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Review

The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications

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Abstract

The highly conserved eukaryotic ubiquitin–proteasome system (UP-S) plays a pivotal role in protein homeostasis and is critical in regulating normal and cancer-related cellular processes. The hierarchical nature of the UP-S provides a rich source of molecular targets for specific intervention and has therefore arisen as a promising approach to innovative anticancer therapies. The first in class proteasome inhibitory agent Bortezomib (Velcade™) has recently obtained regulatory approval for the treatment of multiple myeloma. Ubiquitin-mediated degradation is a complex process that is comprised of well defined steps involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). Although a single E1 activates the ubiquitin conjugation machinery, a large number of E2 conjugating enzymes and E3 ligases are now known to exist. Proteins tagged with ubiquitin are subsequently recognised by the proteasome for digestion and fragmentation. The enzymatic nature, multitude of E3s and their specific substrate recognition predestines them as therapeutic targets. This article will review known inhibitors of the proteasome and their molecular mechanisms as well as ongoing developments and promising avenues for targeting substrate-specific E3 ligases that are likely to yield a new class of therapeutics that will serve and complement the armamentarium of anticancer drugs.

Keywords: Proteasome; Ubiquitination; Cancer; E3 ligases; UP-S inhibition

1. Introduction

1.1. Protein degradation in eukaryotic cells

Nearly all proteins in mammalian cells are continually being degraded and replaced by *de novo* synthesis. The rates of degradation of individual cell constituents vary widely, with half-lives ranging from 10 minutes to several days or weeks [1,2]. Degradation is also a means of eliminating mis-folded, damaged, or mutant proteins with abnormal conformations whose accumulation might be harmful to the cell [1].

Lysosomal and proteasomal degradation are the two major pathways for cellular protein turnover. Cell surface proteins that are taken up by endocytosis are degraded in the lysosome [1–3]. Lysosomal degradation accounts for 10–20% of normal protein turnover. However, the bulk of cellular proteins (80%) is degraded by the proteasome in the cytoplasm and nucleus after being tagged with ubiquitin. In a few exceptions, such as membrane-anchored proteins, ubiquitination could also lead to degradation in the lysosome [1,3,4].

The destruction of regulatory proteins is irreversible and is involved in the physiological regulation of signal

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transduction, transcription, cell cycle and antigen processing, therefore intracellular proteolysis is an essential regulator of protein function [5]. Aberrations in this pathway provide a variety of pathological phenotypes and hence targets for therapeutic intervention in a number of diseases, particularly cancer [3,6].

1.2. The ubiquitination pathway

The ubiquitin–protein ligase system was discovered in the early 1980's prior to the knowledge of the presence of the proteasome. Ciechanover and co-workers [7] described that the presence of three enzymes, termed E1, E2, and E3, were absolutely necessary, along with adenosine triphosphate (ATP), for the conjugation of ubiquitin to proteins and designated these enzymes as the ubiquitin–protein ligase system. E1 (the Ub-activating enzyme) was shown to mediate an ATP-dependent transfer of ubiquitin to an E2 (Ub-conjugating enzyme/Ubc), and the E2 activity involves either the transfer of an activated ubiquitin moiety directly to the substrate or an E3 (Ub-ligase) that can also ubiquitinate specific substrates [7] (Fig. 1(a)). In most instances, polyubiquitinated proteins are degraded by the 26S proteasome [6] (Fig. 1(a)).

However, ubiquitination can also regulate the activity and location of target proteins by means of monoubiquitination or alternate multiubiquitination [8–10]. Commonly, polyubiquitin chains linked *via* lysine residue 48 of ubiquitin mediate destruction of proteins in the proteasome. If ubiquitin is linked through, e.g., Lys63 to a receptor protein, it could function as an internalisation signal [8]. Alternatively, monoubiquitination can lead to nuclear export and translocation of proteins into the cytoplasm [10].

1.3. The proteasome

Proteasome-mediated protein degradation is commonly recognised as an integral part of cellular protein turnover and homeostasis. Proteasomes are localised in the nucleus and cytosol and can constitute up to 1% of the cellular protein content in eukaryotes [11]. The proteasome was first described in 1988, the functional large 26S proteasome assembles from ring-shaped 19S and 20S particles, which are composed of numerous polypeptide subunits (Fig. 1(a)) [11,12].

The 20S catalytic core resembles a cylindrical stack of 4 rings, the two outer rings contain 7α subunits, and the two inner rings are composed of 7β subunits each, together they form a narrow pore (Fig. 1(a)) [11]. This array of 28 subunits has distinct activities, e.g., the MB1 β subunit confers trypsin-like and chymotrypsin-like activity, the delta chain β-subunit has caspase-like (peptidylglutamyl-peptide hydrolysing) effects and can cleave bonds on the carboxyl side of basic, hydrophobic, or acidic amino acid residues [11,13].

These multicatalytic protein degrading complexes were identified as critical compartmentalised components of the highly selective intracellular breakdown of ubiquitin-tagged proteins (Fig. 1) [2,12–14]. Polyubiquitination of protein substrates (Fig. 1(a)) directs them for degradation by the proteasome. ATP-dependent unfolding of the polyubiquitinated substrate allows its translocation into the 20S catalytic pore (Fig. 1(a)). Peptidases located in the subunits of the proteasome then digest the substrate, releasing peptide fragments.

2. Ubiquitination in cancer

Cancer can develop from stabilisation of oncoproteins or, alternatively, from destabilisation of tumour suppressor genes. Cancer-associated proteins that have been reported to be regulated by the ubiquitin–proteasome system include the tumour suppressors, p53 and p27, the cell surface receptors for growth factors, epidermal growth factor receptor (EGFR) or transforming growth factor-β Receptor (TGF-βR) complex (Fig. 2), as well as cell cycle and oncogenic transcription regulators (Fig. 2) [3,6,15,16].

