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Review Article

Isopeptidases in anticancer therapy: looking for inhibitors

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Abstract: Addition of polypeptides belonging to the ubiquitin family to selected lysines residues is a widespread post-translation modification (PTM) that controls many fundamental aspects of cell's life. Specific alterations in the normal turnover of this PTM are frequently observed in tumors. The conjugation/deconjugation cycle of ubiquitin (Ub) or ubiquitin-like (Ubl) proteins influences the activities of oncogenes and tumor suppressor genes. Two families of enzymes work in antagonizing manner to add or remove Ub and Ubl-proteins on target proteins: the E3 ligases and the isopeptidases. These enzymes are the subjects of fervent research with the ambition to comprehend their regulation, their mechanisms of action, their involvement in human diseases, and to develop specific inhibitors for therapeutic intervention. Here we will discuss of isopeptidases, the deconjugating enzymes, with particular emphasis on the pro-apoptotic activities of the relative inhibitors identified so far.

Keywords: Apoptosis, cancer, proteasome, isopeptidase, DUBs, necrosis, caspase, proteasome, USP

Introduction

Cells have evolved a spectacular array of strategies to modulate protein functions in response to environmental stimuli. In multicellular organisms post-translation modifications (PTMs) are well suited to mach rapid cellular responses to new and unexpected needs of the organism. Ubiquitination is a PTM, which consists in the covalent addition of ubiquitin (Ub), a 76-residue polypeptide, to lysine residues of specific target proteins [1]. The carboxyl group of the C-terminal glycine of ubiquitin forms an isopeptide bond with the ε-aminogroup of lysines present on the target protein [2]. Many recent excellent reviews have discussed the complexity of protein ubiquitination [2-4]. This complexity renders the protein-ubiquitination system (UPS) the foremost flexible PTM.

There are two main aspects at the origin of the UPS complexity. First, the Ub linkage is subjected to multiple options such as: mono-ubiquitination, poly-ubiquitination on different lysines of the Ub itself (K6, K11, K29, K48 and K63) or on different lysines of the target protein (poly-mono-ubiquitination) and also the amino-terminal ubiquitination [5]. Second, Ub belongs to a protein family, characterized by 14 members (including 3 putative) and classified as ubiquitin-like (Ubl) proteins. Ubl-proteins share structure, but not sequence, similarities with ubiquitin. Differentially from Ub, Ubl-proteins have only regulative but not degradative activities towards their targets [4].

The spectacular collection of options available to cells to modify Lys residues is reflected in a vast assortment of effects on the target proteins, as we begin to comprehend in the case of Ub. Through poly-ubiquitinations, mono-ubiquitination, poly-mono-ubiquitinations this PTM can govern: the proteasomal-mediated degradation of proteins, their assembly into signaling complexes or their localization into specific subcellular compartments. Not surprisingly the Ub and Ubl-proteins are pivotal for several cellular processes, including: cell cycle, apoptosis, DNA repair, membrane trafficking, autophagy, inflammatory response, ribosomal protein synthesis and both the innate and adaptive immune responses [6-8].

Proteins regulated by Ub or Ubl are in general

selectively modified by the coordinate action of three Ub-ligase or Ubl-ligases known as the E1, E2 and E3 enzymes. E1 and E2 are responsible for activating the ubiquitin molecule for conjugation, whereas E3 acts as matchmakers between the activated Ub-E2 intermediate and substrate proteins [1-3, 8].

Over the past decade this complex molecular machinery has attracted much attention, not only among molecular and cellular biologists, but also among pharmacologists and oncologists. The protease activity and the unquestionable involvement of many Ub-targets in the control of cell proliferation inspired the searching for specific inhibitors of the ubiquitin-proteasome system (UPS), to be used in clinic. The approval of Bortezomib/Velcade/PS-341 for the treatment of multiple myeloma and several ongoing clinical trials using bortezomib or other, more recently developed UPS inhibitors, have proved the importance of the UPS as drug-target for anti-neoplastic therapies [9, 10].

