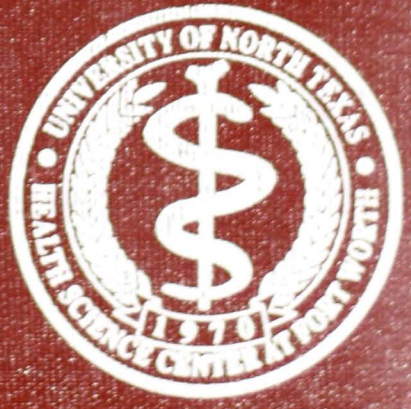
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HEALTH SCIENCE CENTER AT FORT WORTH
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Lu, Min, Investigation of Proteasome Chymotryptic Activities and Effects of Their Inhibition in Rat and Human Natural Killer Cells. Doctor of Philosophy (Biochemistry and Molecular Biology), April, 2003, 185 pp., 3 tables, 32 illustrations, bibliography, 158 titles.

The proteasome is a multicatalytic proteinase complex that is involved in the major extralysosomal pathway responsible for intracellular protein degradation in mammalian cells. This dissertation focuses on investigating proteasome chymotryptic activities and the effects of selective inhibitors of these activities on the function of natural killer (NK) cells. In this dissertation, 20S proteasomes derived from rat RNK16 cells were purified and some of their biochemical and biophysical properties were investigated extensively. The results indicated that RNK16 cell-derived proteasome differ from the proteasome of other origins in many aspects including substrate selectivity, inhibitor specificity, and kinetic regulation, although they may share some common biochemical properties with others.

To investigate the effects of proteasomal inhibition on the function of NK cells, several proteasome inhibitors were used including MG115, MG132, *clasto*-lactacystin-β-lactone, EGCG and LLnL. MG115 and MG132 were shown to induce apoptosis of RNK16 cells, as evidenced by DNA fragmentation, caspase-3 activation and the appearance of sub-G_1 cell populations. Activation of multiple caspases and increased expression of cell surface Fas (CD95) protein were also observed following the treatment of RNK16 cells by these two inhibitors.

This dissertation also tested the hypothesis that different cell types could respond differentially to proteasome inhibitors. The effects of several proteasome inhibitors were determined on the purified 20S proteasomal and 26S proteasomal chymotrypsin-like activity in whole cell extracts and intact YT and Jurkat cells, human NK and T cell lines respectively. Following such treatment, caspase-3 activation occurred much earlier in Jurkat cells than YT cells; cell cycle analysis indicated a sub-G_1 apoptotic cell population in Jurkat cells and G_2/M arrest in YT cells. In addition, accumulation of p27 and IκB-α was detected only in Jurkat cells, but not YT cells. Therefore, proteasome inhibitors appear to act differentially in cell cycle progression and apoptosis signaling pathways between human NK and T cells. These studies indicate that the generation of ideal proteasome inhibitors for the treatment of malignancies could be screened or designed to specifically induce cancer cells to undergo programmed cell death, while having little or no apoptosis-inducing abilities for natural killer cells and other cells of the immune response, thus enhancing the selectivity and specificity of the anti-cancer, apoptosis-inducing capabilities of proteasome inhibitors.

INVESTIGATION OF PROTEASOME CHYMOTRYPTIC ACTIVITIES AND EFFECTS OF THEIR INHIBITION IN RAT AND HUMAN NATURAL KILLER CELLS

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INVESTIGATION OF PROTEASOME CHYMOTRYPTIC ACTIVITIES AND EFFECTS OF THEIR INHIBITION IN RAT AND HUMAN NATURAL KILLER CELLS

DISSERTATION

Presented to the Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

By

Min Lu, M.D., M.S.

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LIST OF ABBREVIATIONS

Ac	N-Acetyl
AFC	7-amido-4-trifluoromethyl-coumarin
AMC	7-amido-4-methylcoumarin hydrochloride
CDK	cyclin-dependent kinase
CHO	aldehyde
CP	core particle
DEVD	Asp-Glu-Val-Asp
DMSO	dimethyl sulfoxide
Dnp	2,4-dinitrophenyl
EGCG	epigallocatechin gallate
FMK	fluoromethyl ketone
GGL	Gly-Gly-Leu
GGR	Gly-Gly-Arg
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IETD	Ile-Glu-Thr-Asp
LEHD	Leu-Glu-His-Asp
LEVDGWK	Leu-Glu-Val-Asp-Gly-Trp-Lys
LLE	Leu-Leu-Glu
LLnL	N-Acetyl-Leu-Leu-Norleu-al

LLVY	Leu-Leu-Val-Tyr
MG115	Z-Leu-Leu-Norvalinal
MG132	Z-Leu-Leu-Leu-aldehyde
PCNA	proliferating cell nuclear antigen
PIPES	1,4-Piperazinediethanesulfonic acid
RP	regulatory particle
SDS	sodium dodecyl sulfate
Suc	succinyl
TPCK	N-p-tosyl-L-phenylalanyl chloromethyl ketone
UPP	ubiquitin-proteasome pathway
VDVAD	Val-Asp-Val-Ala-Asp
VEID	Val-Glu-Ile-Asp
VKM	Val-Lys-Met
WEHD	Trp-Glu-His-Asp
Z	carbobenzyloxy
ZPCK	N-carbobenzyloxy-L-phenylalanyl chloromethyl ketone

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INTRODUCTION

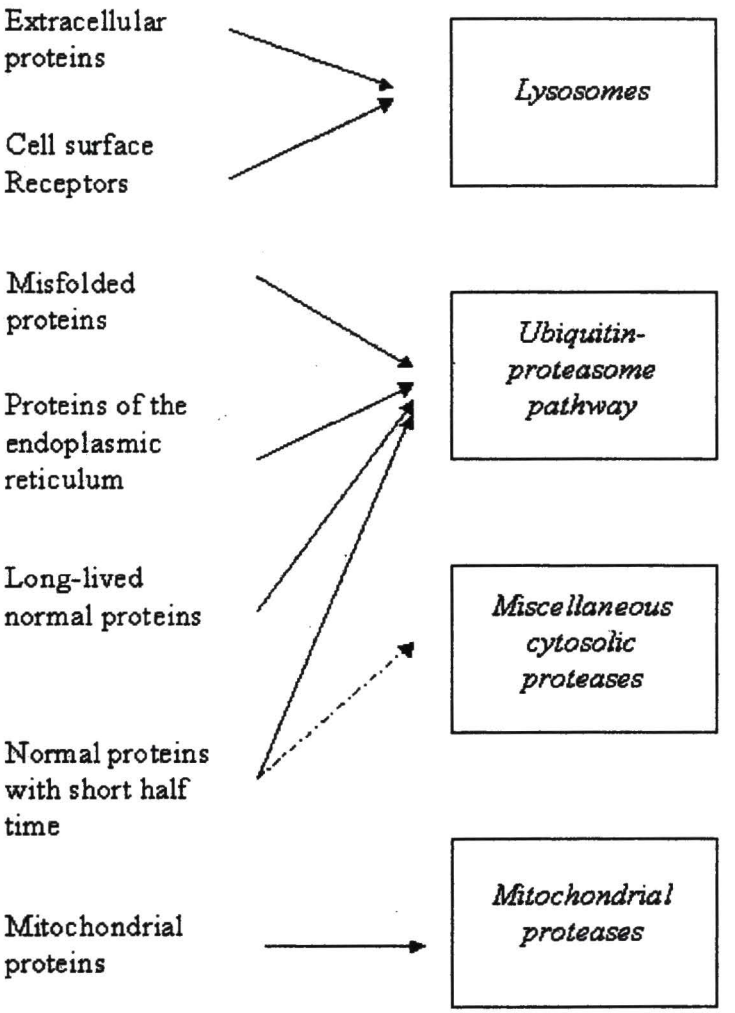
Cellular protein degradation pathways

Protein degradation in cells is a necessity for many reasons: to maintain homeostasis via limited proteolysis while cellular structures are continually rebuilt, in particular during development or in response to external stimuli; to scavenge misfolded proteins resulting from mutations or heat or oxidative stress; to provide a way to terminate the function of many regulatory proteins at distinct times, including cyclins, transcription factors, and components of signal transduction pathways [Coux et al., 1996; Hilt and Wolf, 1996; Varshavsky, 1997]; to generate immuno-competent peptides from the degradation of foreign antigens for the immune system [Goldberg, 1995]. Therefore, all cells contain multiple pathways for protein breakdown (Fig 1).

A. Lysosomal system

Extracellular proteins, such as plasma proteins or hormones and phagocytosed bacteria, are taken up by endocytosis and are completely degraded within lysosomes. Previously, the lysosome was believed to be the only site for protein degradation in cells, but studies

Fig 1. Cellular protein degradation pathways. Figure is adapted from *Journal of Nutrition* 129:227S-237S, 1999 by Stewart H. Lecker, Vered Solomon, William E. Mitch and Alfred L. Goldberg.


Extracellular proteins
Cell surface Receptors
Lysosomes
Misfolded proteins
Proteins of the endoplasmic reticulum
Long-lived normal proteins
Normal proteins with short half time
Ubiquitin-proteasome pathway
Miscellaneous cytosolic proteases
Mitochondrial proteins
Mitochondrial proteases

with weak bases that raise pH or lysosomal proteases ultimately confirmed only a minor role for lysosomes in cytosolic protein degradation [Shah et al., 2002] and no measurable effects of lysosome enzyme inhibitors on cell cycle protein degradation [Tao et al., 2002]. These organelles contain several acid-optimal proteases, including cathepsins B, H, and D, and many other acid hydrolases. Proteolysis within the lysosome also generates peptides that are presented to the immune system in association with MHC class II molecules. Endocytosis of cell surface receptors is followed by their degradation in lysosomes, and this process is often speeded up after ligand binding. Some cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that fuse with lysosomes [Lardeux and Mortimore, 1987], but it seems that proteins entering lysosomes for subsequent degradation must contain peptide sequences related to Lys-Phe-Glu-Arg-Gln (KFERQ) [Dice, 1990]. In some cells, there is a specific mechanism involving HSP70 for transporting certain cytosolic proteins directly into the lysosome [Chiang et al., 1989]. It is possible to determine the contribution of the lysosomes to a proteolytic process by using agents that block lysosomal acidification (e.g., chloroquine and methylamine) or by using inhibitors of the lysosomal cysteine proteases, cathepsins B, H, and L (e.g., leupeptin or E64) [Furuno and Goldberg, 1986]. Use of these inhibitors has demonstrated that the lysosomal pathway is mainly involved in degrading surface membrane proteins and endocytosed, extracellular proteins, rather than having a major role in the normal turnover of cytosolic proteins under normal conditions [Furuno and Goldberg, 1986] [Lowell et al., 1986].

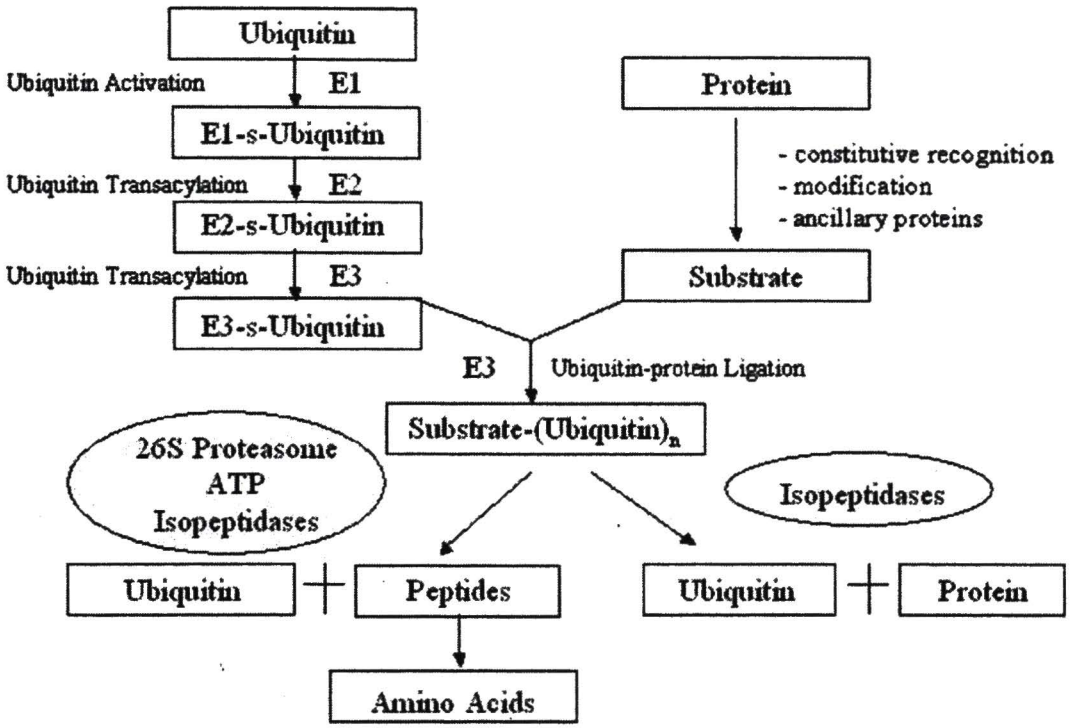
B. Ubiquitin-proteasome system

The ubiquitin-proteasome pathway (UPP) was initially regarded as a simple mechanism of destruction for old or damaged proteins, but it is now emerging as a crucial mechanism in cellular regulation. In most mammalian cells, proteins are ubiquitinated in a cascade reaction involving three classes of ubiquitinating enzymes: ubiquitin-activating enzyme (E1), ubiquitin-carrier proteins (E2) and ubiquitin-protein ligases (E3). The ubiquinated proteins are then recognized by 26S proteasome and degraded inside the cavity of the proteasome (Fig 2). UPP is present in both the nucleus and cytoplasm, regulating various cellular processes, such as cell cycle progression, cell differentiation, signal transduction, stress responses, and apoptosis. This pathway is also the focus of my dissertation and will be reviewed and explained in detail later.

C. Miscellaneous cytosolic proteases

Though the bulk of proteolysis in the cytosol occurs through the ubiquitin (Ub)-proteasome pathway, other cytosolic proteolytic systems also exist in mammalian cells. One Ca^{2+}-activated and ATP-independent proteolytic process involves the cysteine proteases termed calpains [Mellgren, 1987] [Murachi et al., 1980] [Waxman, 1981]. These enzymes appear to be activated when cells are injured and cytosolic Ca^{2+} rises, and therefore may play an important role in tissue injury, necrosis and autolysis [Goll et al., 1992]. Some recent investigations have indicated that they may function in the normal turnover of cell proteins too, e.g., a tumor suppressor gene product p53 can also be a

Fig 2. The ubiquitin-proteasome pathway. Figure is taken from *Annual Review of Medicine* 50:57-74; 1999 by Alan L. Schwartz, Aaron Ciechanover.


Ubiquitin
Ubiquitin Activation
E1
E1-s-Ubiquitin
Ubiquitin Transacylation
E2
E2-s-Ubiquitin
Ubiquitin Transacylation
E3
E3-s-Ubiquitin
Protein
- constitutive recognition
- modification
- ancillary proteins
Substrate
E3
Ubiquitin-protein Ligation
Substrate-(Ubiquitin)n
26S Proteasome
ATP
Isopeptidases
Isopeptidases
Ubiquitin
Peptides
Ubiquitin
Protein
Amino Acids

substrate for cleavage by calpain since a preferential site for calpain cleavage exists within the N terminus of the p53 protein [Kubbutat and Vousden, 1997].

Another important family of cytosolic proteases is the caspases or ICE (interleukin-β converting enzyme)-related proteases. A common feature for these cysteine proteases is a highly restricted cleavage specificity for an aspartic acid P_1 residue. Usually, they are synthesized as inactive precursors and activated in different cell types when those cells are stimulated with agents that promote apoptosis, including Fas mAb [Salvesen and Dixit, 1997]. In response to a variety of toxic stimuli (e.g., DNA damage signals), the various activated caspases lead cells to programmed cell death.

D. Mitochondrial proteases

Mitochondria contain a complete system for protein turnover within the matrix, where there exists an ATP-dependent pathway for breakdown of mitochondrial proteins. In the process of apoptosis, some lethal proteases released by the mitochondria activate caspases whereas others act in a caspase-independent manner, by acting as nucleases (e.g., endonuclease G), nuclease activators (e.g., apoptosis-inducing factor), or serine proteases (e.g., Omi/HtrA2) [Ravagnan et al., 2002].

Proteasome: A protein death machine

Proteasomes are large, multisubunit proteases that are found in the cytosol, both free and attached to the endoplasmic reticulum, and in the nucleus of eukaryotic cells [Coux et al.,

1996]. Their ubiquitous presence and high abundance in these cell compartments reflects their central role in cellular protein degradation. The structure and functions of the proteasomes are reviewed briefly below.

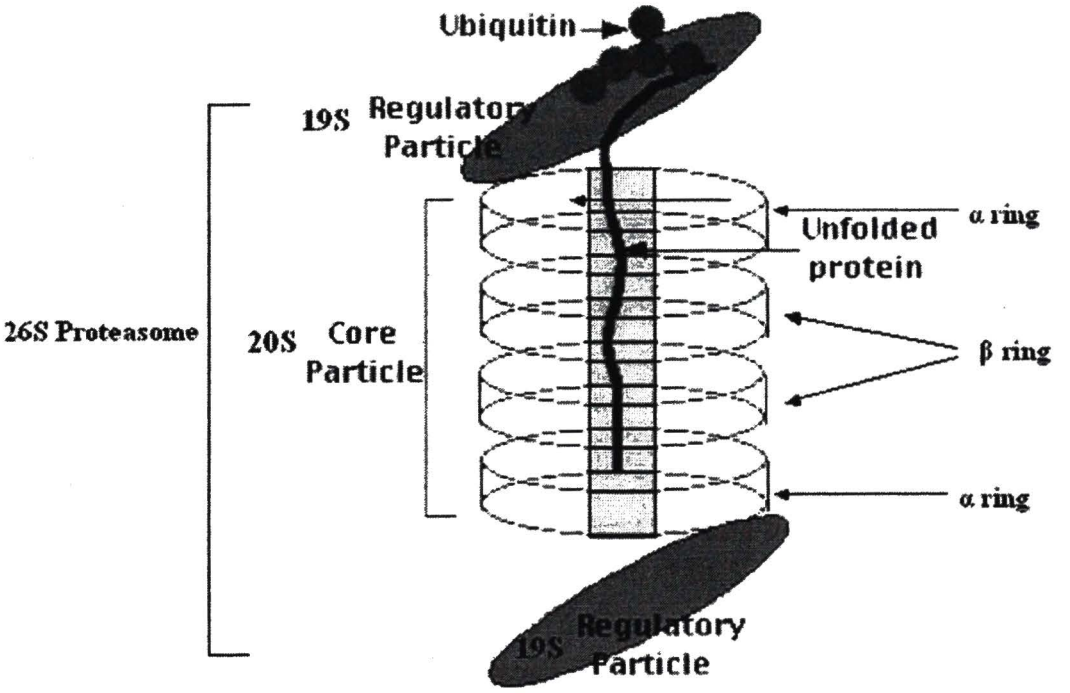
A. 26S proteasome

The proteasome holoenzyme is known as the 26S proteasome, which is a ~2.5 MDa complex made up of two copies each of at least 32 different subunits that are highly conserved among all eukaryotes. The overall structure can be divided into two major subcomplexes: the 20S core particle (CP) that contains the protease subunits and the 19S regulatory particle (RP) that regulates the function of the former (Fig 3). The 20S CP is a barrel-shaped structure made up of four rings of seven subunits each. The two inner β-rings contain the proteolytic active sites facing inward into a sequestered proteolytic chamber [Groll et al., 1997; Lowe et al., 1995]. One or two regulatory particles attach to the surface of the outer α-rings of the 20S CP to form the 26S proteasome holoenzyme (Fig 3).

B. 20S core particle (CP)

This 20S CP is also known as 20S proteasome, imaged as a cylinder 15 nm in length and 11 nm in diameter by electron microscopy. Its structure in yeast as determined by X-ray crystallography showed that the 20S CP is a hollow cylindrical structure composed of four heptagonal rings [Groll et al., 1997]. Each of the two outer rings is composed of seven genetically related and structurally similar α-subunits, and each of the two inner

Fig 3. Structure of 26S proteasome holoenzyme. Figure is modified from *Surgery* 131:595-600; 2002 by Shimul A. Shah, Michael W. Potter, and Mark P. Callery.


Ubiquitin
19S Regulatory Particle
Unfolded protein
α ring
β ring
α ring
26S Proteasome
20S Core Particle
19S Regulatory Particle

rings is comprised of seven similarly conserved β-subunits. The proteolytic activity of the proteasome is found within the β-subunits of 20S CP [Bochtler et al., 1999; Heinemeyer et al., 1997]. In eukaryotes, three of the seven β-subunits have functional threonine protease active sites, meaning that each proteasome has six (3 different) proteolytic active sites. In archaea, all β-subunits are identical, leading to 14 active sites in total. The protease active sites face an inner cavity within the β-rings that can be accessed through a narrow channel leading from the surface of the α-rings [Groll et al., 1997; Kohler et al., 2001]. Furthermore, these active sites have been characterized by three activities with distinct specificities against short synthetic peptides: a chymotryptic-like activity with preference for tyrosine or phenylalanine at the P1 position; a tryptic-like activity with preference for arginine or lysine at the P1 position; and a postglutamyl hydrolyzing (PGPH) activity with a preference for glutamate or other acidic residues at the P1 position [Coux et al., 1996; DeMartino and Slaughter, 1999].

C. 19S regulatory particle (RP)

The 19S RP plays multiple roles in regulating proteasomal activity: selecting substrates, preparing them for degradation, translocating them into the 20S CP, as well as possibly influencing the nature of products generated by the 20S CP. The 19S RP itself can be further dissected into two multisubunit substructures, a lid and a base [Glickman et al., 1998a]. The subunit composition of the RP from different species is remarkably similar [DeMartino et al., 1994; Dubiel et al., 1995; Glickman et al., 1998b; Verma et al., 2000]. The RP is comprised of at least 18 different subunits with a total mass close to 1 MDa

and can assemble at either end of the 20S CP to form the 26S proteasome [Glickman et al., 1998b]. Six of the RP subunits are ATPases found in many multisubunit cellular machines such as translocaters, transporters, membrane fusion complexes, and proteases [Beyer, 1997; Ogura and Wilkinson, 2001] and are designated in yeast as Rpt1–6 (for regulatory particle triple-A protein). The other subunits of the RP are designated in yeast Rpn1–13 (regulatory particle non-ATPase). Purified proteasomes are always found as a mixture of free CP, singly capped (RP_1CP), and doubly capped (RP_2CP) forms [Glickman et al., 1998b]. It seems that the majority of proteasomes are present as doubly capped forms *in vivo* in *S. cerevisiae* [Russell et al., 1999], while in mammalian cells the ratio of RP to CP is lower probably leading the presence of free 20S CP and to proteasomes with a single 19S RP [Brooks et al., 2000].

Interferon-γ inducible immunoproteasome

Upon interferon- γ signaling, a change occurs in the 20S proteasome subunit composition by replacement of the proteolytically active β-subunits with alternative, so-called LMP or βi, subunits. Each of these βi-subunits is genetically homologous to a specific constitutively expressed β-subunit and can be incorporated into the corresponding position within the β-ring of newly assembled proteasomes. β1i/LMP2 replaces its constitutively expressed β1-homolog, β5i/LMP7 replaces β5, and β2i/LMP10 replaces β2 [Belich et al., 1994; Fruh et al., 1994; Groettrup et al., 1996; Hisamatsu et al., 1996; Nandi et al., 1996]. The subunit composition of proteasomal core particles is thus altered upon interferon-γ induction, and they are therefore often referred to as

"immunoproteasomes". Interferon-γ also alters the level of the regulators and induces the binding of the 20S catalytic core of the proteasome to a complex called 11S regulator or PA28, which may further increase the spectrum of peptides generated [Groettrup et al., 1995]. Some studies also indicated that PA28 is necessary for immunoproteasome assembly and is required for efficient antigen processing [Preckel et al., 1999]. Moreover, interferon-γ decreases the level of phosphorylation of proteasome subunits [Bose et al., 2001].

During the process of replacing constitutive β-subunits by interferon-γ inducible β-subunits, there exists a cooperative mechanism which favors the assembly of homogeneous "immunoproteasomes" containing all three inducible subunits, i.e., LMP10 requires LMP2 for efficient incorporation into preproteasomes, and preproteasomes containing LMP2 and LMP10 require LMP7 for efficient maturation [Griffin et al., 1998; Schmidt et al., 1999].

