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# A new structural class of proteasome inhibitors identified by microbial screening using yeast-based assay

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**Abstract**

A yeast-based growth interference assay was developed utilizing a yeast strain in which expression of *Xenopus* cyclin A1 was induced to elevate cell division cycle 28 (Cdc28) kinase activity. Since the hyperactivation of Cdc28 kinase in yeast results in a growth-arrest phenotype, compounds which could rescue the cyclin A1-induced growth arrest might be potential new, antitumor drug candidates acting on the cyclin-dependent, kinase-mediated, cell cycle regulation pathway. In the course of our microbial screening program, the new *Streptomyces* metabolites, belactosins, were identified. As reported previously, belactosin A induced cell cycle arrest at G2/M phase in human cancer cells. However, the molecular mechanism of action was unknown. We herein demonstrate the proteasome inhibition by belactosin A. Belactosin A did not inhibit yeast Cdc28 kinase and human cyclin-dependent kinase *in vitro*. On the other hand, it inhibited the chymotrypsin-like activity of the rabbit 20S proteasome. From the initial SAR studies, we identified a hydrophobic belactosin A derivative, KF33955, which exhibited a 100-fold greater growth-inhibitory activity against HeLa S3 cells than belactosin A, presumably due to its higher cell permeability. The biochemical analysis using KF33955 suggested that the proteasome inhibitory activity of KF33955 were irreversible and required the β-lactone moiety to inhibit the proteasome. KF33955 increased the intracellular levels of protein ubiquitination in NIH3T3 cells. In addition, KF33955 treatment resulted in the accumulation of known proteasome substrates in HeLa S3 cells. These results identify belactosin A as a useful lead compound to target proteasome for the treatment of disease whose etiology is dependent on the unregulated ubiquitin–proteasome pathway.


*Keywords:* Cyclin; CDK; Inhibitor; Proteasome; Belactosin; Lactacystin

## 1. Introduction

Cyclin–cyclin-dependent kinase (CDK) complexes belong to the serine/threonine protein kinase family, and play key roles as positive regulators in cell cycle progression [1]. Overexpression of cyclins or CDKs, and/or decreased levels of endogenous CDK inhibitor proteins, such as p16 (Ink4a) and p27 (Kip1), have been reported in various tumors [2]. Thus, the importance of CDKs in cell cycle regulation and proliferation of cancer cells has encouraged an active search for small molecule inhibitors of cyclin–CDK complexes. Indeed, Flavopiridol, a small molecule inhibitor of cyclin–CDK complexes, showed potent antitumor activity in a series of experimental tumor models and is currently in clinical trials [3].

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*Abbreviations:* Cdc28, cell division cycle 28; CDK, cyclin-dependent kinase; SAR, structure–activity relationship; PGPH, peptidyl-glutamyl peptide hydrolyzing; Boc, *t*-butoxycarbonyl; ICE, IL-1β converting enzyme; Suc, succinyl; AMC, 7-amino-4-methyl-coumarin; Z, benzyloxycarbonyl; Ac, acetyl; HRP, horseradish peroxidase; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide; AcpAla, 3-(2-aminocyclopropyl)-alanine; MG-132, Z-Leu-Leu-Leu-CHO; TNFα, tumor necrosis factor alpha; NF-κB, nuclear factor kappa B; IκB, inhibitor of NF-κB.

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On the other hand, it has been demonstrated that the ubiquitin–proteasome pathway plays a significant role in cell cycle transitions [4]. The ordered and temporal degradation of numerous key proteins (e.g. cyclin B, E, p27 (Kip1), and p21 (Cip1)) is required for cell cycle progression and mitosis [5]. The proteasome is a large intracellular molecule with multicatalytic protease activities. The catalytic core of this complex is found on the 20S proteasome subunit, a multicatalytic protease containing at least three peptidase activities: chymotrypsinc-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities [6]. Both naturally occurring and synthetic inhibitors of the proteasome have been identified and are currently being employed to study the physiological roles of the proteasome [7]. A *Streptomyces* metabolite, lactacystin, was discovered on the basis of its ability to induce neurite outgrowth on the Neuro 2A mouse neuroblastoma cell line [8]. Subsequent work demonstrated that the biological effects of lactacystin result from its ability to acylate and inhibit the proteasome. Lactacystin in aqueous solution can spontaneously undergo an intermolecular reaction to form the active species, *clasto*-lactacystin β-lactone, that modifies a critical threonine residue of the X/MB1 subunit of the 20S proteasome [9–11].

