Effect of TM208 on QGY-7703 xenograft tumor growth

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A newly synthesized dithiocarbamate derivative, 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3, 3-diphenylpropyl ester hydrochloride (TM208), has demonstrated anticancer effects with low toxicity in earlier studies; however, the mechanism has yet to be identified. We explored antitumor effects of TM208 and the possible mechanisms by which it inhibited the growth of human hepatocellular carcinoma cell line QGY-7703 xenograft tumors. Cell proliferation was evaluated with the sulforhodamine B assay *in vitro*. The results suggested that TM208 had slightly antiproliferative activity on QGY-7703 cells. The antitumor effect of TM208 was assessed in nude mice xenografted with QGY-7703 tumors. We found that TM208 significantly inhibited tumor growth but did not cause loss of body weight or leukocytopenia. Western blotting was used to detect the expression of protein kinase Cα, mitogen-activated protein kinase signal pathways, and cell cycle-related proteins. The results showed that TM208 decreased the expression of protein kinase Cα, phospho-extracellular signal-regulated kinase-1/2, phospho-p38, cyclin B1, cell division cycle 2 (cdc2), and phospho-cdc2 (Thr^{161}) and increased the expression of phospho-cdc2 (Tyr^{15}). Taken together, our data show that TM208 has little antiproliferative effect on QGY-7703 cells *in vitro*, whereas it significantly inhibits the growth of QGY-7703 xenograft tumors with low toxicity *in vivo*. The inhibition of mitogen-activated protein kinase signal pathways and the regulation of the G_2/M phase may be responsible for its antitumor effects. *Anti-Cancer Drugs* 19:593–598 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatocellular carcinoma, a common fatal malignancy, is the fifth most common cancer and the third leading cause of cancer-related death worldwide. In China, hepatocellular carcinoma causes more than 100 000 deaths each year, and the incidence has increased dramatically in recent decades [1], which has motivated a great deal of interest in finding more effective chemotherapeutic agents to treat this difficult disease.

Dithiocarbamates, as fungicides, are widely used in agriculture because they are cheap and effective. Some studies have shown that dithiocarbamates can exert antibiotic effects [2], have antiinflammatory properties [3], can remove nitrogen monoxidum from the body [4], and can chelate heavy metals in the body [5]. Others have reported that dithiocarbamates could be used to prevent or treat cancer [6,7]. Recently, a series of new dithiocarbamate derivatives has been synthesized [8,9], including 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208). An earlier study showed that TM208 significantly inhibited the growth of transplanted hepatocyte carcinoma 22 and sarcoma 180 in mice and implanted human gastric carcinoma in nude mice with low toxicity [10]; however, the precise antitumor mechanism of TM208 has not been identified. In this study, we investigated the growth-inhibiting effects of TM208 on an in-vitro proliferation assay and its antitumor effects and the possible mechanism of growth inhibition on QGY-7703 xenograft tumors *in vivo*.

Materials and methods

Cell culture

A hepatocellular carcinoma cell line (QGY-7703) was purchased from the Department of Genetics of Fudan University (Shanghai, China); the characteristics of QGY-7703 cells have been published elsewhere [11]. QGY-7703 cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% heat-inactived fetal calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml). Cells were incubated at 37°C with 5% carbon dioxide at 95% humidity.

Drug preparation

TM208 was provided by Professor Run-tao Li (Peking University) (Fig. 1). TM208 was dissolved in dimethylsulfoxide (DMSO) to 10^{-2} mol/l as stock solution. Adriamycin (ADM) purchased from Shenzhen Main Luck

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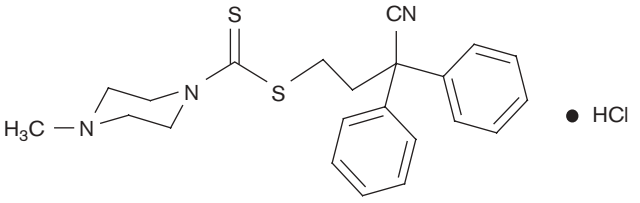
Fig. 1



The structure of 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride. Molecular weight: 432.