A recent study investigated the level of 20S catalytic proteasome core in plasma of healthy individuals *versus* that of patients' plasma having various neoplastic diseases including acute myeloid leukaemia (AML), Hodgkin's disease, chronic myeloproliferative syndromes and solid tumours [17]. The results derived by comparing these individuals showed that the 20S catalytic proteasome core levels in plasma of the pathological states was markedly increased, and was, in fact, up to 1000-fold higher in patients with solid tumours compared with plasma of normal subjects [17].

While the number of cancer-related proteins tagged with ubiquitin and destined for degradation in the proteasome as well as proteasome particles are manifold, little is known about the role of the individual components of the ubiquitin system in tumourigenesis. Only a limited number of the ubiquitin-conjugating enzymes and ligases have been examined for their expression pattern in a large number of human tumours [4,15,18–21].

A single E1 enzyme is responsible for the initial ubiquitin activation in mammalian cells. Although the deletion of the gene has been shown to be lethal, an involvement in cancer has not been described [4,22] (Fig. 1(a)). An E2 conjugating enzyme (UbcH10) and few E3 ligases studied in relation to cancers, were shown to have high expression levels in malignancies [18–21,23,24]. The E2 UbcH10, for which protein expression data are lacking, was found at extremely low RNA levels in normal tissues, while RNA

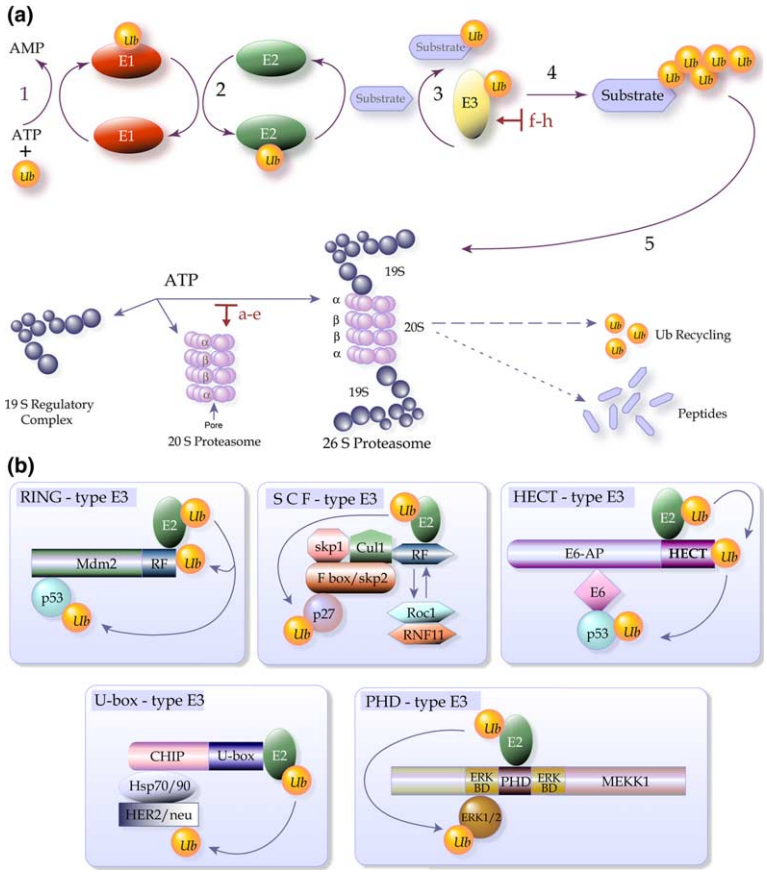

Fig. 1. (a) Overview of the ubiquitin–proteasome pathway and possibilities for intervention. Known and proposed targets are indicated by brown arrow, inhibitors listed in Table 1 are marked at their respective position of interference. 1 = Ubiquitin activation; 2 = Ubiquitin (Ub) transfer from a ubiquitin activating enzyme (E1) via a thioester linkage to a ubiquitin conjugating enzyme (E2), 3 = E3 ubiquitin ligase which facilitates positioning and transfer of ubiquitin from the E2 directly onto the substrate and 4 = ubiquitin chain elongation by conjugation of Ub to lysine residues of ubiquitin at position 48 (or alternatively Lys29 and 63) by an E3 ligase. 5 = Transfer of Ub-tagged proteins for adenosine triphosphate (ATP)-dependent substrate degradation in the 26S proteasome, which assembles from a 20S core and two 19S regulatory complexes. (b) The major types of E3 ligases are shown in Fig. 1 B. The RING-type ligases can either be monomeric such as human Mdm2 or a multisubunit complex (SCF). Mdm2 acting on p53 and SCF acting on one of its prominent substrate, p27, are presented as examples. The components of the SCF complex are labelled. Roc1 and RNF11 may have interchangeable functions as RING-fingers (RF) in the SCF complex. E6-AP is depicted as HECT-type E3 ligase, the presence of HPV oncoprotein E6 is necessary for the ubiquitination of p53. CHIP is shown as an example of an U-box type E3. MEKK1 represents the best-known PHD domain containing protein with E3 ligase activity. MEKK1 ubiquitinates ERK1/2 after binding to one of its ERK binding domains (ERK BD). RING, really interesting new protein; RNF-11, RING-finger protein 11; Mdm2, mouse double minute 2; ERK, extracellular signal-regulated protein kinase; Hsp 70/90, heat shock protein 70/90; CHIP, carboxyl terminus of hsp 70-interacting protein; E6-AP, E6 associated-protein.

expression was high in primary tumours such as lung, stomach, uterus and bladder cancers [20].

