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Screening of Cell Cycle Inhibitors from Microbial Metabolites by a Bioassay Using a Mouse cdc2 Mutant Cell Line, tsFT210

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Abstract—We have established a bioassay system using a mouse cdc2 mutant cell line, tsFT210, to detect inhibitors of the mammalian cell cycle. When cultured at the high temperature, restrictive temperature at 39.4 °C, tsFT210 cells can be arrested at G2 phase and are large in size. Four hours after release from G2 arrest, the cells entered into the G1 phase. At this time, G1 phase cells were easily discriminated from the G2/M-cells by their size under microscopic observation. The cell-morphology-based bioassay utilizing tsFT210 cells is very simple and sensitive for detecting cdc2 kinase inhibitors and also G2/M-phase inhibitors of the mammalian cell cycle. To demonstrate the merits of this bioassay, the effects of protein kinase inhibitors isolated from actinomycetes were investigated. RK-286C and RK-1409, which are structurally related to staurosporine, inhibited cell cycle progression at the G2 phase in both G2-synchronized and nonsynchronized cultures of tsFT210 cells. Another kinase inhibitor, sangivamycin, inhibited cell cycle progression at the G2 phase of cells released from temperature arrest but did not inhibit that of the exponentially growing cells. Using the bioassay system, we carried out screening of the cell cycle inhibitors from the microbial metabolites and have discovered several new inhibitors, including novel compounds such as tryprostatins A, B and acetophthalidin. Thus, this bioassay allowed for the detection of cell cycle inhibitors and provided a convenient and useful method for the screening of new inhibitors from the microbial metabolites. Copyright © 1997 Elsevier Science Ltd

Introduction

Mammalian cdc2 kinase is a key enzyme which regulates the cell cycle transition from G2- to M-phase.[1–3] Cdc2 kinase itself is regulated by a complex series of phosphorylation events[4–6] and by its association with cyclin B.[7,8] The complex of cdc2 kinase and cyclin B is called maturation/M-phase promoting factor (MPF). As MPF plays a central role in the mitotic progression of mammalian cells, it is worthwhile investigating the regulation mechanism of its activation.

In general, the biological function of a particular cellular factor can be elucidated by two major approaches. One approach is genetic through the isolation of defective mutants; the other approach is biochemical through the development or application of specific inhibitors. These two strategies are complementary in understanding the biological function of cellular factors. For example, analysis of tsFT210, a mouse temperature-sensitive mutant with a defect in the *cdc2* gene, demonstrated that the cdc2 kinase is essential for triggering mitosis.[9–11] Similarly, there are also many reports which describe the application of inhibitors in elucidating the regulatory mechanism of the cell cycle.[12–15] Among them, it was shown that a protein kinase inhibitor, staurosporine, inhibits the cell cycle progression at G2 phase through the inhibition of cdc2 kinase.[14,16] Based on these recent findings, we have established a simple and rapid bioassay system utilizing the temperature sensitive mutant, tsFT210 cells, to detect cell cycle inhibitors from the microbial metabolites.

Using this bioassay system, we screened cell cycle inhibitors from microbial metabolites, and we have identified several new microbial inhibitors of the mammalian cell cycle. In this paper, we describe the bioassay method in detail and several new inhibitors discovered from the microbial metabolites by using the present bioassay method.

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Materials and methods

Cell culture

A temperature-sensitive mutant, tsFT210,[9] which was isolated from the mouse mammary carcinoma cell line FM3A, was maintained in suspension culture in RPMI-1640 medium supplemented with 5% calf serum (HyClone Inc., Logan, UT, U.S.A.) at 32 °C. The tsFT210 cells were synchronized at the G2 phase by culture at 39.4 °C (restrictive temperature) for 17 h.

Inhibitors from microbial origin

Sangivamycin was purified from the fermentation broth of *Streptomyces* sp.[17] RK-286C,[18] RK-1409,[19] and RK-1409B[20] were isolated from actinomycetes in our laboratory. Tryprostatins A, B,[21] and acetophthalidin[22] were isolated from the fermentation broth of fungi which were collected in sea sediments. The inhibitors

were dissolved in methanol and used at a final methanol concentration of less than 1%.

Chemicals

Nocodazole (Aldrich Co., Milwaukee, WI, U.S.A.) was dissolved in methanol and added to the culture medium of tsFT210 cells. Giemsa solution, propidium iodide and histone type III-S were products of Sigma Chem Co. Ltd (St Louis, MO, U.S.A.). Wright solution and [γ-^{32}P]ATP were purchased from Wako Pure Chem. (Tokyo, Japan) and ICN Radiochemicals (Costa Mesa, CA, U.S.A.), respectively.

Bioassay procedure for screening of cell cycle inhibitors

In the asynchronous-culture assay, cells cultured at the permissive temperature of 32 °C were seeded into a 12-well plate at a density of 2×10^5 cells/mL in 1 mL of fresh medium. Then cells were treated with inhibitors and cultured for 17 h at 32 °C.

In the synchronous-culture assay, cells were seeded at a density of 2×10^5 cells/mL into a 12-well plate and were pre-incubated at 39.4 °C for 17 h to obtain G2-arrested cells. Then, 10 μL of each sample solution was added, and the cells were incubated at 32 °C for 4 h. After incubation, morphological characteristics of the cells were examined directly by microscopic observation. The cells were also subjected to flow cytometric analysis as described below to confirm the DNA contents in cells.

Flow cytometry

A flow cytometer (Profile II, Coulter Co., Hialeah, FL, U.S.A.) was used to determine the DNA content of tsFT210 cells as described by Noguchi et al.[23] Nuclei of tsFT210 cells were prepared by the treatment with detergent and stained with propidium iodide for 30 min. Cells (5×10^4) were analysed by flow cytometry, and the distribution within the cell cycle was calculated using the computing program, CytoLogic (Coulter). Inhibition rate of the cell cycle for an inhibitor at a concentration was calculated by comparison with the corresponding control data and IC_{50} value for the inhibitor was obtained from the dose–response curve of the inhibitor.

Microscope observation

In routine bioassay, morphology of tsFT210 cells cultured under various conditions in multi-well plates were directly observed under a microscope, while the cells were spread on glass slides and visualized with Wright and Giemsa stains for examining their nuclear characteristics. A microscope (TMD-2, Nikon, Tokyo, Japan) was used for morphological observations. The G2, M and G1 cells were distinguishable through the combination of flow cytometric analysis and microscopic observations.

Measurement of cdc2 kinase inhibitory activity

Cdc2 kinase activity was measured based on the methods of Arion et al.[24] Cell-extracts were prepared from tsFT210 cells (5×10^6) which were synchronized in M-phase. The cells were washed twice with phosphate buffered saline, suspended in 1 mL of homogenizing buffer; 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1% Triton X-100, 5 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM DTT, and disrupted by sonication. A clear supernatant was obtained by centrifugation ($15000 \times g$) at 4 °C for 10 min.

