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Recent Advances in the Discovery of Deubiquitinating Enzyme Inhibitors

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1. INTRODUCTION

In its entirety, the ubiquitin proteasome system (UPS) involves an intricate network of hundreds of enzymes regulating the major mechanism for degrading damaged or unrequired proteins in eukaryotic cells. More simply, it is a process whereby proteins are tagged for 'shredding' by the 26S proteasome. This is a large multi-subunit complex consisting of a 20S proteolytic core and one or two 19S regulatory particles [1]. The tag used for this process is ubiquitin, a 76 amino acid (approximately 8.5 kDa) peptide.

These ubiquitin tags can be attached as monomers or as polyubiquitin chains. The attachment is an isopeptide bond formed between the carboxy-terminal glycine of ubiquitin and the ϵ -amino group of lysine residue(s) of the substrate protein. Ubiquitin itself contains seven exposed lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) which, in addition to its N-terminus, provide eight linking options in situations where it forms part of a polyubiquitin chain. The exact connectivity of these linkages provides a more detailed ‘message on the tag’: Lys11-linked and Lys48-linked polyubiquitin chains are most commonly used to flag protein substrates for proteasomal degradation [2].

Unwanted proteins are ubiquitinated via the sequential action of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases [3]. In man, high substrate specificity is achieved through the selective utilisation of one of over 700 E3 ligases for the final ubiquitin transfer step [4]. This tagging process is kept in balance by a class of proteases which remove ubiquitin from proteins. These are the deubiquitinating enzymes (DUBs). DUB-mediated removal of ubiquitin saves tagged proteins from being degraded, but DUBs are also involved in more subtle editing of the polyubiquitin signal [5], and are necessary to cleave ubiquitin precursors and to recycle ubiquitin monomers [6].

Despite this field having been researched for over 35 years, the full complexity of the UPS continues to emerge [7,8]. Because of its central role in regulating homeostasis, it is involved in many aspects of cellular proliferation and survival. Finding compounds which modulate the UPS has therefore become an active area of research for pharmaceutical companies. It became a validated therapeutic pathway with the approval of the proteasome inhibitors bortezomib (1) and carfilzomib (2) (in 2003 and 2012, respectively) for the treatment of haematological malignancies (Figure 1) [1]. These compounds preferentially inhibit the chymotrypsin-like proteolytic sites of the 20S core. Since the approval of bortezomib, there has been much effort on targeting the protein ubiquitination and deubiquitination processes upstream of the proteasome in the search for new cancer treatments. The most clinically advanced compound to emerge so far is MLN4924 (3) which is an inhibitor of Nedd8-activating enzyme E1 subunit 1. Nedd8 is a ubiquitin-like protein. MLN4924 is currently in Phase I clinical trials for treating haematological malignancies and solid tumours [9]. In addition to cancer [10–16], the utility of DUB and E1/E2/E3 inhibitors is being researched across other therapeutic areas including neurodegeneration, haematology and infectious disease [17–20].

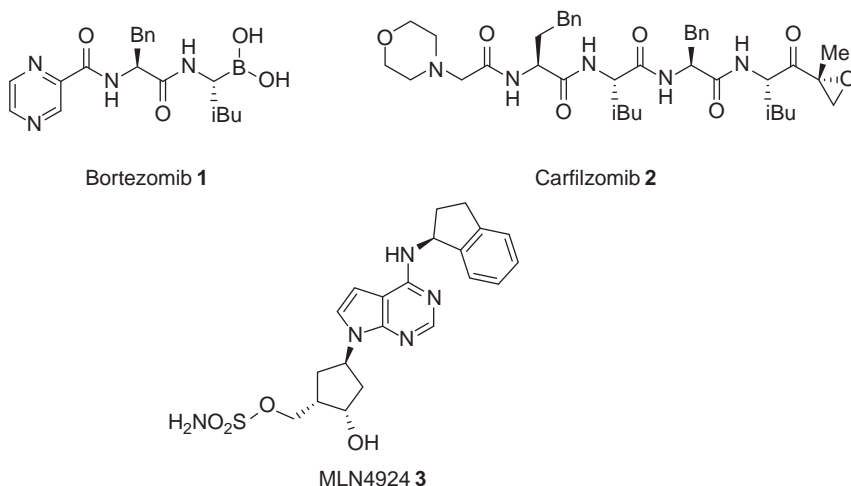


Figure 1 Structures of marketed proteasome inhibitors and MLN4924.

In 2010, Sir Philip Cohen contrasted the research activities in the fields of protein phosphorylation and protein ubiquitination [8]. The theme of this perspective was to predict whether, as happened within the kinase family, the UPS would ever become a major source of drug targets. To help inform this evolving story, in this chapter we review representative small-molecule inhibitors of a range of DUBs covering the last decade. The focus is on human DUBs, although viral DUBs are also discussed in order to illustrate specific points. General trends which emerge from this analysis are discussed in Section 5. Note the DUB inhibitor patent landscape has also been the subject of a recent review [21].

2. FUNCTION AND STRUCTURE OF DUBs

There are approximately 100 DUBs encoded in the human genome. The majority are cysteine proteases, with the remainder being zinc metalloproteases [17,22]. The cysteine protease DUBs are subdivided into four families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado–Joseph disease proteases (MJD, Josephins) and ovarian tumour proteases (OTUs). The zinc metalloprotease DUBs are known as the JAB1/MPN/Mov34 (JAMM) family.

Also shown in the phylogenetic tree in Figure 2 are the sentrin/SUMO-specific proteases (SENPs). These are isopeptidases, closely related to DUBs, which remove small ubiquitin-like modifier proteins (SUMOs). These are

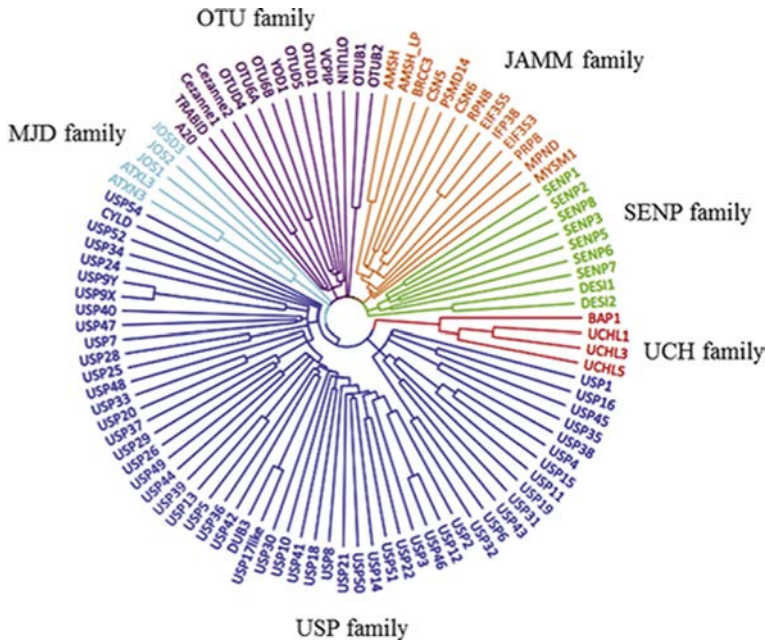


Figure 2 Human DUB and SENP phylogenetic tree.

approximately 10 kDa peptide tags similar to ubiquitin and occur in four isoforms in mammals. In an analogous manner to ubiquitin E1/E2/E3 enzymes and DUBs, the SUMO E1/E2/E3 enzymes and SENPs preserve the balance between sumoylated and unsumoylated proteins in cells. Several studies have implicated SENPs in the development of various diseases, especially prostate cancer. The disease associations and SENP inhibitor developments have recently been reviewed [23].

DUBs are isopeptidases capable of binding ubiquitinated substrates or polyubiquitin chains and then hydrolysing the amide bond between ubiquitin and (most commonly) a lysine side chain (or the N-terminal methionine in the case of linear polyubiquitin chains). Figures 3 and 4 show the reaction mechanisms for hydrolysis by zinc metalloprotease and cysteine protease DUBs, respectively.

The last two C-terminal residues of ubiquitin lack side chains (Gly75–Gly76) resulting in a narrow linker on both sides of the isopeptide bond. Consequently, DUBs are characterised by their long narrow active sites into which the Ub(1–74)–Gly75–Gly76– ϵ –Lys–substrate binds. Although the structure of DUB–substrate complexes cannot be studied without mutating catalytic residues [24], in addition to apo DUB X-ray structures, there are

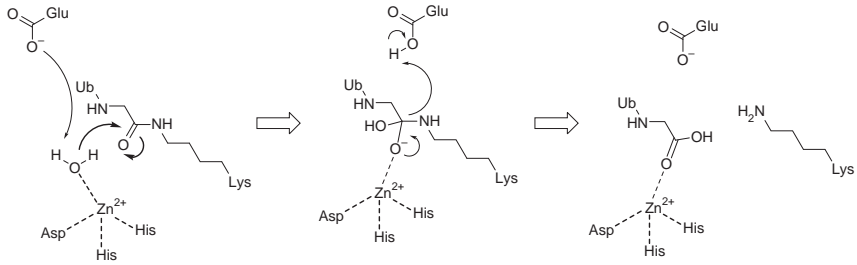


Figure 3 Isopeptide hydrolysis reaction mechanism mediated by a zinc metalloprotease DUB (Ub = ubiquitin1–75).

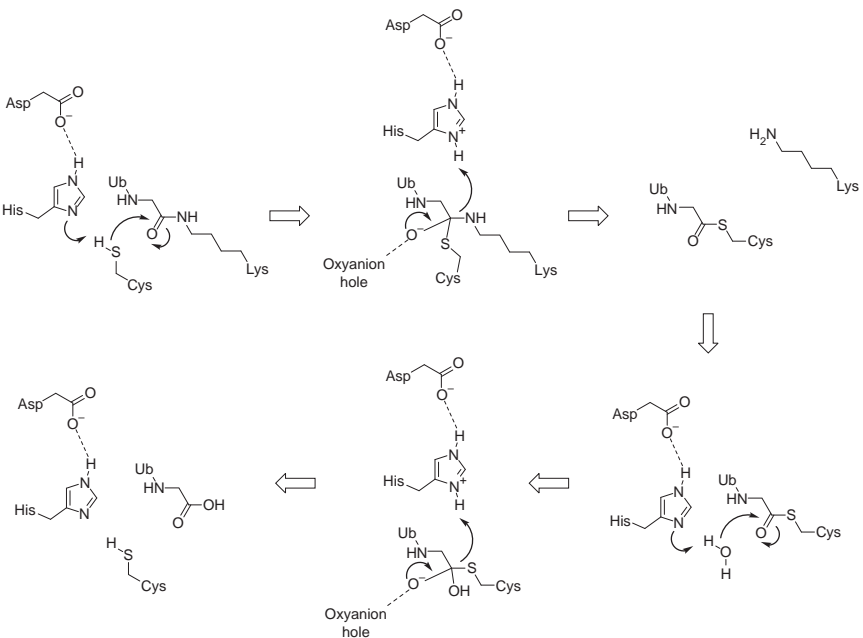


Figure 4 Isopeptide hydrolysis reaction mechanism mediated by a cysteine protease DUB (Ub = ubiquitin1–75).

several X-ray structures of DUB complexes with ‘warheaded ubiquitin’ (i.e. modified ubiquitin in which the carboxy group of Gly76 is replaced by an electrophilic moiety such as aldehyde or vinyl methyl ester (VME)) [25].

