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Deubiquitinases (DUBs) and DUB inhibitors: a patent review

Pershang Farshi[#1], **Rahul R. Deshmukh**[#1,4], **Joseph O. Nwankwo**[5], **Richard T. Arkwright**[1,2], **Boris Cvek**[6], **Jinbao Liu**[7], and **Q. Ping Dou**[1,2,3,4,#]

[1]Barbara Ann Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, Michigan, USA

[2]Department of Oncology, School of Medicine, Wayne State University, Detroit, Michigan, USA

[3]Department of Pharmacology, School of Medicine, Wayne State University, Detroit, Michigan, USA

[4]Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan, USA

[5]Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Federal University, Ndufu-Alike Ikwo, Ebonyi State, Nigeria

[6]Department of Cell Biology & Genetics, Palacky University, Slechtitelu 11, Olomouc 78371, Czech Republic

[7]State Key Lab of Respiratory Disease, Protein Modification and Degradation Lab, Department of Pathophysiology, Guangzhou Medical University, Guangdong 510182, China

[#] These authors contributed equally to this work.

Abstract

Introduction—Deubiquitinating-enzymes (DUBs) are key components of the ubiquitin-proteasome-system (UPS). The fundamental role of DUBs is specific removal of ubiquitin from substrates. DUBs contribute to activation/deactivation, recycling and localization of numerous regulatory-proteins, thus playing major roles in diverse cellular-processes. Altered DUB activity is associated with multitudes of pathologies including cancer. Therefore, DUBs represent novel candidates for target-directed drug development.

Areas covered—The article is a thorough review/accounting of patented compounds targeting DUBs stratifying/classifying the patented compounds based on: chemical-structures, nucleic-acid compositions, modes-of-action and targeting-sites. The review provides a brief background on the UPS and DUBs involvement. Furthermore, methods for assessing efficacy and potential pharmacological utility of DUB inhibitor (DUBi) are discussed.

Expert opinion—The FDA's approval of the 20S proteasome inhibitors: bortezomib and carfilzomib for treatment of hematological malignancies established the UPS as an anti-cancer

[#]Corresponding author: Q. Ping Dou, PhD, Professor, Barbara Ann Karmanos Cancer Institute, and Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, Michigan, USA. doup@karmanos.org.

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target. Unfortunately, many patients are inherently resistant or develop resistance to proteasome inhibitors (PIs). One potential strategy to combat PI resistance is targeting upstream components of the UPS such as DUBs. DUBs represent a promising potential therapeutic target due to their critical roles in various cellular processes including protein-turnover, localization and cellular homeostasis. While considerable efforts have been undertaken to develop DUB modulators, significant advancement is necessary move DUB inhibitors into the clinic.

Keywords

ubiquitin–proteasome system; deubiquitinating enzymes; DUB inhibitors; cancer; drug discovery; molecular targeting

1. Introduction

1.1 Regulation of Protein Degradation by the Ubiquitin-Proteasome System (UPS)

The UPS consists of a series of enzymatic reactions wherein ubiquitin; a 76 amino acid polypeptide, is conjugated onto specific lysine residues of a variety of protein substrates via conjugation [1]. There are approximately 600 ubiquitin E3 ligases identified, with variable specificities to individual protein-substrates [2]. Once a protein is polyubiquitinated by an E3 Ligases it is designated for degradation under unmodified UPS conditions, and is transported to the multimeric 26S proteasome complex which cleaves it into oligopeptides and releases the ubiquitin moieties which are recycled by the UPS [1] [2]. The 26S proteasome consists of a complex of a 20S core particle consisting of heteromeric β-subunits that possess a protease-like activity, and is capped by two 19S regulatory particles on each end of the 20S core[1] [3]. For a detailed description of the UPS and its components please refer the following reviews [4-7].

Importantly, aberrant proteasome activity has been extensively associated with various pathologies, including but not limited to cancer and multiple neurological disorders [8, 9]. This has led to extensive interest in the molecular machinery that regulates the UPS and modulation of protein-ubiquitination states [10, 11]. The fate of ubiquitinated proteins is determined by: the number of ubiquitin residues (ub-chains), the position of conjugations, and the specific branching patterns of poly-ubiquitin chains [12]. Protein ubiquitination (Ub) is a reversible mechanism (deubiquitination) regulated by DUBs, which has emerged as a cardinal regulatory-target for posttranslational modifications [1] [2] which, like protein phosphorylation, adds to the complexity and sensitivity of the UPS. The complex nature of UPS modulation allows the cell to finely tune its response to different physiological states and stimuli by modulating the fate of key molecules in the cells' molecular-machinery/-systems, and biological-pathways. Ubiquitination status is now recognized as a key post-translational modification that plays a plethora of critical roles in protein turnover as well as variety of signal transduction pathways [2].

1.2 Deubiquitinating or Deubiquitylating Enzymes (DUBs)

DUBs are a class of the human family of proteases that remove ubiquitin or ubiquitin-like proteins from a variety of protein-substrates [13]. DUBs selectively cleave the isopeptide bond present at the C-terminus of ubiquitin molecule [13, 14]. Through this process DUBs

regulate the degradation, and consequently the expression and activity of plethora of critical molecular targets and as a result, DUBs regulate diverse cellular processes and functions (Figure 1) including [15] processing or removal of polyubiquitin moieties or chains on proteins, thereby altering the targeted protein's fate with regards to either degradation by proteasome and/or differential subcellular localization [15, 16]. In addition, DUBs regulate gene expression, apoptosis, cell cycle, DNA repair as well as cytokine signaling [17] which will be further discussed in 'DUBs in other diseases' section.

1.2.1 Mechanisms of actions of DUBs—DUBs have three distinct mechanisms of action (MOA):[2]

1) Generation/release of free ubiquitin (De Novo Ub Synthesis)

2) Cleavage of polyubiquitinated chains

3) Complete removal of ubiquitin chains from ubiquitinated proteins

1.2.1. 1 Generation/release of free ubiquitin (De Novo Ub Synthesis): Ubiquitin is transcribed as a linear fusion of multiple ubiquitin moieties by the ribosome; It is the DUBs activity that generates the free ubiquitin and interestingly, this process could be performed concurrent with translation [18]. This fundamental aspect of DUBs activity is in the process of generating de novo Ub for the UPS.

1.2.1.2 Cleavage of polyubiquitinated chains: DUB-mediated cleavage of polyubiquitinated chains works via a similar mechanism as a protein phosphatase to release phosphorylated groups in the kinase-phosphatase network [19]. Thus DUBs and E3 Ligases regulate protein fate in a similar way that kinases and phosphatases regulate protein activity [19, 20]. However, the complexities of the UPS far exceed the kinase/phosphatase dynamic. As mentioned previously ubiquitin chains of different length and/or branching patterns can modulate the localization of degradation of the ubiquitinated proteins [5, 21]. Therefore, specific cleavage along the ubiquitin chain in different locations can have differential effects on the fate of the ubiquitinated protein, which provides the cells with a complex and dynamic system to modulate protein turnover, activity, expression and localization [19, 22].

1.2.1.3 Complete removal of ubiquitin chains: The complete removal of ubiquitin chains from ubiquitinated proteins generally results in protein stabilization by preventing it from UPS degradation [13, 23]. It may also affect the localization of the protein depending on the target protein/ubiquitin system. In either event, the activity of DUBs always releases the Ub moieties (completely or incompletely) which are then recycled by the UPS to maintain a state of ubiquitin homeostasis [13, 24].

1.2.1.4 DUB specificity: There is substantial scientific evidence demonstrating that DUBs exhibit strong substrate selectivity including protein specificity and specificity for specific branching patters of motifs of poly-ubiquitin chains. The key domain of DUBs is their ubiquitin-binding domain (UBD) [13]. The UBD consists of: A ubiquitin-interacting motif (UIM), a zinc finger ubiquitin-specific protease domain (ZnF-UBP domain), and a ubiquitin-associated domain (UBA domain) [25]. Most research indicates that the ZnF-UBP imparts

the ability of the DUBs to specifically target individual proteins and/or structural-motifs, thereby imparting target specificity for the individual DUBs [13]. It has been hypothesized that almost 95 DUBs are encoded by the human genome [26, 27]. DUBs can be subdivided into five subfamilies that consist of four cysteine proteases: Ubiquitin-specific proteases (USPs), Ubiquitin C-terminal hydrolases (UCHs), Ovarion tumour proteases (OTUs), Machado-Joseph domain proteases (MJD), and a zinc metallo-iso-peptidase and the Herpes virus tegument USPs (e.g. JAB1/MPN/MOV34 metalloproteases/JAMM) [27-29]. In addition, $M48^{USP}$, a novel class of proteases that function as a DUBs but are not in the same gene family**,** has also been reported [13]. This suggests that there may be other gene families that act on the UPS to modulate protein ubiquitination status, which should be considered when designing/developing DUB inhibitors and targeted therapies [30]. Among the DUBs, three DUBs are associated with 19S regulatory particle (RP) in humans [31] [30, 32], including Rpn11, Usp14/Ubp6 and Uch37/UchL5.

