3-chloromethylene-6-fluorothiochroman-4-one, A novel DNA Topoisomerase poison

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Abstract: 3-Chloromethylene-6-fluorothiochroman-4-one (CMFT) is a novel thiochromanones derivative that has better anti-tumor activity. In this paper, we will compare the antitumor activity of the cis-trans isomers, and explore their inhibiting effects on human topoisomerase I and topoisomerase II in cell free reaction system. The MTT method was used to study inhibition rates; the AO/EB double staining and TUNEL assay was used to assess proportion of apoptotic cells. The inhibition of CMFT to Topo I and II could be identified by adding CMFT solutions to Topo-DNA reaction mixtures and observing the relative quantities of relaxed strands and supercoils in electrophoresis assay. Results showed that CMFT had dramatic anti-tumor activities at low concentrations and the activity of CMFT trans-isomer is more significant. Use of AO/EB double staining and TUNEL indicated that CMFT induces apoptosis. DNA relaxation assays and DNA cleavage and relegation assays were performed and showed a higher potential to interact with topoisomerase I (Topo I) and topoisomerase II (Topo II) and it was verified that CMFT is a Topo poison which could be one of the mechanisms that induce cell apoptosis. Our results provide preliminary data for further investigation for the mechanism of CMFT of the apoptotic mechanism.

Keywords: 3-chloromethylene-6-fluorothiochroman-4-one (CMFT), cis-and trans-isomers, anti-tumor activity, topoisomerase.

INTRODUCTION

Thiochromanones with unsaturated cyclic ketone as the main structure, have extensive biological activities, like anti-bacterial, anti-inflammatory, anti-tumor ect. It has gained considerable attention because of its diversity in biological activity, such as antifungal activity (Liu Y *et al*., 2008; Fang BL *et al*., 2010). Previous reports also indicated their probable antitumor activities (such as tumors of the breast, endometrium and prostate). There years we synthesized a series of thiochromanones derivatives and evaluated their activities in our lab (Li CN *et al*., 2010; Huang X *et al*., 2012; Zhao YM *et al*., 2014; Yang CL *et al*., 2015). Recently, we reported that (Z)-3-(chloromethylene) -6-flourothiochroman-4-one (Z-CMFT) could inhibit the polymerization of microtubules, triggered caspase cascade and increased the expression of death receptor 3 (DR3) (Zhao YM *et al*., 2014). And the structure of the CMFT was described in the fig 1. Now confirmed thiochroman ketone compounds can inhibit cell proliferation and affect the cell cycle. Based on this study, we tested and analyzed the inhibitory effect and mechanism of CMFT on DNA topoisomerase.

DNA topoisomerases are over expressed in proliferating cancer cells, so they are one of the most promising targets for development of anticancer agents which manipulate the topology of DNA (Cummings J and Smyth JF, 1993; Giles GI and Sharma RP, 2005; Montecucco A *et al.*, 2015). And Topo catalyzes the concerted breaking and rejoining of DNA strands and is involved in producing the necessary topological and conformational changes in DNA (Pendleton M *et al*., 2014; Seo YH, 2015; Xu Y and Her CT, 2015). In humans, there exist topoisomerase type I (Topo I) and type II (Topo II). Topo I breaks and rejoins single-strand of a double helix while Topo II breaks and rejoins double-strand DNA.

In this paper, the anti-tumor activity of the cis-isomer (Z-CMFT) and trans-isomer (E-CMFT) was compared. We have also tested and analyzed the inhibitory effect and mechanism of CMFT on DNA topoisomerase.

MATERIALS AND METHODS

Dulbecco's modified eagle medium (DMEM) was purchased from Beijing Solarbio Science & Technology Inc.; Fetal bovine serum (FBS) was purchased from U.S. HyCloneInc.; Camptothecin (CPT) was provided by Sun Chemical Technology (Shanghai) Co; Etoposide (VP-16) was purchased from TCI (Shanghai) development Co; PBR322 DNA Plasmid, Human topoisomerase I and II were purchased from TopoGen. In Situ Cell Death Detection Kit, fluorescein, Roche, Indianapolis, IN, USA. Fluorescence microscope, TE2000U, Nikon, Tokyo, Japan. CMFT solutions were prepared by dissolving compound in DMSO, and diluted by doubling dilution to required concentrations. These solutions were stored in 4°C before use. The cells were obtained from the Chinese academy of medical science.

