A Target Class Approach to Deubiquitinase Inhibitor Discovery

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Accessibility
A Target Class Approach to Deubiquitinase Inhibitor Discovery

A dissertation presented
by
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to
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in partial fulfillment of the requirements
for the degree of
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in the subject of
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Harvard University
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Abstract

Deubiquitinases (DUBs) have been proposed as therapeutic targets in cancer due to their ability to alter degradation rates of known oncoproteins. However, previous efforts to develop potent and selective DUB inhibitors have been largely unsuccessful. In this work, we develop a target class drug discovery platform targeting DUBs, which incorporates small molecule libraries, enzyme libraries, and a suite of structural and functional activity assays. Using this platform, we demonstrate one of the first examples of potent and selective DUB inhibitor development, targeting USP7 (chapter II). We then use our potent USP7 inhibitors (chapter III) as well as our annotated DUB-wide small molecule library (chapter IV) in a series of cellular assays to identify novel biological function of USP7 (chapter III) and USP10 (chapter IV). Finally, we describe our efforts to improve and expand our current target class library in order to continue to identify novel DUB inhibitors and DUB biology.
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Chapter I: Background
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Portions of this section, in particular I.iii: DUBs and DUB inhibitor strategies, have been adapted from a review manuscript that is currently in preparation.

i. A brief history of cancer chemotherapy

In this manuscript, I discuss my contributions to an evolving hypothesis relevant for targeted cancer therapy: namely, that inhibition of deubiquitinating enzymes (DUBs) will specifically destabilize oncoproteins, leading to slowed tumor growth with limited side effects.\textsuperscript{1,2} As I will discuss in this chapter, this therapeutic hypothesis has been challenged on both biochemical and physiologic grounds. Questions over specificity (of small molecule scaffolds for individual DUBs and of DUBs for individual oncogenic targets) have led some to conclude that DUB inhibition will not have a sufficient therapeutic window for clinical benefit. With these concerns in mind, I begin my discussion of DUB inhibitor development with an introduction to cancer chemotherapy writ large. It bears mentioning here that cancer chemotherapy \textit{in toto} was generally regarded as an unnecessarily dangerous approach until the clinical success of combination chemotherapies (VAMP for childhood ALL and MOMP / MOPP for Hodgkin’s disease) in the late 1960s.\textsuperscript{3–5} While a complete overview of the history of cancer chemotherapy is beyond the scope of this manuscript, I will provide a brief synopsis here in an attempt to situate DUB inhibitor development within the broader context of cancer chemotherapy. I direct the reader to several more comprehensive historical reviews in both the scientific and popular literature.\textsuperscript{5–9}
Chemotherapy (broadly defined as “the use of chemicals to treat disease”) was demonstrated as a viable approach for infectious diseases of bacterial and parasitic origin in the early 20th century thanks to groundbreaking work by Paul Ehrlich, Alfred Bertheim, Sahachiro Hata, and chemists at IG Farben including Gerhard Domagk and Hans Andersag. Although certain chemicals (e.g. inorganic arsenicals and cinchona extracts) had been known to have disease-alleviating properties for centuries, Ehrlich (who coined the term chemotherapy) was the first to employ a systematic approach to chemotherapeutic discovery: 1) correctly characterize the structure of a biologically active small molecule, 2) synthesize a library of analogs, and 3) test those analogs in in vivo animal models of disease for efficacy and toxicity. The discovery of arsphenamine (Salvarsan), widely discussed as the first true antibiotic, exemplifies this approach: Ehrlich and Bertheim correctly characterized the chemical structure of Atoxyl, an organoarsenical in general use since the mid-19th century but only recently demonstrated to possess anti-trypanosomal activity in animal models of infection. Based on the structure of Atoxyl, Ehrlich’s group synthesized a library of structural analogs and began testing them in animal models of infectious disease (albeit with limited success against trypanosomes). Fortuitously, Treponema pallidum was identified as the causative infectious agent of syphilis in 1905, and Hata was able to establish a rabbit model of syphilis in Ehrlich’s laboratory. Hata and Ehrlich screened the organoarsenical library in the rabbit model of syphilis and identified arsphenamine as a potent anti-syphilitic with relatively mild side-effects. Arsphenamine and a more stable analog, neo-arsphenamine, quickly became the gold standards for syphilis treatment, and remained so until their replacement by penicillin in the 1940s.
This systematic approach did not require complex molecular mechanistic knowledge (the precise mechanism of action of arsphenamine is still unknown)\textsuperscript{23,24} but was still capable of producing curative therapies in multiple diseases. For much of the 20\textsuperscript{th} century, progress in chemotherapeutic development was enabled more by advances in small molecule purification, structural characterization, synthetic chemistry, and animal models than by advances in our molecular mechanistic understanding of disease. The early history of cancer chemotherapy is similarly marked by drugs whose development was based on fairly general phenotypic observations: for example, in the wake of World War I, it was found that exposure to mustard gas led to severe myelo- and lymph suppression.\textsuperscript{25} In 1943, Alfred Gilman and Louis Goodman, hypothesizing that nitrogen mustards could limit the growth of myeloid and lymphatic malignancies, demonstrated the efficacy of nitrogen mustards in a mouse model of lymphoma,\textsuperscript{26} leading to the eventual development of cyclophosphamide and other nitrogen mustard DNA alkylating agents.\textsuperscript{27} The discovery of corticosteroids (e.g. prednisone),\textsuperscript{28} anti-folates (e.g. methotrexate),\textsuperscript{29} thiopurines (e.g. 6-mercaptopurine),\textsuperscript{30} and vinca alkaloids (e.g. vincristine)\textsuperscript{31} followed very similar narratives. However, unlike most antimicrobials of the time, none of these compounds was capable of producing prolonged remissions in human patients. Indeed, in the 1950s cancer treatment – be it chemotherapy, radiation therapy, or surgical therapy – consistently produced a low rate of cures, with universal treatment-related morbidity.

In 1964, Howard Skipper’s group at the Southern Research Institute performed careful experiments using a transplantable model of murine leukemia to demonstrate what became known as the fractional kill hypothesis: that a given dose of a chemotherapeutic kills a consistent \textit{percentage} of tumor cells, and the time to disease recurrence is directly related to the number of
tumor cells remaining after treatment.\textsuperscript{32} Skipper and others also demonstrated that a single leukemia cell was capable of killing a mouse,\textsuperscript{32,33} leading to a new therapeutic hypothesis: in order to produce cancer cures, high doses of multiple cancer chemotherapies should be used in order to ensure that every single cancer cell was removed from the host. By the mid-1960s, the cancer chemotherapy toolkit was large enough to test this hypothesis in human patients. In a chemotherapeutic combination known as VAMP, Emil Frei and Emil Freireich at the National Cancer Institute (NCI) applied a combination of vincristine (vinca alkaloid), methotrexate (antifolate, then known as amethopterin), 6-mercaptopurine (thiopurine), and prednisone (a glucocorticoid) in pediatric acute lymphoblastic leukemia (ALL) and demonstrated multi-year remissions in the majority of the treated patients.\textsuperscript{34} Similar combinations (mechlorethamine, vincristine, methotrexate, and prednisone, MOMP; and mechlorethamine, vincristine, procarbazine, and prednisone, MOPP) produced long-term remissions in Hodgkin’s lymphoma,\textsuperscript{35} and by the mid-1970s a number of pediatric and adult leukemias and lymphomas were considered curable with a combination of chemotherapy and radiation.\textsuperscript{36} In the same time frame, the addition of post-operative (adjuvant) chemotherapy to treatment regimens for solid tumors was evaluated and shown to provide clear benefit in the context of breast cancer.\textsuperscript{37} Thus, the current age of cancer therapeutics was born, with localized radiation or surgery used to remove the majority of the tumor burden, then adjuvant chemotherapy combinations used to clear any residual cancer cells.

Since the 1970s, there have been marginal improvements to overall cancer survival, although significant improvements to patient morbidity have been achieved by improvements in surgery, radiation, and chemotherapy.\textsuperscript{38} From a chemotherapeutic perspective, the most important development over the last 40 years has been our improved understanding of human genetics, which
has transformed our understanding of cancer as a general disease of aberrant cell growth to a genetic disease\(^3\) (or, perhaps more accurately, a signaling disease driven by genetic and epigenetic perturbations of specific pathways).\(^4\) While chromosomal instability had been observed in cancer even before DNA was known to be genetic material,\(^5\) it was not until the 1970s that specific cancer-causing alleles (oncogenes) were identified and cloned.\(^6\) Consistently, these genes were identified in viral infection studies as necessary and sufficient for oncogenic transformation, and also consistently, analogs of the viral oncogenes were identified in the host genome.\(^7,8,43,44\) Over the next decade, a number of human proto-oncogenes were shown to have tyrosine kinase activity that was aberrantly activated in cancer.\(^4\)

The most famous and clinically actionable oncogene to date has been c-Abl. Its history begins in 1960 with the discovery of the Philadelphia Chromosome (Ph) as a conserved translocation, t(9;22)(q34;q11), in chronic myelogenous leukemia (CML).\(^9\) In 1970, the Abelson murine leukemia virus was found to be carcinogenic in mice,\(^10\) and in 1978 the v-Abl gene and its protein product P120 was cloned from infected cells.\(^11\) In 1980, v-Abl was shown to be a tyrosine kinase,\(^12\) and in 1983 a human homolog, c-Abl, was identified and localized to the characteristic Ph breakpoint.\(^13\) By 1985, the carcinogenic gene product of the Ph chromosome was found to be a chimeric Bcr-Abl fusion,\(^14\) and in 1990 Bcr-Abl was confirmed to be an oncogene \textit{in vivo}.\(^15\)–\(^18\) This 30-year journey of biological discovery was complemented by a parallel 20-year effort by both academic and industry labs to develop potent tyrosine kinase inhibitors (TKIs),\(^19\) eventually leading to the clinical approval of the c-Abl TKI imatinib for CML treatment in 2001.\(^20\) Imatinib provides not only sustained remissions in CML, but also significantly improved patient morbidities
and lower cost of care, as it is an oral drug that can be dosed at home with relatively mild side effects compared to traditional chemotherapies.

This genetic approach to target discovery in oncology has had a transformative impact on how cancer chemotherapies are developed. In this model, the molecular mechanism of oncogenic transformation is of utmost importance, as drug candidates must be targeted at specific oncogenic drivers. The major benefit of this approach, as described above for imatinib, is a reduction in dose-limiting toxicity and improved morbidity. However, target validation has become a major challenge for drug discovery against putative genetic vulnerabilities in cancer. First, a number of cancer “targets” are proposed and published based on incomplete evidence or unreproducible results, a result that may be best addressed via systematic changes to incentives in scientific publishing. However, clinical resistance to cancer chemotherapy is still commonly observed for drugs that target bona fide oncogenes, and the underlying reasons are still not fully understood.

The current landscape of cancer therapy involves an integrated approach centered on multiple modalities: surgery, radiation, and chemotherapy (including classical agents targeting all rapidly dividing cells, targeted agents that modify specific oncoproteins or signaling pathways, or immunotherapies that enhance T-cell mediated tumor clearance). In general, the goal of therapy is to enhance cancer cell killing (with a specific focus on the proportional kill hypothesis) while minimizing deleterious side effects. Ultimately, this leads to a general reliance on therapeutic combinations and creative dosing schedules. As we gain an improved understanding of the complexity of genetics, signal transduction, metabolism, and immune evasion in cancer, there is a
growing need for novel therapies that will address the shortcomings of our current clinical toolkit and help produce sustained remissions in patients.

ii. The biological motivation for targeting DUBs in cancer

Ubiquitination is a post-translational modification (PTM) involved in a number of important cellular pathways. Ubiquitin (Ub) was first discovered as a histone modifier, but the biochemistry and molecular biology of ubiquitination was largely discovered in the context of ATP-dependent protein degradation. Currently, Ub is largely discussed in relation to its role in proteasomal protein degradation (the so-called Ub proteasome pathway, UPP), but it is important to note that ubiquitination of histones, microtubules, and endosomes clearly plays a key role in the regulation of those cell compartments. Further, non-degradative ubiquitination of cytosolic and nuclear proteins induces significant structural changes and plays a key role in intracellular signal propagation, sub-cellular trafficking, and formation of competent catalytic complexes. Disruption of several Ub-dependent pathways leads to phenotypes that may be relevant for cancer. In particular, the use of small molecule proteasome inhibitors to disrupt the UPP produced phenotypes (cell cycle arrest, apoptosis, disruption of antigen presentation, increased ER stress, and disruption of inflammatory signal transduction – particularly the NF-κB pathway) that suggested a potential therapeutic avenue in B cell malignancies. In the clinic, proteasome inhibitors produced responses in patients with multiple myeloma and mantle cell lymphoma, two B cell tumors that are reliant on NF-κB signaling and have a highly secretory phenotype. These findings were the first examples of approved cancer chemotherapies targeting a Ub-dependent pathway, and they motivated significant research into identifying components of the UPP that
would affect ubiquitination of specific protein targets. For this reason, I will devote most of my discussion in this thesis to the role of deubiquitinases (DUBs) in regulating the UPP (see Figure 1.1a).

Ub is a 76-residue protein that is translated as either an N- to C-linked poly-Ub chain or a mono-Ub-ribosomal protein (RP) conjugate.\(^7\)\(^5\),\(^7\)\(^6\) Ub is a highly stable protein with a globular structure and a 6-residue disordered C-terminal tail (LRLRGG). In higher order eukaryotes, the UPP requires conjugation of multiple Ub residues onto a protein substrate.\(^7\)\(^7\) This process is typically achieved by the formation of an isopeptide bond between the Ub C-terminus and the ε-amino group of a substrate lysine (although other side chains can be ubiquitinated). Because Ub contains seven internal lysine residues, poly-Ub chains consisting of straight or branched chains can also be constructed, leading to significant diversity in Ub topology.\(^7\)\(^8\) The conjugation of Ub to a substrate requires the concerted action of a Ub-activating (E1) enzyme, Ub-conjugating (E2) enzyme, and Ub-ligating (E3) enzyme.\(^6\)\(^2\) In an adenosine triphosphate (ATP)-dependent manner, the E1 active site cysteine forms a Ub-adenosine monophosphate (AMP) bond.\(^7\)\(^9\) This activated Ub-AMP is then transferred to an E2, which makes a direct thioester bond with the Ub C-terminus via its active site cysteine.\(^8\)\(^0\) E3’s facilitate Ub-substrate isopeptide bond formation by one of two mechanisms. Really Interesting New Gene (RING)-type E3’s serve as scaffolding proteins that recruit substrates and facilitate transfer of Ub from the E2 to the substrate side chain.\(^8\)\(^1\) Homologous to E6-AP
carboxy-terminus (HECT) and RING between RING (RBR)-type E3’s, on the other hand, form a Ub-thioester bond at their active site cysteine and transfer Ub directly to the substrate.\textsuperscript{82}

Substrates that are multiply mono-ubiquitinated or poly-ubiquitinated via K48 or mixed K48/K11 chains are then recognized by the regulatory “cap” particle of the 26S proteasome.\textsuperscript{77} The 19S regulatory cap consists of a series of Ub-binding domains as well as a base of six ATPases Associated with diverse cellular Activities (AAA) proteins that prevent general access to the 20S proteasome core particle.\textsuperscript{83} Ub binding allows the AAA ATPases to bind the substrate directly. ATP hydrolysis allows the AAA ATPases to unwind the substrate protein and feed it into the core particle as a linear peptide. The proteasome core contains three sets of threonine proteases (chymotrypsin-like, preferring hydrophobic residues; trypsin-like, preferring acidic residues; and caspase-like, preferring basic residues), and these enzymes degrade the substrate protein into short peptides with an average length of ~7 peptides.\textsuperscript{84} These peptides can then be digested to monomers by cytosolic proteases or loaded onto Class I major histocompatibility complexes (MHC) for antigen presentation.\textsuperscript{85}

DUBs regulate the UPP at several stages. First, DUBs are required for the release of Ub monomers from Ub precursors.\textsuperscript{86} Second, DUBs reverse substrate ubiquitination to prevent proteasomal targeting of specific substrates.\textsuperscript{87} Third, at least one proteasome-bound DUB (USP14, and possibly UCHL5) constitutively removes Ub from transiently proteasome-bound substrates in order to prevent degradation of minimally ubiquitinated substrates.\textsuperscript{88} Fourth, one proteasomal DUB (Rpn11, also known as POH1) constitutively removes Ub from substrates that are already committed to proteasomal degradation.\textsuperscript{89} The combined activity of Rpn11 and precursor-
processing DUBs is essential for maintaining a pool of free Ub monomers that is available for activation by E1’s.\textsuperscript{86} Since the approval of the first clinical proteasome inhibitor, bortezomib, in 2003, researchers have hypothesized that DUB inhibition could provide specific perturbations of the UPP that may prove to be clinically useful. Disruption of proteasomal DUBs may provide a more nuanced approach to modulating global protein processing, and disruption of non-proteasomal DUBs may provide an avenue towards targeted therapy within the UPP: DUB inhibition will increase steady-state ubiquitination of specific substrates, increasing their degradation without affecting global protein levels. In the context of cancer, inhibiting non-proteasomal DUBs is appealing for groups attempting to target oncoproteins and aberrantly active signaling pathways that are not responsive or amenable to direct inhibition with targeted therapies.

In the next section I will discuss some of the major technical challenges to designing potent and selective DUB inhibitors as well as some of the approaches that we and others have taken in order to overcome these challenges. However, it bears mentioning that the above-stated therapeutic hypothesis (DUB inhibition induces degradation of \textit{specific, physiologically important} targets) has been challenged on from the standpoint of human biology on two major fronts. First, given that there are >600 E3 ligases in the human proteome and only ~100 DUBs, it seems likely that even selective inhibition of a single DUB will lead to changes in ubiquitination of multiple substrates, leading to potentially unforeseen on-target effects. Second, studies on the ubiquitination of several important cancer-related proteins (e.g. Ras,\textsuperscript{90–92} Myc,\textsuperscript{93–95} p53,\textsuperscript{96,97} EGFR,\textsuperscript{98–101} etc.) has revealed interactions with multiple DUBs, such that inhibition of one may not be sufficient to induce major changes to equilibrium protein levels. With a combination of promiscuity and redundancy, DUB inhibition may have an insufficient therapeutic window for clinical utility. Unfortunately, there is
still a lack of good \textit{in vivo} models of DUB modulation, and many DUBs have been proposed as therapeutic targets based on suggestive cellular data rather than \textit{in vivo} pathophysiology.\textsuperscript{102} Ultimately, we believe that potent and bioavailable DUB inhibitors will be essential for critically assessing the DUB-focused therapeutic hypothesis on an individual basis \textit{in vivo}, as genetic models may produce phenotypes via non-enzymatic or developmental functions that will not be reproduced with small molecules. With this biological background to DUB inhibition in mind, I turn now to a discussion of the history of DUB inhibitor development.

\textbf{iii. DUBs and DUB inhibitor strategies}

The discovery of the UPP in the early 1980s was built on a series of biochemical assays in cell lysate. The first isolated DUB activity (later attributed to UCHL3) was discovered by monitoring \textit{in vitro} cleavage of a ubiquitin-dithiothreitol (Ub-DTT) adduct in rabbit reticulocyte extract.\textsuperscript{103} Of particular importance in this experiment was the discovery that the DUB cleaving Ub-DTT was a cysteine protease, since a theoretical framework was already in place for inhibiting cysteine proteases by mimicking the reactive intermediate.\textsuperscript{104} With this framework in mind, a reversible intermediate isostere, ubiquitin-aldehyde (UbAl), was synthesized in 1986 and confirmed in 1987 to globally oppose protein deubiquitination in reticulocyte lysate.\textsuperscript{105,106} In the following decade and a half, a number of cysteine-reactive, irreversible ubiquitin-based DUB inhibitors were synthesized, including ubiquitin-vinylmethyl sulfone (Ub-VS),\textsuperscript{107} ubiquitin-vinylmethyl ester (Ub-VME),\textsuperscript{108} and ubiquitin-propargylic acid (Ub-PA).\textsuperscript{109}
The most recent census of mammalian DUBs includes 99 enzymes across seven families (Figure 1.1b,c): 56 ubiquitin-specific peptidase (USPs), 12 Jab1/Mov34/Mpr1 Pad1 N-terminal+ (JAMMs), 17 ovarian tumor (OTUs), 4 Machado-Josephin Domain (MJDs), 4 ubiquitin C-terminal hydroxylase (UCHs), 5 Motif Interacting with Ubiquitin-containing novel DUB family (MINDYs), and ZUP1, which is the sole member of the newest unique DUB family. Six of these seven families are cysteine proteases, while the JAMM family has a Zn metalloprotease active site fold. There has been surprisingly limited development of ubiquitin-based JAMM inhibitors despite a general understanding in the literature for strategies to target Zn metalloproteases.

Still, broad-spectrum ubiquitin-based cysteine DUB inhibitors have been used extensively in cell-free systems to gain a better understanding of DUB activity and function. Because they trap the intermediate state of the DUB active site, structural studies using ubiquitin-based DUB inhibitors have provided significant insight into the mechanism of activation and activity for multiple DUB families. There are currently co-crystal structures of ubiquitin-based DUB inhibitors bound to more than two dozen DUBs in the protein data bank (PDB), and these structures include all known mammalian cysteine DUB families. In addition, ubiquitin-based ABPs have successfully been used to isolate DUB activity from specific sub-cellular compartments and compare DUB activity from different cell types. DUB ABPs continue to be of key importance for discovery of DUB activity: the DUB ABP HALO-Ub-PA enabled the discovery of ZUP1 as a DUB in 2018.
The important insights into DUB structure and function achieved with these ubiquitin-based inhibitors, along with increased recognition of their relevance in disease, led to pursuit of more specific and cell-permeable inhibitors in the early 2000s. Small molecules have dominated the effort and are the focus of my work, but it should be noted that ubiquitin variants with high selectivity for specific DUBs have been achieved.\textsuperscript{126,127} While bioavailability remains a limiting factor for adoption of ubiquitin-based DUB inhibitors physiological studies, improvements to intracellular protein delivery may allow for effective cellular studies of DUB function using ubiquitin variants.

Two strategies were pursued in the early 2000s toward small molecule DUB inhibitors: rational design based on peptide sequence and high-throughput screening of small molecule libraries. The first approach followed an established formula for development of cysteine protease inhibitors: an optimal peptidic substrate is identified using a peptide library, and this hit is optimized for bioavailability by depeptidization.\textsuperscript{128} Unfortunately, early attempts to identify shorter peptides with high affinity for cysteine DUBs produced mixed results.\textsuperscript{129} While DUB inhibition was possible with peptidic molecules, a 12-residue peptide with an irreversible VS group was required for DUB inhibition. This minimum length is likely necessary because cysteine DUBs have either a misaligned or occluded active site in their \textit{apo} state, and substrate interactions, often at distal sites, are required for DUB activation.\textsuperscript{117} While a 12-mer is a conceivable starting point for optimization and depeptidization, the process is not trivial, and we have not encountered follow-up studies on rational design of peptidic and peptidomimetic DUB inhibitors in the literature. Around the same time of these reports, the first DUB-targeted HTS was published.\textsuperscript{130} This was enabled by work from Stein and coworkers, published in 1998, that synthesized the first HTS-
amenable substrate for DUB activity profiling, ubiquitin-7-amino-4-methylcoumarin (Ub-AMC). DUB activity releases AMC from the ubiquitin C-terminus, leading to fluorescence unquenching ($\lambda_{ex} = 345$ nm, $\lambda_{em} = 440$ nm). Lansbury and coworkers screened a library of 42,000 compounds against UCHL1 and Ub-AMC. False positives were eliminated by demonstrating dose dependence and lack of AMC quenching with each of the hit compounds. A structure activity relationship (SAR) targeting one hit scaffold was then performed by testing analogs in dose against both UCHL1 and UCHL3 (as a counter-screen) by Ub-AMC. This study produced LDN-57444, a competitive UCHL1 inhibitor ($K_i = 400$ nM).

This first DUB-targeted HTS was a successful demonstration of the ability of small molecules to bind and inhibit DUBs and the use of the approach in finding chemical matter that target members of the enzyme family. Looking back, we can also appreciate that this initial report demonstrated the relative immaturity of the DUB inhibitor discovery field in 2003: despite the use of confirmatory assays, this study provided no method for orthogonal validation, very little selectivity profiling, and a limited SAR with no guidance from the scaffold:UCHL1 binding mode. In the last 15 years, the DUB small molecule inhibitor discovery field has matured significantly, built largely on improvements in four areas: orthogonal assays, selectivity screens, and structure-guided SAR.

At the time that I began my graduate thesis work, limited examples of such improvements had been published: in 2011, Kessler and colleagues reported the use of a competitive ABP assay to orthogonally validate DUB inhibitors in native cell lysate or live cells. In 2014, Zhuang and colleagues reported the most extensive selectively profiling performed on a DUB inhibitor to date, including in vitro activity assays against 16 DUBs, 72 other proteases, and 451 kinases. And in the same year, Mesecar and colleagues reported the structure-guided optimization of a second-
generation viral DUB (papain-like protease, PLpro) inhibitor.\textsuperscript{134} While there was not evidence at the time that these approaches would be generalizable for all DUBs, subsequent reports have provided evidence for hit-to-lead based discovery of potent and selective DUB inhibitors from HTS.\textsuperscript{135–142}

\textbf{iv. A target class approach to DUB inhibition}

When I first joined the Buhrlage lab in early 2015, a total of 35 small molecule scaffolds had been reported as DUB inhibitors in the literature. Most of these scaffolds were identified by HTS, although some had been discovered in phenotypic screens or assays targeting other enzyme families and were only later annotated as DUB inhibitors.\textsuperscript{143–147} While we recognized the continuing need for discovery of novel DUB inhibitor chemical matter, we determined that the contemporaneous number of reported DUB inhibitors provided a sufficient starting point for the initiation of a target class approach to DUB inhibitor development.

Target class drug discovery has been defined largely in contrast to disease-centric drug discovery, which seeks to modulate a specific molecular target implicated in human disease (Figure 1.2).\textsuperscript{148} Target class drug discovery instead seeks to establish general principles for targeting multiple members of an enzyme family. This approach incorporates several insights gleaned from drug discovery platforms in the genomic age: first, specific enzyme families often have multiple therapeutically relevant targets, and in many cases a consistent toolkit can be employed to develop potent and selective inhibitors of enzymes from the same family. Second, successful drug discovery programs require large amounts of equipment and technical expertise, but this
1.2

a: Phenotypic drug discovery

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b: Target-driven drug discovery

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c: Target class drug discovery

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institutional knowledge is often domain specific and not generalizable from one target screen to the next. Third, disease-centric drug discovery has a binary success v. failure result, making it inherently high-risk. This problem is exacerbated by the high rate of published therapeutic targets that are later demonstrated to not be necessary or sufficient for disease initiation or progression. Target class drug discovery thus represents a model to de-risk drug discovery efforts by producing cost-effective, longitudinal knowledge relevant for multiple therapeutic targets.

Target class drug discovery is built on three pillars of domain-specific principles: enzyme structure, small molecule structure, and generalizable assays for the enzyme family. In the context of DUBs, each of these areas was growing, but still immature when we initiated this project. Although only two DUBs had been co-crystallized with small molecule ligands, a wealth of apo and ubiquitin-bound DUB structures across all five DUB families that were known at the time. This provided preliminary structural information that could potentially be leveraged for inhibitor development, as well as published protocols for DUB purification and crystallization that could be translated into an in-house structural biology program. As mentioned earlier, by 2014 more than 30 DUB inhibitor scaffolds had been published. Many of these inhibitors contained cysteine-reactive electrophilic groups, but otherwise there was no clear evidence that specific molecular scaffolds would have intrinsic affinity for multiple DUBs. There was, however, one published example of successful, albeit limited, target class SAR within the DUB family: a team at Hybrigenics Pharma had converted a broadly DUB active compound (initially described as a USP7...
inhibitor)\textsuperscript{151} into a selective USP8 inhibitor through iterative medicinal chemistry and dual selectivity profiling against USP7 and USP8 by Ub-AMC.\textsuperscript{152} Based on this report, we hypothesized that generalized DUB target class SAR could be achieved and would be greatly enabled by more comprehensive DUB selectivity screening. Thankfully, DUB activity profiling was already a relatively mature area by 2014: Ubiquigent was already operating a commercial \textit{in vitro} Ub-Rho cleavage-based DUB panel with 35 members. As an orthogonal screen to assess DUB inhibitory activity against native di-ubiquitin linkages, we initiated a collaboration with the Trost group to perform small molecule profiling of a panel of 35 purified DUBs against di-ubiquitin, with HT analysis by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). In addition to these two \textit{in vitro} methods, competitive activity-based protein profiling (ABPP)-MS had been demonstrated in native human cell lysate, albeit with only 23 DUBs detected.\textsuperscript{132} We reasoned that these three orthogonal approaches to DUB activity assessment could be leveraged to produce extensive and reliable selectivity profiles of previously reported and newly synthesized DUB inhibitors.

