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Apoptosis induced in L1210 leukaemia cells by an inhibitor of the chymotrypsin-like activity of the proteasome

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Of a number of factors involved in apoptosis, protease activity may play a crucial role. We show that *N*-benzyloxycarbonyl-Ile-Glu(*O*-t-butyl)-Ala-leucinal (PSI), a selective inhibitor of the chymotrypsin-like activity of the proteasome, induces massive apoptosis in murine leukaemia L1210 cells. At 50 nM concentration, PSI induces a block of cytokinesis, while higher concentrations (500 nM) cause S phase block and massive apoptosis. Z-Leu-leucinal, a specific calpain inhibitor, did not induce apoptosis. In contrast to previous reports, TNF-α did not enhance apoptosis when combined with PSI. Our results suggest that proteasome inhibitors may be considered as potential anti-neoplastic agents.

Key words: Apoptosis; experimental cancer therapy; L1210 leukaemia; proteasome, proteasome inhibitor, TNF.

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Introduction

Apoptosis, or programmed cell death, can be induced by a plethora of physical, chemical and biological factors, leading to activation of different signal transduction pathways. However, all these signals converge in a common executioning system, which, most probably, involves the members of the interleukin-1β converting enzyme (ICE)/Ced3 family of cysteine proteases (caspases).[1,2,3]

The ubiquitin-proteasome proteolytic pathway is responsible for proteolysis of various proteins crucial for cell cycle regulation, including cyclins,[4] cyclin-dependent kinase inhibitors[5] and numerous oncogene products (for a review see ref. 6). Proteasomes degrade not only certain unstable or strictly regulated proteins, but also the bulk of cellular proteins.[7] It is therefore conceivable that inhibition of proteasome-dependent proteolysis could affect the cell metabolism, leading to *e.g.*, a cell cycle arrest.[8] It is a common phenomenon that cells which cannot divide either differentiate or succumb to apoptosis.

The role of the ubiquitin-proteasome pathway in apoptosis remains unclear.[3] Lactacystin, a *Streptomyces* metabolite, which is a specific inhibitor of the proteasome, induced apoptosis in human monoblast U937 cells[9] while PSI induced apoptosis in a human leukaemia HL60 cell line.[10] On the other hand, proteasome inhibition prevents apoptosis in thymocytes and neurons.[11,12] Moreover, proteasomes may display ICE-like activity in retinoic acid-treated P19 cells.[13]

In order to verify whether the ubiquitin-proteasome pathway is involved in apoptotic cell death,

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the present study was aimed at testing the effects of *N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal (proteasome inhibitor, PSI), a highly selective inhibitor of the chymotrypsin-like activity of the proteasome[14] on L1210 murine leukaemia cells. We found that PSI is a strong inducer of apoptosis, thus suggesting that proteasome activity may be involved in regulation of programmed cell death.

Material and methods

Cell culture

L1210, a methylcholanthrene-induced murine lymphatic leukaemia cell line,[15] was cultured in RPMI 1640 medium supplemented with antibiotics, 2-mercaptoethanol (50 mM), L-glutamine (2 mM) and 10% heat-inactivated FCS (all from Gibco BRL, Paisley, UK) in a humidified atmosphere with 5% CO_2 at 37°C. The population doubling time was 12 h.

Reagents

PSI (*N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal, proteasome inhibitor) was synthesized as described previously,[14] dissolved in DMSO at a concentration of 5 mM and stored at -70°C. The working concentration was obtained by dilution of the stock solution in fresh culture medium. A similar stock solution was prepared for the control agent Z-leu-leucinal (ZLL), a calpain inhibitor. The final concentration of DMSO in the culture medium was lower than 0.1%. Human recombinant tumour necrosis factor α (TNFα — BASF/Knoll AG, Ludwigshafen, Germany) was diluted with PBS + 1% BSA at a concentration of 10 µg/ml, aliquoted and stored at -70°C, then added to give a concentration of 100 ng/ml in culture medium. All reagents used for SDS-PAGE, Western blotting, DNA purification and electrophoresis were molecular biology grade purchased from Sigma (St. Louis, MO, USA). Anti-ubiquitin rabbit whole antiserum was from Sigma (St. Louis, MO, USA) while anti-rabbit goat antiserum was from BioRad (Hercules, CA, USA).

