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Salinosporamides A and B Inhibit Proteasome Activity and Delay the Degradation of N-end Rule Model Substrates

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The proteasome, which is highly evolutionarily conserved, is responsible for the degradation of most short-lived proteins in cells. Small-molecule inhibitors targeting the proteasome's degradative activity have been extensively developed as lead compounds for various human diseases. An exemplified molecule is bortezomib, which was approved by FDA in 2003 for the treatment of multiple myeloma. Here, using transiently and stably expressed N-end rule model substrates in mammalian cells, we evaluated and identified that salinosporamide A and salinosporamide B effectively inhibited the proteasomal degradation. Considering that a variety of proteasome substrates are implicated in the pathogenesis of many diseases, they have the potential to be clinically applicable as therapeutic agents.

Key Words : Degradation, Ubiquitin, Proteasome, Inhibitor, N-end rule

Introduction

Most intracellular protein degradation in cells are mediated by the ubiquitin-proteasome system (UPS), where proteins are modified with multiple ubiquitin (Ub) moieties and degraded by proteasomes.[1,2] The UPS is also responsible for the protein quality control in the cytoplasm and the nucleus. While protein degradation into small peptides is a favorable reaction in terms of entropy and the lysosomal degradation does not require energy, the UPS actively consumes ATP, suggesting this process is involved in the homeostasis and regulatory mechanism in cells. The proteasome is a ~2.5 MDa holoenzyme complex consisting of structurally and functionally distinguishable regulatory particle (RP, also named 19S) and core particle (CP, also named 20S). The CP contains three proteolytic active sites: chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L).[3,4] Despite the initial skepticism concerning its side effects, it has been proven that proteasome inhibitors can be clinically applicable with significant therapeutic benefits and minimal safety issues, especially to the late-stages of cancer patients.[5-8] For instance, bortezomib (PS-341, Velcade®) was approved by FDA for the treatment of multiple myeloma in 2003, which significantly increased the survival rates of patients from 10% to 40-50%. The proteasome inhibitors appear to have different pharmacological mode-of-actions as anticancer drug than other apoptosis-inducing agents.[9,10]

The type of proteasome inhibitors can be divided into natural products and synthetic compounds. Most of synthetic inhibitors, such as bortezomib derived from MG132 (Z-Leu-Leu-Leu-aldehyde), are a peptide-based molecule with competitive inhibition modes[11,12] (Fig. 1). Bortezomib reversibly inhibits the proteasome's CT-L and C-L proteolytic sites as the boronic acid located on the C-terminus interacts with threonine of the active sites.[13] Natural small-molecule inhibitors of proteasomes, such as salinosporamides purified from gram-positive marine bacteria *Salinispora tropica*, are recently spotlighted as an anticancer agent.[14] Their structures contain unique β-lactone-γ-lactam, similar to that of omuralide with bicyclic ring (Fig. 1). They form an irreversible covalent adduct with the active site threonine in the core particle (CP) active sites and are proven to have excellent specificity and IC_{50} values (low nM ranges).

Kisselev *et al*. used fluorogenic proteasome substrates and purified rabbit 26S proteasomes to compare the inhibitory effects of various proteasome inhibitors.[15] Some inhibitors showed high specificity toward certain proteolytic sites:

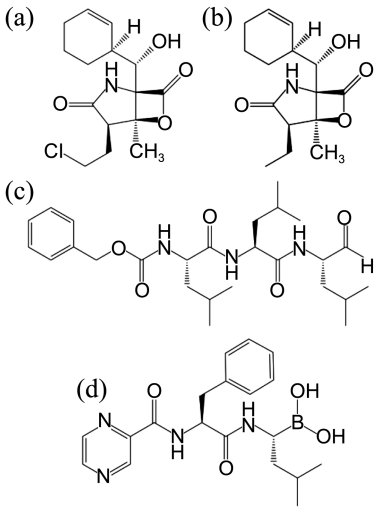

Figure 1. Compound structures of proteasome inhibitors. (a) salinosporamide A, (b) salinosporamide B, (c) MG132 (Z-leu-leu-leu-al), (d) bortezomib (Pyz-Phe-boroLeu). Note that (c) and (d) are N-protected tripeptide and dipeptide, respectively.

