Proteasomes are a target of the anti-tumour drug vinblastine

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Proteasomes, the proteolytic machinery of the ubiquitin/ATP-dependent pathway, have a relevant role in many processes crucial for cell physiology and cell cycle progression. Proteasome inhibitors are used to block cell cycle progression and to induce apoptosis in certain cell lines. Here we examine whether proteasomal function is affected by the anti-tumour drug vinblastine, whose cytostatic action relies mainly on the disruption of mitotic spindle dynamics. The effects of vinblastine on the peptidase activities of human 20 S and 26 S proteasomes and on the proteolytic activity of 26 S proteasome were assessed in the presence of specific fluorogenic peptides and ^{125}I-lysozyme–ubiquitin conjugates respectively. The assays of ubiquitin–protein conjugates and of inhibitory κBα (IκBα), which are characteristic intracellular proteasome substrates, by Western blotting on lysates from HL60 cells incubated with or without vinblastine, illustrated the effects of vinblastine on proteasomes *in vivo*. We also evaluated the effects of vinblastine on the signal-induced degradation of IκBα. Vinblastine at 3–110 μM reversibly inhibited the chymotrypsin-like activity of the 20 S proteasome and the trypsin-like and peptidyl-glutamyl-peptide hydrolysing activities of both proteasomes, but only at 110 μM vinblastine was the chymotrypsin-like activity of the 26 S proteasome inhibited; furthermore, at 25–200 μM the drug inhibited the degradation of ubiquitinated lysozyme. In HL60 cells exposed for 6 h to 0.5–10 μM vinblastine, the drug-dose-related accumulation of polyubiquitinated proteins, as well as that of a high-molecular-mass form of IκBα, occurred. Moreover, vinblastine impaired the signal-induced degradation of IκBα. Cell viability throughout the test was approx. 95 %. Proteasomes can be considered to be a new and additional vinblastine target.

Key words: cytostatic agents, leukaemic cells, proteolysis, ubiquitin conjugates.

INTRODUCTION

In eukaryotic cells most proteins are degraded in the cytoplasmic and nuclear compartments through the ubiquitin/ATP-dependent pathway, which is involved in a substantial number of cellular events. This process is based on two discrete steps: the covalent attachment of multiple ubiquitin molecules to the target protein and the degradation of the polyubiquitinated protein by the 26 S proteasome [1–3]. The latter is a multisubunit particle resulting from the ATP-dependent association of the 20 S proteasome, constituting the catalytic core, and the 19 S complex, acting as the regulatory component.

The 20 S proteasome derives from the assembly of 14 pairs of subunits arranged in four rings. The two external rings are composed of α subunits and the two inner ones of β subunits, giving rise to a hollow cylinder-shaped structure. Eukaryotic 20 S proteasome presents three major activities, namely chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolysing (PGPH) activities, as defined by the character of the P1 cleavage sites of fluorogenic substrates. Only three of the 20 S proteasome subunits (β_1, β_2 and β_5) are proteolytically active and use the hydroxyl group of their N-terminal threonine as the catalytic nucleophile in the cleavage of the peptide bond [4,5]. Mutational studies in yeast have shown that β_1 is responsible for the PGPH activity, β_2 for the trypsin-like activity and β_5 for the chymotrypsin-like activity [6]. The isolated 20 S proteasome acquires significant proteolytic activity only when associated with the 19 S complex, which gives it specificity for polyubiquitinated proteins, ATP dependence and enhanced peptidase activity and is believed to be responsible for the unfolding of the substrate protein and its entry into the catalytic chamber [4,5]. This complex consists of at least 15 subunits, classified into ATPases and non-ATPases [5,7,8], set to form two caps, one at each end of the cylinder.

The 20 S and 26 S proteasomes have a relevant role in maintaining the structural and metabolic integrity of the cell, being responsible for the rapid degradation of old or damaged proteins in addition to rate-limiting enzymes, transcriptional regulators, cell-cycle proteins and tumour suppressors, whose short half-life is crucial for cellular physiology and proper cell cycle progression [3,4,9–12]. In this view it is of interest that proteasome inhibitors are described to block cell cycle progression and to induce apoptosis in certain cell lines [13].

In the present study we were interested in exploring whether vinblastine, a *Vinca* alkaloid used largely as an anti-mitotic agent, also affects proteasomal activity. If this were so, proteasomes would be a new vinblastine target in addition to the mitotic spindle, whose dynamics it disrupts sharply by microtubule stabilization [14–17], thus improving knowledge of the mechanism of action of this drug. It is noteworthy that the anti-tumour drug aclacinomycin A has been shown to inhibit the degradation of ubiquitinated proteins by the selective inhibition of the chymotrypsin-like activity of the proteasomes [18].

