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Original Research

Effects of spin labeled derivatives of podophyllotoxin on cell cycle and macromolecular synthesis in human lymphoid leukemia Molt 4B cells

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KEY WORDS cell cycle; lymphocytic leukemia; cultured tumor cells; spin labels; podophyllotoxin; etoposide

AIM: To examine the effect of the spin labeled derivatives of podophyllotoxin, *N*-podophyllic acid-*N*″-[4-(2,2,6,6-tetramethyl-1-piperidinyloxy)] thiosemicarbazide (GP4) and 4-[4″-(2″,2″,6″,6″-tetramethyl-1″-piperidinyloxy) amino]-4′-demethylepipodophyllotoxin (GP7) on the cell cycle and macromolecular synthesis of human lymphoid leukemia Molt 4B cells *in vitro*. **METHODS**: MTT assay, ^{3}H incorporation, and flow cytometer were used. **RESULTS**: GP4, GP7, and etoposide 0.02 - 100 mmol·L^{-1} cultured for 48 h inhibited the proliferation of human lymphoid leukemia Molt 4B cells. IC_{50} values of GP4, GP7, and etoposide were 0.11, 4.7, and 1.6 mmol·L^{-1}, respectively. DNA and protein syntheses were obviously suppressed by GP4, GP7, and etoposide 10 mmol·L^{-1} for 48 h. After Molt 4B cells were treated with GP4, GP7, and etoposide 10 mmol·L^{-1} for 6 and 12 h, the mitotic index was increased by GP4 and reduced by GP7 and etoposide. According to flow cytometric BrdU/DNA analysis, GP4 slightly retarded S phase and mainly arrested cell cycle progression in G_2/M phase, whereas GP7 similar to etoposide induced cells accumulated at S phase and retarded the cells in G_2 phase. **CONCLUSION**: GP4 and GP7 inhibit the proliferation of Molt 4B cells, but the mechanisms are different.

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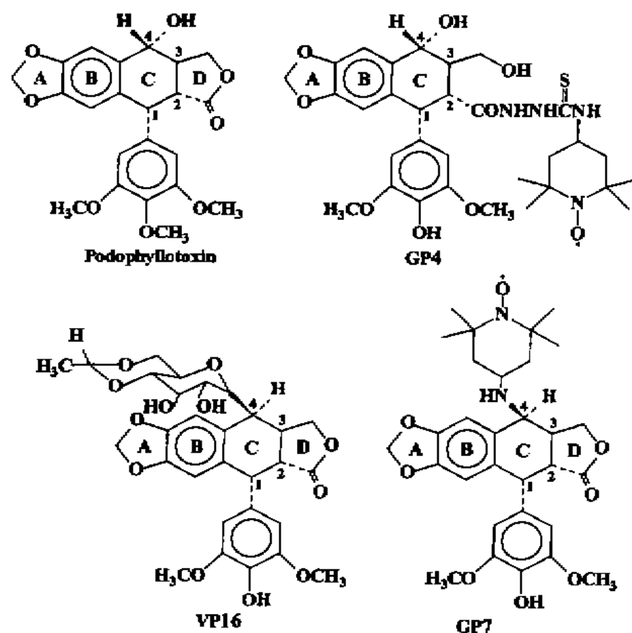
Podophyllotoxin and a number of its derivatives possess antitumor activity. As most antitumor drugs presently in use, including etoposide and teniposide, exhibit a wide range of toxic effect[1,2], it is of interest to find new readily synthesizable drugs with high activity and low toxicity. The spin labeled derivatives of podophyllotoxin, *N*-podophyllic acid-*N*″-[4-(2,2,6,6-tetramethyl-1-piperidinyloxy)] thiosemicarbazide (GP4) and 4-[4″-(2″,2″,6″,6″-tetramethyl-1″-piperidinyloxy) amino]-4′-demethylepipodophyllotoxin (GP7) were synthesized by introducing the nitroxyl radical moiety into podophyllotoxin at different positions[3]. In this paper, we investigated the effects of GP4 and GP7 on cell proliferation, macromolecular synthesis, and cell cycle progression as compared with those of etoposide.



