The therapeutic potential of deubiquitinating enzyme inhibitors

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Abstract
Proteases play a key role in various pathological processes and several protease inhibitors are already available for treatment. DUBs (deubiquitinating enzymes) constitute one of the largest classes of human proteases and are key effectors of the ubiquitin–proteasome system. This pathway regulating cellular protein turnover has been implicated in the pathogenesis of many human diseases, including neurodegenerative disorders, viral diseases and cancer. The therapeutic efficacy of the proteasome inhibitor Velcade® (bortezomib) for treating multiple myeloma and mantle cell lymphoma establishes this system as a valid target for cancer treatment. A promising alternative to targeting the proteasome itself would be to target the upstream, ubiquitin conjugation/deconjugation system, to generate more specific, less toxic anticancer agents. Advances in small molecule-based inhibitors specifically targeting DUBs are presented in this review.

Introduction
Ubiquitin is a 76-amino-acid polypeptide highly conserved in all eukaryotes. Ubiquitin is added to protein substrates by a cascade of reactions involving ubiquitin activation by an E1-conjugating enzyme, followed by its transfer to an internal lysine residue on the substrate, which is mediated by ubiquitin-conjugating enzymes (E2) and E3 ubiquitin ligases [1,2]. The covalent conjugation of ubiquitin molecules to the substrate regulates the distribution and activity of the protein or targets the protein for degradation by the multicatalytic proteasome complex [3]. Ubiquitin conjugation is a reversible process mediated by DUBs (deubiquitinating enzymes), which specifically cleave the isopeptide bond at the C-terminus of ubiquitin. DUBs can act at various points in the ubiquitin pathway, including polyubiquitin chain processing, ubiquitin substrate removal to change the fate of the protein or the removal of residual ubiquitin to facilitate proteasomal degradation and recycle ubiquitin [4,5]. The human genome encodes approx. 95 putative DUBs from the protease superfamily [6]. Most of these DUBs have recently been linked to biological pathways through the generation of the human DUB interactome [7]. These proteases can be classified into six subclasses, five of which correspond to cysteine proteases: USPs (ubiquitin-specific proteases), UCHs (ubiquitin C-terminal hydrolases), MJDs (Machado–Joseph domain proteases), OTUs (ovarian tumour domain-containing protease) and hUSPs (herpesvirus tegument USPs), with M48USP as the recent founding member of this structurally different subclass. The JAMM (JAB1/MPN/MOV34 metalloenzyme) motif proteases correspond to the last subclass and are Zn2+-containing metalloproteases.

USP and UCH are the best characterized enzymes of the DUB family. UCH proteins are principally involved in the processing and recycling of ubiquitin, but their specific functions remain poorly understood. USP proteins constitute the largest family, with more than 60 members. The amino acid sequences of these proteins display a lower level of conservation than those of UCH enzymes. The short well-conserved cysteine and histidine boxes, containing amino acids essential for catalytic activity, are separated by unrelated sequences of 300–800 amino acids in length. Many domains, including ubiquitin-binding, ubiquitin-like or zinc-finger domains, have been identified in the N-terminal and C-terminal parts of the USP catalytic domain [6]. The amino acid divergence observed in USP enzymes probably contributes to their broad substrate specificity [5]. Unlike UCH, USP enzymes appear to be specific for proteins with ubiquitin modifications. Owing to their protease activity and their involvement in several human diseases, DUBs have emerged as potential targets for pharmacological modifications of the ubiquitin regulatory machinery [8].
DUBs as druggable targets: lessons from structural and target validation approaches

