Characterization of Selective Covalent inhibitors of USP7

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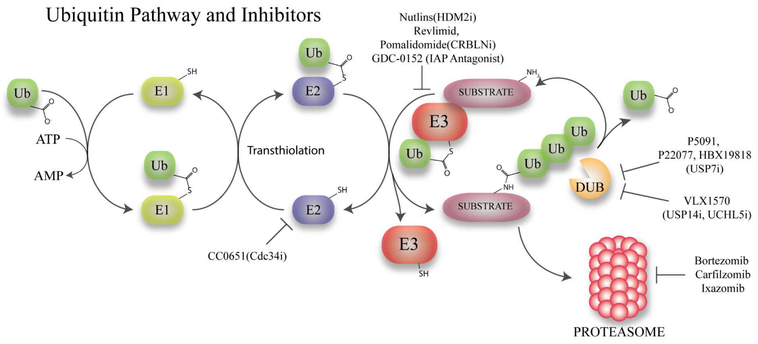
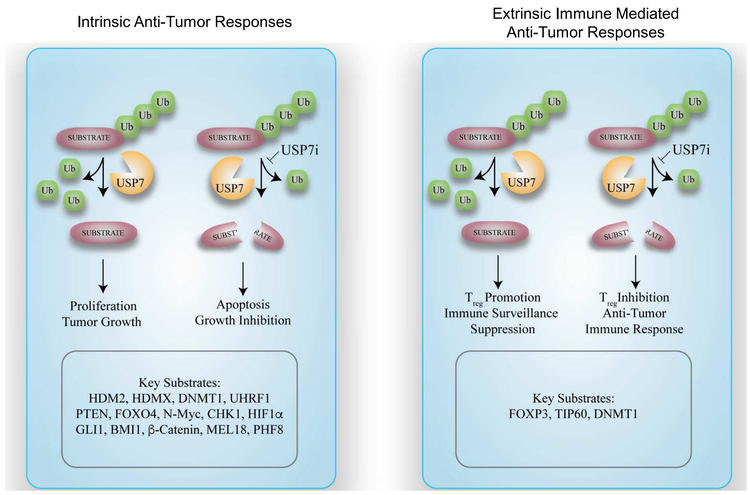
Introduction

The ubiquitin-specific protease 7 (USP7) has emerged as an attractive anti-tumor target due to its critical roles in several cancer signaling pathways as well as its essential role in maintaining Foxp3+ T-regulatory cell (Treg) functions. Pharmacological inhibition of USP7 is therefore expected to have both direct anti-tumor activity and activity in promoting anti-tumor immunity. Previously, we reported a series of selective USP7 inhibitors and demonstrated their anti-tumor activity through both direct anti-tumor and immunotherapy mechanisms. However, the precise mechanism of action of these compounds was not well defined. Using a combination of NMR spectroscopy, mass spectrometry, and single amino acid substitution approaches, in this study, we demonstrated that these inhibitors specifically target the catalytic cleft of USP7 and covalently modify its active site cysteine (Cys223) by forming a covalent adduct. Pharmacokinetic studies revealed sustained USP7 inhibition after short term inhibitor treatment and subsequent changes in the level and ubiquitylation of various pharmacodynamic markers, including the Treg lineage specific transcription factor Foxp3. Detailed knowledge of the mechanism of USP7 inhibition will allow rational design of improved inhibitors as the basis of a new class of anti-cancer therapeutics.

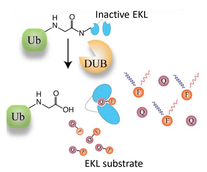
Ubiquitin Proteasome Pathway: The future of unique drugs

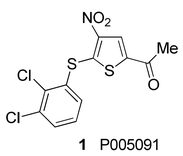


USP7 is a key node of cancer and immune regulatory pathways



Discovery of USP7 Selective inhibitors





1 P005091

•Discovered P005091, a selective USP7 inhibitor using Ub-EKL Reporter Assay System.
•Initial medicinal chemistry optimization resulted in 10 fold increase in potency while maintaining selectivity
•Second generation more potent inhibitors have been developed

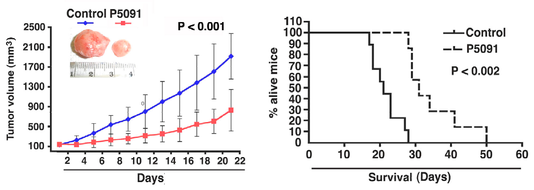
Target	EC_{50} (μM)	
	P5091	P50429
USP2	>31.6	>31.6
USP5	>31.6	>31.6
USP7	4.2±0.9	0.42±0.05
USP8	>31.6	>31.6
USP21	>31.6	>31.6
USP28	>31.6	>31.6
USP47	4.3±0.8	1.0±0.04
Caspase 3	>31.6	>31.6
Cathepsin B	>31.6	>31.6

