

The Theaflavin Monomers Inhibit the Cancer Cells Growth *in Vitro*

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Abstract The inhibition effects of tea theaflavins complex (TFs), theaflavin-3-3'-digallate (TFDG), theaflavin-3'-gallate (TF2B), and an unidentified compound (UC) on the growth of human liver cancer BEL-7402 cells, gastric cancer MKN-28 cells and acute promyelocytic leukemia LH-60 cells were investigated. TFs was obtained through the catalysis of catechins with immobilized polyphenols oxidase. TFDG, TF2B and UC were isolated from TFs with high speed countercurrent chromatography (HSCCC). The results showed that TF2B significantly inhibited the growth of all three kinds of cancer cells, TFs, TFDG and UC had some effect on BEL-7402 and MKN-28, but little activity on LH-60. The inhibition effects of TF2B, TFDG, and UC on BEL-7402 and MKN-28 were stronger than TFs. The relationship coefficients between monomer concentration and its inhibition rate against MKN-28 and BEL-7402 were 0.87 and 0.98 for TF2B, 0.96 and 0.98 for UC, respectively. The IC₅₀ values of TFs, TF2B, and TFDG were 0.18, 0.11, and 0.16 mM on BEL-7402 cells, and 1.11, 0.22, and 0.25 mM on MKN-28 cells respectively.

Key words theaflavin monomer; separation; cancer cells; inhibition

The biological effects of tea and tea constituents have been studied and reviewed in many publications. Tea and its components have been demonstrated to inhibit chemically induced carcinogenesis in animal models, including cancers of the skin, lung, esophagus, stomach, liver, small intestine, pancreas, colon, bladder, prostate, and mammary glands [1]. Most of these studies are focusing on green tea, and limited investigation has been carried out with black tea. Theaflavins complex (TFs, a pigment complex in black tea) is transformed from tea catechins (a colorless component in tea) through oxidation catalyzed by enzyme and chemical condensation. TFs plays an important role in particular color and taste of black tea infusion. It is mainly consisted of the following four components: theaflavin (TF1), theaflavin-3-gallate A (TF2A), theaflavin-3'-gallate B (TF2B), and theaflavin-3-3'-digallate (TFDG).

It has been proved that TFs possesses evident effects such as anti-oxidation, anti-cancer, scavenging or inhibiting free radicals, treatment of blood vessel and heart

diseases [2–8]. Recently, studies on the mechanisms of action of TF2A, TFDG and TF2B to NF- κ B macrophages [9], mice liver cancer cells and human fibrosarcoma HT 1080 cells were reported, and compared with the activity of tea polyphenols [10]. TF3 has been found to have antiviral activity against influenza A and B [11].

In this paper, the inhibition effects of theaflavins complex and monomers on human liver cancer BEL-7402 cells, gastric cancer MKN-28 cells, and acute promyelocytic leukemia LH-60 cells were studied.

Materials and Methods

Preparation of TFs

TFs was prepared by enzymatic oxidation of tea polyphenols (80% purity, provided by Siming Natural Plant Co., Zhejiang, China) using immobilized polyphenol oxidase (PPO). The procedures to produce TFs were similar to that described in the patent applied by Tu *et al.* [12]. Sodium alginate was used for PPO entrapment, 75 ml PPO (1500 u) solution was mixed with 100 ml sodium alginate solution (2%) and entrapped for 5 min, then the enzyme mix-

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ture was injected into 1000 ml 0.1 M CaCl₂ solution by injector. After shaped for 30 min, the particles were taken out and kept in 0.025% glutaraldehyde aqueous for 1 h, an insoluble aggregate between PPO and cross-linking reagent was formed and kept in the pH 5.6 citrate buffer at 4 °C.

The operation of the model tea polyphenol oxidation system was the same as described by Tu *et al.* [12], except that the reaction was carried out at defined pH 5.6 at 37 °C in the incubation and terminated after 30 min.

Preparation of individual theaflavin monomers

An HSCCC instrument equipped with a separation co-column at a distance of 10 cm away from the central axis of the centrifuge (Tauto Biotech Cooperation, Shanghai, China) was used to produce theaflavin monomer in the present study. The column revolves around the central axis of the centrifuge and simultaneously rotates on its own axis at the same angular velocity in the same direction. The column holder is 50 cm long, 2.6 cm diameter and 260 ml capacity. The coils are prepared with PTFE (polytetrafluoroethylene) tubing. The experiment was performed at a revolution speed of 800 rpm. The mobile phase was delivered using ÄKTA explorer system (Amersham Bioscience). An injection loop was used for sample loading and a UV-Vis detector for monitoring the effluent [13].

