Mycotoxin Citrinin Induced Cell Cycle G2/M Arrest and Numerical Chromosomal Aberration Associated with Disruption of Microtubule Formation in Human Cells

Chia-Hao Chang,*,† Feng-Yih Yu,*,† Ting-Shun Wu,*, Li-Ting Wang, and Biing-Hui Liu*†‡

*Department of Biomedical Sciences; and †Department of Medical Research, Chung Shan Medical University Hospital, Taichung 402, Taiwan

†These authors contributed equally to this study.
‡To whom correspondence should be addressed at Department of Biomedical Sciences, Chung Shan Medical University, No. 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan. Fax: +886-4-24757412. E-mail: bingliu@csmu.edu.tw.

Received June 30, 2010; accepted September 10, 2010

As a nephrotoxic mycotoxin, citrinin (CTN) contaminates various foodstuffs and animal feed commodities. In the present study, the effects of CTN on cell cycle arrest and microtubule formation were investigated by applying human embryonic kidney (HEK293) cells as a model. Exposure of HEK293 cells to CTN resulted in an arrest of cell cycle G2/M in a concentration-dependent increase. Administering CTN elevated the expression levels of p53 and p21 proteins, yet attenuated the signals of phosphorylated cell division cycle 2 (cdc2). Furthermore, treating HEK293 with CTN increased both the value of mitotic index and the population of cells recognized by antibody mitotic protein monoclonal 2, suggesting that arrest of CTN-induced cell cycle occurred mainly during the mitotic phase. With the assistance of immunocyto staining of α-tubulin, CTN was found to disrupt the stable microtubule skeleton during the interphase of cell cycle and also interfere with the mitotic spindle integrity during mitosis. Additionally, for either in vivo or in vitro assays, CTN effectively inhibited tubulin polymerization in a concentration-dependent manner. When human peripheral blood mononuclear cells were exposed to CTN, the percentage of cells with numerical chromosome changes was increased by 4.3-fold over that of vehicle-treated group. Results of this study suggest that CTN-activated G2/M arrest primarily arises from the inhibition of tubulin polymerization and associated mitotic spindle formation. Additionally, disruption of microtubule organization by CTN also contributes to the induction of numerical chromosomal aberration in human cells.

Key Words: mycotoxin citrinin; G2/M arrest; microtubule; numerical chromosomal aberration.

CTN is a secondary metabolite generally produced by various fungi, including Penicillium, Monascus, and Aspergillus (Bennett and Klich, 2003). This metabolite frequently occurs concurrently with ochratoxin in food and feed; both of these mycotoxins are suspected as etiological agents of Balkan endemic nephropathy (Vrabcheva et al., 2000). CTN is also detected in Monascus fermentation products, which have received considerable attention recently as natural dietary supplements to prevent cardiovascular disease in addition to prescription drugs (Liu et al., 2005; Wei et al., 2003).

As the kidney is the major target organ of CTN toxicity in animal models, administering lethal doses of CTN in porcine, rats, and rabbits caused acute tubular necrosis and kidney enlargement (Kogika et al., 1993; Kumar et al., 2007). Despite inducing renal adenoma in male F344 rats, CTN displayed no carcinogenic activity in mice and Sprague-Dawley rats. (Arai and Hibino, 1983; Knasmuller et al., 2004).

CTN is assumed to require a complex cellular biotransformation to exert mutagenicity, but results of the mutagenicity and genotoxicity of CTN remain controversial. Different aberrations of the structural chromosome were found in bone marrow cells isolated from CTN-stimulated mice; chromosomal changes included chromosome/chromatid breaks, gaps,acentric fragments, metacentric chromosomes, and ring formations (Bouzilmi et al., 2008; Jeswal, 1996). CTN also induced the formation of micronuclei in various cell cultures, including Chinese hamster V79 cells, human lymphocytes, and HepG2 cells (Donmez-Altuntas et al., 2007; Knasmuller et al., 2004; Pfeiffer et al., 1998). Cellular micronucleus generate whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division, so micronuclei test could be used as a screening method to determine clastogenicity and aneugenicity (Heddele et al., 1991). Both chromosome structural and numerical alterations potentially signal the onset of tumor formation (Masuda and Takahashi, 2002; Oshimura and Barrett, 1986).

