Supporting information

Mitochondrial Protease ClpP is a Target for the Anticancer Compounds ONC201 and Related Analogs

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Supplemental Methods

Methods for ClpP siRNA

Approximately 5 x 10^4 SUM159 cells were seeded in 6-well plates. The following day, cells were mock transfected or transfected with Dharmacon siGENOME human ClpP (8192) siRNA (GAAGGAGCCUGUAGAAGCA) at a final concentration of 33 nM according to the standard Dharmacon protocol. Dharmafect was used at a dilution of 1:50. Cells were incubated with siRNA for 48 hrs and then an additional 24 hrs in the presence of drug before harvesting.

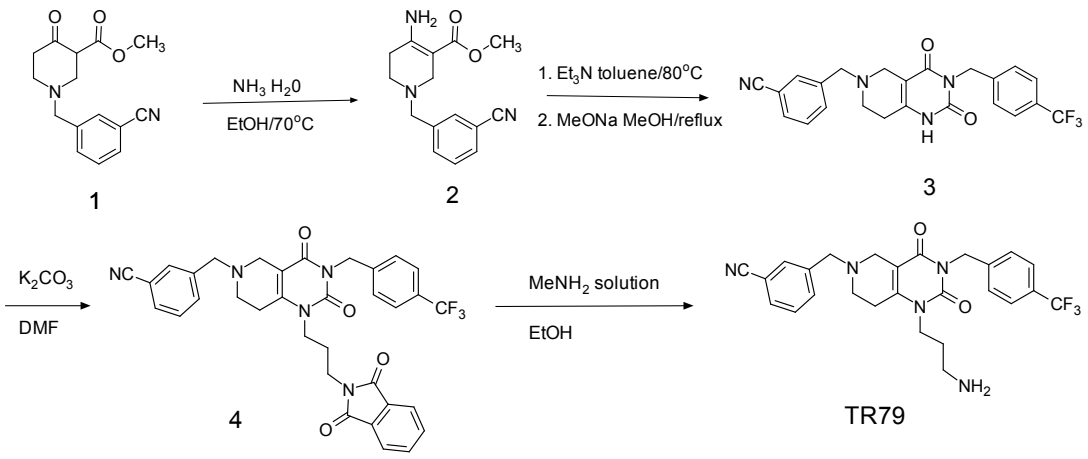
Synthetic Chemistry

The compounds were prepared as given in the following references: [1-3]. For additional clarity we have provided synthetic details below for TR79, TR80 and TR81. 2-(3-iodopropyl) isoindoline-1, 3-dione is available from multiple vendors including Sigma-Aldrich (Aldrich CPR-R465674). In addition, 2-(4-iodobutyl) isoindoline-1, 3-dione is also available from multiple vendors including Sigma-Aldrich (Aldrich CPR-R260312)

Example 1

Synthesis of compound TR79

3-((1-(3-aminopropyl)-2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,2,3,4,7,8-hexahydropyrido[4,3-d]pyrimidin-6(5H)-yl)methyl)benzonitrile



Step 1：A mixture of 1-(3-cyanobenzyl)-4-oxopiperidine-3-carboxylate **1** (8.55 g, 31.4 mmol), and ammonia solution (7 ml, 25%) in ethanol (110 ml) was heated at 70°C for 5 h. The solution was concentrated, extracted with DCM (2 X 300 ml) and washed with brine. The extracts were dried over Na_2SO_4 and evaporated under reduced pressure to give 8 g of 2-((4-amino-3-(methoxycarbonyl)-5, 6-dihydropyridin-1(2H)-yl) methyl)-4- cyanobenzen-1-ide **2** (oil), which was directly used for next step.