The ubiquitin-proteasome pathway in cancer

As indicated above, due to the broad involvement of ubiquitin-proteasome proteolysis in fundamental biochemical processes, this pathway is a potential target for cancer-related deregulation, and alterations of proteasome function have indeed been described in events, such as cellular transformation by oncogenic viruses, multidrug resistance, etc [Spataro et al., 1998]. Here, I briefly review the rapidly increasing body of information

on the role of proteolysis by the ubiquitin/proteasome pathway in various fields of cancer biology.

A. Cell cycle regulation

In the cell cycle, various molecular mechanisms exist to ensure that cellular replication occurs precisely. For example, progression through the cell cycle is promoted by oscillation in the activity of cyclin-dependent kinases (CDKs) and cyclins. Ordered cell cycle progression requires the expression and activation of several cyclins and cyclin-dependent kinases. The ubiquitin-proteasome pathway (UPP) is involved in the proteolysis of many cell cycle regulatory proteins including the G_1 and mitotic cyclins and the mammalian cyclin-dependent kinase inhibitors, $p21^{Cip1\text{-}Waf\text{-}1}$ and $p27^{Kip1}$ [Adams et al., 1999; Pagano et al., 1995]. The p27 is critically regulated post-translationally by proteolysis by the UPP [Hengst and Reed, 1996; Pagano et al., 1995]. Low p27 protein levels had been found in some tumors, such as colorectal carcinomas and breast cancer, and confirmed to be associated with a poor prognosis [Catzavelos et al., 1997; Loda et al., 1997; Porter et al., 1997]. In tumor samples of colorectal carcinomas, it was clearly shown that increased proteasome-dependent degradation was responsible for low p27 levels [Loda et al., 1997]. Like p27, other elements of the cell cycle machinery that are substrates of UPP are potential targets for disregulation in tumors. One of the best characterized transitions in the normal cell cycle is the rapid proteasome-mediated degradation of cyclin B at the exit from mitosis [Glotzer et al., 1991]. Recent evidence suggests that cyclin D1 and E are substrates of the UPP [Clurman et al., 1996; Diehl et

al., 1997], and decreases in their degradation could contribute to the overexpression of these cyclins in tumors.

B. Virus-related malignancies

Human papilloma virus (HPV) was found to be involved in the etiology of the majority of human anogenital carcinomas. The oncogenicity of the human papilloma virus is mediated by up-regulation of the tumor suppressor p53 degradation, which is controlled by UPP. The E6 oncoprotein encoded by high-risk HPV (e.g. HPV-16, -18, -5 and-8) binds to p53 and promotes its degradation by the proteasome [Scheffner et al., 1990]. This property is critical for the immortalization of human cells by HPV. In contrast, low-risk HPVs (e.g. HPV-6 and -11) encode an E6 protein that does not bind to p53 and does not promote its degradation by UPP. The formation of the E6-p53 complex requires a cellular E6-binding protein called E6-AP (E6-associated protein) [Scheffner et al., 1993], which forms thiol ester complexes with ubiquitin in the presence of enzymes of the E2 category, such as UBC4 [Rolfe et al., 1995] or E2-F1 [Ciechanover et al., 1994]. E6-AP acts as an E3 enzyme, which ubiquitinates p53, leading to its rapid degradation by the 26S proteasome.

C. Antigen presentation

The 26S proteasome is responsible for the processing of MHC class I antigens. Peptides derived from endogenously expressed cytoplasmic proteins are carried by MHC class I molecules from the endoplasmic reticulum to the surface for recognition by cytotoxic T

lymphocytes. The proteasome had been postulated to be the proteolytic system that degrades cytosolic antigens, when it was found that the genes encoding proteasome β-subunits LMP-2 and LMP-7 were included in the MHC gene cluster [Beck et al., 1992]. Analysis of mice lacking LMP-7 indicated a decreased surface expression of MHC class I molecules and inefficient antigen presentation [Fehling et al., 1994]. There is strong evidence that MHC class I peptide presentation is modified in tumors and may contribute to escape from immune surveillance. Three different small-cell lung carcinoma lines with low to undetectable levels of mRNA for LMP2 and LMP7 and functional deficiencies in antigen presentation have been described [Restifo et al., 1993]. The mouse T-cell lymphoma line SP-3 displays underexpression of LMP-2 and is defective for antigen presentation, whereas LMP-2 expression and antigen presentation to cytotoxic T lymphocytes are restored upon expression of interferon-γ by transfection [Sibille et al., 1995].

D. Apoptosis

Proteasome inhibition induces apoptosis in many cancer cells. Treatment of human monoblast cells, T-cell leukemia cells, oral squamous carcinoma cells, melanoma cells, prostate cancer cells, and pancreatic cancer cells with various proteasome inhibitors induce apoptosis [An et al., 1998]. Proteasome inhibitors also have enhanced tumor necrosis factor (TNF)-α-induced apoptosis in different tumor types [Mlynarczuk et al., 2001]. Apoptosis induced by proteasome inhibition is believed to occur as a result of many factors, including activation of caspase-3, inhibition of nuclear factor (NF)-κB

activation, and accumulation of cell cycle regulators, like CDK inhibitors (p21 and p27) [Kisselev and Goldberg, 2001].

E. Transcriptional regulation

The proteasome also participates in events that control gene transcription by degrading various oncoproteins or transcriptional regulators either directly or indirectly through signal transduction cascades. Examples include c-Myc, c-Fos, c-Jun, p53, and most widely studied, nuclear factor (NF)-κB/IκB [Ciechanover, 1994]. NF-κB is involved in the activation of genes encoding products such as cytokines, chemokines, growth factors, cell-adhesion molecules and surface receptors in response to a great variety of pathogenic signals. The activation of NF-κB requires two steps of proteasome-dependent proteolysis. The active form of NF-κB is a heterodimer consisting of a p65 and a p50 subunit, which is present in the cytosol as an inactive precursor, p105. UPP is involved first in the biogenesis of the subunit p50 from the precursor p105 and then in the cytoplasmic degradation of the inhibitory factor IκB, which allows the translocation of the active dimer into the nucleus [Palombella et al., 1994].

F. Drug resistance

Many cancers are chemoresistant or acquire resistance after therapy. Studies on POH_1, a novel subunit of the 26S proteasome, indicate a link between UPP and drug resistance [Spataro et al., 1997]. It has been shown by transfection experiments that POH_1 overexpression in mammalian cells can confer multidrug resistance to paclitaxel, 7 -

hydroxystaurosporine, doxorubicin and to ultraviolet radiation [Claret et al., 1996]. Two other subunits of the 19S regulatory complex of the proteasome associated with drug resistance have been identified in fission yeast [Gordon et al., 1993; Gordon et al., 1996]. Since the UPP pathway is highly conserved in mammals, it could confer drug resistance to anti-cancer agents *in vitro* and could potentially be involved in drug resistance in human tumors.

Proteasome inhibitors: A novel type of anti-cancer drug

The ability of proteasome inhibitors to inhibit cell proliferation and selectively induce apoptosis in proliferating cells, together with their ability to inhibit angiogenesis [Oikawa et al., 1998], makes these agents attractive candidates as anti-cancer drugs [Dou and Goldfarb, 2002; Kisselev and Goldberg, 2001]. 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 enzyme's substrate binding pocket in the active site. Since the proteasome utilizes N-terminal threonines of the β subunits as an active site nucleophiles, these pharmacophores in proteasome inhibitors are designed to prefer for the proteasome's N-terminal threonine. They react with the catalytic hydroxyl or thiol groups in the active sites of proteasome to form a reversible hemi(thio)acetal, which resembles a transition state analogue of the enzymatic reaction [Kisselev and Goldberg, 2001]. Based on the pharmacophore, proteasome inhibitors can be divided into several groups: (1) peptide aldehydes; (2)

peptide boronates; (3) natural proteasome inhibitors like lactacystin and its active form β-lactone; (4) peptide vinyl sulfones; and (5) peptide epoxyketones.

Peptide aldehydes are the best characterized and most widely used inhibitors of the proteasome, such as MG132, MG115 and LLnL. These agents are reversible, inhibit the chymotrypsin-like activity of the proteasome [Lee and Goldberg, 1998; Rock et al., 1994], and protein degradation is restored after their removal. An advantage of this type of proteasome inhibitors is their high potency and reversibility. In addition, they block the proteolytic activity of the 26S proteasome without influencing its ATPase or isopeptidase activities [Lee and Goldberg, 1998].

Peptide boronates are much more potent and selective inhibitors of the proteasome than the aldehydes [Adams et al., 1998]. One of the dipeptide boronates, Bortezomib (formerly known as MLN341, LDP-341, PS-341 and Velcade) is currently in phase III clinical trials in patients with multiple myeloma [Dou and Goldfarb, 2002].

Contrary to most proteasome inhibitors synthesized artificially, lactacystin is a natural product isolated originally from actinomycetes that is structurally different from the peptide aldehydes. 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]. X-ray diffraction studies have determined the structure of the lactacystin-proteasome

complex and showed that an acyl enzyme conjugate is indeed an intermediate in catalysis by the proteasome [Groll et al., 1997]. Recently, other natural products have been identified as proteasome inhibitors including epigallocatechin gallate (EGCG) from green tea [Nam et al., 2001]. Structure-activity relationship studies, atomic orbital energy analysis and analysis of the products of EGCG interaction with proteasome strongly suggested that the ester bond in this molecule attacked the proteasome leading to the acylation of the active site threonine. All of these proteasome inhibitors provide a valuable and useful tool to investigate and understand the unique proteolytic mechanism by proteasome.

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CHAPTER II

INVESTIGATION OF PROTEASOME CHYMOTRYPTIC ACTIVITIES IN RAT NATURAL KILLER (RNK16) CELLS

Part of the original results presented in this part was contributed to the following publications:

Kitson RP, Lu M, Siman R, Goldfarb RH. Proteasome inhibitor and lymphocyte function: partial inhibition of cell-medicated cytotoxicity and implication that the lymphocyte proteasome may contain multiple chymotryptic domains. *In Vivo*, 2000, 14(2): 265-268

Reshetnyak YK, Kitson RP, Lu M, and Goldfarb RH. Correlation between conformational and enzymatic changes of proteasome of rat natural killer cells induced by mono and divalent cations. *Journal of Biomolecular Structure*, 2003, submitted

INTRODUCTION

The proteasome is a multicatalytic proteinase complex that is involved in the major extralysosomal pathway responsible for intracellular protein degradation in mammalian cells. Proteasomes catalyze the final step of the ubiquitin-proteasome pathway of protein degradation and the 20S proteasome is the catalytic core of the larger 26S proteasome. The 20S proteasome contains multiple proteolytic activities, defined as the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activity [Coux et al., 1996]. Cell cycle-regulating proteins degraded through ubiquitin-proteasome pathway include cyclins and the cyclin-dependent kinase (CDK) inhibitors, p21 and $p27^{kip-1}$ [Pagano et al., 1995]. Some transcription factors, such as p53, c-Fos and c-Jun, are also degraded by the proteasome [Drexler, 1997]. Several structural investigations on the 20S proteasome have already revealed that it is a hollow cylindrical particle consisting of four stacked rings. The arrangement of 20S proteasome's 28 subunits, which can be classified into two categories α and β, is in the form of $\alpha_7\beta_7\beta_7\alpha_7$; nevertheless, it appears that only the β subunits are catalytically active since all of the known amino acid substitutions which affect proteolysis are located in the β chain [Coux et al., 1996]. In yeast, each β ring of 20S proteasome contains three active sites, which have been identified by X-ray diffraction as the sites of binding of a peptide aldehyde inhibitor [Groll et al., 1997].

Natural killer (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 immunotherapy for cancer patients, NK cells are activated by IL-2 *in vitro* and then transfused back to the patients' body to locate within tumors and kill tumor cells [Basse et al., 1994]. Numerous studies have implicated proteolytic enzymes as crucial to the cytolytic mechanism of these cells [Berke, 1994; Darmon and Bleackley, 1998; Goldfarb, 1986]. 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; Jenne and Tschopp, 1988]. Our studies have focused on proteolytic enzymes other than those found only in cytolytic granules and have indicated that the chymotryptic activity of proteasomes in NK cells might play a role in their cell-mediated cytotoxicity [Goldfarb et al., 1992; Kitson et al., 1995; Wasserman et al., 1994].

In these studies conducted in our laboratory previously, an examination of purified rat NK cell proteasomes by electron microscopy revealed unique proteasome concatemers which have not been reported for other proteasomes [Wasserman et al., 1994]. These proteasomes also appear to contain a subunit composition different than rat liver proteasome as assessed by SDS PAGE and by immunochemical analysis using an anti-rat liver proteasome polyclonal antibody [Wasserman et al., 1994]. While there are only seven different β subunits genes in yeast, there are at least 10 human genes for β subunits [Coux et al., 1996]. Therefore, I hypothesize that NK cell proteasomes may contain more than one site for cleavage of chymotryptic substrates, or in NK cells there may be a two-

site mechanism for proteasomal chymotryptic activity regulation. In this chapter, I report the results of the purification of 20S proteasome from rat natural killer cell line RNK16, comparative investigations of substrate selectivity and inhibitor specificity for proteasome from rat NK and liver cells, and kinetic studies of the RNK16 cell proteasome. These results directly provide us more evidence to understand the difference of proteasome function from different cell types and suggest how the proteasome might be regulated in rat NK cells.

MATERIALS AND METHODS

Materials

The proteasome of rat liver cells was a gift from Dr. A. Jennifer Rivett (University of Bristol, U.K.). RPMI-1640 tissue culture medium, nonessential amino acids, and antibiotics were purchased from Gibco (Grand Island, NY). Sucrose (Ultra-pure) was purchased from Beckman (Fullerton, CA). Ethylenedinitrilotetraacetic acid disodium salt (EDTA) was purchased from Fisher Scientific (Fair Lawn, NJ). Dithiothreitol (DTT), 2-mercaptoethanol, fluorogenic proteasome substrates Suc-AAF-AMC, Suc-LLVY-AMC, and buffers HEPES and PIPES were purchased from Sigma (St. Louis, MO). Amicon® nitrogen pressure-based concentration apparatus and Diaflo® 30 kDa cut-off filters were obtained from Amicon (Beverly, MA). Sephacryl S-400 and heparin-Sepharose CL-6B chromatography media were purchased from Pharmacia (Piscataway, NJ). Glass columns were purchased from Kimble (Vineland, NJ) and the fraction collection apparatus was purchased from ISCO (Lincoln, NE).

Assay of proteasome proteolytic activities

Three major proteasome proteolytic activities, i.e. trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activity, were assayed by using substrates Z-Gly-Gly-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC, and Z-Leu-Leu-Glu-AMC respectively. For trypsin-like activity, reaction buffer 1mM dithiothreitol (DTT) and EDTA, 10 μg/ml BSA, 0.02% Triton X-100 (v/v) in 50 mM Tris/HCl buffer, pH 8.7, was used; for chymotrypsin-like

activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 7.5 was used and for peptidylglutamyl-hydrolyzing activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 8.0, was used. All reaction mixtures contained peptide substrates at 20 μM concentrations unless otherwise noted. The rates of cleavage were determined by taking fluorescent intensity measurements using a Dynatech® fluorescence plate reader. Proteolytic activity was expressed as relative fluorescence units per hour and per gram of protein.

Purification of 20S proteasome from RNK16 cells

All purification procedures were performed at 4 °C and followed the methods described in detail previously [Wasserman et al., 1994]. Briefly, subsequent to harvesting RNK16 cells, postnuclear supernatants were collected after nitrogen cavitation at 325-350 psi for 30-40 minutes. Successively, isopycnic sucrose gradient fractionation, Sephacryl S-400 gel filtration chromatography, and heparin-Sepharose CL-6B chromatography were applied. In each step, the protein concentration and the proteasomal chymotrypsin-like and trypsin-like activities were measured.

Electrophoresis on native or SDS gels

The final preparation of the 20S proteasome from RNK16 cells (after Heparin chromatography) was electrophoresed under nondenaturing conditions in 5% polyacrylamide gel at 20 mA for 6 hours. Under denaturing conditions, the purified 20S proteasome (5 μg) was boiled with 1% SDS in the presence of 5% 2-mercaptoethanol and

electrophoresed. The left lane are the molecular markers, the right lane is the denaturing SDS-PAGE, The molecular weights of the multiple proteasome subunits were determined by loading molecular markers (94, 67, 43, 30, 20 and 14 kDa) in another lane.

Protein concentration assay

Protein concentration was determined by measuring the absorbance at the wavelength of 280 nm by using bovine serum albumin as a standard.

Inhibition assay

The purified 20S proteasome was preincubated at room temperature for 10 min in the presence or absence of various concentrations of each inhibitor, and the remaining activity was measured as described above.

Kinetic assay for proteasome chymotrypsin-like activity

A typical kinetic assay was followed the description previously [Stein et al., 1996]. Briefly, 120 μl of assay buffer (20mM HEPES, 0.5mM EDTA, with/without SDS, pH 7.8) and fluorogenic chymotryptic substrate Suc-Ala-Ala-Phe-AMC were added to a 200 μL fluorescence cuvette. The cuvette was placed in an ISS K2 fluorescence spectrophotometer (ISS, Champagne, IL) and reaction temperature was maintained at 37.0 ± 0.2 °C. After the reaction solution had reached thermal equilibrium, 2.8 μl of the stock proteasome solution was added to the cuvette. Reaction progress was monitored by the increase in fluorescence emission at 440 nm. For each kinetic run, 300-500 data

points, corresponding to (time, fluorescence intensity) pairs, were collected by a microcomputer interfaced to the fluorescence spectrophotometer.

RESULTS

Purification of 20S proteasome from RNK16 cells

The results of the 20S proteasome purification from RNK16 cells showing chymotrypsin-like activity are summarized in Table 1. The cell homogenates containing the 20S proteasome were made in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 10 mM PIPES, pH 7.3) via nitrogen cavitation at 325-350 psi for 30-40 min. Then these homogenates were subjected to the sucrose isopycnic density centrifugation (Fig 1) and fractions containing proteasomal proteolytic activities were collected for further Sephacryl S-400 gel filtration. In the Sephacryl S-400 gel filtration chromatography, the apparent molecular weight of the 20S proteasome to be purified was estimated on a column (1.0 × 30 cm) (Fig 2). The column had been calibrated with thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 44,000), myoglobin (M_r 17,000), vitamine E (M_r 1,350) (Fig 3). The eluted fractions containing proteins around 700 kDa were harvested and then an affinity chromatography, i.e. heparin-Sepharose chromatography, was run. The gel filtration on heparin-Sepharose 6B, the final step of purification, gave a single peak where chymotrypsin-like activity is superimposable on that of trypsin-like activity. From 551.1 mg of the homogenates from RNK16 cells, 0.19 mg of the purified 20S proteasome was isolated, representing 2660-fold purification with a yield of 91.7%, estimated on the basis of chymotrypsin-like activity.

Table 1. Purification of 20S proteasome from RNK16 cells

Group	Total protein (mg)	Activity (pmol/hr/ml)	Total activity	Recovery (%)	Specific activity (pmol/hr/mg)	Overall fold purification
Homogrnate	551.1	325	9588	100	17.4	1
PNS	195.7	623	7476	78.0	38.2	2.2
Peak SG	18.5	302	7248	75.6	394.1	22.6
Peak S-400	8.51	628	7222	75.3	847.7	48.7
Peak Hepain	0.19	10470	8795	91.7	46290	2660.3

*Chymotrypsin-like activity toward Suc-LLVY-AMC was measured in 10 mM Tris/HCl buffer containing 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS at pH7.5 and room temperature.

Fig 1. Isopycnic sucrose density gradient analysis of RNK16 cell-derived proteasomes. The protein concentration, chymotrypsin-like and trypsin-like activities are measured. Z-Ala-Ala-Phe-AMC and Z-Gly-Gly-Arg-AMC are the chymotryptic and tryptic substrates respectively. The bar at the top indicates the fractions pooled for further Sephacryl S-400 gel filtration analysis. The unit for enzyme activity in the figure is pmol/hr/mg while that for protein concentration is mg/ml.

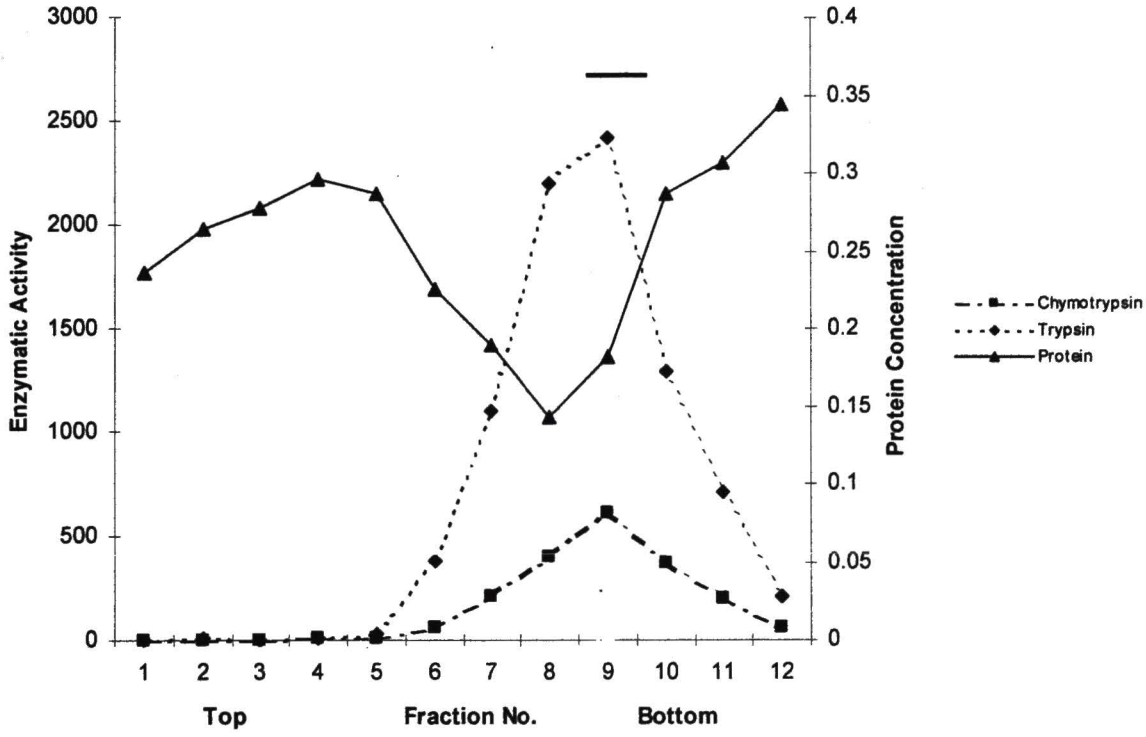

3000
2500
2000
1500
1000
500
0
Enzymatic Activity
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Protein Concentration
1 2 3 4 5 6 7 8 9 10 11 12
Top
Fraction No.
Bottom
Chymotrypsin
Trypsin
Protein

Fig 2. Sephacryl S-400 gel filtration chromatographic analysis of RNK16 cell-derived proteasome. The protein concentration, chymotrypsin-like and trypsin-like activities are measured. Z-Ala-Ala-Phe-AMC and Z-Gly-Gly-Arg-AMC are the chymotryptic and tryptic substrates respectively. The bar at the top indicates the fractions pooled for further heparin-Sepharose affinity filtration analysis. The unit for enzyme activity in the figure is pmol/hr/mg while that for protein concentration is mg/ml.