Although CTN is considered to have aneugenic potential based on the evidence of micronuclei formation, the molecular mechanism underlying this phenomenon is still unclear. Defects in mitotic spindle assembly or in cell cycle checkpoints may be responsible for the chromosome instability of cells. The role of checkpoints, such as p53 and cyclin B1 proteins, in DNA-damaged cells are known to arrest cell cycle transition...
and/or induce cell death (Elledge, 1996; Hartwell, 1992). In the present study, by applying HEK293 cell as a model, we demonstrated that CTN caused the G2/M arrest of cell cycle by interrupting the spindle formation and tubulin polymerization. This study further elucidated the numerical chromosome aberrations in CTN-stimulated human peripheral blood lymphocytes.

**MATERIALS AND METHODS**

**Reagents.** Cell culture medium and serum were obtained from Life Technologies (Grand Island, NY). Antibodies against p53, p21, α-tubulin, and β-actin were purchased from Sigma Chemical Co (St Louis, MO). The antibodies specific to phospho-cell division cycle 2 (cdc2) (Tyr15) and parent cdc-2 kinase were obtained form Cell Signaling (Beverly, MA) and Santa Cruz (Santa Cruz, CA), respectively. Horseradish peroxidase-conjugated goat anti-mouse/anti-rabbit IgG secondary antibodies were obtained from Pierce (Rockford, IL). CTN from Sigma Chemical Co. was dissolved in 25% ethanol in 0.01M PBS at a concentration of 10mM and stored at −20°C.

**Cell cultures.** Human embryonic kidney cell line (HEK293), ATCC number CRL-1573, was obtained from Biosources Collection and Research Center in Taiwan and cultured in medium formulated with minimal Eagle’s medium supplemented with 10% horse serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Human peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood of four healthy subjects (two female and two male ranging in age from 24 to 32 years old) by density-gradient sedimentation over Ficoll-Paque (Amersham Biosciences) and then cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1.5% phytohemagglutinin at 37°C for 48–72 h before toxin treatment.

**Cell viability assay.** HEK293 (1 × 10⁴ cells) were seeded in 96-well plates and treated with vehicle alone (25% ethanol in PBS) or various concentrations (final concentration 0–100μM) of CTN for 24 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, a method applying the mitochondrial metabolic enzyme activity as an indicator of cell viability, was conducted following the protocol described in the report of Liu et al. (2006).

**Flow cytometry.** Cells (3 × 10⁵ cells on a 3.5-mm² culture dish) were cultured for 72 h and then treated with various concentrations of CTN or vehicle (25% ethanol in 0.01M PBS) for 24 h. Following trypanosminization and washes in ice-cold PBS, cells were fixed with ice-cold 80% methanol and stored at −20°C for 24 h. Subsequently, fixed cells were collected, washed in ice-cold PBS, suspended into PBS, and incubated with 0.2 mg/ml of RNase A and 2% Triton X-100 for 1 h at room temperature. For propidium iodide (PI) staining, cells were washed with PBS, suspended into PBS, and incubated with 0.2 mg/ml of RNase A and 0.2 mg/ml of anti-phospho-Ser/Thr-Pro MPM-2 antibody (Upstate, Temecula, CA) for 10 min prior to harvest. The cells were exposed to hypotonic solution (0.56% KCl) and then treated with various concentrations of CTN or vehicle for 24 h. After permeabilized twice with PBS buffer containing 0.2% Triton X-100 for 5 min, the nuclei were recognized by staining for 15 min with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI) at room temperature in dark. After being washed with PBS, the stained cells were visualized and photographed by Zeiss Axiohot fluorescence microscope using a DAPI II filter (magnification ×400). For each sample, a minimum of 500 cells from more than five different random fields of the slide were counted and mitotic cells were scored. Mitotic index was calculated by dividing the total number of examined cells with the number of cells in mitosis.

