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Borrelidin Inhibits a Cyclin-dependent Kinase (CDK), Cdc28/Cln2, of *Saccharomyces cerevisiae*

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We identified borrelidin, a member of macrolide antibiotic, as an inhibitor of a cyclin-dependent kinase of the budding yeast, Cdc28/Cln2. A 50% inhibition concentration (IC_{50}) of borrelidin for Cdc28/Cln2 was 24 μM. In addition, borrelidin arrests both haploid and diploid cells in G1 phase at the point indistinguishable from that of α-mating pheromone, at concentrations not affecting the gross protein synthesis. Although the inhibition of CDK activity may not be a solo cause of the G1 arrest, our results indicate that borrelidin is a potential lead compound for developing novel CDK inhibitors of higher eukaryotes.

The cell cycle progression of eukaryotes is tightly regulated by cyclin-dependent kinase (CDK) family. CDKs are inactive in monomeric form and activated by binding to cyclines, a diverse family of proteins whose levels oscillate during the cell cycle. Regulation of CDK activity occurs at multiple levels, including cyclin synthesis and degradation, activating and inactivating phosphorylation, CDK inhibitor proteins (CKIs) and subcellular localization[1~3]. In mammalian cells, the progression of cells from G1 to S phase is regulated by retinoblastoma gene product (Rb protein), a tumor suppressor gene, that blocks the expression of a set of genes required for enter the S phase[4,5]. CDKs inactivate Rb by phosphorylation and promote cell cycle progression. Because alteration of Rb and/or alteration or absence of CKIs have been associated with many cancers, chemical modulators of CDK activity is an attractive target for cancer chemotherapy. Indeed, two direct CDK inhibitors, flavopiridol (polyhydroxylated flavone) and UCN-01 (purine derivative) were developed and reported to show promising results in early clinical trials[6].

Borrelidin was isolated as an antibiotic in 1949[7]. This drug shows weak antibacterial activity against commonly used test strains but shows antiviral and anti-tumor activity[8,9]. More recently, borrelidin was found to block angiogenesis in rat aorta matrix culture[10]. The major cause of antibiotic activity of borrelidin was reported to be the inhibition of threonyl tRNA synthase[11] and WAKABAYASHI *et al.* observed that the drug inhibits both threonyl tRNA synthase and protein synthesis of cultured rat cells[10]. However, the IC_{50} value for anti-angiogenesis activity of borrelidin was 0.4 ng/ml, while that of protein synthesis was over 20 ng/ml, suggesting some uncharacterized activity of the drug.

We identified borrelidin as a cell cycle modulator of budding yeast, *Saccharomyces cerevisiae*. In this report, we describe our finding that borrelidin is a novel inhibitor of the budding yeast CDK.

Materials and Methods

Strains and Media

S. cerevisiae strains used were W303 (*ura3-1 leu2-3,112*

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trp1-1 his3-11 ade2-1 can1-100) background. W303-1A is a *MATa* strain. YET49 (*MATa GAL1p*::*CLN2-HA*::*LEU2*) and YET50 (*MATa CLB2-HA*::*URA3*) were constructed by one step gene replacement of genomic *CLN2* locus with *Puv*II fragment of pMT209 containing *GAL1p*::*CLN2-HA*::*LEU2*[12] or by integrating the *Spe*I digested pMF914 containing *CLB2-HA*::*URA3*[12] into genomic *CLB2* locus, respectively. Cells were grown at 28°C in liquid YPD (1% yeast extract, 2% peptone, 2% glucose). Because borrelidin contains carboxyl group, pH of the media used in this study was adjusted to pH 4.5 by HCl. Standard genetic methods were used[13].

Materials

Cycloheximide (CHX) and α-mating factor were purchased from Sigma and Peptide Institute Inc., Osaka, respectively.

Flow Cytometry

Overnight culture of yeast in YPD medium were diluted to 5×10^5/ml and allowed for growth to $1.5\sim2\times10^6$/ml before addition of borrelidin. Approximately 1×10^7 cells were withdrawn from these cultures and processed for propidium iodide staining[14]. For each preparation, 20,000 events were analyzed for DNA content using an Epics Elite (Beckman Coulter Co. Ltd.) flow cytometor.

