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Advances in Brief

Eponemycin Exerts Its Antitumor Effect through the Inhibition of Proteasome Function[1]

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Abstract

Cell cycle progression requires the proteasome-mediated degradation of key regulatory proteins such as cyclins, cyclin-dependent kinase inhibitors, and anaphase-inhibitory proteins. Given the central role of the proteasome in the destruction of these proteins, proteasome inhibition has been proposed as a possible cancer therapy. We report here that dihydroeponemycin, an analogue of the antitumor and antiangiogenic natural product eponemycin, selectively targets the 20S proteasome. Dihydroeponemycin covalently modifies a subset of catalytic proteasomal subunits, binding preferentially to the IFN-γ-inducible subunits LMP2 and LMP7. Moreover, the three major peptidolytic activities of the proteasome are inhibited by dihydroeponemycin at different rates. In addition, dihydroeponemycin-mediated proteasome inhibition induces a spindle-like cellular morphological change and apoptosis. These results validate the proteasome as a target for antitumor pharmacological intervention and are relevant for the design of novel chemotherapeutic strategies.

Introduction

The proteasome is an ubiquitously expressed multisubunit complex of M_r 700,000 that serves to degrade proteins that are targeted by the ubiquitination system (1). Proteasome-mediated protein degradation is a highly regulated process that is necessary for a variety of intracellular processes, such as antigen processing (2, 3), nuclear factor κB activation (4), and cell cycle progression (5). Because the proteasome is required for cell growth and division, it has been proposed that proteasome inhibition may serve as an antitumor therapy (6, 7). Here, we provide proof of this concept through the identification of the proteasome as the intracellular target of a demonstrated antitumor agent, eponemycin.

Materials and Methods

Materials. Fetal bovine serum, RNase, trypsin, chymotrypsin, cathepsin B, Triton X-100, SDS, streptavidin-HRP[3], rabbit antimouse antibody-HRP conjugate, streptavidin agarose, sodium citrate, and sodium propidium iodine were purchased from Sigma Chemical Co. (St. Louis, MO).The *in situ* cell death detection kit was obtained from Boehringer Mannheim (Indianapolis, IN). Suc-Leu-Leu-Val-Tyr-AMC and Z-Gly-Gly-Arg-AMC were purchased from Bachem (King of Prussia, PA). Z-Leu-Leu-Glu-AMC, lactacystin, and NLVS, were obtained from Calbiochem (La Jolla, CA). Neutr-Avidin agarose biotinylated protein markers were purchased from Pierce (Rockford, IL) and New England Biolabs (Beverly, MA), respectively. BAECs were generously provided by J. Pober (Yale University, New Haven, CT). Rabbit anti-LMP2 and anti-LMP7 polyclonal antibodies were kind gifts from J. J. Monaco (Howard Hughes Medical Institute, University of Cincinnati, Cincinnati, OH).

Purification of Dihydroeponemycin Binding Proteins. Ten liters of EL4 cells (1×10^6 cells/ml) were harvested and homogenized using a Tekmar Tissumizer homogenizer in 50 ml of lysis buffer [25 mM HEPES (pH 7.4), 5 mM EGTA, and 50 mM NaF] plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each leupeptin, pepstatin, and soybean trypsin inhibitor). After clarification at 100,000 × *g* for 1 h, the supernatant was loaded into a 1-ml streptavidin agarose column to remove endogenous biotinylated proteins. Dihydroeponemycin-biotin (8) was subsequently added to the precleared solution at a final concentration of 8 μM. After a 2-h incubation at 4°C, the lysate was mixed for 10 min with 50 ml of DE52 beads preequilibrated with lysis buffer, subsequently washed twice with 50 ml lysis buffer containing 0.1 M NaCl, and then eluted with 50 ml of lysis buffer containing 0.3 mM NaCl. After SDS was added to a final concentration of 0.5%, the eluant was boiled for 10 min and diluted 2.5-fold with lysis buffer. The diluted solution was next loaded onto a 0.4-ml streptavidin-agarose column. The flow-through fraction was collected and reloaded onto the same column. This was repeated three times. After extensive washes, eponemycin-biotin binding proteins were eluted by boiling the streptavidin agarose in 0.4 ml of Laemmli sample buffer, and the purified protein complexes were separated by SDS-PAGE. Protein bands were excised and identified by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University) using LCQ (mass spectrometry) and automated Edman degradation of internal tryptic peptides.