The separation of theaflavin monomer was performed using HSCCC with a two-phase solvent system composed of hexane/ethyl acetate/*n*-butanol/methanol/acetic acid/water (1:2:1:1:5:1, *V/V*), where the upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. The sample solution was prepared by dissolving TFs in a 1:1 mixture of each phase and loaded into the column by loop injection. For the 260 ml column, 250 mg of TFs in a volume of 20 ml was injected and eluted with the aqueous phase at a flow-rate of 2.0 ml/min. The effluent was monitored with a UV-Vis detector at 280 nm and 380 nm and collected with a fraction collector at 5-min intervals (5 ml per tube). The collections were separated into six parts according to the absorbance values, then, concentrated and dried separately.

High-pressure liquid chromatograph analysis of theaflavins

The concentrations of TFs were detected using an LC-2010 high-pressure liquid chromatograph (HPLC) (Shimadzu Limited, Japan) with a 4.6 mm×250 mm C18 column maintained at 40 °C. The mobile phase consisting of acetic acid/acetonitrile/water (A, 0.5:3:96.5, *V/V*) and mobile phase consisting of acetic acid/acetonitrile/water (B, 0.5:30:69.5, *V/V*) were used with a flow rate of

1 ml/min. 10 µl sample solution was injected and monitored at 280 nm. Authentic standard of TFs was used to identify the peaks and calculate the concentration of TFs in the sample [14].

Cell culture and proliferation

BEL-7402, MKN-28, and LH-60 were resuspended in RPMI 1640 containing 20% heat-inactivated FBS, 100 U/ml of penicillin and 100 mg/ml of streptomycin. After incubation for 3 h at 37 °C in an incubator that was equilibrated with 95% air plus 5% CO₂, the cell solution was harvested in a sterile tube for use.

Inhibition of theaflavins against cancer cells

The cell population was determined using MTT assay according to the method described by Carmichael *et al.* [15]. The LH-60 as described above at a density of 5 million cells per ml was respectively seeded into 96-well plates in 0.1 ml per well, TFs, TFDG, TF2B and an unidentified compound (UC) were added into the 96-well plates with 20 µl MTT separately per well. The final concentrations of TFs, TFDG, TF2B and UC were the same, each with five concentrations: 3.9, 16, 63, 250, and 1000 µg/ml. After incubation for 72 h at 37 °C in an incubator, the incubator was equilibrated with 95% air plus 5% CO₂. Five replicates were set for each treatment. Effects on BEL-7402 and MKN-28 were measured with Sulfur Rodamine B method.

The inhibiting activities of theaflavins were expressed as the mean effective concentrations in mg/ml that inhibited 50% of cell growth amounts (IC₅₀). IC₅₀ was calculated by applying a linear equation using power of regression. The significance of the relationship coefficients between concentration and inhibition rate against MKN-28 and BEL-7402 was tested using *t*-test under the SAS system for windows developed by SAS Institute, Cary, NC, USA.

Results and Discussion

It is hard work to get enough theaflavins monomers because it requires lots of work to separate the monomers of theaflavins using Sephadex LH-20 chromatography or the reversed-phase high-performance liquid chromatography [16]. In order to tackle this problem, in this study, high-speed countercurrent chromatography is used to separate the monomers of theaflavins [13]. It proves to be a better way to get enough theaflavins monomers for the research work.

The inhibiting activities of theaflavins against cancer cells

The results (Fig. 1,2) showed that the inhibiting activities of TFs and individual monomers against BEL-7402 and MKN-28 cancer cells were complied with the first order dynamics equation. The relationship coefficients were greater than 0.86 and were significant for all treatments (Table 1). Particularly, the inhibition effects of UC and TF2B against BEL-7402 were the most significant with relationship coefficients 0.98.

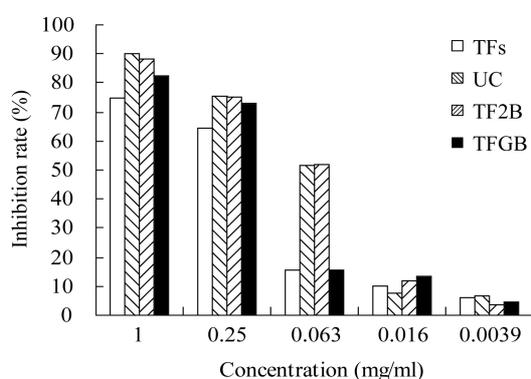


Fig. 1 Inhibition rates against human liver cancer cells by TFs, UC, TF2B, and TFDG

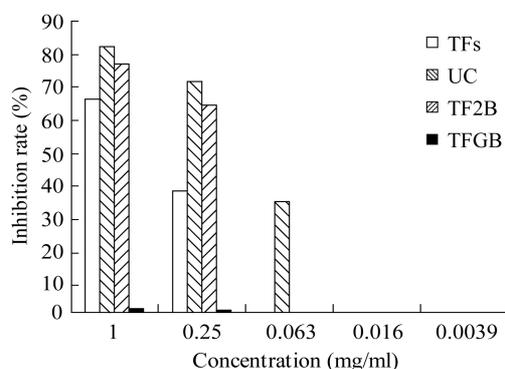


Fig. 2 Inhibition rates of gastric cells by TFs, UC, TF2B, and TFDG

The highest inhibition rates for TFDG, UC, TF2B, and TFs were 82.4%, 90.0%, 88.2%, and 74.7% against BEL-7402, and 76.5%, 80.2%, 75.0%, 64.5% against MKN-28, respectively. This indicated that both TFs and theaflavin monomers were more effective in inhibiting BEL-7402 among the tested cancer cells. UC exhibited the strongest inhibitory effect among the tested compounds against BEL-7402 and MKN-28, whereas TFs had the lowest. It suggests that there are no synergic activities on the growth of those cancer cells among the tested theaflavins when their concentrations are below 1 mg/ml.

