Therapeutic Potential of Targeting Ubiquitin-Specific Proteases in Colorectal Cancer: Challenges and Advances in Drug Discovery

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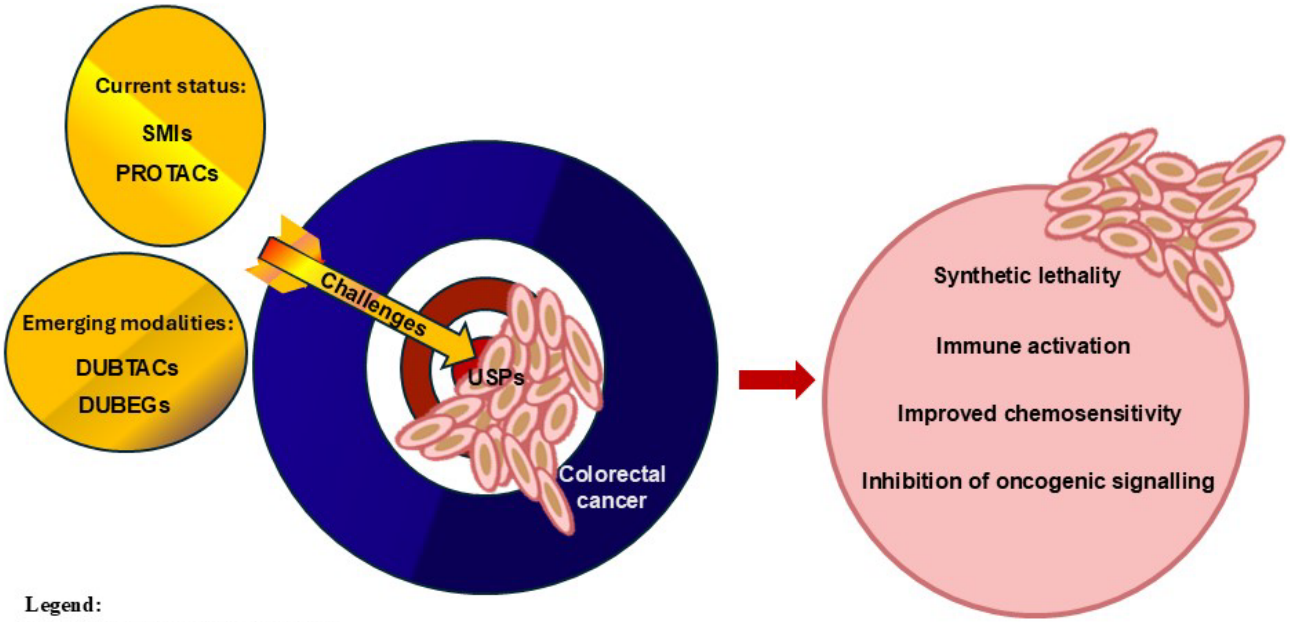
Teaser

Explore how ubiquitin-specific proteases (USPs) offer an attractive reservoir of targets for cancer therapies, with potential that extends beyond traditional pathway-specific modulation, and emerging strategies for targeting them.

Highlights

- USPs present a potential therapeutic strategy for colorectal cancer.
- Developing effective USP inhibitors involves tackling challenges from chemical and biological angles.
- Drug discovery and development efforts are largely focused on USP1 and USP7 as key targets.
- The introduction of PROTAC and emerging strategies like DUBEGs, and DUBTACs represent exciting avenues for targeting USPs

Graphical abstract



Legend:
Ubiquitin-specific proteases (USPs)
Small molecule inhibitors (SMIs)
Proteolysis-targeting chimaeras (PROTACs)
DUB-targeting chimaeras (DUBTACs)
DUB-engaging glues (DUBEGs)

Abstract

Ubiquitin-specific proteases (USPs) are a subset of deubiquitinating enzymes (DUBs) that play crucial roles in regulating key signalling pathways, DNA repair mechanisms, and immune responses by modulating the interactions and stability of proteins, including oncogenes and tumour suppressors in many cancers including colorectal cancer (CRC). USPs present an attractive reservoir of drug targets that could potentially overcome the shortcomings of conventional pathway-specific cancer therapies. This review explores the roles of USPs in CRC, addresses the challenges in discovering and developing USP inhibitors, highlights recent advancements in drug development and discusses the potential of targeted protein degraders and stabilisers including proteolysis-targeting chimaeras (PROTACs), DUB-engaging glues (DUBEGs), and DUB-targeting chimaeras (DUBTACs) as strategies for drugging USPs.

Keywords: Colorectal cancer, ubiquitin-specific proteases, targeted protein degrader, molecular glue, small molecule inhibitor

Introduction

Colorectal cancer (CRC) ranks as the third most common cancer worldwide and is the second leading cause of cancer-related deaths globally[1]. The high mortality rate in advanced CRC is primarily due to limited treatment options. Although chemotherapy has been the mainstay for

advanced CRC, its effectiveness is hampered by the development of resistance and unwarranted toxicity. While immune checkpoint inhibitors (ICIs) are effective in tumours with high microsatellite instability (MSI-h) or mismatch repair deficiency (dMMR), ICIs have shown limited efficacy in microsatellite stable (MSS) tumours which make up 85% of CRC cases [2]. Therefore, developing safe and effective therapies for advanced CRC is an urgent clinical need.

Genomic analyses in CRC have identified significant alterations in pathways such as Wnt–β-catenin, p53, mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK), transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K)/Akt signalling pathways[3].In MSS CRCs, these alterations are closely associated with early clonal driver mutations including adenomatous polyposis coli (APC) (77%), TP53 (73%), Kirsten rat sarcoma virus (KRAS) (48%) and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit-α (PIK3CA) (25%). Epigenetic events including DNA methylation, histone modifications and noncoding RNAs are also crucial players in CRC tumorigenesis. It is noteworthy that while specific mutations can initiate tumorigenesis, the progression and development of tumours are often driven by the complex interplay of multiple genes. Despite extensive research, the specific alterations in CRC patients remain challenging to address with current targeted treatments due to tumour heterogeneity and the complexity of the signalling network, involving multiple feedback loops, crosstalks and compensatory mechanisms. Hence, targeting one specific mutation or pathway may not be sufficient to inhibit tumour growth, thus the need for new therapeutic strategies.

The ubiquitin-proteasome system (UPS) maintains cellular homeostasis by degrading excess or damaged proteins, thereby supporting essential cellular processes. Ubiquitination involves covalent attachment of the 76-amino acid protein ubiquitin to substrate proteins and is a prevalent post-translational modification in eukaryotic cells. The addition of ubiquitin can impact protein localisation, thereby affecting their functions. Beyond marking proteins for degradation by the proteasome or lysosome, ubiquitination also influences protein-protein interactions (PPI) by serving as a signal for proteins with ubiquitin-binding domains, thereby forming protein complexes or initiating signalling cascades. Deubiquitinating enzymes (DUBs) are responsible for removing ubiquitin marks. DUBs contribute to dysregulated cellular processes such as apoptosis and cell proliferation, therefore targeting DUBs has been proposed as a new strategy for anti-cancer therapy [4]. Ubiquitin-specific proteases (USPs), the largest superfamily comprising over 50 members are a subclass of DUBs that modulate protein degradation and stability, thereby regulating cellular signalling and homeostasis.

