9-Methoxycamptothecin from *Nothapodytes foetida* Induces Apoptosis in Murine Sarcoma S180 Cells

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9-Methoxycamptothecin (MCPT) was found to have antitumour activities through topoisomerase inhibition. However, the type of cell death induced in the tumour cells treated with MCPT was not elucidated. In this study, MCPT and camptothecin were isolated from *Nothapodytes foetida* distributed in Hubei Province, China and identified by NMR spectroscopy. MCPT was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay using camptothecin as reference. Annexin V-FITC/propidium iodide double staining and real-time PCR were also performed. The IC_{50} value was (0.385 ± 0.08) μM. The apoptosis rates increased from 9.5% to 17.27%, 30.14%, and 66.46% with an increase in MCPT concentrations from 0, 0.19, 0.38, to 0.95 μM, respectively. The ratio of Bax/Bcl-2 also increased from 1 to 1.61, 2.43, and 4.57, respectively. Bax and Bcl-2 are crucial to the mitochondria pathway. The results indicate that the mitochondria pathway may be involed in MCPT-induced murine sarcoma S180 apoptosis.

Key words: Bcl-2 Family Protein, Camptothecin Derivative, Intrinsic Apoptotic Pathway

Introduction

Apoptosis, also referred to as cell-programmed suicide, is a form of cell death. An abnormality in apoptosis causes neurodegeneration and malignancy, which may lead to uncontrolled cell growth. Therefore, the induction of cancer cell apoptosis is a very useful method of cancer therapy. Apoptosis is a complex endogenous gene-controlled event (Yang *et al*., 2009), and the Bcl-2 family proteins play an important role in the intrinsic apoptotic pathway (mitochondria pathway). Bcl-2 and Bax proteins, which are members of the Bcl-2 protein family, are crucial to this pathway (Youle and Strasser, 2008). The Bcl-2 protein is the first proto-oncogene and is overexpressed in malignancy (Buolamwini, 1999). Bcl-2 reportedly blocks the release of cytochrome c from the mitochondria (Yang *et al*., 1997), arresting the mitochondria pathway. In contrary, The Bax protein shows pro-apoptotic effect in the mitochondria pathway. Under homeostatic conditions, Bcl-2 maintains mitochondrial integrity by preventing Bax from causing mitochondrial damage (Duprez *et al*., 2009). Therefore, the effects of Bax and Bcl-2 in the mitochondria pathway should be considered simultaneously.

Nothapodytes foetida syn. *Mappia foetida* is a species of the family Icacinaceae, which is mainly distributed in India (Govindachari and Viswanathan, 1972) and Taiwan (Wu *et al*., 1996). Its phytochemistry has been extensively researched locally because it is a potential source of anticancer drugs. A number of camptothecinoids (CPTs), other alkaloids, and phytochemicals have been reported from this plant (Wu *et al*., 2008). 9-Methoxycamptothecin (MCPT) is the main antitumour compound in *Nothapodytes foetida* (Hsiao *et al*., 2008). The NCI database confirmed that MCPT has a broad range of antitumour activities against various human tumour cell lines *in vitro* and *in vivo* (http://dtp.cancer.gov/index.html). MCPT was reported to inhibit topoisomerase I (Pommier *et al*., 1991). Generally, potent antitumour coumpounds usually display multiple antitumour mechanisms.

Cell death in mammals can be classified into different types, namely, apoptosis, necrosis, and autophagy (Portugal *et al*., 2009). MCPT is an effective antitumour compound. However, the specific mechanism for inducing cell death in tumour cells treated with MCPT has not been elucidated, trials testing MCPT against murine sarcoma S180 cells have not been performed, and MCPT-induced tumour cell apoptosis has not been reported. In the present study, MCPT and camptothecin (CPT) were isolated from *N. foetida* and their antitumour activities were tested on murine sarcoma S180 cells. Annexin V-FITC/propidium iodide (PI) double staining assays were employed to detect MCPT-induced murine sarcoma S180 cell apoptosis, and real-time PCR was employed to investigate the expression of Bax and Bcl-2 genes.

Material and Methods

Plant material

The stems of *N. foetida* were collected from Hubei Province, China in October 2008. A voucher specimen (Yue Dai 001010) was deposited at the Guangxi Institute of Botany, Chinese Academy of Sciences (Guilin, China).

