

Effect of camptothecin on inducible nitric oxide synthase expression in the colon cancer SW480 cell line

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Abstract. As a topoisomerase I inhibitor, camptothecin (CPT) is regarded as an effective antitumor agent. In an attempt to search for its novel anticancer mechanism, the present study evaluated the effects of CPT on inducible nitric oxide synthase (iNOS) in the human colon cancer SW480 cell line when stimulated with lipopolysaccharide (LPS) and interleukin (IL)-1 β . The data indicated that CPT significantly decreased NO production. Consistent with these observations, the protein and mRNA expression levels of iNOS were inhibited by CPT in a dose-dependent manner. Thus, the inhibitory effects of CPT on LPS/IL-1 β -stimulated NO production were likely mediated via the inhibition of iNOS gene transcription. From these results, we propose that the inhibition of NO biosynthesis by CPT may partially underlie the efficacy of this antitumor agent.

Introduction

Camptothecin (CPT) is a pentacyclic alkaloid first isolated from a Chinese tree, *Camptotheca acuminata*, in the early 1960s (1). CPT is an antitumor agent that is clinically effective, with a broad range of antitumor activities against solid tumors, including breast, ovarian, lung and colorectal tumors (2). The efficacy of CPT is associated with its ability to inhibit the function of DNA topoisomerase I, which is vital for the transcription of supercoiled DNA (3). However, innovations in treatment strategies have been developed, with targets such as oncogenes and elements of cell signaling pathways (4). Tumors are dependent on a switch to an angiogenic phenotype and the resultant formation of new vasculature, and this finding has become the basis of another potential target (5,6). Thus, the inhibition of the blood supply to the tumor is now considered to represent a unique approach for the cessation of tumor growth (7). Certain studies have illustrated that CPT

also produces an inhibitory effect on the development of the vasculature (8). A previous study has also revealed that CPT can induce the apoptosis of cancer cells (9).

A number of studies have indicated that nitric oxide (NO) synthesized by the NO synthase (NOS) enzyme family is significant in tumor growth, invasion and metastasis (10,11). Mammalian systems contain three well-characterized isoforms of the enzyme: Neuronal NOS (nNOS/NOS-1), endothelial NOS (eNOS/NOS-3) and inducible NOS (iNOS/NOS-2). The activity of iNOS has been found to be inducible in response to stimuli such as proinflammatory cytokines or endotoxin (12). In a number of solid tumors, such as colon and breast carcinoma, the overexpression of inducible NOS has been recently documented (13,14). It has also been suggested that the iNOS activity in tumor tissue is associated with the tumor grade and cell differentiation (15-17). A previous study has indicated that CPT affects the expression of iNOS protein and its activity in the virus-transformed mouse macrophage-like RAW264.7 cell line when stimulated with lipopolysaccharide (LPS) plus interferon- γ (18).

The present study investigated whether CPT alters iNOS protein expression in the human colon adenocarcinoma SW480 cell line in order to obtain further insight into the biological effects of CPT on iNOS.

Materials and methods

Chemicals. CPT was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich) at 2 mg/ml, prior to being aliquoted and stored at -20°C. Further dilutions were made in phosphate-buffered saline (PBS; Cellgro, Herndon, VA, USA) to the appropriate concentration just prior to use. The final concentration of DMSO in culture did not exceed 0.1% (v/v), which is non-toxic to cells. LPS was purchased from Fluka Chemical Corporation (Buchs SG, Switzerland) and interleukin (IL)-1 β was purchased from Cytolab Ltd. (Rehovot, Israel). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Life Technologies (Carlsbad, CA, USA). All other chemicals were dissolved in distilled water.

Cell culture. SW480 cells were gained from the Basic Science department at Zhejiang Medical College (Hangzhou, China) and were cultured in DMEM containing 10% fetal calf serum

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(Sijiqing Company, Zhejiang, China) in a humidified incubator at 37°C in an atmosphere containing 5% CO₂. The cells were used between passages 9 and 20.