In addition to the general benefits of target class discovery, we hypothesized at the beginning of this project that a target class approach focused on the DUBs would produce important tools for better understanding DUB biology. Endogenous DUB cellular function has largely been annotated using molecular and cell biology techniques. In particular, affinity-based protein purification (often with ectopic expression of affinity-tagged DUBs) and systematic RNA interference (RNAi) have been the preferred methods for identifying new DUB-substrate and DUB-pathway interactions. These assays have in some cases revealed significant insights into DUB function, but in others they have produced ambiguous phenotypes. Indeed, the most successful instances of
accurate DUB functional assignment have been carried out in targeted, hypothesis-driven experiments assessing DUB activity within specific sub-cellular compartments or complexes.\textsuperscript{89,123,153–155} While cellular knockdown and overexpression studies have played an important role in identifying specific DUB-substrate pairs, they have also shown that these DUB-substrate pairs are dynamically regulated in nuanced ways.\textsuperscript{156,157} In vivo knockout studies have contributed further to our understanding of the systemic consequences of DUB modulation, but there are few examples of conditional or inducible \textit{in vivo} DUB knockouts, which has hampered our ability to understand the phenotypic effects of DUBs that are essential for embryonic development.\textsuperscript{158–161}

Small molecule inhibitors offer the potential to consistently and dynamically perturb systems of varying complexity. We reasoned that a library of well characterized, high quality DUB-targeted small molecule inhibitors would be an invaluable toolkit for assignment of systemic DUB function in cell, organ, and organism models. In this model, currently described broadly as chemical genomics,\textsuperscript{162–164} the small molecule library can be assessed in either a forward (phenotype-driven) or reverse (substrate-driven) chemical genetic screen. If the specific DUB targets of an individual small molecule are known, hits produced by that small molecule can then be assessed in the context of the DUB(s) it inhibits. At the time that we initiated this work, such a study had not been performed for DUBs. But the use of a target class oriented small molecule library for chemical genomic screening in mammalian cells had been formalized in the context of non-selective inhibitors.\textsuperscript{165} We reasoned that the ability to perform chemical genomic screens using our small molecule library would be an added advantage of our DUB target class approach, especially given the significant need for a better understanding of DUB biology.
In the following chapters, I discuss our work developing a target class approach and chemical genomic platform targeting the DUBs. In Chapter II, I discuss the use of our target class platform, specifically incorporating selectivity profiling and DUB:inhibitor crystal structures to guide rational design of a sub-nM USP7 inhibitor. In Chapter III, I discuss a series of cellular assays we used to assess this USP7 inhibitor in the context of its regulation of the tumor suppressor p53 and other putative substrates. In Chapter IV, I discuss the first examples of DUB-targeted forward chemical genomics using our first-generation small molecule library. Finally, in Chapter V, I discuss our initial work designing and building a second-generation DUB inhibitor library, with a particular focus on the incorporation of higher throughput DUB selectivity profiling in native cell lysate.
Chapter II: Structure-guided design of potent and selective irreversible USP7 inhibitors
Author’s note:

This chapter is adapted from work that has been previously published (II.i)\textsuperscript{166} and that is currently in review.\textsuperscript{167} I prepared purified enzyme and small molecule samples for enzymatic analysis and mass spectrometry, prepared cellular lysates for ABPP and chemoproteomics, and performed data analysis for all figures. In addition to my work, several members of the Buhrlage lab and other groups made important contributions to this work: Xiaoxi Liu and Wanyi Hu performed chemical synthesis. Ilaria Lamberto, Rob Magin, Tatiana Mikhailova, and Rebekka Roberts performed USP7 cloning, purification, mutagenesis, and enzymatic assays. Adrian Chan and Scott Ficarro (Jarrod Marto lab) performed MS analysis for purified samples, ABPP samples, and chemoproteomic samples. Roxana Iacob (John Engen lab, Northeastern University) performed hydrogen-deuterium exchange MS assays. Hyuk-Soo Seo and Sirano dhe-Paganon performed crystallography studies and ITC assays. Kyle McKay (Jianing Li lab, University of Vermont) performed molecular dynamics simulations.

i. Identification and structural characterization of a selective scaffold for USP7

As discussed in the previous chapter, one of the major challenges of DUB drug discovery has been the inability to develop potent and selective DUB inhibitors. Here, I discuss our efforts to address this problem by developing potent and selective inhibitors of ubiquitin specific peptidase 7 (USP7). Our decision to pursue USP7 as a target was based on three key factors: the existence of a selective lead compound, our ability to obtain USP7:inhibitor co-crystal structures, and our ability to validate the phenotypic effects of our compounds using a known cellular biomarker. The combination of these three factors made USP7 an ideal target from a rational inhibitor design.
perspective and led to our successful development of selective, sub-nM USP7 inhibitors. I devote more attention to the biological effects of USP7 inhibition in Chapter III, but I note here that USP7 was, even at the time we initiated this project, a well-studied DUB with purported therapeutic relevance. As such, several USP7 inhibitors had been reported at that point, although these compounds were, like other reported DUB inhibitors, generally weak and non-selective.

As part of an effort to identify chemical starting points for development of DUB inhibitors by profiling the inhibitory activity of compounds reported in peer-reviewed and patent literature for activity against large panels of DUBs, we identified a highly selective inhibitor of USP7, Compound 2, reported in a 2013 patent from Hybrigenics (Figure 2.1a). When screened for inhibitory activity across a panel of 38 purified DUBs at a concentration of 100 µM, USP7 was the only DUB substantially inhibited (Figure 2.1b). Dose-response analysis using USP7 catalytic domain (amino acids 208-560) or full-length enzyme (1-1102) and ubiquitin-aminomethylcoumarin (Ub-AMC) as substrate confirmed USP7 inhibitory activity, although potency was weak with IC$_{50}$’s in the double digit micromolar range (Figure 2.1c). Isothermal titration calorimetry (ITC), using catalytic domain, confirmed binding with a K$_D$ of 8 µM (Figure 2.1d). We solved the structure of USP7 bound by Compound 2, which enabled rapid structure-guided development of XL188, a highly potent and selective inhibitor of USP7 (Figure 2.1a).

XL188 inhibits USP7 catalytic domain and full-length enzyme with IC$_{50}$ values of 193 and 90 nM, respectively (Figure 2.1c). The interaction of XL188 with USP7 was confirmed using ITC and differential scanning fluorimetry (DSF). Consistent with the 100-fold improvement in biochemical inhibition of USP7 by XL188 compared to Compound 2, a K$_D$ of 104 nM was measured for USP7
2.1

a

![Compound 2](image)

XL188

![XL203C](image)

b

<table>
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<tr>
<th>DUB</th>
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<th>XL188 (10 pM)</th>
<th>XL203c (10 pM)</th>
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<td>94</td>
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<td>USP11</td>
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USP15 | 90      | 95        | 95      |
USP16 | 92      | 88        | 79      |
USP19 | 92      | 107       | 101     |
USP20 | 83      | 124       | 102     |
USP21 | 93      | 115       | 104     |
USP25 | 91      | 132       | 111     |
USP27X | n.t.    |           |         |
USP28 | 78      | 111       | 94      |
USP30 | 84      | 110       | 110     |
USP35 | 86      | 107       | 108     |
USP46 | 86      | 107       | 102     |
USP45 | 89      | 113       | 90      |
CYLD   | 89      | 96        | 87      |
UCHL1  | 88      | 106       | 90      |
UCHL3  | 86      | 101       | 89      |
UCHL5  | 87      | 97        | 104     |
BAP1   | 81      | 89        | 86      |
OTU1   | 85      | 107       | 116     |
OTUB2  | 72      | 87        | 97      |
OTUD1  | n.t.    |           |         |
OTUD3  | 95      | 97        | 108     |
OTUD6 (p177S) | 100  | 101       | 97      |
OTUD6A | 90      | 112       | 101     |
OTUD6B | 88      | 81        | 92      |
Cecamine | 63      |           | 103     |
VCP1P1 | n.t.    |           | 96      |
AMSHLP | 91      | 97        | 99      |
Alamin-3 | 99      | 111       | 116     |
Alamin-3L | 100     | 109       | 116     |
JOSD1  | 83      | 108       | 121     |
JOSD2  | 92      | 126       | 121     |

![Heat map](image)

K \text{d} = 104 ± 15 nM
n = 1.06 ± 0.01
ΔH = 15.11 ± 0.13 kcal/mol
ΔS = 19.60 cal/mol·K

f

![Heat map](image)

Maximal difference in deacetylation (in Da): compound-bound form v. free USP7
2.1 (continued from previous page)

**Figure 2.1: Validation of XL188 as a selective USP7 inhibitor.** A) structures of Compound 2, XL188, and XL203C. B) profiling of compound 2, XL188, and XL203C at a single dose against a panel of purified DUBs by Ub-Rho (DUBprofiler, Ubiquigent). Values are reported as % activity remaining. C) IC\textsubscript{50} curves of compound 2, XL188, and XL203C against full-length USP7 by Ub-AMC. D) ITC binding of XL188 to USP7. E) Overlay of the co-crystal structure of USP7 bound to XL188 (PDB: 5VS6) and USP7 bound to Ub-Al (PDB: 1NBF, Hu et al. 2002), highlighting the misaligned catalytic triad of USP7•XL188. F) Impact of XL188 binding on USP7 solvent exchange rate by HDX-MS, overlaid onto the USP7•XL188 structure (PDB: 5VS6). G) Ligand interaction diagram of USP7 and XL188. Boxed residues have \geq 80\% homology within the USP family. H) Relative enzymatic activity and compound 2 inhibitory values of a small panel of targeted USP7 catalytic domain mutants. I) Limited SAR with a set of compound 2 analogs.

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<th>Mutation</th>
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<td>Q351S</td>
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<tr>
<td>M407K</td>
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</tr>
<tr>
<td>H456A</td>
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<tr>
<td>H461A</td>
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<tr>
<td>Y514A</td>
<td>&gt; 100</td>
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<tr>
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<tr>
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</table>
catalytic domain using ITC (Figure 2.1d). The selectivity of XL188 was assessed against a panel of 41 purified DUBs, using ubiquitin-rhodamine (Ub-Rho) as substrate. XL188 retained the excellent selectivity for USP7 observed with Compound 2; at a concentration of 10 µM, XL188 exhibited little to no inhibition of any DUBs other than USP7 (Figure 2.1b). In contrast, the enantiomer of XL188, XL203C, showed 80-fold less potent inhibition of USP7 ($IC_{50} = 7.18$ µM, Figure 2.1a,c) and no significant inhibition of other DUBs.

We determined co-crystal structures of Compound 2 and XL188 in complex with purified, recombinant USP7 catalytic domain to 1.9 and 2.2Å, respectively (Figure 2.1e-g). These complex structures revealed unambiguous electron density for the inhibitors in the substrate binding cleft and provided several insights into Compound 2 and XL188 binding. First, the catalytic cysteine and switching loop were in the unproductive conformation, as seen in apo structures, implying that this scaffold binds the inactive conformation of USP7. Second, Compound 2 and XL188 occupy the substrate binding cleft leading to the active site (specifically the S4 and S5 subsites) about 5 Å removed from the catalytic triad, implying that XL188 analogs may be capable of binding the conserved catalytic cysteine of USP7. Third, four inhibitor hetero-atoms common to both compounds make key hydrogen bonds with USP7: the quinazolinone ketone binds the peptide backbone nitrogens of Arg408 and Phe409, the quinazolinone cyclic nitrogen binds the amide side chain of Gln297, the tertiary hydroxy group binds the carboxylic group of Asp295 and the peptide backbone nitrogen of Val296, and the piperidine amide carbonyl binds the side-chain hydroxyl group of Tyr465. Fourth, the phenyl ring of Compound 2 and XL188, as well as the additional methyl group of XL188, engages the S4 pocket via hydrophobic interactions with Tyr514, His456, Phe409, Lys420, Arg408, and Asn460. Finally, nearly all atoms of Compound 2 and XL188 were
buried except the chlorine atom and N-methyl-piperazine sidechain, which we reasoned may serve as linker tethering sites without affecting compound potency.

Crystallographic studies were complemented with hydrogen deuterium exchange mass spectrometry (HDX MS) to monitor changes in protein dynamics (Figure 2.1f). Exchange of backbone amide hydrogens with bulk solvent can be accurately measured upon inhibitor binding. Online digestion of USP7 was performed and 85 peptic peptides covering 85% of USP7 catalytic domain were investigated with HDX MS in the free and bound states. Both XL188 and Compound 2 protected the BL1 and α-4/5 loops, confirming that the observed crystal structure interactions are also relevant in solution. HDX MS results also showed ligand-induced conformational changes and stabilizations distant from the active site: inhibitors stabilized/protected the palm region that is near the catalytic cysteine, including helices α-3/4, consistent with a previously proposed allosteric regulatory mechanism for USP7. Furthermore, a disordered loop between α8/β14 was protected from deuterium incorporation suggesting this region becomes ordered upon inhibitor binding.

We further confirmed the binding mode of this scaffold with USP7 by performing studies aimed at abrogating USP7-inhibitor interaction by modifying the (mutagenesis) or inhibitor scaffold (structure-activity relationship, SAR). First, we generated seven USP7 mutants with a single amino acid substitution and one with a double substitution (Q351S, M407K, M410S, K420A, H456A, H461A, Y514A, and M407K/M410S). Four of the eight mutant proteins retained the ability to cleave the DUB substrate Ub-AMC at wild-type levels, and one of these, USP7Q351S, was highly resistant to both Compound 2 and XL188 (Figure 2.1h). Second, we confirmed by SAR the
importance of several functional groups on XL188 for scaffold binding. USP7 activity was eliminated by a) removing the hydroxypiperidine –OH, which H-bonds D295 and V296, b) removing the amide carbonyl, which H-bonds Y465, c) shrinking of the 6-membered piperidine to a 5-membered ring, d) removing the phenyl ring, which occupies the S4 pocket, or e) shortening the linker between the hydroxypiperidine and phenyl group (Figure 2.1i).

These results were reported in *Cell Chemical Biology* in October 2017. By the start of 2018, three independent groups at Genentech, Forma Therapeutics, and Almac Therapeutics had reported similarly potent and selective USP7 inhibitors. Genentech’s lead inhibitor, GNE-6640, was based on a novel scaffold, but the lead inhibitors from both Forma (FT-671) and Almac (Compound 4) were based on the same Hybrigenics scaffold that inspired development of XL188. The Forma team had also demonstrated that, in principle, a short linker / warhead combination was capable of binding the USP7 active site cysteine. However, Forma’s irreversible inhibitor, FT-827, was less potent than FT-671, implying that conversion to a covalent binding mode had actually weakened the compound’s intrinsic affinity for USP7.

**ii. Investigation of irreversible analogs of Compound 2 and XL188**

For several reasons, we were interested in developing irreversible inhibitors of USP7 that bound the active-site cysteine. First, we were interested in testing the hypothesis outlined above that the USP7 active site cysteine behaves similarly to non-conserved surface cysteines when in its inactive, non-catalytically competent state. Second, as outlined in Chapter III, we observed discrepancies between the cellular phenotypes of XL188 and GNE-6640 that were not easily
explained by DUB activity profiling (both compounds are highly USP7 selective in commercial DUB panels), and we were interested in assessing the proteome-wide binding profile of our scaffold. Chemoproteomics, which identifies proteins bound to a specific chemical scaffold, requires covalent binding of the small molecule to its binding partners via either an irreversible warhead or chemical cross-linking.\(^{176}\) Finally, the structure of XL188 bound to USP7 did not offer clear avenues to inhibitor optimization that would dramatically increase the potency of XL188. Rather than performing unbiased medicinal chemistry on the scaffold, we hypothesized that an irreversible analog could be designed with higher cellular potency than XL188 based on its two-step binding mode.\(^{177}\) For these reasons, we began to systematically investigate irreversible USP7 analogs.

In addition to Compound 2, Hybrigenics has reported two additional USP7 inhibitor scaffolds, a tetrahydroacridine (HBX-19818)\(^{170}\) and an indenopyrazine (HBX-41108).\(^{151}\) In our DUB profiling experiments, we found that HBX-41108 was highly promiscuous, while HBX-19818 was fairly selective (albeit with higher affinity for other DUBs including USP10, as discussed in Chapter IV). However, Hybrigenics had demonstrated that HBX-19818 was capable of irreversibly labeling USP7 C223, the active-site cysteine (with some off-target labeling of the solvent-accessible C315).\(^{170}\) We therefore designed a series of XL188 / HBX-19818 hybrids with varying linker lengths between the two moieties and examined whether these compounds exhibited time- and dose-dependent inhibition of USP7.

The most potent compound in this series was XL041, which contains a 4-carbon aliphatic linker between the XL188 core and the HBX-19818-based warhead (Figure 2.2a). XL041 displayed
Cpd | Structure | USP7 IC₅₀ (nM) |
<table>
<thead>
<tr>
<th></th>
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<td>XL177A</td>
<td><img src="image1" alt="Structure of XL177A" /></td>
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</tr>
<tr>
<td>XL177B</td>
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2.2 (continued from previous page)

Figure 2.2: Validation of XL177A as an irreversible USP7 inhibitor. A) structures of XL041, XL112, and XL177A and B. B) Kinetic parameters of XL041 inhibition of USP7 Ub-AMC cleavage. C) Labeling time course of purified USP7 catalytic domain with irreversible USP7 inhibitors, as determined by MS. D) Impact of XL041 or XL177A binding to solvent accessibility of the USP7 catalytic domain, as determined by HDX. Results are overlaid onto the structure of USP7•XL188 (PDB: 5VS6). E) Limited SAR of a series of XL177A analogs, reporting their activity against USP7. F) IC$_{50}$ of XL177A and XL177B against full-length USP7 cleavage of Ub-AMC. G) MS-MS of XL177A bound to USP7, confirming selective labeling of the catalytic cysteine C223. H) IC$_{50}$ of XL177A against WT, Q351S, and F291N USP7 cleavage of Ub-AMC. I) MD simulation of XL177A (orange) binding to USP7 (green), overlaid with the structure of XL188 (red).
time- and dose-dependent inhibition of USP7, and we were able to establish kinetic parameters for an irreversible binding mode of XL041 ($K_i = 2.8 \, \mu M$, $k_{\text{inact}} = 0.004 \, s^{-1}$, $k_{\text{inact}} / K_i = 1331 \, M^{-1}s^{-1}$) (Figure 2.2b). We also confirmed that XL041 labels USP7’s catalytic domain stoichiometrically in a time-dependent manner using mass spectrometry (MS) (Figure 2.2c). Similar to the scaffold from Forma, XL041 has less intrinsic affinity for USP7 than XL188, although XL041 is both more potent and faster acting than FT827. Based on our knowledge of the USP7-XL188 complex, we hypothesized that the loss of USP7 affinity for XL041 may be related to its loss of the phenyl ring that binds the hydrophobic S4 pocket. Using HDX MS, we confirmed that XL041 binding to USP7 reduces solvent exchange between every region of USP7 stabilized by XL188 except for the S4 pocket (Figure 2.2d). These results suggested that reintroducing the phenyl ring would increase the affinity of XL041 for USP7.

We synthesized XL112, an analog containing a benzyl functional group, and observed a dramatic increase in USP7 inhibition (Figure 2.2a). Our experimental determination of $K_i$ and $k_{\text{inact}}$ is limited by the time between fluorescent read-outs (one minute), and we were unable to observe time-dependent inhibition with XL112, implying that has a $k_{\text{inact}} > 1 \, \text{min}^{-1}$. Instead, we benchmarked the activity of XL112 and other analogs against XL041 by pre-incubating USP7 with inhibitor for 6 hours (long enough to ensure full binding of XL041) and then performing dose-response experiments (Figure 2.2f). We found that XL112 was 25-fold more potent than XL041 (6 nM v. 150 nM IC$_{50}$, respectively). Because we were able to identify enantiomers with differential USP7 activity in our reversible inhibitor series, we purified the (R) and (S) enantiomers of XL112 and found that the (S) enantiomer was ~1,000-fold more active against USP7 than the (R) enantiomer (0.34 nM and 340 nM IC$_{50}$’s, respectively, Figure 2.2g). For the rest of this
manuscript, I refer to the active (S) enantiomer as XL177A and the inactive (R) enantiomer as XL177B (Figure 2.2a).

After confirming the biochemical potency of XL177A, we performed several additional experiments to confirm a covalent binding mode that engages the same P4 / P5 pocket as XL188. Despite extensive efforts, we were unable to obtain a co-crystal structure of XL177A and USP7, and we instead turned to a number of complementary approaches to understand the binding mode of XL177A. First, we confirmed that XL177A selectively labeled the active site cysteine of USP7 catalytic domain (Figure 2.2c,g). Second, we confirmed by HDX MS that, unlike XL041, XL177A is able to stabilize the hydrophobic S4 pocket in addition to the other residues stabilized by XL188 (Figure 2.2d). Third, we showed that XL177A was markedly less potent (≥500-fold) against two XL188-resistant USP7 mutants: USP7 Q351S and USP7 F291N, which Forma reported as an FT-671-resistant mutant and we confirmed as an XL188-resistant mutant as well (Figure 2.2h). Fourth, we performed limited SAR on the XL177A scaffold to confirm that we could eliminate USP7 activity by a) removing the hydroxypiperidine –OH or b) removing the tetrahydroacridine –Cl, which serves as a necessary leaving group for irreversible labeling of C223 (Figure 2.2e). We further confirmed the importance of the interaction between the tetrahydroacridine –Cl and C223 by demonstrating by MS that no adduct formed when either a) WT USP7 was incubated with the minus–Cl XL177A analog or b) USP7 C223A was incubated with XL177A (Figure 2.2c). Finally, we performed molecular dynamics (MD) simulations based on covalent labeling of C223 and H-bonding to Q297 and found that final snapshots after a 150 ns production stage showed a similar binding mode to that of XL188 (Figure 2.2i).
Taken together, these findings demonstrate that XL177A is a potent analog of XL188 that binds USP7 in a similar fashion and extends to and irreversibly labels the active-site cysteine. These findings serve as a proof of concept that a) it is possible to design inhibitors that irreversibly label the conserved active site cysteine in its inactive conformation, and b) that design of irreversible analogs can be used to increase the potency of optimized reversible DUB inhibitors. These are novel findings that have implications for the future of DUB drug development, as will be discussed in Chapter V. However, despite this proof of concept, we had not yet confirmed that XL177A retained selectivity for USP7, and we initiated a series of experiments to confirm its selectivity.

iii. Profiling the DUB- and proteome-wide selectivity of XL177A

One of our major motivations for developing an irreversible analog of XL188 was to enable chemoproteomic profiling of proteins that bind this scaffold, and XL177A served as a potential entry point to enable these studies. In addition to chemoproteomic profiling, the addition of a cysteine-reactive warhead to a molecule can introduce new potential off-targets within the DUB family, especially considering that the targeted cysteine is the active residue. We hypothesized that the non-conserved conformation of USP7’s inactive site would limit the number of DUB off-targets, but this needed to be confirmed experimentally. First, we assessed XL177A’s activity in the same panel of 41 recombinant DUBs used to profile Compound 2 and XL188 and found that it was highly selective for USP7 (with some activity for USP1) at 1 µM (Figure 2.3a). However, this DUB panel represents <50% of known cysteine DUBs, and we were interested in establishing the activity of XL177A against a broader portion of the DUB-ome in a more native context.
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37
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**Figure 3.3: Verification of XL177A selectivity.** A) profiling of XL177A against both a panel of purified DUBs by Ub-Rho at a single dose (column 1) and in dose by competitive ABPP in both HEK293 and MCF7 lysates. Values are reported as percent inhibition of enzymatic activity. B) structures and USP7 inhibitory activity of 041-dtb and 177-dtb. C) proteins detected in 041-dtb-treated but not DMSO-treated HEK293 lysates, ordered by number of unique peptides detected. D) integrated signal of all proteins detected by 041-dtb and 177-dtb-treated HEK293 lysate. E) competition of XL177A with 041-dtb and 177-dtb at 1 or 10 µM. Proteins are ordered on a log scale by signal relative to probe (041-dtb or 177-dtb)-only controls, with negative numbers indicating inhibition of probe binding.
Activity-based protein profiling (ABPP) MS has been used extensively for a number of enzyme families including DUBs.\textsuperscript{179,180} Competitive ABPP MS enables selectivity profiling of small molecules within specific enzyme families by quantitatively comparing protein intensity between null- and compound-treated samples. However, when our studies on XL188 were initiated, only one report of small molecule DUB inhibitor profiling by competitive ABPP MS had been published,\textsuperscript{132} and this study, which used HA-Ub-VME as an ABP, had only identified 23 DUBs. At the time that we initiated our investigations of XL177A, a report using a novel ABP pair, biotin-Ub-PA and biotin-Ub-VME, was published, with 35 DUBs detected.\textsuperscript{181} Based on these reports, we initiated ABPP studies using a series of biotinylated DUB ABPs. We were able to detect up to 58 DUBs with biotin-Ub-PA, 61 DUBs with biotin-Ub-VME, and 53 DUBs with biotin-Ub-VS in native HEK lysate. In a follow-up experiment, we used a combination of biotin-Ub-PA and biotin-Ub-VME to detect up to 59 DUBs and chose to move forward with this pair of ABPs. We performed two competitive ABPP MS experiments using native cell lysate from HEK293 cells, detecting 59 and 58 DUBs, respectively. In both experiments, competition with 0.1, 1, or 10 µM led to partial (0.1 µM) or complete (1 and 10 µM) elimination of USP7 binding without affecting binding of other DUBs. We performed a similar experiment in native lysate from MCF7 cells, which we used for early phenotypic exploration of these compounds, and again confirmed that XL177A was selective for USP7 among 54 identified DUBs (Figure 2.3a).

As discussed earlier, we were interested in performing chemoproteomic profiling of the XL188 and XL177A scaffold. Given that the N-methyl-piperazine of XL188 is solvent exposed, we synthesized a series of XL041 and XL177A analogs that possessed a desthiobiotin (DTB) tag directly conjugated to the piperazine group through hydrophilic linkers. The most potent
compounds, which we termed XL041-DTB and XL177A-DTB, retain USP7 inhibitory activity (IC$_{50}$ = 1.17 µM and 6.75 nM, respectively) (Figure 3.2b). We performed an initial chemoproteomic experiment with XL041-DTB in native HEK293 lysate and identified unique peptides for 356 proteins including USP7 (Figure 3.2c). Given the potential for low affinity interactions to be detected as hits by MS,$^{182}$ we next performed a series of competitive chemoproteomic experiments by pre-treating cell lysates with XL177A and then enriching proteins bound to XL041-DTB or XL177A-DTB. In these experiments, the most highly enriched protein in both the XL041-DTB-only and XL177A-DTB-only samples was USP7, indicating strong on-target probe binding (Figure 3.2d) In both cases, we found that XL177A prevented DTB probe binding to USP7 with similar potency and selectivity to competitive ABPP amongst 876 (XL041-DTB) and 566 (XL177A-DTB) proteins detected (Figure 3.2e). Together, these inhibitor selectivity profiling experiments definitively demonstrated the selectivity of XL177A for USP7.

iv. Conclusions and future directions

The results of these experiments, as well as experiments probing the cellular activity of XL188 and XL177A, were compiled in two published manuscripts and one that is currently in review.$^{167,183,184}$ In this work, we developed a class of highly potent and selective reversible (XL188) and irreversible (XL177A) inhibitors of USP7, as well as structurally matched negative controls for each series (XL203C and XL177B, respectively). We extensively profiled both XL188 and XL177A for selectivity, and in the case of XL177A we have provided to our knowledge the first example of competitive chemoproteomics for a putative DUB inhibitor. Together, these
compounds provide a useful toolkit of small molecules for performing cellular experiments investigating USP7 enzymatic function, as will be discussed in Chapter III.

Our major goal for this scaffold in the near future is to develop bioavailable analogs of our lead inhibitors. Although XL177A has potent USP7 activity in vitro, it is rapidly degraded \( t_{1/2} = 2 \text{ min} \) in mouse liver microsomes, and we have yet to initiate in vivo experiments with this compound due to concerns over its stability. We are currently pursuing exploratory medicinal chemistry to improve the pharmacological properties of the scaffold in order to enable in vivo experiments. These compounds would allow us to not only orthogonal validation of the phenotypic findings in Chapter III, but also to verify USP7 target engagement and selectivity in in vivo disease models. Despite the disadvantages of these compounds for in vivo studies, we found them to be excellent tools for understanding USP7 enzymatic function in vitro.

v. Materials and Methods

General chemical synthesis methods

All commercially available starting materials were purchased from Sigma Aldrich, Fisher Scientific, Oakwood Chemical and Combi Block. All reagents were used as received without further purification. Known compounds were synthesized according to published literature procedures and any modifications are noted. Anhydrous solvents, such as tetrahydrofuran (THF), diethyl ether, dichloromethane (DCM), dimethyl formamide (DMF), dimethylsulfoxide (DMSO), 1,4-dioxane, and toluene (PhMe) were purchased from Fisher Scientific, and used as received. If
necessary, air or moisture sensitive reactions were carried out under an inert atmosphere of nitrogen.

Removal of solvents was accomplished on a Büchi R-300 rotary evaporator and further concentration was done under a Welch 1400B-01 vacuum line, and Labconco FreeZone 6 plus system. Purification of compounds was performed by normal phase column chromatography using Teledyne CombiFlash chromatography system, and/or reversed phase chromatography on Waters Micromass ZQ preparative system with SunFire® Prep C18 OBD™ 5μM column. The purity was analyzed on Waters Acquity UPLC system. Analytical thin layer chromatography (TLC) plates were purchased from Fisher Scientific (EMD Millipore TLC Silica Gel60 F254). Visualization was accomplished by irradiation under UV light (254 nm).

All $^1$H-NMR spectra were recorded at 298K on a Bruker ARX 500 (500 MHz) spectrometer. $^{13}$C-NMR spectra were recorded on a Bruker ARX 500 (126 MHz) spectrometer. Samples were dissolved in CDCl$_3$, DMSO-$d_6$, or CD$_3$OD. The spectra were referenced to the residual solvent peak (chloroform-$d$: 7.26 ppm for $^1$H-NMR and 77.16 ppm for $^{13}$C-NMR; DMSO-$d_6$: 2.50 ppm for $^1$H-NMR and 39.25 ppm for $^{13}$C-NMR, CD$_3$OD: 3.31 ppm for $^1$H NMR and 49.00 ppm for $^{13}$C NMR or tetramethylsilane (TMS) as the internal standard. Chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad peak), coupling constants (Hz), and number of protons. Mass spectrometry (LCMS) data were obtained on Waters Acquity UPLC system in positive ESI mode.

*Synthetic procedures for XL188 and analogs*
2-aminobenzoic acids (10.0mmol) and formamide (1.8g, 40.0mmol) were mixed in pressure tube, which was heated at 150°C overnight. Then the reaction was cooled to room temperature. The solid was suspended in cold water, then collected by vacuum filtration, and dried on high vacuum line. The products 1.6g (-Cl) and 1.8g (-NO₂) were isolated as light brown solid in 88% (-Cl) and 95% (-NO₂) yields with no further purification.

Sodium hydride (60% dispersion in mineral oil) (0.88g, 22.0mmol) was dissolved in 40mL anhydrous DMSO at 0°C under N₂. Trimethylsulfoxonium iodide (4.84g, 22.0mmol) was added into the solution portionwisely. When addition completed, the mixture was warmed up to room temperature, and stirred for 40min. Then 1-Boc-4-piperidone (3.98g, 20.0mmol) was added portionwisely. The reaction mixture was then stirred at room temperature for 1 hour, then at 65°C for another hour. Then the mixture was poured on 100mL ice. Aqueous phase was extracted using EtOAc (50mL×2). Combined organic phase was washed with brine, dried over MgSO₄, filtered, and evaporated under reduced pressure. The crude material was purified by flash column chromatography (50% EtOAc in hexanes) to afford 2.98g product in 70% yield.
Into the solution of S1 (2.13g, 11.8mmol) in 50mL DMF was added S3 (2.78g, 13.0mmol) and Cs₂CO₃ (11.54g, 35.4mmol). The mixture was heated at 80°C overnight. Then the reaction was cooled to room temperature, and diluted with EtOAc. The solution was washed with saturated NH₄Cl (50mL×2) Aqueous phase was extracted with more EtOAc. Combined organic phase was washed with brine, dried over MgSO₄, followed by filtration and evaporation under reduced pressure. The crude material was purified by flash column chromatography (40% to 100% EtOAc in hexanes) to afford 3.94g product in 85% yield.

S4 was taken up in trifluoroacetic acid (TFA) as 1M solution, which was stirred at room temperature for 2 hours. The solution was concentrated under reduced pressure, further on high-vac overnight. S6 was directly used as starting material for the following synthesis without further purification.

