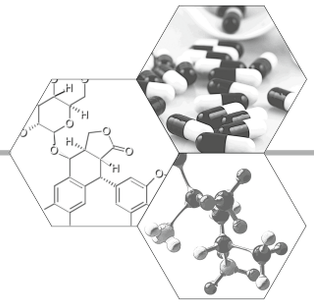


Targeting the ubiquitin–proteasome pathway with inorganic compounds to fight cancer: a challenge for the future

Proteasomes are large multicatalytic complexes endowed with proteinase activity, located both in the cytosol and in the nucleus of eukaryotic cells. The ubiquitin–proteasome system is responsible for selective degradation of most intracellular proteins and therefore plays an essential regulatory role in many critical cellular processes. The proteasomal activity can also contribute to the pathological states of many diseases, including inflammation, neurodegeneration and cancer, through a disregulation in the level of regulatory proteins. These diseases may be targeted by modulating components of the ubiquitin–proteasome pathway, using small molecules as inhibitors. Bortezomib (Velcade®), used for the treatment of relapsed multiple myeloma, is the first and up to now the only proteasome inhibitor approved by the US FDA. Nowadays, the discovery that some metal-based complexes exert their antiproliferative action by affecting proteasomal activities provides the possibility of developing new opportunities in cancer therapy.

Lisa Dalla Via[1], Chiara Nardon[2] & Dolores Fregona*[2]

[1]Department of Pharmaceutical Sciences, School of Pharmacy, University of Padova, Via Marzolo 5, 35131 Padova, Italy
[2]Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy
*Author for correspondence:
Tel.: +39 49 827 5845
Fax: +39 2 700 500 560
E-mail: dolores.fregona@unipd.it

The major component of the nonlysosomal protein degradation pathway is the proteasome, a barrel-shaped multimeric protein complex with proteolytic activities. The proteasome is found in eukaryotes, as well as in prokaryotes, and is involved in a variety of essential biological processes: protein management, antigen presentation, signal transduction, cell cycle control, cell differentiation and apoptosis [1]. In eukaryotes, the nonlysosomal protein degradation is performed by the enzymatic mechanism of the ubiquitin–proteasome pathway [2]. This pathway plays a major role in the degradation of oxidatively damaged and mutated proteins, as well as of proteins involved in cell cycle progression, proliferation and apoptosis [3], producing most of the antigenic peptides presented to the immune system by **major histocompatibility complex** Class I molecules [4]. The ubiquitin–proteasome system degrades short-lived proteins that are involved in critical steps of the control of cell proliferation and cell death [5–8]. Proteins to be degraded through the ubiquitin–proteasome system are tagged with ubiquitin molecules. Ubiquitin is composed of 76 amino acids and attaches to a client protein (ubiquitination) prior to degradation [1]. Ubiquitination requires the sequential actions of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3), which result in the formation of the polyubiquitin chain [9–12]. The polyubiquitinated proteins are then shuttled to the proteasome for degradation in an ATP-dependent manner **(Figure 1)**.

The executioner of the ubiquitin–proteasome pathway is the 26S proteasome, which is built up from the proteolytic core particle (20S proteasome) and two regulatory particles (19S regulatory complex) **(Figure 1)**. The 20S proteasome has a barrel shape characterized by twofold symmetry and contains multiple catalytic centers located within the inner cavity of a molecular cage. It comprises 28 subunits, which are arranged in four seven-membered rings that stack upon each other, yielding an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ complex [13,14]. Compared with archaebacterial proteasomes, which have 14 proteolytically active sites [15], eukaryotic proteasomes contain only three proteolytically active β subunits per β ring (subunits β1, β2 and β5), whereas the other β subunits are inactive [16,17]. The β5, β2 and β1 subunits are responsible for three different catalytic activities of the proteasome: **chymotrypsin**-like, trypsin-like and peptidyl glutamyl peptide hydrolyzing- like, respectively [16,18]. In all three β-subunits, the amino-terminal threonine (N-Thr) is regarded as the catalytically active site [19].

As discussed, the ubiquitin–proteasome pathway is essential for many fundamental cellular processes [20]. Moreover, the proteasome

FUTURE SCIENCE part of fsg

Key Terms

Major histocompatibility complex: Cell surface proteins encoded by a gene family found in all higher vertebrates. Major histocompatability complex proteins help the immune system to recognize foreign substances.

Chymotrypsin: Proteolytic enzyme (serine protease) selective toward peptide substrates containing either aromatic or large hydrophobic amino acid residues, such as Tyr, Trp, Phe, Leu and Met. The corresponding side chains fit into the 'hydrophobic pocket' of the enzyme (S1 enzyme-binding site), thus positioning their C-terminus (P1 substrate position) near the serine (nucleophilic amino acid residue) and therefore leading to the peptide amide bond hydrolysis.

NF-κB: Cytoplasmic transcription factor that plays a crucial role in immune and inflammatory reactions. Activated NF-κB, induces the expression of cytokines, growth factors, transcription factors and immunoreceptors, thus affecting immune responses and cell signaling pathways.

Reactive oxygen species: Highly reactive oxygen containing molecules (free radicals) with unpaired electrons able to trigger oxidative chain reactions, thus leading cell damages.

Inorganic compound: Although the distinction between inorganic and organic compounds is not absolute, any substance in which at least one element different from carbon, hydrogen, oxygen, nitrogen, sulfur and phosphorous is included. This definition is not comprehensive and several exceptions are present.

Multiple myeloma: Malignancy characterized by an hyperproliferation of plasma cells, which are normally responsible for the production of antibodies. Accumulation of abnormal cells in bones, causes bone lesions. Accumulation in bone marrow interferes with the normal production of blood cells.

also contributes to the pathological state of several human diseases [21], including cardiovascular diseases [22], viral diseases [23], neurodegenerative disorders [24] and numerous cancer types, in which some regulatory proteins are either stabilized due to decreased degradation or lost due to accelerated degradation [25–27]. One example is **NF-κB**, whose activation is controlled by the ubiquitin–proteasome pathway [28]. This factor plays a crucial role in many human cancers mainly because of its protective effects against apoptosis.

These diseases may be targeted by modulating components of the ubiquitin–proteasome system and/or the conjugation pathways of several ubiquitin-like proteins using small molecules as inhibitors. So far, a number of natural and synthetic compounds have been reported to inhibit the components of the ubiquitin–proteasome system. Natural products, such as salinosporamide A **(Figure 2)**, isolated from a marine actinomycete, were found to be more effective than the only drug in clinical use, bortezomib, and therefore some of them are now undergoing clinical trials for cancer. Based on the chemical moiety that interacts with a threonine residue in the active site of the proteasome, proteasome inhibitors can be divided into several classes: aldehydes, boronates, β-lactones, epoxyketones, cyclic peptides and macrocyclic vinyl sulfones, with different inhibitory mechanisms. Moreover, proteasome inhibitors can target the proteasome pathway in a reversibly or irreversibly way **(Figure 2)** [12].

In general, the mechanism of action of the antiproteasome drugs has not been wholly elucidated. The apoptosis associated with the inhibition of ubiquitin–proteasome pathway starts inducing a stress condition in the endoplasmatic reticulum and the consequent inhibition of the NF-κB inflammatory pathway, activation of caspase-8 and increase of **reactive oxygen species** (ROS) [29–31].

The therapeutic potential of ubiquitin–proteasome inhibition in cancer has been confirmed by the proteasome inhibitor bortezomib **(Figure 3)**, which was approved by the US FDA in 2003. Moreover, a recent ongoing study on the roles of other components of the ubiquitin–proteasome conjugation pathways has identified several enzymes that could be additional targets for therapeutic molecules [32].

Although the preclinical and clinical efficacy of proteasome inhibitors as anti-tumor agents has been well described, the mechanism by which they lead to cell death of malignant cells *in vitro* and *in vivo* has not been fully elucidated.

Bortezomib reversibly binds the proteasome, inhibiting the proteasomal enzymes. A number of other molecules with different mechanisms of inhibition have been developed to overcome the resistance to bortezomib. Some of these bind irreversibly to the active sites of the proteasome and some others are molecules that are able to inhibit the function of the proteasome by binding the complex outside of the active site [33]. Bortezomib and some metal-based proteasome inhibitors will be discussed in detail below.

Bortezomib

Bortezomib (*N*-acyl-dipeptidyl boronic acid, Velcade®), an **inorganic compound**, is the first and, at present, the only proteasome inhibitor approved by the FDA for the treatment of relapsed **multiple myeloma** (MM) and mantle cell lymphoma. It is a small boronic acid dipeptide molecule that binds reversibly to the chymotrypsin-like β5 subunit of the catalytic cavity of the 20S proteasome [34–36].

Bortezomib was approved in 2003, experiencing an unprecedented 8-year program from lead compound discovery to FDA approval owing to a huge clinical need for MM treatment. In addition [37], it is administered to patients with mantle cell lymphoma who have already attempted other treatments [38]. To date, bortezomib is approved in more than 90 countries and it has been developed and marketed by Millennium Pharmaceuticals, a biopharmaceutical company based in Cambridge (MA, USA) and acquired by Takeda Pharmaceutical Company in 2008 [201].

Throughout its development two steps proved decisive. On the National Cancer Institute's 60-cell line screen, bortezomib showed remarkable cytotoxic activity against several cancer types [39,40]. Besides tumor proliferation inhibition, it induced high apoptosis indexes and overwhelmed drug resistance [30,39]. Notably, large Phase II clinical trials were carried out involving 202 patients with relapsed and refractory MM turning out a 35% total response rate (including 4% of complete remission), 6% of almost complete remission, 18% of partial remission and only 7% of negligible response [30,34]. Concerning drug dosing, the range used in Phase I trials was 0.13–1.56 mg/m^2 [34,38,41]. In a Phase III study, patients with relapsed myeloma were randomly assigned to receive intravenous

bortezomib or high-dose oral dexamethasone. Patients who received bortezomib had better clinical outcomes compared with those who received dexamethasone, with higher complete and partial response rates (38% for bortezomib vs 18% for dexamethasone) and longer 1-year survival [42].

More recently, bortezomib has been used in front-line therapy for myeloma, either as a single agent or in combination with standard or emerging therapies. For example, in the randomized Phase III trial, bortezomib was used as initial standard therapy in MM: patients with newly diagnosed symptomatic myeloma who were not candidates for high-dose therapy and autologous bone marrow transplant were treated with Melphalan (9 mg/m^2) and Prednisone (VISTA trial) with or without bortezomib. This clinical trial on 682 patients demonstrated that the addition of bortezomib improved the time to progression (median time to progression with vs without bortezomib: 20.7 vs 15 months) and also improved overall survival [43,44]. Therefore, the trend in clinical trials with newly diagnosed patients with myeloma is to combine agents that are known to be active against this disease, including bortezomib, to improve response rates [33].

■ Mechanism of action

From the pharmacodynamic point of view, bortezomib anti-tumor activity occurs by a regioselective and reversible proteasome inhibition and reaches its maximum 1 h post-dose [45,46]. In order to explain the regioselectivity, it is worth noting that the subunits β1, β2 and β5 recognize distinguishing side chains of different peptides, thus resulting in specific activity for each catalytic site, whereas the remaining β-subunits are inactive [47]. Concerning the substrate specificity, *in vitro* assays disclosed that proteasomal activities are restricted to only five distinct cleavage preferences: chymotrypsin-like, trypsin-like, peptidyl-glutamyl-peptide-hydrolizing-like (also called postacidic or caspase-like), branched chain amino acid-preferring and small neutral amino acid-preferring activities [47,48]. The first three are assigned to β5, β2 and β1 subunits, respectively. Bortezomib mainly targets the proteasomal β5 active site (β5 > β1 >> β2) even if its binding mode to all three subunits is basically identical [40]. The oxygen atom on the side chain of the *N*-terminus threonine (Thr1O$^\gamma$) of each subunit forms a tetrahedral adduct with the boron atom owing to its Lewis acidity. On the other hand, drug peptide backbone binding mode brings about different affinities to the three proteasomal pockets because of interactions of inhibitor organic moieties with specific functional groups of each enzymatic pocket (topological–morphological and chemical–complementarity). However, in its bound conformation, bortezomib adopts an antiparallel β-sheet in any active site. This conformation is stabilized by direct hydrogen bridges between main chain atoms of the drug and the conserved residues (Gly47N, Thr21N, Thr21O, and Ala49N) of the β-type subunits **(FIGURE 4)** [40].

