Effects of antitumor compounds isolated from *Pteris semipinnata* L on DNA topoisomerases and cell cycle of HL-60 cells[1]

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KEY WORDS *Pteris semipinnata* L; diterpenes; DNA topoisomerase; cell cycle; genistein; HL-60 cells; drug synergism; antineoplastic agents

ABSTRACT

AIM: To study the effect of the antitumor compounds 5F, 6F, and A from *Pteris semipinnata* L on the activities of DNA topoisomerases and cell cycle of HL-60 cells, and the synergism of compound 6F in combination with genistein *in vitro*. **METHODS**: DNA topoisomerases were isolated from HL-60 cell lines, and supercoiled pBR322 DNA was used as substrate to determine the activities of DNA topoisomerase Ⅰ and Ⅱ. Cell cycle was analyzed by flow cytometry (FCM). Cytotoxicity assay was tested by MTT method. **RESULTS**: Compounds 5F, 6F, and A inhibited the activities of DNA topoisomerase Ⅰ and Ⅱ. After exposure of the cells to compound 6F, an increase in cells in the S and G_2/M phases and a decrease in cells in the G_0/G_1 phase of the cell cycle were observed. At low concentrations (57.8 and 115.6 $nmol \cdot L^{-1}$), compound 6F enhanced the cytotoxicity against HL-60 cell line in combination with genistein, q values were > 1.15. The enhancement times of 57.8 and 115.6 $nmol \cdot L^{-1}$ of 6F by genistein were 2.60 and 4.65, respectively. **CONCLUSION**: Compounds 5F, 6F, and A inhibited the activities of DNA topoisomerases of HL-60 cells. Compound 6F increased the number of cells in S and G_2/M phases, decreased the population of G_0/G_1 phase cells, and enhanced the cytotoxicity of genistein, which had synergism with 6F in antitumor action.

INDRODUCTION

Pteris semipinnata L (*Ps*L), a Chinese traditional herb wildly distributed in Southern China, is used to treat hepatitis, enteritis, and snake bite[1]. The ethanolic extract of the whole plant of *Ps*L had an antitumor activity[2]. Our studies have shown that constituents isolated from *Ps*L had a strong cytotoxicity against human promyelocytic leukemia cells (HL-60), gastric adenocarcinoma cell MGC-803, lung adenocarcinoma cell line SPC-A-1, nasopharyngeal carcinoma cell line in low differentiation (CNE-2Z), liver adenocarcinoma cell line BEL-7402, and HePG Ⅱ[3].

DNA topoisomerases are enzymes that alter the topological structure of DNA by strand breakage and rejoining[4]. It is thought that inhibitors of DNA topoisomerases are potential antitumor agents. In recent years, many scholars deemed DNA topoisomerase as a target to study antitumor agents. In this report, we study whether the active constituents of *Ps*L, which are diterpenoid[3], inhibit the catalytic activity of DNA topoisomerases of HL-60 cells, affect the cell cycle distribution, and have a synergism of compound 6F in combination with genistein *in vitro* on HL-60 cells.

MATERIALS AND METHODS

Materials Compounds 5F (ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid), 6F (ent-7α, 9-dihydroxy-15-oxo-kaur-16-en-19, 6β-olide), and A (ent-7α, 11α-dihydroxy-15-oxo-kaur-16-en-19, 6β-olide) isolated from *Pteris semipinnata* were purified and their structures were identified by the Laboratory

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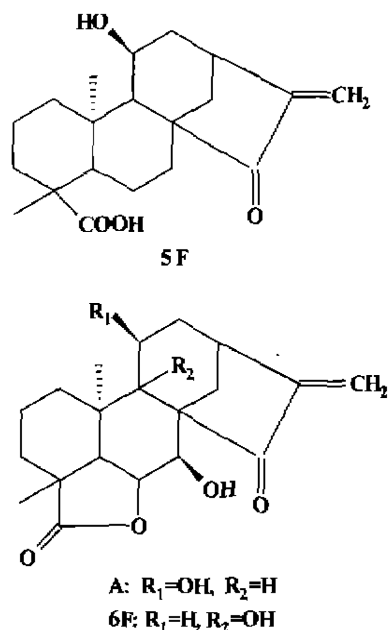

of Phytochemistry, Guangdong Medical College. Genistein, etoposide (Vp-16) camptothecin (Cam), plasmid pBR322 DNA, and phenylmethylsulfonyl fluorid (PMSF) were purchased from Sigma. Tetrazolium salt (MTT) was from Serva.