Amide formation by HATU-catalyzed coupling reaction: S6 was taken up in DMF as 1M solution, and 3 equivalence of Et₃N was added. The carboxylic acid (1.2eq) was pre-mixed with HATU.
(2eq) and Et\textsubscript{3}N (5eq) in DMF at the same concentration, which was stirred at room temperature for 10 min. Two solutions were then mixed together and further stirred at room temperature for 5 hours. The reaction was directly subjected to prep. HPLC purification. The isolated product was then further purified by normal phase flash chromatography to afford product with desired purity for following biological tests.

Reduction of aromatic nitro group: \textbf{S8} (0.49g, 1.08mmol) was dissolved in 10mL AcOH/EtOH (1:1). Iron powder (0.25g, 4.39mmol) was added in one portion. The reaction was then stirred at 50°C for 1 hour. The iron powder was removed by filtration. Filtrate was concentrated under reduced pressure. The crude material was then purified by normal phase flash column chromatography (10% to 40% MeOH in EtOAc), followed by reverse phase HPLC to afford 0.22g product \textbf{S9} in 53% yield.

Installation of solubilizing groups: \textbf{S9} (0.11g, 0.25mmol) was dissolved in 5mL dichloromethane. Et\textsubscript{3}N (0.035mL, 0.25mmol) was added at −20°C 3-bromopropionyl chloride (0.03mL, 0.25mmol) in 1mL DCM was added dropwisely. The reaction was stirred at 0°C for 3h. Then it was quenched by addition of drops of water, then concentrated under reduced pressure. The crude product was used for the next step without further purification.
Crude material from last step (0.06g, 0.1mmol) was dissolved in 1mL DMF. Into the solution was added N-methylpiperazine (0.016mL, 0.12mmol) and Et$_3$N(0.028mL, 0.2mmol). The reaction was stirred at 80°C for 3 hours. The solution was directly subjected to reverse phase HPLC purification, followed by normal phase flash column chromatography (20% to 60% MeOH in EtOAc with 0.5% Et$_3$N) to afford 0.043g product XL188 in 75% yield.

**Synthetic procedures for XL177A and analogs**

![Chemical structure](image)

7-nitroquinazolin-4(3H)-one (1.55g, 8.1mmol) and tert-butyl-1-oxa-6-azaspiro[2.5]octane-6-carboxylate (1.90g, 8.9mmol) were added into 20mL DMF. Cesium carbonate (7.82g, 24.0mmol) was added in one portion. The mixture was heated at 80°C overnight. The mixture was diluted with EtOAc, then washed with sat. NaCl. Combined organic layer was concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc: hexanes: 50%-70%) to afford 2.42g S5 (75%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.54 (d, $J = 2.1$ Hz, 1H), 8.44 (t, $J = 8.6$ Hz, 1H), 8.26 (dd, $J = 8.8$, 2.2 Hz, 1H), 8.20 (s, 1H), 4.10 (s, 2H), 3.88 (s, 2H), 3.14 (t, $J = 11.6$ Hz, 2H), 1.73 – 1.48 (m, 5H), 1.44 (s, 9H). LCMS (ESI) $m/z$ 304.97 (M+H−Boc) [(M+H)$^+$

C$_{19}$H$_{25}$N$_4$O$_6$ calcd for 405.18]
Compound S5 (2.4g, 6.0mmol) was suspended in 20mL solvent (EtOH/AcOH=1:1). 4 eq. of Fe powder was added in portions. The mixture was stirred for 1 hour at 55°C. Then the reaction was cooled down to room temperature, and filtered through a pad of Celite. The filtrate was concentrated under reduced pressure to afford the crude product, which was then purified by flash chromatography (10%MeOH in EtOAc) to afford 2.1g product S10 (93%) ¹H NMR (500 MHz, DMSO) δ 8.04 (s, 1H), 7.79 (d, J = 8.7 Hz, 1H), 6.72 (dd, J = 8.7, 1.9 Hz, 1H), 6.61 (d, J = 2.0 Hz, 1H), 6.09 (s, 2H), 4.87 (s, 1H), 3.89 (s, 2H), 3.64 (d, J = 12.0 Hz, 2H), 3.05 (s, 2H), 1.54 – 1.24 (m, 13H).LCMS (ESI) m/z 374.97 [(M+H)⁺ C₁₉H₂₇N₄O₄⁺ calcd for 375.20]

Compound S10 (2.1g, 5.6mmol) was dissolved in anhydrous 10mL dichloromethane under N₂ at 0°C. 3.0 eq. of Et₃N was added. Then 3-bromopropionyl chloride (1.15g, 6.7mmol) was added dropwisely. The mixture was stirred at 0°C for 1 hour, then quenched with MeOH, and concentrated under reduced pressure. The solid residue was directly used for the following step without further purification. The crude product from last step was dissolved in 10mL MeOH, then 3.0eq of Et₃N was added. Into the stirred mixture was added 1-methylpiperazine (0.67g, 6.7mmol) dropwisely. After the addition completed, the mixture was stirred for 1 hour at 50°C. Then the reaction mixture was cooled down to room temperature, then directly subjected to HPLC purification (MeOH/H₂O with 4‰ TFA) to afford 2.1g product S11 (73% in two steps) ¹H NMR (500 MHz, MeOD) δ 8.28 (s, 1H), 8.20 (d, J = 8.8 Hz, 1H), 8.12 (d, J = 1.9 Hz, 1H), 7.69 (dd, J = 8.7, 2.0 Hz, 1H), 4.11 (s, 2H), 3.82 (d, J = 13.4 Hz, 2H), 3.23 (m, 2H), 2.85 (t, J = 7.0 Hz, 2H),
2.79 – 2.50 (m, 10H), 2.37 (s, 3H), 1.72 – 1.62 (m, 2H), 1.50 (d, \( J = 17.4 \) Hz, 11H). LCMS (ESI) 

\[ m/z \ 529.08 \ [(\text{M+H})^+ \ C_{27}H_{41}N_6O_5]^+ \ \text{calcld for 529.31}] \]

**S11** (0.53g, 1.0mmol) was dissolved in 3mL DCM, then 5mL 4M HCl in 1,4-dioxane was added in portions. The solution was stirred for 1 hour at room temperature. Then the mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual acid. Then the product (0.11g, 0.25mmol) was dissolved in 3mL DMF, and basified by adding 10 eq of Et\(_3\)N. Into the solution was added (\( S \))-2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid (0.11g, 0.35mmol) and HATU (0.16g, 0.4mmol) sequentially. The resultant solution was stirred overnight. Then the mixture was directly subjected to HPLC purification (MeOH/H\(_2\)O with 4‰ TFA) to afford 183mg **S12** (quantitative) LCMS (ESI) \( m/z \ 718.00 \ [(\text{M+H})^+ \ 718.43 \ \text{calcld for C}_{39}\text{H}_{56}\text{N}_{7}\text{O}_{6}^+] \) \( ^1\text{H}-\text{NMR} \) data will be provided before final revision.

**S12** (0.18g, 0.25mmol) was dissolved in 4M HCl in 1,4-dioxane, and stirred for 1h at room temperature. Then the mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual solvent. Then the product (0.16g, 0.25mmol) was dissolved in 5mL DCM with 10 eq. of Et\(_3\)N. Into the solution was added 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid (0.10g, 0.4mmol), and T3P (50% in EtOAc) (0.42g, 1.3mmol). The solution was stirred at room temperature under nitrogen overnight. Then the mixture was concentrated under
reduced pressure, and purified sequentially by flash chromatography and HPLC (MeOH/H$_2$O with 4‰ TFA) to afford 98mg XL177A (46%).

**Compound characterization**

![Chemical structure of XL177A](attachment:image.png)

((R)-N-(3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-4-oxo-3,4-dihydroquinazolin-7-yl)-3-(4-methylpiperazin-1-yl)propenamide (XL188): off-white solid, 75% yield) $^1$H NMR (500 MHz, DMSO) $\delta$ 10.57 (s, 1H), 8.20 (d, $J = 13.1$ Hz, 1H), 8.07 (d, $J = 8.7$ Hz, 1H), 8.02 (d, $J = 1.6$ Hz, 1H), 7.64 (d, $J = 8.7$ Hz, 1H), 7.25 (dd, $J = 12.4$, 6.2 Hz, 4H), 7.13 (dd, $J = 18.7$, 10.4 Hz, 1H), 4.96 (d, $J = 5.8$ Hz, 1H), 4.02 (d, $J = 13.6$ Hz, 1H), 3.90 (q, $J = 14.0$ Hz, 2H), 3.63 (dd, $J = 29.0$, 16.2 Hz, 1H), 3.27 – 3.12 (m, 2H), 2.86 (dd, $J = 17.7$, 14.8 Hz, 1H), 2.69 – 2.61 (m, 2H), 2.61 – 2.52 (m, 3H), 2.48 – 2.22 (m, 8H), 2.16 (s, 3H), 1.58 – 1.26 (m, 4H), 1.20 (d, $J = 6.4$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 170.76, 168.83, 159.92, 159.87, 149.10, 148.73, 146.41, 146.28, 144.06, 127.93, 127.90, 126.97, 126.62, 126.60, 125.69, 125.63, 118.04, 116.26, 114.45, 69.03, 68.98, 54.37, 53.22, 51.96, 45.30, 40.81, 40.71, 39.97, 36.67, 35.95, 35.74, 34.77, 34.64, 34.09, 34.01, 33.95, 21.79, 21.61. LCMS (ESI) $m/z$ 575.32 [(M+H)$^+$; calcd for C$_{32}$H$_{43}$N$_6$O$_4$: 575.33].
((S)-N-(3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-4-oxo-3,4-
dihydroquinazolin-7-yl)-3-(4-methylpiperazin-1-yl)propenamide (XL203C): off-white solid, 26% yield) \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 10.52 (s, 1H), 8.19 (d, \(J = 13.1\) Hz, 1H), 8.08 (d, \(J = 8.7\) Hz, 1H), 8.02 (d, \(J = 2.0\) Hz, 1H), 7.62 (dd, \(J = 8.8, 1.4\) Hz, 1H), 7.25 (dd, \(J = 12.6, 6.3\) Hz, 4H), 7.19 – 7.08 (m, 1H), 4.93 (s, 1H), 4.08 – 3.97 (m, 1H), 3.90 (q, \(J = 14.0\) Hz, 2H), 3.64 (t, \(J = 12.9\) Hz, 1H), 3.30 – 3.09 (m, 2H), 2.95 – 2.81 (m, 1H), 2.69 – 2.60 (m, 2H), 2.61 – 2.52 (m, 3H), 2.47 – 2.25 (m, 8H), 2.15 (s, 3H), 1.58 – 1.26 (m, 4H), 1.24 – 1.14 (m, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 170.76, 168.82, 159.88, 149.11, 148.74, 146.41, 146.29, 144.04, 127.94, 127.91, 127.00, 126.63, 125.69, 125.64, 118.04, 116.28, 114.46, 68.99, 54.43, 53.24, 52.02, 45.38, 40.82, 40.71, 39.96, 36.68, 35.96, 35.75, 34.77, 34.64, 34.03, 21.80, 21.61. LCMS (ESI) \(m/z\) 575.32 [(M+H)+; calcd for C\(_{32}\)H\(_{43}\)N\(_6\)O\(_4\)+: 575.33].

![Chemical Structure](attachment:image.png)

(7-chloro-3-((1-(3-phenylpropanoyl)piperidin-4-yl)methyl)quinazolin-4(3H)-one: white solid, 33% yield) \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.21 (d, \(J = 8.6\) Hz, 1H), 7.93 (s, 1H), 7.70 (d, \(J = 1.9\) Hz, 1H), 7.46 (dd, \(J = 8.6, 2.0\) Hz, 1H), 7.31 – 7.25 (m, 2H), 7.22 – 7.18 (m, 3H), 4.69 (d, \(J = 13.4\) Hz, 1H), 3.88 – 3.73 (m, 3H), 3.00 – 2.92 (m, 2H), 2.91 – 2.82 (m, 1H), 2.60 (dp, \(J = 14.3, 7.3\) Hz, 2H), 2.48 (td, \(J = 13.1, 2.4\) Hz, 1H), 2.17 – 2.04 (m, 1H), 1.70 (d, \(J = 12.9\) Hz, 1H), 1.62 (d, \(J = 12.8\) Hz, 1H), 1.17 (qd, \(J = 12.5, 4.3\) Hz, 1H), 0.97 (qd, \(J = 12.5, 4.2\) Hz, 1H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 169.56, 159.76, 149.58, 148.95, 141.43, 138.88, 128.37, 128.18, 127.28, 126.30, 125.79, 120.37, 50.79, 44.55, 40.74, 38.22, 35.01, 33.98, 30.87, 29.54, 28.84. LCMS (ESI) \(m/z\) 410.29 [(M+H)+; calcd for C\(_{23}\)H\(_{25}\)ClN\(_3\)O\(_2\)+: 410.16].
(7-chloro-3-((4-hydroxy-1-(3-phenylpropyl)piperidin-4-yl)methyl)quinazolin-4(3H)-one: white solid, 13% yield) \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 8.29 (s, 1H), 8.21 (d, \(J = 8.6\) Hz, 1H), 7.69 (d, \(J = 1.9\) Hz, 1H), 7.54 (dd, \(J = 8.6, 2.0\) Hz, 1H), 7.26 (q, \(J = 7.1\) Hz, 2H), 7.21 – 7.13 (m, 3H), 4.11 (s, 2H), 2.97 (d, \(J = 12.0\) Hz, 2H), 2.76 – 2.59 (m, 6H), 1.98 – 1.81 (m, 4H), 1.63 (d, \(J = 13.4\) Hz, 2H). \(^{13}\)C NMR (126 MHz, MeOD) \(\delta\) 161.13, 149.97, 148.77, 140.38, 140.25, 128.24, 128.13, 128.00, 127.52, 126.00, 120.22, 67.60, 56.16, 53.81, 48.21, 32.77, 32.00, 25.90. LCMS (ESI) \(m/z\) 412.39 [(M+H)\(^+\); calcd for C\(_{23}\)H\(_{27}\)ClN\(_3\)O\(_2\): 412.18].

(7-chloro-3-((3-hydroxy-1-(3-phenylpropanoyl)pyrrolidin-3-yl)methyl)quinazolin-4(3H)-one: white solid, 8% yield) \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.29 (s, 1H), 8.17 (dd, \(J = 8.6, 4.3\) Hz, 1H), 7.76 (t, \(J = 2.3\) Hz, 1H), 7.59 (ddd, \(J = 8.7, 7.5, 2.1\) Hz, 1H), 7.29 – 7.19 (m, 4H), 7.16 (t, \(J = 6.8\) Hz, 1H), 5.27 (s, 1H), 4.17 (s, 1H), 4.14 (d, \(J = 4.0\) Hz, 1H), 3.51 (s, 2H), 3.32 (dt, \(J = 23.6, 11.7\) Hz, 1H), 2.84 – 2.74 (m, 2H), 2.59 – 2.51 (m, 2H), 2.47 – 2.40 (m, 1H), 1.95 (ddt, \(J = 39.5, 12.7, 9.3\) Hz, 1H), 1.76 (ddd, \(J = 12.9, 11.0, 5.7\) Hz, 1H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 169.93, 169.76, 160.22, 160.13, 150.33, 149.06, 149.04, 141.55, 141.51, 138.95, 128.45, 128.40, 128.35, 128.22, 127.24, 126.27, 125.81, 120.46, 78.33, 76.84, 55.97, 55.57, 51.10, 50.87, 44.46, 43.77, 36.06, 35.63, 35.06, 34.54, 30.29, 30.23. LCMS (ESI) \(m/z\) 412.29 [(M+H)\(^+\); calcd for C\(_{22}\)H\(_{25}\)ClN\(_3\)O\(_3\): 412.14].
(3-\((1\text{-}acetyl\text{-}4\text{-}hydroxypiperidin\text{-}4\text{-}yl})\text{methyl}\)-7-chloroquinazolin\text{-}4(3H)-one: white solid, 57% yield) \(^1\)H NMR (500 MHz, DMSO) \(\delta 8.40 \text{ (s, 1H)}, 8.15 \text{ (d, } J = 8.6 \text{ Hz, 1H}), 7.75 \text{ (d, } J = 2.0 \text{ Hz, 1H)}, 7.57 \text{ (dd,} J = 8.6, 2.1 \text{ Hz, 1H)}, 4.09 - 3.96 \text{ (m, 4H)}, 3.58 \text{ (d, } J = 13.4 \text{ Hz, 1H)}, 3.33 - 3.20 \text{ (m, 1H)}, 2.97 - 2.85 \text{ (m, 1H)}, 1.98 \text{ (s, 3H)}, 1.56 \text{ (td, } J = 13.3, 4.3 \text{ Hz, 1H)}, 1.49 - 1.34 \text{ (m, 3H)}.\(^{13}\)C NMR (126 MHz, DMSO) \(\delta 167.66, 159.89, 150.25, 148.62, 138.65, 128.19, 126.92, 125.88, 120.05, 68.98, 53.39, 41.40, 36.44, 34.61, 33.93, 20.98.\) LCMS (ESI) \(m/z 336.18 \text{ [(M+H)+]; calcd for C}_{16}\text{H}_{19}\text{ClN}_{3}\text{O}_{3}+: 336.11}\).
145.59/145.56 (conformer), 144.24, 139.97, 139.81/139.75 (conformer),
135.19/135.17 (conformer), 130.07/130.02 (conformer), 128.93/128.79 (conformer),
135.19/135.17 (conformer), 130.07/130.02 (conformer), 128.93/128.79 (conformer),
128.12/128.00 (conformer), 127.43/127.37 (conformer), 127.26/127.20 (conformer),
125.99/125.94 (conformer), 125.78, 125.37/125.32 (conformer), 123.39,
118.25/118.20 (conformer) 116.56/116.45 (conformer), 114.67, 69.16/69.08 (conformer), 54.62,
53.53/53.33 (conformer), 53.44, 52.20, 45.54, 41.47/41.24 (conformer), 40.90/40.68 (conformer),
38.65, 37.01/36.96 (conformer), 35.22, 34.63, 34.47, 34.26, 33.96, 33.56, 30.46, 29.66, 28.98,
27.05, 26.76, 21.86. LCMS (ESI) m/z 860.82 [(M+H)+; C48H58ClN8O5+ calcd for 861.42]

(R)-N-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-
3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-
carboxamide (XL177B). 1H NMR (500 MHz, DMSO) δ 10.49 (d, J = 5.6 Hz, 1H), 8.82 – 8.69 (m, 
1H), 8.46 (d, J = 14.0 Hz, 1H), 8.20 – 7.95 (m, 5H), 7.66 – 7.55 (m, 1H), 7.30 – 7.05 (m, 5H), 4.82
(d, J = 4.8 Hz, 1H), 4.06 (dd, J = 62.9, 12.9 Hz, 1H), 3.95 – 3.75 (m, 1H), 3.61 (m, 2H), 3.31 –
3.21 (m, 4H, overlapped with H2O), 3.12 (m, 2H), 3.04 (m, 2H), 2.95 (m, 2H), 2.83 (m, 1H), 2.79
– 2.70 (m, 2H), 2.70 – 2.60 (m, 4H), 2.53 (m, 3H), 2.20 (s, 4H), 1.94 – 1.81 (m, 4H), 1.70 – 1.34
(m, 5H), 1.34 – 1.00 (m, 4H), 0.40 (dt, J = 12.0, 9.1 Hz, 1H). 13C NMR (126 MHz, DMSO) δ
172.75/172.70 (conformer), 171.45, 165.81/165.77 (conformer), 160.89/160.84 (conformer),
160.58/160.52 (conformer), 149.82/149.78 (conformer), 149.45, 146.09/146.06 (conformer),
144.74, 140.47, 140.32/140.25 (conformer), 135.69/135.67 (conformer), 130.58/130.53
(conformer), 129.43/129.29 (conformer), 128.63/128.51 (conformer), 127.93/127.87 (conformer),
127.77/127.70 (conformer), 126.50/126.44 (conformer), 126.29, 125.87/125.82 (conformer),
123.90, 118.76, 117.07/116.96 (conformer), 115.18, 69.67/69.59 (conformer), 54.98, 54.04, 53.88,
52.52, 45.83, 41.97/41.75 (conformer), 41.41/41.19 (conformer), 39.15, 37.52/37.46 (conformer),
35.73, 35.13, 34.97, 34.74, 34.46, 34.06, 30.97, 30.16, 29.48, 27.56, 27.26, 22.36. LCMS (ESI)
m/z 860.72 [(M+H)\textsuperscript{+} C\textsubscript{48}H\textsubscript{58}ClN\textsubscript{8}O\textsubscript{5}\textsuperscript{+} calcd for 861.42]

N-(4-benzyl-5-((4-hydroxy-4-(((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-
carboxamide (XL112). \textsuperscript{1}H NMR (500 MHz, DMSO) \(\delta\) 10.50 (d, \( J = 5.5\) Hz, 1H), 8.82 – 8.70 (m, 1H), 8.46 (d, \( J = 13.8\) Hz, 1H), 8.22 – 7.93 (m, 5H), 7.60 (dd, \( J = 16.4, 9.4\) Hz, 1H), 7.29 – 7.03 (m, 5H), 4.82 (s, 1H), 4.06 (dd, \( J = 62.6, 12.8\) Hz, 1H), 3.94 – 3.73 (m, 1H), 3.62 (m, 2H), 3.28 (m, 4H, overlapped with H\textsubscript{2}O), 3.13 (t, \( J = 11.0\) Hz, 2H), 3.05 (m, 2H), 2.96 (d, \( J = 11.0\) Hz, 2H), 2.85 (m, 1H), 2.79 – 2.71 (m, 2H), 2.70 – 2.60 (m, 4H), 2.53 (m, 3H), 2.14 (s, 4H), 1.88 (d, \( J = 3.1\) Hz, 4H), 1.71 – 1.34 (m, 5H), 1.33 – 1.03 (m, 4H), 0.39 (m, 1H). \textsuperscript{13}C NMR (126 MHz, DMSO) \(\delta\) 172.74/172.69(conformer), 171.51, 165.80/165.76(conformer), 160.89/160.84(conformer),
160.58/160.52(conformer), 149.82/149.78(conformer), 149.46, 146.10/146.07(conformer),
144.75, 140.47, 140.32/140.25(conformer), 135.68, 130.58/130.53(conformer),
129.43/129.29(conformer), 128.63/128.51(conformer), 127.93/127.87(conformer),
127.77/127.70(conformer), 126.50/126.44(conformer), 126.29, 125.87/125.83(conformer),
123.90, 118.75, 117.07/116.95(conformer), 115.17, 69.67/69.59(conformer), 55.22,
54.04/53.84(conformer), 53.99, 52.82, 46.19, 41.97/41.74,(conformer) 41.41/41.19(conformer),
39.15, 37.51/37.46 (conformer), 35.73, 35.13, 34.97, 34.78, 34.47, 34.06, 30.97, 30.16, 29.48, 27.56, 27.26/27.23 (conformer), 22.36. LCMS (ESI) m/z 860.72 [(M+H)+; C₄₈H₅₈ClN₈O₅⁺ calcd for 861.42]

(4-benzyl-5-(4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-carboxamide. ¹H NMR (500 MHz, DMSO) δ 10.52 (d, J = 4.2 Hz, 1H, conformer), 8.78 (dt, J = 18.3, 5.4 Hz, 1H, conformer), 8.48 (d, J = 18.1 Hz, 1H, conformer), 8.23 – 8.11 (m, 2H), 8.11 – 7.99 (m, 3H), 7.62 (m, 1H), 7.19 (m, 5H), 4.39 (dd, J = 39.4, 12.8 Hz, 1H, conformer), 3.78 (m, 2H), 3.61 (ddd, J = 43.3, 13.4, 7.0 Hz, 1H), 3.29 (m, 2H), 3.13 (s, 1H), 3.06 (m, 2H), 2.99 (m, 2H), 2.88 – 2.71 (m, 2H), 2.71 – 2.59 (m, 4H), 2.58 – 2.52 (m, 3H), 2.39 (m, 4H), 2.15 (s, 3H), 2.00 – 1.83 (m, 5H), 1.74 – 1.35 (m, 6H), 1.23 (m, 1H), 1.05 (td, J = 23.0, 11.6 Hz, 1H), 0.72 (dt, J = 12.3, 8.8 Hz, 1H).

¹³C NMR (126 MHz, DMSO) δ 172.78/172.70 (conformer), 171.53, 165.81/165.75 (conformer), 160.91, 160.17, 149.45, 148.92, 146.06, 144.77, 140.50, 140.33/140.28 (conformer), 135.68, 130.59, 129.58/129.32 (conformer), 128.69/128.51 (conformer), 127.89/127.87 (conformer), 127.54/127.49 (conformer), 126.51/126.44 (conformer), 126.32/126.29 (conformer), 125.87/125.84 (conformer), 123.96/123.91 (conformer), 118.94, 117.03/116.98 (conformer), 115.25, 55.23, 54.00, 52.84, 50.97/50.80 (conformer), 46.20, 45.31, 44.72, 42.09/41.83 (conformer), 41.54, 41.16, 39.01, 35.56/35.39 (conformer), 34.79, 34.07, 30.92, 30.30, 29.98, 29.61, 29.35, 27.56, 27.28/27.18 (conformer), 22.36. LCMS (ESI) m/z 844.82 [(M+H)+ C₄₈H₅₈ClN₈O₄⁺ calcd for 845.43]
N-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-5,6,7,8-tetrahydroacridine-3-carboxamide. 

$^{1}$H NMR (500 MHz, DMSO) $\delta$ 10.50 (d, $J = 4.4$ Hz, 1H, conformer), 8.74 – 8.60 (m, 1H), 8.40 (d, $J = 14.3$ Hz, 1H, conformer), 8.11 (m, 2H), 8.07 – 7.96 (m, 2H), 7.92 – 7.81 (m, 2H), 7.69 – 7.52 (m, 1H), 7.16 (m, 5H), 4.82 (s, 1H, conformer), 4.06 (dd, $J = 61.0$, 12.9 Hz, 1H, conformer), 3.88 (q, $J = 13.9$ Hz, 1H), 3.71 (dd, $J = 88.4$, 13.6 Hz, 3H), 3.27 (m, 2H), 3.13 (m, 2H), 3.03 (dd, $J = 7.9, 4.8$ Hz, 2H), 2.96 (m, 2H), 2.83 (m, 1H), 2.80 – 2.69 (m, 2H), 2.69 – 2.59 (m, 4H), 2.59 – 2.52 (m, 3H), 2.16 (d, $J = 18.6$ Hz, 3H), 1.98 – 1.87 (m, 2H), 1.87 – 1.77 (m, 2H), 1.60 (d, $J = 29.3$ Hz, 1H), 1.43 (m, 4H), 1.35 – 1.00 (m, 4H), 0.40 (t, $J = 10.7$ Hz, 1H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 172.76/172.70 (conformer), 171.52, 166.30, 160.61/160.52 (conformer), 160.32/160.30 (conformer), 149.84/149.79 (conformer), 149.46, 145.88/145.85 (conformer), 144.75, 140.49, 140.25, 134.81, 134.75, 132.73/132.70 (conformer), 129.44/129.30 (conformer), 128.62/128.51 (conformer), 127.78/127.75 (conformer), 127.62, 127.42/127.37 (conformer), 126.49/126.44 (conformer), 124.42/124.38 (conformer), 118.76, 117.07/116.97 (conformer), 115.17, 69.67/69.59 (conformer), 55.23, 54.04/53.84 (conformer), 54.00, 52.84, 46.20, 41.97/41.74 (conformer), 41.40/41.17 (conformer), 39.18, 37.46, 35.72, 35.13, 34.95, 34.79, 34.47, 33.46, 30.97, 30.17, 29.03, 27.31, 23.05, 22.75. LCMS (ESI) $m/z$ 827.61 [(M+H)$^+$ C$_{48}$H$_{59}$N$_8$O$_5$$^+$ calcd for 827.46]
9-chloro-N-(5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-5,6,7,8-tetrahydroacridine-3-carboxamide (XL041). $^1$H NMR (500 MHz, DMSO) δ 10.60 (s, 1H), 8.79 (t, $J$ = 5.0 Hz, 1H), 8.46 (s, 1H), 8.22 (s, 1H), 8.15 (d, $J$ = 8.7 Hz, 1H), 8.06 (dd, $J$ = 14.6, 8.7 Hz, 3H), 7.64 (d, $J$ = 8.8 Hz, 1H), 4.11 – 3.90 (m, 4H), 3.65 (d, $J$ = 13.0 Hz, 1H), 3.38 – 3.20 (m, 6H), 3.07 (m, 5H), 2.96 (m, 4H), 2.77 (m, 6H), 2.36 (m, 2H), 1.88 (s, 3H), 1.55 (d, $J$ = 26.5 Hz, 6H), 1.48 – 1.31 (m, 4H). $^{13}$C NMR (126 MHz, DMSO) δ 170.76, 170.28, 165.81, 160.83, 160.63, 149.94, 149.38, 145.93, 144.60, 140.44, 135.78, 130.56, 127.77, 127.74, 126.26, 125.85, 123.90, 118.84, 117.12, 115.34, 69.80, 53.86, 52.38, 51.73, 49.40, 42.61, 41.48, 39.60, 37.41, 35.58, 34.82, 33.98, 33.18, 32.45, 29.17, 27.54, 22.89, 22.33. LCMS (ESI) $m/z$ 771.47 [(M+H)$^+$; $C_{41}$H$_{52}$ClN$_8$O$_5$$^+$ calcd for 771.37]

**USP7 cloning, expression, and purification**

USP7 full length (amino acids 1-1102) and catalytic domain (208-560) were amplified using Addgene plasmid #16655$^{185}$ as a template and primers listed below and cloned into pET28PP and pET28aLIC, respectively, using InFusion HD EcoDry Cloning Kit (Takara Bio Cat# 121416). Amino acid mutations of catalytic domain were introduced by PCR using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer’s protocol using primers listed below. All DNAs were handled in E.coli 10beta cells (New England Biolabs Cat#C3020K) and transformed in E.coli BL21(DE3) for protein expression.
A construct of human USP7 covering residues 208–560 in the pET28aLIC vector was overexpressed in E. coli BL21 (DE3) in terrific broth (TB) medium in the presence of 50 ug/ml of kanamycin. Cells were grown at 37°C to an OD of 0.8, cooled to 17°C, induced with 500 μM isopropyl-1-thio-D-galactopyranoside (IPTG), incubated overnight at 17°C, collected by centrifugation, and stored at −80°C. Cell pellets were sonicated in buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM Imidazole, and 3 mM BME) and the resulting lysate was centrifuged at 30,000 xg for 40 min. Ni-NTA beads (Qiagen) were mixed with lysate supernatant for 30 min and washed with buffer A. Beads were transferred to an FPLC-compatible column and the bound protein was washed with 15% buffer B (50 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 300 mM imidazole, and 3 mM BME) and eluted with 100% buffer B. Thrombin was added to the eluted protein and incubated at 4°C overnight. The sample was then concentrated and passed through a Superdex 200 16/60 column (GE healthcare) in a buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. Fractions were pooled, concentrated and frozen at −80°C.