SDS-PAGE and Western blotting

Control cells or cells treated for 24 h with 500 nM PSI were washed with PBS and resuspended in sample buffer containing 2% SDS. The concentration of total protein was estimated by the BioRad Protein assay. The samples were diluted to obtain a final protein concentration of 2 mg/ml, supplemented with 10% 2-mercaptoethanol, 1% bromophenol blue and run on 12% SDS-PAGE in a MiniProtean II electrophoresis chamber. The resulting gel was Western blotted with anti-ubiquitin whole antiserum. Amplified alkaline phosphatase goat anti-rabbit immunblot assay kit was used for visualization following the manufacturer's recommendations. The molecular mass marker used was Prestained SDS-PAGE Standard Low Range from BioRad (Hercules, CA, USA).

MTT assay

The cytostatic and/or cytotoxic effects on L1210 cells were tested in a standard MTT assay, as described previously.[16] Briefly, cells were dispensed in a 96-well flat-bottomed microtiter plate (Corning, New York, NY, USA) at a concentration of 2×10^4 cells/100 µl/well. PSI diluted to appropriate concentrations with culture medium was added to a final volume of 200 µl. Following 24 h or 72 h incubation, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution (2.5 mg/ml) was added to each well. Incubation followed for 4 h and the plates were centrifuged (350g/10 min) and 200 µl of supernatant was carefully removed from each well and replaced with 200 µl acid-DMSO. The results were read in an ELISA reader (SLT Labinstrument GmbH, Salzburg, Austria), using a 550 nm filter. Each group was tested five times which allowed the calculations of mean values and standard deviations. Cytostatic/cytotoxic effect was expressed as relative viability of L1210 cells (per cent of control L1210 cultures incubated with medium only) and was calculated as follows: Relative viability = $(A_e - A_b) \times 100 / (A_c - A_b)$, where A_b is background absorbance, A_e is experimental absorbance and A_c is the absorbance of untreated controls. A series of independent experiments were performed and the results presented in the paper are most representative. Statistical significance was de-

termined by Students' *t*-test at $p < 0.05$.

Microscopy

Cells were cultured for 24 h in the presence or absence of PSI or ZLL. Cytospin preparations were made, cells were then fixed with Serra fixative (15 parts of formaldehyde, 15 parts of methanol, one part of glacial acetic acid) at -20°C, stained with Mayer's hemalum and observed under a light microscope.

The cytospin preparations of fixed cells stained with DAPI-Rhodamine 101 were observed in a fluorescence and phase contrast microscope.

DNA electrophoresis

PSI or the control agent were added to cell culture flasks to a final concentration of 500 nM. Cells were washed and the pellet was suspended in TES buffer (pH 7.9, 10 mM Tris, 1 mM EDTA, 0.5% SDS) with 0.4 mg/ml proteinase K. After 24 h digestion, DNA was further purified with a phenol/chloroform extraction and ethanol/NaCl precipitation according to a routine protocol. DNA was suspended in TE buffer (pH 7.9, 10 mM Tris, 1 mM EDTA) supplemented with a stock loading solution (0.25% bromphenol blue, 0.25% xylene cyanol, 15% Ficoll) and loaded on a 1% agarose gel with 0.5 µg/ml ethidium bromide. DNA AmpliSize Standard 50–2000 bp (BioRad, Hercules, CA, USA) was run on a separate lane. The electrophoresis was performed for 45 min at 90 V, and the gel was analyzed by GDS 8000 Complete Gel Documentation and Analysis System (Ultra-Violet Products Ltd., Cambridge, UK). Three independent experiments were performed with similar results.