NLVS for CT-L, Ac-APnLD-al for C-L, and leupeptin for T-L. It appeared that, although all the three proteolytic sites collectively contributed the substrate degradation, their relative contributions varied with the substrates. While bortezomib targets CT-L at low concentrations and CT-L and C-L at higher concentration, salinosporimide A exhibited broader inhibitory spectrum, mainly inhibiting CT-L and T-L activation at its low concentrations.[16] Therefore, salinosporamide A and bortezomib as a combination may block all three proteolytic sites in the proteasome. Moreover, salinosporamide A showed sustainable inhibitory effect up to 7 days when administered in mice with high efficiency. With these pharmacological characteristics, salinosporamide A is considered to have great potential to be clinically applied.[17] In this study, we examined whether the purified salinosporamide A and salinosporamide B inhibit the proteasomal degradation of model N-end rule substrates and whether they exhibit synergic effects with MG132. For this, we generated quantitative stable cell lines expressing proteasome substrates and their controls.

Experimental

Proteasome inhibitors, either commercially available or purified as described,[18] were dissolved in DMSO (dimethyl sulfoxide) at 10 mM concentration and stored at −20 °C before use. DMEM (Dulbecco's Modification of Eagle's Medium, Cellgro) supplemented with 10% FBS (fetal bovine serum, Cellgro), 1% penicillin-streptomycin, and 1% L-glutamine was used for cell culture. Cells were incubated at 37 °C in conditions of 5% of CO_2 with humidity.

To confirm inhibitory effects on proteasome, we used N-end rule pathway model substrates Ub-X-GFPs, which were either transiently overexpressed in HeLa cells or stably expressed in 293 cells. Transfection was performed using 2 μg of plasmids expressing pcDNA3.1(+)-Ub-X-GFP for a 6-well with lipofectamine2000 (Invitrogen).[19] After 24 h incubation, cells were treated with 10 μM of proteasome inhibitors for 4 h, and whole cell extracts were collected in RIPA buffer with protease inhibitor cocktails (0.8 μM aprotinin, 20 μM leupeptin, and 10 μM peptatin A). Collected samples were used for Western blottings.

For RT-PCR, total RNA from cultured cells was prepared using the TRIzol reagent (Invitrogen), followed by further purification through RNeasy mini-column (Qiagen).[20] 250 ng of extracted RNA was used for quantitative RT-PCR. Each mRNA level was normalized to that of GAPDH and the values were plotted as means ± SD of three independent experiments. Primer sequences used were as follows: for GFP, forward (5'-GCAGAAGAACGGCATCAAGGT-3') and reverse (5'-ACGAACTCCAGCAGGACCATG-3'), for GAPDH, forward (5'-GAGTCAACGGATTTGGTCGT-3') and reverse (5'-GACAAGCTTCCCGTTCTCAG-3').

Results and Discussion

To examine the activities of proteasome inhibitors, we first used transiently overexpressed N-end rule model substrates in HeLa cells as reporter substrates. In the N-end rule pathway, which is revolutionarily conserved from bacteria to human, proteins with destabilizing N-terminal residues (N-degron) are short-lived. The N-terminal Met from the start codon AUG is a stabilizing residue unaffecting the substrate's half-life. N-degrons are divided into type 1 (Arg, His, Lys; positively charged residues) and type 2 (Leu, Phe, Trp, Tyr; bulky, hydrophobic). Here we used Ub-Arg-GFP and Ub-Phe-GFP as type 1 and type 2 N-end rule model substrates, respectively (Fig. 2(a)). Stable Ub-Met-GFP was used as control of degradation. Once expressed, the site next to Ub is cleaved by unidentified deubiquitinating enzymes, releasing free X-GFP. By comparing the band intensities before and after the proteasome inhibitor treatments, we identified which and how much inhibitors effectively block the degradation of proteasome substrates. We cotransfected X-GFP with stable V5-LacZ as control of expression.

