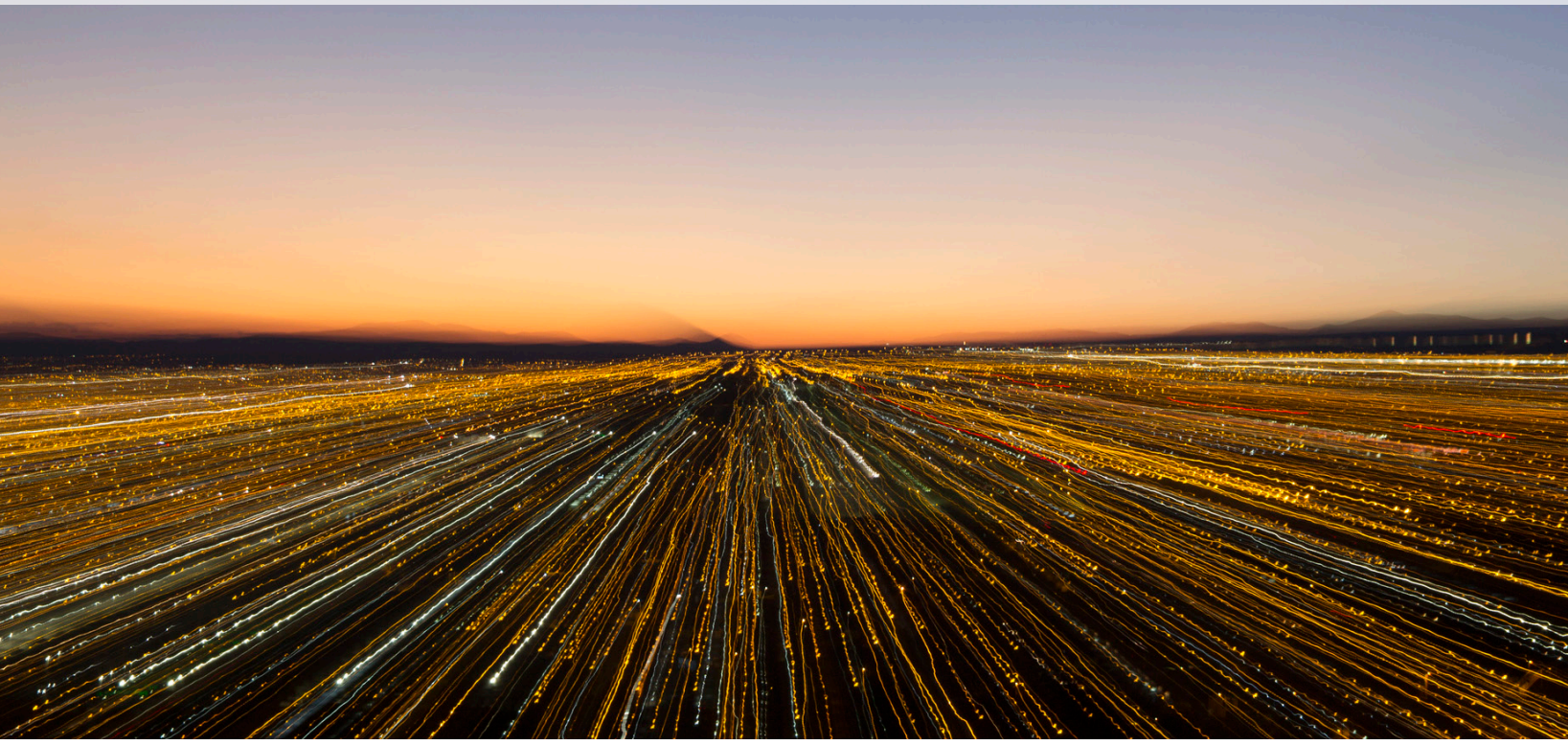

Deubiquitinating Enzymes as Drug Targets

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The functional repertoire of the cellular proteome is greatly enhanced by post-translational modifications (PTMs), which are vital for normal growth and functioning of the cell. Like other PTMs, ubiquitination is reversible and countered by a large group of proteases termed deubiquitinating enzymes, or deubiquitinases (DUBs). While most DUBs can cleave ubiquitin from substrate proteins, edit ubiquitin chains and process ubiquitin precursors[1] , some DUBs and related enzymes are involved in editing or processing ubiquitin-like proteins (UBLs) and their conjugates; prime examples of these being the SENP (sentrin/SUMO-specific protease) proteins that process SUMO precursors and SUMO conjugates.