**Western blot analysis.** Whole-cell protein extracts were prepared according to Liu et al. (2006) and then incubated with Laemmli buffer and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and reacted with primary antibodies specific to p53, p21, phospho-cdc2, cdc2, cyclin B1, α-tubulin, or β-actin at 4°C for overnight. Secondary antibodies conjugated to horseradish peroxidase were subsequently added for another 1 h at room temperature. Bound antibodies on the membrane were detected using an enhanced chemiluminescence detection system according to the manufacturer’s manual (Amersham Pharmacia Biotech, Amersham, UK). The intensities of bands on blots were quantitated using the ImageGauge program Ver. 3.46 (Fuji Photo Film, Tokyo).

**Immunocytochemistry.** Cells seeded on coverslips were cultured for 72 h and then treated with various concentrations of CTN or vehicle for 24 h. After treatment, cells were fixed with 4% paraformaldehyde for 20 min and then washed twice with 0.2% Triton X-100 in PBS for 5 min. After permeabilizing, cells were blocked with 1% BSA containing 0.2% Triton X-100 in PBS at room temperature for 30 min. Then, cells were incubated for 1 h with monoclonal antibody against α-tubulin before further incubation with FITC-conjugated anti-mouse IgG secondary antibody (Zymed) for 2 h in dark. DAPI were added to a final concentration of 10 μg/ml for 15 min at room temperature to recognize the nuclei and then rinse twice with PBS. After the staining period, the cellular microtubules were observed under Zeiss Axiohot fluorescence microscope with a ×100 oil immersion objective. The microtubules condensation and spindle formation in mitotic cells were examined by Zeiss LSM510 META confocal laser scanning microscope equipped with a ×63 oil immersion objective.

**In vivo microtubule polymerization assay.** HEK293 (3 × 10⁵ cells on a 3.5-mm² culture dish) cultured for 72 h were exposed to CTN or vehicle for 24 h. Cells then were rinsed with 0.01M PBS and lysed for 10 min at room temperature with hypotonic buffer (20mM Tris-HCl [pH 6.8] 1mM MgCl₂, 2mM ethylene glycol tetraacetic acid (EGTA), and 0.5% NP-40) and protease inhibitor solution (0.8μM aprotinin, 1μM 4-(2-aminoethyl) benzenesulfonaryl fluoride hydrochloride, 20μM leupeptin, 40μM bestatin, 15μM pepstatin A, 14μM E-64, and 0.2mM phenylmethylsulfonyl fluoride). The supernatant containing cytosolic tubulin was separated by centrifugation at 13,000 rpm for 10 min at room temperature. The pellet representing the polymerized tubulin was collected and resuspended in hypotonic buffer. Lysates were subjected to 10% SDS-PAGE before Western blotting.

**In vitro microtubule polymerization assay.** The effect of CTN on tubulin polymerization was detected using the CytoDYNAMIX Screen TM3 (CDS-03) Kit (Cytoskeleton, Denver, CO) according to the manufacturer’s protocol. In brief, tubulin proteins (> 99% purity) were suspended (300 μg per sample) with 100 μl of G-FEM buffer containing 80mM piperazine-1,4-bis(2ethanesulfonic acid), 2mM MgCl₂, 0.5mM EGTA, 1.0mM GTP (pH 6.9), and 5% glycerol in the present of CTN (50 and 100μM), colchicine (10μM), paclitaxel (10μM), or vehicle. Then, the mixture was transferred to the pre-warmed 96-well plate, and the polymerization of tubulin was measured every minute during 30 min by using Spectra384 microplate reader set at 37°C and 340 nm (Molecular Devices, Sunnyvale, CA).

**Chromosome number analysis.** Purified human PBMC cultures were treated with 50μM CTN for 72 h and then colcemid (0.1 μg/ml) was added 25 min prior to harvest. The cells were exposed to hypotonic solution (0.56% KCl) for 10 min at 37°C and fixed with chilled methanol/acetic acid (3:1). Chromosome spreads were obtained using the standard air-drying techniques and stained with 0.1% Giemsa stain solution. For each experimental point, the chromosome number in each of 100 metaphases was counted at a magnification
of ×1000 with an optical microscope. Three independent experiments were conducted.

Statistical analysis. All statistical analyses were carried out using the software program GraphPad Prism Version 4.0 (GraphPad Software, CA). Experimental data grouped by one variable were analyzed by unpaired two-tailed t-test or one-way ANOVA followed by Tukey posttest. A value of p < 0.05 was considered significant.