FIGURE 5 shows the enzyme–ligand interaction in β5 cleft obtained by means of the software Chimera (PDB code: 2F16) [49]. Besides the reversible covalent bond Boron-Thr, 11 hydrogen bonds are established, according to the crystal structure solved by Groll *et al.* in 2006 [40].

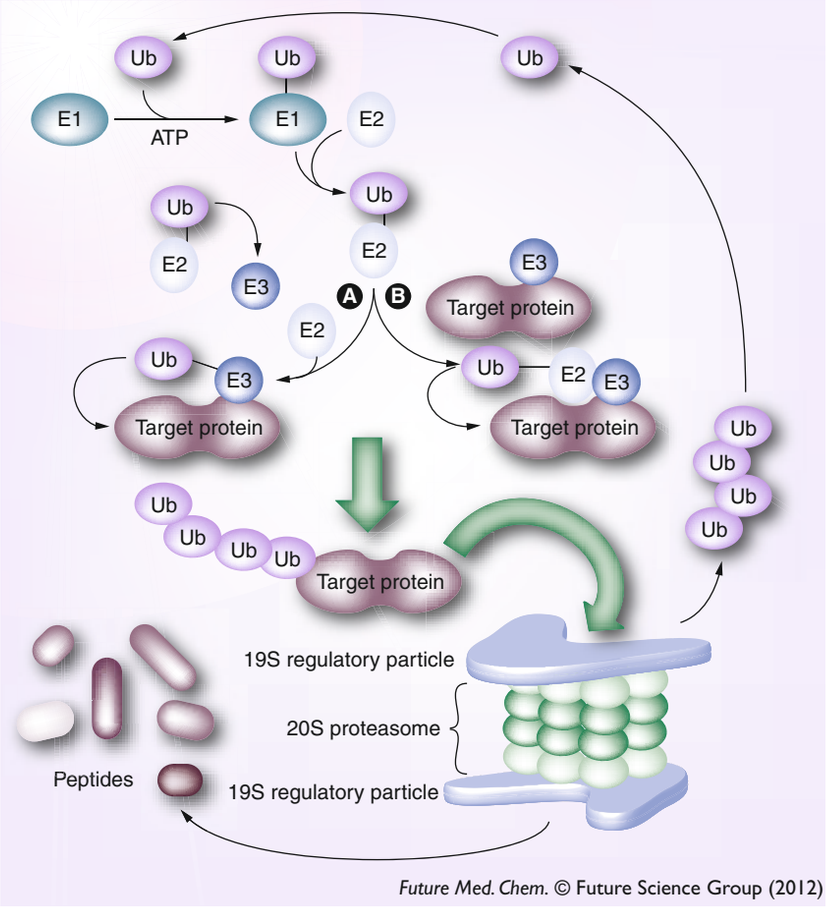


Figure 1. The ubiquitin–proteasome system. Upon attachment of several ubiquitin molecules to the target protein via either pathway A or B, such a substrate is escorted to the proteasome, recognized by the 19S complex, deubiquinated and then degraded by the catalytic 20S core into oligopeptides. The ubiquitin molecules are released and recycled.
Ub: Ubiquitin.

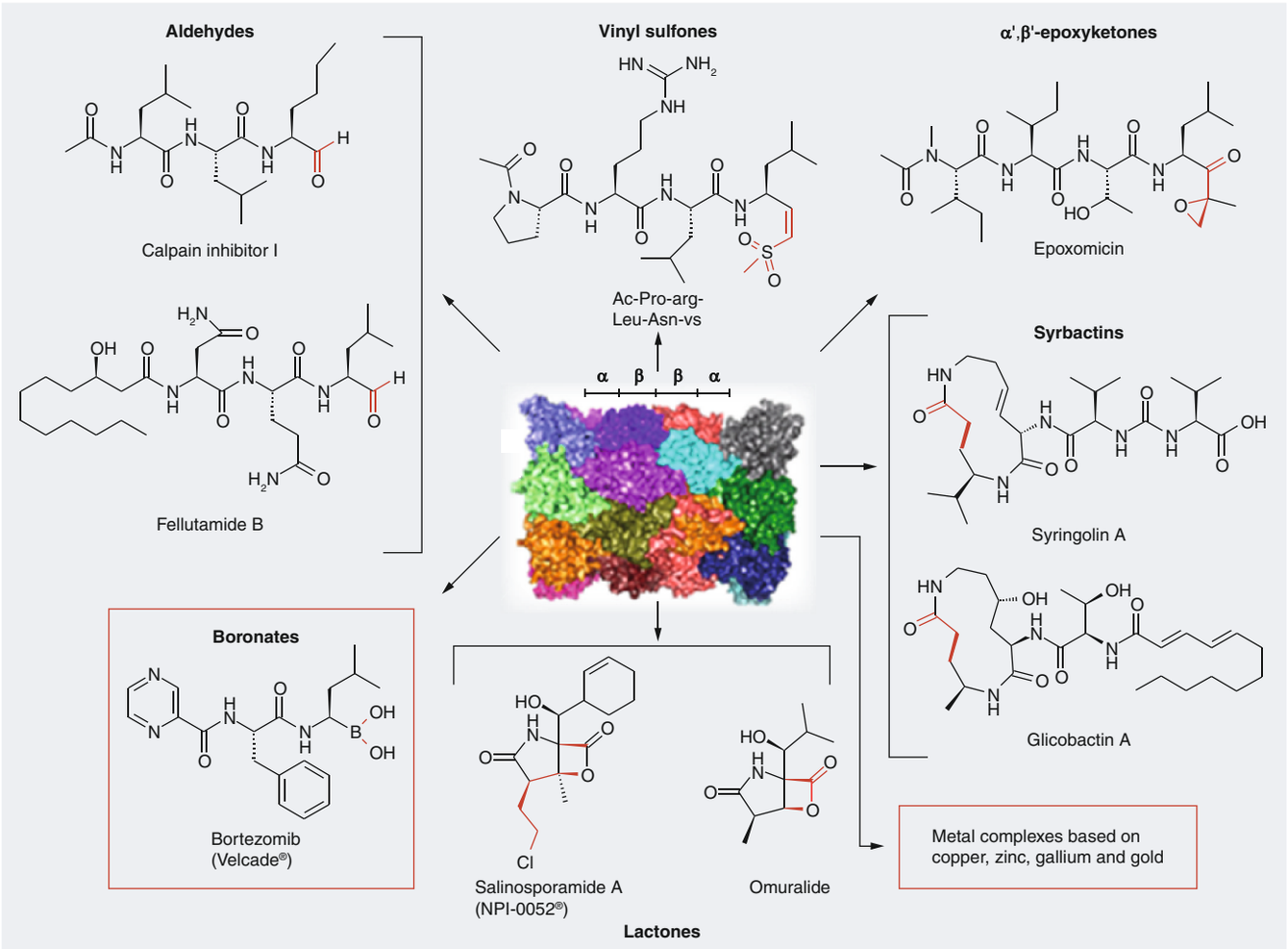


Figure 2. Proteasome inhibitors: selection of natural and synthetic compounds proved to be proteasome inhibitors.
Bortezomib (velcade) is at present, the only drug approved by the US FDA for the treatment of multiple myeloma.
Modified with permission from [14].

Interestingly, it has been shown that chymotrypsin-like pocket targeting is associated with apoptosis induction in cancer cells [50]. In addition to bortezomib's high affinity for the proteasomal β5 subunit under physiological conditions, Ntn-hydrolases targeting may account for its unmatched anti-tumor activity for patients with MM. Bortezomib does not show cross-reactivity toward cysteine or serine proteases. Regarding the former hydrolases, according to the Lewis hard–soft acid–base principle, boron atoms preferentially bind to hard oxygen nucleophiles instead of soft sulfur donors. Nevertheless, boronic acid derivatives are well-known inhibitors of serine proteases [51], turning out a serine–boronate tetrahedral adduct [52,53]. If compared with serine proteases, the proteasomal Thr1O$^{\gamma}$–bortezomib tetrahedral adduct is stabilized by a further tight hydrogen bond between the second acidic boronate hydroxyl moiety (H donor) and the N-terminal threonine nitrogen atom (H acceptor) [40].

β-5 subunit inhibition by bortezomib triggers various effects in signal transduction pathways that regulate cell cycle and proliferation. For instance, the nuclear factor NF-κB mediates tumor cell survival and proliferation, since upon activation it can translocate to the nucleus and elicit transcription of target genes by binding to specific DNA sequences (promoter regions). The genes involved encode for an ever-growing list of species such as cell adhesion molecules, cytokines, anti-apoptotic factors and enzymes. The inhibitor protein I-κB binds to NF-κB in the cytoplasm, thus inactivating and thereby hampering cell growth as a result of downregulation of anti-apoptotic target

genes. However, I-κB can be phosphorylated, ubiquitinated and therefore degradated by proteasome. On the basis of these considerations, proteasome–bortezomib interaction is followed by an increase in I-κB levels that leads to tumor growth inhibition [41,54].

Furthermore, bortezomib determines accumulation of proapoptotic proteins such as NOXA, p53, p27 and BAX and decreased levels of antiapoptotic proteins including Bcl-2, MCL-1 and IAP [6,30,55–60]. Such an altered balance prompts a mitochondrial membrane potential decrease, release of cytochrome c in the cytosol and activation of caspases (-9, -7 and -3) that ultimately cause DNA cleavage and apoptosis [55].

From the pharmacokinetic point of view, in preclinical studies it has been shown that plasma clearance of bortezomib is rapid (the elimination half-time in monkeys was 8–10 h). In fact, blood levels dropped below the detection threshold within minutes [46].

■ Clinical evidence

Despite the clinical success of bortezomib in MM and mantle cell lymphoma [35,42], the majority of patients (even those who respond) eventually develop resistance over time by mechanisms still unknown [61,62]. In addition, the clinical response to bortezomib in solid tumors is very low [63]. For example, in studies where bortezomib was used as a single agent in newly diagnosed patients, 52% did not achieve any response and almost all relapsed refractory patients in the end progressed [33,34,64]. It seems that increased and/or altered proteasome subunit expression may play a role in acquired drug resistance to bortezomib [61,62]. The mechanism of resistance seems to be either at the level of the proteasome or downstream of this enzymatic complex. At present, it is uncertain whether alterations or overexpression of the β5 subunit are responsible for clinical resistance to bortezomib. As matter of fact, its overexpression or mutation were shown to induce bortezomib resistance in several *in vitro* models [65–67]. Moreover, factors downstream of the proteasome activity can mediate resistance to bortezomib [33,68]. Clinical resistance may also be mediated by pharmacokinetic factors that affect the stability, metabolism and tissue delivery of the drug. In such cases, the development of proteasome inhibitors with different pharmacokinetic properties may also be clinically useful for some patients.

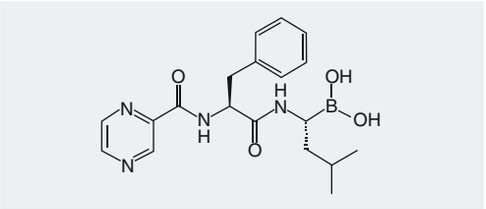


Figure 3. Proteasome inhibitor bortezomib ([(1*R*)-3-methyl-1-({(2*S*)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl}amino)butyl]boronic acid).

In addition to the acquired resistance, bortezomib has a very narrow therapeutic window, which is in relation to the normal functions of the proteasome. The therapeutic dose of bortezomib is 1.3 mg/m^2, whereas 1.5 mg/m^2 produced dose-limiting toxic effects in Phase I studies [35]. Painful peripheral neuropathy, characterized by decreased sensation and paresthesia, is a common dose-limiting toxicity, limiting its use in many patients [69]. Moreover, other side effects frequently associated with the use of the drug, reported in the clinical trials, include cardiovascular disorders, asthenia, nausea, diarrhea, vomiting and hematological toxicity [42,70].