Experiments were therefore devised to determine whether vinblastine inhibits the catalytic activity of purified 20 S and 26 S proteasomes, and whether, in cells cultured with the drug, high-

Abbreviations used: AMC, 7-amino-4-methylcoumarin, IκBα, inhibitory κBα; βNA, β-naphthylamide; PGPH, peptidyl-glutamyl-peptide hydrolysing; Suc, succinyl; Z, benzyloxycarbonyl.

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molecular-mass ubiquitin–protein conjugates accumulate and signal-induced degradation of inhibitory κB (IκB) is decreased, because these two events are hallmarks of proteasome inhibition *in vivo* [19,20].

MATERIALS AND METHODS

Materials

The fluorogenic peptides Suc-LLVY-AMC, Z-LLR-AMC and Z-LLE-βNA (in which single-letter amino acid codes are used and AMC stands for 7-amino-4-methylcoumarin, βNA for β-naphthylamide, Suc for succinyl and Z for benzyloxycarbonyl) were supplied by Bachem Feinchemikalien AG (Bubendorf, Switzerland); chromatographic supports, ECL® plus and Hyperfilm autoradiography films were from Amersham-Pharmacia Biotech (Milan, Italy); Centricon 30 was from Amicon (Millipore Corp., Milan, Italy), vinblastine was from Fluka (Milan, Italy), and PMA and ionomycin were from Sigma (Milan, Italy) and Calbiochem (Milan, Italy) respectively. Buffers and other reagents were of the highest quality from Sigma. ^{125}I-Lysozyme–ubiquitin conjugates were a gift from W. Dubiel (Humboldt University, Berlin, Germany). Antisera against human 20 S proteasome and 19 S complex subunits were from Affiniti (Exeter, Devon, U.K.), monoclonal antibodies against ubiquitin and actin, and polyclonal antibody against IκBα, were from Santa Cruz Biotechnology (Heidelberg, Germany). Calpain was kindly provided by F. Salamino (University of Genova, Genova, Italy).

Purification of 20 S and 26 S proteasomes

The two proteasomes were purified to homogeneity from 2 units of outdated human red blood cells, in accordance with previously published methods [21] with minor modifications [22].

Enzyme assays

The chymotrypsin-like, trypsin-like and PGPH activities of the 20 S and 26 S proteasomes were evaluated by a spectrofluorimetric assay with the fluorogenic peptides Suc-LLVY-AMC, Z-LLR-AMC and Z-LLE-βNA as substrates, as described by Dubiel et al. [23].

The proteolytic activity of the 26 S proteasome was assessed in the presence of ^{125}I-lysozyme–ubiquitin conjugates by the method of Hough et al. [24].

The activities of commercial preparations of trypsin and chymotrypsin were assayed in the presence of the fluorogenic peptides used in this study for the corresponding proteasome activities, and that of papain in the presence of benzoyl-arginyl ethyl ester [25]. The activity of calpain from human red blood cells was assayed as described by Michetti et al. [26].

Enzyme inhibition assays

Vinblastine was dissolved in methanol at a final concentration of 11 mM, aliquoted and stored at -20 °C. Immediately before use, aliquots were dried in a vacuum centrifuge and redissolved in an equal volume of DMSO. To evaluate the effects of the drug on the peptidase activities of the 20 S and 26 S proteasomes and on the proteolytic activity of the 26 S proteasome, aliquots of the two proteasomes were incubated in the presence of fixed substrate (fluorogenic peptides or ubiquinated radioiodinated lysozyme) and increasing vinblastin concentrations. The final drug concentrations used in inhibition assays are reported in the Tables.

Kinetic analysis of enzyme inhibition

The initial velocity of the hydrolytic reaction of the above-reported fluorogenic peptides by the 20 S and 26 S proteasomes was plotted against substrate concentration with and without vinblastine at increasing concentrations. The experimental results were fitted, by non-linear regression analysis [27], to the general equations for competitive, non-competitive and uncompetitive inhibition [28].

Non-denaturing PAGE and substrate overlay

At different purification stages, proteins were analysed by non-denaturing PAGE. Non-denaturing gels consisted of a 2.5 % (w/v) stacking gel and a 4.5 % (w/v) running gel cast in 90 mM Tris/HCl (pH 8.3)/1.6 mM boric acid/0.08 mM EDTA. Samples were subjected to electrophoresis for 300 V·h at 5 °C. Resolved proteins were identified from their peptidase activity by the gel overlay technique as described by Hoffman et al. [21] and then stained with Coomassie Brilliant Blue.

