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# Redifferentiation of human hepatoma cell induced by 6-(*p*-chlorophenyl)-3-[1-(*p*-chlorophenyl)-5-methyl-1*H*-1,2,3-triazol-4-yl]-s-triazolo[3,4-*b*]-1,3,4-thiadiazole (TDZ)

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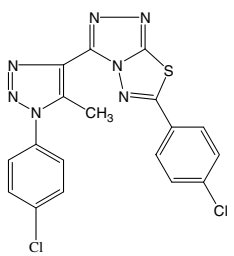
6-(*p*-Chlorophenyl)-3-[1-(*p*-chlorophenyl)-5-methyl-1*H*-1,2,3-triazol-4-yl]-s-triazolo[3,4-*b*]-1,3,4-thiadiazole (TDZ) is a derivative of various substituted s-triazolo[3,4-*b*]-1,3,4-thiadiazoles, which are associated with diverse pharmacological activities. However, the antitumor activity of TDZ is not well understood. To evaluate its role on tumor cell lines, we have examined the effect of TDZ on two tumor lines: human hepatoma cell (SMMC-7721) *in vitro* and Sarcoma180 tumor (S180) *in vivo*. The cytotoxicity of TDZ on human hepatoma cells was assessed using the MTT assay. The inhibition on tumor growth was evaluated by means of trypan blue exclusion test *in vitro*, and using a Sarcoma180 tumor (S180) animal model *in vivo*. A scanning electronic microscope was used to discover the morphological changes on cell surface, cell electrophoresis was employed to determine the changes of cell surface negative charges, and $\alpha$-fetoprotein was applied as a biomarker of hepatoma. The effect of TDZ on DNA synthesis was determined by a [$^3$H]-thymidine incorporation assay, and cell cycle distribution by flow cytometry. The $IC_{50}$ value of TDZ on SMMC-7721 cells was 52.9 $\mu$g/ml (48 h). However, TDZ could inhibit the growth of SMMC-7721 cells at concentrations far lower than the $IC_{50}$ value. Treated with the same low concentrations of TDZ, microvilli on the surface of SMMC-7721 cells decreased obviously, electrophoresis rate of cells reduced from 2.14 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ of control to 1.54 and 1.56 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$, the content of AFP dropped from $205.14 \pm 6.41$ ng $\cdot$ mg$^{-1}$ Pr to $115.68 \pm 3.47$ and $78.57 \pm 2.35$ ng $\cdot$ mg$^{-1}$ Pr, and the DNA replication was inhibited by 26.8% and 45.2%. These results indicated that TDZ may inhibit proliferation of cancer cells by reversing SMMC-7721 cells malignant phenotypic characteristics and inducing redifferentiation. Flow cytometry showed that TDZ-treated cells resulted in a higher proportion of cells in S phase compared with untreated cells, and only when the concentration reached 64 $\mu$g/ml, the apoptosis could happen at the rate of 4.2%. Detection of the inhibition of Sarcoma 180 tumor growth *in vivo* showed that TDZ reduced the tumor weight and 69.08% of the growth was inhibited. TDZ could inhibit the proliferation of tumors *in vitro* and *in vivo*; the possible antitumor mechanism might be inducing redifferentiation at a lower dosage *in vitro*.

## 1. Introduction

Various substituted s-triazolo[3,4-*b*]-1,3,4-thiadiazoles are associated with diverse pharmacological activities, such as antimicrobial, bactericidal, antiinflammatory, antiviral, antihypertensive, anthelmintic and analgesic effects (Dwivedi et al. 1991; Eweiss et al. 1987; Hang et al. 1991; Zhang et al. 1993). 6-(*p*-Chlorophenyl)-3-[1-(*p*-chlorophenyl)-5-methyl-1*H*-1,2,3-triazol-4-yl]-s-triazolo[3,4-*b*]-1,3,4-thiadiazole (TDZ), a new s-triazolo[3,4-*b*]-1,3,4-thiadiazole compound, was synthesized by cyclocondensation of 4-amino-3-(1-*p*-chlorophenyl-5-methyl-1,2,3-triazol-4-yl)-5-mercapto-1,2,4-triazole. It was shown to have antibacterial activity (Sun et al. 1999) and we demonstrate the anticancer activity the first time here.