Figure 5 shows the X-ray structure of the 40 kDa catalytic domain (CD) of USP7 (also known as herpes virus-associated ubiquitin-specific protease (HAUSP)) in complex with ubiquitin aldehyde. This structure highlights the characteristic three subdomains of USP CDs that were likened by

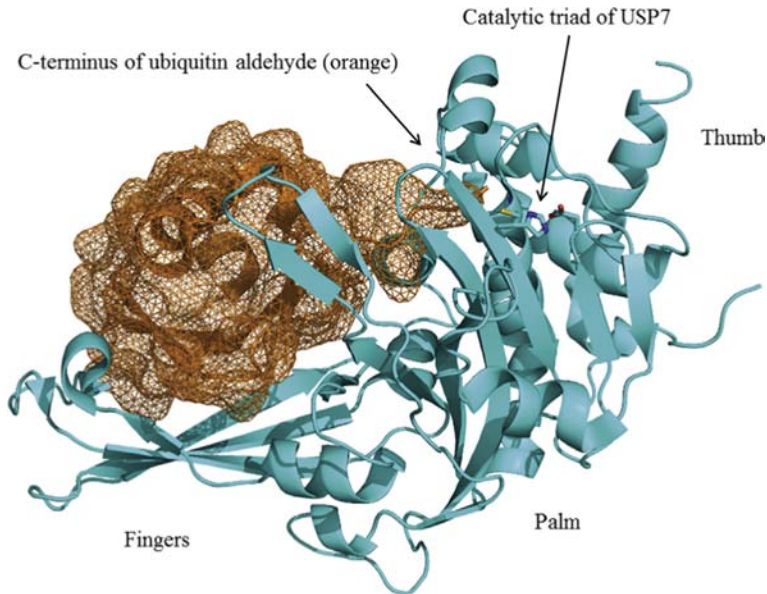


Figure 5 X-Ray structure of USP7 CD in complex with ubiquitin aldehyde (PDB: [1NBF](#)).

Hu *et al.* to the fingers, palm and thumb of a right hand [26]. The C-terminus of ubiquitin aldehyde (orange (grey in the print version)) extends into the narrow active site cleft of USP7 CD (cyan (grey in the print version)). Notably, Hu *et al.* also solved the apo structure of USP7 CD (PDB: [1NBF](#)) and found that ubiquitin binding induces a drastic conformational change of the active site that realigns and ‘activates’ the catalytic triad via movement of a ‘switching loop’ [26,27]. In the absence of ubiquitin binding, the catalytic triad adopts an inactive conformation in which the cysteine is too far away from the histidine (10.2 Å) for the hydrolysis mechanism described in [Figure 4](#) to occur.

Ubiquitin-mediated activation of the catalytic mechanism has been elucidated for several other DUBs, and is especially clear in the case of one of the smallest family members UCHL1 (24.8 kDa). The X-ray structure of apo UCHL1 was first solved by Das *et al.* in 2006 [28]. The active site was found to resemble that of a typical cysteine protease such as papain [29], except that His161 is 7.7 Å away from the catalytic cysteine (Cys90) resulting in a structure that looks incapable of being catalytically active (PDB: [2ETL](#)). In this paper, the team speculated that a substrate-induced rearrangement would be required in order to activate this protease. Four years later, the same team solved the X-ray structure of UCHL1 bound

to ubiquitin VME [30]. This structure revealed that an amino-terminal β -hairpin of ubiquitin pushes into a distal site of UCHL1. This triggers a domino effect, via two phenylalanine side chains, which results in His161 being pushed within 4 Å of Cys90 and thereby activating UCHL1 to react with the VME warhead. Figure 6 shows the resultant structure (PDB: 4KW5) in the same orientation as Figure 5, with ubiquitin (orange (grey in the print version)) and UCHL1 (purple (dark grey in the print version)). The β -hairpin of ubiquitin is highlighted, as is the important 'substrate-filtering' crossover loop of UCHL1 which is a characteristic of the entire UCH family [31].

The domino effect is illustrated in Figure 7 which superimposes the relevant parts of the 2ETL (yellow (light grey in the print version)) and 3KW5 (purple (dark grey in the print version)) structures. Presumably, the push of the β -hairpin of ubiquitin (green (grey in the print version)) causes the side chain of Phe214 to swivel by approximately 180°, which in turn does the same to the side chain of Phe53. This completes the cascade by pushing the basic imidazole ring of His161 nearly 4 Å closer to Cys90.

A ubiquitin-mediated catalytic triad rearrangement is not required for all DUBs. For example, the X-ray structures of apo USP8 and USP14 reveal that their catalytic triads are organised in an active state. However, in both cases, the proteases are auto-inhibited by loops adjacent to the active site folding into the cleft [32,33].

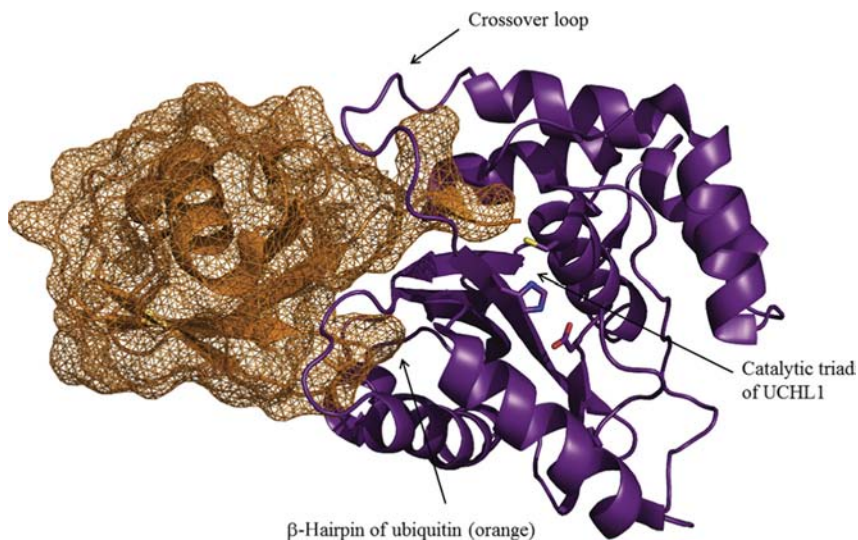


Figure 6 X-Ray structure of UCHL1 in complex with ubiquitin VME (PDB: 3KW5).

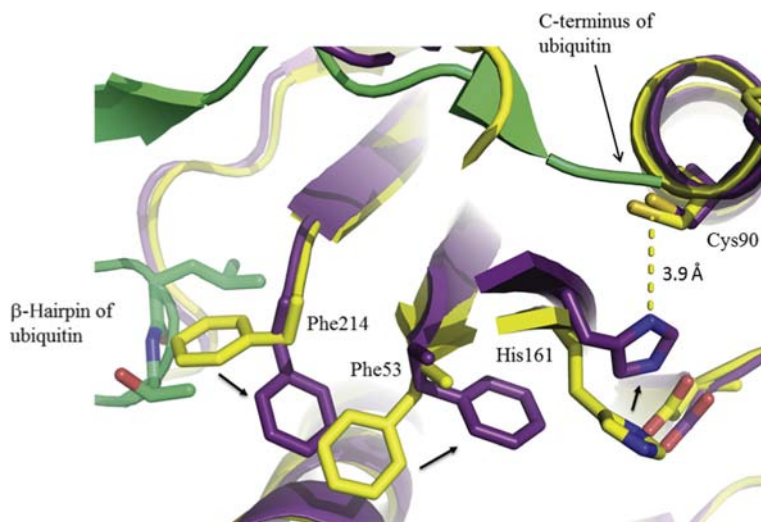


Figure 7 An overlay of X-ray structures of apo UCHL1 (2ETL, yellow (light grey in the print version)) and UCHL1 bound to ubiquitin VME (3KW5, enzyme is purple (dark grey in the print version) and ubiquitin VME is green (grey in the print version)).

An X-ray structure indicates that the USP4 CD likewise has a catalytically competent triad in its apo form, but this time it is allosterically regulated via one of its ubiquitin-like (Ubl) domains [34,35]. Ubl domains are very common within the USP family [36,37]. They are stretches of 45–80 amino acids which share the β -grasp fold of ubiquitin, and can be located either N-terminally, within or C-terminally to the CD. Structural information on how Ubls and additional domains of DUBs interact continues to emerge [38]. This is demonstrated by recent publications on the first characterisation of USP11 domain architecture and the discovery of a new binding site on USP7 (via the second of its five Ubls) [39,40]. The presence of additional domains, as well as cofactors for a few USPs [41,42], in theory provides multiple allosteric options for DUB inhibition. It also results in DUB family members varying greatly in domain architecture and size (ranging from 188 to >2000 amino acids). This ‘cornucopia’ is elegantly analysed and categorised by Komander *et al.* [6].



3. ASSAYS TO IDENTIFY DUB INHIBITORS

Primary assays for screening compound collections against isolated DUBs have evolved rapidly within the past decade. Initially, the most

commonly used substrate was ubiquitin coupled via its carboxy-terminus onto the amino group of the fluorescent tag 7-amino-4-methylcoumarin (Ub-AMC). Fluorescence polarisation detection could then be utilised to test whether compounds were capable of blocking the DUB-mediated cleavage of Ub-AMC to AMC. Although simple, this relatively old technology is prone to picking up fluorescent artefacts [43]. More importantly, Ub-AMC does a poor job of mimicking the natural DUB substrates, since the flat bicyclic AMC group is very different from the narrow side chain of a lysine. Alternative tags which are less prone to fluorescent artefacts were subsequently incorporated (rhodamine/Rho 110 and tetramethylrhodamine/TAMRA), but the resultant Ub-Rho and Ub-TAMRA substrates are no closer to genuine DUB substrates than Ub-AMC.

A significant improvement has been to use substrates in which a lysine residue (linked via the ϵ -amino group) is inserted between ubiquitin and the tag [44–47]. Another technique, known as Ub-CHOP, utilises a reporter enzyme (PLA₂) conjugated onto ubiquitin [48]. PLA₂ is inactive in this conjugated form but becomes catalytically active when the Ub-PLA₂ bond is hydrolysed. Activated free PLA₂ then proceeds to cleave its fluorescent-tagged substrate.

A complication in all isolated cysteine protease DUB assays is the requirement for adding reducing agents such as dithiothreitol (DTT) to prevent cysteine oxidation and to keep the enzymes viable. Researchers at AstraZeneca showed that changing either the concentration or the nature of the reducing agent had a considerable effect on the resultant IC₅₀ values for inhibitors [49]. Hence, experimental conditions need to be considered when comparing potencies of inhibitors between different publications.