Spin labeled derivatives of podophyllotoxin

MATERIALS AND METHODS

Chemicals and cell culture Starting from podophyllotoxin, isolated from a Chinese medicinal herb *Podophyllum emodi* Wall var *chinesis* Sprague, *N*-Podophyllic acid-*N*″-[4-(2, 2, 6, 6-tetramethyl-1-piperidinyloxy)] thiosemicarbazide (GP4) and 4-[4″-(2″, 2″, 6″, 6″-tetramethyl-1″-piperidinyloxy) amino]-4′-demethylepipodophyllotoxin (GP7) were synthesized as described previously[3]. They were dissolved in 1 % Me_2SO before using. Etoposide, as a reference drug, was purchased from Nippon Kayaku Co Ltd, Tokyo, Japan. Human lymphoid leukemia Molt 4B cells were grown in RPMI-1640 medium (Nissui Pharmaceutical Co Ltd, Tokyo, Japan) with 10 % fetal calf serum, benzylpenicillin (50 kU·L^{-1}), and streptomycin (50 mg·L^{-1}) in a 5 % CO_2 incubator at 37 ℃.

Cell proliferation and mitotic index (MI) A suspension of Molt 4B cells (1×10^8 cells·L^{-1}) was grown in 96-well microtiter plates and incubated at 37 ℃ with or without different concentrations of drugs for 48 h. Then, a tetrazolium-based compound (MTT) (Sigma Chemical Co, St Louis MO) was added to each well and incubated for further 4 h. After centrifugation, Me_2SO was used to solubilize the formazan crystals. The absorbance was read at 540 nm[4]. MI was determined after cells were fixed on a slide and stained with Giemsa's solution. According to counting of 1000 cells on each slide under a light microscope, the percentage of cells in mitotic phase was calculated.

Determination of macromolecular synthesis Molt 4B cells were cultured with or without GP4, GP7, and etoposide 10 mol·L^{-1} for 48 h. Then [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine (Biomedicals Inc, Costa Mesa CA) 37 MBq·L^{-1} was added to the culture medium, and the radioactivities in trichloroacetic acid-insoluble materials were measured at different times through a liquid scintillation counter[5].

Flow cytometric BrdU/DNA analysis Molt 4B cells for analysis of BrdU/DNA were exposed to drugs by 2 methods. In method 1, cells were first treated with or without GP4, GP7, or etoposide 10 mol·L^{-1} for 6 and 12 h. Then bromodeoxyuridine (BrdU) (Sigma Chemical Co) 10 mol·L^{-1} was added to the cells and incubated at 37 ℃ for the final 30 min. The cells were washed twice and fixed in cold 70 % ethanol for at least 30 min. The fixed cells were washed and resuspended in HCl 4 mol·L^{-1} for 30 min. After being washed the cells were resuspended in borax 0.1 mol·L^{-1}, pH 8.5. The cells were again washed twice and treated with 0.5 % Tween-20. Then the cells were washed and resuspended in PBS 0.5 μL. Anti-BrdU FITC (Becton Dickinson Co) 20 μL were added and cells were incubated at 22 ℃ for 30 min. The cells were washed twice and resuspended in PBS 1 mL containing propidium iodide (PI) 5 g·L^{-1} (Sigma Chemical Co) for 15 min. The single cells thus obtained were analyzed by flow cytometry. In method 2, the cells were first pulsed with BrdU, washed twice, and then incubated in medium containing drug. At different times, the progression of BrdU labeled cells in cell cycle was analyzed as above.

