VOL. 57 NO. 7, JULY 2004 THE JOURNAL OF ANTIBIOTICS pp. 415 — 420

Fungerin, a Fungal Alkaloid, Arrests the Cell Cycle in M Phase by Inhibition of Microtubule Polymerization

YUKIO KOIZUMI, MASAYOSHI ARAI, HIROSHI TOMODA* and SATOSHI ŌMURA

Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences,
Kitasato University and The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

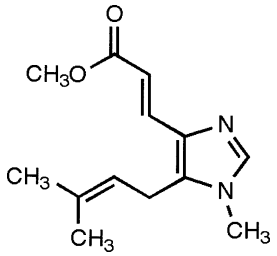
(Received for publication March 26, 2004)

A fungal alkaloid fungerin was found to arrest the cell cycle of Jurkat cells at the G_2/M phase, then to induce apoptosis. Immunoblotting showed that fungerin led to hyperphosphorylation for Cdc25C and dephosphorylation of Cdc2, indicating that the compound arrests the cell cycle at the M phase. Moreover, fungerin inhibited the polymerization of microtubule proteins *in vitro*. It was concluded that fungerin arrests the cell cycle at the M phase through inhibition of microtubule polymerization.

Microtubules are the main component of spindles in the mitotic apparatus of eukaryotic cells, and are also involved in many cellular functions, such as axonal transport, motility and determination of cell shape. Microtubules are cylindrical fibers consisting of a heterodimer of α- and β-tubulin as the major component and microtubule associated proteins (MAPs) as the minor ones[1]. There are a number of natural and synthetic compounds that interfere with the function of microtubules, causing the arrest of cell cycle at the M phase. These compounds are useful for medicinal and agrochemical purposes such as anticancer, antifungal or anthelmintic agents, and for biochemical and cytological probes[1,2].

During the course of searching for microbial metabolites that affect the cell cycle status, we have found that fungerin (Fig. 1) from a culture of *Metarhizium* sp. FKI-1079 arrests the cell cycle at G_2/M phase. Fungerin was originally reported as an antifungal imidazole derivative produced by *Fusarium* sp. and was established as 3-[1-methyl-5-(3-methyl-2-butenyl)-1*H*-imidazol-4-yl]-2(*E*)-propenoic acid methyl ester[3]. Independently, visoltricin, a fungerin isomer with a structure of 3-[1-methyl-4-(3-methyl-2-butenyl)-1*H*-imidazol-5-yl]-2(*E*)-propenoic acid methyl ester, was isolated from a culture of *Fusarium tricinctum* and showed various biological activities such as toxicity to *Artemia salina*, growth inhibition of human tumor cell lines, miotic effect on rabbit eye and anti-cholinesterase activity[4,5]. However, a recent synthetic report proved that visoltricin was identical to fungerin, and the structure of visoltricin was revised[6]. Therefore, the reported biological activities of visoltricin were due to fungerin. In this study, we investigated the mechanism of the cell cycle arrest by fungerin and found that fungerin inhibits the polymerization of microtubule proteins.

Fig. 1. Structure of fungerin.



Materials and Methods

Chemicals

Fungerin was isolated from a culture of *Metarhizium* sp.

* Corresponding author: tomoda@lisci.kitasato-u.ac.jp

FKI-1079. Briefly, the culture broth, which was fermented under stationary condition, was extracted with EtOH. The EtOH extract was concentrated and re-extracted with EtOAc. The resulting extract was subjected to chromatography on silica gel. Then, an active fraction was washed with hexane, and an insoluble fraction was dissolved with $CHCl_3$. The soluble fraction containing fungerin was separated by preparative HPLC (ODS), giving pure fungerin. Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). Colchicine was obtained from Sigma (St. Louis, MO). Other reagents were commercially available analytical grade products.

Cell Culture

Jurkat cells, from a human T cell leukemia, and 3T3 cells, mouse fibroblasts, were cultured in RPMI-1640 medium and DMEM, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified chamber at 37°C containing 5% CO_2.

