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Action of free radical in podophyllic acid piperindyl hydrazone nitroxide radical on its antitumor activity and toxicity[1]

JIA Zheng-Ping, WANG Rui[2] (*Department of Biology, Lanzhou University, Lanzhou 730000, China*)
XIE Jing-Wen, XU Li-Ting
(*Department of Pharmacy, General Hospital of Lanzhou Command of PLA, Lanzhou 730050, China*)

KEY WORDS podophyllotoxin; free radicals; antineoplastic agents; toxicity tests; cultured tumor cells; nucleic acid synthesis inhibitors; cell cycle; mitotic index

ABSTRACT

AIM: To study the action of free radical in the spin-labeled podophyllotoxin derivative, podophyllic acid piperindyl hydrazone nitroxide radical (GP-1) on its antitumor activity and toxicity, by comparison with those of its free radical reduced product, podophyllic acid piperindyl hydrazone (GP-1-H). **METHODS**: After treatment with GP-1 and GP-1-H, the inhibitory effects on the growth of mouse transplantable tumors were determined; MTT formazan formation, [^{3}H]deoxythymidine ([^{3}H]TdR) incorporation, cell cycle progression, and mitotic index of SGC-7901 or L1210 cells were measured; the acute toxicity and immune function of mice were assayed. **RESULTS**: At doses of 1/6 and 1/12 LD_{50}, the inhibitory rates against Lewis lung carcinoma were 60.3 % and 42.1 % (GP-1), 38.9 % and 10.3 % (GP-1-H), respectively; more effective antitumor activity of GP-1 against P388, HePS, and S-180 than that of GP-1-H were found. *In vitro*, GP-1 exhibited more powerful inhibitory effects on the proliferation and DNA synthesis of SGC-7901 and L1210 cells than GP-1-H. GP-1 and GP-1-H arrested the L1210 cells at G_2/M phase with a corresponding decrease of the cells in G_1 phase, and increased the mitotic index of the cells; but the effects of GP-1-H were weaker than those of GP-1. After treatment with doses of 1/4 and 1/8 LD_{50} for 5 d, no significant difference on immune function of mice between GP-1 and GP-1-H was found. **CONCLUSION**: GP-1 had more powerful antitumor activities than GP-1-H. The free radical in the spin-labeled podophyllotoxin derivative, GP-1, played an important role in its antitumor activity.

INTRODUCTION

The nitroxides belong to stable free radicals which are widely used for spin labeling in electron spinning resonance technique[1]. Such compounds trap hydroxyl radical in nonbiological system like aqueous solution[2], and biological system in rat[3]. In recent years, it has been found that introduction of nitroxy radical moiety into some antitumor drugs, such as thiotepa, could result in new agents with pharmacological properties superior to those of the parent compounds[4,5]. Based on these findings, a series of the spin-labeled derivatives of podophyllotoxin had been synthesized by our group, and some of them were proven to have more effective antitumor activity and lower toxicity than VP-16[6-8]. However, it is still unknown what role is acted by the free radical in the synthesized or semi-synthesized spin-labeled derivatives for their antitumor activity and toxicity.

Podophyllic acid piperindyl hydrazone nitroxide radical (GP-1) is one of spin-labeled derivatives of podophyllotoxin synthesized by us[9]. In our previous works[10-12], it was found that GP-1 inhibited the growth of the transplantable mouse tumors, sarcoma 180 (S-180) and Ehrlich carcinoma. *In vitro*, it exhibited remarkable inhibitory effects on the proliferation of human esophageal carcinoma Eca-109

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[2] Correspondence to Prof WANG Rui.
Phn 86-931-891-2567. Fax 86-931-891-3382.
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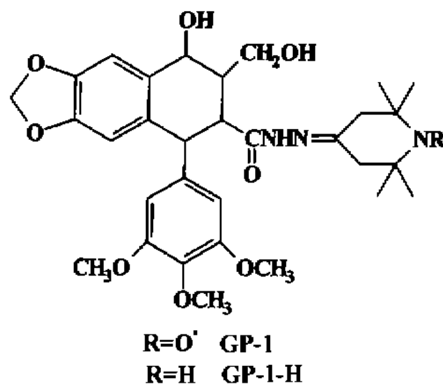
cells and on incorporation of [^{3}H]deoxythymidine ([^{3}H]TdR), [^{3}H]uridine, and [^{3}H]leucine into DNA, RNA, and protein of lymphocyte leukemia L7712 cells. To study the role of the free radical in GP-1 in its antitumor activity and toxicity we synthesized its free radical reduced product, podophyllic acid piperindyl hydrazone (GP-1-H), and compared the antitumor activities and toxicities of GP-1 with those of GP-1-H, so as to find a new way to synthesize new podophyllotoxin derivatives with high antitumor activity and low toxicity.