Many small-molecule inhibitors (SMIs) and proteolysis-targeting chimaeras (PROTACs) have emerged in the drug discovery pipeline over recent years. These inhibitors and degraders have shown promising preclinical efficacy, leading to potent inhibition or degradation of oncogenic proteins, induction of apoptosis, and suppression of tumour growth. Despite tremendous efforts in preclinical development, no USP inhibitor has yet achieved clinical approval. The drug candidates currently in clinical trials have either demonstrated limited benefits or exhibited undesirable toxicity, underscoring the difficulties in targeting this family of proteins for therapeutic purposes. This review aims to provide an overview of the role of USPs in CRC, the status of USP drug candidates in development, challenges and future directions for targeting these enzymes for cancer therapy.

Role of USPs in CRC

USPs are integral to key signalling pathways that regulate cell proliferation, apoptosis, cell cycle progression, cancer stemness, drug resistance, immune response, and DNA damage response (DDR) [5]. USP1 and 7 are the most pursued targets in drug discovery and development due to their unique roles, mechanisms, and implications in cancer. The next section explores their roles in CRC, along with other USPs that have functions beyond their enzymatic activity.

USP1

USP1 plays a key role in DNA repair by deubiquitylating crucial proteins involved in the process, such as proliferating cell nuclear antigen (PCNA) in the translesion synthesis (TLS) pathway and Fanconi anaemia group D2 (FANCD2)/ Fanconi Anemia Complementation Group I (FANCI) in the Fanconi anaemia (FA) pathway [6]. USP1 deubiquitinates the FANCI-FANCD2 complex, which is essential for initiating proper DNA repair in the FA pathway. Hence, inhibiting USP1 disrupts DDR and offers therapeutic potential in tumours with HRD. Mutations in DDR are present in both MSI and MSS CRC [7]. A recent study has identified ARID1A (7.5%), ATM (5.7%), and BRCA2 (2.6%) as the most frequently mutated DDR genes in CRC with pathway mutation frequencies as follows: homologous recombination repair (HRR) at 14.72%, checkpoint pathways (CP) at 7.5%, FA at 5.7%, mismatch repair (MMR) at 4.1%, base excision repair (BER) at 3.8%, and NHEJ at 2.6% [8]. Thus, targeting DDR alterations in clinical practice could potentially expand the therapeutic landscape for CRC through synthetic lethality. Moreover, elevated USP1 levels in CRC are associated with a poor patient prognosis. Knockdown of USP1 leads to G2/M cell cycle arrest while pharmacological inhibition of USP1 sensitises CRC cells to DNA-damaging agents [9]. highlighting its potential as a therapeutic target in CRC. It is noteworthy that while DDR-targeted therapies offer a solid biological basis for treating CRC, their clinical data and application are still in their infancy and require further research. Identifying biomarkers for DDR genomic alterations and predictive biomarkers remains an ongoing challenge [10].

USP7

In CRC, USP7 is overexpressed and associated with tumour progression and poor prognosis [11]. In APC-truncated mice, USP7 deletion blocks tumour growth without harming healthy intestines [12]. USP7 has garnered considerable scientific interest due to its function in stabilising oncogene and tumour suppressor proteins. USP7 stabilises the p53 tumour suppressor protein to maintain cellular homeostasis under oxidative stress or DNA damage [13]. USP7 also stabilises oncogenic proteins like Murine double minute 2 (MDM2) and Murine double minute X (MDMX), which promotes tumour survival [14]. Knockdown or inhibition of USP7 increases p53 levels and reduces the viability of haematological cancer cells [15]. Interestingly, cancer cells with p53 mutations also respond to USP7 inhibitors, indicating that other mechanisms may contribute to their anti-tumour effects [14] The presence of over 20 known substrates for USP7 adds to the complexity of its biological functions. Additionally, compensatory mechanisms like USP22 upregulation and activation of cancer pathways, including c-Myc may present challenges for USP7-targeted therapies [16]. These findings suggest that targeting USP7, alone or in combination, could yield benefits tailored to specific contexts, underscoring the need to identify the appropriate malignancies and patient groups.

Other USPs

Beyond their primary role in cleaving ubiquitin from protein substrates, USPs have broader functions. They interact with various binding partners, including proteins and regulatory molecules, and act as scaffolds to facilitate PPI within signalling complexes. For example, USP3, through its interaction with the long non-coding RNA AC092894.1, enhances deubiquitination of the androgen receptor and promotes apoptosis in CRC cells by activating the MAPK pathway[17]. Similarly, USP4 is upregulated in MSS CRC, where it plays a critical role in modulating immune responses and promoting tumour growth through its enzymatic activity and PPIs. Notably, USP4 deubiquitinates the interferon regulatory factor 3 (IRF3), which enhances the efficacy of anti-PD-L1 therapy in CRC [18]. Furthermore, USP4 stabilises β-catenin and interacts with cortactin (CTTN), an actin-binding protein to enhance cell migration through Src/focal adhesion kinase (FAK) binding [19]. USP6 plays a pivotal role in enhancing Wnt signalling by deubiquitinating Frizzled [20]. Analysis of clinical CRC samples reveals that USP6 is overexpressed and positively associated with cancer invasion and metastasis. Conversely, depleting USP6 reduces these capabilities in CRC cells [21]. Additionally, USP6 confers platinum resistance in CRC through its interaction with circRNA circCYFIP2, leading to the deubiquitination of Golgi phosphoprotein 3 (GOLPH3) and cell survival [22].

Targeting USPs in CRC with small molecules

Historically, SMIs have been the primary candidates targeting USPs. Numerous SMIs have been evaluated in CRC models, targeting specific USPs and employing broad-spectrum approaches, as summarised in Table 1. Here, we review representative SMIs that illustrate key differences in selectivity and mechanisms of action in CRC, and the factors that have hindered their progression beyond the preclinical stage.

Small molecule inhibitors

USPs recognise and bind to ubiquitinated substrates, using a catalytic triad (typically consisting of cysteine, histidine, and aspartate/serine) to perform hydrolysis. This reaction cleaves the isopeptide bond between ubiquitin and the target protein. In traditional drug development efforts targeting USPs, the focus has been on SMIs that bind to either allosteric or active sites on these enzymes. By doing so, these inhibitors hinder the USPs' ability to deubiquitinate substrates, thereby facilitating the degradation of cancer-promoting proteins.

