Supporting Information

Potent and Preferential Degradation of CDK6 via PROteolysis Targeting Chimera Degraders

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Contents:

Synthesis Routines for the molecules

General

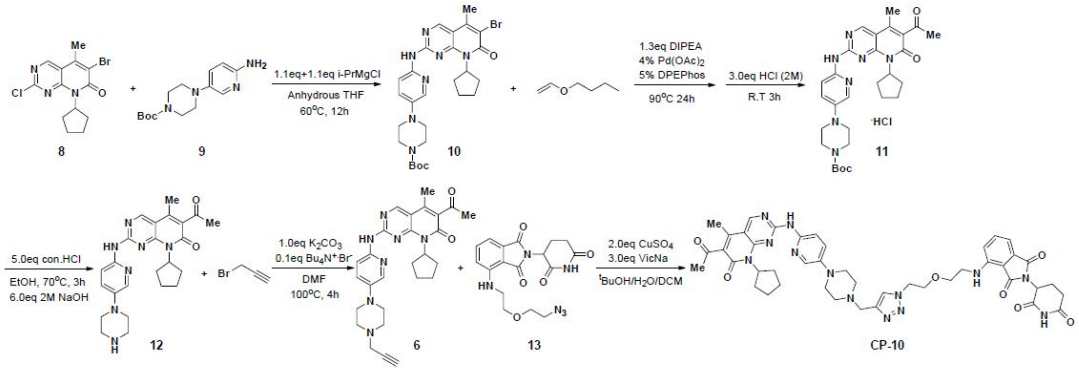
All reactions were carried out under atmosphere or argon. Glassware was oven-dried prior to use. Unless otherwise indicated, common reagents or materials were obtained from commercial source and used without further purification. Flash column chromatography was performed using silica gel 60 (200-300 mesh). Analytical thin layer chromatography (TLC) was carried out on Yinlong silica gel plates with QF-254 indicator and visualized by UV. The 1H and 13C NMR spectra were recorded at 400 MHz, respectively, on Bruker 400 MHz NMR spectrometer.

All NMR spectra were measured at 25 ℃ in CDCl3 or DMSO-d6. Chemical shifts (δ) are reported in parts per million, and coupling constants (J) are reported in hertz. The resonance multiplicities in the 1H NMR spectra are described as “s” (singlet), “d” (doublet), “t” (triplet), “quint” (quintet), and “m” (multiplet), and broad resonances are indicated by “br.” Residual protic solvent of CDCl3 (1H, δ 7.26 ppm; 13C, δ 77.16 ppm), DMSO-d6 (1H, δ 2.50 ppm; 13C 39.50 ppm)was used as the internal reference in the 1H- and 13C-NMR spectra. Low-resolution mass spectral analyses were performed with a Waters AQUITY UPLCTM/MS. Purities of the tested compounds, determined by HPLC, were > 95%. Preparative HPLC was carried out on 250 x 10 mm C-18 column using gradient conditions (1 – 90% B, flow rate = 3.5 mL/min, 30 min). The eluents used were: solvent A (H2O with 0.1% TFA) and solvent B (CH3CN with 0.1% TFA).

Synthetic route of target compound CP-10. As shown in **Scheme 1**. Intermediate 12 was prepared according to the patent WO2014128588A1 [1]. Firstly, compound 10 was synthesized via a substitution reaction between 8 and 9 in presence of isopropylmagnesium chloride. I-PrMgCl was chosen as the base. With this condition, the transformation in step 1, scheme 1 afforded target product in excellent yield.

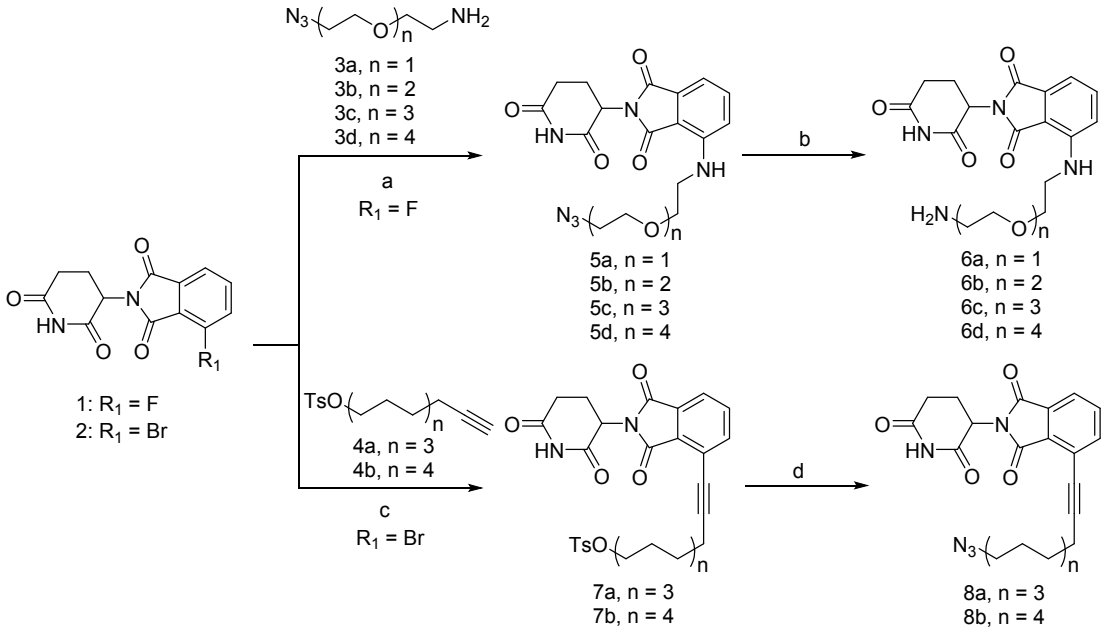
Subsequent intermolecular heck reaction coupled 10 with n-butyl vinyl ether followed by acidolysis led to compound 11. The Boc-protecting group of piperazine was then removed under acid condition to afford 12. An alkynyl group was introduced to 12 by reacting with propargyl bromide, generating intermediate 6. Finally, desired degrader CP-10 was obtained through a click reaction coupling the azide group in 13 with the alkynyl group in 6 [2].

Scheme 1. Synthetic route of target compound CP-10

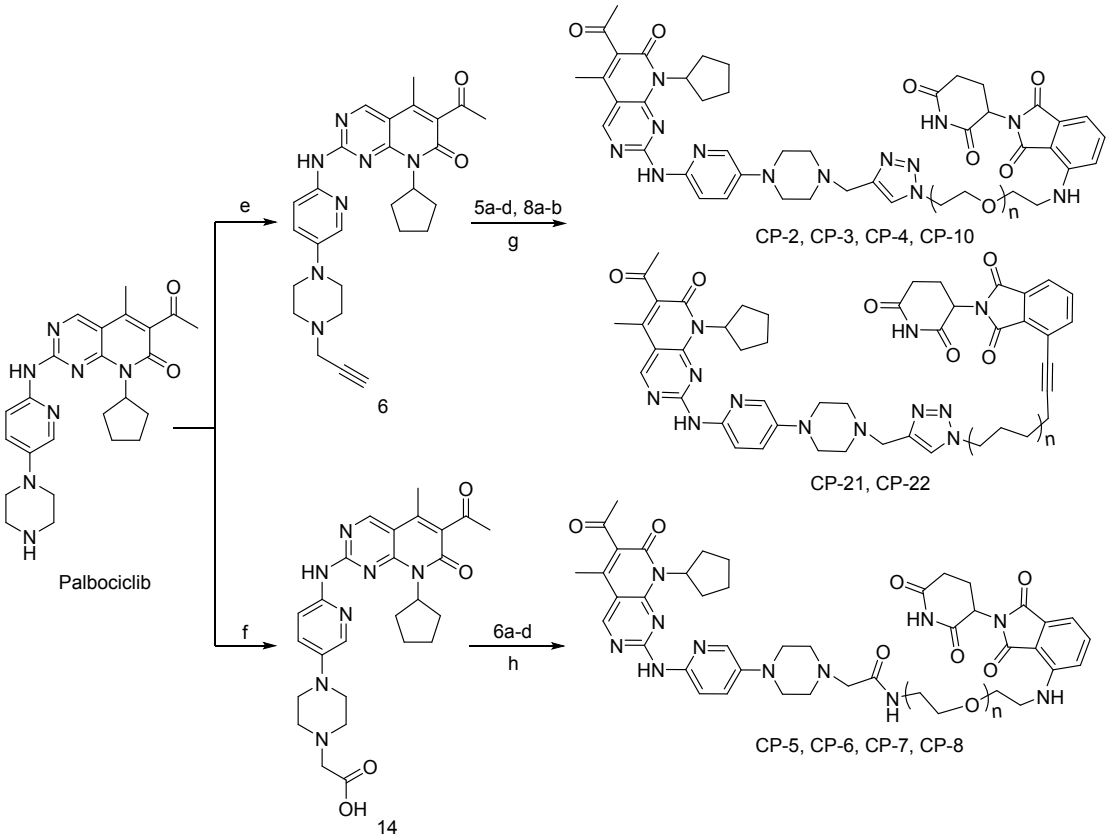


6-acetyl-8-cycolpentyl-5-methyl-2-((5-(4(prop-2-yn-1-yl)piperazin-1-yl)pyridine-2-yl)amino)pyrido[2,3-δ]pyrimidin-7(8H)-one (6). To the solution of Palbociclib (500 mg, 1.12 mmol) in DMF were added 3-bromopropyne (106 µl, 1.23 mmol), K_2CO_3 (136 mg, 1.34 mmol) and TBAB (36 mg, 0.11 mmol). The reaction was stirred at 90 ℃ for 3 h, then washed with water and the organic layer was concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 40:1, vol/vol) give compound 9 (438 mg, 81%) as yellow solid. Rf = 0.4, CH_2Cl_2/MeOH 20:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.38 (s, 1H), 8.16 (d, J = 9.0 Hz, 1H), 8.07 (d, J = 2.8 Hz, 1H), 7.33 (dd, J^1 = 9.1 Hz, J^2 = 3.0 Hz, 1H), 5.92 to 5.83 (m, 1H), 3.39 (d, J = 2.4 Hz, 2H), 3.24 (t, J = 4.8 Hz, 4H), 2.77 (t, J = 4.8 Hz, 4H), 2.54 (s, 3H), 2.37 to 2.33 (m, 5H), 2.29 (t, J = 2.4 Hz, 1H), 2.07 to 2.04 (m, 2H), 1.92 to 1.85 (m, 2H), 1.70 to 1.66 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.84, 161.59, 158.25, 157.40, 155.70, 145.09, 143.73, 14.95, 136.81, 130.87, 126.06, 113.76, 107.88, 78.55, 73.70, 54.19, 51.78, 49.54, 47.07, 31.70, 28.23, 25.91, 14.12, 8.23; LC-MS (ESI^+): m/z calculated for $C_{27}H_{31}N_7O_2$: 486.25 $[M+H]^+$; found 487.6194.