Sepharose beads linked to $p13^{suc1}$ were added to the tsFT210 cell lysate, and the mixture was incubated for 30 min at 4 °C to co-precipitate cdc2 kinase. The supernatant was removed by centrifugation, and the beads were washed twice with the homogenizing buffer. The precipitated beads which contained cdc2 kinase were used for the following reaction. The reaction mixture consisted of 1 mg/mL histone H1, 3.7×10^4 Bq [γ-^{32}P]ATP and various concentrations of an inhibitor in 25 μL. After 30 min incubation, the reaction was stopped by adding SDS-PAGE sample buffer[25] and boiling for 3 min. After the gel electrophoresis, the kinase activity was visualized by autoradiography and quantified by a laser densitometer (Molecular Dynamics Co. Ltd., Sunnyvale, CA, U.S.A.). The inhibition rate of the kinase activity for an inhibitor at a concentration was calculated by comparison with the kinase activity of the control, and the IC_{50} value for the inhibitor was obtained from the dose–response curve of the inhibitor.

Measurement of protein kinase inhibitory activities

Cyclic AMP dependent protein kinase A (PKA), Ca^{2+}/phospholipid-dependent protein kinase C (PKC), and casein kinase I (CK-I) were prepared from HeLa cell lysate by the procedure previously described.[26] Histone H2B, histone H1 and casein were used as substrates for PKA, PKC and CK-I in the kinase assay. IC_{50} values for PKA, PKC and CK-I were obtained by the same manner as described for cdc2 kinase.

Results

Time course of cell cycle transition from G2 to G1 phase of tsFT210 cells

At 32 °C, tsFT210 cells grew normally, with a doubling time of 14 h. When the cells were incubated for 17 h at 39.4 °C, the increase in the cell number stopped, and a significant portion of the cells became arrested at the G2-phase [Fig. 1(a)]. Cells arrested at G2-phase were noticeably larger [23.1 ± 0.7 μm average in diameter, Fig. 1(a)] than corresponding G1-cells [15.8 ± 0.6 μm, Fig. 1(d)], and G2-arrested cells contained 4C DNA [Fig. 1(a)].

After incubation at 39.4 °C for 17 h, the cells were transferred to 32 °C and the morphology of the cells as

well as the distribution of the DNA content were monitored at 0, 1, 2 and 4 h (Figs 1 and 2). Two hours after transfer to 32 °C, mitotic figures appeared (Fig. 2c) and most of the cells completed mitosis after 4 h [Figs 1(d) and 2(d)]. The incubation time and temperature were very important for reproducibility. If the temperature was below 39.4 °C, G2 arrest was incomplete. A longer incubation resulted in the irreversible G2 arrest of tsFT210 cells.

Effects of microbial inhibitors on the cell cycle of asynchronous cultures of tsFT210 cells

To investigate effects of kinase inhibitors on the mammalian cell cycle, inhibitors shown in Chart 1 were added to exponentially growing tsFT210 cells at 32 °C. As shown in Table 1, exposing tsFT210 cells to 32 μM of sangivamycin for 17 h was toxic at 32 °C (Chart 1). Lower concentrations of the inhibitor (lower than

