Journal of Cellular Biochemistry 97:122–134 (2006)

Differential Effects of Proteasome Inhibitors on Cell Cycle and Apoptotic Pathways in Human YT and Jurkat Cells

Min Lu,[1] Q. Ping Dou,[2] Richard P. Kitson,[1] David M. Smith,[2] and Ronald H. Goldfarb[1]*

[1]Department of Molecular Biology and Immunology, Institute for Cancer Research, University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Fort Worth, Texas 76107-2699
[2]Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, 12902 Magnolia Dr., Tampa, Florida 33612-9497

Abstract Herein, we report differential effects of various proteasome inhibitors including *clasto*-lactacystin-β-lactone, (−)-epigallocatechin gallate (EGCG) and *N*-Acetyl-Leu-Leu-Norleu-al (LLnL) on proteasomal activities of YT and Jurkat cells, human natural killer (NK) and T cell lines, respectively. The inhibitory rates of these inhibitors on the purified 20S proteasomal and 26S proteasomal chymotrypsin-like activity in whole cell extracts and intact cells did not show significant differences between the two cell lines. The viability of both cell lines was reduced in the presence of LLnL. Subsequent studies revealed a reduction of the mitochondrial membrane potential and caspase-3 activation in these two cell lines upon treatment with proteasome inhibitors; however, caspase-3 activation occurred much earlier in Jurkat cells. Cell cycle analysis indicated a sub-G_1 apoptotic cell population in Jurkat cells and G_2/M arrest in YT cells after they were treated by proteasome inhibitors. Moreover, pretreatment of YT cells by a caspase inhibitor followed by a proteasome inhibitor did not increase the percentage of G_2/M phase cells. In addition, accumulation of p27 and IκB-α was detected only in Jurkat cells, but not YT cells. In summary, proteasome inhibitors may act differentially in cell cycle arrest and apoptosis of tumors of NK and T cell origin, and may have similar effects on normal NK and T cells. J. Cell. Biochem. 97: 122–134, 2006.

Key words: proteasome; inhibitor; apoptosis; NK cells

The 20S proteasome is the key component of the 26S multicatalytic proteasome complex [Baumeister et al., 1998]. The 20S particle is composed of four stacked rings surrounding a central chamber in which proteins are digested [Voges et al., 1999]. There are three major proteasomal activities: one active site cleaves preferentially after hydrophobic residues (chymotrypsin-like activity), one after basic residues (trypsin-like activity), and one after acidic residues (peptidyl-glutamyl peptide hydrolyzing activity, PGPH) [Loidl et al., 1999]. The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins including p53 [Maki et al., 1996], pRB [Boyer et al., 1996], p21 [Blagosklonny et al., 1996], $p27^{Kip1}$ [Pagano et al., 1995], IκB-α [Verma et al., 1995], and Bax [Li and Dou, 2000].

In the investigation of the unique proteolytic mechanism by proteasome, many proteasome inhibitors provide a very valuable and useful

Abbreviations used: Ac, *N*-Acetyl; AMC, 7-amido-4-methylcoumarin hydrochloride; CHO, aldehyde; DEVD, Asp-Glu-Val-Asp; EGCG, epigallocatechin gallate; LLnL, *N*-Acetyl-Leu-Leu-Norleu-al; LLVY, Leu-Leu-Val-Tyr; MG115, Z-Leu-Leu-Norvalinal; MG132, Z-Leu-Leu-Leu-al; PI, propidium iodide; Suc, succinyl.

Grant sponsor: Robert A. Welch Foundation; Grant number: BK-1396.

Q. Ping Dou's present address is Barbara Ann Karmanos Cancer Institute, 4100 John R, Detroit, MI 48201.

Richard P. Kitson's present address is Sopherion Therapeutics Inc., 100 Overlook Center Suite 100, Princeton, NJ 08540.

David M. Smith's present address is Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

*Correspondence to: Ronald H. Goldfarb, Sopherion Therapeutics Inc., 100 Overlook Center Suite 100, Princeton, NJ 08540. E-mail: rgoldfarb@sopherion.com

Received 2 March 2005; Accepted 26 April 2005

DOI 10.1002/jcb.20543

tool. These proteasome inhibitors are usually short peptides linked to a pharmacophore, generally located at its C-terminus. The pharmacophore interacts with a catalytic residue to form reversible or irreversible covalent adducts, while the peptide portion specifically associates with the proteasome's substrate binding pocket. Based on the pharmacophore, proteasome inhibitors can be divided into several groups: (1) peptide aldehydes; (2) peptide boronates; (3) natural proteasome inhibitors; (4) peptide vinyl sulfones; and (5) peptide epoxyketones [Kisselev and Goldberg, 2001]. Among them, peptide aldehydes are the best characterized and most widely used, such as MG132, MG115, and LLnL. These agents are reversible with high potency, inhibiting the chymotrypsin-like activity of the proteasome [Lee and Goldberg, 1998], and restoring protein degradation after their removal. Contrary to most proteasome inhibitors synthesized artificially, lactacystin is a natural product isolated originally from actinomycetes. It can be converted into its active form, a β-lactone derivative, when placed in aqueous solution [Dick et al., 1996]. It irreversibly binds to subunit X of the proteasome and acylates the active site N-terminal threonine [Grisham et al., 1999]. Recently, other natural proteasome inhibitors have been identified including epigallocatechin gallate (EGCG) from green tea [Nam et al., 2001]. The ester bonds in EGCG attack the active site threonine in the proteasome and lead to its acylation.

Our laboratory has investigated unique aspects of the proteasome in natural killer (NK) cells. NK cells are large granular lymphocytes, which comprise 5%–10% of peripheral blood mononuclear cells. By producing cytokines and exerting cytotoxicity, NK cells participate in resistance against microbial infections and malignant disease. In adoptive therapy for cancer patients, NK cells are activated by IL-2 in vitro and then transfused back to the patient's body to kill tumor cells [Goldfarb et al., 1994]. Numerous studies have implicated proteolytic enzymes as crucial to the cytolytic mechanism of these cells [Goldfarb, 1986; Darmon and Bleackley, 1998]. The most studied proteolytic enzymes in cytotoxic lymphocytes are the granzymes, a series of serine proteases of varying specificities, which are located within the cytolytic granules of these cells [Heusel et al., 1994]. Our studies have focused on proteolytic enzymes other than those found only in lymphocyte cytolytic granules and indicated that chymotryptic activity of proteasome in NK cells might play a role in their cell-mediated cytotoxicity [Goldfarb et al., 1992; Wasserman et al., 1994; Kitson et al., 1995].

During the course of our investigations on proteasome in rat NK cells, we have identified that the proteasome from the NK cell has different biochemical and biophysical properties than that isolated from the rat liver cell [Wasserman et al., 1994]. In addition, we have found proteasome inhibitor-induced apoptosis in rat NK RNK16 cells [Lu et al., 2003]. These findings allow us to hypothesize that there may be differential responses of proteasomes to their inhibitors between NK cells and other cells. In this study, YT cells and Jurkat cells were chosen as models of human NK cells and T lymphocytes, respectively. The proteasome activities in both cell lines were compared after proteasome inhibitor treatment at three different cellular or subcellular levels, that is, purified 20S proteasome activity, 26S proteasome activity in cell extracts and in intact cells. Additional effects of proteasome inhibitors on these two cell lines were also investigated, including effect on cell cycle progression and induction of apoptosis. The results indicated that YT cells and Jurkat cells respond differentially to proteasome inhibitors in cell cycle progression and induction of apoptosis. Our results have an impact in understanding the potential role of proteasome inhibitors in cancer therapy since clinical trials examining proteasome inhibitors have already been conducted in phase I, II, and III [Dou and Goldfarb, 2002; Rajkumar et al., 2005]. Hopefully, ideal proteasome inhibitors can be screened and developed to specifically induce cancer cells to undergo programmed cell death, while they have little or no apoptosis-inducing abilities for the normal living cells, particularly anti-cancer effector cells of the immune response, that is, NK cells.