Substrate-induced conformational changes in DUBs

Despite strong similarities between the active-site cysteine and histidine boxes of different DUBs, the three-dimensional structures of several DUBs have revealed striking differences in accessibility to the catalytic pocket. With the exception of CYLD (cylindromatosis) and PLpro, all resolved DUB structures suggest that the enzyme is in an unproductive conformation before binding to ubiquitin. These inactive states result from the blocking of the active site by loops (USP14 [9] and its yeast orthologue Ubp6, USP8 [10], OTU-2 [11]) or the misalignment of catalytic triads (USP7 [12], suggested for USP2 [13]). Indeed, the active site of the free USP7 core domain exists in an unproductive ‘open’ conformation (which may accommodate large substrates, such as polyubiquitin chains or ubiquitinated proteins), and substrate binding probably triggers a major change in conformation, leading to catalysis. By contrast, the active site of the viral PLpro DUB has been shown to be unobstructed and in a productive conformation in the absence of substrate [14]. CYLD has also been shown to be predominantly preconfigured for catalysis in the absence of substrate. It has been suggested that an extended β12/β13 loop of CYLD contributes to the specificity of this enzyme for Lys63-linked ubiquitin chains [15]. A Lys63-linkage-specific deubiquitination mechanism has also been reported for the AMSH [associated molecule with the SH3 (Src homology 3) domain of STAM (signal-transducing adaptor molecule)] metalloprotease, which has been crystallized in complex with an isopeptide-linked ubiquitin chain [16]. A change in the conformation of USP9 (isopeptidase T) is induced by the presence of ubiquitin in the S1 site, this occupancy being necessary for physiological activity. This requirement may account for the lack of cleavage of polyubiquitinated chains attached to a protein or a peptide [17]. Similar stringency has been observed with UCH1 proteins where additional constraints are exerted by a crossover loop lying directly over the active site and probably preventing the accommodation of highly folded domains in the site, thereby controlling UCH specificity [18].

These ‘inactive’ conformations, which are probably conserved among DUBs, illustrate the importance of substrate-induced conformational changes for enforcing substrate specificity. This structural transition from an inactive to an active conformation may control access to the active site and may be regulated so as to prevent non-specific substrate deconjugation in cells. Cofactors such as DAXX (death domain-associated protein), GMPS (USP7 activators) [19,20], G3BP (USP10 activator) [21], UAF1 (USP1, USP46 and USP12 activator) [22], mono- or di-ubiquitin (UCH-L1 and UCH-L3 inhibitors) [23] and proteasome subunits (USP14 activators) [9] are potentially interesting candidate regulators. Recently it has also been shown that post-translational modifications of DUBs with ubiquitin and ubiquitin-like molecules, such as SUMO (small ubiquitin-related modifier), modulate the catalytic deubiquitinating activity of these enzymes [24–26].

DUBs as targets for the treatment of cancers and viral infections: the case of USP7

Several DUBs have been implicated in various diseases, including neurological disorders, infectious diseases and cancer (see [8] for a review). A genome-wide RNAi (RNA interference) screen of the catalytically active human USPs in cancer-relevant cellular models and phenotypic assays was performed to identify potential USP targets in cancer [27]. The screening results were confirmed with stable cell lines displaying inducible shRNA (small-hairpin RNA) expression and in clonogenic experiments, leading to the identification of USP7/HAUSP (herpesvirus-associated ubiquitin-specific peptidase) and USP8/UBPY as promising targets in cancer. USP7 has been shown to inactivate several tumour suppressors, by degradation (p53 degradation following Mdm2 (murine double minute 2) stabilization [28,29]) or nuclear export (FOXO4 (forkhead box O4) and PTEN (phosphatase and tensin homologue deleted on chromosome 10) inactivation [30,31]). These types of regulation strongly suggest that targeting the deubiquitinating activity of USP7 with small-molecule inhibitors may result in functional tumour suppressors, with the stabilization of p53 through Mdm2 degradation and the reactivation of FOXO4 and PTEN through nuclear relocalization (Figure 1). USP7 overexpression was first reported in prostate cancer and this overexpression was directly associated with tumour aggressiveness [30]. Furthermore, the absence of USP7 in nude mice has been shown to result in significantly smaller tumour volumes [32], consistent with a role for USP7 in cancer cell proliferation.

USP7 was originally identified as a protein interacting with ICP0 (Vmw110), an HSV (herpes simplex virus) immediate-early gene inducing initiation of the viral lytic cycle [33]. ICP0 binding to USP7 leads to the activation of gene expression and viral growth. ICP0 has also been shown to be a RING (really interesting new gene)-containing E3 ligase inducing the proteasome-dependent degradation of a number of cellular proteins, including PML (promyelocytic leukaemia protein) and p53. Recent studies have reported these two enzymes to have reciprocal activities, with a dominant effect of the USP7-mediated stabilization of ICP0 over the ICP0-induced degradation of USP7 during productive HSV-1 (herpes simplex virus-1) infection. These results suggest that the deubiquitination function has been subverted by the virus to protect ICP0 from auto-ubiquitination leading to degradation [34]. ICP0 has also been shown to inhibit the TLR (Toll-like receptor)-dependent inflammatory response, by inducing USP7 translocation from the nucleus to the cytoplasm, thereby playing a key role in HSV pathogenesis and persistence [35]. USP7 has also been shown to interact with another herpesvirus protein, EBNA-1 (Epstein–Barr...
nuclear antigen-1). EBNA-1 plays an important role in the replication and segregation of EBV (Epstein–Barr virus) episomes during latent infection, by binding to the viral latent origin of replication. EBNA-1 also induces the expression of other latency genes and is involved in the immortalization of host cells by EBV. USP7 binding to EBNA-1 may regulate the replicative function of EBNA-1, but has no effect on EBNA-1 stability [36].

