Expanding the PROTAC Toolbox: Targeted Degradation of the Deubiquitinase USP7 in Cancer

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Abstract

Targeting deubiquitinating enzymes (DUBs) has emerged as a promising therapeutic approach in several human cancers and other diseases. DUB inhibitors are exciting pharmacological tools but often exhibit limited cellular potency. Here we report PROTACs based on an ubiquitin-specific protease 7 (USP7) inhibitor scaffold to degrade USP7. The hit compound CST967 caused highly selective degradation of USP7 and inhibited proliferation of USP7-dependent cancer cells. We present the first DUB degrader, which will be a useful tool to deepen our understanding of USP7.
Main text

The recent advancements in therapeutic modalities enable the targeting of a plethora of proteins, which were previously deemed undruggable. At present, proteolysis targeting chimeras (PROTACs) promise a paradigm shift in modern medicinal chemistry and have attracted substantial interest and investment from both academia and pharmaceutical industry. PROTACs are bifunctional compounds comprising a target ligand, an E3 ligase binder, and a linker tethering both moieties. Instead of merely blocking the functional domain of the protein of interest (POI), PROTACs enable target degradation via hijacking of the ubiquitin-proteasome machinery. Recently, PROTACs have shown clinical activity and favourable side effect profile in cancer highlighting the applicability as therapeutics. Deubiquitinating enzymes (DUBs) recognize and cleave the isopeptide bonds linking ubiquitin to proteins and play an important role in maintaining protein stability, homeostasis and signalling in cells. DUBs emerged as promising targets that could, if inhibited or depleted successfully, complement the inventory of drugs for various diseases, especially cancer. USP7 is a DUB known to be associated with a variety of tumour-suppressor genes and proto-oncogenes, pointing to its significance in tumour biology and progression. USP7 exhibits high expression levels in numerous cancer tissues and often correlates with poor prognosis and metastasis. It is a key regulator of the tumour suppressor p53 either via direct interaction, or indirectly by stabilizing mouse double minute 2 homolog (MDM2), the E3 ligase, which promotes ubiquitination and degradation of p53. Inhibition of USP7 promotes MDM2 ubiquitination and proteasomal degradation, ultimately leading to elevated levels of p53 and cell death. Therefore, USP7 represents a validated target for cancer therapy. Several potent and USP7-selective inhibitors were developed in the last decade, of which selected compounds are depicted in Figure 1. Of note, all inhibitors (compounds 1-3) possess a 4-hydroxypiperidine motif despite being developed in separate studies. The USP7 inhibitors FT671 (2) and XL188 (3) were chosen as a possible starting point for PROTAC synthesis. Notably, both scaffolds exhibited high polar surface area (Table 1), accompanied by a moderate logD value of 1–2. As reported in previous work on the discovery of these inhibitors, the (R)-configuration of the methyl or di/trifluoromethyl groups at the right-hand side tail is critical for target binding affinity (Figure 2A).
trifluoromethyl analogue (4) of XL188 was accessible via an asymmetric catalytic reduction of a prochiral cinnamic acid derivative (Scheme S1), and the introduction of the CF$_3$ group increased the lipophilicity. Unexpectedly, the USP7 inhibitory activity of 4 was lower compared to XL188 containing an (R)-CH$_3$ group at this position (Table 1, Figure S1). Compounds 5 and 6 with N-Boc-piperidine instead of the solvent-exposed piperazine handle (Figure 2B) showed notably decreased inhibition at 5 µM with 58% and 53% residual USP7 activities, respectively, whereas 7 inhibited USP7 with an IC$_{50}$ value of 1.7 ± 0.2 µM. Trends of the in vitro data were in agreement with docking results and cell viability profiles (Table 1, Figures S2-S5). Moreover, the nitrogen replacement resulted in the desired increase in logD of the ligand.

