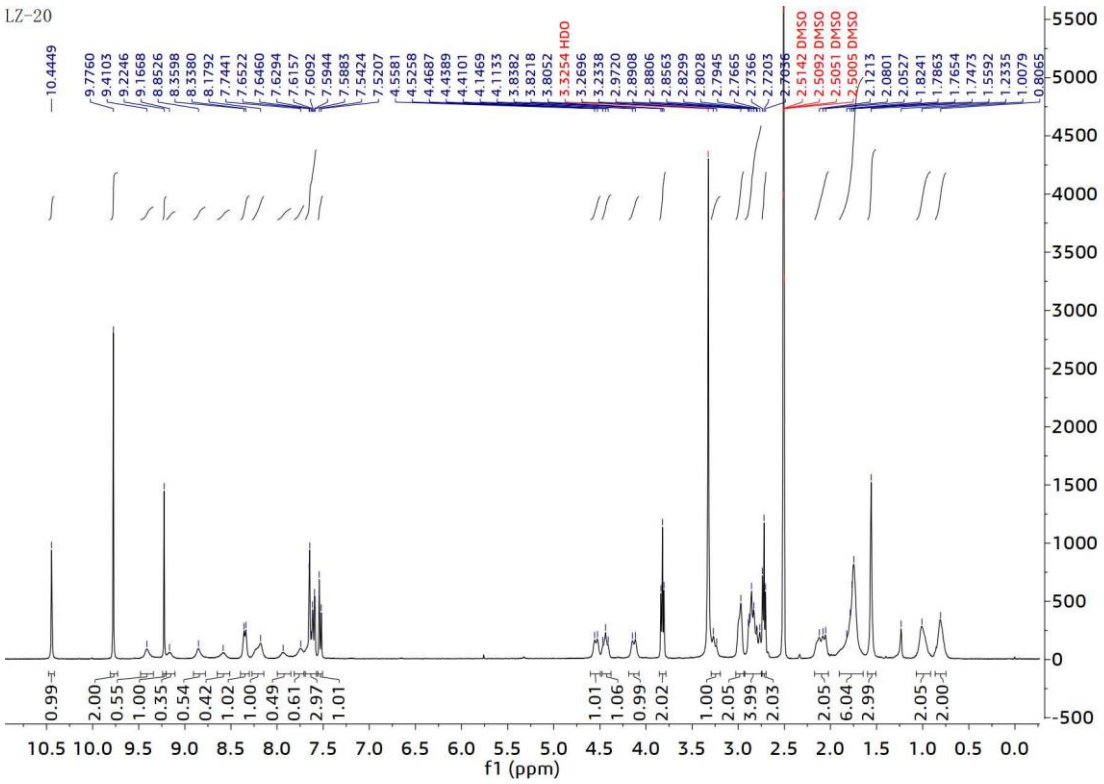
^{1}H-NMR of compound PL-18



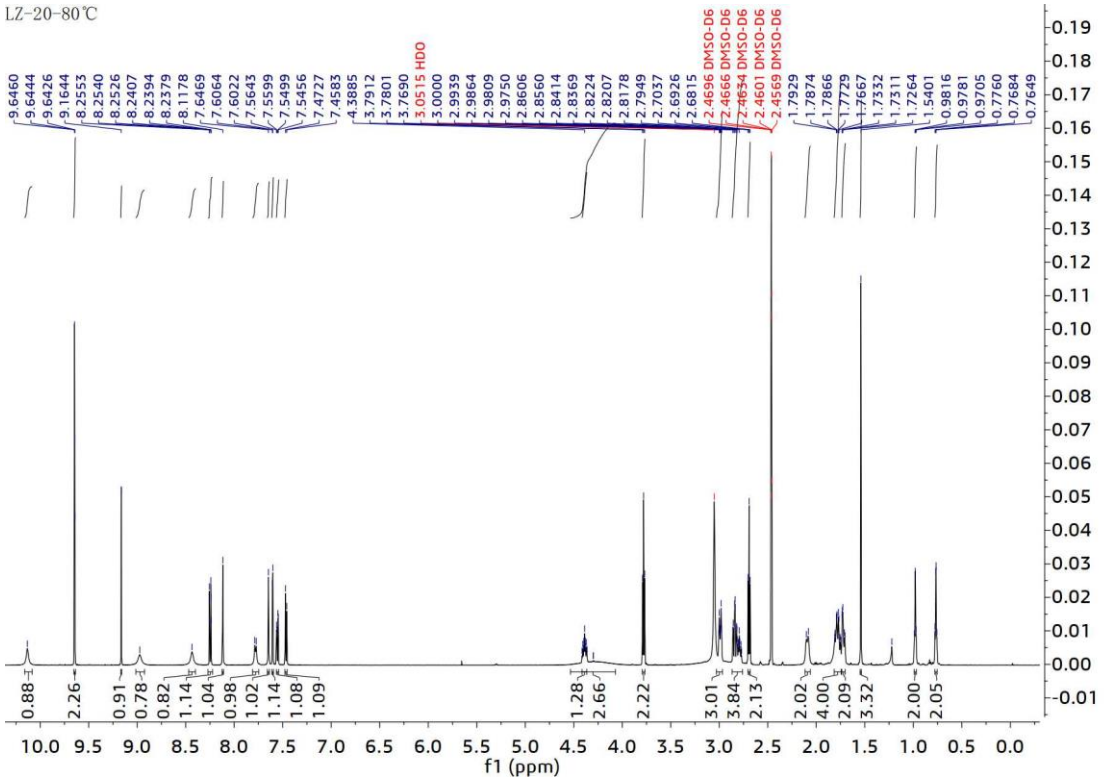
LZ series

By comparing ^{1}H-NMR of LZ-20 under ambient and elevated temperatures, we observed several significant spectral differences, including shifts in chemical shifts, alterations in coupling constants, broadening of peak widths, and relative changes in the number of peaks. These observations have led us to suspect that the LZ series of compounds may exhibit rotational isomerism. Given the instability of the rotational isomers, we have decided against their separation and instead focus on the study of the overall characteristics of the compound.