DUBs and other isopeptidases

As for other PTMs, such as phosphorylation or acetylation, conjugation of Ub or Ubl-proteins to protein substrates is a reversible process. Isopeptidases, a heterogeneous family of proteolytic enzymes, are involved in this task. The isopeptidases family includes deubiquitinating enzymes (deubiquitinases or DUBs), which in principle should be specifically devoted to the rupture of Ub linkages and other proteolytic enzymes, which are dedicated to deconjugate the Ubl-proteins [4, 11]. Generally, they can be viewed as E3 ligase antagonists. Genomic studies have identified 79 human genes encoding for functionally putative DUBs [12].

From a structural point of view isopeptidases can be grouped into five distinct subfamilies [4, 11]. Four of them are cysteine-proteases subdivided into (i) the Ub-C-terminal hydrolases (UCH), (ii) the ubiquitin specific protease (UBP/USP), (iii) the ovarian tumor-related (OTU), and (iv) the Machado-Josephin domain (MJD). The last group includes (v) the JAMM, Zn-metalloproteases (AB1/MPN/Mov34 metalloenzyme). In addition, there are many UBL-isopeptidases that do not fully fit within these categories, but nevertheless they are interesting therapeutic targets.

Isopeptidases deconjugation activity can exert diverse outcomes on the substrates [4, 11, 13]. First they can endorse the processing/maturation of the Ub and Ubl precursors, a necessary step for their subsequent conjugation to the targets. Second, they can antagonize the E3 ligase activities, thus operating as negative regulators of the PTM. Third, DUBs, in particular, can support the degradative phase. Substrate degradation is coupled to its deubiquitination, which is required both for efficient substrate degradation and for recycling the Ub the pool [6].

Curiously, interference with this last function is the manifested consequences of treating cells with non-selective isopeptidase inhibitors (N-SIIs). Accumulation of poly-ubiquitinated proteins in the presence of an active catalytic chamber of the proteasome, is the distinguished trait of these inhibitors [14-16]. Accumulation of poly-ubiquitinated proteins is also elicited by bortezomib, but in this case the catalytic chamber of the proteasome is to be inhibited [10].

Historically much attention has been focused on the role of E3-ligases in the control of protein turnover. Only recently the interest has been manifested towards enzymes that reverse the process. The sprouting of this awareness is witnessed by the rising number of excellent reviews published last year on isopeptidases [13, 17-19].

Isopeptidases and cancer

Isopetidases control the activities of several proteins located at vital nodes of various signaling pathways (**Table 1**). Oncogenes and tumor suppressor genes can be modulated by cycles of Ub or Ubl addiction/removal [17]. The p53 is emblematic, being conjugated with either, Ub and Ubl proteins [20, 21]. Hence, the discovery of mutations in certain DUBs in cancer was not unexpected. Germline mutations in the CYLD gene, which encodes for a DUB that removes 63-linked Ub-chains, are responsible for three different syndromes, which share the predisposition for developing multiple skin tumors of the head and neck [22-24]. Translocations of USP6 gene have recently been found to be causative in most aneurysmal bone cysts, that are benign tumors [25]. Inactivating somatic mutations of TNFAIP3/A20 in different lymphomas have

Figure 1. Structures of the principal non-selective isopeptidase inhibitors (N-SIIs)