Flow cytometric detection of living, apoptotic and necrotic cells

Two methods were used to assay apoptotic and necrotic cells by flow cytometry: modified test of cell membrane permeability to propidium iodide (PI)[17] and test of DNA fragmentation with 4,6-diamino-2-phenylindole (DAPI) on fixed cells.[18]

In the first method, living cells (0.2×10^6) were incubated in a culture medium in the presence of 20 mg/ml of PI for 10 min at 37°C, 5% CO_2 and then analyzed by flow cytometry. During apoptosis the plasma membrane transport function becomes transiently defective prior to total loss of the ability to exclude charged fluorochromes. In the second method, cells were fixed with 1% paraformaldehyde in PBS for 10 min and then transferred to cold (-20°C) 80% ethanol. Then the ethanol was removed and to 10^5 cells at 4°C 1.5 ml of cold DAPI-sulforhodamine 101 solution was added (1 mg DAPI and 20 mg sulforhodamine 101 per ml of Pipes buffer, 2 mM $MgCl_2$, 0.1% Triton X100, pH 6.8). After a short incubation, data were acquired and DNA content per cell was measured. The DAPI-sulforhodamine 101 method was used for cell cycle analysis as well.[18]

Cell fluorescence and light scatter were assayed using FACSCalibur and FACSVantage flow cytometers (Becton Dickinson Immunocytochemistry Systems, San Diego, CA, USA) equipped with an argon ion laser (UV light about 351 nm or/and 488 nm). L1210 cells were separated from other events based on light scatter characteristics (the gate of FSC and SSC). After constructing an L1210 cell gate, the FSC against fluorescence gains (for PI or DAPI) were optimized. List mode data was collected for 8,000 events. The number of living, apoptotic and necrotic cells was determined by comparison of PSI treated and untreated control cells with the use of a software system (CELLQuest, v. 1.2). Both the intensity of fluorescence, forward (FSC) and 90-degree (SSC) light scatter were calibrated to a standard mean channel with the use of CaliBRITE beads.

Results

SDS-PAGE and Western blotting (Figure 1) show that L1210 cells treated for 24 h with 500 nM PSI accumulate high molecular mass ubiquitin-protein conjugates.

In the MTT assay, PSI was found to be cytostatic/cytotoxic with an ED_{50} of approximately 30 nM. The decrease of the relative viability was dose- and time-dependent (Figure 2). ZLL did not cause any statistically significant decrease in the relative viability of the L1210 cells even at a concentration of 5 µM.

Figure 1. Whole cell lysates of untreated L1210 cells (lane C) or cells treated for 24 h with 500 nM PSI (lane ψ) run on 12% SDS-PAGE and Western blotted with anti-ubiquitin serum. The molecular marker set (lane M) was Prestained SDS-PAGE Standard Low Range from BioRad (Hercules, CA, USA). It can be observed that high molecular mass ubiquitin-protein conjugates accumulate in treated cells.

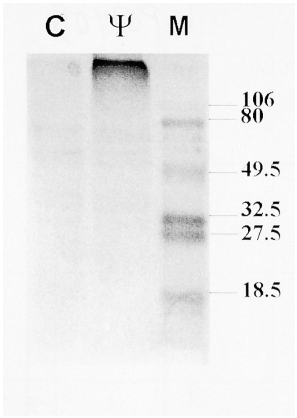


Figure 2. Comparison of cytostatic/cytotoxic PSI effects toward L1210 murine leukaemia cells depending on the time of incubation, which was 24 (-❍) or 72 (-∇-) h. Relative viability, expressed as per cent of control, was measured by the MTT assay. Standard deviations are indicated by vertical bars.

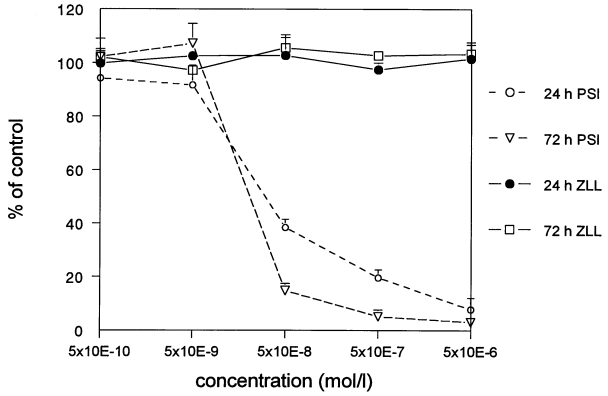
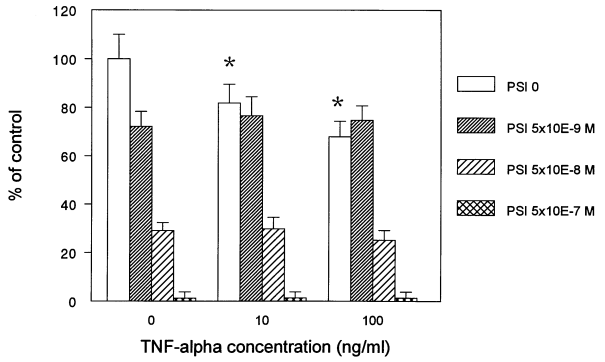


