

Original Article

Experimental Study on Effect of Compounds in Inhibiting HCT-116 Human Colon Cancer Cells: The Preliminary Results

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Key Words

Proliferation;
Colon cancer;
MTT assay

Objective. Several compounds were studied for their growth inhibitory effects on cultured HCT-116 human colon cancer cells.

Materials and Methods. Compounds with anti-inflammation, anti-oxidation, or free-radical scavenging ability were used, including honokiol, emodin, lipoic acid, berberine, diosgenin, resveratrol, rottlerin, pinolo, curcumin, melatonin, and sodium butyrate. Cultured cells were incubated in a serum-free medium with various concentrations of different compounds for 24 and 48 hours in a 5% CO₂ incubator, after which the proliferation of HCT-116 cells was assessed by 3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and the significance of differences was analyzed by Student's t test.

Results. Honokiol, emodin, resveratrol, rottlerin, and curcumin used in this study were more effective than lipoic acid, berberine, diosgenin, pinolo, melatonin, and sodium butyrate. The 50% suppression doses after 48-hour exposure were 18.5 μM for honokiol, 17.3 μM for emodin, 25.3 μM for resveratrol, 6.9 μM for rottlerin, and 22.3 μM for curcumin respectively.

Conclusion. Further investigations should be conducted to elucidate the mechanisms modulating anti-tumor effects on HCT-116 cells for honokiol, emodin, resveratrol, rottlerin, and curcumin.

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Colon cancer is the second leading cause of death for cancer worldwide. In Taiwan, the Bureau of Health Promotion, Department of Health has proclaimed that about 19.5 people per 100 thousand die per year of colorectal cancer. Even though surgical resection is curative for early stage diseases, currently used chemotherapeutic agents for advanced stage colon cancer are palliative. Much research has been undertaken in the battle against colon cancer over the past few decades. However, limited advances have

been obtained in spite of a substantial body of new discoveries about the molecular biology of cancer cells.¹ In addition, side effects of drugs are also potential obstacles to successful chemotherapies. Compounds with anti-inflammation, anti-oxidation, or free-radical scavenging ability have also been demonstrated to possess varied degrees of anti-tumor activity in the literature. One promising approach involves the administration of dietary phytochemicals that possess cancer-preventative activity but with greater safety, better

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availability, and minimal toxicity. Here we selected several candidate compounds with one or more of the aforementioned properties (anti-inflammation, anti-oxidation, or free-radical scavenging ability) for investigating the *in-vitro* anti-proliferative activity against the HCT-116 cancer cell line in culture by 3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.

Berberine is found in plants such as *Berberis*, *Hydrastis canadensis*, and *Coptis chinensis*, usually in the roots, rhizomes, stems, and bark. The traditional clinical applications of berberine include anti-infection² and diabetic control.³ In addition, berberine has been shown to suppress the growth of a wide variety of tumor cells including prostate cancers⁴ and colon cancers.⁵

Diosgenin, a steroid sapogenin, is extracted from the tubers of *Dioscorea wild yam*. Diosgenin is a well-known precursor of various synthetic steroidal drugs.⁶ Over the past decade, much research has been conducted to understand the role of diosgenin on human cancers and diosgenin has been found to have a role in multi-target based chemopreventive or therapeutic properties.⁷

Lipoic acid is a naturally-occurring co-factor present in many enzyme complexes regulating human metabolism. Lipoic acid has been demonstrated to have properties of anti-oxidant activity⁸ and diabetic control.⁹ In addition, because of its free-radical scavenging ability, lipoic acid has the potential to interfere with processes within malignant cells.¹⁰

Pinolo is a nonselective beta-adrenergic blocker, possessing partial beta-adrenergic receptor agonist activity. It also has membrane-stabilizing effects. Clinically, pinolo has been used in angina pectoris, hypertension, arrhythmias, and prophylaxis of acute stress reactions. Several studies have also investigated its role on inhibition of tumor growth.¹¹

