

Proteasome Inhibitors as Therapeutic Agents: Current and Future Strategies

J.G. Delcros ^a, M. Baudy Floch ^b, C. Prigent ^c and Y. Arlot-Bonnemains ^{c,*}

^aGroupe de Recherche en Thérapeutique Anticancéreuse, Faculté de Médecine, Université Rennes 1, 2 avenue du Professeur Léon Bernard, CS34317, F-35043 Rennes Cedex, France

^bLaboratoire de Synthèse et Electrosynthèse Organiques (SESO), UMR CNRS 6510, Institut de Chimie, Université Rennes 1, 263 Avenue du Général Leclerc, CS74205, F-35042 Rennes Cedex, France

^c Groupe Cycle Cellulaire, CNRS UMR 6061 Génétique et Développement, IFR 97 Génomique Fonctionnelle et Santé, Faculté de Médecine, Université Rennes 1, 2 av du Pr Léon Bernard, CS 34317, 35043 Rennes Cedex, France

Abstract: In cells, protein degradation is a key pathway for the destruction of abnormal or damaged proteins as well as for the elimination of proteins whose presence is no longer required. Among the various cell proteases, the proteasome, a multicatalytic macromolecular complex, is specifically required for the degradation of ubiquitinated proteins. In normal cells, the proteasome ensures the elimination of numerous proteins that play critical roles in cell functions throughout the cell cycle. Defects in the activity of this proteolytic machinery can lead to the disorders of cell function that is believed to be the root cause of certain diseases. Indeed, many proteins involved in the control of cell cycle transitions are readily destroyed by the proteasome once their tasks have been accomplished. Moreover, because proteasome inhibitors can provoke cell death, it has been suggested that proteasomes must be continually degrading certain apoptotic factors.

For these reasons, proteasome inhibition has become a new and potentially significant strategy for the drug development in cancer treatment. The proteasome possesses three major peptidase activities that can individually be targeted by drugs. Different classes of proteasome inhibitors are reviewed here. In addition, we present new pseudopeptides with the enriched nitrogen backbones bearing a side chain and a modified C-terminal position that inhibit proteasome activity.

INTRODUCTION

The proteasome is a highly conserved intracellular non-lysosomal multicatalytic protease complex, degrading proteins usually tagged with a polyubiquitin chain. The ubiquitin/proteasome pathway plays a major role in the degradation in many short-lived regulatory proteins, which govern cell division, growth activation, signaling and transcription [1,2]. Tightly ordered proteasomal degradation of proteins critical for the cell cycle control indicates a role for the proteasome in controlling cell proliferation and maintaining cell survival. As this pathway is involved in the destruction of very important regulatory components, the deregulation of the system could lead to an anarchic cell proliferation and to a tumor development [3,4]. Defects in the ubiquitin/proteasome pathway are also involved in inflammatory, autoimmune and other diseases [4,5]. The realization that proteasome activity is implicated in human diseases has prompted research into the design and synthesis of various proteasome inhibitors and the evaluation of their therapeutic potential [4,6].

In recent years, there has been a great deal of interest in proteasome inhibitors as a novel class of anti-cancer drugs. Here we review the progress made to date in this area and highlight the potential advantages and weaknesses of this approach. We will summarize the literature related to human cancer, cell death induction by proteasome, examine the proteasome inhibitors discovered or synthesized over the past years, and evaluate their potential for treating cancer and other human diseases.

THE UBIQUITIN PROTEASOME PATHWAY

Structure of the Proteasome

In the 1970s, a new type of proteolytic pathway requiring ATP hydrolysis and catalyzing the degradation of proteins, was identified [7]. The macromolecular entity with the proteolytic activities called the proteasome, is a multicatalytic proteic complex that is well conserved throughout the evolution, from archaeobacteria to yeast and human. The structure of the proteasome has been determined from the electron micrograph of the complex purified from different organisms [8-10].

The 26S proteasome is composed of two distinct units: a 20S proteolytic cylinder of about 700 kD flanked by two

*Address correspondence to this author at the Groupe Cycle Cellulaire, CNRS UMR 6061 Génétique et Développement, IFR 97 Génomique Fonctionnelle et Santé, Faculté de Médecine, Université Rennes 1, 2 av du Pr Léon Bernard, CS 34317, 35043 Rennes Cedex, France; Fax: +33(0)2.23.23.44.78; E-mail: yannick.arlot@univ-rennes1.fr

19S regulatory caps (also known as PA 700). Another form of proteasome composed of a 20S unit flanked by two 11S subunits is known as the immunoproteasome which plays a role in Class I antigen processing. The 20S proteasome is a large cylindrical structure consisting of four stacked rings, each of them composed of 7 subunits. The eukaryotic proteasome is composed of a stack pile of two outer rings of 7 unique but related α subunits and two inner rings of 7 unique but related β subunits [11,12]. The 19S particle, consisting of 18 subunits, controls the recognition, the deubiquitinylation and the unfolding of the protein substrate prior to its translocation into the catalytic core of the 20S proteasome. This regulatory complex contains polyubiquitin receptors [13], an isopeptidase activity that catalyses the release of free ubiquitin [14], and six ATPases needed in particular for the denaturation of the protein substrates [15]. The entire complex associates with the ends of the 20S core to allow the proteolysis in an ATP-dependant manner.

Proteolytic Activities of the Proteasome

The multimeric 20S proteasome possesses several enzymatic activities working together. The eukaryotic 20S proteasome shows a very low protease activity that is modulated by its association with the 19S and 11S regulatory complexes. The specificity of the different catalytic activities has been characterized using small synthetic substrate peptides. Three major different activity sites are present in the 20S proteasome, distinguished by their active sites, kinetics, pH optima and sensitivities to inhibitors. Currently, the peptidase activities of the proteasome are designed as “chymotrypsin-like” (CT-L), “trypsin-like” (T-L) and “peptidyl-glutamyl peptide-hydrolyzing” (PGPH) also named the caspase-like activity [16]. These three activities are responsible for the cleavage after hydrophobic, basic, and acidic amino acid residues, respectively. Analysis of the 20S subunit reveals that each β subunit can be assigned to a specific activity. The subunit $\beta 1$ is responsible for PGPH [17] activity while $\beta 5$ and $\beta 2$ provide the CT-L and T-L activities respectively [18]. In addition to these well characterized peptidase activities, the 20S proteasome possesses two other activities: a branched chain amino acid preferring (BrAAP) activity, and a small neutral amino acid preferring (SNAAP) activity [19].

Analysis of the proteasome catalytic mechanism has revealed the importance of the amino terminal “Thr-1” as a catalytic nucleophile for the proteolysis. Other amino acid residues such as Ser, Cys, and Asp, which act as nucleophile catalysts in numerous non-proteasomal proteases, were not involved in the proteolysis. X ray analysis of the 20S proteasome complexed with the inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) found the inhibitor to be very close to the hydroxyl group of the amino acid terminal Thr-1, with the formation of a hemi-acetal intermediate group which blocks the proteasome activity [8].

Intracellular Localization

To further understand the functions of any protein, it is essential to know its intracellular location. It has been well

documented that the proteasome is localized both in the nucleus and the cytoplasm of eukaryotic cells. The exact proportion of the nuclear to cytosol proteasome is variable for different cell types. The localization and proportion of the proteasome 26S and the subunits 20S, 19S and 11S also vary from one cell type to another. The different subunits could associate to form 19/20/19 S or 11/20/11 S complexes found in the nucleus and in the cytoplasm with variations dependent on the cell type and the cell cycle stage. However, the relationship between proteasome activity and its intracellular location is poorly understood.

As an example, the 20S proteasome is found in all the nuclei of rat liver and mammalian cultured cells [20]. In HeLa cells, the level of proteasome in cytoplasm is 10 fold higher than in the nucleus [21]. In rat liver cells, only 3% of the proteasome are localized in the nucleus [22]. Some authors have reported a spatial localization dependent on the cell cycle phase. In mitotic cells, as the nuclear envelope disappears, the proteasome localizes around the periphery of the chromosomes and associates with the spindle pole [23]. As the proteasome and its subunits can undergo changes in their localization, it is necessary to consider an active or passive nuclear transport. The proteasome possesses nuclear localization signals (NLS) that are situated on the outer periphery of the α rings ensuring the translocation of the proteasome in an ATP-dependent manner from the cytoplasm to the nucleus.

Ubiquitin Conjugation

The degradation of proteins by the 26S proteasome requires their specific recognition. Consequently, the proteins have to be marked by a special signal that can be detected by the 19S particle of the complex. This signal is a covalent addition of a polyubiquitin chain that is dependent on three types of enzymes. Each enzyme has a specific function and sequentially attaches a ubiquitin via an isopeptidic linkage to a lysine on the substrate, or a lysine on the previously attached ubiquitin [24]. This covalent attachment is a multistep process that begins with the activation of ubiquitin by an enzyme E1, identified as a “ubiquitin activator” and required for all modifications [25]. The second enzyme involved in the process is a ‘ubiquitin carrier protein’ (E2) also called ‘Ubiquitin Binding Complex’ (UBC). The UBC domain contains an essential amino acid Cys responsible for the formation of a ubiquitin thioester intermediate. Many E2s have been identified, especially in yeast, with 13 known E2’s [26]. E2 transfers the ubiquitin to the substrate either by itself or in cooperation with a third enzyme, a ubiquitin ligase (E3). E3 binds directly to the substrate and consequently confers the specificity and high regulation to the ubiquitination mechanism. There are numerous E3 enzymes, which thus permit multiple ubiquitination pathways. Once the ubiquitin protein conjugate begins to be destroyed by the 26S, monomers of ubiquitin are removed from the large polyubiquitin chain, to be recycled for another proteolysis process. Thus, the Ub/proteasome system is a complex machinery that can be targeted at different levels by specific inhibitors.

It is noteworthy that some proteins do not require ubiquitinylation to be degraded by the proteasome [27]. In this case, alternative pathways provide the proteasome recognition/degradation signal. Ornithine decarboxylase (ODC) is a well-studied example [28]. Its degradation is triggered by its association with another protein, the antizyme. This association is responsible for structural changes in the C-terminal region of ODC, which contains basal degradation elements [29,30].

On the other hand, ubiquitination is not an absolute signal for protein degradation. As an example, it has recently been shown that the ubiquitination of Ruk, the regulator of ubiquitous kinase, does not lead to its degradation [31].

UBIQUITIN /PROTEASOME PATHWAY AND DISEASES

The proteasome regulates the levels of proteins involved in cell cycle control such as G1 and mitotic phase cyclins [32,33], cdk inhibitors [34], various oncogene tumor suppressor proteins [35], and other regulatory proteins [36]. As evidenced, ubiquitin/proteasome-dependent proteolysis plays an essential role in controlling cell proliferation and cell death. It also plays a role in immune surveillance [37], the acquisition of long term memory [38], and in regulation of circadian rhythms [39]. The availability of new inhibitors of the proteasome has been instrumental in revealing new and unexpected functions of this activity.

The ubiquitin proteasome pathway can be involved in the development of diseases in two different manners:

- Stabilization and accumulation of normal and abnormal proteins.

This could result from a loss of proteasome activity or the non-degradation of mutant proteins as observed in many cancers and neurodegenerative diseases (see below). Another example is the stabilization of the mutated epithelial sodium channel, which results in the Liddle syndrome, an autosomal dominant form of hypertension [40].

- Abnormal degradation of proteins.

An excess of proteasome activity can induce high levels of degradation of regulatory proteins. Abnormal mutant proteins unable to fold correctly and more susceptible to degradation could be important causes of various genetic diseases. The efficient degradation of the mutant $\Delta F508$ protein leads to a lack of cystic fibrosis transmembrane conductance regulator at the cell surface, resulting in cystic fibrosis [41]. The rapid degradation of a mutated copper-transporting P-type ATPase is responsible for Wilson disease [42]. The cleavage of a misfolded mutated insulin proreceptor resulting in decreased numbers of insulin receptors at the cell surface is involved in a hereditary form of diabetes mellitus [43]. Forced degradation of normal proteins is also employed by viruses to escape from immune surveillance. For example, HIV utilizes the proteasome pathway to down-regulate the CD4 receptor in the infected cells [44]. Similarly the human cytomegalovirus avoids

immune surveillance by down regulating MHC class I molecules used to display peptides from foreign antigens and essential to elicit a cytolytic T cell response [45].

The role of ubiquitin/proteasome pathway in cancer and neurodegenerative diseases is detailed below.

The Proteasome and Cancer

The unregulated proliferation of human cancer cells can be caused by the reduced growth inhibitory activity, and especially the low levels of tumor suppressors. Degradation of the tumor suppressor p53 [24] and the p27^{kip1} inhibitor of cyclin dependent kinase [34] can promote tumorigenesis. Excessive degradation of important cellular proteins such as p53, Bax, NF- κ B precursor p105, by the proteasome ubiquitin pathway also plays an essential role in the development of drug resistance in human cancer. Inhibition of proteolytic degradation using proteasome inhibitors can be expected to contribute to restoring expression of these proteins [46].