MATERIALS AND METHODS

Chemicals GP-1 and GP-1-H (99.6 % purity determined by HPLC) were synthesized by the Department of Chemistry, Lanzhou University. They were dissolved in 5 % Me_2SO before use. [^{3}H]Deoxythymidine ([^{3}H]TdR) was purchased from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) was got from the Fluka (Buchs, Switzerland).

Mice and tumors Five - six week-old Kunming, C57/BL, DBA/2, and Balb/c mice (Grade Ⅱ, medical animal № 14-002) were purchased from the *Experimental Animal Center*, *Biological Products* Institute of Lanzhou. The mice were housed five per plastic cage with wood chip bedding in a standard animal room. All mouse transplantable tumors, Lewis lung carcinoma (Lewis), leukemia P388 (P388), solid carcinoma from ascitic hepatoma (HePS), and S-180, were initially supplied by the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences.

Evaluation of antitumor activity Lewis, HePS, and S-180 tumor cells (2×10^6 cells/mouse) were inoculated sc under axillae of mice. One day after the inoculation, 1/6 and 1/12 of the LD_{50} of GP-1 and GP-1-H were injected ip for 10 consecutive days. The same volume of the solvent was injected ip into the control mice. On the next day after the last ip, the mice were anesthetized and killed, the tumors were weighed. P388 cells (1×10^6 cells/mouse) were inoculated ip into DBA/2 mice. One day after the inoculation, 1/6 and 1/12 of the LD_{50} of GP-1 and GP-1-H were injected ip every other day for 5 times. The *survival time was expressed by* $T/C \times 100$ (%), *where* T was the mean survival time of the treated mice, and C was that of control mice.

Spleen weight, thymus weight, antibody production, and LD_{50} Balb/c mice were immunized ip with 5 % sheep red blood cells (SRBC) 0.2 mL/mouse on d 1. Then 1/4 and 1/8 of the LD_{50} of GP-1 and GP-1-H were injected ip daily for 5 d. On d 6, the mice were bled under anesthesia for serum. The spleen and thymus were weighed and evaluated by spleen index (spleen weight/mouse weight) and thymus index (thymus weight/mouse weight).

To determine the antibody-producing ability of spleen cells, the assay of quantitative hemolysis of SRBC (QHS) was used. The spleen cell suspension (1×10^{10} cells·L^{-1}) of the mice after treatment with the agents was prepared. One mL of 0.2 % SRBC and 1 mL of 10 % guinea pig serum were mixed with 1 mL of cell suspension and incubated at 37 ℃ for 1 h. After centrifugation at $1000 \times g$ for 10 min, the supernatants were assayed with a 721 spectrophotometer at 540 nm[13].

To measure the proliferation of mouse splenic lymphocytes, splenocytes were prepared on ice and suspended in RPMI-1640 medium (2×10^{11} cells·L^{-1}). Following a culture with GP-1, GP-1-H, and Con A in a 5 % CO_2 incubator at 37 ℃ for 56 h, [^{3}H]TdR was added to a final concentration of 37 MBq·L^{-1}. After incubation for another 16 h, the cells were harvested, and the incorporated radioactivity was determined[13].

For determination of LD_{50}, seven logarithmically spaced doses were injected ip into 7 groups of mice (1:1, ♂/♀). The LD_{50} values were calculated.

Cell culture and drug treatment Human

gastric adenocarcinoma SGC-7901 (SGC-7901) and leukemia 1210 (L1210) cells were maintained in RPMI-1640 (Gibco) supplemented with 10 % heat-inactivated newborn calf serum, benzylpenicillin 100 $kU \cdot L^{-1}$, streptomycin 100 $mg \cdot L^{-1}$ in a humidified atmosphere containing 5 % CO_2 at 37 ℃. Exponentially growing cells were exposed to GP-1 and GP-1-H 0.08 – 50 $\mu mol \cdot L^{-1}$ for 24 – 72 h.

