Specific inhibition of the chymotrypsin-like activity of the proteasome induces a bipolar morphology in neuroblastoma cells

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Specific inhibition of the chymotrypsin-like activity of the proteasome induces a bipolar morphology in neuroblastoma cells

Gabriel Fenteany and Stuart L Schreiber

Background: Lactacystin inhibits cell proliferation and induces a distinctive, predominantly bipolar (two-neurite-bearing) morphology in Neuro 2A murine neuroblastoma cells. It binds with high specificity to the multicatalytic 20S proteasome and inhibits at least three of its peptidase activities (chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing), each at a different rate, without inhibiting other known proteases. The chymotrypsin-like and trypsin-like activities of the proteasome are inhibited most rapidly, and irreversibly. In an effort to determine which of the peptidase activities needs to be inhibited for neurite outgrowth to occur, we treated Neuro 2A cells with peptide aldehydes that selectively inhibit different proteasome activities.

Results: Treatment with peptide aldehydes ending in a hydrophobic residue, all of which inhibit the chymotrypsin-like activity, results in a bipolar morphology in Neuro 2A cells, whereas treatment with a peptide aldehyde inhibitor of the trypsin-like activity does not lead to a detectable change in morphology. One of the inhibitors that induces neurite outgrowth has been previously shown to inhibit the chymotrypsin-like activity of the proteasome without inhibiting the other apparently distinct peptidase activities that cleave after neutral residues, the so-called 'branched chain amino acid preferring' (BrAAP) and 'small neutral amino acid preferring' (SNAAP) activities, or the peptidylglutamyl-peptide hydrolyzing (PGPH) activity.

Conclusions: The chymotrypsin-like activity appears to antagonize bipolar-type neurite outgrowth in Neuro 2A cells, while the trypsin-like, PGPH, BrAAP and SNAAP appear not to do so. Selective inhibition of a single peptidase activity, as opposed to general inhibition of the proteasome, appears sufficient to induce a specific cellular process. Selective inhibition might be useful in managing diseases where only one activity is involved without completely inhibiting the proteasome. It is also possible that endogenous regulators of the proteasome could affect cellular processes and that certain peptidase activities of the proteasome may have roles in specifying a given cell fate.

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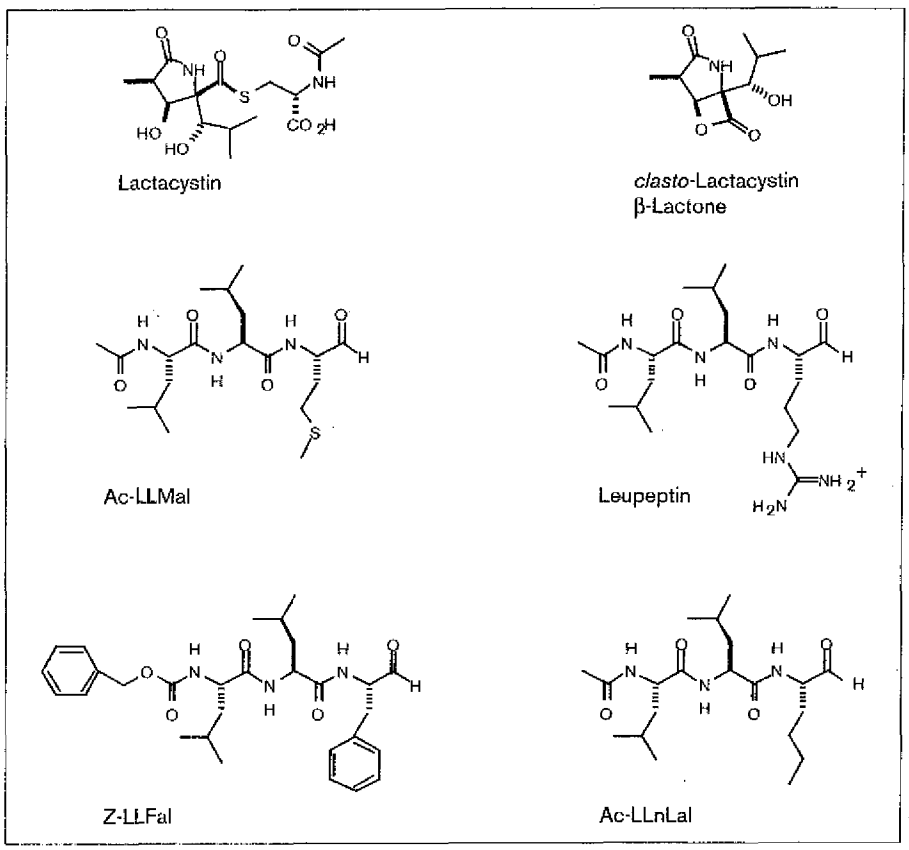
Introduction