Approximately 103 DUBs have been identified in the human genome[2] and based on sequence and domain conservation they are subdivided into six families: USPs (ubiquitin-specific proteases), UCHs (ubiquitin carboxy-terminal hydrolases), MJDs (Machado-Josephin domain-containing proteases), OTUs (ovarian tumor proteases), MINDYs (motif-interacting with ubiquitin-containing novel DUB family) and JAMMs (JAB1, MPN, MOV34 family). SENPs and the first five DUB families are cysteine proteases, whereas JAMMs are zinc metalloproteases.
As proteases DUBs play multi-layered roles in the cells1: (a) generating free ubiquitin molecules from newly transcribed ubiquitin precursor proteins, (b) rescuing ubiquitin-tagged proteins from either proteasomal or lysosomal degradation, leading to stabilization of these proteins, (c) removing non-degradative ubiquitin signal, (d, e) salvaging and recycling ubiquitins from the degradation target, and (f) editing the form of ubiquitin modification by trimming ubiquitin chains (Figure 1).

Dysregulation of DUBs contributes to various sporadic and genetic disorders (Table 1)[3] . In many types of human cancer, mutations in genes encoding DUBs have been detected. Germline mutations in the CYLD gene were identified in kindreds with familial cylindromatosis and sporadic cylindromas and also in patients with Brooke-Spiegler syndrome and familial trichoepithelioma[4] . These are autosomal dominant inherited diseases associated with the development of multiple skin tumors of the head and neck. Mutations are thought to affect the catalytic activity of CYLD enzyme. Various types of alterations have been found in the gene encoding the BAP1 deubiquitinase in various

