Original Article

The Anti-cancer Effects of Resveratrol Combined with 5-fluorouracil Treatment in BALB/c Mice Bearing CT-26 Cells

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Key Words
Resveratrol;
5-fluorouracil;
Cyclooxygenase-2;
Cancer therapy

Abbreviations
ATCC: American Type Culture Collection;
COX-2: Cyclooxygenase-2;
5-FU: 5-fluorouracil;
GSK-3β: Glycogen synthase kinase 3 beta;
PCNA: Proliferating cell nuclear antigen

Purpose. Resveratrol, a naturally phytoalexin, has antioxidant and anti-inflammatory properties and possesses chemopreventive and chemotherapeutic effects. The aim of this study was to undertake an evaluation of the therapeutic effect of resveratrol combined with 5-fluorouracil treatment on BALB/c mice (CT-26).
Methods. Two experiments were used: treatment from day 1 after CT-26 cell implantation; and treatment from day 7 after CT-26 cell implantation with detection of tumor mass. Mice were randomly divided into four groups: feeding with saline; feeding with resveratrol (12.5 mg/kg/day); 5-fluorouracil injection (5-FU 100 mg/kg/week); and resveratrol feeding (12.5 mg/kg/day) combined with 5-FU injection (100 mg/kg/week).
Results. The data showed resveratrol combined with 5-FU inhibited Cyclooxygenase-2 (COX-2) and β-catenin expression, increased GSK-3β expression, suppressed Bcl-2 expression, and suppressed both tumor proliferation and mitosis. There were two major findings in this study. First, resveratrol inhibited COX-2 expression and down-regulated the WNT/β-catenin signaling pathway, which may be an apoptosis pathway. Second, resveratrol combined with 5-FU significantly decreased Bcl-2 expression and inhibited both PCNA expression and tumor proliferation.
Conclusion. Resveratrol might elevate the chemosensitization of tumor cells. Resveratrol combined with 5-fluorouracil demonstrated a synergistic effect for cancer therapy. However, further research on resveratrol is required in order to confirm these findings and to develop new treatment strategies.
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Colorectal cancer is the third most common cancer and is a frequent cause of cancer deaths worldwide.[1,2] The incidence of colon cancer markedly increased from 34.0/10^5 in 2002 to 41.4/10^5 in 2009 in Taiwan (Taiwan Cancer Registry). Surgical resection is the mainstream treatment for localized colon cancer,

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and micrometastasis is treated with chemotherapy. In advanced cancer, chemotherapy is palliative treatment rather than curative.[3] In the search for potential new treatments, clinicians have investigated a number of agents derived from plants to evaluate their ability to both treat and prevent cancer.

A recent study showed that inflammation is a critical component of tumor progression.[4] Therefore, anti-inflammatory treatment has been applied as a novel therapeutic approach in cancer research.[4,5]

The majority of colorectal cancers overexpress cyclooxygenase-2 (COX-2), and this phenomenon is thought to inhibit apoptosis, induce angiogenesis, destroy the immune system, and promote tumor invasion.[6-9] Resveratrol, a naturally occurring phytoalexin, has anti-inflammatory properties that may have the potential for cancer chemoprevention and anticancer therapy.[7,10,11] Kunu et al. reported that resveratrol inhibited TPA-induced COX-2 expression via modulation of the IKK-NF-κB signaling cascade in mouse skin in vivo.[9] Because resveratrol has anti-inflammatory properties that may augment the efficacy of chemotherapy in chemoresistance colon cancer,[12] it may lead to the development of new therapeutic strategies. Therefore, we designed this study to investigate the therapeutic effect of resveratrol combined with 5-fluorouracil treatment on CT-26 colorectal adenocarcinoma cells implanted into BALB/c mice.

