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Research Article

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In vitro and *in vivo* anti-tumor effect of (Z)-3-(chloromethylene)-6-chlorothiochroman-4-one

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ABSTRACT

The anti-tumor agent, (Z)-3-(chloromethylene)-6-chlorothiochroman-4-one, could inhibit tumor cell proliferations. However, the anti-tumor activity in vivo is poorly understood. So we research the anti-tumor activity in vitro and in vivo, and the probable anti-tumor mechanism systematically in this study. The results showed that the compound CMCT was effective with tumor as the concentration-dependent in vitro and in vivo, and the toxicology of CMCT affecting body weight, WBC, organ and bone in mice was lower than DDP. It would be specially mentioned that ILS of the S180 ascites tumor models was 90.22% at the dose of 10 mg/ (kg·d) by peritoneal injection; In addition, the result showed that CMCT could induce cell thapoptosis and inhibit tubulin aggregation to play an efficient role against tumor. All in all, The compound of CMCT which possessed obvious anti-tumor activity and lower toxicity deserved further research on its anti-tumor mechanism deeply.

Keywords: Anti-tumor, In vitro and in vivo, H22, S180, Mechanism

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INTRODUCTION

Tumor remains a deadly disease and there is a great medical need for finding new anti-tumor agents. Thiochromanone is heterocyclic compounds containing sulfur atoms, its extensive biological activities attracted more and more attention recently[1]. Research progress of thiochromanone and its analogues show that it had good anti-tumor activity[2-4]. Mark H. Holshouser pointed out most of 3-substituted-thiochromanones had biological activities such as inhibiting ehrlich ascites tumor growth and P-388 lymphocytic leukemia growth[5,6]. Thiochromanone analogues synthesis of Jiangli Song could inhibit the activity of cathepsin L[7] . And there is a close relationship between the occurrence of cathepsin L and tumor[8,9]. These years，A series of thiochromanones were synthesized in our laboratory[10,11], and these chemicals was reported to be cytotoxic agents against tumor cells in vitro[12,13]. But their anti-tumor activity in vivo have not reported.

Most of the chemotherapy play a role of anti-tumor by inducing cell apoptosis. When the chemotherapy into the body, drugs acted on drug target, such as DNA[14], tubulin etc[15,16], and induced the target cell injury. Then target cell turned the injury into signals that are transmitted to the checking points of cell cycle. Finally, To achieve the purpose of inducing tumor cell apoptosis and inhibiting cell proliferation. In the clinical application, antimitotic agent occupied the important position through controlling the formation of tubulin and the dynamic balance of tubulin protein dimer. Drugs with anti-tumor activity can inhibit the tubulin aggregates, also can promote aggregation. For example, vincristine could inhibit microtubule assembly and induce tubulin self-association into coiled spiral aggregates[17]. And taxol inhibited the growth of tumor by interfering with tubulin structures that help chromosomes to separate during cell division[18,19].

Thiochroman-4-one derivatives are heterocyclic compounds which include a sulfur atom in their molecule structure

and exhibit a wide range of biological activities. (Z)-3-(chloromethylene)-6-chlorothiochroman-4-one (CMCT, Fig. 1) is a new compound synthesized in our laboratory. In current work, we investigated its anti-tumor activity in vitro and in vivo, and the probable anti-tumor mechanism.

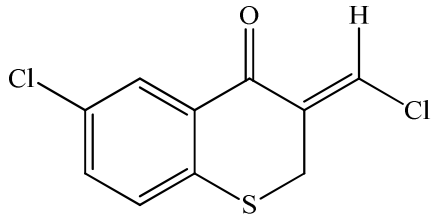


Fig. 1 (Z)-3-(chloromethylene)-6-chlorothiochroman-4-one (CMCT)

EXPERIMENTAL SECTION

Medicines and reagents

Dulbecco's modified eagle medium (DMEM), roswell park memorial institute (RPMI) 1640, and dimethyl sulfoxide (DMSO) were provided by Beijing Solarbio Science & Technology Inc. ; Fetal bovine serum (FBS) was provided by U.S. HyClone Inc. ; Trypsin and 3-[4,5-dimethylthiazol-2-y]-2,5-diphenylterazolium bromide (MTT) were provided by U.S. Aniresco Inc. ; Heparin Sodium was purchased from Tianjin Biochemical Pharmaceutical Inc.; And cisplatin for injection (DDP) was purchased from Qilu pharmacy Inc. ; Phosphate-buffered saline (PBS) was prepared by our self; CMCT was synthesized by our laboratory that was dissolved in DMSO for in vitro studies and in the mixture of cremophor EL- water for in vivo studies.