The product of the tumor suppressor gene p53 is an unstable nuclear protein with a short half-life in normal cells. p53 plays a central role in cellular responses including cell cycle arrest, and cell death in response to DNA damage. In this case, the level of p53 increases due to its stabilization, leading to cell growth arrest or apoptosis. This stabilization is thought to occur via the down regulation of its degradation via the ubiquitin proteasome pathway [47,48]. p53 dysfunction can induce abnormal cell growth, increased cell survival, genetic instability and also the drug resistance. Aberrations of p53 have been reported in numerous human cancers, such as breast [49,50], colon [51], oesophagus [52], head and neck [53], lung [54] and ovary [55] as well as bone cancers [56]. A significant accumulation of p53 protein is often associated with a poor survival of the cancer patient [57]. But the prognostic value of p53 overexpression is controversial. Many studies relate the involvement of p53 in tumor progression and invasiveness of Hepatocellular Carcinoma (HCC) [58]. Recent prospective studies have revealed that p53 mutations or nuclear accumulation could be a valuable marker for predicting the prognosis of HCC after resection [59].

In contrast, the tumor suppressor p53 is very low in uterine cervical carcinoma tumors, caused by high-risk strains of the Human Papilloma Virus (HPV). In the case of HPV-related cancer, the oncogenicity of the HPV involved in the etiology of the majority of human anogenital carcinomas is linked to an increased deregulation of p53 via the Ub/proteasome pathway. In cervical cancer, low levels of p53 were found in subjects with high-risk strains of HPV. p53 is targeted for ubiquitin degradation by the high-risk species of the HPV oncoprotein E6 which provokes a complex hyper ubiquitination of p53 and allows the virus to transform the host cells [60,61]. Low risk strains encoding a slightly different E6 protein, neither transform cells nor target the p53 for degradation.

As previously described, disruption of the G₁/S and mitotic checkpoints, leads to uncontrolled cell growth,

abnormal development and progression of cancer. Overexpression of cell cycle regulators such as cyclin A, D, and E have been correlated with the tumor relapse of human HCC [62]. The p27 protein, which binds to and inhibits cyclin dependent kinase complexes, is a negative regulator of cell cycle progression. The protein p27 plays a central role in a variety of diseases, involving aberrations in cell proliferation particularly in neoplasia. In breast cancer, p27 proteolysis can be an early event in tumorigenesis [63]. Low expression of cdk inhibitor p27 correlates with tumors such as colorectal carcinoma, and breast cancer. A very good correlation was found between low levels of p27 and increased proteasome activity. Degradation of p27 was abolished by the inhibition of proteasome. Other elements of the cell cycle machinery of Ub/proteasome degradation are potential targets for the deregulation in tumors. One of those is cyclin B whose degradation is important for exit from mitosis [32]. Cyclin B is overexpressed in a set of breast cancer cell lines [64]. Similarly, the overexpression of cyclin E and recently of cyclin D1 has been reported in tumors [65,66].

The ubiquitin-proteasome system is an important regulator of cell growth and apoptosis. Proteasome-mediated proteolysis of some apoptotic elements plays an important role in the development of cancer. The potential of specific proteasome inhibitors to act as novel anti-cancer agents is currently under intensive investigation. Several proteasome inhibitors potentially induce apoptosis in tumor cells *in vitro*, exert anti-tumor activity and may prevent metastasis *in vivo* [67]. In addition, tumor cells resistant to traditional therapy are sensitive to proteasome inhibitors, indicating the importance of proteasomal targets. The mechanism of apoptosis induced by proteasome inhibitors is not fully understood and may vary from one cell line to another [5,68]. Inhibition of the 26S proteasome results in the rapid accumulation of p53, p53-inducible p27, MDM2 or Bax [48,69,70] in the cells which readily enter apoptosis [71]. In mammals, cytoplasmic Bcl2 family members such as Bad or Bik have been identified as proapoptotic factors. Inhibition of the proteasome results in the accumulation of Bax, a protein that binds to and inhibits Bcl2 protein, triggering cytochrome c-dependent apoptosis [72].

By inhibiting NF- κ B transcriptional activity, proteasome inhibitors may also prevent angiogenesis and metastasis *in vivo* and further increase the sensitivity of cancer cells to apoptosis. Tumor growth requires the induction of new blood vessels to provide nutrients and oxygen. The process of angiogenesis depends largely on the transcriptional activation of pathways leading to the production of growth factors that initiate angiogenesis. This step is critically dependent on proteasome function. For example, lactacystin has been shown to prevent angiogenesis *in vivo* and *in vitro* offering a possibility of anticancer therapy [73].

The Proteasome and Neurodegenerative Diseases

It has been proposed that the proteasome could contribute to neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer, Parkinson, Huntington and Creutzfeld-

Jacob diseases. Indeed the accumulation of ubiquitin conjugates has been observed in the inclusion bodies of those neurodegenerative diseases [74].

Alzheimer disease (AD) is one of the most common causes of dementia in the elderly. The molecular pathological hallmarks of AD are intracellular neurofibrillary tangles and extracellular amyloid plaques [75,76]. It has been suggested that the proteasome could regulate the intracellular concentration of the holoproteins, presenilins 1 and 2 (PS1 and 2) which seem to play a crucial role in the maturation of the β Amyloid Precursor Protein (β APP) into Amyloid β 40 Protein (A β 40) [77]. Senile plaques are thought to derive from the abnormal levels of A β 40. An interesting study indicated that, *in vitro*, A β 40 could prevent ubiquitin-dependent protein degradation [78]. By controlling intracellular concentrations of PS1 and PS2, the proteasome could act as a regulator of β APP and its maturation fragments. Mutations on PS1 or PS2 are responsible for the onset of the early forms of Alzheimer disease, probably caused by exacerbation of the pathogenic pathway of β APP maturation. Controlling the concentrations of presenilins could have profound repercussions on cell physiology, as suggested by the fact that proteasome inhibitors drastically potentiate the 'normal' or 'pathogenic' presenilin phenotype related to β APP processing. Inhibitors of the proteasome will, therefore, enhance the A β 40 accumulation. Activators of the proteasome would, by contrast, enhance presenilin degradation, and the resulting decrease of their intracellular concentration would reduce the amount of intracellular A β 40 protein.

In Huntington disease, proteins encoded by the Huntington genes aggregate in ubiquitin- and proteasome-positive intranuclear inclusions bodies, suggesting that the accumulation of the protein could be due to its stabilization [79].

The Parkinson disease and the Angelman syndrome illustrate other abnormal functions of the ubiquitin-proteasome pathway. Mutations in the UCH (ubiquitin-carboxyl terminal hydrolase L1), parkin and α -synuclein genes cause an autosomal dominant familial form of Parkinson disease [80]. UCH is thought to regenerate ubiquitin by cleaving ubiquitin chains. Mutations in α synuclein and parkin impair their degradation by the proteasome 26S and their accumulation leads to the formation of proteinaceous Lewy bodies [81-83].

In patients with Angelman syndrome, the UBE3A gene encoding an E3 ligase (called E6-AP) is altered, leading to the accumulation of an unidentified substrate and thus results in a neuropathological disorder [84].

The Proteasome and Muscle Cachexia

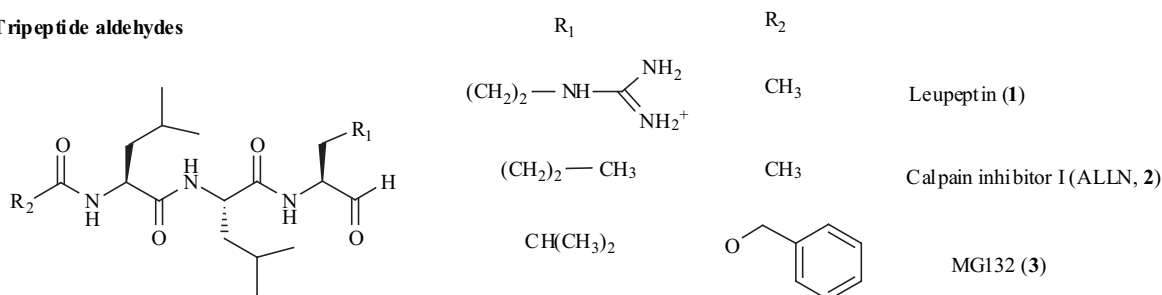
Muscle cachexia is a prominent metabolic consequence of catabolic diseases such as AIDS, cancer, renal disease diabetes and trauma [85]. Cachectic cancer patients also have a considerably reduced response to chemotherapy [86]. Muscle cachexia mainly results from an increased breakdown of muscle protein. Ubiquitin proteasome dependent

proteolysis plays a pivotal role in the loss of muscle mass [87,88] as evidenced by the reduction of soluble microfibrillar protein breakdown observed in septic rats treated with proteasome inhibitors [89]. Mediators of the cachectic process include cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1. Tumor cells also produce some mediators such as the proteolysis-inducing factor [90]. Neutralizing antibodies or specific receptor antagonists should help elucidate the exact role of those mediators and may also have some therapeutic potential.

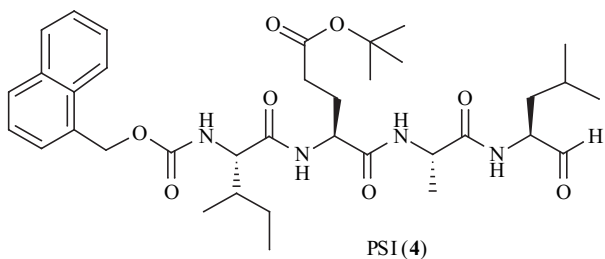
PHARMACOLOGICAL TARGETING OF THE UBIQUITIN-PROTEASOME PATHWAY

The proteolytic machinery of the proteasome has been the main target for pharmacological development. In contrast, very few pharmacological approaches have targeted the upstream ubiquitination pathway. The nucleophile catalyst in the 20S proteasome-mediated proteolysis is the hydroxyl group of the N-terminal Thr of each catalytic β -subunit. The largest family of 20S proteasome inhibitors consists of peptide-based structures with appended functional groups

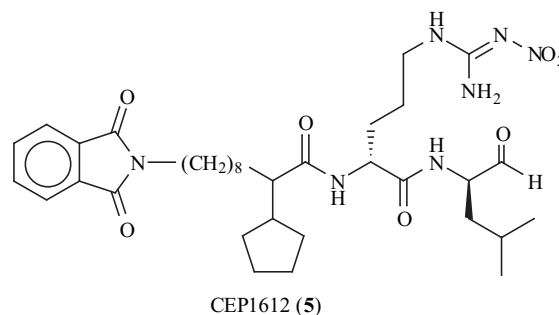
(a) Tripeptide aldehydes



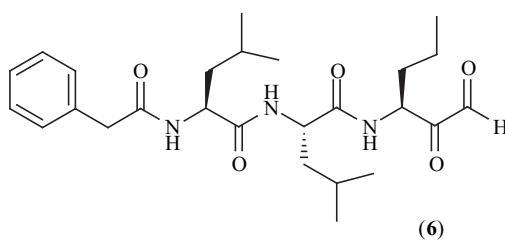
Tetrapeptide aldehyde



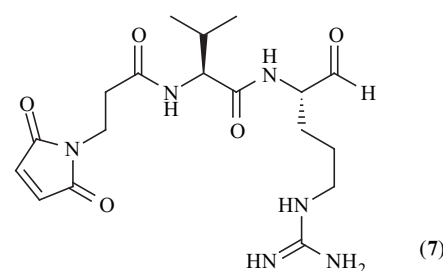
Dipeptide aldehyde



Peptide Glyoxal



Maleoyl- β -alanyl-dipeptide aldehyde



(b)

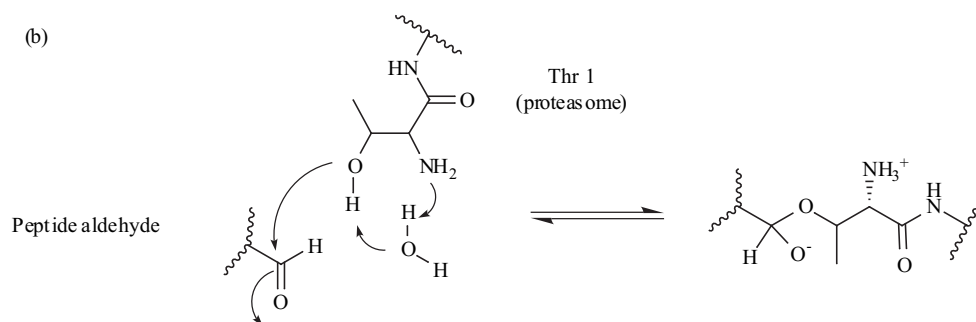


Fig. (1). (a) Proteasome inhibitor family of peptide aldehydes. (b) The proposed mechanism for the inhibition of the 20S proteasome by peptide aldehydes.

receptive to nucleophilic attack by hydroxyl groups. Such peptide-based inhibitors include various synthetic and natural small peptides bearing diverse functional groups such as aldehyde, vinyl sulfone, boronic acid or α',β' -epoxyketone. This group also includes some natural peptide macrocycles. Non-peptidic inhibitors constitute another class of inhibitors. They display a wide variety of scaffolds of core structures and pharmacophores, and will be discussed later.

Targeting the 20S Proteasome with Peptide-Based Inhibitors

Peptide Aldehydes

The first inhibitors of the 20S proteasome were identified among the commercially available tripeptide aldehydes used as reversible inhibitors of serine and cysteine proteinases (Fig. 1a). Leupeptin (1), a peptidyl arginine aldehyde, selectively inhibits the T-L activity while calpain inhibitors I (2, ALLN), a peptidyl norleucine aldehyde, targets the CT-L activity of the 20S proteasome [19,91-93]. The peptide aldehydes form a reversible covalent hemiacetal intermediate between the aldehyde group of the inhibitor and the hydroxyl group of the amino terminal Thr, reminiscent of the tetrahedral intermediate of a protease reaction (Fig. 1b) [8].

