Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(3):664-668

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

The apoptosis induction effect of (Z)-3-(chloromethylene)- 6-methylthiochroman-4-one on Hela Cells

Yitian Wang[1], Yuanyuan Guo[1], Pengpeng Tian[1], Yanping Wang[1], Yuemin Zhao[1], Zhengyue Ma[1,2], Chunliu Yang[1,2] and Yuxin Liu[1,2*]

[1]Laboratory of Cell Pharmacology, College of Pharmaceutical Sciences, Drug Quality Control Key
[2]Laboratory of Hebei Province, Hebei University, 180 East Wusi Road, Baoding, Hebei Province, 071002, China

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ABSTRACT

Previously we reported that (Z)-3-(chloromethylene)-6-methylthiochroman-4-one (CMMT), a novel thiochromanones derivative could exhibit dramatically antitumor activities on 12 human tumor cell lines in vitro. How was CMMT kill the tumor cells? The purpose of this study was to answer this question. Pretreatment of Hela cell cultures with 5, 10, 20μmol/L CMMT for 24h, afforded significant apoptosis induction as judged by Flow cytometry assay, and Hoechst 33258 fluorescence staining. We found that CMMT could cause cancer cell death, in particular apoptotic cell death. Mechanistically, CMMT increased production of cysteinyl aspartate specific proteinase including Caspase-3, Caspase-8 and Caspase-9, and death receptors (DR3) underlay apoptosis induction effect. Taken together, these results suggest that CMMT kills tumor cells by apoptosis induction via increasing production of caspase-3 Caspase-8, Caspase-9, and death receptors 3.

Keywords: (Z)-3-(chloromethylene)-6-methylthiochroman-4-one, thiochromanones, cervical cancer, apoptosis, Caspases, DR3

INTRODUCTION

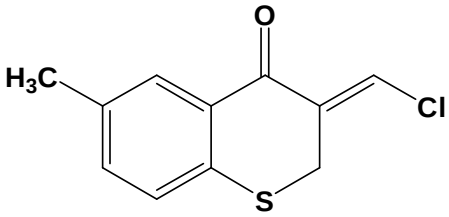
Thiochromanones is a compound of high fat-soluble, low water-soluble with extensive biological activities. Thiochromanones has gained considerable attention because of its diversity in biological activity, such as antifungal activity [1, 2] and anti-platelet aggregative activity [3]. Previous reports also indicated their probable antitumor activities [4] (such as tumors of the breast, endometrium and prostate). In view of these researches and in continuation of our work in thiochromanones, a series of thiochromanones derivatives herein were designed synthesized for the evaluation of their antitumor activities by Yang GL etc. in our lab [5-7].

Previously we reported that (Z)-3-(chloromethylene)-6-methyl-thiochroman-4-one (CMMT) had prominent antitumor activities in vitro [6]. But how this chemical kill the tumor cells are unknown. Herein, we report that CMMT kill the tomor cells by inducing apoptosis.

Apoptosis is the process of automatically end an active life determined by genes in normal tissues. During apoptosis, a cell triggers a process that will allow it to "commit suicide". In this process, the cell undergoes a reduction in size as its cellular components break down and condense [8, 9]. Unlike cell necrosis, cells shrinkage round and appears gathered Karyopycnosis in the process of apoptosis. Fractured chromatin fragments or cell organelles are reflexed, parceled by cell membrane to the formation of apoptotic bodies. Apoptotic bodies are swallowed by adjacent cells, not causing inflammation [10]. Following many extracellular and intracellular activation, a series of cysteine proteases called caspases was activated, causing the apoptosis.

Numerous studies have shown that the incidence of cancer is not only related to abnormal cell proliferation, but also

apoptosis abnormalities; cancer can be usually caused by the lack of apoptosis [11]. In our present research, the results showed CMMT could induce Hela cells apoptosis. And we explored the possible mechanisms and pathway that regulated this apoptosis in Hela cells.



CMMT

Figure 1 Chemical structure of CMMT

EXPERIMENTAL SECTION

1.1. Chemicals

(Z)-3-(chloromethylene)-6-methylthiochroman-4-one (CMMT) was obtained from Drug Quality Control Key Laboratory of Hebei Province. DMEM, DMSO and Bis Benzimide Hoechst 33258 were purchased from Beijing Solarbio Science & Technology Co. Ltd., placental bovine serum(NBS) was purchased from Zhejiang Tianhang Biological Technology Co. Ltd., Trypsin(1:250) was purchased from Amersco Inc. BCA Protein Assay kits was purchased from Beijing Biomed Co. Ltd., Caspase-3,Caspase-8 and Caspase-9 ELISA kits were purchased from Beyotime Institute of Biotechnology, DR3 and TNFR1 ELISA kits were purchased from R&D Systems in Shanghai Yuanye Bio-Technology Co. Ltd., Annexin V-FITC ELISA kits were purchased from Becton, Dickinson and Company.