4-((2-(2-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-10). Compound 6 (15 mg, 0.03 mmol), compound 13 (0.04 mmol, 1.2 eq), sodium ascorbate (17.8 mg, 0.09 mmol) was dissolved in ^{t}BuOH/DCM: 1 ml/0.5 ml, then the solution of $CuSO_4$ (9.6 mg, 0.06 mmol) in 0.5 ml water was added. The resulting mixture was stirred at room temperature for 10 min. After the reaction was completed, the solvent was removed and then dealt with 7 M ammonium hydroxide, organic layer was separated and concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 20:1, vol/vol) give compound CP-10, (0.019 mmol, 63%) as solid. Rf = 0.4, CH_2Cl_2/MeOH 10:1.^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 8.79 (s, 1H), 8.22 (s, 1H), 8.12 (d, J = 9.1 Hz, 1H), 8.02 (d, J = 2.5 Hz, 1H), 7.75 (s, 1H), 7.51 (dd, J^1 = 8.3 Hz, J2 = 7.3 Hz, 1H), 7.30 (dd, J^1 = 9.1 Hz, J2 = 2.5 Hz, 1H), 7.14 (d, J = 7.3 Hz, 1H), 6.90 (d, J = 8.3 Hz, 1H), 6.63 (t, J = 5.3 Hz, 1H), 5.89 to 5.84 (m, 1H), 4.92 to 4.88 (m, 1H), 4.60 to 4.60 (m, 2H), 3.94 to 3.87 (m, 3H), 3.72 to 3.63 (m, 3H), 3.43 to 3.41 (m, 2H), 3.24 to 3.19 (m, 4H), 2.76 to 2.69 (m, 7H), 2.54 (s, 3H), 2.36 to 2.32 (m, 5H), 2.19 to 2.15 (m, 1H), 2.07 to 2.03 (m, 2H), 1.89 to 1.83 (m, 2H), 1.70 to 1.66 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.87, 712.18, 169.99, 169.54, 167.62, 161.58, 158.25, 157.39, 155.68, 147.07, 141.99, 136.42, 132.69, 130.81, 126.09, 124.75, 117.17, 112.30, 110.93, 107.79, 77.39, 69.91, 69.61, 68.12, 54.23, 52.84, 52.49, 50.67, 49.18, 49.06, 42.40, 31.69, 31.55, 29.84, 28.20, 27.06, 25.87, 25.76, 23.20, 14.10; LC-MS (ESI^+): m/z calculated for $C_{44}H_{49}N_{13}O_7$: 437.195 $[M+2H]^{2+}$; found 437.2669. **Purities of CP-10 determined by HPLC, were > 95%**.



Conditions: a. 2.0 eq DIPEA, DMF, 85 ^{o}C, 4 h; b. H_2, Pd/C, MeOH, R.T, 12 h;
c. 5% CuI, 10% $Pd(dppf)_2Cl_2$, 10.0 eq, Et_3N, dry THF, 70 ^{o}C, 12 h; d. 1.3 eq, NaN_3, DMF, 70 ^{o}C, 6 h

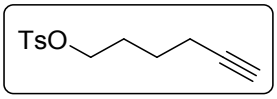


Conditions: e. 1.2 eq 3-bromopropyne, 1.2 eq K_2CO_3, 0.1 eq TBAB, DMF, 90^{o}C, 4 h; f. 1.2 eq Methyl bromoacetate, 1.5 eq K_2CO_3, DMF, 70^{o}C, 3 h, then 3.0 eq LiOH, THF/ MeOH/ H_2O: 1.5 ml/ 3 ml/ 1.5 ml, 50^{o}C, 1 h; g. 2.0 eq $CuSO_4$, 3.0 eq NaVic, ^{t}BuOH/ DCM/ H_2O: 2 ml/ 0.5 ml/ 0.5 ml, R.T, 15 min; h. 5.0 eq DIPEA, 1.1 eq HATU, DMF, R.T, 5 h

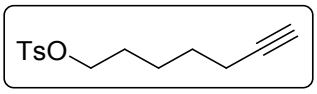
The ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz, respectively, on Bruker 400 MHz NMR spectrometer. All NMR spectra were measured at 25 ℃ in $CDCl_3$ or DMSO-d6. Chemical shifts (δ) are reported in parts per million, and coupling constants (J) are reported in hertz. The resonance multiplicities in the ^{1}H NMR spectra are described as “s” (singlet), “d” (doublet), “t” (triplet), “quint” (quintet), and “m” (multiplet), and broad resonances are indicated by “br.” Residual protic solvent of $CDCl_3$ (^{1}H, δ 7.26 ppm; ^{13}C, δ 77.16 ppm), DMSO-d6 (^{1}H, δ 2.50 ppm; ^{13}C 39.50 ppm)was used as the internal reference in the ^{1}H- and ^{13}C-NMR spectra.

Compound 1, 2, 3a-d were prepared according literature procedures[3] and Palbociclib was commercially available.

General procedure for synthesis of compounds 4a, 4b. To the solution of hex-5-yn-1-ol or hept-6-yn-1-ol (10.2 mmol) in DCM were added tosyl chloride (2.3 g, 12.2 mmol), Et_3N (1.7 ml, 13.2 mmol) and DMAP (125 mg, 1.02 mmol) at room temperature. The reaction was stirred overnight then washed with saturated $NaHCO_3$ solution, the organic layer was separated and concentrated to dryness. Flash column chromatography (petroleum ether/ethyl estate, 15:1, vol/vol) give compounds 4a (2.3 g, 88%), 4b (2.5 g, 91%) as colorless oil. Rf = 0.65, petroleum ether/ethyl estate 5:1.



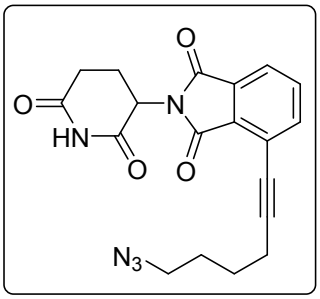
Hex-5-yn-1-yl 4-methylbenzenesulfonate (4a). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.3 Hz, 2H), 4.05 (t, J = 6.3 Hz, 2H), 2.44 (s, 3H), 2.15 (td, J1 = 7.0 Hz, J2 = 2.6 Hz, 2H), 1.91 (t, J = 2.6 Hz, 1H), 1.80 to 1.73 (m, 2H), 1.58 to 1.51 (m, 2H).



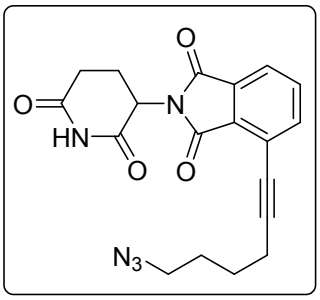
Hept-6-yn-1-yl 4-methylbenzenesulfonate (4b). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 4.02 (t, J = 6.5, 2H), 2.44 (s, 3H), 2.13 (td, J1 = 6.7 Hz, J2 = 2.6 Hz, 2H), 1.91 (t, J = 2.6 Hz, 1H), 1.69 to 1.62 (m, 2H), 1.50 to 1.39 (m, 2H).

General procedure for synthesis of compounds 5a-d. To the solution of compound 3a-d (0.47 mmol, 1.3 eq) and DIPEA (0.73 mmol, 2.0 eq) in DMF was added compound 1 (100 mg, 0.36 mmol). The resulting mixture was stirred at 85 ℃ for 4 h, then washed with water and the organic layer was concentrated to dryness. Flash column chromatography (petroleum ether/ethyl estate, 2:1, vol/vol) give compound 5a (0.13 mmol, 35%), 5b (0.14 mmol, 39%), 5c (0.13 mmol, 35%), 5d (0.12 mmol, 33%) as yellowishandgreen solid. Rf = 0.5-0.4, CH_2Cl_2/MeOH 20:1.

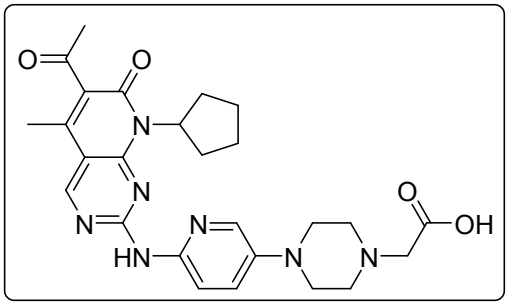
General procedure for synthesis of compounds 8a-b. To the sealed tube were added compound 1 (200 mg, 0.6 mmol), compound 4 (2.98 mmol, 5.0 eq), CuI (23 mg, 20%) and Pd(dppf)Cl_2 (44 mg, 10%), then the tube was degassed and charged with Ar, followed by addition of anhydrous THF and Et_3N (543 μl, 4.2 mmol). The reaction was stirred at 70 ℃ for 12h and concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 50:1, vol/vol) give compounds 8a (265 mg, 87%), 8b (280 mg, 89%) as colorless oil. Rf = 0.4, CH_2Cl_2/MeOH 40:1.



4-(6-azidohex-1-yn-1-yl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (8a). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 1H), 7.78 (dd, J1 = 7.0 Hz, J2 = 1.4 Hz, 1H), 7.70 to 7.64 (m, 2H), 4.98 (dd, J1 = 12.5 Hz, J2 = 5.6 Hz, 1H), 3.36 (t, J = 6.7 Hz, 2H), 2.92 to 2.72 (m, 3H), 2.57 (t, J = 6.8 Hz, 2H), 2.15 to 2.11 (m, 1H), 1.90 to 1.85 (m, 2H), 1.83 to 1.74 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 170.89, 167.95, 166.56, 166.07, 138.50, 133.98, 132.34, 130.80, 122.76, 121.60, 98.59, 76.62, 51.09, 49.36, 31.48, 27.99, 25.47, 22.68, 19.45.

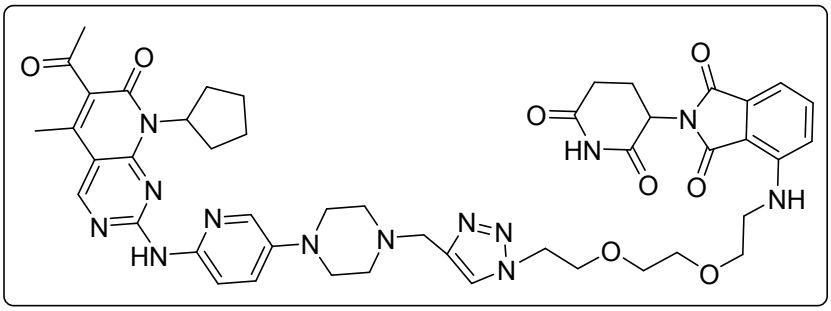


4-(7-azidohept-1-yn-1-yl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (8b). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 1H), 7.77 (dd, J1 = 7.0 Hz, J2 = 1.3 Hz, 1H), 7.70 to 7.62 (m, 2H), 4.97 (dd, J1 = 12.2 Hz, J2 = 5.2 Hz, 1H), 3.30 (t, J = 6.7 Hz, 2H), 2.91 to 2.70 (m, 3H), 2.54 (t, J = 6.6 Hz, 2H), 2.14 to 2.10 (m, 1H), 1.73 to 1.58 (m, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 170.88, 167.94, 166.59, 166.11, 138.57, 133.96, 132.33, 130.76, 122.68, 121.77, 99.14, 76.36, 51.46, 49.36, 31.48, 28.51, 27.89, 25.99, 22.71, 19.76.