The easy access to peptide aldehydes has led to the development of a wide variety of aldehyde inhibitors with an improved potency and/or selectivity toward the CT-L activity of the proteasome such as MG132 (3) or PSI (4). MG132 is as potent as ALLN but is much more selective [94,95]. The more potent inhibitors with enhanced specificity and improved selectivity for CT-L activity over T-L activity were identified in a dipeptide aldehyde series [96]. As an example, CEP1612 (5) inhibits proteasome activity both *in vitro* and *in vivo* at nanomolar

concentrations and shows a much reduced ability to inhibit the activities of cysteine proteases such as calpain I and cathepsin B [97]. Another approach to improve peptide aldehyde potency was to add a ketone moiety at the α position, yielding a glyoxal. Peptidyl α -keto aldehydes share an obvious structural similarity with peptide aldehydes and are 10-fold more potent than their aldehyde counterparts in inhibiting their serine and cysteine protease targets [98]. Some tri-peptidyl glyoxals (e.g. 6, Fig. 1a) are excellent reversible inhibitors of the proteasome with K_i values as low as 3nM [99].

Because CT-L sites cut preferentially after large hydrophobic residues, all potent aldehyde inhibitors of the CT-L are highly hydrophobic and thus are cell-permeant, a major advantage for their use *in vivo*. Most peptide aldehydes are active in cultured cells, and the inhibition of proteolysis is fully reversible upon the removal of the peptides from the medium. The major limitation of those peptide aldehydes remains their lack of specificity since they all inhibit calpains as well as various lysosomal cathepsins in addition to the proteasome [100]. Control experiments are therefore needed to confirm that the observed effects are due to the inhibition of the proteasome. Despite these limitations, these inhibitors remain widely used for *in vitro* and *in vivo* studies.

In contrast, relatively little effort has been made in developing specific inhibitors of the T-L or caspase-like sites, probably because these two proteolytic activities are not considered as important as the CT-L activity. In particular, the CT-L (but not the T-L) activity has been shown to be associated with tumor cell survival [71,101] so that the proteosomal CT-L activity is considered as a prime target for the development of anticancer drugs. In addition, because of the specificity of the T-L and caspase-like proteolytic sites, active inhibitors contain charged residues and consequently, are less cell-permeable. Specific inhibitors of the T-L activity have been designed by following an

Table I. Inhibition of 20S Proteasome by Homo- and Heterobivalent Inhibitors (IC_{50} , μM) [103]

| Inhibitor | PGPH | T-L | CT-L |
|--|------|-------|-------|
| | | | |
| monovalent | | | |
| Ac-Leu-Leu-Nle-H (ALLN) | >100 | >100 | 2.1 |
| Ac-Arg-Val-Arg-H | >100 | 6.4 | >100 |
| 'inter-ring homobivalent inhibitors' | | | |
| $\begin{pmatrix} \text{CO-Leu-Leu-Nle-H} \\ \text{(PEG)}_x \\ \text{CO-Leu-Leu-Nle-H} \end{pmatrix}$ | >100 | >100 | 0.017 |
| $\begin{pmatrix} \text{CO-Arg-Val-Arg-H} \\ \text{(PEG)}_x \\ \text{CO-Arg-Val-Arg-H} \end{pmatrix}$ | >100 | 0.071 | >100 |
| 'heterobivalent inhibitor' | | | |
| $\begin{pmatrix} \text{CO-Leu-Leu-Nle-H} \\ \text{(PEG)}_x \\ \text{CO-Arg-Val-Arg-H} \end{pmatrix}$ | >100 | 0.097 | 0.031 |

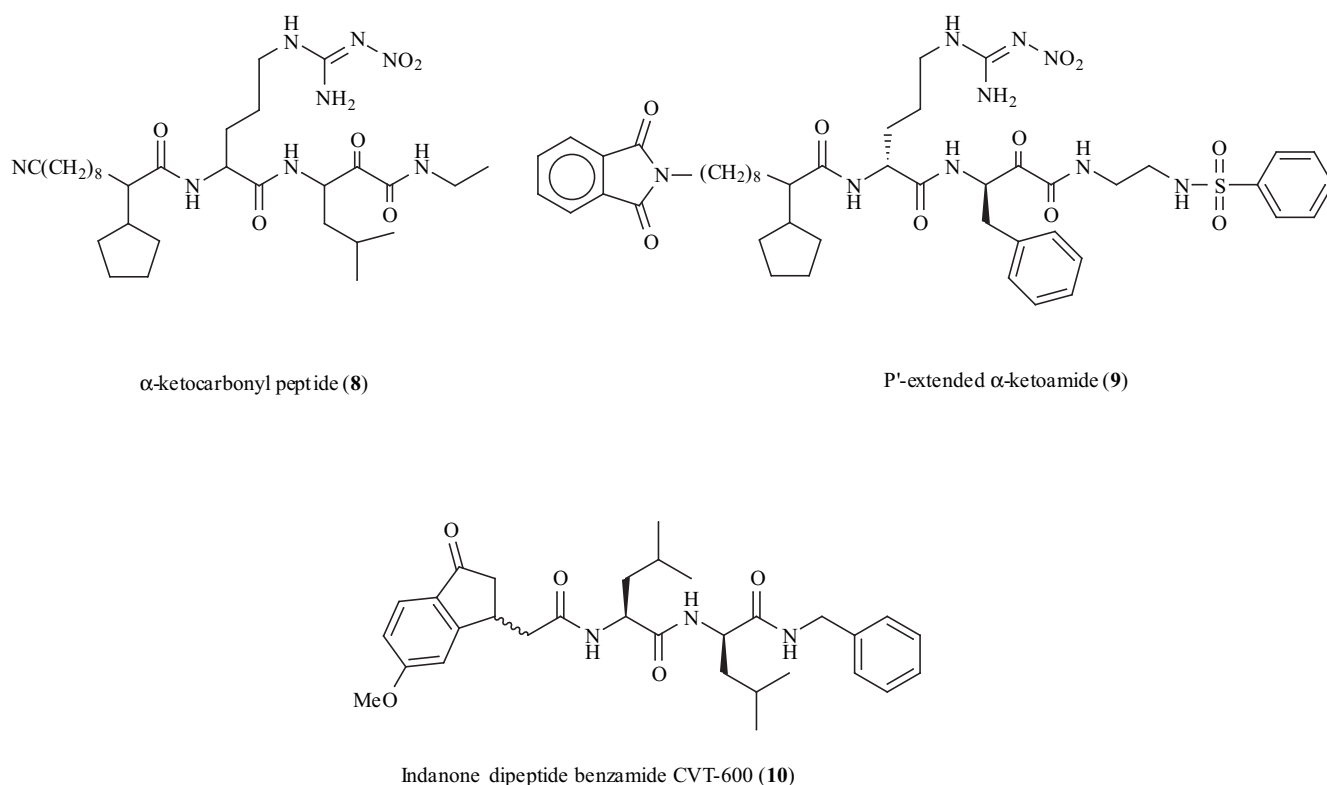


Fig. (2). α -ketocarboxyl and benzamide derived proteasome inhibitors.

original approach exploiting neighboring functionalities. The presence of a Cys residue (β 3 Cys118) near the β 2 active site of the proteasome lead to the development of peptide aldehydes bearing a maleinimide group as a thiol-reactive handle [102]. Some maleoyl- β -alanyl-dipeptide aldehydes (e.g. **7**, Fig. **1a**) were potent and highly selective inhibitors of the T-L activity of the proteasome. However, the presence of the highly thiol-reactive maleinimide group is likely to limit their use to *in vitro* studies.

Loidl and his collaborators have elegantly shown that the unique topography of the six active sites of the proteasome can also be exploited for the design of multivalent inhibitors [103,104]. Two monovalent binding head groups were linked via a polymeric spacer of appropriate length (e.g. polyethyleneglycol) to yield homo- or heterobivalent inhibitor of remarkably enhanced binding affinity for CT-L and/or T-L sites, as illustrated in table I.

Besides the rational design of synthetic inhibitors, microorganisms are also a source of new peptide aldehyde inhibitors. Various species of actinomycetes produce peptidyl arginine aldehydes such as the above-cited leupeptin [105]. Recently, tyropeptins A and B, natural peptidyl tyrosine aldehydes isolated from the culture broth of *Kitasatospora sp.* were reported to be potent inhibitors of the CT-L and T-L activities of 20S proteasome [106,107].

Reversible Non-Aldehydic Peptide Inhibitors

The lack of specificity and the poor stability of peptide aldehydes are due to the presence of the highly reactive aldehyde functional group. To overcome this problem, 'non-reactive' peptide inhibitors were developed by replacing the

aldehyde by groups relatively inert in the absence of a target. A number of aldehyde replacements have been examined. The α -ketocarboxyl derived inhibitor (**8**) shows activity comparable with the corresponding aldehyde [108] and the P'-extended analog of CEP1612 (**9**) is a very potent inhibitor of the CT-L activity with a K_i value of 1.1nM (Fig. **2**) [109]. 5-methoxy-1-indanone-3-acetic acid based di- (e.g. CVT-600, **10**) and tri-peptide benzamides (Fig. **2**) were identified as highly selective and competitive inhibitors for the CT-L activity of the 20S proteasome *in vitro* as well as *in vivo* [110,111]. They do not inhibit the other proteasomal proteolytic activities and calpain.

Among aldehyde surrogates often exploited as serine protease inhibitors, the boronic acid moiety emerged as the more potent pharmacophore (Fig. **3a**). The boronates are much more potent than the aldehydes. The tripeptide boronate MG262 (**11**) and the dipeptide boronate PS341 (**12**) have sub-nanomolar potency ($K_i = 0.03$ and 0.62 nM respectively) [112]. MG262 is 100-fold more potent than its aldehyde analog MG132. Peptide boronates also exhibit extremely high selectivity for the proteasome over common serine proteases. Boronic acids act as transition-state analogues for serine proteinases because the boron can accept the oxygen lone pair of the active site serine residue. These compounds probably react similarly with the catalytic N-terminal Thr residue, forming a tetrahedral adduct. In addition, the boron-Thr¹O γ bond is much more stable than the carbon-Thr¹O γ bond found in the hemiacetal formed between peptide aldehydes and the proteasome (Fig. **3b**), and although the boronates are considered reversible inhibitors, the inhibition is practically irreversible. Bortezomib (PS341) is the first proteasome inhibitor to have entered the clinical trials for the treatment of cancer [113-

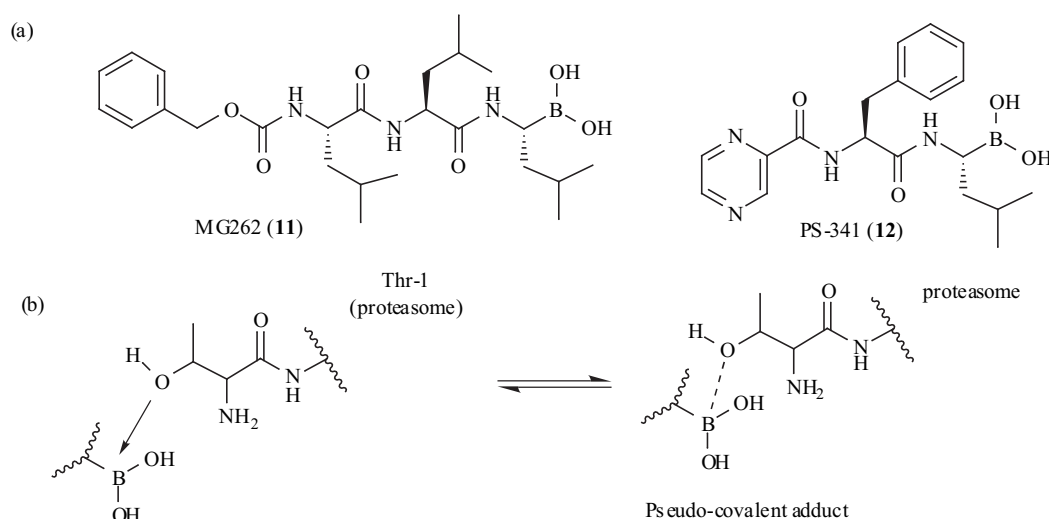


Fig. (3). (a) Synthetic peptide boronic acid inhibitors. (b) Their proposed mechanism for the inhibition of the 20S proteasome.

115]. This agent can induce apoptosis and sensitizes the tumor cells to radiation or chemotherapy. Preclinical studies have shown its activity against a variety of B-cell malignancies and bortezomib is currently in phase II clinical trials.

