# Identification of Preussin as a Selective Inhibitor for Cell Growth of the Fission Yeast *ts* Mutants Defective in *Cdc2*-Regulatory Genes

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Recent studies have revealed that cell cycle control in G1 or G2 phase is the basis for understanding the mechanism for tumorigenesis[1,2]. It is therefore likely that inhibitors of G1 and/or G2 progression are candidates for a new type of chemotherapeutic agents. Since the cell cycle regulators such as cyclins and CDKs are highly conserved from yeast to humans, screening for inhibitors of the yeast cell cycle seems to be one of the promising ways leading to identification of mammalian cell cycle inhibitors. The *cdc* mutants containing temperature-sensitive cell cycle regulators are incapable of progressing through a specific stage of the cell cycle at the nonpermissive temperature[3]. In addition, *cdc* mutants often show altered sensitivity to agents targeting the *cdc* gene products at the permissive temperature[4,5]. We therefore performed a panel screening using several *cdc* mutants of *Schizosaccharomyces pombe* in order to identify compounds to which these *cdc* mutants showed hyper-sensitivity. The mutants we used were *cdc2*, *cdc13*, *cdc25*, and *wee1*, all of which are involved in controlling the M-phase promoting factor associated with *cdc2* kinase activity[6]. The *cdc2* kinase plays a key role in G1 and G2 progression in *S. pombe*[7,8]. *cdc13* encodes a B-type cyclin that is associated with Cdc2 kinase and necessary for its catalytic activity, *cdc25* encodes a protein-tyrosine phosphatase that dephosphorylates the kinase-inhibitory phosphotyrosine-15 residue on Cdc2 kinase and thereby activates Cdc2, and *wee1* encodes a protein-tyrosine kinase that phosphorylates the tyrosine residue and thus acts as a negative regulator for Cdc2. These regulators as well as Cdc2 are highly conserved in all eukaryotes[9].

In the course of our screening program, we found a strong activity in the broth of a fungus *Aspergillus* sp. strain 693, which inhibited the proliferation of *cdc25*, *cdc13*, and *wee1* but did not produce any visible inhibitory zone on the wild-type or other *cdc* strains in the paper disc assay. For purification of the active substance, the producing strain 693 was cultivated in 5 litters of YMPG medium containing glucose 2%, yeast extract 0.5%, malt extract (Difco) 0.5%, and Bactopeptone (Difco) 0.5%, for 5 days at 26.5°C. The active substance was extracted with hexane - ethylacetate (9 : 1) from both the mycelial acetone extract and the cultured supernatant. The concentrated material was dissolved in hexane - ethylacetate (9 : 1) and successively fractionated by a silica gel column (50 × 100 mm, 200 ml) with a solvent system of a gradient of hexane - ethylacetate (from 9 : 1 to 1 : 1) and by silica gel thin layer chromatography (Merck Art. 15389) using a solvent system composed of ethylacetate - methanol (1 : 1) to give a broad spot at Rf 0 ~ 0.25 by iodine visualization. The active compound was recovered from the silica gel plate, giving 5.0 mg of an active compound as yellow wax.

The active compound was soluble in chloroform, ethylacetate, acetone, and methanol, but almost insoluble in hexane and $H_2O$, and was optically active ($[\alpha]_D = +21.8°$ ($c = 0.66$ $CHCl_3$)). FAB mass spectrometries exhibited its ion peak $(M+H)^+$ at $m/z$ 318, suggesting that the molecular formula of this compound is $C_{21}H_{35}NO$. These data together with other spectroscopic analyses indicate that the compound is identical to preussin (synonymous with L-657,398), which was previously reported as an antifungal antibiotic[10,11] (Fig. 1). Preussin is structurally related to anisomycin, an anti-protozoan and anti-yeast compound isolated from *Streptomyces griseolus* and *Streptomyces roseochromogenes*[12]. Anisomycin was reported to be an inhibitor of protein synthesis in certain yeasts and mammalian cells[13]. However, anisomycin caused strong growth inhibition on all the strains tested, suggesting that the target molecule of preussin is different from that of anisomycin (data not shown).