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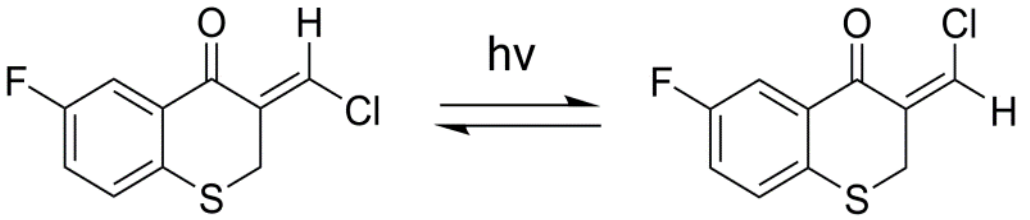

(Z)-3-chloromethylene-6-fluorothiochroman-4-one (E)-3-chloromethylene-6-fluorothiochroman-4-one

Fig. 1: The structures of Z-CMFT and E-CMFT

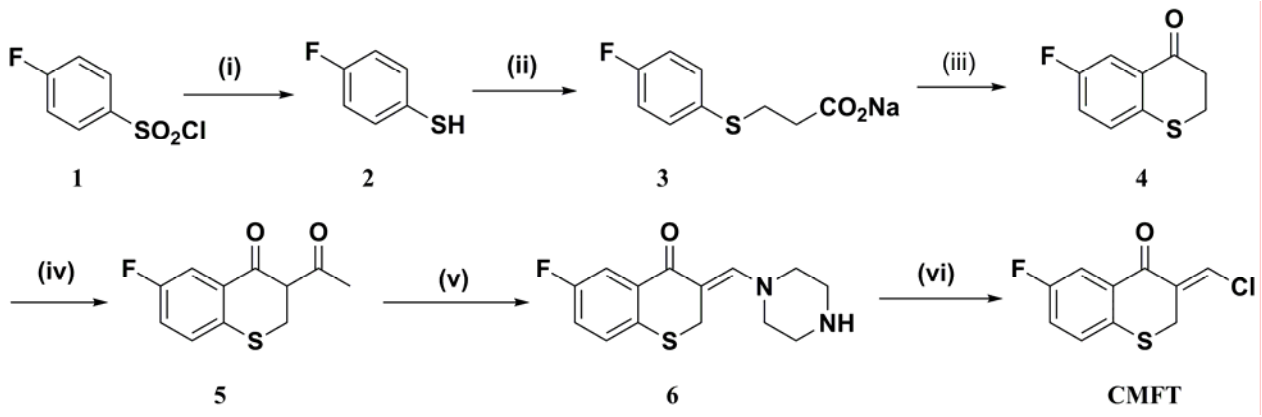

Fig. 2: Synthesis of CMFT. Reagents and conditions: (i) Zn/H_2SO_4, 0°C; (ii) 2M NaOH, $ClCH_2CH_2COOH$; (iii) concd. H_2SO_4, ambient, 24 h; (iv) ethyl formate, MeONa, toluene, 0-10°C, 5 h; (v) anhydrous piperazine, CH_2Cl_2, 0°C, 1 h; (vi) chloroacetyl chloride, CH_2Cl_2, 0°C.

Table 1: IC_{50} values of four kinds of tumor cells by CMFT cis-trans-isomer (unit: μM)

	E-CMFT	Z-CMFT
MGC-803	27.91688 ± 1.129	32.70332 ± 0.278
BV_2	20.61928 ± 0.352	47.88604 ± 2.23
Hela	30.80259 ± 1.135	54.09760 ± 0.835
MCF-7	28.56271 ±0.866	60.31095 ± 5.339

Synthesis of CMFT

The synthetic route of CMFT was described in the patent (Fang BL *et al*., 2012) Briefly, a reduction was carried out by heating 4-fluorobenzene-1-sulfonyl chloride 1 in the presence of Zn/H_2SO_4 to afford 4-fluorobenzenethiol 2. Then 2 was stirred with 3-chloropropionic acid in alkaline solution giving the corresponding carboxylate 3. 3 treated with concd. H_2SO_4 to afford 6-fluorothiochroman one 4. The reaction of 4 with ethyl formate in the presence of MeONa to obtain 5. And then 5 was reacted with anhydrous piperazine to gain 6. 6 was reacted with chloroacetyl chloride to afford the desired compound CMFT (fig. 2).

