Minireview

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Lactacystin, Proteasome Function, and Cell Fate*

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Lactacystin, a microbial natural product that inhibits cell proliferation and induces neurite outgrowth in a murine neuroblastoma cell line, has become a widely used reagent in functional studies of the proteasome, a high molecular weight, multicatalytic protease complex responsible for most non-lysosomal intracellular protein degradation. The proteasome is composed of a 20 S catalytic core and additional subunits that are thought to be involved in the recognition and unfolding of ubiquitinated proteins; the composite structure has a sedimentation coefficient of 26 S. Lactacystin binds certain catalytic subunits of the 20 S proteasome and inhibits the three best characterized peptidase activities of the proteasome, two irreversibly and all at different rates (1). At least one of these catalytic subunits is modified by lactacystin on the side chain hydroxyl of the amino-terminal threonine (1), which appears to function as the catalytic nucleophile in the proteolytic mechanism. Lactacystin also inhibits peptide hydrolysis by the larger 26 S complex and inhibits ubiquitin/proteasome-mediated degradation of short and long lived proteins in the cell (2). This small molecule is currently the only compound known that inhibits the proteasome specifically without inhibiting any other protease yet tested *in vitro* (1, 3); also it does not inhibit lysosomal protein degradation in the cell (2). This is in contrast to other commonly used proteasome inhibitors, such as peptide aldehydes and 3,4-dichloroisocoumarin, which inhibit a wide range of proteases. Lactacystin has been used to implicate proteolysis by the proteasome in a number of fundamental processes from cellular differentiation and apoptosis to the degradation of proteins normally residing in the endoplasmic reticulum. In this review, the putative mechanism of action and some applications of lactacystin are discussed, as well as recent findings on proteasome function.

Lactacystin Inhibits Cell Cycle Progression in Different Cell Types and Induces Neurite Outgrowth in a Murine Neuroblastoma Cell Line

The *Streptomyces* product lactacystin (Fig. 1) was discovered on the basis of its ability to inhibit cell growth and to induce neurite outgrowth in a murine neuroblastoma cell line, Neuro-2a (3). The cytostatic effects of lactacystin are not unique to Neuro-2a cells (4), and lactacystin has been found to inhibit cell cycle progression in both the G_0/G_1 and G_2/M phases of the cell cycle (4, 5). Lactacystin treatment of Neuro-2a cells results in a predominantly bipolar morphology with two long processes emanating from opposite sides of the cell body, maximal between 16 and 32 h after the start of treatment (4). The cells become progressively more multipolar (multiple neurite bearing) with continued exposure, and the neurites become increasingly branched and display the hallmarks of mature neurites (3). The phosphatase inhibitors okadaic acid and calyculin A block lactacystin-induced bipolar morphology but not the formation of branched neurite networks after 3 days (6). This implies that induction of bipolar morphology and subsequent formation of branched neurite networks are separable processes, with only the former being dependent on the activity of phosphatases. Both of these responses to lactacystin require *de novo* protein synthesis, microtubule assembly, and actin polymerization (4).

The predominantly bipolar morphology that follows treatment with lactacystin is distinct from the response to a number of other common treatments leading to morphological differentiation. Neuro-2a cells deprived of serum or treated with a variety of agents that raise intracellular cAMP levels (and thus activate protein kinase A) tend to become predominantly multipolar (4, 7, 8). Treatment of Neuro-2a cells with retinoic acid, natural gangliosides, and synthetic sialyl compounds tends to induce a more unipolar (single neurite bearing) morphology (7, 8).

Lactacystin was initially tested in a variety of cellular and biochemical assays in the hope of shedding light on its mode of action. Lactacystin treatment was found to result in a transient intracellular increase in cAMP levels, peaking at 24 h after the start of treatment, which coincides with the window of maximal bipolar morphology (3). The kinetics of this accumulation of intracellular cAMP are very different from that observed with commonly used agents that increase intracellular cAMP, such as prostaglandin E_1 or adenosine and isobutylmethylxanthine, which result in peak levels within about 15–30 min of treatment in Neuro-2a cells (3). Since there is evidence that secreted proteases and protease inhibitors play roles in regulating neurite outgrowth and nerve regeneration (for review, see Ref. 9), lactacystin was tested for its ability to inhibit two extracellular serine proteases, thrombin and plasminogen activator, and found to have no effect on these proteases (3). Lactacystin was also shown to have no effect on protein kinase C activity, the inhibition of which had also been previously implicated in neuronal differentiation (3).