MATERIALS AND METHODS

Materials

RPMI-1640 tissue culture medium, nonessential amino acids, 2-mercaptoethanol, L-glutamine, and antibiotics were purchased from Gibco (Grand Island, NY). Sucrose (Ultra-pure) was purchased from Beckman (Fullerton, CA). Proteasomal inhibitors lactacystin, *clasto*-lactacystin-β-lactone, *N*-Acetyl-Leu-Leu-Norleu-

al (LLnL), MG132, and highly purified tea polyphenols EGCG (>95%) were purchased from Sigma. Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3 activity), the specific caspase-3 inhibitor Ac-DEVD-CHO, and nuclear stain Hoechst 33258 were also obtained from Sigma. Monoclonal antibodies to $p27^{Kip}$, p53, Bcl-2, Bax, and PCNA were purchased from PharMingen (San Diego, CA), polyclonal antibodies to IκB-α and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Cell Extract Preparation

YT cells (a human NK cell line) and Jurkat cells (a human T cell leukeamic cell line) [Chuang et al., 2000] were kindly donated by Dr. P.A. Mathew, UNT Health Science Center, Fort Worth, TX. These cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% (v/v) MEM nonessential amino acids, 5×10^{-5}M 2-mercaptoethanol, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (growth medium). Cells were treated with either a proteasome inhibitor, or DMSO (solvent). During this process, morphological changes and cellular detachment were monitored. At each time point, cells were harvested, and used for measurement of apoptosis and other biochemical events. A whole cell extract was prepared as described previously [An et al., 1998]. Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Afterward, the lysates were centrifuged at 14,000*g* for 30 min, and the supernatants were collected as whole cell extracts.

Purification of Proteasome

20S proteasome was purified from YT and Jurkat cells following methods that we have described previously [Wasserman et al., 1994]. Briefly, subsequent to harvesting cells, postnuclear supernatants were collected by centrifugation after nitrogen cavitation at 325–350 psi for 30–40 min. Successively, isopycnic sucrose gradient fractionation, Sephacryl S-400 gel filtration chromatography, and heparin-Sepharose CL-6B chromatography were performed. In each step, the protein concentration and the specific activities for proteasomal chymotrypsin-like and trypsin-like activities were measured.

Cell Viability Assay

Cell viability presented as mean ± SE was determined by exclusion of trypan blue. At the indicated time points after treatment with proteasome inhibitors, cells were detached, pelleted, and resuspended in RPMI 1640 solution. After staining with trypan blue, viable cells were counted in five different 200× power fields, and the percentage of treated viable cells to untreated viable cells was determined as a percentage of viability.

Inhibition of Purified 20S Proteasome Activity by Proteasome Inhibitors

The chymotrypsin-like activity of purified 20S proteasome was measured as follows. Purified 20S proteasome (0.3 μg) from YT or Jurkat cells was incubated with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 90 min at 37°C in 100 μl of assay buffer (20 mM Tris-HCl, pH 8.0) with or without a proteasomal inhibitor. After incubation, the reaction mixture was diluted to 200 μl with the assay buffer followed by a measurement of the hydrolyzed free AMC groups using a VersaFluor™ Fluorometer (Bio-Rad) with an excitation filter of 380 nm and an emission filter of 460 nm. The relative activity is defined as a percentage of fluorescence generated from inhibitor-treated reaction to that of control reaction.

Inhibition of 26S Proteasome Activity in Whole Cell Extracts by Proteasome Inhibitors

A whole cell extract (3.5 μg) of YT or Jurkat cells was incubated for 90 min at 37°C with 20 μM Suc-LLVY-AMC, a fluorogenic peptide substrate for chymotrypsin-like activity of the proteasomes, in 100 μl of the assay buffer with or without proteasomal inhibitor *clasto*-lactacystin-β-lactone, LLnL or EGCG at various concentrations. The hydrolyzed AMC groups were quantified as described above.

Assessment of Mitochondrial Membrane Potential ($\Delta\varphi_m$)

This was conducted following the description [Wang et al., 1998]. Briefly, at each indicated

time point, cells were harvested and 2×10^5 cells were incubated with 50 nM 3,3′-dihexyloxacarbocyanine ($DiOC_6$; Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C in PBS. Analysis was then carried out on an EPICS XL-MCL flow cytometer with SYSTEM™ II software (Beckman-Coulter Corporation, Miami, FL) with excitation and emission settings of 488 and 500 nm, respectively. The percentage of cells exhibiting low levels of $DiOC_6$, reflecting reduction of mitochondrial membrane potential, was then determined by WinMDI software.

Assay for Caspase-3 Activities

For caspase-3 activity assay, a YT or Jurkat cell extract (3.5 μg) was incubated for 30 min at 37°C with 20 μM fluorogenic peptide substrate, Ac-DEVD-AMC. After incubation, the reaction mixture was diluted to 200 μl with the assay buffer, and the hydrolyzed AMC groups were quantified as described above.

Western Blot Analysis

The methods for protein extraction and Western blot analysis have been described previously [An et al., 1998]. Briefly, cells were treated with proteasome inhibitors at the indicated concentrations. After treatment by inhibitors for various times, cells were harvested and cell lysates were prepared. Then 50 μg of protein were loaded in each lane and separated by SDS–PAGE on a 15% gel. After transfer to nitrocellulose membrane (Millipore, Bedford, MA), blots were blocked with 5% milk protein, incubated for the indicated time with primary antibody, and then incubated for 2 to 4 h with corresponding horseradish peroxidase-conjugated secondary antibody. Protein–antibody complexes were detected by the Enhanced Chemiluminescence System (Amersham, Piscataway, NJ). Immunoblotting for actin was performed to verify equivalent amounts of loaded protein.

Cell Cycle Analysis

Following proteasome inhibitor treatment, cells were pelleted at 500*g* and fixed in cold (4°C) 70% ethanol for 30 min. The cells were then washed twice in cold PBS and resuspended in 1 ml cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide) at a concentration of 1×10^6 cells/ml. Samples were stored at 4°C in the dark until flow cytometry analysis by using EPICS XL-MCL flow cytometer with SYSTEM™ II software (Beckman-Coulter Corporation).

Nuclear Staining Assay

To assay nuclear morphology, YT and Jurkat cells harvested at each time point were washed with PBS once, then fixed with 70% ethanol for 1 h and stained with Hoechst 33528 (1 mM) for 30 min. The nuclear morphology of cells was visualized by fluorescence microscope (Olympus BH2, Tokyo, Japan). Numbers of cells with apoptotic nuclei were counted under three random microscope fields and ratio of the apoptotic cell numbers to total cell numbers was calculated.

