African Journal of Biotechnology Vol. 7 (8), pp. 1151-1156, 17 April, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Environmental, genetic and cellular toxicity of tenuazonic acid isolated from *Alternaira alternata*

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Accepted 4 December, 2007

***Alternaria alternata*, an important pathogen of many plants, produces tenuazonic acid (TeA) with bioactivity to microbes, plants and animals. TeA is one of the main mycotoxin to humans and other organisms. Using *Chlamydomonas reinhardtii*, *Vicia faba* root tip and three mammalian normal cell lines as target materials, environmental toxicity, genetic toxicity and cytotoxicity of TeA were examined. The growth and chlorophyll concentration of *C. reinhardtii* were inhibited at above 100 µg/ml concentration, with $EC_{50(G)}$ of 310.36 µg/ml and $EC_{50(Chl)}$ of 294.27 µg/ml. The micronuleus test results indicated that MCN‰ was >15‰ only at higher concentrations. TeA inhibited the proliferation and total protein contents of 3T3 mouse fibroblasts (3T3 cells), Chinese hamster lung cells (CHL cells) and human hepatocytes (L-O2 cells) at concentrations ranging from 12.5 - 400 µg/ml. Of the three cell lines, 3T3 cells were the most sensitive to the toxin ($EC_{50(24h)}$ = 41.64 µg/ml), followed by CHL cells ($EC_{50(24h)}$ = 59.33 µg/ml), and L-O2 cells ($EC_{50(24h)}$ = 85.98 µg/ml) had the lowest sensitivity.**

Key words: Tenuazonic acid, *Chlamydomonas reinhardtii*, micronucleus, mammalian cell line, protein content, toxicity.

INTRODUCTION

The genus *Alternaria* contains important pathogens that produce carcinogenic, teratogenic and mutagenic mycotoxins causing deleterious effects on the health of human and animal (Pero et al., 1973; Schrader et al., 2001). These mycotoxins have been found as natural contaminants in spoiled grains (Sauer et al., 1978), diseased plants (Buchwaldt and Jensen, 1991), and some visibly rotten fruits (Schroeder and Cole, 1977; Stinson et al., 1980). Consumption of contaminated food and feedstuffs by humans and animals can cause mycotoxicosis (Prelusky et al., 1994). Among them, tenuazonic acid (TeA) is one of the main toxic components and considered as a possible causal factor of Onyalai, a hematological disorder in man (Steyn and Rabie, 1976). TeA, a tetramic acid derivative, was first isolated as an anti-tumor agent from the culture filtrates of *Alternaria tenuis* (Rosett et al., 1957) and its structure was elucidated by Stickings (1959) with classic methods (Figure 1). TeA also exhibits phytotoxic, insecticidal, zootoxic, cytotoxic, antibacterial and antiviral activity (Chelkowski and Visconti, 1992).

Several toxicology studies of TeA have been conducted because of toxicity concerns. When it was fed orally, TeA induced collapse in the cardiovascular system of mouse, rat, dog and monkey, and caused young mice moderate and severe dysplasia. Moreover, microscopic abnormality in epithelial cells and increase of salivary secretion, nausea and deaths were observed in these animals (Smith et al., 1968; Yekeler et al., 2001). TeA produced mortality in chicken embryos and induced marked microscopic and macroscopic lesions in various tissues of treated young chickens (Giambrone et al., 1978; Griffin and Chu, 1983). The Ames *Salmonella* test showed no mutagenic influence of TeA in TA98, TA100 and TA104 regardless of the presence or absence of S9 (Scott and Stoltz, 1980; Schrader et al., 2001, 2006), but TeA was slightly mutagenic to TA97 and TA102 with the presence of S9 (Schrader et al., 2006).