USP7 full length (amino acids 1-1102) in pET28aLIC was transformed in BL21(DE3) cells. An overnight culture was used to inoculate one liter of TB supplemented with 50 ug/ml kanamycin. Cells were grown at 37°C till they reached optical density (OD) ~0.6 at 600 nm. Protein expression was initiated by the addition of 0.4 mM IPTG. Cells were then grown for 16–20 hours at 17°C prior collection by centrifugation. Cell pellets were washed in PBS and resuspended in 25 mM HEPES pH 7.5, 500 mM NaCl, 10% glycerol and 1mM TCEP, 10 mM Imidazole, 0.1% IGEPAL sonicated and incubated with Ni-Nta beads (Quiagen) for 30 min at 4°C. Beads were washed with
10% buffer B (25 mM HEPES pH 7.5, 500 mM NaCl, 10% glycerol and 1mM TCEP, 250 mM Imidazole,) and eluted with 100% buffer B. Protein containing fractions were concentrated and loaded on Superdex 200 10/300 GL column in a buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. Fractions were pooled, concentrated and frozen at -80°C.

**Primers**

**USP7 208-560**
forward: GTTCCCGTGGGTAGTAAGAAGCACACACAGGCTACGTC  
reverse: CAAGCTTCCGTATCATTCATT CCTGGCCGCTCCTTCCGC

**USP7 1-1102**
forward: AGGAGATATACCATGAACCACCAGCAGCAGCAG  
reverse: GGTGGTGGTGCTCGAGGGTTATGGGATTTTAATGGCCCTT

**USP7 Q351S**
forward: GAAGATTATTATGATATCTCGCTAAGTATCAAAGG  
reverse: CCTTTGATACTTACCGAGATCATCATAATAATCTTCTTC

**USP7 M407K**
forward: CCAGTGTTACATCTACAACGTGAAGAGATTTATGTATGACCC  
reverse: GGGTCATACATAAATCTCTCGAGTTTGATAGATGTAACACTGG

**USP7 M410S**
forward: CTACAACTGATGAGATTAGTTATGACCCTCAGACGGACC  
reverse: GGTCCGTCATGAGGGTCTCATAACTAAATCTCATCAGTTGTAG
**USP7 M407K/M410S**

forward: CCAGTGTTACATCTACAACTGAAGAGATTTAGTTATGACCCTCAGACGGACC

reverse: GGTCCGTCTGAGGGTCATAACTAAATCTCTTCAGTTGTAGATGTAACACTGG

**USP7 K420A**

forward: CCCTCAGACGGACCACAAATATCGCGATCAATGATAGGTTTGAATTCC

reverse: GGAATTCAAACCTATCATGACTGCGATATTTTGGTCCGTCTGAGGG

**USP7 H456A**

forward: CTTTCATGCAGTCTCTGGTTGCTAGTGGAGATAATCATGGTGG

reverse: CCACCATGATTATCTCCACTAGCAACCGAAGGACTGCATGAAG

**USP7 H461A**

forward: CTGGTTCATAGTGGAGATAATGCTGGTGGACATTATGTGG

reverse: CCACATAATGTCCACCAGCAATTATCTCCACTATGAACCAG

**USP7 Y514A**

forward: CGACACTGCACCTAATGCTGCCATGTATGCTACTACATCAAGGG

reverse: CCCTGATGTAGACTAACATGGCAGCATTAGTGACGTGTCG

**USP7 C223A**

forward: GAATCAGGGAGCGACTGCTTACATGAACAGCC

reverse: GGCTGTTCATGTAAGCAGTCGCTCCCTGATTC

**USP7 F291N**

forward: GGAAACTTTAGATAGCAACATGCAACATGATGTTC

reverse: GAACATCATGTGGCATGTTGCTATCTAAAGTTTCC
**USP7 enzymatic profiling by Ub-AMC**

**XL188 and analogs:** USP7 and mutants were tested for their activity in Ubiquitin-AMC assay in presence or absence of inhibitors. For this assay USP7 catalytic domain WT or mutant was used at the following concentrations: 250nM USP7 WT, M407K, M407K/M410S or Q351S, 125 nM H461A, 600 nM Y514A and 10 nM M410S. For the same assay USP7 full length WT and Q351 mutant were used at 50 nM. USP7 variants were pre-incubated with different concentrations of inhibitors or DMSO as a control in 50 mM HEPES pH 7.6, 0.5 mM EDTA, 11 uM ovalbumin, 5 mM DTT. The reaction was incubated 30 min at room temperature prior to the addition of 2 uM Ubiquitin-AMC (Boston Biochem) substrate. The initial rate of the reaction was measured by collecting fluorescence data at one minute interval over 30-minute period using a Clariostar fluorescence plate reader at excitation and emission wavelength of 345 and 445 nm respectively. The calculated initial rate values were plotted against inhibitor concentrations to determine IC\textsubscript{50}s.

**XL177A and analogs:** Full length USP7 was tested for its activity in Ubiquitin-AMC assay in presence or absence of inhibitors. USP7 (5 nM) was pre-incubated for 6 hours at room temperature with different concentrations of inhibitors or DMSO as a control in 50 mM HEPES pH 7.5, 0.5 mM EDTA, 11 uM ovalbumin, and 5 mM DTT. Ubiquitin-AMC (Boston Biochem) was then added to a final concentration of 500 nM. The initial rate of the reaction was measured by collecting fluorescence data at one minute intervals over 30-minute period using a Clariostar fluorescence plate reader at excitation and emission wavelength of 345 and 445 nm respectively. The calculated initial rate values were plotted against inhibitor concentrations to determine IC\textsubscript{50}s.
All the experimental data were plotted using Prism GraphPad. All assays for each compound were performed at least twice for each compound.

To calculate the $k_i$ and $k_{i\text{anct}}$ values for XL-9678-041, the procedure above was used, but different concentrations of inhibitor were incubated with USP7 for different time points (5 min - 3 hours) before adding Ubiquitin-AMC. To determine $k_{o\text{bs}}$, the time course curves were fit to the equation $y = y_{\text{max}}(1 - \exp(-k_{o\text{bs}}x))$. The $k_{o\text{bs}}$ values were then plotted against the inhibitor concentrations and fit to the equation $y = k_{\text{anct}}/(1+(k_i/x))$ to obtain the values for $k_i$ and $k_{i\text{anct}}$.

*Differential scanning fluorimetry*

Differential Scanning Fluorimetry (DSF) experiments were carried out in a RTPCR 7500 Real-Time System (LifeTech) in 96 well plates, a total volume of 20 ul, and with an optimized SYPRO Orange dye concentration (5000x concentration in DMSO, Invitrogen). Compound dilutions in assay buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) were first prepared by an NT8 liquid handler (Formulatrix) and then addition of dye was performed also by the same liquid handler. Sealed plates were heated at 1°C/min from 25°C to 95°C with fluorescence readings every 0.5°C. Tm values were determined as the minimum of the first derivative of the recorded fluorescence intensity versus temperature plot.

*Isothermal titration calorimetry*
The binding affinity of protein/ligand was measured by adding 0.02 mM protein in cell and titrating with 0.2 mM ligand in the syringe using an Auto-ITC200 microcalorimeter (Malvern) at 20°C. Proteins and ligands were prepared within ITC buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, and 2% DMSO. The data were fit using Origin 7.0 software.

Irreversible USP7 labeling by MS

Purified USP7 catalytic domain was diluted to 20 µM in 10 µL labeling buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and incubated for the indicated times with 50 µM (2.5X) compound. After incubation, samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Intact MS Analysis: Intact mass analysis was performed by injecting 5 µg USP7 catalytic domain onto a self-packed reversed phase column (1/32” O.D. x 500 um I.D., 5 cm of POROS 10R2 resin). After desalting, protein was eluted with an HPLC gradient (0-100% B in 4 minutes, A=0.2M acetic acid in water, B=0.2 M acetic acid in acetonitrile, flow rate ~30 µL/min) into an LTQ ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA). Mass spectra were processed using MagTran1.03b2.186

CE-MS Analysis: To identify sites of covalent modification, treated protein was reduced (10 mM dithiothreitol), alkylated (22.5 mM iodoacetamide), and digested with trypsin overnight at 37 °C. Peptides were desalted using SP3,187 dried by vacuum centrifugation, and reconstituted in 1% formic acid/50% acetonitrile with 100 mM ammonium acetate. Peptides were then analyzed by
CE-MS using a ZipChip CE system and autosampler (908 Devices, Boston, MA) interfaced to a QExactive HF mass spectrometer (ThermoFisher Scientific, San Jose, CA). Peptide solution was loaded for 30 seconds, and the mass spectrometer was operated in data dependent mode and subjected the 5 most abundant ions in each MS scan (60k resolution, 3E6 target, lock mass enabled) to MS/MS (15k resolution, 1E5 target, 100 ms max inject time). Dynamic exclusion was enabled with a repeat count of 1 and an exclusion time of 6 seconds. MS/MS data was extracted to .mgf using multiplierz scripts\textsuperscript{188,189} and searched against a forward-reverse human NCBI refseq database using Mascot version 2.6. Search parameters specified fixed carbamidomethylation of cysteine, and variable oxidation (methionine) and XL177A modification (cysteine). Precursor mass tolerance was set to 10 ppm and product ion tolerance was 25 mmu. Spectral validation was performed using mzStudio.\textsuperscript{190}

\textit{Ub-Rhodamine-based selectivity profiling}

Selectivity profiling (DUBProfiler) was performed by Ubiqiiient using the manufacturer’s protocols.

\textit{Crystallization, data collection and structure determination}

One equivalence of compound (from a 100 mM DMSO stock) was mixed with 1 mM protein and crystallized by sitting-drop vapor diffusion at 20 °C in the following crystallization buffer: 30% PEG 3350, 0.2 M NaFormate, 0.1 M NaCitrate-pH 5.5, and 10 mM DTT. Crystals were transferred briefly into crystallization buffer containing 25% glycerol prior to flash-freezing in liquid nitrogen.
Diffraction data from complex crystals were collected at beamline 24ID-C of the NE-CAT at the Advanced Photon Source (Argonne National Laboratory). Data sets were integrated and scaled using XDS.\textsuperscript{191} Structures were solved by molecular replacement using the program Phaser\textsuperscript{192} and the search model PDB entry 1NB8. The ligand was positioned and preliminarily refined using Buster and RHOFIT.\textsuperscript{193} Iterative manual model building and refinement using Phenix\textsuperscript{194} and Coot\textsuperscript{195} led to a model with excellent statistics.

\textit{Hydrogen exchange experiments and mass analysis}

Hydrogen exchange experiments were performed essentially as described in Iacob et al.\textsuperscript{196} A stock solution of USP7 catalytic domain at 50 pmol/μL in 20 mM Hepes (pH 7.5), 200 mM NaCl, 1 mM TCEP, 5% glycerol H\textsubscript{2}O was prepared. Deuterium exchange in USP7 alone was initiated by dilution with 15-fold D\textsubscript{2}O buffer (pD 7.5), at room temperature. At each deuterium exchange time point (from 10 s to 4 hours) an aliquot from the exchange reaction was removed and labeling was quenched by adjusting the pH to 2.5 with an equal volume of quench buffer (0.8% Formic Acid and 0.8M Guanidine Hydrochloride, H\textsubscript{2}O). Quenched samples were immediately injected into the LC/MS system.

For the HDX MS experiments of USP7 bound to compounds, each compound was individually incubated with USP7 at room temperature in a 10:1 ratio (compound:USP7), XL188 for 30 min, XL041 for 60 min, and XL177A for 30 minutes, before dilution with D\textsubscript{2}O. The same time course as for the protein alone was implemented for the compounds work (10sec–4h).
Each sample was analyzed as previously described.197 Briefly, the samples were digested online using a Poroszyme immobilized pepsin cartridge (2.1 mm x 30 mm, Applied Biosystems) at 15 °C for 30 s, then injected into a custom Waters nanoACQUITY UPLC HDX Manager™ and analyzed on a XEVO G2 mass spectrometer (Waters Corp., USA). The average amount of back-exchange using this experimental setup was 20–30%, based on analysis of highly deuterated peptide standards. Deuterium levels were not corrected for back-exchange and are therefore reported as relative.175 All experiments were performed in duplicate. The error of measuring the mass of each peptide averaged ± 0.12 Da in this experimental setup (4). The HDX-MS data were processed using PLGS 3.0 and DynamX 3.0 (Waters Corp., USA).

Molecular dynamics simulations

The models of the XL188-bound USP7 complex were constructed from the co-crystalized PDB crystal structure (PDBID: 5VS6). The model of the XL177A- and XL177B-bound USP7 complexes were constructed from the apo and Ub-Aldehyde bound USP7 crystal structures (PDBIDs: 1NB8 and 1NBF, respectively). With the preparation and simulation protocols tested in our prior studies,198–200 all protein structures were prepared using Protein Preparation Wizard, solvated in SPC water with sodium counter ions by the System Builder, and simulated with the NPT ensemble (300 K, 1 atm, Martyna-Tuckerman-Klein coupling scheme) using the OPLS3e force field201 in Desmond v5.4.202 Each construct underwent minimization, equilibration, and 150-ns production stages with a time step of 2 fs. The Ewald technique with a 9 Å cutoff was used for the Van der Waals and other electrostatic calculations. Hydrogen atoms were constrained using the SHAKE algorithm. At least two replicas were collected for each construct. Trajectory
visualization and data analysis was done with the VMD program\textsuperscript{203} and in-house TCL and Python scripts.

\textit{Activity-based protein profiling and chemoproteomics}

\textbf{ABPP:} DUB profiling was performed using conditions similar to those in Lawson et al.\textsuperscript{181} HEK 293AD cells were lysed using target engagement lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl\textsubscript{2}, 0.5 mM EDTA, 0.5\% NP-40, 10\% glycerol, 1 mM TCEP, protease and phosphatase inhibitors), and the lysate was cleared by centrifugation. Samples were diluted to 2 mg/mL, and 1 mL lysate was incubated with the indicated concentration of XL177A for 5 hours at RT. Excess inhibitor was removed using a 30K Amicon spin filter, then the resulting lysate was incubated with 1 \(\mu\)M each of Biotin-Ub-PA and Biotin-Ub-VME for 90 minutes at RT. SDS was added to a final concentration of 1.2\%, and samples were heated to 80\(^\circ\)C for 5 minutes. After cooling to RT, 1X PBS was added to dilute the final SDS concentration to 0.2\%. 100 \(\mu\)L streptavidin agarose slurry was added to each sample, followed by incubation at RT for 3 hours. After streptavidin enrichment, samples were washed vigorously (2x 0.2\% SDS, 3x PBS, 3x ddH\textsubscript{2}O). After the final wash, all supernatant was removed using a flat-bottom tip, and the resin was flash frozen and stored at -80\(^\circ\)C until workup for TMT labeling.

\textbf{Chemoproteomics:} HEK 293AD cells were lysed as described above, and the lysate was cleared by centrifugation. Samples were diluted to 10 mg/mL, and 200 \(\mu\)L lysate (2 mg protein total) was incubated with the indicated concentrations of XL177A for 4 hours at RT, then 2 \(\mu\)M of WH114A for 4 additional hours. SDS was added to a final concentration of 1.2\% (27.2 \(\mu\)L of a 10\% stock),
and denatured by heating to 80°C for 5 minutes. After cooling to RT, 1125 µL 1X PBS was added to dilute the final SDS concentration to 0.2%. 50 µL streptavidin agarose slurry was added to each sample, followed by incubation at RT for 3 hours. After streptavidin enrichment, samples were washed vigorously (2x 0.2% SDS, 3x PBS, 3x ddH2O). After the final wash, all supernatant was removed using a flat-bottom tip, and the resin was flash frozen and stored at -80°C until workup for TMT labeling.

**Sample Prep for Mass Spectrometry Analysis:** Streptavidin beads were resuspended in 100 mM Tris pH 8.0 and bound proteins were denatured with 0.1% rapigest, reduced (10 mM dithiothreitol), alkylated (22.5 mM iodoacetamide), and digested with trypsin overnight at 37 °C. To remove rapigest, recovered supernatants were acidified with 10% TFA, incubated at 37 °C for 45 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. Peptides were then desalted by C18, and dried by vacuum centrifugation. Dried peptides were reconstituted in 50mM pH8.0 TEAB and labeled with TMT reagent at RT for 1 hour. TMT reactions were pooled and treated with hydroxylamine according to the manufacturer’s instructions. Peptide mixtures were then dried, reconstituted in 50 mM ammonium bicarbonate and desalted by SP3. Eluted peptides were then analyzed by nanoflow LC-MS/MS as described with a NanoAcquity UPLC system (Waters, Milford, MA) interfaced to a QExactive HF mass spectrometer (Thermofisher Scientific, San Jose, CA). TMT labeled peptides were injected onto a precolumn (4 cm POROS 10R2, Applied Biosystems, Framingham, MA), resolved on an analytical column (30 µm I.D. x 50 cm packed with 5 µm Monitor C18) and introduced to the mass spectrometer by ESI (spray voltage = 3.5 kV, flow rate ~30 nL/min). The mass spectrometer was operated in data dependent mode such that the 15 most abundant ions in each MS scan (m/z 300-2000, 120K resolution, target=3E6, lock
mass for 445.120025 enabled) were subjected to MS/MS (m/z 100-2000, 30K resolution, target=1E5, max fill time=100 ms). Dynamic exclusion was selected with a repeat count of 1 and an exclusion time of 30 seconds. MS/MS data was extracted to .mgf using multiplierz scripts\textsuperscript{188,189} and searched against a forward-reverse human NCBI refseq database using Mascot version 2.6. Search parameters specified fixed carbamidomethylation of cysteine, fixed N-terminal and lysine TMT labelling, and variable oxidation (methionine). Additional multiplierz scripts were used to filter results to 1\% FDR and derive protein-level aggregate reporter ion intensities using peptides mapping uniquely into the genome.\textsuperscript{205}

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All biochemical curves and statistical analyses were produced using Prism (GraphPad Software).

**DATA AND SOFTWARE AVAILABILITY**

Structure files and coordinates have been deposited to PDB under these accession numbers: 5VSB for USP7/Compound 2, 5VSK for USP7/XL188-rac, and 5VS6 for USP7/XL188. Supplemental data files including raw data from MS experiments can be accessed via the online versions of the peer-reviewed manuscripts\textsuperscript{166,167} or by directly contacting the author (nathan.schauer@gmail.com).

**AUTHOR CONTRIBUTIONS**
Chapter III. Investigation of the p53-dependent and p53-independent effects of USP7 inhibition
Author’s note:

This chapter is adapted from work that has been previously published\textsuperscript{166,184} and that is currently in review.\textsuperscript{167} I performed cell treatments, Western blots, qPCR, cell proliferation, target engagement assays, flow cytometry, and data analysis. In addition to my work, several members of the Buhrlage lab and other groups made important contributions to this work: Ilaria Lamberto, Rob Magin, and Sumner Perera performed cell treatments and Western blots. Laura Doherty and Sarah Boswell (Peter Sorger lab at Harvard Medical School) performed DGE-RNAseq and preliminary data analysis. Björn Stolte (Kim Stegmaier lab) performed \textit{TP53} knockout in TC32 cells and cell proliferation in Ewing cell lines. The Broad PRiSM platform performed PRiSM profiling and preliminary data processing. Andrew Giacomelli (William Hahn lab) performed Luciferase competition assays in A549 and RKO cells.

i. An introduction to USP7 biology

Ubiquitin-specific peptidase 7 (USP7) is one of the most widely studied DUBs, and it has been associated with multiple substrates, cellular pathways, and disease states. USP7 was first discovered as an interacting partner and stabilizer of the Herpesvirus immediate early (IE) gene product and E3 ligase ICP0.\textsuperscript{206} Since then, USP7 has also been reported to interact with and regulate numerous mammalian E3 ligases, epigenetic modifiers, and transcription factors, among other targets.\textsuperscript{207} Of these potential substrates, USP7’s stabilization of MDM2, the E3 ligase for p53, has garnered the most interest from a mechanistic and therapeutic standpoint given the role of p53 as tumor suppressor across many cancer types. Although many cancers are driven by mutant \textit{TP53}, roughly half of all adult malignancies and 95% of pediatric cancers harbor intact WT \textit{TP53}.
and may benefit from therapeutic approaches that stabilize p53. Indeed, MDM2 inhibitors such as idasanutlin and the dual MDM2/MDM24 inhibitor ATSP-7041 are currently undergoing clinical evaluation, supporting investigation of additional p53 stabilizing strategies such as USP7 inhibition. However, USP7’s promiscuity has raised questions over the relative importance of p53 in its overall cellular function. USP7 has more than 20 reported substrates (see recent reviews), several of which (PTEN, FOXO4, N-Myc, PCNA, Claspin and others) play a key role in proliferation and tumorigenesis. Indeed, USP7 has now been proposed as a therapeutic target independent of TP53 mutational status in multiple cancers including bortezomib-resistant multiple myeloma, neuroblastoma, T-cell acute lymphoblastic leukemia, and acute myeloid leukemia.

These findings have spurred interest in the development of specific USP7 inhibitors, as there now appear to be several cancer indications that may benefit from USP7 modulation. There have been a number of small molecule USP7 inhibitors reported to date, and these compounds have consistently demonstrated the ability to stabilize p53 protein levels in cyto, although they do not exhibit p53-dependent growth suppression. These findings have largely been credited to USP7’s role in other cellular pathways, although they could also be due to unidentified off-targets of these compounds. Given the selectivity of XL188 and XL177A, we sought to apply these compounds to several phenotypic assays in order to better understand the biology of USP7. First and foremost, we wanted to elucidate whether functional p53 signaling is necessary and sufficient for producing an anti-proliferative response to selective USP7 inhibition. We also wanted to systematically assess the cellular response to USP7 inhibition in order to identify potential therapeutic applications of USP7 inhibition.
ii. Selective USP7 inhibition induces MDM2 degradation and p53 stabilization

We treated two TP53-WT cell lines, MM1.S and MCF7, in dose with XL188 and XL203C. In both cases, protein levels of p53 and p21 (a canonical transcriptional target of p53) increased following 16 hours of treatment with cellular concentrations of XL188 in line with the IC$_{50}$ of native USP7 (Figure 3.1a). Consistent with the effects being USP7-dependent, we observed no change in levels of the same proteins across the same range of concentrations with XL203C. However, we did not see any effect on MDM2 protein levels at 16 hours with either compound. This may be explained by the fact that the p53 transcriptional program includes a negative feedback loop that upregulates MDM2. Indeed, when new protein synthesis was blocked by addition of cycloheximide, MDM2 loss was observed in a similar, USP7-dependent manner (Figure 3.1b).

In order to verify that XL188 and XL177A were capable of inhibiting native USP7, we performed a competitive target engagement study in HEK293 lysate using the DUB ABP HA-Ub-VS (Figure 3.1c,d,f). We confirmed not only that XL177A was more potent than XL188, but also that it displayed time-dependent USP7 inhibition in native lysate, supporting an irreversible binding mode. Both compounds were more potent than their enantiomers in this system.

We next sought to validate XL177A and XL177B in MCF7 cells, taking several additional steps to validate the importance of USP7 inhibition for MDM2 loss and p53 induction. Because XL177A and XL177B have a covalent mode of action, their cellular target engagement of USP7 could be assessed via HA-Ub-VS competition (Figure 3.1e,f). We demonstrated that XL177A was not only
3.1

a) Effect of XL188 and XL203C on MDM2, p53, and p21 in MCF7, 16h lysate.

b) Effect of XL188 and XL203C on MDM2, p53, and p21 in MM.1S, 16h lysate.

c) Effect of XL188 and XL203C on HA-UB-VP7 USP7 GAPDH in HEK293T (lysate).

d) Effect of XL188 and XL203C on HA-UB-VP7 USP7 GAPDH in HEK293, 30m (lysate).

e) Effect of XL188, XL177A, and XL177B on HA-UB-VP7 USP7 GAPDH in MCF7, 18h.

f) IC50 values for XL188, XL203C, XL177A, and XL177B in MCF7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Condition</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>XL188</td>
<td>30m (lysate)</td>
<td>904 ± 33</td>
</tr>
<tr>
<td>XL203C</td>
<td>30m (lysate)</td>
<td>&gt; 50.000</td>
</tr>
<tr>
<td>XL177A</td>
<td>30m (lysate)</td>
<td>85 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>4h (lysate)</td>
<td>8 ± 0.1</td>
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<tr>
<td></td>
<td>16h (cells)</td>
<td>39 ± 52</td>
</tr>
<tr>
<td>XL177B</td>
<td>30m (lysate)</td>
<td>&gt; 1.000</td>
</tr>
<tr>
<td></td>
<td>4h (lysate)</td>
<td>796 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>16h (cells)</td>
<td>1938 ± 75</td>
</tr>
</tbody>
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h) Effect of XL177A and XL177B on MDM2, p53, and p21 in MCF7.

i) Effect of XL177A and XL177B on MDM2, p53, and p21 in MCF7, 2h.
Figure 3.1 (continued from previous page): XL188 and XL177A stabilize p53 in cyto. A) MCF7 or MM1.S cells treated for 16 hours with XL188 or XL203C and analyzed by Western blot. B) MCF7 or MM1.S cells pre-treated with XL188 or XL203C for 4 hours, then treated with cycloheximide for 2 hours and analyzed by Western blot. C) XL188 and XL203C target engagement in HEK293T lysate using HA-Ub-VS competition to assess USP7 activity in situ. D) XL177A and XL177B target engagement in HEK293 lysate using HA-Ub-VS competition at different time points to assess USP7 activity. E) HA-Ub-VS labeling of lysates from MCF7 cells treated for 16 hours with XL177A or XL177B. F) Densitometry-based IC\textsubscript{50} determination for XL188, XL203C, XL177A, and XL177B competition with HA-Ub-VS. Reported data are the mean and standard deviation based on two biological replicates for each condition. G) MCF7 cells treated for 16 hours with XL177A or XL177B and analyzed by Western blot. H) MCF7 cells treated with XL177A or XL177B in a 4-hour time course experiment and analyzed by Western blot. I) MCF7 cells treated for 2 hours with XL177A or XL177B and analyzed by Western blot.

more potent than XL188 in cyto, but that induction of p53 and p21 after 18 hours of treatment was directly correlated to the extent of USP7 inhibition (Figure 3.1c,g). Higher concentrations of XL177B were able to both inhibit USP7 and increase p53 and p21 protein levels, lending further support to the necessity of USP7 for this effect. Because XL177A has a rapid $k_{on}$ and high cellular potency, we used it to understand the kinetics of MDM2 / p53 dynamics in MCF7 cells. We found that XL177A (but not equivalent concentrations of XL177B) induces time-dependent loss of MDM2 (Figure 3.1h). By 2 hours after treatment, MDM2 levels are notably lower and p53 levels are notably higher, but p21 remains unchanged, lending support to the hypothesis that p21 induction is due to a transcriptional effect. By 4 hours after treatment, p21 levels have increased slightly, but MDM2 levels have notably increased back to equilibrium levels, with p53 slightly reduced (although still higher than baseline). This result suggests that MDM2 re-stabilization may occur via a non-transcriptional mechanism, although there is some evidence that p53-responsive
MDM2 transcripts are translated more rapidly. In all, these results demonstrate that selective inhibition of USP7 induces transient MDM2 loss, sustained stabilization of p53, and downstream induction of p21. There is some initial evidence that MDM2 loss precedes p53 induction, but we have not verified that MDM2 is necessary for p53 loss in this context given the challenge of producing stable MDM2-KO TP53-WT cell lines.222,223 We further confirmed this result by demonstrating dose-dependent MDM2 loss and p53 grain after 2 hour MCF7 treatment with XL177A, but not XL177B (Figure 3.1i).

iii. Selective USP7 inhibition induces a global p53 transcriptional program

While p53 protein levels do increase after treatment with XL188 or XL177A, the effect is modest. The increase in p21 protein levels is much starker, and we wanted to verify that the observed effect was a downstream consequence of p53 stabilization. First, we tested XL177A in two TP53-mutant cell lines, MKN45 and SKOV3, and found that p21 levels remained unchanged after 18 hours (Figure 3.2a). Second, we confirmed by quantitative real-time polymerase chain reaction (qPCR) that CDKN1A (the gene encoding p21) transcript levels were increased after 6 and 24 hour treatment with XL177A but not XL177B. In parallel to p21, we assessed the levels of several other p53 target genes, including BAX, DDB2, GADD45A, and MDM2 and found that they were all significantly increased after 24 hours, although the dynamics and extent of change was variable for each transcript (Figure 3.2b). Importantly, we noted that TP53 and USP7 transcript levels were unchanged at either timepoint. Having confirmed that p21 transcript and protein levels increase after XL177A treatment, we used propidium iodide (PI) staining to assess the cell cycle effects of XL177A and XL177B treatment. As controls, we confirmed that serum starvation induces G1
arrest and taxol treatment induces G2/M arrest of MCF7 cells. Like serum starvation, treatment
with XL177A significantly increased the proportion of cells in G1, while XL177B had no effect
on cell cycle (Figure 3.2c). These results indicate that selective USP7 inhibition induces p53-
dependent induction of p21 and cell cycle arrest. Interestingly, both PI staining and dual PI /
Annexin V FITC staining showed no change in apoptosis levels of MCF7 cells treated with either
XL177A or XL177B (Figure 3.2d). We also observed no cleavage of PARP or Caspase 3 even

Figure 3.2 (continued from previous page): downstream consequences of USP7 inhibition. A) SKOV3 (TP53-mutant), TOV21G (TP53-WT), MKN45 (TP53-mutant), and NUGC4 (TP53-WT) cells treated for 24 hours with XL177A and analyzed by Western blot for p53 and p21. B) MCF7 cells treated for 6 or 24 hours with 1 µM XL177A or XL177B and analyzed by qPCR. Relative expression is assessed using the 2^−ΔΔCt method with at least two biological replicates and three technical replicates. C) MCF7 cells treated for 24 hours 1 µM XL177A, 1 µM XL177B, serum starvation (G1 arrest), or 1 µM taxol (mitotic arrest), ethanol-fixed, PI-stained, and analyzed by flow cytometry. Cell cycle populations were quantified across three biological replicates using FlowJo’s Cell Cycle Analysis tool. D) MCF7 cells treated for 48 hours with XL177A or XL177B, stained with PI and annexin V-FITC, and analyzed by flow cytometry. Staining intensity was quantified using the instrument software for the BD Fortessa analyzer. E) MCF7 cells treated for 24, 72, or 96 hours with XL177A or 24 hours with MG132 and analyzed by Western blot. F) RNA-seq analysis: Spearman correlation coefficient for all transcripts that pass QC across MCF7 cells treated with Nutlin-3A (10 µM), XL177A (1 µM), XL177B (1 µM), GNE-6640 (10 µM), or P22077 (10 µM) for 24 hours. G) RNA-seq analysis: relative expression of all transcripts or transcripts that are members of the direct p53 target gene set (Fischer et al., 2017) across all samples. H) RNA-seq analysis: GSEA for XL177A, Nutlin-3A, P5091, and GNE-6640, reporting the top 5 most correlated and anti-correlated gene sets for each treatment condition. Significant genes from each treatment group were pre-ranked based on LOG2(relative expression)^−LOG10(p value) and analyzed using GSEA software from the Broad Institute (http://software.broadinstitute.org).
after longer exposures to XL177A (Figure 3.2e). Together, these results suggest that selective USP7 inhibition induces cell cycle arrest but not apoptosis in MCF7 cells, and that this effect is likely related to a p53 transcriptional program.

