while the phenylbutyl derivative **5** selectively inhibited PDE4 (IC_{50} = 1 nM).

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Inhibition of the proteasome as a therapeutic approach

In the late 1970s, research into proteolytic enzymes was considered one of the most uninteresting fields of research. Some of these enzymes worked with amazing specificity, but the destruction of the proteins appeared childishly simple compared with the complexity of other metabolic pathways.

The hydrolysis of a peptide bond is a thermodynamically favored reaction, even though proteolytic reactions requiring ATP hydrolysis have been known for 40 years [Hershko, A. (1996) *Trends Biochem. Sci.* 21, 445–449]. A major breakthrough was the discovery that the energy-dependent proteolytic system required the presence of a small, 76 amino acids long polypeptide, later named ubiquitin [Hershko, A. (1996) *Trends Biochem. Sci.* 21, 445–449; Ciechanover, A., Hod, Y. and Hershko, A. (1978) *Biochem. Biophys. Res. Commun.* 81, 1100–1105]. In 1980, Wilk and Orlowski reported the existence of a pituitary protease of unexpectedly high molecular mass and multiple activities [*J. Neurochem.* (1980) 35, 1172–1182], and that was linked to the ubiquitin system [Eytan, E. *et al.* (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 7751–7755]. Following years have brought the dissection of this proteolytic pathway: it is characterized by energy consumption, it requires ubiquitin, ubiquitin activating and conjugating enzymes, and also the multicatalytic proteinase complex – which has been named the proteasome in 1988 [Arrigo, A.P. *et al.* (1988) *Nature* 331, 192–194].

The 1990s could as well have been named the decade of the proteasome, as it brought a boom of papers regarding the ubiquitin system. Scientists working in different fields, for instance antigen presentation [Tanaka, K. *et al.* (1997) *Adv. Immunol.* 64, 1–38], cell-cycle regulation [Pagano, M. *et al.* (1995) *Science* 269, 682–685; Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) *Nature* 349, 132–138; Hershko, A. (1997) *Curr. Opin. Cell Biol.* 9, 788–799], signal transduction [Pahl, H.L. and Bauerle, P.A. (1996) *Curr. Opin. Cell Biol.* 8, 340–347] and apoptosis [Wójcik, C. *et al.* (1997) *Apoptosis* 2, 455–462], discovered that they were all studying the same proteolytic machinery.

Proteasomes are present in all studied eukaryotic cells and account for up to 1% of total cell protein [Hendil, K.B. (1988) *Biochem. Int.* 17, 471–477]. Their structure was revealed through the study of archeabacterial enzymes. Similar complexes have also been discovered in eubacteria [Tamura, T. *et al.* (1995) *Curr. Biol.* 5, 766–774]. Much of our knowledge of the ubiquitin–proteasome system has been elucidated through the discovery and use of various inhibitors. Proscript (Cambridge, MA, USA) will shortly be initiating clinical trials of proteasome inhibitors for the treatment of malaria and cancer [Featherstone, C. (1997) *Mol. Med. Today* 3, 367; Adams, J. *et al.* (1998) *Bioorg. Med. Chem. Lett.* 8, 333–338]. Proteasome inhibitors are apparently effective anticancer drugs against mouse colon carcinoma cells (T. Stoklosa *et al.*, unpublished) and human Burkitt's lymphoma in nude mice [Orlowski, R.Z. *et al.* (1998) *Cancer Res.* 58, 4342–4348]. A new class of drugs is therefore appearing on the therapeutic horizon.

The ubiquitin–proteasome pathway has recently received attention in several excellent reviews by leading experts in the field [Coux, O., Tanaka, K. and Goldberg, A.L. (1996) *Annu. Rev. Biochem.* 65, 801–847; Varshavsky, A. (1997) *Trends Biochem. Sci.* 22, 383–387; Ciechanover, A. and Schwartz, A.L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2727–2730, (1998) *J. Biochem.* 123, 195–204]. Therefore, only the essentials will be discussed here.

Proteasomes – multicatalytic protease complexes

The 20S proteasome is an ~700 kDa complex, formed by four stacked rings: two inner β rings and two outer α rings, with a set of inner cavities (Fig. 1). The rings are arranged according to a C2 symmetry, so the proteasomes can be divided into two identical halves, each consisting of one α and one β ring. The rings of each type are composed of seven different subunits of the α- and β-family respectively. The yeast 20S proteasome has been crystallized and analysed at a 2.4 Å resolution [Groll, M. *et al.* (1997) *Nature* 386, 463–471].