Irreversible Non-Aldehydic Peptide Inhibitors

Chloromethanes and diazomethanes are reagents used for the alkylation of the catalytic residues of serine or cysteine

proteases. Individual peptidase activities of proteasome can be inhibited irreversibly by a variety of peptidylchloromethanes and peptidyl diazomethanes [116,117]. Examples include Tyr-Gly-Arg-chloromethane and Z-Tyr-Ala-Glu-chloromethane inhibitors of T-L and CT-L activities respectively, while peptidyl diazomethanes containing hydrophobic amino acid residues inhibit PGPH activity. Their rates of inactivation are often very slow and the inhibition incomplete. Nevertheless, radiolabeled forms of these inhibitors have proved to be the useful tools to

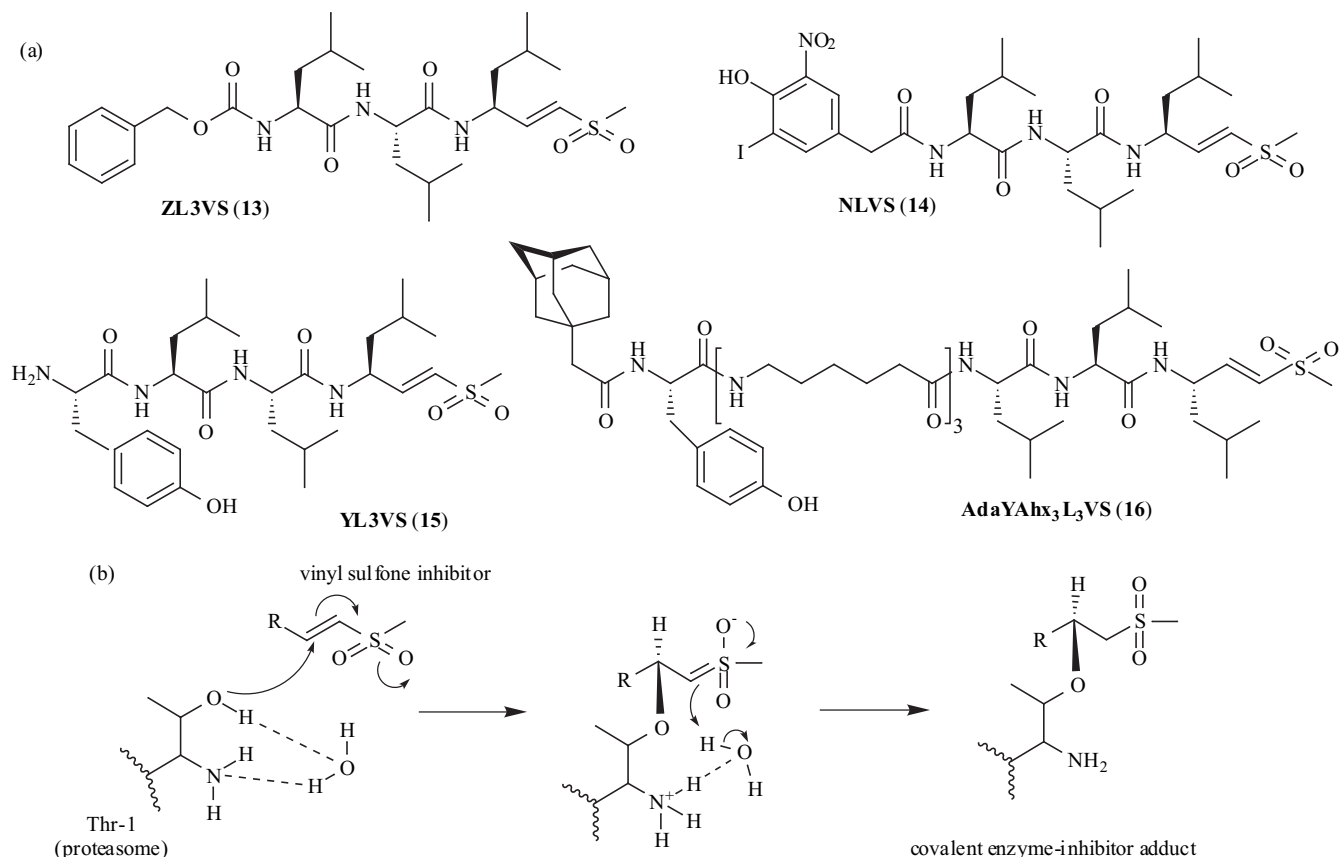


Fig. (4). (a) Synthetic peptide vinyl sulfone inhibitors. (b) Their proposed mechanism for the inhibition of the 20S proteasome by vinyl sulfones.

identify catalytic components associated with each of the three peptidase activities of the proteasome [118].

Synthetic peptide vinyl sulfones (Fig. 4a) are potent irreversible inhibitors of proteasome *in vitro* as well as *in vivo*. Z-L₃VS (**13**), the vinyl sulfone analog of MG132 inhibits the CT-L activity and with less efficiency the T-L and caspase-like activities [119]. Introduction of the trileucine vinyl sulfone core to different substituents at P4 yielded the inhibitors to a preference for β 5 (NLVS, **14**) and β 2 (YL3VS, **15**) [120]. Highly specific inhibitors of β 2 were also identified in positional scanning libraries of peptidyl arginine vinyl sulfones [121]. N-terminal extension of peptide vinyl sulfones has lead to the identification of cell-permeable inhibitors (e.g. AdaYAhx₃L₃VS, **16**) more potent but less selective for the individual subunits, and active *in vivo* [122]. These compounds covalently modify the N-terminal Thr of the catalytic β subunits due to the reaction of the catalytic hydroxyl with the double bond of the vinyl sulfone moiety (Fig. 4b). This mechanism was recently

confirmed by the X-ray structure analysis of crystals of proteasome 20S complexed with peptide vinyl sulfone inhibitors, either selective about β 2 or non-selective (which binds to all the three catalytic subunits) [123]. Certain features of these compounds make them useful also as active site probes. The peptide vinyl sulfones can be conveniently tagged with either biotin for the purposes of affinity chromatography or a nitrophenol moiety for subsequent radiolabeling [119,122]. The ¹²⁵I-labeled vinyl sulfone derivative NLVS has been used for active site labeling of the proteasome in living cells [119]. However, the lack of specificity of these compounds has to be taken into account. Peptide vinyl sulfones were initially developed as the inhibitors of cysteine proteases [124], and in the living cells, the ¹²⁵I-labeled vinyl sulfone derivative NLVS has been shown to label cathepsin S, a cysteine protease [119].

Eponemycin (**17**) and epoxomicin (**18**), natural α',β' -epoxyketone peptides (Fig. 5a) produced by various actinomycetes [125,126], are potent antitumor agents with a

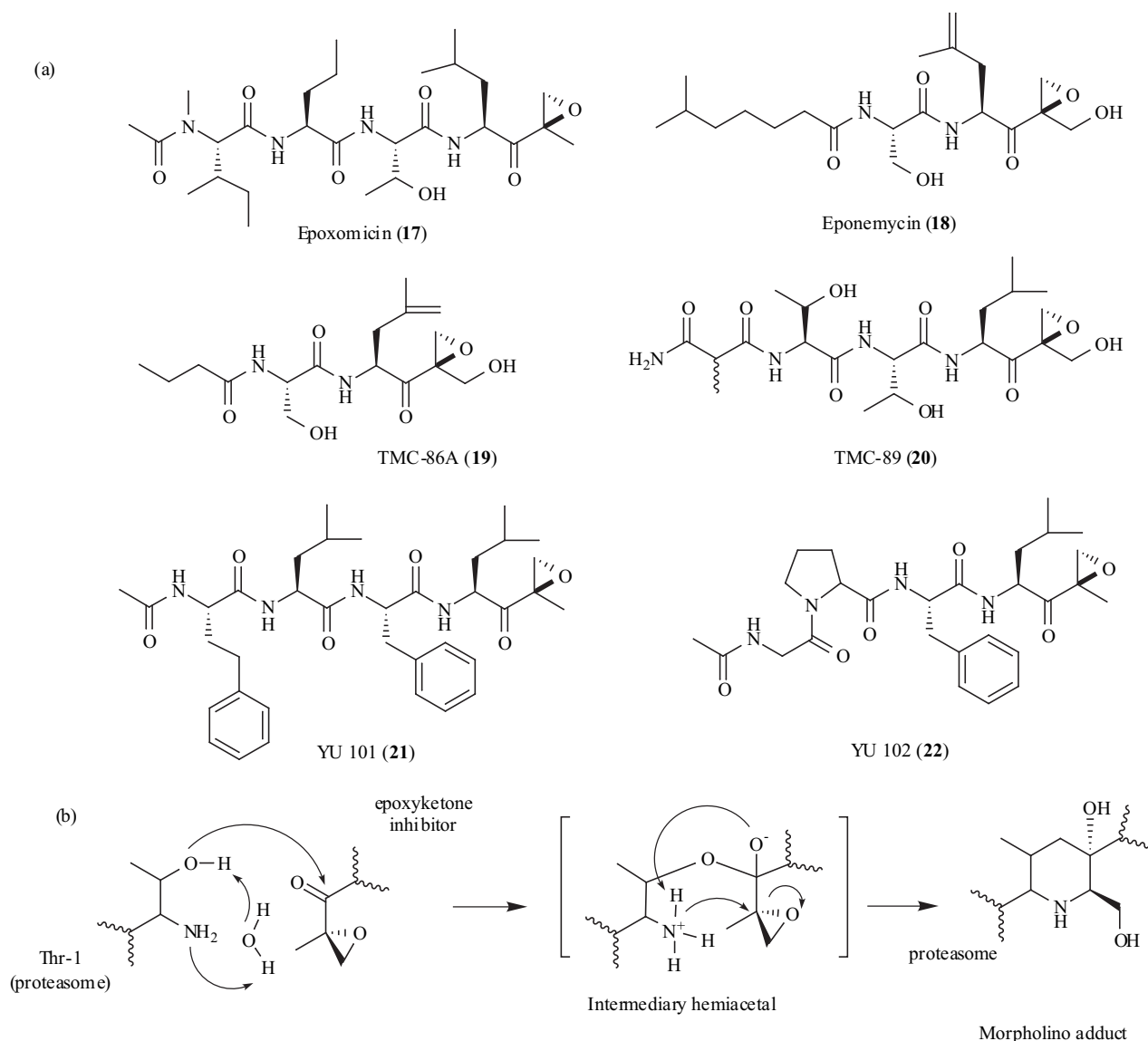


Fig. (5). (a) Natural and synthetic α',β' -epoxyketone peptide inhibitors. (b) Their proposed mechanism for the inhibition of the 20S proteasome.

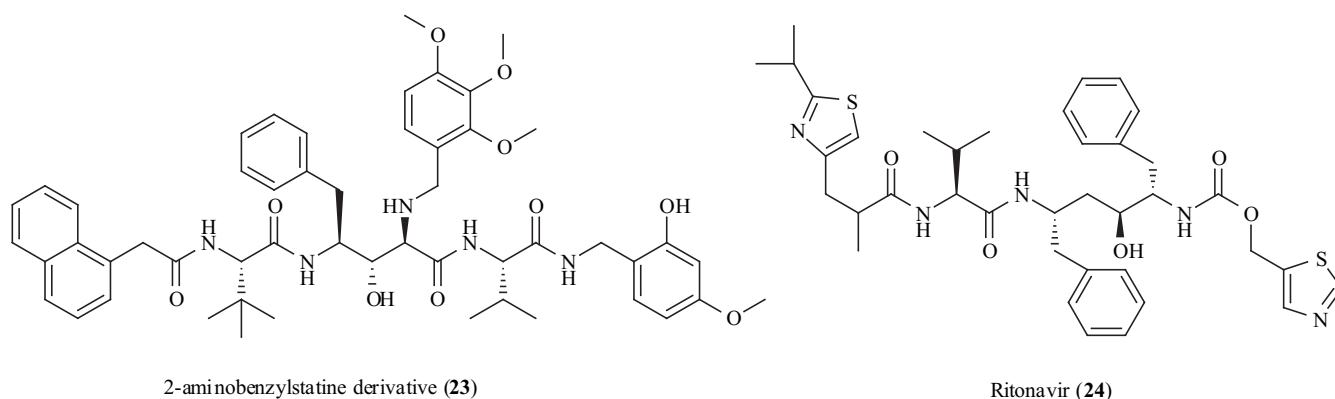


Fig. (6). Pseudopeptide inhibitors.

powerful anti-angiogenic activity [127,128]. They both share the proteasome as a common intracellular target [129,130]. Epoxomicin most potently inhibits the CT-L activity of the 20S proteasome [131] while dihydroeponemycin, a synthetic analog of eponemycin, inhibits the CT-L and caspase-like sites at similar rates [130]. Unlike many proteasome inhibitors, these compounds are specific for the proteasome and do not inhibit other non-proteosomal proteases [128,130]. *In vivo*, epoxomicin effectively inhibits NF- κ B-mediated pro-inflammatory signaling dependent on the proteasome activity [131].

In cells treated with biotinylated derivatives of epoxomicin and eponemycin, the proteasomal subunits are the only cellular proteins covalently modified by epoxyketones [131]. Their high selectivity relies on a unique mechanism based on a two-step reaction of the pharmacophore with both the hydroxyl and amino groups of the catalytic Thr residue. This involves the attack of the carbonyl group of the epoxyketone moiety by the catalytic hydroxyl, followed by the opening of the epoxy ring by the free α -amino group to form a cyclical morpholino ring (Fig. 5b). The crystal structure of the yeast 20S proteasome complexed with epoxomicin confirmed the formation of morpholino adduct between the epoxyketone pharmacophore and the active site N-terminal Thr-1 [132]. Cysteine or serine proteases do not allow such reactions because their catalytic site has no free N-terminus adjacent to the nucleophilic group. More natural proteasome inhibitors with an epoxy- β -aminoketone moiety, e.g. TMC-86A (19) and TMC-89 (20), with micromolar potency, have recently been isolated from the fermentation broth of *Streptomyces sp.* and *Saccharothrix sp.* [133-135]. The search for α' , β' -epoxyketone-based subunit-specific proteasome inhibitors has also led to the synthesis of YU 101 (21), a potent and selective inhibitor of the CT-L activity of the proteasome, and YU 102 (22) which inactivated the caspase-like activity 50-times faster than CT-L activity [136,137].