SDS/PAGE

Aliquots of purified proteasomes or slices excised from non-denaturing gels encompassing the active regions were boiled in reducing sample buffer and analysed on 4 % (w/v) (stacking) and 9 % or 12 % (w/v) (resolving) gels by the method of Laemmli [29]. Proteins were revealed with Coomassie Brilliant Blue and quantified by scanning densitometry with a Bio-Rad GS-690 imaging densitometer.

Western immunoblotting

Proteins resolved by SDS/PAGE were electrotransferred to PVDF membranes by the method of Towbin et al. [30] and Piccinini et al. [31]. Immunodecorated protein bands were detected with an enhanced chemiluminescence system (Amersham) by using Hyperfilm autoradiography films. Immunoreactive bands were quantified densitometrically as described above.

Protein quantification

The protein content of samples separated in each purification step was determined by the method of Bradford [32], with BSA as a standard.

Cell culture

The effect of vinblastine on intact cells was evaluated on human leukaemic HL60 cells, cultured at 2×10^6 cells/ml on six-well plates in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum.

Detection of ubiquitin–protein conjugates and of polyubiquitinated IκBα

Cells were treated for 6 h with vinblastine at increasing concentrations (0.5–10 μM) or with the drug vehicle or with 10 μM lactacystin as a positive control. After incubation, cells were washed with PBS, lysed with 100 μl of non-reducing sample buffer and centrifuged at 100000 ***g*** for 60 min. At the end of the incubation, cell viability was assessed by the exclusion of Trypan Blue.

Clear supernatants from cell lysates were diluted with 1 vol. of reducing sample buffer, boiled for 4 min and subjected to SDS/PAGE (20 μg of protein per lane) followed by Western blotting with antibody against ubiquitin or IκBα. Immuno-

decorated protein bands were detected and quantified as described above.

Effects of vinblastine on signal-induced degradation of IκBα

HL60 cells pretreated (or not) for 6 h with the drug (1, 5 and 10 μM) or with the drug vehicle or with 10 μM lactacystin were stimulated for 2 h with 20 ng/ml PMA plus 3.4 μg/ml ionomycin [33] and then harvested, washed with PBS and processed as described above, with a polyclonal antibody against IκBα.

RESULTS

Proteasomes 20 S and 26 S were purified to homogeneity and identified on the basis of their multicatalytic activity (Table 1), electrophoretic mobility on non-denaturing gels (Figure 1) and immunoreactivity (Figure 2).

As an initial experiment, the two proteasomes were incubated with and without 3–110 μM vinblastine, a concentration range that includes the values described to be present in HL-60 cells cultured with the drug [34–36]. Vinblastine progressively inhibited the chymotrypsin-like, trypsin-like and PGPH activities of the 20 S proteasome (Table 2). It also inhibited the trypsin-like and PGPH activities of the 26 S proteasome and, at 110 μM, the chymotrypsin-like activity (Table 3). In all cases inhibition was reversed by dialysis (results not shown). To characterize the drug-determined inhibition further, the initial velocities of the

Table 1 Multicatalytic activity of purified 20 S and 26 S proteasomes

The assay was performed at 37 °C in samples that contained, in a final volume of 100 μl, 65 μM fluorogenic peptide, 25 mM Tris/HCl, pH 7.7, 4 mM NaCl, 5 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2.5 mM $MgCl_2$ and 10% (v/v) glycerol in the presence or absence of 1 mM ATP. The reaction was started by the addition of 1.45 and 0.2 μg of 20 S and 26 S proteasomes respectively. At defined times the reaction was quenched with 200 μl of cold ethanol. In control samples, run in parallel, the proteasomes were added at the end of the incubation, immediately before ethanol. Samples were centrifuged for 1 min at 10000 ***g*** and then evaluated for ethanol-soluble fluorescence on a PerkinElmer fluorimeter with excitation wavelengths of 380 and 335 nm and emission wavelengths of 440 and 410 nm for AMC and βNA respectively. Fluorescence was quantified with AMC and βNA as a standard. Values are means ± S.D. for three separate experiments. Activities are in pmol of AMC or βNA released/min per μg of protein.