Melatonin is a natural human hormone, produced by the pineal gland. It is essential in the regulation of the circadian rhythms of several biological functions. Melatonin's biological effects are produced through activation of melatonin receptors or through its powerful antioxidant activities.¹² It has been demonstrated to have the properties of antioxidant activities¹³ and prevention of ischemia brain damage.¹⁴ A systematic

review, involving 643 cancer patients, using melatonin found a reduced incidence of death.¹⁵ Moreover, reduced melatonin level has been proposed as a likely carcinogenic factor in night workers.¹⁶

Sodium butyrate, a short-chain volatile fatty acid in a non-toxic short-chain fatty acid, is the product of large bowel microbial fermentation of dietary fiber in the colon. Numerous studies have demonstrated the anti-proliferative effect of sodium butyrate treatments in breast,¹⁷ prostate,¹⁸ and colon cancers.¹⁹

Curcumin is the chief ingredient in both traditional Chinese and Indian medicine and in Indian turmeric spice, which is a member of the ginger family (*Zingiberaceae*). Curcumin has been reported to have anti-inflammatory²⁰ and anti-oxidant²¹ activities. It has been used for thousands of years by Asians in various clinical applications including cancer treatment.²²

Resveratrol is a natural product highly enriched in grapes, red wine, and many other food sources. It has been demonstrated to have anti-inflammatory²³ activities and cancer chemopreventive properties.²⁴

Honokiol is a pure biphenolic compound, present in cones, barks, and leaves of *Magnolia officinalis* extracts, which is used in traditional Chinese medicine. Recent research demonstrated that honokiol has variable biological activities including anti-inflammatory²⁵ and anti-oxidant effects.²⁶

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is the main component in the rhizome of *Rheum palmatum* L. (*Polygonaceae*). Emodin has been demonstrated to have anti-bacterial²⁷ and anti-tumor activities.²⁸

Rottlerin, a compound from Indian tree, is a selective inhibitor of protein kinase C-delta (PKC-delta). The PKC family is a major group of intracellular phosphorylating enzymes which play a role in proliferation, differentiation, as well as tumor promotion and progression.

Materials and Methods

Materials

The compounds (purity > 99%) were obtained

from Alexis Biocompounds (San Diego, CA). Culture medium RPMI-1640, fetal bovine serum (FBS) and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, CA).

Cell culture and treatment

HCT-116 cells are derived from a colon carcinoma [American Type Culture Collection (ATCC), Rockville, MD; ATCC # CCL247] and serve as a useful model for study. The culture medium used was RPMI 1640, containing 10% fetal bovine serum, 20 mmol/L HEPES buffer, and 100 µg/mL gentamicin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The candidate compounds were dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 100 mmol/L and diluted with an FBS-free medium to achieve the designated concentrations. The same concentration of DMSO without any compounds added was used as a control.

MTT viability assay

Cell viability was assessed by MTT assay. HCT-116 cells cultured onto 24-well plates were treated with various compounds. After 24 hours or 48 hours incubation, the cells (10⁴/well) in 24-well plates were washed twice with phosphate-buffered saline (PBS), and MTT (100 µg/0.1 mL of PBS) was added to each well. The cells were incubated at 37 °C for 4 hours. Culture medium was then replaced with an equal volume of DMSO to dissolve formazan crystals. The absorbance was measured at 550 nm by microplate reader (Bio-Tek, Winooski, VT). The cell proliferation inhibition rate was calculated as 1 - (average OD value of wells with administered drug/average OD value of control wells) × 100. All experiments were performed a minimum of 3 times and data was presented as the average value ± the standard error of the mean (SEM).

Statistical analysis

The values given are means ± S.E.M. The significance of difference between the experimental group and control was assessed by Student's *t* test. The dif-

ference is considered significant if the *p* value is < 0.05.