Minimal inhibitory concentrations (MICs) of preussin against *S. pombe cdc* mutants were determined by a conventional agar dilution method. As shown in Table 1, the MICs of preussin for *cdc13*, *cdc25*, *wee1*, and *wee1*-disrupted strains were 24 ng/ml, 12 ng/ml, 391 ng/ml, and 196 ng/ml, respectively. These mutants were 100 to 1000-fold more sensitive to preussin compared with wild-type *S. pombe*, since the MIC of preussin for the wild-type strain was 12.5 μg/ml. To determine whether

Fig. 1. Chemical structure of preussin.

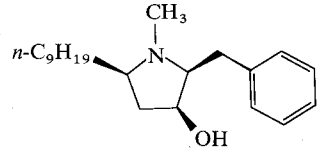


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Table 1. Minimal inhibitory concentrations of preussin against the fission yeast *cdc* mutants.

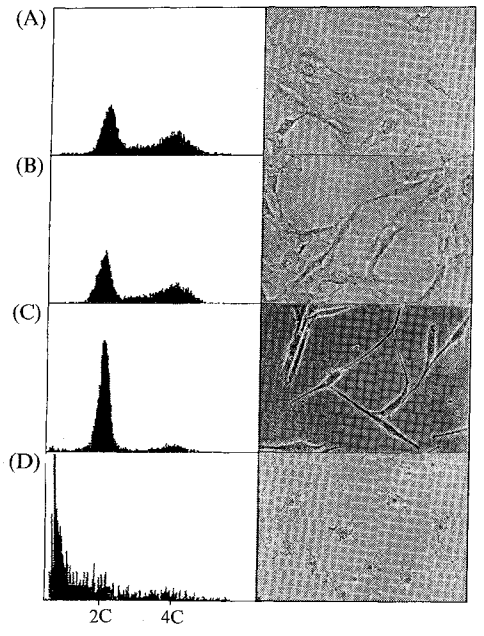
| | MIC (μg/ml) | | MIC (μg/ml) |
|---|---|---|---|
| Wild type | 12.5 | *cdc13* | 0.024 |
| *cdc1* | 12.5 | *cdc14* | 6.25 |
| *cdc2* | 6.25 | *cdc15* | 0.781 |
| *cdc3* | 6.25 | *cdc16* | 12.5 |
| *cdc4* | 0.781 | *cdc17* | 12.5 |
| *cdc5* | 1.56 | *cdc18* | 12.5 |
| *cdc6* | 6.25 | *cdc19* | 6.25 |
| *cdc7* | 6.25 | *cdc20* | 12.5 |
| *cdc8* | 6.25 | *cdc21* | 12.5 |
| *cdc9* (*wee1*) | 0.391 | *cdc22* | 12.5 |
| *cdc10* | 12.5 | *cdc23* | 6.25 |
| *cdc11* | 12.5 | *cdc25* | 0.012 |
| *cdc12* | 6.25 | *Δwee1* | 0.196 |

the supersensitivity of these mutants to preussin is due to the respective *cdc* gene mutations, we analyzed the phenotypes of their tetrads crossed with the wild-type strain. All the *ts* segregants were supersensitive to preussin. These results showed that the *cdc* mutations themselves were responsible for the preussin sensitivity.