The prototypic example of a protein that plays a pivotal role in many cancers and is regulated by substrate-specific E3 ubiquitin ligases is p53. The tumour suppressor protein p53 plays a crucial role in cell cycle control, DNA repair and apoptosis (Fig. 2, reviewed in Refs. [18,19]). Two E3 ligases, namely the RING-type E3 ligase Mdm2 and the HECT-type E3 ligase E6-AP can effect its degradation by the proteasome (Figs. 1(b) and 2) [3,15]. Cervical cancers have been strongly associated with infections by the oncogenic human papilloma virus (HPV) forms 16 and 18. As a result, the E6 and E7 gene products of HPV are detected in these tumours. It is believed that p53 is present at very low levels in cervical cancer cells because it is associated with the E6/E6-AP E3 ligase complex, and is therefore rapidly degraded by ubiquitin-mediated proteolysis (Fig. 1(b))[15].

In the vast majority of tumours, Mdm2 regulates p53 by either inhibiting its transcriptional activity or targeting p53 for ubiquitination and proteasomal degradation [18] (Fig. 1(b)). Thus, overexpression of Mdm2 provides an alternative to p53 mutation and/or deletion leading to development of cancers [9]. Mdm2 is recognised as the major ubiquitin ligase for p53 and has been found overexpressed in several human tumour types. While Mdm2 overexpression by means of gene amplification is mainly seen in soft tissue sarcomas and gliomas, transcriptional/translational overexpression has been found in acute lymphoblastic leukaemia (ALL), melanomas and breast carcinomas [18,24–26]. Mdm2 overexpression was predictive for a poor outcome in sarcomas,

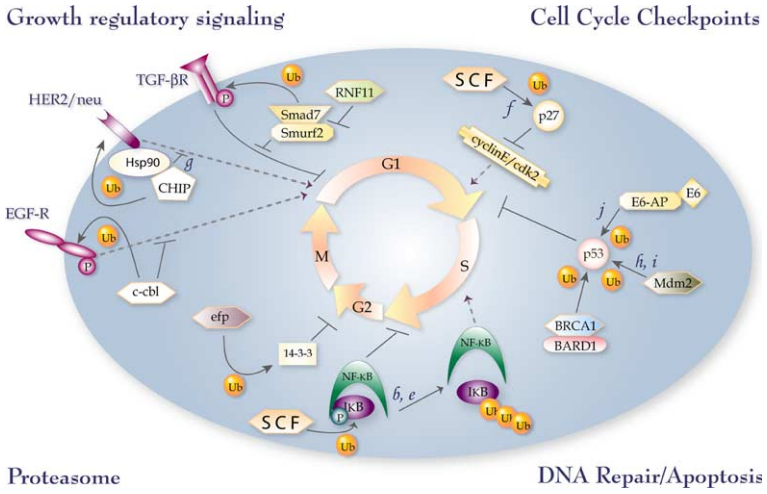

Fig. 2. E3s and their substrates in the cancer cell. Shown is E3-mediated substrate ubiquitination. U-box E3s are shown as pentagons, RING-type E3s as hexagons, and HECT-type E3s as an octagon. Arrows with broken lines indicate stimulatory effects. Strategies for therapeutic intervention listed in Table 1 are indicated as lower case letters. The description of the individual pathway in the figure is included in the text. BRCA1, breast cancer; BARD1 = BRCA1 associated RING domain.

gliomas and ALL, but was identified as a favourable prognostic marker in melanoma and breast cancer patients. Latter studies indicate that Mdm2 might have tissue-specific, p53-independent functions in cancers, for example as a regulator of cell proliferation by targeting tumour suppressor gene(s) as well as other key proteins governing cell growth (s) [26,27].

Another HECT-type E3 ligase that has emerged as being important in certain cancers is Smad ubiquitination regulatory factor-2 (Smurf2). High-level expression of Smurf2 was shown to correlate with a poor prognosis in oesophageal squamous cell carcinoma [21,28]. Smurf2 directs the ubiquitinylation and proteasomal degradation of R-Smads, and the TGF-β receptor complex (Fig. 2) [29]. TGF-β signalling molecules have been shown to play a crucial role in breast and other cancers and it is therefore likely that the Smurf2 HECT-type E3 ligase will also have functions in these tumour types [30].

A RING-type E3 ligase with proven role in breast cancer is BRCA1. Germline mutations in the RING finger of the tumour suppressor gene *BRCA1* predispose women to early onset breast tumours [31]. BRCA1 ubiquitin ligase activity is enhanced when it is dimerised with BRCA1-associated RING domain (BARD1) and has been implicated in p53-associated DNA damage response [32] (Fig. 2).

Together, these findings indicate that cancer cells maintain a higher amount of proteolytic machinery that would likely enable them to cope with the normal cell's proteomic ''repair response'' and either facilitate degradation of tumour suppressor proteins or activation of proto-oncogenes [3].

From this generalised perspective and after considering the fact that cancer remains a largely incurable disease with few long-term treatment options, the ubiquitin–proteasome system evolves tantalisingly with a multitude of opportunities for targeted therapeutic interventions.

3. Possibilities for therapeutic intervention – utilising the proteasome catalytic activities

3.1. Irreversible 20S/26S inhibitors

The 26S proteasome large multi-subunit complex, which is present in both the cytoplasm and the nucleus in all eukaryotic cells, has the arduous task of eliminating all cellular proteins tagged for degradation through the polyubiquitination process (Fig. 1(a)) [3]. Because proteolytic degradation includes a large variety of proteins with crucial function(s) in regulation of cell growth, the proteasome *per se* would seem a rather implausible therapeutic target for a highly selective cancer therapy. However, the development of potent and specific chemical inhibitors of the proteasome has created much excitement about the therapeutic potential of such agents, not only in cancer treatment, but also in inflammatory and immune diseases [6,33].