1.3 DUBs in cancer

Bortezomib is a 20S proteasome inhibitor (PI) approved by the FDA for the treatment of multiple myeloma (MM) and mantle cell lymphoma. Inhibition of the proteasome by bortezomib was the first clinical validation of targeting the UPS in cancer therapeutics [6] [33-35]. However, soon it was observed that extended treatments with bortezomib were associated with toxicities and drug resistance, limiting its efficacy [36]. Furthermore, PIs have failed to show efficacy in solid tumors in humans [37]. Researchers now propose that targeting specific aspects of the ubiquitin–proteasome pathway upstream of the 20S proteasome (e.g. DUBs) might be advantageous with regards to efficacy as well as adverse effects [38]. The aspect of DUB activity that makes them an attractive therapeutic target for cancer and other diseases is the ability to modulate protein fate in a specific or selective manner/fashion [2] [5]. Therefore, targeting this pathway can modulate the signaling status of the cell by modifying specific “Key Aspects” of the pathological pathways to specifically direct the cell towards death or recovering from the pathological state. For example, a DUB that targets a tumor suppressor protein may be a good target for activation in order to increase the expression of the tumor suppressor by decreasing UPS degradation, alternatively the targeting of a DUB may change the localization of a protein and serve to inhibit oncogenic processes. Conversely, DUBs that target oncogenic proteins may be targeted by compounds to inhibit their activity through degradation by UPS. Development and design of an enzyme selective/specific inhibitor is easier than designing and developing an enzyme activator (due to competitive inhibition and modeling of substrates). Therefore researchers emphasize greater efforts towards the development of DUB inhibitors [2][5]. However, it is important to emphasize the benefits of studying the basic science modulating this complex system in order to determine the intrinsic mechanisms that enhance selective DUB activity endogenously and then we could potentially highjack/utilize these process/ properties to design therapies around nature’s stratagem.

USP and UCH subfamilies of DUBs have been relatively well characterized and fortunately the crystal structures of many DUBs in the USP/UBP class, e.g. USP7 and USP2 have been resolved, leading to molecular recognition profiling studies of these proteases in their active (ubiquitin or ubiquitin aldehyde-complexed) states [39]. These types of studies have

supported the rationale to design and develop DUB inhibitors. Furthermore genomic studies have been identified at least 530 additional genes that might be involved in deconjugation of the ubiquitin molecules. It has been proposed that at least 79 genes from this set encode functional DUBs with multiple potential isoforms [26, 40]. The process of ubiquitin conjugation has been studied extensively but in contrast the study of deubiquitnation activity of DUBs is still in its preliminary stages [39].

Interestingly, early studies suggests that a number of DUBs, such as USP4 (UNP), USP6 (Tre-2), USP8 (UBPY), USP14, USP28 and UCHL5 (UCH37) play a prominent role in cancer development and progression [39]. USP14 is thought to be involved in different cancers including; but not limited to, ovarian and colorectal cancer. Screening for genetic abnormalities in ovarian cancer cell lines *in vitro* as well as the 3T3 focus formation assays point to the role of USP14 in ovarian carcinogenesis [41]. Another study in colorectal cancer patients found that upregulated USP14 expression levels are associated with the pathologic stages as well as liver and lymph node metastases [41-43]. CXCR4 degradation and chemotaxis is controlled due to its deubiquitination by USP14 [44]. Another study suggests that UCHL5 regulates Nuclear Factor-κB (NF-κB) as well as TGF-β/Smad signaling [45]. Overexpression of UCHL5 resulted in enhanced cell proliferation and its knockdown led to apoptosis in A549 lung cancer cells [45]. When tumor samples from 111 patients with esophageal squamous cell carcinoma were analyzed, a direct correlation between the upregulated UCHL5 levels and lymph node metastasis was found [46].

Inhibition of the DUBs that act as oncoproteins as well as activation (or degradation inhibition) of DUBs that serve as tumor suppressor could be a promising therapeutic strategy [39]. USP8 (UBPY), has variety of substrates including the epidermal growth factor receptor (EGFR) leading to degradation [47]. Indeed, inhibition of USP8 either by its knockdown or synthetic small molecule led to attenuation of variety of receptor tyrosine kinase (RTK) activities, resulting in the inhibition of cell proliferation in gefitinib-resistant and -sensitive non-small cell lung cancer (NSCLC) cells [47].

Furthermore, it has been shown that USP14 and UCHL5 are highly expressed in MM cells, when compared to normal plasma cells and in addition, knockdown (siRNA) decreases MM cell viability [48]. Both USP14 and UCHL5 bind reversibly to the 19S RP and are implicated in cancer [48]. Therefore, their inhibition could lead to decrease in the uptake for protein substrates that are destined to be degraded [48]. These studies led to identification of various DUB inhibitors, e.g. b-AP15 (Discussed in detail in section 2.1.1) is a novel inhibitor of 19S regulatory particle that selectively blocks deubiquitinating activity of USP14 and UCHL5 without compromising proteolytic activities of the 20S core particle [49].

Although various DUB inhibitors have been reported in last decade, they are still in preclinical phases and can only be used as research tools. Therefore, further in depth studies are warranted on DUB inhibitors and modulators in order to develop them for clinical purposes [49].

1.4 DUBs in other diseases

DUBS have been implicated in numerous other pathologies including: neurological disorders, autoimmunity, inflammation and microbial infections. However, the precise mechanism of how DUBs contribute to neurological disorders is still unclear. For example; Ataxin-3 has been shown to be mutated in humans, and playing a major role in ataxia however the mechanism is not understood [50]. Various studies have shown conflicting results, whether S18Y allele for UCH-L1 gives protection against sporadic Parkinson's disease or is a risk factor for it [51, 52]. It has been shown that elevated USP16, located on chromosome 21 in partly involved in Down syndrome phenotypes [53]. Many bacteria (e.g. Burkholderia mallei, salmonella) and viruses (e.g. SARS coronavirus PLpro, herpes virus) exploit the host cell machinery and express multifunctional proteases that target multiple host Ubls and ubiquitin so that they will have advantage in infection and pathogenesis of host cells [54]. These multifunctional enzymes are structurally different from human DUBs. This quality could be exploited to design and develop selective inhibitors for pathogenic DUBs [17].

Deubiquitination by A20; an OTU domain containing protein, regulates NF-κB activation and signaling, deregulated A20 activities are associated with prolonged inflammation and autoimmune disorders. Interestingly, various mutations in A20 genes have been detected in and associated with autoimmune disorders and human lymphoma [55]. Also, many DUBs, including: BAP1, USP3, USP7, USP21, USP22, USP49 and BRCA-containing complex (BRCC36) regulate chromatin structure and gene expression by targeting histones; mainly H2A and H2B. This evidence suggests that aberrant DUBS activities lead to alterations in gene silencing, cell cycle and repairs in DNA damage [56].

1.5 DUBs inhibitors

An extensive search for highly-potent, irreversible DUB inhibitors resulted in identification of Ubal (ubiquitin aldehyde) and UbVS (ubiquitin vinyl sulfone) [57]. Unfortunately due to their peptidic nature, high molecular weights and lack of specificity, these agents are not pharmacologically viable in the clinic. However, they can still be used as research tools to study DUBs in normal cells, cancers, as well as virally infected cells. Indeed, UbVS and Ubal have been used to image and study the three dimensional structures of functionally active DUBs [58].

UCH proteins have major roles in the recycling of ubiquitin molecules by removing them from the C-terminal adducts [26]. Therefore, efforts were taken to design and develop their inhibitors which led to identification of compounds of isatin O-acyl oximes series (Figure 2, compounds A-C) [58]. They are competitive, reversible and directed compounds targeted against the active site with IC_{50} values in micro-molar range for UCH-L1 with approximately 30-fold higher selectivity over UCH-L3. UCH-L1 inhibits cell proliferation in lung and neuro-blastoma cell lines; when these cell lines were treated with these inhibitors, enhanced cell proliferation was observed, further supporting the anti-proliferative role of UCLH-1 enzymes [59].