More recently, for screening DUBs which can utilise diubiquitin as their substrate, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) has been utilised as the detection technique to identify inhibitors [50]. This has the advantage of using an unmodified substrate. As described later, wide DUB profiling with this technique has led to a re-evaluation of the selectivity of some literature DUB inhibitors.

Activity probe assays have also recently been optimised for DUBs. These measure an inhibitor's ability to prevent a DUB reacting irreversibly with appropriately 'warheaded' ubiquitin or polyubiquitin. These have now been optimised from low-throughput Western blot assays to much higher throughput versions. Potential inhibitors can be tested in whole-

cell and tissue systems (followed by lysing and competing with the non-cell-penetrant activity probes) which is more physiologically relevant than an isolated DUB in solution with an arbitrary reducing agent [46,47,51–53].

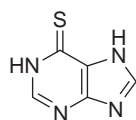


4. DUB INHIBITORS BY TARGET

4.1 Viral DUBs

Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are two of the six known human coronaviruses. Both are highly pathogenic with the potential for human to human transmission [54]. Both contain papain-like cysteine proteases termed SARS-CoV PLpro and MERS-CoV PLpro, respectively. In addition to processing viral polyprotein, these proteases function as DUBs (and also as deISGylating enzymes) removing ubiquitin and ISG15 (another ubiquitin-like peptide) from host cell proteins, resulting in antagonism of the host antiviral immune response [55]. Hence, both SARS-CoV PLpro and MERS-CoV PLpro have been proposed as important antiviral targets.

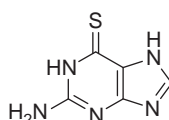
The X-ray structures of both proteases have been solved and found to have most similarity with the USP family of DUBs [25,56–58]. SARS-CoV PLpro has received more attention, with several groups identifying inhibitors. Chou *et al.* discovered that the immunosuppressive thiopurine drugs 6-mercaptopurine (6MP **4**) and 6-thioguanine (6TG **5**) (Figure 8) are weak but ligand-efficient inhibitors of SARS-CoV PLpro [59]. The same group has recently found that the compounds have similar potency against MERS-CoV PLpro [60]. In both cases, the group used



6MP **4**

SARS-CoV PLpro IC₅₀ 21.6 μM

MERS-CoV PLpro IC₅₀ 26.9 μM



6TG **5**

SARS-CoV PLpro IC₅₀ 5.0 μM

MERS-CoV PLpro IC₅₀ 24.4 μM

Figure 8 Structures of thiopurine inhibitors of SARS-CoV PLpro and MERS-CoV PLpro.

computational docking studies to predict similar binding modes to a common cavity near the catalytic triad of the two PLpro enzymes.

The Mesecar and Ghosh group identified low μM inhibitors of SARS-CoV PLpro by high-throughput screening of approximately 50,000 compounds with RLRGG-AMC (the 'C-terminal 5-mer' of Ub-AMC) as the substrate [56]. Compound optimisation resulted in GRL0617 (**6**) which has an IC_{50} of 0.6 μM against SARS-CoV PLpro and has an EC_{50} of 15 μM in an antiviral cell-based assay (Figure 9). An X-ray structure of **6** in SARS-CoV PLpro showed that it binds within the S4 and S3 subsites of the protease (PDB: 3E9S). These equate to the regions in which the side chains of Lys73-Arg74 of ubiquitin bind. Over 6 years, the same team reported in a series of publications [54,61–63] compounds from related series such as **7** which retain the naphthyl group and bind to the same S4/S3 subsites of SARS-CoV PLpro (PDB: 3MJ5). Their most recent publication shows how a further twofold potency improvement was achieved by changing the benzodioxolane for a 3-fluoro phenyl to give compound **8** (IC_{50} 0.15 μM , antiviral EC_{50} 5.4 μM) [54].

The X-ray structure of **8** (purple (dark grey in the print version)) in SARS-CoV PLpro (green (grey in the print version)) is shown in Figure 10 with the same viewing orientation as used for Figures 5 and 6 (PDB: 4OW0). Note that the thiol of the active site Cys112 has become oxidised to a sulfonic acid. This structure shows how compound **8** spans

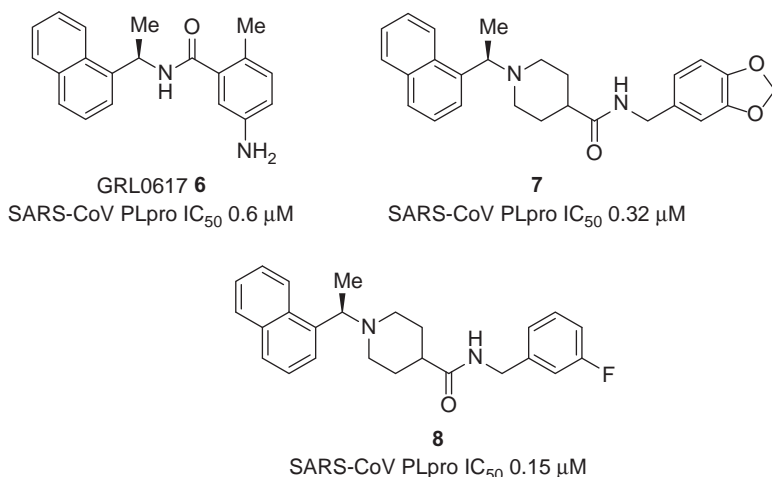


Figure 9 Structures of SARS-CoV PLpro inhibitors from the Mesecar and Ghosh group.

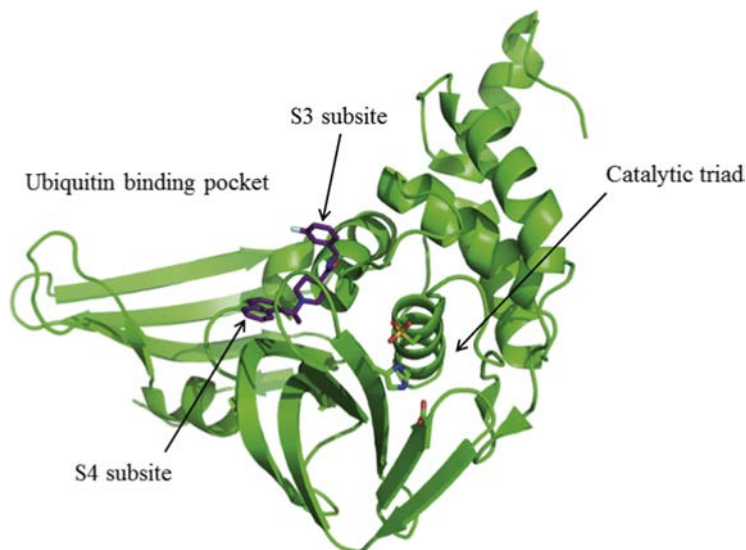


Figure 10 X-Ray structure of compound **8** in SARS-CoV PLpro (PDB: 4OW0).

the S4 and S3 subsites. Although these sites are appreciably different in USPs [25], consistent with the more than 100-fold selectivity of series representatives over USP2/7/8/20/21 [54], it is notable that the binding site of compound **8** (and also of compounds **6** and **7**) is in approximately the same region as the distal binding site of the UCHL1/Ub-VME complex (Figure 6), and also the ligand-binding site described later for USP14.

Although progress in SARS-CoV PLpro inhibitor optimisation has been slow, it is nonetheless encouraging to see inhibitors such as **8**, relatively potent and devoid of toxicophores [64], binding to a protease that has structural similarities with human DUBs. With high lipophilicity and free benzylic positions, compound **8** is, not surprisingly, unstable in mouse liver microsomes with a $T_{1/2}$ of 2.8 min. However, more polar analogues do demonstrate that significant stability improvements can be made without sacrificing too much potency for SARS-CoV PLpro [54].

4.2 Non-selective DUB Inhibitors

The bithiocyanate PR-619 (**9**) (Figure 11) was first described by Altun *et al.* as a broad panel DUB inhibitor in a study that used the compound to inhibit a wide range of DUBs in a cellular activity probe assay [65]. Interestingly, although PR-619 was found to inhibit DUBs from all the families sampled (USP/UCH/MJD as well as ‘PLpro core’) with IC_{50} s around 10 μ M, it was

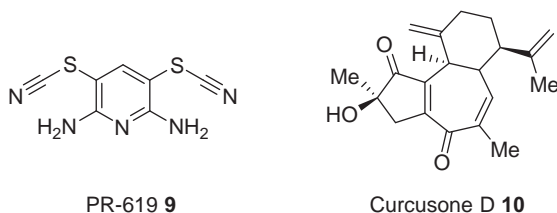


Figure 11 Structures of non-selective DUB inhibitors.

more than fivefold weaker against representative non-DUB cysteine proteases Calpain1 and Cathepsin B. The mode of inhibition has not been described. At *MISSION* Therapeutics, we likewise found that PR-619 acts as a good positive control in most of the more than 30 DUB assays that we run in-house. The recent study on the DUB selectivity of literature inhibitors by MALDI-TOF MS agreed that PR-619 strongly inhibits all DUBs tested (covering USP/UCH/MJD/OTU families) at 5 μ M.

The diterpene curcusone D (**10**) has been described as a broad inhibitor of USPs (with a significant effect on USP5/7/8/14/15/22 in cells at 10 μ M) through a reactive oxygen species, but surprisingly UCHL1 and UCHL3 were unaffected [66].

4.3 Ubiquitin-Specific Proteases

4.3.1 USP1

USP1 has become an anticancer target because of its role in regulating DNA damage response pathways [13,67]. It is one of the DUBs that requires an interacting partner protein for full activation. In the case of USP1, its cofactor is USP1-associated factor 1 (UAF1). High-throughput screening of some nine and a half thousand bioactive compounds with the USP1/UAF1 complex using a Ub-Rho substrate by the Zhuang group identified several low μ M hits [68]. The most potent was the antipsychotic drug pimozide (**11**) (Figure 12). Further screening revealed pimozide to be more than 50-fold selective over other USPs, including USP46/UAF1 which shares the same cofactor. The compound was found to be ‘largely reversible’, not binding at the active site and not acting via USP1/UAF1 complex disruption. However, no further optimisation of this hit has been described. More recent DUB panel profiling of pimozide using the diubiquitin/MALDI-TOF MS technique suggests that the compound is less selective than previously thought, inhibiting many other DUBs with a similar affinity to USP1 [50].