Step 2: To a solution of 2-((4-amino-3-(methoxycarbonyl)-5, 6-dihydropyridin-1(2H)-yl) methyl)-4- cyanobenzen-1-ide (**2**, 2 g, 7.4 mmol) in toluene 20 mL was added 1-(isocyanatomethyl)-4-(trifluoromethyl)benzene (1.6 g, 7.5 mmol) and triethylamine (1.1 g, 10.4 mmol). The solution was heated to 80°C for 8 h. The reaction solution was cooled to rt and concentrated *in vacuo*. The formed white solid was filtered and dissolved in MeOH (20 ml). NaOMe (350 mg) was added and the mixture was refluxed overnight. Then ca 10-15ml of methanol was removed and the precipitate was filtered. The desired product 3-((2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,2,3,4,7,8-hexahydropyrido[4,3-d]pyrimidin-6(5H)-yl)methyl)benzonitrile (**3**) was obtained as a pale yellow solid (0.8 g, 25%).

Step 3：To a solution of 3-((2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,2,3,4,7,8-hexahydropyrido[4,3-d]pyrimidin-6(5H)-yl)methyl)benzonitrile (**3**, 200 mg) in DMF (2 ml) was added potassium carbonate (150 mg) and 2-(3-iodopropyl)isoindoline -1,3-dione (150 mg). The mixture was heated at 100ºC for 12 h. Water (ca 3 ml) was added and the solution was extracted with EtOAc (3 X 5 ml). The combined extracts were washed with brine 3 times (ca 5 ml), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the crude product. The purified product (**4**) was obtained by preparative TLC, 100 mg, Yield 35%.

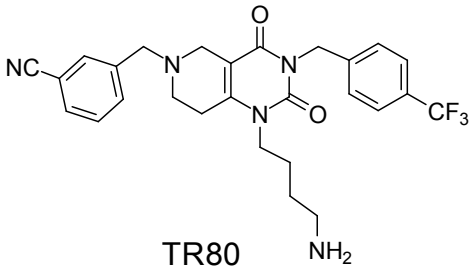
Step 4：To a solution of product (**4**) (100 mg) in EtOH (3 ml) was added methylamine solution (0.25 ml, 30%). The mixture was heated at 80ºC for 4 h. The water was added and the solution was extracted with DCM (3 X 3 ml). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the crufe product, **TR79**. The final product (**TR79**) was obtained by preparative HPLC, 15 mg, Yield 19%.

^{1}HNMR (400MHz, CD_3OD) 2.03 (t, J = 7.2Hz, 2H), 2.99 (t, J = 6.8Hz, 2H), 3.18 (s, 2H), 3.67 (s, 2H), 4.01 (t, J = 6.8Hz, 2H), 4.07 (s, 2H), 4.62 (s, 2H), 5.17 (s, 2H), 7.5-7.57 (m, 4H), 7.69 (t, J = 8Hz, 1H), 7.86-7.93 (m, 2H), 7.99 (s, 1H); LC-MS: m/z = 498.1(M+1).

Example 2

Synthesis of compound TR80

3-((1-(4-aminobutyl)-2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,2,3,4,7,8-hexahydropyrido[4,3-d]pyrimidin-6(5H)-yl)methyl)benzonitrile



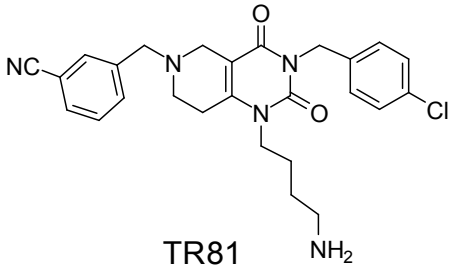
TR80

TR80 is prepared in a similar fashion as example 1. ^{1}HNMR (400MHz, CD_3OD) 1.7 (s, 4H), 2.95 (s, 2H), 3.16 (s, 2H), 3.64 (s, 2H), 3.9 (s, 2H), 4.03 (s, 2H), 4.59 (s, 2H), 5.15 (s, 2H), 7.49-7.57 (m, 4H), 7.67-7.7 (m, 1H), 7.88 (t, J = 8Hz, 2H), 7.98 (s, 1H); LC-MS: m/z = 512.2(M+1).