Except for the Ames test, majority of the animal toxicity studies of TeA cited above focused mainly on the histopathology. In order to completely evaluate the toxicity of TeA, a mammalian cell gene mutation assay, chromosome aberration test, micronucleus test, and even environmental toxicology are required. In the present

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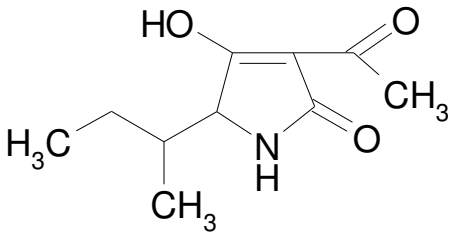

Figure 1. Structure of tenuazonic acid (Stickings, 1959).

work, *Chlamydomonas reinhardtii*, *Vicia faba* root tip and three mammalian normal cell lines were used to examine environmental toxicity, genetic toxicity and cytotoxicity of TeA with the goal of gaining thorough understanding of TeA toxicology.

MATERIALS AND METHODS

Toxin production

TeA was isolated and purified from the culture of *Alternaria alternata* isolate NEW. The fungus strains were inoculated into a 1000 ml flask with 400 ml sterile potato (200 g/L), sugar (20 g/L) and potassium dihydrogen phosphate (1 g/L) liquid medium (PSK), and cultured for 6 days on an orbit shaker (Model 3527X Orbit Environ-Shaker; Lab-Line Instruments, Inc., Ill. USA) at 110 rpm in the dark at 25℃. The filtrate was passed through a macroporous resin DA201 column (Shanghai Yadong Heji Resin Inc., China), and finally the column was eluted with ethanol. The ethanol-diluted extraction was concentrated by rotary evaporator under regular pressure at 80℃. The condensate was extracted three times with the same volume of ethyl acetate. The extract was concentrated by rotary evaporator under regular pressure at 70℃, and then the crude toxin was obtained. The crude toxin was fractioned three times by column chromatography on Silica gel, and then the fractions were subjected to preparative Silica (Qingdao Ocean Chemistry Industry Inc., China) TLC in petroleum ether-ethyl acetate-acetic acid (15:10:1) until the purified toxin was obtained. The purity was determined by TLC and HPLC to be higher than 98%.

Green algae and cell lines

The wild type strain of green algae *C. reinhardtii* purchased from Duke University was grown at 25 ± 1℃ in total darkness in a liquid tris-acetate-phosphate (TAP) medium (Harris, 1989). After 96 h, cells at the late logarithmic phase (750 nm O.D., ~0.75; ~5 ×10^6 cells/ml) were harvested to be used for subsequent steps. Three cell lines, 3T3 mouse fibroblasts (3T3 cells), Chinese hamster lung cells (CHL cells) and human hepatocytes (L-O2 cells), purchased from the Chinese Academy of Science Cell Bank, were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA), supplemented with 10% calf serum (GIBCO BRL, Grand Island, NY, USA) and maintained as monolayers in 80 cm^2 cell culture flasks in a 60% humidified incubator with 5% CO_2 in the air at 37 ± 1℃ for 3 days prior to experiments.

C. reinhardtii environmental toxicity assay

The harvested green algae (*C. reinhardtii*) were inoculated in the TAP media at a 1/9 ratio of algae mother fluid to medium, and then treated with different toxin concentrations 12.5, 25, 50, 100, 200, and 400 µg/ml, dissolved in 0.5% methanol. The treated green algae were cultured in darkness at 25 ± 1℃ for 96 h. The growth rate of the treated *C. reinhardtii* wild type was determined by measuring the optical density of the cells in the TAP culture medium at 750 nm using a UV 751GD type spectrophotometer (Shanghai, China). Chlorophyl (Chl) concentration was determined by suspending the cells in 80% acetone at 40℃ for 20 min. The samples were centrifuged at 8000 × g for 10 min, and the resulting pellet was discarded. The absorbance of the supernatant was measured at 663 and 645 nm using the same spectrophotometer. Chl concentrations were calculated according to the equations of Porra et al. (1989). The treatments of medium and medium with 0.5% methanol were respectively designed as CK0 and CK1, and every treatment was replicated four times.

