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Oxaline, a fungal alkaloid, arrests the cell cycle in M phase by inhibition of tubulin polymerization

Yukio Koizumi, Masayoshi Arai, Hiroshi Tomoda*, Satoshi Ōmura

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

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Abstract

Oxaline and neoxaline, fungal alkaloids, were found to inhibit cell proliferation and to induce cell cycle arrest at the G_2/M phase in Jurkat cells. CBP501 (a peptide corresponding to amino acids 211–221 of Cdc25C phosphatase), which inhibits the G_2 checkpoint, did not affect the G_2/M arrest caused by oxaline, suggesting that oxaline causes M phase arrest but not G_2 phase arrest. The Cdc2 phosphorylation level of oxaline-treated cell lysate was lower than that of the control cells, indicating that oxaline arrests the M phase. Oxaline disrupted cytoplasmic microtubule assembly in 3T3 cells. Furthermore, oxaline inhibited polymerization of microtubule protein and purified tubulin dose-dependently in vitro. In a binding competition assay, oxaline inhibited the binding of [^{3}H]colchicine to tubulin, but not that of [^{3}H]vinblastine. These results indicate that oxaline inhibits tubulin polymerization, resulting in cell cycle arrest at the M phase.

Keywords: Fungal alkaloid; Oxaline; Cell cycle; Microtubule; Tubulin

1. Introduction

Microtubules, which are cytoplasmic fibers with a tubular form composed of a heterodimer of α- and β-tubulin as the major component, and microtubule-associated proteins (MAPs) as the minor ones, are involved in many cellular functions including mitosis, cell morphogenesis and intracellular transport [1]. There are a number of natural and synthetic compounds that interfere with microtubule function to cause arrest of the cell cycle at the M phase. These compounds are useful biochemical and cytological probes, and have proven or potential utility as antitumor drugs [2,3].

Screening for microbial metabolites that have antiproliferative activity and arrest the cell cycle at the G_2/M phase identified oxaline and neoxaline (Fig. 1) as active compounds. Oxaline and neoxaline are alkaloids isolated from the culture broth of *Penicillium oxalicum* and *Aspergillus japonicus*, respectively [4,5]. However, their biological activities are largely unknown [5]. These alkaloids possess several interesting structural features, including an N-OMe group, an unusual coupling of tryptophan and dehydrohistidine, a single carbon atom having three different nitrogen substituents and a reversed isoprenyl group [6–8]. The structure of these alkaloids differs from that of well-known G_2/M arrest inducers such as colchicine, vinblastine and taxol [2,3]. Thus, in addition to their quite unique chemical structures, their biological activity prompted us to study the mechanism of action of these drugs. In this study, we show that oxaline inhibits microtubule protein/purified tubulin polymerization, resulting in arrest of the cell cycle at the M phase.

2. Materials and methods

2.1. Chemicals

Oxaline and neoxaline were isolated from a culture of *Penicillium* sp. FKI-0779 and *A. japonicus* Fg-551, respec-

Abbreviations: MAPs, microtubule-associated proteins; HPLC, high pressure liquid chromatography; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; EGTA, ethylene glycol-*bis*(β-aminoethyl ether); PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate

* Corresponding author. Tel.: +81-3-3444-6161; fax: +81-3-5791-6271.
E-mail address: tomoda@lisci.kitasato-u.ac.jp (H. Tomoda).

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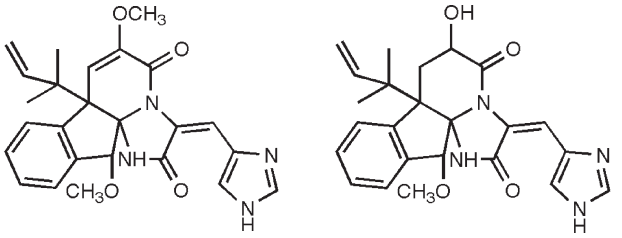

Oxaline Neoxaline

Fig. 1. Structures of oxaline and neoxaline.

tively. Briefly, the ethyl acetate extracts of the culture broths were separated by chromatography on silica gel, and the alkaloids then purified to >99% by reversed-phase HPLC. CBP501 [9] was kindly provided by CanBas Co. Ltd (Shizuoka, Japan). Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). Colchicine was obtained from Sigma (St. Louis, MO). [^{3}H]Colchicine was purchased from PerkinElmer Life Science (Boston, MA) and [^{3}H]vinblastine from Amersham Biosciences (Piscataway, NJ). Other reagents were commercially available analytical grade products.

2.2. 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.