Measurement of Protein Synthesis by [^{35}S]-methionine Incorporation

For analysis of gross protein synthesis, trichloroacetic acid (TCA)-precipitable radio activity were quantitated from pulse-labeled cultures. W303A cells cultured to 2×10^6/ml in synthetic complete (SC) medium[13] minus methionine were treated with various amount of borrelidin, cycloheximide or with drug vehicle (ethanol). After 20-minutes treatment, 100 μl culture was removed and labeled at 28°C for 7 minutes with 5 μCi [^{35}S]-methionine (Amersham) and incorporation was stopped by adding 200 μl of ice cold 20% TCA. After standing on ice for 20 minutes, cells were collected on Whatman filters and serially washed with 10 ml each of 10% TCA and ethanol. Filters were dried and counted by a liquid scintillation counter.

Order-of-function Mapping

A reciprocal shift experiment was performed as described[15]. Samples were taken at 15-minutes intervals, fixed with 3% formaldehyde and scored for cell number and emergence of buds.

Immunoprecipitation and Kinase Assay

YET49 cells grown to mid-log phase in YPD were induced for production of HA-tagged Cln2p by shifting to YPGalactose (YPD containing 2% galactose in stead of glucose) for 3 hours. For preparation of cell lysate, cells were harvested by centrifugation at 4°C and washed twice with ice cold lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM sodium pyrophosphate, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate, 2 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 μg/ml aprotinin), resuspended in the same buffer and broken with glass beads by two bursts of vigorous vortexing for 5 minutes at 4°C. Clude clarified lysates were made by pelleting cell debris at 12,000 *g* for 15 minutes. To 100 μl of the lysate containing ~400 μg of protein, 2 μg of anti-HA mouse monoclonal antibody (clone 12CA5, Boehringer Mannheim) were added and incubated on ice for 1 hour. Twenty microliters of protein G magnetic beads (Protein G BioMag, PerSeptive Biosystems) suspended in lysis buffer were then added and the incubation was continued for 1 hour on ice. The immunoprecipitates were recovered by separating the beads with magnetic separator, serially washed four times and twice with lysis buffer containing 150 mM NaCl and kination buffer (10 mM HEPES-KOH, pH 7.2, 10 mM $MgCl_2$, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.02% Triton X-100), respectively, and then resuspended in 40 μl kination buffer. The reaction mixture (60 μl) containing 30 μl of the beads suspension, 40 μg of histone H1, 2.5 μM ATP, 1.2 μCi [γ-^{32}P] ATP and borrelidin or drug vehicle (ethanol) were incubated at 30°C. At appropriate intervals 10 μl samples were withdrawn, mixed with 2.5 μl of 5× PAGE sample buffer, boiled for 3 minutes and loaded on a 10% SDS-PAGE gel[16]. Phosphorylated proteins were visualized by autoradiography and quantified by a BAS-2000 Bioimaging analyzer (Fuji Photo Film Co.).

Results and Discussion

Borrelidin Blocks G1 Progression

In order to identify novel cell cycle inhibitors, we screened for activity that arrests yeast cell growth in unbudded G1 stage, on culture supernatant of several thousand strains of actinomycestes. Among those, broth of a strain No. SNA24891 showed potent activity. The active compound was purified from a culture broth of this strain by ethyl acetate extraction, silica gel column chromatography and HPLC. UV and NMR spectra indicated that it was identical to borrelidin (Fig. 1, data not shown). The purified drug was dissolved in ethanol at

Fig. 1. Structure of borrelidin.