1.2. Preparation of CMMT

(Z)-3-(chloromethylene)-6-methylthiochroman-4-one (CMMT) was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 8μmol/L As stock solution, stored at 4℃. Dilluted with culture medium to final concentrations (20, 10 and 5 μmol/L), and keep DMSO is 0.2% in every treatment solutions.

1.3. Hela cell culture and treatments

Human cervical carcinoma cells Hela was a precious gift from Dr. Jingxiang Zhao (Academy of Military Medical Sciences), maintained by our laboratory at 37℃ in a humidified atmosphere incubator with 5% CO_2 and 95% air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100U/mL of penicillin, 100μg/mL of streptomycin and 10% placental bovine serum. For experimental purpose, the cells were harvested in log phase and were plated in 6 well sterileplate. 12 h later, the cells were treated with vehicle, 5, 10, 20μmol/L of CMMT. The cells were digested and collected at different time points for ELISA assay, fluorescence staining and flow cytometry (FCM).

1.4. Flow cytometry assay

Collected 24h drug-treated culture, wash them three times with PBS, then the cells were treated using Annexin V-FITC ELISA kits following the manufacturer's instructions; apoptosis inducing effect of CMMT was carried out by Flow cytometry.

1.5. Fluorescence staining by Bis Benzimide Hoechst 33258

Added 1mL of Bis Benzimide Hoechst 33258 per well in 24 h drug-treated cultures, continued to incubate for 30 minute. Removed the medium, and washed two times with PBS. Watched and recorded images by a confocal microscope (Olympus IX81-FV1000, Japan)

1.6. Caspases, DR3 and TNFR1 assaies

Washed 24h drug-treated cultures with PBS, collected cells in the centrifuge tube, and added 150 μl precooling cell lysis solution (RIPA) and 15μl protease inhibitors (PMSF) per tube. Incubated the tube on the ice at 4℃ for 20 minute, centrifuged at 1,000 r/min for 10 min. The supernatants were collected, the level of Caspase-3, Caspase-8, Caspase-9, DR3 and TNFR1 in supernatant was measured according to the manufacturers' instructions. The optical density was read at 405 nm for caspases and 450nm for DR3 and TNFR1 with a Bio-Tek microplate Reader.

1.7. Data processing and statistical analysis

Data are presented as the mean ± standard error of mean (SEM) of independent repeated three experiments. Statistical significance was assessed with ANOVA followed by t-test using SPSS Statistics 17.0. Apoptosis data was assessed

with cell Quest/Pro. A value of $p < 0.05$ was considered significant statistically.

RESULTS

1.8. CMMT-induced apoptosis in Hela cells

Flow Cytometric Analysis of Annexin V/PI double staining showed that CMMT could induce apoptosis in Hela cells in a concentration-dependent manner (Fig.1A). When the concentration of CMMT was greater than 10 μmol/L, apoptosis rate of Hela cells was significant higher than vehicle (Fig.1B).

Hoechst 33258 staining assay also showed that CMMT- treatment induced nuclei condensation or apoptotic bodies formation in Hela cells, but the nuclei of cells were round and homogeneously stained in the control group. (Fig.1C).

1.9. CMMT induced Caspases activation and increased protein level of DR3

To investigate how CMMT induced apoptosis in Hela cells, ELISA assaies were used to detect the enzymatic activities of Caspase-3, Caspase-8 and Caspase-9, and the protein level of DR3 and TNFR1. The results showed that CMMT could activivate Caspase-3, Caspase-8 and Caspase-9 in a concentration-dependent manner. (Fig.2). Enzymatic activities of Caspase-3, Caspase-8 and Caspase-9 were increased by 159.16, 284.90, and 244.90 percent respectively at 20μmol/mL, and was increased by 8.45, 81.13, and 59.18 percent respectively even if at lowest concentration (Fig.2). As shown in Fig.3. CMMT significantly enhanced the protein level of DR3 in higher concentration (20.0 μmol/L), but has no effect on TNFR1, the level of TNFR1 inuced by CMMT(5, 10 and 20μmol/L) was 98.53%, 85.07%, 138.24% of the vehicle group respectively.