Identification of the Target of Lactacystin and Mechanism of Action

Based on studies of neurite outgrowth in Neuro-2a cells and inhibition of cell cycle progression in MG-63 human osteosarcoma cells using a series of analogs of lactacystin, it was determined that an electrophilic carbonyl at the C-4 position was required for activity (4). Of particular interest was the finding that a β-lactone related to lactacystin, *clasto*-lactacystin β-lactone (Fig. 1), retained activity, whereas the corresponding dihydroxy acid, formally the product of hydrolysis of the lactacystin thioester or the β-lactone, did not (4). The C-4 carbonyls of both the thioester and the β-lactone are reactive electrophiles, whereas the carboxylate of the dihydroxy acid is essentially inert to nucleophilic attack. These results suggested that the target may contain a reactive nucleophile that attacks the

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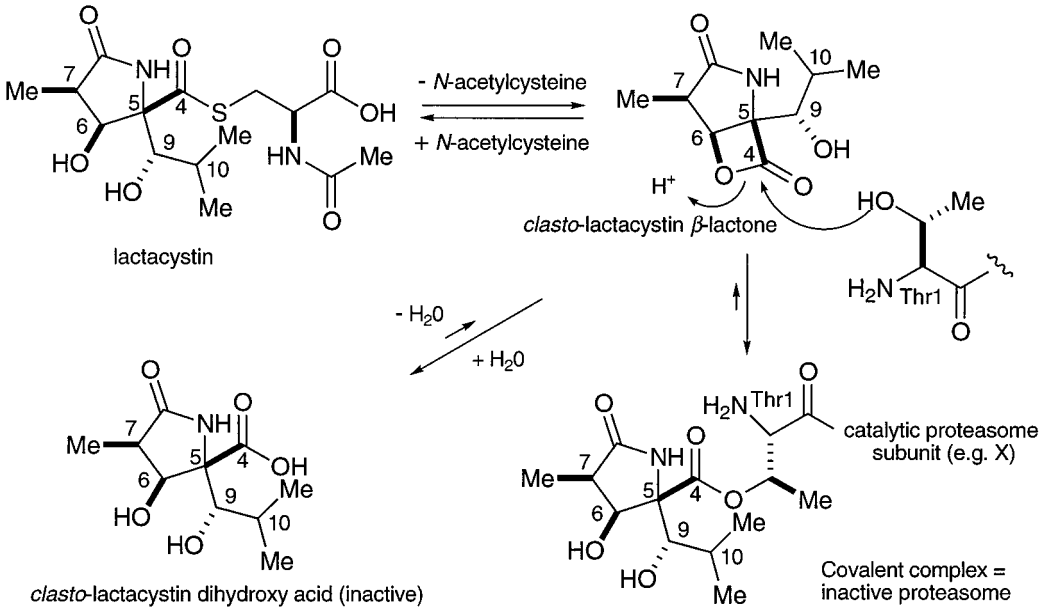

FIG. 1. **Putative mechanism of action of lactacystin (see Refs. 1, 4, and 10).** In addition to *N*-acetylcysteine, glutathione can also react with *clasto*-lactacystin β-lactone to form a thioester analogous to lactacystin, and there is some evidence that only the β-lactone, and not lactacystin itself, is capable of crossing the cell membrane (11). Of eukaryotic proteasome subunits, there is at present direct evidence only for amino-terminal threonine modification of mammalian subunit X (1) and its homolog in yeast, Pre2 (13), although other putative catalytic β-type subunits of the eukaryotic proteasome also appear capable of binding lactacystin (1, 2).

C-4 position and that this reaction may affect the target's activity. The acylation of the target by lactacystin was envisioned to occur possibly through formation of *clasto*-lactacystin β-lactone as an active intermediate, resulting from cyclization of the lactacystin with concomitant loss of the leaving group *N*-acetylcysteine.