Lactacystin (Fig. 1) inhibits cell proliferation and induces a predominantly bipolar (two-neurite-bearing) type of neurite outgrowth in Neuro 2A murine neuroblastoma cells. The neurite outgrowth reaches a maximum 16–32 h after treatment, with the cells becoming progressively more multipolar (multiple-neurite-bearing) and the neurites more branched as time goes on [1,2]. The bipolar morphology induced by lactacystin is distinct from the response to a variety of other common treatments leading to differentiation. For example, depriving Neuro 2A cells of serum or treating them with agents that raise intracellular cyclic adenosine monophosphate (cAMP) levels and thus activate protein kinase A both tend to induce a predominantly multipolar morphology [2–4], whereas treatment with retinoic acid, natural gangliosides or synthetic sialyl compounds tends to induce a more unipolar (single-neurite-bearing) morphology [3,4]. Induction of bipolar morphology in response to lactacystin is inhibited by phosphatase inhibitors, while branched neurite network formation around three days following lactacystin treatment is not inhibited by phosphatase inhibitors [5]. Lactacystin inhibits cell cycle progression in both the G1 and G2 phases of the cell cycle [2,6].

Using radioactively labeled derivatives of lactacystin, the target of lactacystin was purified from bovine brain extracts and identified as the 20S proteasome [7]. The proteasome is a high molecular weight, multicatalytic protease complex that is responsible for ubiquitin-dependent protein degradation within the cell (for reviews, see [8–11]). The proteasome is capable of both complete proteolysis of protein substrates and limited proteolytic processing leading to activation of protein function [12].

Figure 1



Structures of lactacystin and a related β-lactone, *clasto*-lactacystin β-lactone, and of the peptide aldehydes used in this study. Z-LLFal = *N*-benzyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalaninal; Ac-LLnLal = *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; Ac-LLMal = *N*-acetyl-L-leucyl-L-leucyl-L-methional; leupeptin = *N*-acetyl-L-leucyl-L-leucyl-L-argininal.

The proteasome consists of a multi-subunit catalytic core with a sedimentation coefficient of 20S (the 20S proteasome) that can degrade peptide and certain protein substrates independent of the presence of ATP and ubiquitin and 'capping' subunits that, together with the proteolytically active core, form the so-called 26S proteasome which is ATP- and ubiquitin-dependent.

Activities of the proteasome

The 20S proteasome is thought to have at least five distinct catalytic activities. The most firmly established and best characterized of these activities are named the chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing (PGPH) activities. These cleave on the carboxyl side of hydrophobic, basic and acidic residues, respectively. Based on inhibitor, activator, kinetic and genetic studies, these activities appear to arise from separate active sites. Inhibitor studies also provide evidence for the existence of two additional peptidase activities that cleave after amino acid residues with neutral side chains, which are apparently distinct from the chymotrypsin-like activity. One of these cleaves preferentially after amino acid residues with branched side chains (the BrAAP activity), the other between residues with the small neutral sidechains glycine and alanine (the SNAAP activity) [13]. There is evidence to suggest that the BrAAP activity is a major contributor to protein degradation by the proteasome [13].

Lactacystin binds to the 20S catalytic core of the proteasome and inhibits three of its major peptidase activities, the chymotrypsin-like, trypsin-like and PGPH activities, *in vitro* [7]. Lactacystin also binds to the proteasome *in vivo* (data not shown) and inhibits ubiquitin-mediated protein degradation, which occurs via the proteasome, *in vitro* and *in vivo* ([14,15], and J Hagler, O Rando, GF, SLS & T Maniatis, unpublished data). There is an excellent correlation between the ability of a series of analogs of lactacystin to compete with radiolabeled lactacystin for binding to the proteasome, inhibiting its function and their ability to induce neurite outgrowth in Neuro 2A cells [2,7]. Furthermore, lactacystin appears to be highly specific for the proteasome, since no other protease tested, including trypsin, chymotrypsin, papain, calpain and cathepsin B [7], thrombin and plasminogen activator [1], has been inhibited by lactacystin. It also does not inhibit lysosomal protein degradation [15]. Lactacystin is unique in terms of its proteasome

specificity and, as a result, has become a widely used probe of proteasome function.