Figure 3. Comparison of cytostatic/cytotoxic TNFα effects toward L1210 murine leukaemia cells in the absence or presence of increasing PSI concentrations after 72 h incubation. Relative viability, expressed as per cent of control, was measured by the MTT assay. Standard deviations are indicated by vertical bars. Results which differ significantly from control (*i.e.,* the group with TNFα 0 and the respective PSI concentration) as measured by Student's *t*-test are indicated by asterisks.



TNFα caused a dose-dependent cytostatic/cytotoxic effect as measured by the MTT assay. This effect was only slightly pronounced after a 24 h incubation (results not shown), while it was very strong after a 72 h incubation period (Figure 3). When TNFα at both concentrations tested was combined with three different PSI concentrations, there was neither a statistically significant synergism nor an additive effect observed. Moreover, it appeared that the lowest PSI concentration tested (5×10^{-9} M) protected L1210 cells from TNFα.

In order to determine the morphological basis of the cytostatic/cytotoxic PSI effect, cytospin preparations were made and observed. L1210 leukaemia cells treated for 24 h with 500 nM PSI showed massive morphologic signs of apoptosis, while cells cultured under control conditions showed only sporadic apoptotic cells (Figure 4). In treated culture, cell death was evident and numerous apoptotic bodies with typical chromatin condensation were observed.

To further verify whether PSI induces apoptosis in L1210 leukaemia cells, total DNA was isolated and analyzed. A typical ladder was obtained after running the sample isolated from cells treated for 24 h with 500 nM PSI, suggesting internucleosomal DNA fragmentation which is a feature of apoptotic cell death (Figure 5).

PSI-induced apoptosis was also assessed by flow cytometric analysis (Figure 6). The influence of PSI on the relative number of apoptotic and necrotic cells was observed at 500 nM concentration of the inhibitor (Figure 6B). Lower concentrations tested (0.5, 5 and 50 nM) were not effective (Table 1). A similar per cent of apoptotic cells was found with both DAPI and PI methods. The amount of PI within cells, measured as median fluorescence units was about 10 times higher in apoptotic cells and about 200 times higher in necrotic cells compared with living cells.

Cells incubated with 50 nM PSI were blocked at

Table 1. Per cent of living, apoptotic or necrotic cells in a control sample and in samples incubated 24 h with different concentrations of PSI in the culture medium. Flow cytometric analysis with PI or DAPI

Cells	Control	0.5 nM PSI	5 nM PSI	50 nM PSI	500 nM PSI
Living	96	98	98	94	40
Apoptotic	1 (1)*	1	1 (1)*	2 (4)*	14 (12)*
Necrotic	3	1	1	4	47

* Number in brackets is the per cent of apoptotic cells analyzed with DAPI.

Figure 4. Light microscopic images of cytospun L1210 murine leukaemia eiher cells cultured under the standard conditions (**A**) or in the presence of 500 nM PSI for 24 h (**B**). Note the striking difference between these preparations — there is massive apoptosis in the presence of PSI, as revealed by the presence of numerous apoptotic bodies and the absence of normal looking cells. Arrow on (A) points to a single apoptotic cell in the control population.