Pharmaceutical Co. Ltd (Shenzhen, China) was dissolved in phosphate-buffered saline (PBS) to 1 mg/ml as stock solution, and 0.6% DMSO was prepared and used as the solvent control. All drugs were further diluted in PBS for in-vitro experiments under pathogen-free conditions. Cyclophosphamide (CPA) was purchased from Shanghai Hualian Pharmaceutical Co. Ltd (Shanghai, China). TM208 and CPA were then dissolved in normal saline for in-vivo experiments.

In-vitro proliferation assay

Growth inhibition was evaluated by using a sulforhodamine B (SRB) assay. SRB was obtained from Sigma-Aldrich (Sheboygen, Wisconsin, USA). Briefly, QGY-7703 cells were plated in 96-well microplates (2×10^4/well). After 24 h of incubation, the cells were treated with PBS, solvent control (0.6% DMSO), adriamycin (1.5 μg/ml), and TM208 (20, 40, and 60 μmol/l) for 48 h. The cells were then fixed with 10% trichloroacetic acid at 4°C for 1 h. Plates were read at 540 nm for SRB staining by using a FLUOstar OPTIMA microplate muti-detection reader (BMG, Offenburg, Germany). The final DMSO concentration in each well was less than 0.6% (v/v). Background control wells were included in each experiment.

Tumor model and treatment

Beijing Vital Laboratory Animal Technology (Beijing, China) provided male nude mice (5–6 weeks old). Animal procedures were approved by the Department of Laboratory Animal Science of Peking University Health Science Center (Beijing, China). The mice were fed with sterile water and mouse chow. We injected 0.2 ml QGY-7703 cell suspension (1.8×10^8/ml) into the backs of 32 nude mice. After solid tumors developed, the mice were randomized into a normal saline control group (0.1 ml/10 g/day), a CPA group (30 mg/kg/2 days), and two TM208 groups (100 and 200 mg/kg/day), with eight mice in each group. Drugs were administered to the mice intragastrically for 28 days. Each mouse was weighed three times per week. At the end of the experiment, mice were euthanized and tumors were harvested. Meanwhile, blood was collected to assess the effect of TM208 on leukocytes by using Medonic-CA620 Vet Automated Cell Analyzer (Boule Co., Sweden). The tumor inhibition rates were calculated according to the following formula: (mean tumor weight of control nude mice – mean tumor weight of treated nude mice)/mean tumor weight of control nude mice × 100%.

Western blotting

Tumor tissues from nude mice implanted with QGY-7703 cells were cut into small pieces and lysed with a solution containing 10 mmol/l Tris–HCl (pH = 7.4), 1% Nonidet P-40, 150 mmol/l NaCl, 2 mmol/l EDTA, 2 mg/ml leupeptin, 2 mg/ml aprotinine, and 10 mg/ml phenylmethylsulfonyl fluoride. Samples were sufficiently homogenized on ice and then incubated for 30 min. Finally, the tumor lysates were centrifuged at 14 000 rpm at 4°C for 30 min. The supernatants were collected, and their protein concentrations were measured by using the Pierce protein assay reagent (Pierce Biotechnology, Rockford, Illinois, USA). Equal amounts of supernatant protein (60 μg) from normal saline and TM208 groups were denatured by heating at 65°C for 10 min and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After the proteins were transferred to nitrocellulose membranes (Hybond C; Amersham, Little Chalfont, UK), immunoblots were stained with Ponceau red to visualize total proteins contained in each slot, then membranes were blocked with 3% albumin fraction V in Tris-buffered saline with 10% Tween-20 and were probed with primary antibodies specific to protein kinase Cα (PKCα) (Santa Cruz Biotech, Santa Cruz, California, USA), p44/p42 mitogen-activated protein kinase (MAPK), phospho-p44/42 MAPK, p38 MAPK, phospho-p38 MAPK, cyclin B1, cell division cycle 2 (cdc2), phospho-cdc2 (Tyr^{15}), phospho-cdc2 (Thr^{161}) (Cell Signaling, Beverly, Massachusetts, USA), β-actin, cyclin E, and cyclin-dependent kinase (cdk) 2 (Santa Cruz Biotech) at 4°C for 16 h. After being washed three times with Tris-buffered saline-T for 10 min each, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Sigma-Aldrich) for 1 h. The membranes were washed again, and detection was performed by using the enhanced chemiluminescence blotting detection system (Amersham, Biosciences, Little Chalfont, Buckinghamshire, UK).