	Activity (pmol/min per μg)		
Proteasome	Chymotrypsin-like	Trypsin-like	PGPH
20 S	5.3 ± 0.5	7.5 ± 0.4	9.5 ± 0.5
26 S	145.0 ± 5.0	37.9 ± 3.5	28.5 ± 1.5

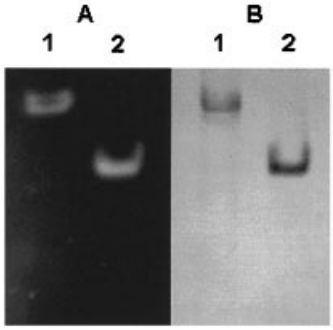

Figure 1 Resolution of the purified 20 S and 26 S proteasomes by non-denaturing PAGE

Samples of purified 26 S (lanes 1) and 20 S (lanes 2) proteasomes were subjected to electrophoresis for 300 V·h at 5 °C on a 4.5% (w/v) (resolving) non-denaturing gel. (**A**) Peptidase activity detected by gel overlay with 250 μM Suc-LLVY-AMC in 50 mM Tris/HCl (pH 7.5)/5 mM $MgCl_2$/10 mM KCl/1 mM dithiothreitol/1 mM ATP. Gels were incubated for 10 min at 37 °C, transilluminated with UV and photographed with a Polaroid Camera equipped with a 475 nm interference filter. (**B**) The same gel after staining with Coomassie Blue.

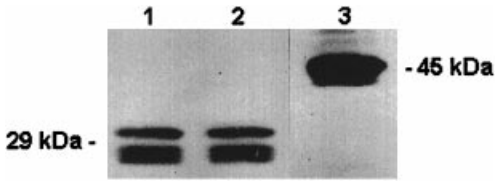

Figure 2 Western blotting of purified 20 S and 26 S proteasomes

Lanes 1 and 2 show 20 S and 26 S proteasomes immunodecorated with anti-human 20 S proteasome polyclonal antibody; lane 3 shows 26 S proteasome probed with anti-(human MSS1) antiserum. PVDF or nitrocellulose membranes were blocked overnight at 4 °C in 5% (w/v) non-fat dried milk powder in PBS containing 0.5% (v/v) Tween 20 (PBST). They were then incubated with primary antibodies in PBST/5% non-fat dried milk powder for 1 h at room temperature. Incubation with secondary peroxidase-linked anti-rabbit antibody was performed under the same conditions.

Table 2 Effect of vinblastine on the peptidase activities of the 20 S proteasome

The activity assays were performed as described in Table 1. Vinblastine was added to obtain the concentrations shown. In control reactions an equal volume of DMSO was added. Values are means ± S.D. for three separate experiments. Activities are in pmol of AMC or βNA released/min per μg of protein.

	Activity (pmol/min per μg)		
Vinblastine (μM)	Chymotrypsin-like	Trypsin-like	PGPH
0	5.3 ± 0.4	7.0 ± 0.5	9.5 ± 0.8
3.4	3.1 ± 0.3	3.8 ± 0.4	8.9 ± 0.6
6.8	2.8 ± 0.2	2.9 ± 0.2	8.1 ± 0.7
13.6	2.4 ± 0.1	2.3 ± 0.3	6.4 ± 0.6
27.2	2.0 ± 0.1	1.6 ± 0.2	2.5 ± 0.3
54.4	1.8 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
110	1.7 ± 0.2	1.6 ± 0.1	1.1 ± 0.1

Table 3 Effect of vinblastine on the peptidase activities of the 26 S proteasome

The activity assays were performed as described in Table 1. Vinblastine was added to obtain the concentrations shown. In control reactions an equal volume of DMSO was added. Values are means ± S.D. for three separate experiments. Activities are in pmol of AMC or βNA released/min per μg of protein.

	Activity (pmol/min per μg)		
Vinblastine (μM)	Chymotrypsin-like	Trypsin-like	PGPH
0	145 ± 5	38 ± 3	29 ± 2
3.4	149 ± 5	27 ± 2	24 ± 1
6.8	159 ± 8	20 ± 3	22 ± 1
13.6	154 ± 1	16 ± 2	17 ± 2
27.2	155 ± 5	11 ± 1	13 ± 1
54.4	132 ± 4	10 ± 1	9 ± 1
110	99 ± 5	9 ± 1	7 ± 1