6-(p-Chlorophenyl)-3-[1-(p-chlorophenyl)-5-methyl-1H-1,2,3-triazol-4-yl]-s-triazolo[3,4-*b*]-1,3,4-thiadiazole (TDZ)

## 2. Investigations and results

### *2.1. Inhibition of cell growth*

As shown in our results, the 50% inhibition ($IC_{50}$) of TDZ was as high as 52.9 $\mu$g/ml (48 h), and at a concentration

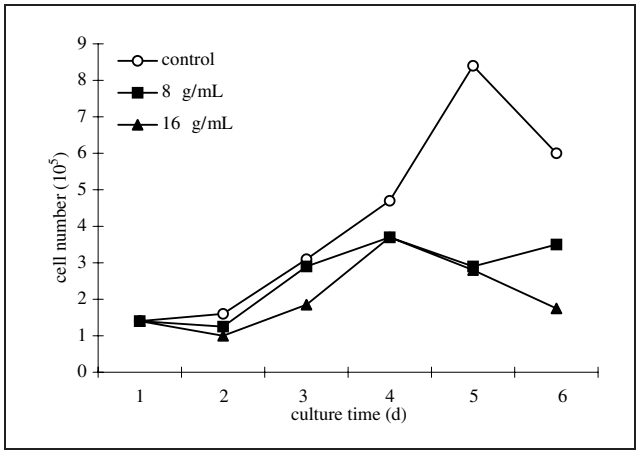



Fig. 1: Effect of TDZ on cell growth curve. To determine the inhibition effect of TDZ on hepatoma cell line, the trypan blue dye exclusion assay was performed in cells after respective TDZ treatments (8 and 16 μg/ml)

**Table 1: Inhibition effect of TDZ on the growth of S180 tumor ($n = 8$, $x \pm s$)**

| TDZ ($mg \cdot kg^{-1}$) | Weight of tumor (g) | Inhibitory rate (%) |
|---|---|---|
| Control | $1.34 \pm 0.15$ | |
| 10 | $0.83 \pm 0.15^{*}$ | $37.72^{*}$ |
| 45 | $0.68 \pm 0.13^{*}$ | $48.88^{*}$ |
| 100 | $0.41 \pm 0.13^{**}$ | $69.08^{**}$ |
| Cyclophosphamide | $0.51 \pm 0.09^{**}$ | $62.13^{**}$ |

Vs Control, $^{*}P < 0.05$, $^{**}P < 0.01$

of 16 μg/ml, the cell viability reaches 90% (data not shown). However, trypan blue results showed that low concentrations (8 or 16 μg/ml) of TDZ inhibit cell growth significantly (Fig. 1). The control cells grew exponentially until the end of the experiment, while the cells treated with 8 or 16 μg/ml of TDZ grew much slower than control. This means that the inhibition of TDZ on the growth of SMMC-7721 cells was not the result of cytotoxicity.

**Table 2: Effect of TDZ on electrophoresis rate**

| Concentration (μg/ml) | Electrophoresis time (s) | Electrophoresis rate ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) | Retardation (%) |
|---|---|---|---|
| 0 | $12.8 \pm 1.6$ | 2.14 | |
| 8 | $17.85 \pm 1.87$ | $1.54^{*}$ | $39.3^{*}$ |
| 16 | $17.67 \pm 0.93$ | $1.56^{*}$ | $37.8^{*}$ |

Vs Control, $^{*}P < 0.05$, $^{**}P < 0.01$

### 2.2. Antitumor activity of TDZ in vivo

After intraperitoneal injection, TDZ and cyclophosphamide ($30\ mg \cdot kg^{-1} \cdot d^{-1}$) had a significant inhibitory effect on the growth of inoculated S180 cells in mice (Table 1). The inhibition rate of $10\ mg \cdot kg^{-1}$ treatment reached 37.72%. When the dosage rose to $100\ mg \cdot kg^{-1}$, the inhibition rate was as high as in the cyclophosphamide group, reaching 69.08%.

### 2.3. Morphological change

On the surface of hepatoma cells, an abundant amount of microvilli was present, with many long and thin microfilament pseudopodia, which extended around the cell (Fig. 2a, 2c). After being treated with 8 μg/ml TDZ, the microvilli and the microfilament pseudopodia on the cell surface were reduced significantly and shortened considerably (Fig. 2b, 2d).