Second generation of USP7 inhibitors

Enzyme/ Comp ID	EC_{50} (μM)							
	P217564	P217649	P217598	P217599	P217640	P218283	P217600	P0050378
USP7	0.6±0.27	0.048±0.025	0.24±0.13	0.3±.18	0.14±0.08	0.082±0.03	0.17±0.12	0.45±0.19
USP47	1.0±0.4	0.46±0.24	0.75±0.25	1.2±0.77	0.20±0.12	0.88±0.36	0.24±0.01	0.53±0.23
USP5	29.0±5.6	15.0±3.6	8.1±4.4	23.4±14	4.7±1.6	46.0±4.4	30.0±11.0	12.1±6.0
USP8 Core	>50	>50	>50	20±3.2	>50	>50	>50	49.4
USP15	>50	32.0±10	31±4.6	36.6±4	28±4.6	>50	28.00	22±1.2
USP21	>50	>50	>50	>50	>50	>50	>50	40.4
USP28	>50	25.70	31.4±4.12	>50	28±4.6	>50	>50	>50
UCHL1	>50	>50	>50	36.6±2.5	>50	>50	>50	>50
Caspase 3	34.2±10	43.80	17.0±7.0	24.0±9.0	37.5±10	27.4±5.4	23.2±11	>50
Calpain-1	>50	>50	>50	>50	>50	>50	>50	ND
Cathepsin K	>50	>50	>50	>50	43.3±4.0	>50	>50	28.00

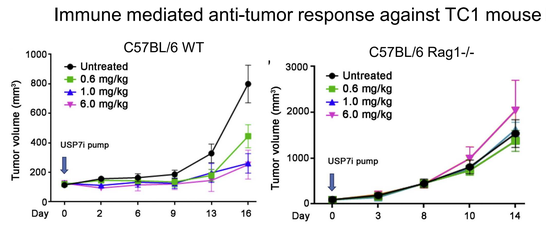
USP7i inhibit tumor growth through direct anti-tumor and immunotherapy mechanism

Direct anti-tumor activity against MM.1S human multiple myeloma



- 2.5x10[6] MM.1S human multiple myeloma cells in RPMI-1640 media, inoculated s.c. in the flank area
- Mice were treated when tumors reached 100-150 mm[3]
- 10mg/kg (biw x3) P005091 *iv* in 4% NMP, 4% tween 80, 92% H_2O
- Chauhan *et al* Cancer Cell (2012) **22**, 345

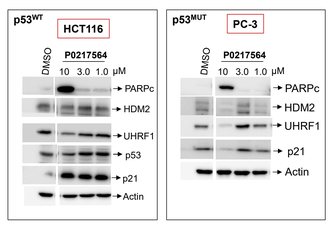
Immune mediated anti-tumor response against TC1 mouse lung adenocarcinoma



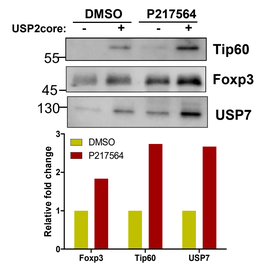
- 1.2x10[6] TC1 mouse lung adenocarcinoma cells were injected s.c.
- Mice were treated when tumors reached ~150 mm[3]
- USP7i (P217564) was delivered through Alzet pump at indicated dosages.
- Wang *et al* EBioMedicine (2016) **13**, 99

Cellular efficacy of second generation potent USP7 inhibitors

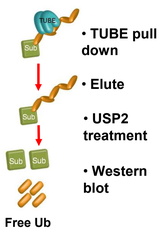
USP7 inhibitors induce apoptosis in $p53^{WT}$ and $p53^{MUT}$ cancer cells



USP7 inhibitor increases substrate ubiquitylation in Tregs

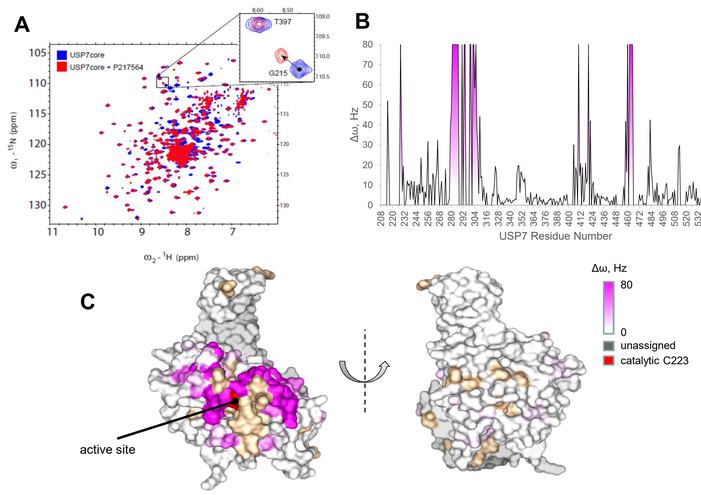


UbiTest

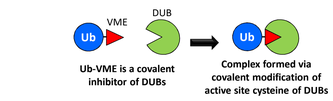


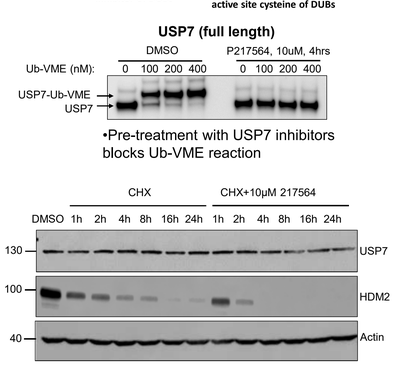
- **TUBE pull down**
- **Elute**
- **USP2 treatment**
- **Western blot**