RESULTS

Effects of Proteasome Inhibitors on Purified 20S Proteasome Activity From YT and Jurkat Cells

Though proteasomes possess three major proteolytic activities, that is, trypsin-like, chymotrypsin-like and peptidylglutamyl-hydrolyzing activities, it is known that inhibition of all multiple active sites in the proteasome is not required to significantly reduce protein breakdown, and inhibition of the chymotrypsin-like site or its inactivation by mutation alone causes a large reduction in the rate of protein breakdown [Chen and Hochstrasser, 1996; Heinemeyer et al., 1997]. In addition, others and we have reported that inhibition of the chymotrypsin-like activity of the proteasome is associated with induction of tumor cell apoptosis [Lopes et al., 1997; An et al., 1998]. Therefore, in this study only the chymotrypsin-like activity was monitored. To determine whether proteasome inhibitors act on proteasomes of YT and Jurkat cells differentially, we performed a cell-free proteasome activity assay by using three proteasome inhibitors, *clasto*-lactacystin-β-lactone, EGCG, LLnL. *Clasto*-lactacystin-β-lactone is the active form of lactacystin, a highly specific and irreversible inhibitor of the proteasome [Fenteany et al., 1995; Dick et al., 1996]. EGCG, a component of green tea, has been shown to inhibit potently and specifically the chymotrypsin-like activity of the proteasome in vitro and in vivo [Nam et al., 2001]. LLnL is a peptide aldehyde, one of the best characterized and most widely used inhibitors of the proteasome. LLnL is a potent and reversible inhibitor of the proteolytic activity of the 26S proteasome, which does not influence the ATPase or

isopeptidase activities of the proteasome [Lee and Goldberg, 1998]. After purification of 20S proteasome from YT and Jurkat cells, 0.3 μg of proteasome was incubated in each reaction for 90 min with Suc-LLVY-AMC, a fluorogenic peptide substrate for the proteasomal chymotrypsin-like activity, in the absence or presence of various concentrations of proteasome inhibitors, *clasto*-lactacystin-β-lactone (Fig. 1A), LLnL (Fig. 1B), and EGCG (Fig. 1C). The released-free AMC groups were measured and the relative activity was determined as a percentage of proteasome activity of the control (reaction without any inhibitors). All of these three inhibitors significantly inhibited the chymotrypsin-like activity of purified 20S proteasome of YT and Jurkat cells in a dose-dependent fashion (Fig. 1A–C). The inhibition curves for the proteasome chymotrypsin-like activity of YT and Jurkat cells were almost congruent in each case, indicating that each of the inhibitors has a similar inhibitory effect on purified 20S proteasome activity of YT and Jurkat cells. Comparison of these inhibition curves also leads us to conclude that *clasto*-lactacystin-β-lactone is the most potent proteasome inhibitor among three of them since it has the lowest IC_{50} under the used experimental conditions (Fig. 1A–C).

Inhibitory Effects of Proteasome Inhibitors on 26S Proteasome Activity in Whole Cell Extracts and Intact YT and Jurkat Cells

We then tested whether these three inhibitors could inhibit the 26S proteasome chymotryptic activity in YT and Jurkat cell extracts similarly. We found that 10 μM EGCG inhibited ~60% of the proteasomal chymotrypsin-like activity in

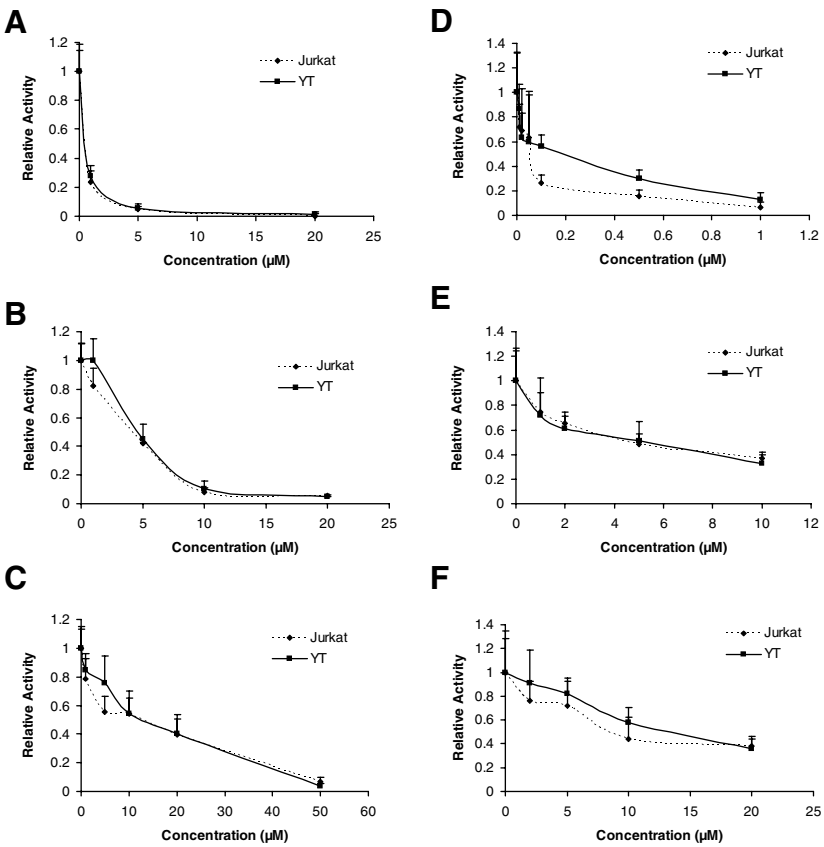

Fig. 1. Inhibitory effects of proteasome inhibitors on the chymotrypsin-like activity of purified proteasome or cell extracts from Jurkat and YT cells. Purified 20S proteasome (0.3 μg/reaction) (**A–C**) or cell extract (3.5 μg/reaction) of YT and Jurkat cells (**D–F**) was incubated for 90 min with LLVY-AMC, a fluorogenic peptide substrate for the proteasomal chymotrypsin-like activity, in the absence or presence of various concentrations of proteasome inhibitors, *clasto*-lactacystin-β-lactone (A and D), LLnL (B and E), and EGCG (C and F). The released free AMC groups were measured and the relative activity was determined as a percentage of proteasome activity of the control (reaction without any inhibitors).

Jurkat cell extract, which is consistent with a previous report [Nam et al., 2001], whereas the proteasomal chymotrypsin-like activity in YT cell extracts was reduced by about 40% (Fig. 1F). However, for the other two inhibitors, LLnL and *clasto*-lactacystin-β-lactone achieved very similar inhibitory effects on the chymotryptic activity in the cell extracts from both YT and Jurkat cells (Fig. 1D,E).

To determine whether proteasome inhibitors get access into the living cells to attack on the proteasome similarly in these two cell lines, both YT and Jurkat cells were first incubated with various concentrations of LLnL or MG132 followed by an additional incubation with Suc-LLVY-AMC. Afterward, the cell medium was collected for the measurement of hydrolyzed free AMCs. By performing this assay, we found that LLnL or MG132 significantly inhibited the proteasomal chymotrypsin-like activity in both intact human Jurkat and YT cells similarly. The IC_{50} of LLnL and MG132 for the proteasome chymotrypsin-like activity in these cell lines are about 30 and 20 μM, respectively (Fig. 2A,B). So far, we confirmed that the proteasomal inhibitors have similar inhibitory effects on the proteasome chymotrypsin-like activities of YT and Jurkat cells at three different levels, that is, purified 20S proteasome, 26S proteasome in cell extracts and intact cells.