### 2.4. Decrease of cell surface negative charge

The average value of the cells electrophoresis rate in the control group was $2.2\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$, whereas, after being treated with 8 or 16 μg/ml TDZ for 48 h, all the electrophoresis rates were decreased (Table 2). Although this effect was easily observable without obvious concentration-dependent relationship, all treatment groups showed that electrophoresis rate decreased compared with the control group.

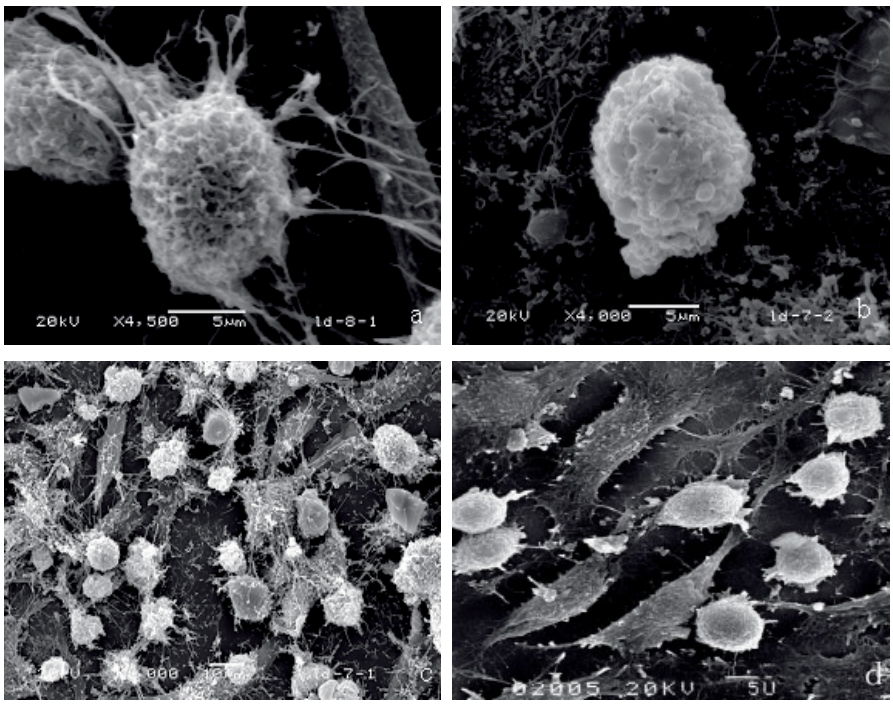



Fig. 2:
SMMC-7721 cells cultured without (a, c) and with (b, d) 8 μg/ml TDZ for 3 days