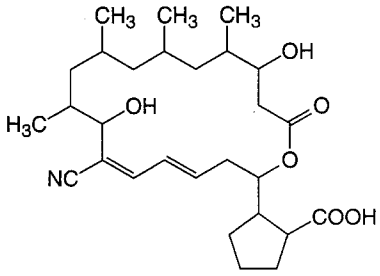
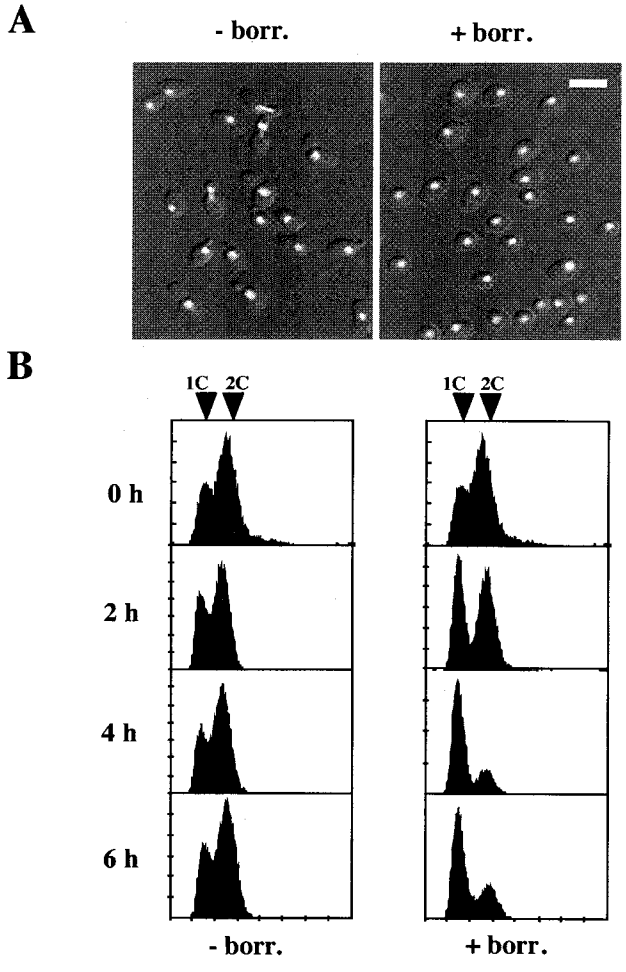


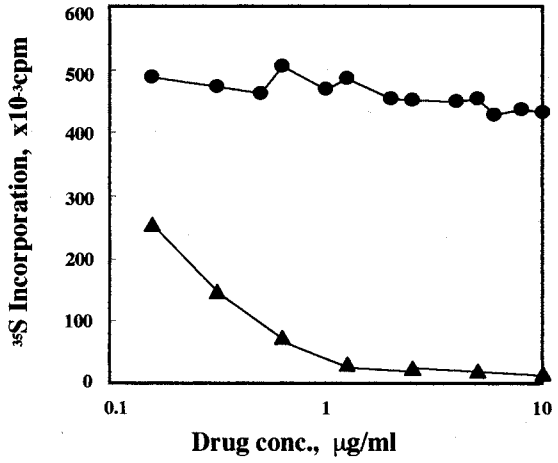
Fig. 2. Effect of borrelidin on the growth of yeast cells.



A: W303-1A cells treated with 4 μg/ml of borrelidin (+borr.) or its vehicle (ethanol, −borr.) for 6 hours were stained with propidium iodide and observed under a microscope. Bar indicates 10 μm.

B: The cells treated as in A were taken at indicated times fixed and stained with propidium iodide. DNA content was analyzed by flow cytometry. Abscissa: fluorescence intensity, ordinate: cell number.

Fig. 3. Effect of borrelidin on [^{35}S]-methionine incorporation.



W303-1A cells grown to early-log phase were treated for 20 minutes with various amounts of borrelidin (closed circle) or cychloheximide (closed triangle) indicated in the figure and pulse labeled with [^{35}S]-methionine for 7 minutes. TCA-precipitable radio activity was counted by a liquid scintilation counter and plotted against the drug concentration. The result is average of two experiments. Standard deviation was within 3%.

5 mg/ml and used for the experiments.

The half-inhibitory concentration of borrelidin on the yeast cell growth estimated by fold increase of cell number during 10 hours incubation was 2 μg/ml (4 μM) and over 90% inhibition was observed at 4 μg/ml. The effect of the drug was fully reversible, *i.e.*, the ratio of colony formation of the cells treated with 10 μg/ml of borrelidin for 10 hours was indistiguishable from that of mock treated cells (data not shown).