■ Next-generation inhibitors

Collectively, these drawbacks prompted the development of alternative proteasome inhibitors that may affect the proteasome in an irreversible fashion. Because they differ in terms of chemical structure and mechanism,

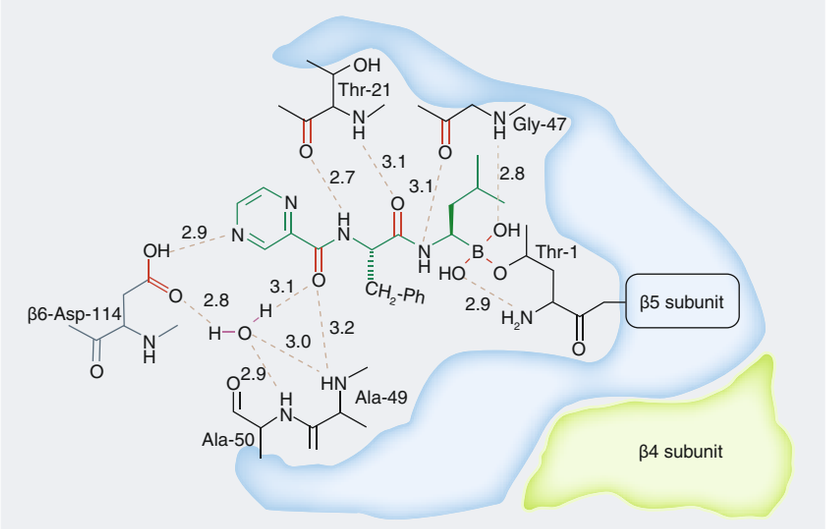


Figure 4. Boron tetrahedral adduct and intermolecular interactions occurring between bortezomib and the proteasomal β5 pocket.

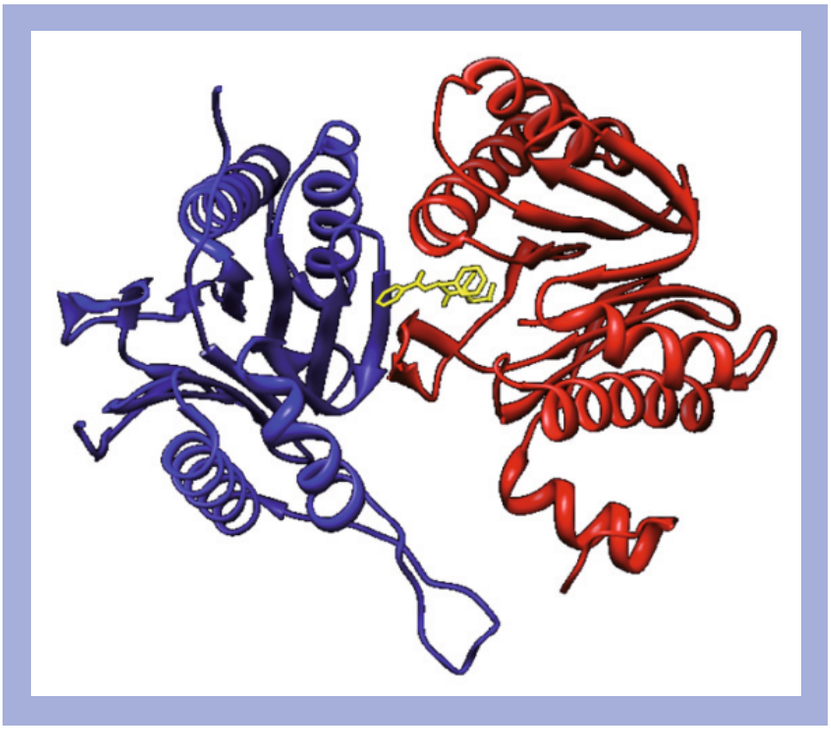


Figure 5. Structure of β5 subunit (red), β6 subunit (blue) and bortezomib (yellow).
Reproduced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, CA, USA.

Key Term

Coordination complex: compound wherein one or more molecules or anions (ligands) donate an electron pair to a central atom or cation, acting as a Lewis acid, thus forming coordinate bonds. Atoms in the ligands that are directly bonded to the central atom/ion are named donor atoms and act as Lewis bases. In the particular case of the metal complexes, the central element is a metal. In the case of the inorganic compound Bortezomib the central atom is the semimetal element boron.

these newer proteasome inhibitors may also possess side-effect profiles that are distinct from that of bortezomib. Examples include proteasome inhibitors that are based on the epoxyketone moyety, such as carfilzomib. Carfilzomib (previously named PR-171) is a novel proteasome inhibitor that is structurally and mechanistically distinct from bortezomib [71]. In comparison to bortezomib, it is an irreversible inhibitor more potent and more selective towards the chymotrypsin-like activity, with less affinity for the trypsin and caspase-like proteases in the 26S proteasome [72,73].

Due to its irreversible interaction, the *ex novo* synthesis of a new proteasome complex is required to reverse the effects of carfilzomib.

Moreover, carfilzomib shows an improved safety profile, thanks to its little or no off-target activity [71]. Preliminary data, presented at the annual meeting of American Society of Hematology in 2008 from ongoing Phase II studies in patients with relapsed and refractory multiple myeloma, showed an encouraging therapeutic index [74].

Metal complexes to target the proteasome

The interest towards the use of metal-based agents as anti-tumor drugs started with the discovery of the antiproliferative effect of cisplatin and, since then, a number of **coordination complexes** have been prepared and studied. In this field, the discovery that some metal complexes are able to exert their cytotoxicity through a mechanism of action that does not involve DNA, constitutes an intriguing possibility to overcome the limiting side-effects and the significant issue of resistance that occurs with cisplatin. In particular, the lack of interaction with DNA can represent an important starting point for the development of anti-tumor drugs endowed with lower or absent genotoxicity. In this connection, the discovery that some metal-based complexes exert their antiproliferative action by affecting proteasomal activities, ascribes to this class of compounds a great potential for the future of cancer therapy [28,75]. These new agents include copper- (Cu), zinc- (Zn), gallium- (Ga) and gold (Au)-containing complexes whose chemical structures and effects on proteasome will be described below.

■ Copper & copper-based complexes

The discovery in 1980 that copper plays a role in angiogenesis stimulated a number of studies on the anti-tumor effects of this metal ion [76]. Angiogenesis is a process that leads to the formation of new blood vessels. In adults, angiogenesis constitutes both a tightly controlled physiological condition, associated with the maintenance of vascularity, female reproductive cycling and wound healing, and a crucial event in tumor growth and metastatic dissemination. VEGF is one of the key factors of angiogenesis because it triggers the network of signaling processes that promotes endothelian cell growth and migration [77,78]. Some studies indicate the ability of copper to stimulate proliferation and migration of human endothelial cells and, more interestingly, to induce VEGF mRNA transcription and protein expression [79–81]. Based on these results, efforts were dedicated to the investigation of the effect of copper chelators, such as disulfiram (DSF) and diethyldithiocarbamate (DDTC) on tumor cells **(Figure 6)**, both characterized by a dithiocarbamate moiety [82,83]. The results highlighted that DSF alone was completely ineffective, while the synthesized DSF–Cu complex was able to induce apoptosis in

human breast cancer cells. The investigation on the mechanism of action responsible for the cytotoxicity demonstrated its ability to inhibit proteasome activity. Taking into account that many types of cancer contain high levels of copper [84–87], it raised the hypothesis that DSF could react with endogenous copper to form a complex able to exert the above biological effects. It was demonstrated that in experimental conditions that mimic the *in vivo* situation (i.e., the presence of high concentrations of copper) DSF induced apoptotic cell death and proteasome inhibition. Moreover, DSF showed the ability to induce apoptosis and inhibition of proteasome activity in tumor tissues [82].

DDTC is one of the major metabolites of DSF and, similarly to the parent compound, is a strong Cu-**chelating agent**. This similarity in structure is accompanied by a similarity in behavior; the DDTC–Cu complex also induces apoptosis on both prostate and breast cancer cells, and potently inhibits proteasomal chymotrypsin-like activity [83].

Dithiocarbamates are a class of metal-chelating compounds, and pyrolidine dithiocarbamate (PyDT) in particular, after binding to copper, inhibits proteasomal chymotrypsin-like activity and cell proliferation, and induces apoptosis in human breast [84] and human prostate [85] cancer cells. A more recent study, where PyDT–Cu(II) and PyDt–Zn(II) complexes were synthesized and studied, highlighted some very interesting results and allowed a proposal for the mechanism of action of this copper-based complex **(Figure 7)** [86]. The $Cu(PyDT)_2$ showed weak inhibition when tested on purified 20S proteasome and at 50 µmol/l only 40% of the proteasome-chymotrypsin-like activity was inhibited. Nevertheless, when tested on intact human breast cancer MDA-MB-231 cells, it caused a significant antiproliferative effect and a strong inhibition of the proteasomal chymotrypsin-like activity with an increase of ubiquinated proteins and proteasome target proteins, IκB-α and p27. Moreover, treatment with $Cu(PyDT)_2$ induces some morphological changes typical of apoptosis, along with an increase in caspase-3/7 activity and the occurrence of the cleaved PARP fragment p85. The capacity of the complex to inhibit cell proliferation and proteasomal activity, and to induce apoptosis, was also demonstrated in other cell lines – two estrogen receptor α-positive breast cancer cell lines (MCF10dcis.com and MCF-7) and on the androgen receptor-independent human prostate cancer cells PC-3. In particular, a comparison between these effects with those obtained on purified proteasome indicates higher activity on intact cells. This result allowed the authors to propose the need for a metabolic activation inside the cell that could modify the chemical structure of the parent compound, thus changing its reactivity towards the target [86].

More recently, the study on the potential anticancer effect of copper complexes was extended by investigation on tridentate [NN′O]-containing ligands with copper [87]. The synthesized complexes are $[Cu(L^I)Cl]$, $[Cu(L^I)OAc]$ and $[Cu(HL^I)(L^I)]OAc$, where HL^I is the 2,4-diiodo-6-((pyridine-2-ylmethylamino)methyl)phenol **(Figure 8)**. The Cu(II) complexes showed a significant and similar ability to induce cell death in human leukemia Jurkat T cells, while neither the ligand alone nor the copper salt were able to induce any effect on cells. In contrast, the *in vitro* evaluation of proteasomal chymotrypsin-like activity showed an inhibitory effect, for both copper chloride salt and the 1:1 Cu chloride:ligand mixture. The ligand alone was unable to induce any inhibition. Interestingly, the assessment of chymotrypsin-like activity on two distinct intact prostate cells (Ca-2B and PC-3) evidenced that both ligand alone and copper salt failed to induce any effect on cells, while in the presence of the complexes, proteasomal inhibition and induction of apoptosis were observed. On the basis of these results, the authors concluded that the combination of the Cu(II) ion and the ligand is a necessary requirement to cross the cell membrane and to reach the proteasome by preventing undesired nonspecific interactions [87,88].

This hypothesis is in agreement with previous studies, which demonstrated that the copper ion inhibits proteasome [84,89]. In particular, the chymotrypsin-like activity of proteasome *in vitro* was assayed in the presence of a series of metal salts by using a Jurkat T cell extract. The metal

Key Term

Chelating agent: Ligands that may bind to the metal by two or more donor atoms (e.g., bidentate and tridentate coordination).

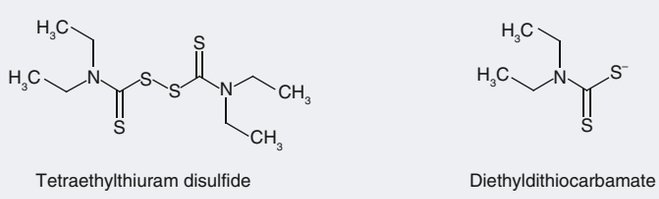


Figure 6. Copper-chelating ligands tetraethylthiuram disulfide (disulfiram, DSF) and diethyldithiocarbamate (DDTC).

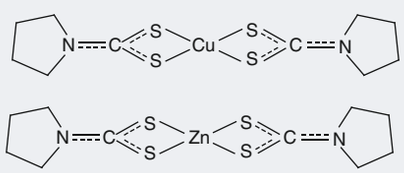


Figure 7. Synthetic compounds $[Cu(PyDT)_2]$ and $[Zn(PyDT)_2]$.

salts taken into consideration have the same common oxidation state or belong to the same period as copper. The results demonstrated that only copper salts were able to inhibit proteasome activity.