2.3. Cell proliferation assay

Cell proliferation was evaluated using the colorimetric 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [10]. The exponentially growing cells were cultured in 96-well plates (Corning Inc., Corning, NY) with drugs at the indicated concentrations at 37 °C for 48 h. After incubation, the cells received 10 μl of 5.5 mg/ml MTT in phosphate buffered saline (PBS), and were then incubated at 37 °C for 4 h. A 90-μl aliquot of extraction solution (40% (v/v) *N*,*N*-dimethylformamide, 2% (v/v) CH_3COOH, 20% (w/v) SDS and 0.03 N HCl) was added, and the mix incubated at room temperature for 2 h. Cell proliferation was determined by measuring optical density at 550 nm.

2.4. Flow cytometry

Jurkat cells (1×10^5 cells) were incubated in 96-well plates with drugs in the presence or absence of CBP501 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 h. DNA content was determined using FACSCalibur analysis (Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed using ModFit LT software for cell cycle profiles.

2.5. Immunoprecipitation and immunoblotting

Jurkat cells (4×10^6 cells) were incubated in the presence or absence of the drugs for 20 h. After incubation, cells were washed twice with PBS 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 min. Cell lysates were clarified by centrifugation, and the resultant supernatants used for protein quantitation using the BCA protein assay reagents (Pierce, Rockford, IL). Fifty micrograms of each cell lysate was reacted with 2 μg/ml of anti-Cdc2 antibody (A17.1.1, Oncogene, Boston, MA) in 250 μl of 5% (v/v) Protein A-Sepharose slurry (Amersham Biosciences) in lysis buffer of one third strength at 4 °C overnight. Immunoprecipitates were then washed extensively with one third strength lysis buffer, eluted by boiling for 3 min in 25 μl of SDS sample buffer and 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), 100 mM NaCl and 0.1% (w/v) Tween 20 (TBS/T) containing 5% BSA for 1 h and then probed with 1 μg/ml of anti-Cdc2 antibody or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY) for 1 h. After washing, the membranes were incubated with 0.2 μg/ml of peroxidase-conjugated anti-mouse antibody (Sigma) for 1 h. Membranes were then washed, and proteins subsequently detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences).

2.6. Immunofluorescence

3T3 cells were cultured on eight-chamber slides for 20 h, and then incubated for 5 h in the presence or absence of the drugs. After removal of the medium, cells were fixed in 3.7% paraformaldehyde for 30 min. After washing, cells were permeabilized with 0.1% Triton X-100 and 4 μg/ml RNase A in PBS for 1 h. Cells were then washed, and incubated with 36 μg/ml of FITC-conjugated anti-α-tubulin antibody (Sigma) and 1 μg/ml propidium iodide in PBS for 1 h in the dark. The cells were washed and mounted with aqueous mounting medium. Microtubules and DNA were observed using a Confocal Laser Scanning Microscope TCS NT (Leica Microsystems AG, Wetzlar, Germany).

2.7. Microtubule protein and purified tubulin polymerization assay

Polymerization assays for microtubule protein and purified tubulin were performed using the CytoDYNAMIX

Screen kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions. Briefly, microtubule protein (2 mg/ml) or purified tubulin (3 mg/ml) was incubated at 37 °C in the presence or absence of the drugs, and polymerization then detected by measuring the change in absorbance at 340 nm.

2.8. Tubulin purification

Microtubule protein was prepared from porcine brain using the polymerization-depolymerization method of Shelanski et al. [11] with a slight modification. Microtubule protein, which was purified by two cycles of polymerization-depolymerization, was dissolved in MES buffer (20 mM MES (pH 6.8), 0.5 mM $MgCl_2$, 1 mM EGTA and 0.1 mM GTP). Tubulin was purified from microtubule protein by phosphocellulose (P11, Whatman, Kent, UK) column chromatography [12].

2.9. Competition assay

A competition assay was performed using the centrifugal gel filtration method [13] with minor modifications. A 100-μl aliquot of 0.5 mg/ml tubulin purified from porcine brain was incubated with 100 nM [^{3}H]colchicine or [^{3}H]vinblastine in the presence or absence of the indicated concentration of drugs for 10 min at room temperature. After incubation, the mixture was loaded onto a 1-ml spin column filled with Sephadex G-50 (Superfine, Amersham Biosciences), and centrifuged at 1500 rpm for 5 min to obtain the tubulin fraction. The radioactivity of [^{3}H]ligand bound to tubulin was measured using the Wallac Micro Beta Trilax (PerkinElmer Life Science) system.

3. Results

3.1. Oxaline and neoxaline have antiproliferative activity and arrest the cell cycle at the G_2/M phase

The antiproliferative activity of oxaline and neoxaline against human T cell leukemia Jurkat cells was evaluated by MTT assay after 48-h treatment. Both alkaloids inhibited cell proliferation in a dose-dependent manner. The IC_{50} values of oxaline and neoxaline were 8.7 and 43.7 μM, respectively, while that of colchicine was 6.8 nM (Fig. 2).