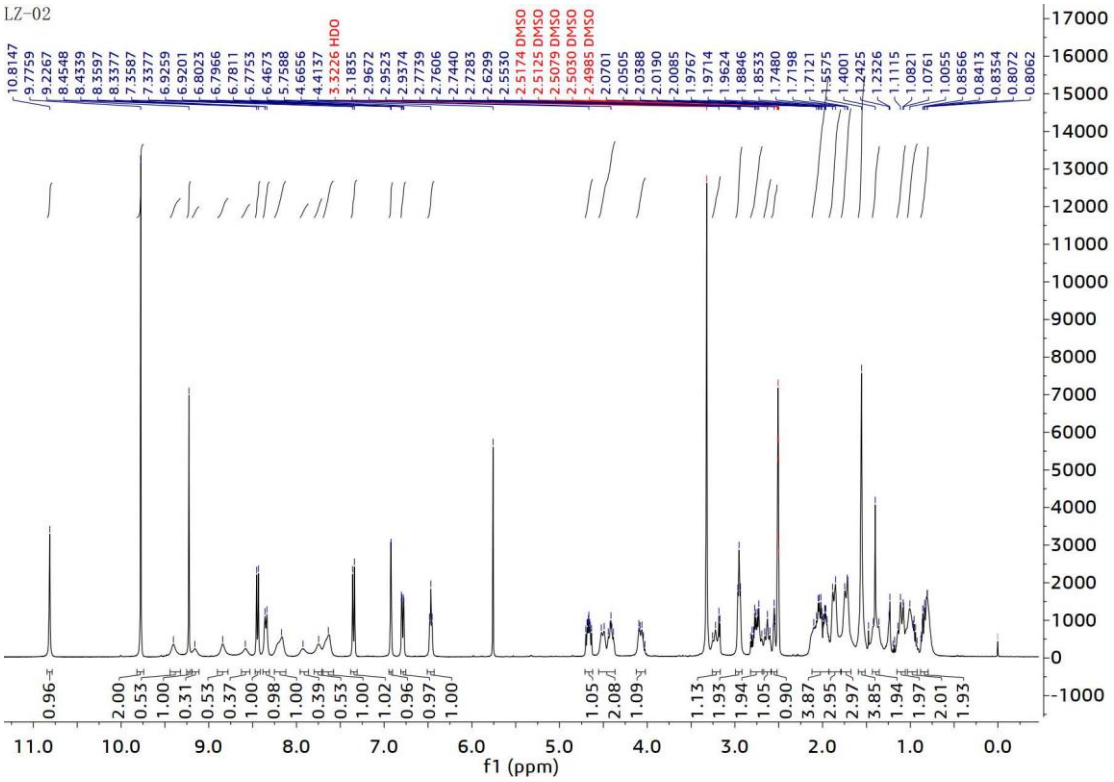
^{1}H-NMR of compound LZ-20



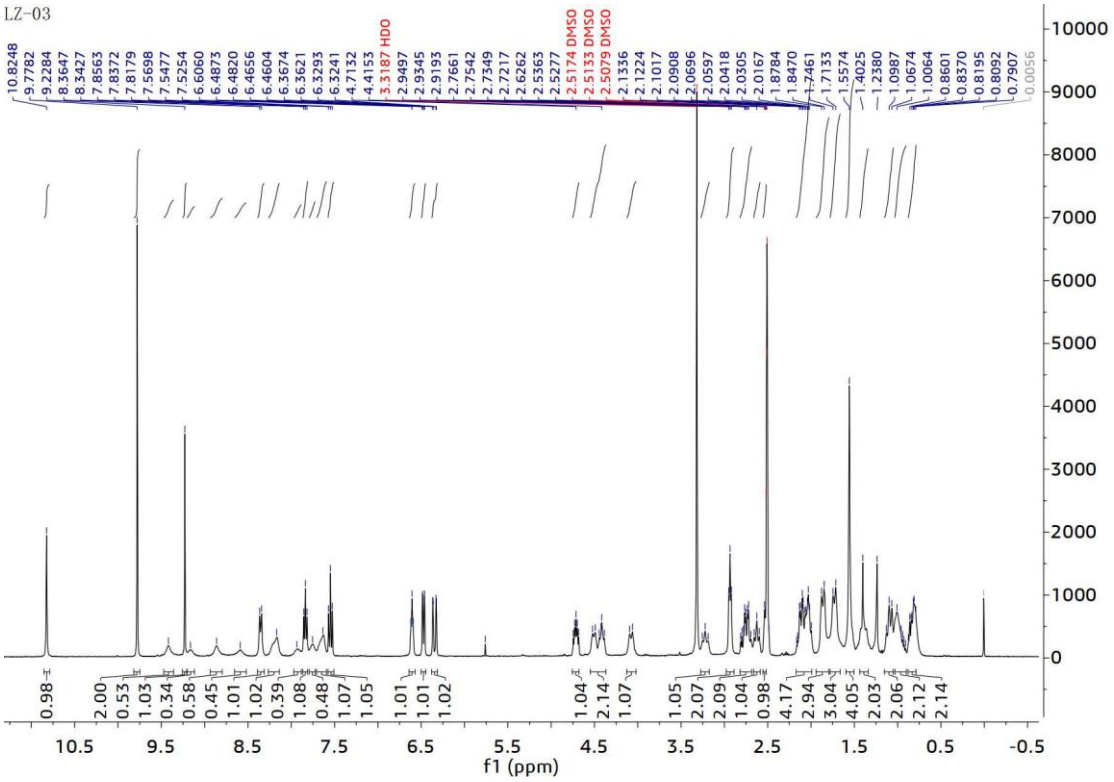
[1]H-NMR of compound LZ-20 **(**High-temperature NMR, temperature = 80°C)