V. faba root tip micronucleus test (MCN)

Commercial seeds of *V. faba*, surface disinfected with 0.1% hydrargyrum chloride (Nanjing Bioindustry Inc., Nanjing, China) for three minutes, were soaked in distilled water for 24 h at room temperature (22 ± □), and then transferred into plates (25 cm × 15 cm) with wet gauze placed on the bottom to germinate. When the roots reached about 2 cm, vigorous *V. faba* seedlings with uniform size were placed in the bud boxes with toxin solution having different concentrations of 12.5, 25, 50, 100, 200, and 400 µg/ml, assuring the root tips were fully immersed in the solutions. The treatments of distilled water and distilled water with 1% methanol were designated as CK0 and CK1, respectively. Each treatment was replicated 3 times. After having grown for another 48 h, the treated seedlings were washed three times with distilled water and then transferred back into distilled water to culture for 24 h. Ten treated root tips from each replication were cut into segments of about 0.5 cm long, and placed in 10 ml-tubes with 5 ml Carnoy's fixative for 24 h at room temperature (22 ± 1℃). The fixed root tips were transferred into 70% ethanol until the next step. The fixed root tips were treated with 2 ml of hydrolysis solution for 15 to 20 min and then softened with 2 ml Carnoy's fixative for 5 min. The tips were washed with distilled water repeatedly until they became white and somewhat sheer. The root tips were cut into 2 mm segments, placed on a microscope glass slide, covered with another slide, and crushed by pressing down on the slide cover. After the covered slide was removed, one or two drops of improved phenol fuchsin dyeing liquor (He and Lu, 2000) was added to the crushed root tips and allowed to stain for 20 - 30 min. A cover slip was placed on the stained root tips Micronuclei were counted in at least 1000 intact well stained cells per treatment with the aid of a light microscope (OLYMPUS BH-2) (× 10).

Mammalian cell toxicity assay

The cells were detached from the 80 cm^2 cell culture flasks with 0.05% (v/v) trypsin (Sigma Inc., USA) and 0.02% (v/v) EDTA (Sigma Inc., USA) in phosphate buffer saline (PBS) (Sigma Inc., USA) and were routinely checked for mycoplasma using the Mycoplasma Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany, Cat. No 1296744). Single cell suspensions were counted using a hemocytometer and re-suspended to 4 × 10^4 cells/ml in cell culture medium. 100 µl of the cell suspension was added to each well of a 96-well microtiter plate (Wuzhou Medicine Inc., Huangyan, Zhejiang, China) and incubated for 24, 48 and 72 h, respectively. The cell culture medium was then replaced with a 150 µl tenuazonic acid solutions which were prepared by dissolving the toxin from 12.5 - 400 µg/ml in fresh cell culture medium with 0.1% v/v DMSO. Water control and solvent control, CK0 and CK1, respectively were