Statistical analysis

Data are expressed as mean ± SD of three determinations. Differences between groups were assessed by using Student's *t*-test. Differences were considered significant at P less than 0.05.

Results

TM208 inhibits the proliferation of QGY-7703 cells *in vitro*

We first determined the effect of TM208 on the proliferation of QGY-7703 cells with the SRB assay. As shown in Table 1, the inhibition rate of TM208 increased gradually with increasing concentration, to a maximum

Table 1 **Effect of TM208 on QGY-7703 cells for 48 h**

Agent	Concentration (μmol/l)	OD value (mean ± SD)	Inhibition rate (%)
PBS	0	0.772 ± 0.08	0
DMSO	0.6[a]	0.729 ± 0.06	5.6
ADM	1.5[b]	0.174 ± 0.04***	77.5
TM208	20	0.561 ± 0.08*	27.4
	40	0.548 ± 0.04*	29.0
	60	0.401 ± 0.05**	48.1

QGY-7703 cells were treated with ADM or TM208 in different concentrations for 48 h and the growth inhibition was determined by the sulphorhodamine B assay. The inhibition rate of TM208 was increased in concentration-dependent manner. These data suggested that TM208 had a slight inhibitory effect on QGY-7703 cell *in vitro*. Values are mean ± SD.
ADM, adriamycin; OD, optical density; TM208, 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride.
[a]0.6% (v/v).
[b]1.5 (μg/ml).
*P<0.05; **P<0.01; ***P<0.001 versus PBS group by Student's *t*-test (n=3).

of 48.1% at the maximum concentration. These data suggested that TM208 exerted a slight inhibitory effect on QGY-7703 cells *in vitro*.

TM208 inhibits the growth of QGY-7703 xenograft tumors with low toxicity

Tumors in the TM208-treated mice weighed significantly less than did tumors in saline-treated mice ($P < 0.05$) (Fig. 2). Although no dose–response was evident, the inhibition rate of TM208 was approximately 50.0% in the highest dose group. All of the TM208-treated mice were still alive at the end of the experiment (after 28 days). TM208-treated mice maintained similar body weights as saline-treated mice, whereas significant body weight loss was observed in CPA-treated mice (Fig. 3), beginning on the fifth day ($P < 0.05$, < 0.01). TM208 treatment had little effect on leukocytes, but total leukocyte count and the percentage of lymphocytes were significantly decreased in CPA-treated mice (Table 2).

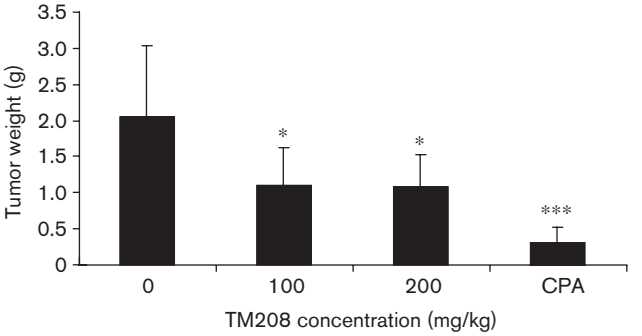
TM208 inhibits the mitogen-activated protein kinase signal pathway

An earlier study showed that TM208 downregulates some signal pathway-related genes. To further elucidate the mechanism of tumor inhibition, we examined the expressions of some MAPK signal pathway-related proteins. We found TM208 decreased expression of PKCα, and the band disappeared completely in the high-dose group. Furthermore, TM208 reduced the expressions of phospho-extracellular signal-regulated kinase (ERK)1/2 and phospho-p38 in a dose-dependent manner, whereas the expressions of total ERK1/2 and p38 were not affected (Fig. 4).