Another set of compounds with dihydro-pyrrole backbone and low micromolar IC_{50} values for UCH-L3 was identified (Figure 2, compounds D-F) [58]. Their probable binding site is the active site with competitive mode of binding. Similarly, few isatin derivatives (Figure 2, compounds G-I) showed higher selectivity for UCH-L3 over UCLH-1 enzymes with compound **H** (UCH-L3, **H**) having IC_{50} as 0.6 μM. When ubiquitin-PEST and Z (benzyloxycarbonyl)-LRGG-AMC (7-amino-4-methylcoumarin) were used as substrates to identify the inhibitors of cellular DUBs (cyclopentenone prostaglandin), resulting compounds induce apoptosis in colon cancer cells with accumulation of ubiquitinated proteins [58, 60]. It should be noted that these compounds lack specificity and can inhibit wide range of DUBs [58].

Commercial sources like Hybrigenics have developed high throughput screening assays, by using ubiquitin derivatives as well as specific USP substrates to screen diverse sets of chemicals in order to find inhibitors for USP7/HAUSP. Although the limiting factor of their work is the lack of the specificity of identified inhibitors, other researchers have shown that selective inhibition of specific DUBs is possible [58].

2. Patents involving DUBs and DUB inhibitors

2.1. Category A: Chemical agents patented as DUB inhibitors

***2.1.1 b-AP15* (WO2013058691)—**b-AP15 (Figure 3A) is a novel proteasome-inhibitory agent that acts by abrogating the DUB activity associated with the 19S RP [61]. It selectively inhibits the activity of two DUBs, UCHL5 and USP14 that are associated with 19S RP while not affecting the non-proteasomal DUBs. The inventors propose that b-AP15 may be effective in treating cancer subtypes that are resistant to bortezomib or other PIs. Furthermore, their research shows that compound b-AP15 may be effective in the treatment of other anti-cancer agent refractory cancers [62].

2.1.1.1 Mechanism of Actions (MOA): b-AP15 induces the accumulation of higher molecular weight polyubiquinated proteins relative to bortezomib, leading to an enhanced unfolded protein response (UPR) [48]. Interestingly, p53 status of the cancer cells or overexpression of anti-apoptotic proteins such as Bcl-2, BAX and PUMA did not affect the induction of apoptosis by b-AP15 [63]. Therefore, b-AP15 MOA differs from bortezomib [48, 64]. b-AP15 also decreases viability in MM cell lines as well as MM cells obtained from patients, even in the presence of bone marrow stroma cells [48]. Importantly, under *in vitro* conditions b-AP15 has been shown to overcome the bortezomib resistance. b-AP15 treatment to MM cells arrested their growth due to the down regulation of CDC25C, CDC2 and cyclin B1 with the induction of caspase-dependent apoptosis and activation of unfolded protein response. b-AP15 is well tolerated in *in-vivo* studies using human MM xenograft models which is associated with inhibition of tumor growth as well as prolonged survival [48]. The combination of suberoylanilide hydroxamic acid, lenalidomide, or dexamethasone with b-AP15 showed synergistic anti-MM activity. b-AP15 induced apoptosis is not dependent on TP53 status or BCL2 expression in cancer cells. This preclinical data demonstrates the efficacy of b-AP15 in MM disease models and validates DUBs as potential

therapeutic targets, providing a basis for future clinical studies on USP14/UCHL5 inhibitors in patients resistant to PIs [48].

2.1.1.2 Applications: b-AP15 treatment leads to inhibition of tumor progression in human cancer cells and mouse *in vivo* models of solid tumors (like breast, lung, colon, and head and neck carcinoma), as well as infiltration in acute myeloid leukemia (AML) model, b-AP15 was predicted to be useful for the treatment of multiple myeloma, and other solid tumor malignancies like breast, lung, colon, pancreas, head and neck prostate, pancreas and ovarian cancer, and therefore may be useful in these contexts [6].

***2.1.2 PX-478* (WO/2005/007828)—**PX-478 (S-2-amino-3-[4′-N,N-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride) or melphalan N-oxide (Figure 3B) is a small molecular weight anticancer agent that inhibits HIF-1α signaling by suppressing the hypoxia inducible factor (HIF-1α) protein levels *via* inhibition of a deubiquitinase activity [65, 66].

2.1.2.1 Mechanism of Actions: The inventors found that PX-478's effect is associated with an increased level of HIF-1α polyubiquitination and degradation, independent of other known pathways of HIF-1α regulation [67]. Inhibition of HIF-1α by PX-478 was also associated with inhibition of a cytoplasmic HIF-1 deubiquitinase activity [65]. The compound decreases the hypoxia-induced increase in HIF-1α protein, inhibits HIF-1 transactivation, and decreases the expression of the downstream target genes such as vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in several cancerous cell lines [68]. The inventors demonstrated that PX-478 acts independently of the Von Hippel Lindeau protein (pVHL) pathway [65]. However, the inventors have not identified the DUB targeted by PX-478 [65].

2.1.2.2 Applications: The importance of HIF-1α to cancer is based on the fact that HIF-1α protein is found in a wide variety of human primary tumors but only at very low levels in normal tissues [69]. This is exemplified in the high incidence of tumors such as renal cell carcinoma, pheochromocytoma and hemingioblastoma of the central nervous system in individuals with loss of function of both alleles of the VHL gene leading to elevated HIF-1α levels [70]. In addition, most cases of sporadic renal cell carcinoma are associated with early loss of function of the HVL gene and increased HIF-1α levels. HIF-1α levels are also increased in cancer cells with mutant or deleted PTEN [71, 72]. HIF-1α was therefore described as an important target for anticancer drug development because of the role it plays in regulating the response of growing tumors to hypoxia and PX-478 as an agent that would be effective against such tumors by being selectively activated in hypoxic environments [65].

***2.1.3 Tricyclic heterocyclics* (WO2011094545)—**This patent involves a group of heterocyclic compounds with tricyclic structure represented by the common structural formula as shown in Figure 3C [73]. The W, X, Y, and Z denote different organic species and substituents as defined by the inventors. Compound IU2-6 as shown in the Figure 3D has demonstrated the highest (74%) inhibition of the Usp14/26S activity (Table 1) [74].

2.1.3.1 Mechanism of action: These compounds enhance proteasome activity by inhibiting the deubiquitinating activity of Usp14, thereby enhancing the degradation of abnormal or misfolded proteins, including those associated with human diseases [73].

2.1.3.2 Application: The inventors suggest that enhancement of proteasome activity could be beneficial in proteinopathies and might also be therapeutic for any disease characterized by deficient proteasome activity, or deficient activity of other components of the ubiquitin-proteasome pathway such as in von Hippel-Lindau disease, Angelman syndrome, spinocerebellar ataxia 1, giant axon neuropathy, inclusion body myopathy with Paget disease of bone and frontotemporal dementia (IBMPFD) [73]. In the case of cancer, it was suggested that variety of oncoproteins are proteasome substrates and by enhancing proteasome activity, their ability to promote tumorigenesis could potentially be inhibited [73].

***2.1.4 Azepan-4-ones* (US20140370528A1)—**Linder et al, 2013 [56] invented a compound of the general structure shown in Figure 3E as a potential novel class of proteasome inhibitors of which the known compound b-AP15 is a representative (Figure 3A). The letters d, R, and X represent chemical configurations and substituents (Figure 3E) as explained by the inventors but a simple representative example is the azepan-4-one (Figure 3F) [75].

2.1.4.1 Mechanism of action: Similar to the mode of action exerted by b-AP15, this set of compounds inhibits activity of two DUBs, UCHL5 and USP14 that are associated with 19S RP, and do not affect non-proteasomal DUBs [75].

2.1.4.2 Application: The inventors describe it as an effective means for the treatment of cancer refractory to conventional chemotherapy due to over-expression of the intrinsic apoptosis-inhibitor Bcl-2 and particularly cancers refractory to bortezomib, or an agent sharing the mechanism of bortezomib [76]. Examples of cancer types amenable to treatment by the compounds are given as multiple myeloma and solid tumor malignancies (like lung, prostate, colon, ovary, pancreas, breast, neck and head cancers) [76].

***2.1.5 WPI 130* (WO2012040527A2)—**The inventors [77] disclose compounds useful as DUB inhibitors and methods for use of same. The compounds may be represented by one example named WPI 130 whose chemical structure is shown as Figure 3G, where Pr indicates an n-propyl group [78].