Preparation of nuclear extracts Nuclear extracts were prepared from $(5-10)\times 10^7$ exponentially growing HL-60 cells[5]. The amount of protein was determined using dye method[6]. The supernatant was stored in the presence of BSA ($1\ g\cdot L^{-1}$) in a mixture of glycerol: nucleus buffer (1:1) at -20 ℃ and used within 2 wk.

Assessment of topoisomerase Ⅰ and Ⅱ in nuclear extracts Approximately 0.3 μg of supercoiled pBR322 DNA was used as a substrate in a final volume of 20 μL. Reaction occured at 37 ℃ for 30 min in a buffer containing Tris-HCl 50 $mmol\cdot L^{-1}$ (pH 7.5), KCl 100 $mmol\cdot L^{-1}$, $MgCl_2$ 10 $mmol\cdot L^{-1}$, edetic acid 0.5 $mmol\cdot L^{-1}$, dithiothreitol 0.5 $mmol\cdot L^{-1}$, and BSA 30 $mg\cdot L^{-1}$. For type Ⅱ DNA topoisomerase ATP 1 $mmol\cdot L^{-1}$ was added[5,7]. Reaction were terminated by addition of SDS, bromophenol blue, and sucrose (1 %, 0.05 %, and 10 % final concentration, respectively). The samples were electrophoresed in 1 % agarose gels at 2 $V\cdot cm^{-1}$ for 10 h in Tris-borate, edetic acid buffer, pH 8.0. Ethidium bromide-stained gels were photographed under UV light. The topoisomerase Ⅰ and Ⅱ activity in a nuclear extract were expressed as the amount of protein required to relax 100 % of the supercoiled pBR322 DNA.

Analysis of cell cycle progression HL-60 cells were plated at a density of 5×10^8 $cells\cdot L^{-1}$ of medium in 60-mm diameter dishes. Cells were taken after 24 h from culture dishes by centrifugation. After washing with ice-cold PBS, cells were suspended in about 0.5 mL of PBS, 0.8 mL of solutions containing 0.1 % Triton X-100, RNase 20 $mg\cdot L^{-1}$, and propidium iodide 50 $mg\cdot L^{-1}$ were added. The cells were kept at 4 ℃ for 30 min. The suspension was filtered through 50 μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL, Coulter Co, Miami FL). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software.

Cytotoxicity For the determination of cytotoxic effect of the tested compounds, MTT method was used[8]. HL-60 cells were resuspended at 2×10^8 $cells\cdot L^{-1}$ in complete medium and then 90 μL aliquots were dispensed into 96-well dishes, 10 μL of drugs were then planted. Each concentration was planted in 4 wells. Plates were incubated at 37 ℃ in a humidified 5 % CO_2-air mixture for 24 h. An aliquot of 20 μL of MTT stock solution ($5\ g\cdot L^{-1}$) was added to each well and the plate was incubated at 37 ℃ for 4 h. To each well 100 μL 20 % SDS and 50 % DMF (dimethyl formamide) solution were added to solubilize the MTT formazan crystal at 37 ℃. After 3 – 5 h, the absorbance (A) of each well was measured with a Microplate Reader (Bio-Rad, model 450) equipped with 570 nm and 450 nm filters. The 570 nm was major filter and the 450 nm was reference filter. The inhibitory rate (%) of agents for cells was calculated:

$$\text{Inhibitory rate (\%)} = 1 - \frac{[A_{570}-A_{450}]\text{treated}}{[A_{570}-A_{450}]\text{control}} \times 100$$

To determine whether there was antagonism, synergism, or enhancement after 2 agents were used together, Jin formula was used to evaluate the q value.

$$q = \frac{E_{A+B}}{E_A + (1-E_A)\cdot E_B}$$

E_{A+B} is the inhibitory rate of 2 agents used in combination. E_A and E_B were the inhibitory rate of the agents used respectively. $q < 0.85$ indicated antagonism, $q > 1.15$ indicated synergism, and $1.15 \geqslant q \geqslant 0.85$ indicated that it had enhancement after 2 agents were used together.