Reduced Viability of YT and Jurkat Cells in the Presence of Proteasome Inhibitors

Since these proteasome inhibitors were confirmed to inhibit the proteasome chymotrypsin-like activities of YT and Jurkat cells similarly, we continued to investigate the effects of the proteasome inhibitors on these two cell lines by incubating cells with 25 μM LLnL for various time and examining the morphological appearance of the cells. After 24 h of treatment, cell shrinkage could be found easily under microscope in both cell lines, especially Jurkat cells. To assess the extent of cell death at indicated time points, cell viability was measured by trypan blue exclusion. We observed that the viability curves for these two cell lines in the presence of LLnL were similar and that the proteasome inhibitor LLnL significantly decreased cell viability in a time-dependent manner (Fig. 3). The viability of both cell lines dropped sharply during their first 3 h with the LLnL treatment; however, during the treatment time from 6 to 20 h, YT cells appeared more resistant

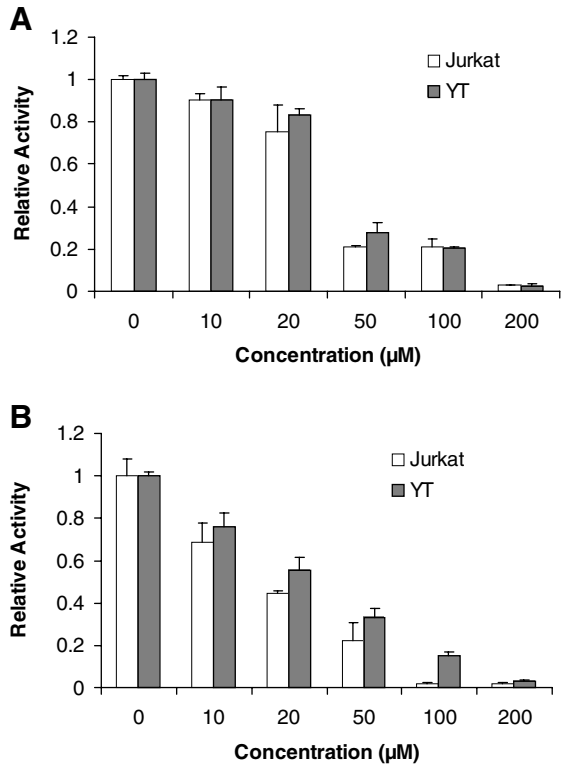

Fig. 2. Inhibition of the proteasome activity by LLnL and MG132 in intact YT and Jurkat cells. YT and Jurkat cells were preincubated for 10 h with either the solvent (indicated by 0) or LLnL (**A**), MG132 (**B**) at the indicated concentrations followed by an additional 2-h incubation with the fluorogenic substrate LLVY-AMC (for the chymotrypsin-like activity of the proteasome). The released free AMC groups were measured and relative activity was determined as described under ''Materials and Methods.'' Most of the data were derived from triplicates in two independent experiments.

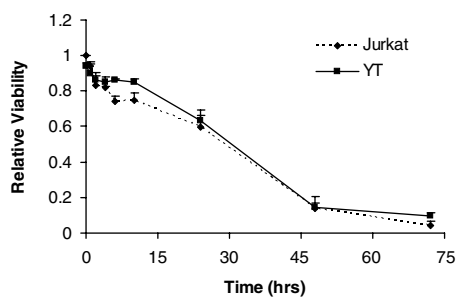

Fig. 3. Viability curve of Jurkat and YT cells during 72 h of treatment with 25 μM LLnL. YT and Jurkat cells were incubated with 25 μM LLnL and harvested at the indicated time points. Viable cells were counted after staining with trypan blue. The viability of untreated control cells was counted as 100%. The viability of treated cells was expressed as a percentage of control cells. Data represent average of three independent experiments.

to LLnL since its viability remained 15%–20% higher than that of Jurkat cells. After 48 h treatment, the viability of both cell lines was below 20% (Fig. 3). These data demonstrated that the viability of both YT and Jurkat cells could be reduced in the presence of the proteasome inhibitor LLnL at 25 μM. Although we have found proteasome inhibitor-induced apoptosis in rat NK RNK16 cells previously [Lu et al., 2003], we continued to investigate whether apoptosis is involved in this process of reduced viability of YT cells.

Involvement of Apoptosis in YT and Jurkat Cells Treated With Proteasome Inhibitors

The first method we used to assess whether apoptosis was involved in the reduction in viability of YT and Jurkat cells in the presence of proteasome inhibitors was the measurement of mitochondrial membrane potential. The induction of apoptosis has recently been attributed to early mitochondrial perturbations, including the loss of mitochondrial membrane potential ($\Delta\varphi_m$) [Zamzami et al., 1995] and/or the release of cytochrome c into the cytoplasm, where cytochrome c acts as a cofactor permitting activation of apoptotic proteases such as caspase-3 [Yang et al., 1997]. Both YT and Jurkat cells were treated with 20 μM MG132 for various times, and mitochondrial membrane potentials measured by detecting the florescence of $DiOC_6$. Cells with low levels of $DiOC_6$ accumulation represent reduction of the mitochondrial membrane potential. As indicated in Figure 4, compared to the untreated controls, a 6-h exposure of both cell lines to MG132 led to a significant left shift of cell population peaks, indicating a reduction of mitochondrial membrane potential ($\Delta\varphi_m$) during the treatment by MG132. However, at earlier time points (e.g., 2 h), small changes (relative to controls) were observed which did not achieve statistical significance (data not shown). This result indicates that there is involvement of early apoptosis events in both YT and Jurkat cells when they were treated with the tripeptidyl proteasome inhibitor MG132 at 20 μM.

Since reduction of mitochondrial membrane potential is an early event in apoptosis, it may cause release of cytochrome c into cytoplasm later and then activate caspases to initiate the execution of apoptosis. Next, we measured caspase-3 activity of both cell lines after they were treated with 25 μM LLnL. After a 4-h

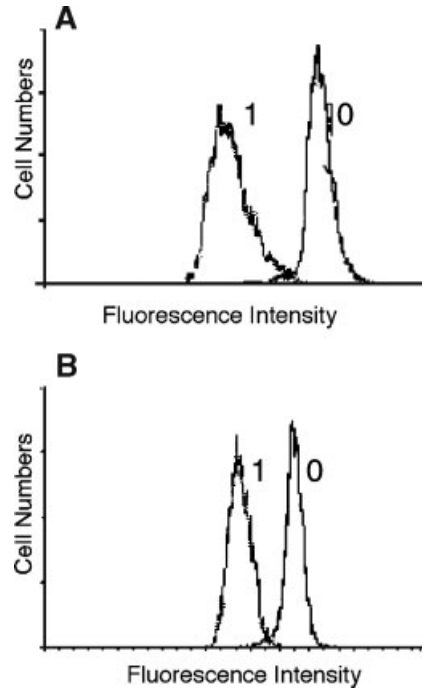

Fig. 4. Reduction of mitochondrial membrane potential in YT and Jurkat cells after treatment with proteasome inhibitor. Following exposure to 20 μM MG132 for 6 h, YT (**panel A**) and Jurkat (**panel B**) cells were pelleted, resuspended, and incubated with $DiOC_6$ as described in the ''Materials and Methods.'' Cells were then subjected to flow cytometry analysis to determine the population of cells expressing ''low'' levels of $DiOC_6$, reflecting reduction of mitochondrial membrane potential ($\Delta\varphi_m$). The maximal value for the X-axis (linear scale) is 1,024, while that for the Y-axis is 256. The curves labeled 0 are the untreated controls, while the curves labeled 1 are cells after a 6-h exposure to MG132.

treatment, a substantial increase in caspase-3 activity was observed in both cell lines (Fig. 5); however, at each time point, LLnL caused significantly higher increases of caspase-3 activity in Jurkat cells than in YT cells. Among the tested time points, the highest caspase-3 activity in Jurkat cells appeared at 10 h while that in YT cells was at 24 h. Thus, maximal activities of caspase-3 occurred earlier and with a greater magnitude in the Jurkat cells as compared to YT cells.