20S Proteasome Purification. The bovine 20S proteasome complex was purified as described (9), with the following two modifications: (*a*) the lysis buffer used was 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, and 0.25 M sucrose; and (*b*) proteins were eluted from the SP-Sepharose FF column (Pharmacia, Piscataway, NJ) with a 180-ml gradient of 0–2 M NaCl.

Murine spleen 20S proteasome was purified as following: 7 g of murine spleen were lysed in 35 ml of lysis buffer and clarified by centrifugation (100,000 × *g*, 1 h). The supernatant was further centrifuged for 5 h at 100,000 × *g*. The resulting pellet was resuspended in 10 ml of 50 mM Tris (pH 7.5) and clarified by centrifugation (20,000 × *g*, 20 min). The supernatant was loaded into a HR5/5 Mono Q column (Pharmacia) and eluted with a 40-ml gradient (0–0.8 M NaCl). Fractions containing proteasome activity, as monitored by Suc-LLVY-AMC hydrolysis, were pooled and loaded into a Superose 6 HR 10/30 gel filtration column (Pharmacia). Purified 20S proteasome was eluted with PBS and stored at 4°C.

Two-dimensional Gel Electrophoresis. Purified mouse spleen proteasome was treated with 1 μM dihydroeponemycin-biotin for 3 h. Two-dimensional gel electrophoresis was performed using a Bio-Rad (Hercules, CA) mini-Protean II two-dimensional system, according to the manufacturer's protocol. After transfer onto a PVDF membrane, biotin-labeled proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and streptavidin-HRP. The same blot was treated with sodium azide to inhibit HRP activity and sequentially analyzed with anti-LMP2 and anti-LMP7 antibodies to confirm the identity of the biotinylated proteins.

Cell Morphological Assay. BAECs were grown on 60-mm culture dishes to 70% confluence. Cells were incubated for 22 h in the presence or absence of dihydroeponemycin (4 μM). Photographs of cells morphology were taken at 100-fold magnification.

TUNEL Assay of Apoptotic Cells. Assays were performed using the *in situ* cell death detection kit (Boehringer Mannheim) according to the manu-

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[3] The abbreviations used are: HRP, horseradish peroxidase; AMC, 7-amido-4-methyl coumarin; NLVS, nitrophenol leucyl-leucyl-leucine vinyl sulfone; BAEC, bovine aortic endothelial cell; PVDF, polyvinylidene difluoride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PGPH, peptidylglutamyl peptide-hydrolyzing.