Unlike other proteases, the proteasome contains an active site N-terminal threonine residue in the MB1 subunits of its β-rings that can be targeted by pharmacophores linked to short peptides (Table 1, Figs. 1 and 3) [11,34]. The first such specific inhibitor discovered to interact with the proteasome was lactacystin (Fig. 3). Lactacystin is a *Streptomyces* metabolite, which interferes with the 20S subunit (Fig. 1(a), Table 1). Three distinct peptidase activities of this enzyme complex (trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide hydrolysing activities) are inhibited by lactacystin, the first two irreversibly, and all at different rates [34].

Table 1
Known inhibitors of the ubiquitin proteasome system. Mdm2, human mouse double minute 2

Target protein	Inhibitory agent(s)	[Reference]
20 S Proteasome core		
	(a) Peptide aldehydes, e.g., MG-132	[36,38]
	(b) PSI	[40]
	(c) Lactacystin	[34,35]
	(d) Epoxomicin	[37]
	(e) Peptide boronic acids, e.g., PS-341(bortezomib, Velcade™)	[36,39]
Substrate-specific E3s		
SCF	(f) Protac-1, methionine aminopeptidase-2 chimera with ovalicin, target proteins to SCF for degradation	[59,60]
CHIP	(g) Geldanamycins	[85,86]
hMdm2	(h) Nutlins (Nutlin-3), compounds 1–3	[54,64]
	(i) Mdm2 antisense oligonucleotides for silencing	[63]
	p53-GAr domain chimeras for gene therapy	[62]
E6-AP/E6	(j) Zinc ejectors (C16)	[76]

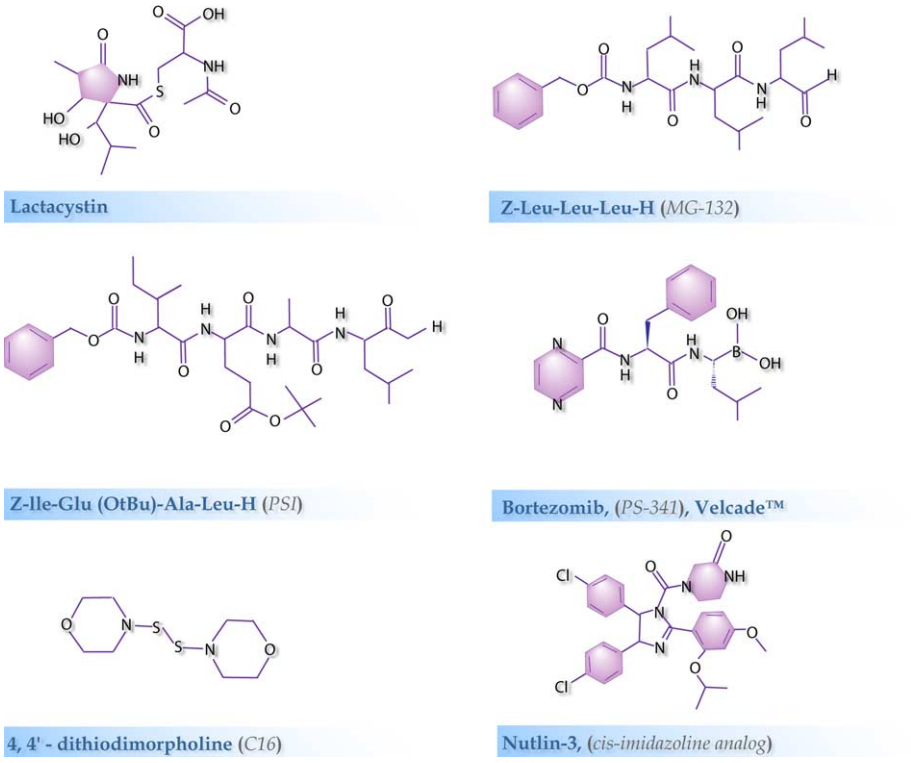

Fig. 3. Molecular structures of experimentally and clinically used proteasome and E3 ligase inhibitors.

Lactacystin also inhibits peptide hydrolysis by the larger 26S complex and thus prevents ubiquitin/proteasome-mediated degradation of regulatory proteins in the cell [35]. Consequently, lactacystin has been found to inhibit cell cycle progression as well as to exert pro-apoptotic effects and thus it seems that transformed cells are much more sensitive to blockade of the proteasome than are normal cells [35,36].

Despite the described selective effects of lactacystin in cancer cells, this agent remains a tool for studying proteasomal processes in *in vitro* systems only, largely owing to its poor metabolic stability and irreversible binding to the proteasomal subunit [6].

Epoxomicin (Table 1) is an *Actinomycetes*-derived, irreversible proteasome inhibitor, which possesses *in vitro* and *in vivo* activities [37]. Epoxomicin binds specifically to the 20S catalytic subunits of the proteasome resulting in inhibition of the chymotrypsin-like activity. It acts several-fold faster and with even greater specificity than lactacystin, unlike some of the reversible inhibitors (see below) [37]. However, its use in humans is restricted for the same reasons described for lactacystin.

Thus, irreversible proteasome inhibitors appear to have no desirable pharmacological features and are therefore currently no longer explored for cancer drug development.

3.2. Reversible 20S/26S inhibitors

The first generation of synthetic proteasome inhibitors was designed to exert reversible inhibitory effects [38]. MG-132 (carbobenzoxyl-leucinyl-leucinyl-leucinal) is the best- known representative of the peptide aldehyde inhibitors (Fig. 3, Table 1). MG-132 is a cell-permeable, potent and reversible inhibitor that reduces the degradation of ubiquitin-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities. However, MG-132 was found to be non-selective because it inhibits other proteases as well, e.g., cathepsin B [39]. Together with lactacystin, MG-132 is the most frequently used compound to study protein degradation/stabilisation experimentally.