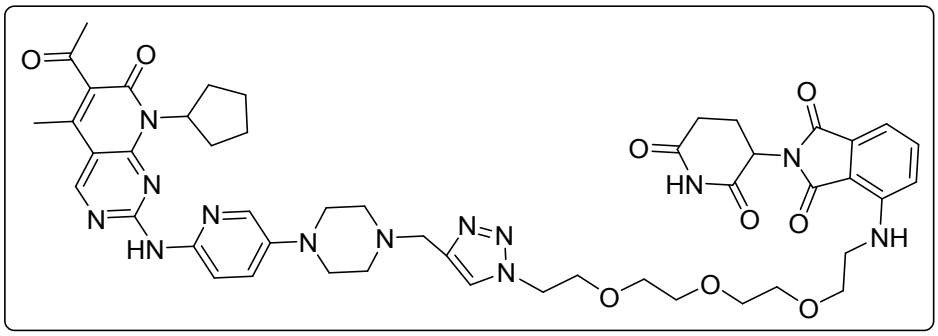
The 2-(4-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-δ]pyrimidin-2-yl)amino)pyridine-3-yl)piperazin-1-yl)acetic acid (14). To the solution of Palbociclib (50 mg, 0.11 mmol), K_2CO_3 (102 mg, 1.01 mmol) in acetone was added methyl bromoacetate (76 μl, 0.8 mmol). The resulting mixture was stirred at room temperature overnight, then concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 20:1, vol/vol) give the intermediate (58 mg, 98%) as brown solid. The intermediate was dissolved in THF/MeOH/H_2O: 1.5 ml/3 ml/1.5 ml, followed by addition of LiOH. The reaction was stirred at 50 ℃ for 1.5 h. Then organic solvent was concentrated in vacuo, resulting solution was acidized to PH<6 with 2 M HCl and the mixture was filtered to get compound 14 (38 mg, 69%); ^{1}H NMR (400 MHz, DMSO-d6) δ 10.23 (s, 1H), 8.96 (s, 1H), 8.11 (d, J = 2.1 Hz 1H), 7.91 (d, J = 9.0 Hz, 1H), 7.57 (dd, J1 = 9.0 Hz, J2 = 2.1 Hz, 1H), 5.85 to 5.80 (m, 1H), 4,16 (s, 2H), 3.45 (s, 8H), 2.42 (s, 3H), 2.31 to 2.24 (m, 5H), 1.89 (m, 2H), 1.78 (m, 2H), 1.58 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d6) δ 202.46, 167.45, 160.75, 158.46, 158.24, 154.79, 145.21, 142.05, 141.90, 129.47, 125.74, 115.08, 106.81, 55.04, 52.97, 51.20, 45.50, 31.33, 27.59, 25.17, 13.67; LC-MS (ESI+): m/z calculated for C26H31N7O4: 506.24 [M+H]+; found 507.7604.

General procedure for synthesis of compounds CP-2, CP-3, CP-4, CP-21, CP-22. Compound 6 (15 mg, 0.03 mmol), compounds 5a-d or 8a-b (0.04 mmol, 1.2 eq), sodium ascorbate (17.8 mg, 0.09 mmol) was dissolved in ^{t}BuOH/DCM: 1 ml/0.5 ml, then the solution of $CuSO_4$ (9.6 mg, 0.06 mmol) in 0.5 ml water was added. The resulting mixture was stirred at room temperature for 10 min. After the reaction was completed, the solvent was removed and then dealt with 7 M ammonium hydroxide, organic layer was separated and concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 20:1, vol/vol) give compound CP-10, CP-2, CP-3, CP-4, CP-21, CP-22 (0.019 mmol, 63%) as solid. Rf = 0.4, CH_2Cl_2/MeOH 10:1.

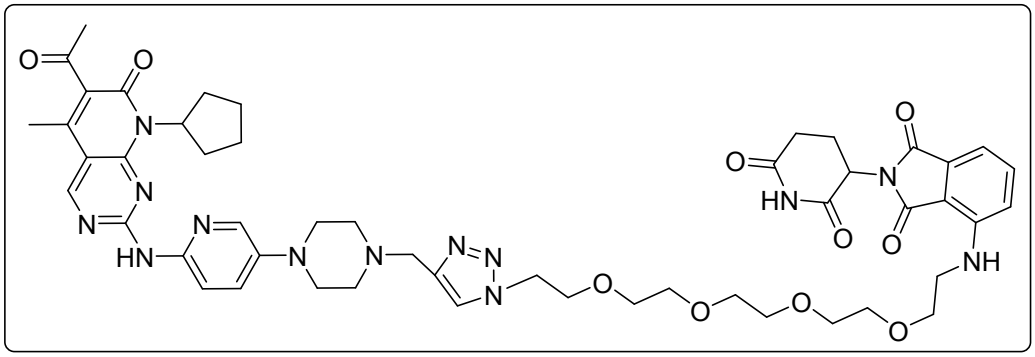


4-((2-(2-(2-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)amino)-2-(2,6-

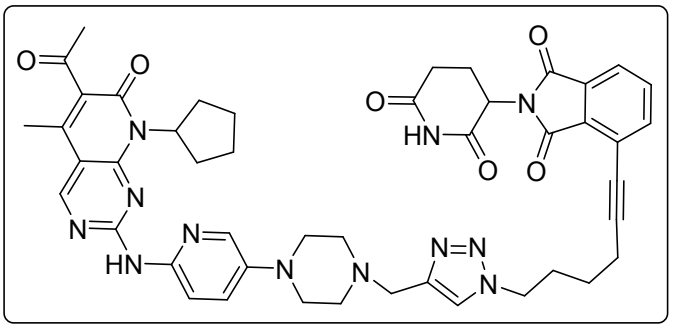
dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.11 (d, J = 8.7 Hz, 1H), 8.04 (d, J = 2.6 Hz, 1H), 7.75 (s, 1H), 7.47 (dd, J1 = 8.6 Hz, J2 = 7.0 Hz, 1H), 7.28 (dd, J1 = 8.7 Hz, J2 = 2.6 Hz, 1H), 7.08 (d, J = 7.0 Hz, 1H), 6.86 (d, J = 8.6 Hz, 1H), 6.54 (t, J = 5.1 Hz, 1H), 5.87 to 5.83 (m, 1H), 4.90 to 4.85 (m, 1H), 4.59 to 4.54 (m, 2H), 3.91 to 3.88 (m, 2H), 3.81 to 3.56 (m, 8H), 3.43 to 3.41 (m, 2H), 3.18 to 3.16 (m, 4H), 2.82 to 2.65 (m, 7H), 2.52 (s, 3H), 2.36 to 2.32 (m, 5H), 2.09 to 2.01 (m, 3H), 1.86 to 1.83 (m, 2H), 1.67 to 1.64 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.79, 172.23, 169.70, 169.68, 167.67, 161.52, 158.21, 157.34, 155.62, 146.75, 144.95, 143.55, 143.27, 141.94, 136.34, 136.20, 132.65, 130.70, 125.93, 124.52, 116.77, 113.73, 111.83, 110.41, 107.66, 77.30, 70.48, 70.44, 69.76, 69.20, 54.18, 52.82, 52.53, 50.28, 49.07, 48.95, 42.30, 31.69, 31.62, 29.77, 28.12, 25.79, 23.15, 14.20, 14.03; LC-MS (ESI$^+$): m/z calculated for $C_{46}H_{53}N_{13}O_8$: 459.205 $[M+2H]^{2+}$; found 459.3080.



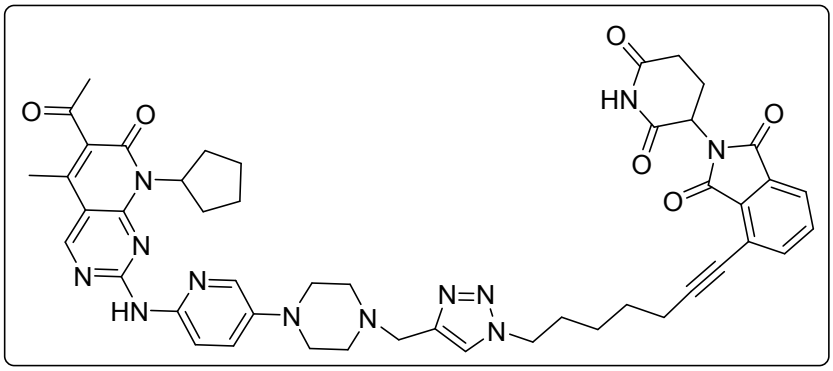
4-((2-(2-(2-(2-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.62 (s, 1H), 8.81 (s, 2H), 8.13 (d, J = 9.1 Hz, 1H), 8.06 (d, J = 2.7 Hz, 1H), 7.65 (s, 1H), 7.47 (dd, J1 = 8.6 Hz, J2 = 7.1 Hz, 1H), 7.30 (dd, J1 = 9.1 Hz, J2 = 2.7 Hz, 1H), 7.08 (d, J = 7.1 Hz, 1H), 6.89 (d, J = 8.6 Hz, 1H), 6.46 (t, J = 8.9 Hz, 1H), 5.89 to 5.84 (m, 1H), 4.93 to 4.88 (m, 1H), 4.53 to 4.51 (m, 2H), 3.88 to 3.85 (m, 2H), 3.74 to 3.59 (m, 12H), 3.47 to 3.43 (m, 2H), 3.19 to 3.17 (m, 4H), 2.91 to 2.70 (m, 7H), 2.53 (s, 3H), 2.35 (s, 5H), 2.13 to 2.01 (m, 3H), 1.87 to 1.85 (m, 2H), 1.68 to 1.65 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.72, 172.52, 169.40, 169.02, 167.64, 161.45, 158.18, 157.28, 155.85, 146.80, 144.89, 143.84, 143.55, 141.85, 136.02, 135.95, 132.56, 130.67, 126.09, 123.84, 116.75, 113.74, 111.71, 110.42, 107.62, 77.29, 70.77, 70.64, 70.58, 69.52, 54.13, 53.18, 52.51, 50.23, 49.18, 48.96, 42.48, 31.55, 29.70, 28.03, 25.70, 22.86; LC-MS (ESI$^+$): m/z calculated for $C_{48}H_{57}N_{13}O_9$: 481.22 $[M+2H]^{2+}$; found 481.2541.



4-((14-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-3,6,9,12-tetraoxatetradecyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.57 (s, 1H), 8.81 (s, 2H), 8.15 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.6 Hz, 1H), 7.69 (s, 1H), 7.48 (dd, J1 = 8.6 Hz, J2 = 6.1 Hz, 1H), 7.31 (dd, J1 = 9.1 Hz, J2 = 2.6 Hz, 1H), 7.09 (d, J = 6.1 Hz, 1H), 6.90 (d, J = 8.6 Hz, 1H), 6.49 (t, J = 9.0 Hz, 1H), 5.90 to 5.85 (m, 1H), 4.94 to 4.89 (m, 1H), 4.55 to 4.52 (m, 2H), 3.88 to 3.85 (m, 2H), 3.76 to 3.59 (m, 16H), 3.48 to 3.44 (m, 2H), 3.20 (s, 4H), 2.92 to 2.70 (m, 7H), 2.36 to 2.32 (m, 5H), 2.14 to 2.04 (m, 3H), 1.88 to 1.86 (m, 2H), 1.68 to 1.65 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.86, 172.67, 169.48, 169.09, 167.79, 161.59, 158.32, 157.41, 155.69, 146.99, 145.00, 143.70, 141.98, 136.15, 136.01, 132.71, 130.83, 126.21, 124.07, 116.92, 113.89, 111.81, 110.54, 77.20, 70.91, 70.83, 70.76, 70.60, 69.65, 54.28, 53.34, 52.62, 50.37, 49.32, 49.08, 42.59, 31.69, 29.85, 28.18, 25.84, 23.02, 14.10; LC-MS (ESI$^+$): m/z calculated for $C_{50}H_{61}N_{13}O_{10}$: 503.235 $[M+2H]^{2+}$; found 503.2952.