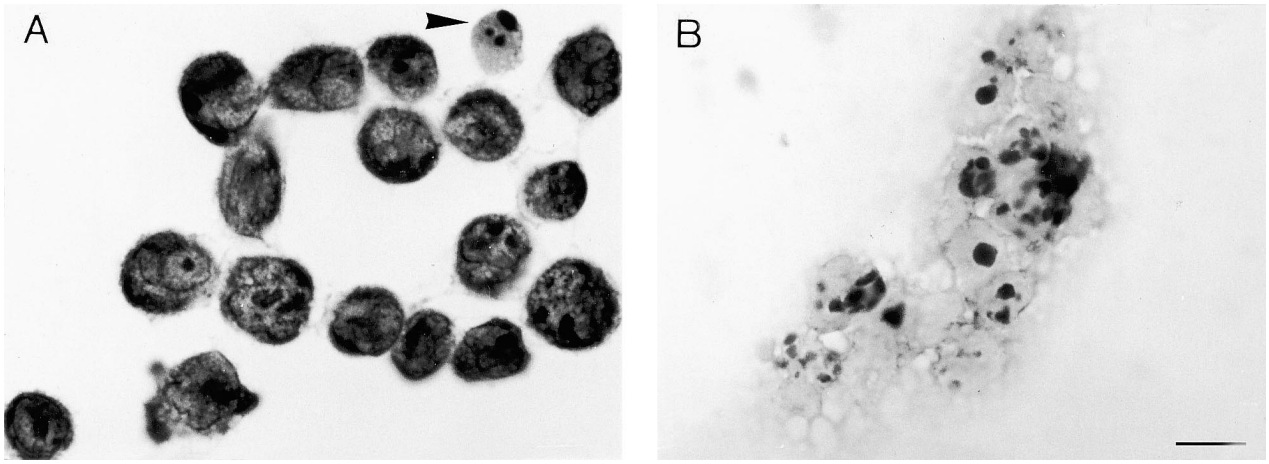
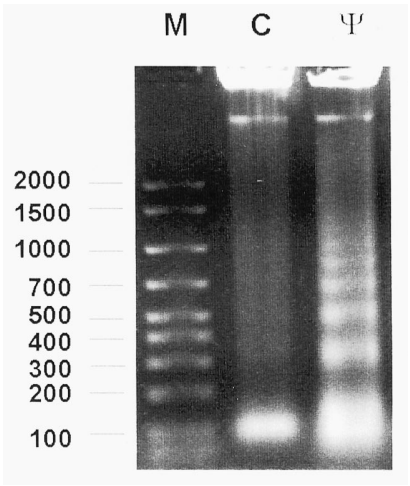


Figure 5. Agarose electrophoresis of DNA isolated from L1210 murine leukaemia cells cultured under standard conditions (lane C) or in the presence of 500 nM PSI for 24 h (lane ψ). Molecular weight marker was loaded on lane M, and the respective molecular weight in base pairs is indicated. Note the DNA fragmentation ladder on lane ψ.