USP7 directly and indirectly regulates protein levels and subcellular localization of multiple transcription factors and epigenetic modifiers including p53, N-Myc, FOXO4, DNMT1, EPOP, and PRC1. In order to confirm the p53 transcriptional effects we had already observed and understand the potential contributions of other USP7 substrates, we evaluated the transcriptome-wide effects of XL177A and XL177B in MCF7 cells. We used a high-throughput 3’ Digital Gene Expression (DGE) RNA-seq assay in a 384-well format, which allowed us to analyze multiple treatment conditions in a single experiment. We treated MCF7 cells for 24 hours with XL177A, XL177B, Nutlin-3A (MDM2-p53 interaction inhibitor), P5091 (USP7 inhibitor from Promega), or GNE-6640 (USP7 inhibitor from Genentech). Overall, we detected transcripts for 7,000-10,000 genes (5387 genes detected across all conditions). A Spearman rank correlation of all genes detected in these samples demonstrated a strong correlation between XL177A and Nutlin-3A ($\rho = 0.62$), while the other three treatment conditions diverged from these treatments and others (Figure 3.2f). In order to look specifically at p53 transcriptional targets we took a list of 350 p53 target genes from a published dataset and assessed the relative expression changes of these genes relative to all detected genes from each sample. We found that the set of p53 target genes was significantly upregulated by Nutlin-3A, XL177A, GNE-6640, and P5091, but not XL177B (Figure 3.2g). These results indicate that, while other reported USP7 inhibitors do induce p53 target gene transcription, their overall transcriptional signature deviates from MDM2 inhibitors, while selective USP7 inhibition closely resembles MDM2 inhibition.
To understand this phenotype more fully, we performed gene set enrichment analysis (GSEA) on all genes whose expression was significantly (p<sub>adj</sub> ≤ 0.05) altered by Nutlin-3A, XL177A, GNE-6640, or P5091 (we did not identify any genes whose expression was significantly altered by XL177B) (Figure 3.2h). We found that the set of direct p53 targets analyzed earlier had the highest enrichment score within the XL177A (normalized enrichment score, NES, = 3.68) and Nutlin-3A (NES = 4.58) data sets and was significantly (FDR ≤ 10%) enriched in the P5091 (NES = 2.09) but not GNE-6640 data sets. A number of additional gene sets associated with DNA damage and cell cycle arrest were also significantly enriched in both the XL177A and Nutlin-3A treatment groups, including additional p53 target gene data sets, upregulated by cisplatin in multiple cell types, and upregulated by ionizing radiation in multiple cell types. Meanwhile, a large number of gene sets associated with proliferation and growth had high negative NES’s across all four data sets, implying that growth and cell cycle arrest is a shared phenotypic consequence of these compounds. Although we identified several genes upregulated by XL177A but not Nutlin-3A, there was an overwhelming correlation between these two data sets: 86 of the 113 gene sets (76%) significantly enriched by XL177A were also enriched by Nutlin-3A.

While we are still pursuing the relevance of additional transcriptional targets of USP7 inhibition, the data from this initial screen pointed to the MDM2-p53 axis as the most important functional consequence of selective USP7 inhibition. We also noted that other previously reported USP7 inhibitors did not produce a strong correlation with Nutlin-3A or point to p53 signaling and the DNA-damage response as particularly relevant, nor did they correlate well with each other. Our current hypothesis is that these compounds are less selective for USP7 and mediate their effects
via multiple targets, complicating deconvolution of their functional consequences. However, it is still possible that, because they possess alternate USP7-binding mechanisms to XL177A, differences in their effects may still be due to on-target USP7 inhibition, which would be an interesting finding. Together, our DGE-RNAseq results encouraged us to pursue a deeper understanding of USP7 inhibitors in the context of functional p53 signaling.

iv. USP7 is a potential therapeutic target in \textit{TP53}-wild-type Ewing sarcoma

As mentioned above, a large proportion of many pediatric tumors have an intact p53 signaling network. Interestingly, many of these same tumors exhibit a high degree of post-translational p53 downregulation (often via MDM2 or MDM4 upregulation), indicating that suppression of p53 signaling may still be necessary for proliferation of these tumors.\textsuperscript{228} Indeed, \textit{TP53} knockout appears to confer a strong growth advantage to \textit{TP53}-WT cancer cell lines. As a follow-up to a genome-scale CRISPR KO screen in 33 cell lines, the Stegmaier lab at Dana-Farber Cancer Institute confirmed that \textit{TP53} knockout confers a strong growth advantage to six of the seven \textit{TP53}-WT cell lines tested.\textsuperscript{229} They also showed that this \textit{TP53} KO response was strongly anti-correlated with several genes in the p53 regulatory network, and that KO of four “druggable” p53 regulatory genes, \textit{MDM2, MDM4, PPM1D,} and \textit{USP7}, was selectively lethal in \textit{TP53}-WT \textit{v. TP53-KO} cells. Because the Stegmaier group studies Ewing sarcoma, and several Ewing lines were included in the original cell line KO experiment, they followed up with individual Ewing lines and confirmed that knockout of \textit{MDM2, MDM4, PPM1D,} or \textit{USP7} was selectively lethal to \textit{TP53}-WT Ewing cell lines. Thus, they established the p53 regulatory network as a potential therapeutic target in the context of Ewing sarcoma.\textsuperscript{184}
Ewing sarcoma is a pediatric cancer that is characterized by fusions of an RNA-binding TET family protein to an ETS family transcription factor, with somatic $EWSR1$-$FLI1$ fusions caused by $t(11;22)(q24;q12)$ the predominant genetic cause. Several preclinical models have shown that EWS-FLI1 is necessary and sufficient for sarcoma formation $in$ $vivo$ and mediates these effects via both DNA- and protein-binding mechanisms. There is evidence that EWS-FLI1 directly suppresses binding of p53 to DNA, which may provide a rationale for the low rate (~5%) of $TP53$ mutations in Ewing sarcoma. Indeed, other than TET-ETS fusions, Ewing sarcoma is characterized as a genetically quiet cancer – the most commonly mutated genes other than $EWSR1$-$FLI1$ are $CDKN2A$ (~25%) and $STAG2$ (~25%). Clinically, Ewing sarcoma is typically diagnosed in ~16-year-old patients as a soft tissue mass (from a progenitor cell of unknown origin) located near a joint. Ewing sarcoma has a 5-year survival rate of ~70 percent, but the prognosis for metastatic or recurrent disease is much worse. In addition, given the “undruggable” nature of EWS-FLI1 and low rate of other mutations, there are currently no targeted therapies available for Ewing patients, and the off-target toxicity (teenage patients are typically rendered infertile upon treatment) and carcinogenicity (Ewing survivors are at significantly elevated risk for developing osteosarcoma secondary to treatment) of chemo- and radiation therapy have a significant impact on disease morbidity. There is thus a significant need to identify new therapeutic targets in Ewing sarcoma.

There are multiple small molecule inhibitors targeting diverse members of the p53 regulatory network, and the Stegmaier lab was able to successfully show that inhibition of MDM2, MDM4, and Wip1 (the phosphatase encoded by $PPM1D$) suppressed growth of $TP53$-WT Ewing sarcoma.
lines. In addition, they performed TP53-KO in two TP53-WT cell lines and demonstrated that the growth suppressive effects of these compounds were p53-dependent. They performed a similar set of experiments with the previously reported USP7 inhibitor P5091 and found results that were suggestive but did not show clear TP53-dependent growth suppression. Given our DGE-RNAseq results and other reports of the effects of P5091 and its analogs, we were not surprised by this result: P5091 is known to signal via both p53-dependent and oxidative stress pathways, and we hypothesized that the non-p53-dependent effects of this compound may be due to non-USP7 targets.

In order to confirm this hypothesis, we tested our USP7 inhibitors, XL188 and XL177A, in the same model systems: a panel of TP53-WT / mutant Ewing sarcoma cell lines and a matched set of TP53-WT / KO cell lines. We tested the control compounds, XL203C and XL177B, in parallel in order to determine whether the growth effects of these compounds were mediated by the extent of USP7 inhibition. For both the reversible and irreversible inhibitors, we found that response to selective USP7 inhibition resembled responses to the dual MDM2/4 inhibitor ATSP-7041: TP53-WT cell lines had increased sensitivity to both compounds relative to TP53 mutants, and that the TP53-KO cells were completely insensitive to USP7 inhibition. Meanwhile, both XL203C and XL177B were significantly less potent than their respective enantiomers, indicating that these effects were due to on-target inhibition of USP7 (Figure 3.3a,b). Interestingly, both XL177A and XL177B demonstrated some generalized cytotoxicity at higher doses (~10 µM). This result suggests that non-specific cysteine labeling may occur with these compounds at high enough concentrations, and care must be taken to ensure a good therapeutic window *in vivo*. In all, these results demonstrate that selective inhibition of USP7 suppresses growth of TP53-WT Ewing
sarcoma cells in a p53-dependent manner. This result, in particular the necessity of intact TP53 for cellular response to USP7 inhibition, is somewhat surprising given that multiple signaling networks have been implicated in USP7 biology, and that USP7 KO is still growth suppressive in a number of TP53-mutant cell lines. We hypothesized that Ewing sarcoma may present the relatively rare case of a single-oncogene-driven cancer, and that other USP7 targets may be more important in more complex disease. In order to test this hypothesis, we assessed the activity of XL177A in a larger panel of cancer cell lines.

v. TP53 status predicts response to USP7 inhibition in multiple cancer cell lines

The Broad Institute recently established a high-throughput small molecule screening assay built on stably bar-coded cancer cell lines treated in a pooled assay format.236 This assay, termed PRISM, allows for bar-code-read-based deconvolution of the proliferative effects of a small molecule probe, in dose, against a panel of 484 cancer cell lines from the cancer cell line encyclopedia (CCLE).237 The use of the CCLE library in this assay format allows integration of the results with the cancer dependency map (DepMap), which contains profiling data on genotype, mRNA expression, protein expression, RNAi response, and KO response for each of these cell lines.238 Todd Golub and colleagues have reported an analysis pipeline that allows for predictive
modeling of potential small molecule therapeutic applications based on comparing PRISM data to what has been described previously in DepMap.236

We tested XL177A and XL177B in dose in the PRISM platform. Due to the relatively weak anti-proliferative potency of XL188, we chose not to assess its activity in parallel, although a future evaluation of XL188 activity at doses up to 100 µM may be worth exploring. In this assay, pools of 20 bar-coded cell lines were treated in parallel in 384-well format for 5 days, then each well was sequenced and benchmarked to a negative (DMSO) and positive (20 µM bortezomib) control. Based on established quality-control criteria, a dose response curve was established for 484 cell lines across 28 lineages. In general, XL177A treatments are highly or moderately correlated at doses above 20 nM, while XL177B doses are only correlated to each other or XL177A at high doses (5 or 20 µM), indicating that USP7 target engagement is likely driving observed growth responses (Figure 3.4a). These dose response curves were integrated to produce an area under the curve (AUC) value for each cell line, and these AUCs were queried against DepMap to produce predictive modeling of responses to XL177A or XL177B based on cell lineage, gene mutational status, mRNA expression, protein expression, RNAi response, or CRISPR response. While we did not test any reported MDM2 inhibitors in our assay, Nutlin-3A is included in the set of PRISM validation compounds, and we were able to perform a post-hoc analysis comparing our compounds to Nutlin-3A. In addition to the general caveats of post-hoc analysis on previously generated data sets, we note here that Nutlin-3A was tested in 6 replicates, while XL177A and XL177B were only tested in 3, giving larger p values for similarly distributed data. Still, we continued to use comparison to Nutlin-3A as a benchmark for XL177A activity.
3.4 (continued from previous page)

Figure 3.4: XL177A induces p53-dependent growth suppression across multiple cell lines. A) PRiSM analysis: heatmap of Spearman correlation coefficients of all cell lines treated with each dose of XL177A or XL177B. B) PRiSM analysis: GSEA of mRNA expression datasets significantly enriched in XL177A or Nutlin-3 sensitive (S) or resistant (R) cells, listing the top 5 gene sets for each compound. Significant genes were pre-ranked based on LOG2(differential expression)−LOG10(p value) and analyzed using the Broad Institute’s GSEA software. C) PRiSM analysis: correlation between XL177A or Nutlin-3A AUC profile and CRISPR knockout profiles from DepMap (AVANA). Positively correlated datasets are reported with positive LOG(Fold-Change) values. D) PRiSM analysis: correlation between XL177A or Nutlin-3A AUC profile and RNAi knockdown profiles from DepMap (DEMETER). Positively correlated datasets are reported with positive LOG(Fold-Change) values. E) PRiSM analysis: correlation between XL177A or Nutlin-3A AUC profile and DepMap mutational profile. Mutations that confer resistance to inhibitors are reported with positive Difference in Means values. F) A549 or RKO cells expressing FF:Renilla or sgTP53-Renilla-Luc were mixed 1:1, treated with sgRNA targeting USP7 or MDM2, and analyzed by comparative luminescence in a time course. G) PRiSM analysis: enrichment of lineage types that are sensitive (negative Difference in Means) or resistant to XL177A. H) TP53-mutant (black) and TP53-WT cells were treated with XL177A for 5 days and analyzed by Cell Titer Glo.
Multiple hypothesis testing generated a list of genes whose expression, mutational status, knockdown, or knockout was correlated or anti-correlated with XL177A or XL177B treatment. In general, cell lines with intrinsically higher expression levels of DNA-damage response (DDR) genes and p53 target genes were enriched in the XL177A-sensitive population. These gene sets were slightly enriched in the XL177B-sensitive population and strongly enriched in the Nutlin-3A-sensitive population, providing further evidence for the importance of intact p53 signaling in response to these compounds. The XL177A-insensitive population was enriched for a number of gene sets associated with more aggressive growth (Figure 3.4b). Few proteins were detected with differential expression between the sensitive and insensitive populations of XL177A or XL177B, so GSEA was unsuccessful for these data sets.

Instead, we focused on correlations between our datasets and datasets generated by genome-scale shRNA knockdown (KD) or Cas9 knockout (KO) in the CCLE. Importantly, we found that USP7 was the most significantly correlated target gene to XL177A in both the KD and KO data sets (Figure 3.4c,d). USP7 was also correlated to XL177B, which indicates that the effects of higher XL177B doses were still on target for USP7. XL177A response also positively correlates to KO and KD of p53 regulatory genes such as MDM2 and MDM4. A negative correlation between data sets implies that cells that are sensitive to one treatment are insensitive to the other, and we hypothesized that TP53-mutant cells would be sensitive to TP53 KD or KO but insensitive to p53 stabilizing agents. Indeed, we found that TP53 KD and KO were strongly anti-correlated to responses to XL177A and Nutlin-3A (Figure 3.4c,d).
We concluded our data analysis by assessing the enrichment of genetic variants or specific lineages in compound responders or non-responders. The only gene whose alteration was significantly associated with response to XL177A or Nutlin-3A was TP53. As observed in the case of Ewing sarcoma, TP53 mutant cell lines were less sensitive to these compounds than TP53-WT cells across the CCLE (Figure 3.4e). This result, along with our consistent observation of p53 regulatory and target genes enriched in the gene expression and gene modulation data sets, led us to conclude that TP53 mutational status is a key predictor of response to selective USP7 inhibition across multiple cancer types. We collaborated with Bill Hahn’s group at Dana-Farber to demonstrate that this effect holds true for USP7 genetic modulation as well: in a dual luciferase-based growth competition assay, they showed that TP53-KO cells significantly outcompeted TP53-WT cells when a mixed population was exposed to USP7 KO (Figure 3.4f).

We identified several lineages (rhabdoid, gastric, and ovarian) that were enriched in the XL177A-responsive population and discovered that the responsive cell lines from these lineages were largely TP53-WT (Figure 3.4g). In the case of ovarian cancer in particular, this finding is largely in opposition to the natural history of the disease, which has a very high rate of TP53 mutations. We collected cell lines from several lineages and were able, in general, to confirm that XL177A suppressed the growth of the TP53-WT cell lines (Figure 3.3h). However, we did not observe any growth suppressive effect of XL177A against any TP53-mutant cell line in our confirmation set including MKN45, the most sensitive mutant cell line in PRISM. These results suggest that TP53 status rather than specific cell lineage is a better predictor of response to selective USP7 inhibition. That being said, we note that many TP53-WT cancers do not have fully intact p53 signaling, so there will be a spectrum of responses to USP7 inhibition depending on the ability of p53
stabilization to suppress growth of cancer cells. As an example, we found that MCF7 cells, despite being quite responsive to XL177A at the protein and transcription level, were relatively resistant to XL177A-induced growth suppression. This finding suggests that USP7 inhibition should be pursued not in all cancers that have intact p53 signaling, but in cancers that are particularly sensitive to disruption of the p53 regulatory network. At the same time we submitted these findings, Charles Roberts and colleagues reported that MDM2 and MDM4 are therapeutic vulnerabilities in malignant rhabdoid tumor (MRT), which may explain why rhabdoid tumors were some of the most sensitive cell lines we found in the PRISM screen.240

vi. Conclusions and Future Directions

Preclinical validation of therapeutic targets requires careful confirmation via rescue experiments and multiple orthogonal assays, and there is a growing awareness that the biomedical research community is being harmed by a tendency toward over-reaching conclusions from limited experiments.57,58 Today, the majority of published therapeutic targets are subsequently invalidated, leading to a reproducibility crisis similar to that experienced by other fields. There are a number of institutional fixes that have been proposed for this issue, but at a day-to-day level one of the most important things we can do as researchers is to exhaustively validate our findings before reporting them as bona fide therapeutic targets. We believe that small molecule studies have an important role to play in these studies because they allow for dose- and time-dependent responses, activity-dependent responses, and orthogonal validation across multiple assay formats using the same tools.241 That being said, poorly characterized small molecules can produce phenotypes due to unknown off-target effects, and it is of utmost importance to understand the nature of the
compound one is working with before using it as a small molecule probe. In the case of USP7, the lack of selective inhibitors has prevented confirmation of p53 as a therapeutically relevant USP7 target, as a number of p53-independent effects of USP7 inhibitors have been reported, leading multiple groups to identify and implicate other substrates.

As discussed in Chapter II, we have gained a deep understanding of the nature of XL177A as a chemical probe. On the positive side, XL177A is exquisitely selective for USP7 proteome-wide, we understand its mode of binding (irreversible labeling of the active-site cysteine), we are able to assess and confirm its cellular activity via ABPs, and we are able to compare it to a matched control compound with significantly less activity against USP7 (XL177B) as well as a reversible USP7 inhibitor with reduced potency (XL188). We thus decided to employ XL177A in a number of assays in order to assess the effects of selective USP7 inhibition. We included both targeted and unbiased assays in order to answer specific questions about the relevance of p53 and explore whether other USP7 targets may be more or less important.

Our findings with XL188 have been published, and our findings with XL177A are in a manuscript that is currently under review. While these findings are limited to a cellular level, they nevertheless clearly demonstrate an important role for p53 in response to USP7 inhibition. Specifically, we found that USP7 inhibition strongly upregulates p53 signaling and suppresses growth of TP53-WT cells across multiple lineages. We also found that specific cancer lineages that are susceptible to inhibition of p53 regulatory genes are also susceptible to our USP7 inhibitors. And, most importantly, we found that intact p53 is necessary for response to USP7 inhibitors in these TP53-WT cells. Although we were not able to obtain stable USP7 KO cell lines
to confirm that the observed phenotypes were USP7-dependent, we leveraged our selectivity profiling data and matched control compounds to increase our confidence that USP7 was mediating the observed effects of these compounds. We also orthogonally validated some of the observed phenotypes using USP7 KO.

As mentioned in the Chapter II, we are currently pursuing the development of bioavailable XL177A analogs that can be used to confirm these effects in vivo. In particular, we are interested in evaluating USP7 inhibition in the context of Ewing Sarcoma and other potentially p53-sensitive malignancies such as MRT. We are also very interested in understanding the tolerability of USP7 inhibition in vivo. We were struck by the difference in overall cytotoxicity of USP7 inhibition or USP7 KD (which were generally well tolerated) versus USP7 KO (which had a growth suppressive effect in virtually all lines tested) in the Broad CCLE panel. Given that USP7 KO is embryonic lethal in mice, we are interested in understanding whether USP7 inhibition will be less toxic in vivo.

From a mechanistic perspective, there is also more to learn about the link between USP7 and p53. Like USP7, stable MDM2 KO cells are difficult to obtain in a TP53-WT background, and we have not been able to confirm that the effects of these compounds are MDM2-dependent. Given the complex p53 regulatory network and the multiple substrates of USP7, we would not be surprised if a mechanism other than MDM2 degradation is important for XL177A-dependent upregulation of p53 signaling. We are currently considering both targeted and unbiased assays to address this question. From a targeted perspective, USP7 is known to localize to nuclear PML bodies, as do many of its best validated substrates, including ICP0, PTEN, FOXO4,
MDM2,\textsuperscript{247} and p53,\textsuperscript{248,249} USP7 KD has been shown to increase total cellular PML protein levels,\textsuperscript{244} and we hypothesize that PML body ubiquitination dynamics may help to explain the downstream effects of USP7 inhibition. Investigation of this relationship would proceed from confirmation of changes to PML levels, followed by microscopy analysis in a PML$^{+/+}$ and PML$^{-/-}$ background. From an unbiased perspective, we propose a combination screen, which would entail genome-scale KO in an XL177A-sensitive line in order to identify deletions that selectively confer a growth advantage in the presence of XL177A.\textsuperscript{250} With these two approaches, we believe that a better understanding of the USP7-p53 axis will be achievable.

Finally, we are still interested in understanding the relevance of the p53-independent effects of USP7 inhibition. To that end, we have initiated quantitative proteomic screens in collaboration with Eric Fischer’s group to evaluate protein-level changes after XL177A exposure. These results have yielded several putative hits (see Sumner Perera’s undergraduate thesis at Harvard College), but we are still in the early stages of validating their authenticity and relevance. In general, the results of this chapter demonstrate that selective USP7 inhibitors are important tools for phenotypic validation and discovery. We report the novel finding that USP7 inhibition acts predominantly through p53, and that p53 is necessary for response to USP7 inhibition. From a DUB-target-class perspective, we provide evidence that selective DUB inhibitors can be used in a range of cellular assays to gain biological understanding of their targets. This particular finding is one that we hope to generalize, and we discuss additional applications of our inhibitor library toward phenotypic discovery in the following section.
vii. Materials and Methods

Cell lines

HEK293T cells were obtained from the James E. Bradner’s laboratory and were not further authenticated. MM.1S cells were obtained from Kenneth C. Anderson’s laboratory and were not further authenticated. MCF7 and MDA-MB-231 cells were obtained from Jean Zhao’s laboratory and were not further authenticated. NCI-H1975 cells were obtained from Pasi Jänne’s laboratory and were not further authenticated. TOV21G and SKOV3 cells were obtained from Alan D’Andrea’s laboratory and were not further authenticated. MKN45 and NUGC4 cells were obtained from Adam Bass’s laboratory and were not further authenticated. HEK 293AD, G401, G402, MESSA, and SNU1 cells were purchased from ATCC.

293T and 293AD cells were grown in DMEM with high glucose (Gibco 11965-118) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco 16140-071), and penicillin/streptomycin (Gibco 15140-122) and maintained in a humidified 37 °C/5% CO₂ incubator. MCF7, MKN45, SKOV3, NUGC4, TOV21G, MM.1S, NCI-H1975, MDA-MB-231, and SNU1 cells were grown in RPMI (Gibco 11875-119) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco 16140-071), and penicillin/streptomycin (Gibco 15140-122) and maintained in a humidified 37 °C/5% CO₂ incubator. A673 cells were cultured in DMEM + 10% FBS + 1 mM sodium pyruvate + 1%PSQ. TC32 and TC71 cells were cultured in RPMI + 10% FBS +1% PSQ. TC138 and CHLA258 cells were cultured in IMDM + 20% Fetal Bovine Serum + 4mM L-Glutamine + 1X ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid).
G401, G402, and MES-SA cells were cultured in McCoy’s Modified Media (ATCC) + 10% FBS. All cell lines were maintained in 10 cm² tissue-culture treated dishes at 37°C in a 5% CO₂ incubator. All cell lines were verified Mycoplasma-free by the MycoAlert test kit.

*Primary cell cultures*

Primary cells were obtained through written consent under approval of the Dana-Farber Cancer Institute Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) from normal individuals were isolated by density gradient centrifugation through Ficoll-Plaque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 400xg for 25 minutes, followed by two washes in PBS. Cells were then maintained in RPMI media, supplemented with 10% FBS, and maintained in a humidified 37 °C/5% CO₂ incubator.

*HA-Ub-VS competition in native lysate*

HEK 293T or MCF7 cells were pelleted, washed with PBS, lysed on ice in Lysis Buffer A (50 mM Tris pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM TCEP, phosphatase inhibitor cocktails (Sigma P5726 and Calbiochem 524624), and protease inhibitors (pepstatin, leupeptin, PMSF, and aprotinin), and clarified by centrifugation. Protein content was quantified by BCA, and 50 ug of lysate was diluted into 30 uL labeling buffer (50 mM Tris pH 7.6, 5 mM MgCl₂, 0.5 mM EDTA, 250 mM sucrose, 1 mM TCEP) and incubated at room temperature with shaking with the indicated inhibitors for 30 minutes. Samples were then supplemented with 1 uM HA-Ub-VS and incubated at room temperature with shaking for 15
minutes. Reactions were quenched with 4x LDS sample buffer (Thermo Fisher B0007) supplemented with 10% BME, vortexed vigorously, and heated to 95°C for 5 minutes. Samples were resolved by SDS-PAGE and analyzed by Western blot with the indicated antibodies.

Cell treatments

Cells were treated with DMSO or different concentrations of the indicated compounds for varying time courses in the presence or absence of cycloheximide. For the experiments in which cycloheximide was used, cells were treated with compounds for 4 (MM1S) or 14 (MCF7) hours prior to the addition of 50 µg/ml of cycloheximide for an additional two hours. At the end of cell treatment, cells were washed in PBS and lysed in either modified RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris, 150 mM NaCl, 1 mM EDTA) or Lysis Buffer A (see HA-Ub-VS section) containing phosphatase inhibitor cocktails 1 and 2 (Sigma), and protease inhibitors. Protein concentrations were quantified using the BCA protein assay kit (Pierce) and samples were probed by immunoblotting using mdm2 (Santa cruz sc-965), p53 (Cell signaling 9282), p21 (Cell signaling 2947), GAPDH (Cell signaling 2118), USP7 (Cell signaling 4833) antibodies.

Peripheral blood mononuclear cell testing

Peripheral blood mononuclear cells (PBMCs) were generously provided by Dr. Steven Treon and Dr. Guang Yang. PBMCs from normal individuals were isolated by density gradient centrifugation through Ficoll-Plaque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 400xg for 25
minutes, followed by two washes in PBS. Cells were then maintained in RPMI media, supplemented with 10% FBS. Primary cells were obtained through written consent under approval of the Dana-Farber Cancer Institute Institutional Review Board. The trypan blue exclusion assay has been previously described and was used for quantification of PBMCs prior to seeding for CellTiter-Glo Luminescent Cell Viability assays (Promega, Madison, WI). These assays were used for proliferation studies and carried out according to manufacturer instructions. Cell viability is reported as percentage of control (untreated) cells, and error bars represent the standard deviation for each data point.

**Quantitative PCR**

BAX (Hs00180269_m1), CDKN1A (Hs00355782_m1), DDB2 (Hs03044953_m1), GADD45A (Hs00169255_m1), GAPDH (402869), MDM2 (Hs00540450_s1), and TP53 (Hs01034249_m1) Taqman probes were obtained from Thermo-Fisher. After cell treatment under the indicated conditions, total cellular RNA was purified using a Qiagen RNEasy kit (Thermo 74104). 1 µg of RNA was then converted to cDNA using SuperScript III First-Strand Synthesis (Thermo 18080051). cDNA from each sample was then combined with the indicated TaqMan probe and 2x Universal MasterMix (Thermo 4440038) in a 96-well Fast RT-PCR plate (Thermo 4346907). qPCR was performed on an Invitrogen 7500 Fast qPCR instrument and gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method on Graphpad Prism.

**Flow cytometry**
For propidium iodide (PI) staining, treated cells (~1 million per condition) were washed with cold PBS, then fixed in 80% ethanol overnight at -20°C. After fixing, cells were pelleted, washed with PBS, and reconstituted in 500 µL FxCycle PI / RNAse A staining solution (Thermo F10797). Cells were stored overnight at 4°C and analyzed using a BD Fortessa flow cytometer. For PI / Annexin V FITC staining, treated cells were washed with cold PBS and prepared using a Thermo Fisher FITC Annexin V/Dead Cell Apoptosis Kit (V13242). Briefly, cells were washed with cold PBS, resuspended in annexin-binding buffer, diluted to 1 x 10^6 cells / mL, then incubated at RT with FITC annexin V and PI. Cells were then placed on ice and immediately analyzed using a BD Fortessa flow cytometer.