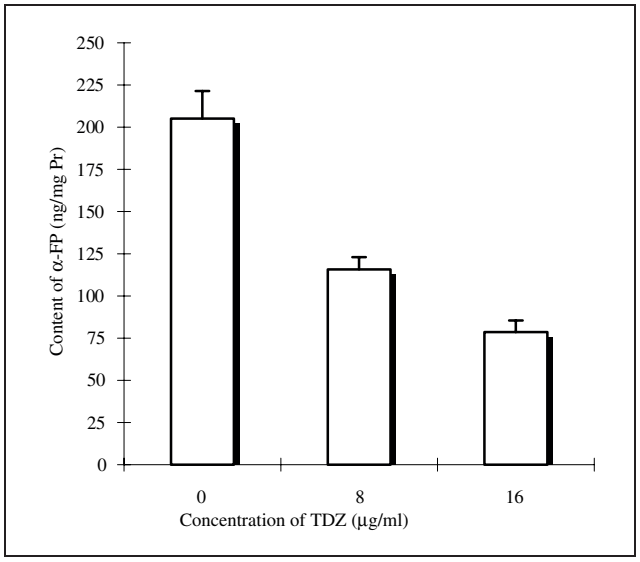



Fig. 3: Effect of TDZ on A-FP content of SMMC-7721 cells

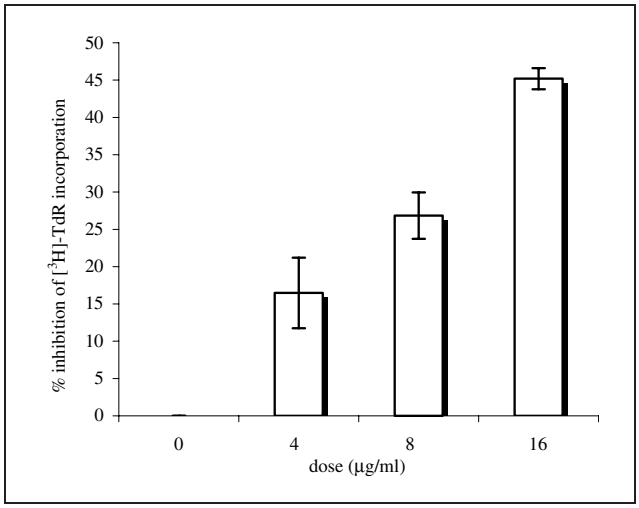



Fig. 4: Effect of TDZ on cells' proliferation

### *2.5. Decrease of α-fetoprotein (α-FP) content*

After being treated with 8 or 16 μg/ml TDZ for 48 h, the content of α-FP dwindled markedly. With the concentration increasing from 8 to 16 μg/ml, the content of α-FP dropped from 205.1 $ng \cdot mg^{-1}$ pr of control group to 115.7 and 78.6 $ng \cdot mg^{-1}$ pr, respectively (Fig. 3).

### *2.6. Inhibition of cells DNA synthesis by TDZ*

Fig. 4 shows that TDZ could significantly inhibit the DNA synthesis of SMMC-7721 cells in a concentration-dependent manner. Compared with control, the incorporation of [$^3$H]-TdR into DNA of SMMC-7721 cells was inhibited by 16.5%, 26.8% and 45.2% after being treated with 4, 8 and 16 μg/ml. This result indicated that TDZ checked cancer cells' proliferation in DNA levels, and our results also showed that TDZ exhibited little cytotoxicity on SMMC-7721 cells at 16 μg/ml concentration determined by the trypan blue exclusion test (the cells viability $>95\%$).

### *2.7. Effect of TDZ on cell cycle distribution*

Cell cycle distribution was analysed by flow cytometry as described previously. As shown in Fig. 5, TDZ-treated cells resulted in a higher proportion of cells in S phase compared with untreated cells, which can readily enter the G2 phase. That means TDZ can block cell cycle progression and then inhibit cancer cells' proliferation. A similar result was found in human oral epithelial cells after treatment with curcumin (Khafif et al. 1998). When the concentration was 64 μg/ml, apoptosis occurred at a rate of 4.2% (Fig. 5).

## 3. Discussion

The SMMC-7721 cell line is a well established human hepatoma cell line which has been commonly used as an *in vitro* model for the purpose of analyzing hepatoma cell differentiation (Li et al. 1997; Li et al. 1990). In the present study, we investigated the anti-proliferative and differentiation-inducing activities of TDZ on human hepatoma cells. From the results of the MTT assay, we have found that the estimated $IC_{50}$ value for TDZ on human hepatoma cells was 52.9 μg/ml (48 h). Interestingly, the observed growth-inhibitory effect of TDZ on human hepatoma cells cannot be attributed directly or solely to the cytotoxic effect of TDZ, as our results show that TDZ exhibited little, if any, cytotoxicity on human hepatoma cells at or below 16 μg/ml concentrations. Moreover, only 8 μg/ml of TDZ could trigger cells to undergo morphological redifferentiation. This concentration is much lower than its $IC_{50}$.

The net charges at the tumor cell surface are more general than those in corresponding normal cells (Hang et al. 1991), thus, the cell electrophoresis rate of tumor cells is higher than that of normal cells, and the low electrophoresis rate has been taken as an appraisal of tumor cell differentiation. Our results reveal that TDZ could decrease the amount of cell surface charge, and make the tumor cell lose its malignant surface character. Again, the drastic reduction in number and size of microvilli induced by the agent, as shown in Fig. 2, may decrease the cell's mechanical fluid resistance, leading to an increase in electrophoretic mobility. However, our results point out the electrophoresis rate decreased dramatically in treatment groups, which mean that the absolute decrease in the surface net charge induced by this agent might be higher than we can show in this experiment, for the electrophoresis rate demonstrated in the experiment included at least two parts: the increase induced by the reduction of microvilli and the decrease induced by the reduction of surface net charge.