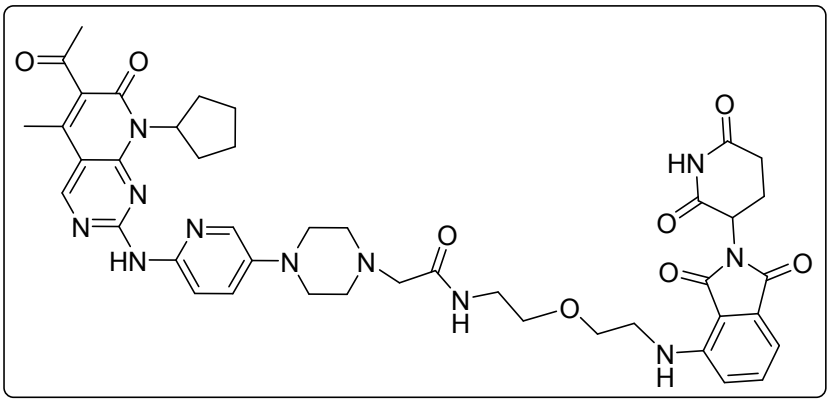


4-(6-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)hex-1-yn-1-yl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-21). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.65 (s, 1H), 8.81 (s, 1H), 8.40 (s, 1H), 8.12 (d, J = 9.0 Hz, 1H), 8.04 (d, J = 2.4 Hz, 1H), 7.81 to 7.77 (m, 2H), 7.68 to 7.66 (m, 2H), 7.30 (dd, J1 = 9.0 Hz, J2 = 2.4 Hz, 1H), 5.90 to 5.82 (m, 1H), 4.97 to 4.93 (m, 1H), 4.64 to 4.57 (m, 1H), 4.48 to 4.40 (m, 1H), 4.0 (d, J = 13.0 Hz, 1H), 3.65 (d, J = 13.0 Hz, 1H), 3.31 to 3.21 (m, 4H), 2.86 to 2.60 (m, 9H), 2.53 (s, 3H), 2.31 to 2.28 (m, 5H), 2.22 to 2.16 (m, 1H), 2.05 to 2.03 (m, 2H), 1.88 to 1.86 (m, 2H), 1.74 to 1.66 (m, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.76, 172.40, 170.03, 166.76, 166.34, 158.13, 157.28, 155.57, 144.88, 143.56, 143.33, 141.86, 138.11, 136.65, 134.13, 132.30, 130.93, 130.70, 125.90, 123.19, 122.90, 121.35, 113.63, 107.70, 98.01, 77.27, 77.16, 54.09, 52.64, 52.54, 50.21, 49.25, 48.30, 31.58, 29.37, 28.98, 28.10, 25.79, 24.96, 23.16, 19.22, 14.00; LC-MS (ESI^+): m/z calculated for $C_{46}H_{48}N_{12}O_6$: 433.69 $[M+2H]^{2+}$; found 433.7517.

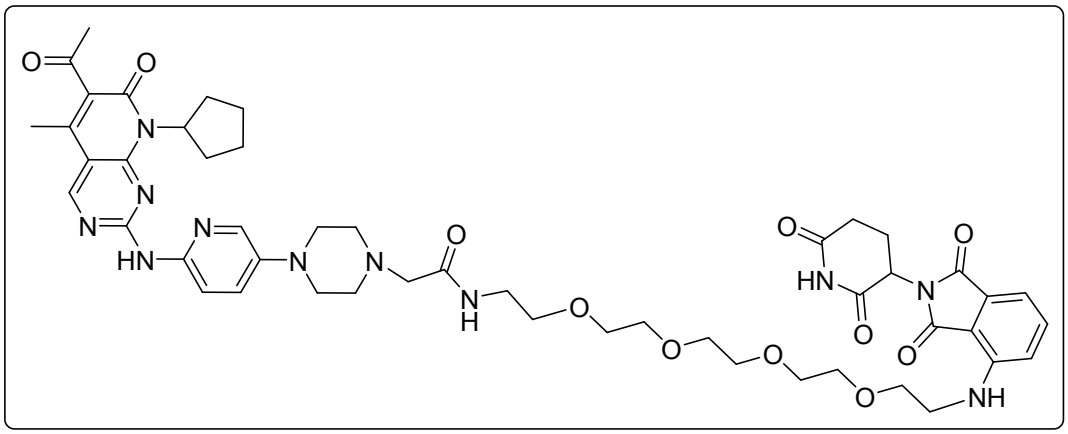


4-(7-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)hept-1-yn-1-yl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (CP-22). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.56 (s, 1H), 8.82 (s, 1H), 8.67 (s, 1H), 8.12 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 2.6 Hz, 1H), 7.78 (dd, J1 = 6.7 Hz, J2 = 1.4 Hz, 1H), 7.71 to 7.64 (m, 3H), 7.31 (dd, J1 = 9.1 Hz, J2 = 2.6 Hz, 1H), 5.90 to 5.82 (m, 1H), 5.00 to 4.95 (m, 1H), 4.55 to 4.84 (m, 1H), 4.39 to 4.32 (m, 1H), 3.86 (d, J = 13.3 Hz, 1H), 3.66 (d, J = 13.3 Hz, 1H), 3.26 to 3.19 (m, 4H), 2.85 to 2.70 (m, 7H), 2.59 to 2.47 (m, 5H), 2.36 to 2.32 (m, 5H), 2.15 to 2.12 (m, 1H), 2.04 (s, 3H), 1.87 to 1.85 (m, 3H), 1.71 to 1.58 (m, 6H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 202.80, 172.05, 169.44, 166.53, 166.52, 161.53, 158.21, 157.34, 155.64, 144.98, 143.57, 143.31, 141.94, 138.61, 136.49, 134.05, 132.34, 130.83, 130.74, 125.97, 123.73, 122.81, 121.65, 113.73, 107.72, 98.75, 77.37, 76.96, 54.17, 52.91, 52.59, 50.70, 49.44, 48.85, 31.69, 31.64, 29.79, 29.61, 28.15, 26.88, 25.83, 25.29, 23.06, 19.28, 14.05; LC-MS (ESI^+): m/z calculated for $C_{47}H_{50}N_{12}O_6$: 440.70 $[M+2H]^{2+}$; found 440.7820.

General procedure for synthesis of compounds CP-5, CP-6, CP-7, CP-8. The solution of compound 14 (19 mg, 0.04 mmol), HATU (16 mg, 0.04 mmol), DIPEA (15 µL, 0.08 mmol) in DMF was stirred at room temperature for 10 min, then compound 6a-d (0.04 mmol, 1.1 eq) was added. The resulting mixture was stireed for 2 h and washed with water, organic layer was separated and concentrated to dryness. Flash column chromatography (CH_2Cl_2/MeOH, 40:1, vol/vol) give compound CP-5, CP-6, CP-7, CP-8 (0.02 mmol, 52%) as solid. Rf = 0.6, CH_2Cl_2/MeOH 10:1.



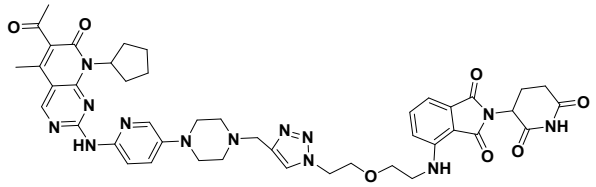
2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethoxy)ethyl)acetamide (CP-5). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.95 (s, 1H), 8,82 (s, 1H), 8.70 (s, 1H), 8.13 (d, J = 9.1 Hz, 1H), 8.03 (d, J = 2.8 Hz, 1H), 7.47 to 7.40 (m, 2H), 7.28 (dd, J1 = 9.1 Hz, J2 = 2.8 Hz, 1H), 7.06 (d, J = 7.0 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 6.46 (t, J = 5.4 Hz, 1H), 5.91 to 5.82 (m, 1H), 4.93 to 4.88 (m, 1H), 3.72 to 3.70 (m, 2H), 3.62 to 3.44 (m, 6H), 3.17 to 3.14 (m, 4H), 3.09 (s, 2H), 2.89 to 2.69 (m, 7H), 2.54 (s, 3H), 2.38 to 2.33 (m, 5H), 2.11 to 2.03 (m, 3H), 1.88 to 1.85 (m, 2H), 1.72 to 1.66 (m, 2H); LC-MS (ESI$^+$): m/z calculated for $C_{43}H_{49}N_{11}O_8$: 425.19 $[M+2H]^{2+}$; found 425.2963.



2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)-N-(14-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)-3,6,9,12-tetraoxatetradecyl)acetamide (CP-8). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.23 (s, 1H), 9.09 (s, 1H), 8,82 (s, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.09 (d, J = 2.6 Hz, 1H), 7.50 to 7.47 (m, 2H), 7.35 (dd, J1 = 9.0 Hz, J2 = 2.6 Hz, 1H), 7.09 (d, J = 7.0 Hz, 1H), 6.89 (d, J = 8.5 Hz, 1H), 6.53 (t, J = 5.1 Hz, 1H), 5.90 to 5.83 (m, 1H), 4.94 to 4.90 (m, 1H), 3.73 to 3.43 (m, 20H), 3.20 (s, 4H), 3.08 (s, 2H), 2.94 to 2.70 (m, 7H), 2.54 (s, 3H), 2.33 to 2.28 (m, 5H), 2.15 to 2.06 (m, 3H), 1.88 to 1.86 (m, 2H), 1.70 to 1.66 (m, 2H); LC-MS (ESI$^+$): m/z calculated for $C_{49}H_{61}N_{11}O_{11}$: 491.23 $[M+2H]^{2+}$; found 491.3246.

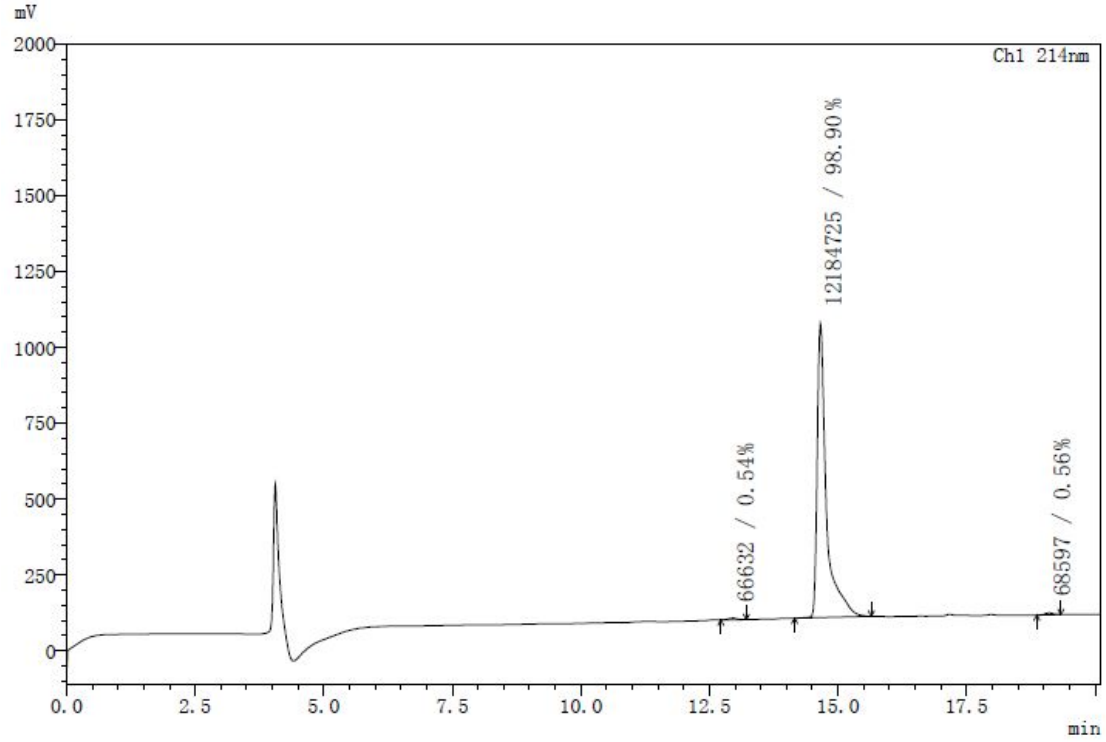
HPLC analysis

CP-10

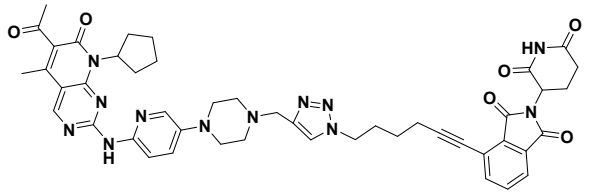


Gradient: 10% - 90% CH_3CN with 0.1% TFA/H_2O with 0.1% TFA over 20 min at a flow rate of 3.5 mL/min.
Wavelength: 214 nm
Report:

Peak	Retention Time/min	Area	Area %
1	12.767	66632	0.54
2	14.659	12184725	98.90
3	18.856	68597	0.56

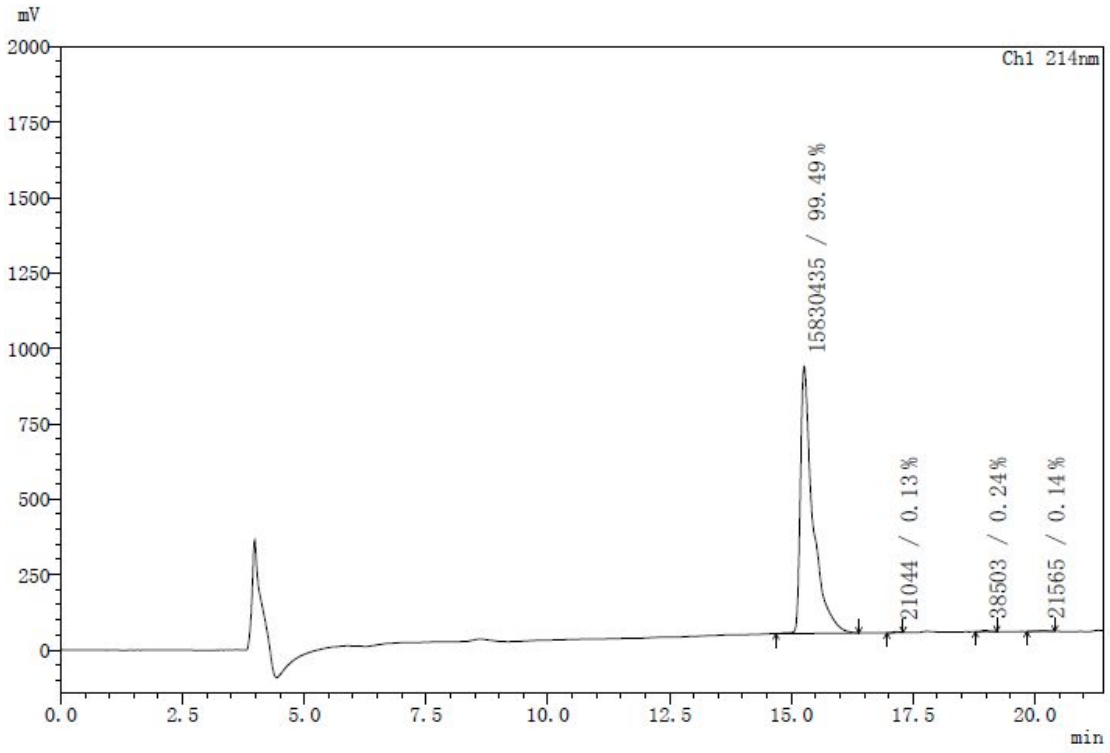


CP-21

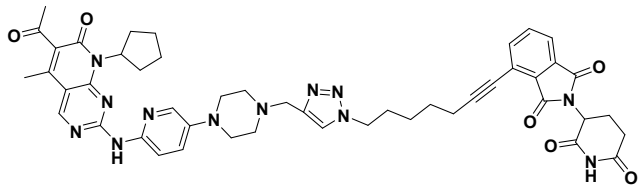


Gradient: 10% - 90% CH_3CN with 0.1% TFA/H_2O with 0.1% TFA over 20 min at a flow rate of 3.5mL/min.
Wavelength: 214nm
Report:

Peak	Retention Time/min	Area	Area %
1	15.261	15830435	99.49
2	17.280	21044	0.13
3	18.975	38503	0.24
4	20.171	21565	0.14



CP-22



Gradient: 20% - 90% CH_3CN with 0.1% TFA/H_2O with 0.1% TFA over 20 min at a flow rate of 3.5mL/min.
Wavelength: 214nm
Report:

Peak	Retention Time/min	Area	Area %
1	14.080	8733441	99.32
2	15.783	3635	0.04
3	17.644	14285	0.16
4	19.277	42055	0.48

Predictive model construction

Molecular Prediction of ligands binding to CDK6/CRBN was done with Schrodinger Suite 2017-1. Processing of the protein structure was performed with the Protein Preparation Wizard. Converting of ligands from 2D to 3D structures was performed using LigPrep. Molecular docking was performed with Glide. Models were constructed with PyMOL.

Details for biological experiments

Cell lines. Human neuroblastoma cell line U251, human Ewing's sarcoma cell line A-673, human leukemia cell line THP-1 and HL-60, human multiple myeloma cell line MM.1S, human mantle cell lymphoma cell line JeKo-1, human medullablastoma cell line DAOY and human embryonic kidney cell line HEK293T were purchased from cell bank (Shanghai) of Chinese Academy of Sciences (www.cellbank.org.cn). Human multiple myeloma cell line RPMI 8226 and human mantle cell lymphoma Mino were kindly provided by Dr. Wanli Liu. Cells were cultured according to providers' manual in humidified incubator under 37℃ and 5% CO2. U251, A-673 were maintained in DMEM supplemented with 10% FBS and Penicillin-Streptomycin (PS). THP-1, HL-60 and RPMI 8226 were maintained in RPMI 1640 supplemented with 10% FBS and PS. Mino and Jeko-1 were maintained in RPMI 1640 supplemented with 20% FBS and PS. MM.1S was maintained in RPMI 1640 supplemented with 10% FBS, PS, Sodium Pyruvate and GlutaMAX. DAOY was maintained in MEM supplemented with 10% FBS, PS, Non Essential Amino Acids, Sodium Pyruvate and GlutaMAX.CDK6 overexpression stable cell lines in A-673 and CDK6 mutant overexpression stable cell lines in MCF-7 were generated via lentivirus infection. Palbociclib-resistant cell lines FAT1CR were generated via CRISPR knockout strategy as previously published[4]. All the cells were identified as mycoplasma-free.

Plasmids. Human CDK4 and CDK6 CDS were cloned from cDNA template. FLAG tag was fused to the N-term or C-term of CDK4 or CDK6 and inserted into regular mammalian expression vector pCS2+ or lentiviral vector pLentiCMVBsd. Mutagenesis of CDK6 was conducted on pLenti-CDK6-FLAG plasmids via Stratagene protocol. All plasmids were verified by sequencing. For transient expression experiments, plasmids were transfected into the cells via lipofectamin 3000 following producers' manual.

Antibodies and Reagents. Primary antibody β-tubulin (KM9003) was purchased from Sungene. FLAG M2 (F3165) was from Sigma. CDK1 (sc-54) were from Santa Cruz Biotechnology. CDK2, CDK4, CDK6, MEK1, EGFR, Cyclin D1, MKK7 and Histone H3 were from Cell Signaling Technology. CDK5 and CDK9 were from Beyotime. ZFP91 was from Abcam. Secondary antibodies were purchased from Thermo with code #31430 (Goat-anti-mouse) and #31460 (Goat-anti-rabbit). Proteasomal inhibitor and Phosphatase inhibitor cocktail were purchased from Bimake. Small molecules carfilzomib, palbociclib and pomalidomide were purchased from Selleck. PROTACs were synthesized as described in the following sections.

TMT Mass spectrometry. For TMT analysis, U251 cells were first seeded on 10 cm plate and allowed to grow to 70%~80% confluency. Then 500 nM CP-10 or vehicle DMSO was introduced into the culture medium. 4 h later, cells were trypsinized, serum terminated and harvested by centrifuge at 4℃, 500 × g. Cells were washed twice with cold PBS and lysed with RIPA buffer on ice for 30 min before centrifugation. Supernatants were collected, quantified (via Thermo BCA kit) and precipated by acetone before regular TMT sample preparation procedure as previously described[5]. Samples were labeled with isobartic tags and subjected to mass spectrometry. The MS2 signals were collected and analyzed with Proteome Discovery 2.0.

***In vitro* kinase assay.** The in vitro kinase assay was performed by ChemPartner. In brief, the kinase inhibitory activities of compound palbociclib and CP-10 against CDK4/CycD3 and CDK6/CycD3 were evaluated by mobility shift assay (280 μM ATP for CDK4 and 800 μM ATP for CDK6). The FAM-labelled peptide derived from CDK4/6 substrate RB was applied. Compounds were tested in consecutive 3-fold dilution starting from 10 μM, 10 points, in duplicate.

Cell proliferation assay. For suspending haematopoietic cell lines, cells were seeded into 96-well plate in triplicates and drug stock was introduced into cell suspension immediately to the required final concentrations. The cells were kept in culture for 84 h before CCK-8 agent addition and absorbance on OD450 nm was measured after 2 h incubation. For adherent MCF-7 cells, cell proliferation was measured as described previously [6].

Supplementary Figures

A

Gene Symbol	mRNA expression level by TPM in U251
CRBN	15.1
cIAP1（BIRC2）	69.2
cIAP2（BIRC3）	17.8
MDM2	20.9
VHL	22.1
CDK4	190.5
CDK6	50.9

B

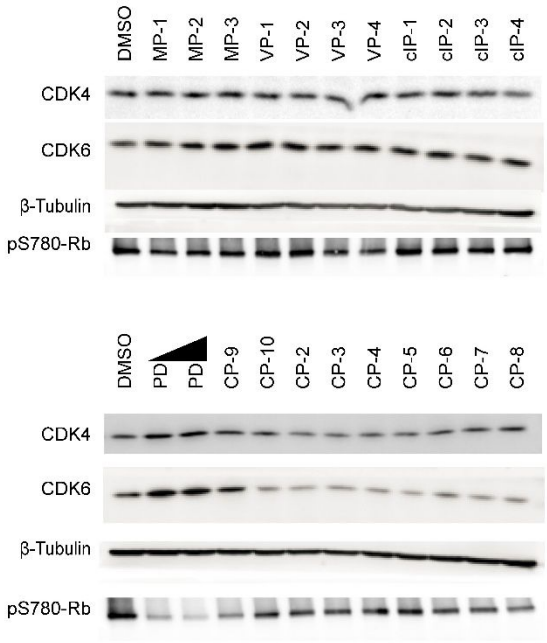


Figure S1. Expressions of tested E3 ligases in U251 and responses to various PROTACs in DAOY cells.

(A) The mRNA expression levels for all the relevant E3 ligases and targets in this study in U251 glioblastoma cells. Data derived from proteinatlas database (www.proteinatlas.org). (B) CDK4/6 levels in medullablastoma DAOY cells after 24 h treatment with different PROTACs (1 μM for all). PD (palbociclib) here was applied as positive control and the concentrations were 500 nM and 1 μM.