Experimental animals and tumor cell

Eight-week-old Kunming mice were purchased from experimental Animal Center of Hebei Medical University. Human lung adenocarcinoma cell line A549, human hepatoma cell line HepG2, human cervical carcinoma cell line HeLa, , humanstomach adenocarcinoma cell line BGC-823, human breast adenocarcinoma cell line MCF-7, human melanoma cell line A375, human gastric cancer cell line MKN45,human hepatocellular carcinoma cell line SMMC-7721 , murine liver cell lines H22 and murine sarcoma cell lines S180 were donated by Chinese Academy of Science Cell Institute.

MTT Assay

Cell viability assays were performed using the MTT method as described by Mosmann. The human tumor cells were grown in PRMI 1640 supplemented with 10% heat-inactivated FBS and 1% penicillin (100 units/mL), streptomycin (100 mg/mL), 2-mercaptoethanol (50 μM), and sodium pyruvate (1 mM) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37°C. The cells were harvested in log phase by washing with PBS, trypsinization and centrifugation , Collected cells was seeded in 96-well plates (10^4 cells /100μL/per well), and incubated for 12 hrs, then treated by adding Serial dilutions of CMCT (last concentration: 0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 μg/mL) or DDP (10μg/mL). after 24 hrs, 20 μL MTT (5 mg/mL) was added and cells were incubated for additional 4 hrs, then 150 μL of DMSO was added. Subsequently, the optical density (O.D.) was read at a wavelength of 490 nm using a plate reader. Each experiment was repeated at least three times. The growth inhibition rate was examined and 50% inhibitive concentration (IC_{50}) was calculated.

H22 tumor-bearing mice assay

Mice were inoculated with H22 cells subcutaneously at an injection volume of 0.2 mL, 2.0×10^6 cells/mouse on day 0, and divided randomly into 5 groups: negative group, positive group(DDP), and 3 CMCT (1,3 and 10mg/kg/Day) groups, each group had 10 mice with half male and half female. Mice were ministered once a day from day 1, and continued for 10 days. The diet, weights and growth of tumor were observed every day. Long diameter (a) and short diameter (b) of tumor were examined by vernier caliper, then tumor volume (TV) was calculated as: $TV= (1/2) \times ab^2$ (mm^3), and the tumor growth curve was drawn.

On day 11, Blood was collected from the retro-orbital veins of the eyes under anesthesia, white blood cell (WBC) was determined by haematometer. Then the thymus, spleen, kidney and tumor were removed and weighed. Afterwards, marrow was extracted from thighbone, with the bone marrow nucleated cell (BMNC) direct count method counting. Finally, the inhibitory rate was calculated as: Inhibitory rate = (1-T/C) ×100%, in which T/C was the rate of average tumor weight of the treated group to that of the negative control group. the organ index of H22 tumor mice were calculated as: Organ index = (Organ Weight /Body Weight) ×100%. And the bone marrow DNA inhibition rate was received as: DNA inhibition rate = (1- O.D. of treated group / O.D. of negative control group) ×100%.

S180 ascites tumor-bearing mice assay

Mice were inoculated with S180 cell intraperitoneal at an injection volume of 0.2 mL (2×10^6 cells). In this study, the mice being divided randomly into 4 groups received a single dose of CMCT once a day from day 1 by peritoneal injection administration or the mixture of cremophor EL-water in the same way as CMCT group, and continued for 10 days. Everyday, the survival status of mice was observed, and the deaths was recorded, it was 30 days when mice survived for more than 30 days. Test was evaluated by calculating ILS of the treated (T) and control (C) groups expressed as ILS= (T/C- 1) ×100%. In the acute toxicity testing, the mice

Apoptotic Cells Analysis by Flow Cytometry

Cells were inoculated in 6-well plates, and then added serial dilutions of CMCT (last concentration: 5.0, 10.0 and 20.0 μmol/L) or DMSO after overnight. 24 hours later, the cells were harvested in log phase by washing with PBS, after that stained by Annexin-V and propidium iodide (PI). At last, the detection results of flow cytometry was analysed using cell Quest/Pro.