Proteasome inhibitors represent a potential active novel anticancer therapy [12,13]. PS-341, one of the potent and selective proteasome inhibitors, shows potent and wide-ranging antitumor activity [14]. There was excellent correlation between intrinsic potency against the proteasome and cell cytotoxiciy among tested proteasome inhibitors [14]. *In vitro* and *in vivo* (murine xenograft) studies show that PS-341 has activity against a variety of malignancies, including myeloma, chronic lymphocytic leukemia, and various solid tumors [15]. PS-341 is the first proteasome inhibitor to enter oncology clinical trials and represents a novel approach to cancer chemotherapy with broad therapeutic potential [16].

The *Xenopus* cyclin A1 could associate with the endogenous Cdc28 in budding yeast to form an active kinase complex with increased Cdc28 kinase activity. Furthermore, induction of cyclin A1 expression in this system caused cell cycle arrest of budding yeast [17]. Using this system we screened for small molecules that could rescue the cyclin A1-induced growth arrest. Belactosins were isolated from culture broth of *Streptomyces* sp. as the active compounds [18]. However, the molecular mechanism of action was unknown. Belactosin A did not inhibit the CDK kinase activity *in vitro*, despite its ability to rescue the growth arrest in the yeast strain and its ability to induced cell cycle arrest in human cancer cells. We herein demonstrate the proteasome inhibition by belactosin A. Our investigation on the proteasome inhibitory activity of belactosins with the preliminary SAR studies suggested that this structurally unique natural product could be a useful lead compound to target proteasome for the treatment of disease whose etiology is dependent on the unregulated ubiquitin–proteasome pathway, such as cancer.

## 2. Materials and methods

### 2.1. Chemicals, enzymes, and antibodies

Belactosin A, B, and C were isolated from the culture broth of KY11780, as described previously [18]. KF33955 or KF44504 was prepared from Boc-belactosin A or B, respectively. $^1$H NMR and FABMAS spectra are in full agreement with the structure proposed. MG-132, lactacystin, and *clasto*-lactacystin β-lactone were purchased from Calbiochem. Rabbit 20S proteasome was purchased from MBL. Cathepsin B and elastase were purchased from Sigma Chemical. Recombinant human IL-1β converting enzyme (ICE) was prepared by heterologous expression in *Eschericha coli* [19]. Suc-LLVY-AMC, Boc-LRR-AMC, Ac-YVAD-AMC, Z-RR-AMC, and Suc-AAA-AMC were purchased from Peptide Institute (Minoh-shi, Osaka, Japan). Z-LLE-AMC was purchased from Calbiochem. Antibodies used in these studies were commercially available. Anti-ubiquitin from StressGen Biotech, and anti-p53 (DO1) and anti-p27 (F-8) were from Santa Cruz Biotech. Anti-mouse or anti-rabbit secondary antibodies conjugated to HRP were obtained from Amersham.

### 2.2. Mammalian cell culture

HeLa S3 human uterine cervix carcinoma and mouse fibroblast NIH3T3 were obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin at 37° in a humidified atmosphere containing 5% $CO_2$.

### 2.3. Yeast-based growth interference assay

The yeast strain carrying the pMR438 in which the *Xenopus* cyclin A1 mutant (destruction box mutant, Δ24–62) was fused to the inducible GAL1 promoter [17] was grown at 30° to stationary phase in glucose medium. Agar plates were prepared by adding 50 μL of the above culture to 50 mL of galactose agar. Paper discs soaked in drugs were placed on the galactose agar plates, the plates were incubated at 30° for 3 days, and the diameters of the zones of growth were measured for quantitative analysis [20].

### 2.4. Histone H1 kinase assay

Histone H1 kinase assay was performed, as previously described [17]. The yeast cell extracts were incubated with anti-cyclin A1 antiserum and supernatants were precipitated with Affi-Gel Protein A (Bio-Rad Lab).

The immunoprecipitates were assayed in 20 μL of reaction mixture (20 mM Tri–HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.2 mM ATP, 10 μCi [$\gamma$-$^{32}$P]ATP, 1 mg/mL histone H1) and incubated for 30 min at 30°, followed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography.

### 2.5. Inhibition of the chymotrypsin-like activity of the 20S proteasome

Inhibitors were mixed with a 10 μM Suc-LLVY-AMC and assay buffer [20 mM Tris (pH 8.0), 0.5 mM EDTA, and 0.035% SDS] in a 96-well plate. Hydrolysis was initiated by the addition of 50 ng of rabbit 20S proteasome. After incubation at 37° for 2 hr, the reaction was quenched with stop buffer [20 mM Tris (pH 8.9) and 0.5% SDS]. Fluorescence (360 nm excitation/460 nm detection) of the reaction mixture was measured using ARVO (Wallac).