The proteasome was characterized by the presence of different proteolytic activities, defined against small synthetic peptides as the chymotrypsin-like (ChTL), trypsin-like (TL), peptidyl-glutamylpeptide-hydrolyzing (PGPH), small neutral amino acids-preferring (SNAAP), and branched chain amino acid-preferring activity (BrAAP) [Orlowski, M. (1990) *Biochemistry* 29, 10289–10297]. These activities depend on the presence of a free N-terminal Thr residue on three of seven β-type subunits. This Thr acts as a nucleophile and is essential in the mechanism of the catalytic activity. In contrast with lower eukaryotes, mammalian genomes encode three more active β subunits, which can be expressed and incorporated into the 20S proteasomes after interferon γ (IFN-γ) stimulation.

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Purified 20S proteasomes degrade only small peptides. To degrade proteins, they must associate with protein complexes called the PA700 activators or 19S caps, thus forming the 26S proteasomes of ~1400 kDa, which are able to degrade ubiquitinated proteins in the presence of ATP. 20S proteasomes can also bind different sets of activators, such as PA28(α,β) or PA28(γ), either alone or in combination with PA700 [Hendil, K.B., Khan, S. and Tanaka, K. (1998) *Biochem. J.* 332, 749–754].

Ubiquitin system

Proteins degraded by the 26S proteasomes must first be recognized and polyubiquitinated by a cascade of enzymes, consisting of ubiquitin-activating enzyme (UBA) or E1, ubiquitin-conjugating enzyme (UBC) or E2, and in some cases an additional enzyme, the ubiquitin ligase or E3. This cascade is depicted on the right-hand side of Fig. 1. A notable exception of this rule is the degradation of ornithine decarboxylase by the 26S proteasome without previous ubiquitination [Murakami, Y. *et al.* (1992) *Nature* 360, 597–599; Seemuller, E. *et al.* (1995) *Science* 268, 579–582]. Ubiquitin is added by a formation of an isopeptide bond between an ε-amino group of an internal Lys of the substrate and the C-terminal Gly of ubiquitin. As ubiquitin itself contains internal Lys residues, additional ubiquitin moieties can be added by the same mechanism (usually to Lys48) [Varshavsky, A. (1997) *Trends Biochem. Sci.* 22, 383–387; Ciechanover, A. and Schwartz, A.L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2727–2730].

The recognition of ubiquitination substrates has recently received much attention. It depends on two different criteria: the presence of at least one internal Lys residue prone for isopeptide bond formation and a signal such as the N-degron, cyclin box, δ box of c-*jun* or a poorly defined secondary and tertiary protein structure. The E2 enzymes form a rapidly growing family of enzymes made up of multi-subunit complexes; for example, the cyclosome (anaphase promoting complex) or the SCF complex [Elledge, S.J. and Harper, J.W. (1998) *Biochim. Biophys. Acta* 1377, M61–M70]. However, the molecular machinery is more complex yet, as the ubiquitinating activity of this E1–E2–E3 cascade is opposed by various deubiquitinating enzymes [Wilkinson, K.D. (1997) *FASEB J.* 11, 1245–1256]. Moreover, another level of complexity is introduced by the presence of ubiquitin-like proteins, which can also be conjugated with proteins. Although they have been implicated in protein targeting, their role in degradation, as yet, cannot be ruled out [Saitoh, H., Pu, R.T. and Dasso, M. (1997) *Trends Biochem. Sci.* 22, 374–376].