The effect of these alkaloids on the cell cycle of Jurkat cells was examined using a flow cytometer. Jurkat cells were treated with various concentrations of alkaloids for 20 h, and stained with propidium iodide for cell cycle analysis. Accumulation at the G_2/M phase was observed by treatment with 20 μM oxaline or 70 μM neoxaline, and the maximum effects of oxaline and neoxaline were observed at 70 and 200 μM, respectively (Fig. 3A). The G_2/M arrest by these alkaloids increased time-dependently from treatment times of 0 to 15–20 h, followed by a decrease in the number of

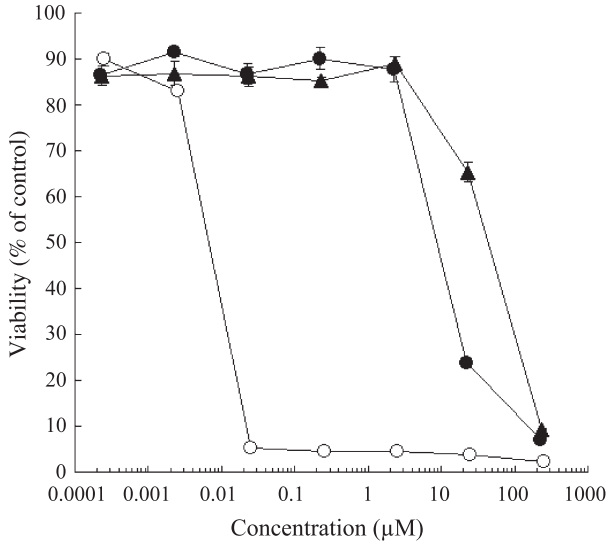

Fig. 2. Antiproliferative activity of oxaline and neoxaline. Jurkat cells were incubated in 96-well plates with oxaline (●), neoxaline (▲) or colchicine (○) at the indicated concentrations for 48 h. After treatment, cell viability was evaluated by MTT assay. Each value represents the mean ± S.D. from triplicate determinations.

G_2/M arrest cells and an increase in sub G_1 cells, indicative of apoptotic cells (Fig. 3B). In contrast, the cell cycle status of control cells remained fundamentally unchanged for 0 to 40 h (Fig. 3B). In these cell assays, oxaline was several times more potent than neoxaline; so further studies were performed using oxaline.

3.2. Oxaline arrests the cell cycle reversibly

We tested whether the G_2/M arrest by oxaline was reversible. As shown in Fig. 3B, the oxaline effect was visible after 5 h of treatment. Therefore, Jurkat cells were pretreated with oxaline for 8 h, washed and then incubated with or without oxaline for 12 h. The proportion of G_2/M phase of oxaline-treated cells was 43.8%, whereas that of oxaline-pretreated cells was 4.0% (Table 1). Although oxaline-pretreated cells underwent apoptosis slightly, most cells recovered from G_2/M arrest to G_1/S phase by removal of oxaline, indicating that the effect of oxaline is reversible.

3.3. CBP501 does not affect the G_2/M arrest caused by oxaline

Although oxaline arrests the cell cycle at the G_2/M phase, it is unclear whether this arrest occurs in the G_2 or M phase. Thus, we used CBP501, a peptide corresponding to amino acids 211–221 of Cdc25C phosphatase, which is an inhibitor of G_2 checkpoint [9]. DNA damage activates a cell cycle checkpoint that prevents the progression to M phase while DNA repair is under way [14]. In the G_2 checkpoint, Cdc25C, which activates Cdc2 involved in G_2/M transition, is phosphorylated at Ser216 by Chk1 or Chk2 and is exported from nucleus to cytoplasm through the binding