α-Fetoprotein is secreted in fetal liver, while largely absent in normal adult liver, and transiently neoexpressed in hepatoma carcinoma according to previous observations. α-Fetoprotein neoexpression is defined a dedifferentiated cellular phenotype during the development of liver cancer (Li et al. 1990). Our results show that after being treated with 8 and 16 μg/ml TDZ, α-fetoprotein content decreased considerably, which confirmed that TDZ could redifferentiate SMMC-7721 cells.

In the current studies, we also demonstrated that TDZ could significantly inhibit the DNA synthesis in SMMC-7721 cells. This result indicated that TDZ inhibited cancer cell's proliferation in DNA levels.

In summary, our results show that TDZ can decrease the electrophoresis rate, which in turn reflects the decline of the amount of cell surface charge, and make tumor cell lose its malignant surface character, so TDZ can induce redifferentiation of the human hepatoma cell. At the same time, flow cytometry also shows that TDZ can block cell cycle progression in S phase. Only when the concentration reaches 64 μg/ml, apoptosis can happen and the rate stays at just 4.2%.

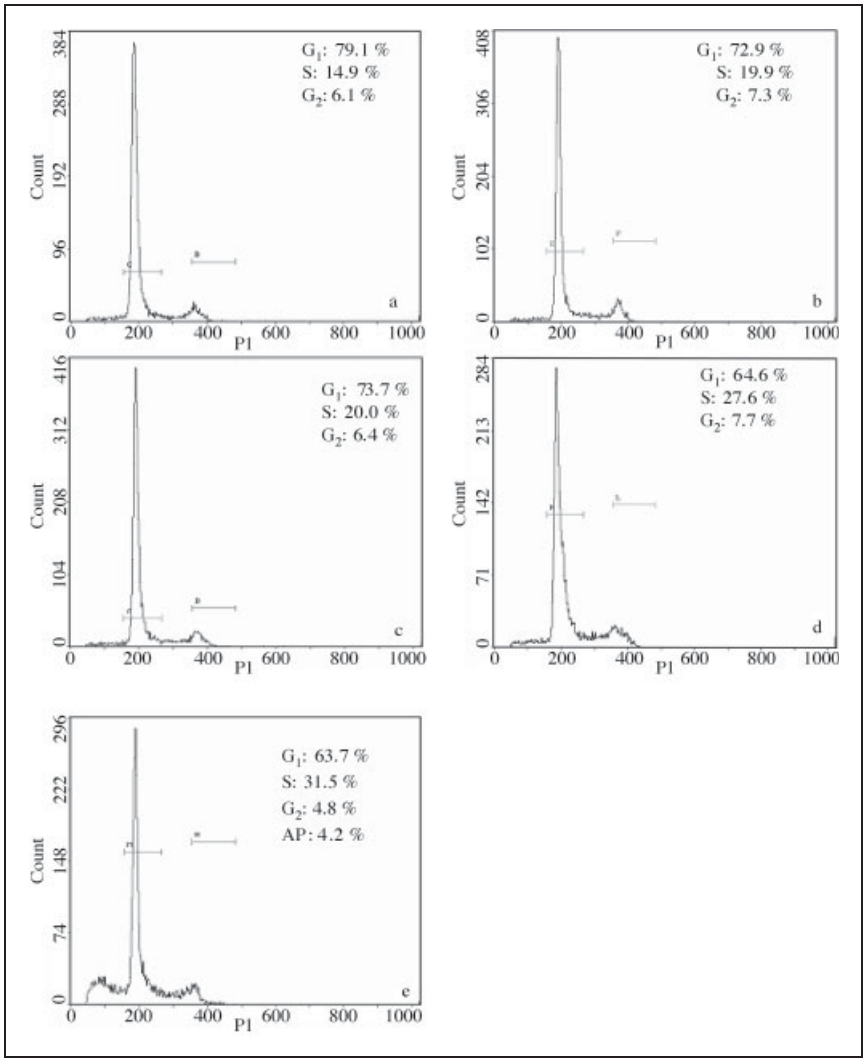



Fig. 5:
The flow cytometry analysis of different phases of tumor cell proliferation after treatment without (a) or with different concentrations of TDZ, b: 8 μg/ml, c: 16 μg/ml, d: 32 μg/ml, e: 64 μg/ml

Our results from *in vivo* studies indicate that TDZ displays significant inhibitory effects on the growth of S180 transplanted into mice. The inhibitory efficiency to all S180 cells is directly proportional to the dosage applied. When the dosage comes to $100\,mg \cdot kg^{-1}$, the inhibition rate reaches 69.08%. And the $LD_{50}$ of the acute toxicity of TDZ to mice is higher than 800 mg/kg (data not shown). This study provides a direct proof that TDZ may be a potent drug for clinical treatment of cancer.