MTT-microculture tetrazolium assay Cell growth and viability were determined using the MTT-microculture tetrazolium assay[14]. Briefly, 200 μL of SGC-7901 cell suspensions (5×10^7 $cells \cdot L^{-1}$) were dispensed into each well of 96-well flat bottom culture plates (Costar). After a 56-h culture with the agents, the cells were incubated with MTT (250 $mg \cdot L^{-1}$) for another 4 h. Then, the medium was removed, and 200 μL of Me_2SO was added to solubilize the MTT formazan produced by the viable SGC-7901 cells. Absorbance at 540 nm (A_{540}) was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR4000). Cell densities at $(2.5 - 80) \times 10^7$ $cells \cdot L^{-1}$ gave rise to a relatively linear range of absorbance values ($r = 0.989$).

[^{3}H]TdR incorporation The L1210 cell suspension was diluted to 1×10^8 $cells \cdot L^{-1}$ and placed into the wells of 24-well tissue culture plates (Hounslow, UK). Following a 12-h incubation, GP-1 and GP-1-H were added to the medium, and the solvent was added to the control wells. Meanwhile, [^{3}H]TdR (37 kBq/well) was added. After a 24-h incubation, the cells were washed twice by centrifugation at $100 \times g$ for 5 min with cold Hanks' solution and precipitated by 20 % trichloroacetic acid, then collected onto glass fiber filters. The filters were dried and transferred to vials containing scintillation fluid 4 mL, and [^{3}H]TdR incorporation was determined by liquid scintillation. The radioactivity measurement which represented the [^{3}H]TdR incorporated into newly synthesized DNA was expressed per 10^5 cells.

Analysis of cell cycle progression The L1210 cells cultured with GP-1 and GP-1-H for 24 h were washed twice with PBS and fixed in cold 70 % ethanol for 24 h at 0 – 4 ℃. After the removal of ethanol, the cells were incubated in PBS containing RNase A 50 $mg \cdot L^{-1}$ at 37 ℃ for 1 h, and stained with PI 50 $g \cdot L^{-1}$ at 4 ℃ for 30 min, then analyzed by a flow cytometer (Becton Dickinson).

Assay of MI After exposure to GP-1 and GP-1-H for 24 h, the L1210 cells were harvested by centrifugation at $100 \times g$ for 5 min, treated with KCl 75 $mmol \cdot L^{-1}$ for 15 min, fixed by methanol and glacial acetic acid, and stained by Giemsa. The number of cells in mitotic phase was counted with microscope.

Statistical analysis The t-test was used.

RESULTS

MTT formazan product MTT formazan produced by viable SGC-7901 cells was inhibited by GP-1 and GP-1-H in a concentration-dependent manner. At concentrations of 0.08 – 50 $\mu mol \cdot L^{-1}$, the inhibitory rates were 13.9 % – 76.9 % (GP-1) and 1.1 % – 56.8 % (GP-1-H) with IC_{50} of 2.8 (95 % confidence limits 1.2 – 4.4) and 18.0 (95 % confidence limits 5.5 – 40.5) $\mu mol \cdot L^{-1}$, respectively. GP-1 had a more powerful inhibitory effect on SGC-7901 cell proliferation than GP-1-H (Fig 1A).

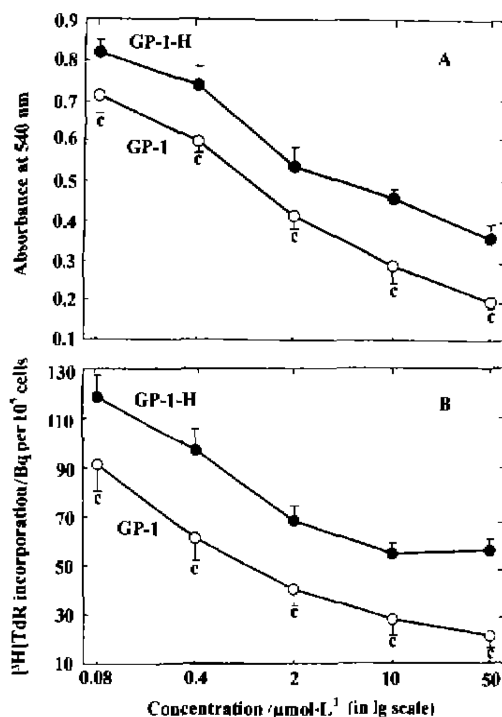


Fig 1. Inhibitory effects of GP-1 and GP-1-H on MTT formazan produced by SGC-7901 cells (A) and [^{3}H]TdR incorporation into DNA of 1210 cells (B) *in vitro*. $n = 6$ wells in each group. $\bar{x} \pm s$. $^cP < 0.01$ *vs* the groups of GP-1-H with corresponding dose.