Tubulin Activity Analysis

Tubulin was extracted and isolated from swine brain in advance, and determine the content of it according to the direction of BCA protein quantitation kit, confirm the purity of tubulin by SDS-PAGE electrophoresis. Referring to previous report [20], ATP solution was put into the purified tubulin (last concentration:100 mol/L), then seeded into 96-well plate and kept under 37°C. Detected the OD data of tubulin per 3 min, and down regulated the temperature to 0°C when the OD data remained unchanged. The plate was read at 350nm per 3min. paclitaxel and colchicine was choosed as positive control.

Statistical Analysis

Results are expressed as the mean ± S.D. of values obtained in triplicate from at least three different experiments. Using SPSS17.0 for Windows statistic software, differences between groups were compared by Student's t test; Results were considered significant statistically if probability of the difference occurring by chance was less than 5 in 100 ($p<0.05$).

RESULTS AND DISCUSSION

The cytoxicity of CMCT on 8 kinds of tumor cell lines

The results of the *in vitro* cytotoxic effects on 8 kinds of tumor cell lines after 48 hrs revealed by MTT assay. Regression analysis was performed on the cell viability data and the resulted equation was used to compute the inhibition concentration required to produce IC50 in Fig. 2.

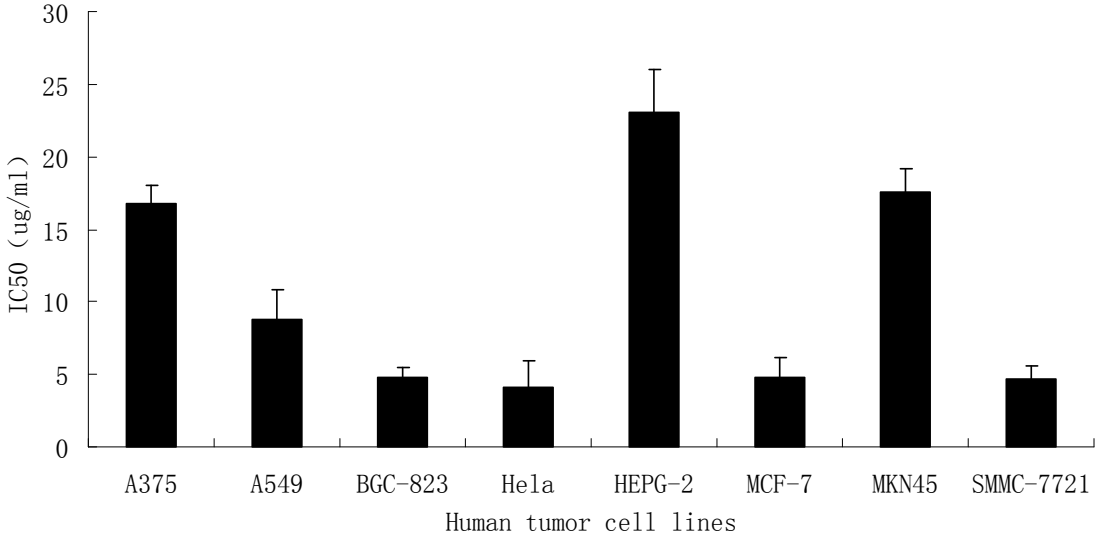


Fig. 2 The IC50 of CMCT on 8 kinds of tumor cells

The experiments were repeated three times and similar results were consistently obtained. Error bars indicate the standard deviations (S.D.)