Pseudopeptide Inhibitors

The 2-aminobenzylstatine core structure was initially used in mimetics of scissile bonds to design HIV protease inhibitors [138,139]. Some of these pseudopeptides (Fig. 6) are potent, selective and non-covalent inhibitors of the CT-L activity of the proteasome 20S. They likely bind to the active site by stacking/hydrophobic interactions [140,141].

A structure-based optimization approach allowed the identification of compounds (e.g. 23) with nanomolar potency [142].

Ritonavir (24), another transition mimetic (Fig. 6), is a potent HIV protease inhibitor successfully used for the treatment of HIV-infected patients and AIDS disease. It also binds to the proteasome in a non-covalent and reversible manner. Interestingly, ritonavir affects differently the three proteolytic activities of the proteasome. It inhibits competitively the CT-L activity whereas the T-L activity is enhanced [143,144]. To explain the complex effects of ritonavir on proteasome activities, Schmidtke *et al.* [145] proposed a « two-site modifier » model which assumes that ritonavir binds to a non-catalytic modifier site in addition to the active site. The existence of non-catalytic modifier sites in the proteasome core opens new perspectives for pharmacological intervention. Ritonavir is the first identified member of a new class of proteasome-targeting agents, referred as 'proteasome modulators'. The observation that ritonavir alters antigen processing [143,144] is consistent with its action as a modulator of proteasome activity *in vivo*.

Cyclic Peptide Inhibitors

The unique metabolites TMC-95A-D produced by *Apiospora montagnei*, consisting of modified amino acid residues forming a heterocyclic ring system (Fig. 7), are specific reversible proteasome inhibitors [146,147]. TMC-95A (25) inhibits the CT-L activity of the proteasome at nanomolar level. It also inhibits the T-L and PGPH activities of the 20S proteasome but has no activity on various serine or cysteine proteases [146]. The structure of the TMC-95A-proteasome complex, determined by X-ray diffraction, indicates a non-covalent linkage of the compound to the active β -subunits with no modification of the nucleophilic Thr-1. The complex is stabilized by a network of specific hydrogen bonds with the main-chain atoms of the proteins [148]. The recent report of the total synthesis of TMC-95A and B [149] opens the door to the development of new TMC-95 based inhibitors. Cyclosporin A (26) is a nonpolar cyclic oligopeptide fungal metabolite (Fig. 7) with immunosuppressant activity, used in organ transplantation and in preventing graft-versus-host disease. Cyclosporin A also exhibits anti-inflammatory properties. Cyclosporin A acts as an uncompetitive inhibitor of the CT-L activity of the 20S proteasome *in vitro* and suppresses *in*

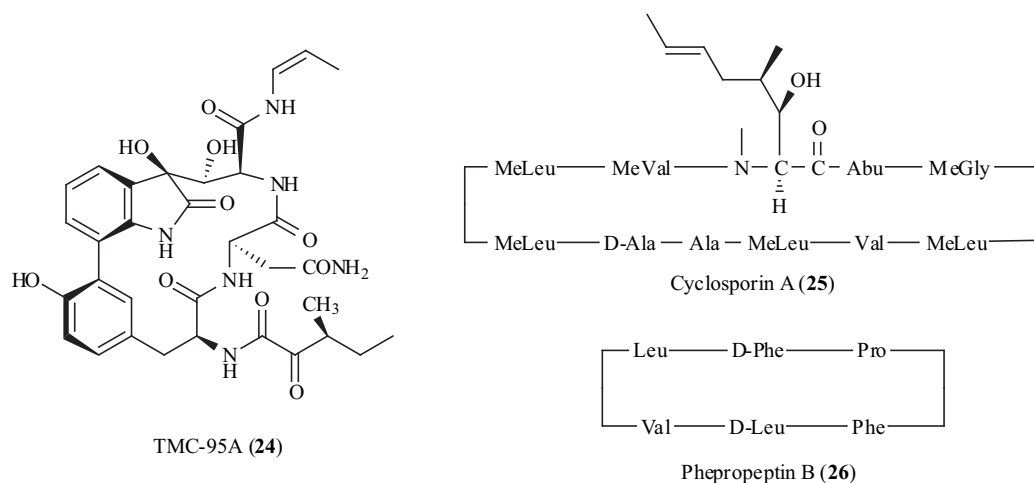


Fig. (7). Cyclic peptide inhibitors.

in vivo the I κ B degradation necessary for signal-induced NF- κ B activation [150]. Recently, Paslaru *et al.* [151] observed in kidney cells, treated with cyclosporin A, an increase in multi-ubiquitinated proteins, a stabilization of p53 as well as the activation of the two heat shock transcription factors HSF1 and 2, known to be activated by proteasome inhibition. These observations argue for an inhibitory action of cyclosporin A on the proteasome *in vivo*.

More recently, new cyclic hexapeptides, phepropeptins A-D (e.g. **26**, Fig. 7), produced by *Streptomyces sp.* were shown to inhibit the proteasomal CT-L activity but not α -chymotrypsin [152].

Targeting the Proteasome with Non Peptidic Inhibitors

β -lactones and Ester-Bond-Containing Polyphenols

The *streptomyces* metabolite lactacystin (**27**) is a highly specific and irreversible inhibitor of the proteasome. It was

initially discovered on the basis of its ability to inhibit cell growth and to induce neurite outgrowth in a murine neuroblastoma cell line [153-155]. The activity of lactacystin relies on its conversion into the intermediate *clasto*-lactacystin- β -lactone (**28**, omuralide), resulting from cyclization of the lactacystin with concomitant loss of N-acetylcysteine. This intermediate is the sole inhibitory species [156]. This β -lactone contains an ester bond responsible for interacting and inhibiting the proteasome. The acylation of the proteasome involves a nucleophilic attack on the carbonyl carbon of the β -lactone with a subsequent ring opening (Fig. 8) [156]. All the three peptidase (CT-L, T-L and caspase-like) activities of bovine brain 20S proteasome are inhibited by lactacystin, the first two irreversibly, and all at different rates [157]. Unlike the peptide aldehydes, lactacystin is more potent against the 26S particles than 20S, and in cells, it inhibits efficiently the ubiquitin-dependent, proteasome-mediated degradation of short- and long-lived proteins [158]. The β -lactone covalently binds only two β -type subunits of the purified brain proteasome [157]. Contrarily to all catalytic β -subunits

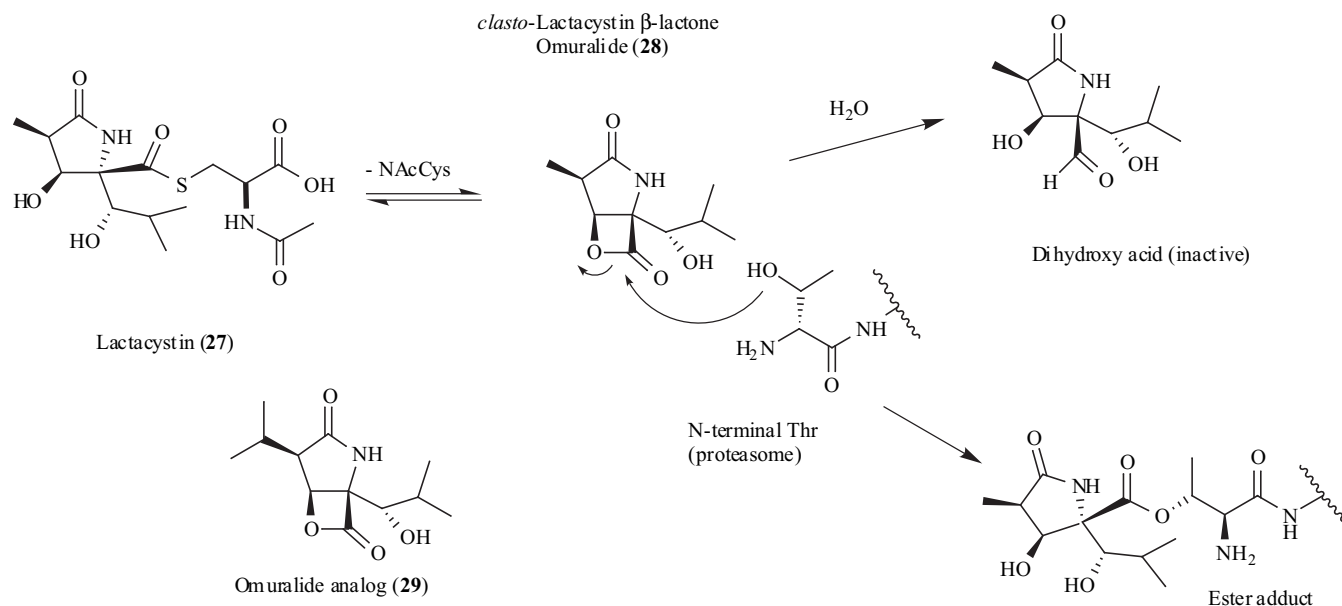


Fig. (8). Mechanism of proteasome inhibition by lactacystin.

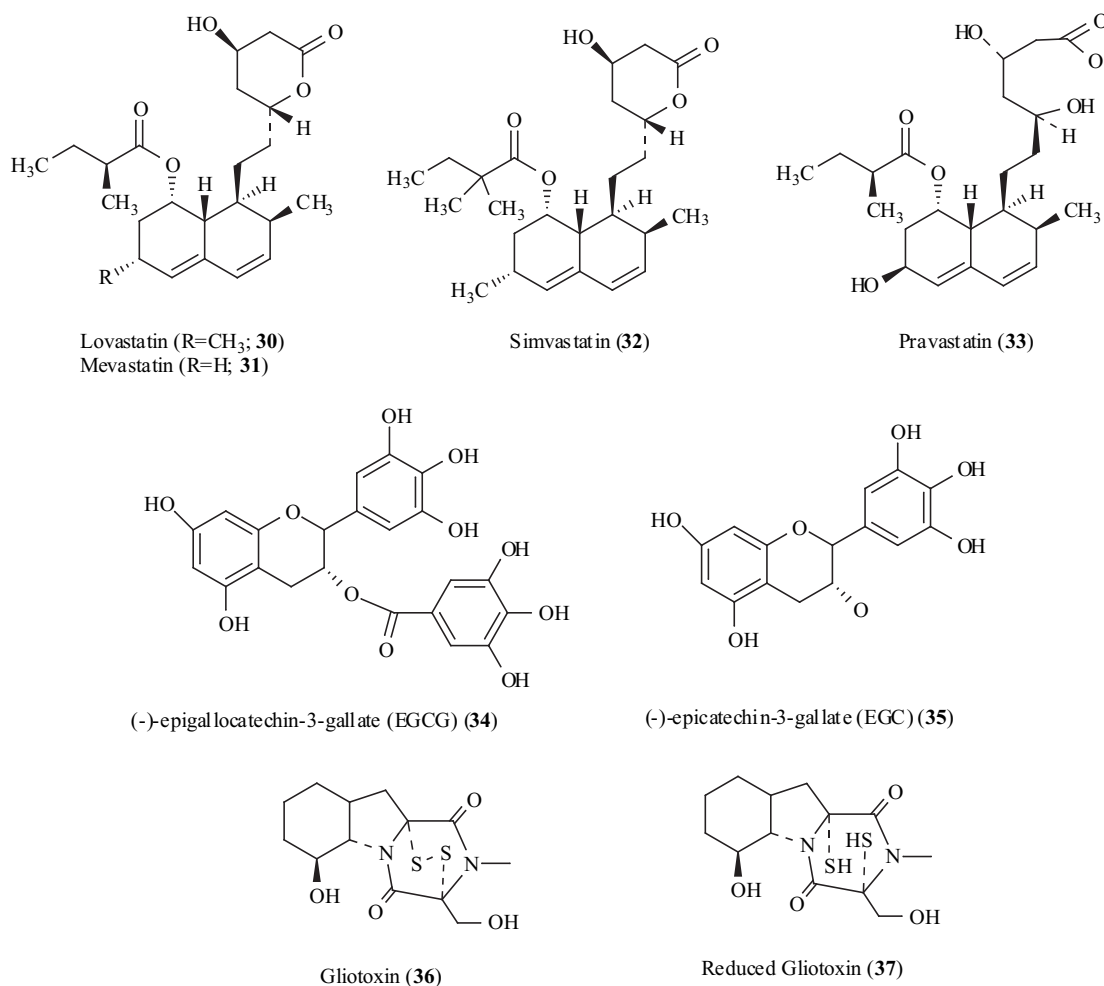


Fig. (9). Non peptidic proteasome inhibitors.

in rabbit muscle proteasome [158]. However, only the catalytic N-terminal Thr of the $\beta 5$ subunit of the eukaryotic 20S proteasome appears to be covalently modified, as confirmed by the analysis of the X-ray structure of yeast proteasome with bound *clasto*-lactocystin [9,157].

Lactacystin has initially been reported to be highly specific for the proteasome. It was found inactive on various serine and cysteine proteases [157] and did not inhibit lysosomal protein degradation [158]. However, the inhibition by lactacystin of other cellular non-proteosomal proteases such as cathepsin A has recently been reported [159,160]. Due to its structural novelty, the β -lactone omuralide is an interesting lead for the development of new synthetic inhibitors. The difficulty of synthesis has now been overcome [161-163]. Numerous analogs were recently synthesized and an elegant structure-activity study revealed that modifications allowed on the core structure are limited [162]. Only the replacement of the C(7)-methyl group by larger substituents (e.g. isopropyl moiety, **29**) results in the doubling of the proteasome inhibitory activity relative to the parent compound.