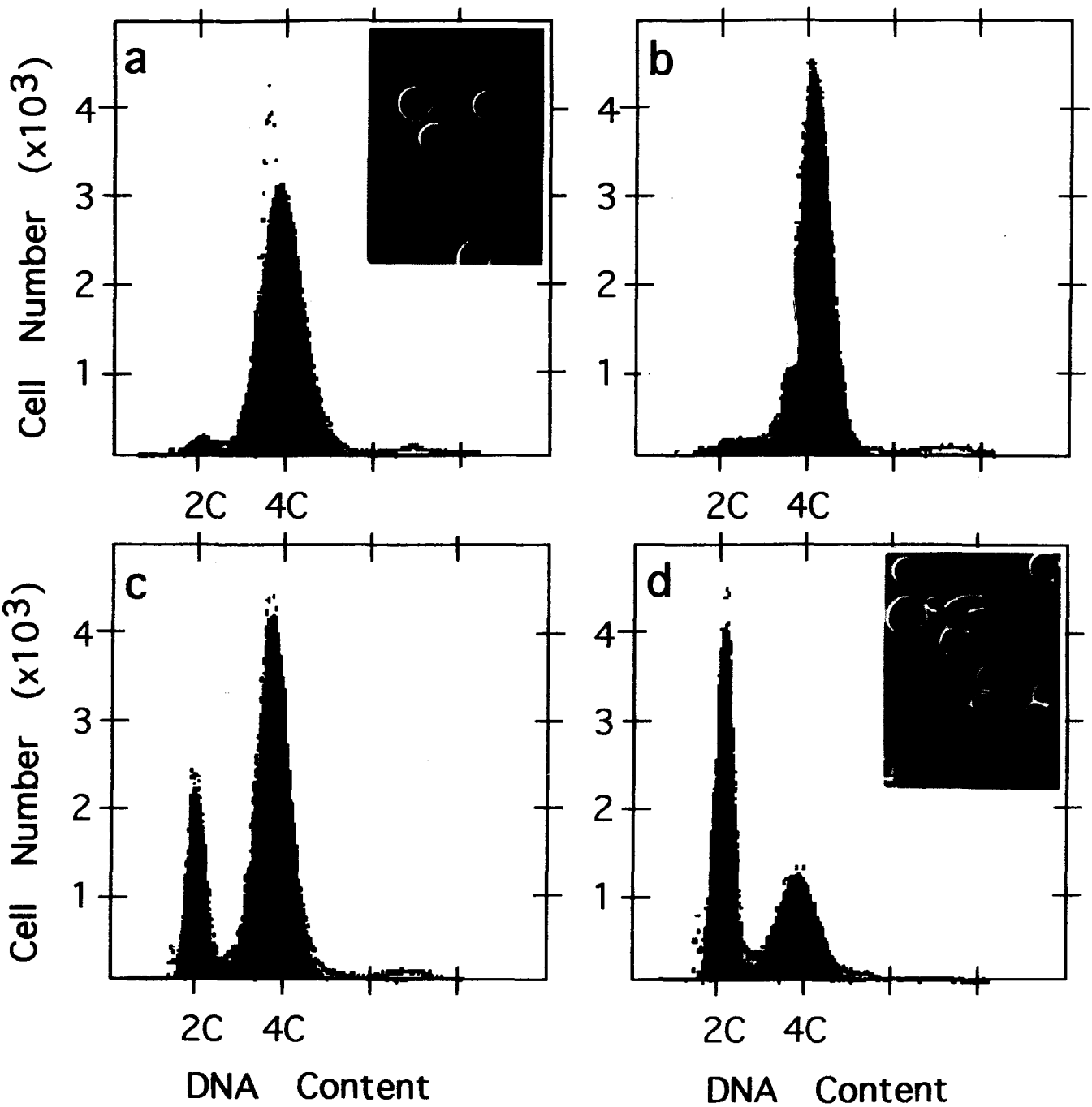


Figure 1. Time course of the cell cycle progression of tsFT210 cells from G2 to G1 phase. The tsFT210 cells were cultured at 39.4 °C for 17 h to synchronize them at the G2 phase. The cells were collected at (a) 0, (b) 1, (c) 2 and (d) 4 h after release from temperature-arrest. The DNA distribution in the cells was determined by flow cytometry. Dot lines indicate the raw data directly obtained from flow cytometry and filled figures indicate the data calculated by a computer software, M-cycle (Coulter). Photographs show morphological characteristics of the corresponding cells directly observed under a microscope without stain of the cell nuclei. Distance between two bars on the photographs is 100 μm.

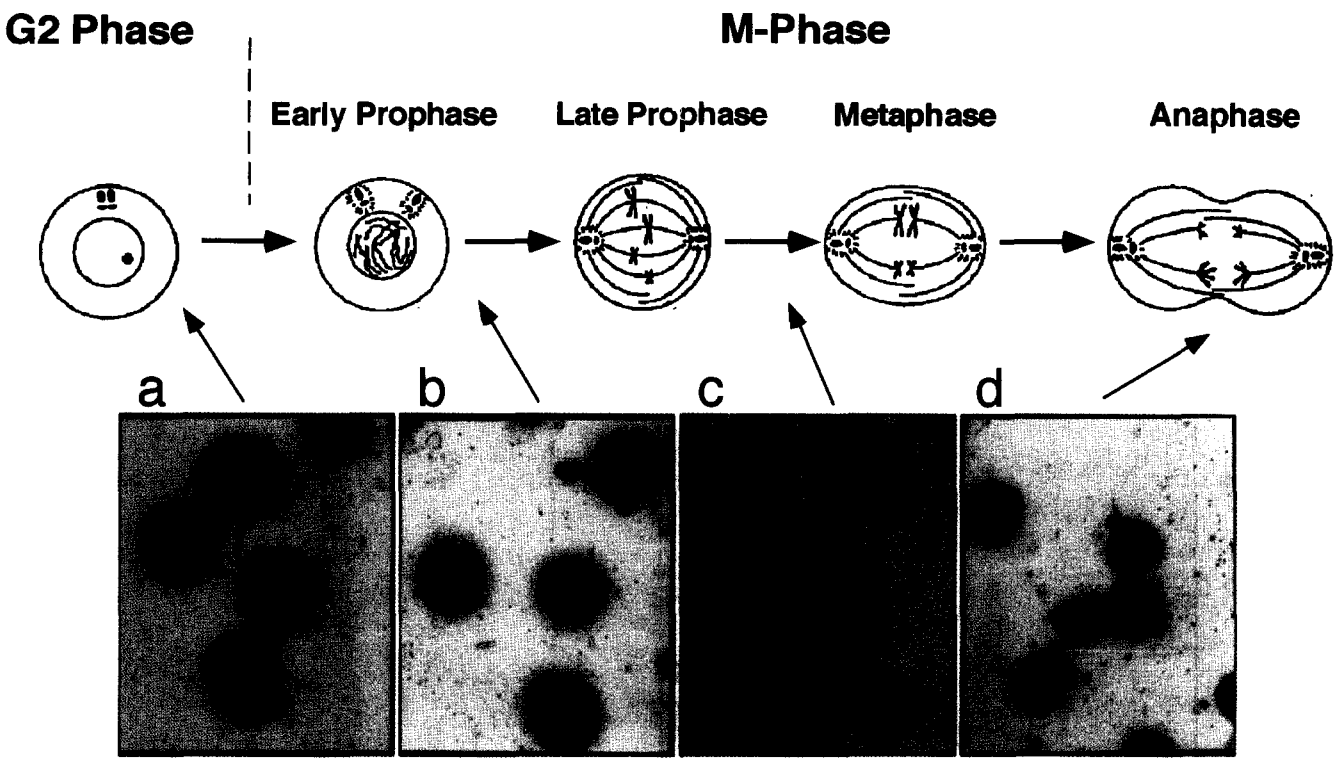


Figure 2. Morphology of tsFT210 cells. The cells were collected at (a) 0, (b) 1, (c) 2 and (d) 4 h after release from temperature-arrest. The mitotic chromosomes were observed under the microscope after visualizing the nuclei with Giemsa stain.

10 μM) retarded the cell growth but did not show a characteristic arresting point in the cell cycle [Fig. 3(b)]. On the contrary, staurosporine, RK-1409, RK-286C and RK-1409B arrested the cell cycle at G2 phase at concentrations lower than their toxic levels (Table 1). Staurosporine and RK-1409 inhibited the cell cycle at G2 phase and these cells contained 4C DNA in the nucleus surrounded by a nuclear membrane [Fig. 3(c)]. The cells treated with RK-286C or RK-1409B (3′-epimer of RK-286C) contained 4C and 8C DNA in their nuclei [Fig. 3(d)]. Morphological observations of the corresponding cells are given in Fig. 3, respectively. As cells treated with the G2 arresting compounds were large in size [Figs 3(c) and 3(d)] compared with the corresponding control cells [Fig. 3(a)], it was easy to detect the G2-arrest by microscopic observation.

Effects of kinase inhibitors on the cell cycle transition from G2 to M-phase of synchronous culture of tsFT210 cells

The cell size of tsFT210 cells cultured at 39.4 °C became large and the cell cycle was arrested at the G2-phase [Figs 2(a) and 4(b)]. When the G2 synchronized cells were transferred to 32 °C, mitosis occurred synchronously within a short time, and the cells moved