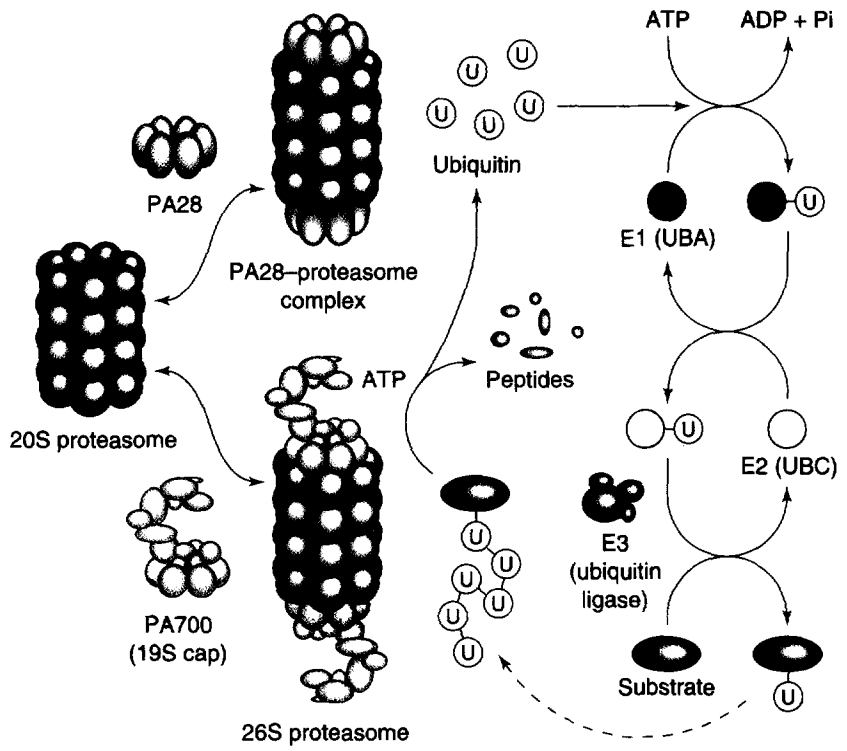
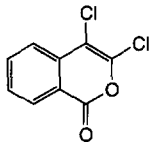


Figure 1. *In the proteasome–ubiquitin system, the 20S proteasome associates with at least two different activators – PA28 and PA700 or 19S cap. The latter complex forms the 26S proteasome, which is involved in ATP-dependent degradation of polyubiquitin tagged substrates with the release of short peptides and free ubiquitin. Ubiquitin is conjugated to the substrates by a cascade of enzymes: E1 or ubiquitin-activating enzymes (UBA), E2 or ubiquitin-conjugating enzymes (UBC) and E3 or ubiquitin ligases. The latter are usually multisubunit complexes specifically recognizing different substrates.*

Peptidyl derivatives as proteasome inhibitors

The ubiquitin-dependent proteolysis can be blocked at various steps; however, most of the attention has been focused on the direct inhibition of proteasome activities. It has revealed that the different activities have different inhibition profiles. A peptide aldehyde transition-state analog of the substrate is a reversible inhibitor of ChTL activity, while leupeptin and sulphydryl blocking agents block TL activity [Wilk, S. and Orlowski, M. (1983) *J. Neurochem.* 40,

842–849]. ChTL activity is also irreversibly blocked by the serine protease inhibitor 3,4-dichloroisocoumarin (**1**) [Orlowski, M. (1990) *Biochemistry* 29, 10289–10297] and reversibly by the peptide aldehydes calpain inhibitors I (*N*-acetyl-Leu-Leu-norleucinal, LLnL, **2**) and II (*N*-acetyl-Leu-Leu-methioninal) [Figueiredo-Pereira, M., Banik, N. and Wilk, S. (1994) *J. Neurochem.* 62, 1989–1894].



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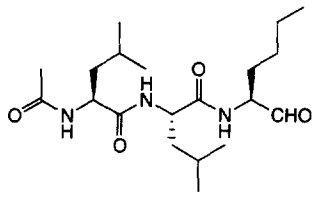

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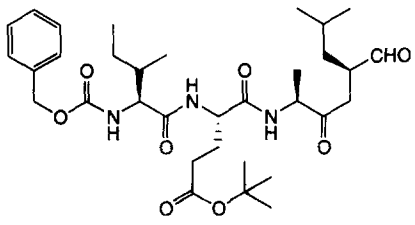
Different groups have since designed peptide aldehydes with increased selectivity for the ChTL activity; for example, PSI [*N*-benzyloxy-carbonyl-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal, **3**] [Figueiredo-Pereira, M., Berg, K. and Wilk, S. (1994) *J. Neurochem.* 63, 1578–1581] or MG115 (Cbz-LLnVal) and MG132 (Cbz-LLLal, **4**) [Rock, K.L. *et al.* (1994) *Cell* 78, 761–771]. Similar compounds have been designed to inhibit other activities of the proteasome, such as the BrAAP-activity-specific Z-GPFL-CHO [Vinitsky, A. *et al.* (1994) *J. Biol. Chem.* 269, 29860–29866]. Inhibitors of this class are cell permeable and cause accumulation of protein–ubiquitin conjugates in cultured cells [Figueiredo-Pereira, M., Berg, K. and Wilk, S. (1994) *J. Neurochem.* 63, 1578–1581; Rock, K.L. *et al.* (1994) *Cell* 78, 761–771]. Such conjugates are not dispersed throughout the cell, but accumulate in discrete aggregates [Wójcik, C. *et al.* (1996) *Eur. J. Cell Biol.* 71, 311–318].