Names	Process/Mechanism	Comments
USP1	DNA repair, UAF1 (USP1-associated factor 1)	Deubiquitinates and inactivates PCNA. Implications in Fanconi's anemia-related cancer [82].
USP2	Cell proliferation. Implications in prostate cancer	Stabilization of FAS (Fatty Acid Synthase). Deubiquitinates and stabilizes MDM2 [83, 84].
USP3	Cell cycle and genomic stability	Regulator of H2A/H2B ubiquitination and involvement in the response to DNA double-strand breaks [85].
USP4	Cell proliferation differentiation. Implications in prostate cancer	Influence on Wnt signalling acting as a suppressor of β -catenin dependent transcription [86].
USP5	p53 pathway	p53 stabilization by USP5 knockdown, accumulation of unanchored polyubiquitin [87].
USP7	p53 and AKT pathways (DNA damage, oxidative stress)	Deubiquitinates and affects stability of both p53 and MDM2 [70-73].
USP8	Endocytic trafficking and receptor Internalization	Regulates abundance of EGFR and several receptor tyrosine kinases (RTKs) [76, 88, 89].
AMSH	Endocytic trafficking and receptor degradation	Impaired AMSH reduces EGFR degradation without affecting internalization [90].
USP9X	Cell cycle, chromosome partition, apoptosis,TGF-β and oxidative stress signaling	Promiscuous, multiple targets [80, 91-94].
USP10	Post-endocytic trafficking/p53 pathway	Deubiquitinates p53, reversing Mdm2 induced nuclear export and degradation [95].
USP11	DNA damage/ NF-kB pathway	Component of the HR double-strand break repair pathway. Negatively regulates NF-kB pathway by targeting IkBα [96, 97].
USP14	Proteasome/protein degradation	Proteasome associated DUB, implicated in colorectal cancer [98].
USP16	Cell cycle and chromatin regulation	Deubiquitinates histone H2A. Its downregulation promotes mitotic phase defects in HeLa cells [99].
USP17	Cell cycle, Ras pathway	USP17 expression blocks Ras membrane localization and inhibits downstream kinases MEK/ERK phosphorylation [100].
USP18	Negative regulator of the interferon response	USP18 downregulation augments interferon induced TRAIL expression and apoptosis [81]. Regulates EGFR protein synthesis [101].
USP20	Receptors recycling, HIF1 regulation, motility	Substrate of von Hippel-Lindau tumor suppressor. Beta2 adrenergic receptor recycling. Deubiquitinates and stabilizes HIF1-alpha [102, 103, 104].
USP21	Chromatin regulation, NF-kB pathway	Deubiquitinates chromatin bound H2A. Inhibition of TNF-induced NF-kB activation through RIP1 deubiquitination [105, 106].
USP22	Chromatin modification	Subunit of the hSAGA transcriptional cofactor complex. Deubiquinates histones H2A/H2B [107].
USP28	DNA damage/cell proliferation	Stabilizes MYC proto-oncogene. Role in the Chk2-p53-PUMA pathway [108, 109].
USP33	Receptors recycling/Motility	Substrate of von Hippel-Lindau tumor suppressor. Beta2 adrenergic receptor recycling [102, 103].
USP39	Chromosome segregation, mitotic spindle checkpoint regulation	Involved in splicing of Aurora B and other mRNAs essential for spindle checkpoint function [110].
USP44	Chromosome segregation, mitotic spindle checkpoint regulation	Deubiquitinates Cdc20 stabilizing the APC-inhibitory complex Mad2-Cdc20 preventing the premature activation of APC itself [111].
CLYD	NF-kB pathway	Inhibits NF-kB signaling by removing K63-liked chains on NEMO, TRAF-2 and TRAF-6 [22, 23, 24, 112].
A20	NF-kB pathway	Replaces the K63 chains of the NF-kB signaling intermediates TRAF6, RIP1, RIP2 and IKK with proteasome-targeting K48 chains [113].
UCHL1	Cell cycle and mitosis	Involved in Parkinson's and Alzheimer's disease and cancer [65, 66, 67].

