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Triptolide Inhibits Cyclooxygenase-2 and Inducible Nitric Oxide Synthase Expression in Human Colon Cancer and Leukemia Cells

Xiangmin TONG[#], Shui'er ZHENG[#], Jie JIN, Lifen ZHU, Yinjun LOU, and Hangping YAO*

Institute of Hematologic Disease, Department of Hematology, First Affiliated Hospital, Medical School, Zhejiang University, Hangzhou 310003, China

Abstract Triptolide (TP), a traditional Chinese medicine, has been reported to be effective in the treatment of autoimmune diseases and exerting antineoplastic activity in several human tumor cell lines. This study investigates the antitumor effect of TP in human colon cancer cells (SW114) and myelocytic leukemia (K562), and elucidates the possible molecular mechanism involved. SW114 and K562 cells were treated with different doses of TP (0, 5, 10, 20, or 50 ng/ml). The cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Results demonstrated that TP inhibited the proliferation of both tumor cell lines in a dose-dependent manner. To further investigate its mechanisms, the products prostaglandin E_2 (PGE_2) and nitric oxide (NO) were measured by enzyme-linked immunosorbent assay (ELISA). Our data showed that TP strongly inhibited the production of NO and PGE_2. Consistent with these results, the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) was up-regulated both at the mRNA level and the protein expression level, as shown by real-time RT-PCR and Western blotting. These results indicated that the inhibition of the inflammatory factor COX-2 and iNOS activity could be involved in the antitumor mechanisms of TP.

Key words triptolide; leukemia; colon cancer; cyclooxygenase-2; inducible nitric oxide synthase

Inducible cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) are important enzymes that mediate inflammatory processes [1]. Recent studies have shown that improper up-regulation of *COX-2* and/or *iNOS* has been associated with pathophysiology of certain types of human cancers [2,3]. The expression of COX-2 and its induced product, prostaglandin E_2 (PGE_2), can be induced by various agents, including inflammatory cytokines, mitogens, reactive oxygen intermediates and many other tumor promoters. Increased expression of *COX-2* and *PGE_2* has been reported in many colorectal tumors and adenocarcinomas [4,5]. iNOS catalyzes the oxidative deamination of *L*-arginine to produce NO, a potent pro-inflammatory mediator. NO has multifaceted roles in mutagenesis and carcinogenesis [6–8].

Triptolide (TP), a purified component of a traditional Chinese medicine, is extracted from a shrub-like vine *Tripterygium wilfordii* Hook F. It has been reported to be effective in the treatment of autoimmune diseases, especially rheumatoid arthritis [9]. TP can also induce antineoplastic activity on several human tumor cell lines. It was able to inhibit transcriptional activation of NF-κB in Jurkat cells and human bronchial epithelial cells [10, 11]. Furthermore, TP has also been shown to down-regulate the expression of various NF-κB-regulated genes or to induce apoptosis [12,13]. It was also reported that TP inhibits vascular endothelial growth factor expression, which is believed to play a role in tumor angiogenesis [14].

We have shown previously that the alleviation of rheumatoid arthritis by TP might involve the inflammatory factors created through the COX-2 and iNOS pathways [15].

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[#] These two authors contributed equally to this study

*Corresponding author: Tel, 86-571-87236582; Fax, 86-571-87236628; E-mail, yaohp2001@yahoo.com.cn

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In the present study, we show for the first time that TP suppression on tumor proliferation is associated with inhibition of COX-2 and iNOS activation in two different tumor cell lines, myelocytic leukemia cell line K562 and human colon cancer cell line SW114. These results pave the way for a comprehensive understanding of TP mechanisms.

Materials and Methods

Cell culture and triptolide preparation

Human myelogenous leukemia cell line, K562 (ATCC), and human colon cancer cell line, SW114 (ATCC), were grown in RPMI 1640 medium (Gibco, California, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) and 2 mM *L*-glutamine (Gibco). All cell lines were kept under sterile conditions at 37 ºC with 5% CO_2. TP (Sigma, New York, USA) was diluted at various concentrations in serum-free culture medium. K562 and SW114 cells were treated with various concentrations of TP (0, 5, 10, 20, or 50 ng/ml) for 24 h.

Assay for PGE_2 and NO production

The levels of PGE_2 in culture supernatants were determined by competitive enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. The lower limit of detection was 36.2 pg/ml.

NO levels in culture supernatants were measured as its oxidized product nitrate. The kits were purchased from R&D Systems, and the lower limit of detection was 1.35 μM.