Flow Cytometry

Jurkat cells (1×10^5 cells) were incubated in 96-well plates with the indicated concentration of fungerin at 37°C for various time periods. After removal of conditioned medium, cells were resuspended in 200 μl of 50 μg/ml propidium iodide, 20 μg/ml RNase A, 0.1% (w/v) sodium citrate and 0.3% (w/v) Nonident P-40, and incubated at room temperature for 2 hours. DNA content was determined using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Immunoblotting

Jurkat cells (3×10^6 cells) were incubated in the presence or absence of the drugs for 20 hours. After incubation, cells were washed twice with PBS (20 mM NaPi and 150 mM NaCl) and lysed in 500 μl of lysis buffer (50 mM Tris-HCl (pH 7.4) and 150 mM NaCl containing 1% (w/v) Nonident P-40, 0.25% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM Na_3VO_4 and 1 mM NaF) at 4°C for 30 minutes. Cell lysates were clarified by centrifugation, and the resultant supernatants were used for protein quantitation using the BCA protein assay reagents (Pierce, Rockford, IL). Twenty μg of each cell lysate was separated by 12.5% SDS-PAGE. After electrophoresis, the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked in 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl containing 0.1% (w/v) Tween 20 and 5% (w/v) BSA for 1 hour and then probed with 1 μg/ml of anti-Cdc25C (H-6, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cdc2 (A17.1.1, Oncogene Research Products, Boston, MA) or anti-actin (C-2, Santa Cruz Biotechnology) antibody for 1 hour. After washing, the membranes were incubated with 0.2 μg/ml of peroxidase-conjugated anti-mouse antibody (Sigma) for 1 hour. Then, membranes were washed, and proteins were subsequently detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence

3T3 cells (5×10^3 cells) were cultured on 8-chamber slides (Nalge Nunc International, Naperville, IL) for 20 hours, and then incubated for 5 hours in the presence or absence of the drugs. After removal of the medium, cells were fixed in 3.7% (w/v) paraformaldehyde for 30 minutes. After washing, cells were permeabilized with 0.1% (w/v) Triton X-100 in PBS containing 1% (w/v) BSA for 1 hour. Cells were then washed, and incubated with 36 μg/ml of FITC-conjugated anti-α-tubulin antibody (Sigma) and 33 nM Alexa Fluor 594 phalloidin (Molecular Probes, Eugene, OR) in PBS for 1 hour in the dark. The cells were washed and mounted with cover glass using aqueous mounting medium (Daido Sangyo, Tokyo, Japan). The cytoskeltons were imaged using a Confocal Laser Scanning Microscope TCS NT (Leica Microsystems AG, Wetzlar, Germany).