Moreover, this inhibition was time-dependent and irreversible (or tight binding). This latter conclusion was derived from the fact that ethylenediaminetetra-acetic acid was unable to reverse the inhibitory effect when proteasome underwent to a long exposure to copper salt [89]. A more recent study investigated on the possible role of the oxidation state of copper in exerting its inhibitory effect on proteasome [90]. For this purpose, since it is necessary to form complexes able to cross the cell membrane to test the effect of copper on intact cells, neocuproine (which is able to form complexes with both CuCl and $CuCl_2$) was used as the ligand. Both the complexes obtained inhibited proteasome activity and induced cell death on leukemia Jurkat cells and on solid tumor cell lines. Nevertheless, Cu(I) complex showed slight higher effects. Similar behavior was also observed when both complexes were incubated with purified 20S proteasome. In particular, the higher capacity of Cu(I) complex to induce ROS generation raised the possibility that ROS were involved in the inhibitory effect. However, the failure of ROS scavengers to remove the observed proteasomal-inhibitory activity, seems to exclude this possibility. Therefore, the proposed mechanism occurred due to direct binding of the Cu(I) compound to the enzymatic complex, likely to be the active site of the proteasome β5 subunit [90].

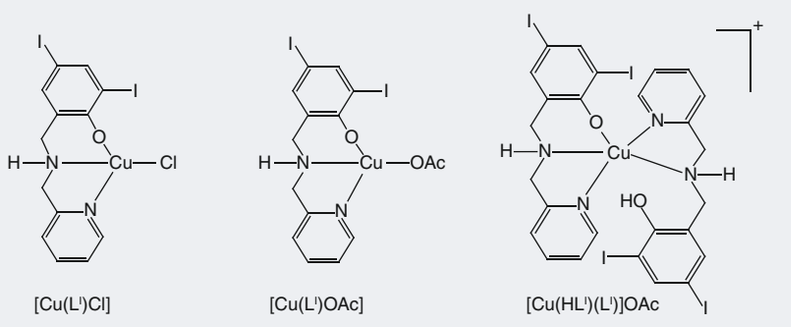


Figure 8. Synthetic compounds $[Cu(L^1)Cl]$, $[Cu(L^1)OAc]$ and $[Cu(HL^1)(L^1)]OAc$.

■ Gallium-based complexes

The use of gallium as an anticancer agent dates from the observation that gallium nitrate exerted anti-tumor effect in animal tumor models and in clinical trials against some types of cancer [91–94]. It has been proposed that the antiproliferative effect of gallium derives from its chemical and biochemical similarities to ferric ion. Indeed, electric charge, ion diameter and electronic configuration, such as the protein binding modes, are similar to that of Fe^{3+} [95,96]. Due to these similarities, gallium interferes with the transferrin-mediated cellular iron uptake and with the intracellular release of Fe from endosome to cellular compartments [97]. Moreover, it was demonstrated that gallium can bind to the ribonucleotide reductase, an enzyme involved in DNA synthesis of rapidly dividing cells. The binding of the ion to the ribonucleotide reductase destabilizes the enzyme and causes the inhibition of DNA synthesis and cellular proliferation [98]. More recently, with the aim of obtaining new potential metal-based anticancer agents, some gallium complexes characterized by two pyridine aminophenolate ligands containing asymmetric NNO′ donor groups were prepared and studied (compounds **1–5**, **Figure 9)** [99].

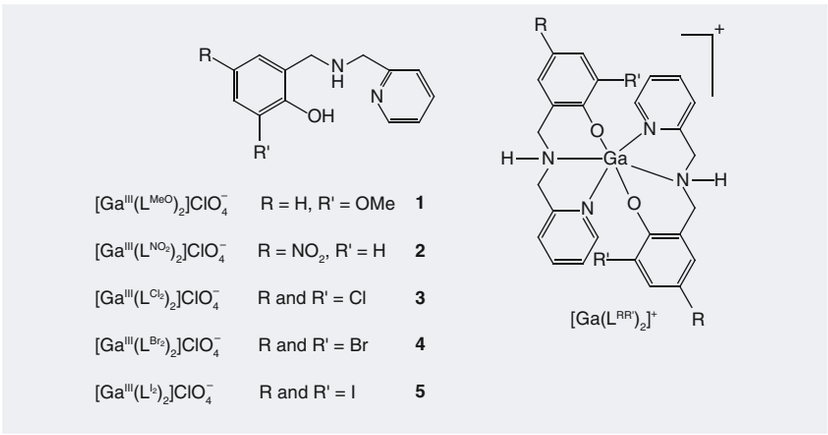
In vitro assays revealed significant antiproliferative activity on tumor cells and, interestingly, these Ga(III) complexes appeared relatively nontoxic on normal human fibroblasts [99]. A study focusing on the investigation of their molecular mechanism of action indicates that compounds containing halogen substituents **(3–5)** were able to induce apoptotic cell death and to inhibit the chymotrypsin-like activity of the proteasome. Indeed, the treatment of various prostate cancer cell lines with these gallium complexes caused the accumulation of ubiquinated proteins as well as the proteasome target protein p27 [100]. In particular, the iodo complex **5** showed the most pronounced effect and interestingly exerts an *in vivo* activity on PC-3 xenografted athymic nude mice by inducing a reduction of tumor growth up to 70% [88]. The presence of a strong electron-donating substituent was proposed as a crucial requirement for the anti-tumor capacity; nevertheless, the molecular mechanism

responsible for proteasome inhibition has not yet been clarified.

■ Zinc-based complexes

Zinc is the second most abundant transition metal ion in the human body and plays a crucial role in many biological functions. Indeed, zinc is essential for the catalytic activity of some proteins (metalloenzymes) and provides the framework for the zinc fingers that regulate gene expression. The concentration of the ion is tightly regulated in the organism and, interestingly, alterations in zinc homeostasis have been reported in some types of cancer [84–86]. Based on the results obtained with copper complexes on proteasome activity and taking into account the proximity of zinc and copper in the periodic table, pyrrolidine dithiocarbammato–Zn(II) (PyDt–Zn(II)) and tridentate [NN′O]-containing ligands with Zn(II) were prepared and studied **(FIGURES 7 & 10)** [86,101].

In particular, both $ZnCl_2$ and a PyDT mixture with Zn(II) showed the ability to inhibit the chymotrypsin-like activity of purified 20S proteasome. Interestingly, the inhibitory effect depended on the mixture ratio because it significantly decreases by increasing the amount of PyDt molecules with respect to a defined concentration of Zn(II) chloride. Furthermore, the PyDt–Zn mixture affects proteasome in intact breast cancer cells, causing an accumulation of ubiquinated proteins and of proteasomal target proteins. The treatment of human breast cancer cells MDA-MB-231 with PyDt–Zn mixture caused the occurrence of apoptotic morphological changes and the appearance of a single cleaved PARP fragment of 65 kDa (p65). This latter band, along with accumulation of p36/Bax and complete disappearance of p21/Bax indicated that the mixture induced apoptosis through a calpain-dependent mechanism. The calpain-mediated events are likely the most important apoptotic pathway induced by the PyDT–Zn mixture, because the lack of a p85 cleaved PARP fragment excluded the involvement of caspase-3/-7 activity. The subsequent synthesis of the $[Zn(PyDT)_2]$ complex **(FIGURE 7)**, confirmed the inhibition of the proteasomal chymotryspin-like activity and apoptosis in intact cells. These effects were not dependent on cell type because they were detected on three breast cancer cell lines (MDA-MB-231, MCF10DCIS.com and MCF-7) and on a human prostate cell line (PC-3) [86].



$[Ga^{III}(L^{MeO})_2]ClO_4^-$	R = H, R' = OMe	1
$[Ga^{III}(L^{NO_2})_2]ClO_4^-$	R = NO_2, R' = H	2
$[Ga^{III}(L^{Cl_2})_2]ClO_4^-$	R and R' = Cl	3
$[Ga^{III}(L^{Br_2})_2]ClO_4^-$	R and R' = Br	4
$[Ga^{III}(L^{I_2})_2]ClO_4^-$	R and R' = I	5

Figure 9. Synthetic gallium(III) complexes with the asymmetric tridentate [NN′O] ligands.

Inside the strategy of developing new metal complexes endowed with [NN′O] tridentate ligands as potential anti-tumor agents, the synthesis of $[Zn(L^I)_2]$ **(FIGURE 10)** was performed and the ability to affect proteasome activity was investigated [101]. The complex significantly inhibited the *in vitro* 26S proteasome activity performed by using an extract of C4–2B prostate cancer cells. A comparable effect was also obtained for $ZnCl_2$. The experiments performed in intact cancer cells showed dose-dependent inhibition of proteasomal activity for the complex, while none significant effect was detected for the salt. The same behavior was obtained also when apoptotic hallmarks were measured: the cleavage of PARP, along with shrinking and blebbing, occurred in the presence of $[Zn(L^I)_2]$, while $ZnCl_2$ showed no effect. These results pointed to the [NN′O] tridentate ligand as an essential carrier that allows crossing of the cell membrane.

Interestingly, the inhibition of proteasomal chymotrypsin-like activity occurred in many

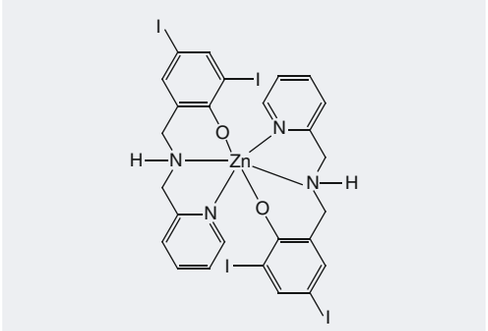


Figure 10. Synthetic Zn(II) complex $[Zn(L^{I,I})_2]$.

types of cancer cells, while normal immortalized cells appeared practically unaffected by the treatment with the complex [101].

■ Au(I,III) compounds

Although cisplatin is the most successful of metals in anti-tumor therapy, many other metal complexes have been synthesized and studied as potential anticancer agents. One such example, gold complexes, constitute an emerging class of metal-based agents endowed with interesting anticancer properties. Owing to their anti-inflammatory and immunosuppressive properties, several Au(I) compounds, such as auranofin, have been employed in the treatment of rheumatoid arthritis and investigated as anti-tumor agents [102]. Auranofin, a Au(I)–thioglucose derivative, and a number of its analogues were evaluated for their *in vitro* cytotoxic activities – they were shown to be active against a panel of tumor cells, but not against solid tumors [103].

Following these encouraging results, a series of Au(I) compounds were synthesized [104] and some of them, such as diAu(I)–phosphino complexes, exhibited improved anti-tumor effectiveness by affecting mitochondrial activity [105,106].

Moreover, over the past few years, a range of Au(III) compounds, which display significant anti-tumor properties, have been synthesized. Overall, their mechanisms of action differ from those of the Pt(II) anti-tumor drugs, having some leading cellular targets other than DNA. In fact, Au(III) compounds have been shown to have many targets in cells that primarily include selenoproteins [107]. Moreover, some interesting Au(III) porphyrin compounds, described by Che and co-workers, have been shown to induce apoptosis through both caspase-dependent and caspase-independent mitochondrial pathways [108,109].

Interestingly, mitochondrial thioredoxin reductase seems the major target for some of these Au(III) compounds, and several of them have been shown to be potent inhibitors of mitochondrial functions [110,111]. Indeed, a variety of Au(III) compounds, including Au(III)–dithiocarbamates, affect either the mitochondrial or cytosolic thioredoxin systems, and thereby cause apoptosis [111,112]. In particular, Au(III)–dithiocarbamates have been demonstrated to affect the thioredoxin system *in vitro*, but to cause a concomitant strong inhibition of proteasome [113]. In particular, Au(III) dithiocarbamates, shown in **Figure 11**, strongly inhibit proteasome activity both *in vitro* and *in vivo* on xenografts and appear very promising agents, thanks to their interesting antineoplastic activity and low toxicity [114,115].