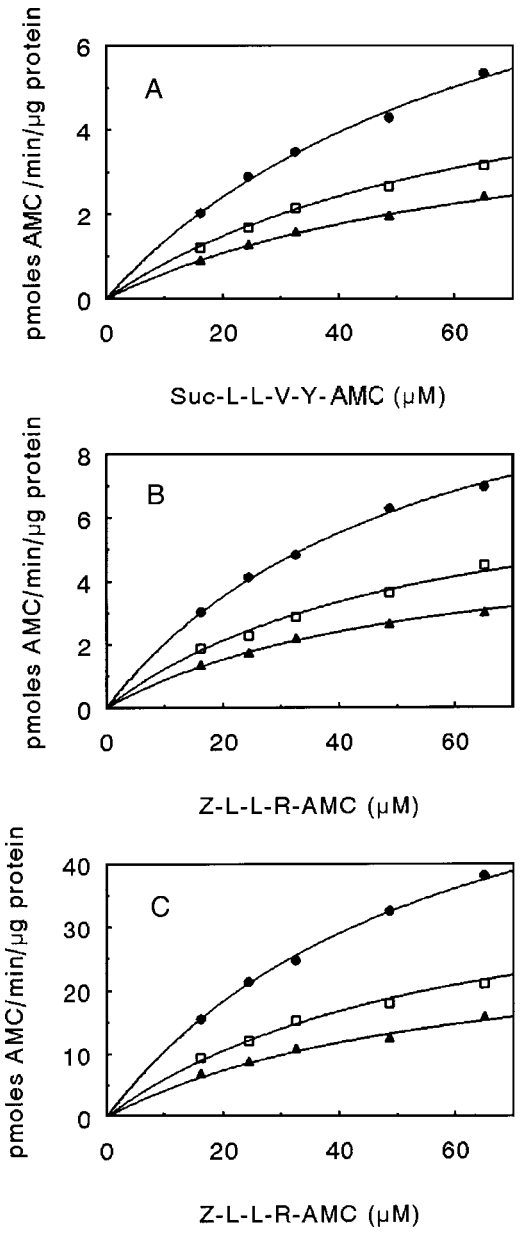

Figure 3 Fitting of the vinblastine-dependent effects on the initial reaction rate of the 20 S (A, B) and 26 S (C) proteasome-catalysed hydrolysis of the fluorogenic peptides Suc-LLVY-AMC (A) and Z-LLR-AMC (B, C) to the general equation for non-competitive inhibition

Vinblastine concentrations were as follows: ●, 0 μM; □, 6.8 μM; ▲, 13.6 μM.

hydrolytic reaction of the three peptides by the 20 S proteasome as well as that of Z-LLR-AMC and Z-LLE-βNA by the 26 S proteasome were plotted against substrate concentration (0–65 μM) with and without vinblastine at increasing concentrations. The results of non-linear regression analysis of the experimental data for the hydrolysis of Suc-LLVY-AMC by the 20 S proteasome and Z-LLR-AMC by both proteasomes are consistent with non-competitive inhibition kinetics (Figure 3 and Table 4). Results on the hydrolysis of Z-LLE-βNA did not fit any of the available models of enzymic inhibition. The selected concentration range of fluorogenic peptides is justified by observations of a progressive substrate inhibition of the chymotrypsin-like activity ([37], and M. Piccinini and M. T. Rinaudo, unpublished work) and also of the PGPH activity (M. Piccinini and M. T. Rinaudo, unpublished work) for fluorogenic peptides above 70 μM.

The effect of vinblastine on the multicatalytic activity of the two proteasomes was confirmed when they were resolved by non-denaturing PAGE and then overlaid with the fluorogenic peptides specific for the chymotrypsin-like and trypsin-like activities in the presence or absence of vinblastine (Figure 4). Fluorescent protein bands obtained in the presence of Z-LLE-βNA were barely detectable and were below the photographic detection limit.

Commercial chymotrypsin, trypsin and papain preparations, and also a preparation of purified calpain from human red blood cells, were exposed to vinblastine. None was affected by the drug at 3–200 μM (results not shown).

The proteolytic activity of 26 S proteasome, tested in the presence of ^{125}I-lysozyme–ubiquitin conjugates, was sharply and progressively inhibited by vinblastine at 25–200 μM, with an IC_{50} of close to 65 μM (Table 5). Inhibition was again reversed by dialysis (results not shown).

Immunoblot experiments performed on lysates from HL60 cells incubated for 6 h with vinblastine at 0.5–10 μM revealed an accumulation of high-molecular-mass ubiquitin–protein conjugates in the range 100–200 kDa (Figure 5). Densitometric evaluation showed that this accumulation was dose-dependent: 34 % for the drug at 0.5 μM, and 44 %, 54 % and 60 % for the drug at 1.0, 5.0 and 10.0 μM respectively. These results are means for three independent experiments; S.D. was less than 5 %. When the lysates were probed with a polyclonal antibody against IκBα, which is a known 26 S proteasome substrate in the phosphorylated polyubiquitinated form [38], a high-molecular-mass immunoreactive band accumulated in a drug-dose-dependent manner, which was presumably a polyubiquitinated form of IκBα (Figure 6) [19]. To prove the ability of vinblastine to inhibit 26 S proteasome-dependent IκBα degradation, we tested the effects of the drug on signal-induced degradation of IκBα by exposing HL60 cells, pretreated or not with increasing drug concentrations, to PMA plus ionomycin, known inducers of IκBα degradation [33]. As shown in Figure 7, IκBα accumulated in drug-treated cells in spite of the depletion of IκBα observed in cells exposed to PMA and ionomycin only.