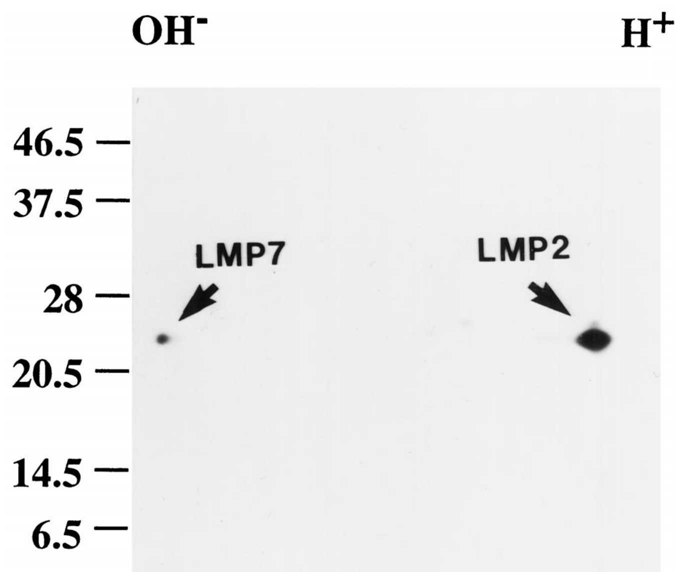

Fig. 1. Identification of dihydroeponemycin-binding proteins. Two-dimensional gel analysis of dihydroeponemycin-biotin binding proteins. Murine 20S proteasome subunits were separated by two-dimensional electrophoresis after incubation with dihydroeponemycin-biotin. Biotinylated proteins were visualized with streptavidin-HRP using enhanced chemiluminescence. Immunoblot analysis using anti-LMP2 and anti-LMP7 antisera confirmed the identities of the spots (data not shown). Molecular weight standards are expressed as $\times 10^3$.

facturer's protocol. Apoptotic cells were detected by flow cytometric analysis (Yale Comprehensive Cancer Center Core Facility).

Proteasome Enzymatic Kinetics. $k_{\text{association values}}$ were determined as follows. Inhibitors were mixed with a fluorogenic peptide substrate and assay buffer [20 mM Tris (pH 8.0), 0.5 mM EDTA, and 0.035% SDS] in a 96-well plate (SDS was omitted in assays for trypsin-like activity). The chymotrypsin-like, trypsin-like, and PGPH catalytic activities were assayed using the fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC and Z-Gly-Gly-Arg-AMC, Z-Leu-Leu-Glu-AMC, respectively. Hydrolysis was initiated by the addition of bovine 20S proteasome, and the reaction was followed by fluorescence (360-nm excitation/460-nm detection) using a fluorescence plate reader (Cytofluor II; PerSeptive Biosystems, Framingham, MA). Reactions were allowed to proceed for 50 min, and fluorescence data were collected every 15 s. Fluorescence was quantified as arbitrary units and progression curves were plotted for each reaction as a function of time. $k_{\text{observed}}/[I]$ values were obtained using Kaleidograph by nonlinear least squares fit of the data to the following equation: fluorescence $= v_s t + [(v_0 - v_s)/k_{\text{observed}}][1 - \exp(-k_{\text{observed}}t)]$, where v_0 and v_s are the initial and final velocities, respectively, and k_{observed} is the reaction rate constant. The range of inhibitor concentrations tested was chosen so that several half-lives could be observed during the course of the measurement. Reactions were performed using inhibitor concentrations that were <100-fold of those of the proteasome assayed.

Results and Discussion

The antitumor compound eponemycin was isolated from a *Streptomyces hygroscopicus* strain on the basis of its ability to significantly prolong the survival time of mice bearing B16 melanoma (10). In addition, eponemycin was shown to inhibit angiogenesis in the chick chorioallantoic membrane assay (11); however, little was known about the molecular mechanisms of action of either of these activities. In the course of our antitumor natural product mode of action studies, we have synthesized an eponemycin-based affinity reagent for use in exploring the mechanisms of eponemycin's biological activities. We hypothesized that eponemycin forms covalent adducts with intracellular proteins, given the importance of the epoxide for biological activity. An eponemycin analogue, dihydroeponemycin, was selected for development as an affinity reagent because of its ease of synthesis (8) and the fact that it possesses similar biological activities to the natural product eponemycin (10). We have previously shown that dihydroeponemycin-biotin covalently and specifically binds a major (M_r 22,000) intracellular cellular protein and a minor (M_r 23,000) protein in bovine endothelial cells (8).