CMCT Inhibiting the growth of H22 solid tumor

Analyzing the results of CMCT in H22 transplanted subcutaneously models on daily intragastric administration, the growth of tumors in the treat group was significantly suppressed ($p<0.05$). With an increasing size of CMCT dose, inhibition of H22 transplanted subcutaneously models of CMCT increased. When CMCT was used as a low dose, it delayed the tumor growth only moderately (Fig.2). The high dose of CMCT had the same effects on tumor growth as DDP initially, but it was slightly lower than DDP finally. Experimental data (Table 1) showed that the inhibition

rates in 3 repeated experiments were 18.26%, 29.21% and 41.30%, respectively.

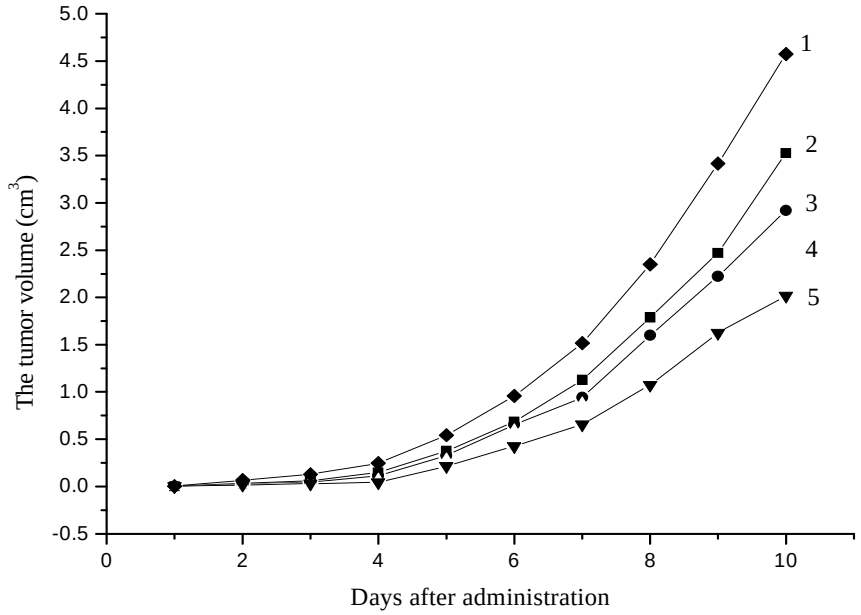


Fig.3 The growth curve of mice implanted tumor H22
1-negative control group, 2-1 mg/kg group, 3-3 mg/kg group, 4-10mg/kg group, 5-positive control group

Table 1: Inhibition of mice implanted tumor H_{22} of CMCT ($\bar{X}$ ±s)

Group	Body weight/g		FBW/ IBW	Tumor Weight/g	Inhibitory rate /%
	IBW	FBW			
negative control	19.16 ± 0.96	25.77 ± 2.15	1.29	2.67 ± 0.63	—
positive control	19.83 ± 0.87	21.14 ± 1.83[b]	1.07	1.30 ± 0.45[b]	51.43
1 mg/kg	19.81 ± 0.81	22.69 ± 1.93[b]	1.14	2.18 ± 0.75	18.26
3 mg/kg	19.38 ± 0.71	24.07 ± 1.32	1.24	1.89 ± 0.61[a]	29.21
10mg/kg	19.53 ± 0.87	25.55 ± 1.29[b]	1.31	1.57 ± 0.54[a]	41.30

n=10; Compared with the negative control group [a]:$p<0.05$, [b]:$p<0.01$
IBW:Initial body weight; FBW:Final body weight

The results (Table 2) indicated that the low dose of CMCT could increase the spleen index a little compared with negative control group. And the results showed that CMCT could notably enhance WBC, the thymus index and the spleen index of H22 transplanted subcutaneously models to different extent compared with positive control group. CMCT could cause bone marrow inhibition, however, BMNC and DNA of CMCT groups increased compared with positive control group.