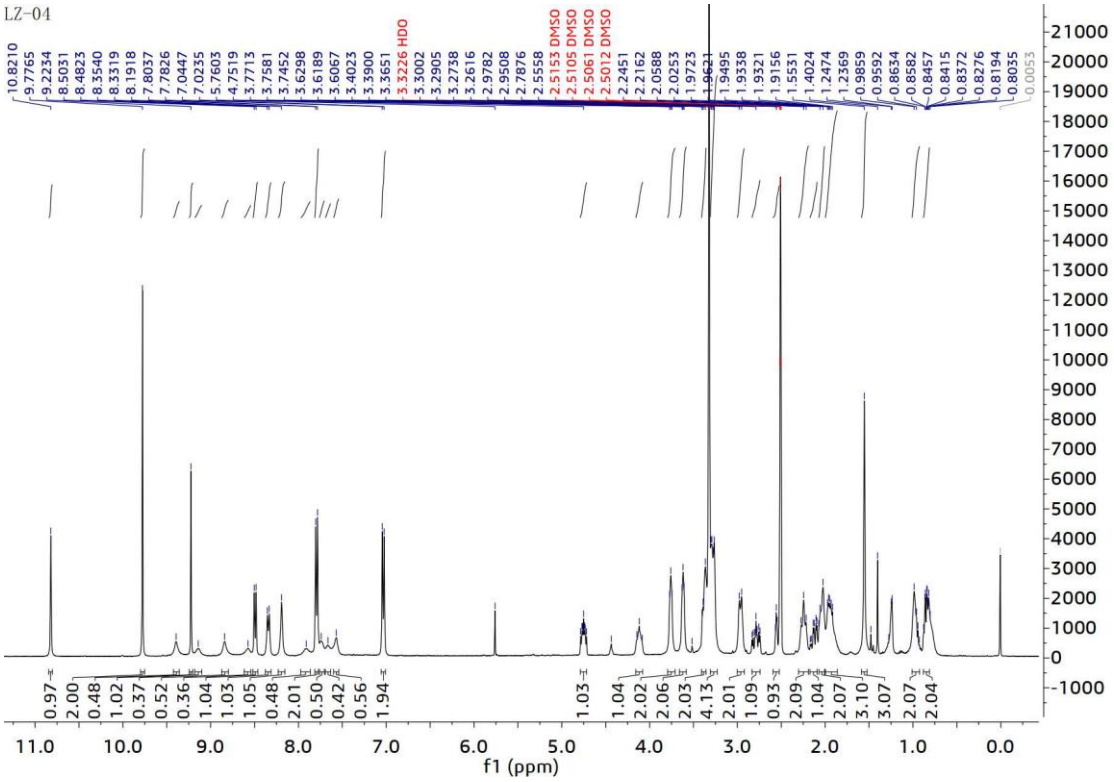
[1]H-NMR of compound LZ-02



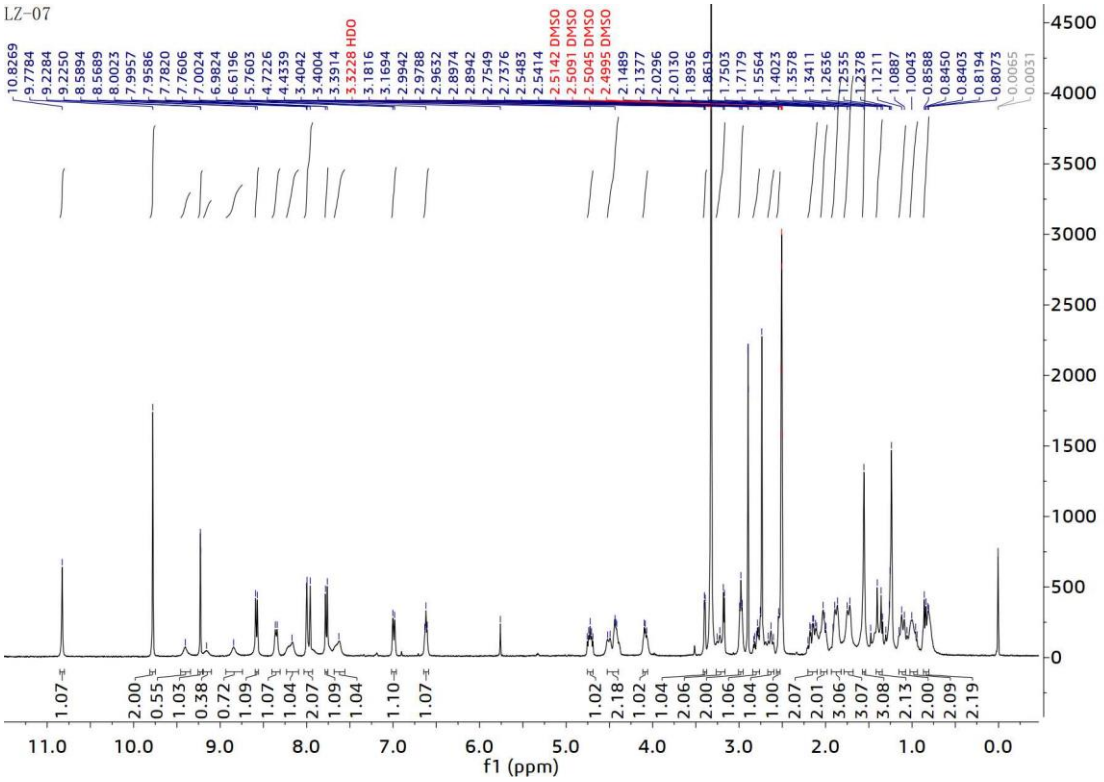
[1]H-NMR of compound LZ-03



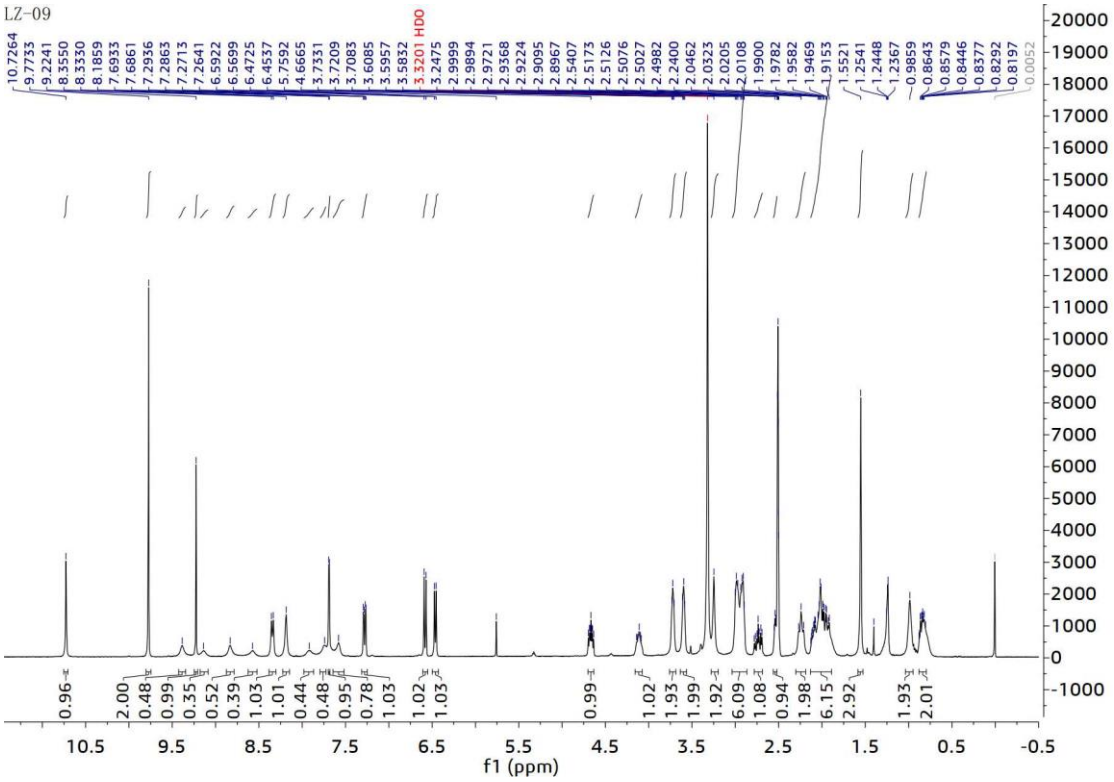
[1]H-NMR of compound LZ-04