USP7i specifically targets the catalytic cleft of USP7



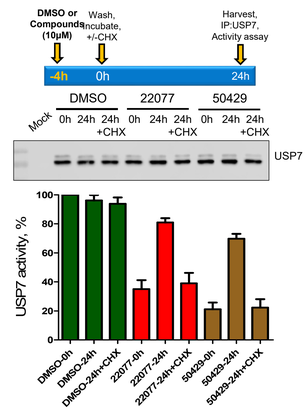
Irreversible/Covalent mode of inhibition





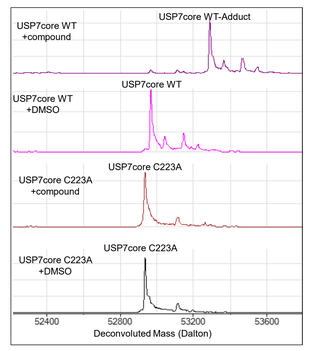
•Pre-treatment with USP7 inhibitors blocks Ub-VME reaction

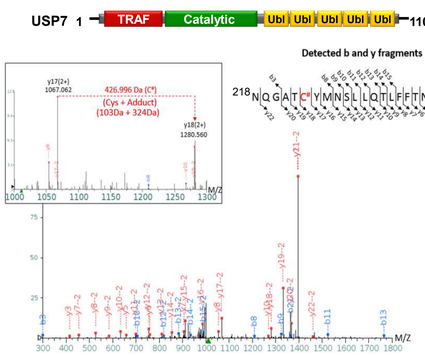
•No detectable decrease of USP7 level was observed in HCT116 cells pre-treated with cycloheximide for 24hrs, indicating that USP7 is a relatively stable protein.



• USP7 isolated from Jurkat cells pre-treated with USP7 inhibitors for 4hrs shows impaired catalytic activity.
• Short treatment of USP7 inhibitors result in sustained inhibition of USP7 activity.
• Recovery of USP7 activity is due to the synthesis of new USP7 proteins.

USP7i selectively target the active site cysteine of USP7





Solvent Accessible Cysteine residues within USP7core

Side chain SASA (Å²)	
Cys 223	13,1
Cys 300	10,6
Cys 315	59,9
Cys 334	0,0
Cys 478	6,7
Cys 488	4,4
Cys 510	46,3

- USP7i is able to form covalent adduct on USP7.
- The conjugation site of inhibitor adduct on USP7 has been mapped to active site cysteine (C223) by LC-MS/MS.
- Cysteine to alanine replacement at USP7 active site completely blocked formation of adduct, suggesting that USP7i selectively target the active site cysteine of USP7.

Summary

- USP7 is an attractive oncology/immuno-oncology target
- Progenra's USP7 inhibitors:
  - are selective covalent irreversible inhibitors
  - specifically target the catalytic cleft of USP7 and covalently modify its active site cysteine (Cys223) by forming a covalent adduct.
  - irreversibly inhibit USP7 in cells and recovery of USP7 activity requires new protein synthesis.
  - show long-lasting efficacy due to the covalent inhibition mechanism and long hald-life of USP7.
  - exhibit direct anti-tumor response against distinct tumor types *in vivo,* including multiple myeloma, T-cell leukemia, and neuroblastoma.
  - show anti-tumor activity against TC-1 (lung tumor) and AE17 (mesothelioma) through impairing Treg functions and unleashing anti-tumor immune response.

References

1. Nicholson, B. and K.G. Suresh Kumar, The multifaceted roles of USP7: new therapeutic opportunities. *Cell Biochem Biophys*, 2011. 60(1-2): p. 61-8.
2. Altun, M. *et al*. Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes. *Chem & Biol*, 2011, 18:1401-1412.
3. Weinstock, J. *et al*. Selective dual inhibitors of the cancer-related deubiquitylating proteases USP7 and USP47. *ACS Med Chem Lett,* 2012, 3 (10): 789-792.
4. Chauhan, D. *et al*. A novel small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes Bortezomib resistance. *Cancer Cell*, 2012, 22: 345-358.
5. Fan, Y.H., *et al*., USP7 inhibitor P22077 inhibits neuroblastoma growth via inducing p53-mediated apoptosis. *Cell Death Dis*, 2013. **4**: p. e867.
6. van Loosdregt, J., et al., Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. Immunity, 2013. 39(2): p. 259-71.
7. Wang, L., et al. Ubiquitin-specific Protease-7 Inhibition Impairs Tip60-dependent Foxp3+ T-regulatory Cell Function and Promotes Antitumor Immunity. *EBioMedicine*, 2016, 13: 99-112