To enable quick access to PROTACs, we applied the racemic compound 6 to screen different linker lengths of PROTAC molecules. As both incorporation of a von Hippel-Lindau or an inhibitor of apoptosis protein E3 ligand would result in USP7 PROTACs with unfavourable high TPSA, we chose cereblon (CRBN) as E3 ligase and synthesized a series of eight PROTACs that are listed in Table S1. From this initial series, the rigidified PROTAC 9 performed best in decreasing USP7 levels in MM.1S cells at a concentration of 1 µM (Table 2 and Figure S6). Subtle structural modifications of this hit compound revealed structure-degradation relationships. Replacement of the racemic CH$_3$ group with an (R)-CF$_3$ substituent in 16 further increased degradation potency, whereas the introduction of an aromatic fluorine atom at the CRBN-binding moiety (17) was detrimental. PROTAC 18, synthesized via reductive amination instead of HATU-mediated amide bond formation as the last synthetic step, had improved physicochemical properties but could not degrade USP7 to the desired extent. Most pronounced degradation was observed with degrader 19 possessing an (R)-CH$_3$ group. PROTAC 19 also showed USP7 inhibition in vitro with an IC$_{50}$ value of 1.6 ± 0.3 µM (Figure S1). Its synthesis is outlined in Scheme 1.

Heterocyclisation of 21 in the presence of formamide gave quinazoline building block 22, which was reacted with 23 that was accessible via a Corey–Chaykovsky epoxidation. Subsequently, piperidinyl compound 24 was deprotected and coupled with the enantiopure acid building block 25. Iron/AcOH-mediated reduction of the nitro group in 26 gave 27, whose anilinic nitrogen was acylated to give PROTAC building block 7. We demonstrated that this compound retained moderate USP7 inhibition activity in
vitro (Table 1), although the exit vector in XL188 was exchanged with a neutral Boc-protected piperidine moiety, which can be employed for the final PROTAC assembly. To achieve frontrunner PROTAC 19, precursor 7 was coupled with CRBN ligand 29 after cleavage of both protecting groups (Scheme 1). Compound 20 (Table 2) represents a CRBN non-binding negative control, as methylation of the imide nitrogen is known to abolish E3 engagement.

Due to the striking USP7 degradation capacity of 19 (CST967), this drug was subjected to extensive biological studies.

Next, the degradation profile of the USP7 PROTAC 19 was determined by western blot analysis performed in the multiple myeloma cell line MM.1S. Increasing the PROTAC concentration enhanced the rate of USP7 degradation, eventually reaching a slight hook effect at 10 µM. Maximal degradation (D$_{\text{max}}$) of 85% at a concentration of 1 µM and DC$_{50/24\text{h}}$ potency of 17 nM was observed. In contrast, no effect on levels of CRBN and IMiD-induced neosubstrates GSPT1 and negligible degradation of IKZF1 was seen (Figure 3). Given the dynamic nature of degradation, a time-course analysis from 3 to 96 hours was performed in MM.1S cells to assess the degradation response over time. We observed maximal degradation at 24 hours which was followed by the gradual recovery of USP7 levels (Figure S7). Furthermore, we noted upregulation of the direct USP7-target p53 and induction of cleaved-PARP, a hallmark of cell death. To discern USP7 protein turnover and cell recovery, a drug washout experiment was performed after 24 hours. USP7 levels reverted 72 hours after recovery. In congruence with this, upregulation of p53 and cleaved-PARP levels were observed (Figure S8). PROTAC-mediated USP7 degradation was abrogated by the proteasome inhibitor MG132 and neddylation activation enzyme (NAE) inhibitor MLN4924 confirming degradation via the ubiquitin-proteasome pathway. Similarly, USP7 degradation was blocked by 100-fold excess pomalidomide, which competes for binding with CRBN (Figure S9), and in a genetically modified MM.1S cell line harbouring CRBN knockout, no USP7 depletion was observed, both corroborating USP7 degradation via the E3 ligase CRBN (Figure S10). A global-proteome analysis of the downstream effects of the USP7 PROTAC was assessed using diaPASEF-based mass spectrometry in MM.1S upon treatment with the PROTAC at 0.1 µM for three hours. Of the total of 7170 unique proteins identified (Figure 4A), USP7 appeared as the most significant downregulated protein, thus further
confirming the degradation ability of the USP7 PROTAC. The only other significantly regulated protein was Cox5A. No changes for CRBN’s known (neo)substrates were observed. Of note, neither in western blots nor in the proteomic data we observed changes of CRBN protein levels. Hence, CRBN deubiquitination and stabilization via USP7 was not evidenced.

To further explore the degrading efficacy and potency of the USP7 PROTAC, we tested a panel of cancer cell lines that are known to be dependent on USP7. Western blot analysis confirmed degradation of USP7 to varying degrees. The USP7 PROTAC led to decreased cell viability in all cell lines, thus underlining the anti-cancer activity of the compound (Figures 4B, S11, S12).

