·Basic Research·

Periplocin extracted from cortex periplocae induces apoptosis of SW480 cells through inhibiting the Wnt/β-catenin signaling pathway

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Grants: National Natural Science Foundation of China (No. 30772752); Natural Science Foundation of Hebei Province (No. C2008000952)

This paper was translated into English from its original publication in Chinese. Translated by:Beijing Xinglin Meditrans Center (http://www.51medtrans.com) and Xiao-Feng Zhu

The original Chinese version of this paper is published in:*Ai Zheng* (*Chinese Journal of Cancer*) 27(5);http://www.cjcsysu.cn/cn/article.asp?id=15501)

Submitted: 2008-10-09
Revised: 2009-01-19

[Abstract] **Background and Objective**: The Wnt/β-catenin signaling pathway plays an important role in the development and progression of human cancers, especially in colorectal carcinomas. This study was to analyze the inhibition effect of periplocin extracted from cortex periplocae (CPP) on proliferation of human colon carcinoma cell line SW480 and the underlying mechanism. **Methods**: Cell proliferation of SW480 cells was measured by MTT assay. Cell apoptosis and cell cycle were analyzed by flow cytometry. Protein expression of β-catenin in total cell lysates, cytosolic extracts, and nuclear extracts were detected by Western blot. Binding activity of the T cell factor (TCF) complex in nucleus to its specific DNA binding site was measured by electrophoretic mobility shift assay (EMSA). Expressions of β-catenin, survivin, c-myc and cyclin D1 mRNA in cells after the treatment with CPP were detected by semi-quantitative RT-PCR. **Results**: CPP significantly inhibited the proliferation of SW480 cells in a time- and dose-dependent manner ($P<0.01$). CPP (0.5 μg/mL) also caused G_0/G_1 cell cycle arrest of SW480 cells and induced cell apoptosis ($P<0.05$). Compared to untreated control cells, after the treatment with CPP, the protein levels of β-catenin in total cell lysates, cytosolic extracts, and nuclear extracts were reduced ($P<0.01$); the binding activity of the TCF complex in nucleus to its specific DNA binding site was suppressed; mRNAs of the downstream target genes survivin, c-myc and cyclin D1 were decreased ($P<0.01$) while β-catenin mRNA remained unchanged. **Conclusion**: CPP could significantly inhibit the proliferation of SW480 cells, which may be through down-regulating the Wnt/β-catenin signaling pathway.

Key words: periplocin extracted from cortex periplocae, colorectal neoplasm, SW480 cells, Wnt/β-catenin signaling pathway, cell cycle, cell apoptosis

The Wnt/catenin signaling pathway is a key pathway regulating cell proliferation and differentiation, which plays important roles in embryonic development, as well as pathogenesis, development and metastasis of tumors. The molecules of this signaling pathway are often up-regulated in many kinds of human tumor tissues, such as colon cancer, melanoma, breast cancer, hepatocellular carcinoma, cervical cancer, medulloblastoma[1] and prostate cancer, and so on. Therefore, developing effective drugs targeting this signal pathway has become a hot topic for the development of anti-tumor drugs.[2]

The main functions of traditional Chinese medicine cortex periplocae (CPP) are anti-arthritis and reinforcement of bones and tendons. It is commonly used in the treatment of rheumatoid arthritis, for weakening ache of lumbus and knee, palpitations or shortness of breath and lower extremity edema and so on.[3] Our previous study showed that CPP had relatively strong anti-tumor activity. Periplocin is a single compound extracted and partially purified from CPP n-butanol[4] that has strong inhibitory effects on the proliferation of different tumor cell lines.[4,5] The purpose of this study was to explore the effect of periplocin on the Wnt/catenin signaling pathway, in order to clarify the mechanism for its anti-tumor effect and provide evidences for the development of new anti-tumor drugs.

Material and Methods

Material and reagents. CPP was extracted and purified in our laboratory, purity >96%. Human colon cancer cell line SW480 was from frozen stock in our lab. RPMI-1640 was purchased from Gibco company. Fetal bovine serum was purchased from Hangzhou Sijiqing company. MTT and PI were purchased from Sigma company. Protein molecular weight marker was bought from Solarbio Company. Nuclear-Cytosol Extraction Kit was purchased from key-GEN Biotechnology Company. Mouse anti-human catenin monoclonal antibody and rabbit anti-human GAPDH polyclonal antibody were from Santa Cruz Company. IRDye ^{800CW} conjugated goat (polyclonal) anti-mouse IgG and IRDye ^{700CW} conjugated goat (polyclonal) anti-rabbit IgG were from LI-COR Company, USA. LightShift chemiluminescent EMSA kit was from Pierce Company, USA. Trizol was from Invitrogen Company and two-step RT-PCR kit was purchased from Promega Company.