Table 2: Effects on immune system of H22 transplanted subcutaneously models of CMCT ($\bar{X}$ ±s)

Group	WBC(10^9/L)	Organ index (mg/g)			BMNC (10^6)	Inhibition rate of DNA(%)
		Thymus index	Spleen index	Kidney index		
negative control	9.71 ± 0.67	3.27 ± 0.59	13.35 ± 3.74	7.52 ± 0.34	10.58 ± 0.50	—
positive control	5.63 ± 0.99[b]	2.31 ± 0.58[a]	10.44 ± 1.57[a]	7.07 ± 0.36	6.02 ± 0.51[b]	18.78
1 mg/kg	9.03 ± 0.62[a]	3.03 ± 0.50	13.49 ± 2.52	7.45 ± 0.63	8.96 ± 0.62[b]	13.17
3 mg/kg	7.24 ± 0.75[b]	2.85 ± 0.67	13.37 ± 1.84	7.36 ± 0.73	8.34 ± 0.46[b]	15.72
10mg/kg	6.36 ± 0.57[b]	2.65 ± 0.46	13.26 ± 2.32	7.26 ± 0.50	7.73 ± 0.59[b]	17.54

n=10; Compared with the negative control group [a]:$p<0.05$, [b]:$p<0.01$

CMCT prolonging survival time of S180 ascites tumor-bearing mice

ILS of the S180 ascites tumor models were 43.61%, 72.18%, 90.22% at the dose of 1, 3, 10 mg/ (kg ·d) by peritoneal injection administration, respectively (Table 3). From the data, CMCT prolonged survival time of the S180 ascites tumor models significantly peritoneal injection administration. Compared with the negative control group, food-taking, weight and mental state in the CMCT group were the same.

Table 3: Effect on survival time of S_{180} tumor-bearing mice ($\bar{X}$ ±s)

Group	MST (days)	Long term survivors 30 days	ILS (%)
negative control	13.3 ± 7.2	1	—
1 mg/kg	19.1 ± 9.1	3	43.61
3 mg/kg	22.9 ± 7.1	6	72.18[a]
10 mg/kg	25.3 ± 6.0	8	90.22[b]

n=10, MST: the survival time of mice, ILS: increase in life span
Compared with negative control group, [a]: p <0.05 , [b]: p <0.01

CMCT inducing apoptosis on HepG-2 Cell Line

These results suggested that different doses of CMCT caused different degrees of apoptosis and inhibition of proliferation. CMCT could induce HepG-2 cells apoptosis in concentration-dependent manners to play an efficient role against the tumor, which you can see by comparing the apoptotic rate.

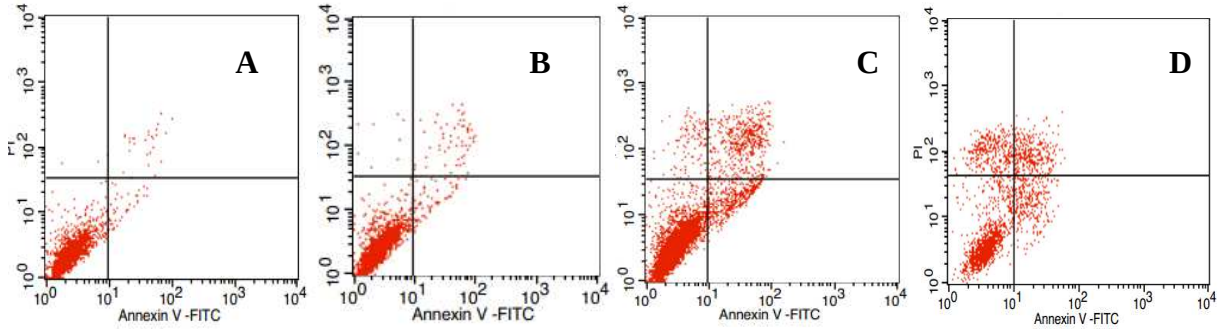
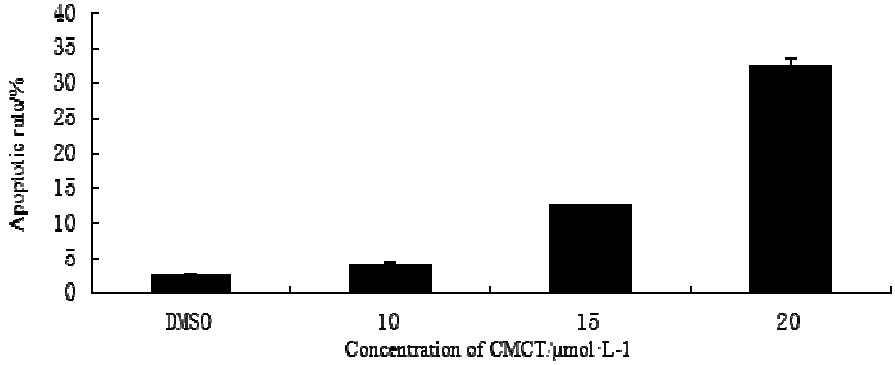