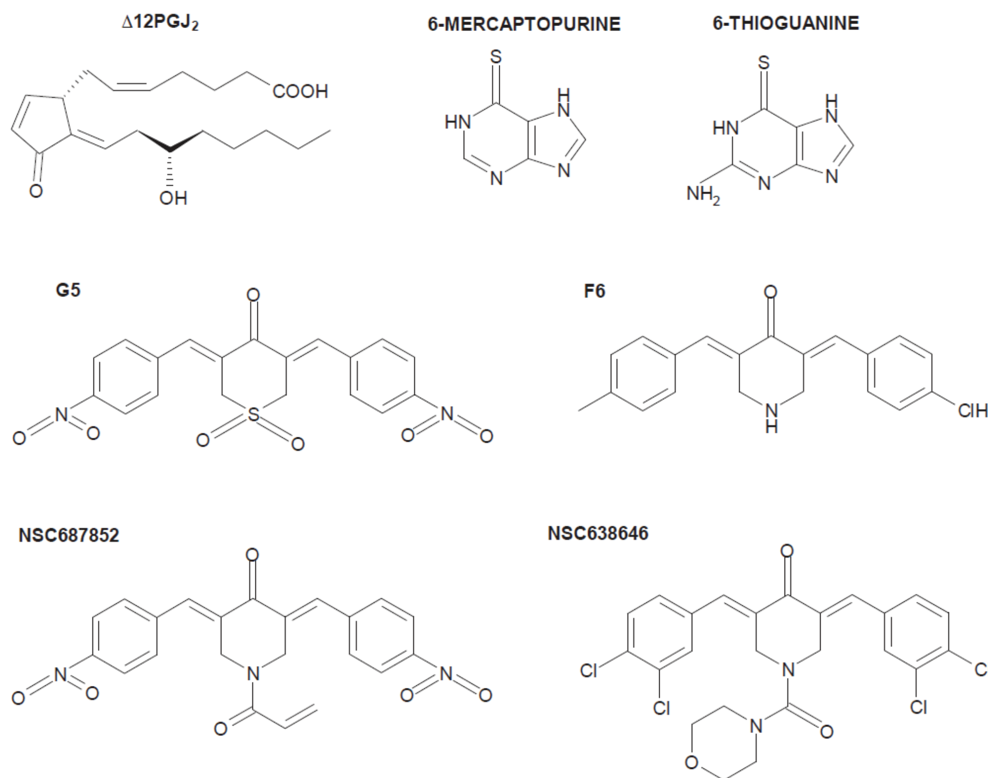

Figure 1. Structures of the principal non-selective isopeptidase inhibitors (N-SIIs).

been recently reported. A20, which contains deubiquitinase and E3 ligase domains, negatively regulates the NF-kB signalling pathway [26]. On the opposite, some isopeptidases show an oncogenic behaviour and are overexpressed in certain tumors [27].

Recent excellent reviews have discussed these aspects in detail [19]. Hence we refer the readers to these reviews.

Isopeptidases inhibitors

A complete eradication of the neoplastic cells, after surgical intervention and pro-apopotic pharmacological strategies, is the best prospect that we can offer to save a patient's life. Unfortunately many incurable tumors show stubborn resistance to die by apoptosis and, as a consequence, an extreme chemoresistance. Generally chemoresistance arises because during tumor progression cancer cells accumulate mutations in critical genes regulating the apoptotic program. Paradoxically, the adverse environment and the host-defense mechanisms, act as a strong selective pressure, which favors the rising of apoptotic resistant clones. Identification and validation of new druggable targets for anticancer therapies is an intense area of the biomedical research, which hopefully will overcome chemoresistance [28, 29].

Due to their protease activity and their involvement in the regulation of important signaling pathways, including apoptosis, isopeptidases are emerging as attractive druggable candidates [10, 19, 30].

As it often happens in the modern biomedical research, serendipity led to the discovery of the first isopeptidase inhibitors. Studies aimed to explore the pro-apoptotic functions of certain prostaglandins and high-throughput screening, using chemical libraries of small compounds, aimed to identify new pro-apoptotic drugs, made possible the discovery of the first N-SIIs [14-16] All these compounds share the same pharmacophore, a sterically accessible, cross-conjugated α,β-unsaturated dienone which confers inhibitory activity toward isopeptidases

(**Figure 1**) [31]. When added to cells, N-SIIs elicit the accumulation of poly-ubiquitinated proteins and the induction of classical markers of the cellular response to proteasomal inhibition [14-16].

These inhibitors are small molecules susceptible to Michael addition reactions with free sulfhydryl groups of cysteines [32]. Consequently, N-SSIs are irreversible inhibitors. Based on their structural characteristics it is difficult to imagine a strong selectivity towards different isopeptidases. In fact, in vitro studies using recombinant enzymes have demonstrated that N-SIIs can inhibit various isopeptidases such as: UCHs, USP2, USP7 and the Ubl-protease SENP2, with comparable IC50 in the low micromolar range [33]. Hence, compounds containing cross-conjugated α,β-unsaturated dienones can be considered N-SIIs.