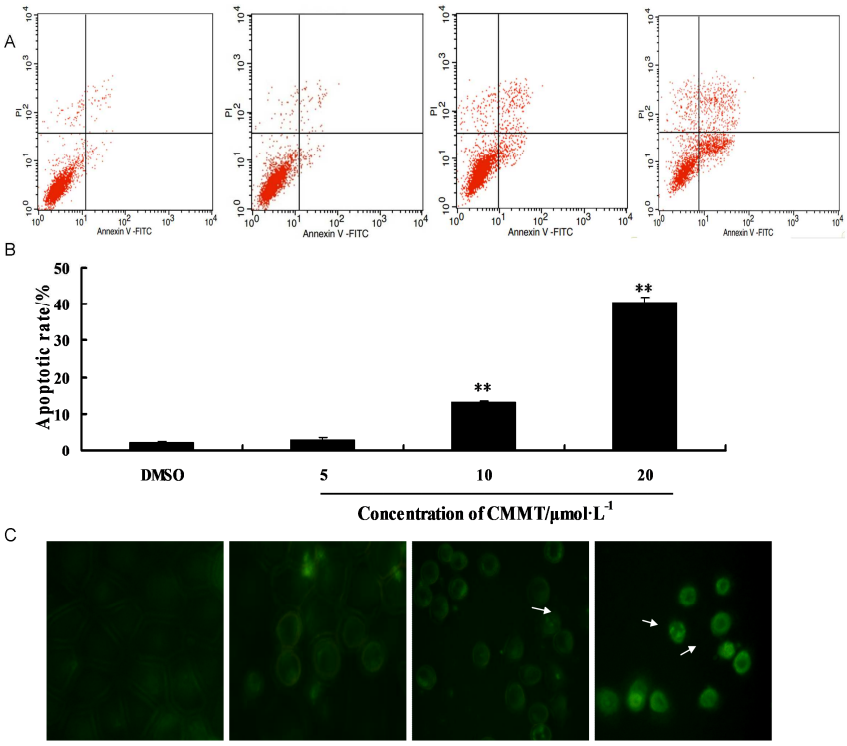


Fig.1 CMMT-induced apoptosis in Hela cells

*(A) DMSO and CMMT-induced apoptosis in Hela cells, early apoptosis cells were tested by AnnexinV, PI is the indicator of late apoptosis cells and necrosis cells. They were DMSO, CMMT (5.0μmol/L), CMMT (10.0μmol/L), CMMT (20.0μmol/L) from left to right respectively. (B) Apoptosis rate on Hela cells induced by DMSO and CMMT. After treated with 5, 10 and 20μmol/L of CMMT for 24 h, apoptosis rate was 3.71%, 13.34%, 41.40% respectively. The results are the mean ± SEM of three independent experiments. **p<0.01 compared with DMSO-treated cells, (C) Different cell-shape in different cultures treated with DMSO and 5,10 and 20μmol/L of CMMT for 24 h, They were DMSO, CMMT(5.0μmol/L), CMMT(10.0 μmol/L), CMMT(20.0 μmol/L) from left to right respectively.*