Remarkably, most of them, in particular Au(III) derivatives of *N*,*N*-dimethyldithiocarbamate and ethylsarcosinedithiocarbamate, ($Au[DMDT]Cl_2$), ($Au[DMDT]Br_2$), ($Au[ESDT]Cl_2$) and ($Au[ESDT]Br_2$), were shown to be from one- to four-fold more cytotoxic than cisplatin and to overcome to a large extent both intrinsic and acquired resistance to cisplatin [116]. More recently, a group of 13 Au(III) complexes, including AUL12 **(Figure 11)**, has been tested according to a specific comparative *in vitro* strategy for the development of new anticancer agents [202]. AUL12 showed very good activity with IC_{50} values in the micromolar range on a panel of 36 human tumor cell lines. Interestingly, from a comparative analysis of 110 reference substances with known modes of action, AUL12 turned out to be the only Au(III) complex covered by this screening study whose mechanism of action did not resemble the mechanism of any of the reference compounds, thus supporting the rationale of this peculiar anticancer drug-design strategy [117]. In fact, the studies on these complexes started from structural and electron considerations. Indeed, Au(III) is isoelectronic with Pt(II), and tetracoordinate–Au(III) complexes are characterized by the same square planar geometries as cisplatin. Interestingly, the complexes showed a very promising biological pattern due to the higher antiproliferative activity with respect to cisplatin, along with increased bioavailability and decreased side effects [115,118,119]. The interest toward these new very active complexes was increased by the demonstration that the Au(III) complexes showed the ability to overcome both intrinsic and acquired resistance to cisplatin. This discovery suggested the involvement of a molecular target different from DNA and consequently prompted further in-depth investigations on the molecular mechanism responsible for their cytotoxicity. The studies devoted to this purpose highlighted for some Au(III) complexes a good ability to interact with calf thymus DNA, nucleosides and their bases [118], but for other complexes, a clear affinity toward S-donor ligands such as glutathione and cysteine, and bovin serum albumin [120].

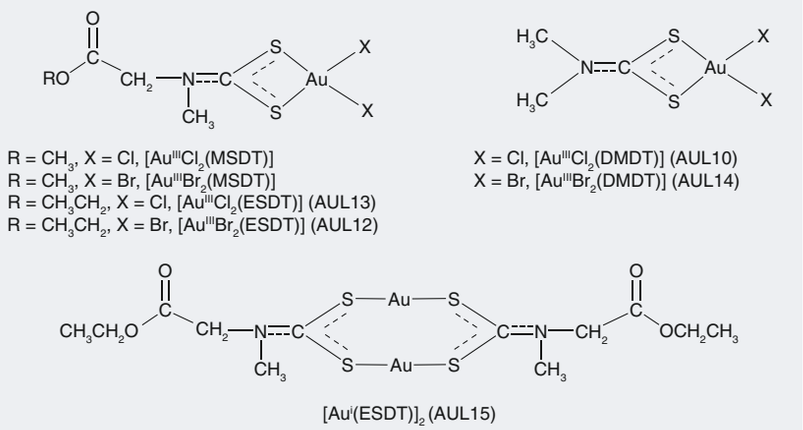


Figure 11. Selected Au(I,III)-dithiocarbamato derivatives.
ESDT: Ethylsarcosinedithiocarbamate ($CH_3CH_2O(O)CCH_2N(CH_3)CSS^-$); DMDT: N,N dimethyldithiocarbamate ($(CH_3)_2NCSS^-$); MSDT: Methylsarcosinedithiocarbamate ($CH_3O(O)CCH_2N(CH_3)CSS^-$); X = Cl, Br.

Overall, these results suggested that protein residues could be the target for these complexes, stimulating a series of studies that culminated more recently in the demonstration that Au(III)–dithiocarbamato compounds inhibit the three peptidase activities (chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing-like) of the 20 proteasomes, especially the chymotrypsin-like [114,121]. It has been reported that the inhibition of this latter proteasomal activity is associated with growth arrest and/or apoptosis induction in cancer cells and, in fact, these compounds showed the ability to induce apoptosis on highly metastatic and invasive estrogen receptor α-negative MDA-MB-231 breast cancer cells and inhibition of tumor growth *in vivo* [113]. Investigations on the molecular event(s) responsible for the proteasome inhibition led the proposal of two possible mechanisms: a direct binding on proteasome and/or the involvement of ROS production [112]. The reversal of AUL12 proteasomal inhibition by the S-donor ligands 1,4-dithio-DL-threitol and *N*-acetyl-L-cysteine (NAC), but not by the antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), suggested the possibility that the sulfhydryl groups on both 1,4-dithio-DL-threitol and NAC promote a redox reaction with the Au(III) complex, thus preventing its direct binding to the proteasome and consequently its inhibitory activity [122]. In fact, the treatment with the hydrophilic form of vitamin E (Trolox) that interacts preferentially with free radicals, such as the peroxyl radicals, was proved not be able to prevent cell death and PARP cleavage [112]. It has been previously reported that some Au(III) complexes are able to bind *S*-donor ligands, such as glutathione and cysteine, cleaving their disulfide bonds [123]. Moreover, an alteration in glutathione levels in cells can also induce an accumulation of ROS, which, in turn, can oxidize and inactivate the proteasome. The demonstration that free radical generating systems provoke oxidation on proteasome and the inactivation of its functionality supports this proposal [124].

Interestingly, gold compounds whose anticancer activity has been already reported in the literature have often shown promising *in vitro* cytotoxicity that, unfortunately, was not confirmed by *in vivo* studies [125]. On the contrary, the *in vivo* anti-tumor activity of Au(III)–dithiocarbamato derivatives is fully consistent with *in vitro* results. For example, it was found that the tested Au(III) dithiocarbamates inhibited the proteasomal chymotrypsin-like activity in MDA-MB-231 whole cell extract in a concentration-dependent manner. Proteasome inhibition by AUL14 was confirmed by decreased proteasomal activity and increased levels of ubiquitinated proteins and the proteasome target protein p27. Most importantly, inhibition of the proteasome activity and accumulation of p27 were also found *in vivo* in MDA-MB-231 xenografts treated with these gold compounds [113].

Analogously, administration of AUL10 (1 mg kg^{-1} every other day) caused an overall 85% reduction of PC-3 prostate tumor xenografts in nude mice after a 19-day treatment (compared with control untreated mice). Notably, in both the treatments, chemotherapy was well-tolerated by treated mice that suffered from minimal systemic toxicity, and histological investigations showed no detectable damage to the animals' main organs [115,119].

All together, these findings clearly indicated that Au–dithiocarbamates can directly target the tumor proteasome *in vivo*. Induction of apoptosis *in vitro* and *in vivo* has been shown by multiple assays that measure characteristic cellular and biochemical hallmarkers (apoptotic morphological changes, PARP cleavage, terminal dUTP nick end labeling, and hematoxylin and eosin staining assays). The data strongly suggested that the proteasome is the primary target for Au(III)–dithiocarbamates and that inhibition of the proteasomal activity is associated with apoptosis in cancer cells [113].

■ Mechanism of action: preliminary studies

The studies on the proteasome inhibition presented by Au(III)–dithiocarbamato anticancer agents confirmed that their antiproteasome activity is associated with apoptosis induction *in vitro* and *in vivo*. Moreover, they exhibit potent antiproliferative activity against different cancer cell lines and anti-tumor activity in nude mice bearing breast and prostate cancer xenografts [119,121]. Recently, an attempt to gain further insights into the biological activity of Au–dithiocarbamato complexes, in which the metal centers can be in different oxidation states, was reported. In particular, in order to clarify the importance of the oxidation state of the gold center and to draft a possible mechanism of action, two Au(III)– and Au(I)–dithiocarbamato derivatives (AUL12 and AUL15) **(Figure 11)**, whose anticancer activity was previously reported, were comparatively investigated [116,118].

The growth inhibitory effect of both gold compounds toward the highly metastatic breast cancer MDA-MB-231 cell line was tested. Even if both compounds inhibited cell proliferation in a dose-dependent manner, Au(I) derivative (AUL15) was less potent than AUL12 with a calculated IC_{50} values of 13.5 µmol/l against 4.5 µmol/l calculated for AUL12 [126]. Contemporaneously to cell death induction, the proteasomal inhibition was measured, demonstrating that both complexes inhibited the proteasomal chymotrypsin-like activity, but at drastically different levels (IC_{50} of 17.7 and 1.13 µmol/l, for AUL15 and AUL12, respectively). Additionally, the treatment for 4 h with Au(I) compound, produced a cell detachment linked with both apoptotic and non-apoptotic death and calpain-mediated PARP cleavage into fragment p65, but in the absence of terminal dUTP nick end labeling-positive cells. This is indicative of a DNA-damage-independent necrosis cell death. However, at 24 h, both apoptosis and necrosis occurred. Therefore, both DNA-damage-independent cell death and apoptosis could be induced by these gold compounds. Moreover, Au(III) had a greater effect, while AUL15 also at higher concentrations induced mainly non-apoptotic cell death. Taken together, the above data suggested that the Au–dithiocarbamato complexes induced various types of cell death, depending on the status of gold compound, concentration and treatment time. Therefore, the possibility that Au(III)–dithiocarbamato complexes are able to trigger cell death, to modify some mitochondrial functions, to increase the levels of phosphorylated ERK1/2 and to induce ROS generation, which might oxidize and inactivate the proteasome, was proposed [113]. On the other hand, the possibility that some proteasome inhibitors could mediate cell death throughout an increase in oxidative stress was already reported by Perez-Galan *et al.* [38]. Moreover, the oxidative modification and inactivation of the proteasome upon exposure to free radicals is well known [124]. The reported data showed that Au(I)– and Au(III)–dithiocarbamato derivatives can inhibit the proteasomal chymotrypsin-like activity and induce cell death, which can be completely reversed by the addition of the reducing agent NAC [126]. To investigate whether ROS were responsible for the observed cell death, cells were treated in the presence of either NAC or the hydrophilic form of vitamin E, Trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Both are good ROS scavengers. NAC is a reducing thiol that helps maintain the cellular thiol redox balance, whereas Trolox interacts preferentially with free radicals, such as the peroxyl radicals. However, co-treatment of cells with NAC or Trolox and Au(III) dithiocarbamato complexes established that while NAC treatment was able to prevent cell death and PARP cleavage, Trolox was not. Indeed, the cells exhibited comparable mortality and PARP cleavage, regardless of Trolox presence [112]. These results rationalize the observations obtained with some Au(III)–methylsarcosinedithiocarbamato complexes, indicating that they both upregulate Bax and downregulate Bcl2 [127], also exerting an inhibitory action on the proteasome system [113]. Hence, proteasome inhibition can also explain the observed long-lasting persistence of phosphorylated ERK1/2 [112].

Moreover, the treatment with Au(I) complex AUL15, at 20 and 30 µmol/l concentrations, was unable to produce reasonably detectable levels of ROS. However, treatment with AUL12 at both concentrations resulted in the enhancement fluorescence, indicative of the production of significant levels of ROS **(Figure 12)**. What is more, the treatment of cells with the Au(I) analogue failed to induce any significant levels of ROS with or without co-treatment with NAC, thus confirming that the Au(I) derivative is redox-inactive. It is remarkable to point out that treatment with Au(I) at 30 µM for 4 h induced approximately 72% of cell death through a non-apoptotic-related pathway, producing very low levels of ROS [126].