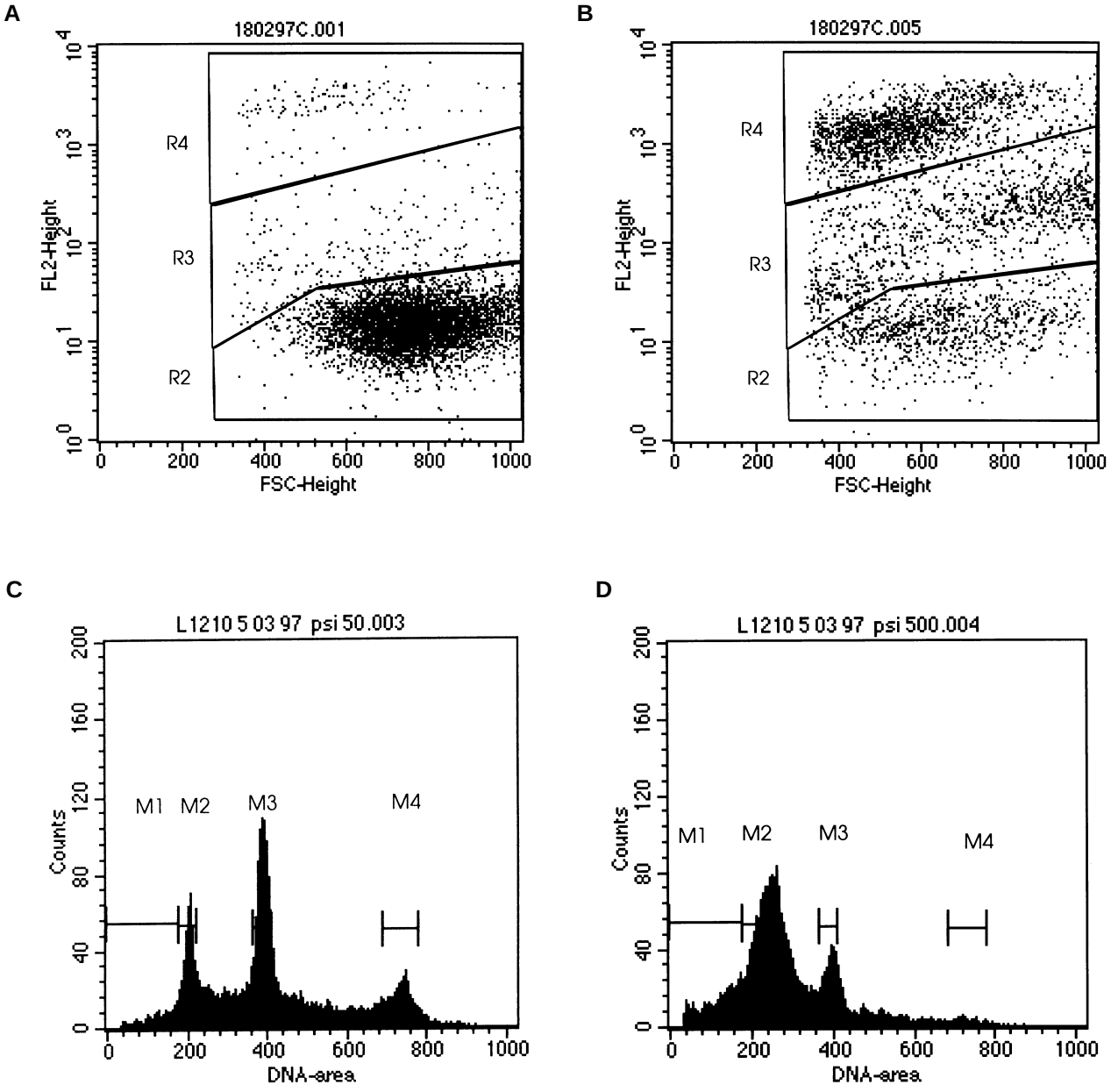


the cytokinesis stage of cell division. About 9% of cells were binucleated (Figure 6C). At 500 nM PSI concentration, S-phase peak was observed (Figure 6D), and was interpreted as a result of S phase block of the cell cycle or/and result of apoptosis of G2 and G4 cells.

Discussion

The results of our experiments demonstrate that PSI induces apoptosis in L1210 leukaemia cells, as determined by observation of morphologic and cytometric changes, DNA fragmentation and metabolic viability. Apoptotic cell death is accompanied by an accumulation of high molecular weight ubiquitin-protein conjugates. Since ZLL, a calpain inhibitor with minimal proteasome inhibitory activity, a peptidyl aldehyde similar to PSI, does not cause any measurable effects, we assume

Figure 6. Living, apoptotic and necrotic cells analyzed by the PI method in a control sample (**A**) and in a sample incubated for 24 h in the presence of 500 nM PSI (**B**). FSC *vs.* FL2 (PI on Y-axis) plot. R2-living cells, R3-apoptotic cells, R4-necrotic cells. Binucleate L1210 cells after 24 h incubation in presence of 50 nM PSI — DNA histogram, binucleate cells stained with DAPI-sulforhodamine 101 (**C**). Apoptotic L1210 cells after 24 h incubation in presence of 500 nM PSI — DNA histogram; M1 — cells with DNA content lower than G1, M2 — G1 cells, M3 — G2/M cells, M4 — tetraploid cells (**D**).



that the induction of apoptosis by PSI is likely a consequence of the inhibition of the chymotrypsin-like activity of the proteasome.