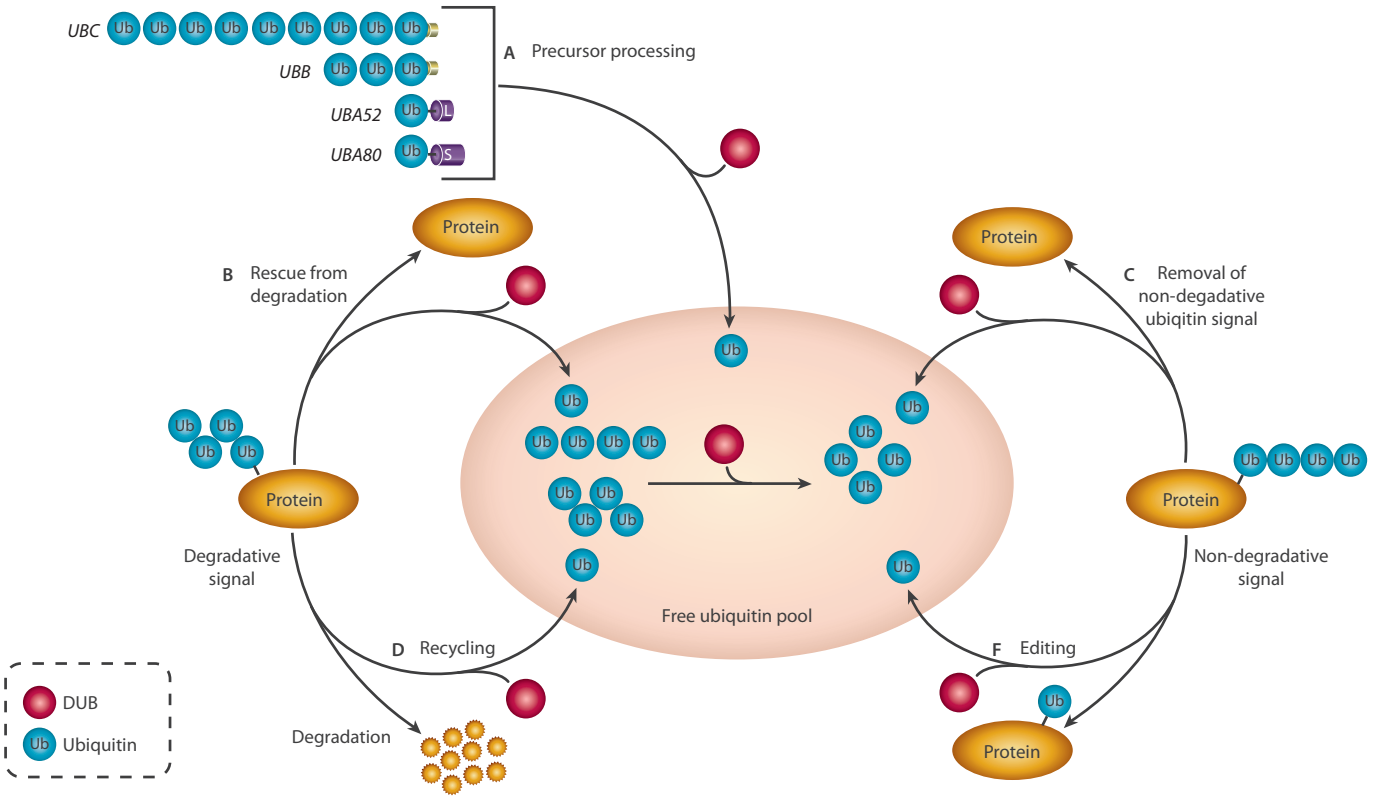


Figure 1: General functions of DUBs

diseases, including lung and breast tumors, clear cell renal cell carcinomas and malignant pleural mesotheliomas[5,6]. A20 (TNFAIP3) is required to terminate NF-κB signaling in response to tumor necrosis factor[7] . Inactivating A20 mutations were reported to be frequently occurring in cases of marginal zone lymphoma. Furthermore, an almost complete loss of A20 mRNA expression has been observed in cases of Non-Hodgkin's Lymphoma[8] . This highlights the possibility that DUBs can contribute to disease not only by mutations, but also by an altered expression or activity. USP14, which is primarily associated with the proteasome 19S regulatory PSMD2 subunit where it gains enhanced DUB activity, is upregulated in non-small-cell lung cancer, especially in adenocarcinoma[9] , and its levels are reportedly elevated in ovarian cancer samples[10]. In line with this, USP14 is connected to several important signaling pathways, for example, as a substrate of AKT mediating intracellular signaling for growth factors and a modulator of Dishevelled proteins, key positive regulators of WNT signaling[11, 12]. Other notable example include: USP28 is overexpressed in colon and breast tumors, and by counteracting the E3 ligase activity of SCF–Fbxw7 it causes the stabilization of cyclin E1 and c-Myc[13]; aberrant USP6 expression, resulting from chromosomal translocation, has been found to be causative in most instances of aneurysmal bone cysts, locally aggressive bone lesions that occur during the first two decades of life[14]; USP9X deubiquitinates and stabilizes the pro-survival protein MCL1, and a correlation between USP9x expression and MCL1 levels was reported in human follicular lymphomas and diffuse large B-cell lymphomas, whose mutations cause developmental disorders and whose expression is dysregulated in cancer[15]; and USP15, amplified in certain glioblastoma, breast and ovarian cancers[16].

Assessment of two DUBs as drug target

Targeting DUBs as an anti-tumorigenic therapeutic strategy has its proof of principle in the use of bortezomib, a broad range inhibitor of the ubiquitin proteasome system, in multiple melanoma treatment[17]. This strategy is however limited by the lack of specificity of bortezomib which results in toxicity[18]. Targeting individual DUBs that play a role in particular diseases is predicted to be a much better strategy, and has seen significant progress in recent years (Table 2)3. However, targeting single DUBs is a very complex challenge due to the high levels of homology, particularly between the catalytically active domains, and promiscuity. Below we discuss the selection of two DUBs, USP7 and USP30, in an effort to establish SignalChem's therapeutic pipeline on targeting the human epigenetic machinery.

Table 1: DUBs connected with human diseases

Oncology				
Process Targeted	DUB	Target	Rationale	Disease association
Proteasome	PSMD14	Many	General protein turnover	Liver cancer
	USP14			Lung and ovarian cancers
	UCHL5			Oesophageal and ovarian cancers

Oncology

DNA repair • Translesion synthesis	USP1 Osteosarcoma	FANCD2, PCNA	• Fanconi anaemia pathway	
	USP4	CTIP	Homologous recombination	Lung, breast and liver cancers
	USP11	PALB2		Breast cancers
	USP9X	Claspin	Replication checkpoint	Sarcoma and tumors of the colon, cervix, kidney, breast, prostate and brain
Oncogenes and tumor suppressors	ATXN3	p53, HDM2	Promotes p53-mediated apoptosis	p53-expressing tumors
	CYLD	NF-κB	Unclear	• Mutated in cylindromatosis and multiple myeloma • Reduced expression in colon and liver cancers and melanoma
	UCHL1	AKT		Osteosarcoma, myeloma and tumors of the colon, breast, lung and kidney
	USP6	–		Translocated in aneurysmal bone cysts
	USP7	p53, HDM2	HDM2-overexpressing tumors	Leukemia and ovarian and lung cancers
	USP8	EGFR	Regulates recycling of receptor tyrosine kinases, including EGFR	• Lung cancer • Mutated in Cushing syndrome
	USP15	Type I TGFβ receptor, R-SMADs	Regulation of TGFβ signaling	Glioblastoma and breast and ovarian cancers
	USP20	HIF1α	Sensitizes hypoxic tumor cells	–
	USP28	FBW7, MYC, JUN, Notch	APC-driven cancers	Colorectal and ovarian cancers
Epigenetics	BAP1	Histone H2A, HCF1	Epigenetic deregulation of tumors	Uveal melanoma, sporadic melanoma, mesothelioma and kidney cancer
	USP22	Histone H2A		Colorectal, breast, oesophageal, lung and pancreatic cancers

CNS disorders

Process Targeted	DUB	Target	Rationale	Disease association
Neurodegeneration	ATXN3	Parkin	Counteracts Parkin autoubiquitination	Expansion of CAG trinucleotide repeats causes Machado–Joseph disease
	USP7	α-Synuclein, REST	• Antagonizes ubiquitination of α-synuclein • Regulates REST signaling and neuronal differentiation	–
	USP8	Parkin, K6-linked Ubiquitin chains	• Regulates mitophagy by removing ubiquitin from Parkin • Regulates TRKA levels in an NGF-dependent manner	–
	USP14	Proteasome substrates	Increased clearance of proteins involved in neurodegeneration (Tau or ATXN3)	Mutations cause ataxia