Materials and Methods

Animals and tumor cell line

Six-week-old male BALB/cByJNarL mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. Two to three mice were housed in a cage and provided with sterilized food and water. The mice were maintained at a constant temperature (22 ± 2 °C) and relative humidity (55 ± 5%) with a 12-h/12-h light/dark cycle. The animal use protocol for the experimental mice was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC Approval No. 98-86). In addition, all procedures adhered to the Guidelines for the Care and Use of Laboratory Animals recommended by Taiwan's Ministry of Health and Welfare.

A CT-26 murine colon carcinoma cell line was purchased from the American Type Culture Collection (ATCC) (Number: CRL-263). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 50 IU/ml of penicillin, 50 mg/ml of streptomycin (Gibco Laboratories, Grand Island, NY) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were routinely passaged by removing the medium and overlaying the cell monolayer with 0.25% trypsin and 0.1% EDTA.

Tumor inoculation and treatment

Two different experiments were designed: treatment on the day 1 after CT-26 cell implantation; treatment from day 7 after CT-26 cell implantation with tumor mass detected. After mice were anesthetized by intraperitoneal injection of ketamine, they were injected subcutaneously in the posterior leg with a 100 μl cell suspension containing 1×10^6 viable CT-26 cells. On day 1, the mice did not have palpable mass and they were randomly divided into 4 groups (10 mice in each group). In the other experiment which started on day 7, all mice which had developed a palpable tumor were further randomly divided into 4 groups (8 in each group). Group I was given saline daily via gastric lavage as the control group; Group II was given resveratrol (12.5 mg/kg/day) via gastric lavage; Group III was injected with 5-FU intraperitoneally (100 mg/kg/ week) (Fluoro-uracil Valeant, VALEANT); and Group IV was given resveratrol (12.5 mg/kg/day) via gastric lavage and injected with 5-FU intraperitoneally (100 mg/kg/week). Tumor growth was monitored every 3 days by measuring the greatest and the least diameters. Tumor volume was calculated using the formula: $V = 0.5 \times a \times b^2$, where a is the greatest diameter, and b is the smallest diameter.

Clinical observations and analysis

The mice were observed daily for clinical signs

and sacrificed after 2 weeks. Body weight was measured every 3 days. Blood was collected to evaluate hematological, hepatic, and renal function parameters under anesthesia at the end of the treatment period. Hematological parameters were measured with an automatic blood cell analyzer (Coulter LH750, Beckman, USA) and hepatic and renal function parameters were measured with an automatic analyzer (Hitachi 7070, Ibaraki, Japan). The tumor specimens were divided into two groups. One group was fixed with 10% formalin and embedded with paraffin. Then the specimens were assessed by hematoxylin and eosin staining. The other group was preserved in a freezer at -80 °C and then PCNA (Proliferating cell nuclear antigen), GSK-3β (Glycogen synthase kinase 3 beta), β-catenin, Bcl-2, and COX-2 (cyclooxygenase-2) were examined by Western blotting.

Statistical analyses

Student's t-test was performed for all analyses using the SAS software, version? (SAS Institute Inc., Cary, NC, USA). All data are expressed as mean ±SE. Values were considered to be significant at $p < 0.05$.

Results

Resveratrol slowed tumor growth

In the early treatment experiment, resveratrol and 5-FU group alone could reduce tumor growth; however after 17 days, the tumor volume increased in the resveratrol and 5-FU alone group, especially in the resveratrol group. But the resveratrol + 5-FU group still significantly inhibited tumor growth (Fig. 1A and a). In the late treatment subgroup with detectable tumor growth, tumor volume was reduced in the resveratrol, 5-FU, and resveratrol combined with 5-FU groups compared with that of the control group (Fig. 1B & b). The time course of responses to treatment revealed significant decreases in tumor size in the 5-FU