The rates of inhibition of the chymotrypsin-like, trypsin-like and PGPH activities of the 20S proteasome by lactacystin are significantly different, and only the two most rapidly inhibited activities, chymotrypsin-like and trypsin-like, are irreversibly inhibited [7]. The chymotrypsin-like activity is inhibited ~20 times faster than the trypsin-like activity (and ~50 times faster than the PGPH activity). A β-lactone related to lactacystin, *clasto*-lactacystin β-lactone, formally the product of elimination of *N*-acetylcysteine from lactacystin, is 15–20 times more potent as an inhibitor of all three peptidase activities than lactacystin, yet is just as specific for the proteasome as is lactacystin [7]. The effects of lactacystin appear to be mediated through formation of the β-lactone as an active intermediate [16].

In 20S proteasome purified from bovine brain, the time-frame of inhibition of the two irreversibly inhibited activities is correlated with binding to one proteasome subunit, subunit X (also known as MB1 or ϵ), with which lactacystin forms a stable covalent complex in a 2:1 lactacystin:protein stoichiometry [7]. Lactacystin modifies the putatively catalytic amino-terminal threonine of the mature, proteolytically processed subunit X, as well as an internal residue [7]. Lactacystin also binds to a second proteasome subunit containing threonine at the amino terminus, now known as subunit Z (or MC14), in bovine brain proteasome preparations; however, binding to this second subunit occurs too slowly to account for inhibition of the chymotrypsin-like and trypsin-like activities, and there is no evidence that the amino-terminal threonine of this secondary lactacystin-binding protein is modified by lactacystin [7].

Studies in other systems provide further evidence that proteasome subunit X and its homologs may have a catalytic role. Subunits X and Z are β-type proteasome subunits, so termed because they are related to the β-subunit of the 20S proteasome from the archaeon *Thermoplasma acidophilum*. The *T. acidophilum* proteasome's autocatalytically processed β-subunit has an amino-terminal threonine that is critical for activity [17]. The X-ray crystal structure of the *T. acidophilum* proteasome with bound peptide aldehyde inhibitor also suggests that this residue is part of the active site and that the sidechain hydroxyl may be involved in nucleophilic attack on the amide carbonyl carbon of peptide substrates [18]. The yeast homolog of subunit X, Pre2, has an amino-terminal threonine as well, and this subunit is required for chymotrypsin-like activity and the degradation of ubiquitinated proteins [19]. The proteasome is therefore a member of the recently recognized amino-terminal nucleophile hydrolase family of proteins. These proteins all appear to be processed from precursor proteins to generate amino-terminal nucleophilic residues (threonine, serine or cysteine) and to have a similar fold in the active-site region, providing the capacity for nucleophilic attack and the possibility for autocatalytic processing [20–22].