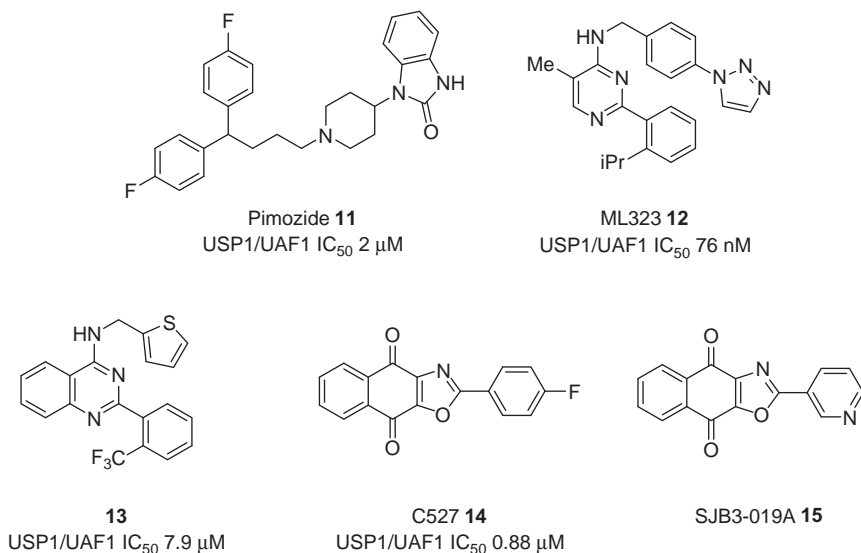


Figure 12 Structures of USP1/UAF1 inhibitors.

The Zhuang group has maintained an interest in finding additional USP1/UAF1 inhibitors and has recently published extensive structure–activity relationships (SARs) for a far more potent pyrimidine-cored compound ML323 (**12**) [69–71]. This compound was the result of optimisation efforts around a weaker quinazoline hit (**13**). Some regions of **13** were found to be alterable without significantly affecting potency, but the ortho-substituent on the pendant phenyl ring was found to be essential. While it was possible to replace the trifluoromethyl group with a variety of substituents, moving it to either the *meta* or *para* positions led to complete loss of USP1/UAF1 activity. More radical changes to the core resulted in the identification of ML323.

By making structural changes to all parts of ML323, the Zhuang group showed that SAR was additive and that the isopropyl group was crucial. Changing this lipophilic group for a more polar ether, alcohol or ketone resulted in a more than 30-fold loss in USP1/UAF1 potency. ML323 is highly selective, showing no effect at 20 μM against 18 DUBs, 70 unrelated proteases and 451 kinases. In the Cerep ‘Lead Profiling Screen 2’, ML323 hit only 7 of the 80 targets.

However, despite the compound’s sub-100 nM USP1/UAF1 IC₅₀, its EC₅₀ in an H1299 cell survival assay was 3.0 μM. This is unlikely to be due to poor cell permeability since the reported Caco-2 permeability of

ML323 is high (P_{app} 23×10^{-6} cm/s with an efflux ratio of 0.9). ML323 was found, however, to sensitise non-small cell lung cancer H596 cells to cisplatin [69]. The team reported 'limited success' at improving metabolic stability in the series. ML323 has a human liver microsome $T_{1/2}$ of 26 min and a rat liver microsome $T_{1/2}$ of 15 min. This is consistent with the high clearance found in rat (>70 ml/min/kg).

In 2013, the D'Andrea group reported the screening of 150,000 compounds in a Ub-Rho USP1/UAF1 assay using DTT as the reducing agent, and identified C527 (**14**) as a sub- μ M inhibitor [72,73]. However, this quinone-containing compound was found to have negligible selectivity over USP5 and UCHL3. Diubiquitin/MALDI-TOF MS DUB profiling also showed that a close analogue of C527 (SJB3-019A **15**) inhibited approximately half of all DUBs screened at 3 μ M. It is likely that **14** and **15** are redox cycling compounds (RCCs) which inhibit USP1 and other DUBs through oxidation of the catalytic cysteine. This has been reported for several structural motifs including quinones, and is especially pronounced in the presence of DTT [74]. This is not an attractive mechanism for protease inhibition since RCCs have promiscuous activity. The Brik group has also uncovered similar quinone-containing RCCs in their screens against USP2. In this case, they were able to confirm oxidation as the mechanism of protease inhibition through MS analysis of USP2 pre- and post-treatment with the compounds. An increase in molecular weight by 32 Da confirmed an oxidation, presumably of the catalytic cysteine side chain from the thiol RSH to the sulfinic acid RSO₂H [75].

4.3.2 USP2

In a 2010 patent publication, Novartis described a small set of 2-cyanopyrimidines including **16** (Figure 13) as USP2 and/or UCHL3 inhibitors for the treatment of proliferative diseases such as cancer [76]. Unfortunately,

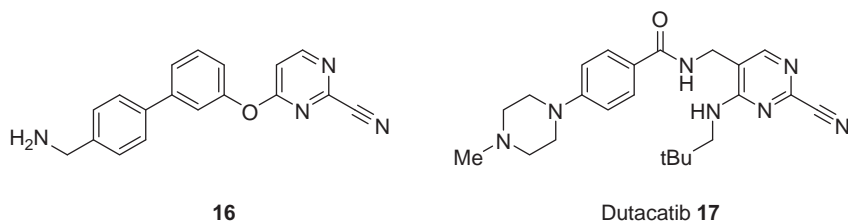


Figure 13 Structures of a representative Novartis USP2 inhibitor and the cathepsin K inhibitor dutacatib.

no potencies are quoted. This series is probably ‘drug-like’ since the 2-cyanopyrimidine warhead, which is known to form reversible covalent bonds with active site cysteine residues [77], has clinical precedent from the Novartis cathepsin K inhibitor dutacatib (AFG495) **17** which reached a Phase I osteoporosis trial [78].

4.3.3 USP7

The design of USP7 (HAUSP) inhibitors has been the subject of more publications over the past 8 years than that of any other DUB. In addition to AstraZeneca’s screening paper [49], Hybrigenics, Progenra, Almac Discovery and Genentech have all published details of their searches for USP7 inhibitors. This is because USP7 has been shown to indirectly regulate the tumour suppressor p53 by deubiquitinating, and hence stabilising, the polyubiquitinated form of the oncoprotein Mdm2. Inhibition of USP7 is expected to increase p53 levels, leading to anti-tumour activity [79,80].

Hybrigenics screened 65,000 compounds using a Ub-AMC assay and identified HBX41108 (**18**) (Figure 14) as a USP7 inhibitor with an IC_{50} of 0.42 μ M [81,82]. The compound was shown to be reversible and not to compete with iodoacetamide, a thiol-alkylating agent, indicating that HBX41108 does not protect the active site cysteine. In 2010, a paper by the same group showed that HBX41108 was more potent against USP8 (96 nM) [83]. This paper also showed that the electron-deficient 2,3-dicyano-pyrazine ring of HBX41108 is highly electrophilic, since dimethylamine displaces the nitrile group *para* to the ketone at room temperature. However, nitrile displacement is unlikely to be involved in the compound’s binding to USP7, since this would be expected to be an irreversible step, inconsistent with the findings of the initial Hybrigenics paper. Nonetheless, this reactivity could explain why HBX41108 was found by

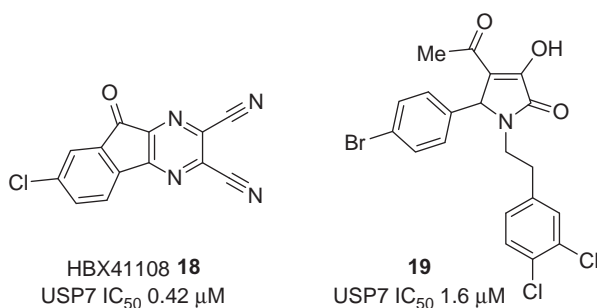


Figure 14 Structures of early USP7 inhibitors from Hybrigenics.

diubiquitin/MALDI-TOF MS DUB profiling to significantly inhibit half the DUBs tested at 1 μM , and all DUBs except the JAMMs at 5 μM [50]. Also in 2010, a Hybrigenics patent was published claiming compounds such as **19** as low μM inhibitors of USP7 [84]. All examples had IC_{50} s of more than 200 μM against USP5, USP8, UCHL1 and UCHL3.

P5091 (**20**) and P22077 (**21**) (Figure 15) from Progenra's thiophene series of USP7 inhibitors were first described in the same paper that used an activity probe assay to show that PR-619 (**9**) is a pan-DUB inhibitor [65,85]. By contrast, at 10 μM P22077 was found to inhibit only USP7 and USP47 appreciably in this cell-based assay. Subsequent optimisation led to compound **22**, which as well as being more potent for USP7 and USP47, is significantly less reactive with glutathione and more stable in plasma [86]. Both of these factors are important for reducing clearance. Although some toxicophores remain in **22** [64], at least the nitro group was found to be non-essential. The nitro to cyano functional group swap was the main factor in markedly improving plasma stability. Nitro groups in similar compounds have been shown to cause unwanted irreversible covalent binding to proteins via a nucleophilic aromatic substitution reaction [87].

Despite the weak μM level potencies of P5091 and P22077 against USP7, both have been utilised *in vivo*. P5091 was administered by intravenous (i.v.) injection at 10 mg/kg twice weekly for 3 weeks to human MM1S multiple myeloma tumour-bearing mice [88]. The compound was found to inhibit tumour growth and prolong survival. P22077 was administered by intraperitoneal (i.p.) injection at 15 mg/kg daily for 3 weeks in an orthotopic neuroblastoma mouse model and showed significant inhibition of xenograft growth [89].

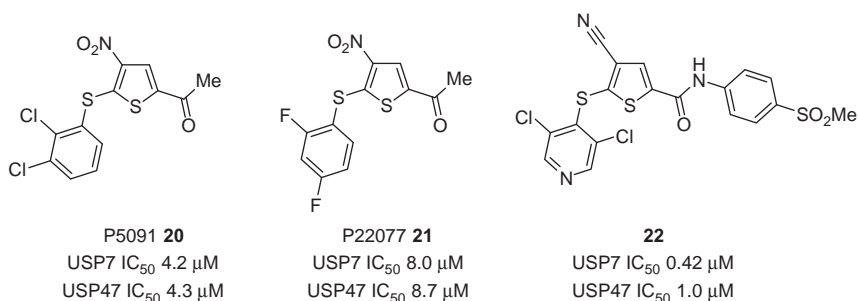


Figure 15 Structures of USP7 inhibitors from Progenra.

Interestingly, P22077 was much more selective than the Hybrigenics compound HBX41108 in the diubiquitin/MALDI-TOF MS DUB profiling [50]. P22077 inhibited only USP7, USP9x, USP10 and USP20 appreciably at 1 μM (USP47 was not tested).