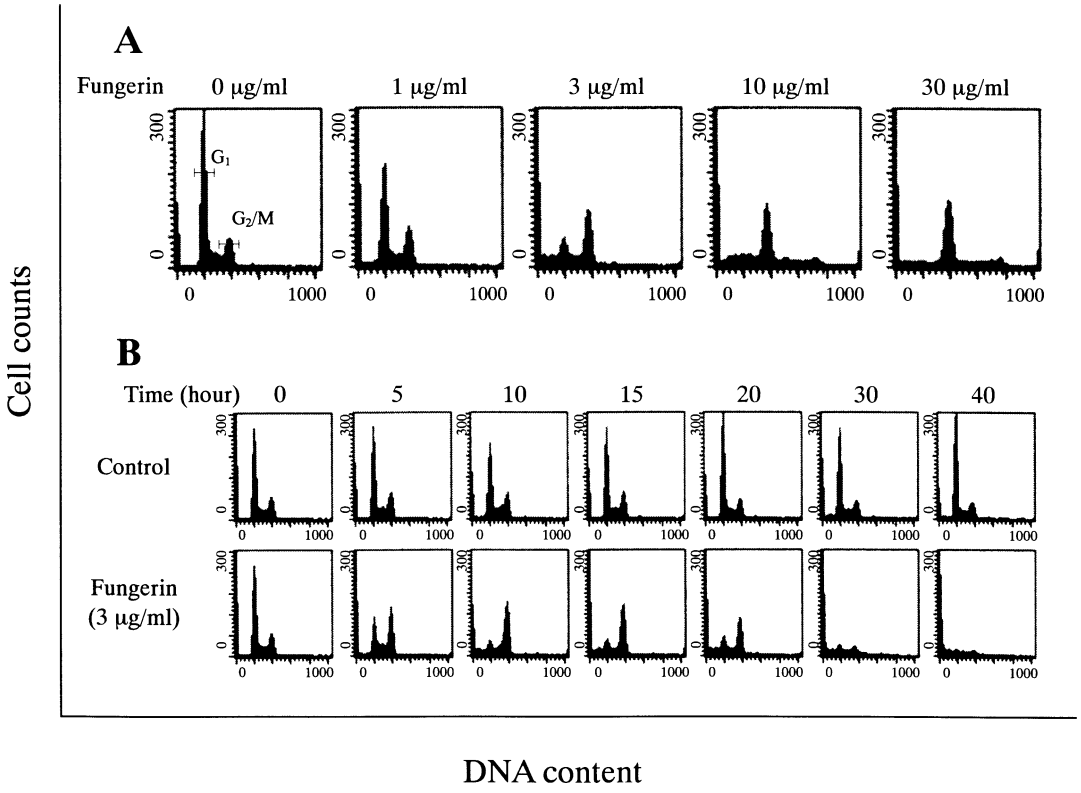
Microtubule Polymerization Assay

Polymerization assays for microtubule proteins were performed using the CytoDYNAMIX Screen kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions. Briefly, microtubule proteins (2 mg/ml) were incubated at 37°C in the presence or absence of the drugs, and polymerization was detected by measuring the change in absorbance at 340 nm.

Results

We examined the effects of fungerin on the cell cycle of human T cell leukemia Jurkat cells using a flow cytometer. Jurkat cells were treated with various concentrations of fungerin for 20 hours, and stained with propidium iodide for cell cycle analysis. Accumulation at the G_2/M phase was observed by treatment with 3 μg/ml fungerin, and the maximum effect of fungerin was observed at 10~30 μg/ml (Fig. 2A). The G_2/M arrest by this alkaloid increased time-dependently from treatment times of 0 to 15 hours,

Fig. 2. Effect of fungerin on cell cycle progression.



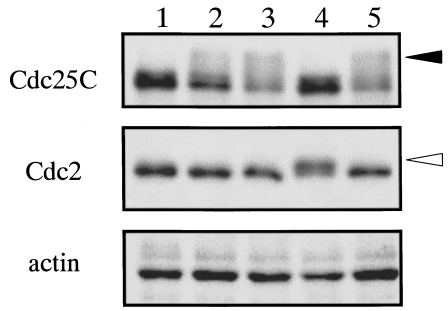
Jurkat cells were incubated in 96-well plates with fungerin at the indicated concentrations for 20 hours (A), or with 3 μg/ml fungerin for the indicated time periods (B). After treatment, cells were stained with propidium iodide, and cell cycle analysis performed using a flow cytometer.

followed by a decrease in the number of G_2/M arrest cells and an increase in sub G_1 cells, indicative of apoptotic cells (Fig. 2B). In contrast, control cells showed almost the same histogram for 0 to 40 hours (Fig. 2B).

Although the cell cycle analysis showed that fungerin arrested the cell cycle at the G_2/M phase, it was unclear whether this arrest occurs in the G_2 or M phase. The progression from G_2 to M phase requires activation of the Cdc2 and cyclin B complex[7]. The activation of Cdc2/cyclin B is regulated by dephosphorylation of Cdc2 catalyzed by Cdc25C phosphatase at the end of G_2 phase[8~12]. The Cdc25C activity is increased during G_2/M phase[13~15], and this activation correlates with hyperphosphorylation, which may in part be attributed to a positive feedback loop involving Cdc2/cyclin B[16~21]. Therefore, the phosphorylation levels of both Cdc2 and Cdc25C can show that the cell cycle status is in either G_2 or M phase. To determine the cell cycle status arrested by fungerin, we examined the electrophoretic mobility reflecting the phosphorylation levels of Cdc2 and Cdc25C using immunoblotting techniques (Fig. 3). Colchicine, a M phase arrest inducer by inhibiting microtubule polymerization, promoted the band shift showing the active hyperphosphorylated form of Cdc25C, whereas bleomycin, a G_2 phase arrest inducer by DNA damage, did not. Fungerin also induced the hyperphosphorylation of Cdc25C as well as colchicine did. Although the treatment of bleomycin showed the retarded mobility of Cdc2 corresponding to the inactive phosphorylated form, the treatments of fungerin or colchicine led to the dephosphorylation of Cdc2. These results indicate that fungerin arrests the cell cycle at M phase.