Reagents and instrumentation

RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from HyClone (Logan, USA). The apoptosis detection kit was purchased from Key Gen Biotech Co. (Nanjing, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, USA). The ReverTra Ace qPCR RT kit was purchased from Toyobo (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) and methanol were purchased from Merck & Co. Inc. (Darmstadt, Germany). Murine sarcoma S180 ascites cells were obtained from the Cancer Research Institute of Hubei Province (Wuhan, China). All other chemicals were purchased from a local chemical market (Wuhan, China).

A Nikon Alphaphot-2 YSZ microscope was used for counting cells (Tokyo, Japan); an enzyme-linked immunosorbent assay meter (Tecan Group Ltd., Mannedorf, Switzerland) for the MTT assay; BD FACSCalibur and CellQuest software (Franklin Lakes, USA) for the cell apoptosis assay; an ABI Prism 7000 Sequence Detector System (Norwalk, USA) for real-time PCR; an AV-400 nuclear magnetic resonance (NMR) instrument (Brucker Co., Fällanden, Switzerland) for NMR spectroscopy assay; an Agilent 1100 HPLC (Santa Clara, USA) instrument, an ESI spectrometer (Agilent G2440 MSD-Trap-XCT ion trap mass spectrometer), and an Agilent chromatographic column (XDB-C_{18}, 4.6 × 150 mm, 5 μm) for liquid chromatography-mass spectrometry (LC-MS).

Extraction and isolation

N. foetida stems were powdered and extracted with methanol. The extracts were concentrated and further fractionated by liquid-liquid extraction with aqueous hydrochloric acid solution (pH 3–4) and dichloromethane. The dichloromethane fraction was concentrated and subsequently separated using silica gel eluted with methanol/dichloromethane (0:100–20:80) to produce two compounds of over 95% purity. Compound identification was performed by NMR spectroscopy and LC-MS.

Cell culture

The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humid atmosphere containing 5% CO_2.

MTT assay

The cytotoxicity assay was performed using the MTT method (Wang *et al*., 2006). The cells were incubated in 96-well plates at a density of $5 \cdot 10^3$ cells per well and exposed for 24 h to a series of varying concentrations of MCPT and CPT. Next, 5 μl of MTT (5 mg/ml) were added to each well. The plates were incubated for another 4 h at 37 °C under 5% CO_2. The murine sarcoma S180 cells were then collected and solubilized for 15 min with 150 μl DMSO. The absorbance at 570 nm was determined using an enzyme mark instrument.

Flow cytometric analysis

The apoptosis-mediated deaths of murine sarcoma S180 cells treated with varying concentrations of MCPT were examined using an Annexin V-FITC/PI apoptosis detection kit (Annexin V-FITC, PI, and binding buffer) according to the manufacturer's instructions. After 24 h of treat-

ment with 0, 0.19, 0.38, and 0.95 μM MCPT, the murine sarcoma S180 cells were collected and washed twice in cold phosphate buffered saline (PBS). Then $2 \cdot 10^5$ cells were collected, added to 500 μl binding buffer, and stained with 5 μl Annexin V-FITC and 5 μl PI for 10 min at 25 °C in the dark. Finally, flow cytometry was used to quantify cell death induced by MCPT. Data acquisition and analysis were performed using BD FACSCalibur and CellQuest software.

Quantitative real-time PCR

Total RNA of murine sarcoma S180 cells treated for 24 h with different concentrations of MCPT were extracted with TRIzol reagent and confirmed by quantitative real-time PCR with the ReverTra Ace qPCR RT kit, following the manufacturer's protocols. Quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detector System. The specific primer pairs included Bcl-2 (forward, 5'-CAT TGG GAA GTT TCA AAT CAG C-3', and reverse, 5'-CTT TGC ATT CTT GGA CGA GG-3'), Bax (forward, 5'-TTG CTT CAG GGT TTC ATC CA-3', and reverse, 5'-CAG CCT TGA GCA CCA GTT TG-3'), and actin (forward, 5'-GTC CAC CGC AAA TGC TTC TA-3', and reverse, 5'-TGC TGT CAC CTT CAC CGT TC-3'). The PCR mixture was initially pre-heated for 2 min each at 50 and 95 °C before undergoing 40 cycles of amplification. The amplification program consisted of incubation at 95 °C for 15 s, 58 °C for 15 s, 72 °C for 45 s, and 72 °C for 10 min. All determinations were performed in triplicate.