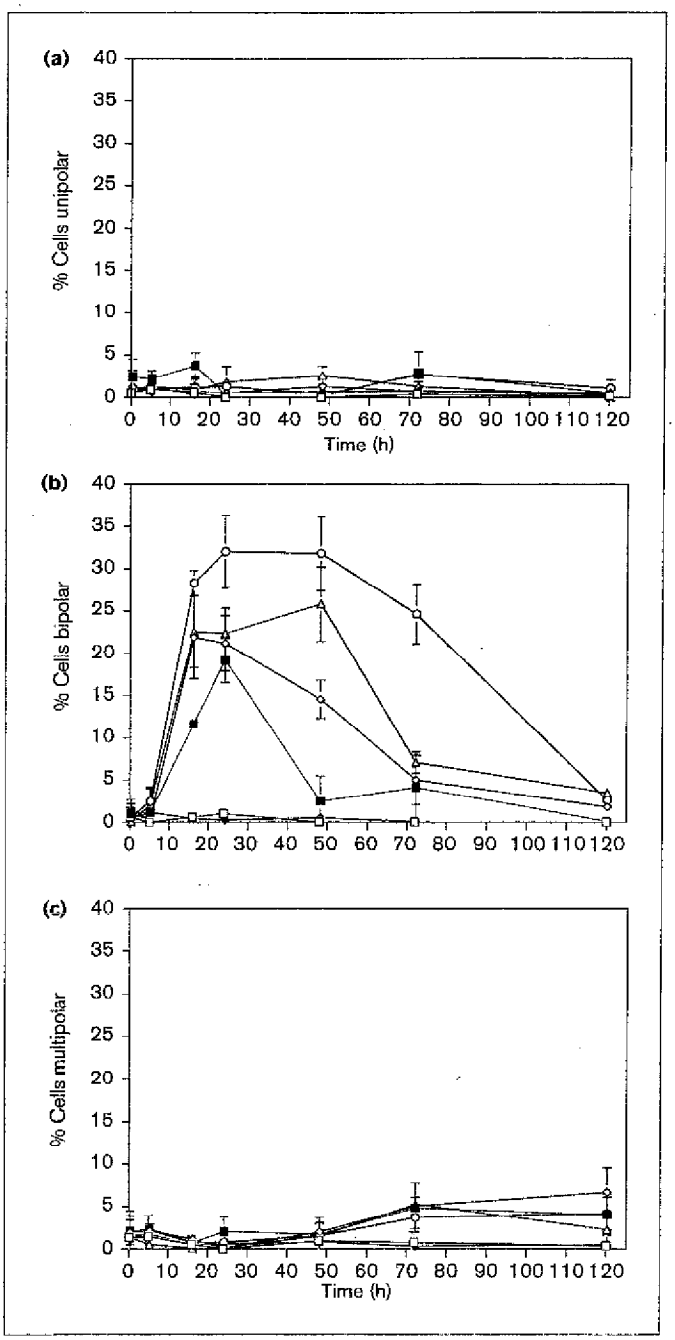
It is now well established that the proteasome is involved in the generation of antigens for class I major histocompatibility (MHC) presentation, and that two proteasome subunits encoded in the class II MHC region, LMP7 (a closely related homolog of subunit X) and LMP2 (related to another subunit termed Y or δ), may be important in the immune response (for reviews, see [9,10,23]). Mice with a targeted deletion of LMP7 exhibit reduced levels of MHC class I expression at the cell surface and inefficient antigen presentation [24]. LMP2 appears to be needed for presentation of certain class I MHC antigens to cytotoxic T lymphocytes [25]. Treatment of mammalian cells with γ-interferon alters the peptidase activities of the proteasome [26–29] and results in down-regulation of subunits X and Y and up-regulation of LMP7 and LMP2 [30–32]. A similar reciprocal relationship also exists between subunit Z and the related γ-interferon-inducible subunit MECL1 (also known as LMP10) [33,34]. These results imply that LMP2 and LMP7 (and possibly MECL1) are partly responsible for optimizing the proteasome for generation of antigenic peptides for class I MHC presentation by inducibly taking over the roles of their normally expressed cognates.

Lactacystin-binding subunits

It has recently been shown that, in addition to subunits X and Z, four other β-type subunits, Y, LMP2, LMP7 and MECL1, all of which have a threonine at their amino-terminus, are capable of binding lactacystin in lymphoblast cells [15], although there is as yet no evidence to suggest that the amino-terminal threonine residues of these other subunits are covalently modified. 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 [7,15].

Lactacystin is uniquely specific for the proteasome versus other proteases; however, it inhibits multiple proteasome peptidase activities [7]. In an attempt to determine which of the peptidase activities of the proteasome needs to be inhibited to induce a bipolar morphology in Neuro 2A cells, we treated cells with cell-permeable peptide aldehydes that inhibit subsets of the proteasome's peptidase activities. We found that treatment with inhibitors ending in hydrophobic residues resulted in bipolar-type neurite outgrowth that was very similar to that induced by lactacystin, while treatment with an inhibitor of the trypsin-like activity did not result in any detectable difference in morphology from untreated control cells. One of the inhibitors tested and found to induce neurite outgrowth, *N*-benzyloxycarbonyl-leucine-leucine-phenylalaninal (Z-LLFal), has been previously shown to inhibit the chymotrypsin-like activity of the

Figure 2



Percentage of Neuro 2A cells exhibiting unipolar **(a)**, bipolar **(b)** or multipolar **(c)** morphology as a function of time after the following treatments: 1 % DMSO (black, □); 10 μM lactacystin (purple, ◇); 1 μM Z-LLFal (red, ○); 5 μM Ac-LLnLal (green, △); 50 μM Ac-LLMal (light blue, ■); 1 mM leupeptin (pink, ◆). Each value is the mean of triplicate samples, and the bars correspond to ± the standard deviation.

proteasome without affecting the two other apparently distinct activities that cleave after neutral residues, BrAAP and SNAAP activities [13], or the PGPH activity [35]. Thus, inhibition of the chymotrypsin-like activity appears to be responsible for the induction of a bipolar morphology in Neuro 2A cells.