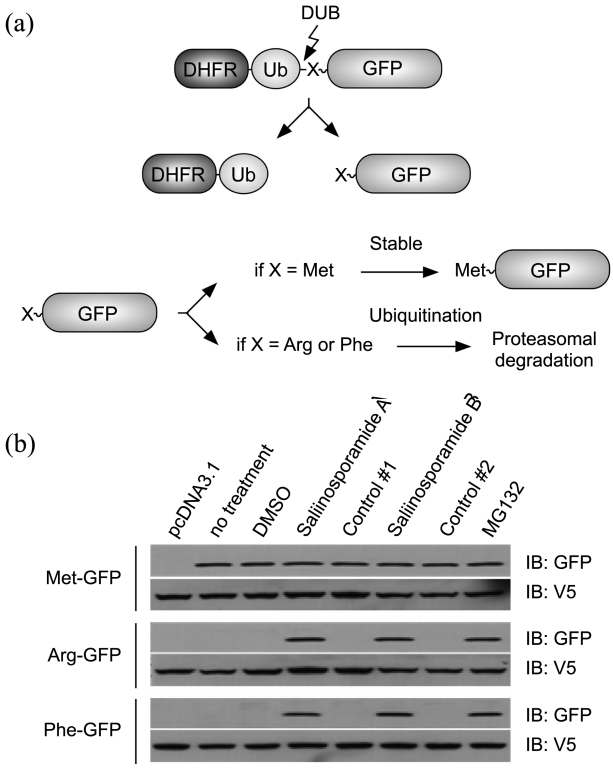

Figure 2. UPS-mediated protein degradation through the N-end rule pathway and its inhibition by various proteasome inhibitors. A, Generation of N-end rule model substrates, Met-, Arg-, and Phe-GFP. DHFR-Ub-X-GFP fusion proteins were transiently expressed in HeLa cells, where co-translational cleavages by deubiquitylating enzymes (DUBs) at the Ub-X junction yields the long-lived DHFR-Ub reference and the X-GFP. B, Arg-GFP and salinosporamide A and B are assigned as np2 and np4, respectively. Controls #1 and #2 are unrelated natural small-molecules from marine bacteria.

Transiently overexpressed Met-GFP proteins were stable in the cells and the levels were unchaned by proteasome inhibitorst as expected (Fig. 2(b), top panel). Arg-GFP and Phe-GFP were very short-lived, which in normal conditions were not detected (Fig. 2(b), middle and bottom panels).

However, when treated with 10 mM salinosporamide A, salinosporamide B or MG132 for 4 h, the levels of these otherwise unstable N-end rule substrates were rapidly accumulated. The control LacZ protein levels were comparable among all conditions, suggesting the exogenous gene expressions did not cause the different protein levels. The stabilization effects of salinosporamides and MG132 on both Arg-GFP and Phe-GFP appeared to be similar.

MG132 is known to mainly inhibits the CT-L and C-L active sites of proteasomes, while salinosporamide A broadly inhibits all three sites. The reason that these inhibitors showed similar activities might be that, at the 10 mM concentrations, the effects were already saturated, achieving the maximum stabilization of the model substrates. This result also suggests that the degradation of N-end rule substrates are not significantly affected by a specific proteolytic activity of proteasome. Therefore it would be interesting to investigate whether the inhibitors, and the N-end rule substrates as well, exhibit protease specificity at low concentrations and whether we may obtain synergistic inhibitory effects on proteasome activity by treating these two types of proteasome inhibitors simultaneously.