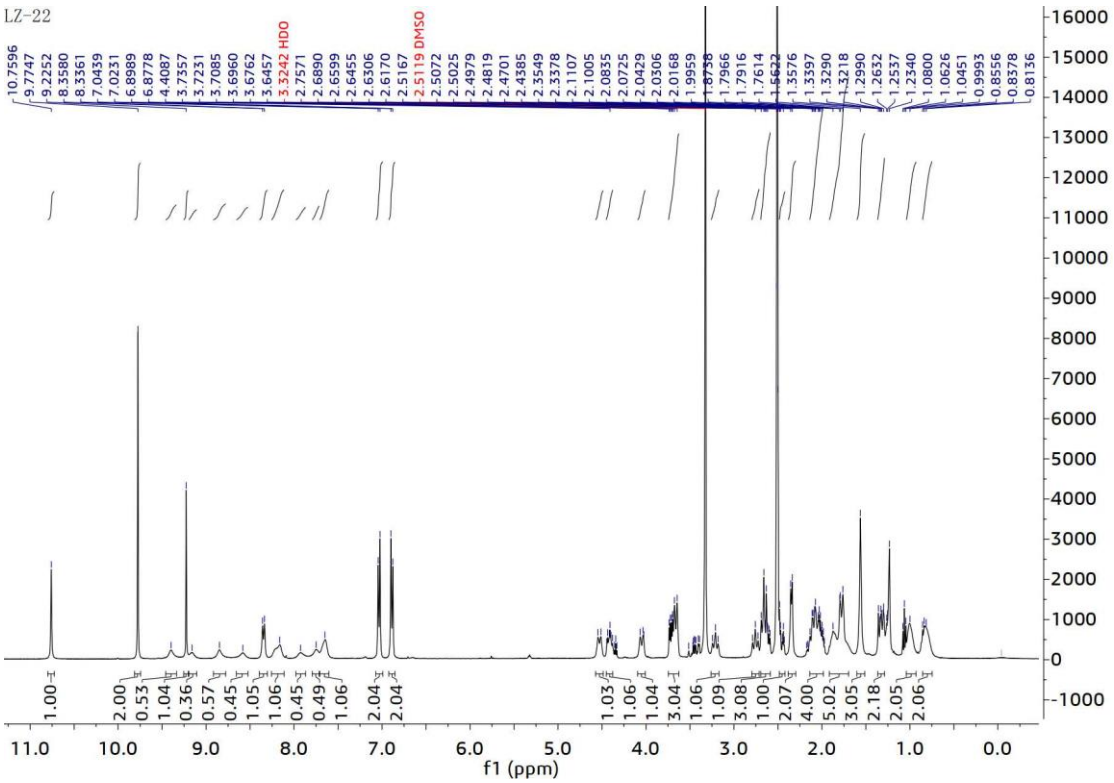
[1]H-NMR of compound LZ-07



[1]H-NMR of compound LZ-09



[1]H-NMR of compound LZ-22



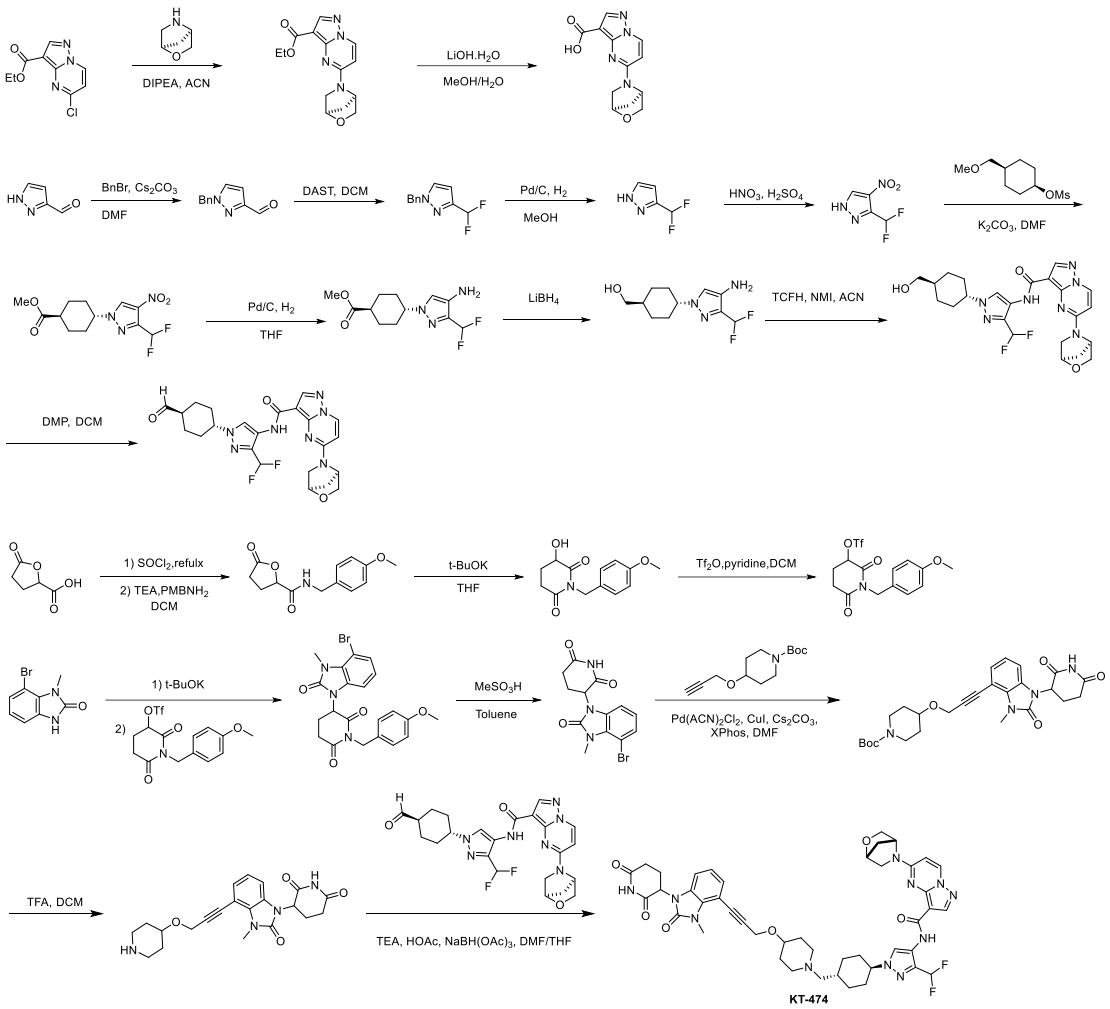


Fig. S1. Synthetic route of KT-474.