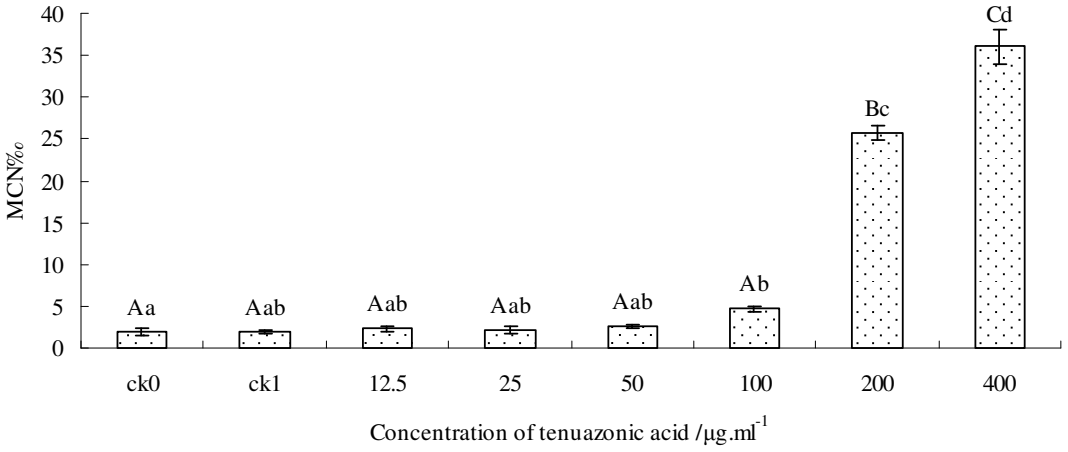

Figure 2. Effects of TeA on MCN‰ of root tip cells in *Vicia faba*. *V. faba* root tips were treated for 48 h with TeA at concentrations from 12.5 – 400 μg/ml. Micronuclei were observed after the end of the treatments. Values represent means ± SE (standard error) of three triplicate determinations. The different small letters in each column indicate significant difference at P <0.05 level; the different capital letters in each column indicate significant difference at P <0.01 level.

Table 1. Effect of TeA on green algae *Chlamydomonas reinhardtii*.

Concentrations of TeA/μg.ml^{-1}	Optical density of cells (750nm)	Chl conc./mg.l^{-1}
CK0	0.75 ± 0.021Cc	19.22 ± 1.63Cc
CK1	0.74 ± 0.035Cc	19.17 ± 1.03Cc
12.5	0.81 ± 0.18Aa	21.65 ± 1.82Aa
25	0.78 ± 0.12Bb	19.89 ± 2.01Bb
50	0.74 ± 0.09Cc	18.00 ± 1.85Dd
100	0.66 ± 0.16Dd	16.29 ± 1.96Ee
150	0.51 ± 0.08Ee	13.35 ± 1.59Ff
200	0.42 ± 0.04Ff	11.98 ± 0.92Gg
300	0.39 ± 0.05Gg	8.57 ± 0.74Hh
400	0.32 ± 0.03Hh	7.42 ± 0.53Ii

Values represent means ± SE (stand error) of four assays. The different small letters in each column indicate significant difference at P <0.05 level; the different capital letters in each column indicate significant difference at P <0.01 level.

also included. The MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide), purchased from Sigma Chemical Company, was dissolved in PBS solution at 5 mg/ml concentration and filtered through a 0.22 μm filter to sterilize and remove insoluble residues. The microtiter plates were incubated for another 24 h, after incubation, the media were discarded and 20 μl of the MTT solution was added to each well of 96 well plates, and incubated for 4 h at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation period, the media were discarded and 150 μl DMSO solution was added to each well of the 96-well microtiter plates. The microtiter plates were placed on an orbital shaker at 80 rpm for 10 min and the absorbance of each well was measured at 490 nm by a microplate reader (BIO-RAD Model 450 Microplate reader, UCE CO., Japan). Inhibition percentage of cell proliferation was calculated as follows: (the mean absorbance value of a control – that of a toxin-group)/that of a control.

The cell culture media were discarded after cells were exposed to tenuazonic acid for 72 h, quickly washed with D-Hanks solution, and 1 ml SDS solution (10 g/l) was added to resolve the cells in each well. Total protein content in culture of three types of cell was measured by the method of Lowry et al. (1951).

Statistical analysis

Data were expressed as mean ± SE. Statistical analyses were carried out using one-way ANOVA followed by Dunnett's test for multiple comparisons. The EC_{50} values were calculated by regression analysis.

RESULTS

Effect of TeA on green algae, *C. reinhardtii*

The growth and total Chl contents of *C. reinhardtii* were not significantly affected by toxin dosages at lower than 50 and 25 μg/ml concentrations (Table 1). However, with increas-ing TeA concentrations, marked inhibition of the growth and chl contents was observed. The 96 h EC_{50} of TeA to green algae *C. reinhardtii* were displayed in the 96h $EC_{50(G)}$ of 310.36 μg/ml and 96 h $EC_{50(Chl)}$ of 294.27 μg/ml.