### 2.6. Specificity assay

Inhibitors were incubated with ICE, elastase, or cathepsin B in the presence of appropriate fluorogenic peptide substrate at 37° for 2 hr under the conditions described previously [19]. The assay mixtures employed here were as follows. For ICE, 10 mM sodium phosphate (pH 6.8), 0.2% BSA, 10 μM Ac-YVAD-AMC. For elastase, 50 mM Tris (pH 7.6), 0.2% BSA, 10 μM Z-RR-AMC. For cathepsin, 25 mM sodium acetate (pH 5.0), 0.2% BSA, 10 μM Suc-AAA-AMC. The fluorescence (360 nm excitation/460 nm detection) of reaction mixture was measured using ARVO.

### 2.7. Enzyme kinetics assays

The rates of association ($K_{association}$) of inhibitors were determined as described [21]. 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 10 μM fluorogenic peptide substrates Suc-LLVY-AMC, Z-LLE-AMC, and Boc-LRR-AMC, respectively. Hydrolysis was initiated by the addition of 50 ng of rabbit 20S proteasome, and the reaction was followed by fluorescence detection by ARVO (360 nm excitation/460 nm detection). Reactions were allowed to proceed for 60 min, and fluorescence data were collected every 3 min. Fluorescence was quantified as arbitrary units and progression curves were plotted for each reaction as a function of time. $k_{observed}/[I]$ values were obtained using Kaleidagraph by nonlinear least squares fit of the data to the following equation: fluorescence $= v_s t + [(v_0 - v_s)/k_{observed}][1 - \exp(-k_{observed} t)]$, where $v_0$ and $v_s$ are the initial and final velocities, respectively, and $k_{observed}$ is the reaction rate constant.

### 2.8. Western blot analysis

Cells were treated with different doses of drugs (in DMSO) for different periods of time, as indicated on the figures. Cells treated with drugs were washed twice with PBS, and then suspended in 30 μL of lysis buffer containing 50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% sodiumdeoxy cholate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 1% Triton X-100, and 0.1% SDS at 4° for 30 min. After centrifugation at 1500 *g* for 10 min at 4°, the supernatants were collected, and protein amount was assessed by a Bio-Rad protein assay kit (Bio-Rad). Equal amounts (10 μg of protein) of lysate were subjected to a SDS–PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane. Evenness of loading was verified by examination of gels stained with Coomassie Blue. The membrane was blocked with 5% nonfat milk in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Triton X-100] solution, and incubated for 2 hr with the corresponding primary antibodies in the blocking solution (1:1000) at room temperature. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 hr, with HRP-conjugated secondary antibody diluted with TBST (1:10,000). The signals of detected proteins were visualized by ECL (enhanced chemiluminescence) system.

### 2.9. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide (MTT) assay

The cytotoxicity of the drugs was determined by measurement of cell viability by use of the MTT assay. Cells were seeded in 96-well tissue culture plates and treated the next day with drugs. After 72-hr exposure, drug-containing medium was removed, and the cells were washed once with PBS. MEM/10% FBS containing 0.5 mg/mL MTT (Sigma) was added to each well. The cells were incubated at 37° for 4 hr, and then an equal amount of solubilization solution (0.01 M HCl, 10% SDS) was added to each well and mixed thoroughly to dissolve the crystals of MTT formazan. After all of the crystals were dissolved, the plates were read on the micro-plate reader at 570 nm. The absorbance for control cells was defined as an MTT activity of 100%.

## 3. Results

### 3.1. Yeast-based growth interference assay and discovery of belactosins

The *Xenopus* cyclin A1 could associate with the endogenous Cdc28 in budding yeast to form an active kinase complex with increased Cdc28 kinase activity. Furthermore, induction of cyclin A1 expression in this system

caused cell cycle arrest of budding yeast [17]. This enabled us to develop a yeast-based, interference assay in which chemical inhibitors of CDK could be detected by their ability to restore the growth of the yeast expressing the non-destructible cyclin A1 mutant. The strain carrying the Δ24–62 cyclin A1 mutant gene controlled by the GAL1 promoter was grown to stationary phase in glucose medium. Agar plates were then prepared by adding the above mycelia to galactose agar. Paper discs soaked in the test compounds were then placed on the plate. Active compounds were those that could suppress the growth arrest and thereby allow a halo of growth around the respective paper discs. A strain of *Streptomyces* from soil was found to produce two active compounds, belactosin A and belactosin C [18]. Spectral analysis revealed that belactosin A possessed a unique, and as yet unknown, structure containing a novel amino acid, 3-(2-aminocyclopropyl)-alanine (AcpAla), and a β-lactone. Belactosin C contained an ornithine instead of the AcpAla with β-lactone. An inactive but related compound, belactosin B, that lacks the β-lactone moiety was also isolated (Fig. 1). 1–10 nmol/disc of belactosin A restored the growth of the yeast strain expressing the mutant cyclin A1 as indicated in Fig. 2A.