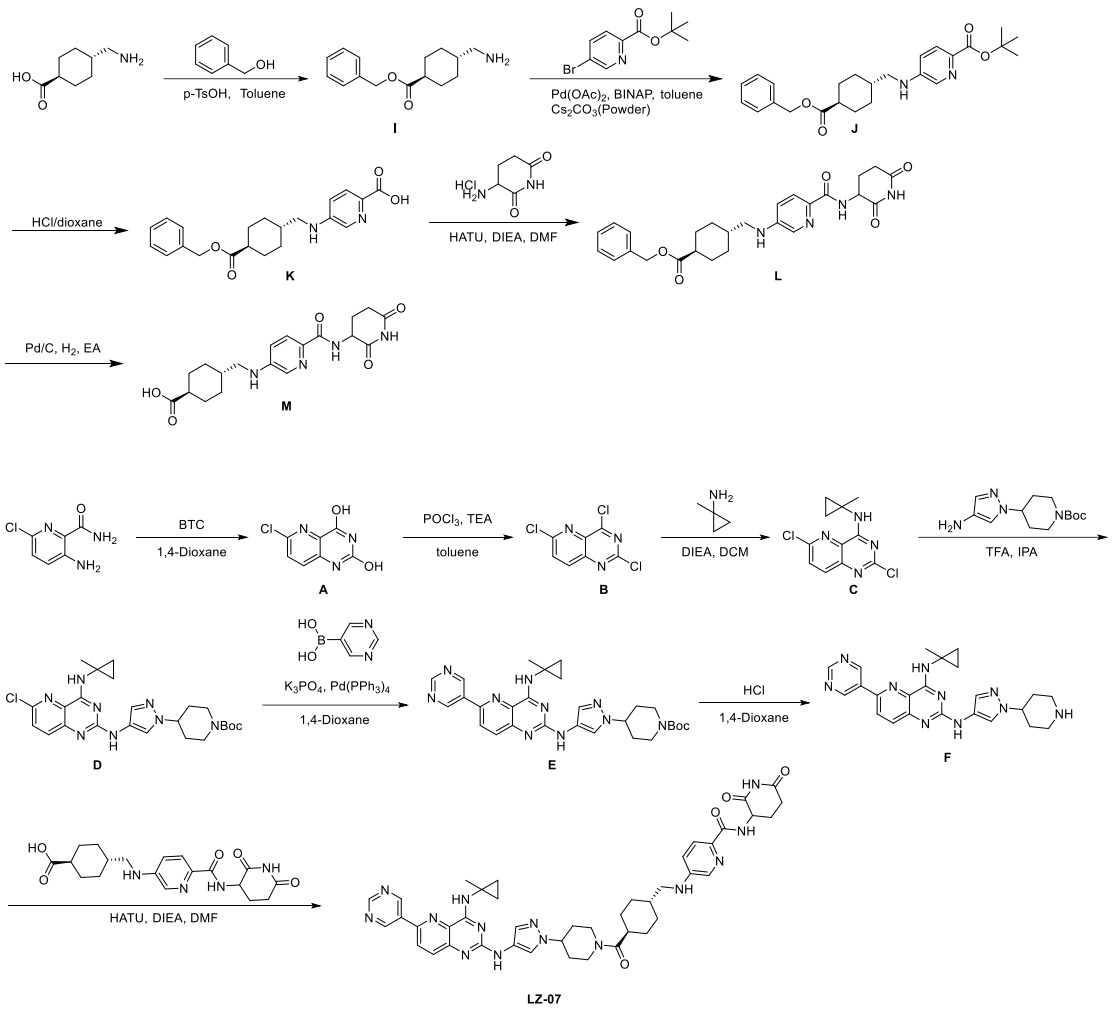


Fig. S2. Synthetic route of LZ-07.

Supplementary information, Data S2

High-content screening

Green Fluorescent Protein (EGFP)-IRAK4-expressing HeLa cells (30 μL) were seeded into Agilent 384-well plates. Subsequently, test compounds were loaded into LDV 384-well source plates, and 30 nL of each compound was transferred to the cell-containing plates using the Echo 550 nanoscale acoustic liquid handler. The plates were then incubated for 16 hours to allow for compound action. Following the incubation period, fluorescence imaging was performed using the Opera Phenix, a laser confocal high-content imaging system. The acquired data were analyzed with Harmony 22 software to quantify fluorescence intensity and perform statistical evaluations. Lastly, a heatmap of the final fluorescence intensities was generated using GraphPad Prism version 9 for data visualization.

Immunoblotting

The human THP-1, TMD8 and DOHH2 cell were provided by Prof. Wanli Liu from Tsinghua University. RPMI 1640, Pen/Strep was purchased from Gibco®. DMSO (Cat. No. D2650-100ML) was purchased from SIGMA®, FBS was purchased from Gemini. THP-1 and DOHH2 cells were cultured in RPMI 1640 media with 10% FBS and 1% penicillin-streptomycin at 37°C incubator containing 5% CO_2. The entire cell culture

environment is subjected to disinfection with a mycoplasma clearance agent (MycAway™ Spray, Cat #40605ES02 ; Yeasen, Shanghai, China).

Cells were suspended in 1 mL of culture medium in 12-well cell culture plate (1×10^6 cells/well), which were treated with compounds at various concentrations. After the indicated time, cells were lysed with 1.2X SDS loading buffer (Cat #20315ES20, Yeasen, Shanghai, China) and heated at 100 ºC for 15 min. 10 μL of the cell lysate were loaded onto 10% SDS-PAGE gel for the protein band separation, and the gel then electrotransfered to PVDF membranes at 4ºC, 100V for 1.5 h. After blocking with 5% milk in TBST, the membranes were incubated with primary antibody at 4℃ overnight. Primary antibodies: IRAK4 (Mouse mAb, Abcam, Ab19942, 1:1000) and IRAK4 (Rabbit mAb, Abclonal, a21779, 1:1000) were purchased from Abcam and Abclonal, respectively. GAPDH (Rabbit mAb, Servicebio, GB11002, 1:1000) was purchased from Servicebio. Secondary antibodies for rabbit (Anti-rabbit IgG, HRP-Linked Antibody, CST, 7074, 1:3000) and mouse (Anti-mouse IgG, HRP-Linked Antibody, ABclonal, AS003, 1:3000) were incubated at room temperature for 1 h. Blots were imaged with M5 Hiper ECL Western HRP Substrate (MF074-05) on AllCap ECL instrument. For grayscale analysis, we use the imageJ 1.50i. GraphPad Prism 9 software was used for calculating DC_{50} by nonlinear regression analysis.

ELISA Assay

The Peripheral Blood Mononuclear Cells (PBMCs) (donor ID: Y1828) were provided by Beijing Muxing Biotech Co., Ltd. (Cat # FPB003F-C), and the PBMCs (donor ID: P122071008C) were provided by Milestone Biological Science & Technology Co.，Ltd (Cat # PB100C). The RPMI-1640 medium (Cat # 8123329) was purchased from Gibco, and the lipopolysaccharide (LPS) (Cat # L6529) was acquired from Sigma-Aldrich. For cytokine measurements, ELISA kits from Invitrogen were utilized, specifically the Human IL-1β Uncoating ELISA kit (Cat # 88-7216), Human IL-6 Uncoating ELISA kit (Cat # 88-7066), Human IL-10 Uncoating ELISA kit (Cat # 88-7106), and Human TNF-α Uncoating ELISA kit (Cat # 88-7346). The compounds under investigation, LZ-07 and KT-474, were tested at a range of concentrations including 10.00 μmol/L, 33.33 μmol/L, 1.11 μmol/L, 0.37 μmol/L, 0.12 μmol/L, 41.15 nmol/L, 13.72 nmol/L, 4.57 nmol/L, and 1.52 nmol/L. All experimental operations and materials, including the ELISA kits, were provided by our collaborators. GraphPad Prism 9 software was used for calculating DC50 by nonlinear regression analysis.