ML323 is the first **selective** USP1/UAF1 inhibitor demonstrating a half-maximal inhibitory concentration (IC_{50}) of sub-100 nM for USP1/UAF1 [23,24]. ML323 allosterically disrupts the active site of USP1[25] and induces replication stress and apoptosis by trapping USP1 on DNA [26]. More recently, Xu et al. reported that USP1 is auto-ubiquitinated in a manner that is dependent on its catalytic function., ML323 destabilises USP1, providing further insight into the mechanism of action of SMIs on USP1[27]. ML323 enhances the cytotoxic effects of DNA-damaging agents like doxorubicin, topoisomerase I/II inhibitors, and poly ADP ribose polymerase (PARP) inhibitors in CRC cells [9]. As a standalone, ML323 did not reduce the growth of HCT116 xenograft tumour, however, ML323 increases sensitivity to anticancer drug-mediated apoptosis *in vivo* [28]. However, ML323 displays a high clearance rate in rats (exceeding 70 ml/min/kg), which may reduce systemic exposure and potentially limit its therapeutic efficacy. Attempts to improve the metabolic stability of compounds in this series have

not been successful, thus limiting the utility of ML323 primarily as a probe molecule in preclinical studies.

FT671 is a non-covalent **selective inhibitor** of USP7, with an IC_{50} of 52 nM for the USP7 catalytic domain and demonstrates good *in vitro* potency at half-maximal growth inhibitory concentration (GI_{50}) of 33 nM for multiple myeloma (MM.1S) cells [29]. It destabilises USP7 substrates, such as MDM2, leading to increased levels of p53 and its target genes but only modestly inhibits tumour growth in an MM.1S mouse model.

P-22077 (USP7 IC_{50} = 8.0 μM; USP47 IC_{50} = 8.7 μM) and P-5091 (USP7 IC_{50} = 4.2 μM; USP47 IC_{50} = 4.3 μM) are **dual inhibitors** of USP7 and USP47 with weak potencies [30]. P-22077 inhibits Wnt signalling and modestly reduces growth in patient-derived organoids with APC mutations [12], while P-5091 moderately suppresses CRC tumour growth and enhances immune responses by increasing the activity of CD4+ and CD8+ T cells and reducing the levels of immune-suppressing Tregs [31]. Additionally, P-5091 inhibits Wnt signalling by promoting the ubiquitination of β-catenin, leading to apoptosis in CRC cells, independent of p53 status [32].

ML364 is a non-covalent inhibitor **dual targeting** USP2 and USP8, exhibiting an IC_{50} of 1.1 μM for the Lys-48-linked substrate and an GI_{50} of 3 μM in the HCT116 cell viability assay [33]. While it has been shown to induce defects in homologous recombination (HR) and may be beneficial for treating cyclin D1-driven cancers, ML364 has a less optimal absorption, distribution, metabolism, and excretion (ADME) profile, which restricts its *in vivo* application.

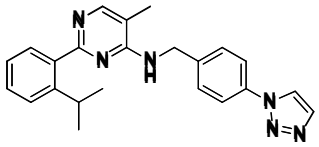
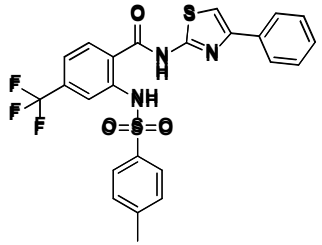
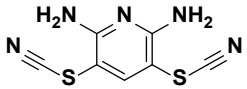
PR-619 is a **broad-spectrum** DUB inhibitor, exhibiting an IC_{50} range of 1-20 μM in cell-free assays [34]. It influences immune checkpoint and ferroptosis pathways and improves therapeutic outcomes when combined with anti-PD-1 therapy in a CRC mouse model [35]. Similarly, parthenolide, another non-selective DUB inhibitor, targets cancer stemness and Wnt signalling to impede CRC tumorigenesis [36,37]. Despite the reported anti-cancer activities of these inhibitors, their lack of selectivity can result in off-target effects, limiting their utility as targeted therapeutics.

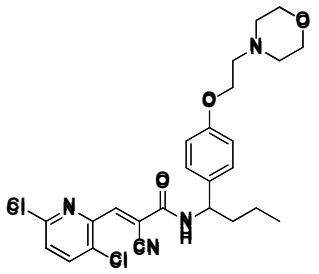
Although these SMIs have not progressed beyond the preclinical stage, the more selective USP inhibitors serve as valuable tool compounds for target validation and benchmarks for developing improved drugs.

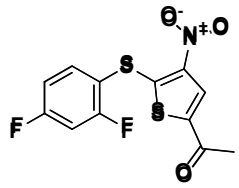
PPI modulators

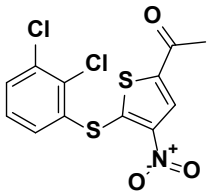
Recent advancements in PPI modulators targeting USPs have demonstrated the potential for disrupting interactions between USPs and their substrates or other cellular proteins. For example, UAT-B, a novel analogue derived from *Streptomyces conglobatus*, can disrupt interactions involving USP25 and Tankyrase (TNKS), thereby blocking Wnt signalling in CRC [38]. PSH-4, a synthetic peptide, has been reported to interfere with the USP25 and shieldin complex subunit 2 (SHLD2), impairing non-homologous DNA end joining (NHEJ) and ultimately promoting cancer cell death [39]. However, the development of PPI modulators remains challenging due to the large, flat binding surfaces involved, which complicate achieving selectivity and potency.

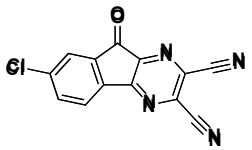
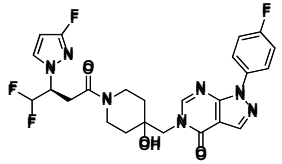
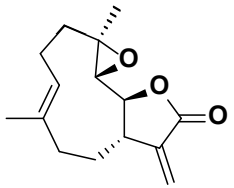
Table 1. Small molecule compounds targeting USPs and their mechanism of action in CRC.

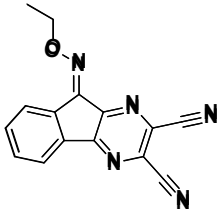
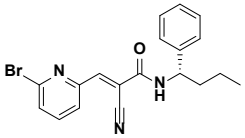
Compound	DUB	Target protein	Mechanism	Regulated pathway	Ref
*ML323  *Potential degrader of USP1	USP1	USP1	Destabilises USP1 and enhances the cytotoxic effects of DNA-damaging agents (doxorubicin, TOPI/II and PARP inhibitors), but not 5-FU in HCT116.	p53 DDR	[9] [27]
ML364 	USP2, 8	Cyclin D1	Degrades cyclin D1, arrests cells at the G1 phase and hampers homologous recombination-mediated DNA repair in HCT116.	DDR Cell cycle	[33]
PR-619 	Broad DUB	GPX4	Degrades Glutathione Peroxidase 4 (GPX4), induces ferroptosis and promotes $CD8^+$ T cells-mediated anti-tumour immunity. Combining PR-619 with anti-PD1 significantly inhibits CT26 tumour growth *in vivo*.	Immune checkpoint Ferroptosis	[35]