Measurement of nitrite. Nitrite production, an indicator of NO synthesis, was measured in the supernatant as described previously (19). The SW480 cells were plated into 96-well tissue culture plates at 2x10⁴ cells/well and grown under standard culture conditions. After 12 h, in order to induce iNOS, old culture medium was replaced by fresh culture medium containing LPS (10 mg/l) plus IL-1β (20 ng/ml). To assay the effect of CPT on nitrite production, CPT (0, 0.032, 0.125, 0.5, 1.0, 2.0 μg/ml) was added in the presence of LPS/IL-1β for 12 h. Nitrite was measured based on the Griess reaction. Cell culture medium (100 μl) was mixed with 100 μl Griess reagent (1% sulfanilamide and 0.1% naphthylendiamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The optical density at 490 nm (OD₄₉₀) was measured with a microplate reader (SmartSpec™ 3000; Bio-Rad Laboratories Inc., Hercules, CA, USA). A standard curve was prepared for calculating the concentration using the OD₄₉₀.

Cell viability. Cell viability was determined by MTT assay. A total of 100 μl culture medium was present per well following the Griess reaction. MTT was purchased from Sigma Aldrich and was dissolved with PBS. MTT solution (5 mg/ml) was added in every well. The cells were incubated for 4 h. The supernatant was discarded and then 150 μl DMSO was added. Agitation was performed for 10 min and the OD₄₉₀ was measured. The viability percentage was calculated.

Reverse transcription-polymerase chain reaction (RT-PCR). The SW480 cells were cultured in 100-ml culture bottles. After 12 h, fresh medium with LPS/IL-1β was added to replace the old medium. Next, two concentrations of CPT (0.032 and 0.125 μg/ml) were added after 12 h, and 24 h later, total cellular RNA was extracted from control and treated cells using TRIzol reagent (Bio Basic Inc., Markham, ON, Canada). Total RNA preparation (2 μg) was mixed with oligo(dT)₁₈ (Invitrogen Life Technologies, Carlsbad, California, USA) for reverse transcription using MMLV to derive the first-strand cDNA. A pair of gene-specific PCR primers (Invitrogen Life Technologies) were designed for iNOS and GAPDH as follows: iNOS, 5'-GATCAATAACCTGAACG-3' and 5'-GCCCTTTTTGCTCCATAGC-3'; and GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACC ATGTTGCTGTAA-3'. PCR was performed with an S1000™ Thermal Cycler (Bio-Rad Laboratories Inc.) at 94°C for 3 min for denaturing, followed by multiple cycles at 94°C for 45 sec, 50°C for 1 min and 72°C for 1 min. For iNOS, 36 cycles were performed and for GAPDH, 35 cycles were performed. The PCR products were separated by electrophoresis and their quantity was determined by Quantity One software (Bio-Rad Laboratories Inc.).

Western blot analysis. The SW480 cells were cultured in 100-ml culture bottles. After 12 h, fresh medium with LPS/IL-1β was added to replace the old medium. Next, two concentrations of CPT (0.032 and 0.125 μg/ml) were added after 12 h, and 24 h later, the cells were solubilized with lysis buffer. A BCA

Table I. Nitrite production and cell viability of SW480 cell in the presence of CPT for 18 h.

Concentration of CPT, μg/ml	Nitrite production, μg/ml	Cell viability after 18 h, %
0.000	26.68±1.15	99.98±2.09
0.032	24.71±1.71	93.93±6.39
0.125	24.16±1.37	94.56±4.91
0.500	23.88±1.40	93.21±8.78
1.000	23.68±0.87	91.04±4.85
2.000	21.63±4.59 ^a	92.58±4.69

Effect of different concentrations of camptothecin (CPT) on lipopolysaccharide/interleukin-1β-induced nitrite accumulation and cell viability in SW480 cells. The cells were exposed to CPT for 18 h. Results are expressed as the mean ± standard deviation of 3 observations. ^aP<0.05 vs. control (0 μg/ml).

Protein Assay Kit (Amersham, Uppsala, Sweden) was applied to detect the concentration of protein. Lysates containing 5 μg protein were separated by SDS-PAGE on 7.5% polyacrylamide gels with perpendicular electrophoretic apparatus (EPS 2A200; Amersham) and transferred onto nitrocellulose membranes with a transmembrane machine (semi-dry transfer unit; Amersham). Subsequent to blocking, the membrane was incubated with rabbit polyclonal anti-iNOS body (1:2,000; Santa Cruz Inc., Dallas, TX, USA) for 2 h at room temperature. Blots were washed with Tris-buffered saline plus Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000; Santa Cruz Inc.) for 2 h at room temperature. Next, immunoreactive bands were detected with an enhanced chemiluminescence mixture (Sigma Aldrich). Thereafter, the same membrane was stripped and reprobbed with rabbit anti-β-actin (1:2,000; Santa Cruz Inc.). The membrane was placed into a magazine with a film. Following exposure, the film was immersed in development agent for 1-2 min, then in fixer. The film was scanned and the OD value was calculated by Quantity One software.