RESULTS

Effect of 5F, 6F, and A on topoisomerase I activity in NaCl 0.35 mol · L^{-1} nuclear extracts Supercoiled (form I) pBR322 DNA was completely relaxed by nuclear extract proteins (0.6 μg) after a 30-min incubation, but the relaxation of pBR322 DNA by topoisomerase was gradually inhibited by compound 6F at the concentration from 0.01 mg · L^{-1} and was inhibited completely at 10 mg · L^{-1}. Compounds 5F and A inhibited the relaxation of pBR322 DNA by topoisomerase gradually at the concentration from 1 mg · L^{-1} and completely at the same concentration as 6F (Fig 1).

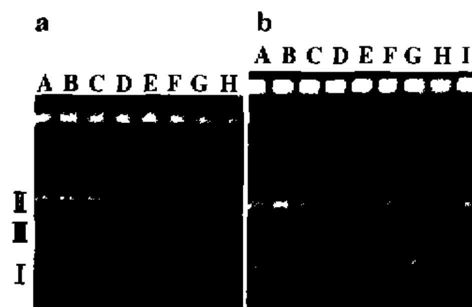

Fig 1. Effect of compounds 5F, 6F, and A on activity of topo I of HL-60 cells. (a) Supercoiled pBR322 DNA 0.3 μg (lane H) was incubated with 600 ng nuclear protein alone (lane G), or in the presence of either 6F 100, 10, 1, 0.1, and 0.01 mg · L^{-1} (lane B-F) or Cam 100 mg · L^{-1} (lane A). (b) Supercoiled pBR322 DNA 0.3 μg (lane I) was incubated with 600 ng nuclear protein alone (lane H) or in the presence of either Cam 100 mg · L^{-1} (lane G) or A 100, 10, 1 mg · L^{-1} and 5F 100, 10, 1 mg · L^{-1} (lane A - F). I, II, and III indicated the position of supercoil, nicked and linear pBR322 DNA, respectively.

Inhibition of DNA topoisomerase II by 5F, 6F, and A There were two forms of antitumor agents affecting topoisomerase II: promoting or repressing the supercoiled DNA relaxation activity or the DNA cleavable reaction that topo II induces. The former repressed the rejoining of cleavable DNA and the later directly suppressed the DNA breaking reaction induced by topo II. Compounds 5F, 6F, and A inhibited the DNA break reaction that DNA topo II catalyzed. 6F 0.01 mg · L^{-1} only inhibited the topo II and when the concentration was increased to 10 mg · L^{-1}, topo II activity was lost completely (Fig 2). It was similar at 5F 100 mg · L^{-1} and A 10 mg · L^{-1}.

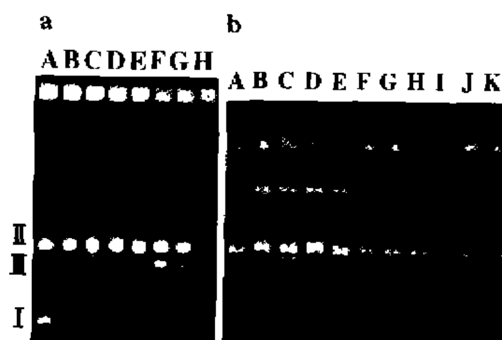

Fig 2. Effect of compounds 5F, 6F, and A on the activity of topo II of HL-60 cells. (a) Supercoiled pBR322 DNA 0.3 μg (lane G) was incubated with 600 ng nuclear protein alone (lane H) or in the presence of either 6F 100, 10, 1, 0.1, 0.01 mg · L^{-1} (lane A-E) or VP-16 100 mg · L^{-1} (lane F). (b) Supercoiled pBR322 DNA 0.3 μg (lane K) was incubated with 600 ng nuclear protein alone (lane J) or in the presence of either VP-16 100 mg · L^{-1} (lane E) or A 100, 10, 1 mg · L^{-1} (line F - I) and 5F 100, 10, 1 mg · L^{-1} (lane A - D). I, II, and III indicated the position of supercoil, nicked and linear pBR322 DNA, respectively.

Effects of 6F on cell cycle of HL-60 DNA content of HL-60 nuclei was measured by flow cytometric analysis after 24 h of exposure to 6F. Compound 6F (57.8, 115.7, 173.4, and 231.2 nmol · L^{-1}) increased the number of cells in S phase from 52.3 % to 58.8 %, 71.4 %, 68.1 %, and 68.3 %, respectively, and in G_2/M from 15.6 % to 20.8 %, 21.6 %, 24.8 %, and 15.3 %, respectively, but comparatively decreased the population of G_0/G_1 cells from 31.8 % to 20.4 %, 7.1 %, 7.1 %, and 16.4 %, respectively. At the concentration of 231.2 nmol · L^{-1}, it had no effect on the percentage of cells in G_2/M phase, while subploidy cells were observed by 19.4 % of total cells. (Tab 1)