CNS disorders				
	USP15	–	Opposes Parkin-mediated mitophagy	Glioblastoma
	USP30	Ubiquitin conjugates at mitochondrial surface, Parkin	Mitochondrial dysfunction, mitophagy	–
Down syndrome				
	USP16	Histone H2A	Antagonizes self-renewal and/or senescence in Down syndrome	–

Inflammation, immunity and infectious disease				
Process Targeted	DUB	Target	Rationale	Disease association
Negative regulation of the immune response	A20	NEMO, RIPK1, TRAF6	Inhibits NF-κB signaling	Expression levels regulated by TNFα, IL-1β and LPS
	CYLD	RIG1, TBK1, IKK		–
	OTULIN	RIPK1, RIPK2, NEMO		–
	USP18	–	• Functions in hematopoietic cell differentiation • Removes ISG15 conjugates • Negative feedback regulator of type I IFN signaling	Expression regulated by IFNγ
	USP25	RIG1, TRAF2, TRAF3, TRAF6	• Negatively regulates IL-17-triggered signaling • Negatively regulates virus-induced type I IFN production	Expression regulated by IFN and IRF7
T_{reg} responses	USP7	FOXP3	• Stabilizes FOXP3 in Treg cells • Negative regulator of TNFα-stimulated NF-κB activity	Expressed and regulated upon viral infections in B and T cells
	USP21	FOXP3	Stabilizes FOXP3 in T_{reg} cells	–
T_H1 and T_H17 responses	CEZANNE	ZAP70	• Positive regulator of T cell receptor signaling • Binds to and deubiquitinates ZAP70	–
	TRABID	JMJD2D	Positive regulator of IL-22 and IL-23 cytokine production	–
	USP4	RORγt, RIG1, TAK1	• Stabilizes RORγt in TH17 cells • Positively regulates RIG1-mediated antiviral response • Negative regulator of TLR–IL-1R signaling • Targets TAK1 to downregulate TNFα-induced NF-κB activation	Highly expressed in $CD4^+$ T cells from patients with rheumatic heart disease
	USP10	T-bet	Stabilizes T-bet in TH1 cells	Highly expressed in PBMCs from patients with asthma

Inflammation, immunity and infectious disease				
	USP17	RORγt, RIG1, IL-33	• Positive regulator of RORγt in TH17 cells • Regulates virus-induced type I IFN signaling • Regulates the stability and nuclear function of IL-33	–
	USP18	TAK1–TAB1 complex	Regulates TAK1–TAB1 interaction required for T_H17 differentiation	Expression induced by cytokines

Ubiquitin-specific protease 7 (USP7)

Among all druggable targets in the DUB family, USP7 has attracted the most attention because of its involvement in multiple oncogenic pathways. Importantly, USP7 is a genetically validated deubiquitinase for MDM2, a ubiquitin E3 ligase that promotes the proteasomal degradation of the tumor suppressor p53. USP7 depletion results in elevated autoubiquitination and subsequent degradation of MDM2, thus stabilizing p53 and promoting cell cycle arrest and apoptosis[19,20].

Dysregulation of USP7 expression has been reported in a number of human malignancies, including human prostate cancer, ovarian cancer, and non-small-cell lung cancer. Furthermore, early studies in human colon cancer xenograft models showed that downregulation of USP7 suppresses cell proliferation and delays tumor growth due to p53 stabilization in the absence of cellular stress.

Human USP7 predominantly localizes in the cell nucleus and is a 135-kDa multi-domain protein. It contains a TRAF-like domain in its N-terminus, a central catalytic USP domain and a 64-kDa C-terminal domain that harbors five ubiquitin-like (UBL) domains. The catalytic domain (AA. 208-560) of USP7 binds ubiquitin and removes it from the substrate, while its TRAF-like (AA. 62-205) and UBL domains specifically recognize its targeting substrates, including p53 and MDM2[21]. Unlike other USPs, the apo form of USP7 is catalytically incompetent but adopts an active conformation upon binding to ubiquitin[22].