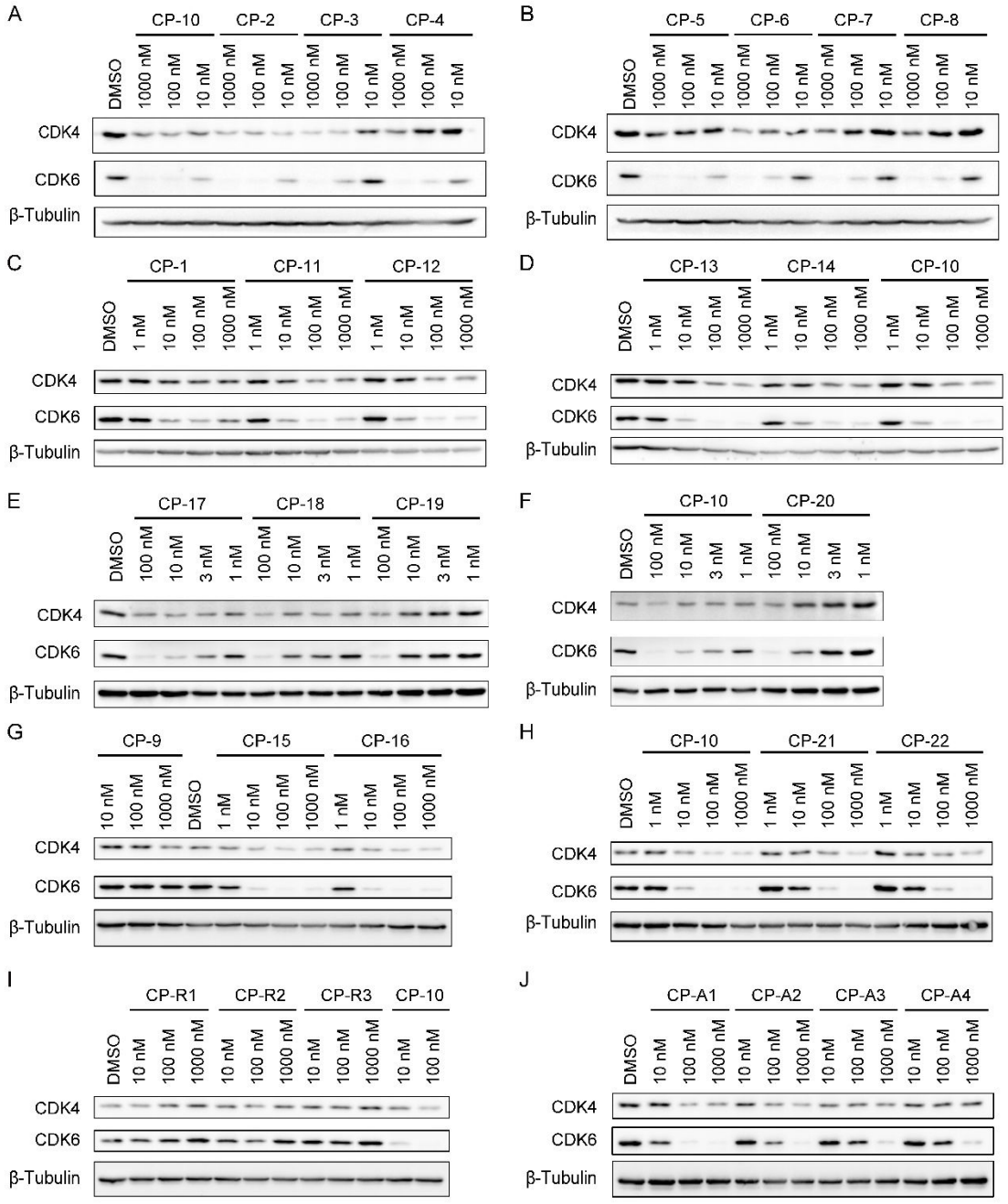


Figure S2. Degradation of CDK4/6 by CRBN-recruiting PROTACs.

(A/B) CDK4/6 levels in U251 cells after 24 h treatment with different concentrations of PROTACs CP-10/2/3/4/5/6/7/8. (C/D) CDK4/6 levels in U251 cells after 24 h treatment with different concentrations of PROTACs CP-1/10/11/12/13/14. (E/F) CDK4/6 levels in U251 cells after 24 h treatment with different concentrations of PROTACs CP-10/17/18/19/20. (G/H) CDK4/6 levels in U251 cells after 24 h treatment with different concentrations of PROTACs CP-9/10/15/16/21/22. (I/J) CDK4/6 levels in U251 cells after 24 h treatment with different concentrations of PROTACs CP-R1/2/3 and CP-A1/2/3/4. CP-R/As were conjugates of ribociclib/abemaciclib with pomalidomide. CP-10 was applied as positive control.

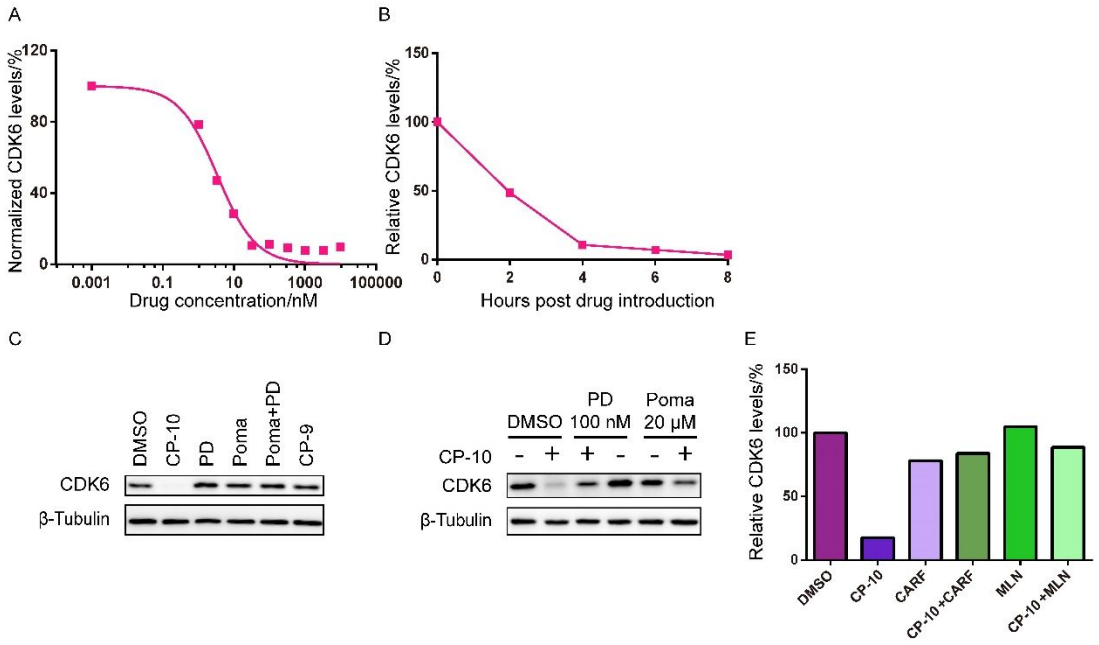
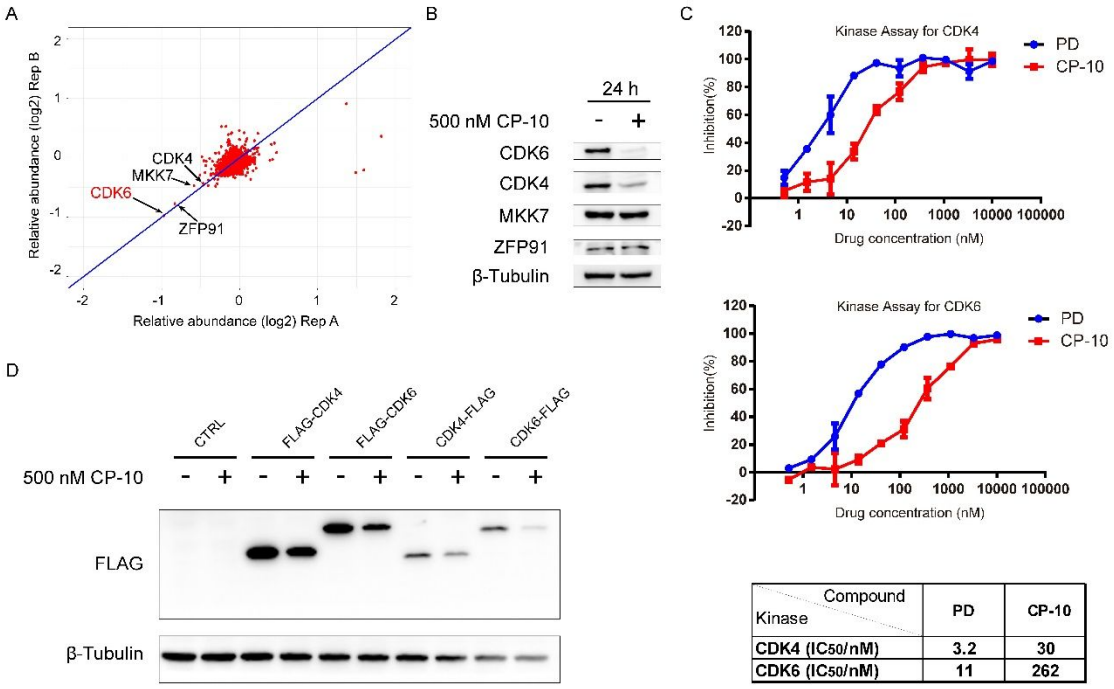


Figure S3. CP-10 induced potent and rapid degradation of CDK6.

(A) Degradation of CDK6 by titration of CP-10 in U251 cells. (B) CDK6 levels in U251 cells rapidly decreased upon 100 nM CP-10 treatment. (C) Single introduction of Palbociclib (PD), Pomalidomide (Poma) or CP-9 (negative control) failed to degrade CDK6. All drugs were applied with 100 nM for 24 h. (D) PD or Poma rescued CP-10 induced CDK6 degradation. U251 cells were treated for 24 h as indicated. CP-10, 100 nM. PD, 100 nM, Poma, 20 μM. (E) Proteasomal inhibitor carfilzomib (CARF, 1 μM) or NAE1 inhibitor MLN4924 (MLN, 200 nM) could reverse the degradation of CDK6 induced by CP-10 (500 nM) in U251 cells. Cells were treated for 8h. All data were representatives of replicated experiments for at least twice.



Compound / Kinase	PD	CP-10
CDK4 (IC_{50}/nM)	3.2	30
CDK6 (IC_{50}/nM)	11	262

Figure S4. CP-10 induced selective/preferential degradation of CDK6 over CDK4.

(A) Dot plots of relative abundance for identified proteins comparing 500 nM CP-10 treated U251 cells to DMSO treated cells. (B) Protein levels of MKK7 and ZFP91 upon CP-10 treatment. U251 cells were treated as indicated for 24 h before harvest. CDK4 and CDK6 were examined here as positive control. (C) *In vitro* kinase assay of CDK4/CDK6 for PD (palbociclib) and CP-10. IC50 values were calculated by the inhibition percentage. (D) CP-10 induced degradation of N-term FLAG-tagged CDK4/6 or C-term FLAG-tagged CDK4/6.Same amount of plasmids were transfected into U251 cells. CTRL, control, no transfection. 500 nM CP-10 or DMSO was introduced into the medium 6 h post transfection and cells were allowed to grow for another 28 h before harvest.