We also examined the effect of the inhibitors on X-GFP expressed in stable cell lines (Fig. 3). We have generated 293-derived cell lines stably expressing Ub-Met-GFP, Ub-Arg-GFP, Cys-GFP, or Ub-Phe-GFP, and confirmed that the model substrates behaved as transiently expressed proteins and *in vitro* synthesized proteins.[20] Similar to the transiently expressed X-GFP, stably expressed Met-GFP was long-lived, while Arg-GFP and Phe-GFP, containing type 1 and type 2 N-degrons, respectively, were highly unstable (Fig. 3). We treated the cells with the proteasome inhibitors with the same condition as the transfection experiment and observed the changes of X-GFP levels. Both salinosporamides A and

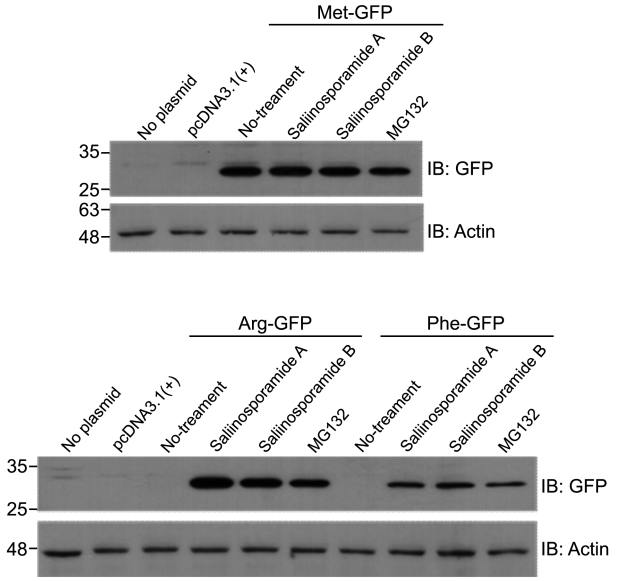

Figure 3. Proteasome inhibitors on the 293-derived stable cell lines expressing N-end rule substrates. The inhibitory effects were similar to those observed on transiently overexpressed proteins. Actin is used as loading control.

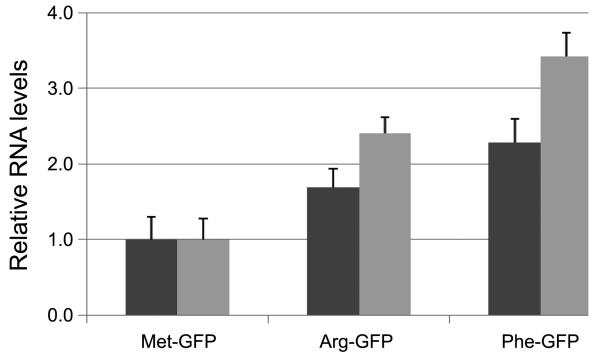

Figure 4. Posttranscriptional modification changed levels of Arg-, and Phe-GFP proteins in the 293-derived stable cell lines. Total RNA was isolated and quantitative RT-PCR was performed in two independent experiments. Relative GFP mRNA levels were normalized by those of GAPDH mRNA.

B had strong inhibition on model substrate degradation. Salinosporamides A exhibited stronger inhibitory effects on Arg-GFP degradation than MG132, but not on Phe-GFP.

It has been frequently observed that the levels of type 2 model substrates such as Phe-GFP were not restored as much as type 1 model substrates by proteasome inhibitors (data not shown). We first reasoned that there could be differential protein expressions between Arg-GFP and Phe-GFP. To compare their mRNA levels in the 293-derived stable cell lines, we performed quantitative RT-PCR (Fig. 4). To the opposite to the protein levels, Phe-GFP mRNA levels were even higher than Arg-GFP mRNA. Met-GFP mRNA levels were the lowest. This observation indicated that N-end rule model substrates might be under the post-transcriptional regulation, instead of controlled at the gene expression levels, and that there might be a compensatory mechanism on gene expression of short-lived proteins such as Arg-GFP and Phe-GFP. The results that Phe-GFP mRNA levels were detected higher than Arg-GFP were probably related with the weaker stabilization of Phe-GFP than Arg-GFP (Fig. 3).