The inhibition rate of TF2B against the growth of LH-60 was about 100% at the concentration 1.0 mg/ml, but only 26.5% at the concentration of 0.25 mg/ml. TFs, TFDG and UC had less than 10% inhibition rate on LH-60 even at the concentration 1.0 mg/ml. It implied that the inhibition of theaflavins against the growth of LH-60 had some selectivity on different cancer cells. On the other hand, the results demonstrated that TF2B had more multifaceted bioactivity functions whereas TFs, TFDG, and UC had less.

IC₅₀ of theaflavins against BEL-7402 and MKN-28

For the convenience of comparison with other literatures, the unit of IC₅₀ values of TFs, TF2B, and TFDG was converted into mM. The IC₅₀ values derived from the corresponding linear equations are listed in Table 1. The IC₅₀ values of TF2B, TFDG, UC, and TFs on BEL-7402 were lower than those on MKN-28, suggesting that these components had better inhibiting activities against BEL-7402 than against MKN-28. The IC₅₀ values (mg/ml) of TF2B and UC on BEL-7402 were the lowest among all the treatments, implying that TF2B and UC were the most effective components among the tested TFs against BEL-7402.

We conjecture that UC may be the isomer of theaflavin-3'-gallate according to the study reported by Lewis *et al.* [17] and the structures of some other tea polyphenol oxide compounds reported in recent years. UC will be purified further and its structure will be identified in our near future work.

In this study all of the theaflavins showed inhibition

Table 1 IC₅₀ of theaflavins on human liver cancer cell and gastric cancer cell

Theaflavins	TF _s (mM)	UC (mg/ml)	TF2B (mM)	TFDG (mM)
BEL-7402	0.18 ($r=0.93^{**}$)	0.08 ($r=0.98^{**}$)	0.11 ($r=0.98^{**}$)	0.16 ($r=0.93^{**}$)
MKN-28	1.11 ($r=0.89^{*}$)	0.14 ($r=0.96^{**}$)	0.22 ($r=0.87^{*}$)	0.25 ($r=0.90^{**}$)

*the relationship coefficients are significant at $P<0.05$ level; **the relationship coefficients are highly significant at $P<0.01$ level.

activity against BEL-7402 and MKN-28, and there was a good negative relationship between the concentration of tested compounds and growth of cancer cells. The tested concentration of theaflavins in this study is lower than the effective concentration of 1 mg/ml in inhibiting mice liver cancer cells of AH109A and anti-bacterium reported by Maeda-Yamamoto *et al.* [10]. Although the EC_{50} values of TF monomers are higher than 20 $\mu\text{g/ml}$, it is still worth exploring and designing natural new anticancer medicine based on TF structure.

The effects of purified TFs and its monomers on BEL-7402, MKN-28, and LH-60 were rarely reported. After comparing the inhibition concentration of TFs with catechins against the cancer cells, we find that TFs concentration is lower than that of catechins. The theaflavins showed a strong inhibitory effect on AP-1 activity with an estimated IC_{50} value of 5 $\mu\text{g/ml}$ for TF2A, TF2B, and TFDG. TF1 was less effective than its gallate derivatives at 20 $\mu\text{g/ml}$ [18]. However, the inhibition concentration of the three most potent green tea components EGCG, GC, and EGC against four different human cancer cell lines such as MCF-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma, and UACC-375 melanoma were 10, 100, and 1000 mM [19]. Lung *et al.* [20] reported that EGCG suppressed the proliferation of the EoL-1 cells in a dose-dependent manner, with an estimated IC_{50} value of 31.5 mM. Yang *et al.* [21] reported that green tea catechins (-)-epigallocatechin-3-gallate (EGCG) and (-)-epigallocatechin (EGC) displayed strong growth inhibitory effects against lung tumor cell lines H661 and H1299, with estimated IC_{50} values of 22 mM, which were much higher than the TFs concentration range mentioned above and the TFs concentration in this test.

TFs is a synthetic form of two B rings. Since there are three OH groups for this structure, it is likely that catechins have scavenging action for radicals and good interaction with ascorbic acid, proline and lysine. The gallic acid moiety is important for TFs to express antioxidative activity and antimutagenicity. Thus, it may be expected that TFs could play an important role in chemoprevention, for which lipid peroxides or active oxygen are important.

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