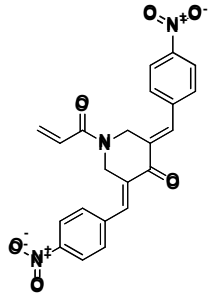
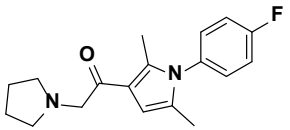
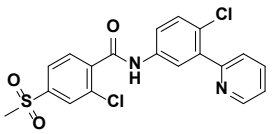
EOAI3402143 	USP9x, 24, 5	PD-1	Destabilises PD-1 and activates $CD8^+$ T cells, suppressing immune evasion and tumour growth. Combining EOAI3402143 and Trametinib or cytotoxic T-lymphocyte associated protein 4 (CTLA-4) blockade enhances the suppression of CT26 tumour growth.	Immune checkpoint [40]

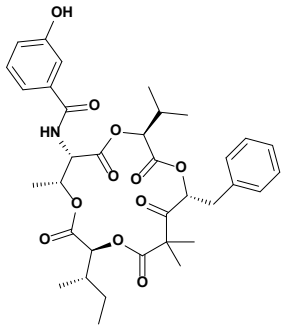
P-22077 	β-catenin	Wnt	
	USP7, 10, 47	Inhibits the growth of APC-truncated patient-derived cancer organoids/ xenografts.	[12]

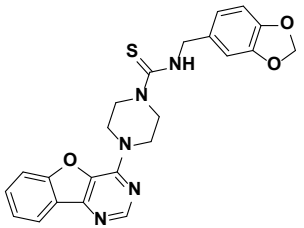
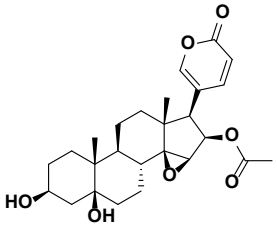
P-5091 	β-catenin	Wnt Immune response	
	USP7, 47	Suppresses tumour growth, decreases IL-10, enhances CD4+ and CD8+ T-cell function by upregulating IFN-γ, and reduces immune suppressor Tregs by lowering FOXP3 expression.	[31] [32]

HBX 41108 	USP5, 7, 8 and UCH-L3	p53, MDM2	Inhibits cell proliferation and induces p53-dependent apoptosis	p53	[41]
FT671 	USP7	SP1, MDM2, p53	Decreases SP1 protein level but upregulates c-Myc in HCT116. Destabilises MDM2, elevates p53 and p21, and inhibits tumour growth without weight loss of cachexia in MM.1S xenograft model.	p53	[16] [29]
Parthenolide 	USP7, 8, 21, 27X, 30, 35, 47 and JOSD2	β-catenin	Diminishes colorectal stem cell (CCSCs) self-renewal and induces apoptosis of CCSCs. Destabilises β-catenin and suppresses cell proliferation and induces apoptosis in HCT116 and SW480.	Cancer stemness Wnt	[36] [37]
DUBS-IN-2	USP8, 48	PDL-1 HMGA2	Elevates PDL-1 and enhances the efficacy of anti-PD-1/PD-L1 in MC38 tumour-bearing immunocompetent mice.	Immune response EGFR	[42] [43] [44]

			Suppresses Caco-2 tumourigenesis and enhances the *in vivo* efficacy of 5-FU by suppressing EGFR and EGFR-mediated signalling pathways. Detabilises high-mobility group AT-hook 2 (HMGA2), inhibits SW480 cell metastasis *in vitro* and *in vivo*.		
WP1130 	USP5, 9X, 14, 37 and UCHL1	MCL-1	Sensitises cancer cells to chemotherapeutic agents including 5-FU and Bcl-2/Bcl-xL inhibitors by degrading MCL-1.	Chemoresistance	[45]
b-AP15	USP14 and UCHL5		Inhibits the activation of NF-κB pathway and decreases resistance to 5-FU in RKO and HCT15 *in vitro* and *in vivo.*	NF-κB Chemoresistance	[46]

					
IU1 		USP14	IDO1	Decreases IDO1 expression, restores cytotoxic T-cell activity, and enhances responsiveness to anti-PD-1 therapy in MC38 syngeneic mouse model USP14 loss in HCT116 cells promotes a senescence-like phenotype not replicated by pharmacologic inhibition using IU1.	Immune response Senescence	[47] [48]
Vismodegib, an inhibitor of Hedgehog signalling pathway 		USP25, 28		Inhibits HCT116 and Ls174T cell proliferation. No observed vismodegib-associated benefit in combination with either FOLFOX or FOLFIRI plus bevacizumab in Phase II clinical trial for previously untreated mCRC (ID: NCT00636610)	Wnt c-Myc Notch	[49] [50]

PSH-4 peptide	USP25	SHLD2	Disrupts USP25-SHLD2 interaction and impairs non-homologous DNA end joining (NHEJ) and increases sensitivity to 5-FU in patient-derived xenograft	DDR	[39]
UAT-B 	USP25	TNKS	Disrupts USP25-TNKS interaction blocks Wnt signalling and inhibits multi-drug resistance CRC progression *in vivo*. Suppresses tumorigenesis and progression in patient-derived xenograft and $APC^{min/+}$ spontaneous CRC models.	Wnt	[38]
MP470	USP31	RUNX1	Disrupts USP31-RUNX1 interaction and decreases RUNX1 transcription of CDC20 and MCM2 expression.	Mitotic checkpoint	[51]

			
Cinobufotalin	c-Myc	c-Myc	
	USP36	Decreases c-Myc stabilization, inhibits tumour growth and metastasis in HCT116 xenografts, and increases sensitivity to Oxaliplatin.	[52]

Challenges in designing specific USP inhibitors: highly conserved residues within the catalytic domain

The development of SMIs targeting USPs presents a formidable challenge in achieving the required selectivity and potency. The analysis of the catalytic binding sites of USPs is conducted to elucidate the conserved regions and key residues involved in substrate recognition and catalysis across this diverse enzyme family (see Supplementary Methods). Understanding the evolutionary relationships and functional similarities among USPs facilitates the prediction of substrate specificity and binding sites for SMs. The multiple sequence alignment (MSA) of 53 USP binding site residues (Figure 1a) demonstrates the presence of highly conserved residues across the USP family. Notably, residues Cys-223, Tyr-224, His-405, Leu-406, Arg-407, Lys-456, and Tyr-514 are conserved in nearly all USP sequences analysed, indicating their critical role in maintaining the structural and functional integrity of the binding site. These conserved residues are likely involved in catalytic activity and substrate recognition, consistent with previous studies highlighting the importance of these amino acids in DUB function [53].