RESULTS

Effects of CTN on Cell Viability of HEK293 Cells

The cell viability of HEK293 cells following treatments with vehicle or various concentrations of CTN was evaluated by MTT assay. When HEK293 cultures were incubated with 100μM CTN for 24 h, the overall viability of the cells slightly decreased to 90.8% of the control levels (Table 1); however, this difference was not statistically significant.

CTN Induced the Cell Cycle Arrest of HEK293

To examine whether CTN-induced growth inhibition was associated with cell cycle regulation, the cell cycle distribution was analyzed by flow cytometry. Treating HEK293 with CTN for 24 h caused an accumulation of cells in G2/M content and a decrease of cells in G1 DNA content in a concentration-dependent manner (Fig. 1A). The cell number in G2/M region after 100μM CTN treatment increased significantly to 53.6 ± 1.2%, in comparison with that of solvent-treated control (23.7 ± 1.4%). Additionally, the percentage of diploid cells was found to be 29.4 ± 3.7% in 100μM CTN-treated group, whereas that was 52.0 ± 2.7% in the control group.

The levels of proteins involved in G2-M transition were further examined by Western blotting analysis. p53 and p21 have been implicated in the control of G2-M checkpoint in addition to G1-S (Bunz et al., 1998). Phosphorylation status of cdc2 kinase governs the activity of cdc2/cyclin B1 complex, which is responsible for the onset of mitosis (Elledge, 1996; O’Connor, 1997). According to Figure 1B, exposure of HEK293 to CTN induced the expression of p53 and p21 proteins, whereas the signal of phospho-Tyr15 cdc2 kinase was downregulated. CTN treatment did not affect the levels of parent cdc2 and cyclin B1 in HEK293.

CTN Induced the Arrest of Mitotic Phase

Both cells in the G2 or mitotic phase possess 4 N DNA content, explaining why analysis of the cell cycle distribution by flow cytometry (Fig. 1A) could not differentiate G2 cells from mitotic ones. Therefore, this study evaluated whether CTN induces the arrest of cell cycle at G2 or mitotic phase, by staining cells with DAPI, a DNA-specific dye, to reveal the organization of the chromatin. In response to CTN concentrations, the number of mitotic cells with condensed DNA substantially increased (Fig. 2A). In Figure 2B, the mitotic index of vehicle-treated cells was around 1.9 ± 0.3%. However, after 100μM CTN and 50nM colchicine stimulation, mitotic index significantly increased to 24.6 ± 1.1% and 33.9 ± 2.8%, respectively. Colchicine, an inhibitor of tubulin polymerization, served as a positive control in this study.

Furthermore, since mitotic entry is accompanied by the accumulation of mitosis-controlling proteins with phosphoepitopes, CTN-treated cells were examined for the levels of phosphoproteins that are specifically recognized by antibody MPM-2. The MPM-2 antibody, an effective tool for studying the regulation of mitosis, specifically binds to a phosphoamino acid-containing epitope present on more than 40 proteins involved in the mitotic process (Davis et al., 1983; Westendorf et al., 1994). Western blotting analysis indicated that signals recognized by MPM-2 were markedly upregulated in a dose-dependent manner (Fig. 3A). Moreover, according to data from flow cytometry, the cell population with both 4 N DNA content and MPM-2 antigen positive, considered as mitotic cells, was significantly elevated in cultures treated with CTN above 50μM (Fig. 3B).

In the control group, 3.4% of detected cells could be recognized by MPM-2. When HEK293 was exposed to 75μM CTN, around 25.6% of the cells were identified as mitotic cells by MPM-2. The cell number in G2 phase, with 4 N DNA that did not react with MPM-2, was also slightly elevated along with the increasing concentration of CTN. Above results suggest that CTN altered the cell cycle distribution mainly in the mitotic phase.