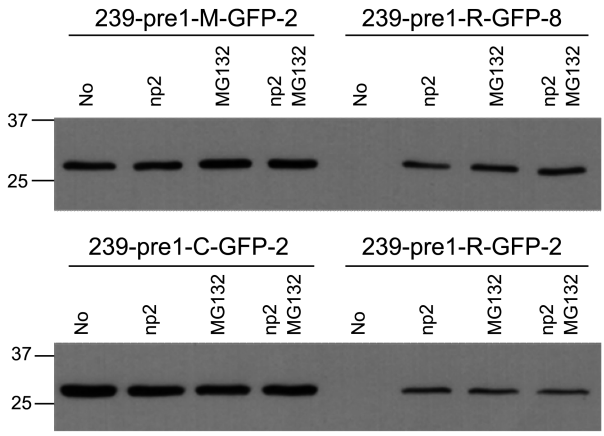

Figure 5. Little synergic inhibitory effects were found in MG132 and salinosporamide A (np2). Combinations of the two proteasome inhibitors were treated to the 293-derived stable cell lines expressing N-end rule substrates. Cys-GFP (C-GFP) was unexpectedly long-lived and was little affected by the proteasome inhibitors as Met-GFP.

However, it remains to be determined whether the weak protein stabilization resulted in gene expression.

Finally, we examined the possible synergic inhibitory effects of MG132 and salinosporamide A on degradation of N-end rule model substrates using the stable cell lines (Fig. 5). While Arg-GFP (type 1) showed only very mild additive effects when using the two inhibitors, the levels of Phe-GFP (type 2) were virtually identical in all inhibitor treatment conditions. The inhibitors affected little on Met-GFP and Cys-GFP levels. N-terminal Cys has been categorized as a tertiary destabilizing residue, modified with oxidation and subsequent arginylation. Therefore, it was expected to be short-lived similar to a type 1 substrate. However, to the contrary we found the stably expressed Cys-GFP in 293 cells are long-lived (Fig. 5), probably reflecting the imbalance between the abundant Cys N-degron and insufficient reactive oxygen species to modify them.

Conclusion

In study, we found that both salinosporamides A and B effectively inhibited the degradation of either transiently or stably expressed N-end rule substrates. However, we could not observe a specificity of the substrates on proteolytic sites in the proteasome or significant synergic stabilization using multiple proteasome inhibitors. The structurally highly similar salinosporamides showed similar inhibitory efficiency, but a more systemic approach is required to evaluate their activities and cooperative function with other proteasome inhibitors. The exceptionally fast degradation rates of N-end rule substrates strongly suggested their high susceptibility on all proteasomal proteolytic sites. Type 1 and type 2 N-degrons interact with two independent recognition components of UBR proteins, the UBR box and the N-domain, respectively. It has been observed that the type 2-N-domain interaction is usually weaker than the type 1-UBR box interaction. Although the molecular mechanism is to be determined, the weaker stabilization of Phe-GFP by proteasome inhibitors compared with that of Arg-GFP (Figs. 3 and 4) suggests that type 1 and type 2 N-end rule substrate may require different strategies to effectively block their degradation.

Because of the identification of a diverse range of physiological roles or the UPS and its pathological implication, there is mounting interest in developing more potent inhibitory methods of proteasome activity. Using inhibitor cocktails targeting different proteolytic sites will exhibit synergic inhibitory effects as bortezomib effect is being actively tested in combination with other medicinal interventions for the treatment of various cancer. This is the first report that both salinosporamides A and B effectively delay degradation of N-end rule model substrates. It would be interesting to test whether they, as themselves or in combination with bortezomib, could be applied to various human diseases that originated from abnormal N-end rule regulation such as cardiac diseases.

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