A nuclear staining assay was then used to examine changes in the nuclear morphology of these cells in the presence of proteasome inhibitors. In contrast to living cells, the nuclei of apoptotic cells have highly condensed chromatin that can be easily stained by Hoechst dyes, which may be visualized by fluorescence microscopy. After treatment with 25 μM LLnL for 6 h, Jurkat cells demonstrated characteristic

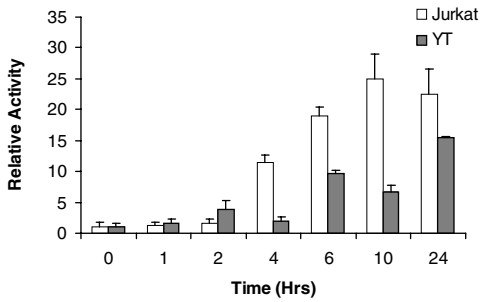

Fig. 5. Caspase-3 activation induced by proteasome inhibitor in YT and Jurkat cells. YT and Jurkat cells were incubated with 25 μM LLnL for the indicated hours and then lysed. Caspase-3 activity was determined by using fluorogenic substrates Ac-DEVD-AMC. The caspase-3 activity of these two cells at 0 h was counted as 100%. Data represent average of three independent experiments.

condensed apoptotic nuclear morphology under the microscope. About 32% and 58% of Jurkat cells have apoptotic nuclei at the time points of 10 and 24 h, respectively (Fig. 6A). However, no YT cells exhibited apoptosis-specific nuclear morphology even after they were treated with same concentration of LLnL for 10 h. At 24 h, only 2% YT cells demonstrated the apoptotic nuclear morphology changes (Fig. 6B). The differences in nuclear morphology changes between YT and Jurkat cells could be due to the temporal difference in the occurrence of caspase-3 activation (Fig. 5).

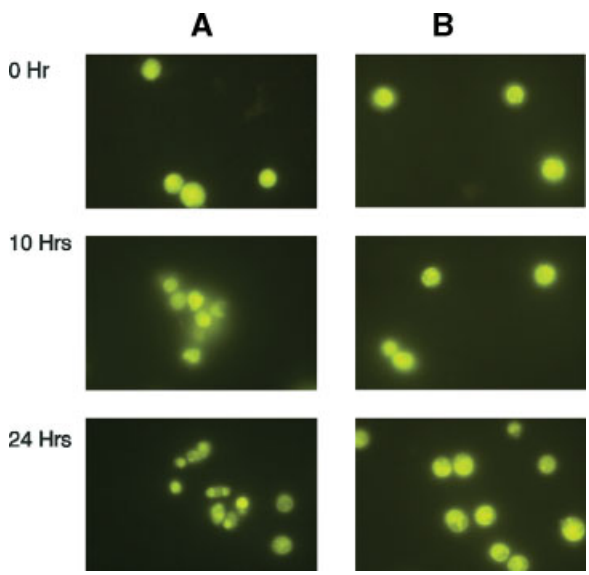

Fig. 6. Nuclear morphological changes of YT and Jurkat cells induced by proteasome inhibitor. Jurkat cells (**A**) and YT cells (**B**) were treated with 25 μM LLnL for the indicated hours, followed by collecting both detached and attached cell populations. Both cell populations were used for nuclear staining assay as described in the Materials and Methods. Original magnification: 200×. Similar results were obtained in two to four independent experiments.

Effects of Proteasome Inhibitors on Cell Cycle Progression

We were also interested in examining the effects of proteasome inhibitors on cell cycle progression in YT cells and Jurkat cells. Flow cytometric analysis of propidium iodide (PI)-stained cells was performed to monitor the effects of proteasome inhibitors on cell cycle progression since the lower DNA content of apoptotic cells stained with PI can be measured by flow cytometry. When Jurkat cells were treated with 25 μM LLnL for 10 h, there was a significant increase in the apoptotic population with sub-G_1 DNA content (Fig. 7A). By comparison, when YT cells were treated under the same condition, no obvious increase in sub-G_1 population was detected (Fig. 7A). Interestingly, YT cells showed an arrest in G_2/M phase after the treatment of LLnL, and the percentage of G_2/M phase cells increased from 25% at time 0 to 46% in YT cells after treatment for 10 h. This cell cycle arrest in YT cells could cause their later cell death via apoptosis, since apoptotic population of YT cells with sub-G_1 DNA content increased substantially after 24 h treatment (data not shown here). To exclude the possibility that the differential induction of apoptosis in YT and Jurkat cells is proteasome inhibitor specific, the two cell lines were treated with 20 μM MG132. Similarly, the results indicated that an 11% increase of sub-G_1 cell population in Jurkat cells when they were treated with MG132 for 8 h (Fig. 7B). However, in the treated YT cells, much less sub-G_1 cells and higher percentage of G_2/M phase cells (Fig. 7B) further indicated the cell cycle arrest was induced by proteasome inhibitors. These results indicate that proteasome inhibitors differentially regulate the cell cycle progression in YT and Jurkat cells.

It has been reported that caspase-3 is a key protease that becomes activated during apoptosis and we had already determined that caspase-3 activation in YT cells is induced by proteasome inhibitors. We further investigated the role of caspase-3 activation in the G_2/M arrest of YT cells induced by proteasome inhibitors, by using the caspase-3 specific inhibitor DEVD-CHO to block the caspase-3 activation. YT cells were pretreated with DEVD-CHO for 2 h at various concentrations,

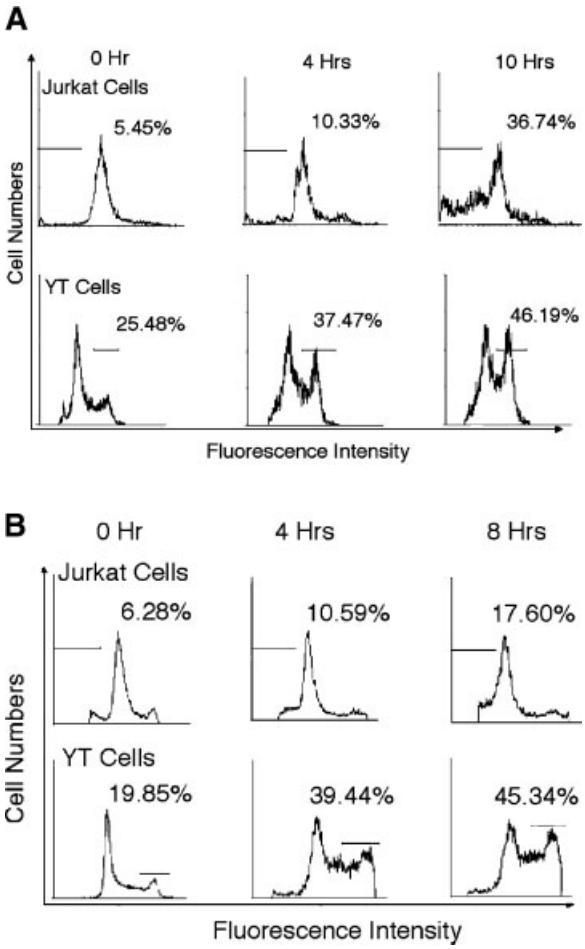

Fig. 7. Effects of proteasome inhibitors on cell cycle progression of YT and Jurkat cells. After treatment of YT and Jurkat cells by 25 μM LLnL (**A**) or 20 μM MG132 (**B**) for the indicated hours, cells were collected and labeled with PI and analyzed by DNA flow cytometry. The data and bars in Jurkat cells indicate the percentage and margin of sub-G_1 cells, respectively, while those in YT cells indicate the percentage and margin of G_2/M phase cells. The maximal value for the X-axis on all graphs (linear scale) is 1,024, while that for the Y-axis is 128. All experiments were performed in duplicate and gave similar results.

and then the percentage of cells in each stage of the cell cycle measured after incubation of cells with 20 μM MG132 for various times. As a result, treatment with increasing concentration of DEVD-CHO in YT cells did not change the percentage of YT cells in G_2/M phase cells significantly after treatment with LLnL (Fig. 8), implying that caspase-3 activation does not contribute to the G_2/M arrest of YT cells.