Cell culture. SW480 cells were cultured using RPMI-1640 medium containing 10% fetal bovine serum, 100 u/mL penicillin and 100 μg/mL streptomycin, in a 37℃, 5% CO_2 incubator. Cells were passaged by 0.25% trypsin digestion and those in logarithmic growth phase were used in the experiments.

MTT assay. SW480 cells were suspended and adjusted to 1×10^5/mL and plated in 96-well plate, 90 μL per well. After cell attached, 10 μL RPMI-1640 medium containing 10% FBS was added into blank control group, while different concentrations of CPP (0.125, 0.25, 0.5, 1.0 or 2.0 μg/mL) was added into the experimental group. After cultured for 24, 48 or 72 h respectively, 20 μL MTT (5mg/mL) was added and cells continued to be cultured for 4 h. Then 150 μL DMSO was added into each well and absorbance values (A value) at 570 nm wavelength of each well were measured on the microplate reader. Inhibitory rate on cell growth was calculated using the following equation and growth curve was plotted.

Inhibitory rate = $(1-A_{\text{experimental group}}/A_{\text{control group}})\times 100\%$.

Detection of apoptosis and cell cycle by flow cytometry. SW480 cells treated by 0.5μg/mL CPP for 0, 6, 12 or 24 h were collected, washed by PBS, fixed using pre-cooled 70% ethanol for 2 h. After centrifugation and discarding fixation solution, cells were re-suspended in PBS for 5 min and filtered through the 300 mesh cell strainer. Then PI staining solution was added and stained at 4℃ in dark for 30 min. Finally, cell cycle distribution and apoptotic rates were analyzed by flow cytometry.

Western blot analysis. 1×10^7 SW480 cells treated by 0, 0.125, 0.5 and 2.0 g/mL CPP respectively were collected and total cellular protein, cytosolic protein and nuclear protein were extracted using total protein extraction kit and Nuclear-Cytosol Extraction Kit. Equal amount of protein was mixed with sample buffer, incubated in 100℃ for 7 min and run on SDS-PAGE electrophoresis gel after cooling. Then proteins on SDS gel were transferred onto PVDF membranes and the membranes were blocked by 1% non-fat milk for 60 min. Mouse anti-human -catenin monoclonal antibody was added, 4℃ overnight, followed by goat anti-mouse IgG and incubated in dark at room temperature for 60 min. Odyssey dual-color infrared fluorescence scanning system was used to

detect and analyze density of protein bands and the relative expression level of target protein was determined by the ratio of target protein band density to GAPDH band density. The above experiment was repeated for 3 times.

Electrophoretic mobility shift analysis (EMSA). Experimental groups were divided following method described in section 1.5 and nuclear protein was extracted. Poly (dI, dC), nuclear protein and biotin-labeled DNA probe (5'-CCCTTTGATCTTACC-3') were mixed,[6] reacted at room temperature for 20 min and run on 6% polyacrylamide native gel in 0.5% TBE electrophoresis buffer. Proteins on gel were then transferred onto nylon membrane using water bath electric blotting (380 mA for 45 min) method. Positively charged nylon membrane was cross-linked under UV light for 10 min, incubated in blocking solution with gentle shaking for 15 min and reacted with streptavidin-enzyme conjugate buffer at room temperature for 15 min with gentle shaking. Then membrane was washed for 4 times, 5 min each and substrate was added to react for 5 min. Finally chemiluminescent substrate was added and X-ray film was developed in dark room.

RT-PCR analysis of -catenin, survivin, c-myc and cyclin D1 mRNA expression levels in SW480 cells. Experimental groups were assigned as above and total cellular RNA was extracted using Trizol reagent. Reverse transcription was performed and its product was used as template for PCR amplification. Appropriate primers were added in each reaction (see Table 1) and PCR products were separated by 1.0% agarose gel electrophoresis. Images were captured by gel imaging and subjected to scanning analysis. The semi-quantitative analysis of -catenin, survivin, c-myc and cyclinD1 mRNA levels were determined by the density values of -catenin/-actin, survivin/-actin, c-myc/-actin and cyclinD1/-actin respectively. All experiments were repeated for 3 times.