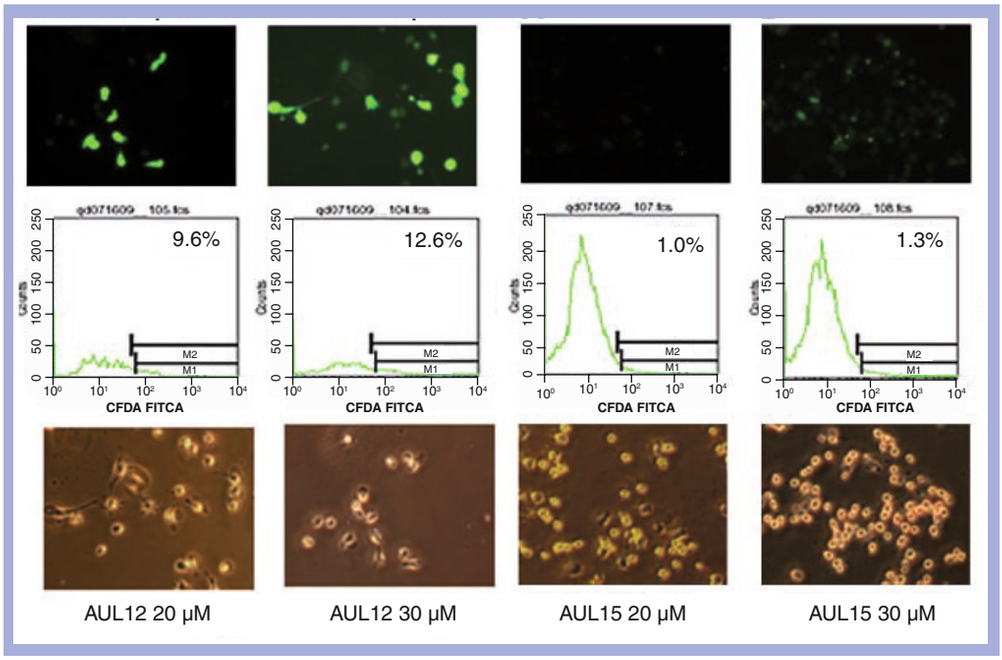


Figure 12. AUL12 (Au[III]) but not AUL15 (Au[I]) induces ROS production in breast cancer cells. ROS formation in MDA-MB-231 cells was determined using the oxidation-sensitive probe (carboxy-H_2DCFDA). Cells were treated with AUL12 or AUL15 at the indicated concentrations for 3.5 h. ROS generation was examined using a Zeiss confocal laser microscope (first rows) and FACS analysis (second rows). The morphological changes of the same cells were also shown (third rows) by phase-contrast imaging.
ROS: Reactive oxygen species.
Modified with permission from [126].

Even if these results could suggest that induction of oxidative stress by Au(III) but not Au(I) is at least partially responsible for its biological activity, which then can be effectively inhibited through the addition of a reducing agent, there are other elements that have to be well thought out.

Since AUL12 and AUL15 possess the same ESDT ligand, this finding suggests that the observed effects could be mediated by the different oxidation states of the gold centers. However, the two gold derivatives show different coordination numbers. In fact, AUL12 is tetracoordinate in a square-planar geometry, and was demonstrated to undergo hydrolysis, thus forming the diaquo complex. This could be the actual Au(III) active species that undergoes a subsequent reduction process within 24 h, leading to the corresponding dinuclear Au(I) analogue, AUL15 [118]. On the contrary, AUL15 is a dinuclear Au(I) complex with a linear structure around the metal center. These differences in coordination geometry, together with the different oxidation state, can affect the stability and the consequent behavior of the two gold species towards the biomolecules.

Moreover, it is well known that sulfur-containing biomolecules, such as cysteine, methionine, glutathione, metallothionein and albumin, play a significant role in metal-based anticancer chemotherapy because of their high affinity to soft metal ions. Sulfur could be involved in the entire metabolic process of metal-derivative drugs, including reactions prior to cellular uptake and deactivation prior to biological target (e.g., proteasome and DNA) binding. Although the role of sulfur compounds is still controversial, the interaction between sulfur-containing antioxidant NAC and the Au(I,III)–dithiocarbamato complexes could inactivate the antiproliferative agents.

Overall, the hypothesis that at least two or three mechanisms could be responsible for the biological activity of these gold compounds, including ROS production, direct metal binding to the proteasome and/or redox processes involving the Au(III) center and some sulfur-containing biomolecules (such as glutathione), have to be considered. For example, it is well established that glutathione is the main antioxidant system in the cell, and

its depletion might facilitate accumulation of ROS in cells treated with anticancer drugs. This accumulation in ROS in turn increases the drug lethality [128]. Nevertheless, Au(III)–dithiocarbamato compounds maintain their antiproliferative activity in the presence of antioxidant agents that do not contain sulfur donors such as Trolox, while the presence of NAC inactivates the drug.

On the other hand, very recent *in vivo* experiments have undoubtedly demonstrated the great anticancer activity of AUL12 and AUL10, accompanied by irrelevant toxic side effects. Subsequent histopathological investigations have confirmed the favorable toxicity profile of the tested Au(III) complexes. In fact, no significant histologically detectable toxicity involving treated animals' heart, liver, spleen, kidney, testicles, pancreas, lung and brain was observed, and SEM evaluation of all examined tissues were considered compatible with normal conditions, with no accumulation of the metal in any of the organs taken into account. These results are in full agreement with the fact that gold seems to be rapidly (i.e., within 48 h) cleared from the body, the large majority being excreted through the feces (>89%) and only approximately 10% via the urinary system [115,119]. This result is extremely positive when compared with the data reported in literature showing the renal toxicity induced by gold derivatives. For instance, it is well known that the anti-arthritic drugs, auranofin and myochrysine, induce proteinuria and kidney disfunctions, and gold nanoparticles are able to penetrate renal cells causing nephrotoxicity [129,130].

The possibilities that Au(III)–dithiocarbamates could directly oxidize and inactivate the proteasome or inhibit the proteasome by a secondary redox effect or even selectively modify surface protein residues, have to be kept in mind for further studies meant to clarify their biological activity. Since up-to-date, pharmacologically employed, platinum-containing compounds are strongly associated with nonspecific toxicity, the alternative of metal complexes (especially gold derivatives), acting as proteasome inhibitors, seems to be a promising approach in cancer therapy.

Conclusion

The proteasome is an intracellular enzyme complex that degrades many cell proteins, including regulatory ones. This process controls protein levels within the cell, thus allowing to maintain cellular homeostasis.

Moreover, the proteasome contributes to the pathological state of several human diseases, in which some regulatory proteins are either stabilized, due to decreased degradation, or lost, due to accelerated degradation. In particular, aberrant proteasome-dependent proteolysis can be associated with the pathophysiology of malignancies, as the pro-apoptotic proteins are ubiquinated and removed by proteasome. As a consequence, tumor cells unhook from metabolic control and escape from apoptosis, allowing the tumor growth.

Bortezomib is the first and, until now, the only proteasome inhibitor approved by the FDA. This drug inhibits the enzyme complex in a reversible manner and has demonstrated clinical efficacy in the treatment of multiple myeloma and mantle cell lymphoma. Nevertheless, despite its undoubted effectiveness, some patients do not respond to bortezomib when it is used as a single agent, and the majority of patients that do respond, ultimately relapse.

In these latter years, in an attempt to overcome these drawbacks, many efforts have focused on the development of new proteasome inhibitors that act through mechanisms that are distinct from that of Bortezomib. A noteworthy example is constituted by carfilzomib, an irreversible proteasome inhibitor, that appears to have clinical efficacy in some patients who have relapsed after Bortezomib treatment.

More recently, the discovery that some metal-based compounds inhibit the proteasoma activity increases the interest in this class of agents; as such, it is an emerging model for the development of new proteasome-inhibitor drugs.

These compounds include, Cu-, Ga-, Zn- and Au-containing complexes.

Since higher concentrations of copper is a common feature of many human tumors, targeting tumor cells with Cu-chelating agents appeared as an interesting anticancer approach. Interestingly, the investigation on their mechanism of action led to the discover that the antiproliferative effect of these agents was related to the inhibition of proteasome activity. Interest in this field led to an innovative class of proteasome inhibitors, constitutes Ga(III), Zn(II) and Cu(II) combined with asymmetric tridentate ligands. In particular, Ga(III) with ligands inspired to some redox-active enzymes, showed 70% tumor growth reduction in prostate PC-3 xenografts, which is related to the inhibition of chymotrypsin-like activity of proteasome. Similarly, interesting results were

also obtained with Zn(II) complexes, able to inhibit proteasomal activity in many types of cancer cells, without significantly affecting normal immortalized cells.

Finally, some Au(III)–dithiocarbamato complexes demonstrated a notable anticancer activity accompanied by irrelevant toxic side effects. Indeed, a model gold compound caused an overall 85% reduction of PC-3 prostate tumor xenografts in nude mice after a 19-day treatment. Interestingly, both *in vitro* and *in vivo* experiments have demonstrated that a principal target of these complexes is the proteasome. However, it is currently not possible to state whether they directly oxidize and inactivate the proteasome or inhibit it by a secondary redox effect, or even modify selective surface protein residues. Taking into consideration that gold compounds also have mitochondria as their target, it is possible that they activate different cellular apoptotic signaling pathways through a pleiotropic mechanism of action.

Overall, the above results strongly indicate that targeting the ubiquitin–proteasome pathway with metal-based compounds is an emerging concept in the development of new anticancer therapeutics.

Future perspective

Nowadays, the field of medicinal inorganic chemistry is receiving considerable attention in the design of anticancer agents. Metal-, and in general, inorganic element-containing compounds, offer many advantages over conventional organic compounds in the development of new therapeutic agents. These advantages are due to their ability to coordinate ligands, thus allowing functionalization of groups that can be customized to target specific biomolecules. Metal-based complexes offer a wide spectrum of coordination numbers and geometries, as well as kinetic properties, which cannot be realized with conventional carbon-based compounds. The oxidation state of a metal and its soft/acid properties are also an important tool in the design of coordination compounds, considering the involvement in the biological redox chemistry. Targeting the ubiquitin–proteasome pathway with metal-based compounds is an emerging concept in the development of new therapeutics.

The positive trend in antiproteasome drug discovery should be continued with in-depth studies to gain further insight into the knowledge of the actual mechanism exerted by inorganic derivatives, taking into consideration that they frequently influence other different cellular targets.

In vivo experiments have already been planned to take place over the next few years, assessing both the antineoplastic activity and toxicity of these innovative classes of antiproteasome compounds.

Acknowledgements

The authors gratefully acknowledge L Calore for technical assistance, and colleagues of Centro Interdipartimentale di Ricerca 'Ruolo degli Ioni Metallici nelle Scienze Biomediche' and of Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici for stimulating discussions.

Executive summary

Background

- The proteasome is found in eukaryotes as well as in prokaryotes and is involved in a variety of essential biological processes.
- The ubiquitin–proteasome system degrades short-lived proteins that are involved in critical steps in both cell proliferation and cell death.
- Inhibition of the components of the ubiquitin–proteasome system is an outstanding pharmacological target.

Bortezomib

- Also known as Velcade® is the first and the only proteasome inhibitor approved by the US FDA for the treatment of multiple myeloma and mantle cell lymphoma.

Mechanism of action

- Bortezomib anti-tumor activity occurs by a regioselective and reversible proteasome inhibition.
- Bortezomib mainly targets the proteasomal β5 active site.

Clinical evidence

- The clinical response to bortezomib in solid tumors is very low. Moreover, some patients develop resistance over time.
- Peripheral neuropathy is a common dose-limiting toxicity.

Metal complexes to target proteasome

- A novel approach to target the proteasome: intriguing metal complexes containing Cu(I)/Cu(II), Ga(III), Zn(II) and Au(I,III).
- Metals possess peculiar properties that are absent in conventional organic drugs.
- Having multiple targets, their mechanism of action could be pleiotropic.

Financial & competing interests disclosure

This work was supported by Fondazione ABO (www.fondazioneabo.org). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

1 Groll M, Bochtler M, Brandstetter H, Clausen T, Huber R. Molecular machines for protein degradation. *Chem. Bio. Chem.* 6, 222–256 (2005).

▪ **Overall description of 26S proteasome shape and activity.**

2 Jung T, Catalgol B, Grune T. The proteasomal system. *Mol. Aspects Med.* 30, 191–296 (2009).

▪▪ **Comprehensive overview of the protein degradation performed by the enzymatic mechanism of the ubiquitin–proteasome pathway.**

3 Nalepa G, Rolfe M, Harper JW. Drug discovery in the ubiquitin–proteasome system. *Nat. Rev. Drug Discov.* 5, 596–613 (2006).

4 Etlinger JD, Auberg AL. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl Acad. Sci. USA* 74, 54–58 (1977).

5 Blagosklonny MV, Wu GS, Omura S, el-Deiry WS. Proteasome-dependent regulation of p21WAF1/CIP1 expression. *Biochem. Biophys. Res. Commun.* 227, 564–569 (1996).

6 Pagano M, Tam SW, Theodoras AM *et al.* Role of the ubiquitin–proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269, 682–685 (1995).