PSI (**P**rotea**s**ome **I**nhibitor, Fig. 3, Table 1) is an inhibitor of the chymotrypsin-like activity of the 20S proteasome. It causes accumulation of ubiquitinated proteins and has been reported to prevent the activation of the transcription factor Nuclear Factor (NF)-κB, while stabilising the newly phosphorylated form of Iκ-B-α bound to NF-κB (Fig. 2) [40]. It has recently been shown that PSI is capable of reducing the tumour load in mice injected with murine 5TGM1 plasmacytoma cells and inhibits the development of osteolytic lesions [41]. However, the good pharmacodynamics of PSI are outweighed by its poor pharmaceutical properties. The peptide compound is insoluble and requires high micromolar concentrations to exert pharmacological effects.

The second-generation proteasome inhibitors were consequently designed to encompass both, the selectivity of the irreversible agents and reversibility of drug effect, while possessing acceptable pharmaceutical and pharmacological properties. Dipeptide boronic acid analogues, in contrast to the peptide aldehyde agents, are potent, selective and reversible proteasome inhibitors. The first in its class compound, namely PS-341, generically known as bortezomib and by its trade name Velcade™ has recently obtained marketing approval by the Food and Drug Administration (FDA) (Fig. 3) [33,39,42,43]. Boronic acids block threonine protease activity by inhibiting the chymotryptic activity of the proteasome resulting in the attenuation of the degradation of cell cycle regulatory proteins, e.g., I-κB, cyclin E, p53 or p27 (Fig. 2) [36,38,39,44]. While affecting multiple proteins, boronic acid treatment leads to inhibition of tumour cell growth and induction of apoptosis [39]. For this class of inhibitors, it has further been shown that inhibition constants (K_i) correlate with *in vitro* and *in vivo* cytotoxicity against tumour cells, and that a broad range of cancer cell types can be inhibited [39].

Bortezomib (Velcade™) is broadly active in cancer cells, yet it has a only a few toxic effects on normal cells. It was shown to be active as a single agent in preclinical human tumour xenografts models and in primary cultures of haematological and solid tumour types such as multiple myeloma, lymphoma, prostate and head and neck cancers [33,36].

The major mechanism of action of bortezomib (Velcade™) seen in preclinical models systems is a time- and concentration-dependent induction of apoptosis and this appears to be cell type-specific. This could be by the blockage of tumour necrosis factor (TNF) α-induced NF-κB activation through inhibition if I-κB phosphorylation and degradation in multiple myeloma cells (Fig. 2) [36,45], or G2-M-phase arrest along with induction of $p21^{cip/waf1}$ or stabilisation of p53 in prostate and lung cancer cells, respectively [46].

Phase I trials in patients with refractory haematological and solid malignancies with bortezomib (Velcade™), showed that the drug was well tolerated, while producing efficacious clinical responses [33,47,48]. In a phase II study in patients with highly-refractory multiple myeloma, this clinical benefit response was confirmed. A 35% overall response rate and 10% complete responses were seen [49]. Since 80% of all cellular proteins are processed by the 26S proteasome, Bortezomib (Velcade™) can barely be considered as a specific molecularly-targeted anticancer agent. It's broad range of activities and few, but significant, grade 3/4 side-effects, suggests that there should be considerable opportunities for improvement. Many possibilities for the development of more specific interventions in the UP-S protein degradation enzymatic cascade exist upstream from the proteasome (Fig. 1) [3,11,36]. The following section will therefore focus on approaches that are currently being investigated.

4. Potential avenues of specific inhibition of protein degradation

Due to the hierarchical nature of the ubiquitination cascade, namely having only one E1 to transfer ubiquitin to E2s, cessation of E1 activity would lead to general, but highly detrimental/lethal, effects on all cells, both normal and diseased [3]. However, blockage of enzymes or recognition motifs downstream of E1, that affect the ubiquitin-involved process only partially, may result in disease-specific targeted anticancer activity.

The multitude of E2s and E3s and their substrates participating in growth regulation and tumour progression, provides a broad spectrum for drug specific-targeted intervention (Fig. 2, Table 2). Therapeutic approaches of various types are currently underway to inhibit individual components of multi-subunit E3 complexes, Mdm2, E6-AP, Efp (Oestrogen-responsive finger

Table 2
Investigational targets for specific therapeutic intervention in the ubiquitination pathway

Pathway	Target	Mechanism of inhibition
SCF: F-box proteins		
	Skp2	Stabilisation of cell cycle regulators and tumour suppressors such as p27, Rb, p130, PTEN
	Fbw7/hCdc4	Phosphorylation-mediated turnover of cyclin E and notch
Ubiquitin E3 ligases		
	E6-AP/E6	Stabilisation of p53 by inhibition of ubiquitination
	Mdm2	Stabilisation of p53 by inhibition of ubiquitination
	Efp	Stabilisation of 14-3-3 cell cycle inhibitory protein
	C-cbl	Activation of cbl confers degradation of RTKs and termination of signalling
	BRCA1/BARD1	Stabilisation of tumour suppressor function/DNA repair
	CHIP	Induction of prodegradation chaperone complex that downregulates HER2/Neu

RTKs, receptor tyrosine kinases.

protein) and CHIP ligases (Figs. 1, 2, Table 2) [18,19]. The E3 Ub ligase family comprises two distinct classes: the HECT-type and adaptor type E3s containing a RING finger, a U-box or a PHD domain [18,31,50–53] (Fig. 1(b)).

Each of these 4 forms of E3 ligases is characterised by similar catalytic mechanisms, and hence provides a unique targeting opportunity (Fig. 1(b)). The first specific and effective, small molecule (Nutlin-3) that prevents the interaction of Mdm2 ligase with its substrate p53 by blocking the binding pocket, thus stabilising the tumour suppressor protein, has recently been described [54].

4.1. Ring-finger ligases

RING-finger like proteins can be either monomeric or members of multisubunit E3 ligase complexes (Fig. 1(b)) [18,31,55].