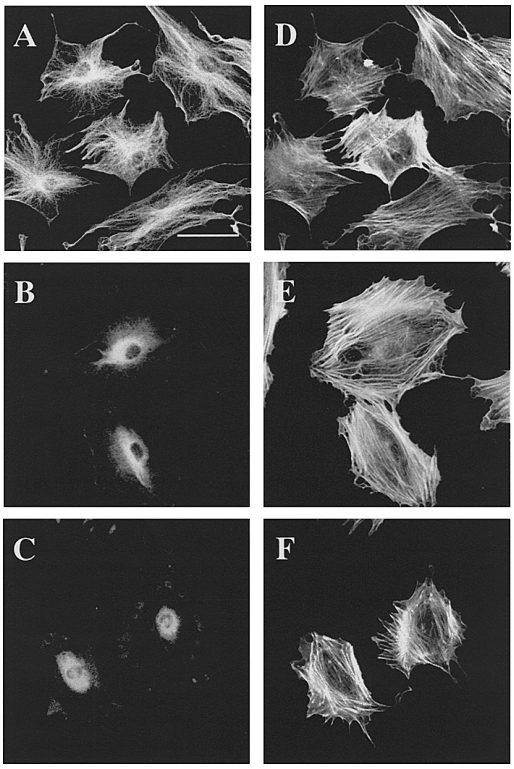
Above results indicate that the effects of fungerin on Jurkat cells resemble those of colchicine. To investigate whether fungerin shows the same action as colchicine, we examined the effect of fungerin on cytoplasmic microtubule assembly in mouse fibroblast 3T3 cells

Fig. 3. Effect of fungerin on the phosphorylation of Cdc25C and Cdc2.



Jurkat cells were incubated without (lane 1) or with 1 μg/ml fungerin (lane 2), 3 μg/ml fungerin (lane 3), 60 μg/ml bleomycin (lane 4) or 0.2 μg/ml colchicine (lane 5) for 20 hours. After incubation, cells were lysed and separated on 12.5% SDS-PAGE followed by immunoblotting with anti-Cdc25C, anti-Cdc2 or anti-actin antibody. Proteins were detected using the ECL system. Filled arrowhead and open arrowhead indicate the hyperphosphorylated form of Cdc25C and the phosphorylated form of Cdc2, respectively.

Fig. 4. Effect of fungerin on microtubule assembly in 3T3 cells.



3T3 cells were cultured on 8-chamber slides for 20 hours, and then incubated for 5 hours without (A and D), or with 10 μg/ml fungerin (B and E) or 0.2 μg/ml colchicine (C and F). After removal of the medium, cells were fixed and permeated. Cells were then incubated with FITC-conjugated anti-α-tubulin antibody and Alexa Fluor 594 phalloidine. Microtubules (A, B and C) and actin filaments (D, E and F) were observed using a Conforcal Laser Scanning Microscope. Bar, 40 μm.

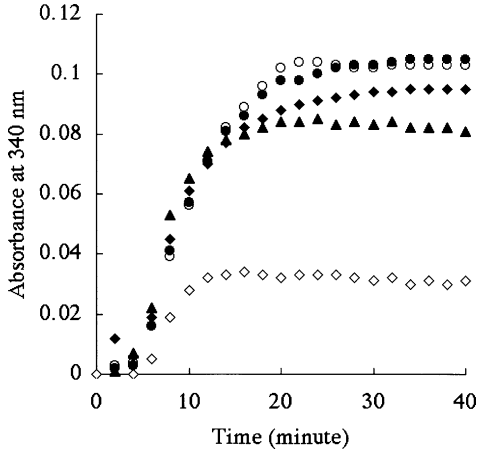
by immunofluorescence staining with FITC-conjugated anti-α-tubulin antibody. The microtubule assembly of control cells was clearly visible (Fig. 4A), whereas fungerin completely disrupted microtubule assembly, as did colchicine (Figs. 4B and 4C). Fungerin did not affect actin filaments, different cytoskeletal proteins, in 3T3 cells (Fig. 4E).

Next, we investigated whether fungerin affects microtubule polymerization *in vitro*. After the addition of fungerin to depolymerized microtubule proteins, we chased the changes in absorbance at 340 nm showing polymerization levels at 37°C (Fig. 5). Fungerin inhibited 7% and 22% at the concentration of 30 μg/ml and 100 μg/ml, respectively, whereas colchicine inhibited 66% at the concentration of 1 μg/ml. Thus, we concluded that fungerin arrests the cell cycle at the M phase through inhibition of microtubule polymerization.

Discussion

It was reported previously that the tumor cell growth was inhibited by visoltricin, which was produced by *Fusarium* sp.[4,5]. In this study, we found that fungerin produced by *Metarhizium* sp. arrested cell cycle at G_2/M phase and, subsequently induced apoptotic cell death. Gel shift mobilities of Cdc2 and Cdc25C that play a critical role in the cell cycle progression from G_2 to M phase indicated that the arrest by fungerin was M phase but not G_2 phase. A number of well-known natural or synthetic M phase inhibitors affect the dynamics of cellular microtubules. Although much higher concentration is required as compared with colchicine or vinblastine, fungerin inhibited the polymerization of microtubules in cytoplasm and *in vitro*. The difference of inhibitory concentrations between two microtubules assays was observed as well as other mitotic inhibitors. Since the concentration required for M phase arrest was almost equal to that of cytoplasmic

Fig. 5. Effects of fungerin on polymerization of microtubule proteins.