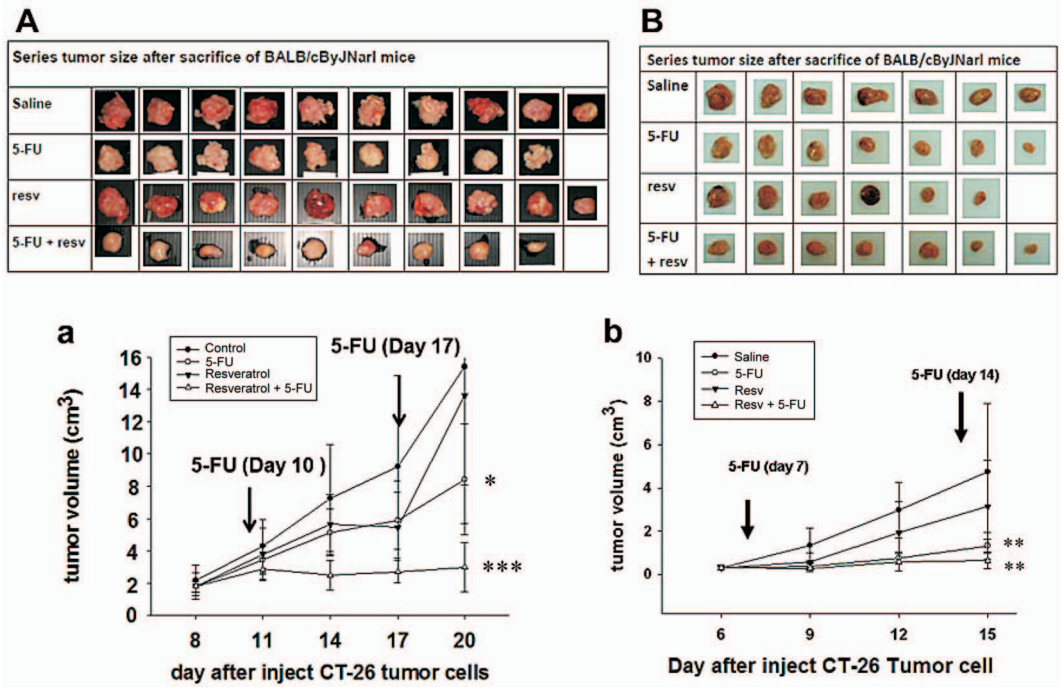

Fig. 1. A. Tumor sizes after sacrifice in the early treatment experiment; B. Tumor sizes after sacrifice in the late treatment experiment; a. Early treatment: Changes in tumor size of BALB/cByJNarl mice; b. Late treatment: Changes in tumor size of BALB/cByJNarl mice. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$, compared to control group.
1. Saline; 2. 5-FU; 3. Resv; 4. Resv + 5-FU. Resv: Resveratrol; 5-FU: 5-fluorouracil; Early treatment: Resv treatment started immediately after injection of CT-26 tumor cells on day 1; Late treatment: Resv treatment started when tumors were detected on day 7.

and resveratrol combined with 5-FU groups at 6, 9, 12, and 15 days as compared to the tumor sizes in the control mice (Fig. 1b).

In the early treatment experiment, measurements of the tumor weights revealed that the tumor inhibition rate increased to 38% in the 5-fluorouracil group alone, and to 75% in the resveratrol + 5-flurorouracil group compared to the rate of the control group (Table 1A). In the late treatment experiment, the assessment of tumor weight revealed that the resveratrol group alone inhibited tumor growth rate to about 36%, and 5-FU group inhibited tumor growth rate to about 57% as compared with the rate of the control group. In the resveratrol combined with 5-FU group, the inhibition rate increased to 69% (Table 1B).

In this study, lab data showed that resveratrol did not affect body weight and did not impair hepatic and renal functions.

Resveratrol inhibited tumor cell proliferation

In the early treatment experiment, H&E staining showed neovascularization, tumor cell proliferation, and mitosis in the saline and resveratrol groups (Fig. 2A). In the late treatment experiment, H&E staining showed tumor cell proliferation and mitosis in the control group, but tumor cell shrinkage and death in the other treatment groups, especially the 5-FU and resveratrol combined with 5-FU groups (Fig. 2B).