RESULTS

All drugs depressed the growth of Molt 4B cells in a concentration-dependent manner. The concentration-response curves were very different. At low concentrations (0.02 – 0.2 mmol·L^{-1}) the inhibiting effect of GP4 appeared to be dependent on the concentration, while at high concentrations (1 – 100 mmol·L^{-1}) the effect of GP4 almost remained at a plateau status. The concentration-response curve of GP7 was a straight line, which was similar to etoposide. At low concentrations, the activity of GP4 was higher than that of GP7 and etoposide. IC_{50} values of GP4, GP7, and etoposide were 0.11 (0.08 – 0.15), 4.7 (3.0 – 7.4), and 1.6 (0.9 – 2.8) mmol·L^{-1}, respectively (Fig 1).

As GP4 and GP7 markedly inhibited the proliferation of cells, it suggested that the mechanism of growth inhibition was attributed to the suppression of DNA and protein syntheses.

After Molt 4B cells were treated with 3 drugs for 6 and 12 h, the cell cycle distribution was markedly changed in a different characteristic (Tab 1).

DNA and protein syntheses in Molt 4B cells were inhibited by treatment of GP4, GP7, and etoposide, but the effect on RNA synthesis was

not obvious (Fig 2).

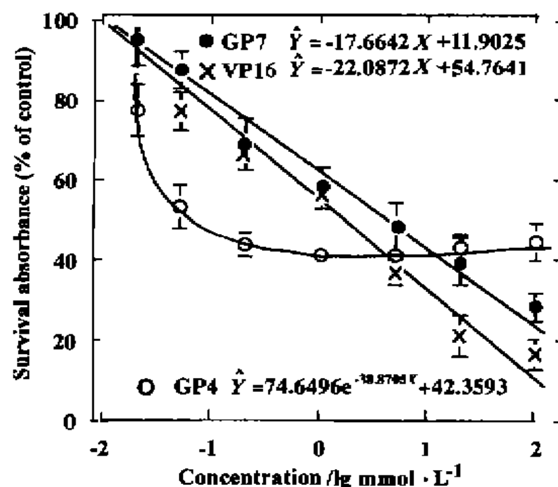


Fig 1. MTT assay following continuous exposures to drugs for 48 h. $\bar{x} \pm s$ of triplicate experiments. $P < 0.01$.

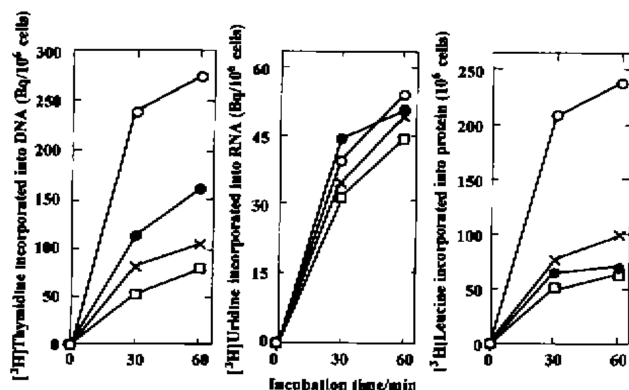


Fig 2. Effect of GP4, GP7, and etoposide on DNA, RNA, and protein syntheses in Molt 4B cells. The cells at initial density of 2.5×10^8 cells · L^{-1} were incubated with GP4 (●), GP7 (×), and etoposide (□) 10 mmol · L^{-1} or without drugs (○) for 48 h before radiolabeled thymidine, uridine or leucine was added. The experiment was repeated twice.

The presence of GP4 during 6- and 12-h incubation resulted in a maximum of the G_2/M accumulation with a slight increase in S phase. The marked increase in S and G_2/M phase was seen after treatment with GP7 and etoposide. GP7, similar to etoposide, not only blocked the cells in S phase but also retarded the cells in G_2/M phase. The data also indicated that GP4 markedly increased the MI of cells, whereas GP7 and etoposide decreased the MI. Molt 4B cells were firstly pulsed with BrdU for 30 min,

Tab 1. Cell cycle distribution and MI after treatment with GP4, GP7, and etoposide. $n = 3$ experiments. $\bar{x} \pm s$.

