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Joining the Army of Proteasome Inhibitors

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An article in this issue of *Chemistry & Biology* (Hines et al., 2008) and a recent study in *Nature* (Groll et al., 2008) establish three natural products as novel proteasome inhibitors. These inhibitors, discovered in an unusual way, reveal a different mechanism of proteasome inhibition and suggest new therapeutic application of its inhibitors.

Targeted degradation of proteins by the ubiquitin-proteasome pathway plays an essential role in the regulation of protein homeostasis and in the regulation of essentially every function of the living cells. The proteasome is a large, multisubunit, proteolytic complex that processively degrades ubiquitinated proteins into small peptides. Numerous inhibitors of this degradation machine, discovered in the past 15 years, serve as excellent tools to determine proteasome involvement in a cellular or physiological process and to determine if a protein of interest is degraded by the proteasomes (Kisselev and Goldberg, 2001). Proteasome inhibitors cause selective apoptosis of malignant cells, and represent a new class of antineoplastic agents (Adams, 2004). One such inhibitor, bortezomib (VELCADE), has been approved by the FDA for the treatment of multiple myeloma and mantle cell lymphoma. Three second-generation proteasome inhibitors, carfilzomib (PR-171) (Demo et al., 2007), salinosporamide A (NPI-0052) (Chauhan et al., 2005), and CEP-18770 (Piva et al., 2008), are in phase I and II clinical trials (Figure 1).

Two recent papers, one in *Nature* (Groll et al., 2008), and one in this issue of *Chemistry & Biology* (Hines et al., 2008)

now report additional proteasome inhibitors. If there are so many proteasome inhibitors already available, why do these compounds deserve special attention? One of them inhibits the proteasome by a mechanism not previously described and the other suggests potential for additional therapeutic applications of these compounds. In addition, these inhibitors were discovered in an unusual way.

Groll et al. (2008) set out to investigate the mechanisms of the Syringolin A (SylA, Figures 1 and 2) virulence factor of the plant pathogen *Pseudomonas syringae*. Treatment of wheat and *Arabidopsis thaliana* with this peptide derivative leads to changes in gene expression profiles that resemble changes occurring in yeast and mammalian cells treated with proteasome inhibitors (i.e., upregulation of transcripts encoding proteasomal subunits and heat shock proteins). This observation allowed Groll et al. to hypothesize that this compound is a proteasome inhibitor. Indeed they found that it irreversibly inhibits all three types of proteasomal proteolytic sites. In order to elucidate the mode of inhibition, they solved the structure of SylA complex with the yeast 20S proteasome. This structure revealed a novel mode of inhibition whereby the hydroxy group of proteasome's catalytic

threonine performs a Michael type 1,4-addition to the vinyl ketone moiety in the 14-membered ring of the inhibitor (Figure 2). This mechanism resembles mechanisms of inhibition by another class of proteasome inhibitors, peptidyl vinyl sulfones (Groll and Huber, 2004). They also found that another microbial metabolite, Glidobactin A (GlbA), inhibited the chymotrypsin- and the trypsin-like activities of the proteasome and reacted with active site threonines in a similar fashion. Both SylA and GlbA blocked proliferation and induced apoptosis of malignant cells, further confirming that they are proteasome inhibitors.

Hines et al. (2008) investigated the mechanism of neurotropic activity of marine fungal metabolite fellutamide B. It was known that treatment of cultured neurons and fibroblasts with this compound induces nerve growth factor (NGF) secretion, but the mechanism leading to this event had not been elucidated. They noticed similarities in the structures of this lipopeptide aldehyde and peptide aldehyde proteasome inhibitor MG132, and tested whether it is a proteasome inhibitor. Indeed they found that fellutamide B is a very potent inhibitor of the chymotrypsin-like sites and that it also inhibits the trypsin-like and caspase-like sites,