Kinase assays

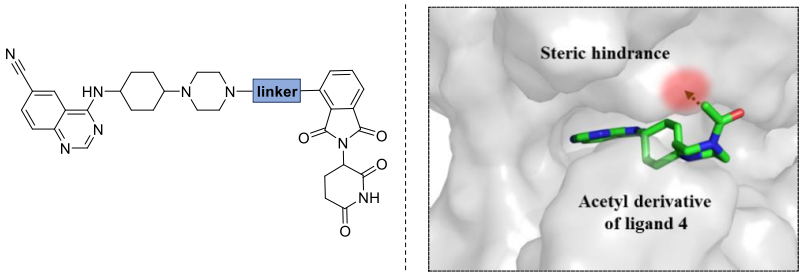
ChemPartner provided kinome screening services, in which LZ-07 was utilized at a concentration of 3 μmol/L to determine the IC_{50} against PI3Kδ with an ATP concentration of 25 μM. Additionally, PI103 served as a reference compound for comparative analysis within our study. The PI3Kδ (Cat # 14-604-M) was purchased from Millipore, and the PI103 (Cat # 2930) was acquired from Tocris. The ADP-Glo Kinase Assay (Cat # v9102/3) was obtained from Promega, and the PIP2 (Cat # PR8982B) was sourced from Life Technologies. The HEPES, pH7.5 (Cat # 11344-041) was bought from Gibco, and the Brij-35 solution (Cat # B4184) was procured from Sigma. The EDTA (Cat # 15575-038) was also obtained from Gibco, and the $MgCl_2$ (Cat # M2670-500g) was purchased from Sigma. The DTT (Cat # D0632-10G) was acquired from Sigma, and the 96 well plate (Cat # 3365) was bought from Corning. The 384 well Echo plate (Cat # PP-0200) was obtained from Labcyte, and the PROXIPLATE-384 PLUS (Cat # 6008289) was procured from PE.

The testing procedure involved preparing the compound through serial dilution to achieve a 100X stock solution in 100% DMSO, from which 100 μL was transferred to a 96-well source plate. This plate also received 100 μL of 100% DMSO for no compound and no enzyme controls. An intermediate plate was prepared by transferring 40 μL of the compound to a new 384-well plate. Echo was used to transfer 50 nl of the compound

in 100% DMSO to the assay plate. The kinase reaction was initiated by preparing a 2x kinase solution and adding 2.5 µL to each well of the assay plate, with the exception of enzyme-free control wells which received 2.5 µL of 1x kinase buffer. Following a shake, a 2x substrate solution was prepared and 2.5 µL was added to each well to start the reaction, which was then shaken again. The assay plate was covered and incubated at room temperature for 1 hour. For kinase detection, ADP-Glo reagent was equilibrated to room temperature, and 5 µL was added to each well to halt the reaction. After a brief centrifugation and slow shaking, the plate was equilibrated for 120 minutes. Then, 10 µL of Kinase Detection Reagent was added to each well, shaken for 1 minute, and equilibrated for 30 minutes before luminescence reading on a plate reader. Data collection was performed on Envision, and curve fitting was done using XLFit excel add-in version 5.4.0.8 to determine IC_{50} values from the log(inhibitor) versus response slope (variable), with the equation Y = Bottom + (Top - Bottom) / (1 + (IC_{50} / X) ^ HillSlope). Percent inhibition was calculated as (max - sample RLU) / (max - min) * 100, where "min" indicates the RLU of the no enzyme control and "max" indicates the RLU of the DMSO control.

Table S1

The degradation potency of compounds derived from ligand **4** in the first-generation IRAK4-targeting PROTAC library.



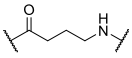
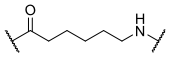
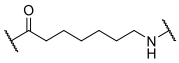
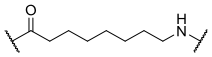
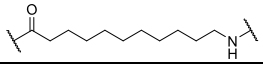
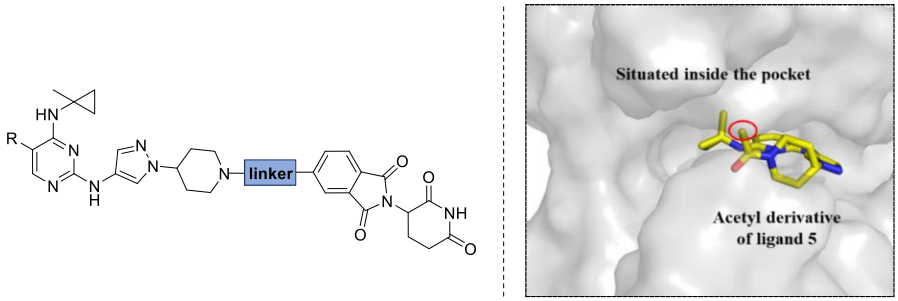
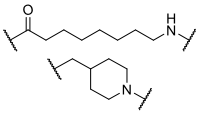
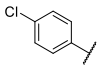
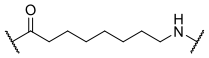
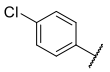
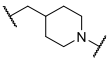
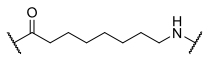
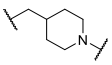
Compounds	Linker	% IRAK4 protein degradation in THP-1 cells (24 h)	
		0.3 µmol/L	3 µmol/L
PL-02		< 5	< 5
PL-03		< 5	< 5
PL-04		< 5	< 15
PL-05		< 5	< 5
PL-06		< 5	< 5
PL-07		< 5	< 5

Table S2

The degradation potency of compounds derived from ligand **5** in the first-generation IRAK4-targeting PROTAC library.



Compounds	R	Linker	% IRAK4 protein degradation in

			THP-1 cells (24 h)	
			0.5 μmol/L	5 μmol/L
PL-08	H		< 5	< 5
PL-09	H		< 5	< 5
PL-10			< 5	< 5
PL-11			< 5	< 5
PL-12			< 5	< 5
PL-13			< 5	< 5

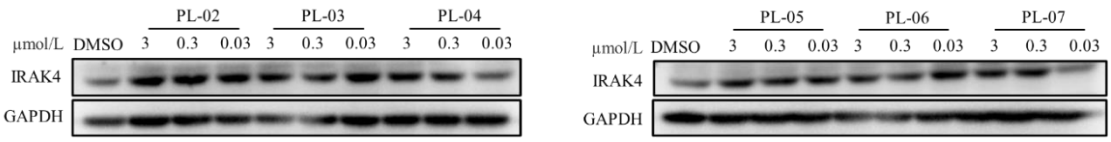


Fig. S3. The degradation assay of compounds derived from ligand **4** in our first-generation IRAK4-targeting PROTAC library. Immunoblots for IRAK4 in THP-1 cells after treatment with compounds for 24 h.