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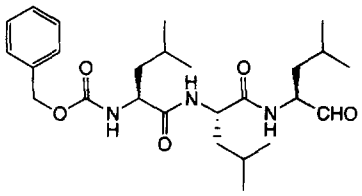

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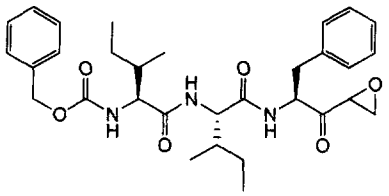
The elucidation of the role of the proteasomes using peptidyl aldehydes boosted the development of even more selective compounds by pharmaceutical companies. GlaxoWellcome developed the tripeptide α′,β′-epoxyketones (**5**) [Spaltenstein, A. *et al.* (1996) *Tetrahedron Lett.* 37, 1343–1346], Cephalon developed α-ketocarbonyl and boronic ester derivatives such as **6** [Iqbal, M. *et al.* (1995) *J. Med. Chem.* 38, 2276–2277],



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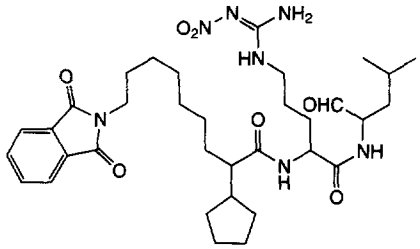

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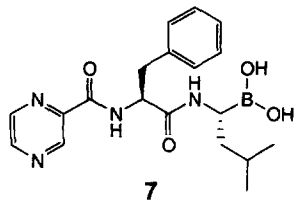
Proscript introduced dipeptidyl boronic acids, exemplified by **7** [Adams, J.M. *et al.* (1998) *Bioorg. Med. Chem. Lett.* 8, 333–338], and CV Therapeutics has investigated indanylamide derivatives such as **8** [Lum, R.T. *et al.* (1998) *Biochem. Pharmacol.* 55, 1391–1397]. Independent researchers also introduced inhibitors with a vinyl sulphone moiety (**9**) [Bogyo, M. *et al.* (1998) *Chem. Biol.* 5, 307–320].



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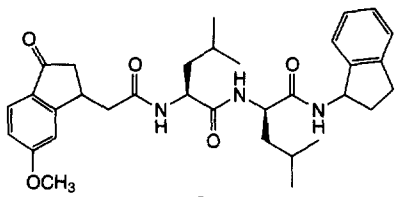

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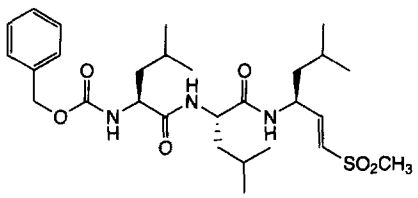
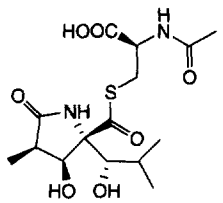


9

Lactacystin – an antiproteasomal antibiotic

All of the above mentioned inhibitors have been developed by improvement of the original transition-state analogs of the proteasome substrates. Meanwhile, Omura's group, while investigating new microbial metabolites, identified lactacystin (**10**), as the *Streptomyces* metabolite that induced neurite outgrowth [Omura, S. *et al.* (1991) *J. Antibiotics.* 44, 113-116].



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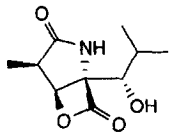
Surprisingly, the target of lactacystin proved to be the proteasome. Lactacystin appeared to modify covalently the active-site Thr of the β-type subunit X(MB1), resulting in an irreversible inhibition of ChTL and TL activities of the proteasome. The active derivative of lactacystin is the clasto-lactacystin β-lactone (**11**) [Dick, J.R. *et al.* (1996) *J. Biol. Chem.* 271, 7273–7276].



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As none of the other five proteases tested was inhibited by lactacystin, it was acclaimed as the most specific proteasome inhibitor known [Fenteany, G. *et al.* (1995) *Science* 268, 726–731]. However, more recently there has been a report that lactacystin also inhibits cathepsin A [Ostrowska, H. *et al.* (1997) *Biochem. Biophys. Res. Commun.* 234, 729–732].