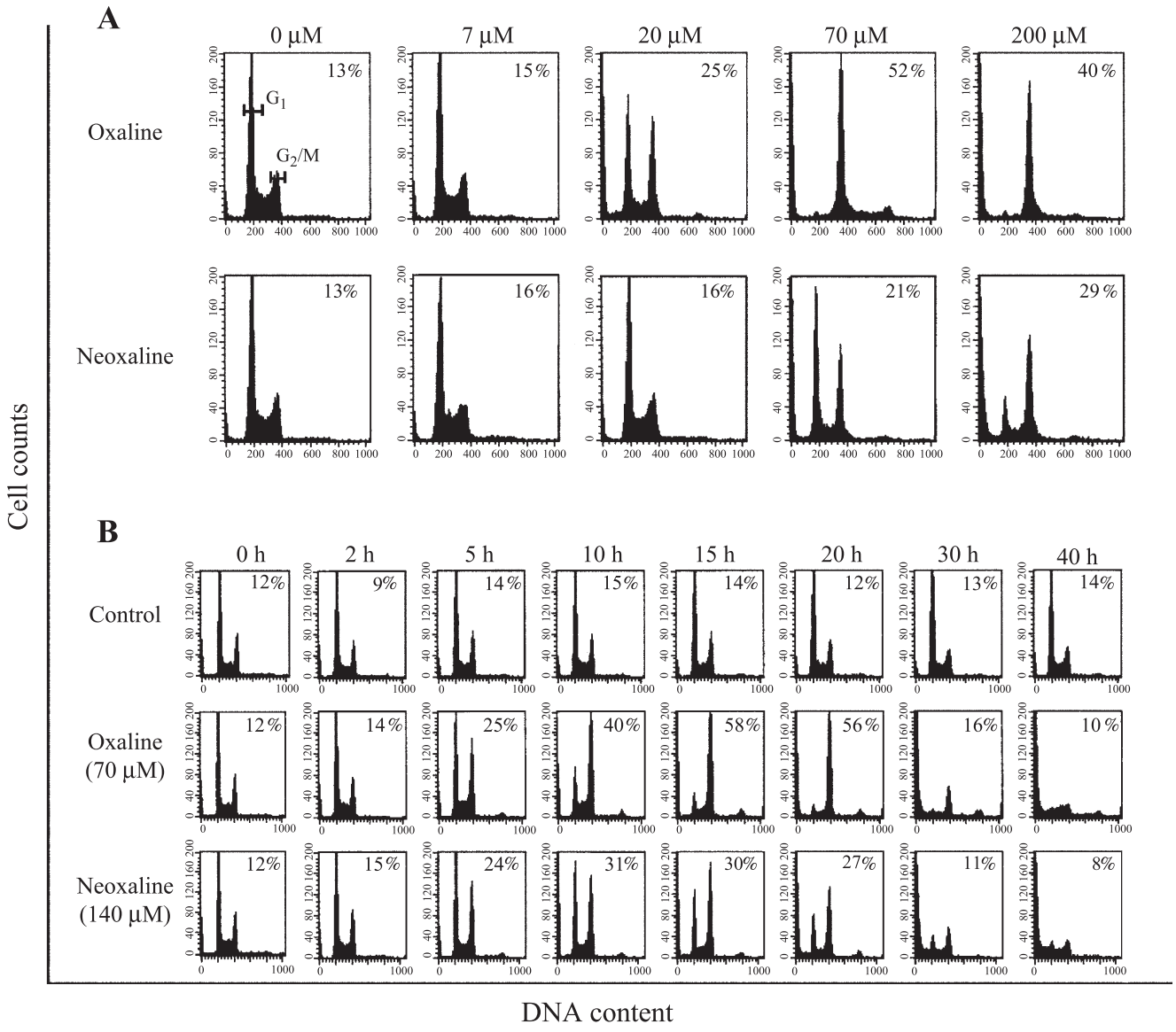

Fig. 3. Effects of oxaline and neoxaline on cell cycle progression. Jurkat cells were incubated in 96-well plates with oxaline or neoxaline at the indicated concentrations for 20 h (A), or with 70 μM oxaline or 140 μM neoxaline for the indicated time periods (B). After treatment, cells were stained with propidium iodide, and cell cycle analysis performed using a flow cytometer. The percentages of cells arrested in G_2/M phase were listed on each histogram.

with 14-3-3, resulting to Cdc2 remaining in an inactive phosphorylation form [15–21]. CBP501 inhibits the DNA damage-dependent phosphorylation of Cdc25C by Chk1 and Chk2 and induces cell death [9]. As shown in Fig. 4, although bleomycin, a potent DNA-damage inducer, and colchicine, a microtubule polymerization inhibitor, arrest the cell cycle at the G_2 phase and M phase, respectively, the histograms of the two drugs show similar cell cycle distribution in G_2/M arrest. In combination with CBP501, G_2 arrest induced by bleomycin was abrogated, and sub G_1 cells increased. In contrast, colchicine-treated cells showed no change (Fig. 4). Oxaline-treated cells also showed no change in combination with CBP501, suggesting that oxaline induces arrest of the cell cycle at the M phase, but not G_2 phase.