In the past 3 years, Hybrigenics and Progenra have described several additional series of USP7 inhibitors (Figure 16). All are weak compared to the thiophene series. By screening a natural product library of marine invertebrate extracts against USP7/Ub-Rho, researchers at Progenra found that the pyrrole alkaloid spongiacidin C (**23**) is an inhibitor with an IC_{50} of 3.8 μM [90]. No binding mechanism is proposed, but **23** does exhibit some selectivity over the small panel tested (USP21CD 16.6 μM , USP2CD and USP8CD >30 μM). HBX19818 (**24**) was discovered by Hybrigenics from a high-throughput USP7/Ub-AMC screen [91,92]. This 4-chloro-pyridine is a weak USP7 inhibitor (28 μM) which binds irreversibly by nucleophilic aromatic substitution onto the electron-deficient heterocycle with consequent loss of the chlorine. MS/trypsin digest studies confirmed this mechanism and interestingly showed that **24** reacted almost entirely with the active site cysteine (Cys223) in preference to other solvent-exposed cysteine residues. This shows that **24** is not an indiscriminate arylating agent and is consistent with the selectivity of the compound (negligible activity at 100 μM against USP2/5/8/20, UCHL1, UCHL3 and SENP1). In a more

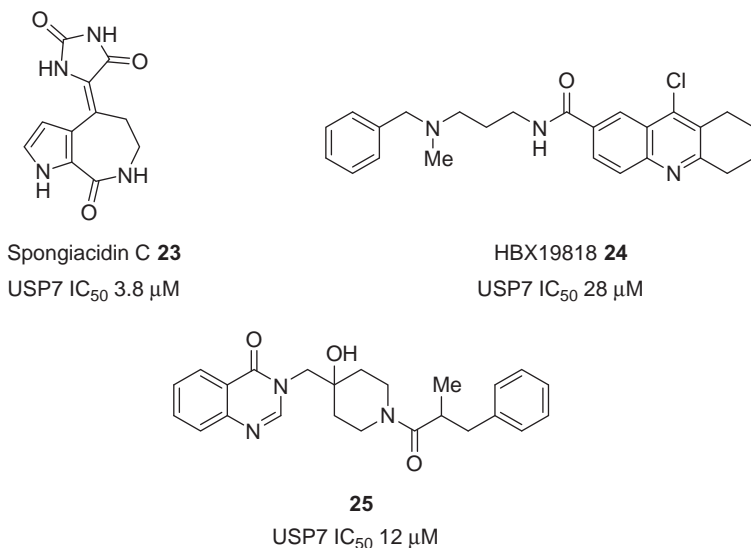


Figure 16 Structures of recent USP7 inhibitors.

recent Hybrigenics patent, a series of 4-hydroxy piperidines are described as weak non-covalent USP7 inhibitors [93]. The most potent is the racemic compound **25** (USP7 IC_{50} = 12 μ M, USP8 IC_{50} > 200 μ M).

In a poster presented in 2014, researchers from Almac Discovery described how they applied a USP focussed fragment screening campaign to six USPs and discovered optimisable hits for USP7 [94]. A fragment set of 2000 compounds was screened at 200 μ M using surface plasmon resonance (SPR) as the detection technique. This gave 34 primary hits which were validated with an orthogonal protein nuclear magnetic resonance (NMR) study. One of these hits (ADC-01) was relatively potent (K_d = 2.2 μ M by SPR and IC_{50} = 12 μ M in a USP7 biochemical assay). This compound was optimised with the assistance of ligand–enzyme complex X-ray structures to a much more potent example ADC-03 (45 nM). Although no structures were shown, ADC-03 was described as being non-covalent, extremely selective (IC_{50} > 100 μ M against 38 other DUBs, including the closely related DUB USP47 which the Progenra compounds do not discriminate over), clean in redox/aggregation assays and very stable to both human plasma and glutathione ($T_{1/2}$ > 6 h). The compound works in a whole-cell HCT116 activity probe assay at around 1 μ M, is non-genotoxic and demonstrates p53 stabilisation. No microsomal, hepatocyte or *in vivo* data were included.

Genentech has also screened fragments against USP7, this time using NMR [95]. Although little detail is known, some fragments were found to bind to a unique site on the palm portion of USP7 some distance from the catalytic triad. This could be consistent with our findings for USP14 (see later).

4.3.4 USP8

In addition to compound **18** (USP8 IC_{50} = 96 nM), Hybrigenics have described several related fused tri- and tetra-cyclic series as USP8 inhibitors for treating a wide range of diseases [83,96,97]. Interestingly, the oxime derivative **26** retained the sub- μ M USP8 potency of **18** but was inactive (IC_{50} > 100 μ M) against USP7. The oxime group is responsible for this selectivity, since the parent ketone **27** has low μ M USP7 potency (Figure 17). Another sub- μ M USP8 oxime **28** was profiled more widely and found to have an IC_{50} of more than 100 μ M against USP5, USP7, UCHL1 and SENP1. Byan *et al.* reported efficacy after administering compound **26** by i.p. injection at 0.2 and 1 mg/kg for 5 days per week in a non-small cell lung cancer mouse xenograft model [98].

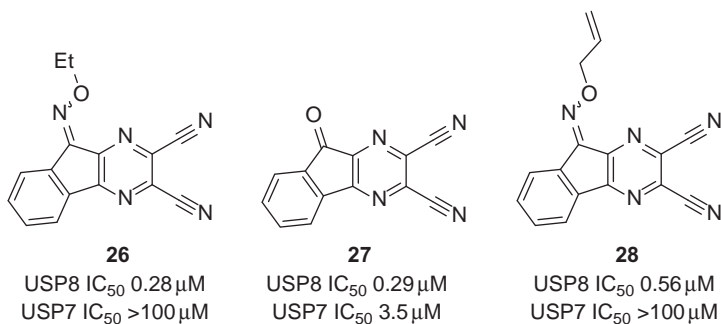


Figure 17 Structures of USP8 inhibitors from Hybrigenics.

In 2014, Kathman *et al.* described how they synthesised a 100-member library of amidomethyl methyl acrylates and screened them against four proteases using an MS assay [99]. They identified compound **29** as a binding hit (Figure 18) albeit extremely weak (30% labelling of USP8 at 100 μM). This is interesting since at least the Michael acceptor group, which may be acting as a covalent warhead, has some clinical precedent from other cysteine proteases. Rupintrivir (**30**), an inhibitor of rhinovirus 3C protease, was progressed to Phase II trials by Agouron (now Pfizer) [100]. In addition, GSK recently reported some Phase I data on another structurally related compound which is a covalent irreversible cathepsin C inhibitor GSK'660 (**31**) [101].

4.3.5 USP9x

WP1130 (**32**) (Figure 19) was initially identified by the Donato group from a cell-based Janus kinase 2 (Jak2) inhibition screen seeking improved Jak2 inhibitors as anti-tumour agents. However, the compound was discovered not to be a direct Jak2 inhibitor, but instead an inhibitor of DUB(s) capable of modulating Jak2 ubiquitination, trafficking and signal transduction [102]. The same group showed, using an activity probe cell-based assay, that WP1130 inhibits USP5, USP9x and the 19S regulatory particle-associated cysteine DUBs USP14 and UCHL5 (UCH37) with an IC₅₀ of between 5 and 10 μM [103,104]. It also inhibits USP5 and USP9x (as well as UCHL1) in isolated DUB assays using Ub-AMC (>80% inhibition at 5 μM). The Donato group used WP1130 (30 mg/kg, oral daily to infected mice) to demonstrate that it has anti-murine norovirus (MNV) activity [105]. They attributed this to the compound's USP14 activity since this DUB is required for optimal norovirus infection. Another group showed

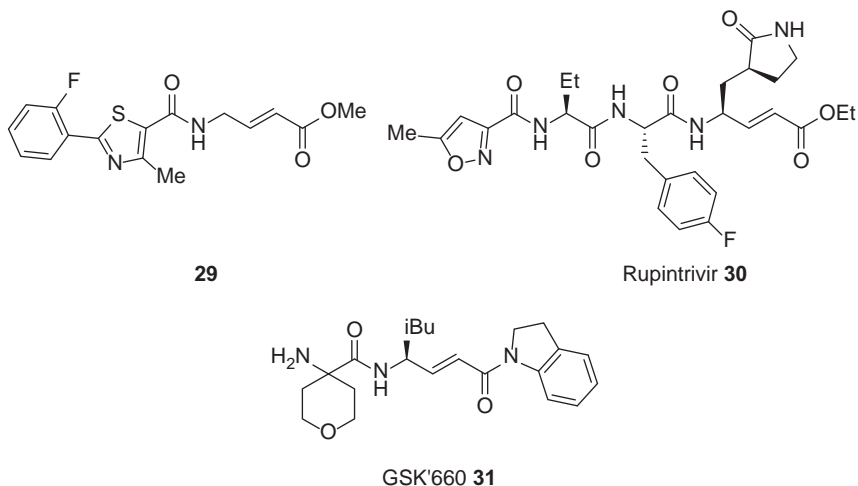


Figure 18 Structures of a USP8 binder and two inhibitors of non-DUB cysteine proteases which advanced to clinical trials.

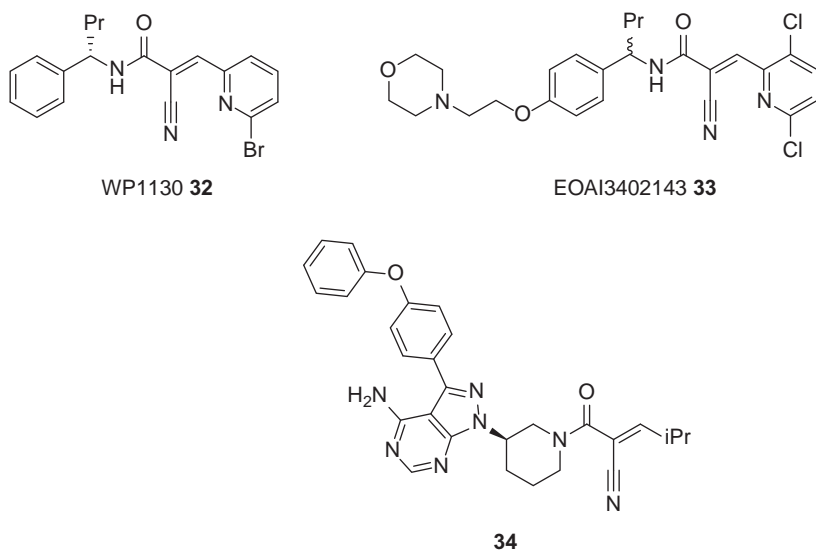


Figure 19 Structures of two compounds which inhibit USP9x (and other DUBs) and a structurally related BTK inhibitor.

that WP1130 depleted E-twenty-six-related gene (ERG) and retarded prostate tumour growth, this time through its USP9x inhibitory activity, by administering it by i.p. injection (40 mg/kg) on alternate days to xenografted mice [106].

A proposed mechanism for WP1130's DUB inhibition has recently been proposed, again by the Donato group [107]. MS work using USP9x shows that the compound binds covalently, but the adduct is lost when the protein–ligand complex is diluted and heated, indicating reversibility, presumably via a retro Michael addition reaction. Using UV absorbance of WP1130, the group demonstrated that the compound bound preferentially to the cysteine residues located within the zinc-finger structural element. When WP1130 was analysed by the diubiquitin/MALDI-TOF MS technique, it was found to inhibit USP9x only modestly at 1 μM . It inhibited more than 10 other DUBs more strongly [50]. Although discrepant from earlier claims of WP1130 being 'partially selective', this wider profiling result would tie in with the fact that approximately half of all DUB's CDs are predicted to have a zinc finger [107].