Here, we identify these dihydroeponemycin targets after purification from the murine thymoma cell line EL4 using affinity chromatography. Pilot experiments failed to purify any dihydroeponemycin-protein adducts from a cellular lysate using avidin-agarose. However, avidin-HRP recognizes dihydroeponemycin-modified proteins when they are denatured and immobilized on a PVDF membrane (8). This suggested that, under nondenaturing conditions, the biotin moiety of the dihydroeponemycin-biotin adduct is inaccessible to avidin, possibly buried deep within a multiprotein complex. As hypothesized, denaturation of protein lysates with SDS after dihydroeponemycin-biotin incubation and subsequent dilution facilitated the purification of these two dihydroeponemycin binding proteins using an avidin-agarose affinity matrix. Following preparative denaturing gel electrophoresis and peptide sequencing of internal tryptic peptides, the major and minor dihydroeponemycin binding proteins were identified as the proteasomal β catalytic subunits LMP2 and LMP7, respectively. This identification was confirmed by two-dimensional gel electrophoresis and immunoblot analyses of dihydroeponemycin-biotin-modified proteins. Murine splenocyte lysates were incubated with dihydroeponemycin-biotin, electrophoresed, and transferred to a PVDF membrane. Subsequent probing with avidin-HRP revealed a major spot and a minor spot with apparent M_rs 22,000 and 23,000 (Fig. 1). These two-dimensional gel electrophoresis results correspond to the size and migration patterns of the murine LMP2 and LMP7 proteins (12). Furthermore, anti-LMP2 and anti-LMP7 antisera recognized spots of the same size and charge, thus confirming these biotinylated proteins as proteasome catalytic subunits (data not shown).

In recent years, several compounds that target the β catalytic subunits have been developed as proteasome inhibitors. Many such inhibitors structurally resemble proteasome substrates but yet have a reactive moiety on the COOH terminus comprising the pharmacophore (*e.g.*, aldehydes, vinyl sulfones, and boronic acids; Ref. 13). Several of these compounds were originally developed as inhibitors of other intracellular proteases and are not specific for the proteasome, thus potentially limiting their therapeutic utility. Unlike several of these other inhibitors, the microbial metabolite lactacystin is highly specific for the proteasome (9, 14). However, lactacystin's potential as

Table 1 *Inhibition of proteasome catalytic activities*[a]

Compound	$k_{\text{association}} = k_{\text{observed}}/[I]$ ($M^{-1} \cdot s^{-1}$)		
	Chymotrypsin-like activity	PGPH activity	Trypsin-like activity
Dihydroeponemycin	66.4 ± 8.9 (20–60)	60.5 ± 8.8 (12.5–50)	4.4 ± 0.43 (125–500)
Lactacystin	675 ± 86.1 (1.0–2.25)	3.71 ± 0.48 (60–100)	29.9 ± 4.21 (50–100)
NLVS	9820 ± 3810 (0.2–0.4)	4.95 ± 1.65 (60–100)	0 (75–100)

[a] The rates of covalent inhibition ($k_{\text{associations}}$) of the three major proteasome catalytic activities were determined for the eponemycin analogue dihydroeponemycin as well as the potent inhibitors lactacystin and the peptide vinyl sulfone NLVS. Ranges of concentrations (given in parentheses in μM) were used to determine the $k_{\text{association}}$ for inhibition of individual enzymatic activities.

a therapeutic agent may be limited due its conversion to the more labile *clasto*-lactacystin β-lactone in aqueous solutions (15).

Because dihydroeponemycin covalently binds to two proteasome catalytic subunits, we investigated the possibility that this compound could represent a family of proteasome inhibitors, different from those currently used. Enzymatic assays using fluorogenic substrates and 20S proteasome purified from bovine brain were performed in the presence of varying concentrations of dihydroeponemycin. Dihydroeponemycin was found to inhibit the proteasome potently, competitively, and irreversibly. Given the covalent and irreversible nature of dihydroeponemycin's inhibitory activity, its rate of proteasome inactivation, $k_{\text{association}}$ (k_{observed}/[I]), was measured for different catalytic activities associated with the 20S proteasome. As shown in Table 1, dihydroeponemycin inhibits the enzymatic activity of all three major activities (trypsin-like, chymotrypsin-like, and PGPH activity) but at different rates. Inhibition of the chymotrypsin-like activity and PGPH activity proceeded >10-fold faster than inhibition of the trypsin-like activity. Because dihydroeponemycin binds LMP2 and LMP7, these findings are consistent with the reported assignment of the PGPH and chymotrypsin-like activities to the LMP2 and LMP7 catalytic β subunits of the proteasome, respectively (16). Interestingly, dihydroeponemycin inhibited PGPH activity at a rate an order of magnitude faster than two of the most potent proteasome inhibitors, the peptide vinyl sulfone NLVS, and lactacystin. Moreover, dihydroeponemycin does not inhibit the nonproteasomal proteases calpain and trypsin, although minor inhibition is observed against cathepsin B and chymotrypsin at higher concentrations of dihydroeponemycin (data not shown). The proteasome-inhibitory activity described here is consistent with the prior report of proteasome inhibition by a peptide containing an epoxyketone moiety similar to the active pharmacophore of dihydroeponemycin (17).