As reported previously, belactosin A inhibited cell cycle progression of human cancer cells at G2/M [18]. Antiproliferative activity of belactosin A against human cancer cells was examined with MTT assay (Table 1). Belactosin A and C inhibited the growth of HeLa S3 cells with $IC_{50}$ values of 51 and 200 μM, respectively. In contrast, belactosin B showed no apparent antiproliferative activity ($IC_{50}$ value >300 μM), which was consistent with the inability of belactosin B to restore the growth of the yeast (Fig. 2B). We next determined whether belactosin A inhibited the cyclin A1–Cdc28 kinase activity. Immunoprecipitated cyclin A1–Cdc28 complex was added to its substrate, histone H1, in the presence of [γ-$^{32}$P]ATP. No reduction of phosphorylated histone H1 was observed at amounts up to 500 μM belactosin A, compared to the control (Fig. 2C). When human CDKs were immunoprecipitated from the human tumor Saos-2 cells by anti-cyclin A1 antibody or anti-Cdc2 antibody, no inhibition at 500 μM belactosin A was observed (data not shown). These results demonstrated that neither the growth restoration in yeast nor the growth inhibition in cancer cells by belactosin A were due to direct inhibition of the kinase activity of the cyclin–CDK complex.

Table 1
Proteasome inhibition and growth-inhibitory activity against human cancer cell lines

| Compound | $IC_{50}$ (μM) | |
|---|---|---|
| | Proteasome inhibition[a] | Growth inhibition[b] |
| Belactosin A | 0.21 | 51 |
| Belactosin B | >10 | >300 |
| Belactosin C | 0.21 | 200 |
| KF33955 | 0.048 | 0.46 |
| KF44504 | >10 | >10 |
| Lactacystin | 0.28 | NT |
| MG-132 | 0.021 | NT |

NT, not tested.
[a] Inhibition of chymotripsin-like activity of rabbit 20S proteasome.
[b] Inhibition of growth in HeLa S3 cells.

### 3.2. Proteasome inhibition by belactosins

Recently, it has been demonstrated that proteolysis by the proteasome is a mechanism of regulation of many cellular processes, including cell cycle progression [4].

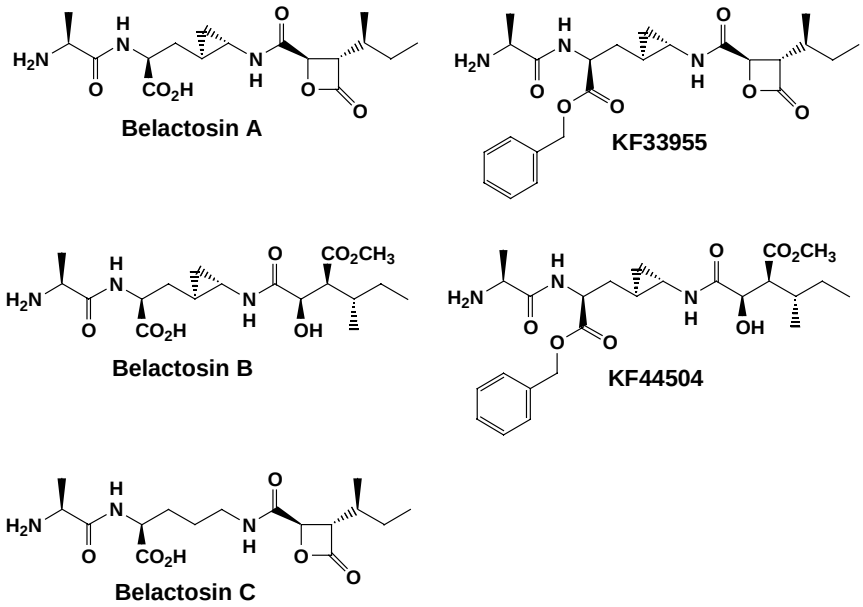


Fig. 1. Structures of belactosins and their analogs represented herein.