The product of acylation was expected to be *clasto*-lactacystin covalently attached to its target. In order to identify the lactacystin-binding molecule, radioactive versions of lactacystin and the related β-lactone were synthesized (1), with the expectation that these compounds would serve as covalent affinity labels. This strategy led to the identification of the 20 S proteasome as the sole observed target of lactacystin (1). Lactacystin binds specifically and covalently to certain putatively catalytic subunits of the proteasome and inhibits multiple proteasome peptidase activities (1), apparently through the intermediacy of the β-lactone (10). In particular, the amino-terminal threonine of the mammalian proteasome subunit X (also known as MB1 or ϵ) is modified on its side chain hydroxyl by *clasto*-lactacystin β-lactone, forming an ester-linked *clasto*-lactacystin/proteasome subunit X adduct (Fig. 1).

The β-lactone can be formed spontaneously from lactacystin by an intramolecular nucleophilic attack of the C-6 hydroxyl on the C-4 carbonyl carbon with displacement of the *N*-acetylcysteine moiety as leaving group. The β-lactone can undergo spontaneous hydrolysis with ring opening to form *clasto*-lactacystin dihydroxy acid, an inactive species (Fig. 1). There appear to be two other major possible fates for the β-lactone, which occur only within the cell (unlike hydrolysis, which can occur inside or outside the cell). The first, as described, is the acylation of the proteasome, which involves a nucleophilic attack on the C-4 carbonyl carbon of the β-lactone with subsequent ring opening. The second is attack by the sulfhydryl of glutathione with ring opening to form a thioester adduct analogous to lactacystin; the resulting species does not directly react with the proteasome but can subsequently undergo recyclization to regenerate the active β-lactone (11). Not only is there evidence to suggest that the β-lactone is the active intermediate in these reactions, but there is also some evidence to suggest that only the β-lactone (and not lactacystin) can enter cultured mammalian cells (11).

Lactacystin as a Probe of Proteasome Function

Lactacystin and *clasto*-lactacystin β-lactone covalently modify two β-type subunits of proteasome purified from bovine brain (1). Lactacystin acylates the amino-terminal threonine on proteasome subunit X, the primary lactacystin-binding protein in bovine brain, as well as an internal residue on this protein, and it inhibits multiple proteasome peptidase activities (1). The amino-terminal threonine residue of this mammalian β-type subunit may therefore function as the catalytic nucleophile in the attack on the amide carbonyl carbon of the substrate, as in the archaeal and yeast proteasomes. Although lactacystin also binds at a much slower rate to proteasome subunit Z in bovine brain proteasome preparations, there is no evidence that the amino-terminal threonine of this protein is modified (1).

Lactacystin inhibits the three well characterized, distinct peptidase activities of the proteasome, chymotrypsin-like, trypsin-like, and caspase-like, the first two irreversibly and all at different rates (1). The β-lactone inhibits each of these activities 15–20 times faster than does lactacystin with the same rank order of effectiveness. The complete, irreversible inhibition of the chymotrypsin-like and trypsin-like activities in the bovine brain proteasome occurs in a time frame in which only subunit X is modified with a 2:1 lactacystin:protein stoichiometry at saturation. This suggests that in bovine brain proteasome covalent modification of subunit X alone accounts for the irreversible inhibition of two distinct peptidase activities. In addition to inhibiting the activities of the 20 S proteasome (1), lactacystin and the β-lactone also inhibit peptidolysis by the 26 S proteasome and the ubiquitin-dependent, proteasome-mediated degradation of short-lived and long-lived proteins in the cell (2). Lactacystin also blocks major histocompatibility complex class I antigen presentation (2, 12).

The recent crystal structure of the *Saccharomyces cerevisiae* 20 S proteasome with *clasto*-lactacystin bound (soaked into the

already crystallized 20 S proteasome for 6 h) reveals that only the side chain oxygen of the amino-terminal threonine of Pre2, the yeast homolog of the mammalian subunit X, is covalently bound to *clasto*-lactacystin (13). Although it is formally possible that the amino-terminal α-amino group of threonine might also attack the β-lactone, the fact that the modified amino-terminal threonine on subunit X is not blocked to Edman degradation (1) and the aforementioned structural data (13) suggest that the side chain hydroxyl is the final, if not only, nucleophile involved in this reaction. *clasto*-Lactacystin displays four hydrogen bonds with the main chain of Pre2, and the dimethyl group on C-10 of *clasto*-lactacystin projects into the hydrophobic S-1 pocket of Pre2 (13). These results are consistent with structure/activity relationships observed using lactacystin analogs (1, 4). The yeast proteasome subunit Pup1, a homolog of the mammalian subunit Z (the secondary lactacystin-binding protein in bovine brain) is not bound to *clasto*-lactacystin in the crystal structure (13).