In 2015, the Donato group described EOAI3402143 (**33**) as a slowly reversible USP9x inhibitor ($\text{IC}_{50} = 1.6 \mu\text{M}$ against USP9x CD, cf. 4.8 μM for WP1130) with an improved aqueous solubility (80 μM as the HCl salt, cf. only 2.3 μM for WP1130) [108,109]. Using an activity probe assay, the group also showed that **33** inhibits USP24, a DUB closely related to USP9x, as well as other DUBs including USP5. Intraperitoneal dosing of MM1S tumour-bearing mice with **33** (2.5–10 mg/kg daily for 2 weeks) revealed that the compound significantly suppressed tumour growth at doses above 5 mg/kg. Despite the modest mouse pharmacokinetic $T_{1/2}$ of 45 min, **33** was shown to sustain inhibition of USP9x *in vivo* for up to 24 h. The authors propose that the covalent nature of **33** enables this dissociation of pharmacokinetics from pharmacodynamics.

In terms of 'drug-likeness', the Michael acceptor group of compounds **32** and **33** is similar to that used by Principia Biopharma for its Bruton's tyrosine kinase (BTK) inhibitors, which achieve prolonged on-target residence times by covalently latching onto a non-catalytic cysteine. Compound **34** is a representative example [110]. Principia Biopharma have recently announced completion of a Phase I clinical trial with their covalent reversible prolonged action BTK inhibitor PRN1008 [111]. Although the exact identity of PRN1008 is unknown, it is likely to be related to compound **34**.

4.3.6 USP10 and USP13

Liu *et al.* used a Ub-AMC assay to characterise spautin-1 (**35**) (Figure 20) as an inhibitor of USP10 and USP13 with IC_{50} values of 0.6–0.7 μM , and with no activity against another USP, CYLD [112].

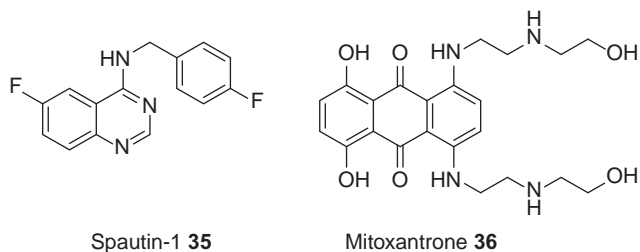


Figure 20 Structures inhibitors of USP10/13 and USP11.

4.3.7 USP11

A high-throughput USP11/Ub-AMC screen of 2000 Food and Drug Administration (FDA)-approved compounds was run to identify inhibitors of this DUB, which plays a key role in DNA double-strand break repair [113]. The most potent hit was the type II topoisomerase inhibitor mitoxantrone **36** (USP11 IC_{50} = 3.15 μ M) (Figure 20). Interestingly, although its structure looks like a potential RCC [74], **36** was found to be active in the USP11 assay even when the reducing agent was switched from DTT to cysteine.

4.3.8 USP14 (and UCHL5)

The 19S regulatory particle of the 26S proteasome contains three DUBs: the metalloprotease RPN11, and two cysteine proteases USP14 and UCHL5 (UCH37). These latter proteases mediate stepwise ubiquitin removal from the distal end of polyubiquitinated substrates [1]. Two groups have reported inhibitors of these enzymes. Proteostasis Therapeutics has targeted USP14 specifically (see later), while the Linder group has sought joint inhibitors of both 19S cysteine protease DUBs.

In a screen for compounds that induce the lysosomal apoptosis pathway, the Linder group discovered b-AP15 (**37**) (Figure 21) as a proteasome inhibitor [114]. In mechanistic investigations, they found that **37** did not inhibit

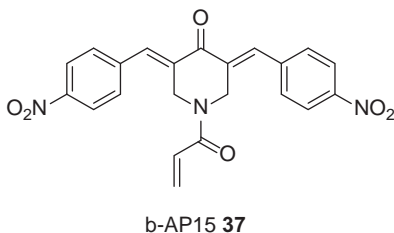


Figure 21 Structure of b-AP15.

the proteolytic activity of the proteasome or cause disassociation. Activity probe assays with either the whole 26S proteasome or the 19S regulatory particle showed that the compound blocked the reaction of both USP14 and UCHL5 with haemagglutinin (HA)-tagged ubiquitin vinyl methyl sulfone (VMS). By using purified 19S in a Ub-AMC assay, **37** was shown to have an IC_{50} of 2.1 μM (6.5 μM in a later version of the assay using Ub-Rho [115]). By measuring recovery of 19S DUB activity following rapid dilution of the enzyme/**37** complex, the inhibitor was shown to be reversible. b-AP15 is not an indiscriminate DUB inhibitor, since in isolated DUB assays it does not inhibit USP2/7/8, UCHL1, UCHL3 or BAP1 at 50 μM . Even though 19S inhibitory potency was modest, the Linder group showed that **37** inhibited tumour progression in four different *in vivo* solid tumour models in mice (2.5–5 mg/kg by subcutaneous injection), validating the 19S regulatory particle as a new anticancer drug target.

Subsequent mouse *in vivo* studies by the same team showed that **37** induces synergistic anti-multiple myeloma activity when combined with suberoylanilide hydroxamic acid, lenalidomide or dexamethasone [116]. A small amount of SAR has been described around **37** by using an HCT116 cell viability assay [115,117,118]. It was found that the acrylamide group was non-essential, and that replacing both nitro groups with fluorine had almost no effect on HCT116 cell viability. Only adding hydroxy groups to both phenyl rings (**38**) caused a significant 10-fold reduction of effect (Figure 22). However, this could be due to poor cell penetration rather than a change in USP14/UCHL5 affinity. A ring-expanded analogue of b-AP15 (VLX1570, **39**) was twofold more potent in this HCT116 assay (EC_{50} = 0.58 μM) and also had improved aqueous solubility making it suitable for i.v. injection [115]. In an isolated 19S assay, **39** had an IC_{50} of 6.4 μM . The Linder group explain the 10-fold loss of potency between cellular EC_{50} and isolated 19S IC_{50} , not through off-target polypharmacology,

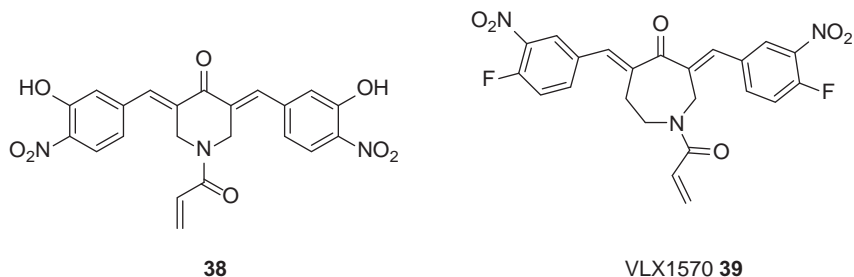


Figure 22 Structures of b-AP15 analogues.

but by enrichment of the compound in cells [117]. They supported their hypothesis by showing that **39** inhibits USP14 at 1 μM in a whole-cell activity probe assay. The labelling of UCHL5 in this assay, however, was 'less consistent'.

Further profiling of **39** against 211 kinases at 10 μM gave negligible hits except CDK4 (77% inhibition). Wide DUB profiling against 41 DUBs at 20 μM gave only one hit (USP5, 50% inhibition), although UCHL5 and USP14 (reconstituted with Ub-VMS treated proteasomes) were also in this panel and showed no inhibition. Poor solubility of the test compound in the assay buffer could be a possible explanation for these surprising results.

In 2014, the Linder group, now in collaboration with Vivolux, announced that the FDA had granted clearance to proceed with VLX1570 to clinical Phase I/II for treatment against relapsed and/or refractory multiple myeloma [119]. This is the first time that a compound has been progressed to the clinic on the basis of its DUB inhibitory activity.

Meanwhile a group from Proteostasis Therapeutics have spent several years optimising series for USP14 inhibition alone. Their first lead compound (initially from Harvard Medical School before the series was licensed to Proteostasis Therapeutics), identified from a high-throughput screen of 63,000 compounds against Ub-AMC/USP14 reconstituted with Ub-VMS-treated proteasomes, was IU1 (**40**) (Figure 23) [120].

The USP14 IC_{50} for **40** is 4–5 μM , compared with a UCHL5 IC_{50} of 700 μM (using proteasomes lacking USP14). Compound **40** was also inactive against USP2/5/7/15, UCHL1, UCHL3 and BAP1 at 17 μM . It was found to be cell permeable and enhanced substrate degradation in cells by blocking the USP14-mediated trimming of polyubiquitinated substrates, including Tau, that have been implicated in neurodegenerative disease.

A close analogue IU1C (**41**) (Figure 24) has no affinity for USP14 even at 1 mM, suggesting that the ketone of **40** might be acting as a cysteine warhead. USP14 potency was also found to be sensitive to the nature and

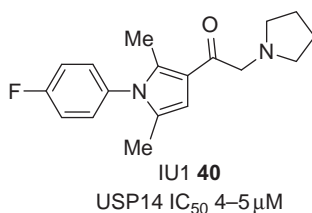


Figure 23 Structure of IU1.

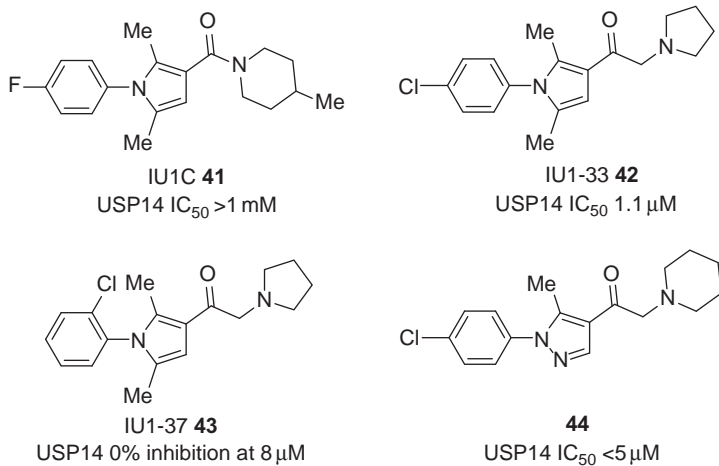


Figure 24 Structures of IU1 analogues.

position of phenyl substituents. For example, although a fluorine for chlorine swap (IU1-33, **42**) increased potency fourfold, moving the chlorine to the *ortho* position (IU1-37, **43**) resulted in complete loss of USP14 activity [121]. Although the N-aryl pyrrole is an unattractive structural motif because it is associated with promiscuous pharmacology [122], this was shown to be replaceable with a range of more attractive heteroaryl cores such as the pyrazole **44** [123].

This continues to be an active area of research and two additional patents were published recently. The first adds an additional therapeutic use for these aryl ketone USP14 inhibitors, as antiviral agents, in response to the finding that UPS inhibition can reduce viral yield [124]. The second is a more than 350-page patent containing many sub-0.5 μM IC₅₀ examples such as **45** and **46** (Figure 25), suggesting that Proteostasis Therapeutics is well advanced in the lead optimisation stage [125]. This is supported by pipeline statements on the company's website [126].

In December 2013, Proteostasis Therapeutics and Biogen entered into a collaboration to research and develop therapeutic candidates based on the inhibition of USP14. The premise is to enhance proteasome activity and thereby increase the clearance from the brain of aggregation-prone proteins associated with Alzheimer's and Parkinson's diseases. In July 2014, Proteostasis Therapeutics announced that it had achieved a key preclinical milestone in this collaboration and had consequently received a multimillion dollar payment [127].