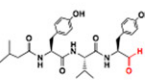
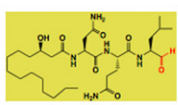
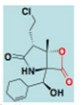
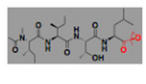
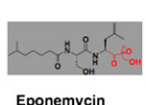
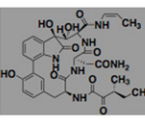
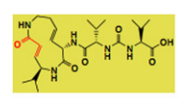
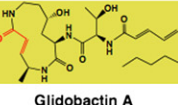
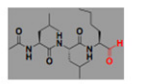
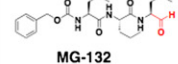
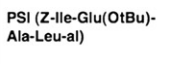
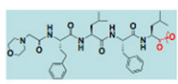
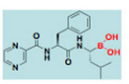
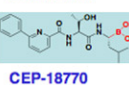
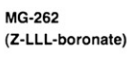
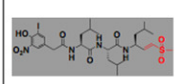
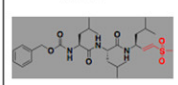
Class Origin	Aldehydes	β -lactones	Epoxyketones	Boronates	Vinyl sulfones	Cyclic peptides**	Macrocyclic vinyl ketones
Natural products	 Tyropeptin A  Fellutamide B	 Omuralide (clasto-lactacystin- β -lactone)  Salinosporamide A (NPI-0052)	 Epoxomicin  Eponemycin			 TMC-95A	 Syringolin A  Glidobactin A
Synthetic	 ALLN  MG-132  PSI (Z-Ile-Glu(OtBu)-Ala-Leu-al)	 PS-519	 Carfilzomib (PR-171)	 Bortezomib  CEP-18770  MG-262 (Z-LLL-boronate)	 NLVS  ZLVS	TMC-95 analogues	

Figure 1. Major Classes of Proteasome Inhibitors and Their Selected Representatives

Novel inhibitors described in this commentary are highlighted in yellow. "Founding members" of the family are highlighted in gray, except for novel macrocyclic vinyl ketones, which are highlighted in yellow. Clinically used inhibitors are highlighted in blue. Pharmacophores (i.e., functional groups of the inhibitors that react with proteasome's catalytic threonines) are red. Cyclic peptides (TMC-95 and its derivatives) are the only inhibitors that do not interact directly with the active site threonines.

albeit at higher concentrations. It also caused accumulation of ubiquitinated proteins in cultured cells. X-ray diffraction revealed that it binds to all three catalytic sites of the yeast 20S proteasome with the formation of a hemiacetal bond. Interestingly, the N-terminal aliphatic tail, which distinguished this compound from other peptide aldehydes reported to date, adopts different conformations at different active sites.

Two other proteasome inhibitors, MG 132 and the peptide epoxyketone epoxomicin, also upregulated NGF secretion, confirming that fellutamide B-induced NGF production is a consequence of proteasome inhibition. It should be noted that another natural product proteasome inhibitor, lactacystin, was originally identi-

fied as a compound that promotes neurite outgrowth, and was later shown to be a proteasome inhibitor (see Kisselev and Goldberg, 2001, for review). Consistent with this earlier observation, conditioned media from epoxomicin and fellutamide B treated cells caused neurite outgrowth in undifferentiated PC12 cells. Secretion of NGF was observed at the same concentration of fellutamide B as were other well-documented effects of proteasome inhibition (e.g., accumulation of ubiquitinated proteins, cell cycle arrest, and cytotoxicity). At the same time, NGF was not produced in response to other cytotoxic treatments, further strengthening the conclusion that NGF production is indeed the consequence of proteasome inhibition. The authors then demonstrated

that this effect is a transcriptional response and identified promoter regions in the NGF gene that are responsible for this response. This suggests that proteasome inhibitors exert their neurotropic effect by stabilizing a short-lived yet-to-be-identified transcriptional factor that regulates NGF gene expression.