To assess the effect of borrelidin on cell cycle progression, we examined the DNA content of the cells by fluorescence-activated cell sorting (FACS) analysis. As shown in Fig. 2, the accumulation of the cells with 1C DNA content was observed in the presence of 4 μg/ml of borrelidin after 4-hours incubation, compared to the culture added with drug vehicle, ethanol. Morphology of the cells and nuclei stained with propidium iodide showed that over 85% of the cells treated with 4 μg/ml of borrelidin for 6 hours have no bud and single nucleus. These observations indicate that the drug arrests the cells in G1 phase of the cell cycle. This effect of borrelidin was observed with both *MATa*- and *MAT*α-mating type haploid cells and also with *MATa*/α diploid cells (data not shown).

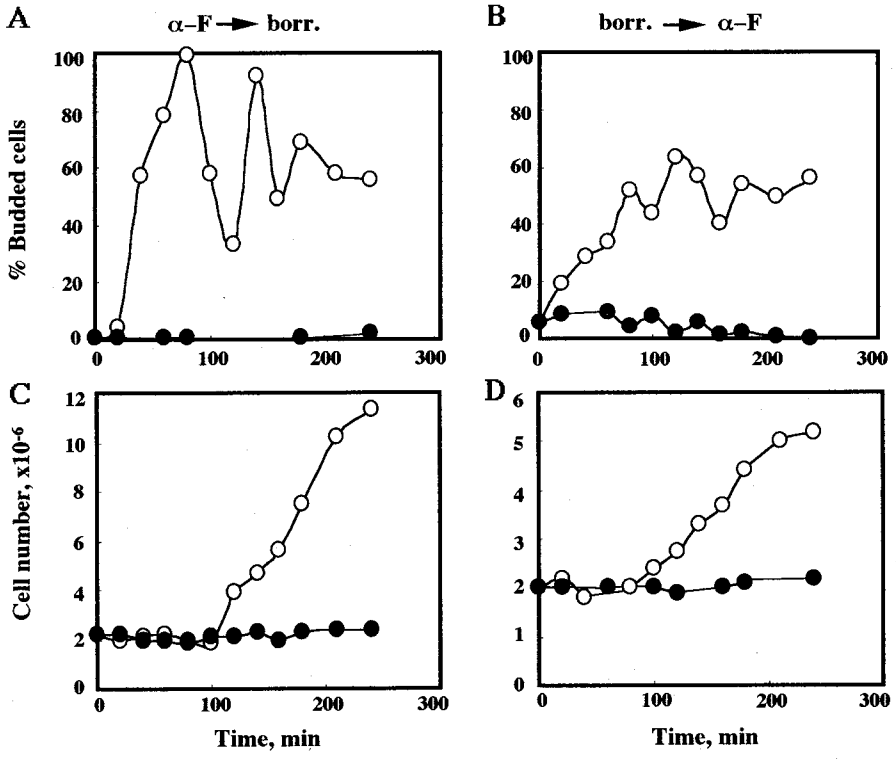
Borrelidin Does not Block Protein Synthesis

Because borrelidin blocks protein synthesis by inhibiting threonine tRNA synthase in both bacterial and mammalian cells[10,11] and because inhibition of protein synthesis in yeast causes an early G1 arrest[17,18], we next examined if the drug affected protein synthesis of yeast by assaying incorporation of [^{35}S]-methionine in the presence of various concentrations of borrelidin. As shwon in Fig. 3, the 20-minutes treatment with borrelidin did not block [^{35}S]-methionine incorporation up to 10 μg/ml, while the treatment with cycloheximide as a control blocked the incorporation nearly completely at the concentration of 1 μg/ml. We also carried out this experiment with longer drug-treatment period (40 minutes) and obtained the same result (data not shown). These results suggest that the G1 block phenotype of borrelidin does not depend on the inhibition of protein synthesis.