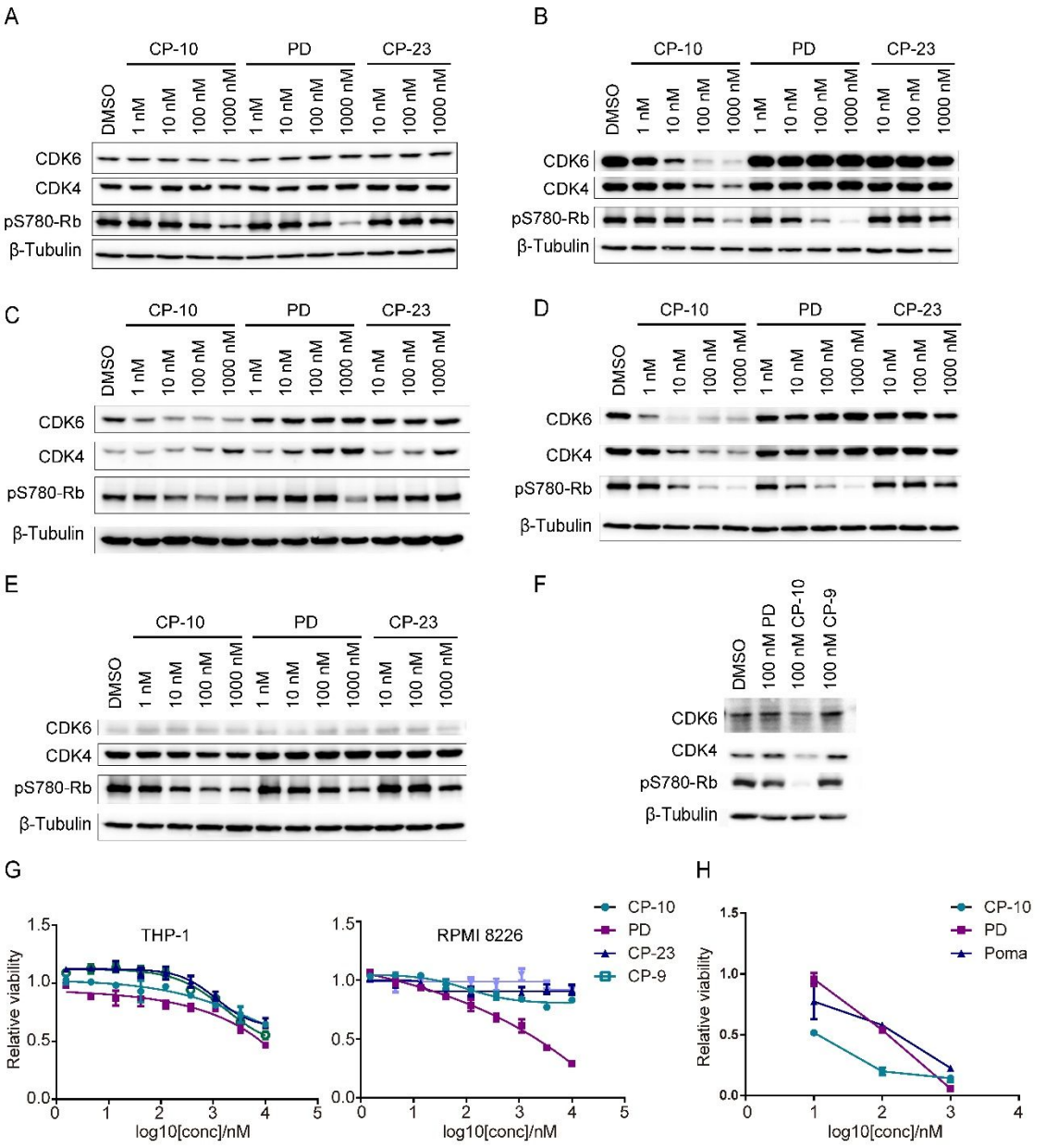


Figure S5. Functional evaluation for CP-10 in various cell lines.

(A-F) Cells were treated as indicated for 24h before subjection to immunoblotting. A, THP-1; B, HL-60; C, RPMI 8226; D, MM.1S; E, Mino; F, Jeko-1. CP-23 or CP-9 was applied as negative control.
(G) Proliferation assays in THP-1/RPMI 8226 upon treatment by serial concentrations of CP-10/PD/CP-23/CP-9.
(H) Proliferation assays in myeloma MM.1S upon treatment by different concentrations of CP-10/PD/Pomalidomide.

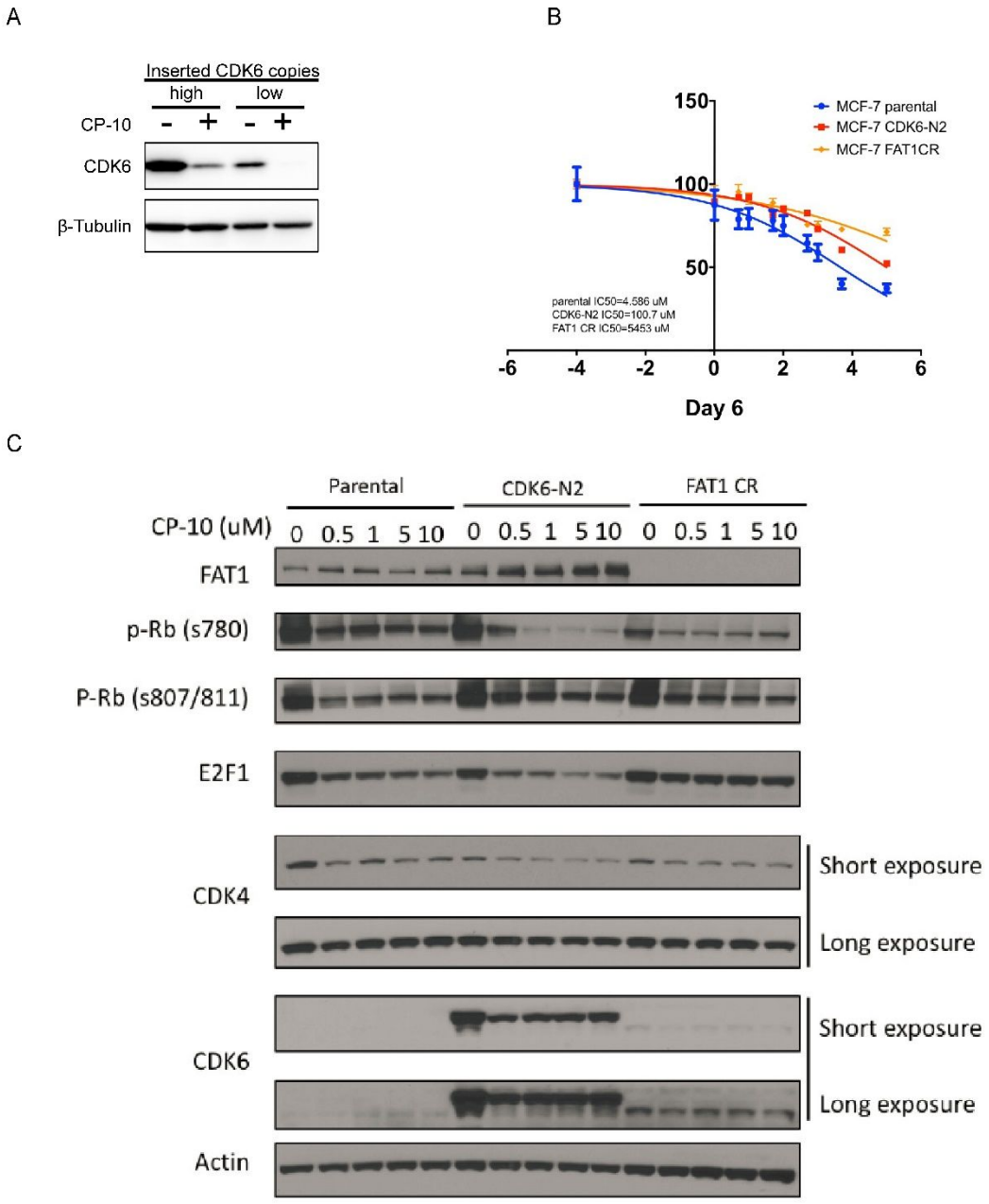
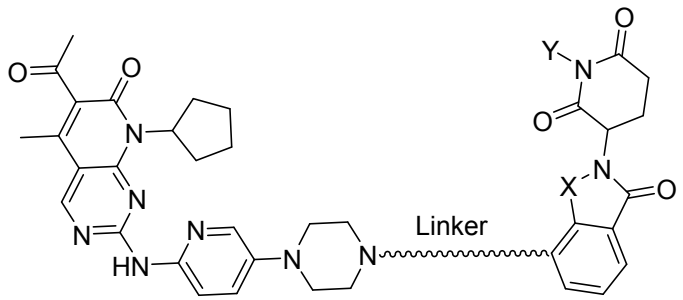


Figure S6. CP-10 induced degradation of over-expressed CDK6 and inhibited growth of palbociclib-resistant breast cancer cell lines.

(A) CDK6 overexpression simulation by inserting additional copies of CDK6 into Ewing's sarcoma cell lines A-673 via lentivirus infection. Cells were treated with 1 μM CP-10 for 24 h before harvest for immunoblotting.
(B/C) Parental cells or resistant cells (CDK6-N2 or FAT1CR) were treated with different concentrations of PROTAC CP-10 for 6 days before determination of cell viability. The expression levels of indicated proteins were shown in panel C.