PSI has already been shown to induce accumulation of ubiquitinated proteins in different cell lines,[19,20] block the activation of NfκB,[21] and induce a double cell cycle block in G2-phase and metaphase and produce aberrant mitosis and apoptotic events in HeLa cells.[8,22] Drexler[10] reported that apoptosis of HL60 cells occurred mainly during the G1 phase of the cell cycle, while L1210 apoptotic cells accumulated mainly in the S and G2 phases of the cell cycle. L1210 cells seem more sensitive than HL60 cells, as apoptosis occurs at a lower PSI concentra-

tion. Moreover, PSI concentrations unable to induce apoptosis induce a block of cytokinesis and accumulation of tetraploid cells.

Inhibition of proteasome-dependent proteolysis in thymocytes and neurons prevents apoptosis, implicating the requirement of some proteasome-dependent event for the apoptotic pathway.[11,12] If the proteasome-dependent event is always required for apoptosis to occur, PSI should prevent apoptosis even if it induces cell cycle block and several metabolic alterations. However, this is not the case, at least with L1210 cells and with U937 human monoblasts.[9] It could therefore be inferred that there is a requirement for a proteasome-dependent step only in some pathways leading to apoptosis.

Apoptosis is induced by various drugs used in tumour chemotherapy. Some of these drugs also inhibit the ubiquitin/proteasome pathway of protein degradation. Among these drugs are cisplatin, 4'-(9-acridynyl-amino) methanesulfon-m-anisidide and mitomycin C, which interfere with the ubiquitination of proteins[23,24] and aclacinomycin A (aclarubicin), which inhibits the ubiquitin system[24] and blocks the chymotrypsin-like proteolytic activity of the 20 S proteasome.[25] All these drugs are potent inducers of apoptotic death, but it is not known to what extent their effect is due to the inhibition of the ubiquitin-proteasome pathway.

TNFα induces apoptosis in various cells, while many other cells are resistant to its action. It was found that this resistance is due, at least in part, to the fact that TNFα also induces activation of NFκB, which somehow protects the cells from the induction of apoptosis.[26,27,28] It has been suggested that proteasome inhibitors should sensitize cells to the cytotoxic action of TNFα,[27] since PSI inhibits NFκB activation.[21] Although TNFα alone was cytostatic/cytotoxic to L1210 leukaemia cells, there was neither an additive nor a synergistic activity of a combination of PSI and TNFα in our system.

As many proteolytic events occur during cell cycle transitions, a block in the cell cycle induced by PSI could be explained on the basis of its ability to interfere with the destruction of cyclins and/or other proteins in a way similar to that described in HeLa cells.[20] The appearance of tetraploid cells requires further study, but could be attributed to the interference with normal spindle function and specifically with the kinetochores.[22]

Apoptosis was induced within the nanomolar concentration range of PSI in L1210 and also in other rapidly proliferating cells like HeLa, B16F10 melanoma and SK-v (unpublished results). However, similar proteasome inhibitors have been demonstrated to prevent apoptosis in poorly- or non-proliferating cells.[12,13] This observation raises the possibility that PSI should most probably affect rapidly dividing cancer cells *in vivo* and therefore could be assessed as a chemotherapeutic agent. This possibility should be verified with L1210 leukaemia and with other experimental tumours.

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References

1. Martin SJ, Green DG. Protease activation during apoptosis: death by a thousand cuts? *Cell* 1995; 82: 349–352.
2. Malejczyk J, Górski A. Editorial review: Apoptosis and its role in immunity. *Apoptosis* 1996; 1: 227–230.
3. Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. *FASEB J* 1996; 10: 587–597.
4. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. *Nature* 1991; 349: 132–138.
5. Pagano M, Tam SW, Theodoras AM, *et al*. Role of the ubiquitin proteasome pathway in regulating abundance of the cyclin dependent kinase inhibitor p27. *Science* 1995; 269: 682–685.
6. Pahl HL, Bauerle PA. Control of gene expression by proteolysis. *Curr Opin Cell Biol* 1996; 8: 340–347.
7. Rock KL, Gramm C, Rothstein L, *et al*. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 1994; 78: 761–771.
8. Wójcik C, Paweletz N, Schroeter D. Localization of proteasomal antigens during different phases of the cell cycle in HeLa cells. *Eur J Cell Biol* 1995; 68: 191–198.
9. Imajoh-Ohmi S, Kawaguchi T, Sugiyama S, Tanaka K, Omura S, Kikuchi H. Lactacystin, a specific inhibitor of the proteasome, induces apoptosis. *Biochem Biophys Res Comm* 1995; 217: 1070–1077.
10. Drexler HCA. Activation of the cell death program by inhibition of proteasome function. *PNAS* 1997; 94: 855–860.
11. Sadoul R, Fernandez PA, Quiquerez AL, *et al*. Involvement of the proteasome in the programmed cell death