Lovastatin (**30**) and mevastatin (**31**), fungal metabolites isolated from various *Monascus*, *Aspergillus* and *Penicillium*, and their synthetic analog, simvastatin (**32**)

(Fig. 9) are hydroxymethyl-glutaryl (HMG)-CoA reductase inhibitors used for the treatment of hypercholesterolemia. These compounds also display antiproliferative properties and *in vivo*, they inhibit tumor growth as well as tumor formation [164,165]. Recently, lovastatin has been reported to decrease the incidence of melanoma in the treated patients [166]. These β -lactones, similar in structure to lactacystin, are pro-drugs which are active on HMG-CoA reductase enzyme in their open-ring forms. Lovastatin, similarly to lactacystin, was found to inhibit the CT-L activity of the proteasome *in vivo* resulting in the stabilization of cyclin-dependent kinase inhibitors and a cell growth arrest in mammary carcinoma cells [167]. Its open-ring form, or pravastatin, an analog with an open ring (**33**), had no effect on proteasome activity. Similarly, the inhibition of proteasome activity by mevastatin but not by pravastatin, was observed in murine neuroblastoma cells [168]. However two independent studies conducted on purified 20S proteasome as well as on colon carcinoma and osteoblastic cells yielded conflicting results. In these studies, the closed ring forms of lovastatin and simvastatin was found to stimulate the CT-L activity both *in vitro* and *in vivo* [169,170], and to inhibit the caspase-like activity of purified proteasome without interfering with the T-L activity [169]. More detailed studies are necessary to elucidate these discrepancies and to determine if the modulation of the

proteasome activity by statins is involved in biological effects independent of their HMG-CoA inhibiting activity, such as their cytotoxic effects.

The cancer preventive properties of green tea polyphenols are well-documented [171,172]. Polyphenols containing an ester bond similar to that found in lactacystine- β -lactone, such as (-)-epigallocatechin-3-gallate (EGCG, **34**, Fig. 9), have been found to inhibit potently the proteosomal CT-L, but not T-L, activity *in vitro* [173]. Related polyphenols lacking the ester bond such as (-)-epicatechin-3-gallate (**35**) are devoid of activity on the proteasome. Tannic acid, present in the bark and fruit of many plants, and which contains multiple similar gallate moieties linked by ester bonds, is also a potent and specific inhibitor of the CT-L activity of purified proteasome [174]. Based on the analysis of atomic orbital energy and a structure-activity relationship study, it was suggested that the N-terminal Thr hydroxyl group of the proteasome attacks the ester bond carbon of EGCG leading to Thr acylation, a mechanism similar to that of lactacystin- β -lactone. EGCG also inhibits proteasome *in vivo* [173] and has an identical effect on apoptosis signaling pathways than lactacystin [175]. In contrast to earlier studies reporting the inhibition of several cancer-related proteins by EGCG, proteasome inhibition was observed at physiological concentrations of EGCG [173], indicating that the proteasome may be a major target for EGCG *in vivo*.

Gliotoxin

The fungal epipolythiodioxopiperazine metabolite gliotoxin (**36**) characterized by a heterocyclic core containing a disulfide bond, have initially been identified as a potent inducer of macrophagocytic apoptosis [176] and an inhibitor of NF- κ B activation [177]. It was recently reported that gliotoxin inhibits the proteasome by an unusual mechanism [178]. Whereas all the known inhibitors bind to the catalytic sites of the proteasome, gliotoxin is a noncompetitive inhibitor of the CT-L activity and probably binds to an unidentified non-catalytic site. The disulfide bridge of gliotoxin, shown to be essential for the majority of its physiological effects, is also involved in proteasome inhibition, suggesting that gliotoxin may target the proteasome by reversible covalent modification involving mixed disulfide bonds at or near the CT-L active site. Gliotoxin inhibits the CT-L activity of purified proteasome with an IC_{50} value of approximately $10\mu\text{M}$, a concentration eliciting the accumulation of proteasome substrates in mononuclear cells. However, the observation that the inhibition of NF- κ B activation is effective at concentrations as low as 100nM [177], raised the question whether or not the proteasome is the prime target of gliotoxin *in vivo*.

Other Non-Peptidic Inhibitors

Proteasome inhibitory activity may contribute to the anticarcinogenic activity of known anti-cancer agents. Aclacinomycin A (Aclarubicin, **38**) and adriamycin (Doxorubicin, **39**) are *Streptomyces* anthracycline antibiotics (Fig. 10), with powerful antitumor properties. Their activity results from their ability to disrupt the structure of DNA as they intercalate into the helices. Earlier studies have shown that aclacinomycin A inhibits the degradation of

ubiquitinated proteins in reticulocyte lysate [179]. Both aclacinomycin A and adriamycin are selective, reversible and noncompetitive inhibitors of the CT-L activity of the 20S proteasome [180,181]. It is thought that these agents bind to an allosteric site causing the distortion of the catalytic site and obstructing its access to the scissile bond. Both the aglycone and sugar moiety of the molecule are required for inhibition of the proteasome. Adriamycin has a high affinity for the proteasome [182] and photoaffinity labeling of L1210 cells with a photoactive adriamycin analogue shows selective binding to the proteasome [183]. Interestingly, it appears that the proteasome is a specific translocator of adriamycin from the cytoplasm to the nucleus [184]. Recent *in vivo* studies have also suggested that proteasome inhibition is involved in the induction of apoptosis by these anti-cancer anthracyclines [181].

Vinblastine (**40**) is a *Vinca* alkaloid (Fig. 10) used as an anti-mitotic agent and is known to disrupt the mitotic spindle dynamics by microtubule stabilization. Vinblastine also inhibits reversibly and noncompetitively the activity of all the three peptidase activities of the proteasome 20S with K_i values around $10\mu\text{M}$. In the proteasome 26S, the T-L and caspase-like activities were more sensitive to inhibition than the CT-L activity. *In vivo*, the drug induced the accumulation of polyubiquitinated proteins and impaired the signal-induced degradation of I κ B [185].

Troglitazone (**41**), a thiazolidinedione derivative (Fig. 10), is a selective ligand of peroxisome-proliferator-activated receptor gamma and is currently used as hypoglycemic agent. A recent study pointed out the anti-proliferative activity of troglitazone, which induces a G1 arrest in gastric cancer cells [186]. This growth arrest involves the induction of p27 (Kip1) whose degradation depends on the ubiquitin-proteasome system. Proteasome activity was inhibited in cells treated with troglitazone, and lactacystin mimics the effects of troglitazone. The anti-proliferative activity of troglitazone is likely mediated by the inhibition of proteasome activity. Whether or not troglitazone directly targets the proteasome core remains to be determined.

The proteasome was also identified to be an intracellular target of 4-hydroxy-2-nonenal (HNE, **42**), a major end product of lipid peroxidation. Under oxidative stress, HNE-modified proteasome was detected in the kidney of mice along with a reduction of both the T-L and the caspase-like but not the CT-L activities. *In vitro*, similar effects were observed on the activities of the proteasome but inhibitory concentrations were much higher than those required for the *in vivo* effects [187]. In addition, HNE was shown to prevent NF- κ B activation in human monocytic cells, but at the concentrations that did not affect proteasome activity [188]. Altogether these results suggest that *in vivo*, proteasome is probably not a direct target for HNE.

Lithium is the most commonly used drug for the treatment of manic-depressive illness. However, its mechanism of action remains still unclear [189]. Lithium chloride was reported to cause synergistic induction of the terminal differentiation of myelomonocytic leukemia cells when combined with all-*trans*-retinoic acid. This effect appears to be mediated by the specific inhibition of the CT-

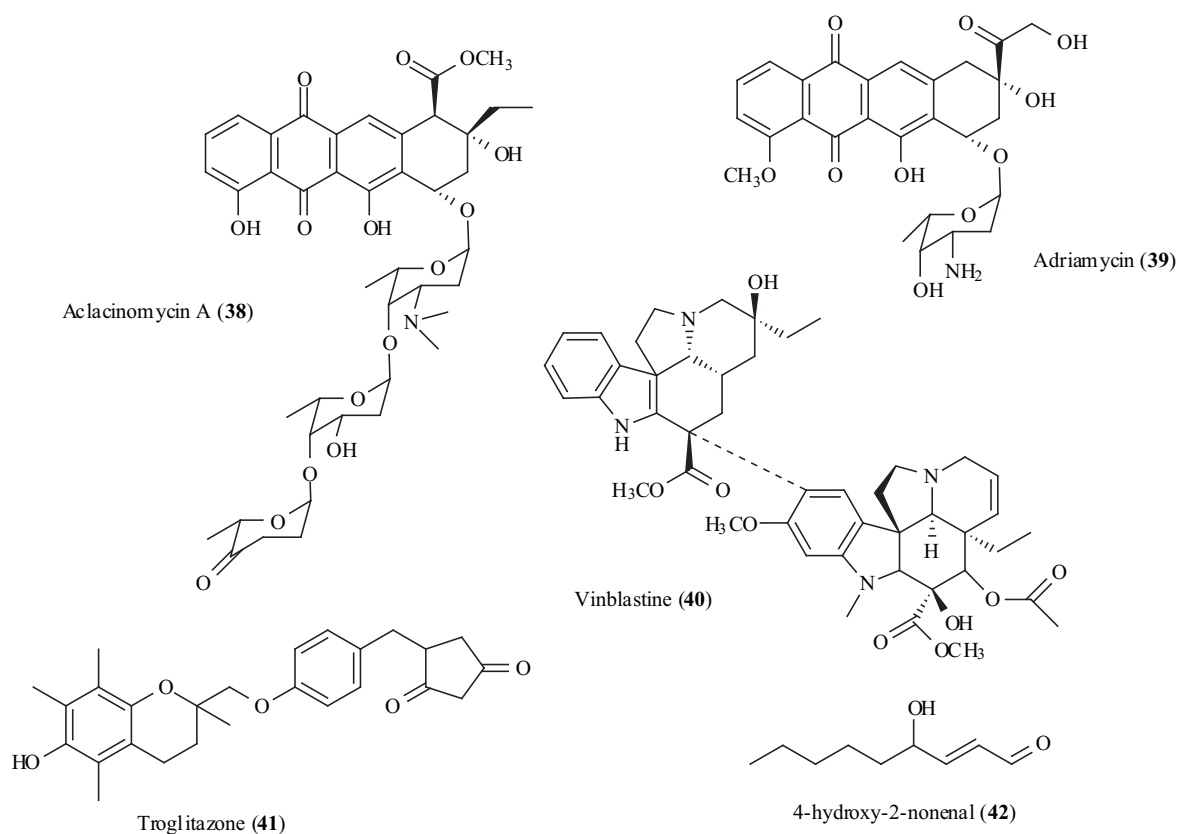


Fig. (10). More non-peptidic proteasome inhibitors.

L activity of both the 20S and 26S proteasome by LiCl which results *in vivo*, in blocking of the degradation of ubiquitinated proteins as for example, ubiquitinated retinoic acid receptor α proteins [190]. The precise mechanism of action of LiCl on the proteasome remains to be identified. However, the observation that proteasome purified from the cells treated with LiCl had a reduced activity, suggests that LiCl affects the proteasome structure irreversibly.

Targeting the Ubiquitination Pathway

Degradation of many eukaryotic proteins requires their prior ligation to polyubiquitin chains, which target substrates to the 26S proteasome [24]. The polyubiquitination of a target protein requires the activity of three enzymes, the ubiquitin-activating enzyme (E1), one of at least 15 ubiquitin carrier proteins (E2) and one of a hundred of substrate-specific ubiquitin-protein ligase (E3). Ubiquitin isopeptidases, which salvage ubiquitin for reuse, are essential to replenish cells with monomeric ubiquitin and regulate the activity of a variety of substrates by altering their ubiquitination status [191]. All these enzymes are potential targets to impair the process of proteasome-mediated degradation of cellular proteins.

Earlier studies have reported the synthesis of various analogs of ubiquitins inhibiting the ubiquitination of proteins. Adenosyl-phospho-ubiquitinol inhibits the ubiquitin activating enzyme [192]. Methylated ubiquitin prevents the formation of polyubiquitin chains [193]. Ubiquitin nitrile [14], ubiquitin aldehyde [194] and

nonhydrolyzable ubiquitin dimers [195] inhibit the ubiquitin isopeptidases. A hexapeptide derived from the carboxyl terminus of ubiquitin was also found to inhibit the ubiquitin activating enzyme [196]. However, if the ubiquitin derivatives are useful tools for the *in vitro* study of the ubiquitin pathway, they are useless for *in vivo* studies and have no therapeutic potential.