A maximum likelihood phylogenetic analysis of USP family members based on their binding site residues explores the conservation of these residues and depicts potential functional similarities among closely related USPs (Figure 1b). Clusters highlighted in blue indicate USPs which may have evolved to interact with similar or related compounds or inhibitors. For instance, the clustering of USP9X and USP9Y with strong bootstrap support suggests that their binding sites are likely very similar, potentially indicating a shared ability to bind to similar molecules. Similarly, the clusters containing USP49 and USP44, USP42 and USP36, USP13 and USP5, USP25 and USP28, USP53 and USP54, USP6 and USP32, USP35 and USP38 could potentially share a common inhibitor profile, given their conserved binding sites. Potent compounds identified for USP28 have also shown significant activity against USP25 [54]. A recent study further supports this finding by highlighting the role of binding-site cleft residues in USP25 and USP28, providing insight into the similar inhibition mechanisms observed for both USPs [55]. In contrast to the clusters described above, both USP1 and USP7 do not cluster with any other USPs in the phylogenetic tree, indicating that their binding site residues are more divergent. This structural divergence suggests that USP1 and USP7 may have evolved with unique binding site configurations, potentially contributing to their specificity for certain inhibitors. For instance, the structural feature that makes USP7 a prime target for inhibition is its allosteric pocket, distinct from the catalytic site, which modulates enzyme activity. Recent studies have identified inhibitors that bind to this allosteric site and induce conformational changes that prevent the enzyme from adopting its active form [56]. The unique structural feature of USP7 harbours an allosteric regulatory interaction site (switching loop) and allows targeting USP7 beyond its catalytic domain [29].

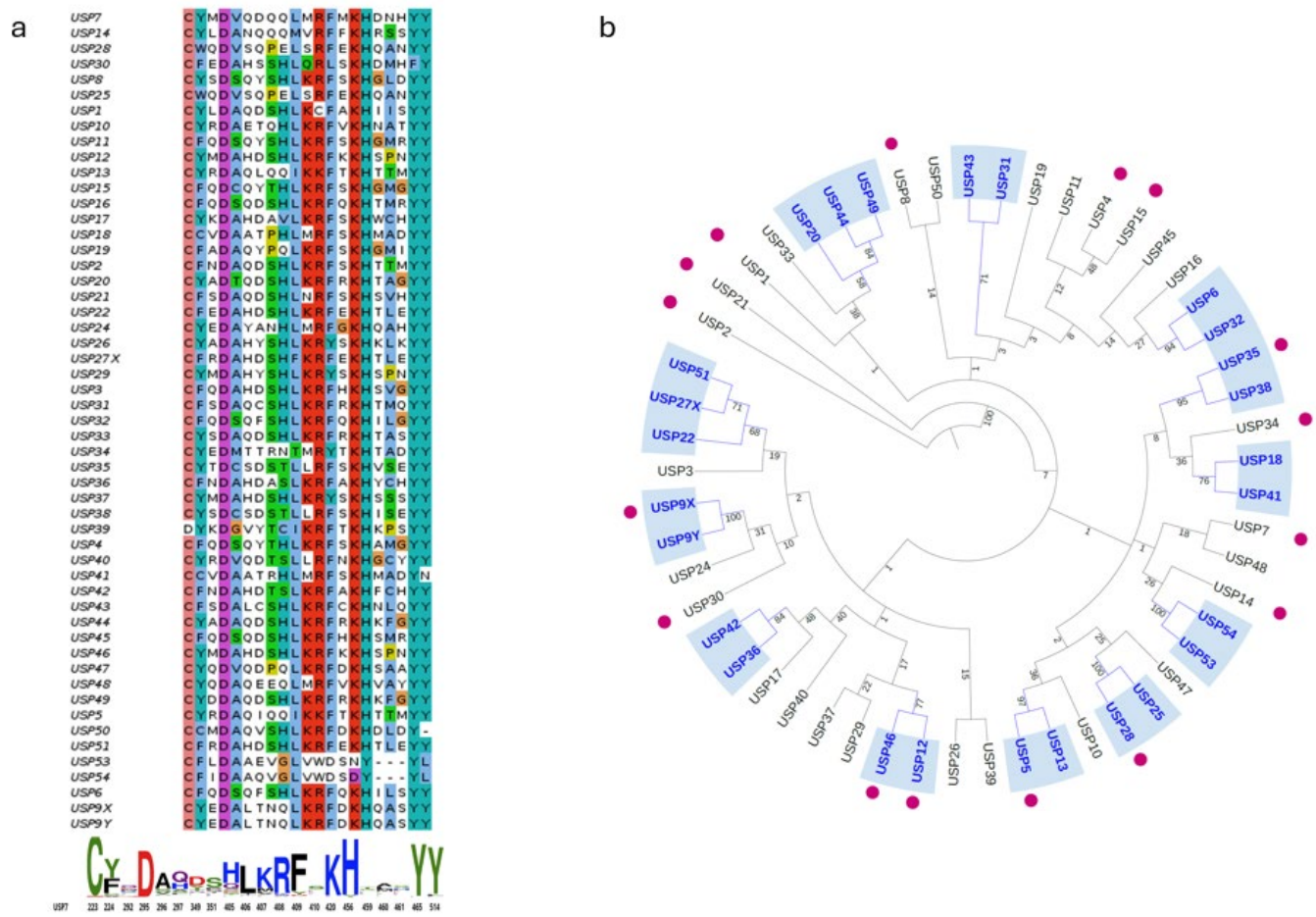


Figure 1: A phylogenetic analysis of USP family members based on their catalytic site residues. a) Multiple sequence alignment (MSA) reveals highly conserved residues, including Cys-223, Tyr-224, His-405, Leu-406, Arg-407, Lys-456, and Tyr-514, which are conserved across most USPs. Variability in other regions of the binding sites is also evident, particularly around residues 292, 410, 459, 460 and 461, indicating potential functional divergence among the USPs. The sequence logo at the bottom of the figure generated using the weblogo3 server (http://weblogo.threeplusone.com/create.cgi) highlights the conservation level at each position, with larger letters representing more conserved residues. The residue numberings are according to the USP7 (PDB:5N9R) structure. b) Sequence branch nodes with bootstrap values greater than 50% (clustered and highlighted in blue) suggest that these groupings are statistically supported with a reasonable level of confidence, in terms of evolutionary relationships. The dark pink dots next to each USPs indicate the availability of X-ray structures as follows USP1(PDB:7AY2), USP2(PDB:5XVE), USP4(PDB:2Y6E), USP5(PDB:3IHP), USP7(PDB:5N9R), USP8(PDB:3N3K), USP9X(PDB:5WCH), USP12(PDB:5L8W), USP14(PDB:6IIK), USP15(PDB:6GH9), USP21(PDB:2Y5B), USP28(PDB:6HEI), USP30(PDB:5OHP), USP34(PDB:7W3U), USP35(PDB:5TXK) and USP46(PDB:5CVM).

The integrated examination of multiple sequence alignments and phylogenetic relationships among residues at USP binding sites offers valuable insights into their evolutionary conservation and functional significance. While many USPs demonstrate notable evolutionary stability and clustering, some with low bootstrap support reveal the diversity present within this enzyme

family. These results enhance our understanding of the evolutionary dynamics within the USP family and highlight the challenges associated with targeting conserved and functionally important residues using SMIs.