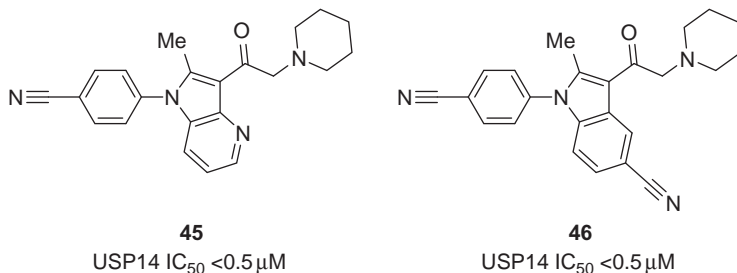


Figure 25 Structures of recent USP14 inhibitors from Proteostasis Therapeutics' aryl ketone series.

Another set of patents [128–130] describes the evolution of a second USP14 inhibitor series from Proteostasis Therapeutics. IU2-6 (**47**) is a reasonably active fragment against USP14 (Figure 26). Small changes to the amino substituent (**48**) or the deletion of the fused cyclohexyl ring (**49**) result in large losses of potency. Conversely, methylene-spaced amide substitution at the 2-position of the pyrimidine ring (**50**) was found to increase USP14 potency.

As part of *MISSION* Therapeutics' research into developing drug-like inhibitors of several DUBs, we have successfully co-crystallised an example from this fused thiophene pyrimidine series into USP14 CD and have a 3.2 Å X-ray structure of the resultant complex. A full description of our findings,

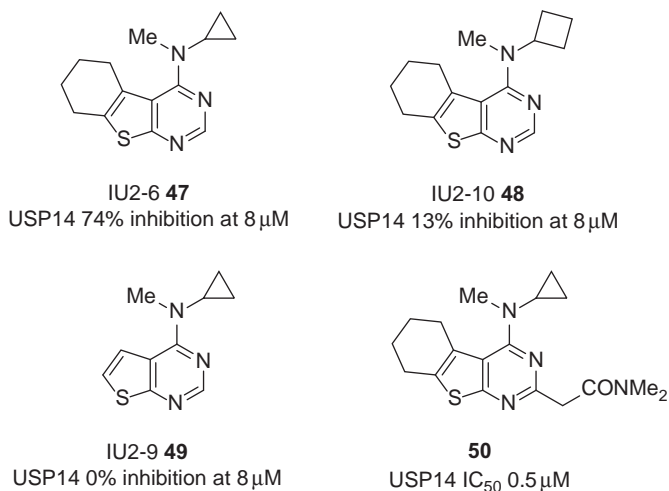


Figure 26 Structures of USP14 inhibitors from Proteostasis Therapeutics' fused thiophene pyrimidine series.

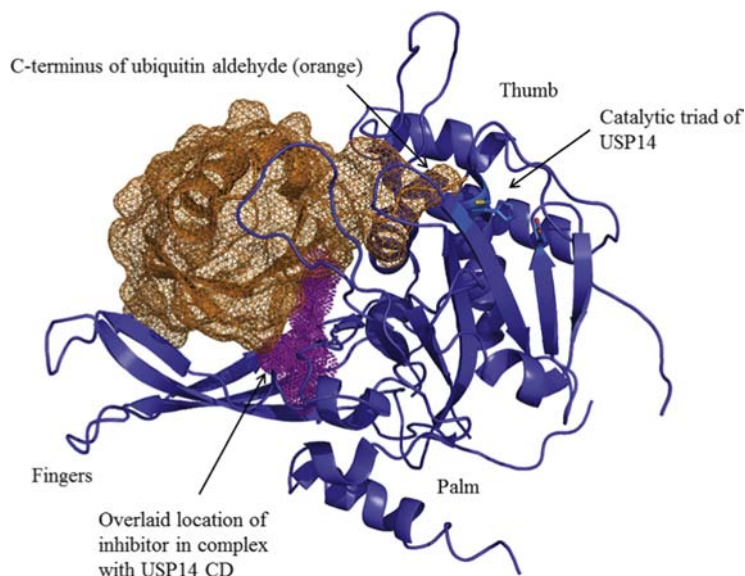
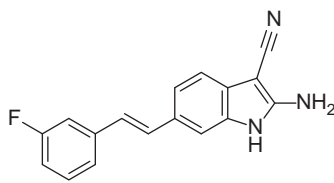


Figure 27 X-Ray structure of USP14 CD in complex with ubiquitin aldehyde (PDB: 2AYO) overlaid with an in-house X-ray structure of a thiophene pyrimidine-cored USP14 inhibitor in complex with USP14 CD (removed for clarity).

along with X-ray structures of other DUB/ligand complexes, will be the subject of a future publication. However, [Figure 27](#) shows the location of the inhibitor (purple (dark grey in the print version)) overlaid with the published structure of USP14CD (blue (dark grey in the print version)) bound to ubiquitin aldehyde (orange (grey in the print version)) (PDB: 2AYO) [33]. The viewing orientation is the same as that used in [Figures 5, 6 and 10](#). The electron density of the USP14 inhibitor overlaps with that of ubiquitin aldehyde from 2AYO, indicating that its inhibitory mechanism is to block the ubiquitin pocket and prevent the ubiquitinated substrate binding. The site of binding, between the fingers and palm regions, is similar to how compound **8** binds into SARS-CoV PLpro, and may also be similar with how the Genentech fragments bind to the USP7 CD (see [Section 4.3.3](#)).

4.3.9 USP20

GSK has presented brief details of its search for USP20 inhibitors. Its oncology interest stems from the finding that USP20 is reported to regulate HIF-1 α stability, and adrenergic receptor recycling and re-sensitisation [131]. Screening a focussed compound library against USP20/Ub-Rho yielded



GSK2643943A **51**
USP20 IC₅₀ 160 nM

Figure 28 Structure of a USP20 inhibitor from GSK.

GSK2643943A (**51**) as a hit with an IC₅₀ of 160 nM (Figure 28). No DUB selectivity data were presented, but the compound was shown to be cell penetrant by its effect on modulating USP33 levels within cells.

4.3.10 USP30

USP30 is a mitochondria-localised DUB. Inhibiting USP30 has recently been proposed by separate groups as an approach to treat Parkinson's disease [132–134] and cancer [135]. Yue *et al.* identified 15-oxospiramilactone (**52**) as a USP30 inhibitor by screening 300 compounds in a cell-based assay (Figure 29) [136]. When tested at 2 μ M, compound **52** was found to induce the elongation of mitochondria in 80% of Mfn1-knockout MEF cells, without affecting cell viability. However at concentrations above 5 μ M, the cells were killed through apoptosis. USP30 was proposed as the molecular target after demonstrating that a biotin-tagged version of **52** bound to streptavidin while complexed with myc-tagged USP30.

4.4 Ubiquitin C-Terminal Hydrolases

4.4.1 UCHL1

UCHL1 (PGP9.5) was first detected as a 'brain-specific protein' in 1981 by Jackson and Thompson [137]. It is probably the most studied DUB, with associations to both neurodegenerative disease and the progression of human malignancies. Although mainly expressed in neurons, high levels of UCHL1 have also been found in non-neuronal tumours, including breast, colorectal and pancreatic carcinomas [138].

The first UCHL1 inhibitors were described by Liu *et al.* in 2003 [139]. A series of isatin oximes was identified by a high-throughput screen of 42,000 compounds using the Ub-AMC substrate. Compound **53** was found to be a competitive and reversible inhibitor, with a UCHL1 IC₅₀ of 0.9 μ M (Figure 30). However, using newer non-AMC assays, at *MISSION*

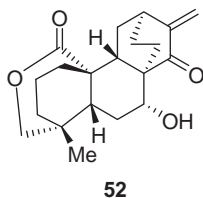


Figure 29 Structure of a USP30 inhibitor.

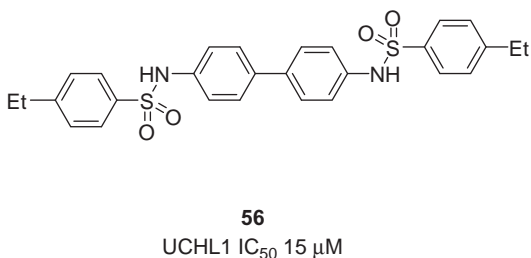
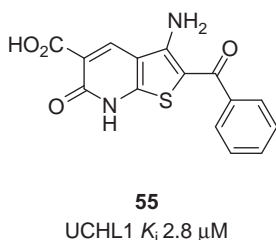
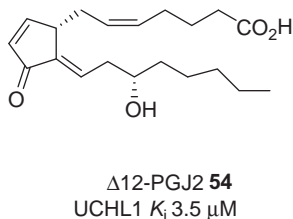
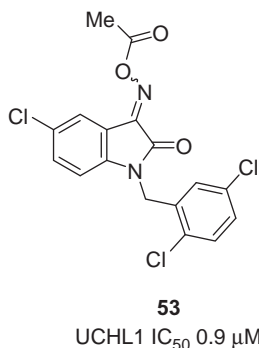


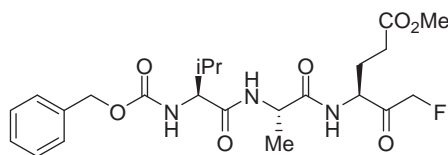
Figure 30 Structures of UCHL1 inhibitors.

Therapeutics we have found **53** to be 50 to 100-fold weaker. The following year, by treating neuronal cultures with prostaglandins, the Michael acceptor-containing Δ 12-PGJ2 (**54**) was found to raise levels of ubiquitinated proteins [140]. Subsequent screening against isolated UCHL1 with Ub-AMC showed that **54** had a K_i of 3.5 μM. Two further micromolar inhibitors of UCHL1 are shown in Figure 30. Compound **55** was identified as an inhibitor with a K_i of 2.8 μM (although in our hands we find it approximately 30-fold weaker) [141], while compound **56** was identified through *in silico* screening of the apo UCHL1 X-ray structure. Subsequent UCHL1/Ub-AMC screening showed it had an IC₅₀ of 15 μM [142].

In 2012, the same group responsible for the X-ray structures of apo UCHL1 and the Ub-VME/UCHL1 complex solved the structure of UCHL1 bound irreversibly to the fluoride-displacement product of a tripeptide fluoromethyl ketone (FMK) [143]. The ligand (Z-VAE(OMe)-FMK, **57**, see Figure 31) is a very weak inhibitor of UCHL1, requiring a concentration of 100 μM to block the reaction of UCHL1 with HA-tagged Ub-VME in an activity probe assay. Notably **57** had no effect on either UCHL3 or UCHL5 at this concentration.