MTT assay

Using the MTT method to determined the cell viability of the cis-trans isomers. The cells were grown in 96-well plates at the density of 10^5 cells per well and treated with different concentrations of Z-CMFT and E-CMFT (last concentration: 0, 12.5, 25, 50 and 100 μM). DMSO at 0.1 % concentration in the culture medium was used as a negative control and cis-platinum was used as a positive control (final concentrations were 0, 12.5, 25, 50 and 100 μM). After 24 hrs, added 10 μL MTT to each well and incubated for another 4 hrs. After that added 100μL DMSO. Then read the absorbance at a wavelength of 490 nm. The growth inhibition rate was examined and 50% inhibitive concentration (IC_{50}) was calculated.

Acridineorange (AO)–ethidium bromide (EB) double staining

Hela cells were seeded into 96-well plates at density of 2×10^4 cells per well and incubated for 12 hrs. Cells were left untreated or treated with different Z-CMFT and E-CMFT concentrations of 20, 40 and 80 μM. The cells were cultured for 24 hrs, then added 20 μL of trypsin to each well. Adding 25μL suspensions to glass slides. Double staining solution (1 μL) were added to each slide and then covered with a cover slip. The solution containing 100 μg/mL AO and 100 μg/mL EB. We used the fluorescent microscope to observe the morphology of apoptotic cells.