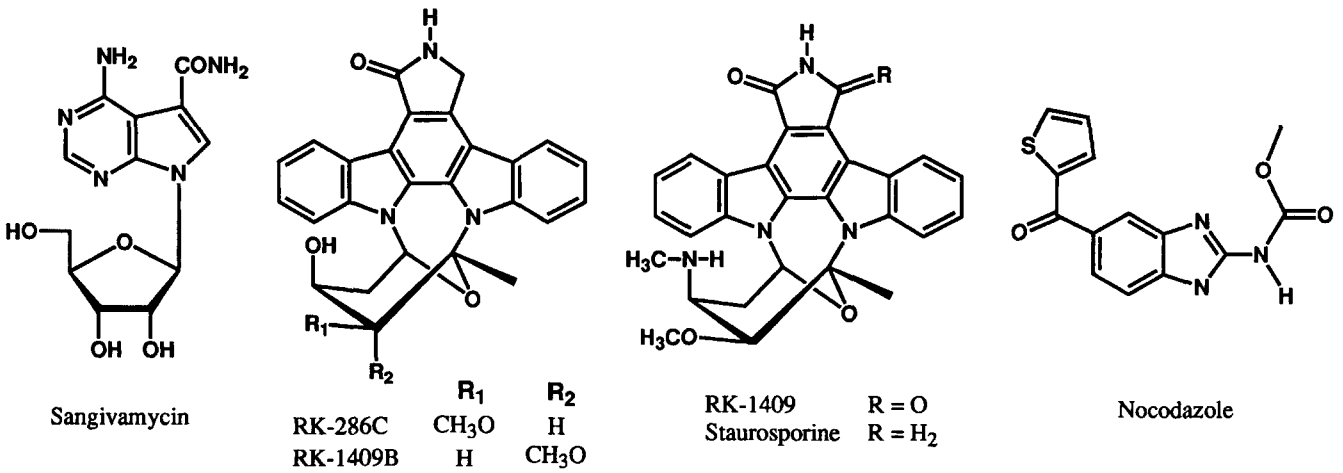


Chart 1. Structures of microbial inhibitors and nocodazole.

through M- to G1-phase [Figs 2(d) and 4(c)]. Utilizing this synchronized culture, we studied the effect of kinase inhibitors on the progression of M-phase by flow cytometry and microscopy (Fig. 4). Sangivamycin (10 μM) had no effect on the cell cycle progression of exponentially growing cells [Fig. 3(b)], however, the same concentration of the inhibitor interfered with the cell cycle transition from G2- to M-phase when arrested cells were released from the G2 block [Fig. 4(d)]. RK-1409 (0.3 μM) and RK-286C (1.6 μM) blocked the cell cycle progression at the G2/M boundary of both exponentially growing cells cultured at 32 °C [Figs 3(c) and 3(d)] and synchronized cells released from 39.4 °C [Figs 4(e) and 4(f)]. A lower concentration (31 nM) of RK-1409 did not inhibit the cell cycle of asynchronized tsFT210 cells (Table 1) but inhibited that of synchronized cells as described in the section of '*Screening for microbial cell cycle inhibitors*'.

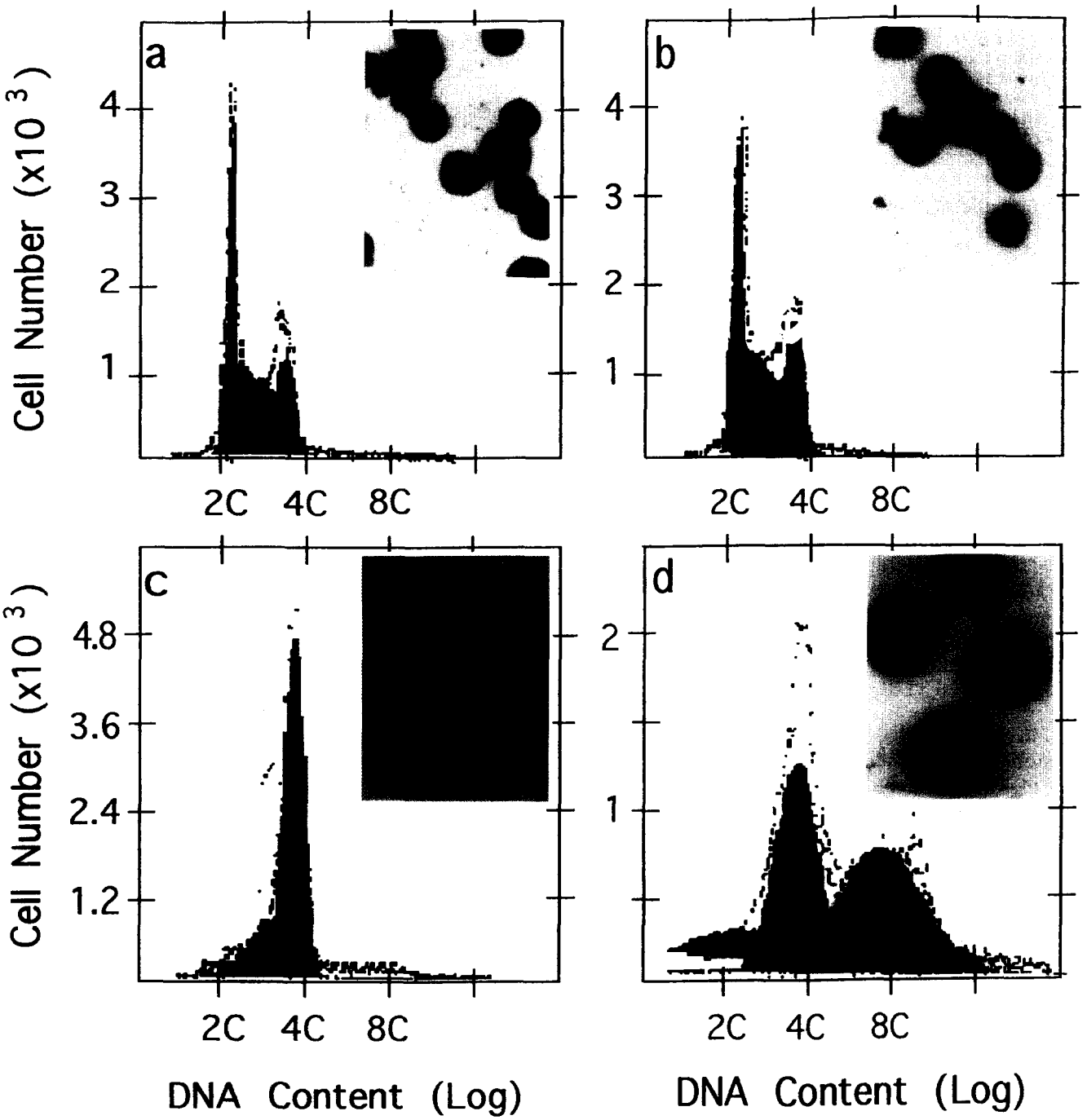


Figure 3. Effects of sangivamycin, RK-1409 and RK-286C on the cell cycle progression of tsFT210 cells cultured at 32 °C. The cells were cultured at (a) (control) 32 °C for 24 h in the presence of (b) 10 μM sangivamycin, (c) 0.3 μM of RK-1409 or (d) 1.6 μM of RK-286C under the same culture conditions. Dot lines indicate the raw data directly obtained from flow cytometry and filled figures indicate the data calculated by a computer software, M-cycle (Coulter). Photographs show morphological characteristics of the corresponding cells observed under the microscope.