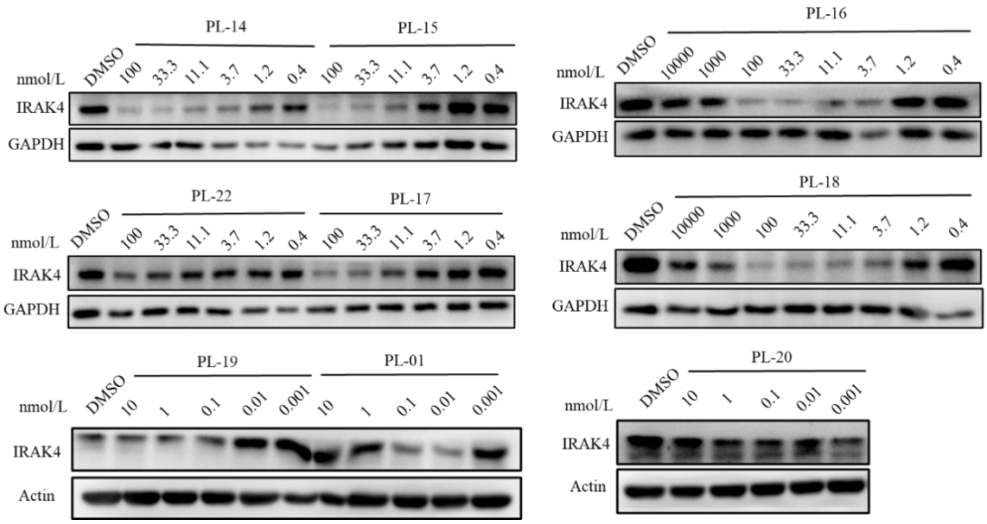


Fig. S4. The degradation assay of compounds derived from ligand **6** in our second-generation IRAK4-targeting PROTAC library. Immunoblots for IRAK4 in THP-1 cells after treatment with compounds for 24 h.

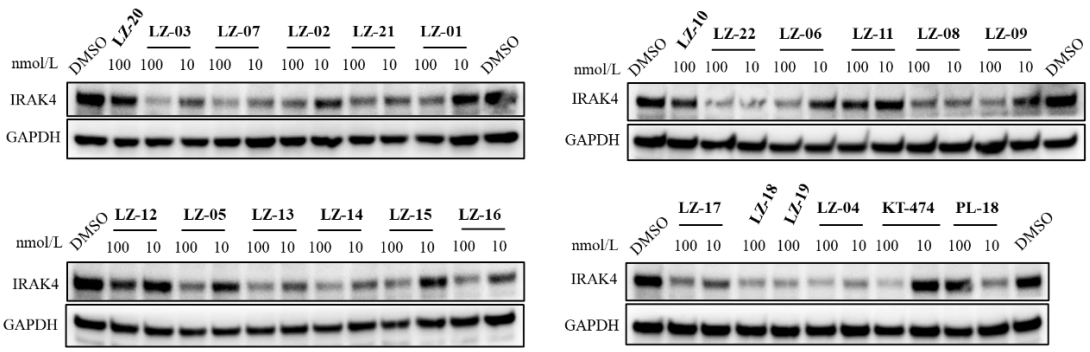


Fig. S5. The degradation assay of compounds based on ligand **6** and simplified CRBN ligands in our third-generation IRAK4-targeting PROTAC library. Immunoblots for IRAK4 in TMD8 cells after treatment with compounds for 16 h.

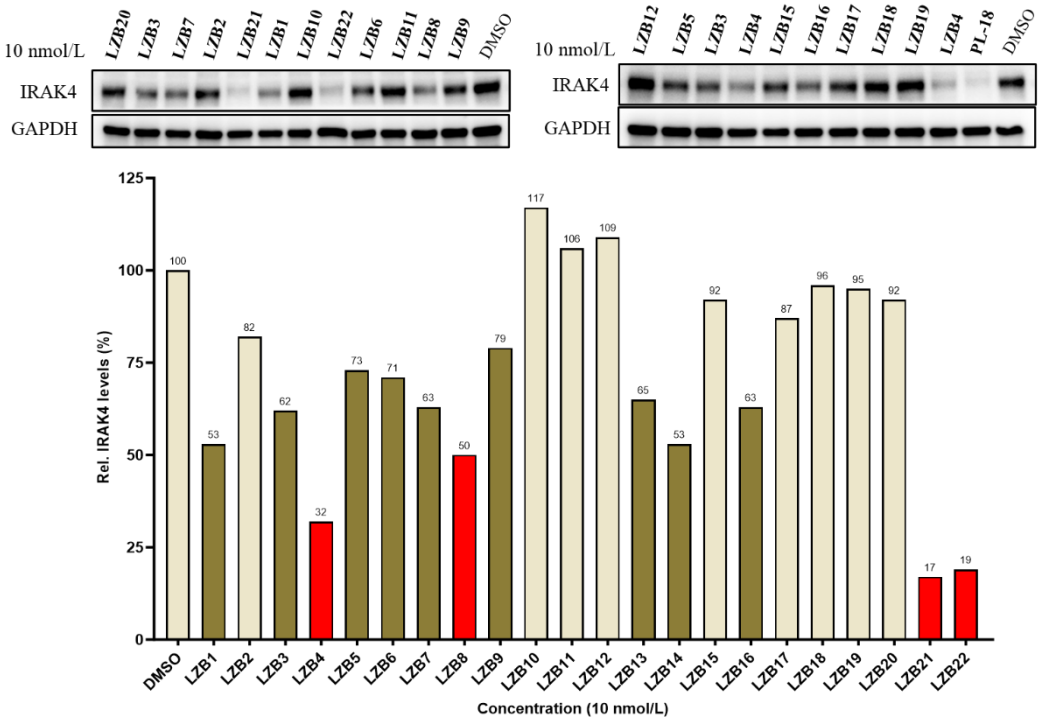


Fig. S6. The degradation assay of compounds based on ligand **6** and simplified CRBN ligands in our third-generation IRAK4-targeting PROTAC library. Immunoblots for IRAK4 in THP-1 cells after treatment with compounds for 16 h.