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[^{3}H]TdR incorporation GP-I exhibited a more potent inhibitory effect on the [^{3}H]TdR incorporation into newly synthesized DNA of L1210 cells than GP-I-H. After treatment with GP-I and GP-I-H 0.08 – 50 μmol · L^{-1} for 24 h, the inhibitory rates were 27.8 % – 83.2 % and 6.3 % – 71.5 % with IC_{50} of 0.6 (95 % confidence limits – 1.2 – 2.4) and 4.9 (95 % confidence limits 3.2 – 6.6) μmol · L^{-1}, respectively (Fig 1B).

Cell cycle progression and MI After exposure to various concentrations of GP-1 for 24 h, the cell cycle distribution was markedly changed. The presence of GP-1 resulted in an accumulation of the cells in G_2/M phases in a concentration-dependent manner; and the cells in G_1 phase and S phase were decreased gradually. The results suggested that GP-I blocked cell cycle progression at the back of M phase. The decrease of the cells in S phase indicated that GP-I also had an inhibitory effect on DNA synthesis of LI2I0 cells. GP-1-H had a similar effect on the cell cycle progression of LI210 cells. However its efficiency of G_2/M arrest was weaker than that of GP-I (Fig 2).

GP-1 and GP-1-H increased MI of L12I0 cells. MI of the cells cultured for 24 h with 0.08 – I μmol · L^{-1} of the compounds were increased by 58.6 % – 713.8 % (GP-1) and –6.9 % – 355.2 % (GP-I-H). (Fig 3).

Antitumor activities against mouse transplantable tumors After treatment with doses of 1/6 and I/I2 LD_{50} for I0 consecutive days, the inhibitory rates of the two agents against Lewis were 60.3 % and 42.I % (GP-I), 38.9 % and 10.3 % (GP-I-H), respectively. A marked difference of inhibitory efficiency was found between GP-I and GP-I-H. GP-1 had also more effective antitumor activities against HePS and S-180 than GP-1-H. The survival time of the P388 mice treated with GP-1 and GP-1-H at doses of 1/6, I/I2 LD_{50} was 2.12, 1.56 (GP-I), and 1.58, I.02 (GP-I-H) times of control (Tab I, 2).

Effects on immune function and acute toxicity in mice Both of GP-1 and GP-I-H, at dose of 1/6 LD_{50}, decreased the spleen index of mice and inhibited the specific antibody formation of mouse splenocytes and the proliferation of mouse splenic lymphocytes activated by Con A *in vitro*. No difference between the two agents was found. At dose

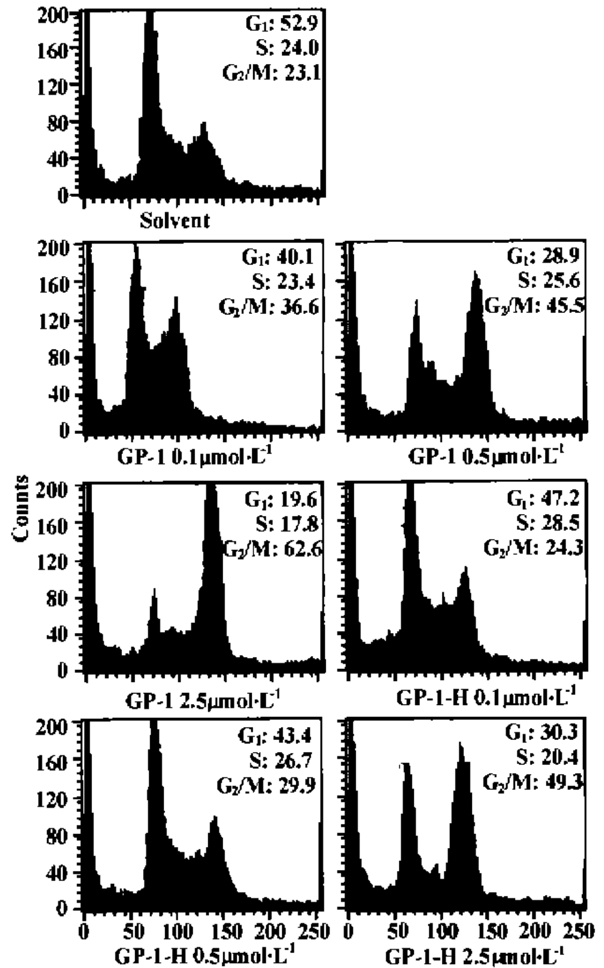

Fig 2. Cell cycle distributions of L1210 cells treated with solvent, GP-1, and GP-1-H.