7 Tsvetkov P, Reuven N, Shaul Y. Ubiquitin-independent p53 proteasomal degradation. *Cell Death Differ.* 17, 103–108 (2010).

8 Chang YC, Lee YS, Tejima T *et al.* mdm2 and bax, downstream mediators of the p53 response, are degraded by the ubiquitin–proteasome pathway. *Cell Growth Differ.* 9, 79–84 (1998).

9 Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* 443, 780–786 (2006).

10 Glickman MH, Ciechanover A. The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* 82, 373–428 (2002).

11 Borissenko L, Groll M. 20S proteasome and its inhibitors: crystallographic knowledge for drug development. *Chem. Rev.* 107, 687–717 (2007).

12 Tsukamoto S, Yokosawa H. Inhibition of the ubiquitin–proteasome system by natural products for cancer therapy. *Planta Med.* 76, 1064–1107 (2010).

▪▪ **Comprehensive overview of small molecules targeting the ubiquitin–proteasome system.**

13 Hegerl R, Pfeifer G, Puhler G, Dahlmann B, Baumeister W. The 3-dimensional structure of proteasomes from *Thermoplasma acidophilum* as determined by electron-microscopy using random conical tilting. *FEBS Lett.* 283, 117–121 (1991).

14 Groll M, Huberb R, Moroder L. The persisting challenge of selective and specific proteasome inhibition. *J. Pept. Sci.* 15, 58–66 (2009).

15 Lowe J, Stock D, Jap R, Zwickl P, Baumeister W, Huber R. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* 268, 533–539 (1995).

16 Groll M, Ditzel L, Lowe J *et al.* Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386, 463–471 (1997).

17 Dou QP, Li B. Proteasome inhibitors as potential novel anticancer agents. *Drug Resist. Updat.* 2, 215–223 (1999).

18 Auberg AL. Functions of the proteasome: the lysis at the end of the tunnel. *Science* 268, 522–523 (1995).

19 Chen D, Dou QP. The ubiquitin–proteasome system as a prospective molecular target for cancer treatment and prevention. *Curr. Prot. Pept. Sci.* 11, 459–470 (2010).

20 Orlowski RZ, Dees EC. The role of the ubiquitination proteasome pathway in breast cancer: applying drugs that affect the ubiquitin–proteasome pathway to the therapy of breast cancer. *Breast Cancer Res.* 5, 1–7 (2002).

21 Ciechanover A, Schwartz AL. The ubiquitin proteasome pathway: the complexity and myriad functions of proteins death. *Proc. Natl Acad. Sci. USA* 95, 2727–2730 (1998).

22 Herrmann J, Ciechanover A, Lerman LO, Lerman A. The ubiquitin–proteasome system in cardiovascular diseases-a hypothesis extended. *Cardiovasc. Res.* 61, 11–21 (2004).

23 Petroski MD. The ubiquitin system, disease, and drug discovery. *BMC Biochem.* 9, S7 (2008).

24 Lehman NL. The ubiquitin proteasome system in neuropathology. *Acta Neuropathol.* 118, 329–347 (2009).

25 Ciechanover A. The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J.* 17, 7151–7160 (1998).

26 Hoeller D, Dikic I. Targeting the ubiquitin system in cancer therapy. *Nature* 458, 438–444 (2009).

27 Xie Y. Feedback regulation of proteasome gene expression and its implications in cancer therapy. *Cancer Metastasis Rev.* 29, 687–693 (2010).

▪▪ **Valuable paper on cellular processes and human diseases affected by proteasome.**

28 Chen D, Milacic V, Frezza M, Dou QP. Metal complexes, their cellular targets and potential for cancer therapy. *Curr. Pharm. Des.* 15, 777–791 (2009).

29 Bazzaro M, Lee MK, Zoso A *et al.* Ubiquitin–proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. *Cancer Res.* 66, 3754–3763 (2006).

30 Hideshima T, Richardson P, Chauhan D *et al.* The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res.* 61, 3071–3076 (2001).

31 Cvek B, Dvorak Z. Targeting of nuclear factor-κB and proteasome by dithiocarbamate complexes with metals. *Curr. Pharm. Des.* 13, 3155–3167 (2007).

32 Bedford L, Lowe J, Dick LR, Mayer JR, Brownell JE. Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets. *Nat. Rev. Drug Discov.* 10, 29–46 (2011).

33 Ruschak AM, Slassi M, Kay LE, Schimmer AD. Novel proteasome inhibitors to overcome bortezomib resistance *J. Natl Cancer Inst.* 103, 1007–1017 (2011).

34 Richardson PG, Barlogie B, Berenson J *et al.* A Phase 2 study of bortezomib in relapsed, refractory myeloma. *N. Engl. J. Med.* 348, 2609–2617 (2003).

35 Fisher RI, Bernstein SH, Kahl BS *et al.* Multicenter Phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. *J. Clin. Oncol.* 24, 4867–4874 (2006).

▪▪ **Description of bortezomib and its activity in clinical trials.**

36 Chari A, Mazumder A, Jagannath S. Proteasome inhibition and its therapeutic potential in multiple myeloma. *Biol. Targets Ther.* 4, 273–287 (2010).

37 Bross PF, Kane R, Farrell AT *et al.* Approval summary for bortezomib for injection in the treatment of multiple myeloma. *Clin. Cancer Res.* 10, 3954–3964 (2004).

38 Pérez-Galán P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood* 107, 257–264 (2006).

39 Adams J, Palombella VJ, Sausville EA *et al.* Proteasome inhibitors: a novel class of potent and effective anti-tumor agents. *Cancer Res.* 59, 2615–2622 (1999)

40 Groll M, Berkers CR, Ploegh HL, Ovaa H. Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. *Structure* 14, 451–456 (2006).

▪▪ **Detailed description of the mechanism of action of bortezomib.**

41 Jackson G, Einsele H, Moreau P, San Miguel J. Bortezomib, a novel proteasome inhibitor, in the treatment of hematologic malignancies. *Cancer Treat. Rev.* 31, 591–602 (2005).

42 Richardson PG, Sonneveld P, Schuster MW *et al.* Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N. Engl. J. Med.* 352, 2487–2498 (2005).

43 San Miguel JF, Schlag R, Khuageva NK *et al.* Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. *N. Engl. J. Med.* 359, 906–917 (2008).

44 Richardson PG, Weller E, Lonial S *et al.* Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. *Blood* 116, 679–686 (2010).

45 Orlowski RZ, Stinchcombe TE, Mitchell BS *et al.* Phase 1 trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies *J. Clin. Oncol.* 20, 4420–4427 (2002).

46 Papandreou CN, Daliani DD, Nix D *et al.* Phase 1 trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer. *J. Clin. Oncol.* 22, 2108–2121 (2004).

47 Huber R, Groll M. Inhibitors of the eukaryotic 20S proteasome core particle: a structural approach. *Biochim. Biophys. Acta* 1695, 33–44 (2004).

48 Groll M, Heinemeyer W, Jäger S *et al.* The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study. *Proc. Natl Acad. Sci. USA* 96, 10976–10983 (1999).

49 Pettersen EF, Goddard TD, Huang CC *et al.* UCSF Chimera – a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612 (2004).

50 An B, Aufarb RH, Siman R, Dou QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. *Cell Death Differ.* 5, 1062–1075 (1998).

51 Walker B, Lynas JF. Strategies for the inhibition of serine proteases. *Cell Mol. Life Sci.* 58, 596–624 (2001).

52 London RE, Gabel SA. Development and evaluation of a boronate inhibitor of g-glutamyl transpeptidase. *Arch. Biochem. Biophys.* 385, 250–258 (2001).

53 Transue TR, Krahn JM, Gabel SA, DeRose EF, London RE. X-ray and NMR characterization of covalent complexes of trypsin, borate, and alcohols. *Biochemistry* 43, 2829–2839 (2004).

54 Mulligan G, Mitsiades C, Bryant B *et al.* Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. *Blood* 109, 3177–3188 (2007).

55 Qin JZ, Ziffra J, Stennett L *et al.* Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res.* 65, 6282–6293 (2005).

56 Li B, Dou QP. Bax degradation by the ubiquitin/proteasome dependent pathway: involvement in tumor survival and progression. *Proc. Natl Acad. Sci. USA* 97, 3850–3855 (2000).

57 Sánchez-Serrano I. Success in translational research: lessons from the development of bortezomib. *Nat. Rev. Drug Discov.* 5, 107–114 (2006).

58 Iijima M, Momose I, Ikeda D. TP-110, a new proteasome inhibitor, down-regulates IAPs in human multiple myeloma cells. *Anticancer Res.* 29, 977–986 (2009).

59 Podar K, Gouill SL, Zhang J *et al.* A pivotal role for Mcl-1 in Bortezomib-induced apoptosis. *Oncogene* 27, 721–731 (2008).

60 Gomez-Bougie P, Wuillème-Toumi S, Ménoret E *et al.* Noxa up-regulation and mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma, *Cancer Res.* 67, 5418–5424 (2007).

61 Fuchs D, Berges C, Opelz G, Daniel V, Naujokat C. Increased expression and altered subunit composition of proteasomes induced by continuous proteasome inhibition establish apoptosis resistance and hyperproliferation of Burkitt lymphoma cells. *J. Cell Biochem.* 103, 270–283 (2008).

62 Ruckrich T, Kraus M, Gogel J *et al.* Characterization of the ubiquitin–proteasome system in bortezomib-adapted cells. *Leukemia* 23, 1098–1105 (2009).

63 Cortes J, Thomas D, Koller C *et al.* Phase 1 study of bortezomib in refractory or relapsed acute leukemias. *Clin. Cancer Res.* 10, 3371–3376 (2004).

64 Dispenzieri A, Jacobus S, Vesole DH, Callandar N, Fonseca R, Greipp PR. Primary therapy with single agent bortezomib as induction, maintenance and re-induction in patients with high-risk myeloma: results of the ECOG E2A02 trial. *Leukemia* 24, 1406–1411 (2010).

65 Lu S, Chen Z, Yang J *et al.* Overexpression of the PSMB5 gene contributes to bortezomib resistance in T-lymphoblastic lymphoma/leukemia cells derived from Jurkat line. *Exp. Hematol.* 36, 1278–1284 (2008).

66 Oerlemans R, Franke NE, Assaraf YG *et al.* Molecular basis of bortezomib resistance: Proteasome subunit b5 (*PSMB5*) gene mutation and overexpression of PSMB5 protein. *Blood* 112, 2489–2499 (2008).

67 Lu S, Yang J, Song X *et al.* Point mutation of the proteasome b5 subunit gene is an important mechanism of bortezomib resistance in bortezomib-selected variants of Jurkat T cell lymphoblastic lymphoma/leukemia line. *J. Pharmacol. Exp. Ther.* 326, 423–431 (2008).

68 Politou M, Karadimitris A, Terpos E, Kotsianidis I, Apperley JF, Rahemtulla A. No evidence of mutations of the PSMB5 (b-5 subunit of proteasome) in a case of myeloma with clinical resistance to Bortezomib. *Leuk. Res.* 30, 240–241 (2006).

69 Moreau P, Pylypenko H, Grosicki S *et al.* Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, Phase 3, non-inferiority study. *Lancet Oncol.* 12, 431–440 (2011).

70 Oakervee HE, Popat R, Curry N *et al.* PAD combination therapy (PS341/bortezomib, doxorubicin and dexamethasone) for previously untreated patients with multiple myeloma. *Br. J. Haematol.* 129, 755–762 (2005).

71 Demo SD, Kirk CJ, Aujay MA *et al.* Anti-tumor activity of PR-171, a novel irreversible inhibitor of the proteosome. *Cancer Res.* 67, 6383–6391 (2007).

72 Jain S, Diefenbach C, Zain J, O'Connor OA. Emerging role of carfilzomib in treatment of relapsed and refractory lymphoid neoplasms and multiple myeloma. *J. Natl Cancer Inst.* 103, 43–57 (2011).

73 Kuhn DJ, Chen Q, Voorhees PM *et al.* Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin–proteasome pathway, against preclinical models of multiple myeloma. *Blood* 110, 3281–3290 (2007).

74 Zangari M, Aujay M, Zhan F. Alkaline phosphatase variation during carfilzomib treatment is associated with best response in multiple myeloma patients. *Eur. J. Haematol.* 86, 484–487 (2011).