4.1.1. Multisubunit E3 complexes

The SCF (Skp1-Cullin-F-box protein) complexes are a prominent family of ubiquitin–protein ligases and ubiquitinylate a broad range of substrates involved in cell cycle progression, signal transduction and transcription (Figs. 1, 2, Table 2) [55]. Deregulation of SCF-dependent proteolysis can likely contribute to neoplastic transformation, because key proteins in this process, e.g., loss of cyclin E ubiquitination and accelerated proteolysis of p27 or I-κB, are known to be modulated by SCF complex [55–57] (Fig. 2). The SCF complexes consist of Cul1, Roc1, Skp1 and a F-box protein unit, the variable F-box protein subunit binds the substrate to be ubiquitinated (Fig. 1(b)) [55]. The large number of F-box proteins in the human genome (at least 38) is believed to allow for the specific ubiquitination of a variety of functionally and structurally diverse substrates [58].

Hence by targeting specific F-box proteins for inhibition either with small molecule drugs, short inhibitory RNAs (siRNA) or antisense oligonucleotides, highly specific stabilisation of many cell cycle regulators and tumour suppressors, such as p27, Rb, or PTEN, can be achieved resulting in the inhibition or reversal of oncogenic processes (Table 2). Experimental strategies that are pursued in preclinical settings are inhibition of $SCF^{\beta-TrCP}$ with "Protacs" which are chimeric molecules that target proteins to the Skp1-Cullin-F box complex (Table 1) [59,60]. Sakamoto and co-workers designed the chimeric compound Protac-1, which – in a 'proof of principle' – has been aimed to recruit MetAP-2 (methionine aminopeptidase-2) to the SCF complex. MetAP-2 catalyses the cleavage of N-terminal methionine from nascent polypeptides and enhances endothelial cell proliferation, it can be inactivated by the angiogenesis inhibitor ovalicin [61]. This "bispecific" compound Protac-1 thus leads to increased degradation/ubiquitination of the proangiogenic peptide MetAP-2 and also inhibits angiogenesis after activation of ovalicin.

4.1.2. Monomeric RING E3-ligases

The monomeric RING-finger family of E3s includes Mdm2, Efp, c-cbl, and BRCA1 (Fig. 2, Table 2) [19,31]. Mdm2 possesses a C-terminal RING-finger domain with demonstrated E3 ubiquitin ligase activity including auto-ubiquitination (Fig. 1(b)). Since, Mdm2 promotes the degradation of p53, it has received much attention for targeted cancer therapy. Initially, Mdm2 antisense oligonucleotides and gene therapy methods were developed, but were hampered by delivery problems to a target tissue [62,63]. Major breakthroughs in small molecule inhibitor design were recently reported [54,64] (Table 1, Fig. 2). Lai et al. [64] identified 3 distinct compounds (compounds 1–3), representing a benzsulfonamide structure, a urea analogue and an imidazolone derivative, which were all capable of selectively inhibiting human Mdm2 (Hdm2) mediated ubiquitination of p53. *cis*-Imidazoline analogues, termed Nutlins, were found to antagonise Mdm2 by binding to the p53-binding pocket and to restore the p53

pathway [54] (Fig. 3). Nutlin-3, in particular, appears to possess good pharmaceutical and pharmacological properties and was shown to markedly inhibit tumour growth in preclinical animal models. Hence, Nutlin-3 represents the first small molecule with antitumour activity that can counteract with a specific E3 ligase and will potentially open the rapid therapeutic exploitation of the ubiquitin conjugation system.

Efp is an oestrogen-inducible RING-finger E3 ligase that ubiquitinates the 14-3-3σ protein. 14-3-3σ has been shown to have tumour suppressor function, and the inhibition of Efp would lead to its stabilisation [19,65]. Efp has been found to be one of the oestrogen receptor (ER) gene targets and has been postulated to play a critical role in proliferation of mammary tumours. Efp has therefore been proposed as molecular target in breast cancer [66,67]. Preclinical studies conducted with the ER-positive human tumour xenograft model MCF-7 showed that Efp-inhibition by antisense oligonucleotides led to reduction of MCF-7 tumour growth [66].

C-cbl is an E3-ligase with a RING-finger and a SH2 domain. It recognises phosphorylated tyrosines on receptor tyrosine kinases through its SH2 domain and negatively regulates signalling by facilitating receptor ubiquitination [15,68]. Cbl involvement in receptor tyrosine kinase signalling makes it a highly attractive target for interdiction and for specific small molecule type cancer therapy (Fig. 2). C-cbl-dependent ubiquitination was shown to be important for the early endosome to late endosome/lysosome sorting step of EGFR downregulation and hence was established as the major endogenous ubiquitin ligase responsible for EGFR degradation [69]. Cbl has also been described as a suppressor of the HER2/Neu oncogene, which belongs to the EGFR superfamily [70]. Overexpression of EGFR, HER2/Neu, and EGF receptor family heterodimer signalling are major contributors to uncontrolled proliferation in many malignant diseases [71]. HER2 antibodies, e.g., trastuzumab (Herceptin™) have been shown to direct HER2 to the c-cbl-regulated pathway, leading to antibody-induced degradation and suggesting that mechanisms underlying immunotherapy might be due to EGF-induced degradation of HER2 by a Cbl-mediated process [72,73].

The BRCA1 RING E3 ligase plays a role in DNA repair and transcriptional control and thus functions as tumour suppressor (Fig. 2, Table 2) [31]. *BRCA1* mutations in its N-terminal RING-finger are associated with familial breast and ovarian carcinomas (reviewed in [31]). BRCA1 mediates its own ubiquitination in vitro, and was recently reported to ubiquitinate p53 in a complex with BARD1 and other partner proteins. However, it remains to be determined whether its E3 activity is critical for its role in DNA repair and induction of apoptosis (Fig. 2) [31,32]. Therapeutic approaches based on replenishment of *wt*-BRCA1 expression have been evaluated in ovarian cancer patients. Although gene therapy utilising viral vectors had shown promise in nude mice xenografts and in initial phase I trials of patients with extensive metastatic cancer, the phase II trials were negative [74]. In view of the successful identification of small molecule Mdm2 antagonists based on its crystal structure and high throughput technology, BRCA1 antagonists following this strategy might soon become available for testing [54].