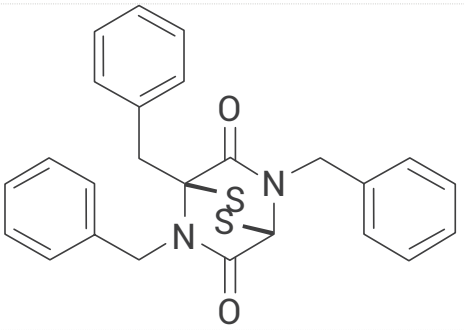
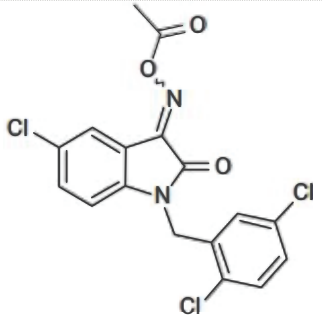
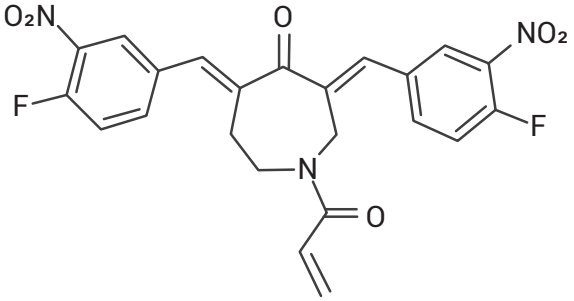
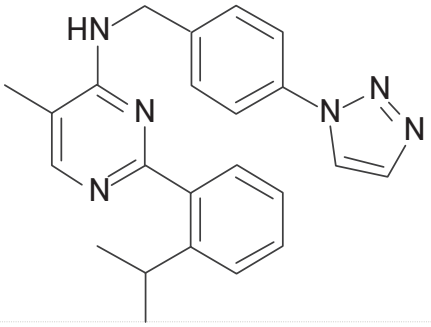
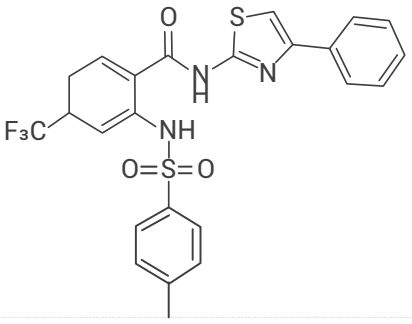
With the advancement of high-throughput screening (HTS) and other novel screening technologies, the candidacy of USP7 as a therapeutic target in anti-cancer drug development has seen great potential in the past decade2[23, 24, 25, 26, 27, 28, 29]. Several small-molecule inhibitors of USP7 have been discovered. For instance, HBX19818 was identified to selectively inhibit USP7 by forming a covalent bond with the catalytic Cys residue in preference to other cysteinyl groups, and to stabilize p53 and promote G1 arrest and apoptosis in cells[24]. Another USP7 inhibitor, P5091 selectively inhibits USP7 both *in vitro* and *in vivo*. Importantly, the cytotoxicity of P5091 was significantly reduced upon USP7 knockout, consistent with its activity being on-target. Furthermore, P5091 induced apoptosis in various multiple myeloma cell lines as well as patient MM cells, including those resistant to prior treatments such as bortezomib, lenalidomide, and dexamethasone[25]. Excitingly, this past year has witnessed several more potent and selective USP7 inhibitors, with the most promising ones exhibiting nanomolar potency with excellent *in vitro* selectivity. The observed target engagement translated into degradation of MDM2, stabilization of p53 and induction of p21 in multiple cell lines2. This important breakthrough in the ability to drug DUBs could signal our entry into the golden state of this enzyme family for therapeutic discovery.

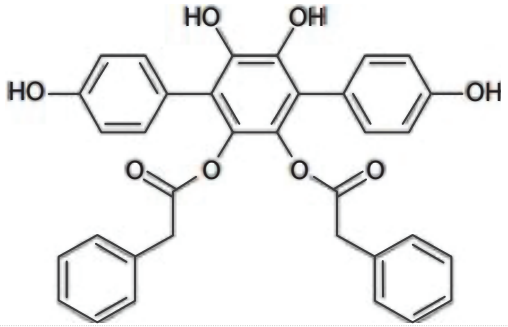
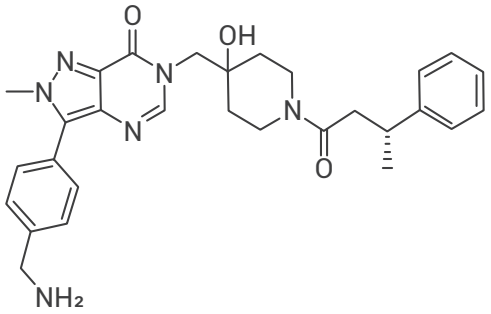
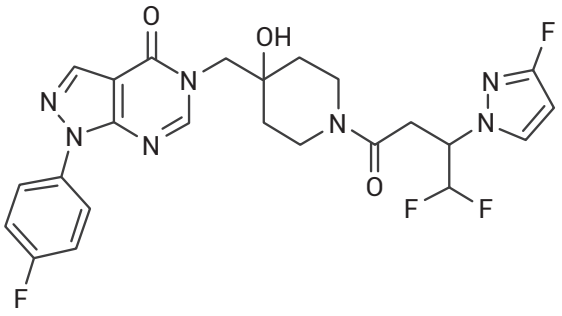
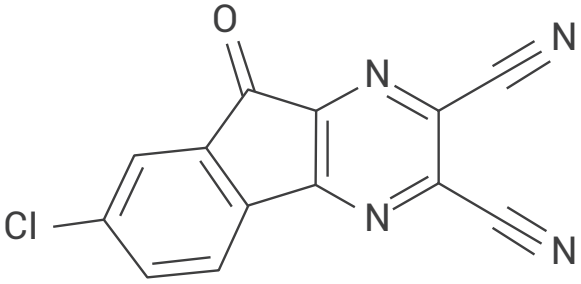
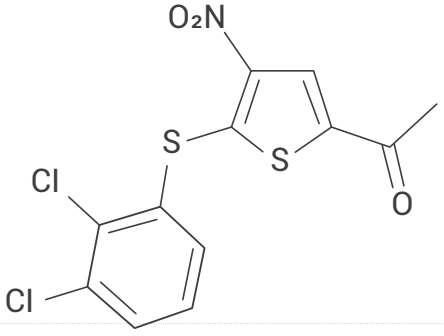
Ubiquitin-specific protease 30 (USP30)