Effect of TeA on *V. faba* root tip micronucleus

Environmental genetic toxicity of TeA was evaluated by *V. faba* root tip micronucleus test. There were few micro-nucleus cells with less than 5‰ MCN observed in *V. faba* root tip cells at the lower concentrations of 12.5 - 100 μg/ml and were similar to the controls (Figure 2). How-ever, at the concentration of 200 and 400 μg/ml, the MCN‰ sharply increased up to 36.0 ‰ (Figure 2).

Effect of tenuazonic acid on the proliferation of mammalian cells

At all treatment concentrations from 12.5 - 400 µg/ml, tenuazonic acid inhibited cell proliferation of the 3T3, CHL and L-O2 cells (Figure 3). Over 80% cell proliferation inhibition of 3T3 cells was observed at 400 µg/ml for 24 h exposure, and 100 µg/ml for 48 and 72 h exposures. On CHL cells, over 80% cell proliferation inhibition was achieved with 200 µg/ml for 24 and 48 h exposures, 100 µg/ml for 72 h exposure. On L-O2 cells, over 80% cell proliferation inhibition was obtained with over 400 µg/ml for 24 h, 300 µg/ml for 48h and 200 µg/ml for 72 h exposures.

Similar trends were observed in dose-response relationships on tested mammalian cell lines at 24, 48, and 72 h exposures, but the 3T3 and CHL cell lines have lower EC_{50} values than that of L-O2 cell line in three exposure duration times which indicates that TeA is more cytotoxic to the 3T3 and CHL cell lines than the L-O2 cell line. The 3T3 cell line was the most susceptible to TeA (Table 2).

Effect of tenuazonic acid on total protein contents of mammalian cells

After 72 h exposure to tenuazonic acid, total protein contents of mammalian cells 3T3, CHL, L-O2 were decreased when measured by Lowry method (Figure 4). Inhibition rate of protein content was from 15.3 - 90.7% for 3T3 cells, from 11.7 - 89.0% for CHL cells and from 10.5 - 87.4% for L-O2 cells over the concentration range. Over 80% cell protein content inhibition of 3T3, CHL, and L-O2 cells was observed at 200, 200, and 400 µg/ml, respectively. The relevant $EC_{50(protein)}$ values were 37.37 µg/ml, 56.28 µg/ml and 68.79 µg/ml, respectively.

DISCUSSION

Algae occur widely and play an important role in the aquatic ecosystem. *C. reinhardtii*, a unicellular freshwater green alga, has been used in the environmental toxicity evaluations through investigation of effects on its ultrastructure, growth, and photosystem (León and Galván, 1999; Morlon et al., 2005). Therefore, *C. reinhardtii* was used in our examination of the environmental toxicity of TeA. Our results suggest that the growth and chlorophyll contents of *C. reinhardtii* were markedly inhibited at toxin concentrations higher than 100 and 50 µg/ml. The 96 h $EC_{50(G)}$ and 96 h $EC_{50(Chl)}$ were 310.36 and 294.27 µg/ml, respectively, and according to the standard that 96 h EC_{50} higher than 3 µg/ml had low toxicity in algae environmental safety evaluation of toxicants (Wang, 1998). It can be concluded that TeA has relatively low toxicity.

V. faba root tip micronucleus test was widely used to determine the mutagenicity of toxicants through scoring MCN frequency (Degrassi and Rizzoni, 1982; Ma, 1982; Ma et al., 1995). Scott and Stoltz (1980) and Schrader et al. (2001) had examined the mutagenicity of TeA using