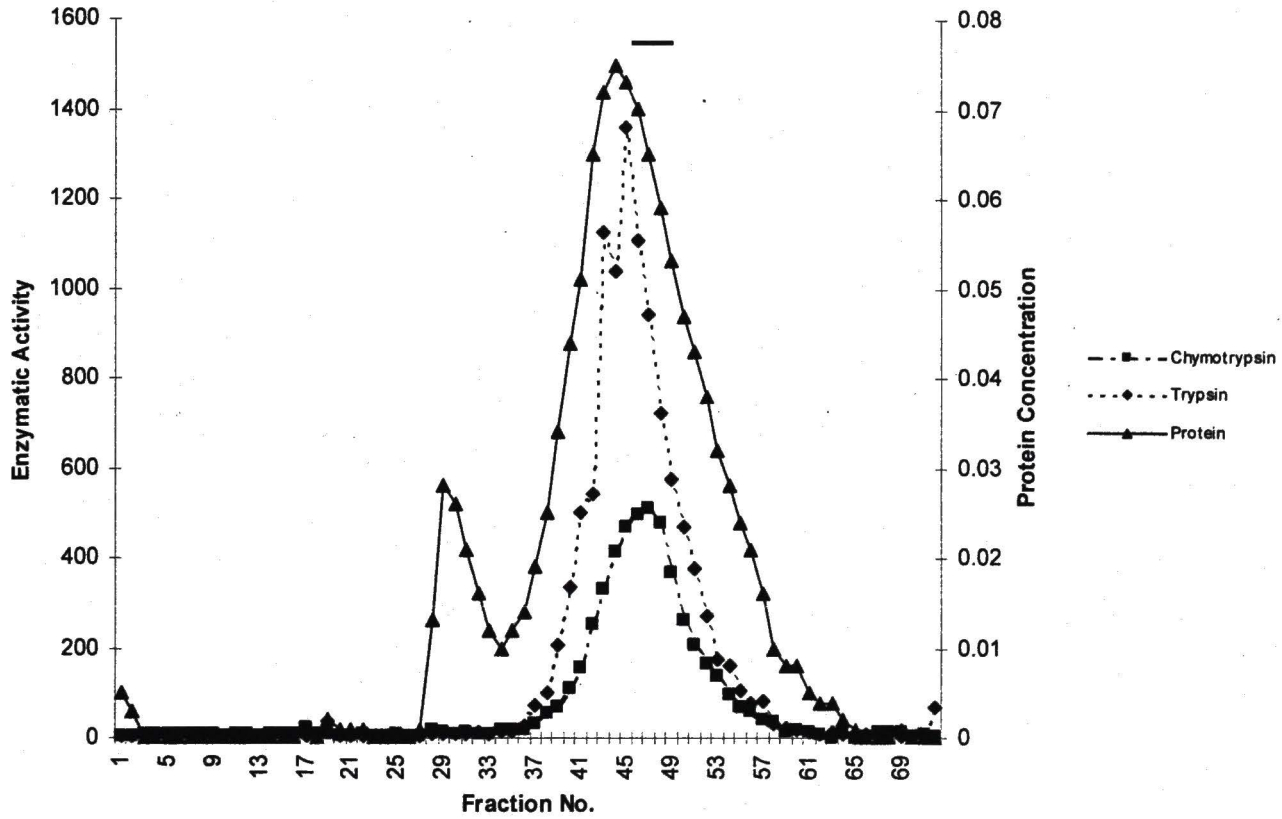

1600
1400
1200
1000
800
600
400
200
0
Enzymatic Activity
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Protein Concentration
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
Fraction No.
Chymotrypsin
Trypsin
Protein

Fig 3. Calibration of the Sephacryl S-400 gel filtration chromatography column by standard protein molecules. The standard protein molecules used are thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 44,000), myoglobin (M_r 17,000), vitamine E (M_r 1,350).

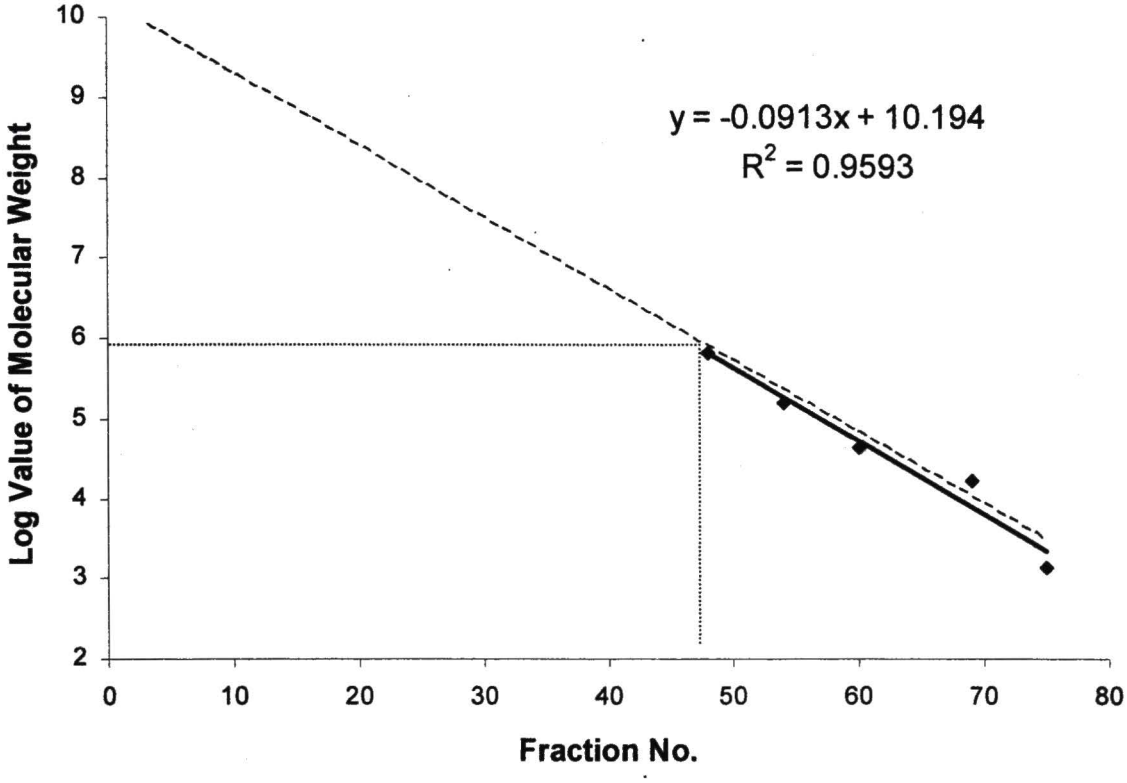
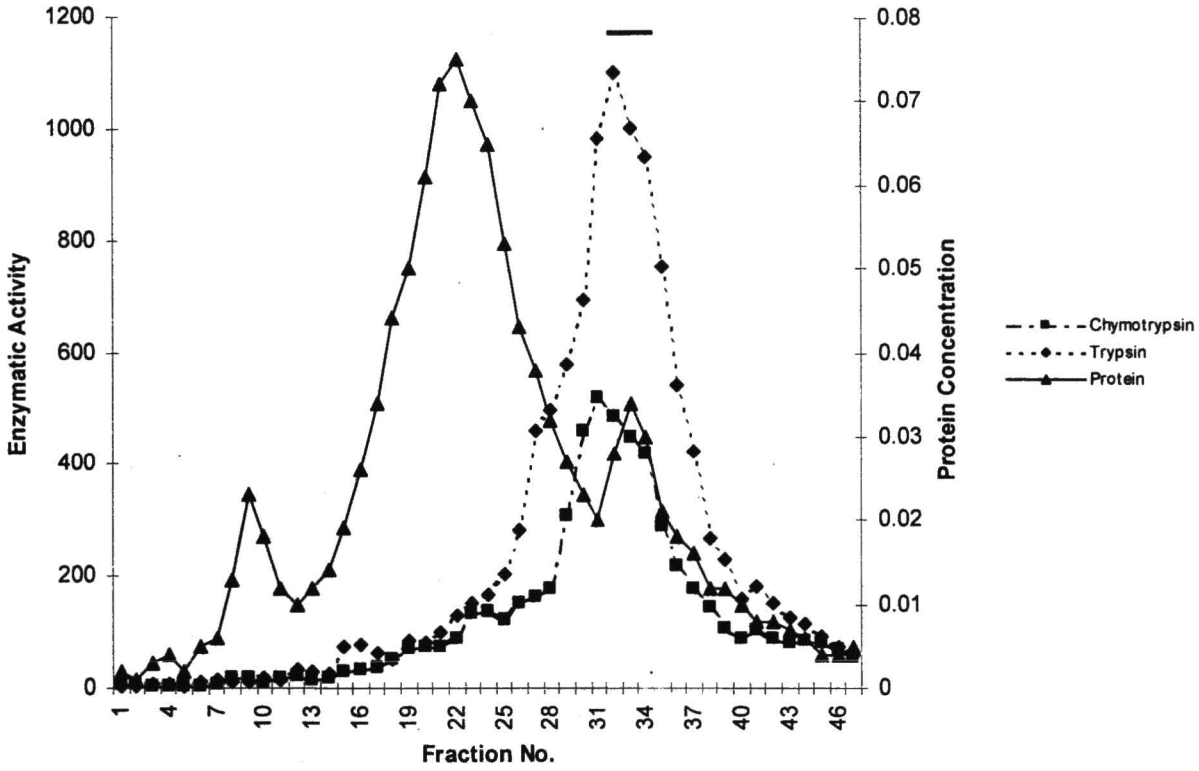

y = -0.0913x + 10.194
R^2 = 0.9593
Log Value of Molecular Weight
Fraction No.
10
9
8
7
6
5
4
3
2
0
10
20
30
40
50
60
70
80

Fig 4. Heparin-Sepharose chromatography of pooled S-400 fractions containing RNK-16 cell-derived chymotrypsin- and trypsin-like activity. The protein concentration, chymotrypsin-like and trypsin-like activities are measured. Z-Ala-Ala-Phe-AMC and Z-Gly-Gly-Arg-AMC are the chymotryptic and tryptic substrates respectively. The bar at the tope indicates the fractions pooled for purified 20S proteasomes. The unit for enzyme activity in the figure is pmol/hr/mg while that for protein concentration is mg/ml.


Enzymatic Activity
Protein Concentration
Fraction No.
Chymotrypsin
Trypsin
Protein
1200
1000
800
600
400
200
0
0.08
0.07
0.06
0.05
0.04
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40
43
46

Native and SDS PAGE analysis of purified 20S proteasome

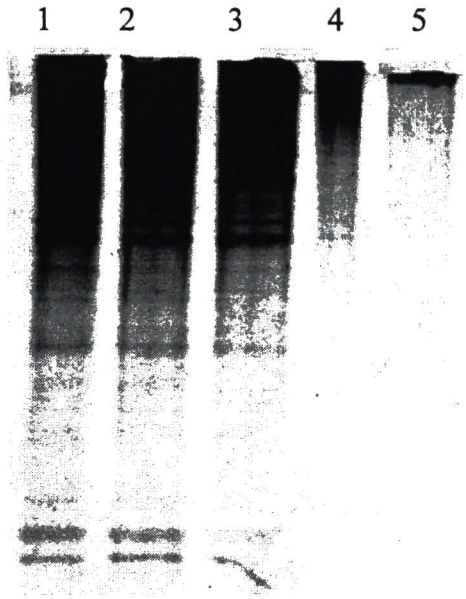
To determine the state of purity of the RNK16-derived proteasome, electrophoresis of purified material after each step, i.e., homogenates, post-nuclear supernatant (PNS), peak fractions after isopycnic sucrose gradient concentrifugation, peak fractions after Sephacryl S-400 gel filtration and peak fractions after heparin-Sepharsoe chromatophy was performed (Fig 5A). On the gel, the final preparation of the proteasome after heparin-Sepharose chromatography showed a single protein band, which migrates as a single entity and suggests its apparent homogeneity (Fig 5A Lane 5 and Fig 5B). When the purified proteasome was subjected to SDS PAGE in 12% gels, at least four bands representing the multiple individual subunits of the 20S proteasome could be distinguished (Fig 5C).

Substrate selectivity of 20S proteasome from rat NK and hepatic cells

Although various substrates are known for the chymotryptic activity of the proteasome, a thorough and comprehensive study of the enzyme with an array of substrates has yet to be accomplished. In this study, I used all commercially available substrates with an AMC leaving group as a reporter of peptide bond cleavage. The 20S proteasome of RNK16 cells showed proteolytic activities toward various substrates preferred by chymotrypsin-like, trypsin-like, and PGPH enzymes (Table 2). Among these substrates, Suc-Leu-Leu-Val-Tyr-AMC and Z-Phe-Ala-Arg-AMC were most efficiently hydrolyzed by the proteasome chymotrypsin-like and trypsin-like activities respectively. These results also indicate the multicatalytic nature of the 20S proteasome.

Fig 5. Electrophoresis of purified 20S proteasome derived from RNK16 cells. (A) The purified proteasomes from RNK16 cells at different stages were electrophoresed under nondenaturing conditions at 20 mA for 1 hour. Lane 1: Homogenates; 2: Post-nuclear supernatant (PNS); 3: Peak fractions after isopycnic sucrose gradient concentrifugation; 4: Peak fractions after Sephacryl S-400 gel filtration; 5: Peak fractions after heparin-Sepharsoe chromatophy. (B) The final preparation of the 20S proteasome from RNK16 cells (after Heparin chromatography) was electrophoresed under nondenaturing conditions in 5% polyacrylamide gel at 20 mA for 6 hours. (C) The purified 20S proteasome (5 μg) was boiled with 1% SDS in the presence of 5% 2-mercaptoethanol and electrophoresed in a 12% gel under denaturing conditions. The left lane are the molecular markers (94, 67, 43, 30, 20 and 14 kDa), the right lane is the denaturing SDS-PAGE, where the multiple proteasome subunits are seen.

A.



B.

C.

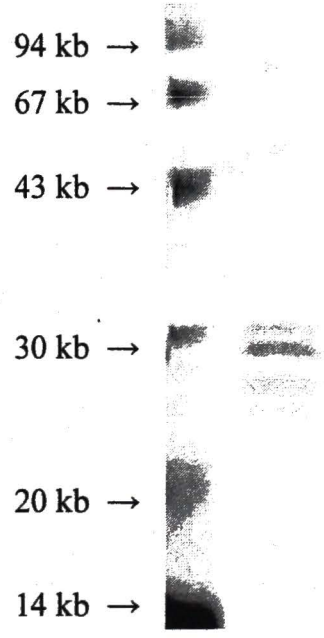


Table 2. Substrate selectivity of 20S proteasome derived from RNK16 cells

Substrates	Concentration (μM)	Activity (%)
Chymotryptic Substrates		
Suc-Leu-Leu-Val-Tyr-AMC	20	100
Suc-Ala-Ala-Phe-AMC	20	70.7
Z-Val-Lys-Met-AMC	20	18.6
Z-Leu-Leu-Leu-AMC	20	56.1
Z-Gly-Gly-Leu-AMC	20	50.4
Suc-Leu-Tyr-AMC	20	17.0
Tryptic Substrates		
Z-Phe-Ala-Arg-AMC	20	100
Boc-Phe-Ser-Arg-AMC	20	68.6
Suc-Ala-Phe-Lys-AMC	20	34.3
Z-Gly-Gly-Arg-AMC	20	27.1
Gly-Pro-Arg-AMC	20	10.6
Substrates for PGPH Activity		
Z-Leu-Leu-Glu-AMC	20	100
Suc-Ala-Glu-AMC	20	31.0

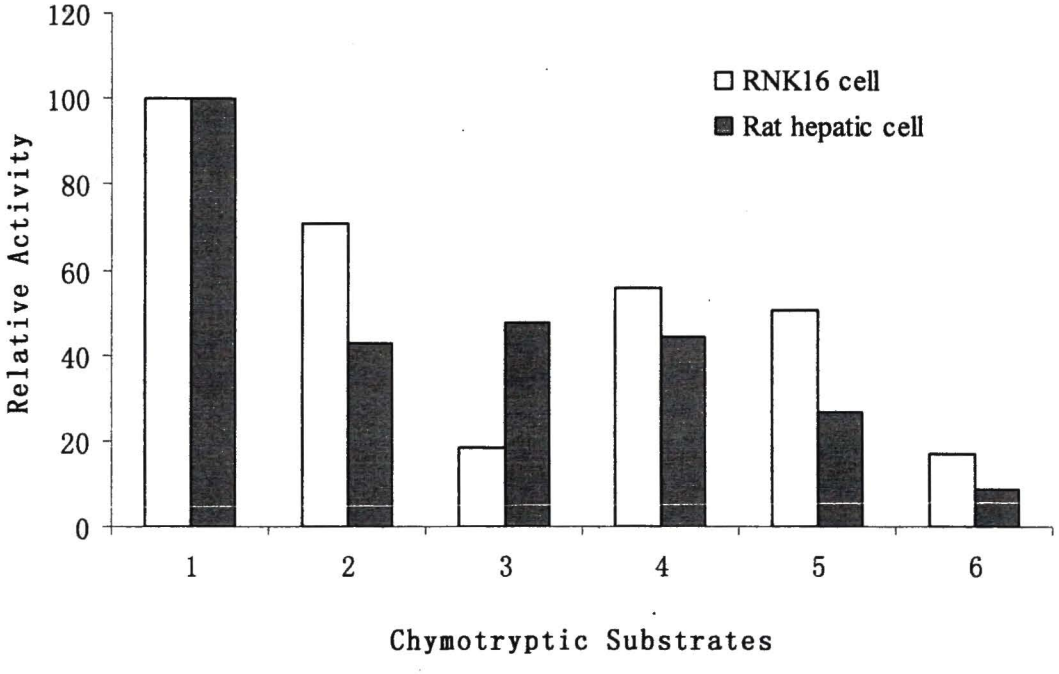
* Samples of 0.5 μg of purified 20S proteasome were used for assay. The activities for Suc-Leu-Leu-Val-Tyr-AMC, Z-Phe-Ala-Arg-AMC and Z-Leu-Leu-Glu-AMC were defined as 100% for the proteasomal chymotrypsin-like, trypsin-like and PGPH activities respectively.

It is also important to know to what extent these preferences for specific peptide bonds may vary among proteasomes of different cellular origin [Peters et al., 2002]. Next I compared the difference of the substrate selectivity of the proteasome between RNK16 cells and rat hepatic cells based on the proteolytic activity on Suc-LLVY-AMC, since Suc-LLVY-AMC is the most efficient hydrolyzed chymotryptic substrates for proteasomes of both cell lines. As a result, proteasomes from RNK16 and rat hepatic cells showed a different preference on some substrates (Fig 6). However, proteolytic activities for the proteasomes derived from RNK16 cells and rat hepatic cells were found to be dependent on a couple of common parameters. First, a minimal substrate length of the polypeptide was required for cleavage. For proteasomes from two different types of cells, activities decreased in a similar trend from Suc-LLVY-AMC to Suc-LY-AMC. There are reports that fluorogenic substrates with one amino acid are not hydrolyzed by the proteasome [Ozaki et al., 1992]. Second, the amino acid at the S_1 site is critical. This is shown by comparing the activities toward Suc-Leu-Leu-Val-Tyr-AMC and Suc-Ala-Ala-Phe-AMC and as well as toward Z-Val-Lys-Met-AMC. Third, the amino acids at the S_2 or S_3 sites may contribute to the recognition of the substrate. The substrate Z-Leu-Leu-Leu-AMC showed more efficient degradation by proteasome than another substrate Z-Gly-Gly-Leu-AMC, which differs on the positions S_2 and S_3.

Inhibitor specificity of 20S proteasome of rat NK and hepatic cells

The effects of various protease inhibitors on the chymotrypsin-like activities of purified 20S proteasome of RNK16 cells were also determined. Totally 10 protease inhibitors

Fig 6. Comparative studies on the chymotryptic substrate selectivity of RNK16 cell-derived and rat hepatic cell-derived proteasome. Chymotryptic substrates used are: 1, Suc-Leu-Leu-Val-Tyr-AMC; 2, Suc-Ala-Ala-Phe-AMC; 3, Z-Val-Lys-Met-AMC; 4, Z-Leu-Leu-Leu-AMC; 5, Z-Gly-Gly-Leu-AMC; 6, Suc-Leu-Tyr-AMC. In the reactions, activities toward Suc-Leu-Leu-Val-Tyr-AMC are defined as 100% for the proteasomes derived from both RNK16 cells and rat hepatic cells.


120
100
80
60
40
20
0
Relative Activity
RNK16 cell
Rat hepatic cell
1
2
3
4
5
6
Chymotryptic Substrates

were tested and 4 of them were found to achieve more than 80% inhibition on the proteasomal chymotryptic activity at the concentration of 5 μM (See Fig 1 in Chapter III). The proteasomal inhibitors, MG115 and MG132, were among these four inhibitors. Comparative studies on the inhibitor specificity of 20S proteasome of RNK16 cells and rat hepatic cells were also performed. The most effective inhibitor of the RNK16 cell-derived proteasomal chymotrypsin-like activity is Z-Leu-Leu-Leu-aldehyde (Fig 7). This inhibitor was less potent for inhibition of the RNK16 cell proteasome (87.6%) versus the rat hepatic cell proteasome (94.6%). While some inhibitors achieved similar inhibition rates in the rat NK and hepatic cells-derived proteasomal chymotrypsin-like activities, some failed and showed significantly different potency on the proteolytic activities.

To further test the hypothesis that NK cell proteasome may have more than one chymotryptic catalytic site, cleavage activities of the purified 20S proteasome toward several different substrates in the presence of inhibitors were also determined. Impressively, while TPCK, a chymotryptic inhibitor, could inhibit the LLVY, AAF, and VKM cleaving proteasomal activity in a dose-dependent manner, it was unable to inhibit the GGL cleaving proteasomal activity (Fig 8). Indeed, previous studies with the Jurkat cell line have indicated that a single synthetic selective chymotryptic proteasome inhibitor, CEP1612, has differential effects on two chymotryptic substrates indicating the possibility of two different chymotryptic active sites [Iqbal et al., 1995].

Fig 7. Comparative studies on the inhibitor specificity of 20S proteasome derived from RNK16 cells and rat hepatic cells. The chymotryptic substrate used was Suc-LLVY-AMC and the inhibitors were: 1, TPCK; 2, ZPCK; 3, H-Ala-Ala-Phe chloromethyl ketone; 4, L-Leucine chloromethyl ketone; 5, lactacystin; 6, MG115; 7, Z-Ile-Glu-Ala-Leu-aldehyde; 8, Z-Leu-Leu-Phe-aldehyde; 9, MG132; 10, TLCK. Reaction buffer was added to the final concentration of inhibitors at 5 μM.