3.4. Tyrosine of Cdc2 prepared from oxaline-treated cells are dephosphorylated

The progression from G_2 to M phase requires activation of the Cdc2 and cyclin B complex. The activation of Cdc2/cyclin B is regulated by dephosphorylation of Cdc2 at Thr14 and Tyr15 catalyzed by Cdc25C [21–26]. Therefore, since the inhibitory phosphorylation of Cdc2 at G_2 phase is dephosphorylated with the progression to M phase, the Cdc2 phosphorylation level becomes the index that shows that the cell cycle status is in either G_2 or M phase. To determine the phosphorylation level of Cdc2, we used immunoprecipitation and immunoblotting techniques. Drug-treated or untreated cell lysates were immunoprecipitated using anti-Cdc2 antibody, followed by detection by

Table 1
Cell cycle profile of cells treated or pretreated with oxaline

	Cell count (%)			
	Sub G_1	G_1	S	G_2/M
Control cells	2.2	45.7	32.0	13.9
Oxaline-treated cells	35.0	0.8	11.4	43.8
Oxaline-pretreated cells	15.3	34.9	39.7	4.0

Jurkat cells were pretreated with oxaline (70 μM) for 8 h, washed and then incubated with or without oxaline (70 μM) for 12 h. After incubation, cells were stained with propidium iodide, and DNA content was determined using a flow cytometer. Data analysis was performed by ModFit LT for cell cycle profiles.

immunoblotting with anti-Cdc2 or anti-phosphotyrosine antibody (Fig. 5). Cdc2 levels in each lysate were almost identical. However, bleomycin increased the phosphotyrosine level of Cdc2, indicating that bleomycin arrested the cell cycle in the G_2 phase. In contrast, oxaline, as well as colchicine, induced the dephosphorylation of Cdc2, and the phosphotyrosine levels were lower than those of the control cells. Thus, Jurkat cells treated with oxaline arrest in the M phase.

3.5. Oxaline disrupts cytoplasmic microtubule assembly in 3T3 cells

These results indicate that the effects of oxaline on Jurkat cells resemble those of colchicine. Colchicine inhibits the polymerization of microtubules, resulting in arrest of the cell cycle at the M phase. We examined the effect of oxaline on cytoplasmic microtubules in mouse fibroblast 3T3 cells by immunofluorescence staining with FITC-conjugated anti-α-tubulin antibody. The microtubule assembly of control cells was clearly visible (Fig. 6A), whereas oxaline completely disrupted microtubule assembly, as did colchicine (Fig. 6B and C). Oxaline did not affect actin filaments, which are different cytoskeletal proteins, in 3T3 cells (data not shown).

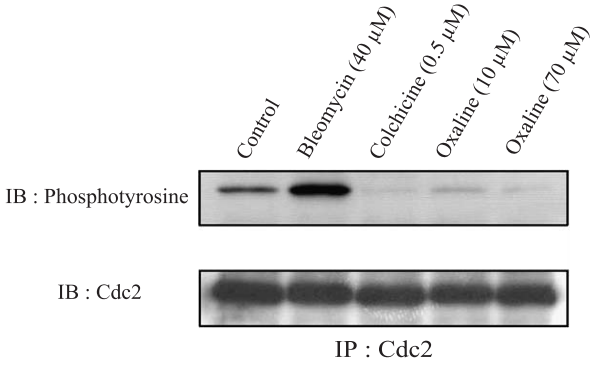

Fig. 5. Effect of oxaline on the phosphorylation of Cdc2. Jurkat cells were incubated with or without 40 μM bleomycin, 0.5 μM colchicine, or 10 or 70 μM oxaline for 20 h. After incubation, cells were lysed and immunoprecipitated with anti-Cdc2 antibody. Immunoprecipitates were separated on 12.5% SDS-PAGE followed by immunoblotting with anti-Cdc2 antibody or anti-phosphotyrosine antibody. Proteins were detected using the ECL system.

3.6. Oxaline inhibits polymerization of microtubule protein and purified tubulin in vitro

Next, we investigated whether oxaline affects microtubule polymerization in vitro. After treatment of unpoly-