Statistical analysis. All data were analyzed using statistical analysis software SPSS13.0. Results were presented as ± s and $p<0.05$ was set as statistically significant difference.

Table 1 Primer sequences of β-catenin, survivin, c-myc and cyclin D1

Gene		Primer	Product size
β-catenin	Upstream:	CGCATGGAGGAGATAGTTG	332 bp
	Downstream:	CGAAAGCCGTTTCTTGTAG	
Survivin	Upstream:	AGCCCTTTCTCAAGGACCAC	363 bp
	Downstream:	GCACTTTCTCCGCAGTTTCC	
c-myc	Upstream:	CCTACCCTCTCAACGACAGC	494 bp
	Downstream:	GTTGTGTGTTCGCCTCTTGA	
Cyclin D1	Upstream:	GATGCCAACCTCCTCAACGAC	171 bp
	Downstream:	CTCCTCGCACTTCTGTTCCTC	
β-actin	Upstream:	TGAGACCTTCAACACCCCAG	312 bp
	Downstream:	GCCATCTCTTGCTCGAAGTC	

Results

Inhibitory effect CPP on proliferation of SW480 cells. After SW480 cells were treated with CPP, the cell proliferative rate was significantly decreased compared to the control group ($P<0.01$) and this inhibitory effect was dose- and time-dependent. 2.0 g/mL CPP treating cells for 72 h had the strongest inhibitory effect (91.71%) (Fig. 1). The IC_{50} of 24 h CPP treatment on SW480 cells was 0.46 g/mL. According to IC_{50} value of CPP, 0.5 g/mL was used for subsequential experiments.

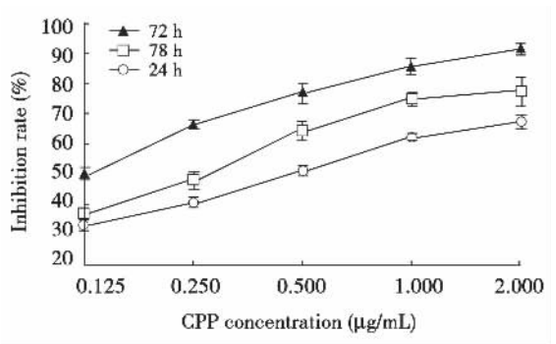


Figure 1 Inhibition effects of periplocin extracted from cortex periplocae (CPP) on proliferation of SW480 cells

Effect of CPP on cell cycle distribution and apoptosis in SW480 cells. SW480 cells were treated with CPP at a final concentration of 0.5 g/mL for different times (0, 6, 12 and 24 h), the cells in G_0/G_1 phase increased significantly (from 17.54% to 37.87%) and apoptotic rate of SW480 cells gradually increased ($P<0.05$) (Table 2).

Table.2 Effect of 0.5 μg/mL periplocin extracted from cortex periplocae (CPP) on cell cycle and apoptosis of SW480 cells

Treatment time (h)	Apoptotic rate (%)	Cell cycle distribution (%)		
		G_0/G_1	S	G_2/M
0	3.89±0.64	17.54±1.40	58.63±1.12	23.83±0.51
6	13.38±0.83[a]	25.87±2.00[a]	51.63±1.28[a]	22.50±1.37
12	21.73±2.02[a]	31.60±1.84[a]	47.83±1.61[a]	20.53±1.26
24	33.60±3.24[a]	37.87±0.57[a]	42.70±1.35[a]	19.43±1.00[a]

[a]P<0.05 vs. 0 h.

Effect of CPP on -catenin expression in SW480 cells. With western blot analysis, the results showed that -catenin expressions among total cellular protein, cytoplasmic protein and nuclear protein in SW480 cells all significantly reduced with increased CPP concentrations (Fig. 2).