Blocking the cell cycle

Three major cell-cycle transitions – entry into S-phase, separation of sister chromatids and exit from mitosis – require degradation of specific proteins by the ubiquitin–proteasome pathway [Hershko, A. (1997) *Curr. Opin. Cell Biol.* 9, 788–799]. These transitions probably occur by inhibition of the degradation of mitotic [Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) *Nature* 349, 132–138] and G1 phase cyclins [Diehl, J.A., Zindy, F. and Sherr, C.J. (1997) *Genes Dev.* 11, 957–972], cdk inhibitors [Pagano, M. *et al.* (1995) *Science* 269, 682–685], various oncogene and tumor suppressor products, such as p53 [Scheffner, M. *et al.* (1993) *Cell* 75, 495–505], and other regulatory proteins [Hershko, A. (1997) *Curr. Op. Cell Biol.* 9, 788–799; Elledge, S.J. and Harper, J.W. (1998) *Biochim. Biophys. Acta* 1377, M61–M70]. As proteasome inhibitors therefore block cell cycle progression at different phases of the cell cycle, this antiproliferative effect may be exploited against various forms of malignant growth [Wójcik, C. *et al.* (1996) *Eur. J. Cell Biol.* 70, 172–178].

Inducers or inhibitors of apoptosis?

Proteasome inhibitors such as lactacystin [Imajoh-Ohmi, S. *et al.* (1995) *Biochem. Biophys. Res. Comm.* 217, 1070–1077] and PSI [Wójcik, C. *et al.* *Apoptosis* (1997) 2, 455–462; Wójcik, C. *et al.* (1996) *Eur. J. Cell Biol.* 70, 172–178] induce apoptosis in various cancer cell lines. Surprisingly, the very same drugs prevent apoptosis in terminally differentiated and non-proliferating cell lines, such as neurons or thymocytes [Sadoul, R. *et al.* (1996) *EMBO J.* 15, 3845–3852; Grimm, L.M. *et al.* (1996) *EMBO J.* 15, 3835–3844]. These agents appear to act on an early step in the apoptotic pathway, prior to the mitochondrial permeability transition and caspase activation [Hirsch, T. *et al.* (1998) *J. Immunol.* 161, 35–40]. These properties increase their value as potential anticancer drugs, because they should mainly target proliferating cancer cells while having no effect in normal tissues or perhaps even protecting them against other damaging agents, such as γ-irradiation or other chemotherapeutics. As well as affecting neoplastic tissue, the proteasome inhibitors will most probably affect rapidly dividing normal tissues in a similar way to other antimitotic drugs.

Antigen presentation

Proteasomes have also been implicated as the processing protease for intracellular antigens, which provides the peptides required for loading onto MHC class I molecules. Indeed, proteasome inhibitors prevent the effective presentation of certain antigens [Rock, K.L. *et al.* (1994) *Cell* 78, 761–771]. In certain MHC alleles, IFN-γ induces the expression of three additional proteasome subunits (LMP2, LMP7 and MECL1) that are incorporated into the proteasome and alter proteolytic specificity [Gaczynska, M., Rock, K.L. and Goldberg, A.L. (1993) *Nature* 365, 264–267]. IFN-γ also induces the proteasome activator PA28 [reviewed by Tanaka *et al.* (1997) *Adv. Immunol.* 64, 1–38]. Proteasome inhibitors may therefore impair the presentation of tumor-specific antigens by MHC class I. This would not be beneficial in cancer therapy, but could be potentially advantageous in the treatment of autoimmune diseases. It has been shown recently that an alternative processing pathway, for at least some antigens, must exist [Vinitsky, A. *et al.* (1997) *J. Immunol.* 159, 554–564]. It is interesting that synthetic activators of the proteasome have also been developed [Wilk, S. and Chen, W.E. (1997) *Mol. Biol. Rep.* 24, 119–124] – such activators may enhance antigen presentation.

Goodbye to aspirin?