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA in the treated K562 and SW114 cells were isolated with Trizol reagent (Gibco) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was prepared by RT of 2 μg total RNA using oligo dT_{18} and 200 U superscript II reverse transcriptase (Invitrogen, California, USA) at 42 ºC for 70 min according to the manufacturer's instructions.

Quantitative RT-PCR was carried out by LightCycler technology (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green I detection. In all assays, cDNA was amplified using a standardized program (10 min for the denaturing step; 55 cycles of 5 s at 95 ºC, 15 s at 65 ºC, and 15 s at 72 ºC; melting point analysis in 0.1 ºC steps, the final cooling step). Each LightCycler capillary was loaded with 1.5 μl DNA Master mix, 1.8 μl $MgCl_2$ (25 mM), 10.1 μl H_2O and 0.4 μl of each primer (10 μM). The final amount of cDNA per reaction corresponded to the 2.0 ng of RNA used for RT. Relative quantification of target gene expression was carried out using a mathematical model, which was also recommended by Roche Molecular Biochemicals. The following primers were use for the experiment: *COX-2* (product size: 756 bp), sense, 5′-CAGCACTTCACGCATCAGTT-3′, antisense, 5′-TCTGGTCAATGGAAGCCT-3′; *iNOS* (product size: 237 bp), sense, 5′-TCTTGGTCAAAGCTGTGCTG-3′, antisense, 5′-CATTGCCAAACGTACTGGTC-3′; β-*actin* (product size: 619 bp), sense, 5′-CGCTGCGCTGGTC-GTCGACA-3′, antisense, 5′-GTCACGCACGATTTCC-CGCT-3′.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analysis

Cell lysates were prepared for Western blotting analysis of iNOS and COX-2 by using whole cellular protein extraction kits (Active Motif, California, USA). The concentration of protein in each cell lysate was determined using a BCA protein assay it (Pierce, Rockford, USA) with bovine serum albumin (BSA) as the standard. An identical amount of protein (40 μg) from each sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes (0.45 μm; S&S, Dassel, Germany). Nitrocellulose membranes were blocked with 5% BSA (Sigma) in TBS (25 mM Tris-HCl, 150 mM sodium chloride, pH 7.2) for 1 h at room temperature. Blots were incubated with anti-COX-2, anti-iNOS or anti-β-actin specific rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:500 dilution at 37 ºC for 2 h. Blots were washed three times then incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2000 dilution) for 2 h at room temperature. All blots were developed using enhanced chemoluminescence reagents (Super signal dura kit; Pierce) following the manufacturer's instructions. Blots were scanned and analyzed for the measurement of the band intensities. Results were calculated as relative ratios of a specific band and the β-actin one.

Cell viability

In vitro, the growth inhibition effect of TP on K562 and SW114 cells was determined by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) dye absorbance of living cells. Briefly,

Table 1 Effect of triptolide (TP) on PGE_2 and nitric oxide (NO) production in human colon cancer (SW114) and myelocytic leukemia (K562) cells

Group	SW114-PGE_2 (μg/L)	SW114-NO (μM)	K562-PGE_2 (μg/L)	K562-NO (μM)
Control	38.02±2.17	109.48±4.71	16.78±1.71	73.00±6.35
TP 5 ng/ml	35.02±3.28	104.30±2.89	14.29±2.37	53.73±1.79
TP 10 ng/ml	19.73±1.16 *	83.56±2.38 *	9.63±0.98 *	43.16±3.23 *
TP 20 ng/ml	13.52±2.31 **	38.57±2.89 **	5.38±0.86 **	23.11±3.22 **
TP 50 ng/ml	6.01±0.23 ***	12.83±3.11 ***	2.82±0.51 ***	13.72±3.38 ***

PGE_2 and NO production were measured in the supernatants of the culture. SW114 and K562 cells were cultured for 24 h with increasing concentration of TP (0–50 ng/ml). *P<0.01 versus control; **P<0.05 versus TP groups at concentration of 0–10 ng/ml; ***P<0.01 versus TP groups at concentration of 0–20 ng/ml.

cells (1×10^5 cells per well) were seeded in 96-well microtiter plates. After exposure to the drug (0, 5, 10, 20, or 50 ng/ml) for 48 h, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37 ºC. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 μl of dimethyl sulfoxide (DMSO) was added to each well before absorbance at 570 nm was measured. Each assay was carried out three times.

Results

Effects of TP on PGE_2 and NO production

The treatment of K562 and SW114 cells with the presence of TP reduced both the PGE_2 and NO production. As it was shown in **Table 1**, the response was dose-dependent, and the effect was significant when the concentration of TP was above 20 ng/ml.