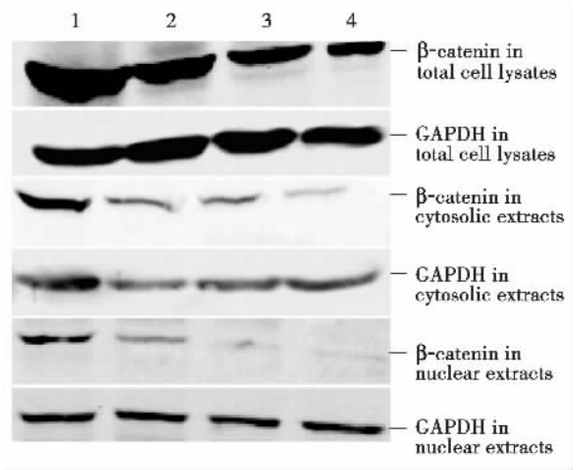


Figure 2 Expression of β -catenin in SW480 cells after incubation with different concentrations of periplocin extracted from cortex periplocae (CPP) for 24 h

1: control; 2: 0.125 μg/mL CPP; 3: 0.5 μg/mL CPP; 4: 2.0 μg/mL CPP.

Effect of CPP on the binding activity of SW480 cell nuclear TCF complex with its specific-binding DNA sequence. The results with EMSA showed that obvious blocking band appeared in untreated group and free DNA band was weak, indicating the adequate binding of nuclear protein and DNA. On contrary, after CPP treatment, blocking band became weak while free DNA band became stronger, suggesting that CPP treatment weakened the binding between nuclear protein and its specific-binding DNA sequence (Fig. 3).

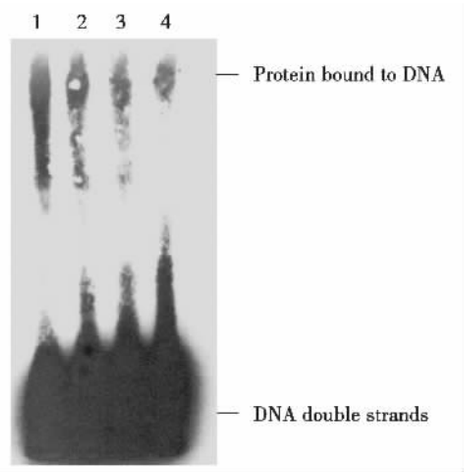


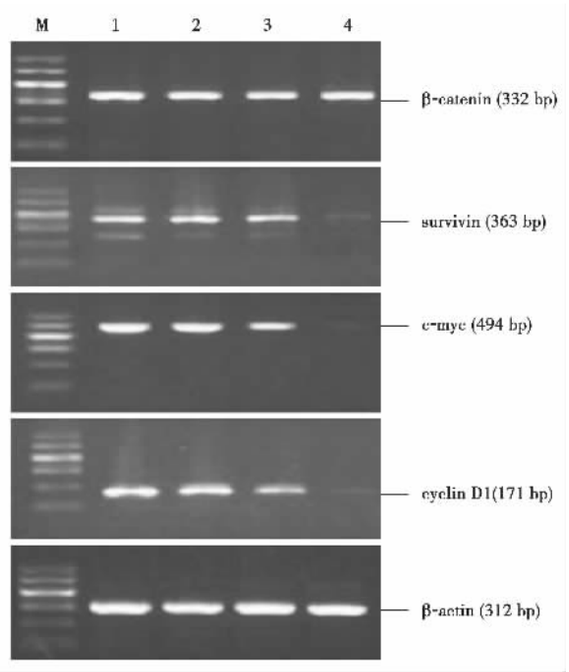
Figure 3 Effect of periplocin extracted from cortex periplocae (CPP) on the binding activity of the TCF complex in nucleus to its specific DNA binding site in SW480 cells after 24 h of treatment

1: control; 2: 0.125 μg/mL CPP; 3: 0.5 μg/mL CPP; 4: 2.0 μg/mL CPP.

Effect of CPP on mRNA expressions of -catenin, survivin, c-myc and cyclinD1 in SW480 cells. After treated by different concentrations of CPP for 24 h, the mRNA expression levels of -catenin in SW480 cell had no significant change compared with the control group ($p>0.05$). However, the mRNA expression levels of -catenin/TCF downstream target genes such as survivin, c-myc and cyclin D1 significantly decreased with increased CPP concentrations. After treated by 2.0 g/mL CPP for 24 h, the mRNA expression levels of survivin, c-myc and cyclin D1 were (0.15 ± 0.03), (0.07 ± 0.02) and (0.15 ± 0.04) respectively, which were significantly lower than that of the control group (0.95 ± 0.13), (1.02 ± 0.04) and (0.96 ± 0.15) ($p< 0.01$) (Fig. 4).