Four additional β-type proteasome subunits, subunit Y and the γ-interferon-inducible subunits LMP2, LMP7, and MECL1, appear capable of binding lactacystin in other tissues, as determined by labeling with radioactive compound followed by two-dimensional gel electrophoresis (2). Therefore, six putatively catalytic proteasome subunits, falling into three groups of related and reciprocally regulated subunits, appear to be able to bind lactacystin, and none of the other subunits of the proteasome bind lactacystin (1, 2). However, there is as yet no evidence to suggest that the amino-terminal threonine residues of these other subunits are modified.

Lactacystin is highly specific for the proteasome, unlike peptide aldehyde inhibitors often used in proteasome studies. Rock *et al.* (14) reported the use of protease inhibitors to study the role of the proteasome in the degradation of various proteins; however, the peptide aldehyde inhibitors used in these studies were also shown to inhibit potently the cysteine proteases calpain and cathepsin B. As demonstrated in initial studies, lactacystin does not inhibit the serine proteases thrombin or plasminogen activator (3). Lactacystin was later shown to have no effect on any other protease tested, including the serine proteases trypsin and chymotrypsin and the cysteine proteases papain, calpain I, calpain II, and cathepsin B (1). Furthermore, lactacystin does not inhibit lysosomal protein degradation (2). Lactacystin therefore appears to interact with structural elements unique to certain β-type catalytic subunits of the proteasome. These results demonstrate that, unlike peptide aldehyde inhibitors often used in proteasome studies, lactacystin is highly specific for the proteasome and thus seems a more useful reagent for the study of the proteasome's involvement in biological processes.

Lactacystin has been used to study the degradation of proteins that normally reside in the endoplasmic reticulum. The cystic fibrosis transmembrane conductance regulator undergoes maturation in the endoplasmic reticulum, during which time much of the wild type and all of a mutant form of the cystic fibrosis transmembrane conductance regulator precursor protein are degraded; this degradation occurs at least in part by proteasome-mediated proteolysis of the cytoplasmic domain, as demonstrated using lactacystin and peptide aldehydes (14, 15). Other transmembrane proteins have also been found to be degraded in this fashion (16–19). The degradation of abnormal and unassembled proteins localized to the endoplasmic reticulum lumen is also dependent upon the function of the proteasome (20–23).

The Proteasome and Cell Fate

The proteasome appears to be the sole or at least main physiologically relevant target of lactacystin. There is a perfect correlation between the ability of a series of structural and stereochemical analogs of lactacystin to inhibit the proteasome's function and to compete with radiolabeled lactacystin for proteasome binding and their ability to induce neurite outgrowth in Neuro-2a cells and to inhibit cell cycle progression in MG-63 cells (1, 4). There is evidence that regulation of ubiquitination may be involved in determining cell fate (for review, see Ref. 24), and it is also possible that regulation of the proteasome itself may be involved in determining cell fate and morphology over the course of development.

The idea that the proteasome may be involved in neuronal differentiation is not entirely unprecedented, although the prior evidence to support this notion is not compelling. The peptide aldehyde protease inhibitor, *N*-benzyloxycarbonyl-Leu-Leu-leucinal, and certain other peptide aldehydes induce neurite outgrowth in PC12 rat pheochromocytoma cells, and purification of the *Z*-Leu-Leu-Leu-7-amido-4-methylcoumarin-degrading activity reveals the proteasome as the main target (25). Lactacystin exhibits dose-dependent toxic effects on PC12 cells; however, it does not appear to cause PC12 cells to differentiate (4). The differentiation of PC12 cells induced by such peptide aldehydes may be the result of inhibition of a protease or proteases other than the proteasome, such as the calpains, since these inhibitors are not specific for the proteasome. Alternatively, lactacystin may inhibit a different subset of the proteasome's peptidase activities than *N*-benzyloxycarbonyl-Leu-Leu-leucinal, and this may somehow abrogate neurite outgrowth in PC12 cells.

