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In vitro cytotoxicity of salvicine, a novel diterpenoid quinone[1]

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KEY WORDS quinones; cultured tumor cells; tetrazolium salts; flow cytometry; vincristine; etoposide

ABSTRACT

AIM: To study the *in vitro* cytotoxicity of 4,5-seco-5,10-friedo-abieta-3,4-dihydroxy-5(10),6,8,13-tetraene-11,12-dione (salvicine), a novel diterpenoid quinone compound on human tumor cell lines and its effect on cell cycle progression. **METHODS**: Growth inhibition of human tumor cells was measured by microculture tetrazolium assay (MTT). Cell cycle was analyzed by flow cytometry. **RESULTS**: Exposing tumor cell lines tested to salvicine for 72 h, in comparison with reference drugs vincristine (VCR) and etoposide (VP-16), salvicine was as cytotoxic as VP-16 and weaker than VCR in 3 leukemia cell lines. For 12 solid tumor cell lines, salvicine exhibited cytotoxic activities and was over 5.41- and 4.15-fold stronger than VCR and VP-16, respectively. Salvicine presented better activities especially against gastric and lung carcinoma cell lines. Exposing K562 leukemia cells to 9 graded concentrations of salvicine (from 0.39 to 100 $\mu mol \cdot L^{-1}$) for 24 h and to salvicine 10 $\mu mol \cdot L^{-1}$ for 7 different periods (from 1 to 48 h), the growth inhibition of cells was enhanced along with increased concentration or prolonged exposure. Cell cycle analysis demonstrated that salvicine arrested K562 cells in G_1 phase and this effect was also heightened with increased concentration or extended exposure. **CONCLUSION**: Salvicine exhibited potent cytotoxic activities against various human tumor cell lines, and blocked K562 leukemia cells in G_1 phase of cell cycle.

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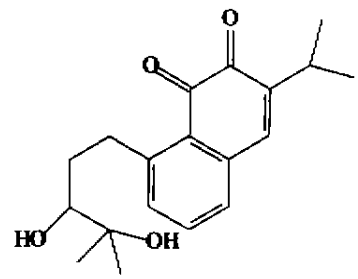
INTRODUCTION

4,5-Seco-5,10-friedo-abieta-3,4-dihydroxy-5(10),6,8,13-tetraene-11,12-dione (salvicine) is a novel diterpenoid quinone compound obtained by structural modification of a natural product lead isolated from a Chinese medicinal plant *Salvia prionitis* Hance (Labiatae). We have isolated more than 40 diterpenoidal compounds from this plant[1-8] and systematic chemical modification of some compounds led to the preparation of pharmacologically active derivatives. Salvicine, one of the derivatives, showed high *in vitro* cytotoxicity on murine leukemic P388 and human lung cancer A549 cells and distinct *in vivo* antitumor activities against murine S180 sarcoma and Lewis lung carcinoma in preliminary tests. Presently, salvicine has been chosen as a candidate compound of antineoplasm in preclinical research stage.



Salvicine

Current study was undertaken to further test the *in vitro* cytotoxicity of salvicine, especially on those human solid tumor cells. We measured the cytotoxic activities of salvicine on 15 human tumor cell lines representing different histopathologies of 7 categories of human tumors, and chose plant-derived anticancer drugs vincristine (VCR) and etoposide (VP-16) as positive controls to compare the cytotoxic potency among them. Using human K562 leukemia cell line as model, we also determined the dose- and time-response relationships of salvicine action and furthermore influence on cell cycle progression.

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MATERIALS AND METHODS

Cell lines The origins and histological characteristics of the cell lines examined are listed in Tab 1. All cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10 % heat-inactivated bovine serum, *L*-glutamine 2 mmol·L^{-1}, benzylpenicillin 100 kIU·L^{-1}, streptomycin 100 mg·L^{-1} and HEPES 10 mmol·L^{-1}, pH 7.4 in a humidified atmosphere of 95 % air + 5 % CO_2 at 37 ℃.

Tab 1. Human tumor cell lines for measuring the cytotoxicity of salvicine.