**DGE-RNAseq**

The DGE RNA sequencing was performed following the published method\(^{252,253}\) with modifications as described below. MCF7 cells were seeded in a 384 well plate at 2500 cells per well, allowed to adhere for 24 hours, then treated with two doses in triplicate of XL177A, XL177B, Nutlin-3, or DMSO. After 24 hours of treatment, an EL405x plate washer (BioTek) was used to aspirate the media and wash once with PBS. Cells were lysed in the plate with 10 µl of lysis buffer (1x Qiagen TCL, 1% BME) for 5 minutes at room temperature, then stored at -80°C. Automated liquid handling was performed with assistance from ICCB-Longwood Screening Facility. A BRAVO Automated Liquid Handling Platform (Agilent) was used for RNA extraction as follows. The lysate was mixed and 10 µl was transferred to a 384 well PCR plate. 28 µl of homemade SPRI\(^{254}\) (solid-phase reversible immobilization) beads were added to the lysate and mixed. After 5 minutes, the beads were pulled down by placing the plate on a magnet, then the beads were
washed twice with 80% ethanol. Beads were air dried for 1 minute, then 20 µl of nuclease free water was added, the plate was removed from the magnet, and the beads were resuspended. After 5 minutes, the plate was placed back on the magnet to pull down the beads. The supernatant was transferred to a fresh 384 well plate. RNA quantity was checked with the Qubit Fluorometer and RNA quality was assessed using the Agilent BioAnalyzer RNA 6000 Pico Kit. The BRAVO platform was again used to transfer 5 µl of supernatant was transferred to a fresh 384 well plate with RT master mix and 1 µl of barcoded E3V6NEXT adapters for reverse transcription and template switching. The plate was incubated for 90 minutes at 42˚C, then cDNA was pooled, purified with the QIAquick PCR purification kit. The full-length cDNA was treated with Exonuclease I to remove excess primers for 30 minutes at 37˚C then amplified x5 cycles with Advantage 2 PCR Enzyme System using the SINGV6 primer. The amplified full-length cDNA was purified with Agencourt AMPure XP magnetic beads and quantitated by Qubit Fluorometer. The sequencing library was prepared using the Nextera DNA kit following the manufacturer’s instructions. Briefly, 55ng of cDNA was tagmented for 5 minutes at 55 ˚C then purified using Zymo DNA Clean & Concentrator-5 column, then amplified x7 cycles and purified using a 0.9x ratio of AMPure XP magnetic beads. The library size distribution was assessed by Agilent BioAnalyzer HS DNA Kit before it was quantified by qPCR and sequenced on an Illumina NextSeq at the Biopolymers Facility at Harvard Medical School.

**Analysis:** The bcbio-nextgen single cell RNA-seq analysis pipeline (https://bcbio-nextgen.readthedocs.io/en/latest/) was used to deconvolve the well barcodes and convert reads to counts. Any detected barcode that differed by more than one base from an expected barcode was removed. Unique reads were identified using unique molecular identifiers (UMIs) in order to
remove PCR duplicates. The reads remaining after these filters were aligned to the transcriptome (GRCh38) using RapMap. The R package DESeq2 version 1.20.0 (R version 3.5.1) was used for differential expression analysis. The Molecular Signatures Database (v6.2) was used to compute overlap for the significantly upregulated and downregulated genes (adjusted p value < 0.05 and fold change > 1.5) for XL177A and Nutlin-3 at both doses.

Lentivirus production and transduction

Lentivirus was produced by transfecting HEK-293T cells with the pLentiV2 vector (Addgene plasmid 52961) and the packaging plasmids pCMV8.9 and pCMV-VSVG according to the FuGENE 6 (Roche) protocol. For lentiviral transduction, Ewing sarcoma cells were incubated with 2 ml of virus and 8 μg/ml of polybrene (Sigma-Aldrich). Cells were selected in puromycin (Sigma-Aldrich) 48 h after infection for single knockout experiments. For dual knockout experiments PPM1D, USP7, and MDM4 sgRNA sequences were cloned into a LentiV2 vector with a blasticidin selection marker (Addgene plasmid 83480).

sgRNA sequences

sgRNAs were designed using the Broad Institute’s sgRNA design tool. The following sequences were used as control or to target the respective genes: control sgRNA, 5’-GTAGCGAACGTGTCGCAGC-3’; sgMDM2 2: 5’-AGTTACTGTGTATCAGGCAG-3’; sgMDM2 5: 5’-AGACACTTATACTATGAAAG-3’; sgMDM4 4: 5’-AGATGTTGAACACTGAGCAG-3’; sgMDM4 6: 5’-AAGAATTCCACTGAGTTGCA-3’; sgUSP7 1:
Deletion of TP53 in A549 cells using CRISPR-Cas9

A549 cells were seeded in 6-well dishes (Costar, Corning, NY, USA) in normal culture media (DMEM containing 10% FBS, 1% penicillin/streptomycin, 2X L-glutamine) and transiently-transfected with a Cas9 expression vector (pLX311-Cas9) along with one of several sgRNA expression vectors (pXPR003) at a 10:1 w:w ratio using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA). 48 h after transfection, cells were trypsinized and re-plated in media containing 2.5 μM nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA). Cells were expanded under nutlin-3 selection for 4 weeks to enrich for cells that had deleted WT p53. Thereafter, cells were maintained in media lacking nutlin-3. Stable p53NULL populations were derived from three independent sgRNAs, one of which was chosen for subsequent rescue experiments and genetic screens (sgTP53-4, 5'-CCCCGGGACGATATTGAACAA-3'). Using the Tracking of Indels by DEcomposition algorithm\textsuperscript{260} (TIDE - https://tide.deskgen.com), we confirmed the presence of single base insertions (57.5% of sequences, P = 0.0), and deletions of one base (10.2%, P = 3.5 \times 10^{-59}), two bases (9.8%, P = 4.4 \times 10^{-55}), or four bases (18.3%, P = 3.6 \times 10^{-180}), all of which lead to premature termination codons. Loss of endogenous p53 protein expression was determined in
all populations by immunoblot. Although we could have selected a single clone for these studies, the use of cell populations decreases the likelihood of studying clone-specific effects.

*Drug synergy analysis using Chou-Talalay combination index for Loewe additivity*

Loewe Additivity is a dose-effect approach that estimates the effect of combining two drugs based on the concentration of each individual drug that produces the same quantitative effect\textsuperscript{261}. Chou and Talalay\textsuperscript{262,263} showed that Loewe equations are valid for enzyme inhibitors with similar mechanisms of action, either competitive or noncompetitive toward the substrate. They introduced the combination index (CI) scores to estimate the interaction between the two drugs. If CI < 1, the drugs have a synergistic effect, and if CI > 1, the drugs have an antagonistic effect. CI = 1 means the drugs have an additive effect.

*Cell viability*

Cells were plated in 384-well culture-treated plates and allowed to settle overnight. After drug treatment and appropriate incubation time, cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Luminescence was read on a Fluostar Omega Reader (BMG Labtech).

*PriSM cancer cell line profiling*
Cancer cell line profiling was performed using the Broad’s PRiSM platform. Cell treatment and data analysis were performed as described in Yu et al. Raw Luminex signal was converted to AUC values for each cell line as described. The resulting AUC profile for each compound was modeled against DepMap using the limma package in R. Because hotspot mutation analysis was performed against DepMap, TP53 annotations in Figures 6C and 6D are directly from DepMap. TP53 annotation using the functional scores in Giacomelli et al. also produced significant differences between the functional WT and functional mutant populations (XL177A: mean diff. = 0.072 ± 0.016, p < 0.0001; Nutlin-3: mean diff. = 0.165±0.011, p < 0.0001).

Computational cancer profiling

The Broad Institute’s public DepMap portal was accessed via www.depmap.org/portal/interactive. The top 100 gene profile correlations for USP7 CRISPR (Avana) and Combined RNAi (Broad, Novartis, Marcotte) were downloaded directly from the DepMap portal. TP53 profiling was performed offline after downloading the CRISPR (Avana) and Combined RNAi (Broad, Novartis, Marcotte) gene dependency sets. TP53 annotation was performed manually using the methods in Giacomelli et al., with cell lines with functional scores > 0 annotated as TP53 functional WT and cell lines with functional scores < 0 annotated as TP53 functional mutant. Parametric, unpaired, two-tailed t-tests were performed using GraphPad PRISM.

Dual reporter luciferase competition assay
p53\textsuperscript{WT} and p53\textsuperscript{NULL} A549 cells constitutively expressing firefly luciferase or Renilla luciferase have been described.\textsuperscript{265} Each cell line was infected with lentivirus encoding \textit{S. pyrogenes} Cas9 under control of the human EF1alpha promoter (pLX311) and selected in blasticidin (InvivoGen) (1 mg/mL) (10 µg/mL). To perform the competition assay, Cas9-expressing p53\textsuperscript{WT} cells were mixed at a 1:1 ratio with complementarily-labeled Cas9-expressing p53\textsuperscript{NULL} cells and seeded at 2,500 cells/well in 96-well dishes in 200 µL of normal culture media. The following day, cells were infected with an array of sgRNA-expressing lentiviruses (pXPR003). Twenty-four hours thereafter, the supernatant was removed and fresh media containing puromycin (InvivoGen) (1 µg/mL) was added to select for infected cells. Two days later, cells were split into two new replica plates, and incubated for four more days. One replica plate was subjected to a dual luciferase assay\textsuperscript{266} and luminescence readings were obtained using a Wallac EnVision (Perkin-Elmer). Readings from wells infected with experimental sgRNAs were normalized to wells infected with control sgRNAs, and firefly:Renilla luminescence ratios were calculated to estimate the relative effects of sgRNAs on p53\textsuperscript{WT} versus p53\textsuperscript{NULL} cells within a well. To continue the assay, the second replica plate was passaged at a 1/16 dilution.\textsuperscript{265} The process of reading and re-plating the cells was repeated every 4 days.

\textit{QUANTIFICATION AND STATISTICAL ANALYSIS}

All biochemical curves and statistical analyses were produced using Prism (GraphPad Software).

\textit{DATA AND SOFTWARE AVAILABILITY}
Supplemental data files including raw data from MS experiments can be accessed via the online versions of the peer-reviewed manuscripts\textsuperscript{166,167,184} or by directly contacting the author (nathan.schauer@gmail.com).

\textbf{AUTHOR CONTRIBUTIONS}

Chapter IV. Chemical genomic evaluation of a DUB-targeted library in AML
Author’s note:

This chapter is adapted from work that has been previously published. Ellen Weisberg (Jim Griffin lab) performed the initial Ba/F3 screen in collaboration with Sara Buhrlage, and Dr. Weisberg and I performed assay validation experiments together when I first joined the Buhrlage lab. Specifically, we performed cell treatments, Western blots, proliferation assays, and flow cytometry. I also performed qPCR, cellular target engagement, and lysosome inhibitor rescue assays. Dr. Weisberg also performed co-immunoprecipitation assays. In addition to Dr. Weisberg and myself, several members of the Buhrlage lab and other groups made important contributions to this work: Ilaria Lamberto, Jing Yang, Laura Doherty, Chengcheng Meng (Griffin lab), and Renee Wright (Griffin lab) also performed cell treatments and Western blots. Atsushi Nonami (Griffin lab), Ilaria Lamberto, and Jing Yang performed shRNA knockdown experiments. Ilaria Lamberto and Jing Yang also performed overexpression experiments and co-immunoprecipitation assays. Sophia Adama (Griffin lab) performed flow cytometry. Shruti Bhatt (Anthony Letai lab) performed BH3 priming experiments. Alexandra Christodolou (David Weinstock lab) and Amanda Christie (Weinstock lab) performed \textit{in vivo} and \textit{ex vivo} assays using patient-derived xenografts. Hong Tiv (Prafulla Gokhale lab) performed \textit{in vivo} bioluminescence assays. Maria Stella Ritorto (Matthias Trost lab at University of Dundee) and Virginia de Cesare (Trost lab) performed DUB selectivity profiling by di-ubiquitin MALDI. Hyuk-Soo Seo and Sirano dhe Paganon purified USP10, and Ilaria Lamberto performed enzymatic assays.

\textbf{i. Forward chemical genomics for identification of novel targets in AML}
In the context of genetic phenotypic discovery, forward genetic screens assess the viability or functionality of a number of genetic mutants in a defined phenotypic assay. Sequencing after recovery of the viable or functional mutant population allows the identification of key genes that modulate the defined phenotype. Reverse genetic screens, on the other hand, systematically introduces mutations in a single coding region and then assesses the function and viability of these variants in a battery of assays. Forward genetics is often referred to as the canonical or classical approach to genetic screening (Mendel’s famous pea crossing experiment is an example of forward genetics), but reverse genetics has become increasingly utilized as molecular biology techniques have been improved.268,269

Small molecule screens can be similarly used to identify target-phenotype links in a process known as chemical genomics.162–164 Forward chemical genomic screens assess the activity of a library of bioactive small molecules in a defined phenotypic assay, then deconvolute the small molecule targets to establish a link between a specific protein and the observed phenotype. Meanwhile, reverse chemical genomics entails the development of potent and selective inhibitors of a specific protein target, followed by phenotypic discovery using these compounds in a battery of assays. While small molecule screens have been employed in biological systems for more than a century, the chemotherapeutic discovery experiments of Ehrlich and others are not truly forward chemical genomic screens: forward chemical genomics requires the establishment of a specific link between a target and a phenotype, and the molecular mechanism of many early chemotherapeutics are still not well understood.270,271 Unfortunately, unlike forward genetic screens, target deconvolution after a forward chemical genomic screen is typically not feasible via a standard genetic approaches, and until recently there have been few examples of successful forward chemical genomic
screens. On the other hand, reverse chemical genomics is an important part of most small molecule drug discovery efforts. The USP7 inhibitor development outlined in chapters II and III is a clear illustration of reverse chemical genomics, in which selective inhibitors are developed and characterized, then assessed in a number of phenotypic assays in order to establish a clear relationship between a target (USP7) and a phenotype (p53-dependent growth suppression). A similar approach has been employed in a number of different settings, and the vast majority of small molecule drugs with known biological mechanisms have been discovered using reverse chemical genomics.

One of the potential benefits of target class drug discovery is it allows for the generation of small molecule libraries that are both biased toward a specific enzyme family and profiled for their selectivity within that family. In theory, the molecular targets of hits from these targeted libraries can be deconvoluted using a smaller set of defined assays rather than having to look proteome-wide for the relevant targets (as has been done for compounds such as rapamycin). Recently, this approach has even been formalized into a predictive modeling program based on the phenotypic responses to kinase inhibitor libraries with known selectivity profiles. One of the major findings of this approach is that inhibitor libraries need not be selective as long as their target class selectivity is well annotated and the library as a whole inhibits a significant portion of the target class. This finding raised the possibility that our first-generation DUB inhibitor library, which includes many non-selective compounds, may be sufficient to enable forward chemical genomic screens, and we embarked on a phenotypic discovery effort in collaboration with James Griffin.
Receptor tyrosine-kinase (RTK)-driven tumors are common in acute myeloid leukemia (AML) and many other cancers.\textsuperscript{280-282} In the context of AML, RTK mutations render them constitutively active without exogenous ligand, leading to a range of aberrant hyperproliferative signaling. In general, many RTK-driven tumors display signs of oncogene addiction (strong growth suppression after inhibition or KO of the driver mutation and a high rate of acquired RTK gatekeeper mutations in resistant tumors), which has created significant interest in the development of selective RTK inhibitors.\textsuperscript{283-285} The most commonly activated RTK in AML is Fms-like tyrosine kinase 3 (FLT3), which dimerizes and trans-autophosphorylates upon binding of its endogenous FLT3 ligand (FLT3-L).\textsuperscript{286} Both point mutations (D835Y) and disruption of the intracellular kinase autoinhibitory domain (juxtamembrane domain internal tandem duplication, ITD) lead to constitutive activation of FLT3 and are associated with poor prognosis.\textsuperscript{287} The overall 5-year survival rate of adults with AML is only 24 percent, so FLT3-ITD AML represents a major area of need in targeted therapy.\textsuperscript{233} FLT3 tyrosine kinase inhibitor (TKI) development has followed a similar path to that of epidermal growth factor receptor (EGFR) inhibitors: first generation inhibitors (e.g. midostaurin) produce transient clinical responses and characteristic gatekeeper mutations (F691L and N676D), second generation inhibitors (e.g. gilteritinib) produce more durable responses but still meet with acquired resistance, and third generation inhibitors are able to overcome intrinsic resistance mutations but still fall short of cures, likely due to extrinsic activation of RTK-Ras signaling.\textsuperscript{288} Thus, while there are three FDA-approved FLT3 TKIs and two more that are currently in FDA review, the clinical benefits of FLT3 inhibition have been limited, and there is waning interest in the continued development of increasingly selective FLT3 TKIs.
Instead, there is a growing interest in the identification of novel targets in FLT3-driven AML that may circumvent drug resistance or be efficacious in combination with direct FLT3 inhibition. Because several assays have already been developed to evaluate the FLT3-dependent effects of TKIs, there is also a good set of tools for evaluating new chemical matter in chemical genomic screens. In particular, the Griffin lab has established a murine Ba/F3 model of FLT3-driven AML. While Ba/F3 growth is usually dependent on exogenous IL-3 for growth, they can be transformed to growth-factor independence by ectopic expression of a potent oncogene. Consequently, FLT3-ITD or FLT3-D835Y expression transforms Ba/F3 cells to IL-3-independent growth, while Ba/F3-FLT3-WT are still IL-3-dependent. Performing a dual forward chemical genomic screen in Ba/F3-FLT3-ITD cells with or without IL-3 can help deconvolute growth suppressive phenotypes that are FLT3-dependent (IL-3 rescuable) as opposed to generally cytotoxic (no IL-3 rescue).

In addition to the existing assay platforms for FLT3, we reasoned that DUB inhibition may be a good avenue to regulating constitutively activated FLT3. Phosphorylated RTKs are recognized by the Cbl family of E3 ligases (c-Cbl, Cbl-B, and Cbl-c), which ubiquitinate the RTK to initiate endosomal sorting and desensitize cells to ligand exposure. Cbl proteins are required for proper RTK endocytosis and degradation, and inactivation of their RING E3 ligase domain leads to expression of dominant negative (DN) alleles that prevent RTK degradation. DN Cbl has also been identified as a classical oncogene: the NS1 murine retrovirus encodes v-cbl, a truncated Cbl analog that has no RING domain and induces B cell lymphoma formation in vivo. DN mutations in c-Cbl have been identified in human AML, and these mutant alleles have been shown in vitro to drive AML progression via FLT3. While constitutively phosphorylated FLT3
mutants are often mislocalized, it still appears that c-Cbl mediates FLT3-ITD ubiquitination, and c-Cbl DN mutants act cooperatively with FLT3-ITD to enhance tumorigenicity. We therefore reasoned that increasing FLT3-ITD ubiquitination via DUB inhibition would oppose FLT3-ITD tumorigenicity by enhancing FLT3-ITD degradation. Although several DUBs have been implicated in the endosomal sorting pathway (e.g. USP8 and AMSH / STAMBＰ), we do not know which DUBs regulate FLT3-ITD, and we reasoned that this mutant would be a good candidate for a DUB-targeted forward chemical genomic screen.

ii. HBX-19818 and P22077 induce the degradation of mutant FLT3

To identify targets and compounds that regulate the protein homeostasis of oncogenic FLT3, we used a whole-cell phenotypic screen of 29 reported small-molecule DUB inhibitors representing the majority of reported DUB inhibitors, annotated for inhibitory activity across a broad panel of DUBs with oncogene-dependent and control cell lines, followed by hit validation, target deconvolution and translational studies. We evaluated compounds for their ability to selectively kill growth-factor-independent Ba/F3 cells expressing FLT3-ITD mutant protein and Ba/F3 cells expressing FLT3-D835Y mutant protein versus IL-3-dependent parental Ba/F3 cells. Two chemically distinct hits from the screen, HBX19818 and P22077, both previously reported as USP7 inhibitors, were confirmed to inhibit the proliferation of mutant-FLT3-positive Ba/F3 cells with values of effector concentration for a half-maximum response (EC50) in the single-digit micromolar range. The effects were partially rescued by IL-3, indicating that the growth suppression resulted from impaired FLT3 function (Figure 4.1a). The antiproliferative activity of HBX19818 and P22077 correlated with the loss of FLT3 in FLT3-ITD-expressing Ba/F3 cells at
4.1

(a) Chemical structures of HBX19818 (used as a source of IL-3) following 72 hr of treatment. (n=2).

(b) Effects of P22077 on mutant and wt FLT3 cells following 24 hr of treatment.

(c) Effects of HBX19818 and P22077 on mutant and wt FLT3 cells following 72 hr of treatment. (n=2).

(d) Effects of P22077 on mutant and wt FLT3 cells following 22 hr of treatment.

(e) Effects of HBX19818 on mutant and wt FLT3 cells following 24 hr of treatment.

(f) Western blot analysis of FLT3 phosphorylation and expression in MOLM13 and MOLM14 cells treated with HBX19818.

(g) qPCR analysis of FLT3 ubiquitination and expression in MOLM13 and MOLM14 cells treated with HBX19818.

(h) Western blot analysis of FLT3 expression in Ba/F3-FLT3-ITD cells treated with HBX19818 and Chloroquine.

(i) qPCR analysis of FLT3 expression in Ba/F3-FLT3-ITD cells treated with HBX19818 and P22077.
the same concentrations, and with a more modest loss of FLT3 in FLT3-D835Y-expressing Ba/F3 cells (Figure 4.1b). Consistent with this, flow cytometry showed a loss of cell-surface expression of FLT3-ITD after treatment with HBX19818 (Figure 4.1c). In contrast, FLT3 levels were unchanged in inhibitor-treated Ba/F3 cells that expressed wild-type FLT3 (Figure 4.1b). Owing to a lack of FLT3-D835Y cell lines, in our subsequent studies we focused on the FLT3-ITD mutant.

We confirmed that the effects of HBX19818 and P22077 on cells that expressed mutant FLT3 were not unique to the Ba/F3 system. Both compounds suppressed the growth of the FLT3-ITD AML cell lines MOLM13-luc+, MOLM14 and MV4,11 in a dose-dependent manner with selectivity toward mutant-FLT3-expressing cells versus cells that expressed the wild-type protein.
or FLT3-null cells (Figure 4.1d). It should be noted, however, that several human hematopoietic cell lines not driven by oncogenic FLT3 showed relative sensitivity to P22077, which can probably be attributed to the multi-targeted nature of this agent. Treatment with HBX19818 and P22077 also led to increased apoptotic priming and induction in cells expressing mutant FLT3 relative to those expressing wild-type or no FLT3 (Figure 4.1e). Consistent with data for the Ba/F3 system, HBX19818 and P22077 strongly induced FLT3 degradation in the FLT3-ITD lines MOLM13-luc+ and MOLM14 at 20 µM but had little to no effect on FLT3 levels in leukemia cell lines expressing wild-type FLT3 (Figure 4.1f). As anticipated, we observed inhibition of total cellular tyrosine phosphorylation in HBX19818-treated mutant-FLT3-positive cells, consistent with drug-induced degradation of mutant FLT3 (Figure 4.1f). Treatment with HBX19818 or P22077 did not lead to the degradation of signaling molecules downstream of FLT3, including AKT and ERK1/ERK2, which suggests that the compounds’ induction of mutant-FLT3 degradation was selective (Figure 4.1f).

Consistent with DUB inhibition being required for the reduction in FLT3-ITD protein levels, we observed increased FLT3-ITD ubiquitination 4–8 h after HBX19818 treatment (Figure 4.1g). Ubiquitinated FLT3 can be degraded via both proteasomal and lysosomal pathways, and we attempted to further demonstrate ubiquitin-dependent FLT3 degradation by inhibiting both of these pathways. Unfortunately, proteasome inhibition leads to increased lysosomal degradation of FLT3, and we were unable to find proteasome inhibitor treatment conditions that enabled FLT3 rescue. However, we were able to establish that both HBX19818- and P22077-induced FLT3-ITD degradation are partially rescued by lysosome inhibition (Figure 4.1h). Meanwhile, we confirmed by qPCR that FLT3 transcript levels remain unchanged in FLT3-ITD cells after HBX-19818
treatment. P22077 treatment leads to a slight decrease in FLT3 transcription at 20 µM, which may contribute to its observed protein-level effects on FLT3-ITD at higher concentrations (Figure 4.1i).

iii. HBX19818 and P22077 inhibit USP10

As mentioned above, HBX19818 and P22077 (Figure 4.2a) have both been reported as irreversible inhibitors of USP7. Using di-ubiquitin as substrate, we profiled the compounds in vitro against a panel of 33 recombinant DUBs at a concentration of 10 µM and identified USP10 as the DUB most potently inhibited by each compound (Figure 4.2b). HBX19818 and P22077 inhibited USP10 with IC_{50} values of 14 and 6 µM, respectively, and USP7 with IC_{50} values of 57 and 10 µM, respectively, when tested for dose response in the same assay. We used lysate labeling with HA-Ub-VS to confirm that both compounds inhibited native USP10 in live cells at µM concentrations (Figure 4.2c). In addition, we showed that HBX19818 and P22077 induced loss of Beclin-1 (a previously reported USP10 substrate) protein levels in both Ba/F3-FLT3-ITD and MOLM14 cells (Figure 4.2d). We note that although they have now been validated as USP10 and USP7 inhibitors, both compounds exhibited at least some degree of inhibitory activity against other DUBs, and they may possess additional activity against non-DUB targets, which probably contributed to the antiproliferative effects observed at concentrations below the point at which USP10 was well inhibited.

USP10 has been reported to regulate the localization and stability of the tumor suppressor p53. Because a drug that degrades wild-type p53 could be undesirable from a cancer therapeutic
4.2

b

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standpoint, we sought to determine whether pharmacological USP10 inhibition affects p53 levels in AML cell lines that express the transcription factor. Treatment of MOLM13-luc+ and MOLM14 cells with HBX19818 or P22077 did not result in decreased p53 levels; in fact, if anything, a modest increase in p53 levels was observed (Figure 4.2d). USP10 shRNA KD did, however, result in decreased p53 protein levels (Figure 4.3a). The USP7-inhibitory activity of these compounds may counteract any potential effects on p53 degradation by USP10.

As a first assessment of a potential role for USP10 in FLT3-mutant AML, we evaluated a small series of HBX19818 analogs for inhibitory activity toward USP10 in a biochemical assay, effects on FLT3 protein levels, and antiproliferative effects against Ba/F3 cells expressing FLT3-ITD.
We observed good correlation between USP10 inhibition, FL3 protein levels, and growth suppression, supporting the idea that USP10 is the relevant target of HBX19818. For example, Compound 9, which inhibited USP10 similarly to HBX19818 but did not inhibit USP7 (IC\textsubscript{50} >> 100 µM), suppressed cell growth and induced a loss of FLT3 at similar concentrations. Compound 9 also retained the ability to selectively prime FLT3-mutant cells for apoptosis (Figure 4.2g). The more potent USP10 inhibitor Compound 3 had a lower anti-proliferative EC\textsubscript{50} and induced FLT3 degradation at lower concentrations compared with HBX19818. Compound 3 also maintained specificity for FLT3-mutant cell lines and induced a loss in cell-surface FLT3 expression (Figure 4.2h). Meanwhile, Compound 4 showed little inhibition of USP10 in a purified enzyme assay (IC\textsubscript{50} >>100 µM), a considerably right-shifted antiproliferation curve, and no effect on FLT3 levels at the same concentrations at which HBX19818 degraded FLT3. Together, these results suggest that USP10 inhibition mediates the cellular response to HBX19818.

iv. USP10 directly interacts with FLT3-ITD and selectively affects FLT3-ITD degradation

Having identified USP10 as a potential candidate for involvement in mutant-FLT3 degradation by HBX19818 and P22077, we further investigated its role in FLT3-mutant AML via KD with three separate shRNAs targeting each DUB. USP10 KD with each shRNA resulted in the robust degradation of FLT3-ITD, as well as substantial growth inhibition in FLT3-ITD cells (MOLM13-luc+ and MOLM14), as compared with treatment with the scrambled control shRNA (Figure 4.3a,b). As was observed for treatment with HBX19818 and P22077, USP10 KD had little to no effect on signaling molecules downstream of FLT3, including AKT and ERK1/2 (Figure 4.3a). Effective USP10 KD by the same shRNAs did not suppress the growth of transformed human
4.3

(a) Cell counts (Trypan Blue exclusion assay) determined approximately 1 week after puromycin selection of USP10 shRNA expressing K562, KU812F, and U937 cells.

(b) Effects of USP10 KD on FLT3 expression in wt FLT3 and p53 protein levels in K562 (wt), KU812F (p53), and U937 (p53) cells. (c) Association of endogenous USP10 with FLT3, AKT, and p53 protein levels in MOLM14 and MOLM13-luc+ cells. (d) Association of endogenous USP10 with FLT3 and p53 protein levels in Ba/F3-FLT3-ITD and wt FLT3.

(e) Relative cell count (h) HBX-19818, Cpd 2. (f) Competitive binding assay of Compound 2 against HBX-19818.

(g) Effects of USP10 KD on FLT3 expression in MOLM14 cells overexpressing FLT3-ITD and wt FLT3.

(h) Immunoblot shown is representative of 3 additional experiments for which similar results were observed (additional experiments are shown in Supplementary Figure 7A).

(i) Immunoblot shown are representative of 3 independent studies for which similar results were observed.

(j) USP10 wt and catalytically inactive USP10, USP10C424S.

(k) Effects of USP10 KD on FLT3 expression in MOLM14 cells overexpressing FLT3-ITD and wt FLT3.

(l) Immunoblot shown is representative of 3 additional experiments for which similar results were observed (additional experiments are shown in Supplementary Figure 7A). (g) Association of endogenous USP10 with FLT3, AKT, and p53 protein levels in MOLM14 and MOLM13-luc+ cells. (d) Association of endogenous USP10 with FLT3 and p53 protein levels in Ba/F3-FLT3-ITD and wt FLT3.

(c) Cells are representative of 3 additional experiments for which similar results were observed.
hematopoietic cell lines not driven by oncogenic FLT3 (K052, K562, KU812F and U937) and, similar to USP10-targeted small-molecule inhibition, did not modulate levels of wild-type FLT3 (Figure 4.3a,b). In contrast to the results of USP10 KD, we observed little to no change in levels of FLT3 or Beclin-1 in FLT3-ITD MOLM14 cells after USP7 KD, and transduction with the USP7-targeting shRNAs had little to no effect on cell viability compared with treatment with the scrambled control shRNA (Figure 4.3c,d). USP7 KD was demonstrated to be selective, as levels
of USP10 decreased in USP10-KD cells but remained unchanged in USP7-KD cells. Furthermore, pharmacological inhibition of USP7 with the selective USP7 inhibitor Compound 2 had less of an effect than HBX19818 on cell viability and did not lead to reduced FLT3 levels in FLT3-ITD-expressing Ba/F3 cells at concentrations up to 20 µM (Figure 4.3e,f).