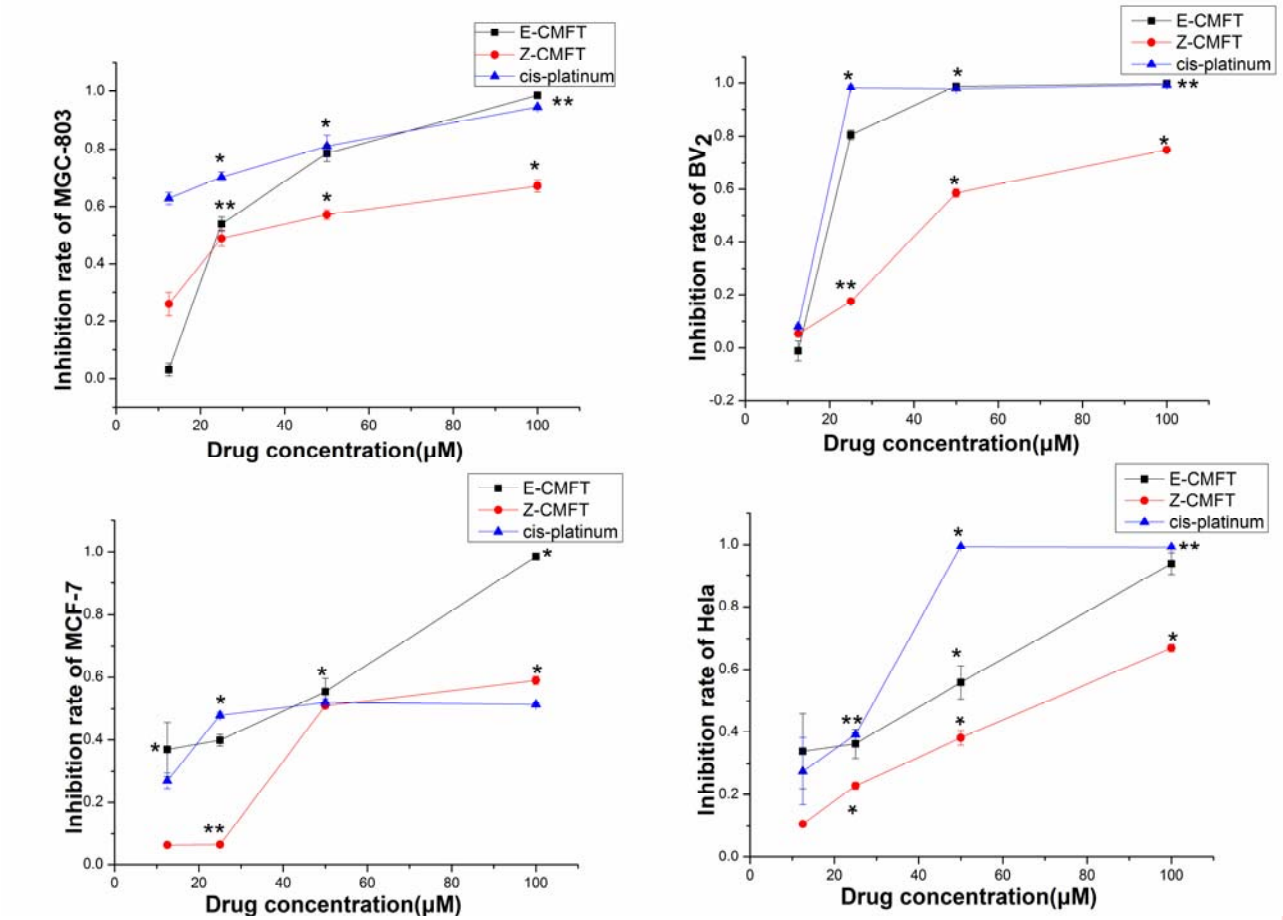

Fig 3: The cytotoxicity of CMFT (*cis*-, *trans*-) and cis-platinum on MGC-803, BV_2, Hela and MCF-7 cells, **P*<0.05, ***P*<0.01

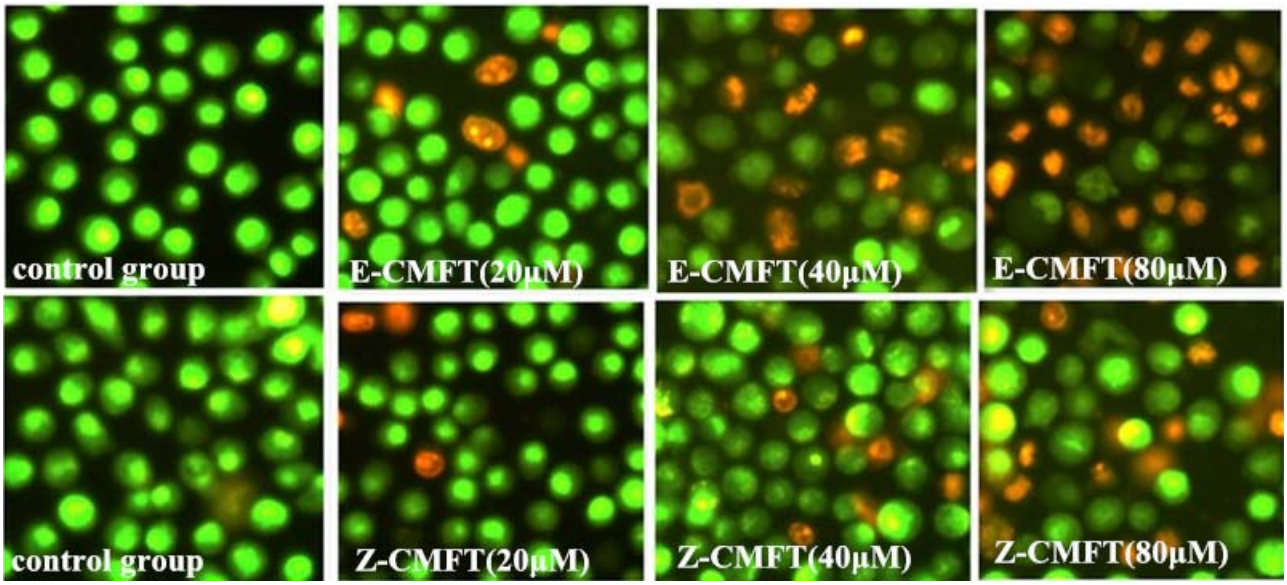

Fig. 4: The Hela cells were stained with AO/EB, and then were photographed by fluorescence microscopy (20, 40 magnification)