CTN Disrupted the Microtubule Organization of HEK293 Cells

Based on the above findings, we hypothesize that CTN induces mitotic arrest by effectively suppressing microtubule dynamics. First, we investigated how CTN affects cytoskeleton components during interphase by immunofluorescent staining of the microtubule. Normal microtubule distribution, which was detected by the antibody specific to α-tubulin, was observed in vehicle-treated HEK293 cells (Fig. 4A). However, exposure with various concentrations of CTN resulted in the appearance of diffuse stain and irregular microtubule fragments throughout the cytosol, which was similar to the microtubule changes caused by colchicine.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Cell Viability of HEK293 Cells after CTN Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTN (μM)</strong></td>
<td><strong>Cell viability (%)</strong></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>105.14 ± 0.73</td>
</tr>
<tr>
<td>75</td>
<td>97.99 ± 1.13</td>
</tr>
<tr>
<td>100</td>
<td>90.83 ± 1.99</td>
</tr>
</tbody>
</table>

Note. Data are given as the mean ± SEM from four independent experiments.

*a*Cell cultures were treated with solvent or various concentrations of CTN for 24 h.

*b*Cell viability was expressed as percentage of solvent-treated control.
An attempt was also made to elucidate whether CTN exerts a similar effect on the mitotic stage of cells, by using a fluorescent confocal microscope to examine the structure of spindle assembly from microtubule building blocks. Figure 4B illustrates representative results showing the microtubule network in response to CTN. The microtubule organization with a shape of spindle remained intact in the vehicle or 25 µM CTN-treated group (data not shown). Nevertheless, after the CTN concentration increased to 50 µM or higher, the mitotic spindles were in disorganization structures; signals of microtubule were distributed at the membrane and perinuclear cytoplasmic regions.

**CTN-Inhibited Tubulin Polymerization In Vivo and In Vitro**

Because CTN interfered with the microtubule organization in situ, we further examined whether CTN modulates the dynamic polymerization of tubulin units in HEK293 cultures (in vivo) and in test tubes (in vitro). When HEK293 cells were exposed to various concentrations of CTN for 24 h, the centrifuged pellets of cell lysate harboring tubulin polymers were collected and applied to immunoblots with antibodies specific to α-tubulin and β-actin (Fig. 5). CTN at concentrations of 50 and 100 µM significantly downregulated the signal of tubulin protein but did not modulate the levels of β-actin, i.e., a major component of cytoskeleton microfilament. This finding suggests that CTN specifically suppresses the polymerization of tubulin in vivo.

*In vitro* tubulin polymerization was conducted to examine whether CTN acts directly on microtubule formation in the absence of cellular components. As presented in Figure 6, commercially purified tubulins in the control group were polymerized in the presence of GTP and a steady state was reached after 25 min of incubation at 37°C. Notably, CTN ranging from 50 to 100 µM substantially inhibited tubulin polymerization in a concentration-dependent manner. For comparison, behaviors of tubulins after exposing to microtubule polymerization inhibitor (10 µM colchicines) and stabilizer (10 µM paclitaxel) were also demonstrated.

**CTN Induced Numerical Chromosome Change in PBMC**

Microtubule disruption may lead to chromosome loss and/or mitotic nondisjunction (Bakhoum et al., 2009), so the chromosome number in CTN-treated PBMC was counted. PBMC, a primary cell culture collected from healthy donors, was adopted herein owing to its better chromosome stability than permanent cell line HEK293. Most (93.5%) of the metaphases from vehicle-exposed cultures had a diploid (2N = 46) number of chromosomes; only 6.5% of the metaphases had changes in chromosome number, which was near the diploid number (Table 2). After PBMC was treated with 50 µM CTN for 72 h, the percentage of abnormal cells increased up to 29.2%. Three of the 400 CTN-treated cells exhibited the tetraploid and near tetraploid number of chromosomes. These observations imply that CTN administration results in numerical chromosomal aberration that is attributed to its ability to interfere with microtubule formation.

**DISCUSSION**

Microtubules, which consist of αβ-tubulin heterodimer, are necessary for a stable microtubule network during interphases and for proper mitotic spindle assembly and chromosome...
segregation during mitosis (Bakhoun et al., 2009). This study and Pfeiffer et al. (1998) have showed that CTN had the ability to block tubulin polymerization in test tube without active cellular components. Nevertheless, results from test tubes are not always applicable to cell cultures, so we also conducted in vivo tubulin polymerization and found that the level of tubulin polymer in HEK293 cells was significantly decreased by CTN (Fig. 5). Additionally, CTN suppressed the microtubule organization and spindle formation in interphase and metaphase of HEK293 when the antibody specific to tubulin was used as a probe for in situ hybridization (Figs. 4A and 4B).