The Borrelidin Block Point is in Late G1 at START

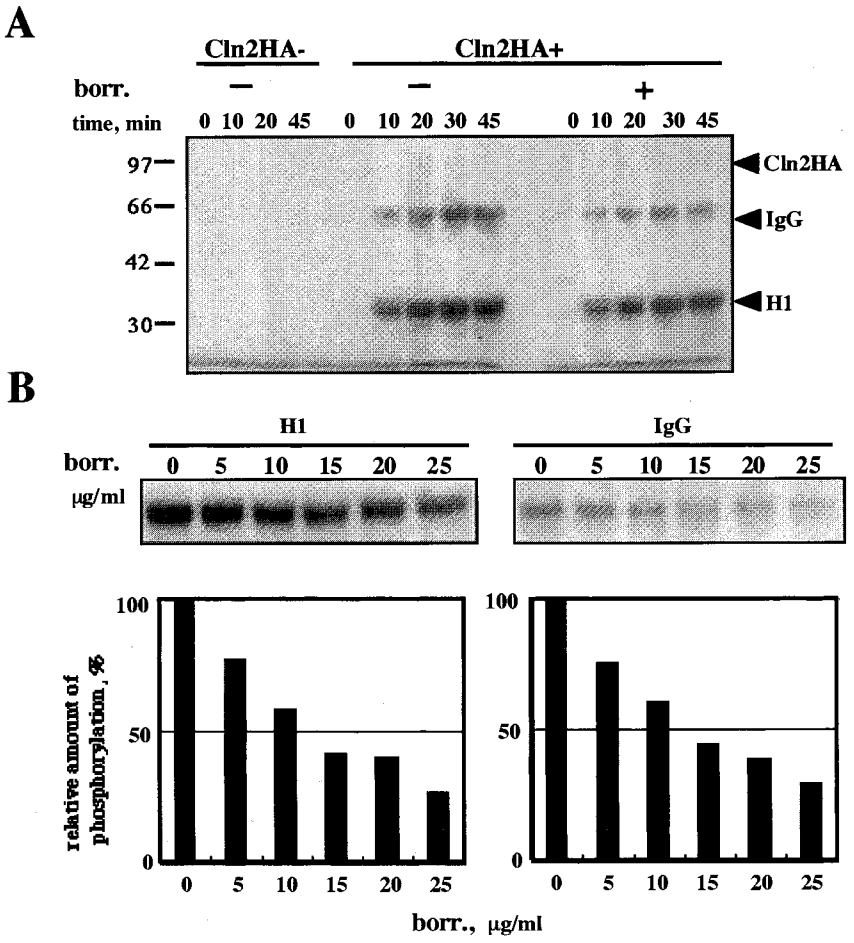
To determine the borrelidin block point within G1, we performed an order-of-function (reciprocal shift) analysis using the mating pheromone α-factor[15]. This maps the borrelidin block point relative to the α-factor arrest point, START. Following release from α-factor block, the cells added with drug vehicle emerged new bud and synchronously divided by 120 minutes. On the other hand, the cells added with 4 μg/ml borrelidin did not form new bud during 240-minutes incubation (Fig. 4, A and C). This result indicated that the block point of borrelidin is after or similar to that of α-factor. Interestingly, in the borrelidin-to-α-factor shift experiment, the cells released from borrelidin were fully sensitive to α-factor and formed shmoo (Fig. 4, B and D, data of shmoo formation not shown). Therefore, the block point of borrelidin was concluded to be similar to that of α-factor.

Fig. 4. Reciprocal-shift analysis on the arrest point of borrelidin within G1.



W303-1A cells treated with 10 μg/ml of α-factor (A and C) or 4 μg/ml of borrelidin (B and D) were washed and released to drug free medium (containing 0.08% ethanol, open circle) and medium containing 4 μg/ml borrelidin (A and C) or 10 μg/ml of α-factor (B and D) (closed circle), respectively. At indicated times, aliquots were withdrawn, fixed with 3% formaldehyde and scored for cell number and bud emergence under a microscope. The experiment was carried out twice with good consistency.

Fig. 5. Effect of borrelidin on Cdc28/Cln2 kinase activity.



A: Anti-HA immunoprecipitates from the Cln2-HA-expressing (Cln2HA+) or not expressing (Cln2HA−) cells were incubated with [γ-^{32}P] ATP, histone H1 (H1) and 5 μg/ml of borrelidin (borr. +) or drug vehicle (borr. −), and portions were taken at indicated times. Phosphorylated proteins were separated through SDS-PAGE gels and visualized by autoradiography. Numerals on the left side of the autoradiogram indicate molecular weight markers in kDa.