Resveratrol inhibited of COX-2 and β-catenin expression

Western blot study revealed that resveratrol alone

Table 1. A. Tumor weight in BALB/cByJNarl mice after sacrifice was comparable with the tumor inhibition rate in the early treatment experiment; B. Tumor weight in BALB/cByJNarl mice after sacrifice was comparable with the tumor inhibition rate in the late treatment experiment

A

	Tumor weight (g)	Tumor inhibition rate (%)
Saline	4.68 ± 0.33	-
5-FU	2.92 ± 0.21***	38
Resv	4.24 ± 0.44	9.4
Resv + 5-FU	1.16 ± 0.13***	75

B

	Tumor weight (g)	Tumor inhibition rate (%)
Saline	2.53 ± 0.39	-
5-FU	1.07 ± 0.18**	57
Resv	1.60 ± 0.34	36
Resv + 5-FU	0.78 ± 0.15***	69

** $p < 0.01$; *** $p < 0.001$, compared with saline.
Tumor inhibition rate (%) = (S − T)/S × 100%.
S: Saline; T: other tumor group.

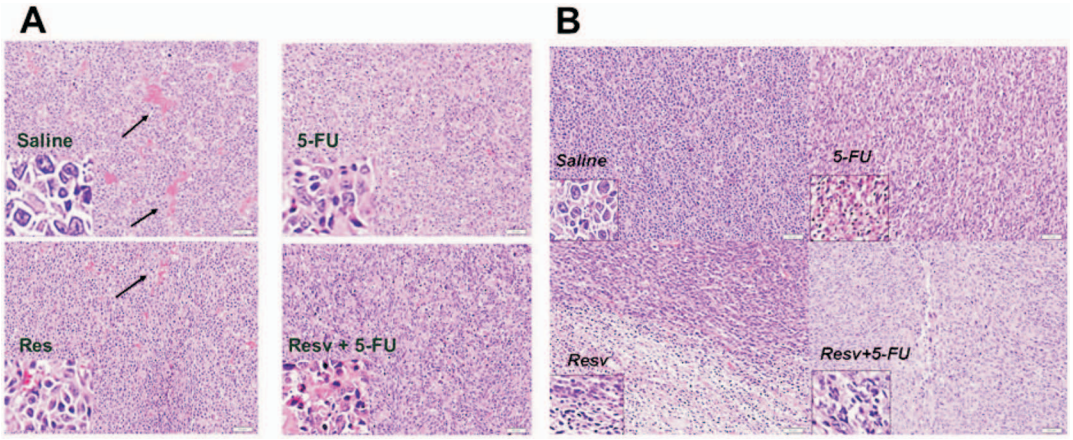

Fig. 2. A. Histopathology of tumor mass cells in BALB/cByJNarl micein the early treatment experiment; B. Histopathology of tumor mass cells in BALB/cByJNarl micein the late treatment experiment.
1. Control (Saline) group; 2. 5-FU group; 3. Resv group; 4. 5-FU + Resv group; hematoxylin and eosin stain (200X); Resv: Resveratrol; 5-FU: 5-fluorouracil; Early treatment: Resv treatment started immediately after injection of CT-26 tumor cells on day 1; Late treatment: Resv treatment started when tumors were detected on day 7.

and 5-FU alone inhibited COX-2 expression when compared with that of the control group. COX-2 expression was also significantly inhibited in the resveratrol combined with 5-FU group (Fig. 4A).

In the Western blot analysis, the data showed β-catenin expression was similar to COX-2 expression, which was significantly inhibited in the resveratrol combined with 5-FU group (Fig. 4A).

Resveratrol inhibited PCNA expression

Western blot study showed tumor proliferation and mitosis were suppressed in the 5-FU group and the resveratrol combined with 5-FU group (Fig. 3A).