2.1.5.1 Mechanism of Action: WPI 130, a small molecule with DUB inhibitory activity, rapidly induces ubiquitination of Bcr-Abl, resulting in its re-localization from the cytoplasm into compact, intracellular protein complexes called aggresomes. This modification results in the loss of downstream Bcr-Abl oncogenic signaling. The compound also directly inhibits Usp9x, a deubiquitinase recently reported to regulate the stability of Mcl-l, an anti-apoptotic protein expressed in many tumors, including hematological malignancies. Mcl-l is associated with drug resistance and survival in hematopoietic malignancies. WPl 130-mediated Usp9x inhibition is associated with reduced Mcl-l levels, together with blocked Bcr-Abl kinase signaling, resulting in the rapid onset of apoptosis. The inventors' results with WPI 130 suggested that targeting specific ubiquitin cycle regulators may emerge as a

novel therapeutic approach to inhibit oncoprotein signaling and reduce elevated apoptotic thresholds.

2.1.5.2 Application: The disclosed compounds and methods are useful in inhibiting a DUB, inhibiting a UCH catalytic domain, inhibiting or preventing a pathogenic infection, inhibiting survival or proliferation of a cell, treating a neurodegenerative disorder, treating one or more symptoms of a neurodegenerative disorder, treating one or more symptoms of a genetic disorder, and compounds that can inhibit a DUB. The methods and compounds are also useful in treating cancer, e.g., preventing, inhibiting and/or ameliorating a cancer or symptom of cancer. In some cases, the method of treating the cancer comprises inhibiting of a DUB, e.g., a DUB involved in survival or proliferation of the cancer.

***2.1.6 6-Amino-pyrimidines* (WO201405952A3)—**The invention provides a method of synthesizing compounds with the general formula as represented in Figure 3H [79] wherein R^1 is selected from aryl, heteroarylaryl, heteroaryl, and heterocyclyl, and these are optionally substituted with 1 to 5 substituents selected from halo, alkyl, alkoxy, trifluoromethyl, cyano, aryl, or optionally substituted aryl, heteroaryl, heterocyclyl, methylenedioxy, and cycloalkyl. R_2 is similarly multi-substituted as in R_1 with minor variations. R_3 is selected from hydrogen and alkyl, and R_4 is selected from hydrogen, alkyl, alkoxy, amino, alkylamino, dialkylamino, alkylthio, and halo substituents. The compounds function as inhibitors of the USP1/UAF1 deubiquitinases complex [79].

2.1.6.1 Mechanism of Action: The USP l /UAF l complex has been found to deubiquitinate monoubiquitinated PCNA (proliferating cell nuclear antigen) and monoubiquitinated FANCD2 (Fanconi anemia group complementation group D2), which are proteins that play important functions in translesion synthesis (TLS) and the Fanconi anemia (FA) pathway, respectively. Among the human USPs, USP1 occupies a special position as it has been implicated in DNA damage response. Because USP1 plays important roles in the two essential DNA damage response pathways, it represents a promising target for small molecule intervention to improve the efficacy of the commonly used DNA damaging drugs by modulating cells' ability of repairing or tolerating DNA lesions. The compounds of the invention exhibit selectivity for USP1/UAF1 versus USP2, USP5, USP7, USP8, and USP 12/46.

2.1.6.2 Application: The invention is claimed to provide a method of inhibiting a heterodimeric deubiquitinase complex in a cell, which method comprises administering to the cell an effective amount of a compound of the invention. In accordance with a certain embodiment, the heterodimeric deubiquitinase complex is USP1/UAF1. It also provides a method of treating cancer in a mammal in need thereof, comprising administering to the animal a compound or salt of the invention. In accordance with these embodiments, the compound or salt of the invention is administered to the mammal by itself, i.e., without co-administration of an anticancer agent, radiation, or bio therapeutic agent.

Alternatively, the invention provides a method of enhancing the chemotherapeutic treatment of cancer in a mammal undergoing treatment with an anti-cancer agent, which method comprises co-administering to the mammal an effective amount of a compound of the

invention. In certain embodiments, the anti-cancer agent is a DNA damaging agent. The DNA damaging agent can be any suitable DNA damaging agent. In a preferred embodiment, the DNA damaging agent is cisplatin. The DNA damaging agent can also be radiation or a biotherapeutic agent such as antibody.

2.2 Category B: Patents on nucleic acids with activity against DUBs

***2.2.1 Use of siRNA against DUBs* (WO1997006247A2)—**The inventors [80] describe nucleic acid inhibitors of DUBs in the form of siRNAs sufficiently complementary to a USP1 and a UAF1 gene sequences to direct RNA interference against their expression when administered to a cell. In another embodiment, they describe the DUB inhibitor as a combination of a USP1 siRNA or UAF1 siRNA and amifostine, ubiquitin aldehyde, β-lapachone, biomol AP401 and RK-682 [80].

2.2.1.1 Mechanism of action: The invention provides siRNA directed against USP1 gene, causing an increase in the level of PCNA-Ub or FANCD2-Ub in the cell. With increased level of PCNA-Ub or FANCD2-Ub, or both, DNA repair activity in the cell nucleus is increased, which increases the viability of a cell exposed to genotoxic agents such as ionizing radiation, ultraviolet light, and antineoplastic agents.

2.2.1.2 Application: The inhibitors can be used to treat or prevent cancer, bone marrow failure, and damage to cells or DNA resulting from genotoxic agents such as antineoplastic agents, including chemotherapeutic agents and radiation. The inhibitors can also be used to enhance cell survival if administered either before or after radiation exposure. Methods are also provided to enhance chemotherapy or radiotherapy of cancer and to enhance DNA repair. Other embodiments and variations of use for the siRNAs against USP1 and UAF1 genes are described in the patent application.

2.3 Category C: Ubiquitin vinyl sulfone (UBVS), protein-based, site-directed probes for identification and inhibition of deubiquinatinases

2.3.1 Method for site-directed probes for identification and inhibition of deubiquinating enzymes (WO2003091411A2)—Authors of this invention describe a method for identifying and designing site-directed inhibitors for enzymes involved in metabolism of ubiquitin and ubiquitin-like proteins of the cell [81]. The invention also covers methods for identification and modulation of such enzymatic pathways for treatment of disorders associated with this pathway, and compositions for investigation of the biological function of the pathway [81].

2.3.1.1 Mechanism of Action: DUBs are subdivided into ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs) [82]. UCHs and UBPs are thiol proteases with specificity for ubiquitin. Introduction of a suitable electrophile at the C-terminus of ubiquitin allows irreversible trapping of UCHs and UBPs as covalent adducts. Labeling such probes with ^{125}I enabled direct visualization of active DUBs and other proteins involved in ubiquitination and deubiquitination [82]. Vinyl sulfones were employed as the appropriate electrophiles since they are versatile functional groups ideally suited to inhibit thiol proteases [82].

2.3.1.2 Application: Employing the [^{125}I]-UbVS probe, a modification of 6 out of the 17 known and putative yeast deubiquitinating enzymes was achieved, namely Yuh1p, Ubp2p, Ubp6p, Ubp12p and Ubp15p [81]. In mammalian cells, a greater number of polypeptides were labeled, most of which were DUBs, including a DUB that associates with the mammalian 26S proteasome, novel protein USP14, a mammalian homolog of yeast Ubp6p [81].

The authors expatiate on the fact that the ubiquitin pathway has been shown to play an important regulatory role in processes such as cell cycle control, signal transduction and immune response, and has been implicated in the development of cancers and neurodegenerative diseases [83]. Therefore, the provided patent represents methods for identification and modulation of the ubiquitin pathways would serve for investigation of and treatment of disorders associated with this pathway [83].

2.4 Category D: Assay methods for DUB Inhibitors

***2.4.1 Fluorescence detection of DUB activity* (WO2011157982A1)—**The inventors, Komander and Ye [84], of this assay method describe a substrate for measuring the activity of a DUB, comprising a diubiquitin molecule, in which an ubiquitin monomer is labeled with a fluorescent tag. The method also serves as an assay for DUB enzymes using such substrates [84].

2.4.1.1 Mechanism of Action: The mechanism of action for determining enzyme activity depends on a substrate comprising of a fluorescently-labeled diubiquitin molecule wherein cleavage of the diubiquitin molecule is accompanied by fluorescence anisotropy, also referred to as fluorescence polarization, or by Forster Resonance Energy transfer (FRET) [84]. Fluorescence anisotropy measures the tumbling rate of a fluorescent molecule in solution and the latter depends on the size and shape of the molecule. If the molecule changes in size, as when a diubiquitin molecule is cleaved by DUB, the rate of tumbling will change. A reduction in size of the molecule by cleavage will change the tumbling rate, and change the degree of polarization in the emitted light, which may be readily measured [84].