Synergism of 6F with genistein The cytotoxic effect of antitumor compound 6F in combination with genistein on HL-60 cells was

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Tab 1. Effect of 6F on cell cycle of HL-60 cells after 24-h treatment. $n=3$ experiments. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

6F/nmol·L^{-1}	% G_0/G_1	% S	% G_2/M
Control	31.8±3.2	52.3±1.6	15.6±1.4
57.8	20.4±0.5^b	58.8±0.8^c	20.8±0.7^b
115.7	7.1±0.5^c	71.4±2.0^c	21.6±2.5^b
173.4	7.1±1.9^c	68.1±3.3^c	24.8±5.3^b
231.2	16.4±3.9^c	68.3±3.4^c	15.3±1.0^a

determined. The inhibitory rate of 6F 57.8 and 115.6 nmol·L^{-1} on HL-60 were 10.7 % and 41.7 %, respectively after treatment for 24 h. When 6F treated cells in combination with genistein, cytotoxicity rates on HL-60 cells were increased, q value >1.15. (Tab 2)

Tab 2. Synergism of 6F in combination with genistein. $n=3-4$ experiments. $\bar{x} \pm s$. Values q in brackets.

Genistin μmol·L^{-1}	Inhibitory rate/% 6F 0	57.8 nmol·L^{-1}	115.6 nmol·L^{-1}
0	0	10.7±1.0 (–)	41.7±1.5 (–)
11.57	5.10±0.14	14.8±0.8 (1.5)	38.0±1.1 (0.8)
23.13	–0.6±0.4	26.8±1.0 (1.8)	36.2±1.0 (0.9)
46.26	2.4±0.5	33.2±0.6 (2.6)	53.9±1.5 (1.2)
92.52	13.1±1.1	55.1±1.6 (2.5)	68.7±1.4 (1.4)
185.04	58.1±0.4	78.9±1.1 (1.3)	86.9±0.8 (1.2)

DISCUSSION

In mammalian cells, two major topoisomerases, type I and II, have been identified, which function by forming enzymes-bridge strand breaks that act as transient gates for the passage of other DNA strands[9,10]. Topo I plays a crucial role in DNA replication, transcription and rejoining, it only breaks the single DNA strands and is an ATP-independent enzyme, and physiological concentration of ATP can inhibit its activity. Topo II can break single or double strands and rejoin DNA strands, it needs ATP to provide energy in the reaction catalyzed[11,12]. It is an essential enzyme for eukaryotic cell survival and plays an important role in eukaryotic cell growth and division, and is required for DNA replication, transcription, recombination, and mitosis for chromosome condensation and chromatid disjunction and may be involved in the removal of DNA supercoils. In recent years, many topoisomerase inhibitors have been found, most of them have been used as antitumor agents clinically.

The most commonly used antineoplastic topoisomerase inhibitors inhibited the function of enzyme through stimulating the cleavable complex formation of topoisomerases and DNA and stabilizing the cleavable complexs. Compounds 5F, 6F, and A inhibited the activity of both topo I and topo II, but the fashion in which they inhibited topo II is different from other antitumor drugs, such as adriamycin (ADM), daunomycin (DNR), etoposide (Vp-16), and so on. They do not promote but suppress the formation of cleavable complexes between DNA topoisomrase II and DNA. This suggests that compounds 5F, 6F, and A are catalytic inhibitors of topo II, which appears to exhibit toxicity by blocking the activity of the enzyme.

It is known[13] that genistein can inhibit the activity of topo II, and further to induce DNA topoisomerase II-mediated double strand DNA breaks and stabilize this intermediate DNA-enzyme "cleavable complex". It was reported[14] that genistein was also able to induce apoptosis and arrest HL-60 cells in G_2/M phase. Our experiments demonstrate that antitumor compound 6F can repress DNA topo II activity, arrest HL-60 cells in S phase strongly and in G_2/M phase lightly, and induce apoptosis of HL-60 cells (data will be shown in other papers). Maybe this is just the reason why 6F has synergism with genistein.

Compounds 5F, 6F, and A are diterpenoids which belong to the type of Kaurane and possess α, β-methylene cyclopentanone moiety. The preliminary study on the cytotoxic activity suggested[15] that α, β-methylene cyclopentanone was attributable to Micheal-type addition reaction with thiol-containing enzyme in cells, and resulted in the loss of activity of enzyme. It is reported that there were essential thiols in topo I. This suggests that it is possible that compounds 5F, 6F, and A inhibit the activity of topo I by direct binding to the enzyme.