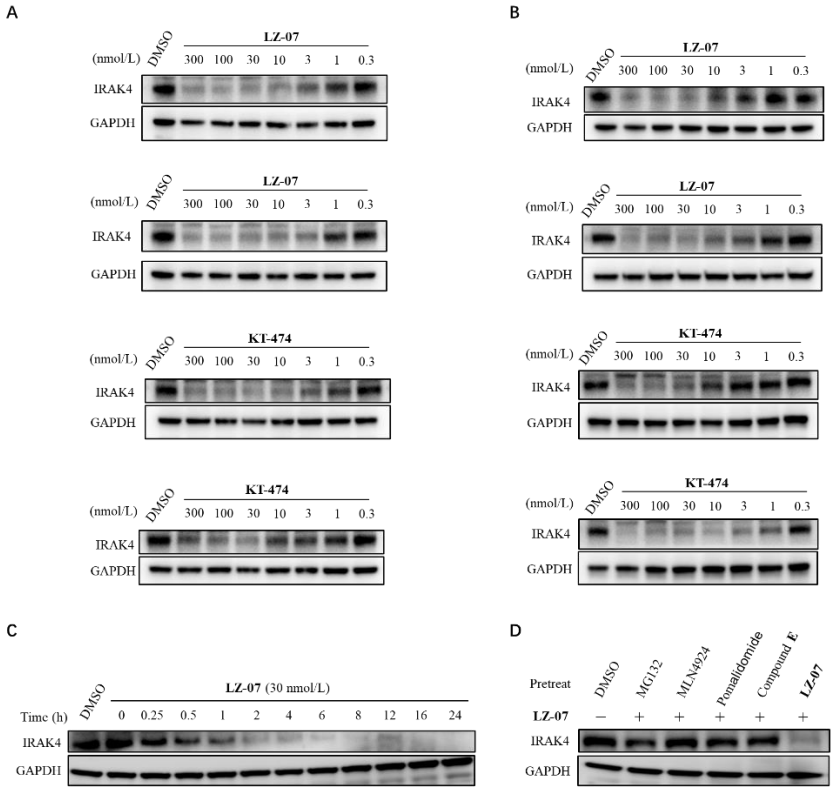


Fig. S7. The degradation evaluation and preliminary mechanism study of LZ-07. (A) Immunoblots and statistical analysis of IRAK4 levels in TMD8 cells following 16-hour treatment with LZ-07 and KT-474. (B) Immunoblots and statistical analysis of IRAK4 levels in DOHH2 cells following 16-hour treatment with LZ-07 and KT-474. (C) Immunoblots for IRAK4 in DOHH2 cells treated with 30 nmol/L LZ-07 at indicated time. (D) Immunoblot analysis of IRAK4 in DOHH2 cells pre-treated with DMSO, MG132 (0.5 μmol/L), MLN4924 (0.5 μmol/L), Pomalidomide (1 μmol/L) and Compound **E** (1 μmol/L) for 2 h in DOHH2 cells, and then treated with LZ-07 (30 nmol/L) for 8 h.

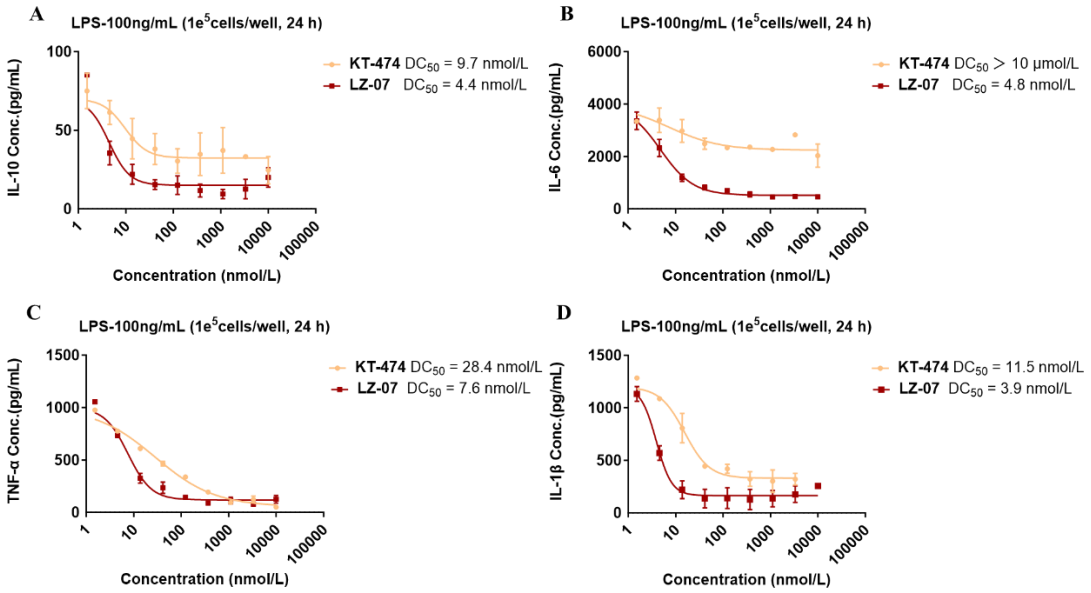


Fig. S8. Comparison of the anti-inflammatory efficiencies of LZ-07 and KT-474 on PBMCs derived from a health donor (donor ID: Y1828). PBMCs were pretreated with LZ-07 and KT-474 for 2 hours, followed by stimulation with 10 μL lipopolysaccharide (LPS, 100 ng/mL) for 24 hours. The levels of cytokines (IL-10, IL-6, TNF-α, and IL-1β) were determined using ELISA technology. (A)-(D) show the inhibitory efficiencies of LZ-07 and KT-474 on the levels of IL-10, IL-6, TNF-α, and IL-1β respectively. Data are presented as mean ± standard deviation (SD) (n = 2). The final IC_{50} was generated by GraphPad Prism 9.

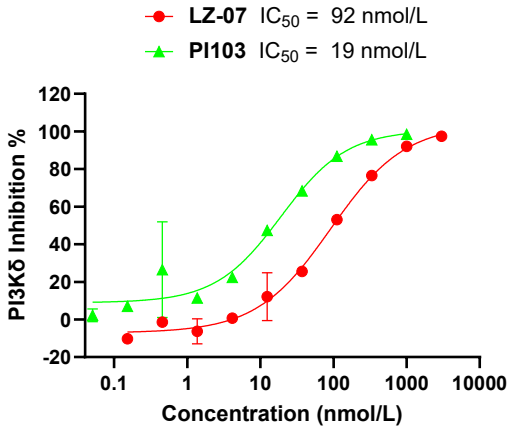


Fig. S9. Enzymatic inhibition of PI3Kδ after treatment with LZ-07, with PI103 serving as a control for PI3Kδ inhibition. Data are presented as mean ± standard deviation (SD) (n = 2).

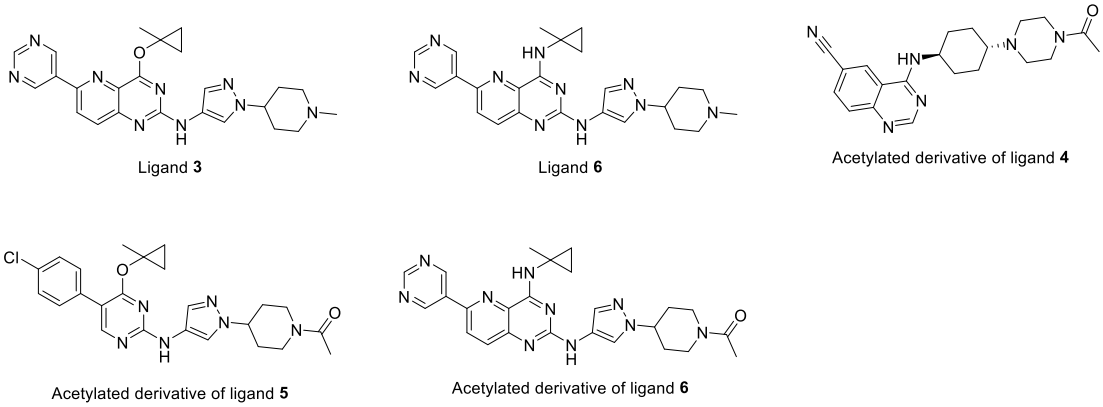


Fig. S10. Compound structures for molecular docking.

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[4] Y. Liu, X. Sun, Q. Liu, C. Han, Y. Rao. J. Am. Chem. Soc. 2024, https://doi.org/10.1021/jacs.4c11930