Mitochondrial dysfunction is crucial for neuronal degeneration in Parkinson's disease (PD)[30]. PINK1 protein kinase and Parkin ubiquitin ligase act together to remove damaged mitochondria from the cell by mitophagy and maintain healthy mitochondria[31]. In PD related to PINK1 or Parkin gene mutations, defective mitophagy participates in dopaminergic neurons' degeneration. USP30, the only DUB constitutively associated with the mitochondrial membranes[32], antagonizes parkin-mediated mitophagy by removing polyubiquitin chains from damaged mitochondria and promotes neurodegeneration . USP30 knockdown is sufficient to restore mitophagy in neuronal cells expressing a dysfunctional parkin mutant and rescue motor function and mitochondrial defects in fly models of PD devoid of a fully functional PINK1–parkin pathway[34]. Thus, inhibiting USP30 should enhance mitophagy and help to clear dysfunctional mitochondria.

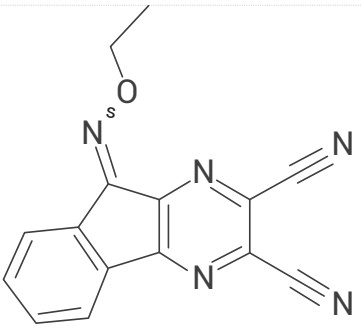
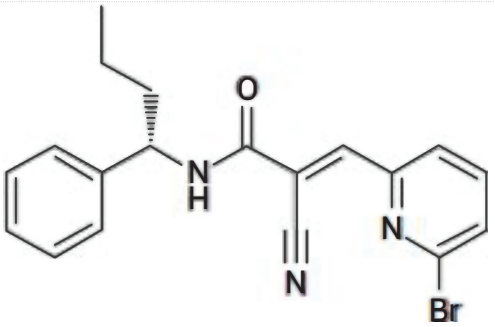
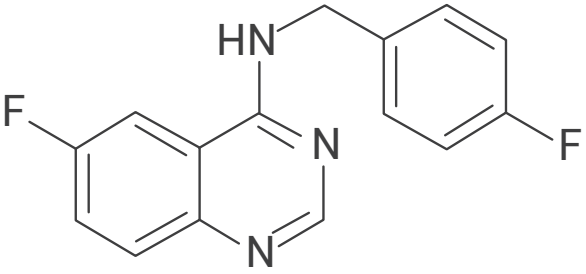
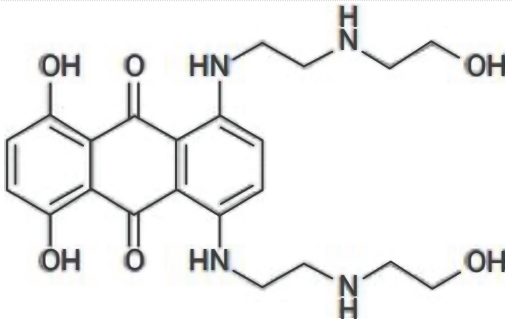
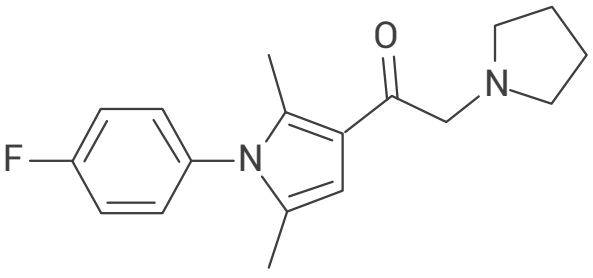
Despite the promising results obtained by the manipulation of USP30 activity, pharmacological proof of concept remains lacking. The potential of USP30 inhibitors in maintaining normal mitochondrial functions has only been unveiled when the diterpenoid analog 15-oxospiramilactone was found to potently induce mitochondrial fusion accompanied by restoration of mitochondrial network and oxidative respiration[35]. More recent effort in a structure–activity relationship (SAR) study has led to the identification of several potent and highly selective inhibitors of USP30, which showed <30% inhibition against a panel of 22 USP30-related DUBs at up to 10 μM. One of these inhibitors, MF-094, has an IC50 of 0.12 μM. In terminally differentiated C2C12 myoblasts, MF-094 increased protein ubiquitination on the mitochondrial outer membrane and accelerated mitophagy[36].