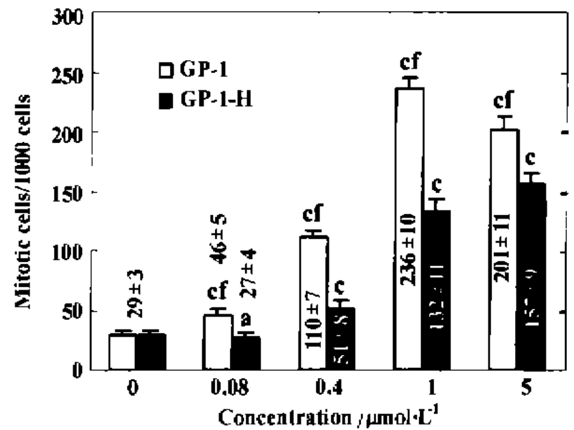

Fig 3. Effects of GP-1 and GP-1-H on mitotic index of L1210 cells. $n = 3$ wells of the cells. $\bar{x} \pm s$.
$^aP > 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* GP-1-H at corresponding dose.

Tab 1. Antitumor activities of GP-1 and its free radical reduced product, GP-1-H on mouse transplantable tumors. 1/6 and 1/12 LD_{50} of the agents were injected ip for 10 d. n = 10 mice. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control. $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* GP-1-H at corresponding dose.

Group	Drugs/ mg·kg^{-1}	Body weight/g (beginning/end)	Tumor weight/ g ($\bar{x}\pm s$)	Inhibitory rate/%
Lewis lung carcinoma				
Control		20.3/25.7	1.26±0.30	
GP-1	63.5	20.0/21.1	0.50±0.18^{ce}	60.3
	31.8	19.8/24.6	0.73±0.17^{cf}	42.1
GP-1-H	49.4	19.9/21.5	0.77±0.24^{c}	38.9
	24.7	20.4/25.0	1.13±0.22^{a}	10.3
Solid carcinoma of ascitic hepatoma, HePS				
Control		21.0/27.9	1.69±0.51	
GP-1	63.5	20.7/22.8	0.76±0.30^{cd}	55.0
	31.8	21.2/26.6	1.01±0.38^{ce}	40.2
GP-1-H	49.4	21.1/22.4	1.18±0.29^{b}	30.2
	24.7	20.8/27.2	1.53±0.45^{a}	9.5
Sarcoma 180				
Control		20.2/26.5	1.42±0.43	
GP-1	63.5	20.1/22.7	0.58±0.24^{ce}	59.2
	31.8	20.5/25.2	0.89±0.30^{cd}	37.3
GP-1-H	49.4	20.4/23.1	0.83±0.23^{c}	41.5
	24.7	19.9/26.0	1.20±0.41^{a}	15.5

Tab 2. Antitumor activities of GP-1 and GP-1-H on mouse transplantable tumor leukemia P388. 1/6 and 1/12 LD_{50} of the agents were injected ip into the mice every other day for 5 times. n = 10 mice. $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* GP-1-H at corresponding dose.

Group	Dose/mg·kg^{-1}	Survival time/d	T/C
Control		11.2±1.1	100
GP-1	63.5	23.8±1.5^{cf}	212.5
	31.8	17.5±0.9^{cf}	156.2
GP-1-H	49.4	17.7±1.3^{c}	158.0
	24.7	11.5±1.4^{a}	102.7

of 1/12 LD_{50}, the decreasing effect of GP-1 on WBC of mice was weaker than that of GP-1-H. The LD_{50} of GP-1 and GP-1-H ip into Balb/c mice was 381 (95 % confidence limits 339 – 423) and 296 (95 % confidence limits 261 – 331) mg·kg^{-1}, respectively (Tab 3).