Supplementary Tables

Table S1. Chemical structure of CDK6-targeting PROTACs



Palbociclib CRBN-recruiting ligand

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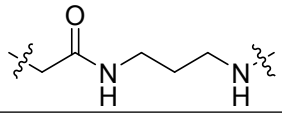
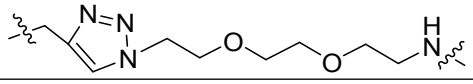
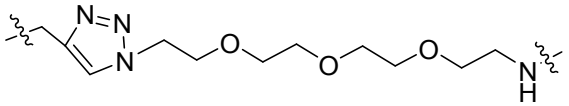
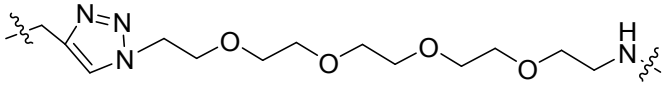
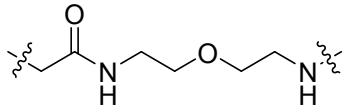
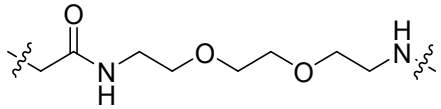
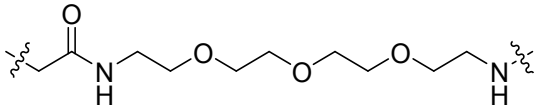
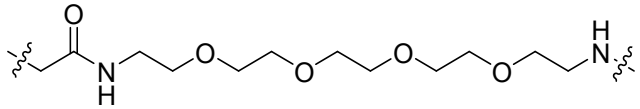
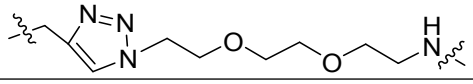
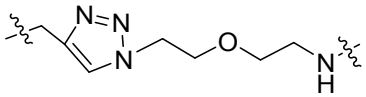
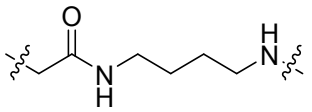
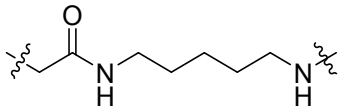
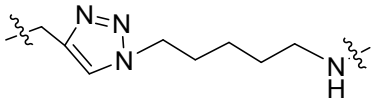
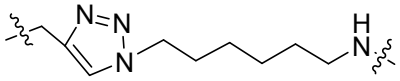
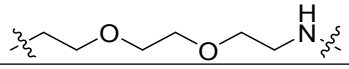
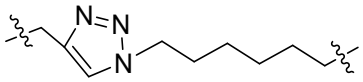
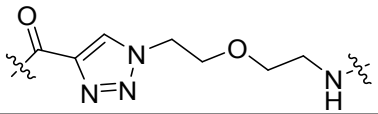
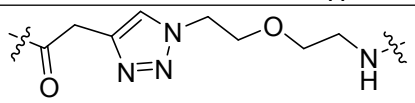
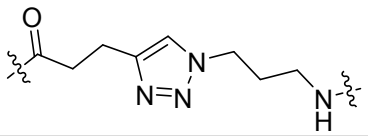
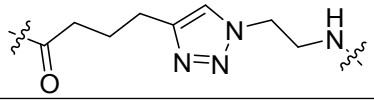
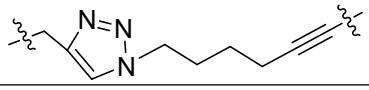
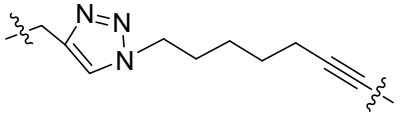
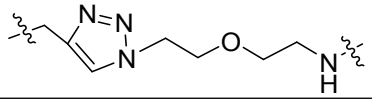
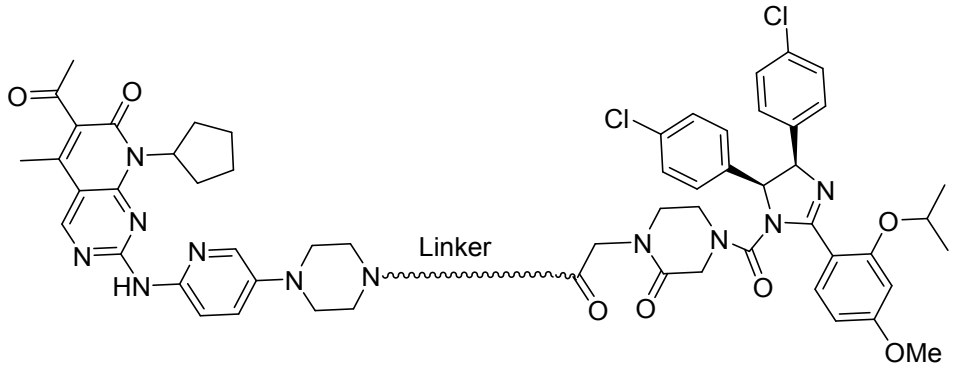
CP-1		CO	H
CP-2		CO	H
CP-3		CO	H
CP-4		CO	H
CP-5		CO	H
CP-6		CO	H
CP-7		CO	H
CP-8		CO	H
CP-9		CO	CH_2CH_3
CP-10		CO	H

CP-11		CO	H
CP-12		CO	H
CP-13		CO	H
CP-14		CO	H
CP-15		CO	H
CP-16		CH_2	H
CP-17		CO	H
CP-18		CO	H
CP-19		CO	H
CP-20		CO	H
CP-21		CO	H
CP-22		CO	H
CP-23		CO	CH_2CH_3



Palbociclib MDM2 recruting (nutlin-3b)

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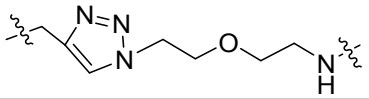
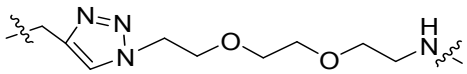
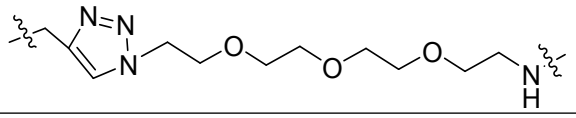
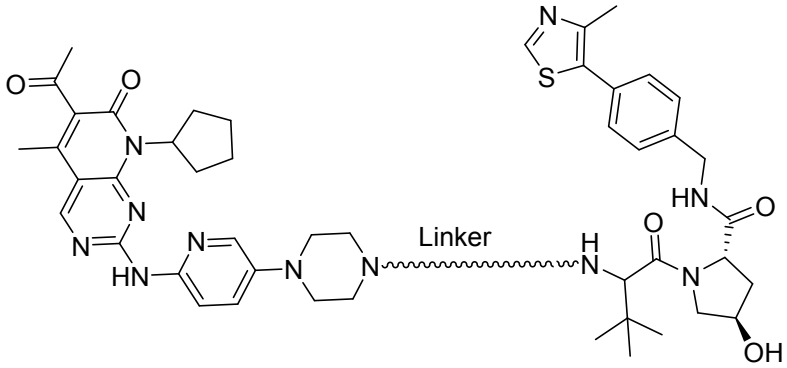
MP-1	
MP-2	
MP-3	



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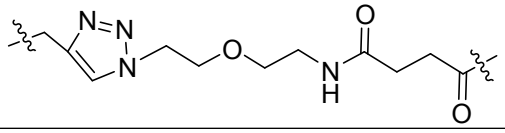
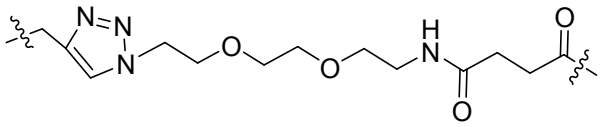
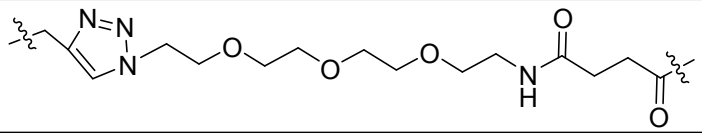
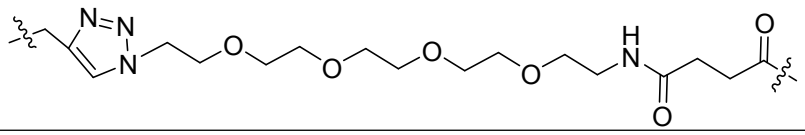
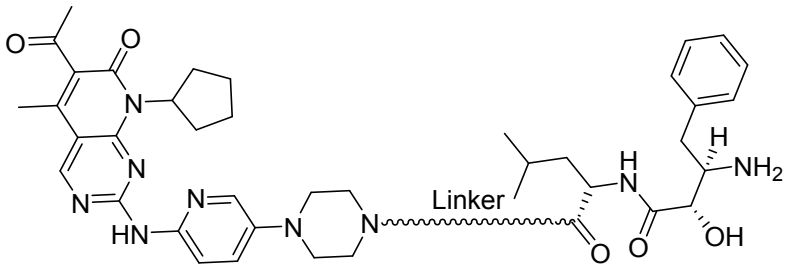
VP-1	
VP-2	
VP-3	
VP-4	



Palbociclib cIAP recruiting (bestatin)

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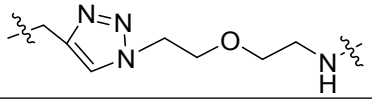
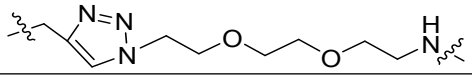
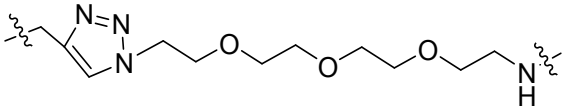
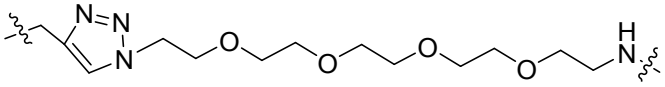
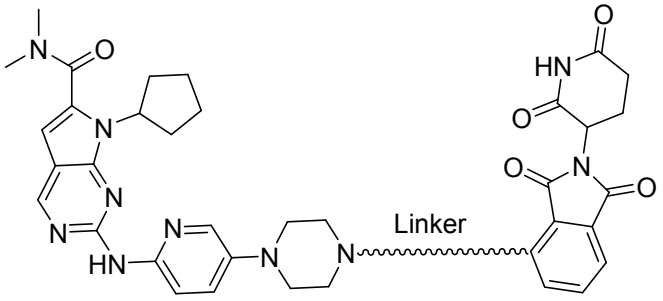
cIP-1	
cIP-2	
cIP-3	
cIP-4	



Ribociclib CRBN recruiting (Pomalidomide)

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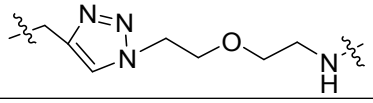
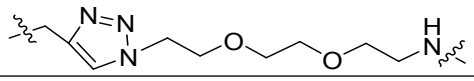
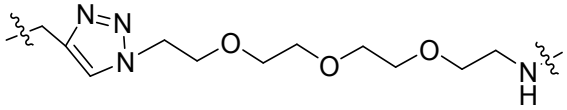
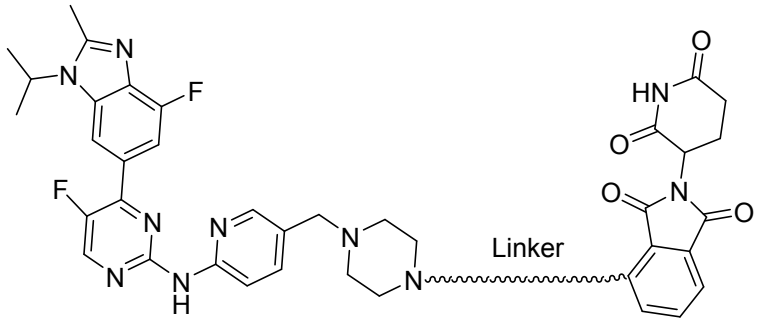
CP-R1	
CP-R2	
CP-R3	



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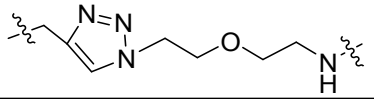
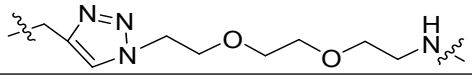
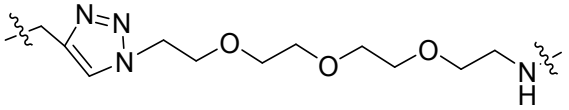
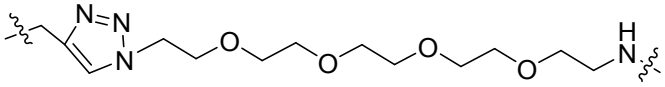
CP-A1	
CP-A2	
CP-A3	
CP-A4	

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Abbreviation used

AR, Androgen Receptor; BRD4, Bromodomain Containing 4; BTK, Bruton Tyrosine Kinase; cIAP, cellular Inhibitor of Apoptosis; CP, Palbociclib-derived CDK-targeting PROTAC; CP-A, abemaciclib-derived CDK-targeting PROTAC; CP-R, ribociclib-derived CDK-targeting PROTAC; CRBN, Cereblon; CRL4 Cullin-RING ubiquitin ligase 4 complex; DC50, the drug concentration that results in 50% protein degradation; E2, Enzyme 2 (conjugating enzyme) for ubiquitin-proteasome system;E3, enzyme 3 (ubiquitin ligase) for ubiquitin-proteasome system; FLAG, FLAG epitope; FAT1, FAT Atypical Cadherin 1. IMiD, Immunomodulatory Drug; MAP2K7 (MKK7), Mitogen-Activated Protein Kinase Kinase 7; MDM2, Mouse Double Minute 2 homolog; PD, palbociclib; PROTAC, proteolysis targeting chimera; VHL, Von Hippel-Lindau tumor suppressor; ZFP91, ZFP91 Zinc Finger Protein.