Results and discussion

Specificity of peptide aldehyde inhibitors

Z-LLFal has previously been shown to inhibit the chymotrypsin-like activity without detectably inhibiting the two other neutral amino acid preferring activities (BrAAP and SNAAP) [13] or the PGPH activity [35]. *N*-Acetyl-L-leucyl-L-leucyl-L-norleucinal (Ac-LLnLal) and *N*-acetyl-L-leucyl-L-leucyl-L-methional (Ac-LLMal) also end in hydrophobic residues and selectively inhibit the chymotrypsin-like activity of the proteasome in *in vitro* studies [35,36]. However, unlike Z-LLFal, these two compounds also appear to inhibit, at least partially, the BrAAP and SNAAP activities [13]. Leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal) inhibits the trypsin-like activity of the proteasome with a K_i of about 1 μM *in vitro* [37] without detectably inhibiting any other proteasome peptidase activity [13,37,38]. Unfortunately, currently available inhibitors of the BrAAP, SNAAP and PGPH activities are not very selective.

Z-LLFal inhibits the proteasome's chymotrypsin-like activity with a K_i of 0.46 ± 0.14 μM (mean value ± standard error) *in vitro* [35] , which is ~100-fold lower than its K_i for the trypsin-like activity, but does not detectably inhibit the PGPH, BrAAP or SNAAP activities [13,35]. Ac-LLnLal inhibits the chymotrypsin-like activity of the bovine pituitary 20S proteasome *in vitro* with a K_i of 5.7 ± 0.16 μM [35], while Ac-LLMal inhibits this activity with a K_i of 33.4 ± 6.8 μM [35]. Rock *et al.* reported lower K_i values of 0.14 μM for Ac-LLnLal and 1.0 μM for Ac-LLMal for the rabbit muscle 20S proteasome, and K_i values of 0.67 μM and 28 μM, respectively, for rabbit muscle 26S proteasome [36]. Both Ac-LLnLal and Ac-LLMal also inhibit the trypsin-like and PGPH activities of the proteasome, though with higher K_i values than for the chymotrypsin-like activity [35,36], and at least partially inhibit the BrAAP and SNAAP activities of the proteasome [13]. It therefore appears that, of the peptide aldehydes ending in hydrophobic residues, Z-LLFal is by far the most specific inhibitor of the chymotrypsin-like activity.

Peptide aldehydes ending in either hydrophobic or basic residues appear cell-permeable, although a higher concentration of a peptide aldehyde ending in a basic residue seems necessary to inhibit protein degradation. Studies using radioactively labeled leupeptin demonstrate that leupeptin can penetrate into the cytosol of cultured cells and inhibit the calcium-activated neutral protease calpain [39]. Leupeptin has been shown to inhibit 80–85 % of lysosomal and ~15 % of non-lysosomal protein degradation in cultured cells at a concentration of 0.3 mM [40]. The peptide aldehydes Ac-LLnLal and Ac-LLMal have also been shown to inhibit protein degradation in cultured

cells [36]. It is reasonable to presume that Z-LLFal, which is hydrophobic and highly active in inducing bipolar morphology, can also penetrate the cell.