DISCUSSION

The results of this article showed that the podophyllotoxin spin-labeled derivative, GP-1 exhibited more potent inhibitory effects on the growth of mouse transplantable tumors *in vivo*, the proliferation of SGC-7901 cells, and [^{3}H]TdR incorporation into new synthesized DNA of L1210 cells than its free radical reduced product, GP-1-H. GP-1 had a bigger LD_{50} and a weaker effect on WBC of mice, at 1/12 LD_{50}, than GP-1-H. These results indicated that the free radical in GP-1 had an important role in its antitumor activity and toxicity. Both GP-1 and GP-1-H increased MI of L1210 cells, and arrested the L1210 cells at G_2/M phase with a corresponding decrease of the cells in G_1 phase, which suggested that the antitumor mechanism of GP-1 might be unaffected strongly by the introduction of the free radical.

The steady free radical is a potent antioxidant[2,3]. Not only can it restrain microsome lipid peroxidation induced by carbon tetrachloride and endogenous substances, but also reduce oxygen consumption. Many compounds with antioxidative activity inhibit the

Tab 3. Toxicities of GP-1 and GP-1-H on WBC and immune function of mice. 1/4 and 1/8 LD_{50} were injected ip daily for 5 d. n = 10 mice. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control. $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* GP-1-H at corresponding dose.

Group	Drugs/ mg·kg^{-1}	WBC/ ×10^7·L^{-1}	Spleen index/ mg·g^{-1}	Thymus index/ mg·g^{-1}	QHS/A at 540 nm per 10^7 splenocytes	[^{3}H]TdR incorporation/ Bq per 10^5 lymphocytes
Control		1.31±0.16	13.1±1.7	2.13±0.55	0.56±0.06	46.8±7.7
GP-1	95.3	0.77±0.10^{cd}	9.9±1.2^{cd}	1.86±0.43^{ad}	0.39±0.06^{cd}	31.1±6.5^{cd}
	47.6	1.26±0.15^{af}	12.6±1.8^{ad}	2.11±0.71^{ad}	0.53±0.05^{ad}	45.3±7.1^{ad}
GP-1-H	74.1	0.79±0.09^{c}	9.2±1.1^{c}	1.92±0.66^{a}	0.37±0.05^{c}	30.2±5.7^{c}
	37.0	0.91±0.13^{c}	12.7±1.6^{a}	2.15±0.57^{a}	0.58±0.07^{a}	44.6±6.0^{a}

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experimental animal tumors induced by chemicals, and avoid harm from different carcinogen. On the other hand, the steady free radical can form additives with other vivacious free radical and eliminate the free radical[2,3]. In the biological system involving insaturated fatty acid and oxygen, free radical eliminator have a similar function with antioxidant to some extent. The effects of the compounds semi-synthesized or synthesized spin-labeled derivatives of antitumor agents on their antitumor activity and toxicity may be related to these functions of free radical. However, the details of the mechanism require further investigations.

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571-576 22

自由基在鬼臼酰肼哌啶腙氮氧自由基抗肿瘤及毒性中的作用[1]

贾正平, 王 锐[2]
(兰州大学生物系, 兰州 730050, 中国)
谢景文, 徐丽婷
(兰州军区总医院药材科, 兰州 730050, 中国)

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关键词 鬼臼毒素; 自由基; 抗肿瘤药; 毒性试验; 培养的肿瘤细胞; 核糖核酸合成抑制剂; 细胞周期; 有丝分裂指数

目的: 探索鬼臼酰肼哌啶腙氮氧自由基(GP-1)中自由基对其抗肿瘤作用及毒性的影响. **方法**: 采用小鼠移植肿瘤及体外培养的肿瘤细胞系, 检测GP-1及其自由基还原物GP-1-H的体内外抗肿瘤作用, 毒性及对细胞周期, 有丝分裂指数和小鼠免疫功能的影响. **结果**: GP-1 1/6, 1/12 LD_{50}给药10 d, 对小鼠Lewis肺癌, S180, P388和HePS生长的抑制作用, 在体外对SGC-7901细胞增殖和L1210细胞DNA合成的抑制作用均比GP-1-H强. 其对L1210细胞作用环节在细胞周期的M期之后. GP-1-H对细胞周期的影响与GP-1相似, 但作用较弱. **结论**: GP-1中的自由基在增强其抗肿瘤作用中起着重要作用.

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