Because both dihydroeponemycin and lactacystin irreversibly inhibit proteasomal catalytic activities, it is possible that these two natural products may bind to the same or nearby sites on the proteasome. Lactacystin has been shown to covalently bind and inhibit the proteasome subunit X/MB1 through interaction with the NH_2-terminal catalytic threonine residue (14). To determine whether dihydroeponemycin binds similarly, we incubated 20S proteasome with lactacystin before challenging it with dihydroeponemycin-biotin. Pretreatment with a 5 equivalent excess of lactacystin prevents subsequent dihydroeponemycin-biotin adduct formation to both LMP2 and LMP7 (data not shown), suggesting that the same or neighboring amino acid is acting as the nucleophilic adduct forming residue for both natural products.

To examine the biological consequences of proteasome inhibition by this natural product, dihydroeponemycin was added to BAECs at differing concentrations. After 22 h, control cells treated with vehicle alone exhibited a cobblestone-like morphology using phase-contrast microscopy, characteristic of endothelial cells in culture (Fig. 2*a*). However, dihydroeponemycin-treated cells displayed a dramatic morphological change. As shown in Fig. 2*b*, a 22-h treatment with 4 μM dihydroeponemycin induced cells to become increasingly elongated, bipolar, and spindle-like in shape. In addition, many cells became round and highly refractile, detaching from the culture dish upon longer incubation. Although similar morphological changes have been observed upon inhibition of the chymotrypsin-like activity of the proteasome by lactacystin and peptide aldehyde inhibitors in neuroblastoma cells (18), to our knowledge, this is the first report of this phenomenon in nonneuronal cells.

Because the refractile nature of cells observed on longer exposure to dihydroeponemycin and their detachment from culture dishes are suggestive of apoptosis, we investigated the possibility that dihydroeponemycin induces apoptosis. Initial fluorescent cell counting using