Statistical evaluation. The results are expressed as the mean ± standard deviation. Statistical comparisons were made between groups using a one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of CPT on nitrite production in SW480. As illustrated in Tables I and II, when the cells were stimulated with LPS/IL-1β, the ability of CPT to cause changes in nitrite production was dependent on the concentration of CPT and the duration of incubation with CPT. As shown in Table I, when the cells were incubated in the presence of CPT for 18 h, nitrite production was significantly reduced only at concentration of 2 μg/ml. As shown in Table I, all concentrations of CPT did not affect the cell viability of the SW480 cells, even at 2 μg/ml. This result indicates that the inhibi-

Table II. Nitrite production and cell viability of SW480 cells in the presence of CPT for 24 h.

Concentration of CPT, $\mu\text{g/ml}$	Nitrite production, $\mu\text{g/ml}$	Cell viability after 24 h, %
0.000	31.26 \pm 2.49	100.00 \pm 3.98
0.032	24.57 \pm 1.48 ^a	93.67 \pm 6.46
0.125	24.02 \pm 1.93 ^a	92.70 \pm 5.31
0.500	23.29 \pm 1.08 ^a	83.41 \pm 5.68 ^a
1.000	22.79 \pm 3.39 ^a	60.58 \pm 8.33 ^a
2.000	24.71 \pm 1.36 ^a	41.02 \pm 7.26 ^a

Effect of different concentrations of camptothecin (CPT) on lipopolysaccharide/interleukin-1 β -induced nitrite accumulation and cell viability in SW480 cells. The cells were exposed to CPT for 24 h. Results are expressed as the mean \pm standard deviation of 3 observations. ^aP<0.05 vs. control (0 $\mu\text{g/ml}$).

Table III. Optical density ratio of RT-PCR in the presence of CPT for 24 h.

Concentration of CPT, $\mu\text{g/ml}$	Optical density ratio (iNOS/GAPDH)
0.000	1.284 \pm 0.008
0.032	1.179 \pm 0.003 ^a
0.125	1.071 \pm 0.017 ^a

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of inducible nitric oxide synthase (iNOS) mRNA expression in lipopolysaccharide/interleukin-1 β (LPS/IL-1 β)-activated SW480 cells with camptothecin (CPT). The cells were treated with LPS plus IL-1 β in the co-existence of various concentrations (0, 0.032 and 0.125 $\mu\text{g/ml}$) of CPT for 24 h. Band intensities were quantified by Quantity One software. This experiment was repeated three times with similar results. ^aP<0.05 vs. control (0 $\mu\text{g/ml}$).

tion of nitrite production by 2 $\mu\text{g/ml}$ CPT was not due to cell death. However, significant inhibition of nitrite production by CPT was observed at all concentrations after 24 h incubation and these effects occurred in a dose-dependent manner. Additionally, there was no significant difference in cell viability among the cells treated with vehicle and 0.032 or 0.125 $\mu\text{g/ml}$ CPT (Table II). This result indicates that lower concentrations of CPT (0.032 and 0.125 $\mu\text{g/ml}$) could reduce nitrite production, but not cause cell death.

Effect of CPT on iNOS mRNA expression. To examine whether CPT could inhibit iNOS mRNA expression, RT-PCR was performed, as shown in Fig. 1. The house-keeping gene, GAPDH, was also amplified from each RNA preparation to enable comparisons of the PCR productions in different samples. As shown in Fig. 1 and Table III, iNOS mRNA was significantly suppressed in the presence of CPT, while GAPDH mRNA was not inhibited significantly. This result indicates that CPT inhibits NO production at the transcription level.

Table IV. Optical density ratio of western blotting in the presence of CPT for 24 h.