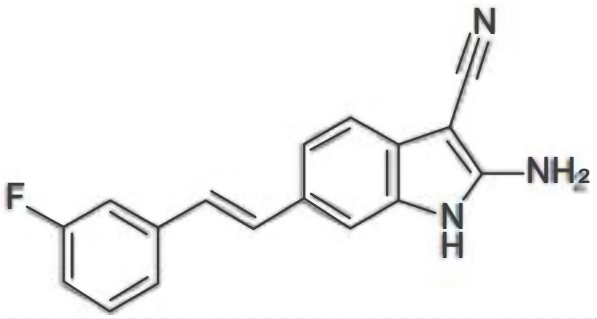
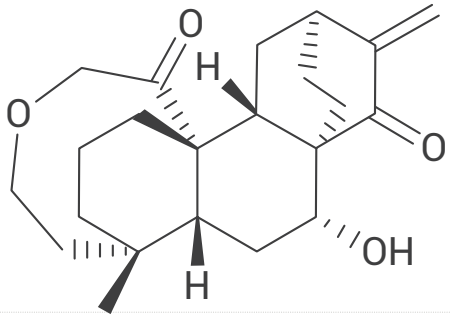
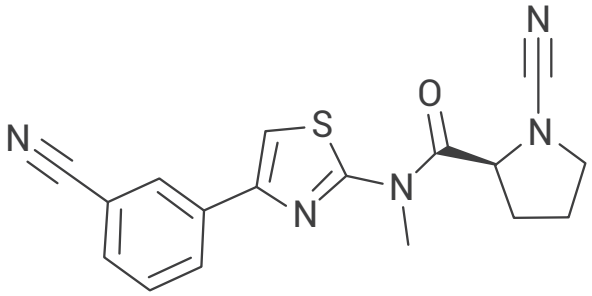
Table 2: DUB inhibitors in development

DUB	Inhibitor	Structure	Company/ Institution	Disease indication	Stage of development
PSMD14	SOP11		Caltech and Amgen	Oncology	Preclinical
UCHL1	LDN-57444		Brigham and Women's Hospital and Harvard Medical School	Oncology	Preclinical
UCHL5 and USP14	VLX1570		Vivolux	Oncology	Clinical trial phase (now suspended)
USP1	ML323		University of Delaware and National Institutes of Health	Oncology	Preclinical
USP2	ML364		National Institutes of Health	Inflammation	Preclinical

DUB	Inhibitor	Structure	Company/ Institution	Disease indication	Stage of development
USP4	Vialinin A		Tokyo University of Agriculture and Shanghai Institutes for Biological Sciences	Inflammation and oncology	Preclinical
USP7	Compound 4		Almac Discovery	Oncology, Immuno-oncology	Preclinical
	FT671		CRUK Therapeutic Discovery Laboratories and FORMA Therapeutics	Oncology, Immuno-oncology	Preclinical
	HBX41108 (shown right), HBX19818		Hybrigenics	Oncology, Immuno-oncology	Preclinical
	P5091 (shown right), P22077, P50429		Progenra	Oncology, Immuno-oncology	Preclinical