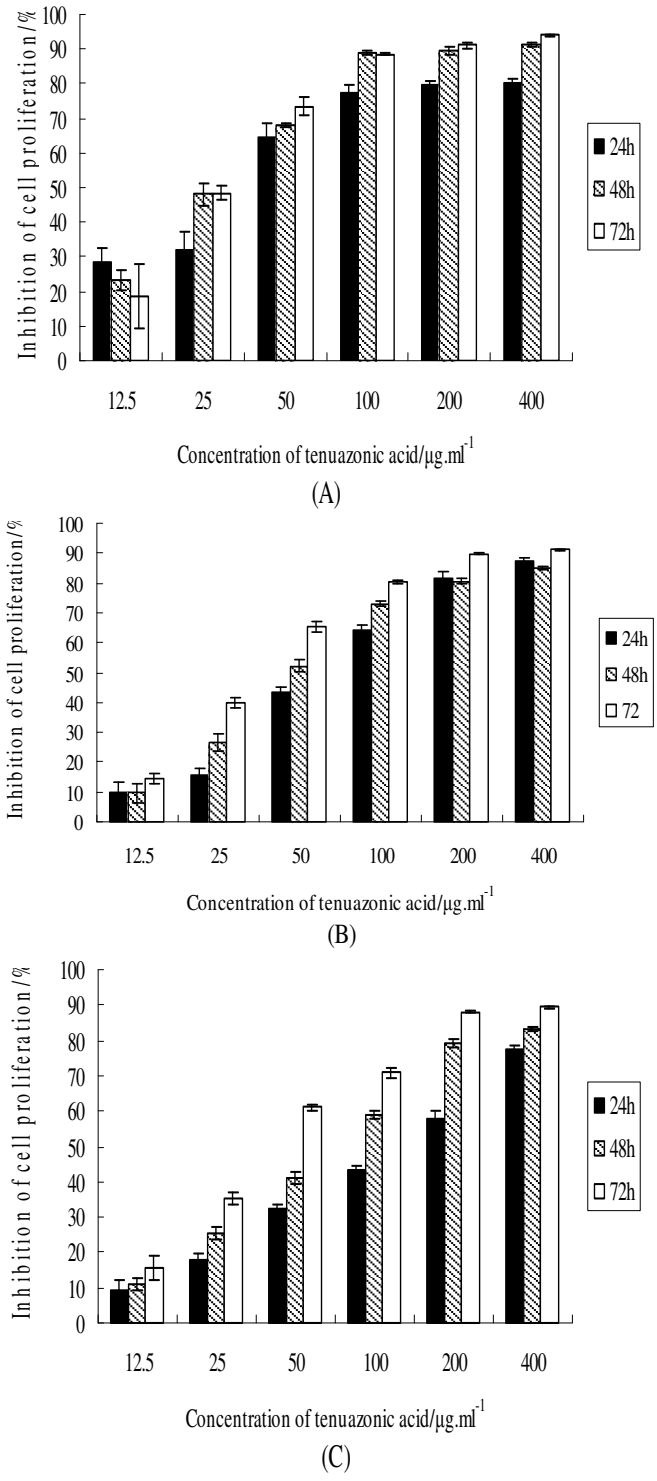

Figure 3. Inhibition of mammalian cell 3T3 (A), CHL (B) and L-O2 (C) proliferation by tenuazonic acid. The experiment was carried out by the MTT method, the cell lines were exposed to tenuazonic acid at concentrations from 12.5 to 400 µg/ml for 24, 48, 72 h.

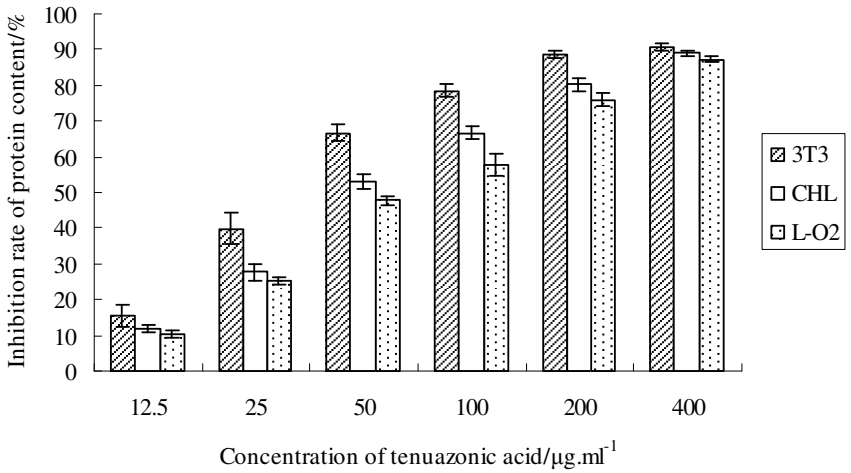

Figure 4. Effect of tenuazonic acid on total protein contents of the 3T3, CHL, and L-O2 mammalian cells exposed 72 h to tenuazonic acid at concentrations from 12.5 to 400 μg/ml. At the end of the treatments, total protein contents were measured by Lowry (1951) method.