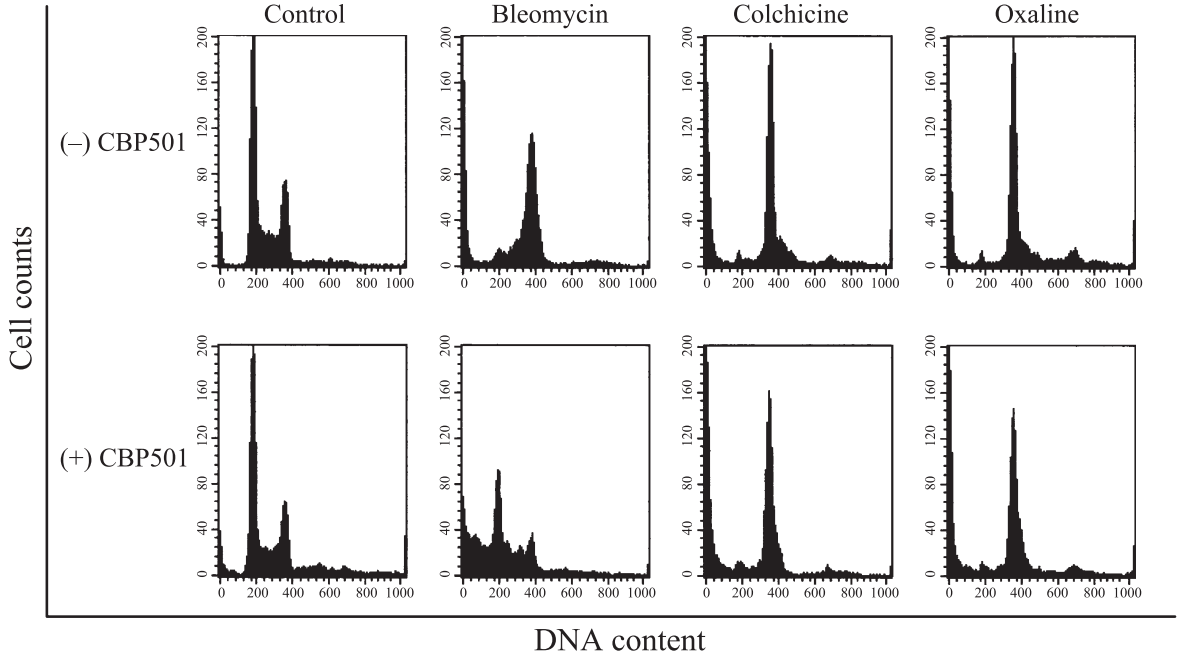

Fig. 4. Effect of G_2 checkpoint inhibitor on cell cycle progression of oxaline-treated cells. Jurkat cells were incubated in 96-well plates with or without 40 μM bleomycin, 0.5 μM colchicine or 70 μM oxaline in the presence or absence of 30 μg/ml CBP501 for 20 h. After treatment, cells were stained with propidium iodide, and cell cycle analysis performed using a flow cytometer.

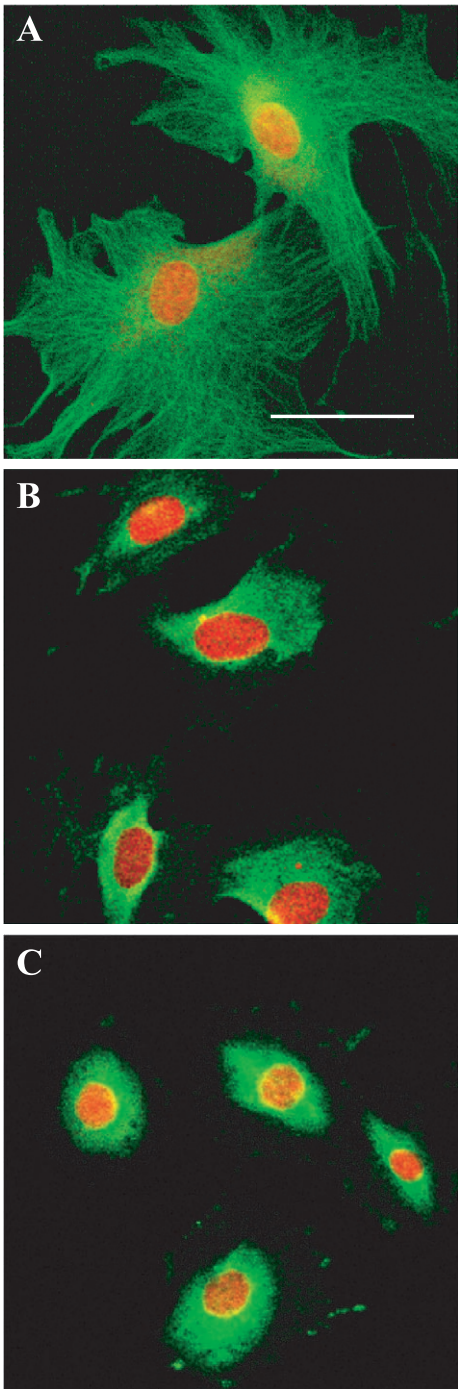

Fig. 6. Effect of oxaline on microtubule assembly in 3T3 cells. 3T3 cells were cultured on eight-chamber slides for 20 h, and then incubated for 5 h without (A), or with 0.5 μM colchicine (B) or 70 μM oxaline (C). After removal of the medium, cells were fixed and permeated. Cells were then incubated with FITC-conjugated anti-α-tubulin antibody and propidium iodide. Microtubules (green) and DNA (red) were observed using a Confocal Laser Scanning Microscope. Bar, 50 μm.