REFERENCES

1 Ding HS. The Chinese Medicinal Crytogam. 1st ed. Shanghai: Shanghai Publishing House of Science and Technology; 1982. p 104.
2 Cui L, Liang NC, Chen ZD, Song ZJ. Studies on the anticancer effect and acute toxicity of *Pteris semipinnata*. J Chin Medicinal Materials 1996; 19: 29 – 32.
3 Zhang X, Cui L, Tanaka N, Liang NC. The active constituents and antitumor action of *Pteris semipinnata*. Chin Pharm J 1997; 32: 37 – 8.
4 Brown PO, Cozzarelli NP. A sign inversion mechanism for enzymatic supercoiling of DNA. Science 1979; 206: 1081 – 3.
5 Markovits J, Larsen AK, Segal-Bendirdjian E. Inhibition of DNA topoisomerases Ⅰ and Ⅱ and induction of apoptosis by erbstatin and tyrphostin derivatives. Biochem Pharmacol 1994; 48: 549 – 60.
6 Bradfork MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 – 54.
7 Ellis AL, Altschuler E, Bales E, Hinds M, Mayes J, Soares L, *et al*. Phorbol regulation of topoisomerases Ⅰ and Ⅱ in human eukemia cells. Biochem Pharmacol 1994; 47: 387 – 96.
8 Hansen MB, Nielsen SE, Berg K. Reexamination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989; 119: 203 – 10.
9 Wang JC. DNA topoisomerases. Annu Rev Biochem 1985; 54: 665 – 97.
10 Slichenmyer WJ, Rowinsky EK, Donehower RC, Kaufmann SH. The current status of camptothecin analogues as antitumor agents. J Natl Cancer Inst 1993; 85: 271 – 91.
11 Osheroff N. Biochemical basis for the interaction of type Ⅰ and type Ⅱ topoisomerase with DNA. Pharmacol Ther 1989; 41: 223 – 41.
12 Castrora FJ, Kelly WG. ATP inhibits nuclear and mitochon-drial type Ⅰ topoisomerase from human leukemia cells. Proc Natl Acad Sic USA 1986; 83: 1680 – 4.
13 Markovits J, Lirassier C, Fosse P. Inhibitory effect of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase Ⅱ. Cancer Res 1989; 49: 5111 – 7.
14 Spinozzi F, Pagliaccl MC, Migliorati G The natural tyrosine kinase inhibitor genistein produces cell cycle arrest and apoptosis in jurkat T-leukemia cells. Leuk Res 1994; 18: 431 – 9.
15 Hall IH, Lee KH, Mar EC. Antitunor agents, a proposed mechanism for inhibition of cancer growth by tenulin and helenalin and related cyclopentenones. J Med Chem 1977; 20: 333 – 7.

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半边旗抗肿瘤有效成分对 HL-60 细胞 DNA 拓扑异构酶活性及其细胞周期的影响[1]

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关键词 半边旗; 二萜; DNA 拓扑异构酶; 细胞周期; 金雀异黄素; HL-60 细胞; 药物协同作用; 抗肿瘤药

目的: 研究半边旗(*Pteris semipinnata* L, *Ps*L)有效成分 5F、6F、A 对 HL-60 细胞 DNA 拓扑异构酶活性和细胞周期的影响. **方法**: 应用 pBR322 质粒 DNA 作为底物测定酶的活性; 细胞周期用流式细胞仪测定; 应用噻唑蓝(MTT)法测定药物对细胞生长的抑制率. **结果**: 5F, 6F, A 均能够抑制 DNA 拓扑异构酶Ⅰ, Ⅱ的活性. 化合物 6F 作用细胞 24 h 后,可升高 S 期和 G_2/M 期细胞, 同时降低 G_0/G_1 期细胞. 低浓度 6F (57.8 和 115.6 $nmol \cdot L^{-1}$)与金雀异黄素(Gen)合用可增强它对 HL-60 细胞的杀伤作用, $q > 1.15$. **结论**: 5F, 6F 和 A 明显抑制 HL-60 细胞 DNA 拓扑异构酶的活性; 6F 阻断细胞于 G_2/M 期, 增强 Gen 对 HL-60 细胞的杀伤作用.

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