Result

To determine the optimal conditions for cytotoxicity in cultures of HCT-116 colon cancer cells, a variety of compounds were used in different concentrations for the indicated time periods. These compounds shown in Fig. 1 include honokiol, emodin, lipoic acid, berberine, diosgenin, resveratrol, rottlerin, pinolo, curcumin, melatonin, and sodium butyrate. Cell viability at each time point was then assessed by MTT assay.

The HCT-116 human colon cancer cells were treated with various compounds at different concentrations in 10% FBS for 48 hours. Incubation with lipoic acid, berberine, diosgenin, pinolo, melatonin, and sodium butyrate had no significant anti-tumor effect on HCT-116 cells, while honokiol, emodin, resveratrol, rotterlin, and curcumin caused a significant (**p* < 0.05) reduction in total cell numbers (Fig. 1).

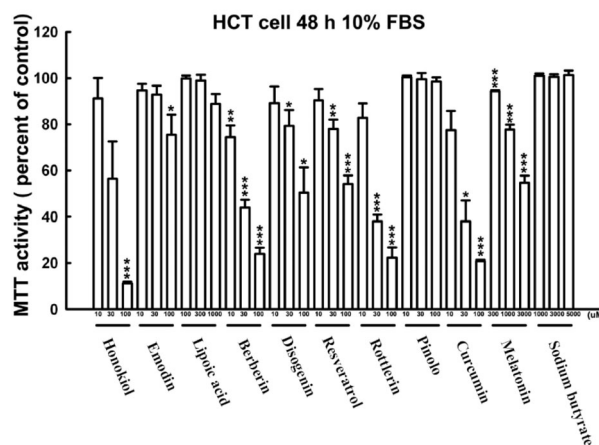


Fig. 1. HCT-116 cells were treated with various concentrations (3, 10, 30 µM) of different compounds, in complete medium with 10% fetal bovine serum (FBS), for 48 hours as described in the text. The cell viability of cells was counted by MTT assay. The results represented the mean ± S.D. of three independent experiments and the significant difference was established at **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with the control group. DMSO served as the solvent control. Each column showed growth inhibition after normalizing untreated cells to 100%.

Subsequently, HCT-116 cells were treated with 3, 10, and 30 μM of the five potentially cytotoxic compounds (honokiol, emodin, resveratrol, rottlerin, and curcumin) for 24 and 48 hours in serum-free medium (Figs. 2 and 3). The results showed that the inhibitory effect of honokiol, emodin, resveratrol, rottlerin, and curcumin on HCT-116 cell proliferation was both time-dependent and concentration-dependent (Fig. 3).

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. According to the criteria of the American National Cancer Institute, values of IC_{50} lower than 30 $\mu\text{g}/\text{mL}$ suggest a compound worth further purification.²⁹ After 24 hour treatment, the IC_{50} values were 18.0 μM for hono-