2.4.1.2 Application: The invention provides a method for assaying the activity of a deubiquitinating enzyme, by exposing a test substrate to a DUB and monitoring the cleavage of the substrate by measuring fluorescence polarization anisotropy or FRET [84]. Other embodiments of the invention exist and were sufficiently detailed by the inventors, including one in which a trimer, tetramer or other polymer of ubiquitin may be used [84]. This variation is stated to be advantageous where for example; the DUB to be assayed is inhibited by ubiquitin dimmers [85]. The invention is further described as satisfying the need for an assay of DUB enzymes, which measures the cleavage of a natural ubiquitin linkage, to better reflect the linkage specificity in DUB enzymes [84, 86].

2.4.2 Assay method for modulators of HIF-1α and activity of VDU1 (WO2004064711A2)—Bernards [87] describe an invention of assay methods for identifying modulators of HIF-1α, where the assay involves identifying substances which bind to and /or modulate an activity of VDU1. The invention also relates to the treatment of

cylindromatosis and generally to the modulation of other conditions associated with activation of the transcription factor NF-kB, such as inflammation [87].

2.4.2.1 Mechanism of action: Generally, the assay consists of bringing the putative modulator into contact with a test system comprising VDU1, VHL and HIF-α, and determining the effect of the putative modulator on the stability and/or state of ubiquitination of HIF-α [87]. Numerous aspects and variations of this basic test system were provided by the inventors to serve several related goals of determining HIF-α modulators [87]. Product results were determined in a variety of ways according to the system in use and included fluorescence resonance energy transfer (FRET), reporter gene assays, scintillation proximity assay, etc.

VDU1 activity was reported as a novel target for the control of HIF-α since loss of VDU1 leads to a decrease in HIF-α and a reduction in the responses mediated by HIF, while conversely, increasing VDU1 activity would stabilize HIF-α and lead to an increase of HIF-mediated responses [88]. The finding that HIF-α stability can be regulated by VDU1 thus provides for a novel assay method for the development of new agents for human or animal therapy [87].

The inventors also identified a regulatory pathway present in cells which directly links the action of CYLD to the suppression of NF-kB. Loss of CYLD reportedly leads to an increase in NF-kB activity, which causes an increase in anti-apoptotic gene function [89]. Such disruption in the balance of pro- and anti-apoptogenic gene regulation in skin cells leads to the growth of the benign tumors associated with cylidromatosis [90].

2.4.2.2 Application: Assaying for modulators of the transcription factor HIF would be useful since the system is a key regulator of responses to hypoxia and crucial to oxygen homeostasis in a wide range of organisms [87]. The inventors mention for instance, the large number of transcriptional targets that have been identified, with critical roles in angiogenesis, erythropoiesis, energy metabolism, inflammation, vasomotor function, and apoptotic/proliferative responses [87]. HIF is also important in cancer, in which it is commonly up-regulated, and has major effects on tumor growth and angiogenesis. The invention also provides a method of treating an individual with cylindromatosis by administering to the individual an effective amount of an NF-kB inhibitor [87][87][87][87].

2.4.3 Di- and poly-ubiquitin deubiquitinase substrates and uses thereof (US8518660B2)—

The present invention relates to the field of detecting the activity of isopeptidases. More specifically, the present invention provides materials and methods for improved sensitivity in the fluorescent (e.g., internally quenched fluorescence technology) detection of isopeptidases such as deubiquitinating enzymes and other ubiquitin-like protein deconjugases by use of diubiquitin protein substrates [91] and for measuring the isopeptidase activity of a deubiquitinase.

2.4.3.1 Application: A diubiquitin for measuring the isopeptidase activity of a deubiquitinase comprising: A) A first ubiquitin molecule operably linked to at least one first energy transfer pair member; and B) a second ubiquitin molecule operably linked to at least

one second energy transfer pair member; wherein said first ubiquitin molecule is operably linked to said second ubiquitin molecule by an isopeptide bond from the C-terminus of said first ubiquitin molecule to the side chain of a lysine residue of said second ubiquitin molecule, wherein said first and second energy transfer pair members are individually either a fluorescent group or a quenching group [91].

2.4.3.2 Mechanism of action: Ubs suitable for the methods and substrates of the present invention can come from any species including, without limitation, human and yeast. Any ubiquitin can be used in the substrates and methods of the present invention for detecting activity of a cognate isopeptidase [91]. In particular embodiments, the Ub moieties of the diubiquitin are the mature form of the protein, i.e., the form of the protein after the precursor has been processed by a hydrolase or peptidase. In particular embodiments, the Ub is a mammalian ubiquitin, more particularly, a human ubiquitin [91].

2.4.4 Novel DUB Fluorescence Assay Substrates (isopeptidases) (US20040265931A1)—

The inventors disclose di-and poly-ubiquitin deubiquitinases substrates and uses thereof: More specifically, the invention provides materials and methods for improved sensitivity in the fluorescent (e.g., internally quenched florescence technology) detection of isopeptidases such as deubiquitinating enzymes and other ubiquitin-like protein deconjugases by use of diubiquitin protein substrates [92].

2.4.4.1 Mechanism of Action: The diubiquitins employed are for measuring the isopeptidase activity of a deubiquitinase. In particular embodiments, the diubiquitin comprises a first ubiquitin molecule operably linked to at least one first energy transfer pair member and a second ubiquitin molecule operably linked to at least one second energy transfer pair member, wherein said first ubiquitin molecule is operably linked to the second ubiquitin molecule by an isopeptide bond from the C-terminus of the first ubiquitin molecule to the side chain of a lysine residue of the second ubiquitin molecule [92]. The first and second energy transfer pair members may be individually either a fluorescent or a quenching group. The methods comprise contacting the sample with at least one diubiquitin of the instant invention and detecting fluorescence in the sample, wherein a change in the fluorescence is indicative of isopeptidase activity. These methods are performed in the presence and the absence of a test compound to screen for the compounds ability to modulate activity of at least one isopeptidase [92].

2.4.4.2 Application: The invention provides methods and corresponding reagents for improved methods for fluorescent detection of isopeptidase activity [92]. Methods and substrates of the invention can be used to detect/measure the activity or presence of a wide variety of enzymes, particularly isopeptidases. More than one isopeptidase substrate may be used for isopeptidase activity detection. Isopeptidases include deubiquitinating enzymes and ubiquitin-like protein (Ubl) specific proteases (Ulp) [92]. In a particular embodiment, the isopeptidase is a deubiquitinase. Methods of the present invention are practiced using a sample in which the activity of an isopeptidase will be detected. Samples may be from a variety of sources including animal or plant cell or cellular lysates, in vitro reaction mixtures, such as for drug screening purposes, including solutions and/or mixtures

containing recombinantly produced isopeptidase, and bodily fluids or tissue samples taken from an animal such as a human (e.g., biological samples) [92].

The invention also provides methods for diagnosing a disease or condition associated with a particular Ub- or Ubl-specific isopeptidase, where a sample from a subject suspected of having the disease or condition is contacted with an appropriate isopeptidase substrate of the invention, followed by detection of fluorescence [92]. The level of fluorescence is indicative of the isopeptidase activity in the sample. The amount of isopeptidase activity in the sample can be compared to the amount of isopeptidase activity in a corresponding sample from a healthy control, wherein a modulation (e.g., increase or decrease) in the isopeptidase activity in the sample compared to healthy controls is indicative of the presence of a disease or disorder [92].

2.5 Category E: Patents on ubiquitin-specific proteases

2.5.1 Ubiquitin-Protease 1 (UBP1) **(WO1991017245A1)—**Varshavsky [93], the inventors of UBP1, describe it as an ubiquitin-specific protease which cleaves ubiquitin from any non-ubiquitin protein or peptide to which the ubiquitin is joined. Significantly, they report that the cleavage targets any ubiquitin fusion (except polyubiquitin) without upper or lower limits on the size of the non-ubiquitin portion of ubiquitin fusion [93].

2.5.1.1 Mechanism of action: UBP1 cleaves the peptide bond on ubiquitin fusion protein between the carboxy-terminal residue of an ubiquitin moiety and the α-amino group of any non-ubiquitin protein or peptide to which it is joined. It also recognizes and cleaves what they described as “sandwich” ubiquitin fusions in which the ubiquitin moiety is located between a first and second non-ubiquitin moiety [93].

2.5.1.2 Application: Projected application pertains to those proteins or peptides whose functional activity is inhibited or otherwise modified by the presence of an ubiquitin moiety [93]. In such circumstances, the ubiquitin moiety can be used as a temporary inhibitor (or modifier) of the activity of a protein or peptide, with the ubiquitin-specific protease employed to restore, by deubiquitination, the original activity of the protein or peptide [93]. The invention also pertains to recombinant vectors expressing the UBP1 protease, to cells transformed with such vectors, and to specific versions of ubiquitin-protein fusions that facilitate isolation and manipulation of non-ubiquitin portions of these fusions using the UBP1 protease [93].