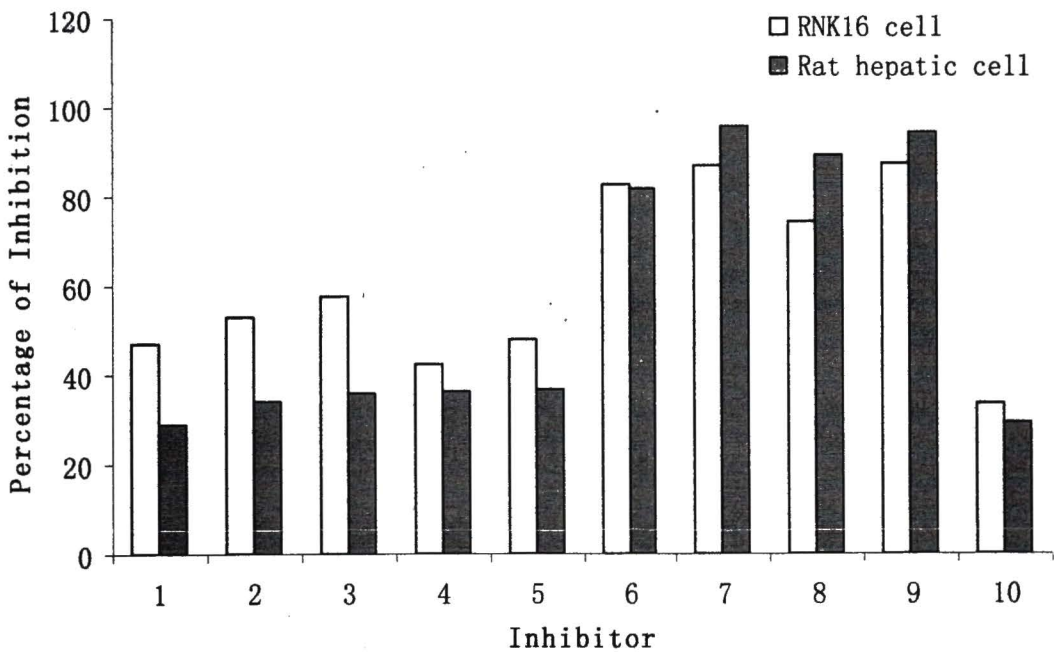
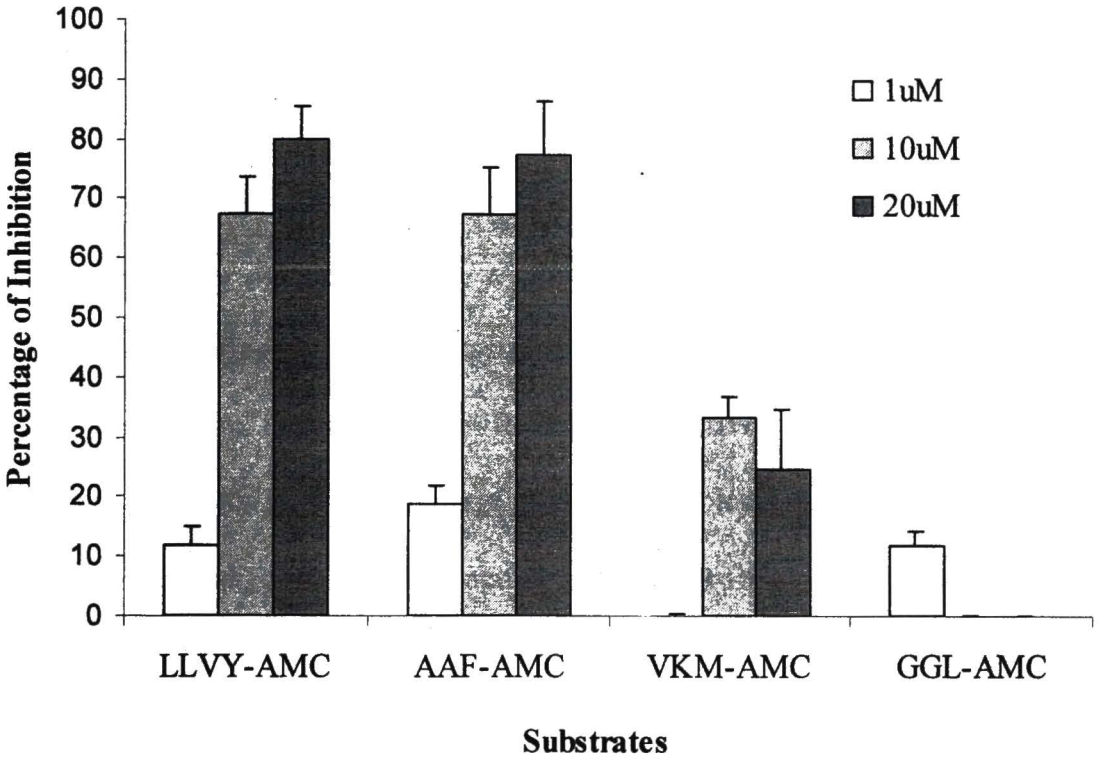

RNK16 cell
Rat hepatic cell
Percentage of Inhibition
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Inhibitor

Fig 8. Effects of chymotryptic inhibitor TPCK on RNK16 cell proteasomal cleavage of various chymotryptic substrates. Various concentrations of TPCK were used, i.e., 1 μM, 10 μM, and 20 μM, to detect their inhibitory effects on RNK16 cell-derived proteasome cleavage of chymotryptic substrates LLVY-AMC, AAF-AMC, VKM-AMC and GGL-AMC (All substrate concentrations were 20 μM).


100
90
80
70
60
50
40
30
20
10
0
Percentage of Inhibition
1uM
10uM
20uM
LLVY-AMC
AAF-AMC
VKM-AMC
GGL-AMC
Substrates

Kinetic studies

To understand the enzymatic mechanism of the 20S proteasome derived from RNK16 cells, I sought to determine the substrate concentration-dependence of the proteasomal chymotrypsin-like activity. In the course of experiments, reaction progress curves at substrate concentrations ranging from 4 to 300 μM were recorded and the slope of each curve represents the cleavage rate toward the substrate. As illustrated in Fig 9, when the concentration of the chymotryptic substrate Suc-LLVY-AMC was greater than 30~40 μM, there was an inhibition of proteasome chymotrypsin-like activity. Thus, the substrate inhibition would lead to a non-Michaelis-Menten kinetic. Mostly, non-Michaelis-Menten kinetics for a single substrate enzyme can be due to a hysteretic or to a modifier mechanism. Therefore, the RNK16 cell-derived proteasomes demonstrated positive cooperativity in which binding of the first molecular of substrate may cause a conformational change to allow a second substrate to bind more tightly. These data further implies that RNK16 cell proteasome may contain multiple chymotryptic catalytic or binding sites.

Effect of SDS on the proteolytic activity of 20S proteasome of RNK16 cells

SDS activation of the proteasome has been described in literature [Dahlmann et al., 1993; Yamada et al., 1995], where reports have generally been qualitative treatments of stopped-time assays. In the kinetic studies of 20S proteasome of RNK16 cells mentioned above, I also used SDS as a component of the assay buffer. To evaluate the effect of SDS on the proteolytic activity of 20S proteasome of RNK16 cells, a detailed kinetic analysis

of the 20S proteasome chymotrypsin-like activity with or without SDS in the assay buffer was performed. The primary data from these experiments are shown in Fig 9 and 10. At each same concentration of the substrate, 20S proteasome showed much lower chymotrypsin-like activity without SDS in assay buffer than that with SDS in assay buffer (Fig 10 vs. Fig 9). Thus, the RNK16 cell 20S proteasome showed latency in the chymotrypsin-like activity to break down oligopeptides under environment without SDS. In other words, SDS can stimulate the latent form of proteasomes to the active form.

Fig 9. Dependence of proteasomal cleavage velocity on substrate concentration for the RNK16 cell-derived 20S proteasome-catalyzed hydrolysis of Z-Ala-Ala-Phe-AMC with low concentration of SDS (0.035%) in assay buffers. Assay buffer, chymotryptic substrate AAF-AMC were added to a cuvette placed in the ISS K2 fluorescence spectrophotometer. After reaching thermal equilibrium, proteasome was added and reaction progress was monitored by the increase in fluorescence emission at 440 nm. The final concentration for 20S proteasome at various substrate concentrations is 2 nM.

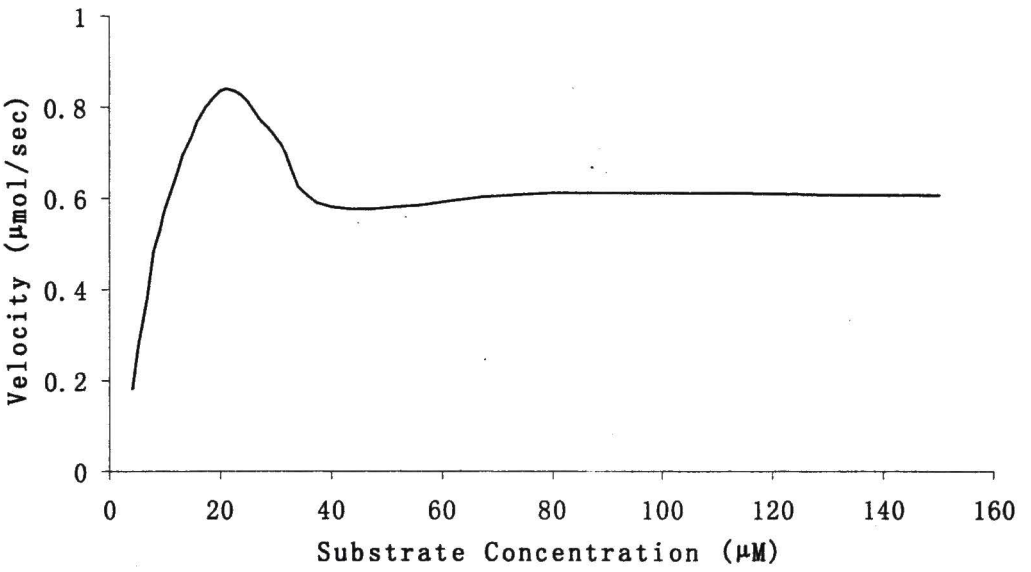
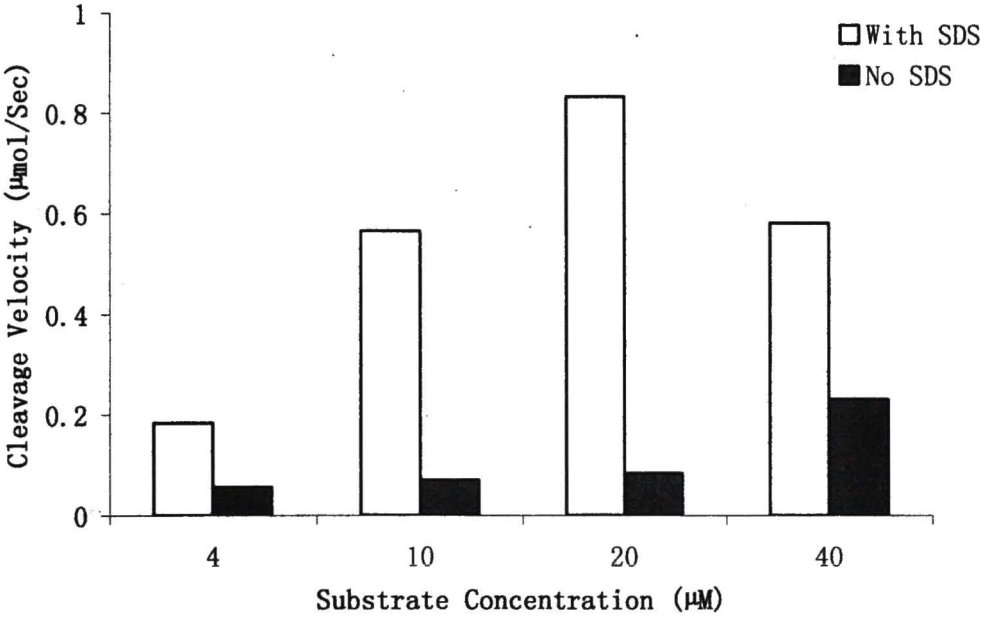

Velocity (μmol/sec)
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
Substrate Concentration (μM)

Fig 10. SDS may stimulate RNK16 cell-derived 20S proteasome from latent to active status. Assay buffer with or without SDS (0.035%), chymotryptic substrate Z-Ala-Ala-Phe-AMC were added to a cuvette placed in the ISS K2 fluorescence spectrophotometer. After reaching thermal equilibrium, proteasome was added and reaction progress was monitored by the increase in fluorescence emission at 440 nm. Cleavage velocities were determined from the progress curves and compared between these two conditions, i.e., with or without SDS in the assay buffer.


1
0.8
0.6
0.4
0.2
0
Cleavage Velocity (μmol/Sec)
With SDS
No SDS
4
10
20
40
Substrate Concentration (μM)

DISCUSSION

The multicatalytic proteinase complex, also known as the 20S proteasome, is a ~700 kD multisubunit proteolytic complex found in all cells including eukaryotic cells [Coux et al., 1996; Rivett et al., 1997]. Studies with the proteasome isolated from yeast have indicated that each of the three major activities (chymotrypsin-like, trypsin-like, and PGPH activity) is controlled by one particular β subunit of which there are two copies in each proteasome molecule [Heinemeyer et al., 1997]. However, previous studies in our laboratory implied that more than one proteasome active site for chymotrypsin-like substrates may exist in NK cells. In this study, 20S proteasome was purified from rat natural killer RNK16 cells, and the substrate selectivity, inhibitor specificity were investigated. Comparison of these biochemical properties of proteasome indicated that many of them are different from RNK16 cells to rat hepatic cells. Kinetic mechanism and effects of SDS on the chymotrypsin-like activity of RNK16 cell-derived proteasome were also investigated. The results indicated a hysteretic or a modifier mechanism in the regulation of the chymotrypsin-like activities of RNK16 cell proteasome. In addition, latent form of RNK16 cell-derived proteasomes is confirmed to be stimulated to active form by SDS. Thus, studies in this section extended the proteasome research in our laboratory and further support our previous hypothesis that in NK cells there may be a two-site mechanism for proteasomal chymotryptic activity regulation.

Besides the research in our laboratory, there is some other evidence showing the possibility that some proteasomes may contain multiple sites for chymotryptic substrates. For example, while there are only seven different β subunits genes in yeast, there are at least 10 human genes for β subunits [Coux et al., 1996]. Studies with peptide aldehyde inhibitor Ac-Leu-Leu-Nle-H were consistent with binding to multiple sites on the proteasome [Stein et al., 1996]. Most recently, the crystal structure of the bovine 20S proteasome was determined at a 2.75 Å resolution. The structures of α2, β1, β5, β6, and β7 subunits of the bovine enzyme were different from the yeast enzyme but enabled the bovine proteasome to accommodate either the constitutive or the inducible subunits. A novel N-terminal hydrolase activity has been proposed for the beta7 subunit [Unno et al., 2002].

In this study, the substrate selectivity and inhibitor specificity of proteasomes were compared between RNK16 cells and rat hepatic cells. The significance of these comparative studies is to allow us to investigate a possible relationship of their chymotrypsin-like catalytic sites [Arribas and Castano, 1993]. But more importantly, in this study the application of comparative studies is to seek the differences of these biochemical properties of proteasomes between two different cell types. Since the spectra of peptides generated by the proteasome may differ when the particle was incubated under different conditions [Akopian et al., 1997], the proteolytic reactions was restricted under the same condition in this study. Even so, the RNK16 cell proteasomes showed some differences in the substrate selectivity and inhibitor specificity with those of rat

hepatic cell proteasome. Because of the secretion of interferon-γ by NK cells, this self-produced cytokine may induce the higher content of immunoproteasome in NK cells. This accumulation of inducible immunoproteasome may contribute to the differences in the substrate selectivity and inhibitor specificity observed in this study. A recent study indicated that when switching from the constitutive proteasome to the immunoproteasome, there is a significant change in the cleavage probabilities and procession rates of some observed cleavage sites in proteasomes [Peters et al., 2002].

Two forms of proteasome are confirmed in RNK16 cells in this study. It is known that proteasomes can be isolated in both active and latent forms with respect to proteolytic activity [McGuire et al., 1989; Mykles and Haire, 1991]. Latent proteasome can be activated reversibly by addition of polycations [Tanaka et al., 1986], detergents and fatty acids [Dahlmann et al., 1985], or irreversibly by dialysis against water [McGuire et al., 1989]. However, the physiological significance of any of these activated forms remains unclear. The structural basis of latency has not been defined, since these treatments have not been reported to cause detectable changes in the subunit electrophoretic pattern of proteasomes, suggesting a conformational change may result in activation of the latent form [Weitman and Etlinger, 1992]. This hypothesis was tested by some biophysical investigations in our laboratory, in which I was involved. Investigations of steady-state tryptophan fluorescence and light scattering properties of the RNK16 cell proteasome demonstrated that SDS, mono- and bivalent cations can induce conformational changes in

proteasome, which led to the activation of chymotrypsin-like activities at pH7.5 (Paper submitted to Journal of Biomolecular Structure).

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CHAPTER III

ACTIVATION OF MULTIPLE CASPASES AND MODIFICATION OF CELL SURFACE FAS (CD95) IN PROTEASOME INHIBITOR-INDUCED APOPTOSIS OF RAT NATURAL KILLER CELLS

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Journal of Cellular Biochemistry, 2003, 88: 482-492

Running title: Caspases activation and Fas modification in NK cells

ABSTRACT

The proteasome is a multisubunit protease complex that is involved in intracellular protein degradation in eukaryotes. Previously, we have reported that selective, synthetic chymotryptic proteasome inhibitors inhibit A-NK cell-mediated cytotoxicity by approximately 50%; however, the exact role of the proteasome in NK cell-mediated cytotoxicity remains unknown. Herein, we report that proteasome inhibitors, MG115 and MG132, decreased the proteasome chymotrypsin-like activity in the rat natural killer cell line RNK16 by 85% at a concentration of 5 μM. The viability of RNK16 cells was also reduced in the presence of these inhibitors. Both inhibitors induced the apoptosis of RNK16 cells, as shown by DNA fragmentation, caspase-3 activation and the appearance of sub-G_1 cell populations. An increase in the fraction of apoptotic cells was observed in a dose- and time-dependent manner in our studies. In addition, the activity of caspase-1, -2, -6, -7, -8, and -9, was increased following the treatment of RNK16 cells with these inhibitors. Further investigation revealed that the expression of Fas (CD95) protein on the RNK16 cell surface was increased after the treatment by MG115 or MG132, indicating that apoptosis induced by proteasome inhibitors in RNK16 cells might be mediated through the Fas (CD95)-mediated death pathway as well. Our studies indicate, for the first time, that proteasomal chymotryptic inhibitors can reduce natural killer cell viability and therefore indirectly inhibit cell-mediated cytotoxicity via the apoptosis-inducing properties of these agents.

INTRODUCTION

The proteasome is a multicatalytic proteinase complex that is involved in the major extralysosomal pathway responsible for intracellular protein degradation in mammalian cells. It is characterized by the presence of multiple proteolytic activities, defined as the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activity [Coux et al., 1996]. Cell cycle-regulating proteins degraded through ubiquitin-proteasome pathway include cyclins and cyclin-dependent kinase (CDK) inhibitors p21 and $p27^{kip-1}$ [Pagano et al., 1995]. Some transcription factors, such as p53, c-Fos and c-Jun, are also degraded by the proteasome [Drexler, 1997]. Dysregulation of the degradation of such proteins has profound effects on cell cycle control and can cause cells to undergo apoptosis [An et al., 1998]. In recent years, a number of proteasome inhibitors were reported to induce apoptosis in actively proliferating cell lines such as HL-60 cells [Drexler, 1997] and Ewing's sarcoma cells [Soldatenkov and Dritschilo, 1997]. These compounds, by preventing degradation of cyclins, CDK inhibitors, tumor suppressor proteins and transcription factors, ultimately lead to deregulation of cell cycle progression and apoptotic cell death.

Natural killer (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 immunotherapy for cancer patients, NK cells are activated by IL-2 *in vitro* and

then transfused back to the patients' body to locate within tumors and kill tumor cells [Basse and Goldfarb, 1994]. Numerous studies have implicated proteolytic enzymes as crucial to the cytolytic mechanism of these cells [Berke, 1994; Darmon and Bleackley, 1998; Goldfarb, 1986]. 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; Jenne and Tschopp, 1988]. Our studies have focused on proteolytic enzymes other than those found only in cytolytic granules and have indicated that the chymotryptic activity of proteasomes in NK cells might play a role in their cell-mediated cytotoxicity [Goldfarb et al., 1992; Kitson et al., 1995; Wasserman et al., 1994].

Due to their ability to inhibit cell proliferation and induce apoptosis, together with their ability to inhibit angiogenesis, proteasome inhibitors have recently become attractive candidates as anti-cancer drugs. A boronate inhibitor MLN-341, previously called PS-341 (developed by Millennium Pharmaceuticals), after showing impressive anti-proliferative effects in several animal model systems and cell culture, is currently in NCI-sponsored human clinical trials for the treatment of several types of cancer [Kisselev and Goldberg, 2001]. As indicated in our previous studies, proteasomal chymotryptic inhibitors substantially inhibit A-NK cell-mediated cytotoxicity against both NK-sensitive and -resistant targets [Kitson et al., 2000]. Therefore, with the spreading usage of the proteasome inhibitors in the clinical field, we considered it of importance to determine their potential toxicities on NK cells since unwanted inhibition of NK cells by such

inhibitors could adversely impact on the overall potential benefit of such agents. In this study, we found that two proteasome inhibitors, MG115 and MG132, could induce the apoptosis of the rat NK cell line RNK16. In this proteasome inhibitor-induced apoptosis, activation of multiple caspases and modification of cell surface Fas (CD95) protein was observed. These results suggest a mechanism by which proteasomal chymotryptic inhibitors could reduce NK cell-mediated cytotoxicity due to their apoptosis-inducing properties.

MATERIALS AND METHODS

Reagents and Chemicals

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). Ethylenedinitrilotetraacetic acid disodium salt (EDTA) was purchased from Fisher Scientific (Fair Lawn, NJ). Dithiothreitol (DTT), 2-mercaptoethanol, fluorogenic proteasome substrates Z-GGR-AMC, Suc-LLVY-AMC, Z-LLE-AMC, proteasome inhibitors MG115 and MG132, protease K, propidium iodide, and buffers HEPES and PIPES were purchased from Sigma (St. Louis, MO). Fluorogenic caspase substrates Ac-WEHD-AMC, Ac-VDVAD-AFC, Ac-DEVD-AMC, Ac-LEVDGWK(Dnp)-NH_2, Ac-VEID-AMC, Ac-IETD-AMC, Ac-LEHD-AFC were purchased from Bachem Bioscience (King of Prussia, PA). Amicon® nitrogen pressure-based concentration apparatus was obtained from Amicon (Beverly, MA). Sephacryl S-400 and heparin-Sepharose CL-6B chromatography media were purchased from Pharmacia (Piscataway, NJ). PE-conjugated Jo2 Fas antibody was purchased from Pharmingen (San Diego, CA).

Cell culture and treatment with proteasome inhibitors

Rat RNK16 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 (vehicle). 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.

Purification of proteasome

20S proteasome was purified from RNK16 cells following methods described previously [Wasserman et al., 1994]. Briefly, subsequent to harvesting RNK16 cells, postnuclear supernatants were collected after nitrogen cavitation at 325-350 psi for 30-40 minutes. Successively, isopycnic sucrose gradient fractionation, Sephacryl S-400 gel filtration chromatography, and heparin-Sepharose CL-6B chromatography were applied. In each step, the protein concentration and the specific activities for proteasomal chymotrypsin-like and trypsin-like activities were measured.

Cell viability assay

To quantitate cell death, cell viability was determined by exclusion of trypan blue. At the indicated times after treatment with proteasome inhibitors, cells were detached, pelleted, and resuspended in RPMI 1640. After staining with trypan blue, viable cells in five random fields of view were counted. The percentage of treated viable cells was determined as a percentage of control viability.

DNA fragmentation assays

At each indicated time point, RNK16 cells were washed in PBS and resuspended in 0.7 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, and 200 μg/ml protease K. The cell mixtures were incubated at 55 °C for 2 hours and then treated with 25 μg/ml RNase at 37 °C for 1 hour. After incubation, DNA was precipitated with 1.5 volume of ethanol and resuspended in TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA). The prepared DNA samples were analyzed in a 1% agarose gel containing ethidium bromide.

Detection of apoptotic cells by flow cytometry

Apoptosis can be detected by measuring the sub-G_1 population with flow cytometry. Cells were fixed in 70% ethanol and treated with 10 μg/mL of RNase. They were stained with 25 μg/mL of propidium iodide and then subjected to analysis on an EPICS XL-MCL flow cytometer with SYSTEM^{TM} II software (Beckman-Coulter Corporation, Miami, FL).

Whole cell extracts and protein determination

RNK16 cells were lysed in ice-cold homogenization buffer [20 mM Tris/HCl (pH7.4) / 0.1mM EDTA / 1 mM 2-mercaptoethanol / 5 mM ATP / 20% (v/v) glycerol, 0.04% (v/v) Nonidet P-40]. The buffer was supplemented with pepstatin (0.5 μg/ml), leupeptin (1.25 μg/ml), and phenylmethylslfonyl fluoride (0.5 mM) to minimize activity of proteases other than the desired ones. The resulting lysates were placed in microfuge tubes and

centrifuged at 13000 ×g at 4 °C for 15 min to remove insoluble material. The resulting high-speed supernatant fractions were placed on ice and assayed for protein concentration by measuring O.D. at 280 nm with bovine serum albumin as standard.

Assay of proteasome proteolytic activities

Three major proteasome proteolytic activities, i.e. trypsin-like, chymotrypsin-like, and peptidylglutamyl-hydrolyzing activity, were assayed by using substrates Z-Gly-Gly-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC, and Z-Leu-Leu-Glu-AMC respectively. For trypsin-like activity, reaction buffer 1mM dithiothreitol (DTT) and EDTA, 10 μg/ml BSA, 0.02% Triton X-100 (v/v) in 50 mM Tris/HCl buffer, pH 8.7, was used; for chymotrypsin-like activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 7.5 was used and for peptidylglutamyl-hydrolyzing activity, 1 mM DTT and EDTA, 10 μg/ml BSA, 0.02% SDS in 10 mM Tris/HCl buffer, pH 8.0, was used. All reaction mixtures contained peptide substrates at 20 μM concentrations unless otherwise noted. The rates of cleavage were determined by taking fluorescent intensity measurements using a Dynatech® fluorescence plate reader. Proteolytic activity was expressed as relative fluorescence units per second and per gram of protein.