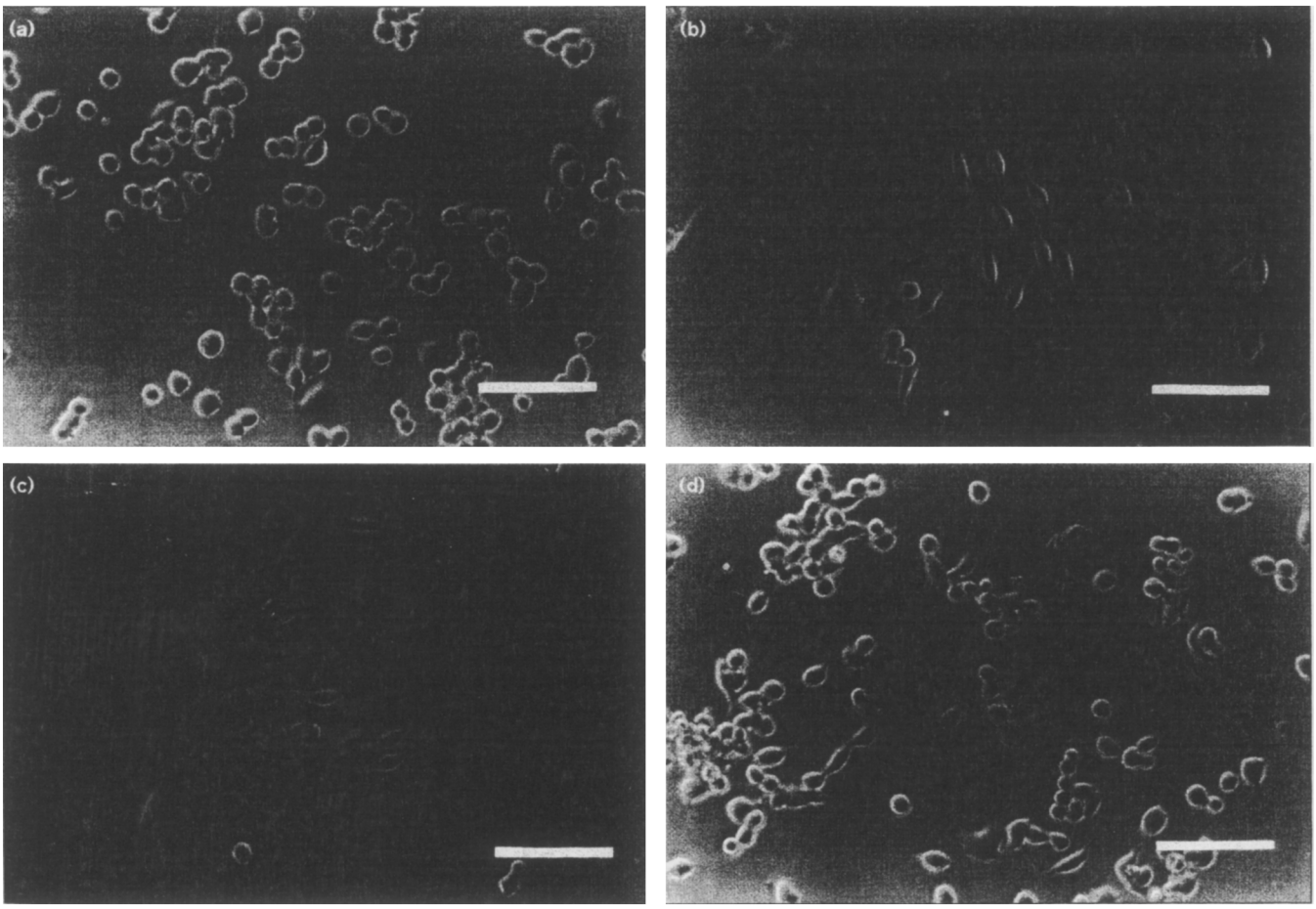
Effects of peptide aldehyde inhibitors

Treatment of Neuro 2A neuroblastoma cells with any one of the peptide aldehydes that end with hydrophobic residues (Z-LLFal, Ac-LLnLal and Ac-LLMal) results in reduced cell proliferation and the induction of a bipolar morphology (Figs 2b,3), in which two neurites extend from opposite sides of the cell body. The cells become progressively more multipolar with prolonged exposure to these peptide aldehydes as with lactacystin (Fig. 2c), while the proportion of unipolar cells in each case is low at every time point (Fig. 2a). The morphology induced by treatment with Z-LLFal (or by Ac-LLnLal or Ac-LLMal) is indistinguishable from that induced by lactacystin (Fig. 3).