Current landscape of USP-targeted drugs and their development progress

This section examines the evolving landscape of USP-targeted drugs and highlights their advancements within the pharmaceutical industry. Notably, PROTACs have emerged as a new modality within the drug discovery pipeline that can potentially address some of the challenges SM therapeutics face. PROTAC binds to a ligandable site on the target protein, recruits an E2/ E3 ubiquitin ligase and brings it into proximity with the target protein, allowing for targeted protein degradation (TPD) with greater selectivity. As of 1st October 2024, Pharmaprojects® reports continued interest in both SMIs and PROTACs, primarily targeting USP1 and USP7 (Table 2).

USP1

Drugs targeting USP1 are designed to exploit the concept of synthetic lethality. ML323 serves as a tool compound for developing improved USP1 inhibitors, including KSQ-4279 (Roche). Like ML323, KSQ-4279 binds to the same cryptic site on USP1 but alters the protein structure slightly differently [57]. VRTX531 (VRise Therapeutics), LAE-120 (Laekna Therapeutics Shanghai), and another unnamed USP1 inhibitor (Aigen Sciences) are currently being evaluated as monotherapies and in combination with a PARP1 inhibitor for homologous recombination deficient (HRD) tumours. Currently, KSQ-4279 (Roche, NCT05240898) and another selective USP1 inhibitor, XL-309 (Exelixis, NCT05932862), are being investigated in Phase I clinical trials as monotherapies or in combination with olaparib for patients with solid tumours exhibiting HRD. The Phase I/II clinical trial for TNG348 (Tango Therapeutics, Inc.), both alone and in combination with olaparib, in patients with BRCA1/2 and other HRD cancers, was recently terminated due to grade 3 and 4 liver function abnormalities. Notably, many of these USP1 inhibitors target USP1 through a single allosteric site. Consequently, drug developers may need to investigate alternative strategies to improve efficacy and reduce toxicity. Recently, Ubiquigent Ltd. has introduced a USP1-targeting PROTAC into its drug discovery pipeline, offering a new approach for targeting USP1 with improved selectivity and specificity.

USP7

The complexity of USP7's role in cancer has not diminished its appeal as a drug target. In the past decade, SMIs aimed at USP7's deubiquitinating activity have been developed using various strategies: (1) indirect methods that do not involve direct binding (e.g., P-5091); (2) covalent binding approaches (e.g., P-22077, HBX19818, HBX41108); and (3) non-covalent binding techniques (e.g., GNE-6640, GNE-6676) [58]. Despite promising results in both *in vitro* and *in vivo* models, many of these inhibitors have not advanced beyond the preclinical stage due to off-target effects, insufficient target specificity, or limited efficacy *in vivo* [59,60]. The wide range of USP7 substrates and the limited potency and selectivity of earlier inhibitors have posed challenges for developing reliable pharmacodynamic biomarkers and identifying suitable patient populations.

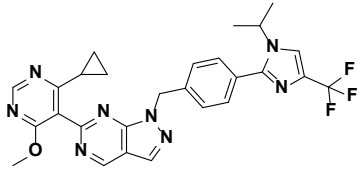
Although evidence supports the potential of USP7 inhibitors as standalone treatments and in combination with other therapies, the high attrition rate highlights the difficulties in advancing these compounds and navigating the complex underlying biology. Promising candidates such as OAT-4828 (Molecure) and USP7 inhibitors from Almac Discovery/Roche and Medivir/Ubiquigent remain active in preclinical development for immuno-oncology indications.

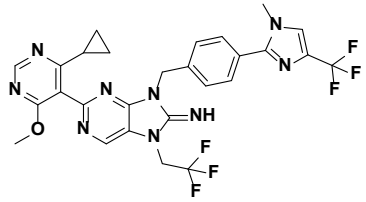
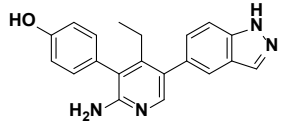
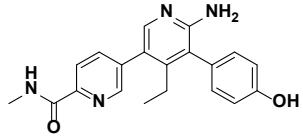
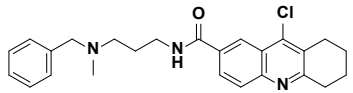
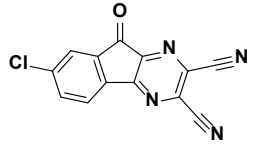
Other USPs

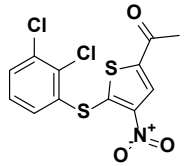
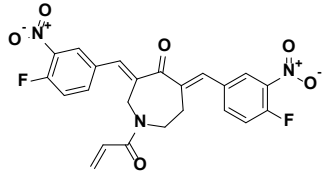
Several inhibitors targeting other USPs have shown potential, though many are still in the early stages of discovery or have been discontinued. For instance, the small molecule HBX-96819 (Hibrigenics) was explored as a treatment for EGFR-resistant lung cancer targeting USP8, but it has since ceased in preclinical development. Similarly, USP9X inhibitors (Ensemble Therapeutics) were investigated for various cancers, including melanoma, pancreatic, brain, and breast cancer, but their development has also been discontinued. VLX-1570 (Vivolux), an SMI against USP14 and Ubiquitin C-Terminal Hydrolase L5 (UCHL5) showed initial promise in treating relapsed/refractory multiple myeloma (RRMM) patients. However, the Phase II trial was discontinued after two study-related deaths occurred due to severe lung toxicity at doses where early signs of anti-tumour activity were noted [61].

USP21 inhibitors which target cancer metabolism are still active in the early stages of drug discovery (Molecure). In contrast, other USP21-targeting therapies (Mission Therapeutics) have been discontinued. Similarly, while USP25, USP28, USP30, USP33, and USP34 inhibitors have shown initial promise, they have ceased development. Translating these early-stage findings into clinical candidates remains a significant challenge.

Table 2. USP-targeting drug development status according to Pharmaprojects®.

USP	Drug	Modality	Disease	Development status as of 1st October 2024	Highest status reached	Company
USP1	ISM-0003091/XL-309	Small molecule	Cancer, unspecified	Active	Phase I Clinical Trial ID: NCT05932862	InSilico Medicine (Originator) Exelixis (Licensee)
USP1	KSQ-4279/ RO7623066 	Small molecule	Breast, ovarian, and solid cancers	Active	Phase I Clinical Trial ID: NCT05240898	KSQ Therapeutics (Originator) Roche (Licensee)
USP1	USP1i DUBi	Small molecule	Cancer, unspecified	Active	Preclinical	Ubiquigent (Originator)
USP1	USP1 inhibitor	Small molecule	Cancer, unspecified	Active	Preclinical	Satya Pharma (Originator)
USP1	USP1 DUB-targeting PROTAC	PROTAC	Cancer, unspecified	Active	Preclinical	Ubiquigent (Originator)
USP1	IMP-13	Small molecule	Cancer, unspecified	Active	Preclinical	Impact Therapeutics (Originator)
USP1	XZP-6924	Small molecule	Cancer, unspecified	Active	Preclinical	XuanZhu Pharma -part of Sihuan Pharmaceutical (Originator)
USP1	LAE-120	Small molecule	Cancer, unspecified	Active	Preclinical	Laekna Therapeutics Shanghai (Originator)
USP1	USP1 inhibitor	Small molecule	Cancer, unspecified	Active	Preclinical	Aigen Sciences (Originator)
USP1	TNG-348	Small molecule	Breast, ovarian, prostate and solid cancers	Ceased	Phase I ID: NCT06065059	Tango Therapeutics (Originator)