Statistical analysis

All data were expressed as means ± SD. Statistical analyses were performed using an unpaired, two-tailed Student *t*-test. All comparisons were made relative to untreated controls, and significance differences were indicated by $*P < 0.01$.

Camptothecin (CPT, **1**): Yellow powder. – ESI-MS: m/z = 349 $[M+1]^+$. – ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 5.287 (2H, s, H-5), 8.694 (1H, s, H-7), 8.130 (1H, d, J = 8 Hz, H-9), 7.716 (1H, ddd, H-10), 7.870 (1H, ddd, H-11), 8.175 (1H, d, J = 8.4 Hz, H-12), 7.353 (1H, s, H-14), 5.435 (2H, s, H-17), 0.890 (3H, t, H-18), 1.877 (2H, m, H-19), 6.549 (1H, s, OH). – ^{13}C NMR (DMSO-d_6, 400 MHz): δ = 153.02 (C-2), 145.95 (C-3), 50.69 (C-5), 130.29 (C-6), 132.01 (C-7), 128.42 (C-8), 128.97 (C-9), 128.11 (C-10), 130.85 (C-11), 129.50 (C-12), 148.4 (C-13), 97.16 (C-14), 150.46 (C-15), 119.53 (C-16), 157.29 (C-16a), 65.72 (C-17), 8.24 (C-18), 30.76 (C-19), 72.84 (C-20), 172.93 (C-21).

9-Methoxycamptothecin (MCPT, **2**): Yellow powder. – ESI-MS: m/z = 379 $[M+1]^+$. – ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 5.143 (2H, s, H-5), 8.740 (1H, s, H-7), 7.088 (1H, s, H-10), 7.670 (1H, ddd, H-11), 7.713 (1H, dd, H-12), 7.278 (1H, s, H-14), 5.420 (2H, s, H-17), 0.902 (3H, t, H-18), 1.871 (2H, m, H-19), 6.515 (1H, s, OH), 4.014 (3H, s, OMe). – ^{13}C NMR (DMSO-d_6, 400 MHz): δ = 152.93 (C-2), 145.82 (C-3), 50.81 (C-5), 129.32 (C-6), 126.31 (C-7), 120.31 (C-8), 155.24 (C-9), 106.26 (C-10), 130.94 (C-11), 121.42 (C-12), 149.13 (C-13), 97.13 (C-14), 150.37 (C-15), 119.44 (C-16), 157.21 (C-16a), 65.72 (C-17), 8.22 (C-18), 30.81 (C-19), 72.83 (C-20), 172.92 (C-21), 56.53 (OMe).

Results and Discussion

Structure elucidation of the compounds

The NMR spectra of compounds **1** and **2** isolated from *N. foetida* were identified as those of CPT and MCPT (Zhou *et al.*, 2000). The *N. foetida* samples from Hubei Province, China are identical to *N. foetida* found in India (Srinivas and Das, 2003).

Antitumour activity in vitro

The MTT assay was used to examine the inhibitory effect of MCPT on tumour cells; CPT

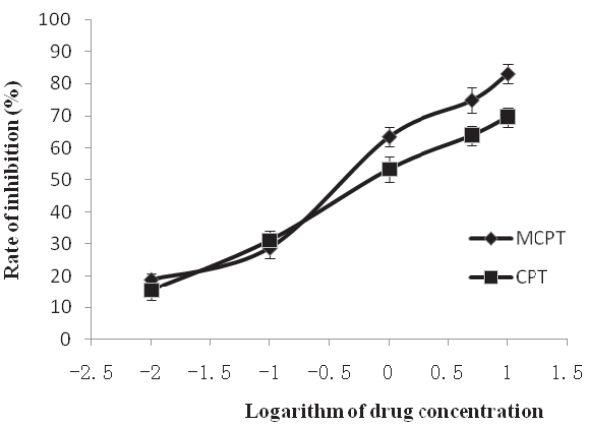

Fig. 1. Comparison of the inhibitory effects of MCPT and CPT on murine sarcoma S180 cells. Data are shown as means ± SD (n = 3).