Table 1. Effects of protein kinase inhibitors on cell cycle progression of tsFT210 cells at 32 °C

Inhibitors	Minimal Effective Concentration (μM)	
	Cell cycle arrest	Cytotoxicity
Sangivamycin	[a]	32
Staurosporine	0.2	0.63
RK-1409	0.3	2.2
RK-286C	1.1[b]	33
RK-1409B	8.3[b]	104
Nocodazole[c]	1.0	330

[a]Sangivamycin retarded cell growth but did not arrest cells at a specific point in the cell cycle.
[b]The inhibitors gave polyploidal cells containing 4C and 8C DNA in the nuclei of tsFT210 cells at these concentrations. Five fold higher concentrations of the inhibitors gave G2 arrested cells with 4C DNA.
[c]Nocodazole is not a protein kinase inhibitor but a chemically synthesized compound that destroys microtubule formation.

Inhibitory effects of inhibitors on protein kinases

The inhibitory activity of sangivamycin, RK-1409 and RK-286C upon various protein kinases which might be involved in the cell cycle regulation was examined (Fig. 5, Table 2). Sangivamycin showed the complete inhibition of cdc2 kinase [Fig. 5(A), lane 3] and PKC [Fig. 5(C), lane 3] activities but not PKA [Fig. 5(B), lane 3] activity at a concentration of 10 μM, which inhibited the cell cycle progression only of the synchronized tsFT210 cells. As shown in lane 9 of Fig. 5, RK-1409 at a concentration of 31 nM which inhibited also the cell cycle progression only of the synchronized tsFT210 cells showed similar inhibitory profile against these kinases like 10 μM sangivamycin. The activities of cdc2 kinase, PKA and PKC were completely inhibited by RK-1409 (Fig. 5, lane 7) and RK-286C (Fig. 5, lane 12) at concentrations of 0.3 and 3.3 μM, respectively. At the same concentration, RK-1409 and RK-286C inhibited the cell cycle both of the synchronized and asynchronized tsFT210 cells.

The effects of staurosporine and other new cell cycle inhibitors on the protein kinase activities are summarized in Table 2. Staurosporine inhibited cdc2, PKA, PKC and CK-I kinase activities at low concentrations, which inhibits cell cycle at the G2-phase, while the newly found cell cycle inhibitors, tryprostatins A and B, fumitremorgin C, demethoxyfumitremorgin C and acetophthalidin, did not show any inhibitory effects on the kinase activities examined although they inhibit the cell cycle at the M-phase.[21,22]

Screening for microbial cell cycle inhibitors

Cell cycle inhibitory effects of the inhibitors were judged by morphological characteristics of the cells observed under the microscope without any stain of the cell nuclei. In the cell-morphology-based assay, G0/G1 cells arrested by G0/G1-arresting compounds, such as cycloheximide, reveromycin, hydroxyurea and actinomycin, appeared to be small and homogenous in size [Fig. 6(d)] compared with the corresponding control cells cultured at 32 °C [Fig. 6(a)], while G2/M-cells arrested by G2/M-arresting agents to be large and homogenous in size [Figs 6(e) and 6(f)]. The results of the screening carried out by the bioassay procedure mentioned in the present paper for confirming the present assay with known inhibitors and for finding new inhibitors are summarized in Table 3. The G0/G1-arresting effects resulting in the characteristic small cells shown in Table 3 could be detected only by the asynchronous-culture assay, while the G2/M-arresting activity could be examined both by the asynchronous- and synchronous-culture assays.

The protein phosphatase inhibitor tautomycin did not show a clear effect on asynchronously growing tsFT210 cells; rather, tautomycin inhibited the cell cycle progression at metaphase in temperature synchronized tsFT210 cells resulting in the characteristic large cells (Table 3). The synchronously cultured tsFT210 cells were more sensitive to inhibitors than randomly cultured tsFT210 cells. Moreover, the cells synchronized in G2/M boundary were homogenous and large in size compared with G1 cells (Fig. 6).

In our bioassay, nocodazole (1–30 μM) which is an inhibitor of microtubule polymerization inhibited the cell cycle progression at M-phase both of asynchronous (Table 1) and synchronous [Fig. 6(e)] tsFT210 cells. The morphology-based bioassay utilizing tsFT210 cells was very effective to detect cdc2 kinase inhibitors, for example, RK-1409 [see Table 2, lanes 6–10 of Figs 5(A) and 6(f)], as well as the mitosis inhibitors which did not directly inhibit cdc2 kinase activity, for instance, nocodazole [Fig. 6(e)] and other new inhibitors shown in Tables 2 and 3.

Based on the above results, we carried out screening of cell cycle inhibitors from the microbial metabolites using this bioassay. During the screening, we have discovered several new inhibitors (**1–9**) from the fungal metabolites as shown in Chart 2. The biological activities for **1–9** are given in Tables 2–4. Compounds **6–9** were recently isolated from an unidentified fungal strain, and they were identified as pyripyropenes E (**6**), F (**7**), gliotoxin (**8**) and genistein (**9**), respectively, according to their physicochemical properties and spectral data. The MIC and IC_{50} values for **6–9** on the cell cycle progression of tsFT210 cells are given in Table 4. Gliotoxin (**8**) inhibited the cell cycle at very low concentration.

Discussion

M-phase promoting factor (MPF), which is a complex of cdc2 kinase and its regulatory protein cyclin B, is involved in the transition from the G2- to M-phase in the mammalian cell cycle.[2,27] Cdc2 kinase plays an essential role in the G2/M-transition as demonstrated by the fact that inhibition of cdc2 kinase activity in the mouse cell line tsFT210 by exposure to the nonpermissive temperature, which contains a temperature-sensitive mutation in cdc2 kinase, was sufficient to block cell