2.5.2 Vif1 and vif2: Peptide inhibitors of HAUSP deubiquitinase (US20140073585A1)—

The inventors disclose two vIRF4 (Kaposi’s sarcoma-associated-herpesvirus vIRF4) peptides, vif1, corresponding to aa202-216 of vIRF4, and vif2, corresponding to aa220-236 of vIRF4, as potent and selective HAUSP antagonists. They further demonstrated that vif1 and vif2 peptides robustly suppress HAUSP DUB enzymatic activity, ultimately leading to p53-mediated anti-cancer activity [94].

2.5.2.1 Mechanism of Action: HAUSP (Herpes virus-associated ubiquitin-specific protease) is an ubiquitin specific protease or a deubiquitylating enzyme that cleaves ubiquitin from its substrates [94]. HAUSP plays pivotal roles in the stability of p53 and

MDM2, raising HAUSP as a potential therapeutic target for tuning p53-mediated anti-tumor activity. HAUSP is most widely known as a direct antagonist of MDM2 [94]. Normally, p53 levels are kept low in part due to MDM2-mediated ubiquitination and degradation of p53. Interestingly, in response to oncogenic insults, HAUSP can deubiquitinate p53 and protect p53 from MDM2-mediated degradation of p53 in response to stress. It was also reported, however, that HAUSP is required for p53 destabilization and disruption of HAUSP stabilizes p53 [94]. Hence, vif1 and vif2 peptides significantly suppressing HAUSP DUB enzymatic activity, ultimately leads to p53-mediated anti-cancer activity [94].

2.5.2.2 Application: The present disclosure provides a method of inhibiting the growth of an acute lymphoblastic leukemia (ALL) cancer cell, comprising contacting the cell with an effective amount of one or more of any of the above vIRF4 peptide fragments, polynucleotides or compositions, thereby inhibiting the growth of the cancer cell. Any of the above peptides can further comprise, or alternatively consist essentially of, or yet further consist of, a cell penetrating domain, which for example, can comprise a HIV TAT peptide [94]. In further embodiments, the treatment comprises administration of polynucleotides encoding the peptides, of the present disclosure, antibodies that specifically bind to the peptides of the present disclosure [94]. Therefore, the vif1 and vif2 peptides, along with their homologues, are useful in treating cancer, in particular acute lymphoblastic leukemia (ALL) through regulation of p53 activity in a cancer cell [94].

For a summary of DUBs inhibitor patents reviewed, see Table 1.

3. Other compounds with DUB-inhibitory activities

3.1 Diethyldithiocarbamate-copper complexes targeting both 19S proteasome deubiquitinases and 20S proteolytic peptidases

It has been shown that copper, a biologically active metal and other metals such as gold could be used for the treatment of variety of pathological conditions although with diverse modes of action, including but not limited to inhibition of the UPS [95-97]. Due to their ability to bind copper and inhibit the cellular proteasome, many copper-chelating compounds could induce apoptosis in tumor cells [96, 98-100]. Similarly, diethyldithiocarbamate (EtDTC) when coupled with copper and zinc could inhibit purified 20S proteasome and intact 26S proteasomal activities in the cancer cells. Interestingly, zinc- and copper-$(EtDTC)_2$ complexes were better inhibitors of 26S proteasome than 20S proteasome [95, 98, 101]. Hence, it has been proposed that the copper or zinc complex act as proteasome inhibitors although their mechanism might be different from clinically approved proteasome inhibitor bortezomib. It is possible that these complexes could inhibit the 19S rather than the 20S proteasome, although this needs to be further assessed [95, 98].

Pyrithione (PT) is an excellent metal chelator and its copper complex (CuPT) could inhibit proteasome-specific DUBs such as UCHL5 and USP14 and 20S proteasomal peptidases resulting in cytotoxicity in vitro in cancer cells as well as in vivo in tumor xenograft studies. CuPT could be a new class of dual inhibitors of DUBs [102], although the exact mechanism for is not clear yet [103, 104].

3.2 Clinically used antirheumatic agent auranofin is a proteasomal deubiquitinase inhibitor and inhibits tumor growth

Proteasomes are attractive emerging targets for anti-cancer therapies. Auranofin (Aur) is a gold-containing compound that has been used for the treatment of rheumatic arthritis since 1985 [105]. Researchers have shown that it has anticancer potential too [106-108]. Therefore, it was recently approved for Phase II clinical trial to treat cancer by US Food and Drug Administration (http://clinicaltrials.gov/ct2/show/NCT01419691). Although, the exact mechanism of action for Aur is not clear, quantitatively it is equally effective as bortezomib in terms of proteasome inhibitory properties but mechanistically it differs from bortezomib as it inhibits proteasome-associated deubiquitinases (DUBs) like UCHL5 and USP14 but not the 20S proteasome [102]. Additionally, UCHL5 and USP14 inhibition is required for its cytotoxic effects. There are several reports that indicated that it has other targets as it showed inhibition of DNA, RNA, protein synthesis as well as reactive oxygen species (ROS), mitochondrial thioredoxin reductase, glutathione-S-transferase, and cathepsin B. Furthermore, *in vivo* in animal models, Aur showed inhibition of tumor growth. Aur also induced cytotoxicity in the cancer cells that were isolated from acute myeloid leukemia patients making it promising anti-cancer drug [105, 109].

4. Conclusions

Due to the high prevalence of intrinsic and acquired resistance to PIs, in addition to their prevalent and extensive adverse effects (*e.g.* peripheral neuropathy), there is an urgent necessity for furtherer development of novel UPS-targeted inhibitors which could be more specific and potent as well as could circumvent PI resistance. The UPS consists of a profound and dynamic regulatory system that controls the fate of specific proteins thereby coordinating the dynamic signaling status and fates of the cells. DUBs play a prominent and specific role in regulation of the UPS and therefore are plausible therapeutic targets. DUBs regulate the ubiquitination state of many crucial molecules and thereby regulate cellular pathways modulating homeostasis and fate. Crystal structures of various DUBs have been resolved which provide additional evidence that DUB inhibitors may be useful in the treatment of proteopathies, viral infections, as well as cancer. Although progress has been made in our understanding of DUB biology/mechanisms, there is considerable work to be done in order to move DUBs into the clinic. Basic research is crucial to discovering the natural regulatory mechanism in a cell in order to identify candidate pathways and targets which are required for regulation of DUB expression and activity that may be modulated pharmacologically. Furthermore, basic science is yet to fully understand system wide pharmacodynamic ramifications of targeting of DUBS. Rigorous, well-controlled studies must be performed in order to fully understand the specificity, selectivity, activity and stability of the various endogenous DUBs in normal and pathological biology in order to determine the best targets for future DUB-based drug developments.

5. Expert Opinions

The approval of bortezomib in 2008 for the treatment of MM patients and hematologic malignancies validated the UPS as an established and potent chemotherapeutic target, in the

treatment of multiple cancers. However, further research and studies have revealed weaknesses in the “direct” targeting of the proteasome. These studies have revealed a high prevalence of inherent and induced direct PI resistance in patients, in addition to a lack of efficacy in the treatment of solid tumors, as well as multiple adverse effects. As a result additional approaches for targeting the proteasome and/or the components of the proteasome system must be pursued.

A canonical strategy implemented to combat generalized drug resistance has been to target another component of the specific pathway (*i.e.*, upstream of proteasome degradation) clinicians often achieve this through the use of multiple chemotherapeutics in hopes of synergy or through differential targeting of the pathway. This review has detailed the significant and diverse roles that DUBs play in protein turnover, localization and cellular homeostasis. The review has also detailed a number of patents that have been filed for compounds that target DUBs through a multitude of mechanisms. This evidence suggests that “Specific” DUBs may be ideal therapeutic targets and or provide valuable diagnostic and prognostic value. This review has also viewed the considerable efforts that have been undertaken in order to develop novel DUB modulators; however, it is clear that further understanding in the basic biology and specificity/selectivity of DUBs is needed before specific DUB inhibitors can be identified and developed for the clinic.