Example 3

Synthesis of compound TR81:

3-((1-(4-aminobutyl)-3-(4-chlorobenzyl)-2,4-dioxo-1,2,3,4,7,8-hexahydropyrido[4,3-d]pyrimidin-6(5H)-yl)methyl)benzonitrile



TR81

TR81 is prepared in a similar fashion as example 1.

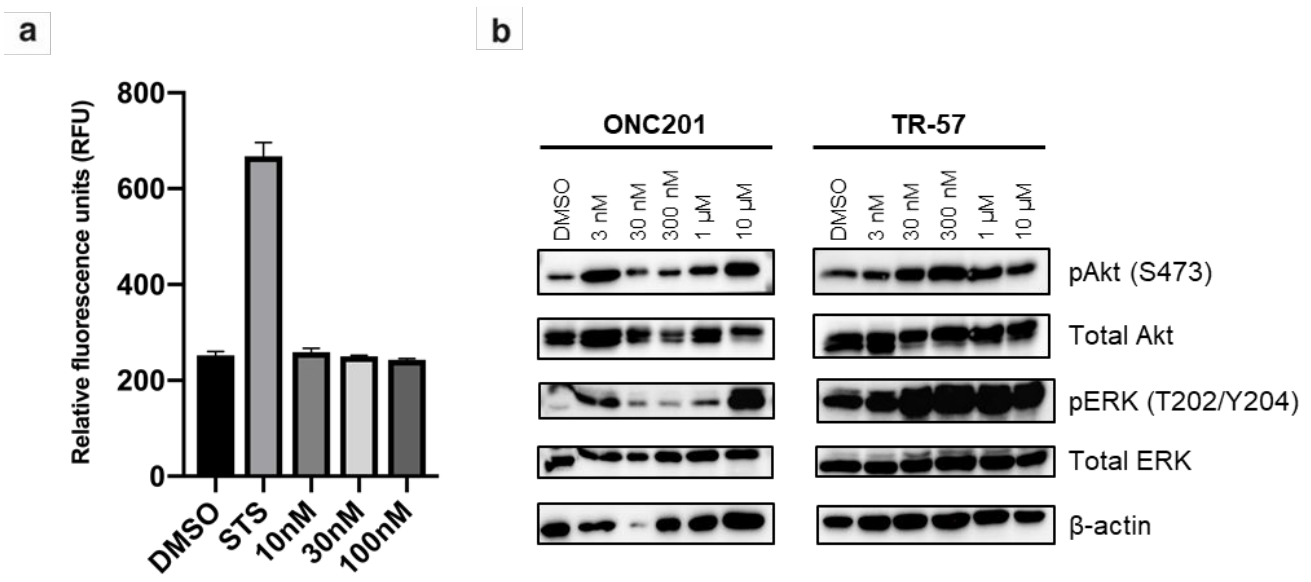
^{1}HNMR (400MHz, CD_3OD) 1.72 (s, 4H), 2.98-2.99 (d, 2H), 3.15-3.17 (d, 2H), 3.61 (t, J=5.6Hz, 2H), 3.91-3.93(d, 2H), 4.01 (s, 2H), 4.57 (s, 2H), 5.08 (s, 2H), 7.28-7.3 (d, 2H), 7.35-7.37 (d, 2H), 7.71 (t, J=7.6Hz, 1H), 7.9-7.92 (d, 2H), 7.99 (s, 1H).