	Cell cycle phase/%			
	G_1	S	G_2/M	MI
After treatment 6 h				
Control	55.9±5.2	34.7±2.6	9.4±2.9	2.8±0.3
GP4	45.1±6.0	35.5±1.1	19.3±5.0	9.5±0.7
GP7	43.9±5.0	40.7±2.5	15.4±3.8	2.3±0.5
Etoposide	42.1±6.6	45.3±4.5	12.6±3.9	0.5±0.2
After treatment 12 h				
Control	50.5±2.2	39.0±2.5	10.5±0.8	3.1±0.2
GP4	11.4±6.0	45.1±3.0	46.5±4.0	15.7±0.8
GP7	21.7±2.6	59.0±6.6	19.3±2.5	1.2±0.4
Etoposide	20.4±5.2	66.1±3.5	13.5±3.2	0.3±0.2

washed, and then incubated in medium containing drug for various times indicated. Cell cycle progression was determined (Fig 3).

DISCUSSION

As shown in Fig 1, the concentration-response curves of GP4 and GP7 on Molt 4B cells were very different. This means that the mechanism of GP4 and GP7 on Molt 4B cells is also different. GP4, GP7, and etoposide were indicated to inhibit the DNA and protein syntheses. These results are in accordance with those about VP16[6].

Compared with the result of Fig 3, the BrdU labeled cells in control progressed from S phase to G_2/M and G_1 phase at 6 and 12 h, and from G_2/M and G_1 to S phase again at 24 h. One cell cycle was completed in 24 h. In GP4 treated cultures, as time elapsed, the BrdU-labeled cells passed S phase rapidly and accumulated in G_2/M phase. As MI in GP4 treated cells markedly increased, the accumulation of the cells in G_2/M phase was mainly due to the increment of the cells in M phase. In GP7 and etoposide treated cultures, the BrdU-labeled cells were arrested in S and G_2/M phase. As GP7 and etoposide decreased MI, the cells accumulated in G_2/M phase mainly consisted of cells in G_2 phase. The present study showed that GP4 allowed cells to progress through the cell cycle until they were stopped in G_2/M phase. GP7 and etoposide retarded the cells at G_2 and S phase. Although cells treated with GP7 and etoposide were able to complete DNA synthesis, they could not proceed