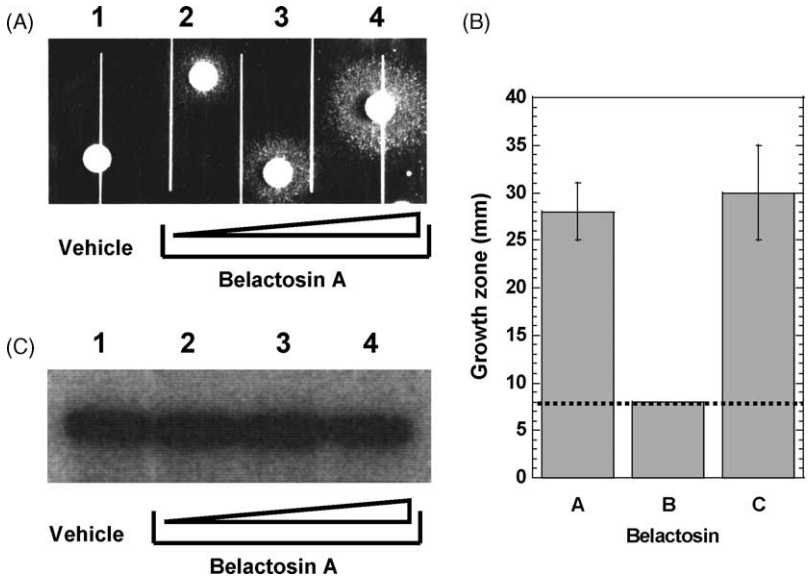


Fig. 2. Belactosin A restored growth of the yeast strain expressing a non-destructible cyclin A1 mutant. The paper discs soaked in the drugs were placed on the plates seeded with the mutant yeast strain. After 3 days at 30°, the plates were photographed and the diameters of the growth zone were measured for quantitative analysis. (A) Photograph of the assay plate. Drugs assayed: 1, 3, and 10 nmol/disc of belactosin A (lanes 2–4). (B) The diameters of the growth zone induced by 10 nmol/disc of respective belactosins. The diameter of the paper disc itself (8 mm) was represented as a dashed line in the graph. (C) Histone H1 kinase assay with immunoprecipitated cyclin A1–Cdc28 complex. Anti-cyclin A1 immunoprecipitates were added to the assay mixture containing histone H1 and [$\gamma$-$^{32}$P]ATP in the absence (lane 1) or presence of 5, 50, and 500 μM of belactosin A (lanes 2, 3, and 4, respectively).

Thus, we hypothesized that the target molecule of belactosin A might be the proteasome. To test this hypothesis, we examined the inhibitory activity of belactosins against the 20S proteasome *in vitro*. Belactosin A inhibited the chymotrypsin-like activity of rabbit 20S proteasome with $IC_{50}$ value of 0.21 μM, using 10 μM of Suc-LLVY-AMC as the peptide substrate (Table 1). Both belactosin A and C showed comparable inhibitory activities to that of lactacystin, whereas belactosin B showed weaker activity than belactosin A. The lower potency of belactosin B on the inhibition of the proteasome was consistent with the smaller effect of belactosin B on the yeast strain and HeLa S3 cells (Fig. 2B and Table 1).

### 3.3. KF33955, a cell-permeable analog of belactosin A

Belactosin A exhibited growth-inhibitory effect against HeLa S3 with relatively lower potency ($IC_{50} = 51$ μM, Table 1) than the proteasome inhibitory activity *in vitro* ($IC_{50} = 0.21$ μM). This low potency of growth-inhibitory activity was probably due to its low permeability into human tumor cells, because its growth-inhibitory activity was improved when intracellular uptake was enhanced by electroporation (data not shown). Introduction of the hydrophobic benzyl group significantly increased its potency. The benzyl ester derivative, KF33955, exerted potent growth-inhibitory activity against HeLa S3 cells with an $IC_{50}$ value of 0.46 μM (Table 1). Proteasome inhibition by KF33955 ($IC_{50} = 0.048$ μM ) was more potent than belactosin A itself. In contrast, the benzyl ester derivative of belactosin B, KF44504, showed even weaker activity ($IC_{50} > 10$ μM). To determine the selectivity of KF33955 and other inhibitors, we tested their ability to inhibit other proteases. As shown in Fig. 3A, KF33955 had no effect or weak effect on other proteases, such as elastase (serine protease), ICE (cysteine protease), or cathepsin B (cysteine protease). These results demonstrated that KF33955 was a cell-permeable proteasome inhibitor and the proteasome inhibition by KF33955 was not nonspecific effect.