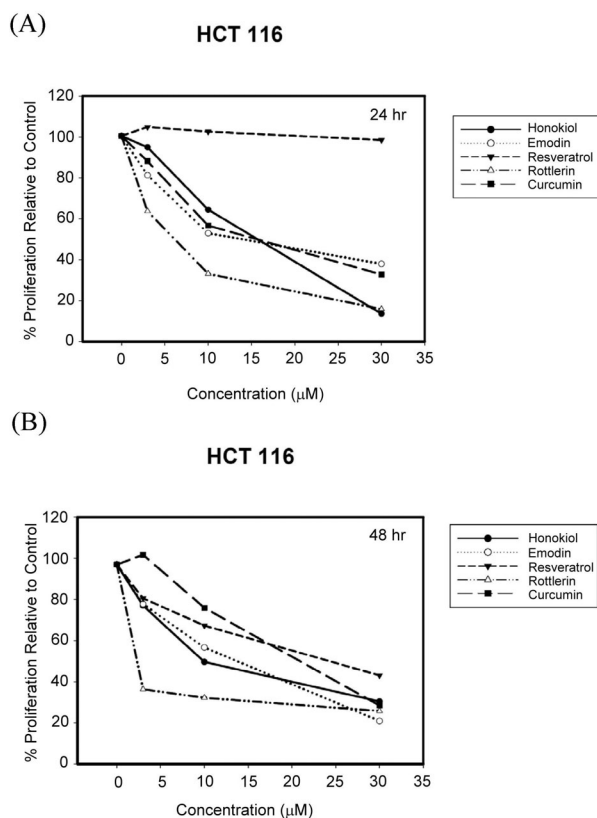


Fig. 2. HCT-116 cells were treated with various concentrations (3, 10, 30 μM) of different compounds (honokiol, emodin, resveratrol, rottlerin, and curcumin), in serum-free medium, for 24 and 48 hours respectively, depicted in the linear regression mode. (A) Viable cells were detected by proliferation assay using MTT assay after 24-hour exposure. (B) Viable cells were detected by proliferation assay using MTT assay after 48-hour exposure.

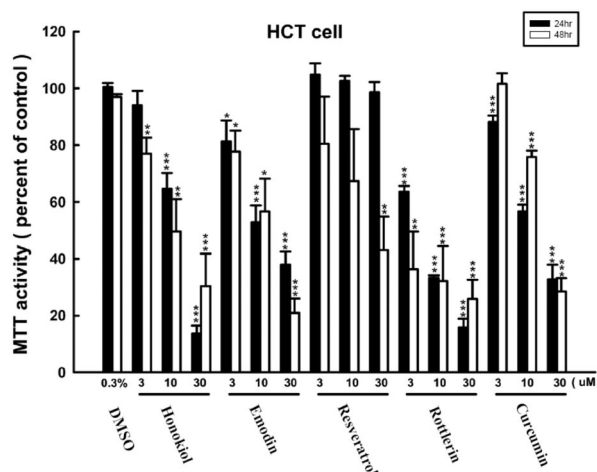


Fig. 3. Merged from Fig. 2A and 2B. HCT-116 cells were treated with various concentrations (3, 10, 30 μM) of different compounds (honokiol, emodin, resveratrol, rottlerin, and curcumin) in serum-free medium, for 24 (black columns) and 48 hours (white columns) respectively, measured by MTT viability assay. Each column showed growth inhibition after normalizing untreated cells to 100%. The results represented the mean \pm S.D. of three independent experiments and the significant difference was established at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the control group for the indicated time. DMSO served as the solvent control.

kiol, 21.5 μM for emodin, infinity for resveratrol, 13.1 μM for rottlerin, and 20.8 μM for curcumin (Table 1). However, only the IC_{50} of rottlerin significantly decreased after 48 hour treatment, while the IC_{50} values of honokiol, emodin, resveratrol, and curcumin had no significant changes (Table 1).

Discussion

In Cragg's study,³⁰ over 50% of the drugs in clinical trials for anticancer activity were isolated from natural sources or were related to them. Recently the search for natural compounds with active anti-tumor properties has been a popular research topic. In this study, the cytotoxicities of the ten candidate compounds were tested against the human colon carcinoma (HCT-116) cell line, using the thiazolyl blue test (MTT) assay. Honokiol, emodin, resveratrol, rottlerin, and curcumin significantly inhibited the growth of the

Table 1. Effective doses (μM) of various compounds (honokiol, emodin, resveratrol, rottlerin, and curcumin) that inhibited cell growth to 50% of control (IC₅₀) in different treatment time period (24 hours and 48 hours)

Compounds	Incubation time	
	24 hrs	48 hrs
Honokiol	18.0 \pm 5 μM	18.5 \pm 11 μM
Emodin	21.5 \pm 5 μM	17.3 \pm 11 μM
Resveratrol	inginity	25.3 \pm 11 μM
Rottlerin	13.1 \pm 1 μM	6.9 \pm 14 μM
Curcumin	20.8 \pm 2 μM	22.3 \pm 2 μM

2×10^4 /ml cells were incubated in the presence of various compounds at different concentrations (0, 3, 10, 30 μM) for 24 and 48 hours, respectively. Anti-tumor effect on the proliferation was measured with MTT test. IC₅₀ was calculated with the Sigmaplot software.