Polymerization assay for microtubule proteins was performed using commercial kits. Microtubule proteins (2 mg/ml) containing 2% (v/v) DMSO were incubated at 37°C with or without fungerin. Polymerization was detected by measuring the change in absorbance at 340 nm. Final concentrations of fungerin and colchicine were 0 (○), 10 (●), 30 (◆) or 100 μg/ml (▲) and 1 μg/ml (◇), respectively. The value of IC_{50} of colchicine was 1.8 μg/ml in this assay (data not shown).

microtubule disassembly, fungerin may arrest at the M phase by the prevention of the function of spindle that mainly consists of microtubules. The binding site of fungerin in microtubules will be reported in due course.

Fungerin is the unique structure where an isoprene unit and a methyl group bind to methyl ester derivative of urokanic acid, probably derived from histidine. Although some (iso)prenylated fungal metabolites that inhibit microtubules polymerization were reported recently (phenylahistin[22,23], tryprostatin[24,25] and oxaline[26,27]), fungerin is the simplest structure in them. Since the presence of an isoprene unit is necessary for the expression of biological activities for phenylahistin and tryprostatin[28,29], it might be that the existence of the isoprene unit for fungerin is also crucial for the activity. Moreover, it was reported that the methylation at N1 of imidazole ring of phenylahistin decreased the activities[28], so it will be interesting to synthesize N1-demethyl fungerin and to test its biological activities.

This report indicated the mechanism of action by which fungerin, which has a simple structure, arrests the cell cycle at the M phase. Further study to optimize the inhibitory activity of microtubules polymerization by fungerin might lead to the development of medicinal or agrochemical drugs.

Acknowledgements

The authors wish to thank Dr. SHIGEO IWASAKI for valuable discussion. This study was supported in part by a grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1) IWASAKI, S.: Antimitotic agents: chemistry and recognition of tubulin molecule. Med. Res. Rev. 13: 183～198, 1993
2) CORREIA, J. J.: Effects of antimitotic agents on tubulin-nucleotide interactions. Pharmacol. Ther. 52: 127～147, 1991
3) KATO, Y.; H. KOSHINO, J. UZAWA & K. ANZAI: Fungerin, a new antifungal alkaloid from *Fusarium* sp. Biosci. Biotech. Biochem. 60: 2081～2083, 1996
4) VISCONTI, A. & M. SOLFRIZZO: Isolation, characterization, and biological activity of visoltricin, a novel metabolite of *Fusarium tricinctum*. J. Agric. Food. Chem. 42: 195～199, 1994
5) VISCONTI, A. & M. SOLFRIZZO: Visoltricin, a novel biologically active compound produced by *Fusarium tricinctum*. Food Addit. Contam. 12: 515～519, 1995
6) RIEDER, J. M. & J. LEPSCHY: Synthesis of visoltricin and fungerin: imidazole derivatives of *Fusarium* sp. Tetrahedron Lett. 43: 2375～2376, 2002
7) SMITH, V. A. J. & R. H. MEDEMA: Checking out the G_2/M transition. Biochim. Biophys. Acta 1519: 1～12, 2001
8) STRAUSFELD, U.; J. C. LABBÉ, D. FESQUET, J. C. CAVADORE, A. PICARD, K. SADHU, P. RUSSELL & M. DORÉE: Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex *in vitro* by human CDC25 protein. Nature 351: 242～245, 1991
9) DUNPHY, W. G. & A. KUMAGAI: The cdc25 protein contains an intrinsic phosphatase activity. Cell 67: 189～196, 1991
10) GAUTIER, J.; M. J. SOLOMON, R. N. BOOHER, J. F. BAZAN & M. W. KIRSCHNER: cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell 67: 197～211, 1991
11) MILLAR, J. B. A.; C. H. MCGOWAN, G. LENAERS, R. JONES & P. RUSSELL: $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. EMBO J. 10: 4301～4309, 1991
12) LEE, M. S.; S. OGG, M. XU, L. L. PARKER, D. J. DONOGHUE, J. L. MALLER & H. PIWNICA-WORMS: $Cdc25C^+$ Encodes a protein phosphatase that dephosphorylates $p32^{cdc2}$. Mol. Biol. Cell 3: 73～84, 1992
13) DUCOMMUN, B.; G. DRAETTA, P. YOUNG & D. BEACH: Fission yeast cdc25C is a cell-cycle regulated protein. Biochem. Biophys. Res. Commun. 167: 301～309, 1990
14) KUANG, J.; C. L. ASHORN, M. GONZALEZ-KUYVENHOVEN & J. E. PENKALA: cdc25 is one of the MPM-2 antigens involved in the activation of maturation-promoting