In our proof-of-concept study, we describe the first degrader of the deubiquitinase USP7. Our lead PROTAC CST967 is highly potent and selective and consistently shows activity in USP7-dependent cancer cell lines. Quantitative proteomic studies confirmed its high target selectivity. We propose that such a tool will be invaluable to unravel novel substrates and (patho-) physiological functions of USP7. While the early generation of USP7 inhibitors only partially and temporally modulates protein function, our degrader leads to a full loss of the protein, which could unveil the full potential of USP7 targeted therapies.27

Acknowledgements

This work was supported by the DFG (Kr-3886/2-1 and SFB-1074 to JK). We acknowledge the support by M. Frelih (University of Ljubljana), C. Ennenbach, and M. Schneider (University of Bonn). The authors appreciate the kind donation of the Walphos ligand SL-W008-2 by Solvias AG. We thank K. Donovan, E. Fischer and the Fischer Lab Degradation Proteomics Initiative for the collection of the global proteomics data supported by NIH CA214608 and CA218278.
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Figures and Figure legends

**Previous work:**

USP7 Inhibitors

![Chemical structures](image)

1 (cmpd 5)  
2 (FT671)  
3 (XL188)

**This work:**

USP7 PROTACs

CRBN

USP7

**Figure. 1** Targeting USP7 with inhibitors and PROTACs.
Figure 2  (A) The crystal structure of USP7 in complex with XL188 (PDB: 5VS6) reveals a possible exit vector for linker attachment; (B) chemical structures of modified USP7 inhibitors along with candidates suitable for linker attachment.
**Figure. 3** PROTAC 19 induces degradation of USP7. Western blot analysis of USP7, IKZF1, GSPT1 and CRBN protein levels in MM.1S cells treated with compounds for 24 h (left). DC$_{50}$ values were obtained by fitting $D_{max}$ values to a variable slope dose–response model (middle). CellTiter-Glo luminescent cell viability assay (right) showing effect of USP7 PROTAC upon 96 h treatment. Data are shown as mean±s.d. (n=2,3 respectively).
Figure 4  (A) diaPASEF-based mass spectrometry was performed in MM.1S cells upon treatment with USP7 PROTAC 19 at a concentration of 0.1 µM for 3 h. A total of 7171 identified proteins were plotted as log2 fold change (USP7 PROTAC / DMSO) versus –log10 of p-value with a threshold of 1% FDR; (B) western blots showing degradation of USP7 in A549 and LNCAP cell lines upon treatment with USP7 PROTAC 19 for 24 h (above). CellTiter-Glo Luminescent Cell Viability Assay upon a 96-hour treatment with USP7 PROTAC 19 in the respective cell lines (below). Data are shown as mean±s.d. (n=3).
Scheme 1  Synthesis of the USP7 degrader 19 (CST967).
Tables

Table 1  Overview of physicochemical properties, experimental and computational USP7 binding data, and impact on cell viability of USP7 inhibitors 2–7.

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<th>TPSA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>logD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PPB&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>MM-GBSA ΔG Bind&lt;sup&gt;e&lt;/sup&gt;</th>
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<sup>a</sup> Topological polar surface area is given in Å<sup>2</sup>.<br><sup>b</sup> Experimental partition coefficient.<br><sup>c</sup> Plasma protein binding (PPB) values were estimated by an HPLC-based method.<br><sup>d</sup> Inhibition data obtained with isolated USP7 catalytic domain using Ub-AMC as a substrate. Residual enzyme activities (RA) at 5 µM of compounds 5–6 are given. The IC<sub>50</sub> value for FT671 is in accordance with literature data,<sup>23</sup> whereas in our assay, XL188 showed marginally lower USP7 inhibition than reported previously.<sup>21</sup><br><sup>e</sup> An estimate for the binding affinity derived from computational docking (see Supporting Information). A more negative value indicates stronger binding.<br><sup>f</sup> Remaining cell viability after 24 h treatment of MM.1S cells with 10 µM of each compound.<br><sup>g</sup> not determined.<br><sup>h</sup> Mean of (R)- and (S)-isomers.
Table 2  Overview of physicochemical properties and cellular activities of rigidified USP7-targeting PROTACs.

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<th>%USP7 depletion&lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup> Topological polar surface area is given in Å². <sup>b</sup> Experimental partition coefficient. <sup>c</sup> USP7 depletion after 24 h treatment of MM.1S cells with 1 µM of each compound.