Cyclopentenone prostaglandins such as $\Delta 12$ -PGJ₂ (**43**, Fig. 11), which possess antitumor and anti-inflammatory activity [197,198], are potent inhibitors of ubiquitin isopeptidase. $\Delta 12$ -PGJ₂ also causes apoptosis independently of p53-mediated gene transactivation [199]. Because the disruption of p53 is associated with chemoresistance [200,201], identification of antineoplastic agents effective with dysfunctional p53 pathways is of particular interest. Structure-activity relationship studies in a series of prostaglandins has led to the identification of a molecular determinant shared by prostaglandins, inhibiting the ubiquitin isopeptidases and causing p53-independent cell death: a cross-conjugated α,β -unsaturated dienone with two sterically accessible electrophilic β -carbons [199]. A search in the NCI database of compounds bearing this pharmacophore allowed the identification of the diterpene shikoccin (**44**) as a highly potent inhibitor of cellular isopeptidase and p53-independent cell death inducers. The synthetic compound benzylideneacetone (**45**) and the natural dye and dietary spice, curcumin (**46**) also share these properties [202]. In this regard, it is noteworthy that curcumin, known to possess anti-inflammatory and anti-cancer activities [203-205], down-regulates the transactivation of NF- κ B by preventing the degradation of

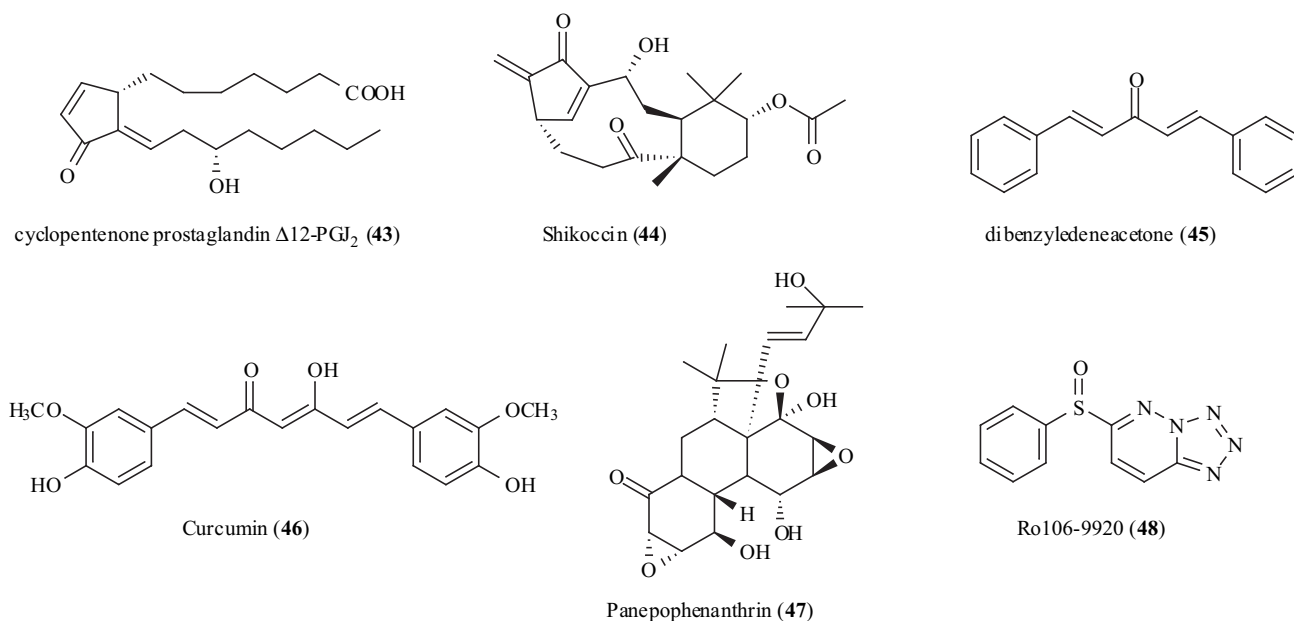


Fig. (11). Inhibitors of the ubiquitination pathway.

$\text{I}\kappa\text{B}\alpha$. Curcumin does not inhibit the 20S proteasome [202], but the inhibition of the ubiquitin isopeptidase could alter the ubiquitinylation of $\text{I}\kappa\text{B}\alpha$, preventing thereby its degradation. The recent synthesis of new cytotoxic curcumin analogues [206] should doubtlessly lead to the discovery of new isopeptidase inhibitors.

A screening of microbial secondary metabolites lead recently to the identification of panepophenanthrin (47), a hexadecahydrophenanthrylene derivative (Fig. 11), as the first potent non-proteinic specific inhibitor of the ubiquitin-activating enzyme [207]. The distinctive structure and activity of panepophenanthrin should provide motivation to contemplate a total synthesis of this compound adaptable for preparing analogs.

Targeting the ubiquitination of a specific protein may prove to be a more difficult task. The specificity is carried by E3 ubiquitin ligases, which recognize specific motifs in their specific substrates, and the eukaryotic cells may contain hundreds of E3 enzymes. However, a recent report demonstrates that a specific E3 ubiquitination activity can be blocked by small molecules [208]. An elegant strategy

employed to discover molecules with anti-inflammatory properties that would target the ubiquitination of $\text{I}\kappa\text{B}\alpha$ led to the identification of the sulfone analog Ro106-9920 (48, Fig. 11). Ro106-9920 is a selective inhibitor of $\text{I}\kappa\text{B}\alpha$ ubiquitination, which targets an unidentified E3 ligase essential for $\text{TNF}\alpha$ - and LPS-induced $\text{I}\kappa\text{B}\alpha$ degradation and NF- κB activation. Ro106-9920 is active *in vivo*: it prevents $\text{I}\kappa\text{B}\alpha$ degradation in cells and inhibits cytokine production in rats.

Targeting Upstream Pathways

The elucidation of signaling pathways involved in the control of the ubiquitin/proteasome pathway may lead to the identification of targets for the development of new therapeutic strategies. For example, $\text{TNF}\alpha$ is implicated in the signaling of muscle wasting [209]. Anti-TNF treatment reverses the increased muscle ubiquitin gene expression in tumor-bearing rats [210]. Xanthine derivatives like pentoxifylline (49, Fig. 12) and torbafylline (HWA 448) suppress the activation of ubiquitin-proteasome-dependent proteolysis, in particular by impairing $\text{TNF}\alpha$ production

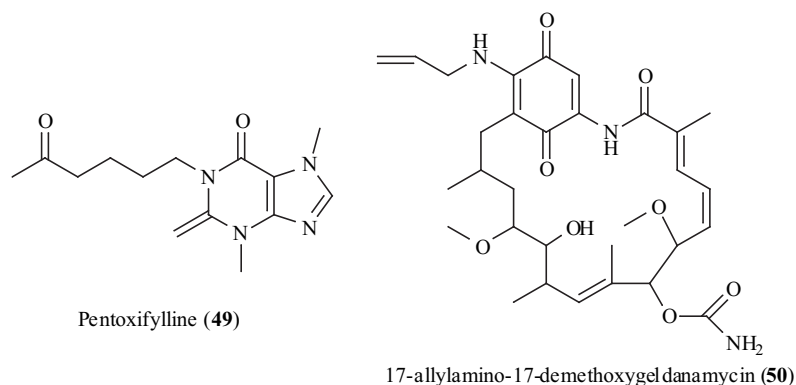


Fig. (12). Compounds targeting upstream pathways.

and are potent inhibitors of muscle wasting *in vivo* [211,212].

The selective destruction of proteins such as oncoproteins, by targeting their interaction with molecular chaperones is an interesting strategy with therapeutic potentials [213]. Heat shock protein 90 (Hsp90) is a molecular chaperone whose association is required for the stability of multiple mutated and over-expressed signaling proteins that promote the growth and/or the survival of cancer cells [214]. ErbB-2, a co-receptor tyrosine kinase, is an example of Hsp90 client protein which is overexpressed in numerous breast and ovarian tumors [215]. The benzoquinoid ansamycin, geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin (17AAG, **50**, Fig. 12) inhibit the hsp90-based chaperone system and induce the dissociation of ErbB-2 from its membrane chaperone, which is readily ubiquitinated and is degraded by the proteasome [214,216,217]. 17AAG is undergoing clinical trials.

PEPTIDOMIMETICS

Although many peptides have been identified as proteasome inhibitors, there are fundamental limitations associated with the development of peptides as therapeutics. The inherent conformational flexibility results in a myriad of conformations adopted by a small peptide. In addition, there are a number of metabolic limitations restricting the use of peptides as therapeutics. Proteolytic degradation, rapid clearance and in some cases, poor solubility and a tendency to aggregate, contribute to low oral availability of peptide-based therapeutics [218,219]. Also, the lack of specific transport systems restrict the efficient passage of peptides to the desired site of action [220]. The susceptibility of externally administered peptides to proteolytic degradation in the gastrointestinal tract, blood, and other tissues results in rapid clearance, thus significantly reducing the effectiveness of these peptides in inducing a satisfactory response. The peptide must therefore survive in the pharmacologically active form under the conditions of exposure to various proteolytic enzymes in the digestive and circulatory systems. Given that the therapeutics are usually administered far from the site of action, there is a clear need for the enhanced metabolic stability.

The use of peptidomimetics to overcome the limitations inherent in the physical characteristics of peptides has become an important strategy for improving the therapeutic potential of peptides [221]. Peptidomimetics can mimic a natural peptide without changing its main biological effect [222], but at the same time improve its undesirable therapeutic characteristics [223]. The term peptidomimetic is a broad term referring to any compound designed to perform the function of a peptide. Such functions include eliciting a specific biological effect through inhibition or agonist/antagonist activity. Generally, peptidomimetics are derived from a lead peptide sequence where the structural modifications have been incorporated to improve binding affinity and/or metabolic resilience. The alteration of peptides to peptidomimetics encompasses side-chain manipulation, turn-mimics [224,225], amino acid extension [226,227], and backbone modifications [221,228-233]. The

pseudopeptide approach seems particularly to be promising since the structure and conformation of the original peptide should largely be preserved in the resulting biomimetic polymer.

Pseudopeptides

The most common manipulation involving the α -carbon atoms of peptides include the inversion of configuration to yield D-amino acids [234]. The importance of this substitution in affording compounds with improved biological potencies, altered conformational properties [235]; increased resistance to enzymatic degradation has been widely recognized and exploited [236]. Replacements of the α -hydrogen of the common amino acids by a methyl group or any other substituent are further examples of α -alkyl modification [237]. Substitutions of the α -carbon atom isoelectronically by a trivalent nitrogen yield azapeptides or azatides [232,238-244]. Peptoids [245-250], azapeptoids [244], ureapeptoids [251,252], amino oxypeptoids [253], retropeptoids [248], and β -peptoids [254] belong to a new conceptual class of pseudopeptides in which the side chains are shifted on nitrogen atoms of the backbone (Fig. 13). This class of pseudopeptides lacks chirality in the α -position and can be considered intermediate in the configuration between D- and L-amino acids [240,255].

Peptoids (**52**), one of the earliest pseudopeptides [245,246], are oligomers of N-substituted glycines. The side chain has been shifted from the chiral α -carbon atom in a peptide (**51**) to the achiral nitrogen. Such analogs have been extensively studied for their biological functions [256], and structural features [249,250,257]. Recently, it was found that peptoids with aminoethyl side chains could transfer DNA into cells and thus may potentially be employed as a novel type of a gene delivery vehicle [258].

Comparison of a portion of a peptide chain to a peptoid chain shows that the direction of the peptide bonds must be reversed in the peptoid ("retro sequence") if the carbonyl group contributes to the binding of the target molecule. In retropeptoids (**53**), the relative orientation of the carbonyl groups to the R groups is maintained and this better resemblance to the parent peptides may be responsible for a somewhat higher biological activity of retropeptoids [246,247].

Azapeptides (**54**) are peptide analogues in which the α -CH groups of one or more amino acid residues has been substituted by a nitrogen, bearing an appropriate side-chain. An azatide (**55**) is an oligo or a pure azapeptide [232]. Azapeptoids (**56**) are the hybrids of peptoids and azapeptides [245]. This modification of the natural peptide bond, from an amide to a urea, produces marked changes in its chemistry and biochemistry. Theoretical studies of diacylhydrazines indicated that, in contrast to azaglycine, N-substituted azapeptides should be more rigid than their conventional peptide counterparts [259]. As a consequence, azapeptides and azapeptoids could be regarded as conformationally restrained, and therefore, as templates that have the potential to induce biological selectivity in pseudo bio oligomers. The aza carbonyl bond is much more