Prep HPLC conditions

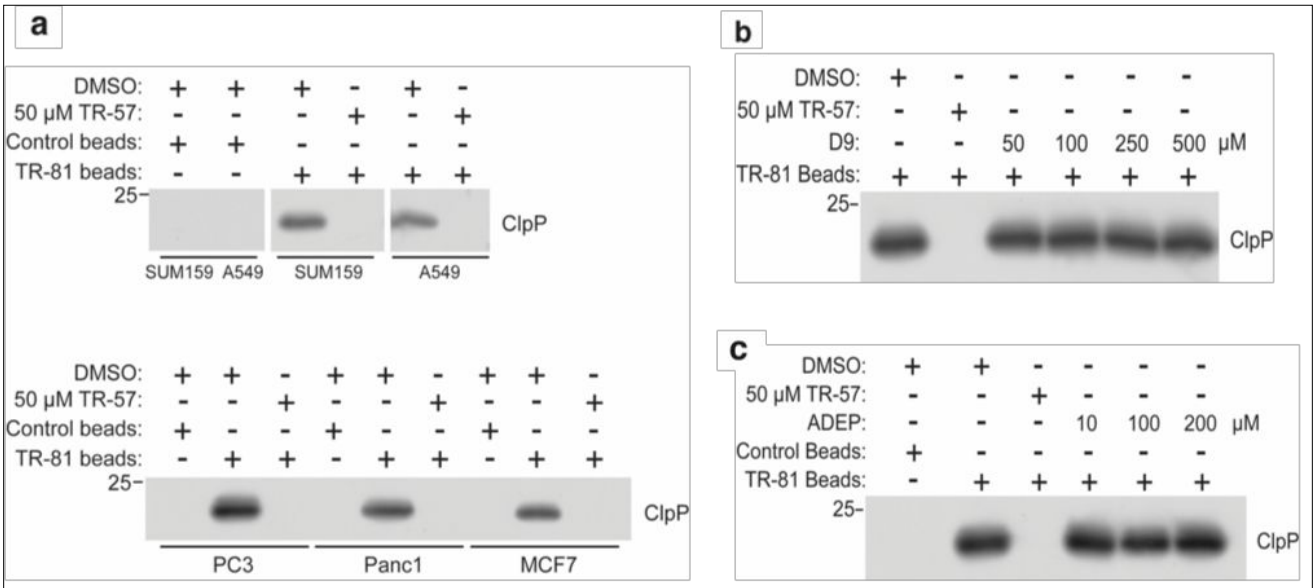
Column:	Waters T3 Prep C18, 5um 19*100mm
PREP	Gilson 215
Mobile Phase(A) :	0.1%FA/Water
Mobile Phase(B):	Acetonitrile
Flow Rate:	20 mL/minute
Detection:	220 nm
Run Time:	12min
Injection Volume:	100uL
Diluent:	Methanol
Gradient	Time A (%) B (%) 0.00 90 10 8.00 50 50 8.50 5 95 10.00 5 95 10.50 90 10 12.00 stop

References

1. Xu, R. L., Y. , Imidazo-Pyrimidine Compounds, and Preparation Methods and Application Thereof. *Chinese Patent 104860948* **2015**, 100.
2. Iwanowicz, E. J., Protein Kinase Regulators. *Patent Application WO2018/031987* **2018**, 92.
3. Iwanowicz, E. J., Protein Kinase Regulators. *Patent Application WO2018/031990* **2018**, 81.

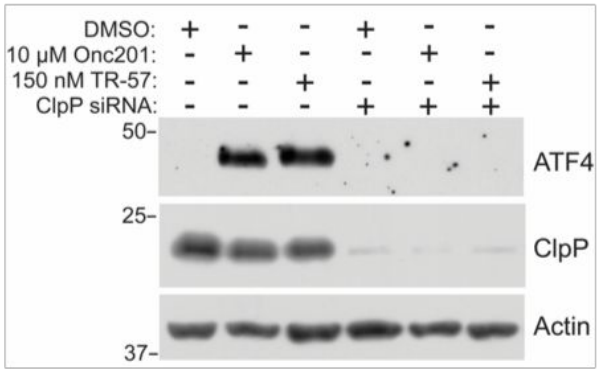


Supplemental Figure 1. ONC201 and TR-57 do not activate caspase or inhibit Erk/AKT in SUM159 cells. (a) Measurement of caspase 3/7 activity in SUM159 cells after 24-hour treatment with 20 nM staurosporine, 10, 30, or 100 nM of TR-57, or DMSO. Graphs shown are representative of 2 biological replicates. Error bars represent standard error of the mean (SEM) from replicates. (b) Immunoblot showing changes in phosphorylation of indicated proteins in SUM159 cells after treatment with either ONC201, TR-57, or DMSO for 24 hours.