The phenotypes associated with USP7 silencing and the links between USP7 and essential viral proteins and oncogenic pathways, such as the p53/Mdm2 and PI3K (phosphoinositide 3-kinase)/PKB (protein kinase B; also called Akt) networks, strongly suggest that targeting USP7 with small-molecule inhibitors may be potentially useful for the treatment of viral diseases and cancer.

**Small-molecule inhibitors of DUBs**

Potent irreversible inhibitors of DUBs, such as Ubal (ubiquitin aldehyde) and UbVS (ubiquitin vinyl sulfone), have been characterized [37]. Ubal has been widely used as a research tool for resolving the three-dimensional structure of DUB/Ubal complexes. These studies revealed that the conformation of the protease core changes upon inhibitor binding. The C-terminally modified vinyl sulfone derivative of ubiquitin, UbVS, has proved useful for directly visualizing active DUBs. This tool has been used to discover and characterize novel ubiquitin/ubiquitin-like proteases and to profile active DUBs in normal, virus-infected and malignant cells [38]. However, owing to their high molecular mass, nature (peptides) and their lack of specificity for the targeting of DUBs, these inhibitors are not considered as good candidates for the development of therapeutic agents.
Small-molecule inhibitors of UCH
UCH proteins belong to the DUB family and are involved principally in the removal of ubiquitin with C-terminal adducts for ubiquitin recycling [6]. The UCH-L1 protein, which is thought to be involved in Parkinson’s disease and cancer, was the first DUB to be neutralized by small-molecule inhibitors [39]. This first class of small-molecule UCH-L1 inhibitors belonged to a chemical series of isatin O-acyl oximes (compounds 1–3; Figure 2A) active against UCH-L1 with micromolar IC₅₀ values and a 30-fold preferential selectivity for UCH-L1 over UCH-L3. Several O-acyl oxime compounds have been shown to promote the proliferation of lung and neuroblastoma cell lines producing UCH-L1, consistent with an antiproliferative effect of UCH-L1 in these cells. A reversible, competitive and active site-directed mechanism was characterized for this class of compounds. More recent studies have identified another family of UCH-L1 inhibitors (compound 4; Figure 2A) with a different mechanism of action, as these compounds bind to the Michaelis complex but not to free UCH-L1 enzyme and the resulting inhibition is non-competitive [40]. This chemical series belongs to a class of 3-amino-2-oxo-7H-thieno[2,3-b]pyridin-6-one derivatives with selectivity for UCH-L1 over five other cysteine proteases tested. Small-molecule inhibitors of UCH-L3 have been identified by in silico structure-based drug design, using the crystal structure data for UCH-L3 and a virtual compound library [41]. A chemical series with similar dihydro-pyrrole skeletons was identified with IC₅₀ values for UCH-L3 of 100–150 μM (compounds 5–7; Figure 2A). These compounds are competitive inhibitors that probably bind to the active site of UCH-L3. Some isatin derivatives were also identified as selective UCH-L3 inhibitors in the UCH-L1 screen, with compound 10 being shown to have an IC₅₀ value of 0.6 μM (compounds 8–10, Figure 2A, [39]). Inhibitors of cellular DUBs (cyclpentenone prostaglandin) have been also identified, using ubiquitin-PEST and Z (benzyloxycarbonyl)-LRGG-AMC (7-amino-4-methylcoumarin) as substrates, and have been shown to induce the accumulation of polyubiquitinated proteins within cells and the apoptosis of colon cancer cells (reviewed in [37]). Cell death was found to be correlated with the inhibition of DUB activity. However, no results concerning the specific inhibition of the various members of the DUB family have been published in this study. A molecular determinant conferring activity has been identified in this class of compounds, leading to
Figure 3 | Hybrigenics pipeline focusing on the development of USP inhibitors

Drug-discovery efforts led to the recent identification of a new USP7-specific series. (A) Improvements in specificity are shown here as the inhibitory effect of selected compounds (4–6 μM) from series numbers 939, 273 and 159 on a wide-ranging panel of cysteine proteases. (B) More detailed specificity profiling of USP8-specific (HBX 90,397) and USP7-specific (HBX 28,231) compounds.