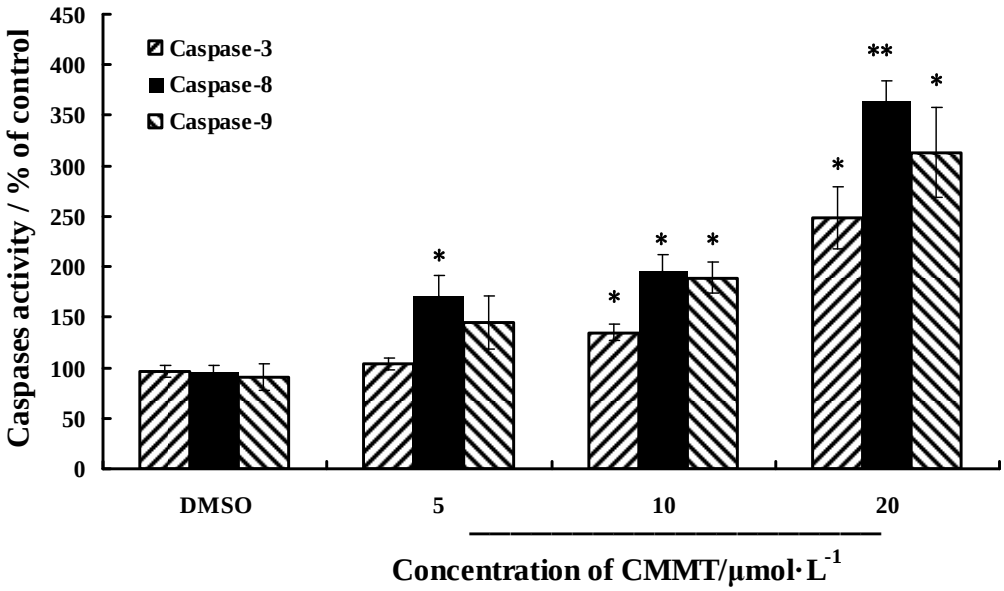


Fig.2 Caspases Activation induced by DMSO and CMMT. Cultures were pretreated with DMSO and CMMT
*Cells were harvested at 24 h for Caspases detection. The results are the mean ±SEM of three independent experiments. *p<0.05, **p<0.01 compared with DMSO-treated cells.*

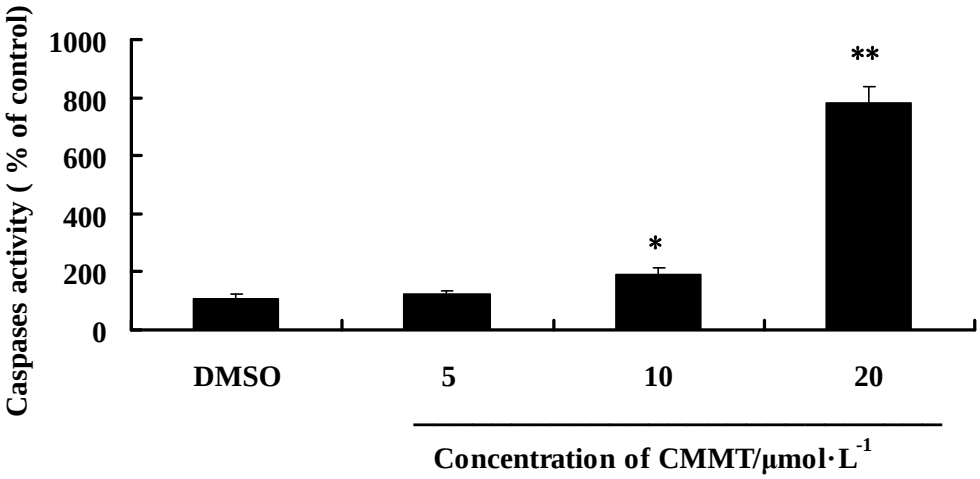


Fig.3 Effect of CMMT on DR3 expression
*Cells were treated with DMSO and 5, 10, 20μmol/L of CMMT followed by 20 min RIPA/PMSF (150μl/15μl) treatment, and then harvested for DR3 assay. The results are mean ±SEM from three independent experiments. *p<0.05, **p<0.01 compared with DMSO-treated cells.*

DISCUSSION

We investigated the cell apoptosis induced by CMMT in Hela cells, and showed the apoptosis from the cell-morphology by confocal microscope. In addition, we explored the possible induce- apoptosis mechanism of CMMT by using ELISA kits, and certificated CMMT could significantly elevate the activation of Caspase-3, Caspase-8, Caspase-9 and the expression of DR3 .

Caspase, as a class of proteases in the cytoplasm, play a key role in the process of apoptosis. Caspase family can be divided into two subgroups, one for the apoptosis initiator such as Caspase-2, Caspase-8, Caspase-9, Caspase-10, can be activated by self-splicing after receiving the stimulation to cause Caspase cascade; Another for apoptosis performer, such as Caspase-3, Caspase-6, Caspase-7, which can be activated by upstream Caspase protein. Caspase activation may induce ICAD/DFF45 (a protective protein for cell) losing the cell-protective function to activate CAD (Caspase-activated deoxyribonuclease), CAD is released into the nucleus. Previous trial had elucidated that CAD can degrade DNA, and then leads to cell apoptosis rapidly [12]. Apoptosis is a complex process. As the center molecule of the apoptosis, the activation of Caspase-3 is the intersection of Caspase-dependent pathways. In present research, 24 hours after CMMT treated Hela cells, the effect was found that Caspase-3 protein was significantly increased compared with the control group, indicating apoptosis induced by CMMT is Caspase-dependent pathway in Hela cells.