In principle, N-SIIs could also attack other proteins or cellular thiols including glutathione (GSH). Although these possibilities cannot be ruled out, we have observed that G5, a compound belonging to this class of inhibitors, when used at a concentration capable of promoting the accumulation of poly-ubiquitinated proteins cannot inhibit caspases, which are cystein proteases [34].

Treatment of cells with GSH depleting agents shows an additive pro-death effect with G5, thus suggesting that glutathione could be involved in its detoxification. However GSH depletion cannot explain N-SIIs pro-apoptotic activities [35].

It is evident that further studies aimed to characterize these inhibitors are needed. In particular it will be important to prove their anti-tumor activity in vivo, using animal models. Notwithstanding they represent valuable leads for further chemical optimization.

The thiopurines 6-mercaptopurine (6MP) and 6-thioguanine (6TG) probably represent another group of N-SIIs (**Figure 1**). These compounds are indicated for the treatment of the acute lymphoblastic leukemia and of Crohn's disease, an inflammatory disorder [36]. It has been reported that these compounds can inhibit the SARS-coronavirus (CoV) papain-like protease (PLpro), a cysteine protease with deubiquitinating and deisgylating activity [37]. Deisgylases are involved in the deconjugation of the Ubl-protein ISG15. The expression of this Ubl and of the conjugation/deconjugation system (including the relative E1, E2 and E3 enzymes) is under the control of interferons. Protein isgylation plays important roles in the modulation of the immune response [38].

Structure comparison studies suggest that 6MP and 6TG are also potential inhibitors of USP14 [36]. Hence, these compounds could belong to a new class of N-SIIs.

Death-pathways activated by non-selective isopeptidase inhibitors

Cells can efficiently eliminate themselves through a sophisticated genetic program known as apoptosis. Proteolytic processing of selected cellular proteins, as operated by a unique family of cystein-proteases called caspases, is necessary to induce apoptosis. Two main apoptotic pathways, the extrinsic and the intrinsic, keep in check caspase activation. The extrinsic pathway controls procaspase-8 maturation and it is triggered by the engagement of death receptors placed at the cell surface. By contrast, the intrinsic pathway, which is responsible for procaspase-9 maturation, is triggered by the release of killer mitochondrial proteins into the cytosol, after MOMP (mitochondrial outer membrane permeabilization) [39].

MOMP is emerging as the point of no return for many death pathways. In the presence of massive MOMP, the cellular demise could take place alternatively, as an effect of the mitochondrial metabolic failure or by means of some still mysterious caspase-independent deaths [40]. MOMP and the release of the mitochondrial pro-apoptotic factors into the cytosol are under the antagonistic control of the master regulators of the apoptotic process: the members of the Bcl-2 family [41].

N-SIIs are potent inducers of apoptosis in various cancer cell lines [14-16, 31, 35]. This apoptotic response, as suspected, shares many traits with apoptosis induced by bortezomib and proteasome inhibitors, including p53 independence, induction of ER stress, activation of the extrinsic pathway, eliciting of MOMP [14, 15, 34, 42]. Interestingly, both bortezomib and N-SIIs can efficiently activate effector caspases in the presence of a defective apoptosome [15,

43]. It seems that a double effect of these inhibitors, on the extrinsic pathway on one side and, on the IAP antagonist Smac, by stabilization of its cytosolic mitochondrial released form, on the other side, allows the activation of caspase-3/-7 also in absence of active caspase-9 [43, 44].