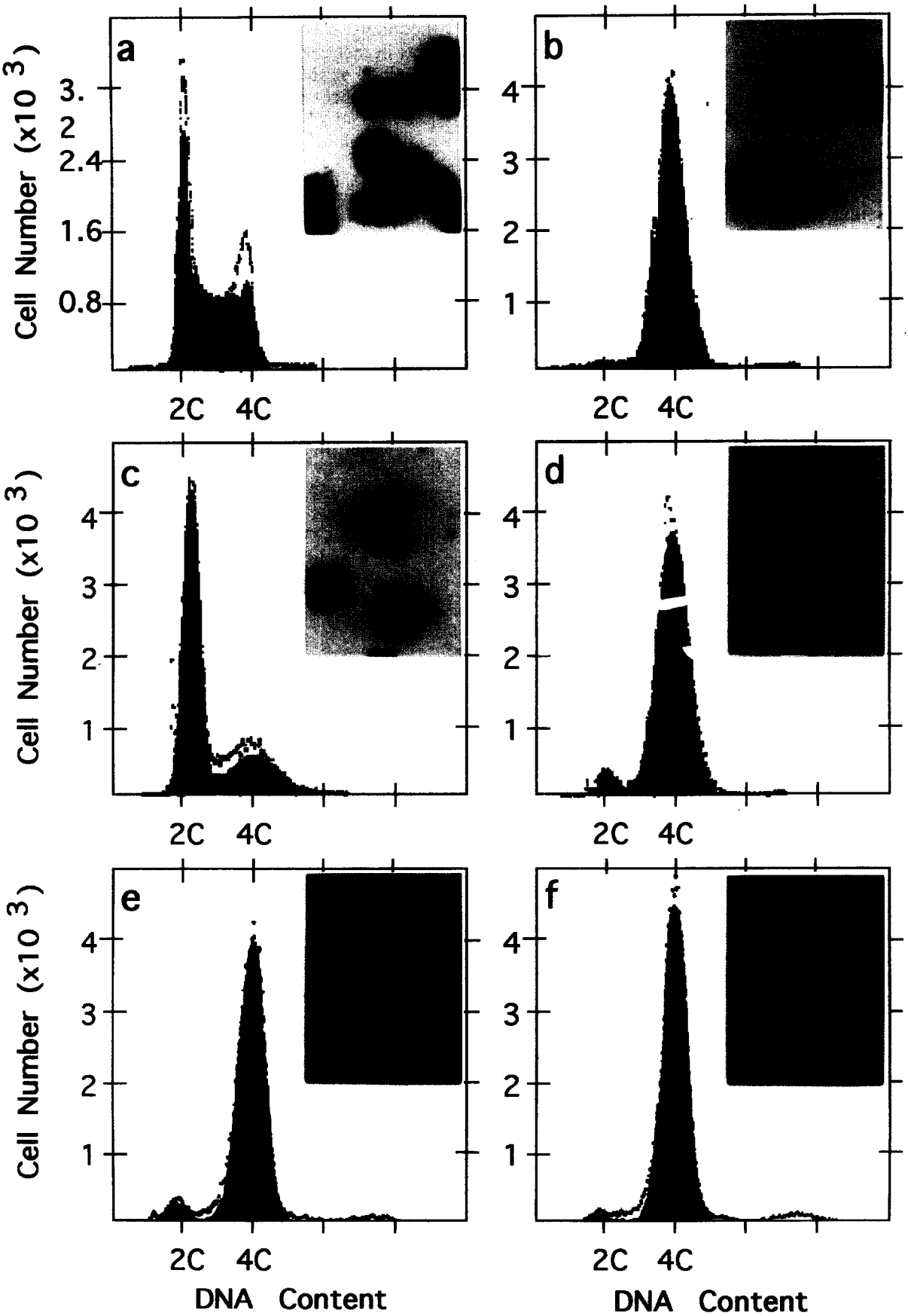


Figure 4. Effects of sangivamycin, RK-1409 and RK-286C on the cell cycle progression from G2- to G1-phase. Asynchronized tsFT210 cells cultured at (a) 32 °C were synchronized at the G2 boundary by incubation at (b) 39.4 °C for 17 h. The cells were then shifted to 32 °C to release them from G2 arrest. The cells passed through M phase and entered G1 (c) after 4 h. When the cells were simultaneously released from the temperature-arrest in the presence of (d) 10 μM sangivamycin, (e) 0.3 μM RK-1409 and (f) 1.6 μM RK-286C, the cell cycle progression from G2- to M-phase was inhibited. Photographs show morphological characteristics of the corresponding cells observed under the microscope.

cycle progression at the G2-phase.[9,10,28] Since one of the main problems of the cancer cells is dysregulation of cell cycle regulation, it is worth exploring inhibitors of MPF.

There have been several reports describing the discovery of cdc2 kinase inhibitors.[14,16,29] In those reports, purified cdc2 kinase was used for the bioassay or flow cytometry was used. In this report, we established a simple bioassay method to detect inhibitors of cdc2 kinase in tsFT210 cells according to the size difference between G2 cells and G1 cells. Cell-morphology-based bioassays are sometimes criticized for possible inaccuracies, however, the morphology-based bioassay is a very effective tool to identify new microbial products because primary screening of large numbers of microbial products requires a simple and rapid method. After the preliminary screening, more sophisticated bioassays using purified enzyme or flow cytometry can be used to confirm the initial results.

In this paper, we demonstrated a correlation between cell size and cell cycle progression. As the temperature-sensitive mutation of tsFT210 cells is known to be in the *cdc2* gene,[11] this bioassay system should be useful for detecting cdc2 kinase inhibitors as well as the inhibitors of cdc2 activation at the G2/M-phase boundary. The synchronous culture of tsFT210 cells was in fact very useful for analysing the progression of M-phase as shown in Figures 1 and 2. In this condition, it took longer time to pass the M phase compared with the normal growing condition because the G2-arrested cells required the reactivation of cdc2 kinase.

We have described the inhibitory spectra of several inhibitors against various protein kinases and against the tsFT210 cell cycle for the first time. There may be several protein kinases such as cdc2 activating protein kinase (CAK)[30] or p40^{MO15}[31] involved in G2 phase in preparation for entry into M-phase.[13,16] There may be some redundancy among the protein kinases which act on the G2/M0-transition allowing for functional compensation. Therefore, an inhibitor with a broad inhibitory spectrum against kinases can block the G2/M-phase of the cell cycle when tsFT210 cells are asynchronously cultured at 32 °C, whereas an inhibitor with a narrow inhibitory spectrum can only inhibit the cell cycle of G2 synchronized tsFT210 cells. New protein synthesis was not necessary for the G2 to the G1 phase because cycloheximide did not inhibit the progress of G2 to the G1 phase (Table 3). As shown in Figure 5 and Table 2, PKA was sensitive to RK-1409 (310 nM) and RK-286C (1.6 μM), but resistant to