TM208 regulates the expression of G_2/M cell cycle-related proteins

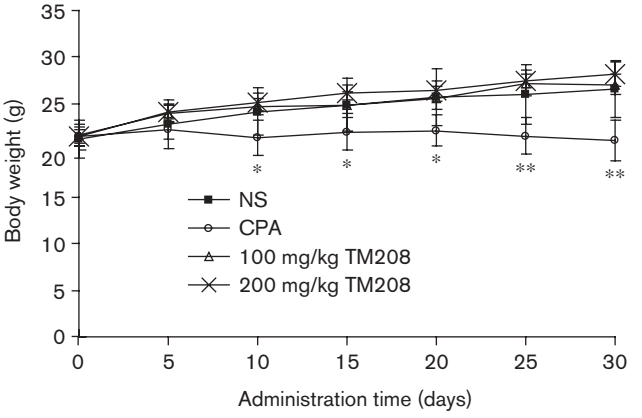
To further investigate the underlying reasons, we examined some cell cycle-related proteins that are targets of the ERK1/2 signal pathway [12], including cyclin E, cdk2, cyclin B1, cdc2, phospho-cdc2 (Tyr^{15}), and phospho-cdc2 (Thr^{161}). Levels of cyclin B1, cdc2, and phospho-cdc2 (Thr^{161}) decreased in a dose-dependent manner, whereas the level of phospho-cdc2 (Tyr^{15}) increased (Fig. 5). No alteration was observed in cyclin E and cdk2 (data not shown).

Fig. 2



Effect of 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208) on QGY-7703 cells xenograft tumor. To the back of nude mice 0.2 ml of QGY-7703 cell suspension (1.8×10^8/ml) was inoculated subcutaneously. After tumor formation, the mice were treated with normal saline (NS) (0.1 ml/10 g/day), TM208 (100 and 200 mg/kg/day) and cyclophosphamide (CPA) (30 mg/kg/2 days) for 28 days. At the end of the study, tumors were harvested and measured. TM208 significantly inhibited the growth of QGY-7703 cells xenograft tumor. The inhibition rate of both TM208 groups and the CPA group was 45.8, 47.0, and 85.6%, respectively. The results are presented as the mean tumor weight ± SD obtained from eight mice in each group. ***P<0.001; *P<0.05 versus NS group by Student's *t*-test (n=8).

Fig. 3



The change of body weight in QGY-7703 cells xenograft tumor model. 4-Methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208) groups had little effect on the body weight, whereas the cyclophosphamide (CPA) group produced body weight loss. Significant differences were observed between normal saline (NS) group and CPA group. *P<0.05; **P<0.01 versus the NS group by Student's *t*-test (n=8).

Discussion

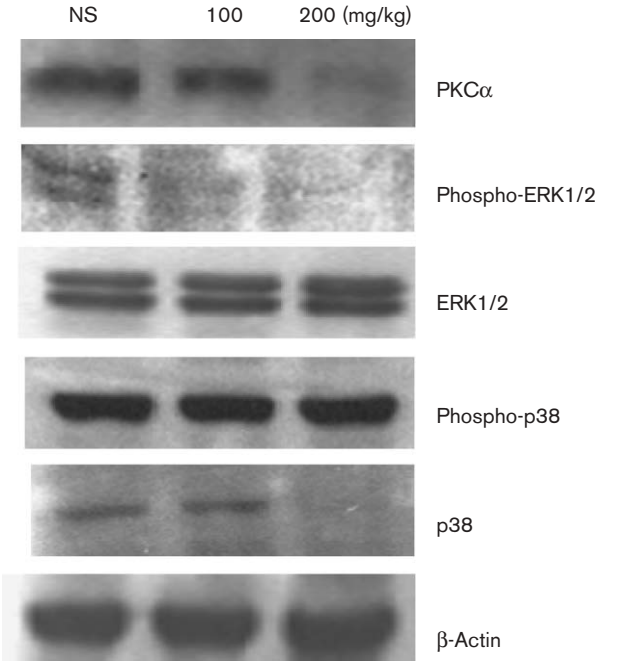
TM208, a new structure of dithiocarbamate derivative, exhibited antitumor effect on several tumor types in mice