Effects of TP on the transcription of *COX-2* and *iNOS* at mRNA level

As described previously, PGE_2 is synthesized by COX-2, and NO is synthesized by iNOS. The results of the specific RT-PCR analysis corresponded well to the level of PGE_2 and NO in supernatants (**Figs. 1** and **2**). The inhibition pattern between COX-2 and iNOS did not show any significant difference. It was observed that TP markedly inhibited *COX-2* and *iNOS* mRNA expression in K562 cells at 20 ng/ml and 50 ng/ml, respectively. In contrast, marked inhibition of expression in SW114 cells was observed at 5 ng/ml.

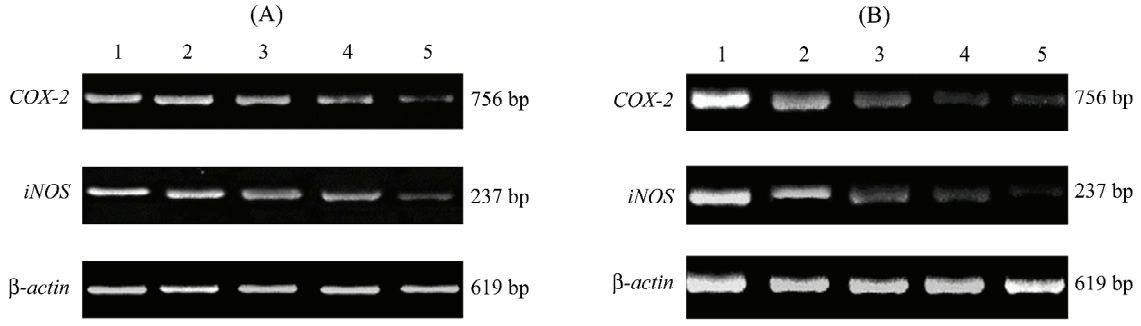

Fig. 1 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA for *COX-2* and *iNOS* in K562 (A) and SW114 cells (B)

Cells were treated with different concentrations of triptolide (0–50 ng/ml) for 24 h. *COX-2* and *iNOS* mRNA were examined with RT-PCR. PCR products were separated in 1.5% agarose gels and analyzed after ethidium bromide staining. Housekeeping gene β-*actin* was used as the control for densitometric analysis. 1, control; 2, 5 ng/ml; 3, 10 ng/ml; 4, 20 ng/ml; 5, 50 ng/ml.

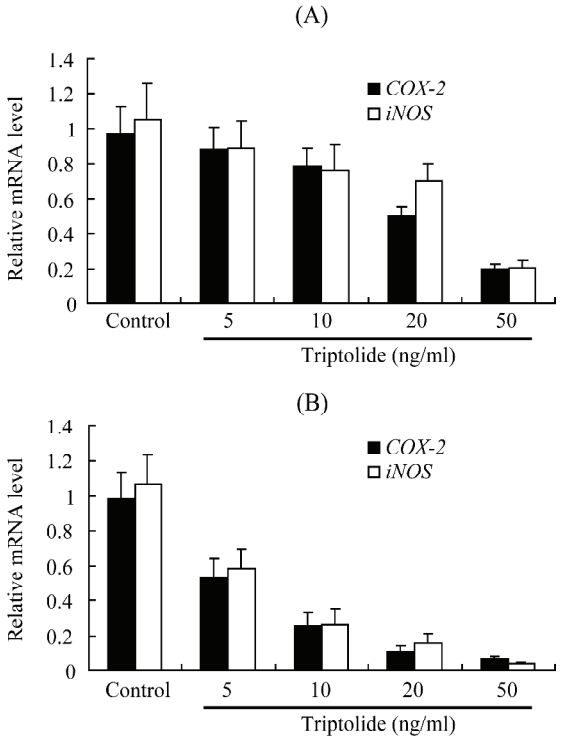

Fig. 2 *COX-2* and *iNOS* expression in relation to *β-actin* mRNA expression in K562 (A) and SW114 cells (B)

Densitomtric analysis was performed on ethidium bromide-stained agarose gel (**Fig. 1**) by software program Kodak Digital Science. The net intensity of each band was compared to that of the housekeeping gene *β-actin* and their ratios are reported. Data were presented as mean±SD.