merized microtubule proteins, the changes in absorbance at 340 nm that indicated polymerization levels were measured at 37 °C (Fig. 7A). Oxaline inhibited polymerization of microtubules dose-dependently. The microtubule proteins used here contained approximately 90% tubulin and 10% MAPs. Colchicine is known to inhibit tubulin polymerization specifically to block microtubule polymerization. On the other hand, some compounds (griseofluvin, estramustine phosphate, tryprostatin A) inhibit microtubule polymerization as a result of interfering with the interaction between tubulin and MAPs [27–31]. To test whether oxaline affects tubulin directly or the interaction with tubulin-MAPs, we examined the effect of oxaline on purified tubulin polymerization. As shown in Fig. 7B, oxaline, as well as colchicine, inhibited tubulin polymerization. The IC_{50} value of oxaline was 107 μM. Thus, we

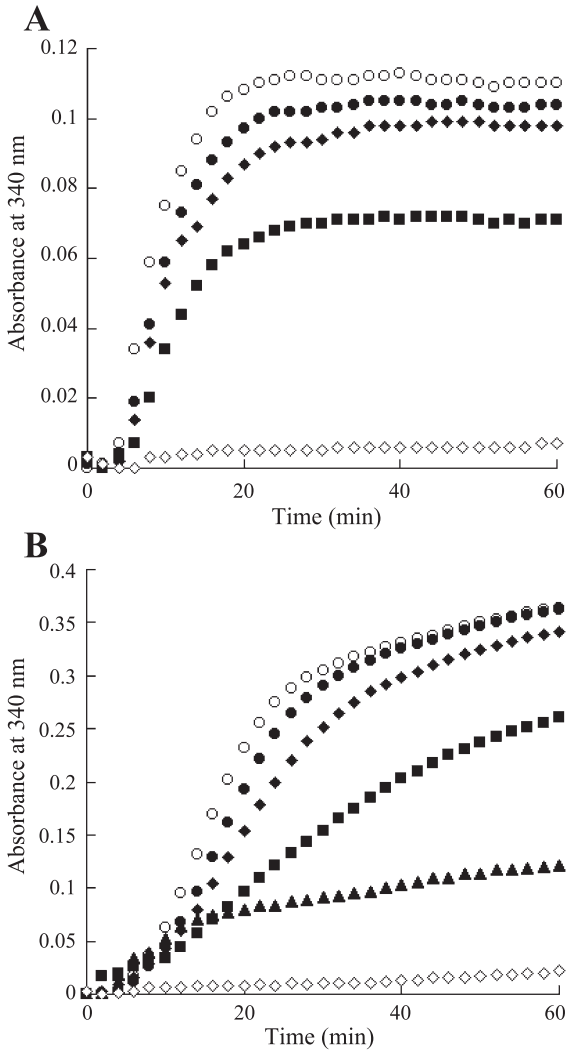

Fig. 7. Effects of oxaline on microtubule protein and purified tubulin polymerization. Polymerization assays for microtubule protein (A) and purified tubulin (B) were performed using commercial kits. Microtubule proteins (2 mg/ml) or purified tubulin (3 mg/ml) was incubated at 37 °C with 2% (v/v) DMSO. Polymerization was detected by measuring the change in absorbance at 340 nm. Final concentrations of oxaline and colchicine were 0 (○), 10 (●), 20 (♦), 70 (■) or 130 μM (▲) and 10 μM (◇), respectively. The values of IC_{50} of colchicine were 2–5 μM in these assay (data not shown).