Differential Molecular Modulation of YT and Jurkat Cells to Proteasome Inhibitors

Lastly, we attempted to demonstrate the reduced viability, apoptosis induction, cell cycle

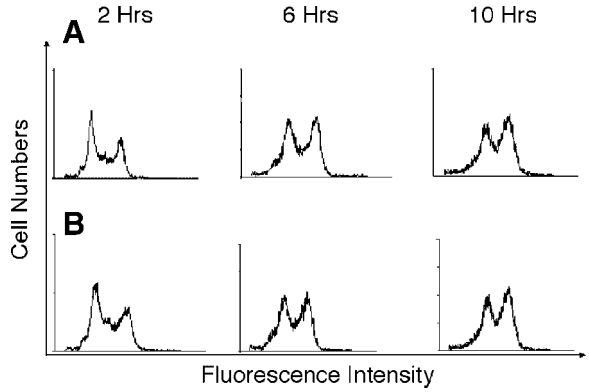

Fig. 8. Caspase-3 independent pathways may contribute to the G_2/M arrest in YT cells induced by proteasome inhibitor. YT cells were pretreated with 1 μM (**A**) and 10 μM (**B**) caspase-3 inhibitor DEVD-CHO for 2 h, then 20 μM MG132 was added and cells were harvested at the indicated time points. The harvested cells were labeled with PI and analyzed by DNA flow cytometry. The maximal value for the X-axis on all graphs (linear scale) is 1,024, while that for the Y-axis is 128. All experiments were performed in duplicate and yielded similar results.

arrest correlated with changes in biomarker (e.g., p27) expression. Previously, it has been reported that p27 was involved in the process of proteasome inhibitor-induced apoptosis [An et al., 1998] and that there was an accumulation of $p27^{Kip1}$ and IκB-α in tumor cells treated with EGCG [Nam et al., 2001]. Since there were differences in nuclear morphology and cell cycle progression during the treatment with proteasome inhibitors between YT and Jurkat cells in our current study, we continued the investigation to explore whether there are differences in the expression of cell cycle regulatory proteins between these two cell lines when they are treated with proteasome inhibitors. Cells were treated either with 10 μM lactacystin or 50 μM EGCG and harvested at each indicated time point. Cell extracts were made and Western blotting conducted for a number of cell cycle regulatory proteins including $p27^{Kip1}$ and IκB-α. For Jurkat cells, a 6-h treatment with 10 μM lactacystin increased IκB-α and p27 significantly (Fig. 9A), and the same treatment with 50 μM EGCG caused a dramatic accumulation of IκB-α and p27 in cells (Fig. 9B). In contrast to the accumulation of $p27^{Kip1}$ and I.B-α in Jurkat cells, there was no significant accumulation of these two proteins in YT cells after they were treated with either 10 μM lactacystin or 50 μM EGCG (Fig. 9A,B).

Our results also indicated that p53 level remains at the same level during the treatment

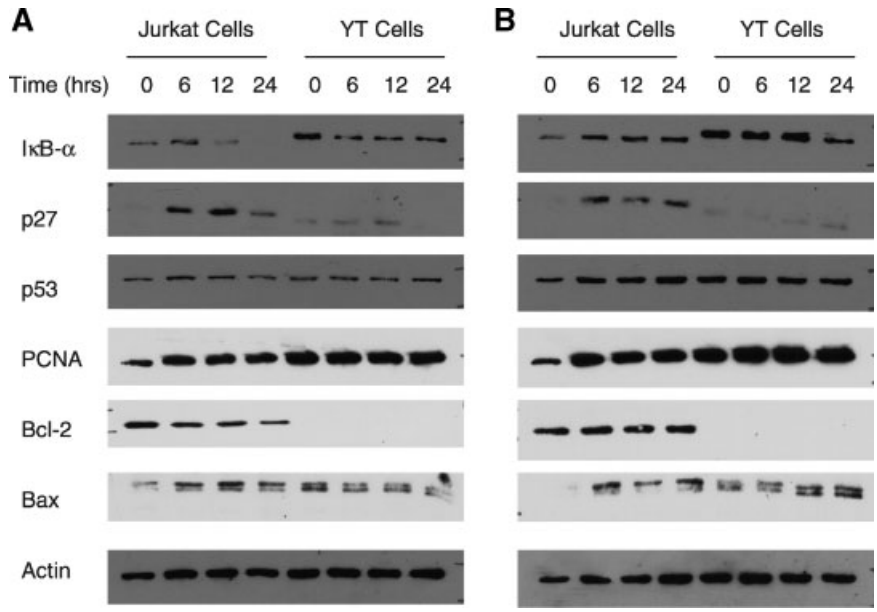

Fig. 9. Differential molecular effects by lactacystin and EGCG in YT and Jurkat cells. Time course experiments in which YT and Jurkat cells were treated with 10 μM lactacystin (**panel A**) or 50 μM EGCG (**panel B**), were performed. After 6, 12, 24 h of treatment, cell lysates were evaluated for levels of I.B-α, p27, p53, PCNA, Bcl-2, and BAX expression by Western blotting as described in Materials and Methods. Actin was measured as a standard.

of cells with proteasome inhibitors lactacystin or EGCG (Fig. 9) in these two cell lines. This implies that apoptosis induced in human Jurkat cells and cell cycle arrest caused in YT cells during their first 24 h treatment by proteasome inhibitors is p53-independent. PCNA, an important index marker for cell proliferation, was also tested and showed no significant changes during the treatment by proteasome inhibitors in either cell line (Fig. 9). This result excluded the possibility that differential molecular modulations and cell cycle progression were related to the cell proliferation.

In the process of apoptosis, release of cytochrome c can be induced by proapoptotic members of Bcl-2 family (such as Bax, Bad, and Bid), but inhibited by anti-apoptotic Bcl-2 family members (such as Bcl-2 and Bcl-XL) [Green and Reed, 1998; Gross et al., 1999]. The ratio of proapoptotic to anti-apoptotic proteins, therefore, is involved in determination of cellular fate [Li and Dou, 2000]. With the investigation of Bax and Bcl-2 in these two cell lines after they were treated with lactacystin or EGCG, we found the ratio of Bax to Bcl-2 was increased in human Jurkat cell with the increasing treatment time (Fig. 9). This is consistent with the apoptosis induced by proteasome inhibitors in human Jurkat cells. Interestingly, in YT cells there was no Bcl-2 protein expressed and no significant accumulation of Bax after the treatment of lactacystin or EGCG (Fig. 9). The role of Bcl-2 family in the G_2/M arrest of YT cells induced by proteasome inhibitors needs to be studied further.