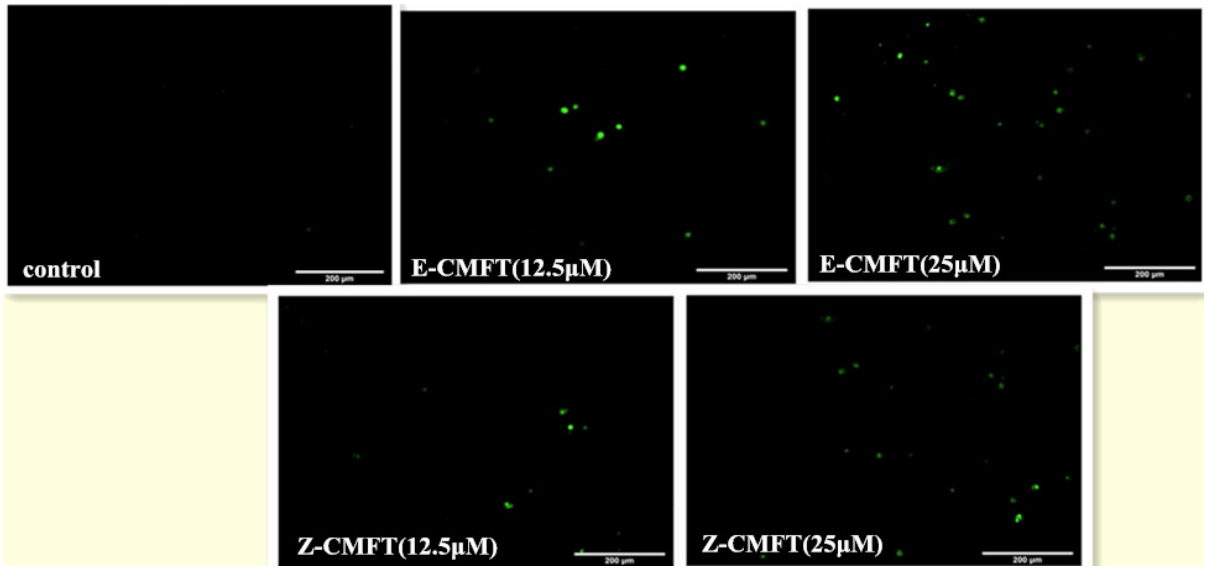

Fig 5: TUNEL staining to detect apoptosis in Hela cells

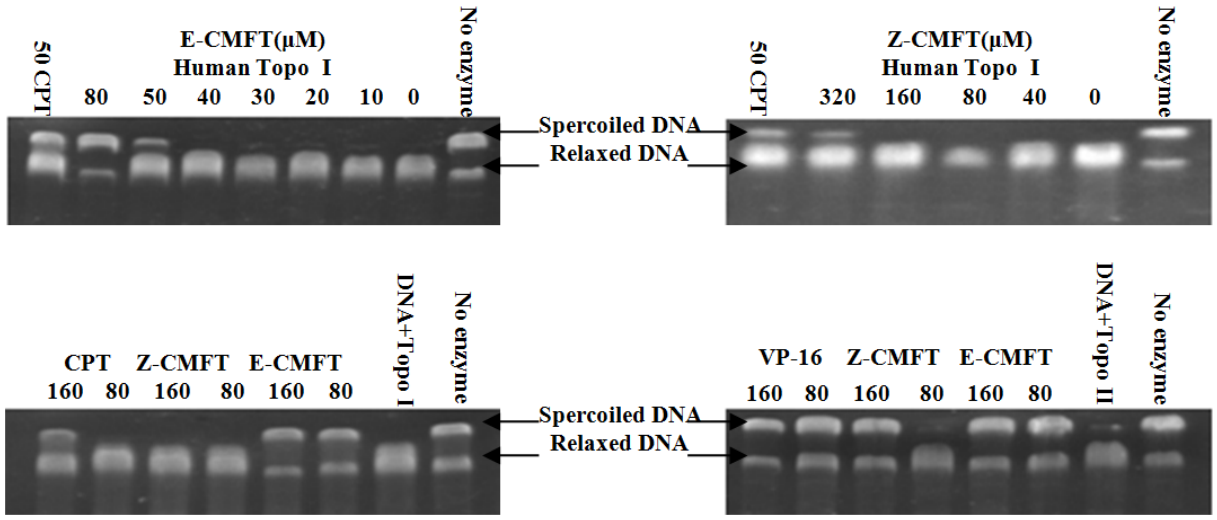

Fig 6: The comparison of relaxation inhibition effects of Z-CMFT and E-CMFT

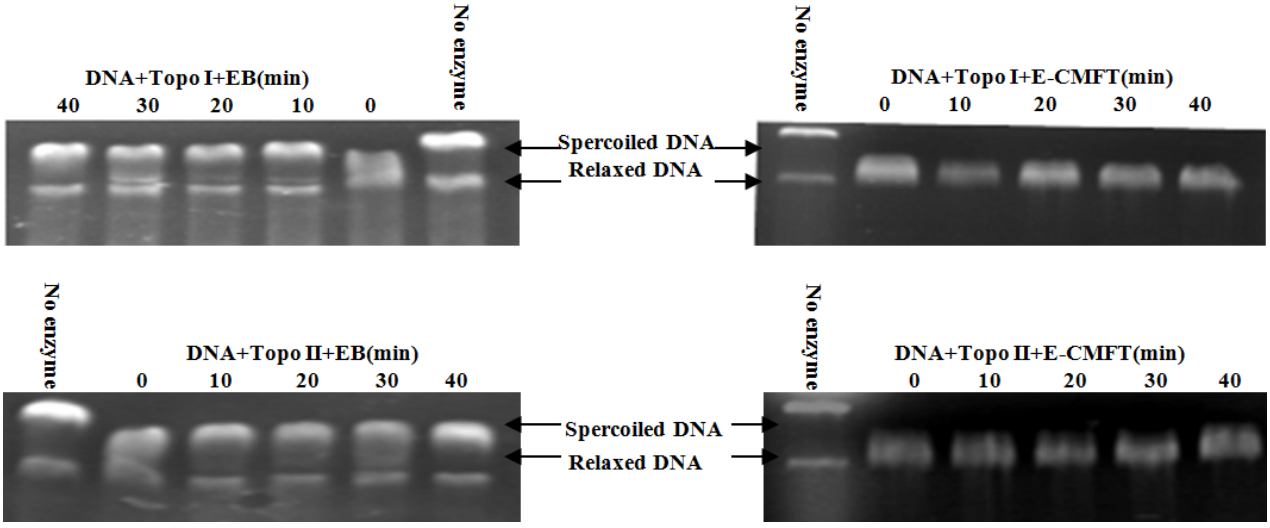

Fig 7: The religation inhibition effects of EB and CMFT to Topo I and Topo II

TUNEL assay

The TUNEL assay is used to detect the cells DNA fragmentation using a commercially available kit, and the theory is that the DNA of its free 3-OH ends connected to fluorescein conjugated dUTP by the enzyme terminal deoxynucleotidyl transferase. (Sharma R *et al*., 2013; Payan-Carreira R *et al*., 2013) Adjust the cell suspension at a concentration of 10^5/mL, seeded in 24-well plates, with a coverslip placed which polylysine-treated inner plates and incubated at 37°C, after12 hrs, adding serial dilutions of Z-CMFT or E-CMFT (last concentration: 12.5 and 25μM). After the cells were incubated for additional 24 hrs, the following operation was accorded to the kit manual. The cells were fixed with 4 % (w/v) para formaldehyde for 1 hour. Then used the PBS to wash the fixed cells three times and then added the penetrating fluid (0.1% (v/v) Triton X-100 containing 0.1 % (w/v) sodium citrate) for 2 min on ice. Adding 50μL of TUNEL reaction mixture to the samples and incubated at 37°C in a dark atmosphere for 1 hrs. The cells were observed under the fluorescence microscope.