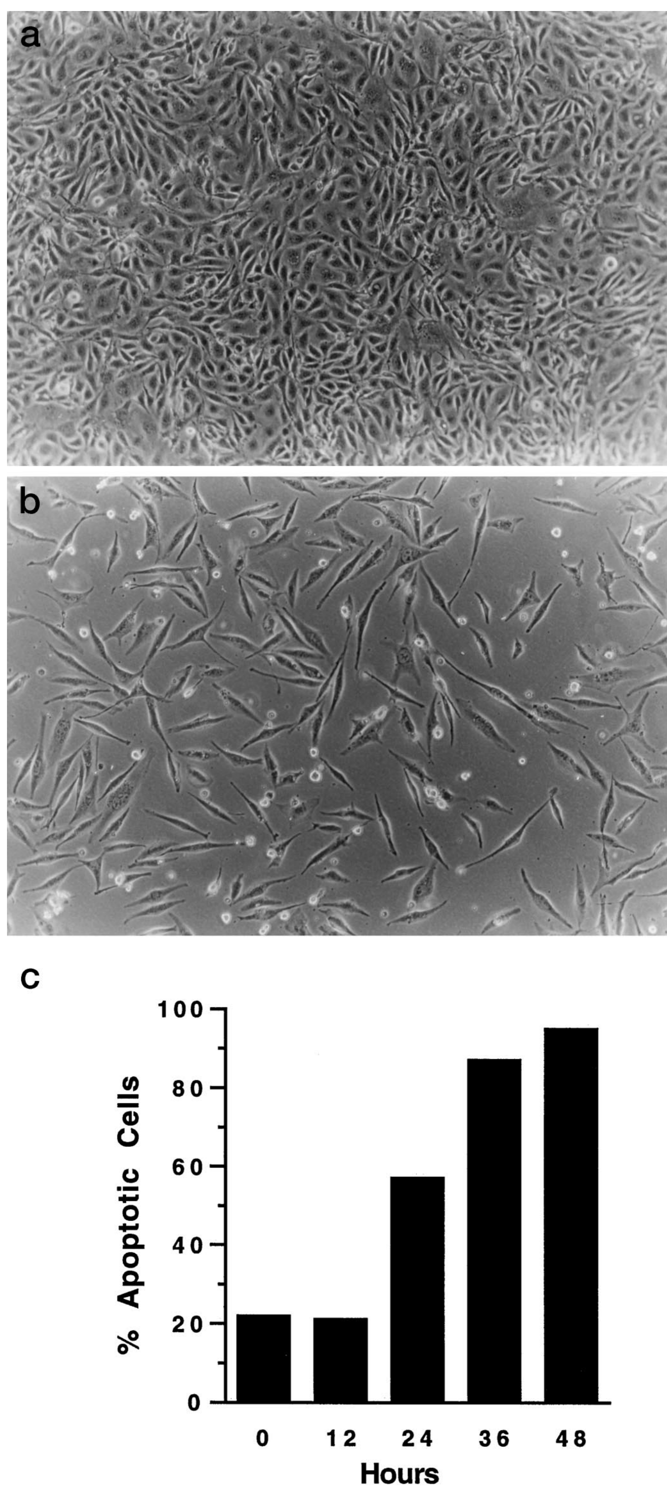

Fig. 2. Morphological and apoptotic induction by dihydroeponemycin. *a*, control BAECs. *b*, BAECs were incubated for 22 h with 4 μM dihydroeponemycin [magnification (*a* and *b*), ×100]. *c*, apoptosis was detected by the TUNEL method, which was quantitated by flow cytometry. Similar results were obtained in three separate experiments.

the DNA intercalating dye propidium iodide revealed an increase in the number of cells with DNA content less than 2 N, indicating DNA fragmentation (data not shown). EL4 cells were treated for various times over a 48-h period at a concentration 2.5-fold higher than dihydroeponemycin's antiproliferative IC_{50} (2 μM), and TUNEL assays were performed to quantitate dihydroeponemycin-induced DNA fragmentation. As shown in Fig. 2*c*, DNA fragmentation occurred in

>95% of cells over a course of a 48-h incubation with dihydroeponemycin. These results are consistent with previous reports of apoptotic induction in human tumor cell lines resulting from peptide aldehyde- (19) and lactacystin-mediated proteasome inhibition (20–22).

The results presented here suggest that targeting the proteasome is a valid antitumor strategy. Proteasome inhibition has already been shown to be an effective part of a combinatorial approach to killing cancer cells *in vitro*. Proteasome inhibition by peptide aldehyde or lactacystin addition greatly sensitizes human tumor cells to the cytotoxic action of tumor necrosis factor-α (22). Moreover, in the course of preparing this manuscript, it was reported that a single dose of a peptide aldehyde proteasome inhibitor prolonged the survival of severe combined immunodeficient mice injected with Burkitt's lymphoma (23). This confirms the findings reported here and together suggest that proteasome inhibitors have a sufficient *in vivo* therapeutic index to warrant further development as antitumor agents. The identification of the 20S proteasome as the target for the potent antitumor agent dihydroeponemycin further confirms the proposed central role of the proteasome in cell cycle regulation and suggests that additional proteasome inhibitors may be effective antitumor agents.

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