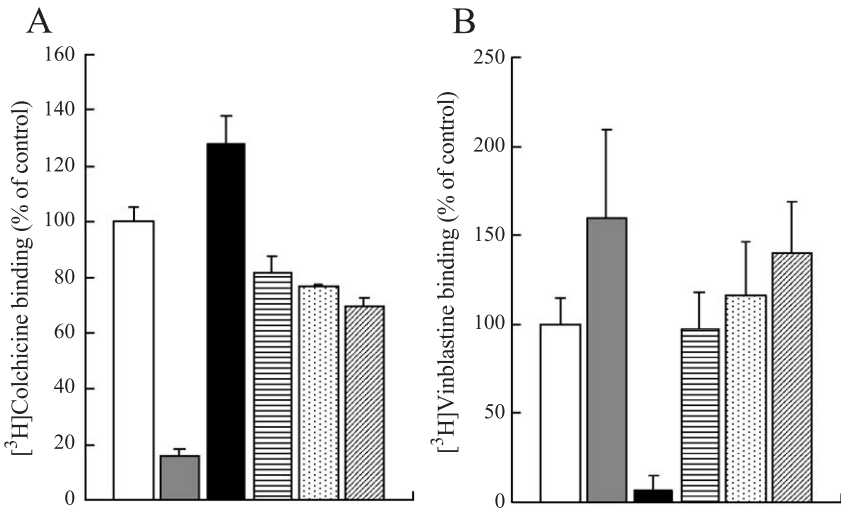

Fig. 8. Effect of oxaline on binding of [^{3}H]colchicine and [^{3}H]vinblastine to purified tubulin. One-hundred-microliter aliquot of 0.5 mg/ml purified tubulin was incubated with 100 nM [^{3}H]colchicine (A) or [^{3}H]vinblastine (B) in the absence (□) or presence of colchicine (100 μM, ▩), vinblastine (100 μM, ■) or oxaline (100 μM, ▤; 300 μM, ⊠; 1 mM, ▨) for 10 min at room temperature. After incubation, the mixture was applied to a 1-ml column of Sephadex G-50, and centrifuged at 1500 rpm for 5 min to obtain the tubulin fraction. The radioactivity of [^{3}H]ligand bound to tubulin was measured using the Wallac Micro Beta Trilax system. Each value represents the mean ± S.D. from triplicate determinations.

concluded that oxaline arrests the cell cycle at the M phase through inhibition of tubulin polymerization.

3.7. Oxaline inhibits the binding of [^{3}H]colchicine to tubulin

Studies on the binding sites of tubulin polymerization inhibitors have revealed that colchicine and vinblastine have different sites of action on tubulin [3]. To determine the binding site of oxaline on tubulin, we further examined the effect of oxaline on binding of [^{3}H]colchicine and [^{3}H]vinblastine to purified tubulin (Fig. 8). Unlabelled colchicine and vinblastine decreased the binding of [^{3}H]colchicine and [^{3}H]vinblastine, respectively. Oxaline weakly inhibited [^{3}H]colchicine binding to tubulin at 10,000-fold concentration (30% inhibition), while no effect of oxaline was observed on binding of [^{3}H]vinblastine even at the highest concentration.

4. Discussion

Although a large number of bioactive compounds with a variety of chemical structures have been discovered from natural resources such as microorganism and plants, their target molecules or functions in cells or living organisms have not been well defined in most cases. Oxaline and neoxaline were originally isolated as fungal alkaloids with a highly unique structure, as shown in Fig. 1 [4–8]. Meleagrin and glandicolins are known analogs, with an unusual coupling of tryptophan and dehydrohistidine, a single carbon atom having three different nitrogen substituents and a reversed isoprenyl group, as seen in oxaline and neoxaline [32–34]. However, the biological activities of oxaline and neoxaline have not been fully determined [5]. In the present study, we found first that these alkaloids inhibit cell proliferation and arrest the cell cycle at the G_2/M phase in Jurkat cells. The result of both the effect of a G_2 checkpoint abrogator and the decrease in Cdc2 phosphorylation level showed that oxaline arrests the cell cycle in the M phase but not in the G_2 phase. There are a number of natural and synthetic compounds that cause cell cycle arrest at the M phase. In particular, colchicine, vinblastine and taxol have been extensively investigated for their practical uses, as well as for use as biochemical and cytological probes. These compounds are well known to inhibit microtubule functions [2,3,35]. Oxaline also disrupted cytoplasmic microtubule assembly and inhibited the polymerization of microtubule proteins and purified tubulin as well as the above compounds. The result of the binding competition assay using [^{3}H]colchicine or [^{3}H]vinblastine is unclear, as oxaline inhibited [^{3}H]colchicine binding to tubulin by only approximately 30% at 10,000-fold higher concentration. It may be that oxaline binds near the colchicine binding site in tubulin or that the interaction between oxaline and tubulin is unstable compared to colchicines, even if oxaline has the same binding site as colchicine.

Oxaline is biosynthesized by fungal strains from the precursor diketopiperazine composed of tryptophan and the dehydrohistidine, roquefortine [36–38]. Recently, two diketopiperazines, tryprostatin A and (−)-phenylahistine, have been reported as inhibitors of microtubule protein polymerization [30,31,39]. The former is composed of tryptophan and proline, and the latter of phenylalanine and dehydrohistidine. Although these compounds have a similar structural motif, the mechanisms of action are different. Tryprostatin A inhibits the interaction between

tubulin and MAP2/tau, and (−)-phenylahistine binds to tubulin at the colchicine binding site. However, roquefortine has no effect on the cell cycle distribution in Jurkat cells even at 250 μM (data not shown). These observations bring us the interest to the investigation on the structure–activity relationship of diketopiperazines.

This is the first demonstration of the mechanism of action of fungal oxaline, which has a unique structure. Further study of the structure–activity relationship among oxaline analogs might lead to the development of a new type of antitumor drug.

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