Lower potency of belactosin B in proteasome inhibition suggested that the mechanism of action of belactosin A and KF33955 was to acylate the proteasome as was reported for lactacystin. When KF33955 was preincubated for 1 hr with the 20S proteasome, the extent of inhibition by KF33955 increased, in a time-dependent and irreversible manner (data not shown). Given the covalent and irreversible nature of KF33955's inhibitory activity, its rate of proteasome inactivation, $K_{association}$ ($k_{observed}/[I]$), was measured for the different catalytic activities of the 20S proteasome. As shown in Table 2, KF33955 inhibited all three major activities (chymotrypsin-like, trypsin-like, and PGPH activity), but at different rates. Inhibition of the chymotrypsin-like activity proceeded >10-fold faster than the inhibition of the trypsin-like and PGPH activities. Furthermore, KF33955 inhibited the trypsin-like and PGPH activities to greater extents than the *clasto*-lactacystin β-lactone.

### 3.4. Effect of KF33955 on the ubiquitination of cellular proteins

Since previous studies have shown that proteasome inhibitors cause a marked increase in the intracellular

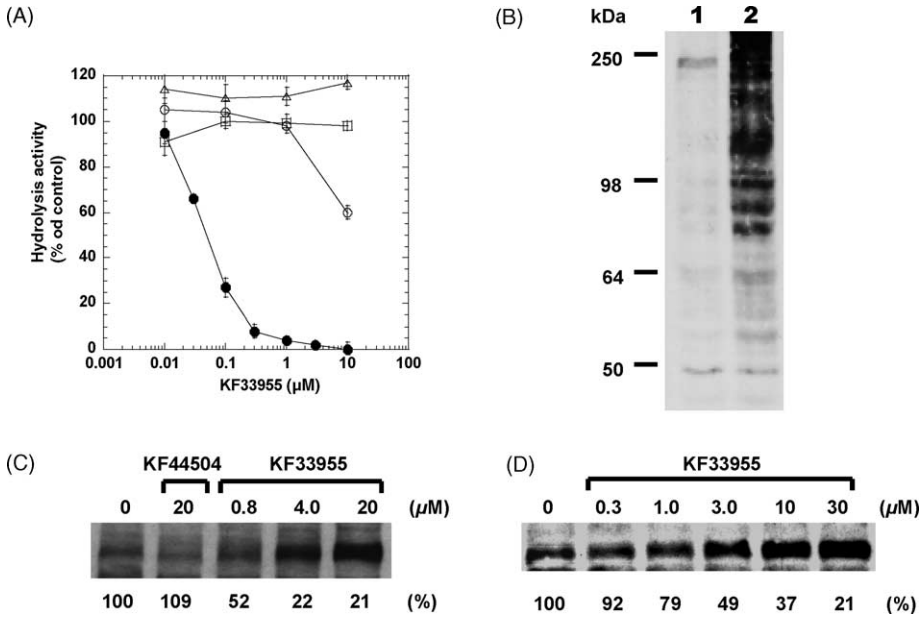


Fig. 3. Proteasome inhibition by KF33955. (A) Effect of KF33955 on the chymotrypsin-like activity of the 20S proteasome or other proteases was examined as described in Section 2. Closed circle, open circle, open square, and open triangle indicate 20S proteasome, cathepsin B, elastase, and ICE, respectively. The assay was performed as described in Section 2. (B) Effect of KF33955 on the ubiquitination of cellular proteins. NIH3T3 cells were treated with 0.1% DMSO (lane 1) or 1 μM KF33955 (lane 2) for 24 hr. Equivalent amounts of total cellular proteins were separated by SDS–PAGE and immunoblotted with anti-ubiquitin antibody. (C) Accumulation of p53 in response to KF33955. HeLa S3 cells were treated for 4 hr with increasing concentrations of KF33955 and KF44504 as indicated at the top of each lane. Chymotrypsin-like activity of cellular proteasome treated with KF33955 or KF44504 was indicated at the bottom of each lane. The protein extract used in immunoblotting for p53 detection was diluted with assay buffer and subjected to measurement of the Suc-LLVY-AMC hydrolysis activities as described in Section 2. (D) Accumulation of p27 in response to KF33955. HeLa S3 cells were treated for 8 hr with increasing concentrations of KF33955 as indicated at the top of each lane. Chymotrypsin-like activity of cellular proteasome treated with KF33955 was indicated at the bottom of each lane. The protein extract used in immunoblotting for p27 detection was diluted with assay buffer and subjected to measurement of the Suc-LLVY-AMC hydrolysis activities as described in Section 2.

