

Introduction Session 13

PROTACS: CHIMERIC MOLECULES TO TARGET PROTEINS FOR UBIQUITINATION AND DEGRADATION

(taken from KM Sakamoto, *Mol. Genetics and Metabolism* 77 (2002) 44-56)

To circumvent the problem of transducing cells at high efficiency, we sought to target deliberately a protein to the SCF complex by developing a chimeric compound, known as **proteolysis targeting chimeric molecule (PTCM)**. We first tested whether the PTCM could recruit methionine aminopeptidase-2 (MetAP-2) to the SCF ^{β -TRCP} for ubiquitination and degradation in vitro. A PTCM was synthesized that contained at one end the minimal 10 aa phosphopeptide sequence of I κ B that is recognized by the F-box protein β -TRCP and at the other end, the MetAP-2 binding compound, ovalicin. MetAP-2 binds to ovalicin. MetAP-2 binds to ovalicin covalently. We performed ubiquitination experiments with lysates from 293T cells transfected with Flag tagged β -TRCP and Flag tagged CUL1. The SCF complex was immunoprecipitated using Flag affinity beads followed by addition of purified E1, E2, ATP, and ubiquitin. Our results demonstrate that the PTCM could recruit the MetAP-2 to the SCF ^{β -TRCP} complex resulting in ubiquitination. Addition of PTCM also resulted in degradation of MetAP-2 in *Xenopus* extracts.

To determine whether PTCM could be generalized to other ubiquitin ligases, we performed ubiquitination assays with Cbl. Cbl is a monomeric ubiquitin ligase that attaches ubiquitin to signaling molecules and receptor tyrosine kinases resulting in proteolysis. We generated a PTCM that consisted of ovalicin and the Zap70 phosphopeptide, which binds Cbl. Ubiquitination reactions were performed with purified Cbl, various E1s, Ubch4 (E2), ubiquitin, ATP, MetAP-2, and the Zap70-ovalicin PTCM. We demonstrated that PTCM promotes ubiquitination of MetAP-2 by Cbl in vitro. These results suggest that PTCM can be generalized to other ubiquitin ligases. Future work will focus on testing other targets that promote tumorigenesis, e.g., androgen receptor in prostate cancer cells. If cell permeable PTCMs prove to increase turnover and degrade proteins in cells, this would lead to potential therapeutic applications in patients with cancer and other diseases.

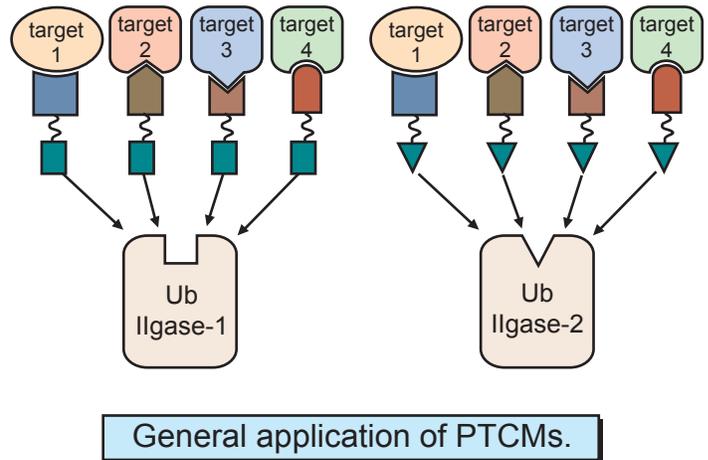


Figure by MIT OCW. After Sakamoto, KM. Ubiquitin-dependent proteolysis: its role in the human diseases and the design of therapeutic strategies." *Mol Genet Metab* 77 1-2 (2002) 44-56.

General application of PTCMs. A schematic representation of how different disease-promoting proteins might be recruited to E3 ligases for ubiquitination and degradation by specific PTCMs.