Measurement of caspase activities

The following synthetic substrates were used to measure caspase activity: caspase-1, Ac-WEHD-AMC; caspase-2, Ac-VDVAD-AFC; caspase-3, Ac-DEVD-AMC; caspase-4, Ac-LEVDGWK(Dnp)-NH_2; caspase-6, Ac-VEID-AMC; caspase-8, Ac-IETD-AMC; and

caspase-9, Ac-LEHD-AFC. All caspase substrates were prepared as stock solutions of 10 mM in 100% DMSO. Before the assay, all caspase substrates were diluted, yielding final concentrations of 20 μM. To each well, 20 μl RNK16 cell lysates, 80 μl reaction buffer, and 100 μl of substrate solution were added and fluorescence of the cleavage product measured over time at room temperature in a microplate spectrofluorometer. For AMC substrates, excitation wavelenth was 360 nm, emission 460 nm; for AFC substrates, excitation was 400 nm and emission 505 nm. Activity was normally measured after incubating 2 hours and was expressed as percentage of activity compared with the activity at t=0 hours. Mean ± SDs from triplicate wells are shown from experiments performed on three separate occasions.

Analysis of cell surface Fas

After treatment with proteasome inhibitors, cells were harvested and washed once by PBS. Then cells were resuspended in staining medium (1% FBS, 0.1% sodium azide in PBS) and incubated with PE-conjugated Jo2 antibody (PharMingen, San Diego, CA) for 30 min at room temperature. After the second wash in staining medium, cells were fixed by 1% paraformaldehyde in PBS and analyzed on an EPICS XL-MCL flow cytometer with SYSTEM^{TM} II software (Beckman-Coulter Corporation, Miami, FL).

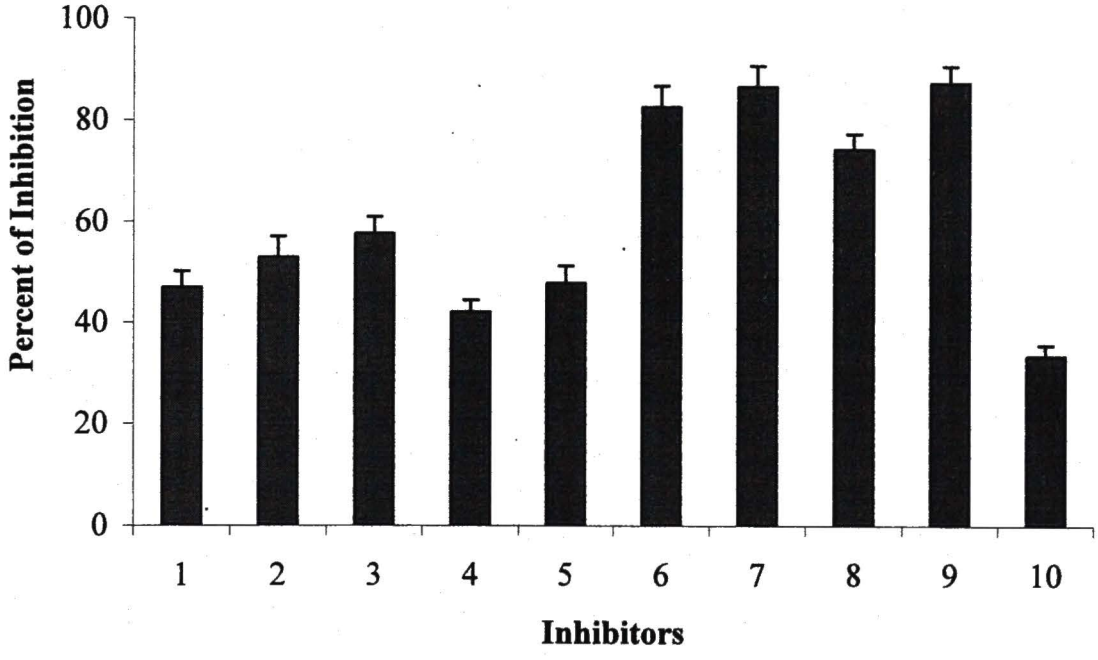
RESULTS

Effects of protease inhibitors on proteasomal proteolytic activities *in vitro*

After purification of 20S proteasome from RNK16 cells, we determined the specificity of some protease inhibitors on the RNK16 cell proteasome. In total 10 protease inhibitors were tested and 4 of them were found to achieve more than 80% inhibition on the proteasomal chymotryptic activity at the concentration of 5 μM (Fig 1). The proteasomal inhibitors, MG115 and MG132, were among these four inhibitors.

Next we investigated the inhibitory effects of these two inhibitors on the major three proteolytic activities of RNK16 cell proteasome, i.e. trypsin-like, chymotrypsin-like and peptidylglutamyl-hydrolyzing activities. It is known that inhibition of all multiple active sites in 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 rates of protein breakdown [Chen and Hochstrasser, 1996; Heinemeyer et al., 1997; Rock et al., 1994]. In our studies, we incubated MG115 or MG132 with the proteasome purified from RNK16 cells to test their inhibitory effects on the three major proteasomal proteolytic activities. Three specific substrates, GGR-AMC, LLVY-AMC, and LLE-AMC were employed to determine proteasomal trypsin-like, chymotrypsin-like and peptidylglutamyl-hydrolyzing (PGPH) activities respectively. At a concentration of 10 μM, MG132 inhibited the trypsin-like, chymotrypsin-like and peptidylglutamyl-hydrolyzing activity of proteasome by 95.6%, 94.2% and 90.1%

Fig 1. Inhibitory effects of various protease inhibitors on the chymotrypsin-like activity of proteasome from RNK16 cells *in vitro*. 0.5 μg purified RNK16 cell proteasome was preincubated with various protease inhibitors for 10 minutes: 1, TPCK; 2, ZPCK; 3, H-Ala-Ala-Phe chloromethyl ketone; 4, L-Leucine chloromethyl ketone; 5, lactacystin; 6, MG115; 7, Z-Ile-Glu-Ala-Leu-aldehyde; 8, Z-Leu-Leu-Phe-aldehyde; 9, MG132; 10, TLCK. Then reaction buffer was added to the final concentration of inhibitors at 5 μM.


Percent of Inhibition
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Inhibitors

respectively, while the inhibition rates of MG115 were 90.8% 91.4% and 88.2% (Table 1).

Effects of proteasome inhibitors on proteasomal proteolytic activities in cultured cells

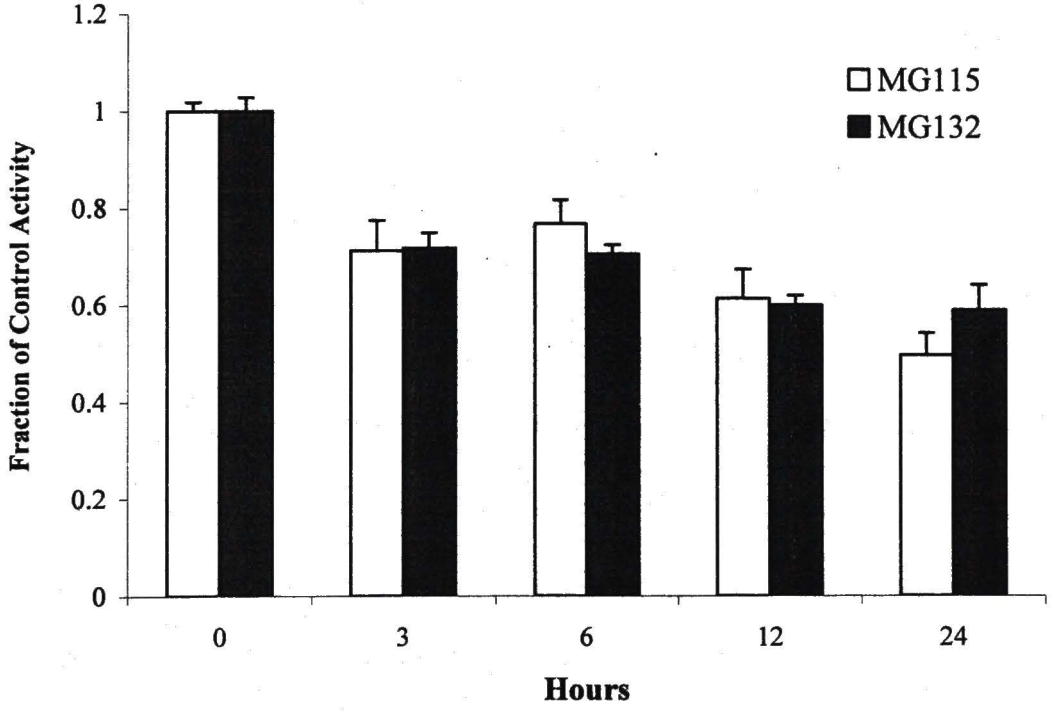
The proteasome inhibitors, MG115 and MG132, used in the present study were reported to efficiently block proteasomal activity in eukaryotic cells [Palombella et al., 1994]. To confirm the inhibitory effects of these proteasome inhibitors on proteasome in cultured NK cells, proteasomal proteolytic activities were assayed after RNK16 cells were incubated with inhibitors MG115 or MG132 in culture media. After lysates of incubated cells at indicated time points were collected, substrates GGR-AMC, LLVY-AMC, and LLE-AMC were employed to determine any changes of the major three proteasomal proteolytic activities occurring during the incubation of RNK16 cells with these proteasome inhibitors. For the chymotrypsin-like activity, incubation of RNK16 cells with either 20 μM MG115 or MG132 did not cause as great a reduction in enzymatic activity as incubation of purified proteasome with either 10 μM MG115 or MG132 in the *in vitro* assay. After incubation for 12 hours, MG115 caused 39% reduction in the chymotryptic activity while MG132 achieved 41% reduction of this activity (Fig 2). Both MG115 and MG132 also inhibited the proteasomal trypsin-like and peptidylglutamyl peptide hydrolyzing (PGPH) activity (data not shown). Indeed, we have also previously reported that certain selective inhibitors of proteasomal chymotryptic activities (e.g., CEP-1508) also substantially inhibit NK cell-mediated cytotoxicity [Kitson et al., 2000].

Table 1. Effects of proteasome inhibitors MG115 and MG132 on proteasomal proteolytic activities *in vitro*.

Inhibitors	MG115	MG132
Trypsin-like activty	90.80%	95.60%
Chymotryptic activity	91.40%	94.20%
PGPH activity	88.20%	90.10%

0.5 μg purified RNK16 cell proteasome was incubated with 10 μM MG115 or MG132 alone in the reaction buffer for 1 hour at 37 °C. The proteasomal trypsin-like, chymotrypsin-like, and PGPH activities were assessed by using 20 μM GGR-AMC, LLVY-AMC, and LLE-AMC in reaction buffer as substrates respectively.

Fig 2. Effects of proteasome inhibitors on proteasomal proteolytic activities in cultured cells. The chymotryptic activity of the proteasome was measued by Suc-LLVY-AMC hydrolysis. RNK16 cells were incubated with 20 μM MG115 or MG132 for 3, 6, 12 and 24 hours. The fluorescence was measured after 1 hour of incubation with the substrates. Activity was expressed as a percentage of activity without inhibitor treatment at each time point. Means ± SDs of two independent experiments, each performed in triplicate, are shown.


1.2
1
0.8
0.6
0.4
0.2
0
Fraction of Control Activity
MG115
MG132
0
3
6
12
24
Hours

These results indicate that these two proteasome inhibitors are able to penetrate RNK16 cells and inhibit the proteasome.

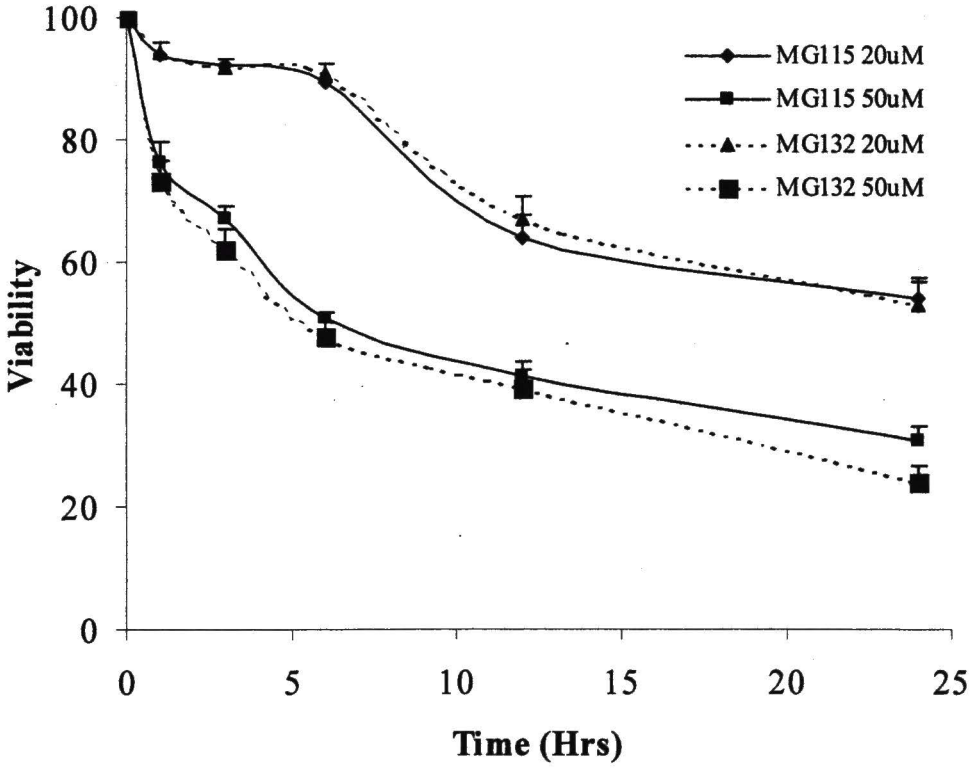
Reduced viability of RNK16 cell in the presence of proteasome inhibitors

To investigate any effects of these proteasomal inhibitors on RNK16 cells, the cells were incubated with proteasome inhibitors and morphological appearance of RNK16 cells were examined next. Although RNK16 cells in culture exist in an equilibrium between attached cells and cells in suspension, with the increasing treatment time more cells detached and were observed floating in the medium, indicating that these compounds upset this balance. After 24 hours treatment, cell shrinkage could be found easily under microscope. To assess the extent of cell death at indicated time points, cell viability was measured by trypan blue exclusion test. The viability of RNK16 cells was reduced to around 60% after 12 hours with either 20 μM MG115 or MG132 alone, while 50 μM MG115 or MG132 caused a similar decrease in viability at 3-6 hours (Fig 3). These data demonstrate that proteasome inhibitors reduce the viability of RNK16 cells in a time- and dose-dependent manner.

Apoptosis of RNK16 cells induced by proteasome inhibitors

To determine the mechanism of reduced viability of RNK16 cells in presence of proteasome inhibitors, we investigated whether cell death was due to apoptosis. Initially, we used a DNA fragmentation assay to see any RNK16 cells undergoing apoptosis. Fragmentation of the genomic DNA is an irreversible event that commits the cell to die.

Fig 3. Reduced viability of RNK16 cell in the presence of proteasome inhibitors. RNK16 cells were incubated for 1, 3, 6, 12, and 24 hours with MG115 or MG132 alone at 20 μM or 50 μM. Viable cells were counted after staining with trypan blue. The viability of untreated control cells at each time point was counted as 100%. The viability of treated cells was expressed as a percentage of control cells at various times. Data represent average of three experiments.


100
80
60
40
20
0
Viability
0
5
10
15
20
25
Time (Hrs)
MG115 20uM
MG115 50uM
MG132 20uM
MG132 50uM

These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit. An agarose gel of RNK16 cell DNA indicated that DNA fragmentation occurred in these cells after they were treated with proteasome inhibitors, MG115 and MG132, at 20 μM for 10 hours (Fig 4).

We also performed flow cytometry analysis of propidium iodide stained cells to measure the DNA content and integrity of proteasome inhibitor treated RNK16 cells. The lower DNA content of apoptotic cells stained by a propidium iodide could be measured by flow cytometry. Our results showed that the percentage of apoptotic cells, characterized by the sub-G_1 peak, increased with longer incubation times with the proteasome inhibitors MG115 or MG132 (Fig 5). When the concentration of MG132 was increased from 20 μM to 50 μM, the sub-G_1 cell population increased significantly at the indicated various time points. These results indicate that RNK16 cells undergo apoptosis in a time- and dose-dependent manner during their incubation with proteasome inhibitors.

Caspase-3 or DEVD-cleaving activity is an early marker of cells undergoing apoptosis. Ac-DEVD-AMC is a synthetic tetrapeptide fluorogenic substrate reported to be specific for caspase-3. By using this substrate, we identified that treatment of RNK16 cells with MG115 or MG132 led to a 7.8 or 7.2 fold respective increase in caspase-3 activity at 12 hours (Fig 6). This increase was not due to a direct effect of MG115 or MG132 on

Fig 4. DNA fragmentation of RNK16 cells during incubation with proteasome inhibitors, MG115 and MG132. RNK16 cells treated with 20 μM MG115 (Lane 1) or MG132 (Lane 2) alone for 10 hours display DNA laddering analyzed by agarose gel electrophoresis. M: DNA size markers.

M 1 2

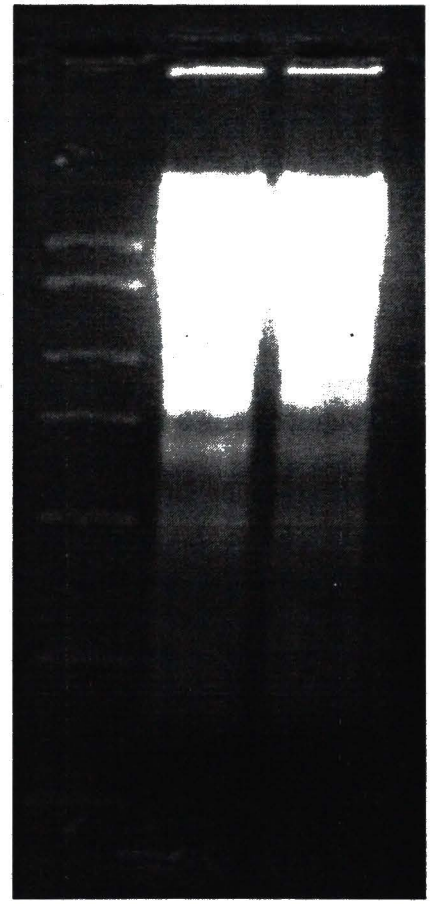


Fig 5. Apoptosis of RNK16 cells induced by proteasome inhibitors evidenced by sub-G_1 cell population in flow cytometry analysis. Exponentially grown RNK16 cells (0 hr) were treated with 20 μM MG115 (Column A) or MG 132 (Column B), or 50 μM MG132 (Column C) for indicated time duration, followed by measurement of sub-G_1, G_1, S and G_2-M cell populations (see Materials and Methods). The units of the y-axis represent cell numbers (in total 10,000 events) and those of the x-axis represent cellular DNA content. The data indicate the percentage of sub-G_1 cells in the cell cycle. All experiments were performed twice independently and gave similar results.

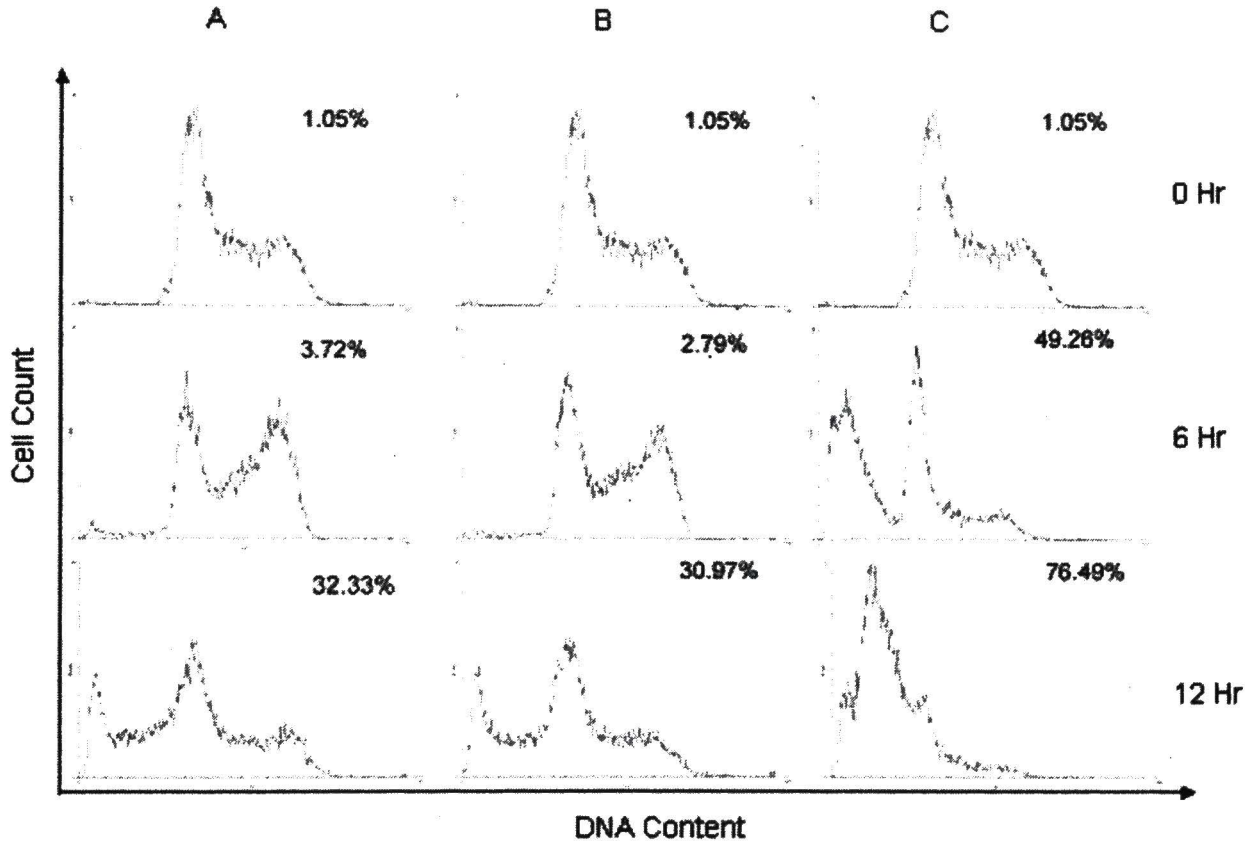
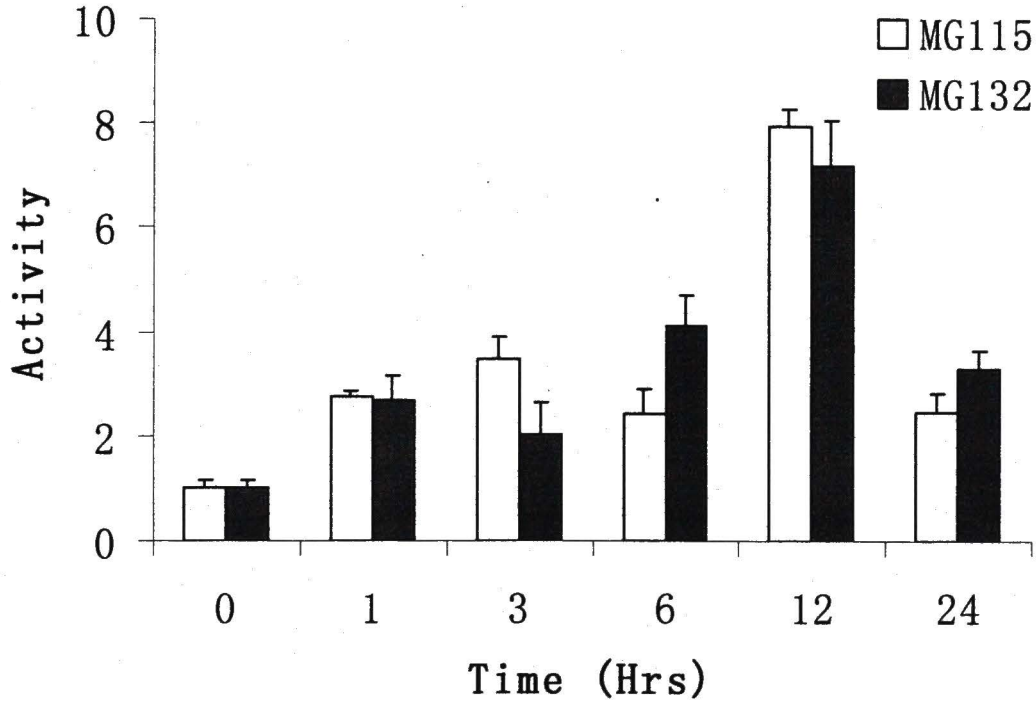

A
B
C
1.05%
1.05%
1.05%
0 Hr
3.72%
2.79%
49.26%
6 Hr
32.33%
30.97%
76.49%
12 Hr
Cell Count
DNA Content

Fig 6. Activation of caspase-3 of RNK16 cells during incubation with proteasome inhibitors, MG115 and MG132. RNK16 cells were incubated with the inhibitors for the times indicated, lysed, and caspase-3 activity was determined using Ac-DEVD-AMC, as described in Materials and Methods. The caspase-3 activity of cells at 0 hours was counted as 100%. Data represent average of three experiments.