levels of protein ubiquitination [22], we examined the effect of KF33955 on protein ubiquitination. NIH3T3 cells were treated with 1 μM KF33955 for 24 hr and the intracellular levels of ubiquitin conjugated proteins were estimated by immunoblotting with an anti-ubiquitin antibody. KF33955 caused a significant increase in the protein ubiquitination of intracellular proteins (Fig. 3B). The tumor suppressor gene product p53 and the endogenous CDK inhibitor p27 (Kip1) are known to be proteasome substrates. Based on these findings, we examined the effect of KF33955 on the intracellular protein level of p53 and p27 in HeLa S3 cells by immunoblotting. Treatment of HeLa S3 cells with KF33955 for 4 hr resulted in a significant increase in the level of p53 protein (Fig. 3C). In contrast, incubation with the inactive derivative, KF44504, resulted in no increase in the p53 level. Accumulation of p27 was also observed in cells treated with KF33955 (Fig. 3D). In addition, a reduction of the chymotrypsin-like activity in whole cell lysates was observed (Fig. 3C and D). This was inversely correlated with the levels of p53 and p27. Taken together, these results support the conclusion that KF33955 is a *bona fide* proteasome inhibitor.

## 4. Discussion

Ectopically expressed *Xenopus* cyclin A1 mutant (non-destructible cyclin A1 mutant Δ24–62) could associate with endogenous Cdc28 to form a constitutively active Ser/Thr kinase complex and that yeast harboring such a mutant

Table 2
Kinetics of inhibition of 20S proteasome activity by KF33955

| Compound | $K_{association} = k_{observed}/[I]$ ($M^{-1}$ $s^{-1}$) | | |
|---|---|---|---|
| | Chymotrypsin-like activity | PGPH activity | Trypsin-like activity |
| KF33955 | 6690 ± 334 (0.1 μM) | 237 ± 17 (3 μM) | 602 ± 78 (10 μM) |
| *clasto*-Lactacystin β-lactone | 5410 ± 162 (0.1 μM) | 61.0 ± 1.8 (10 μM) | 161 ± 24 (10 μM) |

The rates of covalent inhibition ($K_{association}$) of the three major proteasome catalytic activities were determined for KF33955 and *clasto*-lactacystin β-lactone. For proteasome inhibition assays, commercially available purified rabbit 20S proteasome and peptide-AMC substrates were used. 10 μM Suc-LLVY-AMC, Z-LLE-AMC, and Boc-LRR-AMC were used to assay the chymotrypsin-like, trypsin-like, and PGPH activities, respectively.

cyclin was growth arrested as reported previously [17]. This growth-arrest phenotype was confirmed to be due to the elevated Cdc28 kinase activity because non-CDK binding cyclin A1 did not induce this phenotype and new alleles of the Cdc28 mutation that reduced the binding of cyclin A1 were identified as suppressors [17]. This enabled us to develop a yeast-based, interference assay in which chemical inhibitors of CDK could be detected by their ability to restore the growth of the yeast expressing the non-destructible cyclin A1 mutant. Using this system we isolated chemical compounds that were capable of overcoming this growth arrest. Two such compounds designated belactosin A and C were identified as active compounds in the course of our microbial screening program.

Unexpectedly, belactosins turned out to be proteasome inhibitors rather than CDK inhibitors. However, it remains to be determined how belactosins restore the growth of yeast in this assay. The proteasome inhibition is likely to be one of the ways to relieve growth arrest in this system, because another proteasome inhibitor, MG-132, showed a similar growth-restoration activity, whereas conventional DNA-damaging drugs, such as adriamycin, cisplatin, and mitomycin C, did not. However higher amount of belactosin A (>1000 nmol/disc) resulted in no appearance of the halo of growth around paper disc (data not shown). These observations suggest that strong inhibition of the proteasome would block the growth of yeast as efficiently as it blocks growth of cancer cells and the intermediate doses can allow the indestructible cyclin A1-expressing strains of yeast to grow. Proteasome inhibitors are known to upregulate the levels of endogenous CDK inhibitors in cells, such as p27 and p21 in human cells and Sic1 in budding yeast. Since phosphorylation of Sic1 is necessary for its proteasome-dependent degradation, Sic1 may be a target of elevated Cdc28 kinase to reduce the level of Sic1 [23]. It is possible that proteasome inhibition by intermediated doses of belactosin A might upregulate the level of Sic1 protein or other cell cycle regulatory factors to restore the basal endogenous level of Cdc28 kinase activity for driving cell cycle. We obtained another hit compound unrelated to belactosin, that did not show CDK inhibitory or proteasome inhibitory activity.[1] Such a compound might regulate another step in the ubiquitin–proteasome pathway. Further investigations by using these hit compounds might be useful to reveal the mechanisms of growth restoration in the yeast.