The mechanism used by UPS inhibitors in general and by N-SIIs in particular to sustain the extrinsic pathway is not clear. UPS inhibitors have several possibilities of interference at different levels of the signaling cascade. The caspase-8 substrate Bid once cleaved can be targeted to proteasome [45]. FLIP, which different isoforms display antagonistic effects on caspase-8 activation, can also be regulated by the UPS [46-49]. Caspase-8 activation itself is tunable by poly-ubiquitination. The CUL3 ubiquitin-ligase complex mediates caspase-8 poly-ubiquitination. Next, the ubiquitin-binding protein p62/sequestosome-1 promotes aggregation of CUL3-modified caspase-8, which drives enzyme activation [50]. Since caspase-8 poly-ubiquitination can be reversed by the DUBs A20, this represents another site of intervention for the N-SIIs.

A further possibility of interference for N-SIIs is at the level of membrane trafficking. Localization in sub-membraneous compartments and vesicle trafficking can influence the activity of death receptors and of their ligands [51]. It is well known that membrane trafficking is modulated by addition of Ub and Ubl-proteins and that the action of specific isopeptidases elicits a tight control over this process [17, 18]. Here again, a possible effect of the N-SIIs on the extrinsic pathway can be envisaged.

Certain tumors become treacherously resistant to chemotherapy-induced apoptosis. Interestingly, N-SIIs, in cells resistant to apoptosis, can activate a peculiar necrotic response, which depends on the re-organization of the actin cytoskeleton [34]. The effect on actin cytoskeleton can be observed at nanomolars concentration of the N-SII G5. In glioblastoma cells the effect on actin cytoskeleton is coupled to a drastic reduction of cell motility, thus suggesting another possible therapeutic window [35]. N-SII can affect not only cancer cells survival but also their invasive and metastatic properties.

The dramatic and variegated effects of N-SIIs on cell survival are probably a consequence of the simultaneous alterations of multiple cellular functions, normally controlled by different isopeptidases, which renders the stress unmanageable for the cells. In fact, microarray analysis of cells treated with prostaglandin J2 (PGJ2) that contains α,β-unsaturated carbonyl groups, revealed the up-regulation of genes of the heat shock and stress response, including proteasomal components, genes controlling protein folding, detoxification and cysteine metabolism [52]. Perturbation of mitochondrial function with a rapid drop of the mitochondrial membrane potential and lysosomal membrane permeabilization [16, 34, 52], although probably late events of the cellular response to N-SIIs are important markers that further prove the induction of unmanageable cellular stress.

Specific isopeptidases inhibitors

The development of more specific isopeptidase inhibitors could enormously improve the opportunities for pharmacologic interventions. The vast majority of isopeptidases are cystein-proteases. As recently highlighted by Daviet and Colland, it is not simple to develop cysteine-proteases inhibitors exhibiting the mandatory clinical properties [30, 53]. More selective inhibitors should minimize toxicities, which is an important limit for clinical applications for these inhibitors. Unfortunately specificity cannot be an easy task. We are dealing with enzyme families that include many different members in which catalyitic cores show extensive homologies [53].

The catalytic core depends on two or three crucial amino acid residues, constituting a catalytic diad or triad, respectively [11, 17, 30, 53]. All isopeptidases with cystein-protease activity utilize a similar catalytic mechanism, which has been described in detail with studies on the papain family of proteases. A His side chain residue, which is positioned next to the catalytic Cys lowers the pKa of Cys, thus enabling the nucleophilic attack on isopeptides linkages [54].

Another important aspect that must be kept in mind is that DUBs catalytic site can be under conformational regulation. Structural studies have revealed that USPs exhibit a conserved three-domains architecture, comprising Fingers, Palm, and Thumb. Fingers specifically manage the interaction with Ub, whereas the catalytic

centre lies at the interface between the Palm and Thumb sub-domains [55]. Some isopeptidases are constitutively active, whereas in others substrate binding modulates the enzymatic activity. In the absence of the substrate (without Ub binding) the catalytic domain is in an inactive conformation. Substrate binding induces an allosteric change that activates the catalytic domain [17, 55-58]. In USP7/HAUSP (Herpesvirus associated USP) for example Ub binding elicits the alignment of the catalytic Cys to the His residue [55, 56]. In contrast, the catalytic site of USP14 is perfectly aligned also in the absence of the substrate. Inhibition is obtained by steric hindrance of the active site, as operated by the ubiquitin-binding surface loops. Ubiquitin binding leads to translocation of these loops, allowing access of Ub substrates to the active site [17, 57, 59].