Topoisomerases inhibition assay

The catalytic activity of Topo I/II was determined by detecting the supercoiled plasmid DNA in its nicked form. The Topo I reaction mixture containing PBR322 DNA (250ng), 2 units Topo I, 10mM Tris-HCl (pH 7.9), 150 mM NaCl, 5% glycerol, 1mM EDTA, 0.1 % bovine serum albumin (BSA) and 0.l mM spermidine. The reaction mixtures incubated at 37°C for 30 min, and then added the terminated liquid containing 1 % SDS and 1 mg/mL proteinase K digestion. Before the reaction mixture were subject to gel electrophoresis, added 2 μL of loading buffer. The agarose gel was stained with ethidium bromide (EtBr) and visualized under UV light. The activity of Topo II was analyzed as the same manner as Topo I except that the reaction mixture.

DNA cleavage and religation assay

The reaction mixtures containing (Oppegard LM. *et al*., 2012) 1μL Topo I/ II relaxation buffer, 1μL Topo I/ II and 1μL PBR322 were incubated at 37 °C for 6 min, established a balance of plasmid DNA "break and reconnect" (Byl J. AW *et al*., 2001; Byl J. AW *et al*., 1999). Then 1 μL EB or 1 μL solution of CMFT dilutions of different concentrations was added. The reaction mixtures were incubated at 0 °C for 10 min, 20 min, 30 min, 40 min, respectively. The following experimental procedures were as the same as the topoisomerases inhibition assay.

RESULTS

Cytotoxic effect of CMFT cis-and trans-isomer on tumor cells

As the picture showed that (fig. 3), the CMFT has significant antitumor activity, and can significantly inhibit the proliferation of the four tumor cells, and also behave in concentration dependence, namely the inhibition effect was enhanced with the increase of drug concentration; and the half maximal inhibitory concentration (IC_{50}) values were between 20-60μM. To summarize the data processing, the calculated IC_{50} values are shown in Table 1. From the table 1 we can see that the cis-isomer (Z-CMFT) and trans-isomer (E-CMFT) have significantly different on the cytotoxicity of the four kinds of tumor cells. The cytotoxicity of the E-CMFT is stronger than the Z-CMFT. Compared the IC_{50} value of the four tumor cells, the E-CMFT had the lowest IC_{50} value for the cell of BV_2, and the inhibition effect was strongest. In contrast, the Z-CMFT to the four kinds tumor cells in vitro was significantly lower. And the inhibition effect of MCF-7 cells was the weakest.

Acridineorange (AO)–ethidium bromide(EB) double staining

We used the AO/EB double staining to detect the Hela cells morphology of different physiological stages. AO is used for viable cells and it can emit green fluorescence. EB is used for nonviable cells: we distinguished the early apoptotic, late apoptotic and necrotic cells from the basis observation of color and morphological. They were different in these areas such as the membrane blebbing, the cell volume and the nucleus fragmentation.

Cells were treated with 20, 40 and 80 μM of Z-CMFT and E-CMFT for 24 h, followed by AO/EB staining to examine the cells morphological changes. The control cells did not appear significant changes in cell nuclei and cell membrane integrity. Few number of necrotic cells were noticed in addition to apoptotic cells (early and late) at doses of 20μM with the E-CMFT, but in the group that have the same concentration of Z-CMFT, only a few cells apoptosis. And its number significantly high especially at the doses above 40μM of E-CMFT. The number of necrotic cells were also increased with higher doses of Z-CMFT. As the concentration continued to increase, more and more cells were dead. The results showed that E-CMFT treatment significantly inhibited the growth of cells when the cells were cultured with a concentration over 20μM for 24 hrs. At the great same concentration, the inhibition to the cell of E-CMFT had better effect than that of Z-CMFT. This experiment proved that the Z-CMFT and E-CMFT has different degrees of cytotoxicity to Hela cells, while the cytotoxicity of the trans-isomer is more effective and more obvious.

TUNEL assay

The TENEL staining established that the apoptotic cells appeared green fluorescence under the light microscope. CMFT can induce apoptosis of Hela cells. With the increase of concentration (12.5, 25μM), apoptotic cells were more and more, and showed a dose-dependent manner, and apoptotic cells were significantly more than

the control group. From the fig. 5 we can see, with the same concentration of CMFT, E-CMFT can induced a high proportion of cells apoptosis than the Z-CMFT.