Discussion

The Wnt/-catenin signaling pathway is involved in embryonic development, as well as pathogenesis and development of tumor. In recent years, the molecules in the Wnt pathway have been extensively studied and anticancer drug development targeting the Wnt pathway has been a hot topic.-catenin is an important molecule in the Wnt signal pathway.[7] In the absence of the Wnt signaling, cytosolic -catenin protein forms



β-catenin、survivin、c-myc 和 cyclin D1 mRNA 的表达

Figure 4 mRNA expressions of β-catenin, survivin, c-myc and cyclin D1 in SW480 cells after incubation with different concentrations of CPP for 24 h

M: marker; 1: control; 2: 0.125 μg/mL CPP; 3: 0.5 μg/mL CPP; 4: 2.0 μg/mL CPP.

complex with many other proteins such as adenomatous polyposis coli protein (APC), AXIN, casein kinase (CK) 1a/1e and glycogen synthase kinase (GSK-3β). GSK-3 phosphorylates the 41, 37 and 33 residues on -catenin and CK1 further phosphorylates 45 amino acid of -catenin at serine/threonine sites, a process that activates ubiquitin-dependent degradation of -catenin. WNT protein can bind to transmembrane receptor Frizzleds and co-receptor low-density lipoprotein receptor related protein (LRP-5, LRP-6) to activate Wnt pathway and inhibit the formation of -catenin-AXIN-APC-GSK3 complex, therefore reduces the activity of GSK3 and inhibits the phosphorylation of -catenin.[8,9] Unphosphorylated -catenin translocates into nucleus and activates the expression of downstream target genes including survivin,[10] c-myc,[11] cyclin D1[12] and FGF18[13] and so on through Lymphocyte enhancer-binding factor (LEF)/ T cell factor (TCF), which plays important role in the pathogenesis and development of tumor. -catenin has abnormal expression in many human tumor tissues. For example, abnormal -catenin signal transduction activity was detected in 90% of human colon cancer tissues. Therefore, human malignant tumor cell line SW480 was selected as our experimental model to study -catenin signal transduction in the regulation of growthrelated target genes.

Preliminary date in our lab confirmed that CPP had significant anti-tumor effects. This study explored the effect of periplocin on the Wnt/-catenin signal pathway in SW480 cells, in order to further clarify the molecular mechanism of its anti-tumor effect. Our results showed that CPP had significantly inhibitory effect on the proliferation of SW480 cells, in a time- and dose-dependent manner within a certain concentration range. Only 0.5 g/mL CPP could significantly affect the cell cycle and induce cell apoptosis. With CPP treatment time increased, the proportion of SW480 cells in G_0/G_1 markedly increased while these in S phase decreased. Apoptotic peak gradually increased and the apoptotic rate after 24h treatment reached 36.52%. CyclinD1 and C-Myc can accelerate the progress of G_1/S phase in cell cycle.[14] Our results showed that after CPP treatment, the expression levels of cyclin D1 and c-myc mRNA decreased, consistent with the prediction that SW480 cells could be arrested in G_0/G_1 phase by CPP. Survivin plays an important anti-apoptotic role.[15] The increase in SW480 cell apoptosis after CPP treatment might be related with the reduced expression of survivin mRNA.

The final effect of the Wnt signaling is achieved through activation of target gene transcription after binding of -catenin and TCF4 in the nucleus. Our results showed that after CPP treatment, -catenin protein expression levels in SW480 cell total protein, cytosolic protein and nuclear protein all significantly reduced, while the mRNA expression level of catenin remained unchanged, suggesting that the regulation of -catenin by CPP is not at mRNA level, but likely through protein degradation. After CPP treatment, the expression level of -catenin protein decreased among cytosolic proteins, causing the decrease of -catenin translocation into the nucleus and decreased binding activity between -catenin/TCF-4

complex and its specific-binding DNA sequence. Therefore, the activation of downstream target gene expression is lost and the ability of the cell to maintain viability decreased. After CPP treatment, the mRNA expression of its downstream target genes decreased including survivin, c-myc and cyclin D1, which is consistent with the inhibitory effect of CPP on Wnt pathway. Thus, it is likely that the inhibitory effect of CPP on the the Wnt signaling pathway is one of the molecular mechanisms for its anti-tumor effect.

In summary, through inhibition of the Wnt signaling transduction pathway in SW480 cells, CPP induced apoptosis and inhibited cell proliferation. How does CPP inhibit the Wnt signaling pathway and the in-deep mechanism for its in vivo anti-tumor effects should be further explored, so that it can be developed for cancer treatment as an effective inhibitor of the Wnt pathway.

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