HCT-116 cell line in a concentration- and time-dependent manner. However, the anti-tumor activity of lipoic acid, berberine, diosgenin, pinolo, melatonin or sodium butyrate on HCT-116 cells was not significant in the present study.

Extensive research over previous decades demonstrated that curcumin has potent cancer-killing activity *in vitro* against various types of cancers including colon, prostate, and breast.³¹ Mahmoud et al proved that dietary curcumin could suppress intestinal carcinogenesis in a mouse model of familial adenomatous polyposis.³² Ushida et al confirmed that curcumin could prevent esophageal carcinogenesis in mice.³³ Huang et al demonstrated that curcumin could reduce the incidence lymphomas and leukemias in rats.³⁴ Our results demonstrated that proliferation of HCT-116 cells in the presence of curcumin was inhibited in a concentration- and time-dependent manner. On the other hand, we also demonstrated that 24 hour treatment was optimal for curcumin-induced cytotoxicity in cultures of HCT-116 colon cancer cells, because the IC₅₀ value had no significant change after 48 hour treatment. Our results are consistent with other research that curcumin is cytotoxic for HCT-116 human colon cancer cell line.^{35,36} Moreover, curcumin is also cytotoxic against many other types of cancer cells.³⁷⁻³⁹ Taken together, this suggests that curcumin may have broad applications in cancer chemoprevention, although we did not determine whether curcumin was

selectively cytotoxic for neoplastic cells, Watson et al demonstrated that the viability of normal human dermal fibroblasts was not altered following 72 hour exposure to lower concentrations (10 and 20 μM) of curcumin.⁴⁰ This result is also consistent with Chen's research.⁴¹

Resveratrol has been shown to have growth-inhibitory activity in several human cancer cell lines including glioma,⁴² colorectal cancer,⁴³ and epidermoid carcinoma.⁴⁴ In this study, we found that a 24 hour resveratrol exposure did not significantly alter the number of viable cells, suggesting that the anti-proliferative effect was not associated with obvious cell death at this exposure time. However, a 48 hour exposure to 30 μM resveratrol resulted in a significant (** $p < 0.01$) decrease in cell viability. This result might be indicative for delayed apoptotic death in HCT-116 cells exposure to resveratrol. No previous investigations reported any resveratrol-induced HCT-116 cancer cell death. However, in Wolter's study⁴⁵ about resveratrol-induced Caco-2 human colon cancer cell death, the growth inhibitory results were similar to ours.

Honokiol also exhibited apoptosis induction and growth inhibition in some studies including lung,⁴⁶ prostate,⁴⁷ and colon cancer cells.⁴⁸ In this study, we demonstrated that proliferation of HCT-116 cells in the presence of honokiol was inhibited in a concentration- and time-dependent manner with an IC₅₀ value of 18.5 μM (48-hour treatment) and 18.0 μM (24-hour treatment) in serum-free condition. The sole research investigating the anti-tumor effect of honokiol on human colon cancer cell (HCT-116) also demonstrated similar results with an IC₅₀ value of 23 μM in 10% FBS condition.⁴⁸ The different IC₅₀ values may be due to the presence or absence of serum in medium. Recent study⁴⁸ also elucidated that the underlying mechanism is through blocking of Nuclear Factor-kappa B.