The underlying mechanisms for the growth-inhibitory effect of TDZ on cancer cells remain unclear. Our present work shows that TDZ can induce hepatoma cells' redifferentiation, but fail to induce apoptosis in SMMC-7721 cells under lower dosage *in vitro*, as judged by morphologic criteria (data not shown) and by flow cytometry analysis. Despite these findings, the molecular mechanisms by which TDZ can exert its anti-proliferative effect on human hepatoma cells remain a subject to be further elucidated.

## 4. Experimental

### *4.1. Chemical and reagents*

TDZ was provided by Dr. Xiaowen Sun (Sun et al. 1999), and was stored at 4 °C. RPMI 1640 medium was purchased from Gibco (USA). Fetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). MTT, dimethylsulphoxide (DMSO) and propidium iodium (PI) were purchased from Sigma-Aldrich Co. (USA). RNase A was purchased from Shanghai Sangon Biotech Co (Shanghai, China).

### *4.2. Tumor cells culture and animals*

Human hepatocellular carcinoma SMMC-7721 cell line was obtained from the Second Military Medical University, Shanghai, China. Cells were cultured in RPMI-1640 medium supplemented with 10% inactivated calf serum, $100\,U \cdot ml^{-1}$ penicillin, 100 μg/ml streptomycin and $2.0\,g \cdot L^{-1}$ $NaHCO_3$, at 37 °C in the presence of 5% $CO_2$. Cells were routinely seeded by $4 \times 10^5$ cells $\cdot\, ml^{-1}$ in 10 ml mediums. Sarcoma 180 (S180) was provided from Gansu Province Cancer Hospital, Lanzhou, China.

Kunming mice were purchased from the Laboratory Centre For Medical Science, Lanzhou Medical College, Gansu province. The mice were acclimatized under laboratory conditions (21–23 °C, humidity 60%, 12 h light/dark cycle) for 1 week before subject to study. Mice were weighed and randomly assigned to six groups.

### *4.3. Determination of cell survival rate in vitro*

TDZ was dissolved in dimethylsulphoxide (DMSO) and diluted in culture medium. 96-well plates were seeded $2 \times 10^4$ cells $\cdot\, ml^{-1}$ for 24 h in complete medium. And then, cells were treated with or without different concentrations (2.5, 5, 10, 20, 40, 60, 80, 100 μg/ml) of TDZ. After 48 h incubation, culture medium was removed, and MTT solution ($0.5\,mg \cdot ml^{-1}$ in complete medium) $100\,\mu l \cdot well^{-1}$ was added to each well and incubated for a further 4 h at 37 °C. The formazan product was dissolved by the addition of $100\,\mu l \cdot well^{-1}$ DMSO. The optical density of each well was measured using a Bio-Rad Model 3550 plate reader at 595 nm with reference at 655 nm. Wells containing culture medium and MTT but no cells used as blanks. Cell survival was expressed as an absorbance (A) percentage defined as:

$$S = A_{drug-blank} \div A_{control-blank} \times 100\,.$$

### *4.4. Determination of cell growth curve*

$1 \times 10^5$ cells $\cdot\, ml^{-1}$ was cultured in 25 ml culture flasks. The culture flask contained 5 ml of cellular suspension. Low cytotoxic concentration of

TDZ, determined by MTT assay, was performed after subculturing for 24 h, and the culture medium was replaced once every day during incubation. One flask cells from treatment or control group was collected every day in the first 5 days, the viable and dead cells were counted by using trypan blue dye exclusion method, respectively.