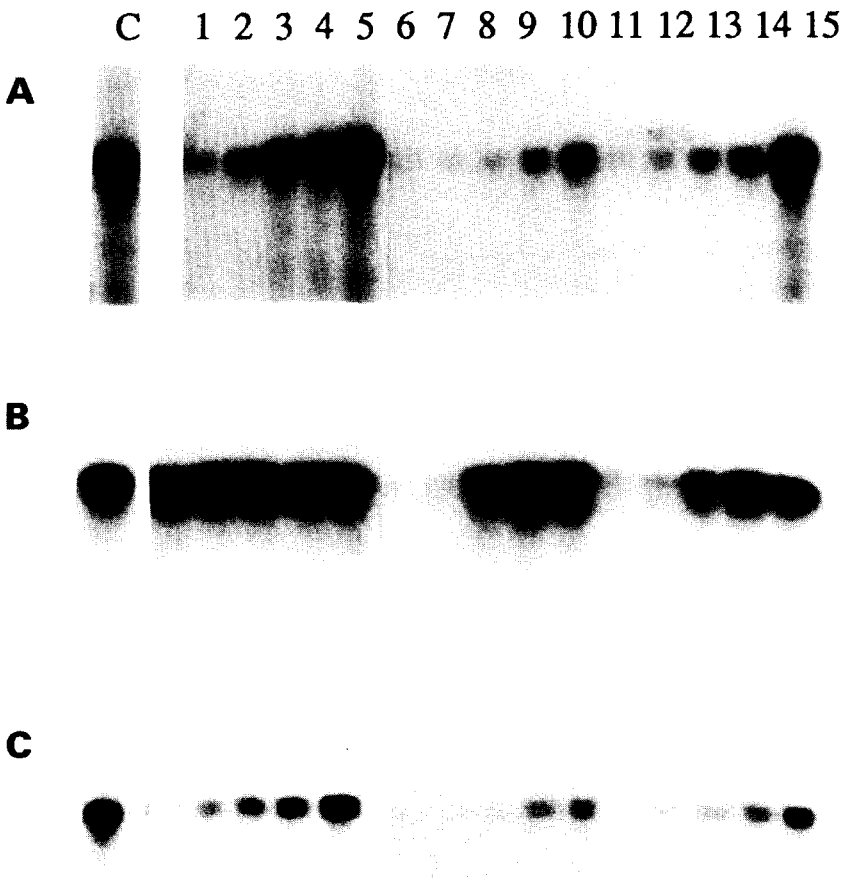


Figure 5. Inhibition of protein kinases by microbial inhibitors. Protein kinases, (A) cdc2 kinase, (B) PKA and (C) PKC, were purified from HeLa cells. Histone H1 type III was the substrate for cdc2 kinase and PKC. Histone H2B was the substrate for PKA. Lane C in each panel is the control without inhibitors. Sangivamycin was added to lanes 1 to 5 at concentrations of 100, 33, 10, 3.3 and 1.0 μM, respectively. RK-1409 was added to the lanes 6 to 10 at concentrations of 1040, 310, 104, 31 and 10 nM, respectively. RK-286C was added to lanes 11 to 15 at the concentrations of 11, 3.3, 1.1, 0.3, and 0.1 μM, respectively.

sangivamycin (10 μM) and RK-1409 (31 nM). The former two at these concentrations inhibited the cell cycle both of asynchronized [Figs 3(c) and 3(d)] and synchronized [Figs 4(e) and 4(f)] tsFT210 cells, but the latter two inhibited the cell cycle of only synchronized cultures [see Figs 3(b) and 4(d) for 10 μM snagivamycin, Table 1 and Fig. 6(f) for 31 nM RK-1409].

Table 2. Effects of protein kinase inhibitors and new cell cycle inhibitors

Inhibitors	50% Inhibitory concentration (nM)			
	cdc2	PKA	PKC	CK-I
Sangivamycin	480	>5000	940	1900
Staurosporine	32	70	3.6	150
RK-1409	95	89	2.3	700
RK-286C	600	690	37	5000
RK-1409B	1200	1050	320	1150
Tryprostatin A	$>10^5$	$>10^5$	$>10^5$	$>10^5$
Tryprostatin B	$>10^5$	$>10^5$	$>10^5$	$>10^5$
Fumitremorgin C	$>10^5$	$>10^5$	$>10^5$	$>10^5$
Demethoxyfumitremorgin C	$>10^5$	$>10^5$	$>10^5$	$>10^5$
Acetophthalidin	$>10^5$	$>10^5$	$>10^5$	$>10^5$

Inhibitors were added to the reaction mixture with the kinase and ^{32}P ATP. After the kinase reaction, the phosphorylated substrate was separated by SDS-PAGE and monitored by autoradiography as shown in Figure 5. Phosphorylated bands were quantified by laser densitometry and 50% inhibitory concentrations were calculated by comparison with the control.

Here, we have illustrated the merits of the bioassay using tsFT210 cells arrested at the G2/M-boundary which are sensitive to various inhibitors. The bioassay was very effective to detect not only cdc2 kinase inhibitors but also other mitosis inhibitors wihch interfere with phosphatase activity and microtubule polymerization. In other words, the bioassay is very sensitive but not specific to cdc2 kinase inhibitors. It should be emphasized, therefore, that cdc2 kinase inhibitors could be detected by the bioassay but novel inhibitors detected by this assay are not always specific cdc2 kinase inhibitors.

Using this bioassay, we have identified several new cell cycle inhibitors **1**–**9** (Chart 2). Among them, **1**, **2** and **5** are novel compounds, respectively named tryprostatins[21] A (**1**), B (**2**) and acetophthalidin[22] (**3**) according to their structural characteristics.

Kinase assays carried out for **1**–**5** showed that **1**–**5** did not inhibit cdc2, PKA, PKC and CK-I kinase activities (Table 2) or the autophosphorylation of EGF receptor (data not shown). These results, coupled with the morphological characteristics of cell nuclei treated by **1**–**5**, revealed that the inhibition target for **1**–**5** is likely to be the mitotic apparatus (Table 3). Further studies on their mechanism of action are currently being undertaken, and the results will be reported elsewhere.

Pyripyropenes are a new group of natural products discovered in recent years as the inhibitors of acyl