Although resveratrol alone showed no significant inhibition of PCNA expression, compared with the control group, but the resveratrol group did show a trend toward decreased PCNA expression (Fig. 3A).

Resveratrol increased GSK-3β and decreased P-GSK-3β expression

Resveratrol alone showed increased GSK-3β and decreased P-GSK-3β expression. The results showed significant differences in the 5-FU and resveratrol combined with 5-FU group as compared with that of the control group (Fig. 4B).

Resveratrol suppressed Bcl-2 expression

Resveratrol and resveratrol combined with 5-FU groups showed significantly suppressed Bcl-2 expression as compared with that of the control group (Fig. 3B).

Our results showed that resveratrol inhibited CT-26 tumor cell growth and COX-2 expression, and increased tumor cell apoptosis.

Discussion

In cancer treatment, tumors might develop resistance to chemotherapeutic agents.[12] Therefore, clinicians have turned to nutraceutical compounds derived from plants with a view to improving the sensitivity of tumor cells to chemotherapy.[13] Resveratrol, a naturally occurring compound present in grapes, inhibited tumor cell activities associated with tumor initiation, promotion, and progression.[14] It has been used as a chemopreventive agent and in chemotherapeutic treatment. When combined with 5-fluorouracil, a widely used chemotherapeutic drug, it could help to chemosensitize previously chemoresistant tumor cells.[12,15,16] Our result shows that the resveratrol alone group failed to control tumor growth in the early experi-

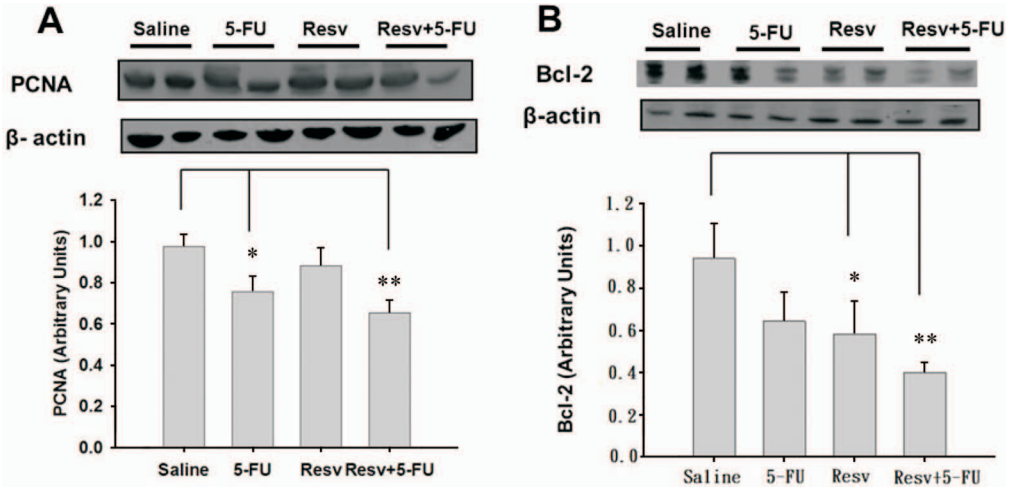

Fig. 3. A. Western blot analysis of PCNA expression in tumor mass cells of BALB/cByJNarl mice; B. Western blot analysis of Bcl-2 expression in tumor mass cells of BALB/cByJNarl mice. * $p < 0.05$, or ** $p < 0.01$, compared to control group.
1. Control (saline) group; 2. 5-FU group; 3. Resv group; 4. Resv group + 5-FU.