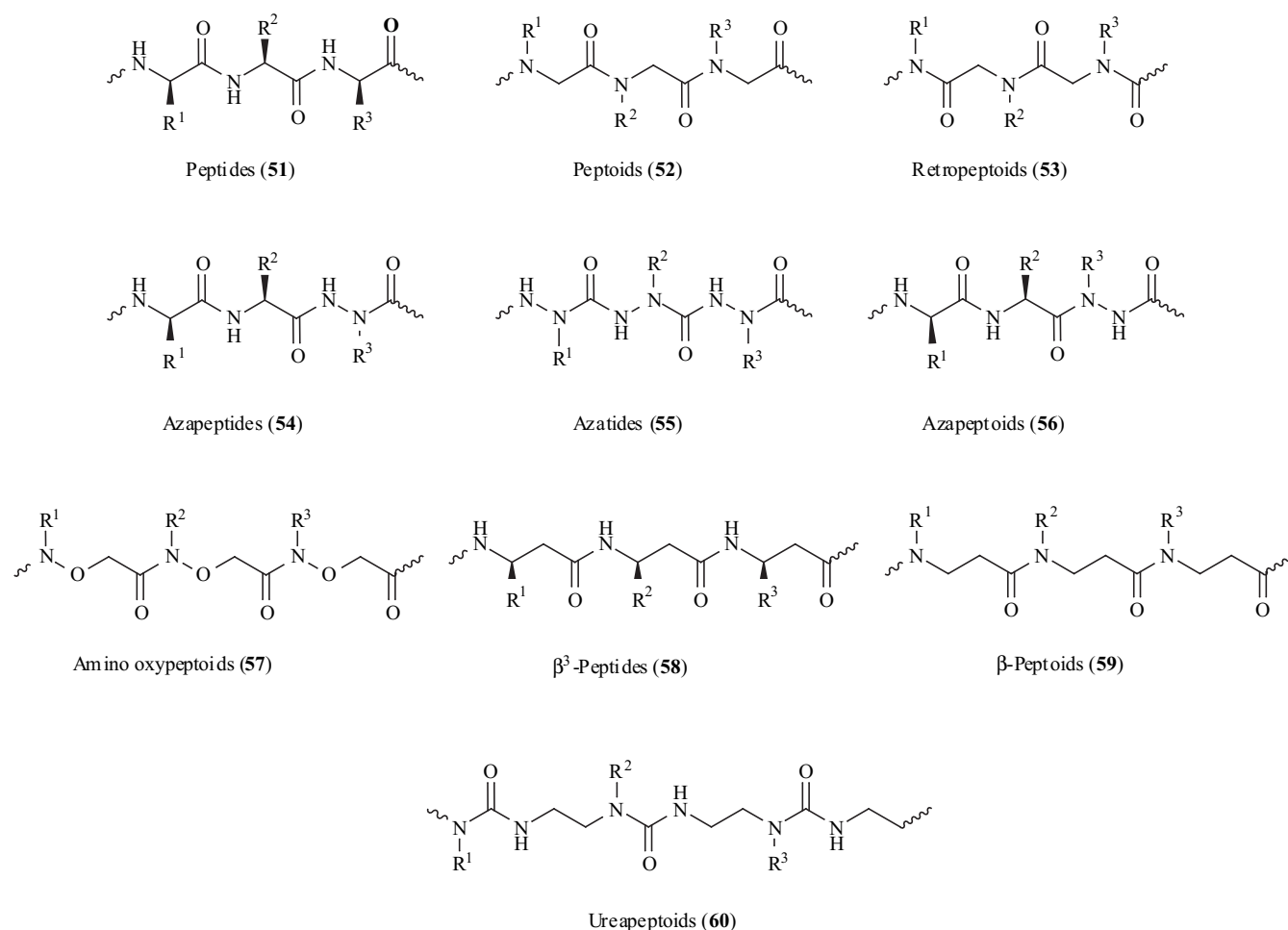


Fig.(13). Comparison of segments of peptide and pseudopeptide chains.

resistant to both chemical and enzymatic hydrolysis than the natural peptide bond [260,261]. Studies have demonstrated azapeptide resistance to amino and carboxy peptidases, and they have also been used as prodrugs [262]. Several groups have recently reported their use as inhibitors of serine, cysteine and aspartyl proteases [263-266].

Amino oxypeptoids (57) are potential peptidomimetics, in which side chains are attached to the nitrogen atom of aminoxy acid monomer. Aminooxypeptoids have been synthesized in order to provide more structural diversity and screening for biological activities is in progress [253].

Another peptidomimetic approach with significant potential that emerged in recent years is the use of β -amino acids. β -amino acids are similar to α -amino acids in that they contain an amino terminus and a carboxyl terminus. However, two carbon atoms separate these functional termini. β -peptides (58), oligomers of β -amino acids, have been shown to exhibit residue controlled secondary structures [267] and to have remarkable stability toward proteases [268]. Recently, β -peptoids (59) have been synthesized to improve the metabolic stability of β -peptides as the tertiary amides of peptoids providing a backbone structure more stable to hydrolysis and less polar than the typical peptide amide bonds [254].

Ureapeptoids (60) can be considered as hybrid of peptoids and urea peptidomimetic. They are a particular attractive class of unnatural oligomers, because the urea group has interesting biological and hydrogen-bonding properties [251,252].

Hydrazino Aza and N-Aza Peptoids

The calpain inhibitor I and numerous peptides modified on C-terminal position have already been described to inhibit the activity of the proteasome [19]. No oligomeric inhibitors such as peptoids have been reported to act on the degradation activity of the proteasome. Azapeptides have been evaluated as potential inhibitors of serine and cysteine proteases and displayed time-dependant inactivation of cathepsin B and calpain. β - and γ -peptides with proteogenic side chains are not degraded by either common proteases or the 20S proteasome [269]. The proteolytic stability of such peptidomimetics is a prerequisite for their use as drugs.

By combining these classes of peptidomimetics, the diversity of compounds can be increased enormously thus obtaining "hybrid" peptidomimetics, which might be very useful for the development of lead compounds from peptides. The hydrazino azapeptoids (61) and N-azapeptoids (62), which have no chiral center with fixed configuration, combine a C-terminal aza α -amino acid unit (aza) or a N-

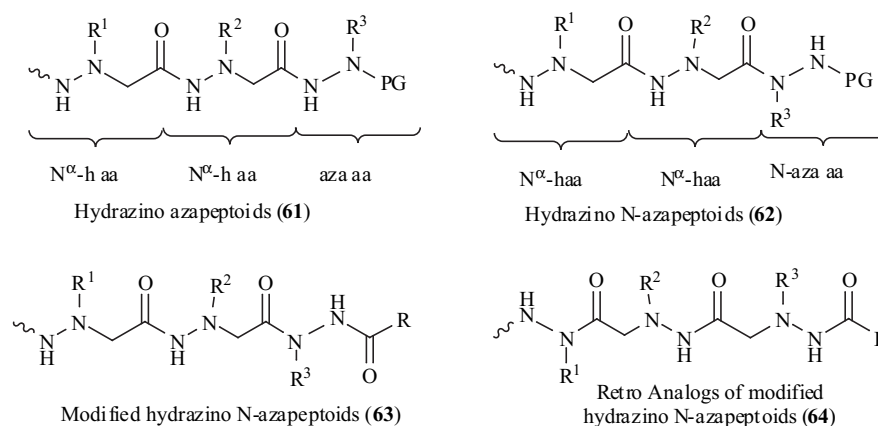


Fig.(14). General structure of new peptidomimetics.

substituted azaglycine (N-aza) with one or more aza- β^3 -amino acid unit (N $^{\alpha}$ h) (Fig. 14) [270]. This new class of “hybrid” peptidomimetics can be considered as a hybrid of peptoid, azapeptide and β^3 -peptidomimetic.

Shifting the side chain from the α -carbon atom of the peptide chain to the nitrogen atom in these new

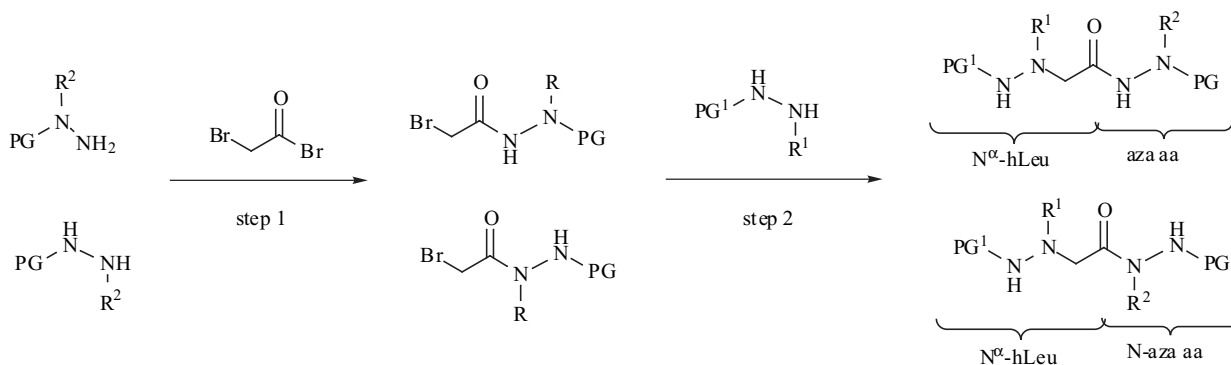


Fig.(15). Submonomer synthesis of hydrazino azapeptoids and N-azapeptoids.

peptidomimetics affords potentially bioactive pseudopeptides with the following advantages:

- Metabolic stability, since natural proteases cannot cleave the N-substituted peptide bond.
- Large variability of side chains, mimicking proteogenic or non-proteogenic amino acids.
- Simple submonomer synthesis, in which two types of building blocks are used to construct each residue: N, N'-disubstituted hydrazines and bromo acetyl bromide (Fig. 15).
- Orthogonally protected, these hydrazino aza and N-azapeptoids could be selectively and easily deprotected and refunctionalized on the C or N terminal position leading to modified hydrazino aza and N-aza peptoids (63) or their retro analogs (64). The incorporation of a variety of groups bearing functionality permits the access of compounds capable of reversible or irreversible interaction with the protease's active site.

- Spectroscopic and crystallographic studies indicated that, even short aza β^3 -amino peptides are capable of adopting eight-membered, hydrogen-bonded turns (hydrazino or N-N turns-Fig. 16) [271]. Their ability to self-organize through intramolecular hydrogen bond reduces the conformational flexibility and therefore could increase the bioselectivity.

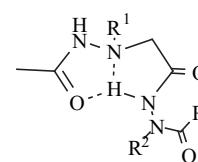


Fig.(16). Hydrazino or N-N turn mimetic of hydrazino azapeptoids.

Hydrazino aza and N-aza Peptoids Designed for Proteasome Inhibition

A series of new pseudopeptides,^{1,2} have been synthesized to mimic ALLN (Ac-Leu-Leu-Nleu-H, 2) or MG132 (Z-Leu-Leu-Leu-H, 3) sequence (Fig. 17). These pseudodipeptides are C- or N- modified hydrazino aza and N-azapeptoid based

¹ Arlot, Y.; Bouget, K.; Baudy-Floc'h, M.; Le Grel, P. *Patent*, N°01/11120, CNRS, France, 27 August 2001

² Arlot, Y.; Bouget, K.; Baudy-Floc'h, M.; Le Grel, P.; Aubin, S. *Patent*, N°PCT/FR02/02935, CNRS, France, 27 August 2002

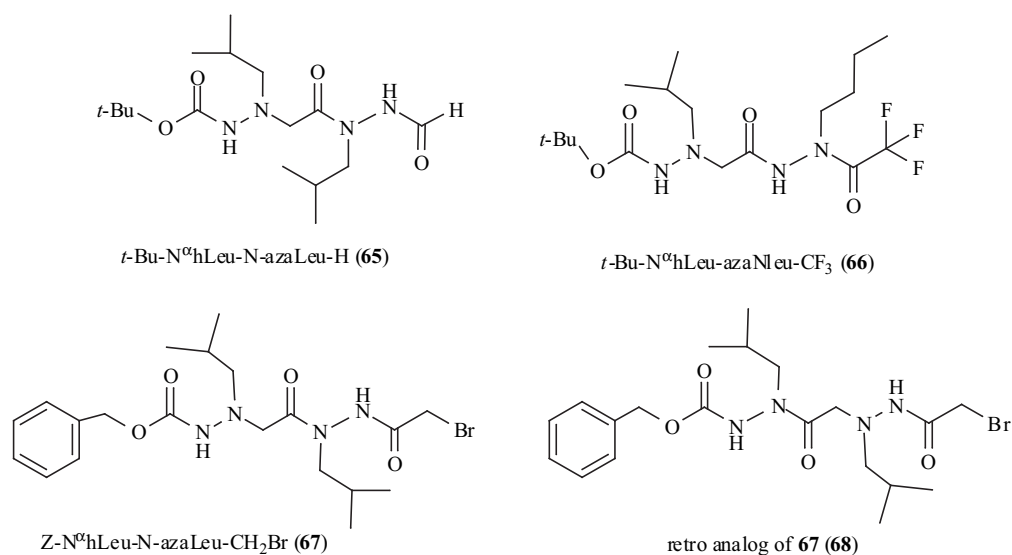


Fig. (17). Hydrazino aza and N-aza peptoids designed to target the proteasome.

compounds bearing diverse electrophilic functional groups such as aldehyde (**65**), ketoester, boronic acid and chloromethyl. Electrophilic pharmacophores never used before in proteasome peptide inhibitors, such as trifluoromethyl (**66**), bromomethyl (**67**) and cyanomethyl, were also introduced. Retro analogs of those peptoids (e.g. **68**) have also been prepared. These new analogs are expected to have a greater metabolic stability and an improved potency toward the enzymatic activities of the proteasome. Their biological evaluations are under progress.

CONCLUSIONS

Proteasomes constitute the degradative machinery of the ubiquitin/ATP-dependent proteolytic pathway, which is involved in many cell functions, including immune response, apoptosis, virus maturation and infectivity, and cancer. Aberrant expression of signal transduction molecules in pathways controlling cell survival, proliferation, death, or differentiation is a common feature of all tumors. The central role of the proteasome in controlling the expression of regulators of cell proliferation and survival has led to an interest in developing proteasome inhibitors as novel anticancer agents.

Different classes of peptidic or nonpeptidic inhibitors have been designed to target proteasome activity. Due to their lack of specificity or their low cell permeability, many of these molecules are useless *in vivo*, but remain helpful tools for *in vitro* studies. *In vitro* and *in vivo* studies have shown that proteasome inhibition is a valuable approach to cancer therapy. Some proteasome inhibitors are active against a variety of tumor types. One of these agents, the dipeptide boronic acid analog PS-341 has entered clinical trials in humans. These inhibitors also have impressive potential for the treatment of inflammatory diseases. A new group of pseudopeptides described in this review, the hydrazino-aza and N-aza peptoid wearing different pharmacophores, may also interact more specifically with the

catalytic site of the proteasome. These could constitute a new class of therapeutic agents.

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