DISCUSSION

Although a number of studies have implicated the proteasome in apoptosis, its exact role and the form of the complex which is involved in this process in various cell types is not yet understood [Brophy et al., 2002]. Proteasome activities have been reported to decrease [Beyette et al., 1998], stay the same or even increase markedly [Dallaporta et al., 2000] during apoptosis. Furthermore, proteasome inhibitors can have pro- or anti-apoptotic effects depending on the cell type and death stimulus [Grimm et al., 1996; An et al., 1998; Awasthi and Wagner, 2004]. Recently, we have found that proteasome inhibitors induced apoptosis of rat NK RNK16 cells [Lu et al., 2003]. Combined with our previous findings that there are some distinct properties of proteasome in NK cells [Wasserman et al., 1994; Kitson et al., 2000], we carried out the present studies to provide further evidence for the apoptosis induced by

proteasome inhibitors in human NK cells and determine if the effects of proteasomal inhibition in NK cells could be differentiated from those in other cell types.

The 20S proteasome is a cylindrical particle consisting of 28 subunits in four stacked rings, of which two outer rings comprise α-subunits while inner two rings composed by β-subunits form a central cavity containing proteolytic activities [Groll et al., 1997; Baumeister et al., 1998]. Theoretically, substrates enter the channel leading to the interior chamber through a narrow constriction in the central portion of the α-ring. In cells, the 26S proteasome is a complex of the 20S core and either one or two regulatory particles [Coux et al., 1996]. The regulatory particles confer ATP dependence and recognition of polyubiquitinated protein substrates, leading to substrate unfolding, deubiquitination, and translocation of the substrate into the 20S core. While the participation of proteasomes in protein turnover is well established, the relative contribution of 20S and 26S subtypes is not so clear. This may raise a question whether 20S and 26S proteasomes differ in their cleavage site selection for substrates, thereby affecting the degradation rate of protein substrates in cells. In addition, a recent study comparing in vitro enzymatic specificities of 26S and 20S proteasomes from human erythrocytes suggested that the actions of 20S and 26S proteasomes are carried out independently [Emmerich et al., 2000].

This study mainly sought to find the differential effects of proteasome inhibitors on human NK cells versus T lymphocytes. Differences in morphological change in the nucleus between YT and Jurkat cells were identified during their incubations with proteasome inhibitors. In contrast to Jurkat cells, no YT cells exhibited apoptosis-specific nuclear morphology after treatment for 10 h and only a small fraction of YT cells demonstrated the apoptotic nuclear morphology changes after 24 h treatment. We also determined the caspase-3 activities in these two cell lines following the treatment with proteasome inhibitors and found that a temporal difference in the occurrence of caspase-3 activation by proteasome inhibitors existed between these two cell lines. A recent study reported a precaspase-activated step in the apoptotic cascade, resulting in specific morphological features showing the presence of a step prior to caspase activation in the cells [Tomioka et al., 2002]. Furthermore, pro-caspase-3 had been shown to sensitize cancer cells to proteasome inhibitor-induced apoptosis [Tenev et al., 2001]. Therefore, the differences in nuclear morphology changes between YT and Jurkat cells could be due to the temporal difference in the occurrence of caspase-3 activation.

We also measured the differential effects of proteasome inhibitors on these two cell lines at molecular levels. Some cell cycle regulatory proteins did display differences including p27 and I.B-α. $p27^{Kip1}$ is a powerful cyclin-dependent kinase inhibitor (of CDK4 and CDK2) whose overexpression arrests cells in G_1 [Toyoshima and Hunter, 1994]. Reduced expression has been shown in various carcinomas to be the result of increased proteasome degradation rather than altered gene expression [Catzavelos et al., 1997; Loda et al., 1997]. Accumulation of $p27^{Kip1}$ and I.B-α due to treatment with proteasome inhibitors may happen in many cell types [Nam et al., 2001; Yin et al., 2005]. In our study, the accumulation of these two proteins and combined effects of other factors induced by proteasome inhibitors, resulted in the rapid induction of apoptosis in Jurkat cells. However, neither $p27^{Kip1}$ nor I.B-α was accumulated in YT cells after they were treated with proteasome inhibitors. Therefore, proteasomes in YT cells might process other target proteins favorably other than $p27^{Kip1}$ and I.B-α. The p53 protein was one of the first cell cycle regulators found to be degraded by the proteasome [Maki et al., 1996; Chen et al., 2000]. Studies of the role of p53 in cell cycle with proteasome inhibitors have shown varying results. Increases in protein levels of p53 were seen during the apoptosis following proteasome inhibition in human T-cell lymphoma cells [Shinohara et al., 1996] and bladder tumor cells [Kamat et al., 2004]. Others, though, have shown cell cycle arrest and apoptosis from proteasome inhibition to be independent of p53 accumulation [An et al., 1998; Kim et al., 2004]. In this study, p53 protein did not show any changes in these cell lines after they were treated by proteasome inhibitors.

In summary, our studies have indicated that proteasome inhibitors act differentially in cell cycle progression, apoptosis induction and molecular effects between YT cells and Jurkat cells, tumors of NK and T cell origin, and these inhibitors may have similar effects on normal NK and T cells. This has indicated that different

cell types may respond differentially to the proteasome inhibitors. This is of great physiological significance since proteasome inhibitors have been tested in phase I, II, and III clinical trials to treat cancers [Dou and Goldfarb, 2002; Rajkumar et al., 2005]. It is therefore possible and a worthwhile goal to screen for ideal proteasome inhibitors, which meet a key prerequisite for optimal therapeutic application for treatment of malignant tumors, that is, selectivity and specificity for malignant cells and the sparing of normal cells.

ACKNOWLEDGMENTS

This study was supported by grant BK-1396 from the Robert A. Welch Foundation to R.H.G.

REFERENCES

An B, Goldfarb RH, Siman R, Dou QP. 1998. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 5:1062–1075.

Awasthi N, Wagner BJ. 2004. Interferon-gamma induces apoptosis of lens alphaTN4-1 cells and proteasome inhibition has an antiapoptotic effect. Invest Ophthalmol Vis Sci 45:222–229.

Baumeister W, Walz J, Zuhl F, Seemuller E. 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell 92:367–380.

Beyette J, Mason GG, Murray RZ, Cohen GM, Rivett AJ. 1998. Proteasome activities decrease during dexamethasone-induced apoptosis of thymocytes. Biochem J 332: 315–320.

Blagosklonny MV, Wu GS, Omura S, el-Deiry WS. 1996. Proteasome-dependent regulation of p21WAF1/CIP1 expression. Biochem Biophys Res Commun 227:564–569.

Boyer SN, Wazer DE, Band V. 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res 56:4620–4624.

Brophy VA, Tavare JM, Rivett AJ. 2002. Treatment of COS-7 cells with proteasome inhibitors or gamma-interferon reduces the increase in caspase 3 activity associated with staurosporine-induced apoptosis. Arch Biochem Biophys 397:199–205.

Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI, Slingerland JM. 1997. Decreased levels of the cell-cycle inhibitor p27Kip1 protein: Prognostic implications in primary breast cancer. Nat Med 3:227–230.

Chen P, Hochstrasser M. 1996. Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. Cell 86:961–972.

Chen F, Chang D, Goh M, Klibanov SA, Ljungman M. 2000. Role of p53 in cell cycle regulation and apoptosis following exposure to proteasome inhibitors. Cell Growth Differ 11:239–246.