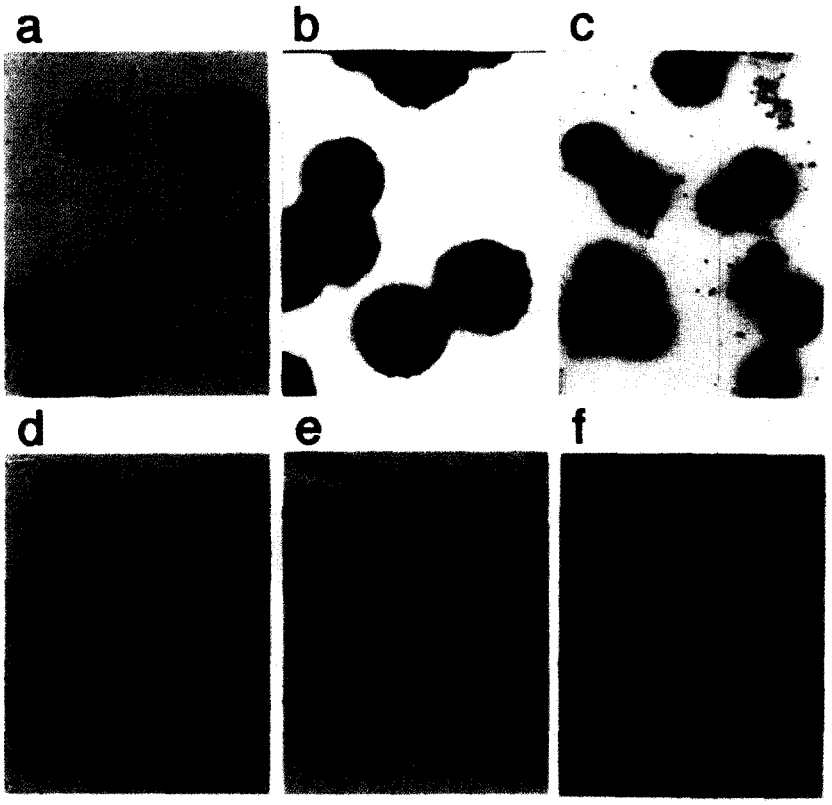


Figure 6. Effects of the inhibitors on the cell size of tsFT210 cells. The tsFT210 cells cultured at 32°C for 17 h (a) in the absence or (d) in the presence of 100 μM reveromycin. (b) The tsFT210 cells were cultured at 39.4 °C for 17 h. The cells were released from temperature arrest (c) in the absence or (e) in the presence of 1 μM nocodazole and (f) 31 nM RK-1409 for 4 h.

Table 3. Screening results of cell cycle inhibitors

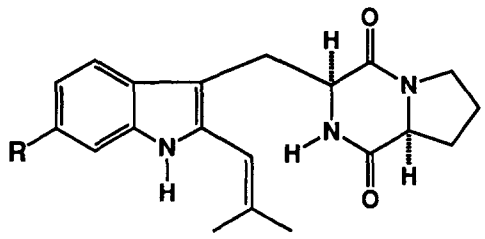
Inhibitors	Inhibition target	Cell size[a]
Cycloheximide	Protein synthesis	S
Reveromycin	EGF-signal transduction	S
Hydroxyurea	DNA synthesis	S
Actinomycin D	RNA synthesis	S
RK-286C, 1409, 1409B	Kinase	L
Sangivamycin	Protein kinase C	L
Genistein	Tyrosine kinase	L
Tautomycin	Phosphatase	L
Nocodazole	Microtubule	L
Tryprostatin A, B	Mitotic apparatus?	L
Demethoxyfumitremorgin C	Mitotic apparatus?	L
Acetophthalidin	Mitotic apparatus?	L
Pyripyropenes	ACAT/other?	L
Gliotoxin	Farnesyl transferase/other?	L

[a]Characters, S and L, are the abbreviations of the words, 'small; average size, 15–18 μm in diameter' and 'large; average size, 21–25 μM', respectively.

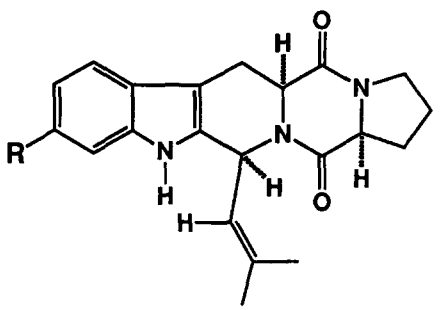
CoA-cholesterol acyltransferase (ACAT).[32,33] In our bioassay, pyripyropenes E (**6**), F (**7**) and other two minor unidentified pyripyropenes were detected as inhibitors of the mammalian cell cycle. Gliotoxin (**8**) which had been reported to be a farnesyl-protein transferase inhibitor (IC_{50}; 1.1 μM)[34] inhibited the cell cycle progression of tsFT210 cells with an IC_{50} value (Table 4) lower than that for inhibition of farnesyl-protein transferase. Even though **6–9** have been reported to show other activities,[34] the present result is the first report describing the inhibitory effect of **6–9** on the cell cycle progression of mammalian cells.

On the other hand, genistein (**9**) which is known as a tyrosine kinase inhibitor showed a strong inhibitory effect (Table 4) on the cell cycle progression of tsFT210 cells and was widely detected in fermentation broths using soybean meals by the present bioassay.

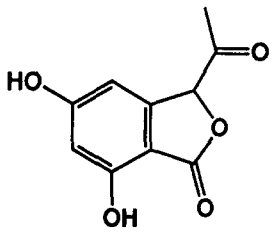
These results showed that it was possible by using the present bioassay to identify rapidly cdc2 kinase inhibitors and also inhibitors of the mammalian cell cycle. The present bioassay provides a convenient and practical method for screening new mammalian cell cycle inhibitors from the microbial metabolites.



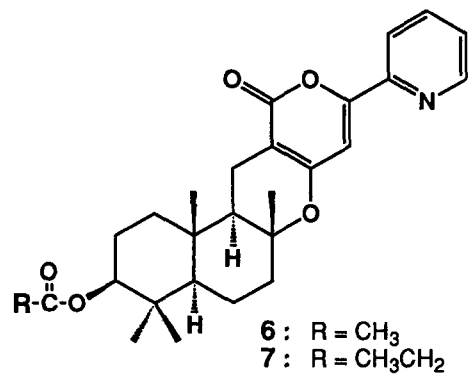
1: R = OCH_3
2: R = H



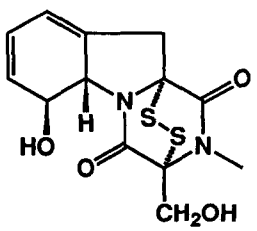
3: R = OCH_3
4: R = H



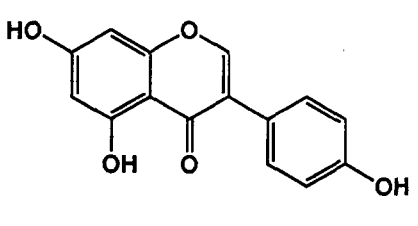
5



6: R = CH_3
7: R = CH_3CH_2



8



9

Chart 2. Structures of mammalian cell cycle inhibitors isolated from the fungal metabolites by using the present bioassay.

Table 4. Effects of newly isolated inhibitors on the cell cycle progression (entry into G1-phase from G2-arrest) of tsft210 cells

Inhibitors	Cell cycle arrest in the G2/M-phase	
	MIC (μM)	IC_{50} (μM)
Pyripyropene E	3.5	6.9
Pyripyropene F	3.4	6.7
Gliotoxin	1.9×10^{-3}	3.8×10^{-3}
Genistein	0.72	4.4

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