In contrast, treatment with leupeptin, which inhibits the trypsin-like activity of the proteasome without inhibiting any of its other peptidase activities [13,37,38], does not lead to any detectable difference in cell morphology from untreated control cells even at the elevated concentration of 1 mM (Figs 2,3). Although it is not clear at this point how important the trypsin-like activity of the proteasome is in determining overall rates of protein breakdown, our results show that inhibition of this activity — whatever its normal physiological function — does not result in neurite outgrowth. Furthermore, since leupeptin inhibits the cytosolic protease calpain [39] and many extracellular and lysosomal proteases [41,42], it is unlikely that inhibition of such proteases could mediate the induction of bipolar-type neurite outgrowth in Neuro 2A cells.

Inhibition of the chymotrypsin-like activity of the proteasome, without inhibition of the other two neutral amino acid preferring activities, appears to be sufficient to induce neurite outgrowth in Neuro 2A cells, since Z-LLFal inhibits only the chymotrypsin-like activity of the three neutral amino acid preferring activities [13]. Z-LLFal does

Figure 3



Photomicrographs of Neuro 2A cells 24 h after treatment with: **(a)** 1 % DMSO; **(b)** 10 μM lactacystin; **(c)** 1 μM Z-LLFal; **(d)** 1 mM leupeptin. The length of the bars corresponds to 100 μm.

not inhibit the PGPH activity and significantly inhibits the trypsin-like activity only at elevated concentrations [35], while treatment with leupeptin, which only inhibits the trypsin-like activity of the proteasome's activities, does not lead to neurite outgrowth (Figs 2,3). Taken together with previous results using the proteasome-specific inhibitor lactacystin [2,7], these results imply that inhibition of the chymotrypsin-like activity is responsible for the neurite outgrowth that results from proteasome inhibition. Since it appears not to be necessary to inhibit the other peptidase activities of the proteasome for neurite outgrowth to occur, only the chymotrypsin-like activity appears to antagonize neurite outgrowth.

Z-LLFal is the most potent inducer of neurite outgrowth on the basis of the concentration required for activity, followed by Ac-LLnLal and then Ac-LLMal (Fig. 2 and data not shown). This order of effectiveness also corresponds to the order of potency of these peptide aldehydes for inhibition of the proteasome's chymotrypsin-like activity [35,36]. Since the more potent inducers of neurite outgrowth are also the more potent inhibitors of the chymotrypsin-like activity, the neuritogenic potency of a peptide aldehyde may be determined by its effectiveness as an inhibitor of the proteasome's chymotrypsin-like activity.

Relevance of the cellular response

Bipolar-type neurite outgrowth in response to lactacystin or the peptide aldehydes ending in hydrophobic residues does not appear to be a non-specific cellular response to stress. Treatment of Neuro 2A cells with cytotoxic concentrations of various compounds leads to rounding up of cells and ultimately to detachment from the substratum without any induction of bipolar morphology at any point (data not shown). Although elevated concentrations of lactacystin and the neuritogenically active peptide aldehydes are cytotoxic, lower concentrations sufficient to induce bipolar morphology do not result in rounding up and death of cells (Fig. 2 and data not shown). In contrast, sub-cytotoxic concentrations of a variety of other growth-inhibitory compounds do not result in bipolar-type neurite outgrowth (data not shown). Lactacystin and the neuritogenically active peptide aldehydes examined are alone among inhibitors of cell proliferation and inducers of differentiation in that treatment results in a mainly bipolar type of neurite outgrowth. While inhibition of the chymotrypsin-like activity of the proteasome may represent a specific and limited kind of cellular stress, the response of the cells is unique in terms of the predominant morphology induced. It is therefore reasonable to assume that the chymotrypsin-like activity of the proteasome is required either to destroy a protein that directly or indirectly promotes neurite outgrowth, or to activate a protein that antagonizes neurite outgrowth.

Two of the peptide aldehydes that induce neurite outgrowth, Ac-LLnLal and Ac-LLMal, have also been shown to inhibit cathepsin B and calpain [36]. Lactacystin, however, inhibits neither of these proteases [7]. It is, of course, possible that bipolar-type neurite outgrowth in response to the peptide aldehydes occurs by a different mechanism than with lactacystin. But as the observations show that the morphology of the cells treated with these compounds is comparable to that of cells treated with lactacystin, and as neuritogenic potency of these compounds correlates with their ability to inhibit the proteasome's chymotrypsin-like activity, it seems more likely that the mechanism involved is similar.