As a positive control, in all experiments, cells were treated with the specific proteasome inhibitor lactacystin [39], which caused a similar but more substantial accumulation (90 %) of polyubiquitinated proteins and also of the high-molecular-mass form of IκBα with respect to vinblastine-treated cells. The same applied to the signal-induced degradation of IκBα. In all blotting experiments actin was used as an internal standard for equal protein loading.

Cell viability throughout the test was approx. 95 %.

DISCUSSION

This study demonstrates that vinblastine adversely affects proteasomal function in terms of peptidase and proteolytic activity. The three major peptidase activities of the 26 S proteasome are inhibited by the drug and the same applies to the corresponding activities of the 20 S proteasome. The two proteasomes share the same catalytic subunits, so these subunits, namely β_1 β_2 and β_5, should constitute the vinblastine target. The close K_i values obtained by kinetic analysis of the trypsin-like activities of the two proteasomes support this conclusion (Figure 3 and Table 4). Interestingly, the chymotrypsin-like activity of the 26 S proteasome was profoundly less sensitive than that of

Table 4 Kinetic parameters of the dose-dependent effect of vinblastine on the peptidase activities of the 20 S and 26 S proteasomes

The non-competitive inhibition model was fitted to initial reaction rates by unweighted non-linear regression analysis. Graphical results of the fitting procedures are shown in Figure 3. *V* is in pmol of AMC released/min per μg of protein. Percentage ranges of variations are given in brackets. The sum of squared residuals (SSR) for 20 S proteasome activities was 3-fold and 7-fold lower than that obtained with the competitive and uncompetitive models respectively; for the trypsin-like activity of the 26 S proteasome it was 4-fold and 3.5-fold lower than that obtained with the competitive and uncompetitive models respectively. Competitive and uncompetitive models were also affected by wider ranges of variations and did not allow acceptable fits.

	20 S proteasome chymotrypsin-like activity		20 S proteasome trypsin-like activity		26 S proteasome trypsin-like activity	
Parameter	Value	Range (%)	Value	Range (%)	Value	Range (%)
V (pmol/min per μg)	10.97	10.1	12.90	8.7	70.52	9.2
K_m (μM)	71.47	16.3	53.42	15.5	83.51	16.1
K_i (μM)	10.91	6.8	10.57	7.0	9.34	6.4
SSR	0.07	–	0.15	–	5.07	–

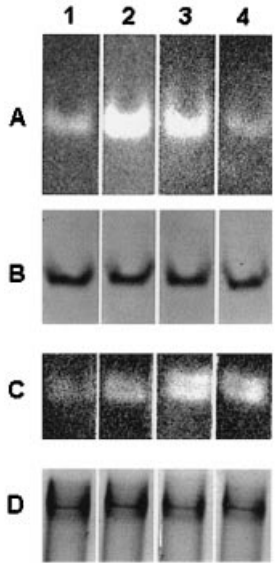

Figure 4 Inhibition of the chymotrypsin-like and trypsin-like activities of the 26 S proteasome revealed on a non-denaturing polyacrylamide gel

Purified 26 S proteasomes was resolved by nondenaturing PAGE [4.5% (w/v) gel] at 300 V·h. At the end of the electrophoretic run the gel was cut along the edges of the lanes and each lane was overlaid with a mixture containing the fluorogenic peptide specific for the trypsin-like [(**A**), lanes 1 and 2] or chymotrypsin-like [(**A**), lanes 3 and 4] activities of the two proteasomes added with the drug vehicle (lanes 2 and 3) or the drug (lanes 1 and 4) at final concentrations in the gels of 20 μM (lane 2) and 110 μM (lane 4). (**B**) The same gel after staining with Coomassie Blue. Comparable results were obtained in two other separate experiments.

Table 5 Inhibition assay of degradation of ^{125}I-lysozyme–ubiquitin conjugates by vinblastine

Samples contained, in a final volume of 100 μl, 25 mM Tris/HCl, pH 7.7, 4 mM NaCl, 5 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5 mM $MgCl_2$ 10% (v/v) glycerol, 2 mM ATP, 5 μg of the purified 26 S proteasome, the radiolabelled substrate (25000 c.p.m.) and vinblastine at the indicated concentrations. The reaction was started by the addition of the purified proteasome, left to proceed at 37 °C for 3 h and blocked in an ice bath. BSA (1%, w/v) (0.8 ml) in 5 mM Tris/HCl, pH 7.2, was added to each sample, followed by 0.1 ml of trichloroacetic acid. After a further 30 min in an ice bath, samples were centrifuged; supernatants and pellets were counted separately. Results are means ± S.D. for two separate experiments. Rates of degradation of conjugate were estimated as acid-soluble radioactivity formed as percentages of total radioactivity. Control reactions were performed in the absence of vinblastine.