Concentration of CPT, $\mu\text{g/ml}$	Optical density ratio (iNOS/ β -actin)
0.000	1.051 \pm 0.024
0.032	0.934 \pm 0.078 ^a
0.125	0.366 \pm 0.016 ^a

Inhibition of inducible nitric oxide synthase (iNOS) protein expression in activated SW480 cells with camptothecin (CPT). The cells were treated with lipopolysaccharide plus interleukin (IL)-1 β in the co-existence of various concentrations (0, 0.032 and 0.125 $\mu\text{g/ml}$) of CPT for 24 h. Band intensities were quantified by Quantity One software. This experiment was repeated three times with similar results. ^aP<0.05 vs. control (0 $\mu\text{g/ml}$).

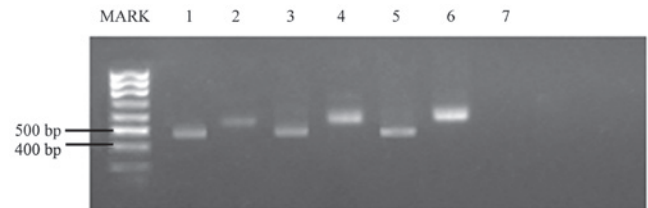


Figure 1. Reverse transcription-polymerase chain reaction analysis of inducible nitric oxide synthase (iNOS) mRNA expression in lipopolysaccharide/interleukin-1 β (LPS/IL-1 β)-activated SW480 cells with by camptothecin (CPT). The cells were treated with LPS plus IL-1 β in the co-existence of various concentrations (0.125, 0.032 and 0 $\mu\text{g/ml}$) of CPT for 24 h. Lanes 1, 3 and 5 show GAPDH expression with the three concentrations of CPT, respectively. Lanes 4 and 6 show iNOS mRNA expression in cells with CPT concentrations of 0.032 and 0 $\mu\text{g/ml}$, and lane 2 shows iNOS mRNA expression in cells with CPT at concentrations of 0.125 $\mu\text{g/ml}$.

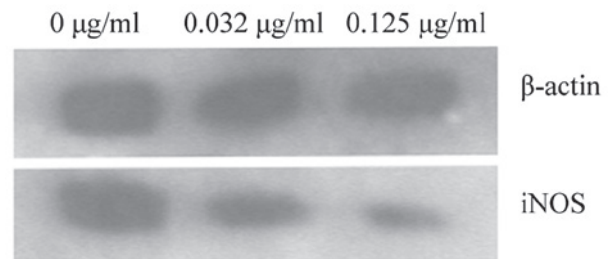


Figure 2. Inhibition of inducible nitric oxide synthase (iNOS) protein expression in activated SW480 cells with camptothecin (CPT). The cells were treated with lipopolysaccharide plus interleukin-1 β in the co-existence of various concentrations (0, 0.032 and 0.125 $\mu\text{g/ml}$) of CPT for 24 h.

Effect of CPT on iNOS protein expression. To determine whether the NO inhibitory effect of CPT was due to the inhibition of iNOS protein expression, western blot analysis was performed. The inhibition of 142-kDa iNOS protein expression by CPT is shown in Fig. 2. Marked suppression was observed at the two concentrations. As β -actin protein was not markedly affected, cell viability was not changed (Fig. 2; Table IV). This result indicates that the inhibition of iNOS protein expression is one of the mechanisms of NO inhibition.

Discussion

A number of the anticancer mechanisms of CPT have previously been revealed. Studies have shown that the antitumor activity is associated with the inhibition of topoisomerase I (3), and that CPT may exhibit an inhibitory effect on the development of the vasculature (8) and the apoptosis of cancer cells (9). In the present study, it was shown that CPT can effect the nitrite production of SW480 cells in a process that is independent of cytotoxicity. In order to further understand the mechanisms of action for NO inhibition, the levels of iNOS mRNA and protein expression were determined. RT-PCR and western blotting data showed that interference with iNOS mRNA and protein expression may be a factor contributing to the inhibitory effect of CPT on iNOS enzyme activity and NO production in SW480 cells. This result has not been reported in previous studies.

iNOS is overexpressed in colon tumors, and NO is important during the progression of colon carcinoma. The associated mechanisms involve inhibiting apoptosis, improving angiogenesis and enhancing the expression of proto-oncogenes. This suggests that iNOS can be the target of anticancer agents. As an effective anticancer agent, CPT can inhibit colon tumors. From the present experiments, it can be concluded that the inhibition of nitrite production should be a novel mechanism underlying the effect of CPT against colon cancer.

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