of NGF-deprived sympathetic neurons. *EMBO J* 1996; 15: 3845– 3852.

12. Grimm LM, Goldberg AL, Poirier GG, Schwartz LM, Osborne BA. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J* 1996; 15: 3835–3844.
13. Kobayashi T, Shinozaki A, Momoi T, Arahata K, Tsukahara T. Identification of an interleukin-1β conferring enzyme-like activity that increases upon treatment with the retinoic acid as the proteasome. *J Biochem* 1996; 120: 699–704.
14. Wilk S, Figueiredo-Pereira M. Synthetic inhibitors of the multicatalytic proteinase complex (proteasome). *Enzyme Protein* 1993; 47: 306–313.
15. Kawiak J, Skórski T, Ciechanowicz A, *et al*. Cytochemical characterization of mouse L1210 leukemia. *Immunol Invest* 1988; 17: 543–550.
16. Lasek W, Giermasz A, Kuc K, *et al*. Potentiation of the anti-tumor effect of actinomycin D by tumor necrosis factor alpha in mice: correlation between in vitro and in vivo results. *Int J Cancer* 1996; 66: 374–379.
17. Zamai L, Falcieri E, Marhefka G, Vitale M. Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation. *Cytometry* 1996; 23: 303–311.
18. Darzynkiewicz Z, Li X, Gong J. Assays of cell viability: discrimination of cells dying by apoptosis. In: Darzynkiewicz Z, Robinson JP, Crissman HA, eds. *Flow Cytometry, Second Edition.* New York, NY (USA): Academic Press, 1994: 15–38.
19. Figuereido-Pereira M, Berg KA, Wilk S. A new inhibitor of the chymotrypsin-like activity in the Multicatalytic Proteinase Complex (20S proteasome) induces accumulation of ubiquitin-protein conjugates in a neuronal cell. *J Neurochem* 1994; 63: 1578–1581.
20. Wójcik C, Schroeter D, Wilk S, Lamprecht J, Paweletz N. Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. *Eur J Cell Biol* 1996; 71: 311–318.
21. Traenckner EB-M, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NFκB and stabilizes a newly phosphorylated form of IκBα that is still bound to NFκB. *EMBO J* 1994; 13: 5433–5441.
22. Paweletz N, Wójcik C, Schroeter D, Finze EM. Are proteasomes involved in the formation of the kinetochore? *Chromosome Research* 1996; 4: 436–442.
23. Isoe T, Naito M, Hirai R, Tsuruo T. Inhibition of ubiquitin-ATP-dependent proteolysis and ubiquitination by cisplatin. *Anticancer Res* 1991; 11: 1905–1910.
24. Isoe T, Naito M, Shirai A, Hirai R, Tsuruo T. Inhibition of different steps of the ubiquitin system by cisplatin and aclarubicin. *Biochim Biophys Acta* 1992; 1117: 131–135.
25. Figueiredo-Pereira M, Chen WE, Li J, Johdo O. The antitumor drug aclacinomycin A, which inhibits the degradation of ubiquitinated proteins, shows selectivity for the chymotrypsin-like activity of the bovine pituitary 20 S proteasome. *J Biol Chem* 1996; 271: 16455–16459.
26. Beg AA, Baltimore D. An essential role for NFκB in preventing TNFα-induced cell death. *Science* 1996; 274: 782–784.
27. Wang CY, Mayo MW, Baldwin Jr AS. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NFκB. *Science* 1996; 274: 784–787.
28. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNFα-induced apoptosis by NFκB. *Science* 1996; 274: 787– 789.