In addition to its effects on cell morphology in Neuro-2a cells, lactacystin has been found to affect cell fate in other cell types. Treatment with lactacystin blocks morphological changes of *Trypanosoma cruzi* at two separate stages in the life cycle of this intracellular protozoan parasite (26). Lactacystin has also been found to prolong survival of sympathetic neurons deprived of nerve growth factor, implying that apoptosis of these cells upon nerve growth factor deprivation involves the proteasome and that the proteasome acts upstream of the interleukin-1β-converting enzyme/Ced-3-like cysteine protease, a protease previously known to be involved in apoptosis (27). Lactacystin also prevents apoptosis in non-proliferating thymocytes, although prolonged exposure to proteasome inhibitors actually results in increased cell death in thymocytes even in the absence of other inducers of apoptosis (28). On the other hand, lactacystin treatment induces rather than inhibits apoptosis in human monoblast cells (29). Inhibition of the proteasome also results in apoptosis in proliferating rat fibroblast cells (but not in quiescent fibroblasts) and in both proliferating and non-proliferating, differentiated PC12 cells (30). Therefore, inhibition of the proteasome may result in cell survival or death, depending on the cells examined, and the response may be partly, though clearly not exclusively, determined by the proliferation state of the cell. On a more mundane level, the response may also simply depend on concentration of inhibitor used and the duration of its effects (and consequently on the level of inhibition of the proteasome over time). This is in basic agreement with findings that the cytostatic and neuritogenic effects of lactacystin and peptide aldehydes give way to cell death with increased dosage (4, 31).

The induction of bipolar morphology by lactacystin in Neuro-2a cells appears to involve inhibition of the chymotrypsin-like activity of the proteasome, since of all the peptidase activities of the proteasome only this activity seems to "antagonize" neurite outgrowth (31). The chymotrypsin-like activity may be responsible for either the proteolytic activation of a protein that antagonizes neurite outgrowth or the degradation of a protein that promotes neurite outgrowth. This activity

could either specifically cleave the protein substrate after a critical hydrophobic residue or else be a rate-limiting initial step in the degradation of the relevant protein substrate(s). The potential importance of the chymotrypsin-like activity as a modulator of cell fate is further demonstrated by the finding that inhibition of this activity in rat fibroblast and PC12 cells results in apoptosis (30).

Since phosphatase inhibitors appear to block lactacystin-induced bipolar neurite outgrowth (6), the immediate target of the proteasome may help regulate the phosphorylation state of critical proteins involved in the process of neurite outgrowth. For instance, the proteasome may directly or indirectly inactivate a phosphatase that promotes neurite outgrowth, or it may directly or indirectly activate a kinase that antagonizes neurite outgrowth. The inhibition of such activities by lactacystin's action on the proteasome could lead to decreased phosphorylation of certain proteins, resulting in neurite outgrowth in Neuro-2a cells.

Neurite outgrowth resulting from inhibition of the chymotrypsin-like activity of the proteasome could reflect a normal mechanism for the induction of a differentiated state in Neuro-2a cells, although this is not clear at present. It is possible that neurite outgrowth through lactacystin-mediated proteasome inhibition mimics a normal mechanism involved in cellular differentiation. A number of endogenous inhibitors of the proteasome have been discovered, some of which affect multiple activities, whereas others are more specific (32–34). Endogenous regulators of proteasome activities might influence cell physiology and fate.

Lactacystin is the only known truly specific inhibitor of the proteasome, and its utility has been demonstrated in a wide range of systems. Lactacystin provides a means to rapidly and specifically inactivate the proteasome and thereby provides a powerful new tool for exploring proteasome function. Lactacystin's action is analogous to the product of a temperature-sensitive allele, except that lactacystin can remove function more rapidly and without resulting in a stress response. Its use is highly portable, even to systems that are less tractable to molecular biology, such as tissues and multicellular organisms.

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