10
8
6
4
2
0
Activity
MG115
MG132
0
1
3
6
12
24
Time (Hrs)

caspase-3, because *in vitro* incubation with MG115 or MG132 had no effect on Ac-DEVD-AMC hydrolysis (data not shown).

Proteasome inhibitors induce the processing of multiple caspases in RNK16 cells

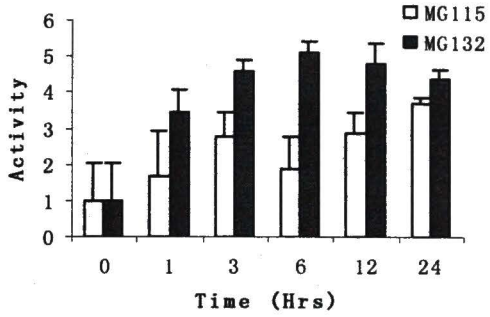
Despite the diversity of signals which can induce apoptosis, these pathways can share several features in their execution. One mechanism which has been consistently implicated in apoptosis is the activation of a series of cytosolic proteases, the caspases. To examine processing of caspases in cell apoptosis mediated by proteasome inhibitors, we sought to measure the proteolytic activity of caspases -1, -2, -6, -7, -8, and -9 by fluorogenic assay. The results indicate that all six of these caspases were induced to active status by proteasome inhibitors at different amplitudes. Most caspases achieved their activity peaks around 12 hours after exposure of RNK16 cells to the proteasome inhibitors (Fig 7). Some caspases showed earlier activation by MG132 than that by MG115. Caspase-9 showed greater amplification of activity than that of caspase-8, achieving a 23.4-fold increase after 12 hours of treatment by MG115 and 29.5 fold after treatment by MG132 compared to 0 hour respectively.

Modification of cell surface Fas by proteasome inhibitors

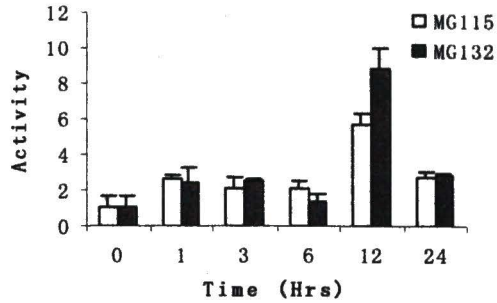
Since the activity of caspase-8 was also seen to increase significantly by the treatment of RNK16 cells with MG115 or MG132, we examined the possible upregulation of Fas (CD95) on cell surface induced by proteasome inhibitors. RNK16 cells were incubated with 20 μM MG115 (Fig 8, panel A) or MG132 (Fig 8, panel B) for 3 and 20 hours and

Fig 7. Activation of multiple caspases in RNK16 cells during their incubation with 20 μM MG115 or MG132. Fluorogenic substrates used for these caspases were Ac-WEHD-AMC (A; caspase-1), Ac-VDVAD-AFC (B; caspase-2), Ac-LEVDGWK(Dnp)-NH_2 (C; caspase-4), Ac-VEID-AMC (D; caspase-6), Ac-IETD-AMC (E; caspase-8), and Ac-LEHD-AFC (F; caspase-9). Activity is expressed as percentage of activity at t = 0 hours (control activity). Means ± SD of three separate experiments are shown.

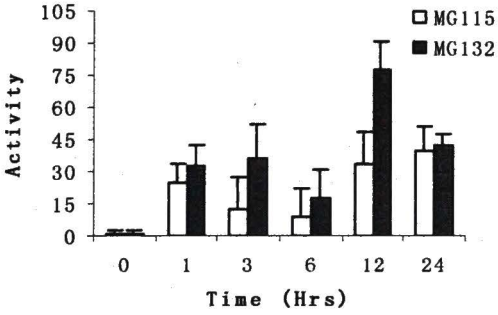
A


MG115
MG132
Activity
0
1
2
3
4
5
6
0
1
3
6
12
24
Time (Hrs)

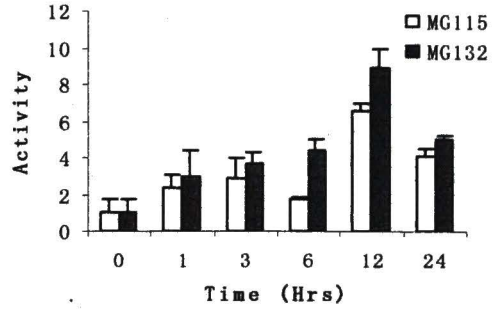
B.


MG115
MG132
Activity
0
2
4
6
8
10
12
0
1
3
6
12
24
Time (Hrs)

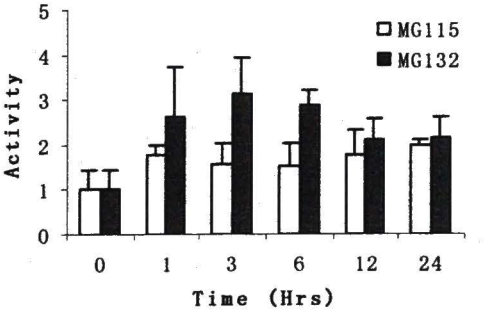
C.


MG115
MG132
Activity
0
15
30
45
60
75
90
105
0
1
3
6
12
24
Time (Hrs)

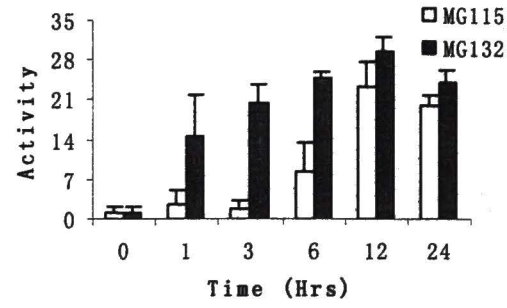
D.


MG115
MG132
Activity
0
2
4
6
8
10
12
0
1
3
6
12
24
Time (Hrs)

E.

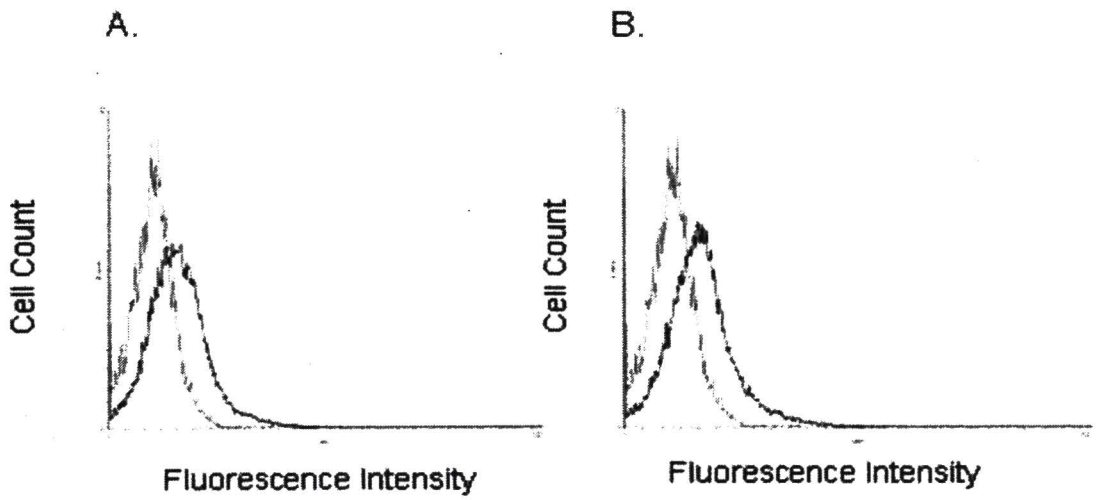

MG115
MG132
Activity
0
1
2
3
4
5
0
1
3
6
12
24
Time (Hrs)

F.


MG115
MG132
Activity
0
7
14
21
28
35
0
1
3
6
12
24
Time (Hrs)

then the level of cell surface Fas protein was determined by flow cytometry after staining with PE-conjugated monoclonal Jo2 which recognizes mouse Fas. Compared with untreated cells, cells treated for 20 hours with MG115 or MG132 showed distinct shifts in the distribution of fluorescence for Fas (Fig 8), while the curves for cells treated for 3 hours with same concentration of inhibitors almost overlaid with that of control cells (data not shown). The data indicate that proteasome inhibitors increased cell surface Fas of rat NK cells and imply that proteasome inhibitor-induced apoptosis of RNK16 cells might be mediated by the Fas-mediated pathway as well.

Fig 8. Modification of cell surface Fas (CD95) of RNK16 cells by proteasome inhibitors, MG 115 and MG132. RNK16 cells were incubated for 0 (line) and 20 hours (bold line) with 20 μM MG115 (Panel A) or MG132 (Panel B). Cells were harvested and stained live for Fas on cell surface with PE-conjugated Jo2 antibody. The fluorescence was determined by flow cytometry.


A.
Cell Count
Fluorescence Intensity
B.
Cell Count
Fluorescence Intensity

DISCUSSION

The proteasome is a 700-kDa protease multi-component complex that is thought to be responsible for turnover of defective proteins in all cells including eukaryotic cells [Coux et al., 1996; Rivett et al., 1997]. It consists of 28 subunits which are arranged in a set of four stacked rings. To date the proteasome has been found to cleave peptides following basic amino acids (trypsin-like), hydrophobic amino acids (chymotrypsin-like), glutamic acid, branched chain amino acids and small neutral amino acids [Coux et al., 1996]. Unlike any other protease, all the proteolytic sites in the proteasome utilize N-terminal threonines of the beta subunits as active site nucleophiles. Most proteasome inhibitors have been designed to attack this N-terminal threonine by reacting with the catalytic hydroxyl or thiol groups in the active sites of the proteasome to form a reversible hemi(thio)acetal, which resembles a transition state analogue of the enzymatic reaction [Groll et al., 1997; Lowe et al., 1995]. Among these activities, the cleavage by the chymotrypsin-like sites appears to be rate-limiting in protein breakdown. Studies have indicated that the inhibition of the chymotrypsin-like site or its inactivation by mutation alone can cause a large reduction in the rates of protein breakdown [Chen and Hochstrasser, 1996; Heinemeyer et al., 1997; Rock et al., 1994], while inactivation of trypsin-like or peptidyl-glutamyl peptide hydrolyzing (PGPH) activities has little effect on overall proteolysis [Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997; Kisselev et al., 1999]. In this study, the potency of MG115 and MG132 was also measured against the trypsin-like and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities with

purified proteasomes of RNK16 cells, in addition to chymotrypsin-like activity. Our results indicate that these two inhibitors act on these three major active sites in the proteasome from RNK16 cells. In addition, with the highly hydrophobic properties of most chymotrypsin-like inhibitors, these two inhibitors were consequently confirmed to be cell-permeable.

By preventing degradation of cyclins, CDK inhibitors, tumor suppressor proteins and transcription factors, proteasome inhibitors lead to deregulation of cell cycle progression and apoptotic cell death. However, some studies have indicated that proteasome inhibition may be protective against some apoptotic stimuli in differentiated cells such as thymocytes [Drexler, 1997; Grimm et al., 1996]. Even though there have been an increasing numbers of reports demonstrating pro- or antiapoptotic effects of proteasome inhibitors, their effects on NK cells have not been fully investigated [Kitson et al., 2000; Wasserman et al., 1994]. The present study sought to examine the potential effects of proteasome inhibitors on NK cells. By investigating effects of proteasome inhibitors MG115 and MG132 on proteasomal activities *in vitro* and *in vivo*, their inhibition of the proteasome of NK cells was confirmed. In addition, this inhibition by the proteasome inhibitors MG115 and MG132 was shown to cause apoptosis in cultured RNK16 cells, as evidenced by appearance of the apoptotic population with sub-G_1 DNA content, internucleosomal DNA fragmentation, and activation of caspase-3.

In mammals, programmed cell death can be initiated by three distinct pathways [Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998]: (1) the extrinsic pathway, which can be triggered by the ligation of death receptors, such as Fas (also termed CD95 or APO-1), tumor necrosis factor receptor, or TRAIL receptor, and subsequent mitochondria-independent caspase-8 activation [Li et al., 1998; Luo et al., 1998]; (2) the intrinsic pathway, which is initiated by diverse apoptotic stimuli and followed by activation of caspase-9; or (3) the granzyme B pathway, where the cytotoxic cell protease granzyme B is delivered to sensitive target cells. Each of these pathways converges to a common execution phase of apoptosis that requires the activation of caspase-3 from its inactive zymogen form to its processed, active form. In this study, we have shown that the induction of cell death by proteasome inhibitors involves the activation of multiple caspases. In this report, we show the processing of caspases -1, -2, -3, -4, -6, -8, and -9 after the treatment of RNK16 cells with MG115 or MG132. Therefore, RNK16 cells seem to undergo apoptosis through a caspase-dependent pathway. However, some studies have observed that there is also caspase-independent apoptosis, led by the blocking of the ubiquitin-dependent pathway, in which accumulation of p53, p27, and cyclins D1 and B1 was seen [Monney et al., 1998]. So, it is therefore important to also examine the changes of signaling molecules in the apoptosis of NK cells induced by proteasome inhibitors. The use of caspase inhibitors allows us to clarify whether caspase-independent pathways are involved in the proteasome inhibitor-induced apoptosis of rat NK cells. For instance, Z-DEVD-FMK is a potent inhibitor of caspase-3, but it can inhibit other caspases as well

when used in cell-culture experiments [Villa et al., 1997]. Such studies are currently under investigation in our laboratory.

Besides the controversy of the role of caspases in apoptosis, there is also contradictory evidence for the role of each individual caspase in proteasome inhibitor-induced apoptosis. In some studies, caspase 8 activation is not a critical step in the killing cascade and to date the role of endogenous death ligand/receptor interactions has not been reported [Wagenknecht et al., 1999]. However, in other studies, proteasome inhibitors did not influence expression of procaspase-8, procaspase-3, but did upregulate Fas and FADD [Kim, 2001], and the interaction of Fas with the ubiquitin-conjugating enzyme may be directly involved in ubiquitin-dependent degradation of Fas by proteasome [Becker et al., 1997; Wright et al., 1996]. Our results appear to be consistent with the latter studies. We suppose that the different findings regarding caspase-8 activation might possibly be caused by the different phenotypes and genotypes of these cell lines.

We have previously determined that some proteasomal inhibitors selective for chymotryptic domains of NK cell proteasome can inhibit NK cell viability (e.g., CEP-1612) [Kitson et al., 2000]. The current study extends and classifies this phenomenon. Indeed, this study reveals at least one mechanism by which apoptosis-inducing proteasomal chymotryptic inhibitors can contribute to reduced NK cell-mediated cytotoxicity.

A key prerequisite for optimal therapeutic application of proteasome inhibitors for treatment of malignant tumors should be the sparing of NK cells, i.e., immune effector cells which recognize and kill tumor cells. In this regard, this study also provides a potential approach towards clinical guidance for therapeutic application of proteasome inhibitors in malignant tumors, i.e., the negative effects on killer cells, should also be considered to minimize unwanted adverse consequence that might arise through untoward inhibition of NK cell function.

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CHAPTER IV

DIFFERENTIAL EFFECTS OF PROTEASOME INHIBITORS ON CELL CYCLE REGULATION AND MOLECULAR MODULATION IN HUMAN NATURAL KILLER CELLS AND T LYMPHOCYTES

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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 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 progression and apoptosis signaling pathways between human NK and T cells.

INTRODUCTION

The 20S proteasome is the key component of the 26S multicatalytic proteasome complex [Baumeister et al., 1998; Goldberg, 1995; Groll et al., 1997]. 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) [Groll et al., 1997; Loidl et al., 1999]. The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins [Hochstrasser, 1995], since many cell cycle and cell death regulators, 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], have been identified as targets of the ubiquitin-proteasome-mediated degradation pathway.

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 patients' body to kill tumor cells [Goldfarb et al., 1994]. Numerous studies

have implicated proteolytic enzymes as crucial to the cytolytic mechanism of these cells [Berke, 1994; Darmon and Bleackley, 1998; Goldfarb, 1986]. 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; Jenne and Tschopp, 1988]. 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; Kitson et al., 1995; Wasserman et al., 1994].

During the course of our investigations on proteasome in rat natural killer 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]. These distinct characteristics allow us to hypothesize that there may be differential responses of proteasomes to their inhibitors between NK cells and T 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, i.e. 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]. 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, i.e. natural killer 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 epigallocatechin gallate (EGCG) (>95%) were purchased from Sigma. Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC[3] (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 natural killer cell line) and Jurkat cells (a human T cell line) 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 minutes. 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 ± S.D. 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. 0.3 μg of purified 20S proteasome 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[3], 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 ($\Delta\psi_m$)

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^{TM} II software (Beckman-Coulter Corporation, Miami, FL) with excitation and emission settings of 484 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 hours 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 minutes. 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^{TM} II software (Beckman-Coulter Corporation, Miami, FL).

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 hour and stained with Hoechst 33528 (1 mM) for 30 minutes. The nuclear morphology of cells was visualized by fluorescence microscope (Olympus BH2; Tokyo, Japan). Numbers of cells with apoptotic nuclei were counted under 3 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

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, epigallocatechin gallate (EGCG), N-Acetyl-Leu-Leu-Norleu-al (LLnL). Clasto-lactacystin β-lactone is the active form of lactacystin, a highly specific and irreversible inhibitor of the proteasome [Dick et al., 1996; Fenteany and Schreiber, 1998; Fenteany et al., 1995]. 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 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 1 A, B, 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 lead 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 1 A, B, C).

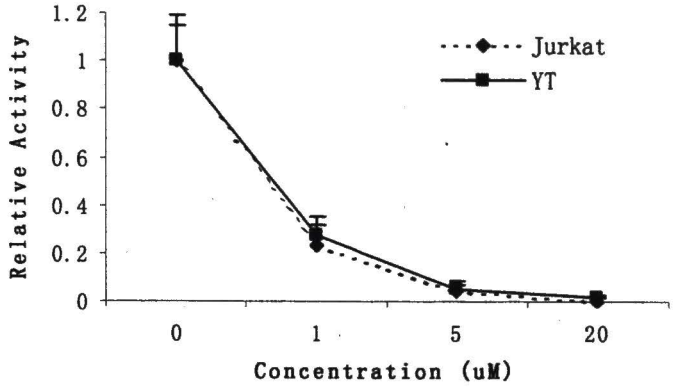
Inhibitory effects of proteasome inhibitors on 26S proteasome activity in whole cell extracts and intact YT and Jurkat cells

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 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 2 C). 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 2 A and B).

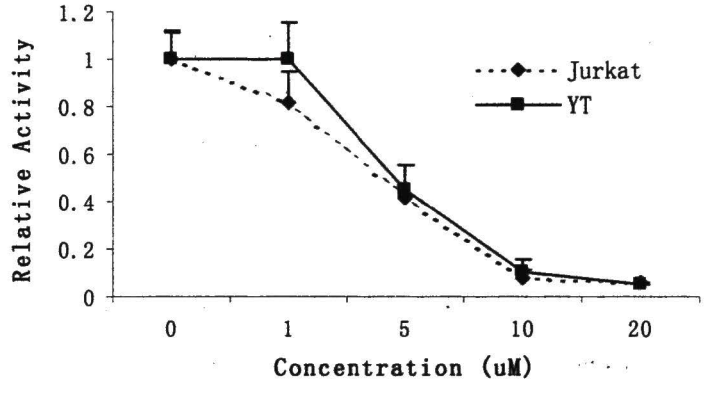
To determine whether proteasome inhibitors get access into the living cells to attack on the proteasome similarly in these two cell lines, both YT and Jurkat cells were first incubated with various concentrations of LLnL or MG132 followed by an additional

Fig 1. Inhibitory effects of proteasome inhibitors on the purified proteasome from Jurkat and YT cells. Purified 20S proteasome from YT and Jurkat cells (0.3 μg/ reaction) 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), LLnL (B), and EGCG (C). 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).

A.


Relative Activity
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
1
5
20
Concentration (uM)

B.


Relative Activity
1.2
1
0.8
0.6
0.4
0.2
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Jurkat
YT
0
1
5
10
20
Concentration (uM)

C.

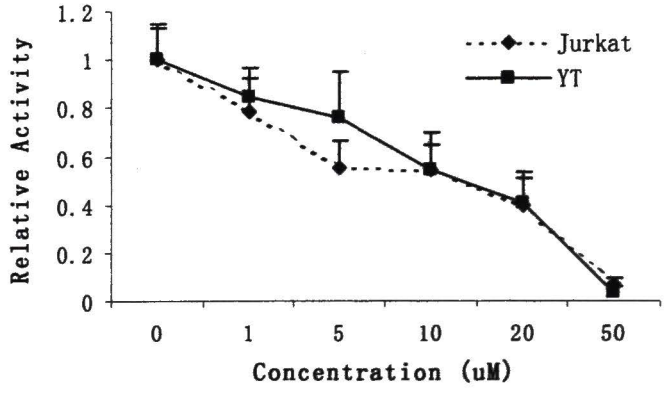
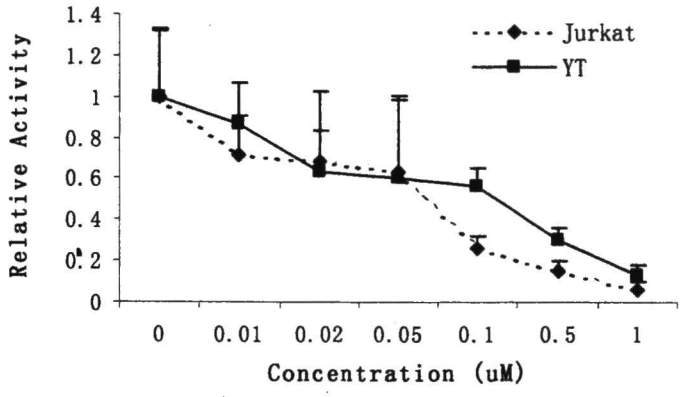
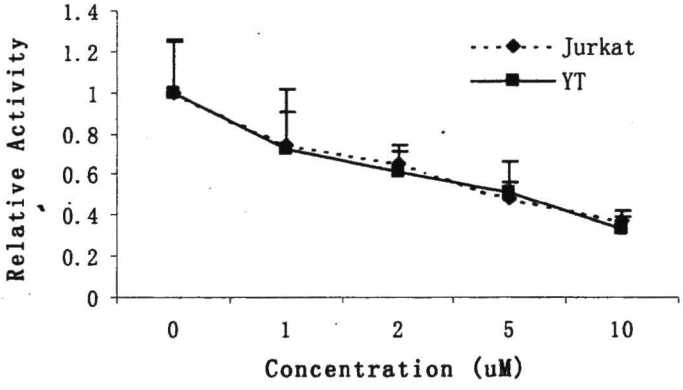

Relative Activity
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
1
5
10
20
50
Concentration (uM)

Fig 2. Inhibitory effects of proteasome inhibitors on proteasomal activity in cell extracts from Jurkat and YT cells. A cell extract (3.5 μg/ reaction) of YT and Jurkat cells 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), LLnL (B), and EGCG (C). 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).

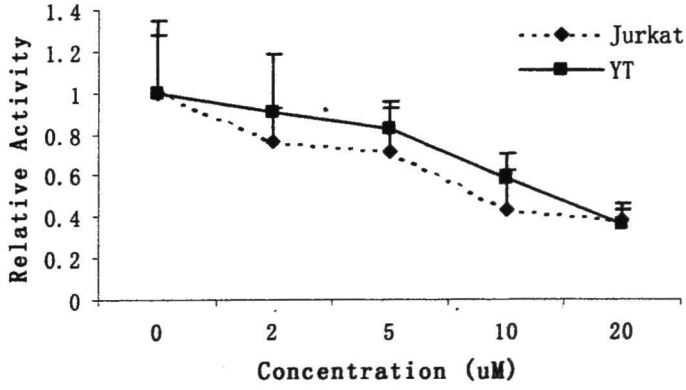
A.