was used as positive control. Fig. 1 shows that following 24 h of treatment, MCPT effectively decreased the viability of murine sarcoma S180 cells, as shown by an IC_{50} value of (0.385 ± 0.08) μM. After the same duration of treatment, CPT showed an IC_{50} value of (0.904 ± 0.14) μM. These results indicate that MCPT can significantly inhibit the growth of murine sarcoma S180 cells in a dose-dependent manner and has better antitumour activity than CPT. The antitumour activities of CPT derivatives have attracted substantial attention for years. Extensive studies on the synthesis of CPT analogues and their structure-activity relationships have emerged and suggest that C9- or lipophilic substitution of CPT can increase the antitumour activity (Huang *et al.*, 2007; Van Hattum *et al.*, 2000). MCPT is a C9-substituted lipophilic derivative of CPT, and its remarkable activity confirms the structure-activity relationship of CPT.

MCPT-induced apoptosis in murine sarcoma S180 cells

To confirm the apoptosis induced by MCPT, Annexin V/PI staining was performed. As shown in Fig. 2, the number of early and middle apoptotic cells increased from 9.5% to 17.27%, 30.14%, and 66.46% after 24 h of treatment with 0, 0.19, 0.38, and 0.95 μM MCPT, respectively. In contrast, the number of early and middle apoptotic cells only increased from 7.17% to 10.9%, 12.84%, and 17.59% after 24 h of treatment with 0, 0.45, 0.91, and 2.7 μM CPT, respectively (data not shown).

The major hallmark of apoptosis is translocation of phosphatidylserine from the inner leaflet of the phospholipid bilayer to the cell surface (Zeng *et al.*, 2009). The Annexin V/PI staining assay is based on this phenomenon. Flow cytometry data indicate that MCPT induced the translocation of phosphatidylserine from the inner leaflet of the phospholipid bilayer to the cell surface. These results indicate that MCPT induced apoptosis in murine sarcoma S180 cells. The apoptosis rate induced by MCPT was by 66.46% higher than that induced by either CPT or its derivatives (Ju *et al.*, 2007; Togano *et al.*, 2009; Wang *et al.*, 2008). Similar to CPT and its derivatives, MCPT is a topoisomerase I inhibitor (Pommier *et al.*, 1991; Lauria *et al.*, 2007), but yields better apoptosis rates. Therefore,