4.2. HECT-Type E3 ligases

Another important class of E3 ligases is defined by the presence of a HECT domain, an active site region first identified in the **h**uman **E**6-AP **C**OOH **t**erminus (Fig. 1(b)). HECT-type E3 ligases contain a C-terminal conserved cysteine residue to which the activated ubiquitin moiety from an E2 conjugate is transferred, whereas the N-terminal domain seems to be involved in substrate specificity and recognition [3,15]. The E6-AP E3-ligase was the first one to be described in targeting p53 for rapid ubiquitinylation (Figs. 1(b) and 2) [3,18]. This process is initiated in the presence of E6, the gene product of HPV-16 (Figs. 1(b) and 2) [75]. The relevance of the E6-AP HECT-type E3 ligase is therefore limited to cancers with causative links to oncogenic HPV infections, such as cervical carcinomas [15]. Possibilities for the inhibition of the E6/E6-AP association have been investigated. The most notable approach is the use of zinc ejecting compounds [76]. Zinc binding is a requirement for E6 interaction with E6-AP, based on the unique zinc finger motif of E6. Zinc ejector 4,4′-dithiodimorpholine (C16) was discovered as an agent that is devoid of antiproliferative effects in HPV-positive normal cells or HPV-negative cancer cell lines, it can, however, selectively inhibit E6-AP activity in HPV-positive tumour cells by removing zinc atoms from the E6 protein. Thereby indicating that it is possible to target a particular zinc finger motif with a small molecule (Fig. 3) [76]. The possibility to specifically interact with the E6 zinc finger motif, while conferring differential cellular toxicity, opens the way to attack the various E3 RING ligases described before, owing to the fact that the RING domain is a specialised zinc finger [77].

A HECT-type ubiquitin ligase, which can be linked to malignant disease, is Smurf2 ubiquitin E3-ligase [21]. Smurf2 was found to negatively regulate Smad signalling, Smad proteins serve as key signalling effectors of the TGF-β superfamily of growth factors [3,18]. TGF-β signalling requires the action of Smad proteins in association with other co-activator and co-repressor proteins to modulate target gene transcription. Smad2 and Smad3 both associate with the c-Ski and Sno oncoproteins to repress the transcription of Smad target genes [78]. Smurf2 ligase displays preferences for Smad2 compared with other receptor-activated Smads (R-smads)

[79]. Smurf2 ubiquitinylation of Smad2 and its co-repressor Sno reduces its ability to promote transcription and hence regulates the competence of a cell to respond to TGF-β signalling (Fig. 2) [18,23,79].

We have recently identified a small RING-H2-finger protein RNF11 that is associated with Smurf2 E3 ligase (Figs. 1 and 2) [23,80,81]. RNF11 binding to Smurf2 in mammalian cells suggests that similar to Smads 2 and 3, it may also recruit targets for destruction by Smurf2 E3 ligase [23]. The two most interesting RNF11/Smurf2 targets, which have been reported are ZBRK1, and AMSH [81]. ZBRK1 is a zinc-finger protein and is known to repress target gene transcription in a BRCA1-dependent manner. Interestingly, ZBRK1 was screened for expression in 61 primary breast cancers and found to be underexpressed in 28 (45.9%) of the cases [82]. Since both Smurf2 ligase and the RNF11 protein were found strongly expressed in Head and Neck (H&N) tumours, and RNF11 is also expressed at exquisitely high levels in breast cancers, a tumour type known to be governed by TGF-β signalling, finding modes of specifically regulating Smurf2 and RNF11 would provide unique opportunities for therapeutic intervention in breast and H&N carcinomas [21,23,28].

4.3. U-box E3 ligases

U-box proteins have only been recently discovered as a new E3 ligase family, mainly because of their similarity with the three-dimensional structure of the RING ligases [50,83]. Hatakeyama and colleagues demonstrated that U-box proteins in conjunction with E1 and E2, but in the absence of other E3 proteins could mediate ubiquitination, whereas deletions or point mutations in the U-box domain would abolish this activity [83]. The best characterised U-box ligase, is CHIP. It contains two tetratricopeptide repeat (TPR) domains that facilitate interaction with the carboxyl terminus of the molecular chaperones Hsp70 or Hsp90 (Fig. 1(b)). The combination of CHIP with Hsp90 mediates the ubiquitination of the glucocorticoid receptor and acts as a classical E3 ligase [50]. While the U-box domain executes the E3 ligase activity, the TPR motif associates with heat shock proteins and the CHIP/Hsp90 complex acts as a protein quality control ubiquitin ligase that is able to selectively lead abnormally folded proteins, recognised by the molecular chaperones, towards degradation by the proteasome (Fig. 1(a) and 2) [84]. CHIP is currently attracting great excitement as a cancer target, because it was shown that it mediates a degradative pathway for "abnormal proteins" which can impact upon the development and/or aggressiveness of several types of adenocarcinomas, including breast and ovarian cancers *via* the proto-oncogene *HER2/Neu* [71,85]. Because of HER2/Neu persistence and resistance to degradation and because of its propensity for dimerisation to other HER2 family members, its overexpression is associated with a poor disease prognosis [73]. Thus, modulating HER2/Neu interaction with CHIP could enhance its destabilisation and affect HER2 signalling. Moreover, the CHIP/HER2 interaction can be enhanced by geldanamycins such as geldanamycin (GA) and 17-AAG (17-allylamino-geldanamycin), the first in its class antitumour ansamycin, which is currently in phase I/II clinical trials (Tables 1, 2, Fig. 2) [85–88]. The CHIP ubiquitin E3 ligase controls both the association of Hsp90 chaperones with HER2 and its down-regulation induced by Hsp90 inhibitors, thus supporting the concept that modulation of substrate-specific E3 ligases can lead to targeted specific tumour inhibition.