factor. Mol. Biol. Cell 5: 135～145, 1994

15) HARTLEY, R. S.; R. E. REMPEL & J. L. MALLER: *In vivo* regulation of the early embryonic cell cycle in *Xenopus*. Dev. Biol. 173: 408～419, 1996

16) KUMAGAI, A. & W. G. DUNPHY: Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell 70: 139～151, 1992

17) IZUMI, T.; D. H. WALKER & J. L. MALLER: Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. Mol. Biol. Cell 3: 927～939, 1992

18) HOFFMANN, I.; P. R. CLARKE, M. J. MARCOTE, E. KARSENTI & G. DRAETTA: Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. 12: 53～63, 1993

19) IZUMI, T. & J. L. MALLER: Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. Mol. Biol. Cell 4: 1337～1350, 1993

20) STRAUSFELD, U.; A. FERNANDEZ, J. P. CAPONY, F. GIRARD, N. LAUTREDOU, J. DERANCOURT, J. C. LABBE & N. J. LAMB: Activation of $p34^{cdc2}$ protein kinase by microinjection of human cdc25C into mammalian cells. Requirement for prior phosphorylation of cdc25C by $p34^{cdc2}$ on sites phosphorylated at mitosis. J. Biol. Chem. 269: 5989～6000, 1994

21) GABRIELLI, B. G.; J. M. CLARK, A. K. MCCORMACK & K. A. ELLEM: Hyperphosphorylation of the N-terminal domain of Cdc25 regulates activity toward cyclin B1/Cdc2 but not cyclin A/Cdk2. J. Biol. Chem. 272: 28607～28614, 1997

22) KANOH, K.; S. KOHNO, T. ASARI, T. HARADA, J. KATADA, M. MURAMATSU, H. KAWASHIMA, H. SEKIYA & I. UNO: (−)-Phenylahistin: A new mammalian cell cycle inhibitor produced by *Aspergillus ustus*. Bioorg. Med. Chem. Lett. 7: 2847～2852, 1997

23) KANOH, K.; S. KOHNO, J. KATADA, J. TAKAHASHI & I. UNO: (−)-Phenylahistin arrests cells in mitosis by inhibiting tubulin polymerization. J. Antibiotics 52: 134～141, 1999

24) CUI, C.-B.; H. KAKEYA, G. OKADA, R. ONOSE, M. UBUKATA, I. TAKAHASHI, K. ISONO & H. OSADA: Tryprostatins A and B, novel mammalian cell cycle inhibitors produced by *Aspergillus fumigatus*. J. Antibiotics 48: 1382～1384, 1995

25) USUI, T.; M. KONDOH, C.-B. CUI, T. MAYUMI & H. OSADA: Tryprostatin A, a specific and novel inhibitor of microtubule assembly. Biochem. J. 333: 543～548, 1998

26) NAGEL, D. W.; K. G. R. PACHLER, P. S. STEYN, P. L. WESSELS, G. GAFNER & G. J. KRUGER: X-Ray structure of oxaline: a novel alkaloid from *Penicillium oxalicum*. J. Chem. Soc. Chem. Comm. 1021～1022, 1974

27) KOIZUMI, Y.; M. ARAI, H. TOMODA & S. ŌMURA: Oxaline, a fungal alkaloid, arrests the cell cycle in M phase by inhibition of tubulin polymerization. Biochim. Biophys. Acta Mol. Cell Res. *In press*

28) KANOH, K.; S. KOHNO, J. KATADA, J. TAKAHASHI, I. UNO & Y. HAYASHI: Synthesis and biological activities of phenylahistin derivatives. Bioorg. Med. Chem. 7: 1451～1457, 1999

29) SANZ-CERVERA, J. F.; E. M. STOCKING, T. USUI, H. OSADA & R. M. WILLIAMS: Synthesis and evaluation of microtubule assembly inhibition and cytotoxicity of prenylated derivatives of cyclo-L-Trp-L-Pro. Bioorg. Med. Chem. 8: 2407～2415, 2000