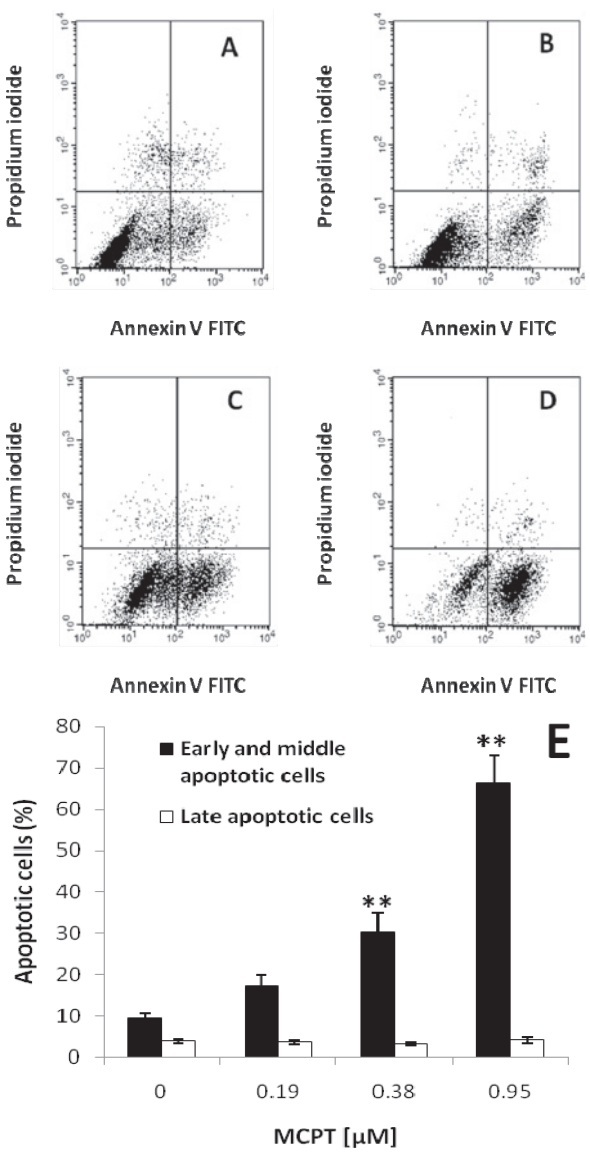

Fig. 2. Annexin V/PI double-staining assay of murine sarcoma S180 cells treated with (A) 0 μM MCPT, (B) 0.19 μM MCPT, (C) 0.38 μM MCPT, (D) 0.95 μM MCPT. The *Y*-axis represents the PI-labeled population, whereas the *X*-axis represents the FITC-labeled annexin V positive cells. The lower left portion of the fluorocytogram (An-, PI-) shows normal cells, whereas the lower right portion of the fluorocytogram (An+, PI-) shows early and median apoptotic cells. The upper right portion of the fluorocytogram (An+, PI+) shows late apoptotic cells. (E) The apoptotic rates of murine sarcoma S180 cells induced by MCPT. **, Significantly different from the corresponding mean value of untreated control groups at $P < 0.01$.

MCPT may be involved in other mechanisms aside from topoisomerase I inhibition.

MCPT-induced Bax protein level increase in murine sarcoma S180 cells

Quantitative real-time PCR was performed 24 h after the murine sarcoma S180 cells were treated with MCPT. The results show that the RNA levels of Bcl-2 in the treatment groups were lower than those in the control groups. The RNA levels of Bax increased from 1 to 1.13, 1.82, and 3.79 after treatment with 0, 0.19, 0.38, and 0.95 μM MCPT (Fig. 3), resulting in dose-dependent Bax/Bcl-2 ratios of 1, 1.61, 2.43, and 4.57, respectively.

Apoptosis is a form of cell death that involves the mitochondria pathway and extracellular pathways. The mitochondria pathway is activated by the Bcl-2 family of proteins, which includes anti-apoptotic genes, such as Bcl-2, and pro-apoptotic genes, such as Bax (Duprez *et al.*, 2009). There is evidence that an increase in the Bax/Bcl-2 ratio results in activation of apoptosis (Wiebe *et al.*, 2010). The results suggest that the genes Bax and Bcl-2 activate apoptosis induced by MCPT on murine sarcoma S180 cells. They also indicate that the mitochondria pathway is involved in apoptosis.

The CPT derivatives irinotecan and SN-38 have been applied clinically as topoisomerase inhibitors. Irinotecan and SN-38 are usually combined with other drugs to obtain desired levels of antitumour activity (Miettinen and Ylikomi, 2009). These synergistic drugs function by increasing the Bax/Bcl-2 ratio, which in turn triggers tumour cell apoptosis (Chen *et al.*, 2003). MCPT, a topoisomerase inhibitor, does not require combination with other drugs to increase the ratio of Bax and Bcl-2 successfully.

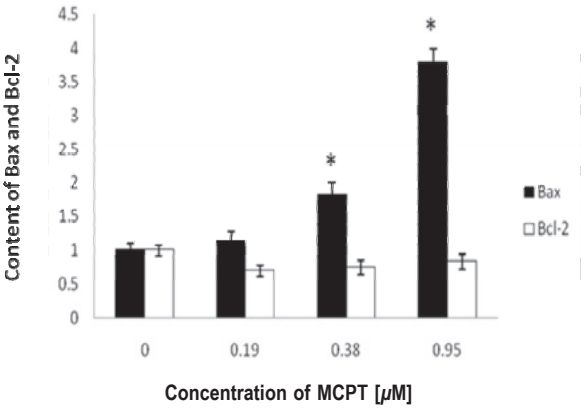

Fig. 3. Changes in the expression of Bax mRNA and Bcl-2 mRNA in murine sarcoma S180 cells treated with MCPT. The results are expressed as means ± SD (n = 3). $P < 0.01$, compared with 0 μM.

Conclusion

MCPT isolated from *N. foetida* showed good antitumour activity and induced a high apoptotic rate in murine sarcoma S180 cells. MCPT also increased the Bax/Bcl-2 ratio in these cells. This mechanism is also exhibited by other topoisomerase inhibitors in combination with other antitumour agents. The mechanisms of Bax- and Bcl-2-mediated apoptosis induced by MCPT, however, are not yet fully understood, and further studies are necessary. These findings indicate that the mitochondria pathway is involved in MCPT-induced murine sarcoma S180 apoptosis.

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