Relative Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
0.01
0.02
0.05
0.1
0.5
1
Concentration (uM)

B.


Relative Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
1
2
5
10
Concentration (uM)

C.


Relative Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
2
5
10
20
Concentration (uM)

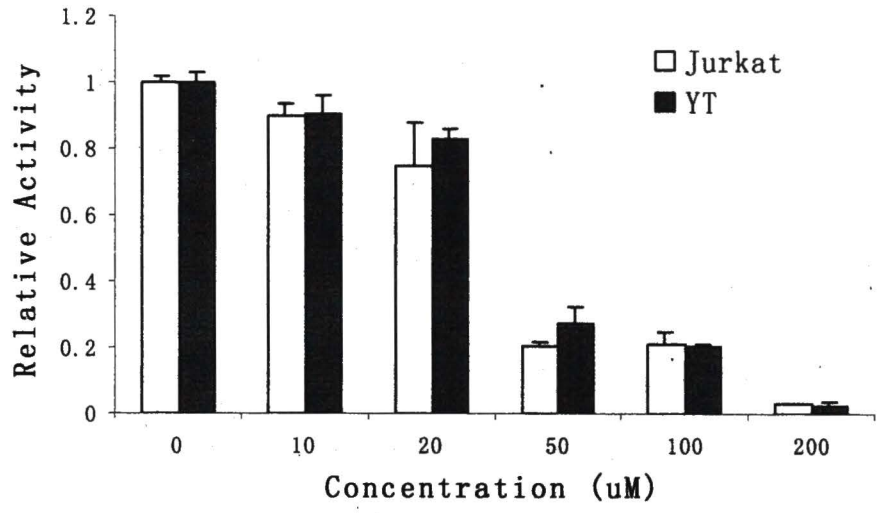
incubation with Suc-LLVY-AMC. Afterward, the cell medium was collected for the measurement of hydrolyzed free AMCs. By performing this assay, we found that LLnL or MG132 significantly inhibited the proteasomal chymotrypsin-like activity in both intact human Jurkat and YT cells similarly. The IC_{50} of LLnL and MG132 for the proteasome chymotrypsin-like activity in these cell lines are about 30 μM and 20 μM respectively (Fig 3 A and 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, i.e., 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 hours 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 4). The viability of both cell lines dropped sharply during their first 3 hours with the LLnL treatment; however, during the treatment time from 6 hr to 20 hr, YT cells

Fig 3. 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 "Material and Methods". Most of the data were derived from triplicates in two independent experiments.

A.


Relative Activity
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
10
20
50
100
200
Concentration (uM)

B.

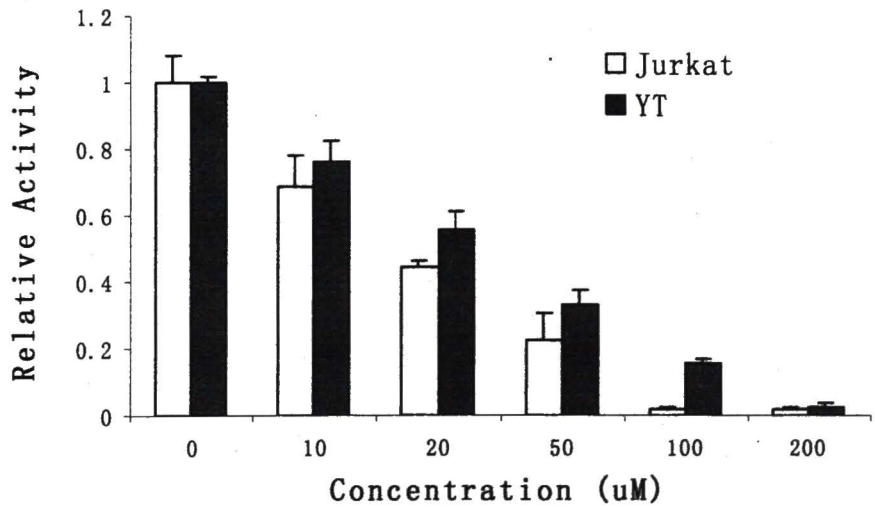
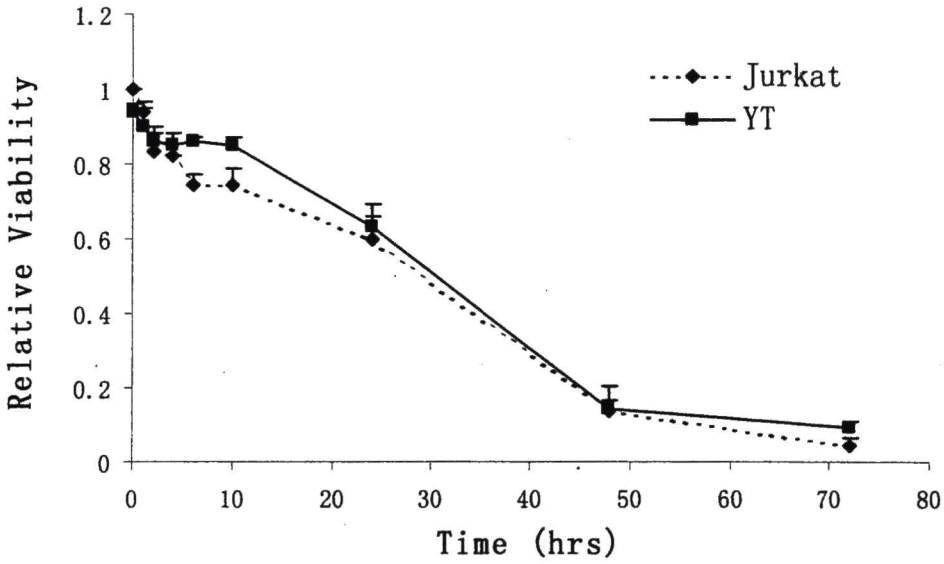

Relative Activity
1.2
1
0.8
0.6
0.4
0.2
0
Jurkat
YT
0
10
20
50
100
200
Concentration (uM)

Figure 4. Viability curve of Jurkat and YT cells during 72 hours of treatment by 25 uM 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.

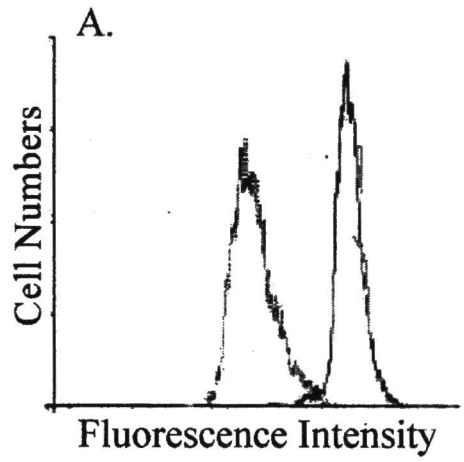

Relative Viability
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
Time (hrs)
Jurkat
YT

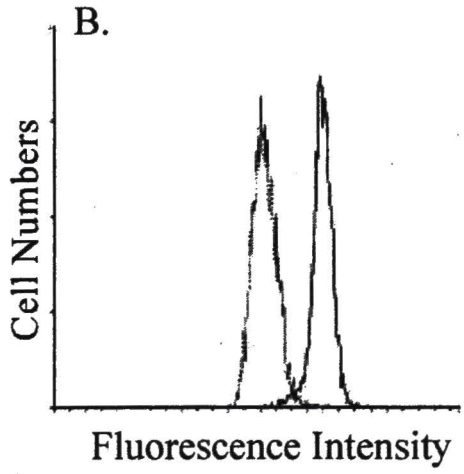
appeared more resistant to LLnL since its viability remained 15-20% higher than that of Jurkat cells. Statistical analysis indicated significant differences exist between these two cell lines at 6 and 10 hrs (P values are 0.010 and 0.033 respectively). After 48 hours treatment, the viability of both cell lines was below 20% (Fig 4). 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 natural killer 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 by 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 ($\Delta\psi_m$) [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]. YT 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 Fig 5, compared to the untreated controls, a 6-hour exposure of both cell lines to

Fig 5. Reduction of mitochondrial membrane potential in YT and Jurkat cells after treatment by 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 "Material 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 ($\Delta\psi_m$).


A.
Cell Numbers
Fluorescence Intensity

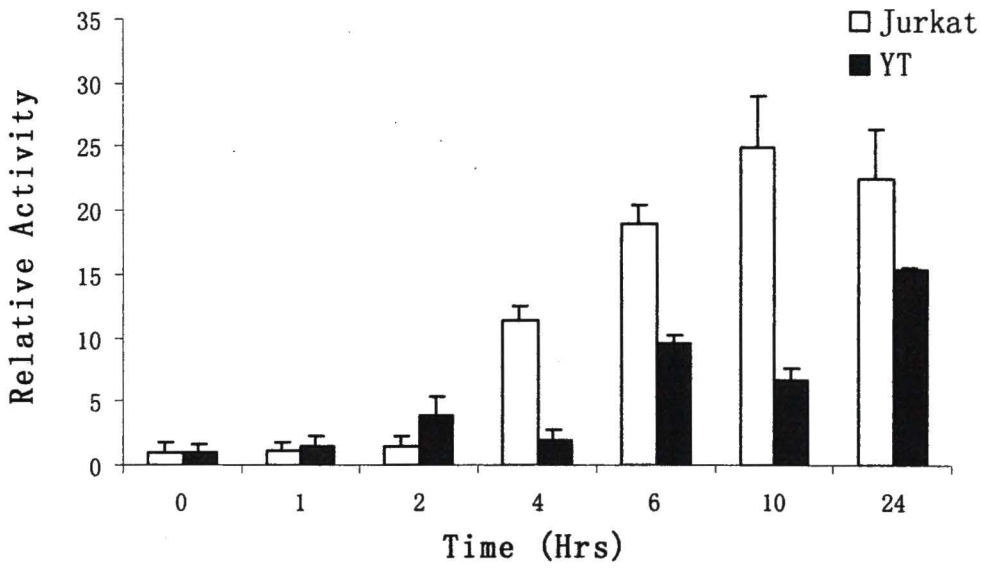

B.
Cell Numbers
Fluorescence Intensity

MG132 led to a significant left shift of cell population peaks, indicating a reduction of mitochondrial membrane potential ($\Delta\psi_m$) during the treatment by MG132. However, at earlier time points (e.g. 2 hr), 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 by 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 hour treatment, a substantial increase in caspase-3 activity was observed in both cell lines (Fig 6); 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 hours while that in YT cells was at 24 hours. 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

Fig 6. 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 flurogenic substrates Ac-DEVD-AMC. The caspase-3 activity of these two cells at 0 hours was counted as 100%. Data represent average of three independent experiments.


Jurkat
YT
Relative Activity
35
30
25
20
15
10
5
0
0
1
2
4
6
10
24
Time (Hrs)

LLnL for 6 hours, Jurkat cells demonstrated characteristic condensed apoptotic nuclear morphology under the microscope. About 32% and 58% of Jurkat cells have apoptotic nuclei at the time points of 10 hours and 24 hours respectively (Fig 7A). However, no YT cells exhibited apoptosis-specific nuclear morphology even after they were treated with same concentration of LLnL for 10 hours. At 24 hours, only 2% YT cells demonstrated the apoptotic nuclear morphology changes (Fig 7B). 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 6).

Effects of proteasome inhibitors on cell cycle progression

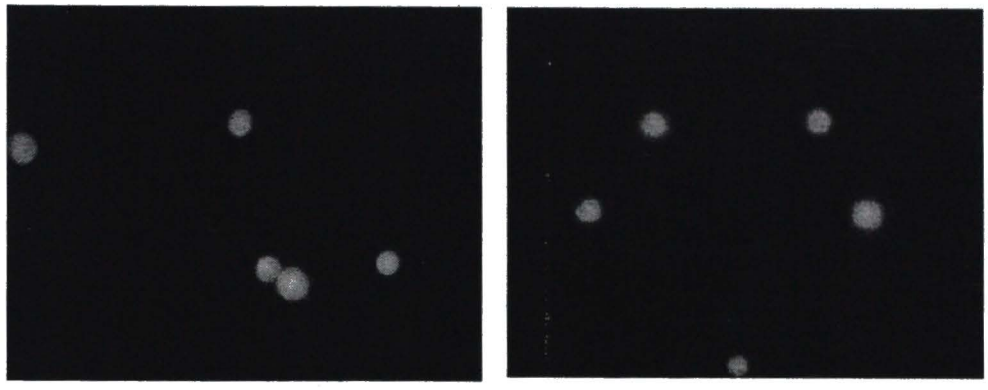
We were also interested in examining the effects of proteasome inhibitors on cell cycle progression in YT cells and Jurkat cells. Flow cytometric analysis of propidium iodide (PI) -stained cells was performed to monitor the effects of proteasome inhibitors on cell cycle progression since the lower DNA content of apoptotic cells stained by PI can be measured by flow cytometry. When Jurkat cells were treated with 25 μM LLnL for 10 hours, there was a significant increase in the apoptotic population with sub-G_1 DNA content (Figure 8A). By comparison, when YT cells were treated under the same condition, no obvious increase in sub-G_1 population was detected (Figure 8A). 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 hours. 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

Fig 7. Nuclear morphological changes of YT and Jurkat cells induced by proteasome inhibitor. YT and Jurkat cells 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 Material and Methods. Original magnification: × 200. Nuclear morphology was examined twice for control cells and cells treated by 25 μM LLnL for 6 hrs, while four times for cells treated for 24 hrs. Similar results were obtained from these independent experiments.

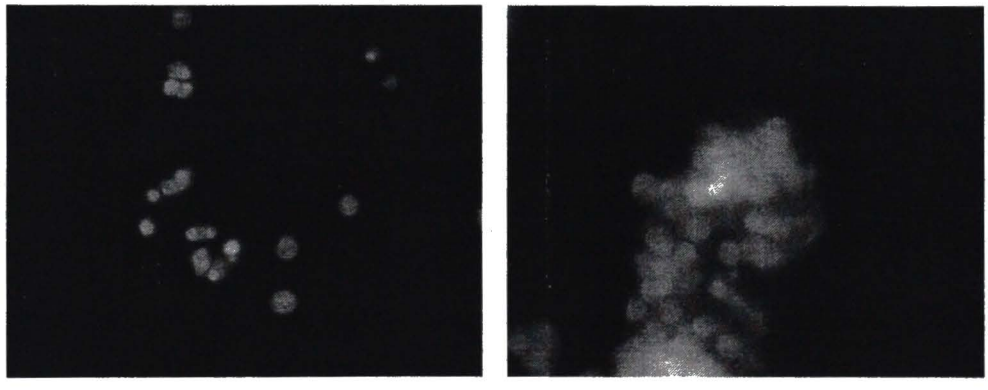
A

B

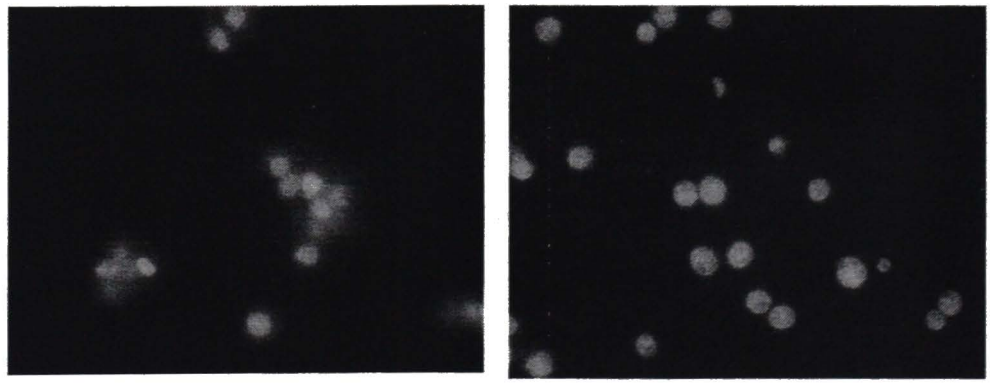
0 H



10 H



24 H

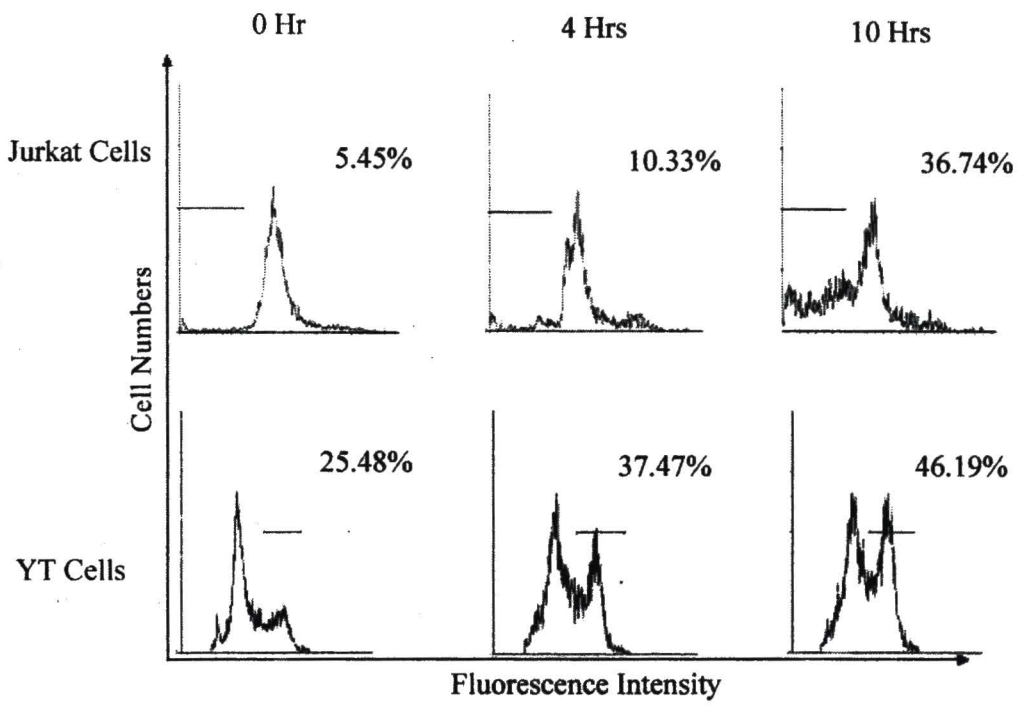


substantially after 24 hr treatment (data not shown here). To exclude the possibility that the differential induction of apoptosis in YT and Jurkat cells is proteasome inhibitor specific, the two cell lines were treated with 20 μM MG132. Similarly, the results indicated that an 11% increase of sub-G_1 cell population in Jurkat cells when they were treated with MG132 for 8 hours (Fig 8B). However, in the treated YT cells, much less sub-G_1 cells (Fig 8B) and higher percentage of G_2/M phase cells 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 hrs at various concentrations, 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 9), implying that caspase-3 activation does not contribute to the G_2/M arrest of YT cells.

Fig 8. Effects of proteasome inhibitors on cell cycyle 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. All experiments were performed in duplicate and gave similar results.

A.


0 Hr
4 Hrs
10 Hrs
Jurkat Cells
5.45%
10.33%
36.74%
Cell Numbers
25.48%
37.47%
46.19%
YT Cells
Fluorescence Intensity

B.

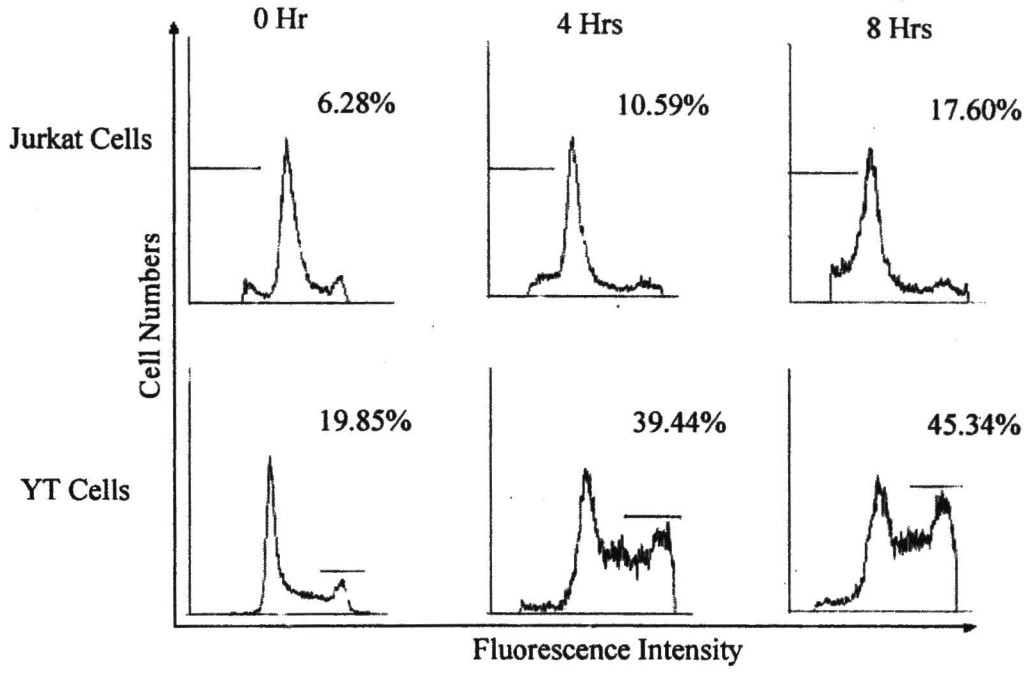
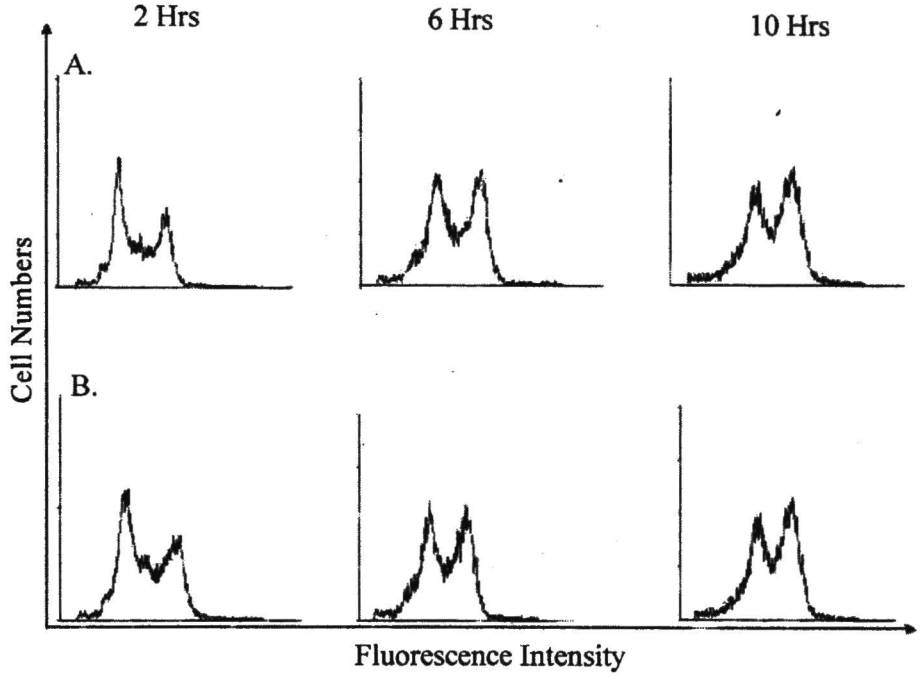

0 Hr
4 Hrs
8 Hrs
Jurkat Cells
6.28%
10.59%
17.60%
Cell Numbers
YT Cells
19.85%
39.44%
45.34%
Fluorescence Intensity

Fig 9. 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. All experiments were performed in duplicate and yielded similar results.