Table 2. EC_{50} values of cytotoxicity of the mammalian cell lines to TeA.

Exposure time (h)	EC_{50} values (μg/ml)		
	3T3	CHL	L-O2
24	34.7536	63.4454	117.7735
48	26.3754	50.1649	65.1928
72	26.1273	33.7054	38.6640

Ames *Salmonella* test. In those studies, TeA did not exhibit mutagenicity at concentrations ranging from 1 – 100 μg/plate, but the mutagenicity of TeA at higher concentration than 100 μg/plate was not examined. This is the first time that *V. faba* root tip micronucleus test had been used in the mutagenicity examination of TeA. TeA caused few micronuclei at concentrations ranging from 1 - 100 μg/ml as such was consistent with earlier reports with the Ames test, but at higher concentrations, TeA caused micronucleus formation. A possible mechanism for TeA-induced micronucleus may involve the formation of free radicals which could attack nucleic acids and result in base substitution and breakage of DNA, and eventually induce mutation (Higashi, 1988; Sandermann, 1988). Recently, Schrader et al. (2006) observed that TeA was somewhat mutagenic to TA97 and TA102 with the presence of S9 at concentrations ranging from 1 - 100 μg/plate in the Ames *Salmonella* test. The difference between the results suggested that the materials and methods maybe affect the effects.

Cytotoxic effects of mycotoxins on cultured cells can be assayed by measuring the metabolic activity of the cells (Mosmann, 1983; Hanelt et al., 1994). In the present study, the MTT bioassay showed that TeA inhibited the proliferation of 3T3 mouse fibroblasts (3T3 cells), Chinese hamster lung cells (CHL cells) and human hepatocytes (L-O2 cells), which had a dose-dependent effect on the metabolic activity resulting in s-shaped curves, and the $EC_{50(72h)}$ values were 31.22, 35.73 and 41.84 μg/ml, respectively. Moreover, cell proliferation inhibition was dependent on exposure time; inhibition was enhanced with the extension of toxin exposure. The Lowry bioassay showed that TeA decreased the total protein content in the three cell lines after 72 h toxin exposure, and the $EC_{50(72h)}$ values were 37.37 μg/ml, 56.28 μg/ml, and 68.79 μg/ml, respectively. Based on the above results, it is concluded that TeA was most cytotoxic to 3T3 cells, followed by CHL cells and L-O2 cells. At lower concentrations, TeA had lower cytotoxicity to the human hepatocyte, which suggests that human hepatocyte may tolerate TeA at low concentrations. In other studies, Shigeura and Gordon (1963) reported that TeA inhibited the growth of Ehrlich ascites tumor cells and rat liver cells, mainly through inhibiting the incorporation of amino acids into protein. Umetsu et al. (1974) observed that TeA inhibited the growth of suspension cultured cells of rice and soybeans through inhibition of amino acids incorporation into protein and nucleic acid fractions. The results in this study also support the opinions that TeA inhibited protein biosynthesis in the cells.

ACKNOWLEDGEMENTS

The authors wish to thank the anonymous reviewers for thoughtful criticisms on earlier versions of this manuscript, which substantially improved this manuscript. This work was financially supported by the 863 Hi-tech Research Program (2006AA10A214), the National Science & Technology Pillar Program (2006BAE01A02-8), the Research Foundation of Doctoral Program of the

Ministry of Education of China (No. 20050307002) and the National Natural Science Foundation (30600409).

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