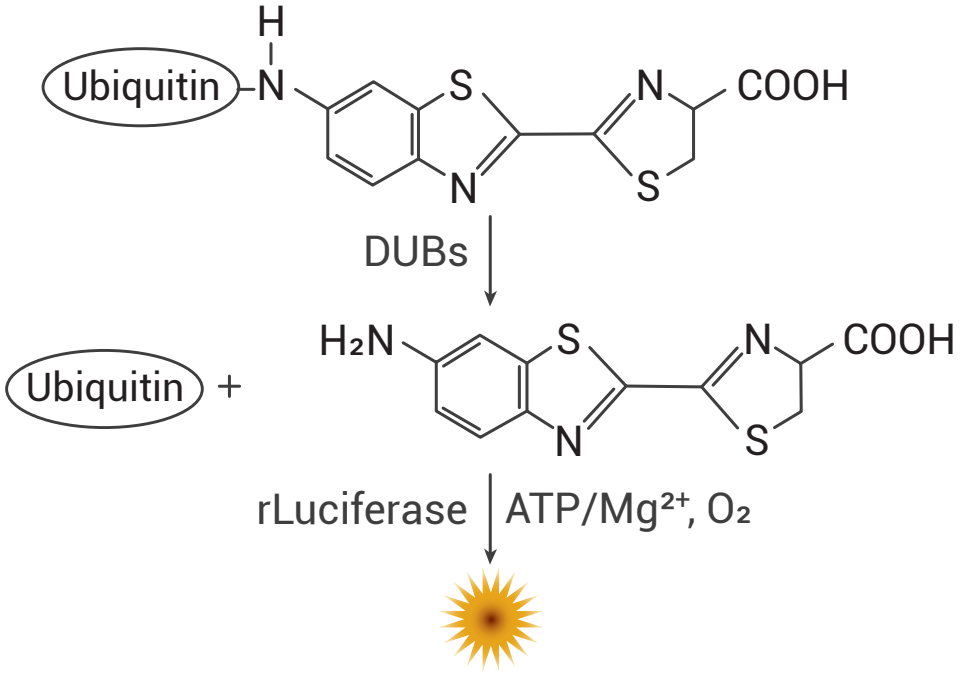
SignalChem

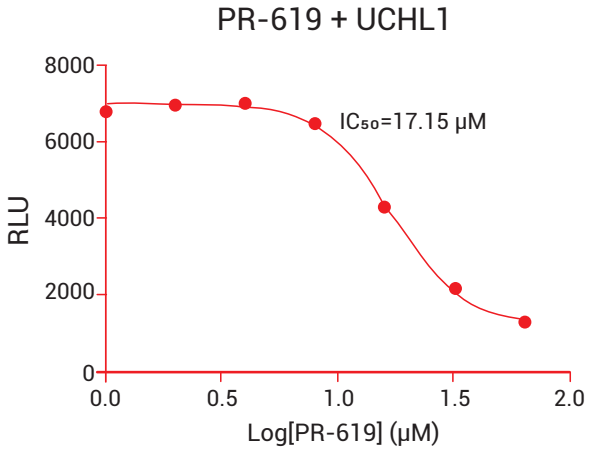
DUB	Inhibitor	Structure	Company/ Institution	Disease indication	Stage of development
USP8	9-(Ethoxyimino)-9H-indeno[1,2-b] pyrazine-2,3-dicarbonitrile		Hybrigenics	Oncology	Preclinical
USP9X	WP1130		University of Michigan	Oncology	Preclinical
USP10 and USP13	Spautin 1		Shanghai Institute of Organic Chemistry and Harvard Medical School	Inflammation	Preclinical
USP11	Mitoxantrone		Thomas Jefferson University	Oncology	Preclinical
USP14	IU1 and analogues		Harvard College and Proteostasis Therapeutics	Neurodegeneration	Preclinical

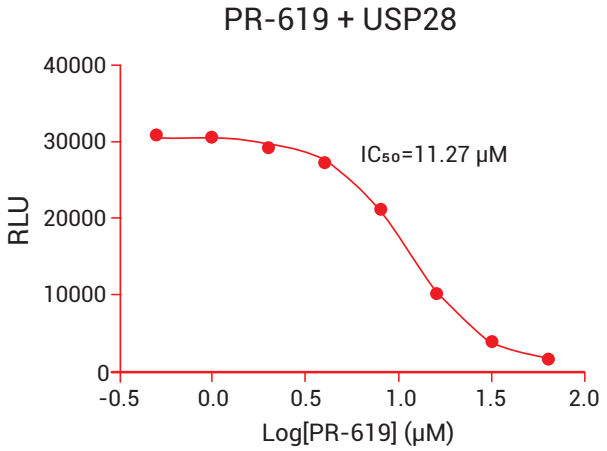
DUB	Inhibitor	Structure	Company/ Institution	Disease indication	Stage of development
USP20	GSK2643943A		GSK	Oncology	Preclinical
USP30	15-oxospirami-lactone		Chinese Academy of Sciences	Neurodegener-ation	Preclinical
	N-cyano pyrro-lidine		Mission Therapeutics	Neurodegener-ation	Preclinical

Screening technologies

In many screening laboratories DUB enzymatic activity are currently measured with a steady-state fluorescence-based assay method that was established two decades ago. This method utilizes fluorogenic substrates such as ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC), or ubiquitin C-terminal rhodamine 110 (Ub-Rho). These substrates are efficiently cleaved or hydrolyzed by various DUBs, releasing a highly fluorescent moiety. While this assay has been used in various DUB inhibitor screens, for example to identify USP1 and USP7 inhibitors[37, 38], one significant drawback, particularly with Ub-AMC, is that it is prone to fluorescence interference exhibited by many small molecules[39]. This interference usually results in poor signal-to-background ratio, as well as a narrow dynamic range with assay.







To overcome these obstacles present in DUB activity assays, SignalChem has recently developed a novel method based on chemically synthesized luminogenic DUB substrates (Figure 2). Catalytic activity of DUBs liberates from substrates a small molecule called aminoluciferin (AML), which reacts with recombinant luciferase to generate bright luminescence in the presence of ATP and $MgCl_2$. The bioluminescent assay has been used to detect activity of all six families of DUBs representing both cysteine and metalloprotease classes and showed wider dynamic range, greater sensitivity and lower detection limits when compared with standard fluorescent assay. This platform has proven to be HTS-compatible for DUB inhibitor screening and therefore is ready to address the needs to assess large library of compounds designed for DUB targeting.

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