Certain tripeptide aldehydes, including *N*-benzyloxycarbonyl-Leu-Leu-leucinal (Z-LLLal), induce neurite outgrowth in PC12 rat pheochromocytoma cells, and purification of the *N*-benzyloxycarbonyl-Leu-Leu-Leu-7-amido-4-methylcoumarin-degrading activity reveals that the proteasome is the main target [43]. Lactacystin, however, does not have any detectable neuritogenic effect on PC12 cells but does exhibit a dose-dependent toxic effect [2]. It is therefore possible that the actual target that mediates neurite outgrowth in PC12 cells in response to Z-LLLal is a protease other than the proteasome. It is also possible that lactacystin, though uniquely specific for the proteasome versus other proteases, may inhibit a broader range of the proteasome's peptidase activities than does Z-LLLal. For example, if the proteasome normally activates a protein that antagonizes neurite outgrowth, a second proteasome activity might be required in PC12 cells to ensure its continued activity. If both activities are inhibited by lactacystin, but only the first is inhibited by Z-LLLal, then Z-LLLal, but not lactacystin, would induce neurite outgrowth in these cells.

Inhibition of the proteasome's chymotrypsin-like activity may promote bipolar neurite outgrowth in Neuro 2A cells by either preventing the degradation of a protein that promotes bipolar neurite outgrowth or preventing the proteolytic activation of a protein that antagonizes bipolar neurite outgrowth. The results presented here suggest that the chymotrypsin-like activity is involved, directly or indirectly, in the degradation or proteolytic activation of the putative substrate(s) of the proteasome that either positively or negatively regulates the induction of a bipolar morphology. There are two simple explanations for the importance of the chymotrypsin-like activity in the regulation of neurite outgrowth, although more complicated models can also be envisioned. The chymotrypsin-like activity may be solely responsible for the relevant cleavage of the substrate, most plausibly leading to an activation of the substrate's function. Alternatively, the chymotrypsin-like protease activity may be required for the first step in the processive degradation of the substrate involved in neurite outgrowth. In the first case, it is the

inhibition of a specific cleavage(s) of the substrate by the chymotrypsin-like that leads to neurite outgrowth. In the second case, inhibition of the chymotrypsin-like activity prevents or significantly retards proteolysis of the substrate by not only the chymotrypsin-like activity itself but also the other activities. This could prevent either an inactivating proteolysis of the substrate by any site or an activating cleavage by another site. In this case, it is necessary to suppose that inhibition of the trypsin-like activity does not prevent proteolysis of the substrate by the chymotrypsin-like or other activities.

It is not clear whether neurite outgrowth in response to inhibition of the chymotrypsin-like activity of the proteasome reflects a normal, endogenous mechanism for the induction of differentiation or not. Endogenous modulators of the proteasome's activities have been discovered (for review, see [8]). Some affect multiple peptidase activities, while others appear more specific. It is possible that such regulators are expressed in a cell-specific manner and help to determine the fate of a cell during development.

Significance

The proteasome's chymotrypsin-like activity, but not its trypsin-like, PGPH, BrAAP or SNAAP activities, appears to antagonize neurite outgrowth in Neuro 2A neuroblastoma cells. Other cellular processes may also be promoted or inhibited by selective inhibition of a given peptidase activity of the proteasome. Such selective inhibition may be useful not only in research but also in managing disease where one proteasome activity is primarily involved. This might reduce the toxicity expected to result from the general inhibition of the proteasome. The results reported herein demonstrate that modulation of a specific proteolytic activity can be responsible for a cellular phenotype. Unique peptidase activities of the proteasome may have roles in determining the physiology and fate of a cell.

Materials and methods

Neuro 2A cells were cultured in minimal essential medium containing 10 % fetal bovine serum, L-glutamine and non-essential amino acids (complete medium). The cells were plated to a density of ~1×10^4 cells per well in 12- or 24-well polystyrene dishes in a volume of 1 ml of complete medium and grown for 24 h prior to treatment. All the peptide aldehydes were purchased from BACHEM Bioscience Inc. (King of Prussia, PA), except for Z-LLFal, which was prepared by the SynPep Corporation (Dublin, CA). Short-term stock solutions of peptide aldehydes were stored as high concentration solutions in dimethyl sulfoxide at −20° C, thawed prior to use and added to cell culture dishes. Cell density and the percentage of cells exhibiting unipolar, bipolar or multipolar morphology were determined using phase-contrast microscopy at various time points following treatment.

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