						
USP7	OAT-4828	Small molecule	Cancer, unspecified	Active	Preclinical	Molecure (Originator)
USP7	USP7 inhibitors	Small molecule	Cancer, solid, unspecified	Active	Preclinical	Medivir (Originator) Ubiquigent (Licensee)
USP7	USP7 inhibitors	Small molecule	Cancer, unspecified	Active	Preclinical	Almac Discovery (Originator) Roche (Licensee)
USP7	GNE-6640 	Small molecule	Cancer, haematological, solid, unspecified	Ceased	Preclinical	Roche (Originator)
USP7	GNE-6776 	Small molecule	Cancer, haematological, solid, unspecified	Ceased	Preclinical	Roche (Originator)
USP7	HBX-19818 	Small molecule	Cancer, leukaemia, chronic lymphocytic	Ceased	Preclinical	Hybrigenics (Originator)
USP7	HBX-41108 	Small molecule	Cancer, unspecified	Ceased	Preclinical	Hybrigenics (Originator)
USP7	MN-4	Small molecule	Cancer, head and neck, sarcoma, unspecified	Ceased	Preclinical	Mission Therapeutics (Originator)

USP7	OPAL-0012	Small molecule	Cancer, lung, non-small cell	Ceased	Preclinical	Valo Health (Originator)
USP7	USP7 inhibitor	Small molecule	Cancer, solid, unspecified	Ceased	Preclinical	Shouyao Holdings (Originator)
USP7	USP7 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	Canthera Discovery (Originator)
USP7	USP7 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	Hibrigenics (Originator) Servier (Licensee)
USP7	USP7 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	RAPT Therapeutics (Originator)
USP7, USP47	P-5091 	Small molecule	Cancer, myeloma	Ceased	Preclinical	Progenra (Originator)
USP8	HBX-96819	Small molecule	Cancer, lung, unspecified	Ceased	Preclinical	Hybrigenics (Originator)
USP9X	USP9x inhibitors	Small molecule	Cancer, brain, breast, melanoma, pancreatic	Ceased	Preclinical	Ensemble Therapeutics (Originator)
USP14	VLX-1570 	Small molecule	Myeloma, ~~Ewing's sarcoma~~	Ceased	Phase II Clinical Trial ID: NCT02372240	Vivolux (Originator)
USP21	USP21 inhibitor	Small molecule	Cancer, unspecified	Active	Preclinical	Molecure (Originator)
USP21	USP21 targeting therapy	Small molecule	Cancer, unspecified	Ceased	Preclinical	Mission Therapeutics (Originator)
USP25	USP25-targeting therapy	Small molecule	Cancer, unspecified	Ceased	Preclinical	Mission Therapeutics (Originator)

USP28	USP28 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	Ensemble Therapeutics (Originator)
USP28	OPL-0015	Small molecule	Cancer, unspecified	Ceased	Preclinical	Valo Health (Originator)
USP30	MTX-325	Small molecule	Cancer, unspecified	Ceased	Preclinical	Mission Therapeutics (Originator)
USP30	USP30 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	Ensemble Therapeutics (Originator)
USP33	VDU1 inhibitors	Small molecule	Cancer, unspecified	Ceased	Preclinical	TopoTarget -now Valerio Therapeutics (Originator)
USP34	P-009492	Small molecule	Cancer, unspecified	Ceased	Preclinical	Progenra (Originator)
USP47	USP47/USP37 targeting therapy	Small molecule	Cancer, unspecified	Ceased	Preclinical	Mission Therapeutics (Originator)

Source: Pharmaprojects® | Citeline, 2024

Emerging strategies in USP-targeted cancer therapies

Modalities of induced proximity are appealing strategies for treating diseases like cancer, where specificity is crucial for efficacy and safety (Figure 2). The emerging approach of targeting undruggable proteins by modulating their half-life marks a significant advancement in drug discovery. Targeted protein stabilisation represents an innovative therapeutic strategy for diseases by addressing challenging protein targets that are subject to aberrant degradation. DUBTACs are heterobifunctional molecules consisting of a covalent recruiter for DUBs linked to a ligand that specifically binds to the target protein, thereby preventing its degradation for therapeutic benefit. Although still relatively underexplored, the use of DUBTACs for targeting DUBs is gaining momentum as these molecules offer potential in treating diseases where the degradation of essential proteins, such as tumour suppressors, contributes to tumorigenesis. Although DUBTACs are often less drug-like based on Lipinski's rule of five, these compounds covalently bind to DUBs, which can be advantageous for ensuring effective DUB engagement. However, the covalent attachment can also permanently occupy the DUB and potentially disrupt its function and recycling.

Molecular glues offer several advantages over PROTACs, including smaller molecular weight, enhanced oral bioavailability and their ability to degrade unligandable proteins (e.g., intrinsically disordered proteins). Additionally, molecular glues, such as lenalidomide, have shown precedence in clinical success and therapeutic benefits in cancer treatment [62]. Although the design of molecular glues has historically been challenging and largely serendipitous, rational design strategies are beginning to emerge [63]. However, the caveat is that while molecular glues usually function by interacting at the interface between a DUB and an E3 ligase to promote selective degradation of DUBs, there is a possibility that the DUB could counteract the E3 ligase's activity by deubiquitinating itself [64]. Addressing this possibility is crucial to refine the molecular glue strategy for targeting USPs.

Identification of molecular glues that elevate tumour suppressor protein levels by interacting with specific DUBs (DUB-engaging glues, or DUBEGs) is likely to broaden the landscape of drug discovery and improve the drug-likeness of compounds designed to restore protein functions. Recent research utilising small-molecule microarray technology has identified bromocriptine (BC) as a molecular glue that binds both p53 and USP7, thereby increasing P53 stability [65]. Although its pharmacokinetic properties still require improvement, this proof-of-concept study introduces a strategy for upregulating p53 and marks a significant step forward in developing molecular glues that target USPs.