The X-ray structure (PDB: 4DM9) shows that the electrophilic FMK group reacts with the active site Cys90 residue to displace the fluoride and form a thioether linkage. The tripeptide motif binds on the prime side of the active site, away from the ubiquitin binding site. Of most interest is the observation that the three residues of the catalytic triad are unmoved from their positions in apo UCHL1 (PDB: 2ETL). Figure 32 shows the same region of UCHL1 shown in Figure 7. Overlaid in Figure 32 are the structures from 2ETL (yellow (light grey in the print version)) and 4DM9 (UCHL1 in blue (dark grey in the print version) and tripeptide FMK in green (grey in the print version)).

The lack of movement of His161 may suggest that the highly electrophilic warhead of this tripeptide FMK is capable of binding to the inactivated form of UCHL1. However, through our X-ray work at MISSION Therapeutics, we have several in-house structures of UCHL1 bound to ligands bearing less reactive, more drug-like warheads. Like 4DM9, these also reveal the catalytic triad to be in the same inactivated state. Hence, another model which would be consistent with these structures is that UCHL1 exists partially in its activated state, even in the absence of ubiquitin binding. This activated state binds covalent inhibitors and then ‘relaxes back’ to its former inactivated state, but with the inhibitor still attached to Cys90.



Z-VAE(OMe)-FMK **57**

Figure 31 Structure of a weak tripeptide FMK UCHL1 inhibitor.

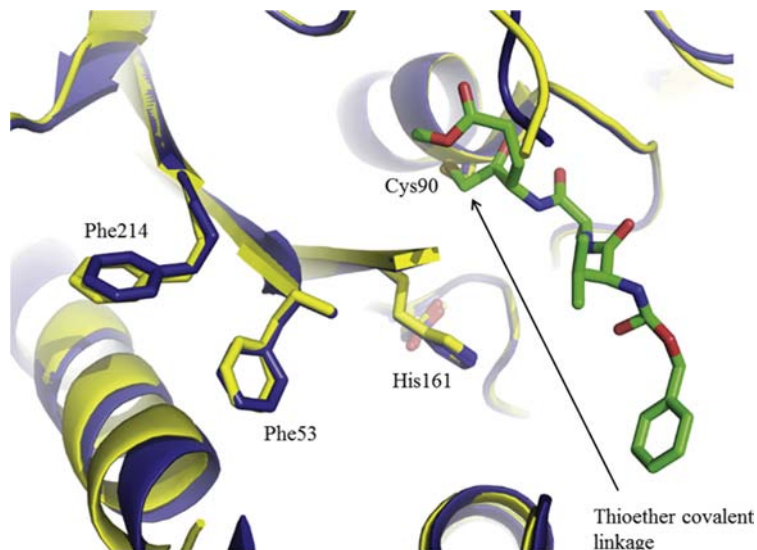


Figure 32 Overlay of the X-ray structures of apo UCHL1 (yellow (light grey in the print version)) and the product of UCHL1 reacting with Z-VAE(OMe)-FMK (**57**) (enzyme in blue (dark grey in the print version) and ligand in green (grey in the print version)).

4.4.2 UCHL3

The same *in silico* screening group responsible for compound **56** applied their technique to UCHL3, a DUB known to be involved in programmed cell death [144]. The hits they found, such as **58** (Figure 33), are very weak (IC_{50} s of 100–150 μ M), contain toxicophores [64] and are structurally very similar to some early Hybrigenics USP7 hits such as **19**. Ohayon *et al.* describe using a UCHL3/Ub-AMC/DTT screen to identify quinone-containing inhibitors such as **59**, but, as discussed previously in Section 4.3.1, these are likely to be RCC false positives [45]. See the USP2 section (Section 4.3.2) for discussion of the Novartis USP2/UCHL3 inhibitor series.

4.5 Ovarian Tumour Proteases

4.5.1 TRABID

TRABID, a putative anticancer target, is a member of the OTU family. Shi *et al.* performed *in silico* screening on a model of the active site of TRABID derived from the X-ray structure of another OTU, A20 [145]. Unfortunately, the only hits identified, again from a DTT-containing assay, were probably RCC false positives such as **60** (Figure 34).

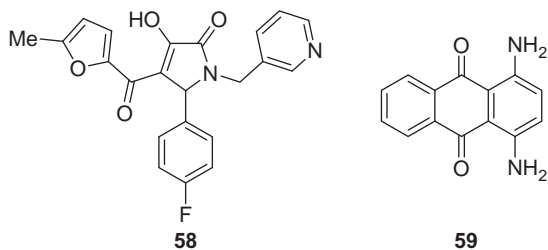


Figure 33 Structures of UCHL3 inhibitors.

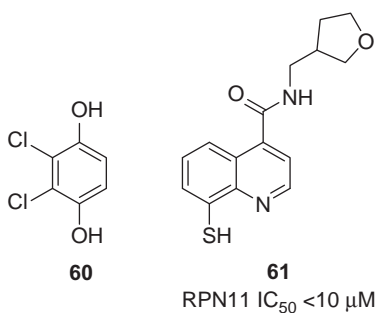


Figure 34 Structures of TRABID and RPN11 inhibitors.

4.6 JAB1/MPN/Mov34 Proteases (JAMMs)

4.6.1 RPN11

RPN11, the zinc metalloprotease DUB associated with the 19S regulatory particle, is essential for substrate degradation. RPN11 inhibitors are expected to exert effects comparable to the marketed inhibitors of the proteolytic 20S core (see [Section 1](#)) [146]. Cleave Biosciences has published a series of patents describing compounds containing well-known bidentate zinc-binding motifs [147]; an example is the thiol-substituted quinoline **61** ([Figure 34](#)) [148–150].

5. SUMMARY

From this analysis of the past decade's research into DUB inhibitors, two things become clear. Firstly, in general terms for the family overall, finding efficient and 'drug-like' inhibitors has proved relatively difficult compared to other protease families. Secondly, a closer inspection reveals that not all DUBs are created equal. Some are definitely more amenable to small-molecule intervention than others. This is not surprising given the wide variety of size, tertiary structure and reliance on extra domains among this diverse family.

The lack of any sub- μM ‘warheaded’ di- or tripeptide-derived inhibitors is notable when compared, for example, with the numerous examples described as inhibitors of the cysteine protease members of the cathepsin family [151,152]. The assumption, in the absence of X-ray structures for the vast majority of DUBs, is that the active sites of DUBs do not commonly contain ‘small-molecule-shaped’ binding pockets since they have been optimised instead to recognise the narrow Ub(1–74)-Gly75-Gly76- ϵ -Lys-R motif of their substrates (see Section 1). This is supported by several studies which illustrate that the C-terminus of ubiquitin, without the remainder of ubiquitin present, has little DUB-binding affinity. Stein *et al.* showed with a range of peptide-AMC substrates based on the C-terminus of ubiquitin that USP5 (IsoT) requires at least the pentapeptide Z-Arg-Leu-Arg-Gly-Gly-AMC (residues 72–76 of ubiquitin) for appreciably efficient catalysis. The k_c/K_m values dropped more than 1000-fold when the substrate was truncated to Z-Gly-Gly-AMC [153]. Likewise Borodovsky *et al.* found that the 12-mer peptide-VMS containing residues 65–76 of ubiquitin was required to prevent a variety of USP enzymes in cell lysates from reacting with [^{125}I]-Ub-VMS. The corresponding 10-mer and smaller VMS-capped peptides were unable to inhibit the USP enzymes at concentrations up to 100 μM [154].

In addition to the active site’s shape, the large conformational changes exhibited by some DUBs upon ubiquitin binding suggest that the flexibility of their active sites may pose an additional challenge for optimally fitting an inhibitor. Associated with this is the fact that, for some DUBs, the active site cysteine may not be nucleophilic in the absence of ubiquitin-mediated activation. This would be expected to pose a problem for active site binders bearing ‘drug-like’ warheads with limited electrophilicity. However, as stated in Section 4.4.1, we have found that this is not the case. This suggests that the binary activated/inactivated model suggested by X-ray studies for UCHL1 is too simplistic to explain the good inhibitory properties of compounds containing ‘drug-like’ warheads. Nonetheless, for other DUBs, it is evident from the inhibitors described in this review that highly reactive compounds have been required to covalently block (presumably) the active site cysteine. These approaches are unattractive because such high levels of reactivity usually lead to poor *in vivo* exposure and selectivity. These include the probable oxidative examples (14, 15, 36, 59 and 60), alkylating examples (39, 52, 54 and 57) and arylating examples (18 and 24). We recognise that this analysis of ‘drug-likeness’ and attractiveness is subjective, dependent on the unmet need of the disease in question,

and also built on assumptions. For example, despite containing electrophilic enone groups, it may be that compounds **37** and **39** are too sterically hindered to react covalently with proteins. This would be consistent with the reported reversibility of **37**, and the fact that *in vivo* efficacy can be achieved with low doses.

Some DUB inhibitors do contain warheads which are closely related to warheads utilised by non-DUB cysteine protease inhibitors in the clinic. Where these exist (compounds **16**, **29** and **33**), we have highlighted the clinical precedent. However, there are very few and they are weak. Conversely, during the course of our research on several therapeutically important DUBs [46,47,155,156], we have discovered covalent active site series which are ‘drug-like’, unrelated to any previously described DUB inhibitor, exhibit good oral bioavailability and have EC₅₀s less than 100 nM in cell-based assays. They can be selectively optimised for a range of DUBs and have yielded co-crystal X-ray structures to facilitate structure-based drug design. Publications describing these series from *MISSION* Therapeutics will be submitted shortly.

The other recent trend in identifying more ‘drug-like’ DUB inhibitors stems from the advances made with non-covalent series for certain USPs. The USP14 inhibitors from Proteostasis Therapeutics such as **50** appear to be high-quality leads, especially since the company’s most recent patent covering this series suggests that the cyclohexyl group, which may prove metabolically vulnerable, can be exchanged for heterocycles [130]. The collaboration with Biogen is also an indicator of confidence in the developable nature of the series. The X-ray structure of an example from this series in USP14 CD (Figure 27) shows that it binds in a ‘small-molecule drug-shaped’ cavity between the palm and finger regions. Given the structural similarity between these USP14 inhibitors and the USP1 inhibitor ML323 (**12**), it is tempting to speculate that this equally drug-like lead may bind into a similar region of the USP1/UAF1 complex.

In June 2015, Genentech and Almac Discovery announced a collaboration on an undisclosed USP target, whereby Almac Discovery’s small-molecule leads would provide the starting point for a two-year joint research programme. Almac Discovery received an upfront payment of \$14.5 million and is eligible for up to \$349 million in further payments if it achieves pre-determined milestones [157]. Given their shared interest in USP7 (see Section 4.3.3), it is likely that this USP is the target. Since Genentech found fragments that bind in the palm of USP7, this may again be evidence that

more generally USPs have druggable sites in this palm/finger region. The co-crystallised X-ray structures of the SARS-CoV PLpro leads such as **8** (see [Figure 10](#)) add further weight to this theory, since structurally this viral DUB is similar to the USP family [25].

As Sir Philip Cohen concluded in his 2010 review of the field, ‘predicting the future is notoriously difficult’ [8]. It appears to us that, although not all DUBs are likely to yield to ‘drug-like’ small-molecule inhibition, emerging trends from the area and from our in-house work suggest that a significant proportion have very good potential.

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