In these experiments, colchicines, which served as a positive control, demonstrated a similar inhibitory pattern as CTN did. As is well known, colchicine exerts its inhibitory effect on the microtubule assembly by binding at the interphase of α- and β-subunits of the tubulin heterodimer, called colchicine-binding site (Bhattacharyya et al., 2008). Many compounds have been grouped as colchicines site inhibitors, some with structures resembling to those of colchicines, whereas others with diverse features. Whether CTN satisfies the criteria for ducking into the colchicine-binding sites on tubulin is unknown because the size and chemical structure of CTN appear to markedly differ from colchicine and its analogs.

Highly dynamic mitotic spindles are among the most potential targets for anticancer therapy. Suppressing microtubule dynamics by anticancer drugs and compounds generally results in cell cycle arrest, subsequently leading to apoptotic cell death (Jordan and Wilson, 2004; Xiao et al., 2003). CTN at lower concentrations, such as 50 μM, triggered the G2/M phase arrest in HEK293 (Fig. 1). However, when HEK293 was incubated with CTN at a concentration exceeding 150 μM, instead of cell cycle arrest, the apoptosis phenomenon was found in this study, including the increase of sub-G1 phase, elevation of caspase 3 activity, and formation of DNA ladder (data not shown). CTN at concentrations ranging from 20 to 100 μM has been reported to induce mitochondrial-dependent apoptosis in various cell cultures, including mouse embryonic stem cells and human promyelocytic leukemia cells (HL-60) (Chan, 2007; Yu et al., 2006). Comparing with these cell lines, HEK293 seems to show a higher resistance to CTN-induced cell death by halting the cell cycle at G2/M phase.
CTN treatment arrested HEK293 at the stage of G2/M (Fig. 1A); it is supported by our previous finding that CTN induced the messenger RNA expression of GADD45 gene in HEK293 cultures (Chang et al., 2009). Induction of GADD45, a p53 regulated and DNA damage–inducible protein, has a role in contributing to both G2 and mitotic arrest in response to antimicrotubule agents (Jin et al., 2002; Mullan et al., 2001). On the other hand, CTN was able to decrease the amounts of phospho-Tyr15 cdc2 kinase in HEK293, indicating that CTN promotes the entry of cells into mitosis (Fig. 1B); dephosphorylated cdc2 is known to convert the cdc2/cyclin B1 complex into an active form, subsequently driving cells from G2 into mitosis (Stark and Taylor, 2006). Furthermore, HEK293 cells stimulated with CTN significantly accumulated

**FIG. 4.** Effect of CTN on the organization of the microtubule cytoskeleton in HEK293. Cells were incubated with vehicle, CTN, and colchicines (chol, 50nM) for 24 h. With the assistance of immunocytochemistry applying anti-α-tubulin antibody, FITC-conjugated anti-mouse secondary antibody, and DAPI staining, the cellular microtubule network during interphase was observed under fluorescence microscope (×100 oil immersion objective) (A), and the microtubules condensation in the mitotic cell was examined by confocal laser scanning microscope (×63 oil immersion objective) (B).

**FIG. 5.** CTN decreased the levels of tubulin polymer. HEK293 cells were treated with CTN or colchicine (chol, 50nM) for 24 h. Polymeric tubulin in the pellet of cell lysate was subjected into 10% SDS-PAGE. Immunoblots were shown using anti-α tubulin or β-actin antibodies as probes. The relative ratio of tubulin over actin levels shown in the lower panels are the mean ± SEM obtained from four independent experiments. *Significantly different compared with controls (*p < 0.05; **p < 0.01).

**FIG. 6.** CTN inhibited tubulin polymerization in vitro. Commercially purified tubulins in reaction buffer were incubated at 37°C in the presence of vehicle (control), the indicated concentration of CTN, paclitaxel (10μM), or colchicine (10μM). The kinetic reaction of microtubule assembly was measured by spectrophotometry as described in the “Material and Methods” section. Data are representative of three independent experiments.
phospho-proteins or mitotic cells, which were recognized by MPM-2 antibody, an effective tool for studying the regulation of mitosis (Figs. 3A and 3B). Therefore, it suggests that CTN-induced cell cycle arrest should primarily occur during the M phase but not at the G2 phase.