Vinblastine concentration (μM)	Activity (% of control)
25	80.0 ± 2.0
50	57.5 ± 2.5
100	31.5 ± 1.5
200	2.5 ± 0.5

the 20 S proteasome because it required at least a 40-fold higher drug concentration for a comparable inhibition. Because the 26 S proteasome results from docking of the 19 S complex with the 20 S proteasome, presumably this event decreases the sensitivity of β_5, the subunit responsible for chymotrypsin-like activity, to the drug. It is noteworthy that this activity is the one most potentiated by the 19 S complex (Table 1) [22].

In addition, other chemotherapeutic agents, namely aclacinomycin A [18], and cyclosporine A [40], which are cytostatic and immunosuppressive drugs respectively, induce a reversible inhibition of the chymotrypsin-like activity of the 20 S proteasome. As with aclacinomycin A, vinblastine behaves as a non-competitive inhibitor; however, in contrast with the former, vinblastine's inhibition is unaffected by the presence in position P1 of an uncharged polar residue. Moreover, vincristine, another cytostatic *Vinca* alkaloid, which impairs microtubule assembly, inhibits the multicatalytic activity of the two purified proteasomes in much the same way as vinblastine (results not shown). The two *Vinca* alkaloids, and the above drugs, might therefore constitute a class of reversible, non-peptide, cell-permeable proteasome inhibitors targeting the proteasomal catalytic core. However, the effect of vinblastine on the two proteasomes is more specific than that of aclacinomycin A because, unlike the latter, it is ineffective against various serine and cysteine proteases.

The proteolytic activity of the 26 S proteasome is also adversely affected by vinblastine. In fact, *in vitro* the degradation of polyubiquitinated lysozyme by the purified proteasome is sharply decreased by the drug at 25–200 μM; *in vivo*, in HL60 cells exposed to the drug at 0.5–10 μM, the accumulation of high-molecular-mass ubiquitin–protein conjugates, a hallmark of intracellular proteasomal inhibition [19], takes place and is related to drug dose (Figure 5). A similar accumulation is also a feature of cells exposed to lactacystin, a highly specific proteasome inhibitor [13,39]. The dose-related accumulation of a high-molecular-mass form of IκBα, a well-known proteasome substrate, also observed in lactacystin-treated cells, is a further indication of the specificity of vinblastine-induced proteasome inhibition (Figure 6). In addition, the fact that high-molecular-mass ubiquitinated material accumulates at increasing vinblastine

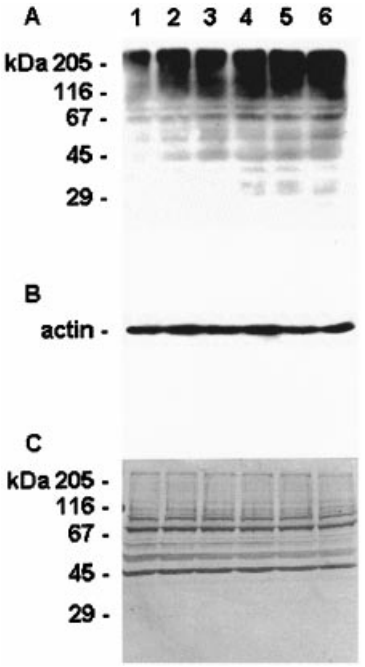

Figure 5 Accumulation of ubiquitin–protein conjugates in HL60 cells exposed to vinblastine or lactacystin

Equal quantities of total proteins (20 μg) from cell lysates were subjected to PAGE [10% (w/v) gel], electrotransferred to PVDF and probed with monoclonal antibody directed against ubiquitin (**A**). The same blot was reprobed with an anti-actin monoclonal antibody (**B**). Total proteins were detected by staining of the membrane with Coomassie Blue R-250 [0.1% in 50% (v/v) methanol] (**C**). Lanes 1, lysate from control cells cultured for 6 h in RPMI medium; lanes 2–5, lysate from cells cultured for 6 h in the presence of 0.5, 1, 5 and 10 μM vinblastine respectively; lane 6, lysate from cells cultured for 6 h in the presence of 10 μM lactacystin. The molecular mass markers were rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (98 kDa), BSA (67 kDa), ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa).