BORTEZOMIB (also PS-341 or Velcade): A novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers

(reviewed by P.G. Richardson *et al. Cancer control* (2003) 10: 361-369)

(A full text PDF of this article is available at <http://www.moffitt.usf.edu/pubs/ccj/v10n5/pdf/361.pdf>)

Cancer cells seem to be more sensitive to the proapoptotic effects of proteasome inhibition than are normal cells. It has also been shown that **proteasome inhibition enhances the sensitivity of cancer cells to traditional anticancer agents** in both *in vitro* and *in vivo* preclinical studies.

The 20S core is a cylindrical complex made up of four stacked rings. The two outer rings bind to the 19S regulatory particles, and the two inner rings each contain three active sites. These active sites account for the three major proteolytic activities of the proteasome, which have been described as chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolytic (PGPH).

Synthetic inhibitors of the proteasome include peptide aldehydes such as Z-Leu-Leu-Leu-al (MG132), Z-Ile-Glu(Obut)-Ala-Leu-al (PSI), Ac-Leu-Leu-Nle-al (ALLN), and peptide vinyl sulfones. Natural proteasome inhibitors include lactacystin, epoxyketones and the TMC-95 cyclic peptides. All of these compounds bind to and directly inhibit active sites within the 20S core particle. However, most primarily interfere with the chymotrypsin-like activity of the core particle (the rate-limiting step in proteolysis) and appear to have little effect on the other proteolytic activities. Many of these inhibitors also **lack specificity or exhibit unfavorable kinetics** for clinical use. For instance, peptide aldehyde inhibitors dissociate rapidly from the proteasome and are inactivated by oxidation, being removed from the cell by the multidrug transporter system. Furthermore, they are also inhibitors of serine and cysteine proteases, including calpains and cathepsins, which can be undesirable for their use in patients. Others, such as the peptide vinyl sulfones and natural inhibitors bind the 20S irreversibly, which can also be detrimental in the long run.

These problems were overcome, however, by replacing the aldehyde group of the synthetic peptide inhibitors with boronic acid. The **peptide boronates** differ from their aldehyde analogs in that they dissociate more slowly from the proteasome, conferring **stable inhibition**. Further, the weak interaction between boron and sulphur means that

the peptide boronates **do not inhibit thiol proteases**. The peptide boronic acids are also up to **100-fold more potent** than their peptide aldehyde analogs. Dipeptide boronic acid bortezomib is of particular interest from a clinical perspective. This small, water-soluble compound is a potent and selective proteasome inhibitor, which offers the additional advantages of **low molecular weight and ease of synthesis**. Bortezomib is the first molecule in this class to reach clinical trials in cancer patients.

(Image removed for copyright reasons. See Figure 1b in Richardson, 2003.)

Multiple myeloma is a hematologic malignancy typically characterized by the accumulation of clonal plasma cells at multiple sites in the bone marrow. Although the majority of patients respond to initial treatment with chemotherapy and radiation, most eventually relapse due to the proliferation of resistant tumor cells; despite the advent of high-dose chemotherapy with stem-cell transplantation, MM remains incurable. This cytotoxic resistance reflects both the inherent characteristics of the MM cell and the protective interactions between the tumor and the bone marrow microenvironment.

There is strong evidence that **the cell death induced by proteasome inhibition is apoptotic**. For example, the cell death observed in MM cells exposed to bortezomib *in vitro* involved caspase-3 activation and annexin V binding. Further, gastric cancer cells treated with MG-132 exhibited signs of apoptosis, such as cytoplasmic and nuclear shrinkage, chromatin condensation and fragmentation, DNA laddering, upregulation of the proapoptotic protein Bax, release of mitochondrial cytochrome c, and caspase activation. In laboratory studies, MM cell lines were significantly more sensitive to the proapoptotic effects of bortezomib proteasome inhibition than were bone marrow cells or peripheral blood mononuclear cells from healthy individuals. Similarly, other proteasome inhibitors induced apoptosis in chronic lymphocytic leukemia cells and oral squamous cell carcinoma cells at doses that had no effect on normal human lymphocytes or oral epithelial cells, respectively. It has also been noted in preclinical studies that actively dividing cells are

more sensitive to proteasome inhibition than are quiescent or differentiated cells.