Both structural chromosome changes (e.g., translocations, chromosome gaps) and numerical chromosome changes (aneuploidy and polyploidy) contribute to the genetic load responsible for carcinogenesis (Kirsch-Volders et al., 2002). Induction of structural chromosome aberration by CTN has been well documented (Bouslimi et al., 2008; Jeswal, 1996), whereas few direct evidence points out the ability of CTN to trigger numerical aberration of chromosomes. Knasmuller et al. (2004) and Pfeiffer et al. (1998) observed that CTN-induced micronuclei were predominantly centromere positive in HepG2 or Chinese hamster V79 cells, suggesting that whole chromosomes are located within the micronuclei, which is an indirect evidence of chromosome loss in cells. In this manuscript, direct evidence of CTN-induced chromosome instability was provided by counting the chromosome number in CTN-treated human primary culture (Table 2). After treating PBMC with CTN for 3 days, 29.2% of cells have abnormal chromosome number ranging from 37 to 92. As chromosomal instability is often attributed to mal-oriented attachment of chromosomes to spindle microtubules, CTN may initially interfere with the tubulin polymerization, subsequently leading to chromosome loss and/or mitotic nondisjunction. Similar mechanism is found in other mycotoxins; zearalenone and deoxynivalenol, isolated from Fusarium species, have been identified to cause aneuploidy in pig oocytes via interrupting the normal organization of meiotic spindle (Malekinejad et al., 2007).

Chromosome aneuploidy is closely associated with malignant diseases and neoplastic transformations (Heng et al., 2006; Koyama et al., 2000). Clinically, most solid tumors are aneuploid, with many of them frequently missegregating chromosomes (Ai et al., 1999; Bakhourm et al., 2009). A previous report demonstrates that colcemid, an analog of colchicine, induces aneuploidy as well as morphological transformation of cells in culture (Tsutsui et al., 1984). Diethylstibestrol, another microtubule disruptor also exhibits neoplastic transformation of cells (McLachlan et al., 1982; Tsutsui et al., 1983). However, when HEK293 were exposed to 20 or 50μM of CTN for 3 and 7 days, respectively, the cells did not display any positive results in cell migration and soft agar colony formation assays (data not shown). It suggests that under the experimental conditions of this study, CTN does not have the cell-transforming ability.

CTN is a mycotoxin frequently found in dietary staple and supplements, especially Monascus fermentation products. The concentration of CTN in commercial Monascus products varies between 0.2 and 17.1 ppm (mg/kg) (Liu et al., 2005; Sabater et al., 1999). According to our results, CTN at 50μM (corresponding to 12.5 ppm) was not cytotoxic to examined human cultures but interfered the microtubule organization and even led to the instability of chromosomal number in human blood cells (Fig. 4, Table 2). It has been shown that when mice were administrated with 0.9 mg/kg body weight (single ip injection) or 0.1 mg/kg body weight of CTN (intubation twice a week for 8 weeks), the bone marrow cells were induced a high frequency of structural chromosome abnormalities, including chromatid gaps and breaks (Bouslimi et al., 2008; Jeswal, 1996). Nevertheless, it is difficult to correlate the toxin concentration applied in cell cultures to the dose used in whole animals because of the toxicokinetic variation.

Although acute exposure to a massive amount of CTN is rare today, long-term consumption of food with low levels of lipophilic CTN remains problematic. This study clearly demonstrates the molecular mechanism and aneuploid potential of CTN. The induction of chromosome loss and/or nondisjunction by CTN in human cells is especially relevant for calculating the risk of carcinogenicity.

### FUNDING

This work was financially supported by the National Science Council of the Republic of China, Taiwan, under Contract No. NSC 97-2313-B-040-004-MY3.

### REFERENCES

Ai, H., Barrera, J. E., Pan, Z., Meyers, A. D., and Varella-Garcia, M. (1999). Identification of individuals at high risk for head and neck carcinogenesis...
using chromosome aneuploidy detected by fluorescence in situ hybridization.