Cell lines	Origins*	Histological sources and types
K562	ATCC	Chronic myelogenous leukemia
HL-60	ATCC	Promyelocytic leukemia
U-937	ATCC	Monocytic leukemia
A-549	NCI	Lung adenocarcinoma
SPC-A4	SIMM	Lung adenocarcinoma
NCI-H23	NCI	Lung adenocarcinoma
NCI-H522	NCI	Lung adenocarcinoma
SGC-7901	SIMM	Gastric adenocarcinoma
MKN-28	JFCR	Gastric adenocarcinoma
HO-8910	SIMM	Ovarian epitheloid carcinoma
3AO	SIMM	Ovarian epitheloid carcinoma
Bel-7402	SIMM	Hepatocellular carcinoma
Bel-7404	SIMM	Hepatocellular carcinoma
HCT-116	NCI	Colon adenocarcinoma
Hela	ATCC	Cervical squamous cell carcinoma

*Abbreviation used: ATCC: American Type Culture Collection. NCI: National Cancer Institute, USA. SIMM: Shanghai Institute of Materia Medica, Chinese Academy of Sciences. JFCR: Japanese Foundation for Cancer Research.

Compounds Salvicine presenting tangerine color crystalloid was provided by Phytochemistry Department of our Institute. Reference drugs VCR and VP-16 were purchased from Guangdong Mingxing Pharmaceutical Factory and Pudong Pharmaceutical Factory of Shanghai Institute of Pharmaceutical Industry, respectively. Salvicine was solubilized at 10 mmol·L^{-1} in solvent containing 50 % Me_2SO and 50 % normal saline solution (NS) as stock solution and Me_2SO contained in the maximal tested concentration (salvicine 50 μmol·L^{-1}) did not exceed 0.25 %. VCR and VP-16 were prepared at 10 mmol·L^{-1} with NS as stock solution. All stock solutions were stored in aliquot at −20 ℃ and thawed just before the test and diluted with complete medium.

Measurement of growth inhibition of cells Growth inhibitory effect of salvicine on tumor cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co) assay[9,10] with minor modification. Briefly, adherent tumor cells were seeded into 96-well microculture plates and allowed to adhere for 24 h before drug addition, while nonadherent cells were seeded just before drug addition. The cell densities were selected based on the results of preliminary tests to maintain the control cells in an exponential phase of growth during the period of the experiment and to obtain a linear relationship between the optical density (OD) and the number of viable cells. Each tumor cell line was exposed to salvicine and reference drugs at 0.16, 0.8, 4, 20, and 100 μmol·L^{-1} for 72 h and each concentration was tested in triplicate wells. At the end of exposure, 20 μL of MTT 5 g·L^{-1} was added to each well and the plates were incubated at 37 ℃ for 4 h, then "triplex solution (10 % SDS-5 % isobutanol-HCl 12 mmol·L^{-1})" was added and the plates were incubated at 37 ℃ for 12 − 20 h. The OD was read on a plate reader at a wavelength of 570 nm. Media and Me_2SO control wells, in which salvicine was absent, were included in all the experiments. The cytotoxicity of salvicine on tumor cells was expressed as IC_{50} (the drug concentration reducing by 50 % the absorbance in treated cells, with respect to untreated cells) that was calculated by Logit method. Finally, the mean IC_{50} ± SD was counted according to the data from 2-6 replicate tests.

Determination of dose- and time-response relationship Human K562 leukemia cells were exposed to salvicine at concentrations of 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 μmol·L^{-1} for 24 h for concentration-response detecting and at concentration of 10 μmol·L^{-1} for 1, 3, 6, 10, 24, 34, and 48 h for testing time-response action. The inhibition of cell proliferation was determined by MTT assay. The inhibitory rate of cell proliferation was calculated by:

$$\text{Growth inhibition (\%)} = \frac{OD_{control} - OD_{treated}}{OD_{control}} \times 100$$

Results were expressed as percentage of growth

inhibition obtained from 2 separate experiments *versus* concentrations or durations of exposure of salvicine.