There are two main Caspase-dependent apoptosis pathways, one is death receptor pathway, inducing apoptosis through Caspase-8 activated by extracellular signal; another is mitochondrial pathway, releasing enzyme activator of apoptosis to active the Caspase-9 through the mitochondria. Previous report has indicated that the caspase-9 G allele was related to the development of the pancreatic cancer, it could enhance susceptibility to this cancer [13]. As death receptors in the surface of cell, CD95, TNFR1, DR3, etc. can directly activate effector molecule Caspase protease to induce apoptosis cascade after combination with a specific ligand for a few seconds, induce apoptosis within few hours [14, 15], so the death receptor-induced apoptosis has been the focus of the researches. 24 hours after the treatment with different concentrations of CMMT, Hela cells elevated the protein content of Caspase-8, Caspase-9 apparently. It suggested that induction of apoptosis in Hela cells may be co-regulated by death receptor pathway and mitochondrial pathway.

In the detection of death receptors (TNFR1, DR3), with the increasing concentration of CMMT, only DR3 content increased significantly, the expression of TNFR1 remained almost the same. There was a significant difference compared with the control group when the concentration of CMMT was 20μmol/L. It indicated that CMMT can activate death receptor DR3, but not TNFR1.

A series of thiochromanones derivatives with anti-tumor activity have been synthesized and screened in our laboratory, such as (cis)-3-(chloromethylene)-5-methylthiochroman-4-one (CMTK) . The chemical structure of CMTK is similar to CMMT. And Huang Xin etc. [9] have preliminarily clarified the mechanism of tumor cell apoptosis induced by CMTK. Our present experiment results showed that CMMT induce apoptosis in a Caspase-dependent death receptor pathway. And Caspase-9 also participates to the regulation in the mitochondrial pathway, the mechanism is similar to CMTK.

If methylene chloride is substituted with another group in the structure of CMMT, the anti-tumor property of new substance would be reduced significantly [16]. Thus, we speculate that methylene chloride may be the active group, methylene chloride can react with many biologically active molecules in cells, induce apoptosis.

As one of the thiochromanones derivatives, CMMT can induce apoptosis in Hela cells. The underlying mechanisms rest with enhancement of Caspase-3, Caspase-8, Caspase-9 and DR3. In conclusion, under our continuous research, CMMT induced Hela cells apoptosis, shedding light for that a therapeutic agent for the treatment of cancer such as cervical cancer might exist.

Acknowledgements
Our research project was supported by a grant from The National Major Scientific and Technological Special Project for “Significant New Drugs Development”(2012ZX09103-101-057), the Key Technologies R & D Program of the Ministry of Science and Technology of Hebei Province (12276403D), and the Science Research Projects of Hebei University (2014-04, 2014-09).

REFERENCES

[1] Philipp, et al. US Patent, **1997**, 4060619.
[2] Talley, et al. US Patent, **1997**, 5670532.
[3] MC Murguı´a, LM Machuca, MC Lurá, MI Cabrera, RJ Grau. *J Surfactants Deterg*, **2008**, 11, 223-30.
[4] A Billich, P Nussbaumer, E Schreiner, et al. Chromanone and thiochromanone compounds.US Patent, **2001**, US6346626BI.
[5] Tian, ZY Ma, GL Yang, BL Fang, G Wang, LB Li. *Organic Chem: An Indian J*, **2010**, 6, 8-12.
[6] CN Li, CL Yang, YX Liu, GL Yang, TF Liu, Q Lu. *Chinese Sci Bull*, **2010**, 55, 3027-31.
[7] BL Fang, ZY Ma, GL Yang, G Wang, W Tian, LB Li. *IntJ Chem*, **2010**, 2, 143-6.
[8] J F R Kerr, A H Wyllie, A R Currie. *British journal of cancer*, **1972**, 26(4), 239.
[9] J F R Kerr, C M Winterford, B V Harmon. *Cancer*, **1994**, 73(8), 2013-2026.
[10] DL Vaux, A Strasser. The molecular biology of apoptosis. Proceedings of the National Academy of Sciences, **1996**, 93(6), 2239-2244.
[11] CB Thompson. *Science*, **1995**, 267(5203), 1456-1462.
[12] M Enari. *Nature*, **1998**, 391, 43.
[13] G E Theodoropoulos, N V Michalopoulos, S G Panoussopoulos, et al. *Pancreas*, **2010**, 39(7), 976-980.
[14] Y Otsuki. *Acta Histochem. Cytochem*, **2000**, 33(4), 235-241.
[15] CJ Norbury, LD Hickson. *Annu Rev Pharmacol Toxicol*, **2001**, 41, 367-401.
[16]SY Cui, YX Liu, GL Yang, BL Fang, W Tian. *China Pharmacist*, **2010**, 13(5), 612-615.