A number of research has demonstrated the growth inhibitory effect of emodin on cancer cells such as ovarian cancers,⁴⁹ colon cancers,⁵⁰ and breast cancers.⁵¹ Here, proliferation of HCT-116 cells in the presence of emodin was inhibited in a concentration- and time-dependent manner with an IC₅₀ value of 17.3 μM (48 hour treatment) and 21.5 μM (24 hour

treatment) in serum-free condition. No previous investigations reported any emodin-induced HCT-116 cancer cell deaths. However, several articles also demonstrated similar anti-tumor effects on human colon cancer cells other than HCT-116.^{50,52}

Some research has already demonstrated the inhibitory effects of rottlerin on cancer cells.^{53,54} In the present study, we have shown that rottlerin inhibits HCT-116 cell proliferation in a concentration-dependent and time-dependent manner both in 24 hour and 48 hour exposures. Although there has been no research about the anti-tumor effects of rottlerin on the HCT-116 cell line, a few articles also demonstrated the significant growth inhibition of rottlerin on various human colon cancer cell lines.^{55,56}

Progression of colon cancer is associated with activation of multiple signaling pathways. Cell death is an essential event in normal life and in the pathophysiological processes which lead to diseases. A pattern of cell death has emerged where each of several distinct organelles (plasma membrane, mitochondrion, nucleus, endoplasmic reticulum, lysosome) give rise to signals which induce cell death. Endoplasmic reticulum (ER) is a central organelle engaged in protein production, folding and maturation. Various toxic insults can perturb ER function and result in ER stress.⁵⁷ There is increasing evidence that ER stress plays an important role in the regulation of cell death.⁵⁸ Until now, only one study reported about ER stress-mediated HCT-116 cell death in the literature.⁵⁹ In the future, treatment of HCT-116 human colon carcinoma cells with these five potential compounds (honokiol, emodin, resveratrol, rottlerin, and curcumin) will be conducted to elucidate the induction of signature ER stress markers: phosphorylation of eukaryotic initiation factor-2 α (eIF-2 α), up-regulation of glucose-regulated protein (GRP)-78, and up-regulation of CCAAT/enhancer-binding protein homologous protein (CHOP).

Conclusion

Here, we only presented the preliminary anti-tumor results of selected candidate compounds on HCT-116 human colon cancer cells. Further investiga-

tions should be conducted to elucidate the underlying mechanisms modulating anti-tumor effects on HCT-116 cells for honokiol, emodin, resveratrol, rottlerin, and curcumin.

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原 著

藥物對 HCT-116 人類大腸癌細胞株 生長抑制的實驗：初步結果報告

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目的 分析數種藥物對於 HCT-116 人類大腸癌細胞株生長抑制的影響。

材料及方法 一些具有抗發炎或抗氧化或具有自由基清除作用之藥物被選用，包括厚朴酚 (honokiol)，大黃素 (emodin)，硫辛酸 (lipoic acid)，黃連素 (berberine)，皂甘 (diosgenin)，白藜蘆醇 (resveratrol)，呂宋揪莢粉素 (rottlerin)，pinolo，薑黃素 (curcumin)，褪黑激素 (melatonin)，以及丁酸鈉 (sodium butyrate)。把 HCT-116 細胞培養在無血清環境之培養盤，分別加入上述藥物，分別以不同藥物濃度之條件下，於百分之五的二氧化碳濃度處理二十四或四十八小時。接著，再以 3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay 分析 HCT-116 細胞生長受影響的情形。

結果 厚朴酚、大黃素、白藜蘆醇、呂宋揪莢粉素、薑黃素，這些藥物對於 HCT-116 細胞生長有明顯的抑制效果。經藥物處理四十八小時後，百分之五十的生長壓制劑量分別是厚朴酚為 18.5 μM ，大黃素為 17.3 μM ，白藜蘆醇為 25.3 μM ，呂宋揪莢粉素為 6.9 μM ，薑黃素為 22.3 μM 。

結論 厚朴酚、大黃素、白藜蘆醇、呂宋揪莢粉素、薑黃素，這些藥物對於 HCT-116 細胞生長有明顯的抑制效果。對於已篩選出的這些藥物，應當設計進一步的實驗探討牽涉在其中的機轉。

關鍵詞 增生、大腸癌、MTT 分析。