Table 2 Effect of TM208 on leucocyte in the QGY-7703 cells xenograft tumor

Group	Dosage (mg/kg)	Leukocyte count/l ($X \pm SD$)	The percent of lymphocyte ($X \pm SD$)
NS	/	9.38 ± 3.19	47.86 ± 23.20
CPA	30	1.11 ± 0.52***	25.66 ± 6.32**
TM208	100	8.43 ± 2.86	46.33 ± 10.28
	200	11.35 ± 3.05	47.14 ± 8.46

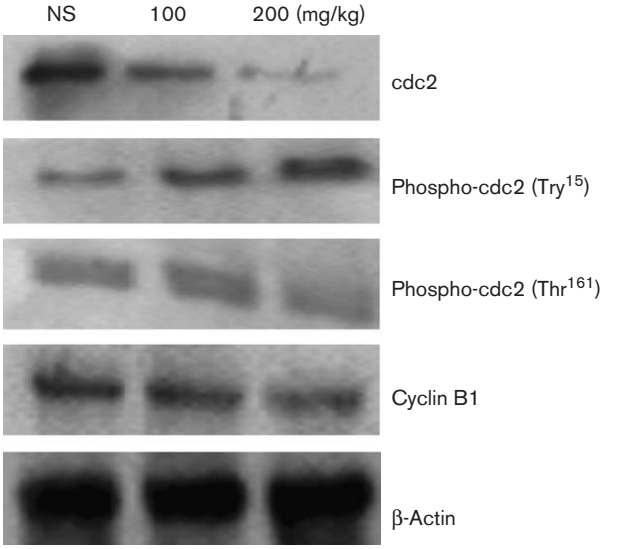
At the end of the study, the mice were euthanized and blood was collected to evaluated the change of leukocyte by using Medonic-CA620 Vet Automated Cell Analyzer. TM208 had little effect on leukocytes, whereas CPA significantly decreased white blood cell total count and the percent of lymphocyte. Values are mean ± SD.
CPA, cyclophosphamide; NS, normal saline; TM208, 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride.
**$P<0.01$; ***$P<0.001$, versus the NS group by Student's t-test ($n=8$).

Fig. 4



Expression of signal pathway related proteins on QGY-7703 cells xenograft tumor treated with 4-methylpiperazine-1-carbodithiocacid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208). Cellular lysate protein (60 μg) from tumors *in vivo* was loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies specific for protein kinase Cα (PKCα), phospho-extracellular signal-regulated kinase (ERK)1/2 and phospho-p38. TM208 led to the downregulation of PKCα, phospho-ERK1/2 and phospho-p38, but the expression of total ERK1/2 and p38 was not affected. Beta actin level was examined as a loading control. The results shown were representative blot of three animals/group, except for phospho-ERK1/2, which was performed in duplicate. NS, normal saline.

Fig. 5



Regulation of G_2/M cell cycle-related protein expression on QGY-7703 cells xenograft tumor treated with 4-methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208). Cellular lysate protein (60 μg) from tumors *in vivo* was loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies specific for cdc2, phospho-cdc2 (Thr^{161}), cyclin B1 and phospho-cdc2 (Tyr^{15}). TM208 downregulated the expression of cdc2, phospho-cdc2 (Thr^{161}) and cyclin B1 and upregulated the expression of phospho-cdc2 (Tyr^{15}) in a dose-dependent fashion. Beta actin level was examined as a loading control. The results shown were representative blot of three animals/group, except for phospho-cdc2 (Thr^{161}), which was performed in duplicate. cdc, cell division cycle protein; NS, normal saline.