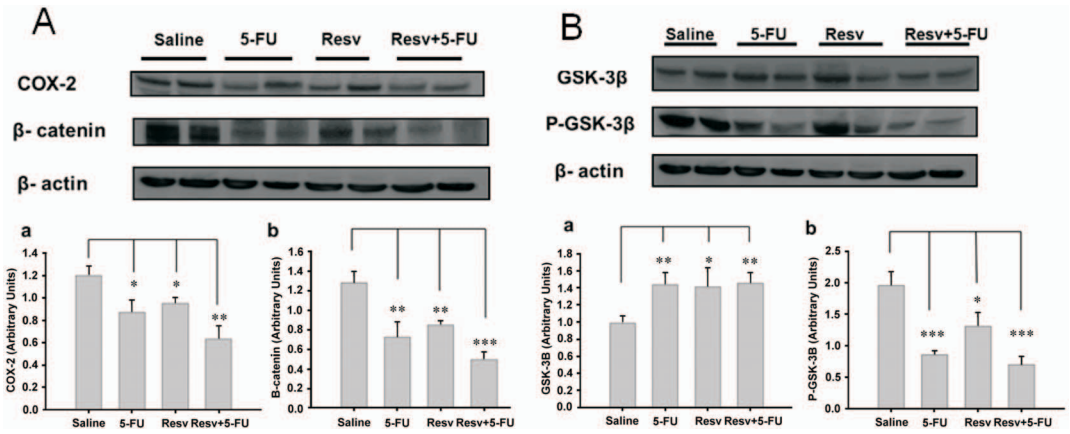

Fig. 4. A. Western blot analysis of COX-2 expression and β-catenin expression in tumor mass cells of BALB/cByJNarl mice; a. COX-2 expression; b. β-catenin expression. B. Western blot analysis of GSK-3β expression and P-GSK-3β expression in tumor mass cells of BALB/cByJNarl mice. a. GSK-3β expression; b. P-GSK-3β expression. * $p < 0.05$,** $p < 0.01$, or *** $p < 0.001$, compared to control group.
1. Control (Saline) group; 2. 5-FU group; 3.Resv group; 4. Resv group + 5-FU.

ment, whereas the 5-fluorouracil could slow tumor growth by about 38% compared with the control group. When resveratrol was combined with 5-fluorouracil, the inhibition rate increased to approximately 75% (Table 1A, Fig. 1A & a). In the late treatment experiment, the resveratrol alone group inhibited tumor growth rate to about 36% as compared with that of the control group. When resveratrol was combined with 5-fluorouracil, the inhibition rate increased to 69% (Table 1B, Fig. 1B & b). Thus, the resveratrol group demonstrated elevated chemosensitization and an improved chemotherapeutic effect.

Recently, other related reports in the literature have highlighted the potential of resveratrol to exert anti-cancer effects through a variety of different pathways, including the induction of apoptosis,[17,18] promotion of antioxidant activity,[19] and inhibition of cyclooxygenase.[20]

In this study, we focused on two major issues: 1) activation of a possible apoptosis pathway by resveratrol treatment, and 2) inhibition of Bcl-2-induced apoptosis by resveratrol combined with 5-FU treatment.

Some studies showed COX-2 inhibitors and nonsteroidal anti-inflammatory drugs could reduce the development and growth of adenoma and adenocarcinoma.[21,22] Resveratrol inhibited tumor growth directly by targeting COX-2 expression [23] and inhibiting the WNT signaling pathway.[9,24] Therefore, our first hypothesis was that resveratrol could inhibit COX-2 expression and downregulate the WNT/β-catenin signaling pathway.[24-26]

In the absence of WNT signaling, β-catenin is phosphorylated by a cytoplasmic protein complex containing GSK-3β. Phosphorylation of β-catenin is degraded in the proteasome. In the presence of WNT signaling, the Axin complex is disrupted, causing the phosphorylation and inactivation of GSK-3β and preventing phosphorylation of β-catenin. Then β-catenin accumulates in the cytosol and translocates to the nucleus, increasing transactivation of TCF/LEF expression and promoting tumor cell proliferation and survival.[24]

In order to test this hypothesis, we analyzed β-catenin, GSK-3β, and P-GSK-3β via Western blot. The results showed resveratrol significantly inhibited β-catenin expression, increased GSK-3β expression, and decreased P-GSK-3β expression (Fig. 4B, a & b).