Effects of TP on the expression of COX-2 and iNOS protein

As expected, *COX-2* and *iNOS* expression in tumor cells at the protein level, under different doses, was parallel with that at the mRNA level, suggesting that no post-translational modifications of the mRNA transcript are necessary to account for the effect. The inhibition effect of TP on tumor cell proliferation was dose-dependent (**Figs. 3** and **4**). We observed that TP markedly reduced the expression of COX-2 and iNOS protein in K562 and SW114 cells at

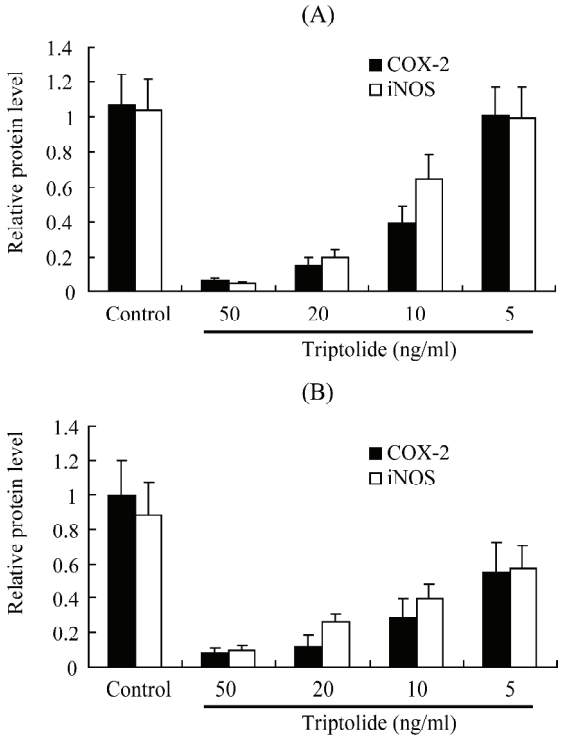

Fig. 4 COX-2 and iNOS expression in relation to β-actin protein expression in K562 (A) and SW114 cells (B)

Densitomtric analysis was performed by software program Kodak Digital Science (**Fig. 3**). The net intensity of each band was compared to that of the housekeeping gene β-actin and their ratios are reported. Data were presented as mean±SD.

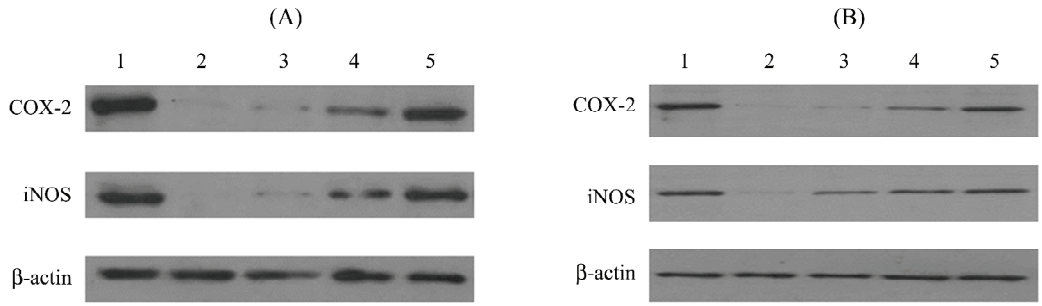

Fig. 3 COX-2 and iNOS protein expression of K562 (A) and SW114 (B) cells determined by Western blotting analysis

Effect of triptolide on the protein expression of COX-2 and iNOS in human myelogenous leukemia cells (K562) and colon cancer cells (SW114). The harvest cells were subjected to Western blotting analysis for COX-2 and iNOS. Forty micrograms of protein was loaded per lane. 1, control; 2, 50 ng/ml; 3, 20 ng/ml; 4, 10 ng/ml; 5, 5 ng/ml.

concentrations of 10 ng/ml and 5 ng/ml, respectively.

Cell viability assay of SW114 and K562 cells

The cytotoxic effect of TP on SW114 and K562 cells was examined by exposing the cells to different concentrations of TP for 48 h. The resulting growth curves (**Fig. 5**) show that TP has a concentration-dependent inhibitory effect. The inhibitory effects of TP were more pronounced at higher doses. The TP IC_{50} was approximately 25–35 ng/ml for SW114 and 40–50 ng/ml for K562. The results show that SW114 was more sensitive than K562 to the treatment by TP. To determine if the inhibition effect of TP on tumor cell proliferation was associated with the COX-2 and iNOS pathways, we studied its effect in RPMI-8226 cells (low *COX-2* and *iNOS* expression) and found a similar cytotoxic effect (data not shown).