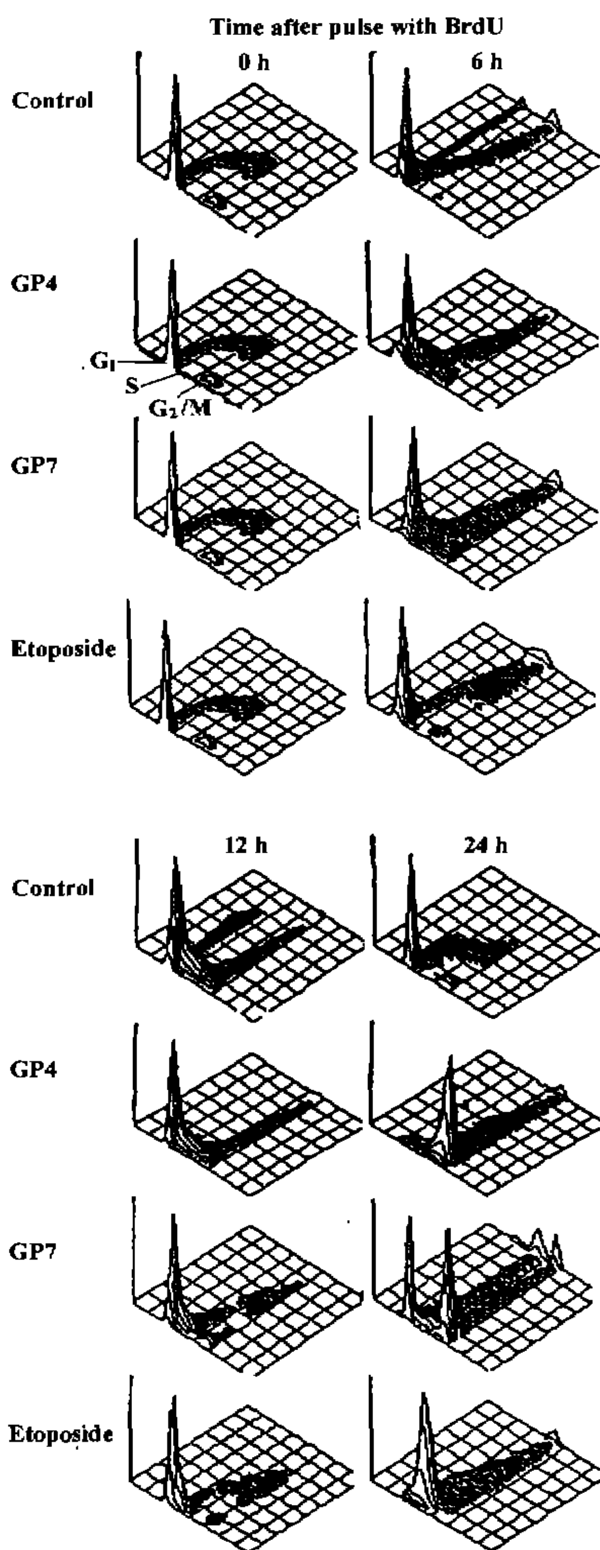


Fig 3. Effect of GP4, GP7, and etoposide 10 mmol · L^{-1} on cell cycle progression. X (PI fluorescence), Y (cell number), and Z axis scale (anti-BrdU fluorescence). The experiment was repeated twice.

to chromosome condensation and mitosis[6].

It has been reported that chemical structur of antitumor drugs are closely correlated to the active mechanisms[7]. Emanuel *et al* reporte that when an iminoxyl radical was introduced in the molecule of an antitumor drug thio-TEPA, tl antitumor activity of the spin labeled drug wa enhanced and the toxicity was much reduced[8] Similar results were also reported in the study new spin labeled derivatives of podophyllotoxin antitumor agents[5,9]. It was reported that GF and GP7 could inhibit the mouse solid tumo Sarcoma-180 and Hepatoma-A *in vivo* ar appeared a lower toxicity than etoposide[3] Since GP4 and GP7 are more readily synthesize than etoposide, they are hopeful of becomir promising antitumor drugs. As the substitutiv position of GP7 is the same as etoposide, it ma be the reason why the action of GP7 is similar etoposide. As GP4 increased MI, it is evider that the chemical modification on D ring podophyllotoxin still reserved the antimitoti activity of podophyllotoxin. These results ma provide insights into structure-activity relationshi and the design of novel derivatives podophyllotoxin useful in cancer chemotherapy.

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自旋标记鬼臼毒素衍生物对人淋巴性白血病 Molt 4B 细胞周期和大分子合成的影响

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关键词 细胞周期; 淋巴细胞性白血病; 培养的肿瘤细胞; 自旋标记物; 鬼臼毒素; 依托泊苷

目的: 证实鬼臼毒素自旋标记物 GP4 和 GP7 体外对 Molt 4B 细胞周期和大分子合成的抑制作用. **方法**: MTT、同位素标记法和流式细胞分光光度仪 BrdU/DNA 分析法. **结果**: GP4, GP7 和依托泊苷(0.02 – 100 mmol·L^{-1}, 48 h)抑制细胞生长量效曲线不同, 其 IC_{50}值分别为 0.11, 4.7, 1.6 mmol·L^{-1}, 并降低 DNA、蛋白质合成. GP4 (10 mmol·L^{-1} 12 h)增加有丝分裂指数, 为对照组的 5 倍; GP7 和依托泊苷都降低有丝分裂指数, 分别为对照组的 1/3 和 1/10. GP4 主要阻止细胞于 G_2/M 期, 而 GP7 与依托泊苷相似, 将细胞阻滞在 S 和 G_2 期. **结论**: GP4 和 GP7 抑制 Molt 4B 细胞生长和大分子合成, 其作用机制不同.

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