The same characteristics that make DUBs intriguing and promising targets in cancer therapy also makes prediction of the downstream consequence of DUB-targeting extremely complicated. DUBs exhibit an extraordinarily dynamic and complex system for regulating cellular activities and fate. Therefore, we must better understand the roles of these molecules in order to determine which DUBs are specific to cancers and what roles they are playing in the pathways such that we can design intelligently crafted clinical targeting “Schemes” based on the unique pathologies of each individual cancer type. For this reason, libraries of DUBs and E3-ligases, sumoylation, kinases, phosphatase and countless other molecular-modulators of key pathway inhibitors must be identified and screened for selectivity, specificity, efficacy and most importantly safety.

It is known that cancers rely on the “perversion” of normal cellular processes. In many of these oncogenic pathways critical components could be targeted by chemotherapeutic drugs. Theoretically, many of these same targets may be regulated by specific DUB or other members of the UPS in order to modulate the pathways endogenously. Researchers must identify the specificity of these UPS components (E1, E2, E3 and DUBs) and determine which are specifically associated with individual cancers. Once these key Oncogenic DUBs have been identified work can begin on identifying selective inhibitors of these targets in order to kill those cancer cells selectively.

A better understanding of DUB functions, differential regulation in normal physiological processes as well as their role in various pathological conditions to delineate between drivers and bystanders. DUBs can act as a tumor suppressor or promoter. As a result of DUBs’ dynamic activity, target promiscuity and differential regulation, the complete understanding of DUBs functions has been elusive [56]. Hence, there is a dire need for an in depth characterization of DUBs’ activities, expression profiles, selectivity, and specificities MOAs

in order to identify the roles of DUBs in normal and pathological contexts. In this way DUB expression profiles might be able to identify patient groups that might respond better to specific DUB inhibitors.

In addition to cancer, DUB-targeted therapies may also benefit other diseases such as pathogenic disease. Identification of new and unique members in the DUB family present in pathological microorganisms is structurally different than host DUBs. This suggests that it may be possible to differentially target DUBs in pathogenic organisms that are inert to the host. With the increasing prevalence of antibiotic resistance bacteria and microorganism DUBs may be utilized as novel and specific anti-biotics if DUB inhibitors could be developed to specifically target the pathogenic DUBs sparing any untoward effects in the host. Due to the lack of effective anti-infectious agents and emergence of resistant strains, inhibitors for DUBs in pathological microorganism pose potential therapeutic targets that could be exploited to treat plants, animals and human subjects [110].

Development of the assays (e.g. biophysical) that study DUB-substrate interaction should be done in a high throughput manner in order to design DUB regulators and refine them to enhance specificity and delivery of these compounds. Small molecule inhibitors could be used to interfere with the interactions between DUB and their substrates or to inhibit the catalytic activity of specific DUBs. Unfortunately, the majority of current small molecule DUBs inhibitors are not selective and target multiple DUBs, although it is yet to be proved if compounds that target only one specific DUB could be clinically useful. It is very likely that resistance might quickly become a problem once again even to these selective and specific inhibitors. However, it is possible that DUB inhibitors that intentionally lack specificity might be more beneficial therapeutically by targeting multiple critical proteins in the same or multiple pathologic pathways. DUBs inhibitors could either be used in the prevention, diagnosis and possibly as a first line therapy or in combination with other established therapeutic agents [111].

High throughput methods for screening available compounds could yield a library of compounds from which researchers may identify key compounds to modify and enhance their pharmacodynamic and pharmacokinetic profiles. Once we have a library of selective compounds we may be able to screen individual patient tumors' in order to determine which combinations of UPS (and other) modulating targets/compounds would be best to interrupt the most prevalent and active pathways in a given cancer. In this way the cell's own proteasomal and cellular machinery could be utilized to destroy these "crucial targets" and thus kill the cancer cells by dys-regulating the "high jacked signaling pathways" that cancers rely on to drive unregulated cellular processes. This stratagem and approach would be a path towards the development of truly personalized medicine for the treatment of cancers.

It is our hope is that this text could serve as a reference for investigators interested in DUB-targeting therapies to understand what compounds are available for DUBs research as well as their properties, and to suggest some techniques that may be useful for developing the next generation DUB inhibitors.

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Article highlights

- DUBs are a key component of ubiquitin-proteasome system
- DUBs control protein turnover, stability and subcellular localization
- Dysregulation of DUB activities is involved in pathological conditions such as: cancer, inflammation, neurological disorders and microbial infections
- DUB inhibitors have potential as novel therapeutic agents for diverse range of diseases
- DUB inhibitors might be able to help overcome or circumvent resistance to classical 20S proteasome inhibitors

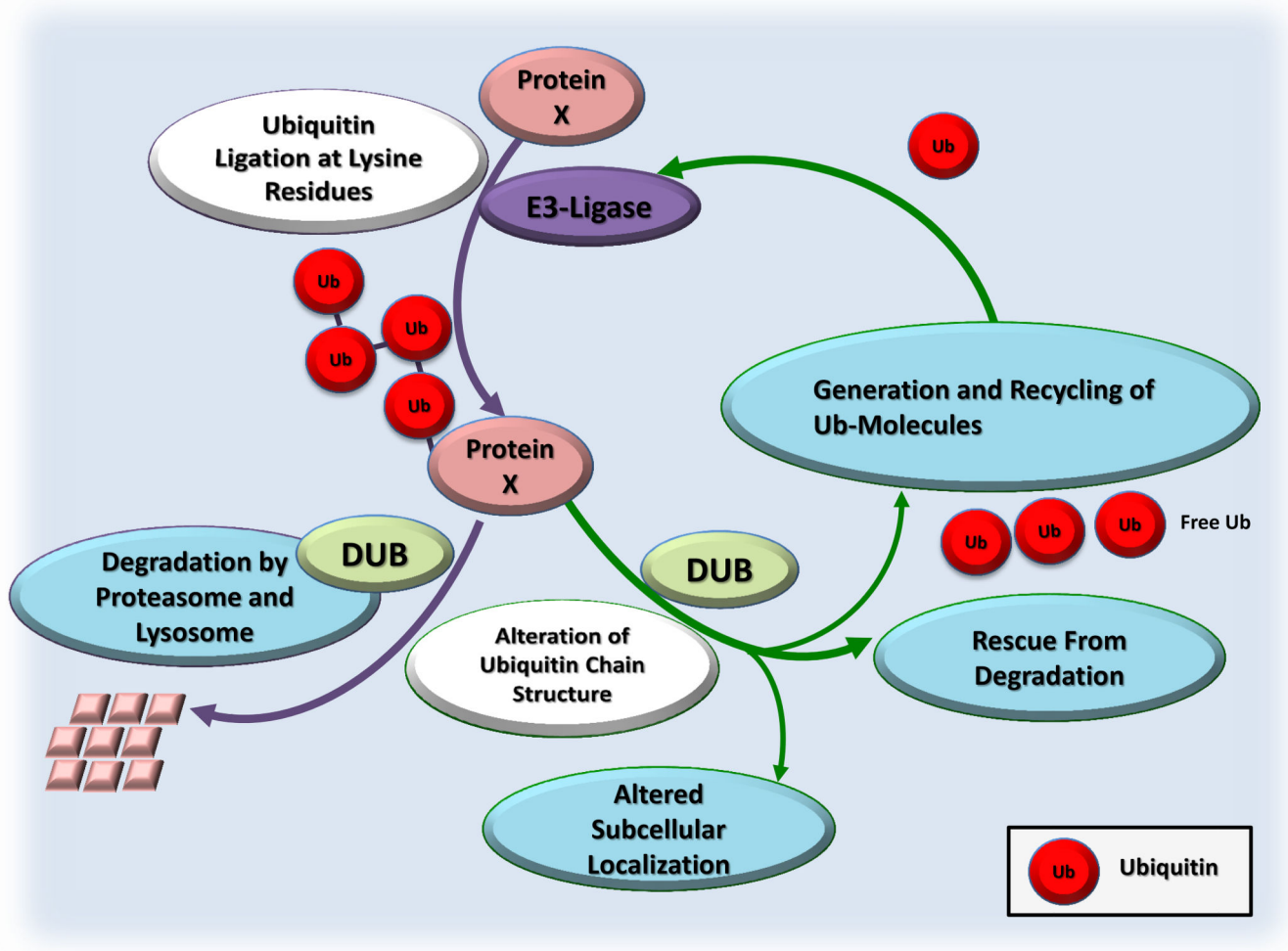

Figure 1. Different functions of deubiquitinase family of proteases

Various proteins are post-translationally modified with either single or multiple residues of ubiquitin. Ubiquitinated proteins could be either degraded by proteasomal - lysosomal pathway or deubiquitinated by DUBs. Their deubiquitination results in precursor processing, generation and recycling of ubiquitin molecules or they are rescued from their degradation and or their subcellular localization is altered.