A promising approach to identify specific isopeptidase is the development of high-throughput screening (HTS) of small compounds libraries. In order to augment specificity and potency, HTS are followed by chemical modification of the most promising hits. A limit of the HTS is the availability of simple and specific assays to measure isopeptidase activities. This weakness is testified by the sprouting of new assays, as described in the recent literature [60-63]. Although the sensitivity and specificity of the assays could be further improved, several research groups have already utilized HTS to isolate isopeptidase inhibitors.

With this approach *O*-acyloxime derivatives of isatins have been isolated as inhibitors of UCH-L1, in the in low micromolar range (Table 2). Compounds structure analysis allowed the generation of further isoforms specific, UCH-L1 or UCH-L3 inhibitors, showing increased inhibitor potency and specificity [64]. The mechanism proposed for this class of compounds is a reversible competitive inhibition of the active site. The compounds were also able to inhibit the growth of different tumor cell lines, mimicking the effect of UCH-L1 specific siRNA.

UCH-L1 is a protease involved in Parkinson's disease (PD) and Alzheimer's disease (AD) as well in promoting cancer cell phenotypes [65-67]. Interestingly, whereas PD and AD are associated with reduced levels of UCH-L1 the tumor progression is dependent on the isopeptidase activity. Compounds inhibiting UCH-L1 should be beneficial for cancer treatment, whereas an enzyme activator could be effective toward PD and AD.

Derivatives of 33-Amino-2-keto-7H-thieno[2,3-b] pyridin-6-one, with a Kiaap of 2.8 μM against UCH-L1 have been recently developed with a similar screening strategy [68]. These inhibitors seem to be specific, since at the same concentration they are non-effective against other DUBs and cystein proteases. Differentially from the previous case the mechanism is a non-competitive inhibition of the Michaelis complex rather than of the free enzyme (Table 2).

Following a different strategy, an in silico virtual drug screening based upon the crystal structure data of UCH-L1, an enzyme potentiator and several inhibitors, the best one with an IC50 of 15 μM, were identified [69]. No data are available about specificity or in vivo effects of these compounds.

HTS approaches, using Ub-AMC (Ub- ubiquitin-7-amido-4-methylcoumarin) as substrate, have been used to isolate specific inhibitors of USP7.

USP7/HAUSP destabilizes p53, by antagonizing the autoubiquitination activity of MDM2 [70, 71]. Silencing of USP7 has p53-dependent growth suppressive effects in tumor cells. USP7 can deubiquinate additional substrates including FOXO4 and PTEN. In both cases USP7 promotes nuclear export and inactivation of the two proteins which leads to the reinforcement of the PI3K/PKB signaling pathway [72, 73].

After the HTS and subsequent optimization, a cyano-indenopyrazine derivative, HBX 41,108 was isolated as USP7 inhibitor [74]. This compound shows an IC50 0.42 μmol/L on Ub-AMC and further analysis suggest for a non-competitive reversible mechanism of inhibition. When tested against other cystein-proteases such as Cathepsin D, L, S or UCH-L1, an inhibitory activity can be measured (IC50>1μmol/L). Curiously, it was less effective in inhibiting the SUMO-protease SENP1 (IC50>10μmol/L). When added to colon cancer cell lines HBX 41,108 can trigger a p53-dependent apoptotic response. After this first discovery, a second-generation of more specific USP7 inhibitors would be on the way [30, 75].

