Eponemycin Exerts Its Antitumor Effect through the Inhibition of Proteasome Function

Lihaoy Meng, Benjamin H. B. Kwok, Ny Sin, and Craig M. Crews

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 $k$B 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 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, $\beta$-galactosidase, 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; PGGH, peptidylglutamyl peptide-hydrolyzing.

Received 3/11/99; accepted 4/28/99.

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1 This research was supported by NIH Grant CA74967-01. C. M. C. is a Burroughs Wellcome Fund Young Investigator and a Donaghue Foundation New Investigator.

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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; PGGH, peptidylglutamyl peptide-hydrolyzing.

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-$\gamma$-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.
OH⁻

H⁺

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. Immuno blot analysis using anti-LMP2 and anti-LMP7 antisera confirmed the identities of the spots (data not shown). Molecular weight standards are expressed as ×10^5.

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 (M₆, 22,000) intracellular cellular protein and a minor (M₆, 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-gel. 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-gel 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 Mₛ, 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).

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>
<thead>
<tr>
<th>Compound</th>
<th>Chymotrypsin-like activity</th>
<th>PGPH activity</th>
<th>Trypsin-like activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydroeponemycin</td>
<td>66.4 ± 8.9 (20–60)</td>
<td>60.5 ± 8.8 (12.5–50)</td>
<td>4.4 ± 0.43 (125–500)</td>
</tr>
<tr>
<td>Lactacystin</td>
<td>675 ± 86.1 (1.0–2.25)</td>
<td>3.71 ± 0.48 (60–100)</td>
<td>29.9 ± 4.21 (50–100)</td>
</tr>
<tr>
<td>NLVS</td>
<td>9820 ± 3810 (0.2–0.4)</td>
<td>4.95 ± 1.65 (60–100)</td>
<td>0 (75–100)</td>
</tr>
</tbody>
</table>

* The rates of covalent inhibition (kₐssociation) 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 kₐssociation for inhibition of individual enzymatic activities.
a therapeutic agent may be limited due to its conversion to the more labile clasto-lactacytin 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. Dihydroepo- nemycin 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, \( k_{\text{association}} \), a 22-h treatment with 4 \( \mu \)M 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 lactacytastin and peptide aldehyde inhibitors in neuroblastoma cells (18), to our knowledge, this is the first report of this phenomenon in nonneuronal cells.

Because both dihydroeponemycin and lactacytin irreversibly in- hibit proteasomal catalytic activities, it is possible that these two natural products may bind to the same or nearby sites on the protea- some. Lactacytin 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 lac- tacystin before challenging it with dihydroeponemycin-biotin. Pretreatment with a 5 equivalent excess of lactacytin prevents subse- quent 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. 2a). However, dihydroeponemycin-treated cells displayed a dramatic morphological change. As shown in Fig. 2b, a 22-h treatment with 4 \( \mu \)M 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 lactacytastin 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 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 \( \mu \)M), and TUNEL assays were performed to quantitate dihydroeponemycin-induced DNA fragmentation. As shown in Fig. 2c, DNA fragmentation occurred in
toxic action of tumor necrosis factor-
lactacystin addition greatly sensitizes human tumor cells to the cyto-
lar.

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 cyto-
toxic 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 dihydroepotecin further confirms the proposed central role of the proteasome in cell cycle regulation and suggests that additional proteasome inhibitors may be effective antitumor agents.

Acknowledgments

We thank J.J. Monaco for generously providing anti-LMP2 and anti-LMP7 antisera as well as Gabriel Fenteany and Peter Cresswell for helpful comments on the manuscript.

References

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