We observed that levels of USP10 were generally higher in most cell lines that expressed higher levels of FLT3, including MOLM14 and MV4,11, consistent with a stabilizing role for USP10 in the regulation of FLT3 (Figure 4.3g). Furthermore, we found that USP10 overexpression correlated with stronger stabilization of FLT3-ITD than of wild-type FLT3 in stably transfected MOLM14 cells and transiently transfected HEK293T cells (Figure 4.3g,h). We note that, similar to their effects in oncogenic-FLT3-driven AML cells, both HBX19818 and P22077 were able to induce the degradation of FLT3 in HEK293T cells, although approximately twofold-higher concentrations were needed to replicate the effects observed with both compounds in mutant-FLT3-driven cells. The introduction of catalytically inactive USP10 (C424S) into MOLM14 cells resulted in reduced stabilization of mutant FLT3 compared with that of the wild-type protein, confirming the importance of USP10’s catalytic activity in regulating FLT3-ITD protein levels (Figure 4.3i). Taken together, the result of our structure–activity relationship, knockdown and overexpression studies strongly support the theory that USP10 is the critical regulator of FLT3-ITD stability; however, they do not address whether this effect is direct or indirect. To answer this, we examined whether USP10 and FLT3 are in a complex in cells that express mutant FLT3. We observed robust co-immunoprecipitation of USP10 with FLT3 in FLT3-ITD-expressing Ba/F3 cells; reverse co-immunoprecipitation studies confirmed the association of FLT3 with USP10 (Figure 4.3j). We found a similar interaction between USP10 and FLT3 in HEK293T cells.
engineered to exogenously express these proteins. We observed that HBX19818 at 2, 4 and 6 h and P22077 at 4 and 6 h blocked the interaction between USP10 and FLT3-ITD (Figure 4.3k).

The observed differential effects on WT and mutant forms of FLT3 with USP10 pharmacological inhibition and knockdown, as well as enzyme overexpression, are in agreement with reports that activated FLT3 is more prone to ubiquitin-mediated degradation. We analyzed the half-lives of WT FLT3 and FLT3-ITD with and without overexpression of USP10 and in the absence and presence of HBX19818 to see whether differences in protein stability might play a role in the differential responsiveness of the two proteins to DUB-inhibitor treatment. In Ba/F3 cells, HBX19818 shortened the half-life of FLT3-ITD from 3–4 h to around 2 h, and shortened the half-life of FLT3-ITD to a greater extent than it did that of wild-type FLT3 (Figure 4.3l). These data suggest that this differential responsiveness to HBX19818 between wild-type FLT3 and FLT3-ITD might be due to modest differences in the inherent overall stability or half-lives of these proteins.

v. Inhibition of USP10 may be a useful therapeutic approach in AML

We sought to confirm that ubiquitin-mediated degradation could be advantageous compared with FLT3 TKI in terms of the ability to override drug resistance. As expected, Ba/F3 cells expressing both FLT3-ITD and a range of tyrosine kinase domain (TKD) point mutations displayed resistance to multiple FLT3 TKIs, validating previous reports of differential resistance to these inhibitors. In contrast, HBX19818 and P22077 treatments were equipotent against FLT3-ITD-expressing Ba/F3 cells with and without the TKD point mutations, but less potent toward Ba/F3 cells engineered to
overexpress wild-type FLT3 (Figure 4.4a). In addition, HBX19818 and P22077 induced the degradation of FLT3 in cells resistant to FLT3 TKI at concentrations that were ineffective in promoting FLT3 degradation in Ba/F3 cells expressing wild-type FLT3 (Figure 4.4b,c). In addition, HBX19818 and P22077 showed similar potency toward parental MOLM13 cells and MOLM13 cells rendered resistant to the FLT3 kinase inhibitor midostaurin after prolonged culture in the presence of the drug (Figure 4.4d). Midostaurin-resistant MOLM13 cells have been characterized as having high overexpression of FLT3, which is believed to contribute to their resistance.308

To further assess the therapeutic potential of USP10 inhibition, we investigated the ability of DUB inhibitors and FLT3 TKIs to interact synergistically. Specifically, we used a median-drug-effect analysis in which we calculated a combination index (CI) from growth-inhibition curves using CalcuSyn software (Biosoft, Cambridge, UK). Dual treatment of Ba/F3-FLT3-ITD, MOM13-luc+, and MOLM14 cells with HBX19818 and either midostaurin or crenolanib at a fixed-ratio serial dilution resulted in decreased cell growth compared with treatment with either agent alone (Figure 4.4e). CI analysis indicated synergistic antiproliferative effects (values less than 1 indicate synergy) at 25%, 50%, 75% and 90% growth inhibition for MOLM13-luc+ cells and Ba/F3 cells, and at 50%, 75% and 90% growth inhibition for MOLM14 cells after concomitant treatment with either kinase inhibitor and HBX19818.

We next evaluated the therapeutic potential of our lead USP10 inhibitor series by testing their growth-inhibitory effects on primary patient tumor samples and patient-derived xenografts (PDXs) ex vivo. USP10 inhibitors HBX19818 and P22077 and selected HBX19818 analogs all caused a
4.4 a) Combination Indexes corresponding to co-treatment of Ba/F3-F
midostaurin, HBX19818, and P22077 on proliferation of MOLM13 and midostaurin-resistant MOLM13 cells. (n=2).

b) Effects of combination of HBX19818 with FLT3-ITD AML primary cells


c) Effects of HBX19818 and P22077 on FLT3-ITD cells or Ba/F3-F

(d-e) Effects of combination of HBX19818 with FLT3-ITD AML primary cells

(f-h) Effects of combination of HBX19818 withFLT3-ITD AML primary cells

(i) Effects of combination of HBX19818 with FLT3-ITD AML primary cells

(j) Effects of combination of HBX19818 with FLT3-ITD AML primary cells

(k) Effects of combination of HBX19818 with FLT3-ITD AML primary cells
Figure 4.4: **USP10 is a potential therapeutic target in FLT3-ITD AML.** A) Relative cell counts of Ba/F3 cells expressing a FLT3 mutants (including several point mutations that confer resistance to FLT3 TKIs) treated for 24 hours with the FLT3 TKI midostaurin, HBX-19818, or P22077. B,C) Ba/F3 FLT3-ITD cells expressing an additional point mutation that confers resistance to FLT3 TKIs (A627T, F691L, or G697R) were treated for 24 hours with HBX-19818 (B) or P22077 (C) and analyzed by Western blot. D) Relative cell counts of parental MOLM13 cells or MOLM13 cells with acquired midostaurin resistance (Weisberg et al., 2011) treated for 24 hours with midostaurin, HBX-19818, or P22077. E) Combination indices of Ba/F3-FLT3-ITD and MOLM14 cells treated with a combination of HBX-19818 and either midostaurin or crenolanib at multiple concentrations based on relative cell count. F) Relative cell count of primary FLT3-ITD AML, primary FLT3-ITD+D835Y AML, or healthy patient-derived bone marrow cells treated for 72 hours with the indicated DUB inhibitors. G) Primary FLT3-ITD+D835Y AML was treated for 24 hours with HBX-19818 and analyzed by Western blot to assess FLT3 levels. H) a primary AML sample was directly engrafted into female NSG mouse, which was treated *in vivo* with P22077 (15 mg/kg, IP QD). Bone marrow was collected 21 days after treatment and analyzed for FLT3 expression by Western blot. I) Relative cell counts of Ba/F3-FLT3-ITD and Ba/F3-FLT3-ITD-luc+ cells treated for 22 hours with HBX-19818 or P22077. J) Ba/F3-FLT3-ITD-luc+ were treated for 26 hours with HBX-19818 or P22077 and analyzed by Western blot. K) Parental Ba/F3 or Ba/F3-FLT3-ITD-luc+ were treated for 24 hours with P22077, stained with a CD135-PE antibody, and analyzed by flow cytometry.
Figure 4.4 (continued from previous page). L) Female NCR-nude mice were engrafted with Ba/F3-FLT3-ITD-luc+ by tail vein injection, then treated with P22077 (50 mg/kg, IP BID) for 4 days. Bone marrow was isolated from the mice and analyzed by flow cytometry either by monitoring luminescence or using a CD135-PE antibody. M) Female NCR-nude mice were engrafted with Ba/F3-FLT3-ITD-luc+ by tail vein injection, then treated with P22077 (50 mg/kg) either IP (BID) or PO (QD). Whole body luminescence was analyzed (Armstrong et al., 2003), and total flux bioluminescence was determined for each treatment arm.

dose-dependent reduction in survival in two each of FLT3-ITD-positive patient samples and PDXs (Figure 4.4f). HBX19818 was less potent toward two donor peripheral blood mononucleated cell (PBMC) samples from healthy donors, and P22077 was less potent toward one of two PBMC samples it was tested against. The selective USP7 inhibitor Compound 2 had little to no effect on the survival of these samples. We obtained enough cells from one PDX to analyze FLT3 levels via immunoblotting, and the results indicated a strong reduction in the amount of FLT3 after 21 h of treatment with either HBX19818 or P22077 at a concentration of 20 µM (Figure 4.4g).

We undertook an in vivo investigation of the effects of USP10 inhibition. Although we had more confidence in the selectivity of the HBX-19818 scaffold for USP10, none of its analogs had not previously been used in vivo, unlike P22077. We determined that, despite its potential off-target effects, P22077 would be a better candidate for in vivo investigation of USP10 inhibition. We administered either vehicle (n = 3) or P22077 (15 mg/kg, n = 3) to FLT3-ITD+ or FLT3-D835Y+ PDX mice intraperitoneally (IP) once per day (q.d.) after disease was detected by flow cytometry. Immunoblot analysis of protein lysates from mouse bone marrow cells, collected after 21 d of treatment and pooled respectively from each treatment group, showed a strong FLT3 signal in vehicle-treated mice that was undetectable in P22077-treated mice, suggesting drug-induced FLT3
degradation in vivo (Figure 4.4h). We note that the mice generally tolerated 15 mg/kg P22077 well over the course of the 21-d treatment period, with little change in weight (approximately 2–3 g of weight lost on average in both vehicle-treated and P22077-treated mice; none of the mice lost more than 15% of their body weight).

In order to evaluate changes in tumor burden in vivo, we administered P22077 in a non-invasive in vivo bioluminescence model of AML. First we confirmed that Ba/F3 cells expressing luciferase-tagged FLT3-ITD (FLT3-ITD–luc+) responded to midostaurin and P22077 similarly to non-luciferase-expressing cells in terms of growth suppression and FLT3 degradation and DUB-inhibitor-induced loss of surface FLT3 expression (Figure 4.4i–k). In a small pilot study, we treated female NCR nude mice harboring Ba/F3-FLT3-ITD–luc+ with 50 mg/kg P22077 twice daily via intraperitoneal injection (n = 4) for 4 d. At the end of the treatment period, bone marrow from P22077-treated mice showed approximately twofold lower FLT3 expression compared with that in bone marrow extracted from vehicle-treated mice (n = 4), as measured by flow cytometry with a phycoerythrin (PE)-conjugated antibody to CD35 (Figure 4.4l). Aliquots of the bone marrow samples showed a similar (approximately two-fold) reduction in luciferase signal in P22077-treated mouse bone marrow samples compared with that in controls. Taken together, these results suggest a reduction in tumor burden via on-target effects. We then carried out a larger, three-arm (n = 8 per arm) study in which we administered 50 mg/kg P22077 twice daily via IP injection, P22077 once daily via oral gavage, or vehicle to female NCR nude mice harboring FLT3-ITD–luc+ Ba/F3 cells. P22077 treatment led to the death of FLT3-ITD-expressing cells in vivo as measured by in vivo bioluminescence, with a statistically significant decrease in leukemia burden compared with that in vehicle-treated mice after 4–6 d of treatment (Figure 4.4m). We did
not observe any significant difference in weight between vehicle- and drug-treated mice after up to 11 d of treatment. There was also generally no evidence of vital organ toxicity in the mice.

vi. Conclusions and Future Directions

This work, published in 2017, represents to our knowledge the first example of a successful forward chemical genomic screen focusing on DUBs as a target class. In this work, we established principles for screening validation and target deconvolution that we have continued to apply in other projects. The advantage of the Ba/F3 screening modality is that it allows for selection of hits with apparent target-specific activity, which helps filter out compounds with broad toxicity. Once on-target phenotypic effects are observed, validation experiments focus on confirming that ubiquitin-mediated target degradation drives this phenotype. Again, because the Ba/F3 system focuses on specific transforming oncproteins, validation in this format is straightforward because we can focus on one molecular target. In the case of FLT3-ITD, we first confirmed that compound treatment led specifically to decreases in FLT3 protein (as opposed to transcript) in a ubiquitination and lysosome / proteasome-dependent manner. These findings allowed us to state with high confidence that the effects of these compounds were DUB-mediated. At that point, we were able to initiate target deconvolution studies in order to establish a connection between FLT3 stabilization and a specific DUB. Our target class approach allowed us to rapidly identify candidate targets without the need for additional experiments, and fortuitously both hit compounds had a common target that was not shared by any other compound in our library (including the reported USP10 inhibitor Spautin 1, which was inactive in our assays). Validation of USP10 as the relevant target in this system began with target engagement studies after live cell treatment. Once
we established that both HBX19818 and P22077 inhibited USP10 *in cyto*, we used targeted KD and overexpression studies to confirm that gene-level *USP10* modulation also induced the expected phenotype of FLT3-ITD degradation. In the end, we were able to state with high confidence that USP10 deubiquitinates FLT3-ITD.

As a next step, we performed several exploratory studies to demonstrate the clinical applicability of USP10 inhibition. These studies began to reveal the drawbacks of our DUB target class library: most compounds are non-selective and weakly potent, which limits one of the major advantages of forward chemical genomics: the ability to rapidly translate screening hits into therapeutically relevant tool compounds. Thus, there was a small therapeutic window between mutant FLT3-expressing cells and WT FLT3-expressing cells, and high compound doses were required for meaningful phenotypes. In addition, the pharmacological properties of the compounds in our DUB targeted library have not been optimized, and we found ourselves in a position where our best-in-class *in vitro* scaffold (HBX19818) was not a good candidate for *in vivo* studies. We instead elected to use P22077, an analog of the reported USP7 inhibitor P5091, and while this compound did produce apparently on-target effects in xenograft models, these experiments are not themselves sufficient to suggest USP10 as a therapeutic target in FLT3-driven AML. We believe that confirmation with more potent and selective bioavailable USP10 inhibitors is still necessary.

The observed drawbacks of our existing DUB inhibitor library for immediate translation after forward chemical genomic screens led us to explore improvements to our existing library. In chapter V, I will discuss recent efforts to expand our annotated DUB inhibitor library.
vii. Materials and Methods

Chemical compounds and biologic reagents

DUB inhibitors HBX19818 and P22077 were purchased from Medchem Express and dissolved in DMSO to obtain a 10 mM stock solution. HBX19818 analogs were purchased from ChemDiv and dissolved in DMSO to obtain a 10 mM stock solution. UPLC-MS analysis of all compounds was consistent with reported purity and molecular weight. Serial dilutions were then made, to obtain final dilutions for cellular assays with a final concentration of DMSO not exceeding 0.1%.

Antibodies

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): total AKT (rabbit, #9272) and total p44/42 MAPK (Erk1/2) (3A7) (mouse, #9107) were used at 1:1000. Anti-GAPDH (D16H-11) XP (R) (rabbit mAb, #5174) was used at 1:1000. Beclin-1 (rabbit, #3738) was used at 1:1000. USP10 (D7A5) (rabbit, #8501) was used at 1:1000. P53 (rabbit, #9282) was used at 1:1000. β-tubulin (rabbit, #2146s) was used 1:1000.

FLT3/Flk-2 (C-20) (sc-479) and Ub (P4D1) (mouse, sc-8017) were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX) and used at 1:1000 for immunoblotting. Anti-pTyr (mouse, clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY) was used at 1:1000. Anti-HAUSP/USP7 antibody (rabbit, ab4080) and anti-ubiquitin antibody (rabbit, ab7780) were purchased from Abcam (Cambridge, MA) and used at 1:1000.
Cell lines and cell culture

FLT3-ITD- or FLT3-D835Y-containing MSCV retroviruses were transfected into the murine hematopoietic cell line Ba/F3 (IL-3-dependent) as previously described. Nomo-1, P31-FUJ, and NB4 were obtained from Dr. Gary Gilliland. MV4,11 cells were obtained from Dr. Anthony Letai. Hel, K562, THP, U937, TF-1 and K052 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The human FLT3-ITD-postive AML line, MOLM14, was obtained from Dr. Scott Armstrong, Dana Farber Cancer Institute (DFCI), Boston, MA. The human FLT3-ITD-positive AML line, MOLM-13 (DSMZ (German Resource Centre for Biological Material), was made to express luciferase fused to neomycin phosphotransferase (pMMP-LucNeo) as previously described.

All cell lines were cultured at a concentration of 2x10^5 to 5x10^5 in RPMI (Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum (FBS) and supplemented with 2% L-glutamine and 1% penicillin/streptomycin. Exceptions include TF-1 and OCI-AML5 cells, which were cultured in RPMI media with 10% FBS and supplemented with 2% L-glutamine and 1% pen/strept and human GM-CSF (2 ng/mL). Parental Ba/F3 cells were cultured in RPMI with 10% FBS and supplemented with 2% L-glutamine and 1% penicillin/streptomycin and 15–20% WEHI (as a source of IL-3). All cell lines were passaged in 5% CO_2 at 37°C.

Cell lines used in this study were submitted for cell line authentication within 6 months of manuscript preparation through cell line short tandem repeat (STR) profiling (DDC Medical,
Fairfield, OH and Molecular Diagnostics Laboratory, Dana Farber Cancer Institute). All cell lines matched ≥80% with lines listed in the ATCC or DSMZ Cell Line Bank STR and were confirmed to be virus- and Mycoplasma-free.

PBMCs were generously provided by Dr. Steven Treon and Dr. Guang Yang.

Immunoblotting and immunoprecipitation

Preparation of protein lysates, immunoblotting, and immunoprecipitation were carried out as described previously.\textsuperscript{251}

Labeling with HA-ubiquitin-vinylmethylsulfone (HA-Ub-VS)

MOLM14 cells were treated for three hours with P22077 and Ba/F3-FLT3-ITD cells were treated for 7 hours with HBX-19818. Cells were harvested, washed with PBS, and lysed in 1% NP-40, 10% glycerol, 2% sodium orthovanadate, and HALT protease inhibitor cocktail (ThermoFisher). Lysate was diluted to 50 μg in 30 μL lysis buffer with 1 mM DTT and incubated on ice for 15 minutes. 0.25 μg HA-Ub-VS was added, and the sample was gently rocked at room temperature for 30 minutes, then denatured with LDS sample buffer. 12 μg lysate was separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked in milk, and treated with a USP10 antibody ((D7A5) (rabbit, #8501) (Cell Signaling, Danvers, MA). After washing, the membrane was treated with a 780-nm IRdye goat anti-rabbit IgG (Licor) and imaged using an Odyssey scanner (Licor).

Quantitative polymerase chain reaction (qPCR)
Ba/F3 cells were treated with the indicated compounds for 23 hours, then harvested and washed with PBS. mRNA was extracted using the RNEasy Mini Kit (Qiagen) and converted to cDNA using SuperScript III reverse transcriptase (ThermoFisher) and a SimpliAmp thermal cycler (ThermoFisher). Real-time PCR was carried out in a 96-well plate using TaqMan probes and a 7500 FAST Real-Time PCR system (ThermoFisher). Relative gene expression was calculated by comparison to a GAPDH reference probe.

Chloroquine rescue

Cells were plated in 24-well plates and 25 uM chloroquine was added. After 60 minutes, the indicated concentration of HBX-19818 or P22077 was added. After 3 or 7 hours for P22077 or HBX-19818, respectively, cells were harvested, washed with 1x PBS, and lysed. 30 ug lysate was separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked in milk, and treated with a FLT3 antibody (Santa Cruz). After washing, the membrane was treated with a horseradish peroxidase-conjugated goat anti-rabbit IgG, incubated with Peirce ECL Western Blotting Substrate (ThermoFisher) and imaged in a dark room.

Proliferation studies

The trypan blue exclusion assay was used for quantification of cells prior to seeding for CellTiter-Glo Luminescent Cell Viability assays (Promega, Madison, WI) and carried out as described previously.\textsuperscript{251} CellTiter-Glo Luminescent Cell Viability assays, carried out according to
manufacturer instructions, were used for proliferation studies. Cell viability is reported as percentage of control (untreated) cells, and error bars represent the standard deviation for each data point.

AML patient cells

Mononuclear cells were isolated from mutant FLT3-positive AML patient samples. Cells were tested in liquid culture (DMEM, supplemented with 20% FBS) in the presence of drug. All blood and bone marrow samples from AML patients were obtained under approval of the Dana Farber Cancer Institute Institutional Review Board. Primary AML 1: Female; 59 years old; <5% bone marrow blasts; 2.6K WBC count; crit: 30; 1% peripheral blasts; previous therapy: 3+7 chemotherapy; cytogenetics: normal; mutations: IDH2 (5%), RUNX1 (15%), SRSF2 (16.8%), FLT3-ITD (24 aa). Primary AML2: Male; 69 years old; 90% bone marrow blasts; 23K WBC count; crit: 24; 5% peripheral blasts; previous therapy: azacytidine, cytarabline, high dose Ara-c; cytogenetics: normal; mutations: SRSF2 (54%), ASXL1 (46%), RUNX1 (39.4%), TET2 (ins) (46%), TET2 (point mutation) (2.8%), TET2 (del) (3.5%), FLT3-ITD (51 aa).

PEI transfection of 293T cells

HEK 293T cells were cultured in DMEM containing 10% FBS, at 37°C, 5% CO2 incubator and transfected using Polyethylenimine (PEI) (Polysciences) according to the manufacturer’s instructions. Ba/F3-FLT3-ITD and MOLM14 cells were maintained in RPMI 1640 medium containing 10% FBS, at 37°C, 5% CO2 incubator.
For the endogenous ubiquitination assay, Ba/F3-FLT3-ITD or MOLM14 cells were treated with HBX19818 or P22077 or DMSO control for 4 or 24h at 0, 5, 10, 20uM, cells were collected and then lysed. Immunoprecipitation was carried out using an anti-FLT3 antibody. Immunoblots were analyzed using anti-ubiquitin or anti-FLT3 antibodies.

**Drug combination studies**

For synergy studies, cell viability was initially determined using the trypan blue exclusion assay to quantify cells for cell seeding. Following this, the CellTiter-Glo Luminescent Cell Viability assay (Promega, Madison, WI) was carried out to measure cell growth. Single agents were added simultaneously at fixed ratios to cells. Cell viability was expressed as the function of growth affected (FA) drug-treated versus control cells, and data were analyzed by CalcuSyn software (Biosoft, Ferguson, MO and Cambridge, UK). The CalcuSyn program was utilized for synergy measurement and based on isobologram generation and the method of Chou-Talalay,\textsuperscript{312} which utilizes the median effect principle to quantify drug combination effects to determine whether the effects of agents administered together are greater than that expected from a simple addition of their individual effects. After determining the ED\textsubscript{50} or IC\textsubscript{50} of each drug, combinations were studied where the concentrations were multiples, or fractions, of the ED/IC\textsubscript{50}. Specifically, concentrations of DUB inhibitor and kinase inhibitor were tested alone and combined as follows: 0.25x IC50, 0.5x IC50, IC50, 2x IC50, and 4x IC50. CalcuSyn program-generated combination index (CI) values allow for a quantitative measurement of synergism, where synergism is defined
by a CI<1, an additive effect is defined by a CI=1, and antagonism is defined by a CI>1. Statistical analysis is automatically part of the computations.

Protein expression and purification

A construct of human USP10 covering residues 376–798 in the pET28a vector was over-expressed in E. coli BL21 (DE3) in TB medium in the presence of 50 mg/ml of kanamycin. Cells were grown at 37°C to an OD of 0.8, cooled to 17°C, induced with 500 μM isopropyl-1-thio-D-galactopyranoside, incubated overnight at 17°C, collected by centrifugation, and stored at −80°C. Cell pellets were sonicated in buffer A (50 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, 10 mM Imidazole, and 3 mM BME) and the resulting lysate was centrifuged at 30,000 xg for 30 min. Ni-NTA beads (Qiagen) were mixed with lysate supernatant for 30 min and washed with buffer A. Beads were transferred to an FPLC-compatible column and the bound protein was washed with 15% buffer B (50 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, 300 mM Imidazole, and 3 mM BME) and eluted with 100% buffer B. Thrombin was added to the eluted protein and incubated at 4°C overnight. The sample was then passed through a HiPrep 26/10 desalting column (GE Healthcare) pre-equilibrated with buffer A without imidazole, and the eluted protein was subjected to a second Ni-NTA step to remove His-tag and Thrombin. The eluent was concentrated and passed through a Superdex 200 10/300GL column (GE Healthcare) in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM DTT. Fractions were pooled, concentrated to 20 mg/ml, and frozen at −80°C.

Ubiquitin-AMC assay
USP10 activity assay. Recombinant USP10, residues 376–798, was tested for its activity in a Ubiquitin-AMC assay in presence or absence of inhibitors. For this assay, 10 nM USP10 were pre-incubated with different concentrations of inhibitors or DMSO as a control in 50 mM HEPES pH7.6, 0.5 mM EDTA, 11 uM ovalbumin, 5 mM DTT. The reaction was incubated for 6 hours at room temperature prior to the addition of 2 uM Ubiquitin-AMC (Boston Biochem) substrate. The initial rate of the reaction was measured by collecting fluorescence data at one minute interval over 30-minute period using a Clariostar fluorescence plate reader at excitation and emission wavelength of 345 and 445 nm respectively. The calculated initial rate values were plotted against inhibitor concentrations to determine IC₅₀ values.

**MALDI TOF DUB assays**

31 human DUBs were freshly diluted in the reaction buffer (40mM Tris–HCl, pH 7.6, 5mM DTT, 0.005% BSA) at different concentrations. Ubiquitin topoisomers (K63, K48, K11 and M1) were diluted to 0.2 μl/μg in dimer buffer (40mM Tris–HCl, pH 7.6, 0.005% BSA) and used as substrates at a fixed concentration (1.5 μM). The enzymes were pre-incubated with the compounds for 30 min at room temperature at 10 μM final concentration. 0.48 μl of di-ubiquitin topoisomers were added to the reaction mixture to initiate the reaction. The reaction was sealed and incubated for 30 min at room temperature and stopped by adding TFA to a final concentration of 2% (v/v). 1.050 μl of each reaction was copied in a fresh plate and spiked with 0.15 μl of 16 μM ¹⁵N-ubiquitin as internal standard and mixed 1:1 with 2.5 DHAP matrix freshly prepared (7.6 mg of 2,5 DHAP in 375 ml ethanol and 125 ml of an aqueous 12 mg/ml diammonium hydrogen citrate). Reaction and
matrix were mixed and 200 nl of mixture was spotted in duplicate onto MTP AnchorChip 1,536 TF (600 mm anchor, Bruker Daltonics).

Mass spectrometry data was acquired on an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics) with Compass 1.3 control and processing software. The sample carrier was taught before each analysis to optimize and centre laser shooting. Internal calibration was performed before each analysis using the $^{15}\text{N}-\text{Ub}$ peak [M+H]$^+$ average = 8,569.3). Samples were analysed in automatic mode (AutoXecute, Bruker Daltonics). Ionization was achieved by a 2-kHz smartbeam-II solid state laser with a fixed initial laser power of 60% (laser attenuator offset 68%, range 30%) and detected by the FlashDetector at detector gain of x10. Reflector mode was used with optimized voltages for reflector-1 (26.45 kV) and reflector-2 (13.40 kV), ion sources (IonSource-1: 25.0 kV, IonSource-2: 22.87 kV) and pulsed ion extraction (320 ns). An amount of 3,500 shots were summed up in ‘random walk’ and with ‘large’ smartbeam laser focus. Spectra were automatically calibrated on the $^{15}\text{N}-\text{Ub}$ m/z and processed using smoothing (Savitzky–Golay algorithm) and baseline subtraction (‘TopHat’) for reproducible peak annotation on non-resolved isotope distributions: one cycle, 0.2 m/z for the width. For area calculation, the complete isotopic distribution was taken into account. An in-house made script was used to report - $^{15}\text{N}$ and mono-ubiquitin areas; plotting of graphs, calculation of standard deviation and coefficient of variation (%) were processed in Microsoft Excel.

_DUB dilutions and di-Ub topoisomer used in MALDI assays_

USP1: 240 ng/µL, K63
USP2: 60 ng/µL, K63
USP6: 3 ng/µL, K63
OTUB2: 30 ng/µL, K63
USP8: 144 ng/µL, K63
USP5: 24 ng/µL, K63
USP20: 60 ng/µL, K63
OTUD1: 6 ng/µL, K63
CYLD: 240 ng/µL, K63
OTUD5: 300 ng/µL, K63
AMSH: 60 ng/µL, K63
AMSH-LP: 24 ng/µL, K63
USP7: 30 ng/µL, K11
USP27x: 120 ng/µL, K11
Cezanne: 12 ng/µL, K11
USP21: 58.4 ng/µL, K11
USP9x: 170 ng/µL, K11
USP28: 60 ng/µL, K11
OTUD3: 60 ng/µL, K11
USP25: 30 ng/µL, K11
USP10: 240 ng/µL, K11
USP36: 750 ng/µL, K11
USP30: 430 ng/µL, K48
Otulin: 1.2 ng/µL, M1
VCPIP: 500 ng/µL, K48
A20: 60 ng/µL, K48
TRABID: 240 ng/µL, K48
OTUB1: 300 ng/µL, K48
USP4: 120 ng/µL, K48
USP16: 60 ng/µL, K48
USP15: 16 ng/µL, K48
USP30: 3.6 ng/µL, K11

Dynamic BH3 profiling (DBP)

To determine drug-induced changes in mitochondrial priming, we performed dynamic BH3 profiling as previously described. Briefly, 0.4×10^6 cells/well were exposed to drug treatment for 14 hours. At the end of incubation time, cells were washed in PBS, pelleted at 500xg for 5 min and resuspended in MEB buffer. 15µl of cell suspension was added to each well of 384 well plate containing 15µl of MEB buffer containing 20 µg/mL digitonin and BH3 peptides at twice their final concentration and incubated for 60 min at 26°C to allow mitochondrial depolarization. Peptide exposure was then terminated by adding 10µl 4% formaldehyde in PBS for 15 min, followed by neutralization with N2 buffer (1.7M Tris, 1.25M glycine, pH 9.1) for 10 min. To determine cytochrome C levels, anti-cytochrome C clone 6H2.B4 conjugated to Alexafluor 647 (BD Bioscience) was diluted 1:50 in 10X staining buffer (10% BSA, 2% Tween-20 and 0.02% sodium azide in PBS) and 10µl of this antibody containing buffer was added to each well for a
final dilution of 1:400. Cells were stained overnight at 4ºC in dark and data was acquired on BD LSR Fortessa analyzer (BD Biosciences). Priming change (Δ) is calculated by comparing cytochrome C abundance in treated cells to that of DMSO treated control cells.

**shRNA infection and knockdown**

pLKO.1puro lentiviral shRNA vector particles against *USP10* and *USP7* were purchased from Sigma-Aldrich (St. Louis, MO). Cells were incubated with the viral particles in the presence of 8 μg/ml Polybrene for 24 hours, and the cells were selected with 1–2 μg/ml puromycin for 72 hours. Following selection, cells were used for the studies described.