PSI prevents activation of nuclear factor κ-binding (NF-κB) by stabilizing the newly phosphorylated form of the NF-κB inhibitor I-κBα [Pahl, H.L. and Bauerle, P.A. (1996) *Curr. Opin. Cell Biol.* 8, 340–347; Traenckner, E.B., Wilk, S. and Bauerle, P.A. (1994) *EMBO J.* 13, 5433–5441]. As NF-κB is the transcription factor responsible for the expression of major pro-inflammatory cytokines in the immune system such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), IL-2, IL-6 and IL-8, its inhibition causes a strong anti-inflammatory effect. Indeed, proteasome inhibitors proved to be effective in the treatment of delayed hypersensitivity in mice as well as in an arthritis model in rats [Adams, J. and Stein, R. (1996) *Annu. Rep. Med. Chem.* 31, 279–288]. The inhibition of the presentation of antigens to MHC class I, discussed previously, may

also offer additional advantages in the treatment of some chronic inflammation states caused by autoimmune reactions, such as rheumatoid arthritis [Gaczynska, M., Rock, K.L. and Goldberg, A.L. (1993) *Nature* 365, 264–267].

Wasting prevention

In various catabolic states, including metabolic acidosis, diabetes, sepsis, starvation and cancer there is an increase in protein catabolism in striated muscle. This increase leads to the mobilization of amino acids into the bloodstream and associated wasting and cachexia. Such conditions are often fatal, aggravating the prognosis of any primary disease. In all such cases it has been shown that increased muscle proteolysis is associated with upregulation of the ubiquitin–proteasome pathway. Proteasome inhibitors may therefore have use in preventing undesirable wasting in catabolic disease states [Attaix, D. *et al.* (1998) *Reprod. Nutr. Dev.* 38, 153–165].

Are proteasome inhibitors a panacea?

In addition to the applications described above there are other, perhaps more surprising, reports of the potential uses of proteasome inhibitors. As ubiquitin-dependent proteolysis has been shown to be responsible for the remodeling of parasites such as *Plasmodium* species, proteasome inhibitors are being evaluated as potential drugs in the treatment of malaria [Featherstone, C. (1997) *Mol. Med. Today* 3, 367]. PSI has also proved to be an effective hypotensive in the treatment of deoxycorticosterone salt-induced hypertension in rats [Takaoka, M. *et al.* (1998) *Life Sci.* 63, PL65–PL70]. Lactacystin has also been shown to prevent angiogenesis both *in vivo* and *in vitro*, offering an additional advantage in anticancer therapy [Oikawa, T. *et al.* (1998) *Biochem. Biophys. Res. Commun.* 246, 243–248].

Most probably, before this *Profile* appears in print, several new reports on the action of proteasome inhibitors will appear. Albeit not a panacea, proteasome inhibitors will undoubtedly enter the market during the next ten years for the treatment of a variety of disorders. However, as they inhibit the degradation of all substrates of the ubiquitin–proteasome pathway, their main disadvantage will be their poor selectivity. The future belongs to inhibitors of ubiquitin-conjugating enzymes and ubiquitin ligases that will selectively inhibit the degradation of specific proteins.

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In short...

Maitake Products (Ridgefield Park, NJ, USA) is a pioneering company that has introduced the products and the health benefits of the Japanese maitake mushroom in the USA. The products have been received well according to the company, in spite of a long-held scepticism for traditional remedies held in the West. With the recent backing by the FDA to approve IND Phase II clinical studies of the effect of a maitake mushroom extract on patients with advanced breast and prostrate cancer, it appears that such scepticism is waning.

Based on previous formal studies in Japan and treatments by mostly non-traditional health practitioners, it is believed that the orally ingested extract, called D-fraction, will both prevent tumour growth and reduce its size. D-fraction is thought to activate T cells and natural killer cells, which can then fight cancerous cells. There is evidence that the extract not only contributes to tumour reduction without a loss of any white blood cells, but also reduces pain, hair loss, nausea and other side effects usually associated with chemotherapy.

Michael Rothkoff, an oncologist with Metabolic Associates (Florham Park, NJ, USA) will treat 14 patients with either advanced breast or prostate cancer over 6–12 months. Although this is not the first study of a natural ingredient for use in medicine, it is one of only a few to have been FDA-approved.

Maitake Products has marketed D-fraction as a dietary supplement to health food stores and alternative medicine practitioners since 1995, but without the seal of approval by the FDA it cannot be marketed as an anticancer agent. Mike Shirota, the company's CEO and founder, believes that D-fraction will ultimately be found efficacious in other cancers and diseases such as AIDS and chronic fatigue. He says, 'Neutraceuticals, as opposed to pharmaceuticals, will lead the way to an enlightened new century'.