Cdc2 is a serine/threonine kinase that acts as a major regulator of the eukaryotic cell cycle. In *S. pombe*, *cdc2* regulates the initiation of both S and M phases and its timing of activation is strictly regulated by *cdc13*, *cdc25*, and *wee1* gene products[6]. We therefore tested whether preussin directly inhibited Cdc2 kinase activity of *S. pombe*. The active Cdc2/Cdc13 complex was extracted by using $p13^{suc1}$ beads[14], and the effect of preussin on the kinase activity was determined by incorporation of [$^{32}$P] into histone H1 from [$^{32}$P-$\gamma$] ATP, as described previously[15]. The catalytic activity of Cdc2 was not inhibited by preussin even at very high concentrations (*i.e.*, greater than 250 μg/ml, data not shown). The effects on Cdc25 phosphatase and Wee1 kinase were determined by using recombinant proteins of their human homologs[16,17], since they possess essentially the same activity as those from *S. pombe*[9]. The C-terminal kinase domain of human Wee1 was produced in *E. coli* and used for Wee1 kinase assay[17]. The recombinant protein produced as an inclusion body was solubilized with 6 M urea, separated by DEAE chromatography, and refolded with a step-wise dilution of urea. The activity of Wee1 kinase was assayed by phosphorylation of human Cdc2-cyclin B complex or auto-phosphorylation. Human Cdc25 was expressed as a fusion protein with glutathion *S*-transferase (GST) in *E. coli*, and purified with a glutathion-Sepharose column (12 × 100 mm)[16]. Similarly to the effect on Cdc2 kinase, preussin did not inhibit their activity *in vitro* at high concentrations more than 50 μg/ml (data not shown). We therefore concluded that Cdc2 kinase, Cdc25 phosphatase, or Wee1 kinase is not the direct target of preussin. However, it is still possible that preussin may interfere with the *in vivo* activation of these regulators. It is also possible that preussin may affect the stability of the multi-protein complex containing Cdc2 and the *ts* regulators.

Fig. 2. Effect of preussin on 3Y1 cells.

A, control cells without treatment; B, 1 μg/ml preussin; C, 10 μg/ml preussin; D, 100 μg/ml preussin.




Normal rat fibroblast 3Y1 cells arrested at the early S phase were released by removing hydroxyurea and treated with various concentrations of preussin for 24 hours. After taken the phase contrast photographs of each culture under a light microscope (right panel), the cells were collected and the cellular distribution of DNA contents was measured with a flow cytometer (left panel).

Preussin caused growth inhibition not only of *S. pombe* but also mammalian cells. The $IC_{50}$ of preussin for normal rat fibroblast 3Y1 cells was determined to be 5 μg/ml on the basis of the total cell number after treatment for 3 days. Strong cytotoxic activity was observed when more than 31.7 μg/ml of preussin was added to the exponentially growing culture of 3Y1 cells. The effect of preussin on the mammalian cell cycle was analyzed by determining the distribution of cellular DNA contents in the early S phase synchronous culture of 3Y1 cells by flow cytometry[18~20] (Fig. 2). Fig. 2A shows a typical profile of DNA content distribution obtained with a control culture 24 hours after hydroxyurea removal. While the cytogram obtained with the culture treated with 1 μg/ml of preussin was almost the same as that of control, accumulation of the cells with 2C DNA content was observed when the cells had been treated with 10 μg/ml of preussin (Fig. 2B, C). Cell elongation was observed in the arrested cell culture. Exposure to 100 μg/ml of preussin caused marked fragmentation of nuclei, suggesting that the high concentration of preussin induces apoptosis in rat 3Y1 fibroblasts. These results indicate that preussin inhibits G1 progression of the mammalian cell cycle in a relatively narrow concentration range and

causes a cytotoxic effect at a high concentration.

The relationship between selective growth inhibition in *S. pombe cdc* mutants and the G1 arrest in mammalian cells is unclear. In mammalian cells, there are multiple cyclin-dependent kinases (CDKs) structurally similar to Cdc2, and most of them are required for G1 progression[2,21,22]. It is therefore conceivable that G1 cyclin/CDKs-regulatory cascade is more sensitive to preussin than G2 cyclin/Cdc2 cascade in mammalian cells. Further studies are needed for elucidation of molecular mechanism by which preussin induces selective growth inhibition of *S. pombe cdc* mutants and G1 arrest in mammalian cells.

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