2 Hrs
6 Hrs
10 Hrs
A.
B.
Cell Numbers
Fluorescence Intensity

Differential molecular modulation of YT and Jurkat cells to proteasome inhibitors

Lastly, we attempted to demonstrate the reduced viability, apoptosis induction, cell cycle 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 by proteasome inhibitors. Cells were treated either by 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 10 A and B), and the same treatment with 50 μM EGCG caused a dramatic accumulation of IκB-α and p27 in cells (Fig 11 A and B). In contrast to the accumulation of $p27^{Kip1}$ and IκB-α in Jurkat cells, there was no significant accumulation of these two proteins in YT cells after they were treated by either 10 μM lactacystin or 50 μM EGCG (Fig 10 A and B, Fig 11 A and B).

Our results also indicated that p53 level remains at the same level during the treatment of cells by proteasome inhibitors lactacystin or EGCG (Fig 10 C, Fig 11 C) 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 24hrs 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 10 D, Fig 11 D). 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 antiapoptotic Bcl-2 family members (such as Bcl-2 and Bcl-XL) [Green and Reed, 1998; Gross et al., 1999]. The ratio of antiapoptotic to proapoptotic proteins, therefore, is involved in determination of cellular fate [Li and Dou, 2000]. With the investigation of Bcl-2 and Bax in these two cell lines after they were treated by lactacystin or EGCG, we found the ratio of Bcl-2 to Bax decreased in human Jurkat cell with the increasing treatment time (Figure 10 E and F; Figure 11 E and F). The ratio decreased from 4.15 at 0 hr to 0.67 at 24 hr when Jurkat cells were treated by lactacystin, and from 30.69 at 0 hr to 1.21 at 24 hr when they were treated by EGCG. This is consistent with the apoptosis induced by proteasome inhibitors in human Jurkat cells. Interestingly, in YT cells there were no Bcl-2 protein expressed and no significant accumulation of Bax after the treatment of lactacystin or EGCG (Fig 10,11 E and F). The role of Bcl-2 family in the G_2/M arrest of YT cells induced by proteasome inhibitors needs to be studied further.

Figure 10. Differential molecular modulations by lactacystin in YT and Jurkat cells. Time course experiments in which YT and Jurkat cells were treated with 10 μM lactacystin, a specific proteasome inhibitor, were performed. After 6, 12, 24 h of treatment, cell lysates were evaluated for levels of IκB-α (A), p27 (B), p53 (C), PCNA (D), Bcl-2 (E), and BAX (F) expression by Western blotting as described in Material and Methods. Actin (G) was measured as a standard.

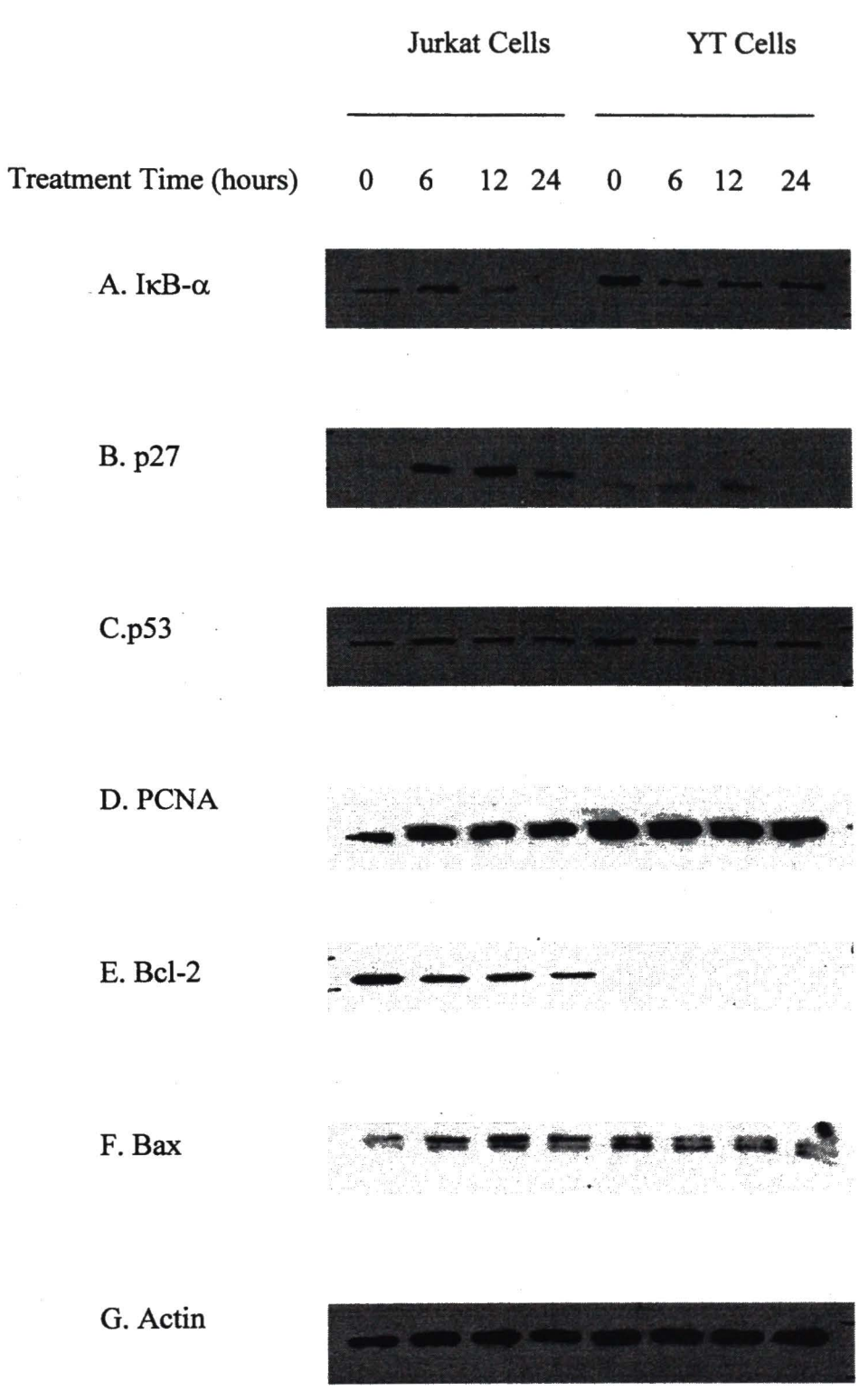
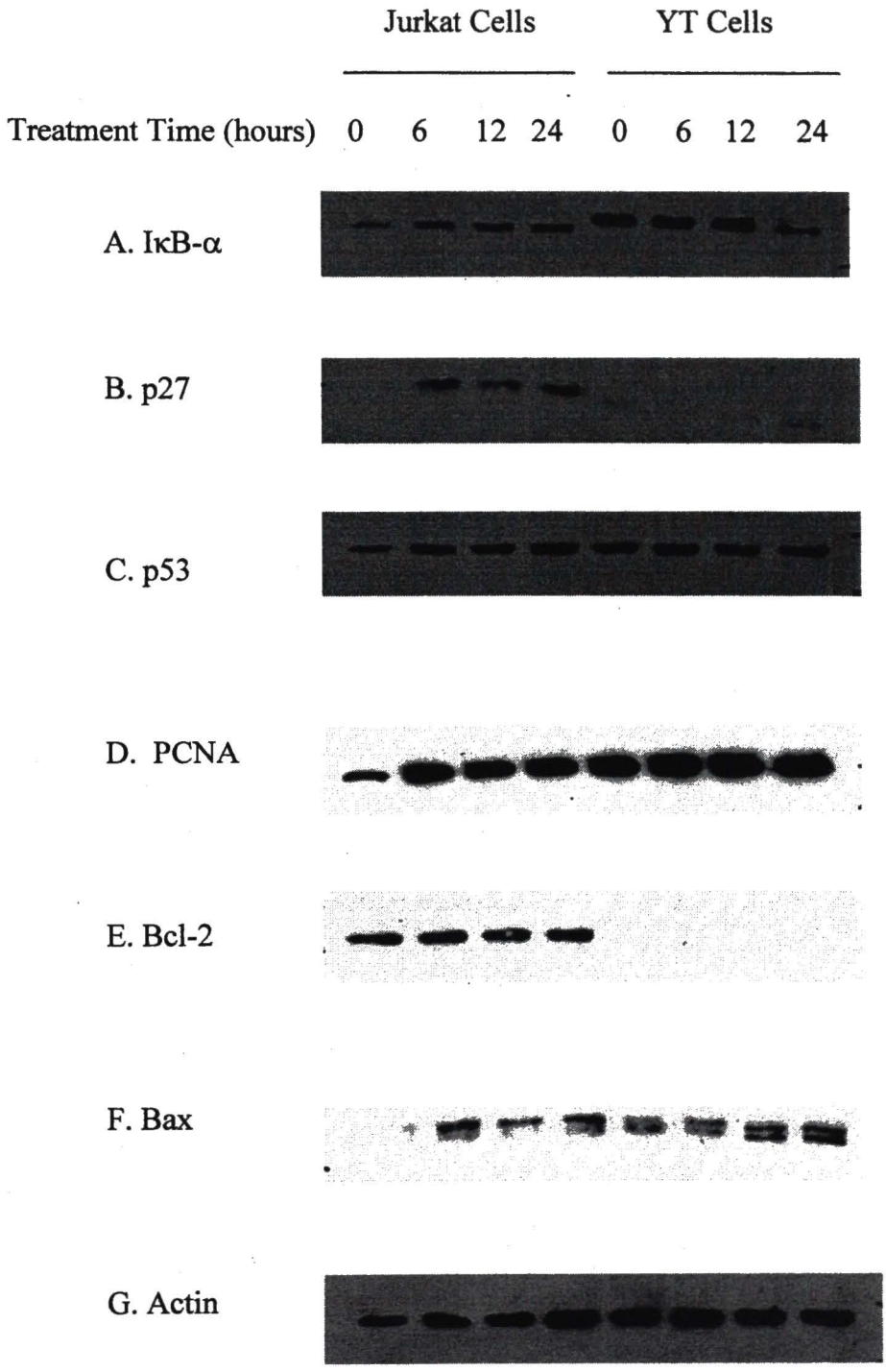

Jurkat Cells
YT Cells
Treatment Time (hours)
0 6 12 24 0 6 12 24
A. IκB-α
B. p27
C.p53
D. PCNA
E. Bcl-2
F. Bax
G. Actin

Fig 11. Differential molecular modulations by EGCG in YT and Jurkat cells. Time course experiments in which YT and Jurkat cells were treated with 50 μM EGCG, a proteasome inhibitor confirmed recently, were performed. After 6, 12, 24 h of treatment, cell lysates were evaluated for levels of IκB-α (A), p27 (B), p53 (C), PCNA (D), Bcl-2 (E), and BAX (F) expression by Western blotting as described in Material and Methods. Actin (G) was measured as a standard.


Jurkat Cells
YT Cells
Treatment Time (hours) 0 6 12 24 0 6 12 24
A. IκB-α
B. p27
C. p53
D. PCNA
E. Bcl-2
F. Bax
G. Actin

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 and proteasome inhibitors can have pro- or antiapoptotic effects depending on the cell type and death stimulus [An et al., 1998; Grimm et al., 1996; Sadoul et al., 1996]. Recently, we have found that proteasome inhibitors induced apoptosis of rat natural killer RNK16 cells [Lu et al., 2003]. Combined with our previous findings that there are some distinct properties of proteasome in NK cells [Kitson et al., 2000; Wasserman et al., 1994], 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 [Baumeister et al., 1998; Groll et al., 1997]. 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 turn-over 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. Morphologically, we identified differences in morphological change in the nucleus between YT and Jurkat cells during their incubations with proteasome inhibitors. In contrast to Jurkat cells, no YT cells exhibited apoptosis-specific nuclear morphology after treatment for 10 hours and only a small fraction of YT cells demonstrated the apoptotic nuclear morphology changes after 24 hours treatment. We also determined the caspase-3 activities in these two cell lines following the treatment by 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 pre-caspase-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 target molecules did display differences including p27 and IκB-α. $p27^{Kip1}$ is a powerful cyclin dependent kinase inhibitor (of CDK4 and CDK2) whose overexpression arrests cells in G_1 [Sudakin et al., 1995; 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; Kudo et al., 2000; Loda et al., 1997]. In our study, the accumulation of p27 protein and combined effects of other factors induced by proteasome inhibitors, resulted in the rapid induction of apoptosis in Jurkat cells. The p53 protein was one of the first cell cycle regulators found to be degraded by the proteasome [Chen et al., 2000; Chowdary et al., 1994; Maki et al., 1996]. 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]. Others, though, have shown cell cycle arrest and apoptosis from proteasome inhibition to be independent of p53 accumulation [Adams et al., 1999; An et al., 1998; Herrmann et al., 1998]. 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 modulation between YT cells and Jurkat cells. This has indicated that different cell types could 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 cancer [Dou and Goldfarb, 2002]. A key prerequisite for optimal therapeutic application of proteasome inhibitors for treatment of malignant tumors should be their selectivity and specificity for malignant cells and the sparing of normal cells. Our studies therefore indicates that the generation of ideal proteasome inhibitors for the treatment of malignancies could be screened to specifically induce cancer cells to programmed cell death, while they have little or no apoptosis-inducing abilities for natural killer cells and other cells of the immune response.

REFERENCE

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CHAPTER V

CONCLUSION AND FUTURE DIRECTION

This dissertation focuses on investigating proteasome chymotryptic activities and the effects of selective inhibitors of these activities on the function of natural killer cells. The proteasome is a multicatalytic proteinase complex that is involved in the major extralysosomal pathway responsible for intracellular protein degradation. Proteasomes catalyze the final step of the ubiquitin-proteasome pathway of protein degradation. The 20S proteasome is the catalytic core of the larger 26S proteasome. The 20S proteasome contains multiple proteolytic activities, defined as the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activity [Coux et al., 1996]. Among these activities, the chymotrypsin-like activity currently appears to be biologically highly significant and is one of the best characterized activities of the proteasome. Inhibition of the chymotryptic activity by mutation alone causes a large reduction in the rates of protein breakdown [Chen and Hochstrasser, 1996; Heinemeyer et al., 1997]. In contrast, inactivation of trypsin-like or peptidyl glutamyl peptide hydrolase-like sites had little effect on overall proteolysis [Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997; Kisselev et al., 1999]. Previous studies performed in our laboratory have indicated

involvement of the proteasomal chymotryptic activity in the function of NK cells, and has implied potential differences in subunit composition between the rat liver cell proteasome and the NK cell proteasome. An examination of purified rat NK cell proteasomes by electron microscopy revealed unique proteasome concatemers which have not been reported for other proteasomes [Wasserman et al., 1994]. These proteasomes also appear to contain a subunit composition different than rat liver proteasome as assessed by SDS PAGE and by immunochemical analysis using an anti-rat liver proteasome polyclonal antibody [Wasserman et al., 1994]. In this thesis, the investigation of purified 20S proteasome derived from rat natural killer RNK16 cells was extended.

Comparative studies of 20S proteasome from rat NK and hepatic cells indicated that they have some differences in substrate selectivity and inhibitor specificity. One specific chymotryptic inhibitor, TPCK, has differential effects on the hydrolysis of various chymotryptic substrates indicating the possibility of more than one different chymotryptic active site in the NK cell proteasome. Kinetic studies using various concentrations of substrates suggested involvement of a hysteretic or a modifier mechanism in the regulation of proteasome chymotryptic activities in NK cells. Latent and active forms of proteasome were confirmed to exist in NK cells and SDS was found to be able to convert the former status to the latter one. Therefore, while RNK16 cell-derived proteasomes share some common biochemical properties with the proteasome of other cell types, they also differ from others in many aspects including substrate selectivity, inhibitor specificity, and kinetic regulation. This continues to provide support for the hypothesis

that NK cell proteasomes may contain more than one site for cleavage of chymotryptic substrates. These investigations have also suggested that in NK cells there may be a two-site mechanism for the regulation of proteasomal chymotryptic activity.

Since many cell cycle--regulating proteins including cyclins, cyclin-dependent kinase (CDK) inhibitors p21 and $p27^{kip-1}$ [Pagano et al., 1995], and some transcription factors, such as p53, c-Fos and c-Jun [Drexler, 1997], are degraded through ubiquitin-proteasome pathway in the cells, disregulation of the degradation of such proteins has profound effects on cell cycle control and can selectively cause cells (e.g., tumor cells) to undergo apoptosis [An et al., 1998]. Recently, a number of proteasome inhibitors have been reported to induce apoptosis, inhibit angiogenesis and tumor cell proliferation, thereby becoming attractive candidates as anti-cancer drugs [Kisselev and Goldberg, 2001]. Our laboratory was among the first to show that treatment of tumor cells with proteasome inhibitors triggers selective apoptosis of tumor cells. This line of research has identified several anti-cancer drugs that are undergoing clinical evaluation for treatment of human cancer. However, our previous study also indicated that selective, synthetic chymotryptic proteasome inhibitors inhibit A-NK cell-mediated cytotoxicity by approximately 50% [Kitson et al., 2000]. The exact role of the proteasome in NK cell-mediated cytotoxicity has remained only fragmentary. In this study, the effects of two well characterized proteasome inhibitors, MG115 and MG132, on rat natural killer RNK16 cells were investigated. These two inhibitors decreased the proteasome chymotrypsin-like activity in the rat natural killer cell line RNK16 by 85% at a concentration of 5 μM. The viability of

RNK16 cells was also reduced in the presence of these inhibitors. Both inhibitors induced the apoptosis of RNK16 cells, as shown by DNA fragmentation, caspase-3 activation and the appearance of sub-G_1 cell populations. In our studies, an increase in the fraction of apoptotic cells was observed in a dose- and time-dependent manner in our studies. In addition, the activity of caspase-1, -2, -4, -6, -8, and -9, was increased following the treatment of RNK16 cells with these inhibitors. Further investigation revealed that the expression of Fas (CD95) protein on the RNK16 cell surface was increased after the treatment by MG115 or MG132, indicating that apoptosis induced by proteasome inhibitors in RNK16 cells might be mediated through the Fas (CD95)-mediated death pathway as well. Our studies indicate, for the first time, a mechanism for how proteasomal chymotryptic inhibitors can reduce natural killer cell viability and therefore indirectly inhibit cell-mediated cytotoxicity via the apoptosis-inducing properties of these agents. It seems likely that a key prerequisite for optimal therapeutic application of proteasome inhibitors for treatment of malignant tumors should be the sparing of NK cells, i.e., immune effector cells which recognize and kill tumor cells. This study also provides a potential approach towards clinical guidance for therapeutic application of proteasome inhibitors in malignant tumors, i.e., the negative effects on killer cells, should also be considered to minimize unwanted adverse consequence that might arise through untoward inhibition of NK cell tumor cell-killing function.

From the evidence in this investigation, some special biochemical and biophysical properties of NK cell-derived proteasome were determined and various proteasome

inhibitors were confirmed to be able to induce NK cell apoptosis. Then, the potential differential effects of proteasomal chymotryptic inhibitors on cell cycle regulation and molecular modulation between natural killer cells and T lymphocytes were examined. YT and Jurkat cells were selected as model cell types to represent human natural killer cells and T lymphocytes respectively. Proteasome inhibitors *clasto*-lactacstin β-lactone, epigallocatechin gallate (EGCG), N-acetyl-leu-leu-norleu-al (LLnL) were found to inhibit the purified 20S proteasome chymotrypsin-like activity of human YT and Jurkat cells to a similar extent. Their inhibitory effects on 26S proteasome chymotrypsin-like activity in whole cell extracts and intact cells did not show significant differences between these two cell lines. The viability of both types of cells was reduced in the presence of the proteasome inhibitor LLnL. Continuing studies indicated the involvement of apoptosis in these two cell lines treated by proteasome inhibitors, as evident by reduction of mitochondrial membrane potential and activation of caspase-3. However, a temporal difference in the occurrence of caspase-3 activation was also observed in these two cell lines treated by a well characterized chymotryptic proteasomal inhibitor LLnL. Following such treatment, caspase-3 activated much earlier in Jurkat cells than in YT cells. This difference may be the cause of the differential exhibition of typical apoptotic nuclei morphology changes in these two cell lines. 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 with proteasome inhibitors. Moreover, pretreatment of YT cells by the caspase-3 inhibitor DEVD-CHO did not increase the percentage of G_2/M phase cells when cells were treated with proteasome inhibitor. This implies that the caspase-3 independent

pathway may contribute to G_2/M arrest induced by proteasome inhibitor in YT cells. Furthermore, accumulation of a few target proteins including p27 and IκB-α, which are degraded through ubiquitin-proteasome pathway, was seen in Jurkat cells while not in YT cells.

In summary, this dissertation provides evidence that proteasome inhibitors act differentially in cell cycle progression, induction of apoptosis and molecular modulation in human natural killer cells and T lymphocytes. The reasons for these differential effects could be due to the unique properties of the proteasome in NK cells. Indeed there is a strong possibility that proteasome inhibition could occur through multiple steps in the cell cycle [Dietrich et al., 1996; Koepp et al., 1999; Machiels et al., 1997; Wang et al., 1998]. The possibility also exists that different cells could inactivate proteasomal chymotryptic inhibitors *in vivo* at different rates.

The findings in this dissertation also set the stage for future directions to elucidate the complex properties of proteasomal chymotryptic activities in NK cells and molecular mechanisms through which the inhibition of proteasomal chymotryptic activities modulate the functions of natural killer cells. As mentioned elsewhere in this dissertation, NK cell may contain high levels of immunoproteasome, which results from the induction by interferon-γ produced by NK cells. Interferon-γ, in addition to other effects on cells, has three major effects on proteasome complexes [Brophy et al., 2002]: (1) it induces the expression of three nonessential subunits (LMP2, LMP7, and LMP10) and thereby

increases the level of immunoproteasomes, (2) it alters the level of the regulators for proteasomes by increasing PA28, (3) it decreases the level of phosphorylation of proteasome subunits [Bose et al., 2001]. One study has indicated that treatment of COS-7 cells with interferon-γ attenuated the increase in caspase-3 activity in apoptosis [Brophy et al., 2002]. This suggests that cells with more immunoproteasome may be more resistant to apoptotic stimuli; therefore, the role of immunoproteasomes in regulating caspase-3 activation in the apoptosis of NK cells induced by proteasome inhibitor is an important issue for further studies.

Secondly, localization of proteasomes appears to be important in the regulation of proteolysis. In yeast, a discrete localization is observed at the nuclear periphery for cells undergoing mitotic growth. This localization is clearly important as degradation by the ubiquitin-proteasome pathway is impaired in mutants that mislocalize proteasomes [Gordon, 2002]. In mammalian cells, proteasomes are present throughout the cell and the localization of the different proteasome subpopulations is distinct but overlapping [Brooks et al., 2000]. The investigation of the subcellular distribution of proteasomes in NK cells undergoing apoptosis could yield interesting insights. It is possible that the subcellular distribution of proteasome in NK cells underlying apoptotic stimuli may be distinct from T cells or unstimulated NK cells.

In addition, there are a number of signal transduction pathways which could be involved in the apoptosis of NK cells induced by proteasome inhibitors. Cell survival is believed to

be governed by a family of regulatory proteins, some of which are degraded by proteasomes. Together, these proteins determine whether cells undergo differentiation, proliferation, or apoptosis, depending on the specific downstream pathways affected, i.e., stress-activated protein kinase (SAPK) or mitogen-activated protein kinase (MAPK). There is accumulating evidence that the dynamic balance between the proapoptotic JNK (c-Jun N-terminal kinase) / p38 (SAPK) pathway and the growth and differentiation-associated ERK (extracellular receptor kinase; MAPK) pathway represents an important determinant of cell survival or death [Vrana and Grant, 2001; Xia et al., 1995]. Therefore, by using specific inhibitors of these pathways, such as curcumin for blocking JNK/AP-1 pathway and SB203580 for inhibiting p38/RK pathway, the involvement of these pathways in the apoptosis of NK cells induced by proteasome inhibitor could be evaluated.

In sum, the studies in this dissertation have compared and contrasted the properties of the proteasome of NK cells with that of other cell types. In addition to the elucidation of intriguing biochemical and molecular differences, these studies have also shed light on an important clinical issue related to the advent of the development of apoptosis-inducing proteasomal inhibitors for the treatment of human malignant diseases [Dou and Goldfarb, 2002]. Our studies indicate that the generation of ideal proteasome inhibitors for cancer treatment could be screened or designed to specifically induce cancer cells to undergo programmed cell death, while having little or no apoptosis-inducing abilities for natural killer cells and other cells of the immune response, thus enhancing the selectivity

and specificity of the anti-cancer, apoptosis-inducing capabilities of proteasome inhibitors by sparing NK cells and thus preserving their anti-tumor cell cytolytic properties that also contribute to tumor destruction.

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