Chuang SS, Kim MH, Johnson LA, Albertsson P, Kitson RP, Nannmark U, Goldfarb RH, Mathew PA. 2000. 2B4 stimulation of YT cells induces natural killer cell cytolytic function and invasiveness. Immunology 100: 378–383.

Coux O, Tanaka K, Goldberg AL. 1996. Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem 65:801–847.

Dallaporta B, Pablo M, Maisse C, Daugas E, Loeffler M, Zamzami N, Kroemer G. 2000. Proteasome activation as a critical event of thymocyte apoptosis. Cell Death Differ 7:368–373.

Darmon AJ, Bleackley RC. 1998. Proteases and cell-mediated cytotoxicity. Crit Rev Immunol 18:255–273.

Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL, Stein RL. 1996. Mechanistic studies on the inactivation of the proteasome by lactacystin: A central role for *clasto*-lactacystin beta-lactone. J Biol Chem 271:7273–7276.

Dou QP, Goldfarb RH. 2002. Bortezomib: Millennium pharmaceuticals. IDrugs 5:828–834.

Emmerich NP, Nussbaum AK, Stevanovic S, Priemer M, Toes RE, Rammensee HG, Schild H. 2000. The human 26 S and 20 S proteasomes generate overlapping but different sets of peptide fragments from a model protein substrate. J Biol Chem 275:21140–21148.

Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL. 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268:726–731.

Goldfarb RH. 1986. Role of neutral serine proteases in tumor cell lysis by natural killer cells (large granular lymphocytes) and activated macrophages. Semin Thromb Hemost 12:308–309.

Goldfarb RH, Wasserman K, Herberman RB, Kitson RP. 1992. Nongranular proteolytic enzymes of rat IL-2-activated natural killer cells. I. Subcellular localization and functional role. J Immunol 149:2061–2068.

Goldfarb RH, Basse P, Ohashi M, Kitson RP, Brunson KW. 1994. Preclinical studies of IL-2 activated natural killer effector cells for locoregional therapy of metastatic cancer. J Infus Chemother 4:77–82.

Green DR, Reed JC. 1998. Mitochondria and apoptosis. Science 281:1309–1312.

Grimm LM, Goldberg AL, Poirier GG, Schwartz LM, Osborne BA. 1996. Proteasomes play an essential role in thymocyte apoptosis. Embo J 15:3835–3844.

Grisham MB, Palombella VJ, Elliott PJ, Conner EM, Brand S, Wong HL, Pien C, Mazzola LM, Destree A, Parent L, Adams J. 1999. Inhibition of NF-kappa B activation in vitro and in vivo: Role of 26S proteasome. Methods Enzymol 300:345–363.

Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R. 1997. Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386:463–471.

Gross A, McDonnell JM, Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899–1911.

Heinemeyer W, Fischer M, Krimmer T, Stachon U, Wolf DH. 1997. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. J Biol Chem 272:25200–25209.

Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 76:977–987.

Kamat AM, Karashima T, Davis DW, Lashinger L, Bar-Eli M, Millikan R, Shen Y, Dinney CP, McConkey DJ. 2004. The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors in vivo. Mol Cancer Ther 3:279–290.

Kim OH, Lim JH, Woo KJ, Kim YH, Jin IN, Han ST, Park JW, Kwon TK. 2004. Influence of p53 and p21Waf1 expression on G2/M phase arrest of colorectal carcinoma HCT116 cells to proteasome inhibitors. Int J Oncol 24: 935–941.

Kisselev AF, Goldberg AL. 2001. Proteasome inhibitors: From research tools to drug candidates. Chem Biol 8: 739–758.

Kitson RP, Miller CA, Goldfarb RH. 1995. Non-granular proteolytic enzymes of rat interleukin-2-activated natural killer cells. III. Enhancement of A-NKP 1, 2, and 3 proteolytic activities (cleaving after Arg, Phe and Pro) in response to interleukin-2. Nat Immun 14:286–294.

Kitson RP, Lu M, Siman R, Goldfarb RH. 2000. Proteasome inhibitor and lymphocyte function: Partial inhibition of cell-mediated cytotoxicity and implication that the lymphocyte proteasome may contain multiple chymotryptic domains. In Vivo 14:265–268.

Lee DH, Goldberg AL. 1998. Proteasome inhibitors: Valuable new tools for cell biologists. Trends Cell Biol 8:397–403.

Li B, Dou QP. 2000. Bax degradation by the ubiquitin/proteasome-dependent pathway: Involvement in tumor survival and progression. Proc Natl Acad Sci USA 97: 3850–3855.

Loda M, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM, Pagano M. 1997. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat Med 3:231–234.

Loidl G, Groll M, Musiol HJ, Huber R, Moroder L. 1999. Bivalency as a principle for proteasome inhibition. Proc Natl Acad Sci USA 96:5418–5422.

Lopes UG, Erhardt P, Yao R, Cooper GM. 1997. p53-dependent induction of apoptosis by proteasome inhibitors. J Biol Chem 272:12893–12896.

Lu M, Kitson RP, Xue Y, Goldfarb RH. 2003. Activation of multiple caspases and modification of cell surface fas (CD95) in proteasome inhibitor-induced apoptosis of rat natural killer cells. J Cell Biochem 88:482–492.

Maki CG, Huibregtse JM, Howley PM. 1996. In vivo ubiquitination and proteasome-mediated degradation of p53. Cancer Res 56:2649–2654.

Nam S, Smith DM, Dou QP. 2001. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. J Biol Chem 276:13322–13330.

Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269:682–685.

Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. 2005. Proteasome inhibition as a novel therapeutic target in human cancer. J Clin Oncol 23:630–639.

Shinohara K, Tomioka M, Nakano H, Tone S, Ito H, Kawashima S. 1996. Apoptosis induction resulting from proteasome inhibition. Biochem J 317:385–388.

Tenev T, Marani M, McNeish I, Lemoine NR. 2001. Procaspase-3 overexpression sensitises ovarian cancer cells to proteasome inhibitors. Cell Death Differ 8:256–264.

Tomioka M, Sameshima M, Nakano H, Kubo T, Shinohora K, Seyama Y, Kawashima S, Tone S. 2002. A possible intermediate step during apoptotic execution. Hum Cell 15:43–51.

Toyoshima H, Hunter T. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78:67–74.

Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. 1995. Rel/NF-kappa B/I kappa B family: Intimate tales of association and dissociation. Genes Dev 9:2723–2735.

Voges D, Zwickl P, Baumeister W. 1999. The 26S proteasome: A molecular machine designed for controlled proteolysis. Annu Rev Biochem 68:1015–1068.

Wang Z, Su ZZ, Fisher PB, Wang S, VanTuyle G, Grant S. 1998. Evidence of a functional role for the cyclin-dependent kinase inhibitor p21(WAF1/CIP1/MDA6) in the reciprocal regulation of PKC activator-induced apoptosis and differentation in human myelomonocytic leukemia cells. Exp Cell Res 244:105–116.

Wasserman K, Kitson RP, Rivett AJ, Sweeney ST, Gabauer MK, Herberman RB, Watkins SC, Goldfarb RH. 1994. Nongranular proteolytic enzymes of rat IL-2-activated natural killer cells. II. Purification and identification of rat A-NKP 1 and A-NKP 2 as constituents of the multicatalytic proteinase (proteasome) complex. J Cell Biochem 55:133–145.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. 1997. Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science 275:1129–1132.

Yin D, Zhou H, Kumagai T, Liu G, Ong JM, Black KL, Koeffler HP. 2005. Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). Oncogene 24:344–354.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182: 367– 377.