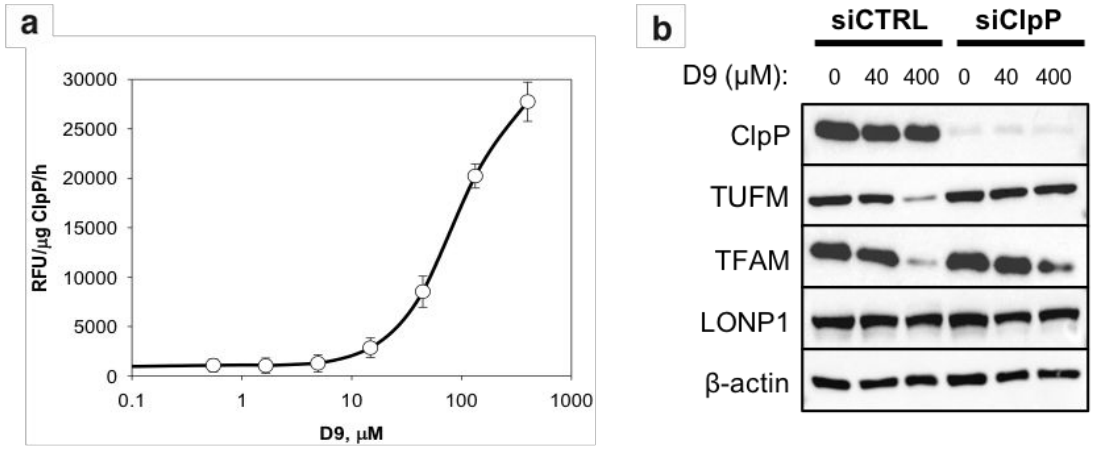


Supplemental Figure 2. Ability of TR-57, but not D9 and ADEP to compete for ClpP binding. Lysates were prepared from the indicated cell lines (a), SUM159 (b) or HeLa cells (c) and mixed with the beads shown in the presence of the indicated compounds. ClpP was detected by immunoblotting.

a



Supplemental Figure 3. ATF4 induction by ONC201 and TR-57 is prevented by ClpP knockdown. (a) SUM159 cells were mock or ClpP siRNA transfected and treated with DMSO, ONC201 or TR-57 for 24 hrs and immunoblotted for the indicated proteins. Blots shown are representative of 3 experiments in multiple cell lines.



Supplemental Figure 4. Effects of D9 are prevented by ClpP knockdown in SUM159 cells (a) Purified, recombinant human ClpP was incubated with the indicated concentrations of D9 and ClpP peptidase activity was measured **(b)** Immunoblot of lysates from SUM159 cells transfected with siCTRL or siClpP, and treated with DMSO or D9 for 24 hours. Blots shown are representative of 3 biological replicates

Protein Name	Database Accession ID	MW (Da)	Peptide Count	Ion Score
ATP-dependent Clp protease proteolytic subunit OS=Homo sapiens GN=CLPP PE=1 SV=1	CLPP_HUMAN	30161	8	435
Peroxiredoxin-1 (Fragment) OS=Homo sapiens GN=PRDX1 PE=1 SV=1	A0A0A0MSI0_HUMAN	18964	2	47

Supplemental Table 1. Identification of ClpP through PID analysis Mass spectrometry protein identification from HELA cell lysate after incubation with TR-80 beads and TR-57 elution.