75 Frezza M, Hindo S, Chen D *et al.* Novel metals and metal complexes as platforms for cancer therapy. *Curr. Pharm. Des.* 16, 1813–1825 (2010).

76 McAuslan BR, Reilly W. Endothelial cell phagokinesis in response to specific metal ions. *Exp. Cell Res.* 130, 147–157 (1980).

77 Risau W. Mechanism of angiogenesis. *Nature* 386, 671–674 (1997).

78 Hicklin DJ, Ellis LM. Role of the vascular endothelian growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol.* 23, 1011–1027 (2005).

79 Hu GF. Copper stimulates proliferation of human endothelial cells under culture. *J. Cell Biochem.* 69, 326–335 (1998).

80 Frangoulis M, Georgiou P, Chrisostomidis C *et al.* Rat epigastric flap survival and VEGF expression after local copper application. *Plast. Reconstr. Surg.* 119, 837–843 (2007).

81 Sen CK, Khanna S, Venojarvi M *et al.* Copper-induced vascular endothelial growth factor expression and wound healing. *Am. J. Physiol. Heart Circ. Physiol.* 282, H1821–H1827 (2002).

82 Chen D, Cui QC, Yang H, Dou QP. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. *Cancer Res.* 66, 10425–10433 (2006).

83 Pang H, Chen D, Cui QC, Dou QP. Sodium diethyldithiocarbamate, an AIDS progression inhibitor and a copper-binding compound, has proteasome-inhibitory and apoptosis-inducing activities in cancer cells. *Int. J. Mol. Med.* 19, 809–816 (2007).

▪ ***In vitro* evaluation of copper and zinc complexes that work as protesome inhibitors.**

84 Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Res.* 7, R897–R908 (2005).

85 Chen D, Peng F, Cui QC *et al.* Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate copper complex is associated with suppression of proliferation and induction of apoptosis. *Front. Biosci.* 10, 2932–2939 (2005).

86 Milacic V, Chen D, Giovagnini L, Diez A, Fregona D, Dou QP. Pyrrolidine dithiocarbamate-zinc(II) and -copper(II) complexes induce apoptosis in tumor cells by inhibiting the proteasomal activity. *Toxicol. Appl. Pharmacol.* 231, 24–33 (2008).

87 Hindo SS, Frezza M, Tomco D *et al.* Metals in anticancer therapy: copper(II) complexes as inhibitors of the 20S proteasome. *Eur. J. Med. Chem.* 44, 4353–4361 (2009).

88 Verani CN. Metal complexes as inhibitors of the 26S proteasome in tumor cells. *J. Inorg. Chem.* 106, 59–67 (2012).

89 Daniel KG, Gupta P, Harbach RH, Guida WC, Dou QP. Organic copper complexes as a new class of proteasome inhibitors and apoptosis inducers in human cancer cells. *Biochem. Pharmacol.* 67, 1139–1151 (2004).

90 Xiao Y, Chen D, Zhang X *et al.* Molecular study on copper-mediated tumor proteasome inhibition and cell death. *Int. J. Oncol.* 37, 81–87 (2010).

91 Adamson RH, Canellos GP, Sieber SM. Studies on the anti-tumor activity of gallium nitrate (NSC-15200) and other group IIIa metal salts. *Cancer Chemother. Rep.* 59, 599–610 (1975).

92 Hart MM, Adamson RH. Anti-tumor activity and toxicity of salts of inorganic group 3a metals: aluminum, gallium, indium, and thallium. *Proc. Natl Acad. Sci. USA* 68, 1623–1626 (1971).

93 Einhorn L. Gallium nitrate in the treatment of bladder cancer. *Semin. Oncol.* 30, 34–41 (2003).

94 Warrell RP Jr, Coonley CJ, Straus DJ, Young CW. Treatment of patients with advanced malignant lymphoma using gallium nitrate administered as a seven-day continuous infusion. *Cancer* 51, 1982–1987 (1983).

95 Green MA, Welch MJ. Gallium radiopharmaceutical chemistry. *Int. J. Rad. Appl. Instrum. B* 16, 435–448 (1989).

96 Bernstein LR. Mechanisms of therapeutic activity for gallium. *Pharmacol. Rev.* 50, 665–682 (1998).

97 Chitambar CR, Seligman PA. Effects of different transferrin forms on transferrin receptor expression, iron uptake, and cellular proliferation of human leukemic HL60 cells. Mechanisms responsible for the specific cytotoxicity of transferrin-gallium. *J. Clin. Invest.* 78, 1538–1546 (1986).

98 Chitambar CR, Narasimhan J. Targeting iron-dependent DNA synthesis with gallium and transferrin-gallium. *Pathobiology* 59, 3–10 (1991).

99 Shakya R, Peng F, Liu J, Heeg MJ, Verani CN. Synthesis, structure, and anticancer activity of gallium(III) complexes with asymmetric tridentate ligands: growth inhibition and apoptosis induction of cisplatin-resistant neuroblastoma cells. *Inorg. Chem.* 45, 6263–6268 (2006).

100 Chen D, Frezza M, Shakya R *et al.* Inhibition of the proteasome activity by gallium(III) complexes contributes to their anti prostate tumor effects. *Cancer Res.* 67, 9258–9265 (2007).

▪ **Antiprotesomal activity of anticancer gallium(III) complexes.**

101 Frezza M, Hindo SS, Tomco D *et al.* Comparative activities of nickel(II) and zinc(II) complexes of asymmetric [NN'O] ligands as 26S proteasome inhibitors. *Inorg. Chem.* 48(13), 5928–5937 (2009).

102 Ward JR. Role of disease-modifying antirheumatic drugs versus cytotoxic agents in the therapy of rheumatoid arthritis. *Am. J. Med.* 85, 39–44 (1988).

103 Kostova I. Au coordination complexes as anticancer agents. *Anticancer Agents Med. Chem.* 6, 19–32 (2006).

104 Vergara E, Casini A, Sorrentino F *et al.* Anticancer therapeutics that target selenoenzymes: synthesis, characterization, *in vitro* cytotoxicity, and thioredoxin reductase inhibition of a series of Au(I) complexes containing hydrophilic phosphine ligands. *Chem. Med. Chem.* 5, 96–102 (2010).

105 Sadler PJ, Sue RE. The chemistry of Au drugs. *Met. Based Drugs* 1, 107–144 (1994).

106 McKeage MJ, Maharaj L, Berners-Price SJ. Mechanisms of cytotoxicity and anti-tumor activity of Au(I) phosphine complexes: the possible role of mitochondria. *Coord. Chem. Rev.* 232, 127–135 (2002).

107 Bhabak KP, Bhuyan BJ, Mugesh G. Bioinorganic medicinal chemistry: aspects of Au(I)-protein complexes. *Dalton Trans.* 40, 2099–2111 (2011).

108 Che CM, Sun RWY, Yu WY, Ko CB, Zhu N, Sun H. Au(III) porphyrins as a new class of anticancer drugs: cytotoxicity, DNA binding and induction of apoptosis in human cervix epithelioid cancer cells. *Chem. Commun.* 14, 1718–1719 (2003).

109 Sun RWY, Che CM. The anticancer properties of Au(III) compounds with dianionic porphyrin and tetradentate ligands. *Coord. Chem. Rev.* 253, 1682–1691 (2009).

▪▪ **Review of Au(III) porphyrin compounds with anticancer activity.**

110 Bindoli A, Rigobello MP, Scutari G, Gabbiani C, Casini A, Messori L. Thioredoxin reductase: a target for Au compounds acting as potential anticancer drugs. *Coord. Chem. Rev.* 253, 1692–1707 (2009).

111 Berners-Price SJ, Filipovska A. Au compounds as therapeutic agents for human diseases. *Metallomics* 3, 863–873 (2011).

▪▪ **Valuable review of Au(I, III) complexes.**

112 Saggioro D, Rigobello MP, Paloschi L *et al.* Au(III)–dithiocarbamato complexes induce cancer cell death triggered by thioredoxin redox system inhibition and activation of ERK pathway. *Chem. Biol.* 14, 1128–1139 (2007).

113 Milacic V, Chen D, Ronconi L, Landis-Piwowar KR, Fregona D, Dou QP. A novel anticancer Au(III) dithiocarbamate compound inhibits the activity of a purified 20S proteasome and 26S proteasome in human breast cancer cell cultures and xenografts. *Cancer Res.* 66, 10478–10486 (2006).

114 Ronconi L, Fregona D. The Midas touch in cancer chemotherapy: from platinum to Au-dithiocarbamato complexes. *Dalton Trans.* 48, 10670–10680 (2009).

115 Marzano C, Ronconi L, Chiara F *et al.* Au(III)–dithiocarbamato anticancer agents: activity, toxicology and histopathological studies in rodents. *Int. J. Cancer* 129, 487–496 (2011).

116 Ronconi L, Giovagnini L, Marzano C *et al.* Au dithiocarbamate derivatives as potential antineoplastic agents: design, spectroscopic properties, and *in vitro* anti-tumor activity. *Inorg. Chem.* 44, 1867–1881 (2005).

117 Casini A, Kelter G, Gabbiani C *et al.* Chemistry, antiproliferative properties, tumor selectivity, and molecular mechanisms of novel Au(III) compounds for cancer treatment: a systematic study. *J. Biol. Inorg. Chem.* 14, 1139–1149 (2009).

118 Ronconi L, Marzano C, Zanello P *et al.* Au(III) dithiocarbamate derivatives for the treatment of cancer: solution chemistry, DNA binding, and hemolytic properties. *J. Med. Chem.* 49, 1648–1657 (2006).

119 Cattaruzza L, Fregona D, Mongiat M *et al.* Anti-tumor activity of Au(III)–dithiocarbamato derivatives on prostate cancer cells and xenografts. *Int. J. Cancer* 128, 206–215 (2011).

120 Fricker SP. A screening strategy for metal anti-tumor agents as exemplified by Au(III) complexes. *Met. Based Drugs* 6, 291–300 (1999).

121 Ronconi L, Aldinucci D, Dou QP, Fregona D. Latest insights into the anticancer activity of Au(III)–dithiocarbamato complexes. *Anticancer Agents Med. Chem.* 10, 283–292 (2010).

▪ **Review of Au(III)–dithiocarbamato complexes.**

122 Milacic V, Fregona D, Dou QP. Au complexes as prospective metal based anticancer drugs. *Histol. Histopathol.* 23, 101–108 (2008).

123 Marcon G, Messori L, Orioli P, Cinellu MA, Minghetti G. Reactions of Au(III) complexes with serum albumin. *Eur. J. Biochem.* 270, 4655–4661 (2003).

124 Szweda PA, Friguet B, Szweda LI. Proteolysis, free radicals, and aging. *Free Radic. Biol. Med.* 33, 29–36 (2002).

125 Ott I. On the medicinal chemistry of Au complexes as anticancer drugs. *Coord. Chem. Rev.* 253, 1670–1681 (2009).

126 Zhang X, Frezza M, Milacic V *et al.* Inhibition of tumor proteasome activity by Au–dithiocarbamato complexes via both redox-dependent and -independent processes. *J. Cell. Biochem.* 109, 162–172 (2010).

127 Aldinucci D, Lorenzon D, Stefani L, Giovagnini L, Colombatti A, Fregona D. Antiproliferative and apoptotic effects of two new Au(III) methylsarcosinedithiocarbamato derivatives on human acute myeloid leukemia cells *in vitro*. *Anticancer Drugs* 8, 323–332 (2007).

128 Troyano A, Fernandez C, Sancho P, de Blas E, Aller P. Effect of glutathione depletion on anti-tumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells, the role of intracellular oxidation. *J. Biol. Chem.* 276, 47107–47115 (2001).

129 Cheriathundam E, Alvares AP. Species differences in the renal toxicity of the antiarthritic drug, Au sodium thiomalate. *J. Biochem. Toxicol.* 11, 175–181 (1996).

130 Sereemaspun A. Effect of Au nanoparticle on renal cell: an implication for exposure risk. *Ren. Fail.* 30, 323–325 (2008).

■ Websites

201 Millenium. The Takeda Oncology Company. www.millennium.com

202 Oncotest GmbH. www.oncotest.de/drug-screening/index.php (Accessed 15 January 2010)