USP8 is a regulator of membrane trafficking and

Table 2. Selective isopeptidase inhibitors

Compound	Isopeptidase	Activity	Specificity	Mechanism	Cell effects	Ref.
O-acyl oxime derivatives of isatins	UCH-L1	IC50 0.80-0.94 μM	IC50 for UCH-L3 17-25 μM	Reversible competitive active site directed	Promote proliferation of H1299 and SHSY5Y cells.	[64]
3-Amino-2-keto-7H -thieno[2,3-b] pyridin-6-one derivatives	UCH-L1	Ki 2.8 μM	Not active for UCHL3, and various cystein proteases	Uncompetitive substrate/ enzyme complex directed	Not reported	[68]
N,N0-4,40- Biphenyldiylbis(4 Ethylbenzenesulfo namide)	UCH-L1	IC50 15 μM	Not reported	Active site directed	Not reported	[69]
2-methyl- N -[1-(2-naphthyl) ethyl] benzamide derivative	PLpro	IC50 0.6 μM	IC50>100 for various DUBs and cystein proteases	Reversible competitive directed active site	Antiviral activity in Vero E6 cells, EC50=10-15 μM	[78]
Cyanoideno-pyrazine derivative	USP7	IC50 0.42 μM	Partial specificity	Reversible, non competitive	Induction of p53 activity, inhibition of cell proliferation and induction of p53 dep. apoptosis	[74]
Not reported HBX 28,231 HBX 28,218	USP7	Low μmolar range	No activity against various DUBs and cystein proteases.	Not reported	Induction of p53 activity, inhibition of cell proliferation and induction of p53 dep. Apoptosis.	[30] [75]
9-Oxo-9h-indeno [1,2-b]pyrazine-2,3 -dicarbonitrile analogues	USP8	IC50 0.98-0.56 μM	No activity against various DUBs and cystein proteases, partial activity against UCH-L3.	Not reported	Affected viability of cancer cells lines HTC116 and PC-3 IC50 0.5-1.5 μM	[77]

endocytosis and it interacts with the epidermal growth factor receptor (EGFR) [76]. One of the USP7 inhibitor identified in the HTS screening of Colland [74], the 9-oxo-9h-indeno [1,2-b] pyrazine-2,3-dicarbonitrile was active in the low micromolar and submicromolar range against USP7 and USP8 respectively. Chemical modification allowed the development of a derivative without activity toward USP7, but with submicromolar activity against USP8 [77]. The resulting compound was inactive against other cystein-proteases and DUBs with the exception of a partial activity toward UCH-L3. The inhibitor series were also able to affect cancer cell lines viability.

GRL0617, an inhibitor of the papain-like protease (PLpro), has been recently isolated by means of HTS. GRL0617, after synthetic optimization, shows an IC50 of 0.6 μM for the Plpro activity and an IC50 of 15 μM versus viral replication. Structural studies have evidenced that it binds within the S4-S3 subsites of the enzyme and induces a loop closure that shuts down catalysis at the active site [78]. Interestingly, USP7/HAUSP, USP18, UCH-L1 and UCH-L3 are not inhibited by GRL06017.

A patent as been recently filled for compounds, identified in a fluorescence polarization assay, active against USP2 and UCH-L3. The reported IC50 range from 100 nM to 50 μM and from 100 nM to 100 μM for UCH-L3 and USP2 respectively [79].

Conclusions

Isopeptidases in general and deubiquitinases more in specific, exhibit many characteristics of a promising therapeutic target. Different biotech companies are investing on this class of enzymes and specific inhibitors are emerging from different kinds of screening. The searching for specific inhibitors of isopeptidases is a growing business. Perhaps we should ask to ourselves whether in fact highly specific inhibitors would be able to trigger apoptosis in cancer cells [80]. Or whether more broad inhibitors, hitting groups of isopeptidases at the same time, might be favored to kill tumor cells highly resistant to apoptosis.

Recently, we silenced 53 different isopeptidases using a small siRNA library, in cancer cells. We found that down-regulation of certain isopeptidases synergistically enhanced the pro-apoptotic activity of known anti-tumor drugs. However, the simple down-regulation of a single isopeptidase was insufficient to trigger a robust apoptotic response [81].

A final consideration regards what we know about isopeptidases. For many aspects our knowledge in this field are at an early stage. In order to develop anti-cancer strategies, based on isopeptidase inhibition is of paramount importance understanding in detail the role played by individual isopeptidase in cell cycle, apoptosis and growth-related pathways. New pieces of information are accumulating, but further work is waiting for us.

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