While **active division** does appear to increase sensitivity to proteasome inhibition, it is likely that other mechanisms contribute to the anticancer activity of proteasome inhibitors.

Inhibition of proteasomal activity results in the accumulation of numerous regulatory proteins within the cell. Proteins stabilized by proteasome inhibition include the tumor-suppressor protein p53 and the cell cycle proteins p21 and p27. It is possible that proteasome inhibition triggers the apoptotic cascade, in part, by **causing the rapid accumulation of incompatible regulatory proteins** within the cell.

The NF- κ B pathway is constitutively active in some cancer cells and is associated with resistance to anticancer therapy. Specifically, MM tumor cells and the bone marrow of MM patients show enhanced NF- κ B activity, and chemoresistant MM cells have increased NF- κ B activity compared with chemosensitive lines. **Proteasome inhibitors** have been shown to stabilize I κ B and **prevent the activation of NF- κ B**. In MM cells, bortezomib increased the level of phosphorylated I κ B protein and inhibited constitutive NF- κ B activity. Further, bortezomib inhibited the tumor necrosis factor α (TNF- α)-induced activation of NF- κ B in MM cells. This is a significant finding, since TNF- α is present in the bone marrow microenvironment and is known to activate NF- κ B-dependent gene expression and proliferation in MM cells. **Proteasome inhibition** has also been shown to **block chemotherapy- and radiotherapy-induced activation of NF- κ B**, resulting in enhanced sensitivity to these tumoricidal agents and increased apoptosis in cancer cells in vitro. However, the direct inhibition of I κ B α phosphorylation is insufficient to completely inhibit the proliferation of MM cells, suggesting that bortezomib does not act through NF- κ B blockade alone.

Proteasome inhibitors may overcome drug resistance in vivo by **interfering with the protective interaction between cancer cells and the bone marrow**. In MM, the adherence of tumor to bone marrow stromal cells (BMSC) provides protection against apoptosis, promotes tumor cell survival and progression, and confers protection against chemotherapeutic drugs. Adhesion to fibronectin protects cells from common chemotherapeutic agents as well as radiation. Of the 52 genes found to

be upregulated more than 2-fold in fibronectin-bound MM cells, 11 are known to be NF- κ B responsive. Importantly, pretreatment with either MG-132 or bortezomib reversed adhesion-mediated drug resistance and sensitized these cells to cytotoxic agents. Further studies of bortezomib activity found that proteasome inhibition decreased the binding of MM cells to BMSCs by 50%. Under normal circumstances, some MM cells produce TNF- α , a cytokine that enhances cell-cell interactions by increasing the secretion of IL-6 from BMSCs and upregulating the expression of the adhesion molecules VLA-4 and LFA-1 on MM cells and the corresponding receptors VCAM-1 and ICAM-1 on BMSCs. **Bortezomib** prevented the TNF- α -induced, NF- κ B-dependent upregulation of IL-6 and therefore **reduced cell adhesion**. Moreover, the **proliferation of the remaining adherent MM cells was also inhibited** by bortezomib.

(Image removed for copyright reasons. See Figure 3 in Richardson, 2003.)

Preclinical studies show that bortezomib is highly toxic against a broad range of cancer cell lines in vitro. Bortezomib may be equally effective against solid tumors and hematological malignancies. It also inhibits angiogenesis and when used in combination with conventional tumoricidal agents, it enhances the sensitivity of cancer cells to these agents.

A study was undertaken in patients with relapsed and refractory MM disease in the United States. Of 202 enrolled patients, 193 were evaluable; 92% had been treated with 3 or more of the major classes of drugs commonly used for myeloma, and 91% were refractory to their most recent therapy. The response rate (complete, partial, or minor response) to bortezomib was 35%. Four percent of patients had complete responses. The median overall survival was 16 months, with a median duration response of 12 months. Also noted were improved quality-of-life parameters, improved levels of normal immunoglobulins, decreased transfusion requirements, and improvements in hemoglobin levels. Given the severity of this cancer, although these results may at first sight seem modest, they actually represent a significant advance in the continuous quest for a successful treatment for this and other cancers and related-diseases.