B: Kinase reaction was carried out as described in A in the presence of various amounts of borrelidin indicated in the figure. The amount of phsphorylation of H1 and IgG was quantified by a BAS-2000 Bioimaging analyzer and indicated as percent of the values with drug vehicle alone (0.5% ethanol, lanes of borr. 0 μg/ml), in bar graphs below each autoradiogram. The experiment was carried out for three times with good consistency. Typical autoradiograms were sited. Standard deviation was within 5%.

Borrelidin Inhibits Cdc28/Cln2 Kinase Activity

The α-mating factor induces G1 arrest by reducing the activity of cyclin-dependent kinase (CDK), Cdc28/G1 cyclin complex[19]. The result obtained in the previous section suggested a possibility that borrelidin possesses an ability to inhibit this kinase. *S. cerevisiae* contains three major G1 cyclins, Cln1 Cln2 and Cln3. Among them, Cln1 and Cln2 function in the execution of START redundantly by binding to Cdc28[20]. To assess if borrelidin could inhibit Cdc28/Cln2 kinase, we isolated the kinase by immunoprecipitation from the cells expressing hem agglutinine (HA)-tagged Cln2 under the control of *GAL1* promoter. Under the reaction using histone H1 as a substrate, strong, weaker and faint phosphorylation of H1, IgG and Cln2-HA, respectively, occurred in time dependent manner with the immunoprecipitates from Cln2-HA expressing cells, but not with that of Cln2-HA minus cells (Fig. 5, A). The addition of 5 μg/ml borrelidin to the reaction decreased the phosphorylation of both H1 and IgG

by 25%. The reactions with increasing amount of borrelidin revealed that IC_{50} of the drug was 12 μg/ml (24 μM) for both substrates (Fig. 5, B). These results clearly indicate that borrelidin is a novel inhibitor of yeast CDK. However, considering the fact that the drug can block yeast cell growth at 4 μg/ml, inhibition of CDK activity is not likely to be a solo cause of the cell cycle arrest. Because G1 cyclins are very unstable proteins, one might argue that a little inhibition of protein synthesis by borrelidin might cause G1 arrest by decreasing the amount of G1 cyclins. Nevertheless, the addition of 1 μg/ml of cycloheximide only caused slight accumulation of G1 cells in our strain background, while the drug inhibited [^{35}S]-methionine incorporation nearly completely at this concentration (Fig. 3, data not shown). It is possible that modest inhibition of CDK in addition to a little perturbation of protein synthesis in the presence of 4 μg/ml borrelidin cooperatively arrested cells in G1. Alternatively, borralidin have other function(s) such as to inhibit other kinase(s) involved in cell cycle regulation. We also tested the effect of borrelidin on Cdc28/Clb2 kinase, a major CDK functioning in the G2/M phase of the cell cycle, by using immunoprecipitates from the cells expressing HA-tagged Clb2. The addition of 25 μg/ml borrelidin into the reaction decreased the phosphorylation of H1 by 15% (data not shown). The inhibitory activity of borrelidin on Cdc28/Clb2 kinase seemed to be weaker than that on Cdc28/Cln2 kinase. Further analyses will be required to understand the effect of borrelidin on yeast CDKs as well as on the mechanisms of the drug action on cell growth.

There are several small molecular CDK inhibitors that have so far been characterized: the purine-based compounds (olomoucine and its analogues), butyrolactone, flavopiridols (flavopiridol and deschloroflavopiridol), staurosporines (staurosporine and UCN-01), polysulfates (suramin), 9-hydroxydllipticine and paullones[6]. Each is either a natural product or derivative of one with a distinct chemical structure. However, no member of macrolides was reported as CDK inhibitor to date. CDKs are conserved through evolution and many CDK inhibitors inhibit both human and yeast CDKs[21]. Our results suggest that borrelidin is a potential lead compound to develop potent anti-tumor agents.

Acknowledgments

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