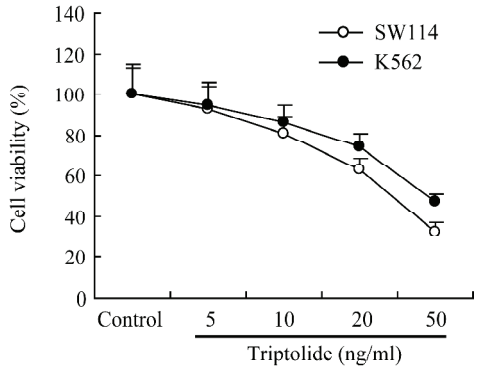

Fig. 5 Cell viability assay of K562 and SW114 cells

The viability of K562 and SW114 cells was tested by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay after exposure to triptolide for 48 h. The IC_{50} of K562 and SW114 were shown to be 25–35 ng/ml and 40–50 ng/ml, respectively.

Discussion

Elevated PGE_2 production can stimulate epithelial cell growth and promote cellular survival. A number of previous experimental studies support a role for products of COX and iNOS activity in the pathogenesis of cancer [16,17]. NO mediates DNA damage or hinders DNA repair, and is thus potentially carcinogenic. NO can stimulate tumor growth and metastasis by promoting migratory, invasive, and angiogenic abilities of tumor cells, which might also be triggered by activation of COX-2 [18]. Up-regulation of both PGE_2 and NO has been reported in a variety of different malignancies [19–22], including colorectal cancer and leukemia. Our results revealed that TP indeed inhibited the production of PGE_2 and NO in SW114 and K562 cells in a dose-dependent manner, assessed by ELISA. These studies suggest the reduced products of NO or PGE_2 might contribute to the inhibition effects of TP on tumor growth.

The up-regulation of PGE_2 and NO results from the production of COX-2 and iNOS. COX-2 and iNOS products have been implicated in the regulation of the immune system, in tumor cell apoptosis, and involved in many human carcinogeneses, including chronic myeloid leukaemic cells [23–25]. Ozel *et al*. [26] demonstrated that *iNOS* expression might act in the first steps of carcinogenesis, whereas *COX-2* expression was seen in more advanced tumors. Cianchi *et al*. [27] showed a prominent role of NO in stimulating COX-2 activity in colorectal cancer. For further study, we therefore decided to test the effects of TP on two types of cell lines to see if this compound would suppress the expression of *COX-2* and *iNOS*.

The major focus of this study was to investigate the chemopreventive efficacy of TP as a possible inhibitor of *COX-2* and *iNOS* expression using SW114 and K562 cells. Selection of TP for study as a chemopreventive agent was, in part, based on the evidence that TP has an inhibitory effect on arachidonic acid-induced inflammation and on its inhibition of arachidonic acid metabolism through the inhibition of cyclooxygenase. The outcome of this study is significant as it clearly emphasizes that TP has the potential to specifically inhibit the expression of *COX-2* and *iNOS* at the mRNA and protein level in SW114 and K562 cells. The inhibitory effect of TP is concentration-dependent. Furthermore, our results also revealed that SW114 was more sensitive than K562 in the inhibition of *COX-2* and *iNOS* expression by TP.

We also addressed the possibility that the down-regulated PGE_2 and NO by TP might be the result of the suppression of COX-2 and iNOS instead of the inhibition of tumor cell proliferation. We have found a similar growth inhibitory effect in RPMI-8226 cells (low *COX-2* and *iNOS* expression) with TP treatment (data not shown) [28].

The results of this study confirm that TP is an inhibitor of COX-2 and iNOS and their products PGE_2 and NO in SW114 and K562 cells. Because COX-2- or iNOS-dependent mechanisms are involved in carcinogenesis and tumor progression [29], these findings provide a new uncovering mechanism about antitumor effects of TP.

However, TP does have one major drawback as an antitumor agent, namely its toxicity. Pyatt *et al*. [30]

demonstrated that therapeutic concentrations of TP exerted a significant hematotoxic effect by inhibiting growth factor response in $CD34^+$ bone marrow cells. However, it should be pointed out that our clinical data showed different results. As early as the 1980s, our clinical department and other departments in China have used TP to treat acute leukemia in clinical trials [31]. The effective dose is 30 μg/kg for day 1–7. No toxicity of heart, liver, kidney, or gastrointestinal tract were observed, and the hematological toxicity was also mild.

In conclusion, we have demonstrated that TP could inhibit COX-2 and iNOS activity, highlighting the potential clinical value of TP in the treatment of colon cancer and leukemia. These data suggest that further evaluation of the pharmacological effect of TP is needed to develop a new therapeutic strategy for treating cancer.

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Edited by
Jae Hong SEO