[10]. Our results confirmed that TM208 exerted little inhibitory effect on QGY-7703 cell *in vitro*, whereas it significantly inhibited tumor growth in a xenograft tumor model with QGY-7703 cells. Furthermore, TM208 did not cause weight loss or leukocytopenia in treated mice, although CPA, a common anticancer drug, did. An earlier study revealed that TM208 might undergo the process of metabolism *in vivo* to exert its antitumor effect [13]. In our studies, we used the QGY-7703 xenograft tumor model to investigate its possible mechanism of growth inhibition.

To clarify the mechanism by which TM208 inhibits tumor growth, we investigated the expression of some MAPK signal pathway-related proteins. Our results revealed that TM208 significantly decreased the expression of PKCα, phosph-ERK1/2 and phosphor-p38 in a dose-dependent manner. The MAPK signal pathway participates in a wide range of cellular functions, including the modulation of gene expression, mitosis, proliferation, and programmed cell death [14]. Three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases, and p38-MAPKs. The Ras/Raf/MEK/ERK cascade activated by growth factors is central to regulating cell proliferation, growth, and differentiation [15]. Meanwhile, it also has a closed relationship with the development of tumors. Inhibiting the Ras/Raf/MEK/ERK cascade may inhibit the growth of several human

carcinoma cell lines [16,17]. PKC, as a substantial role in cellular signal transduction, could activate the Ras/Raf/MEK/ERK cascade in two ways. PKC phosphorylates Ras directly or cooperates with Ras to transmit the ERK1/2 signal pathway [18–22]; it also activates ERK by interacting with Raf or MEK, instead of with Ras [23–26]. In addition, blocking the p38-MAPK pathway inhibits multiple myeloma growth *in vivo* [27]. Our findings indicated that the downregulation of PKCα, phosph-ERK1/2, and phosphor-p38 might help suppress tumor growth.

To further elucidate the mechanism of tumor inhibition, we examined the expression of some cell cycle-related proteins. The results showed that TM208 induced the accumulation of phospho-cdc2 (Tyr^{15}) and the down-regulation of cdc2, phospho-cdc2 (Thr^{161}) and cyclin B1. Deregulated cell cycle progression underlies the growth and development of cancer. Cell cycle progression is regulated by the activity of CDKs in noncovalent association with their regulatory subunits, the cyclins [28]. Cdc2 is a universal inducer of mitosis, and its activity is controlled by binding to cyclin B1 and phosphorylation at three highly conserved residues [29]. Phosphorylation on Thr161 is necessary for the activation of cdc2 kinase. In contrast, phosphorylation on either Thr14 or Tyr15 dominantly inhibits its activation. Activated cdc2 facilitates a complex formation with cyclin B1 and drives the cell through the G_2/M checkpoint transition. Furthermore, overexpression of the ERK1/2 MAPK signal pathway could alter the expression of many cell cycle-related proteins [30]. Inhibiting the ERK1/2 MAPK signal pathway may block cell cycle progression at the G_1/S phase or G_2/M phase [31,32]. Our results implied that blocking the PKCα/ERK1/2 MAPK signal pathway might contribute to G_2/M arrest on QGY-7703 xenograft tumor treated with TM208.

In summary, TM208 displays slightly antiproliferative effect *in vitro*, whereas it significantly inhibits the growth of xenograft tumors with QGY-7703 cells and is associated with little effect on leukocytes or body weight. The mechanism by which TM208 inhibits tumor growth is related to the inhibition of the PKCα/ERK1/2 MAPK signal pathway and the deactivation of cdc2 and cyclin B1. Further research is needed to clarify the precise effect on the PKCα/ERK1/2 MAPK signal pathway and the specific relationship between the PKCα/ERK1/2 MAPK signal pathway and cell cycle arrest. This preliminary study indicates that TM208 may be a candidate for clinical therapeutic protocols and merits further investigation.

Acknowledgements

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