Inflammation increases COX-2 expression and leads to high levels of β-catenin in the cytoplasm, promoting tumor cell proliferation.[9] Resveratrol can inhibit COX-2 and downregulate β-catenin activity; therefore, β-catenin and COX-2 should have similar levels of protein expression. In this study we showed that resveratrol inhibited COX-2 and β-catenin ex-

pression. Moreover, resveratrol combined with 5-FU significantly inhibited β-catenin expression (Fig. 4A, a & b).

The Bcl family proteins are key regulators of apoptosis.[27] Several studies have shown that resveratrol induces apoptosis, but it has also been found that the overexpression of Bcl-2 attenuates resveratrol-induced apoptosis. Therefore, overexpression of Bcl-2 might attenuate the efficacy of resveratrol.[28-30] Resveratrol combined with 5-FU treatment significantly decreased Bcl-2 expression, inhibited PCNA expression, and suppressed tumor proliferation (Fig. 3A & 3B).

In summary, there were two major findings in this study. First, resveratrol inhibited COX-2 expression and downregulated the WNT/β-catenin signaling pathway, which may be an apoptosis pathway. Second, resveratrol combined with 5-FU significantly decreased Bcl-2 expression, and inhibited PCNA expression and tumor proliferation. Resveratrol might elevate the chemosensitization of tumor cells. Resveratrol combined with 5-fluorouracil demonstrated a synergistic effect in cancer therapy. However, further research on resveratrol is required in order to confirm these findings and to develop new treatment strategies.

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原　著

5-氟尿嘧啶合併白藜蘆醇治療 BALB/cByJnarl 荷瘤小鼠之療效加成效應

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目的　白藜蘆醇是一種植物防禦素 (phytoalexins) 具有抗氧化及抗發炎之作用，及化學藥物預防 (chemoprevention) 和抗腫瘤之效果。因此我們設計白藜蘆醇合併 5-氟尿嘧啶治療 BALB/cByJnarl 荷瘤小鼠，來研究是否白藜蘆醇經由增加化學抗藥性腫瘤細胞之化學治療效果而加強 5-氟尿嘧啶之治療。

方法　本研究進行以下兩項實驗大腸直腸癌之早期治療於 ① 實驗第 1 天時皮下接種 CT-26 腫瘤細胞後開始。② 實驗第 7 天腫瘤形成後開始給藥。
實驗分成 4 組：A. 每日餵食逆滲透水 0.2 ml，B. 5-FU 腹腔注射 5-FU (100 mg/kg/week)，C. 每日餵食以逆滲透水稀釋之白藜蘆醇 (12.5 mg/kg/day)，D. 每日餵食以逆滲透水稀釋之白藜蘆醇 (12.5 mg/kg/day)，並給與 5-FU 腹腔注射 (100 mg/kg/week)。

結果　實驗結果顯示單獨使用白藜蘆醇可抑制腫瘤增生，抑制 COX-2 及 β-catenin 表現，增加 GSK-3β 蛋白表現，抑制 Bcl-2 表現。而白藜蘆醇合併 5-FU 治療，更明顯抑制 COX-2 及 β-catenin 表現，增加 GSK-3β 蛋白表現，抑制 Bcl-2 表現。

結論　本研究有兩個主要發現，第一點白藜蘆醇抑制 COX-2 表現並調節 WNT/β-catenin 傳導路徑，誘使細胞凋亡。第二點白藜蘆醇合併 5-FU 治療，抑制 Bcl-2 表現，抑制腫瘤增生。5-FU 合併白藜蘆醇治療可藉由抑制 Bcl-2 之表現，提高腫瘤細胞化學治療之敏感性，而產生加成作用。

關鍵詞　白藜蘆醇、5-氟尿嘧啶、COX-2、癌症治療。