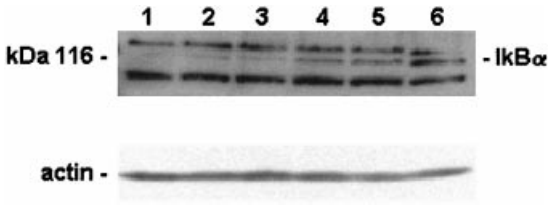

Figure 6 Accumulation of polyubiquitinated IκBα in HL60 cells exposed to vinblastine or lactacystin

Equal quantities of total proteins (20 μg) from cell lysates were subjected to PAGE [10% (v/v) gel], electrotransferred to PVDF, probed with polyclonal antiserum directed against IκBα (**A**) and reprobed with an anti-actin monoclonal antibody (**B**). Lanes and molecular mass markers are as in Figure 5.

concentrations shows that protein ubiquitination, in contrast with proteasomal function, is presumably not affected by vinblastine.

We can therefore conclude that vinblastine inhibits proteasomal activity both *in vitro* and *in vivo* and that *in vivo* it does so at concentrations close to those reported to be attained in the extracellular fluids of treated patients (0.1–0.5 μM) [34]. However, because vinblastine accumulates in cells up to 100-fold [34], its eventual intracellular concentration could be considerably

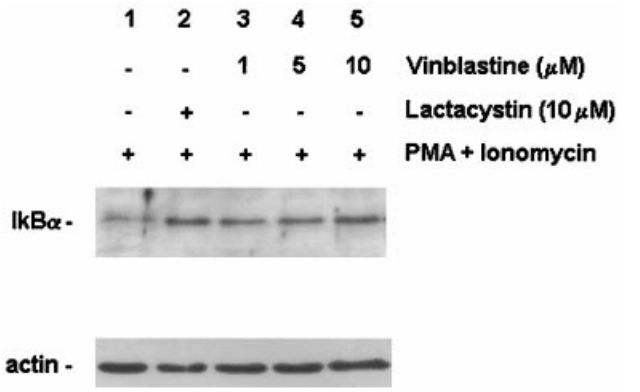

Figure 7 Vinblastine-dependent inhibition of signal-induced degradation of IκBα

HL60 cells, pretreated or not for 6 h with increasing vinblastine concentrations or with lactacystin, were exposed to PMA plus ionomycin for 120 min. Cells were then lysed, electrophoresed and blotted as described in the legend to Figure 5. Blotted proteins were immunodecorated with antibodies directed against IκBα. The same blots were stripped and reprobed with a monoclonal antibody directed against actin as a control for equal protein loading.

higher than that outside and might therefore be close to that at which the drug is active *in vitro* on polyubiquitinated substrates as well as on the chymotrypsin-like activity of 26 S proteasome.

Vinblastine blocks cell proliferation by arresting mitosis at the transition from metaphase to anaphase [14–17]; the main mechanism involved is the disruption of spindle dynamics after microtubule stabilization, owing to a lower rate of tubuline addition and loss at microtubule ends [15,17]. Whether a link exists between this mechanism and inhibition of proteasomal function is difficult to say yet, although the inhibition of proteasomal activity might be expected to lead to an accumulation of proteins that would hamper spindle dynamics. Alternatively, proteasome inhibition could lead to the accumulation of other 26 S proteasome substrates not directly involved in spindle dynamics (anaphase inhibitors, cyclin B1), whose coordinated degradation is essential for anaphase progression and cell exit from mitosis [11,12,41–44]. If this were correct, an additional mechanism by which vinblastine behaves as a cytostatic agent in anti-tumour therapy would have been uncovered. The accumulation of high-molecular-mass polyubiquitinated IκBα, as well as the inhibition of the signal-induced degradation of IκBα, in cells cultured in the presence of vinblastine raises the question whether the action of vinblastine on proliferating cells might cover a wide array of mechanisms going well beyond that of blocking microtubule dynamics. In fact, IκBα inhibits the translocation to the nuclear compartment of the transcriptional factor NF-κB, in which the latter regulates the expression of a number of genes involved in immune functions, inflammation responses and apoptosis control [38,45]. The same accumulation also suggests the existence in these cells, when in proliferation, of an active IκBα turnover, apparently in the absence of pro-inflammatory stimuli, which is slowed down concomitantly with the drug-mediated inhibition of IκBα degradation and cell proliferation.

Improving the identification of the ubiquitin conjugates, that accumulate in vinblastine-treated cells, might provide useful information on the actual target(s) of vinblastine-dependent proteasomal inhibition in the transition from anaphase to

metaphase, as well as on the involvement of this inhibition in other cellular processes.

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