#### *4.5. Tumor growth measurement*

After 24 h tumor transplantation, animals were randomly divided into 6 groups. The groups with TDZ were treated with 10, 45 and 100 mg/kg bw, respectively. The positive control group was treated with 30 mg/kg bw cyclophosphamide. The negative control group received 0.9% normal saline. All test drugs were given through intraperitoneal injections 24 h after tumor transplantation (or inoculation), and once per day for a total of 8 consecutive days. All animals were killed 24 h after the treatments. The inhibition rate of tumor was calculated as follows:

$$\frac{(\text{Mean tumor weight in control} - \text{mean tumor weight in experiment})}{\text{Mean tumor weight in control}} \times 100\% .$$

#### *4.6. Assay for α-fetoprotein (α-FP)*

The cells treated with different concentrations of TDZ 0, 8 and 16 μg/ml for 48 h were collected and washed with phosphate buffer twice, and then resuspended at a density of $5 \times 10^7$ cells $\cdot$ ml$^{-1}$. The cells were left to lyses by ultrasonic on ice for 2 min before centrifugation for 15 min at $3000 \times$ g, supernatant was collected. The α-FP in the supernatant was determined by α-FP reagent kit (Biological Reagent Research Institute, Lanzhou, China) in the way of ELISA biantibody with α-FP. A Ceres900HDi Microfilter Plate Reader (Biotek Instruments) was used to determine absorbance at the wavelength of 450 nm.

#### *4.7. Assay for cells electrophoresis rate*

The cells treated with different concentrations of TDZ (0, 8 and 16 μg/ml) for 48 h were collected and washed with D-Hanks' solution twice, and then resuspended at a density of $2 \times 10^6$ cells $\cdot$ mL$^{-1}$. The cell electrophoresis determination was performed with a round glass tube electric-bridge filled with KCl 9% – agar 0.8% at a direct current voltage 40 V at 24 °C, taking sucrose 9% as the electrophoretic medium and using a microcapillary electrophoresis apparatus (Shandong University, China). The electrophoresis time was expressed by the average time (second) during which a cell moved over a distance of 200 μm and electrophoresis rate was expressed as μm $\cdot$ s$^{-1}$ $\cdot$ V$^{-1}$ $\cdot$ cm. 40 cells in each group were determined. The experiments were repeated three times with similar results. Thus, the result of one experiment was used as the criterion.

#### *4.8. Scanning electron microscopy*

Cells were grown on glass tissue culture cover slips and were fixed in 0.1 M phosphate buffered 2.5% glutaraldehyde (pH 7.4) at 4 °C for 1 h, and 1% osmic acid for 20 min, and then dehydrated with ethanol, dried in a critical point drier (Balzers Union), and finally coated with gold using a sputter coater (Balzers Union). The specimens were examined with a Hitachi s-520 scanning electron microscope.

#### *4.9. [$^3$H]-Thymidine incorporation assay*

The [$^3$H]-Thymidine ($^3$H-TdR) incorporation assay was used to measure the anti-proliferative effect in cancer cells by Lung et al. with a little modification (Lung et al. 2002). SMMC-7721 cells were incubated with 5 ml medium at 37 °C in the absence or the presence of various concentrations of TDZ for 48 h. The cells were then pulsed with 5 μCi [$^3$H]-Thymidine (Shanghai Institute of Nuclear Research) for 4 h. The cells were washed two times with cold 0.9% normal saline and incubated with 5% cold trichloroacetic acid to remove excess thymidine. The cells were lysed and placed in scintillation vials containing liquid scintillation cocktail. The radioactivity, in counts per minute (cpm), was measured by the liquid scintillation counter (Wallac, Finland). Aliquots were taken to measure protein concentration. The results were expressed as cpm $\cdot$ mg pr$^{-1}$. The percentage inhibition of [$^3$H]-TdR incorporation was calculated as follows, using the untreated cells as a control:

$$\text{Inhibition } \% = [1 - (\text{cpm} \cdot \text{mg pr}^{-1})_{\text{test}} / (\text{cpm} \cdot \text{mg pr}^{-1})_{\text{control}}] \times 100\% .$$

#### *4.10. Flow cytometry analysis*

After treatment with TDZ for 48 h, the percentages of cell cycle phase were assessed by flow cytometry. In brief, about $10^6$ cells were harvested, washed with cold PBS fixed with cold 70% ethanol, and then kept 24 h at −20 °C. Cells were rinsed with PBS and stained with 10 μg/ml propidium iodium (PI) working solution (PBS solution containing 0.2% Triton X-100, 0.1 mM EDTA and 100 μg/ml RNase A) for 30 min at room temperature in the dark. Then they were washed with PBS to remove PI, and the determined by using flow cytometer (EPICS XL, U.S COULTER). The data were analyzed by Cellquest version 1.2.2 software (B-D).

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