It has been demonstrated that the ubiquitin–proteasome pathway plays a significant role in cell cycle transitions [4]. The ordered and temporal degradation of numerous key proteins (e.g. cyclin B, E, p27 (Kip1), and p21 (Cip1)) is required for cell cycle progression and mitosis [5]. Inhibition of cell cycle progression by lactacystin strongly support importance of the proteasome in cell growth. In the case of lactacystin, generation of its active species, *clasto*-lactacystin β-lactone, is necessary to acylate and inhibit proteasome function [11]. Thus, we hypothesized that the target molecule of belactosin A might be the proteasome. Belactosin A inhibited the chymotrypsin-like activity of rabbit 20S proteasome with $IC_{50}$ value of 0.21 μM (Table 1). Both belactosin A and C showed comparable inhibitory activities to that of lactacystin, whereas belactosin B showed weaker activity than belactosin A. The lower potency of belactosin B on the inhibition of the proteasome was consistent with the smaller effect of belactosin B on the yeast strain and HeLa S3 cells, suggesting these activities of belactosins in cells were mediated thorough the proteasome inhibition.

The hydrophobic benzyl ester derivative KF33955 had a 100-fold more potent growth-inhibitory activity against HeLa S3 cells than belactosin A. KF33955 treatment increased the cellular concentration of ubiquitinated proteins (Fig. 3B). The E6 protein of human Papilloma virus-16 induces degradation of p53 protein through the ubiquitin–proteasome pathway in HeLa S3 cells [24]. The CDK inhibitor p27 is continuously destroyed by the ubiquitin–proteasome pathway in most cycling cancer cells [25,26]. Substrates of the proteasome, such as p53 and p27, were also accumulated in human cancer cells treated with KF33955 (Fig. 3C and D). On further examination with the luciferase reporter systems, TNFα-induced degradation of IκB and subsequent transcriptional activation of NF-κB were inhibited by KF33955 in 293 cells (see footnote 1). Belactosin B and its benzyl derivative KF44504 both of which are containing a cleaved β-lactone did not inhibit the proteasome *in vitro*. In addition, neither growth restoration in the yeast nor growth inhibition in cancer cells was not induced sufficiently by these inactive analogs. Base on these results, we propose that growth inhibition against several cancer cells by KF33955 is mediated through the proteasome inhibition.

Several proteasome inhibitors have been reported so far and these inhibitors represent a novel therapeutic potency. Lactacystin was identified as the *Streptomyces* metabolite that induced cell growth arrest and subsequent neurite outgrowth [8]. The active species generated from lactacystin, *clasto*-lactacystin β-lactone appeared to covalently modify the active-site threonine of the β-type subunit X (MB1), resulting in an irreversible inhibition of the proteasome [9–11]. The α′,β′-epoxyketone containing natural product epoxomicin was isolated from an *Actinomycetes* strain based on its antitumor activity [27]. Despite its potent activity the mechanism of action had remained unknown. Recently, Meng *et al*. identified epoxomicin as a potent and selective proteasome inhibitor [21]. Using a synthetic biotinylated affinity derivative, they showed that epoxomicin covalently bound to the several catalytic β-subunits. Belactosin A has a β-lactone and inhibited the proteasome irreversibly. In conjunction with the finding that the inactive analog, belactosin B, does not have a

[1] Unpublished results.

β-lactone moiety, the β-lactone moiety of belactosin A probably binds covalently to the same or nearby sites on the proteasome as lactacystin or epoxomicin. We are planning to analyze the binding site with the radio/biotin-labeled belactosins. Such binding studies with cells and tissue extract would provide useful data to compare the activity of belactosin A and other inhibitors. In addition to being useful research tools for dissecting the roles of the cellular proteasome, proteasome inhibitors are eliciting appreciable interest for potential applications in therapeutic uses for cancer and other diseases. PS-341 is the first proteasome inhibitor to enter oncology clinical trials and represents a novel approach to cancer chemotherapy with broad therapeutic potential [16]. In our preliminary evaluation, KF33955 suppressed growth of human colon carcinoma WiDr in a murine xenograft model (see footnote 1). Evaluation of antitumor activity of KF33955 *in vivo* is in progress, and further SAR studies for optimizing its efficacy are underway.

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