In medical neurology there is an urgent need for drugs to treat neuronal injury caused by stroke, ischemia, and neurodegenerative diseases. One possibility is to develop compounds that stimulate NGF production. Can proteasome inhibitors be used for these purposes? Given the overall cytotoxicity of proteasome inhibitors and high sensitivity of neuronal tissue to accumulation of misfolded proteins, their application for the treatment of chronic neurodegenerative diseases appears unlikely at the moment. However, in situations where a single short treatment is sufficient to prevent neuronal injury, therapeutic applications of proteasome inhibitors are certainly possible. Specifically, proteasome inhibitors are effective in prevention of reperfusion injury in animal models of cerebral ischemia (Phillips et al., 2000). The explanation for this effect is that this type of

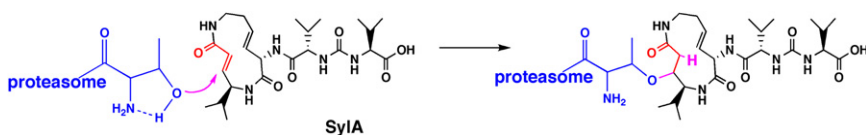


Figure 2. Simplified Mechanism of Syringolin A Reaction with Proteasome Active Site Threonines

Proteasome atoms are blue; inhibitor is black except for pharmacophore, which is red. Newly formed bonds are purple.

brain injury is caused by the development of inflammation at the site of infarction. Proteasome inhibitors block inflammation by preventing proteasome-dependent activation of transcription factor NF- κ B. The study from the Crews group demonstrating neurotropic properties of proteasome inhibitors (Hines et al., 2008) suggests that an increase in NGF production can also contribute to this effect. This should stimulate the interest in continuation of clinical testing of proteasome inhibitors in stroke patients.

Finally, we would like to draw the readers' attention to the fact that all the inhibitors discussed in these articles are natural products, as are the well-known proteasome inhibitors lactacystin, epoxomicin, and salinosporamide A, and the less famous eponemycin, tyropeptin A (Momose et al., 2005), and TMC-95. If proteasome inhibitors are classified based on chemical mechanisms, by which they inhibit the proteasome, seven major classes can be distinguished (Figure 1). Classes represented by natural products outnumber those developed by organic synthesis.

Indeed, four of these classes (β -lactones, peptide epoxyketones, cyclic peptides, and macrocyclic peptide vinyl ketones) were discovered as natural products. Natural products are represented in the fifth class, peptide aldehydes, although these compounds (e.g., MG132) were initially developed by chemical synthesis. Only two classes of inhibitor (peptide boronates, e.g., bortezomib, and peptide vinyl sulfones) do not yet have natural products among them. Clearly, micro-organisms learned of the importance of the proteasome to their hosts long before scientists discovered this fascinating particle. We predict that this trend of discovery of proteasome inhibitors among natural products will continue, and hold hope that some of new inhibitors will open novel therapeutic applications for these compounds as the study by the Crews group suggests.

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Computational Design of Enzymes

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The de novo design of enzymes with activities not found in natural biocatalysts is a major challenge for molecular biology. Sophisticated computational methods have recently led to impressive progress in this exciting and rapidly evolving field (Röthlisberger et al., 2008; Jiang et al., 2008).

Natural evolution has yielded enzymes with well-defined active sites in which virtually all metabolic reactions are catalyzed with high efficiency and specificity. It has been a major goal of biochemistry for the past century to understand the chemical and molecular principles of these extremely precise and exquisite molecular machines. Recently, technical advances in molecular biology have led to a renaissance in enzymology by enabling researchers to modify at will the activities and stabilities of many

naturally occurring enzymes. This rapidly emerging field of “enzyme design” has provided new insights in the structure-function relationships of molecular biocatalysts. Moreover, these approaches have facilitated the generation of stabilized enzymes with increased turnover numbers and altered substrate- and stereo-selectivities to be used in industrial processes (Toscano et al., 2007).

Until now, the most impressive results in enzyme design have been obtained by

“directed evolution.” In this two-step approach random mutagenesis is used to create large enzyme repertoires, from which optimized variants are then isolated using either selection or screening techniques (Bloom et al., 2005). In contrast to directed evolution, the alternative approach of “rational” enzyme design requires a detailed knowledge of a specific enzyme structure and catalytic mechanism (Woycechowsky et al., 2007). Although occasionally successful, rational