The comparison of relaxation inhibition effects of Z-CMFT and E-CMFT

Topo inhibitors are very rare, the most widely studied and characterized being camptothecin, a Topo I poison, and Etoposide and doxorubicin, Topo II poisons (Hsiang YH *et al*., 1985). Camptothecin (CPT) is a typical Topo I poison, and was used as a positive control in Topo I assay; and Etoposide (VP-16) is a typical Topo II poison, and was used as a positive control in Topo II assay. Since Topo I/II could break and reconnect PBR322 to relax its supercoils (Burden, DA and Osheroff N, 1998), and the relaxed strands showed low migration speed in electrophoresis. The inhibition of CMFT to Topo I/II could be identified by adding CMFT dilutions to Topo I/II-DNA reaction mixtures and observing the relative quantities of relaxed strands and supercoils in electrophoresis assay.

Take CPT and VP-16 were used as positive control, the supercoil relaxation ability of Topo I/Topo II to PBR322 DNA was inhibited by CMFT apparently. It exhibited that the higher the concentration of CMFT was, the less the relaxed strands were. According to the calculated values given in fig. 6, E-CMFT showed an excellent inhibition effect when the concentration is up to 50 μM, Z-CMFT could not inhibit Topo I activity in the low concentrations (data not shown) , When the concentration increased to 320 μM, Z-CMFT had the ability to restrain the activity of Topo I .

Under the same condition, with 40μM and 160μM Z-CMFT and E-CMFT on the Topo I (CPT as the positive control) and Topo II (VP-16 as the positive control). Gel electrophr-erogram showed that trans-isomer could inhibit Topo I/II activity in both low and high concentrations, but the Z-CMFT in 40μM concentration and 160μM concentration are not showed inhibitory effect on Topo I. And in the assay of the supercoil relaxation inhibition of CMFT compared with the positive control CPT on Topo I, the inhibition effects of E-CMFT were a little stronger than CPT in low concentrations as shown in the fig. As shown, E-CMFT and VP-16 showed good activity on the concentrations of 40μM and 160μM and the Z-CMFT are not exhibited inhibitory activity on the concentrations of 40μM. And that the inhibitory effect of E-CMFT on the activities of DNA Topo I and II is stronger than that of Z-CMFT.

In this paper, we investigated the mechanisms that CMFT suppresses cancer cell growth. It was revealed that the inhibition of DNA topoisomerases activities is one of the reasons.

DNA cleavage and religation assay

As is known, Topo I can break DNA duplex in mild temperature, but their religation activities remained in low temperature (<4°C) and EB can accelerate this reaction. So EB mediated cleavage and religation assay was applied to identify whether E-CMFT is a catalytic inhibitor or a poison. Since poisons can interfere Topoisomerases to reconnect cleaved DNA strands to supercoils, if E-CMFT was a poison, the nicked DNA would increase, and if E-CMFT was a catalytic inhibitor, the supercoiled DNA wouldn't decrease largely.

Reaction mixtures containing Topo I/II were placed in 0°C, then EB was added, and DNA religation appeared in 40 minutes in the assays. In 40 minutes, with the increase of EB effect time, light show increasing concentration to super helical DNA electrophoresis banding, visibly, Topo I/II gradually reconnection to DNA, DNA reconnection with EB can promote the reaction, the experiment results are consistent with the reports.

However, the Topo I/II inhibition mechanism of CMFT to DNA cleavage and religation were different from EB mediated cleavage and religation reaction that under the same experiments conditions according to fig. 7. The bands have no differences in 40 minutes which indicated E-CMFT had inhibited the religation activity of Topo I/II and the inhibition effects became more conspicuous with time. So it indicated E-CMFT is a Topo I and Topo II poison.

DISCUSSTION

In summary, this study demonstrates that E-CMFT is more critical than Z-CMFT in cytotoxic effect to tumor cell lines. CMFT showed a higher potential to interact with topoisomerase I (Topo I) and topoisomerase II (Topo II), and it was verified that E-CMFT is a Topo I and Topo II poison. Furthermore, the present study also showed that E-CMFT is more effective than Z-CMFT when act on DNA topoisomerases. This work has laid the experimental foundation for the further research.

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