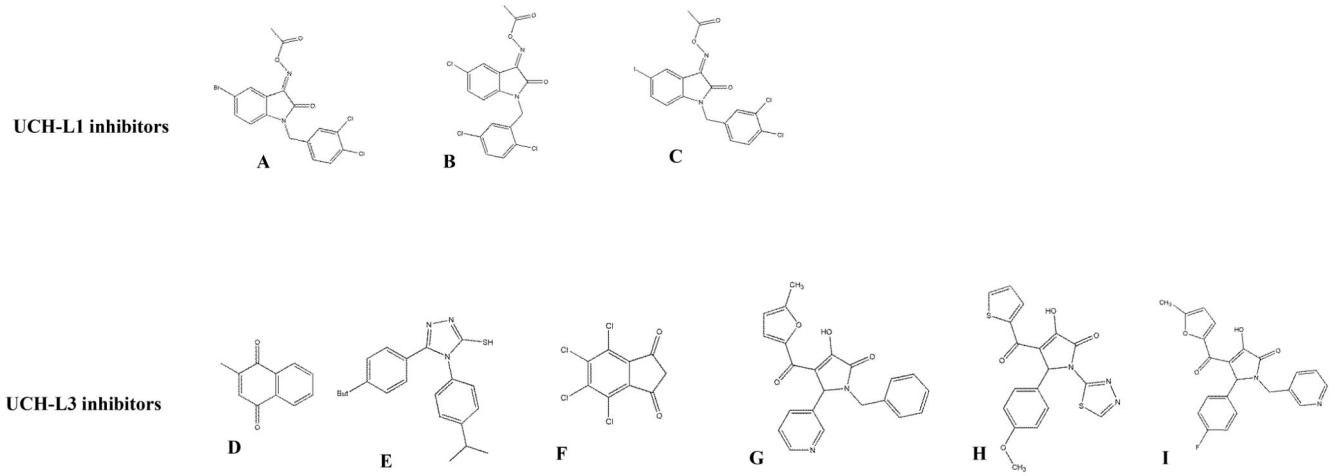

Figure 2. Chemical structures for UCH inhibitors

UCH-L1 inhibitors **A, B, C** and UCH-L3 inhibitors **D,E,F,G,H,I**

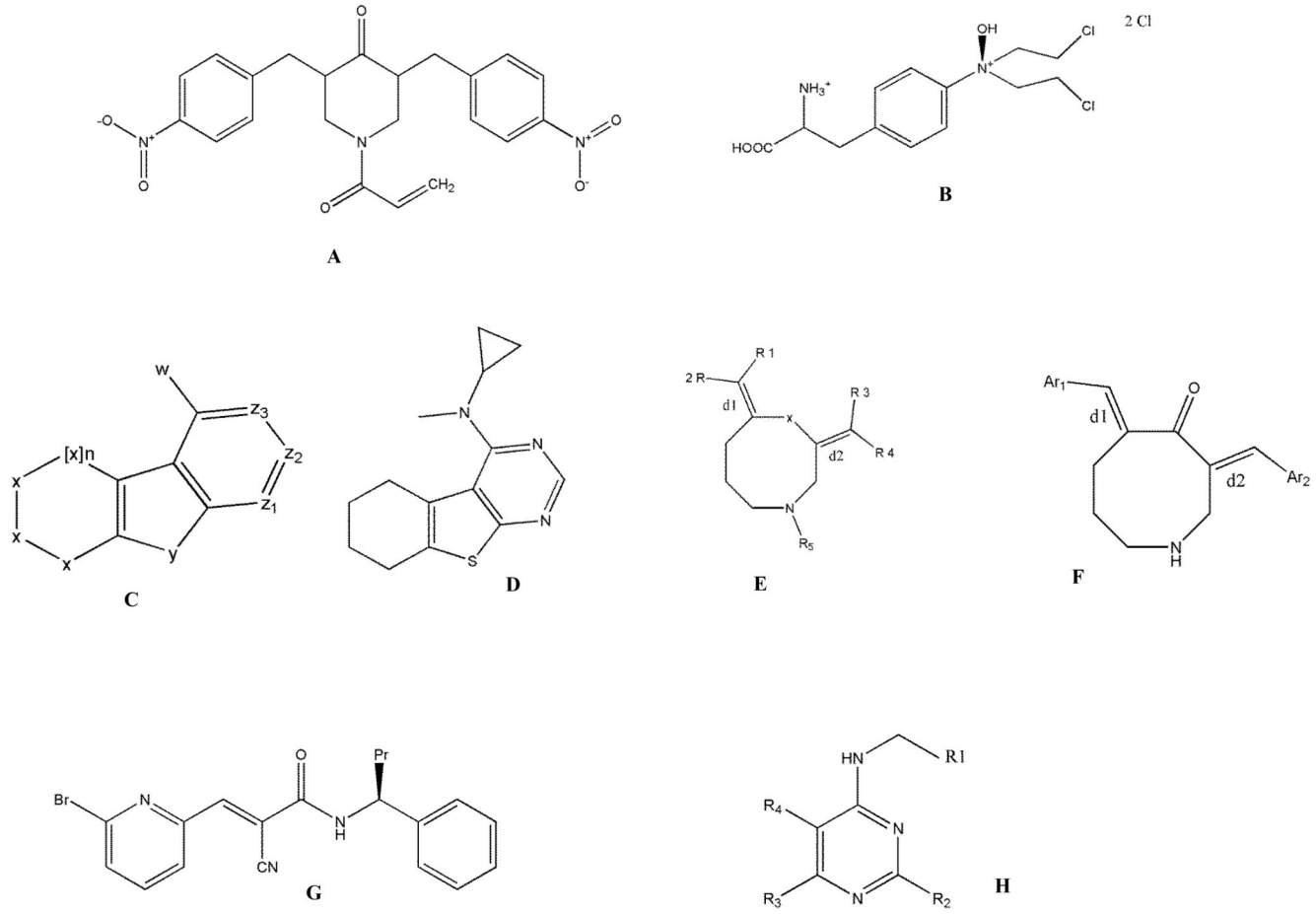

Figure 3. Chemical structures for patented DUB inhibitors

A (b-AP15), **B** (PX-478), **C** and **D** (Tricyclic heterocyclics), **E** and **F** (Azeptan-4-ones), **G** (WPI130B), **H**(aminopyrmindinase)

Table 1
Summary of DUBs inhibitor Patents Reviewed

No.	Patent ID	Invention	Application	Invention Type	Reference number
A		**Chemical Agents**			
2.1.1	W020130586 91	b-AP15	Inhibition of deubiquitinase activity of 19S RP DUBs	DUB Inhibitor	75
2.1.2	W0/2005/00 7828	N-oxides	Regulation of HIF levels in cells via UB/26S proteasome mechanism	DUB Inhibitor	65
2.1.3	W020110945 45	Tricyclics	Inhibition of DUB USP14	DUB Inhibitor	74
2.1.4	US20140370 528A1	Azepan-4-ones	Inhibition of two RP DUBs, UCHL5 and USP14	DUB Inhibitor	62
2.1.5	W020120405 27 A2	WPI 130	DUB inhibition. Additionally inhibiting a UCH catalytic domain	DUB Inhibitor	78
2.1.6	W02014059 52A3	6-Amino-pyrimidines	Inhibition of the USP1/UAF1 deubiquitinase complex	DUB Inhibitor	79
B		**Nucleic Acids**			
2.2.1	W019970062 47 A2	siRNA	Inhibition of USP1 DUBase enzyme complex	DUB Inhibitor	80
C		**protein-based, site-directed probes**			
2.3.1	W020030914 11 A2	UBVS	Protein-based, site-directed probes for ID and inhibition of DUBases	DUB Inhibitor	81
D		**Assay Methods**			
2.4.1	W020111579 82 A1	Fluorescence	Fluorescence detection of DUB activity	Assay	84
2.4.2	W020040647 11 A2	Fluorescence, etc	Regulatory pathway assay for modulators of HIFá/plus activity of VDU1	Assay	87
2.4.3	US8518660 B2	Fluorescence	Di- and poly-ubiquitin deubiquitinase substrates and uses thereof	Assay	91
2.4.4	US20040265 931A1	Fluorescence	Novel DUB substrates (isopeptidases) for improved sensitivity in fluorescent detection of DUB activity	Assay	92
E		**Proteases/Peptides**			
2.5.1	W019910172 45 A1	UBP1 Protease	Ubiquitin-specific protease	DUB Inhibitor	93
2.5.2	US 20140073585 A1	Vif1 and vif2	Peptide inhibitors of HAUSP deubiquitinase	DUB Inhibitor	94