the characterization of additional inhibitors with similar activities, such as DBA (dibenzyldieneacetone), curcumin and shikoccin (NSC-32979) [42]. Prostaglandins, including Δ12-PGJ2 (prostaglandin J3) in particular, have also been shown to affect UCH-L1 and UCH-L3 deubiquitinating activity, with \( K_i \) values of 1–10 μM, to reduce the viability of neuronal cells and to induce an accumulation of ubiquitinated proteins [43]. Additional compounds holding the same pharmacophore as Δ12-PGJ2 were also shown to inhibit several DUB and deSUMOylase enzymes with IC₅₀ values of 10–45 μM [44,45]. The various UCH inhibitors described here thus provide tools for the investigation of UCH-L1 and UCH-L3 functions and may be useful in the development of drugs for treating UCH-L1- and UCH-L3-associated diseases [37,46].

Small-molecule inhibitors of USP

The PLpro protein is a papain-like protease from the coronavirus responsible for SARS (severe acute respiratory syndrome). A screening of 50080 compounds followed by synthetic optimization led to the identification of a chemical series in which the most potent compound (GRL0617, compound 13; Figure 2B) had an IC₅₀ value of 0.6 μM for PLpro [47]. This compound was shown to act through a non-covalent competitive mechanism and to be selective for PLpro (IC₅₀ > 100 μM for NL63 PLP2, UCH-L1, UCH-L3, USP7 and USP18). The X-ray structure of PLpro-GRL0617 revealed a non-classical mode of inhibition through the induction of a loop closure preventing deubiquitinating activity. Some of the members of this chemical series displayed antiviral activity in Vero cells, with EC₅₀ values ranging from 10 to 15 μM.

Advanced HTS (high-throughput screening)-compatible assays with optimized USP substrates, including various ubiquitin derivatives (ubiquitin precursor and branched ubiquitin chains), and specific, physiological substrates were developed at Hybrigenics for the screening of a chemically diverse library for activity against the USP7/HAUSP...
DUB. Primary hits were run through a lead optimization programme to improve potency, selectivity, cellular activity and pharmacokinetic profile. This pioneering programme identified two different cysteine protease inhibitor series (series 939 and 273; Figure 3A). One of our lead compounds from series 939, HBX 41,108, reversibly inhibited USP7 deubiquitinating activity, with an IC50 value in the sub-micromolar range (compound 14, Figure 2B). This optimized cyano-indenopyrazine derivative was shown to stabilize and activate p53 in a non-genotoxic manner, inhibiting cell growth and leading to p53-dependent apoptosis [48]. Two related compounds derived from series 939 (compounds 15 and 16, Figure 2B) were found to exhibit USP8 selectivity in vitro (Figures 3A and 3B). Both compounds also had antiproliferative and pro-apoptotic activities, with a submicromolar GI50 (concentration giving half-maximal growth inhibition) in various cancer cell lines. They also markedly increased total cell protein ubiquitination, recapitulating the phenotypes observed in USP8-knockdown cells.

A pharmacophoric model derived from our previous series was used to assess the role of inhibitor specificity for USP7. The positive hits that docked and had the highest scores were then assessed in our biochemical assays, leading to the recent identification of a USP7-specific series (series 159) (Figures 3A and 3B; [49]). These compounds, active in the low micromolar range only against USP7, stabilized p53 in a non-genotoxic manner, leading to cell cycle arrest through G1 block and apoptosis. A new screening programme for the identification of molecules active against USP7 deubiquitinating activity recently led to the identification of a promising two-generation USP7-specific series (series 258) that is currently under investigation.

Conclusion

One of the main problems hindering the development of cysteine protease inhibitors has been their lack of specificity. Work carried out over the last few years has led to the identification of inhibitors with selective action against various DUB targets, demonstrating the feasibility of selective targeting of DUBs. Salvesen’s group recently determined the sequence preferences of representative unrelated DUBs (OTU-2, UCH-L3, USP5 and PLpro) for the C-terminal part of ubiquitin [50]. Various DUBs were found to display tolerance for different residues in their active-site clefts, strongly suggesting that small molecules targeting the active site may inhibit the deubiquitinating activity of the DUB of interest in a selective manner. There is increasing evidence to suggest that DUB inhibition is potentially useful for the treatment of viral disease, neurological disorders and cancer treatment. Current attempts to develop specific and effective drugs targeting DUBs will facilitate subsequent investigation of the role of this class of molecular targets in normal and disease states, and will provide a structural basis for drug development.

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