4.4. PHD domain-containing proteins with E3 ligase activity

The plant homeodomain (PHD) proteins – like the U-box E3 ligases – resemble the RING finger domain [52,53]. PHD motifs are a specialised form of zinc finger and have been identified in over 400 eukaryotic proteins. They have initially been known to be involved in chromatin-mediated transcriptional regulation. However, several Karposi's sarcoma-associated new viral PHD-containing proteins have been discovered that are targeted to cellular membranes, namely the modulator of immune recognition (MIR) proteins [52,53]. The latter were found to have E3-like function because MHC (major histocompatibility complex) class I chains undergo ubiquitination in presence of MIR1 or 2 and mutations in MHC class I lysine residues or the zinc-coordinating residues of the PHD domain block ubiquitin addition [52]. In accordance with these findings, the mitogen activated protein (MAP) kinase kinase kinase MEKK1, which harbours a PHD domain (Fig. 1(b)), was found to exhibit E3 ligase activity towards ERK1/2 [53], and this activity could be abolished by mutations of conserved cysteines in the PHD domain [53]. ERK1/2 is widely expressed in tumour cells and provides a protective effect against apoptosis, while constitutive MEKK1 activation is sufficient to cause apoptosis and downregulate ERK1/2 survival signals through UP-S degradation pathways [53,89]. Hence, targeting PHD domain-containing E3 ligases by either means of activation (e.g., MEKK1) or inhibition (MIR) could lead to specific treatments for AIDS-related Karposi's Sarcoma and tumours highly dependent on the mitogen-activated protein kinases (MAPKs) ERK1/2, such as malignant melanoma [90].

5. Clinical perspectives and impact on cancer research

After the release of the sequence of the human genome, it became quickly evident that the proteome is

several orders of magnitudes larger than originally speculated, and that many diseases are caused by post-translational modification. Thus, today's efforts in biomedical research are primarily focused on the proteomic challenge. It is a delicate network of thousands of proteins and their interactions in the human cell are tightly regulated; imbalances in this network lead to diseases like cancer and various genetic disorders. The proteasome and its upstream system of ubiquitin-conjugating enzymes is responsible for 80% of the cell's protein degradation, and thereby has a major role in cellular homeostasis.

The E3 ubiquitin ligases confer substrate specificity and their activity in a cell decides the fate of its cognate proteins. To date, only limited numbers of E3 ligases and their specific targets are known. Among the protein–protein interactions that have been elucidated, there are a number of ligases and their protein partners that appear as key players in certain types of cancers, and are addressed in this review. Nonetheless, there remains a network of some 500 estimated E3 ligases and their substrates to be identified, characterised and placed into functional relationships with each other. The ultimate manipulation of the UP-S will certainly be difficult, but critical targets can be established within this pathway that can be highly specific and will be essential in order to combat a pleiotropic disease like cancer that is influenced by the production of ''abnormal'' proteins and their cellular processing. It is likely, and perhaps even obvious, from the currently available data, that different normal and tumour cell-types will be utilising different ubiquitin ligase systems.

While target specificity is clearly very desirable from a point of view of efficacy and tolerability of a new anticancer drug and hence, inhibiting E3 ligases in particular have great potential. However, the price to pay in turn might be the emergence of specific cellular resistance. As seen in the case of imatinib (Gleevec™; Glivec™), a selective inhibitor of the BCR-ABL fusion oncoprotein for the treatment of chronic myelogenous leukaemia, point mutations and overamplification of *BCR-ABL* as well as loss of dependence from the disease-causing genetic aberrations, can occur – demanding ever new treatment strategies [91,92]. A similar scenario cannot be ruled out for E3 ligase inhibitors and other modulators of the UP-S. In fact, it has recently been reported that expression of heat shock protein 27 renders tumour cells resistant to the proteasome inhibitor bortezomib (Velcade™), whereas Hsp27 antisense treatment restored apoptotic response to the drug in resistant cells [93].

Nonetheless, among the many hypothesised targeted treatments that have been postulated and tested for clinical activity in cancer patients in recent years, the proteasome inhibitor bortezomib (Velcade™) was one of few such compounds that led to a unique and efficacious drug, which received rapid regulatory approval. In addition, bortezomib (Velcade™) has a high overall response rate in refractory multiple myeloma patients of 35%, and a complete response rate of 10%, while showing acceptable normal tissue toxicity. This characteristic was somewhat unexpected, owing to its broadly functioning and essential molecular target, namely the chymotryptic activity of the 20S proteasome. Indeed its success illustrates that treatment of a tumour type such as multiple myeloma, which is regulated by the inactivation of a growth suppressing protein, like I-κB, or, conversely, by the activation of a proto-oncogenic/oncogenic protein, like NF-κB, in the proteasome, can result in a good tumour response and improved survival.

Overall, specific UP-S inhibition appears to be a very promising avenue to stabilise tumour growth suppressing proteins. It is therefore likely that a battery of UP-S inhibitors will soon become available for clinical studies.

6. Concluding remarks

The proteasome–ubiqutination pathway is a central part of the cell's homeostatsis, the proteins of all vital compartments: cytoplasm, nucleus and membrane are kept under its regulatory control. In a normal cell, protein destruction is necessary and for the most part is irreversible. The rewards of developing agents that can specifically interfere with the E3 ligase system and its interaction with its substrates will remarkably be high in the area of cancer treatment, given the need for a tight regulation of oncogenic and tumour suppressing proteins. Thus, the understanding of the complex ubiquitination systems and its explicit interactions and mechanisms should lead to more innovative cancer therapies that will offer the hope for better and perhaps greater curative successes in malignant and other diseases.

Conflict of interest

None declared.

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