Repeat *USP10* knockdown studies in MOLM14 cells: Viral particles were produced co-transfecting pLKO.1 containing shRNA or scramble (purchased from Sigma-Aldrich) together with psPAX2 (addgene#12260) and pMD2.G (addgene#12259), concentrated using LENTI-X concentrator (Clontech). MOLM14 cells were then infected in presence of 5 ug/ml polybrene and selection was started 48h post infection using 1 ug/ml puromycin.

**Overexpression of USP10 wild-type and mutant in MOLM14 cells**

FLAG-HA-USP10 was a gift from Wade Harper lab [Addgene (#22543)]. This construct was used to create the corresponding USP10 catalytic dead construct (USP10 C424S) using site directed mutagenesis according to the manufacturer’s instruction. Viral particles were produced by co-transfecting USP10 WT, C424S or control vector together with GAG/POL and VSV-G
containing vectors in 293T cells, and concentrated using LENTI-X concentrator (Clontech). MOLM14 cells were then infected in presence of 5 μg/ml polybrene and selection was started 48h post infection using 1 μg/ml puromycin. Expression of exogenous USP10 was confirmed by HA blot.

*Patient-derived xenograft study*

All animal studies were carried out according to protocols approved by the Dana-Farber Cancer Institute’s Institutional Animal Care and Use Committee.

Female NSG mice (6 weeks of age, Jackson Laboratories, Bar Harbor, ME) were administered either vehicle (10% DMSO, +90% D5W IP QD) (n=3) or P22077, 15 mg/kg IP QD (dissolved in 10% DMSO, + 90% D5W) (n=3) for a total of 21 days once leukemia burden reached the following levels as determined by percent double positive CD45+CD33+ cells in the peripheral blood: 2E#0 (vehicle) (3.07%), 2E#1 (vehicle) (0.34%), 2E#30 (vehicle) (1.63%), 2D#0 (P22077, 15mg/kg) (4.68%), 2D#1 (P22077, 15mg/kg) (1.5%), 2E#10 (P22077, 15mg/kg) (0.29%). Mice were sacrificed on day 21 of treatment. Bone marrow was flushed from mouse femurs, and spleens and livers were dissected and preserved first in formalin, followed 24 hours later by preservation in 70% ethanol.

All AML PDX samples used in the studies in this manuscript were obtained through the Public Repository of Xenografts (proxe.org).
**Flow cytometry**

Flow cytometry was carried out as previously described, according to standard protocols.\(^{308}\) Briefly, a Fortessa flow cytometry machine equipped with FACSDiva analytical software was used for analyzing the percentage of FLT3-positive cells.

**Non-invasive in vivo bioluminescence study**

All animal studies were performed according to protocols approved by the Dana-Farber Cancer Institute’s Institutional Animal Care and Use Committee.

Bioluminescence imaging was performed as described previously.\(^{316}\) Briefly, for administration to female NCR-nude mice (6–8 weeks of age; Taconic, NY), virus- and *Mycoplasma*-free Ba/F3-FLT3-ITD-luc+ cells were washed and resuspended in 1X PBS and administered via IV tail vein injection (0.5 × 10^6 cells/250 uL). A sample size of no less than 8 mice per treatment group was chosen to ensure statistical significance. Anesthesized mice were imaged two days following IV-injection of Ba/F3-FLT3-ITD-luc+ cells to generate a baseline used to establish treatment cohorts with matched tumor burden (mice were randomized and investigators were blinded to group allocation), and total body luminescence was measured as previously described.\(^{311}\) Drug treatment commenced two days after cell injection. Mice were treated with vehicle (10% DMSO in 90% [20%] HPBCD, IP BID) (n=8), P22077 (50 mg/kg, 10% DMSO in 90% [20%] HPBCD, IP BID) (n=8), P22077 (50 mg/kg, 10% NMP in 90% PEG300) for the indicated times. Note: One vehicle mouse that showed ≥ 10-fold lower leukemia burden than the other 7 vehicle mice in the vehicle
treatment group across all time points was removed as an outlier from the final statistical analysis.

One P22077 (PO, QD)-treated mouse died prematurely due to technical complications unrelated to treatment and consequently was not imaged with the other 7 mice from this treatment group.

For *in vivo* assessment of FLT3 protein levels in vehicle-treated and P22077-treated mice, 8 female NCR-nude mice (6–8 weeks of age; Taconic, NY), were administered Ba/F3-FLT3-ITD-luc+ cells via tail vein injection as described above. Mice were imaged and randomized 2 days later to generate a baseline used to establish treatment cohorts with matched tumor burden. At this point, mice were treated with vehicle (10% DMSO in 90% [20%] HPBCD, IP BID) (n=4) or P22077 (50 mg/kg, 10% DMSO in 90% [20%] HPBCD, IP BID) (n=4) for a total of 4 days. Bone marrow cell suspensions were then analyzed for FLT3 levels by flow cytometry using a CD135-PE conjugated antibody (Cat. # IM2234U, Beckman Coulter, Marseille, France). Flow cytometry was carried out as previously described, according to standard protocols. Briefly, a FACS Fortessa flow cytometry machine equipped with FACSDiva analytical software was used for analyzing the percentage of FLT3-positive cells.

The statistical significance in bioluminescence between two groups was determined by using the two-tailed Student’s *t*-test. A *P* < 0.05 was considered to be statistically significant. The data had similar variance, and met the assumptions of the tests.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All biochemical curves and statistical analyses were produced using Prism (GraphPad Software).
DATA AND SOFTWARE AVAILABILITY

Supplemental data files including raw data can be accessed via the online versions of the peer-reviewed manuscript or by directly contacting the author (nathan.schauer@gmail.com).

AUTHOR CONTRIBUTIONS

Chapter V. Conclusions and Future Directions: toward an expanded DUB target class library
Author’s note:

The following chapter contains some material that is adapted from a review article that is currently in preparation. Compounds described in this chapter were either purchased from commercial sources or synthesized by me or ChemPartner. I cloned and purified UCHL1 with assistance from Hyuk-Soo Seo, Sirano dhe Paganon, and Ilaria Lamberto. I also performed enzymatic assays, target engagement studies, and sample prep for ABPP. Adrian Chan and Scott Ficarro (Marto lab) performed MS and preliminary data analysis for ABPP selectivity profiling. Virginia de Cesare (Trost lab) performed di-Ub selectivity profiling, and Ubiquigent’s DUBprofiler team performed Ub-Rho selectivity profiling. Xiaoxi Liu, Wanyi Hu, Bin Hu, and Anthony Varca performed chemistry for the development of the current DUB inhibitor library, and Adrian Chan is currently performing ABPP selectivity profiling with those compounds.

i. Future directions: the motivation and framework for an expanded DUB target class library

In Chapters II, III, and IV, we have validated DUBs as druggable targets and demonstrated the utility of DUB-targeted small molecule libraries for both identifying novel inhibitors and discovering novel DUB function. In this chapter, I discuss our preliminary efforts to expand our current target class DUB inhibitor library. By expanding our in-house library, we believe that we will be able to curate a larger list of hit compounds that can be employed in both forward and reverse chemical genomic studies in order to better elucidate our understanding of DUB biochemistry and biology.
We have employed a library v. library screening format to annotate the selectivity of previously reported DUB inhibitors. Specifically, we collected a library of 29 published DUB inhibitors and tested their activity against a library of 32 purified DUBs using a di-Ub MALDI activity assay.\textsuperscript{173} Using this screening format, we have now collected data on 50 published inhibitors against 42 DUBs, but we are interested in collecting data on hundreds of compounds against as many DUBs as technically feasible. With the insights gleaned in our development of XL177A, for our first-generation library we decided to focus our small molecule library on cysteine-reactive scaffolds and to assess the selectivity of these compounds using competitive ABPP in native lysate from human cell lines.

Our decision to focus on cysteine-reactive scaffolds for inhibitor development has been largely inspired by our preliminary SAR around the HBX-19818 warhead. As discussed in Chapter II, we were able to conjugate the 9-Cl-1,2,3,4-tetrahydroacridine scaffold onto a USP7-binding moiety in order to establish a highly potent and selective inhibitor of USP7. In addition to this bivalent approach,\textsuperscript{177} in Chapter IV we also demonstrated that limited SAR around the HBX-19818 scaffold itself affected selectivity of the scaffold for USP7 v. USP10. This finding is similar to one demonstrated previously for the reported USP7 inhibitor HBX41108,\textsuperscript{151} which was modified to tune selectivity for USP8 v. USP7.\textsuperscript{152} This initial evidence for target class SAR inspired us to develop an exploratory library of analogs to recently reported covalent DUB inhibitors. In particular, we focused on two scaffolds reported by Mission Therapeutics, one of which was reported as selective for UCHL1 and USP30,\textsuperscript{317} and one of which was reported as a cell-penetrant and broadly active USP inhibitor.\textsuperscript{318}
Our decision to focus on competitive ABPP for DUB library selectivity profiling was also inspired by our work developing XL177A. In Chapter II, we showed that competitive ABPP expanded our DUB library to 50+ DUBs and allowed us to profile XL177A activity in a native context. In addition, tandem mass tag (TMT) labeling allows us to analyze up to 11 samples in one experiment, enabling comparative and dose-responsive competitive ABPP analysis in parallel. Through a collaboration with Jarrod Marto at Dana-Farber Cancer Institute, we are now able to rapidly perform competitive ABPP in house, and the increased throughput of 11-plex TMT analysis allows us to perform library v. library DUB selectivity profiling at relatively high throughput in-house. In the following section, I provide a brief overview of our first-generation DUB target class library and its early validation.

**ii. Early inhibitor library development and validation**

As part of our ongoing effort to synthesize and profile all reported DUB inhibitors, we synthesized a handful of compounds (which I refer to here as the F series) inspired by a Mission Therapeutics patent targeting UCHL1. These compounds were chosen based on an analysis of the SAR reported in the patent (Figure 5.1a), and in-house enzymatic profiling of UCHL1 validated our hypotheses for the requirements of UCHL1 inhibition with this scaffold (Figure 5.1b). Specifically, we found that a) the N-cyano group, b) 3-(S) stereochemistry for the β-proline, and c) a nitrogen at the 3-position of the 2-amino thiazole or 2-amino imidazole were all required for potent UCHL1 activity. As briefly discussed in Chapter II, target-driven SAR is greatly enabled by structural information regarding enzyme:inhibitor binding as well as cellular biomarkers for target inhibition. Unfortunately, we were unable to obtain UCHL1:F series co-crystal structures or
identify a clear cellular biomarker for UCHL1 inhibition, preventing our ability to perform extensive target-driven SAR for this series. However, given our previous target class SAR studies with the HBX-19818 scaffold, we prioritized the F series for selectivity profiling in order to establish target class SAR for this scaffold.

We profiled the F series compounds by di-Ub MALDI and/or Ub-Rho depending on the availability of each assay (Figure 5.1c). From this series, we identified compounds with good selectivity for UCHL1 (e.g. F70 and F72), compounds that were more multi-targeted (e.g. F21 and F22), and compounds with apparent selectivity toward other DUBs (e.g. F41). In general, we found that relatively small changes to the noncovalent binding portion of the compound conferred significant differences in the target spectrum of the scaffold. These findings agreed with our findings with the HBX-19818 scaffold, where the DUB target spectrum of the scaffold could be changed by relatively small chemical adjustments.

In order to assess the robustness of our competitive ABPP platform, we performed competitive ABPP with a selective (F70) and a non-selective (F22) UCHL1 inhibitor at three doses in HEK293 lysate (Figure 5.1c). Our data with F70 agreed strongly with in vitro profiling, as the three DUBs identified by competitive ABPP were the three most potently inhibited targets of F70 in Ub-Rho profiling. In contrast, F22 appeared significantly less active by competitive ABPP than by Ub-Rho profiling. Of the 11 DUBs that F22 inhibits in Ub-Rho profiling, seven were detected by ABPP, and only two of these targets were significantly inhibited by F22 in our assay.
5.1

required for UCHL1 activity

![Chemical structure](attachment:structure.png)

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In general, we credit the observed difference to concentration effects: first, F22 was profiled in vitro at high a high concentration (100 µM), which may lead to false positives due to non-specific cysteine labeling. In subsequent experiments, we profiled the F series at 10 µM, which may contribute to the increased selectivity observed for compounds such as F70. Second, higher concentrations of compounds are required for robust inhibition of target DUBs in competitive ABPP experiments. For instance, while XL177A completely inhibits USP7 at 10 nM in vitro, by competitive ABPP concentrations closer to 1 µM are required for complete USP7 inhibition. This observed potency is closer to that of XL177A in cyto, which is affected in part by the more complex milieu of the cellular environment. We similarly find that a higher concentration (10 µM) of both F22 and F70 is required for potent UCHL1 inhibition, suggesting that other DUB targets of these compounds may be identified at higher concentrations. In addition to these concentration effects, we have observed several DUBs (including the F70 targets JOSD1 and USP30) that are either not consistently detected by ABPP or detected with only a single unique peptide, which may lead to competition findings in single experiments that are not robust or reproducible. As we are still in the early stages of using our competitive ABPP assay, we have not yet developed hard and fast rules for which DUBs to include or exclude close the detection limit. We anticipate that, as we
perform more competitive ABPP experiments, we will establish a threshold for robust data that will improve our confidence in the bona fide selectivity profile of the compounds we assess.

Based on our results with the F series, we have initiated a combinatorial chemistry program aimed at synthesizing novel compounds with DUB inhibitory activity. The F series is synthesized via a late stage amidation reaction between two fragments (one of which contains the cysteine-reactive N-cyano group), and we are generally approaching the synthesis of this library using a similar approach. It bears mentioning that we employed a similar approach to synthesize XL177A, and thus the USP7 recognition element as well as the HBX-19818 warhead are included in our combinatorial library. In addition to these chemical series, we also explored another scaffold reported by Mission Therapeutics that was demonstrated to inhibit multiple DUBs in cyto. This chemical series also consists of two fragments (one cysteine-reactive) assembled by late-stage amidation, allowing us to include both fragments in our combinatorial library.

In order to further increase the diversity of electrophiles in our library, we analyzed a set of electrophilic compounds which had been previously profiled for their ability to label cysteines proteome-wide. The 52-member library was split between compounds with an α-halo-amide warhead and compounds with an acrylamide warhead. Cravatt and colleagues had performed competitive ABPP with this library using a generally cysteine-reactive iodoacetamide (IA) probe, and we identified several compound-DUB pairs that had been identified in these experiments. We purchased or synthesized 10 compounds from the report and confirmed that five compounds (BC-02, BC-04, BC-08, BC-11, and BC-43) are active against at least one DUBs at a high concentration (500 µM) (Figure 5.2a). All five of these compounds possess an α-Cl-amide
5.2

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b

Fragment Library A + Fragment Library B → Combinatorial Library

HEK293 Lysate, divided

DMSO | Compound 1 | Compound 2 | Compound 3
---|---|---|---

DUB enrichment with biotin-Ub-PA/VME

Streptavidin pulldown, trypsin digest, sample clean-up

TMT 126 | TMT 127N | TMT 127C | TMT 128N | TMT 128C | TMT 129N | TMT 129C | TMT 130N | TMT 130C | TMT 131

LC-MS/MS

Anticipated Data

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warhead, and two (BC-04 and BC-11) have some structural similarity to the broadly DUB-active Mission scaffold. BC-02 and BC-08 are pseudo-stereoisomers, differing only in amide positioning and number of methoxy groups. Given that these five compounds were identified and confirmed as DUB-active, we included their core electrophilic moieties in our combinatorial library as well.

Several members of the Buhrlage group have contributed and are continuing to contribute to the development of our novel target class library. Xiaoxi Liu and Wanyi Hu have synthesized a number of compounds for profiling, and Adrian Chan is performing ABPP with compounds as they are synthesized. We expect that this experimental pipeline (Figure 5.2b) will yield a number of interesting DUB-reactive compounds that may serve as lead scaffolds for future experiments.

iii. Overall conclusions

In conjunction with other groups, we have validated DUBs as a targetable enzyme class, and we can now make rapid progress toward DUB inhibitor development by employing focused libraries
and coupling them with physiologically relevant assay platforms. By leveraging DUB selectivity profiling with structural information, SAR, and orthogonal validation of compound activity, we developed a potent and selective inhibitor of USP7 (Chapter II). Our ability to perform comprehensive DUB and proteome-wide selectivity profiling enabled a reverse chemical genomics discovery experiment in which we employed our selective USP7 inhibitors to identify p53 status as the major predictor of response to USP7 inhibition (Chapter III). We also demonstrated that the DUB inhibitor library we had compiled could be used for forward chemical genomic screens, identifying USP10 as a DUB that selectively regulates levels of FLT3-ITD (Chapter IV). Acknowledging that structural information was a major limiting factor for further development of potent DUB inhibitors, we are developing a target class SAR approach in order to identify potential candidate leads for novel DUBs (Chapter V). In all, these results establish an in-house platform for DUB target class drug discovery, which has already enabled significant biological discovery and will, we hope, yield many new insights into DUB function in human health.

The DUB field is relatively young – the first DUB was discovered 35 years ago, and the first small molecule DUB inhibitor published 15 years ago – but it has matured rapidly and is in the midst of a transformation. As few as two years ago, significant questions still remained as to whether DUB inhibition by a small molecule would be a feasible and generalizable approach. However, in the last several years, potent and selective inhibitors of DUBs spanning multiple DUB families have been reported from multiple sources in industry and academia.\textsuperscript{136–138,142,183,322} Today, the major remaining question is whether targeting DUBs will ultimately prove to be a therapeutically viable strategy. Answering this question requires advancing our biological understanding of DUB
function, and there is evidence from studies targeting DUBs\textsuperscript{167,267,323} and other enzyme families\textsuperscript{324} that potent and selective small molecule probes will be useful tools for furthering this effort. In addition, there is now significant preliminary evidence that a target class approach to DUB inhibitor drug discovery is feasible, which means that DUB-targeted drug discovery platforms are already well positioned to begin targeting new enzyme family members once a new clinically relevant phenotypes is discovered. In the coming years, we anticipate several current strategies to DUB inhibitor discovery to become formalized, routinized, and commercialized in a way that will accelerate the field.

\textit{New substrates for chemical screens:} Access to chemically modified ubiquitin is now feasible using several strategies.\textsuperscript{325} While early DUB screening assays utilized substrates that contained non-native ubiquitin-neo-substrate linkages, in recent years several small molecule discovery efforts have been based on the cleavage of the ubiquitin C-terminus from a substrate peptide.\textsuperscript{322,326} While this screening platform requires some understanding of the DUB’s native function, it provides the benefit of a more physiologically relevant system. In some cases, ubiquitin–peptide screening may also allow for the discovery of inhibitors that alter DUB activity toward a specific substrate by screening against multiple ubiquitinated substrate peptides. From a technical standpoint, ubiquitin–peptide release assays are amenable to detection of cleavage events using systems with significantly improved signal-to-noise and a lower false positive rate, including Förster resonance energy transfer (FRET) and alphaLISA.\textsuperscript{327–330} Ubiquitin–peptide probes have also been adapted to be used as ABPs, providing the possibility of assessing DUB activity in a native milieu.\textsuperscript{331} A number of novel DUB substrates are now available from commercial sources, including FRET probes for all possible di-ubiquitin linkages and fluorescent K63 tetra-ubiquitin
probes for assessing MINDY DUB activity.\textsuperscript{332,333} We anticipate that access to advanced substrates for DUB activity screening will continue to grow, allowing for the adoption of large-scale inhibitor discovery efforts by multiple groups.

\textit{Target class approach}: Given the possibility of target class SAR for DUB-targeted small molecules, routine selectivity screening has become an increasingly important quality control step for DUB inhibitor discovery. Thus far, reports of DUB selectivity screening have been focused on the orthogonal validation of targeted lead compounds. However, we anticipate that in the coming years, selectivity screening will be used to guide the modification of existing chemical matter towards potent inhibitors of novel DUBs. While the conceptual groundwork for this target class approach has already been laid, several resources now exist that will help routinize annotation of DUB off-target screening. Commercial assays now exist for profiling small molecules against a panel of 41 purified DUBs (Ubiquigent DUBprofiler, www.ubiquigent.com). We are also actively using a similar \textit{in vitro} DUB library for an orthogonal, MALDI-based DUB selectivity di-ubiquitin cleavage selectivity screen.\textsuperscript{173} In addition, our group and others have developed platforms for performing competitive ABPP-based orthogonal validation of DUB selectivity.\textsuperscript{112,136,137,167} We have routinely been able to detect 50+ cysteine DUBs in native HEK lysate, providing the added benefit of profiling DUB activity in a native environment. MALDI and ABPP-MS-based DUB selectivity profiling have yet to be commercialized, but as these technologies mature they will provide multiple orthogonal routes to validating DUB inhibitor selectivity. Ubiquigent, which operates the commercial DUBprofiler \textit{in vitro} screening platform, is currently collaborating with the University of Dundee to develop an \textit{in cyto} DUB selectivity profiling assay, and Zhihao Zhuang’s group has recently published its own cell-permeable DUB ABP.\textsuperscript{334} Such an approach
would complete an important toolkit for DUB-targeted selectivity screens. In addition to providing selectivity data for known DUB inhibitors, selectivity profiling can be used to guide the development of new inhibitors and provide more nuanced SAR results on non-selective scaffolds.

**Toward a unified theory of DUB inhibition:** Over the years, the molecular principles governing targeted inhibition of several enzyme families previously thought to be “undruggable” have been elucidated.\(^{335,336}\) There are now multiple examples of selective DUB inhibitors, but these compounds do not yet provide collective insight into the fundamental principles of targeted DUB inhibition. We anticipate that, as more DUB target class SAR and DUB:inhibitor structures become available, several guiding principles of DUB inhibitor may emerge. Ubiquitin-binding induces dynamic rearrangements to the DUB active site.\(^{337-339}\) In general, two rearrangements appear to be highly recurrent: a misaligned catalytic triad brought into a productive conformation on ubiquitin binding, and a blocking loop occluding active site access being displaced on ubiquitin binding. We anticipate the discovery of chemical motifs targeting these structural activation mechanisms and others, leading to the trapping of target DUBs in an inactive conformation (a mechanism analogous to Type II kinase inhibitors).\(^{340}\) However, this strategy remains a hypothesis and requires the generation of a significant amount of new chemical matter for validation. As new DUB inhibitor scaffolds are developed, the molecular basis for DUB inhibition will become increasingly accessible, leading to the development of increasingly potent and tunable DUB inhibitors. In turn, improved DUB inhibitors will enable further biological discovery, including both mechanistic insights into DUB substrate preference and redundancy as well as translational insights into DUBs as therapeutic targets in specific disease settings. Ultimately, we are optimistic
that target class inhibitor discovery will allow DUB inhibitors to enter the clinic for cancer and other diseases.

iv. Materials and Methods

General chemical synthesis methods

All commercially available starting materials were purchased from *Sigma Aldrich*, *Fisher Scientific*, *Oakwood Chemical* and *Combi Block*. All reagents were used as received without further purification. Known compounds were synthesized according to published literature procedures and any modifications are noted. Anhydrous solvents, such as tetrahydrofuran (THF), diethyl ether, dichloromethane (DCM), dimethyl formamide (DMF), dimethylsulfoxide (DMSO), 1,4-dioxane, and toluene (PhMe) were purchased from *Fisher Scientific*, and used as received. If necessary, air or moisture sensitive reactions were carried out under an inert atmosphere of nitrogen.

Removal of solvents was accomplished on a Büchi R-300 rotary evaporator and further concentration was done under a *Welch* 1400B-01 vacuum line, and *Labconco FreeZone 6* plus system. Purification of compounds was performed by normal phase column chromatography using Teledyne CombiFlash chromatography system, and/or reversed phase chromatography on Waters Micromass ZQ preparative system with SunFire® Prep C18 OBD™ 5μM column. The purity was analyzed on Waters Acquity UPLC system. Analytical thin layer chromatography (TLC) plates
were purchased from Fisher Scientific (EMD Millipore TLC Silica Gel60 F254). Visualization was accomplished by irradiation under UV light (254 nm).

All $^1$H-NMR spectra were recorded at 298K on a Bruker ARX 500 (500 MHz) spectrometer. $^{13}$C-NMR spectra were recorded on a Bruker ARX 500 (126 MHz) spectrometer. Samples were dissolved in CDCl$_3$, DMSO-$d_6$, or CD$_3$OD. The spectra were referenced to the residual solvent peak (chloroform-$d$: 7.26 ppm for $^1$H-NMR and 77.16 ppm for $^{13}$C-NMR; DMSO-$d_6$: 2.50 ppm for $^1$H-NMR and 39.25 ppm for $^{13}$C-NMR, CD$_3$OD: 3.31 ppm for $^1$H NMR and 49.00 ppm for $^{13}$C NMR or tetramethylsilane (TMS) as the internal standard. Chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad peak), coupling constants (Hz), and number of protons. Mass spectrometry (LCMS) data were obtained on Waters Acquity UPLC system in positive ESI mode.

**F series synthesis**

F-21, F-22, F-41, F-42, F-43, F-44, F-46, F-58, F-70, F-71, F-72, and F-73 were synthesized by Shanghai ChemPartner Co., Ltd. through a research contract. Synthetic intermediates were assessed by LC/MS, and final products were purified by HPLC, then analyzed by LC/MS and NMR. The general synthetic route to F-22 and its analogs is outlined below. Compounds were dissolved at 10 mM or 100 mM in DMSO and maintained at -20°C for assays.
Cravatt fragments

BC-02 (CAS#57368-84-0, sc-345083), BC-11 (sc-354895), BC-21 (CAS#103951-51-5, sc-279681), BC-32 (CAS#668980-81-2, sc-354613), and BC-43 (CAS#66932-96-5, sc-307626) were purchased from Santa Cruz. BC-03 (CAS#790-75-0, JS-092C) was purchased from Key Organics. BC-04 (T142433-10mg) was purchased from Sigma Aldrich. BC-08 (CAS#111631-72-2, H33763) was purchased from Alfa Aesar. BC-05 and BC-20 were synthesized in-house from commercial starting materials following the synthetic scheme reported by Backus et al.\textsuperscript{320} Compounds were dissolved in DMSO as 50 mM stocks and maintained at -20°C for experiments.
**BC-05**: Acryloyl chloride (80 µL, 1 mmol) was dissolved in anhydrous DCM (4 mL) and cooled to 0°C. A solution of 4-bromophenylaniline (124 mg, 0.5 mmol) and N-methylmorpholine (160 µL, 1.5 mmol) in DCM (2 mL) was then added dropwise. The reaction was stirred for 1 hr at 0°C then allowed to warm to room temperature slowly. After TLC analysis showed disappearance of starting material (5 hr), the reaction was quenched with saturated NaHCO₃ and extracted with DCM. The combined organic layers were dried over anhydrous Na₂SO₄, concentrated in vacuo, and purified by flash chromatography (70% hexanes, 30% EtOAc) as a white solid (50 mg, 33% yield). [M+H] calculated for C₁₅H₁₃BrNO: 302.0175; ESI (m/z): 302.08.

![Chemical structure of BC-05](image)

**BC-20**: α,α-diphenyl-4-piperidinomethanol (911 mg, 3 mmol) was dissolved in anhydrous DCM (15 mL) and cooled to 0°C. To this, anhydrous pyridine (600 µL, 7.5 mmol) was added in one portion, then chloroacetyl chloride (600 µL, 7.5 mmol) dropwise, and the reaction was monitored by TLC until the complete disappearance of starting material and conversion to product was detected (7 hr). The reaction was quenched with H₂O, diluted with DCM, and washed twice with saturated NaHCO₃. The organic layer was concentrated in vacuo and purified by flash chromatography as a white solid (690 mg, 67% yield. [M+H] calculated for C₂₀H₂₃ClNO₂: 344.1412; ESI (m/z): 344.18.

**UCHL1 expression and purification**
A construct of full-length human UCHL1 (residues 1-223) in the pGEX-6P1 vector (which encodes an N-terminal 3C-cleavable GST tag) was overexpressed in E. coli BL21 (DE3) in terrific broth (TB) medium in the presence of 50 μg/ml of ampicillin. Cells were grown at 37°C to an OD of 0.7, cooled to 17°C, induced with 500 μM isopropyl-1-thio-D-galactopyranoside (IPTG), incubated overnight at 17°C, collected by centrifugation, and stored at −80°C. Cell pellets were sonicated in buffer A (25 mM NaPO₄ pH 7.4, 500 mM NaCl, 10% glycerol, 20 mM Imidazole, and 14 mM BME) + 0.1% IGEPAL + 2 mM EDTA, and the resulting lysate was centrifuged at 30,000 xg for 30 min. Glutathione (GSH) beads (Pierce) were mixed with lysate supernatant for 90 min and washed with 20 column volumes of buffer B (20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM TCEP). The beads were then subjected to on-bead PreScission cleavage using 50 μM Human Rhinovirus 3C protease in 6 mL buffer B with overnight rocking at 4°C. Protease solution was eluted from the resin, concentrated, and passed through a Superdex 200 16/60 column (GE healthcare) in a buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. Fractions were pooled, concentrated and frozen at −80°C.

**Ub-AMC**

Ub-AMC experiments were performed as previously described.¹⁶⁷,¹⁸³ For these experiments, we used 5 nM purified UCHL1 and 400 nM Ub-AMC.

**Ub-Rho profiling**
DUB profiling by Ub-Rho was performed by Ubiquigent in their commercial DUBprofiler platform.

MALDI-TOF DUB profiling

DUB profiling was performed as described in Ritorto et al.\textsuperscript{173} UCH DUBs generally have low activity toward di-Ub, and BAP1, UCHL1, UCHL3, and UCHL5 were assessed using Ub-W (ubiquitin with a C-terminal tryptophan extension) as a substrate.

Competitive ABPP profiling

Sample preparation and MS analysis was performed as described in Schauer et al.\textsuperscript{167}

HA-Ub-VS profiling

BC compounds were assessed at a single dose (500 µM) in Ramos and MDA-MB-231 lysates. Ramos cells were obtained from Nathanael Gray’s group and used without further authentication. MDA-MB-231 cells were obtained from Jean Zhao’s group and used without further authentication. Cells were maintained in a 5% CO\textsubscript{2} incubator at 37°C in RPMI-1640 media (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin / streptomycin. Cells were lysed and treated with compounds and HA-Ub-VS according to the protocol in Lamberto et al., Weisberg et al., and Schauer et al.\textsuperscript{166,167,341} Lysates were resolved by SDS-PAGE and analyzed by Western blot.
for either USP7 (CST 4833), USP10 (CST 8501), UCHL3 (CST 8141), or USP16 (Abcam 189838) using GAPDH (CST 2118) as a loading control.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All biochemical curves and statistical analyses were produced using Prism (GraphPad Software).

**DATA AND SOFTWARE AVAILABILITY**

Supplemental data files including raw data and compound validation data may be obtained by contacting the author (nathan.schauer@gmail.com).
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