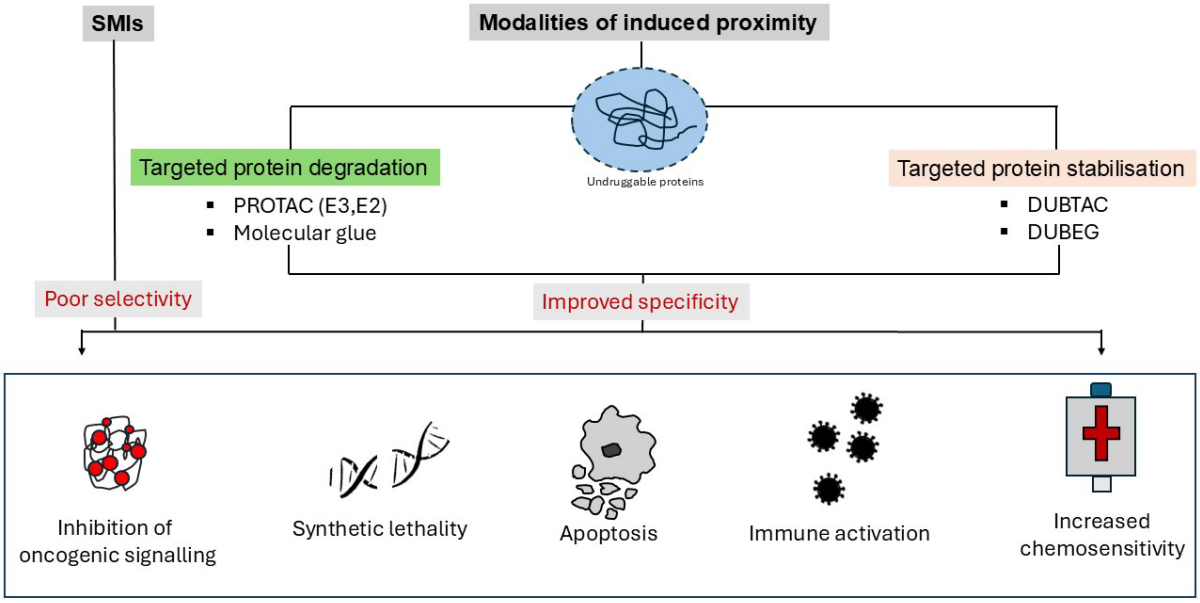


Figure 2. Modalities targeting USPs in cancer and their mechanisms of action. Traditional small molecule inhibitors (SMIs) block the enzymatic activity of USPs. Modalities of induced proximity offer a targeted therapeutic approach with higher specificity than SMIs. Proteolysis Targeting Chimeras (PROTACs) bind USPs to an E2 or E3 ubiquitin ligase while molecular glue facilitates the interaction between USP and an E3 ubiquitin ligase, leading to USP degradation via the proteasome; Deubiquitinase Targeting Chimeras (DUBTACs) and Deubiquitinase Enhancing Glues (DUBEGs) recruit deubiquitinase enzymes to specific target proteins, preventing their degradation. Targeting USP provides a potential therapeutic advantage by disrupting critical oncogenic pathways, inducing synthetic lethality and apoptosis, enhancing the immune response against cancer, and promoting chemosensitivity or overcoming chemoresistance.

Concluding remarks and perspectives:

USPs play a key role in various aspects of tumour biology, such as protein stability, signalling pathways, and DNA repair, offering a broader therapeutic impact and potentially addressing some limitations of single-target approaches. While overcoming challenges like tumour heterogeneity and compensatory mechanisms is crucial, particularly in CRC and other solid tumours, the involvement of USPs in CRC, especially through Wnt and p53 pathways highlights their potential as promising targets. The development of drugs targeting USPs in CRC has shown significant promise in both *in vitro* and *in vivo* studies, however, translating these results into effective clinical therapies remains a major challenge and warrants deep biology understanding.

Although most USPs share a fundamental mechanism for deubiquitinating activity, some USPs exhibit non-catalytic functions that are more common than previously thought [66]. Pharmacological inhibition of USP or genetic knock-out of USP can result in different functional and physiological effects [48], suggesting that USPs may have scaffolding functions beyond their enzymatic activity. This highlights the complexity of targeting USPs, where both catalytic and

non-catalytic functions must be considered. The choice to inhibit USP activity, disrupt PPI or employ TPD could impact therapeutic outcomes. While SMs and PPI modulators are limited to inhibiting protein function, degraders eliminate the protein, effectively targeting both its catalytic and non-catalytic roles. Ultimately, biological understanding is crucial in determining whether degradation or other modalities are preferred for effective therapeutic strategies.

The challenge in targeting USP also lies in its interactions with numerous substrates, which complicates predicting the full impact of its inhibition. This broad substrate interaction can lead to unintended side effects and toxicity because disrupting one USP may have cascading effects on multiple pathways regulated by its various substrates. Additionally, a substrate can be regulated by multiple USPs or other DUBs and E3-ligase complexes [13], thus inhibiting one USP might not be sufficient to alter the substrate's function significantly, leading to potential resistance or insufficient therapeutic effects. Dual inhibitors can potentially address these issues.

Modalities like PROTACs, DUBTACs and DUBEGs offer exciting opportunities to revolutionise cancer treatment, each presenting distinct advantages and challenges. These approaches promise to improve the specificity and effectiveness of therapies, addressing some of the limitations seen with SMIs. However, they also present complexities in drug design and pharmacokinetics. Continued research is crucial to overcome these challenges and optimise their clinical use. Nevertheless, these strategies offer renewed hope for patients and represent a significant step forward in advancing precision medicine.

Supplementary Methods

Sequence retrieval and multiple sequence alignment:

The catalytic domain sequences of 53 human ubiquitin-specific proteases (USPs) were retrieved from the UniProt database and downloaded in FASTA format. (https://www.uniprot.org/). The USP23 gene is currently known as USP21 in the UniProt database, hence USP23 was excluded from the analysis. Multiple Sequence Alignment (MSA) of the catalytic domains was performed using the T-Coffee web server with default settings (https://tcoffee.crg.eu/apps/tcoffee/do:regular). The output alignment file generated was further subjected to manual curation to improve the quality of the alignment using Jalview (version 2.11.2.4). The catalytic binding site residues within 5 Angstrom cut-off were identified using the USP7 co-crystal structure (PDB:5N9R)[56]. These identified residues were then mapped to the alignment file to extract the corresponding binding-site residues for all the USPs.

Phylogenetic tree construction

The maximum-likelihood phylogenetic tree of USP family members based on binding site residues. The final MSA comprising binding-site residues from 53 USP sequences and analysed using the PHYLIP package (version 3.697). An unrooted maximum-likelihood (ML) tree was generated by bootstrapping the MSA 100 times using SEQBOOT while maintaining the original sequence order. The Jones-Taylor-Thornton (JTT) substitution model [67] was applied to compute the ML tree, facilitating an in-depth exploration of the tree topology with the highest likelihood using the PROML program. The final consensus tree was constructed from 100 ML trees using the CONSENSE program. The resulting phylogenetic tree was visualised and clustered using iTOL (https://itol.embl.de/).

Declaration of interests

The authors declare that there are no conflicts of interest concerning the publication of this article.

CRediT authorship contribution statement

Chern Ein OON: Conception and design of the article, writing the original draft, review and editing, and visualisation.

Padmanabhan ANBAZHAGAN: Writing the original draft and computational analysis

Chong Teik TAN: Critical review and scientific discussion

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this article, the author(s) used ChatGPT to explore various ways of phrasing arguments to improve the flow and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and took full responsibility for the content of the published article.

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