Fig.3 CMCT induced apoptosis of HepG-2 cells detected by flow cytometry



A: DMSO对照; B: CMCT 10.0 μmol/L ; C: CMCT 15.0 μmol/L ; D: CMCT20.0 μmol/L

CMCT inhibiting tubulin polymerization

It is clearly observed that CMCT decreased the O.D. of the reaction system. The result suggested that CMCT could strongly inhibit tubulin polymerization in a concentration-dependent manner, that was better than DMSO.

Several previous studies had shown that thiochromanone derivatives exhibited a wide range of biological activities, especially anti-tumor effect. In our laboratory, a series of its analogs, with antibacterial and anti-tumor activity, were designed and synthesized[2,10]. However this study was the first to experimentally demonstrate that the compound could inhibit tumor growth in subcutaneous transplanting tumor model of H22 and ascitic tumor model of S180.

Firstly, we observed the effects of the anti-tumor pharmacodynamic action of CMCT through the experiments of inhibiting cell proliferation of the tumor cell in vitro. The results showed CMCT possessed of inhibitory action to 8 kinds of tumor cell lines.

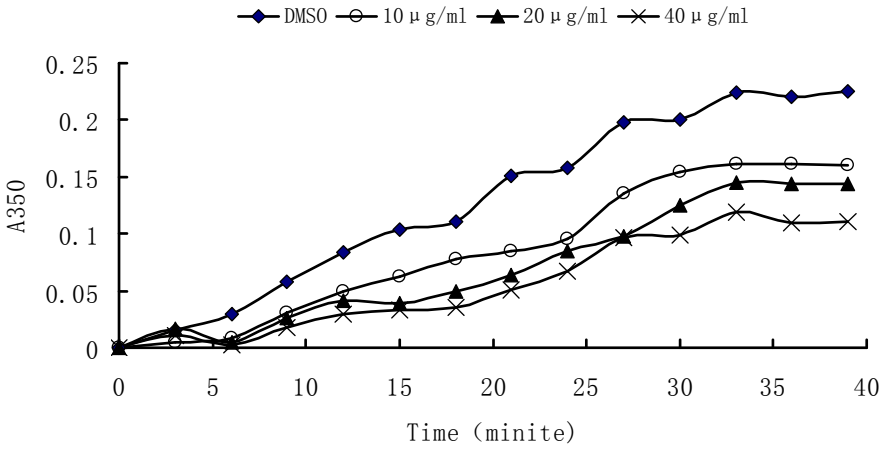


Fig. 4 The effect of CMCT on tubulin polymerization

Secondly, animal experiments results indicate that CMCT significantly inhibited the growth of transplanted solid tumor H22, and there was no discomfort to any of the CMCT group animals compared with negative control group, although there were some changes in WBC, visceral organ, and bone marrow. And prolonged the survival time of mice with ascites tumor S180. Particularly necessary to point out that ILS of the S180 ascites tumor models was 90.22% at the dose of 10 mg/ (kg·d) by peritoneal injection, that was due to the very well effect against tumor of CMCT. And then the anti-tumor effect of CMCT may come from its inhibiting tubulin polymerization，which caused proliferation suppression and apoptosis induction of tumor cell line. According to other literatures, vincristine[17] and taxol [18,19]worked on tubulin that have been shown to have anti-tumor effects. So CMCT inhibiting tubulin polymerization may play an efficient role in Its tumor inhibition effect. And we chose tubulin as the main researching direction. Through the analysis of experiment results, CMCT may influence the changes of cell cycle and induce apoptosis by the pathway of tubulin polymerization inhibition which is able to lead to chromosome mitotic.

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