Analysis of cell cycle K562 leukemia cells of 3×10^5 were exposed to salvicine at concentrations of 0.03, 0.3, 3.13, 6.25, 12.5, 25, and 50 $\mu mol \cdot L^{-1}$ for 24 h or to salvicine 25 $\mu mol \cdot L^{-1}$ for 1, 3, 6, 10, 24, 34, and 48 h. Cells were then fixed by 70 % ethanol (vol/vol), washed twice in phosphate-buffered saline solution (PBS)- and stained with PBS containing RNase 10 $mg \cdot L^{-1}$ and propidium iodide (PI) 50 $mg \cdot L^{-1}$ for 30 min at 25 ℃ in the dark. For each sample, at least 1×10^4 cells were analyzed by flow cytometry (FACSalibur, Becton Dickinson, USA). Results were analyzed by software of CELLQUEST and ModFIT LT for mac V1.01 (Becton Dickinson, USA) and expressed as the percentage of cells found in the each phase of cell cycle.

RESULTS

Cytotoxicity for tumor cells For 3 leukemia cell lines, the mean IC_{50} of VCR, VP-16, and salvicine were 0.88, 7.77, and 7.66 $\mu mol \cdot L^{-1}$, respectively, indicating that *in vitro* cytotoxic activity of salvicine was the same as that of VP-16 and weaker than that of VCR on leukemia cell lines. On the other hand, the mean IC_{50} of salvicine for 12 solid tumor cell lines including 4 lung cancers, 2 gastric cancers, 2 ovarian carcinomas, 2 hepatic carcinomas, 1 colonic cancer, and 1 cervical cancer was 18.48 $\mu mol \cdot L^{-1}$, while those of VCR and VP-16 were over 100 and 76.62 $\mu mol \cdot L^{-1}$, showing that salvicine was over 5.41 and 4.15 times more potent than VCR and VP-16. In addition, the mean IC_{50} for each histological type of solid tumor cells revealed that salvicine had better activities on gastric and lung cancer cell lines. On the average, the salvicine was 10.81- and 7.68-fold more active than VCR, 9.03- and 3.24-fold than VP-16 for gastric and lung cancer cell lines, respectively (Tab 2).

Dose-response relationship Dose-response experiment demonstrated that the growth inhibition of K562 cells was not observed at concentrations below 3.13 $\mu mol \cdot L^{-1}$ of salvicine. From 3.13 to 50 $\mu mol \cdot L^{-1}$, the growth inhibition of K562 cells was enhanced along with increased concentration of salvicine. During this course, the growth inhibition of K562 *versus* the concentration of salvicine exhibited a linear relationship between 3.13 and 12.5 $\mu mol \cdot L^{-1}$. This effect approximated maximum at 50 $\mu mol \cdot L^{-1}$ (Fig 1).

Tab 2. Cytotoxic activities of salvicine and reference drugs on human tumor cell lines.

Cell lines	IC_{50}[a] / $\mu mol \cdot L^{-1}$ VCR	VP-16	Salvicine
Leukemia			
K562	0.19 ± 0.10	14.22 ± 0.59	7.82 ± 2.81
HL-60	0.47 ± 0.31	2.16 ± 0.56	6.83 ± 2.91
U-937	1.99 ± 1.85	6.93 ± 1.80	8.34 ± 0.28
	0.88[b]	7.77[b]	7.66[b]
Solid tumors			
Lung			
A-549	> 100	16.82 ± 3.52	9.70 ± 4.03
SPC-A4	> 100	45.14 ± 2.09	11.92 ± 7.89
NCI-H23	> 100	28.98 ± 23.93	13.64 ± 3.02
NCI-H522	> 100	77.97 ± 0.28	16.82 ± 11.10
	> 100[c]	42.23[c]	13.02[c]
Stomach			
SGC-7901	> 100	> 100	7.79 ± 6.67
MKN-28	> 100	67.13 ± 2.10	10.70 ± 0.53
	> 100[c]	> 83.57[c]	9.25[c]
Ovary			
HO-8910	> 100	51.51 ± 5.36	12.20 ± 0.57
3AO	> 100	52.90	24.90
	> 100[c]	52.21[c]	18.55[c]
Liver			
BEL-7402	> 100	63.51 ± 3.81	14.67 ± 3.16
BEL-7404	> 100	> 100	43.84 ± 10.85
	> 100[c]	> 81.76[c]	29.26[c]
Miscellaneous			
HCT-116	> 100	> 100	10.48 ± 7.11
HELA	> 100	> 100	30.34 ± 0.57
	> 100[d]	> 76.62[d]	18.48[d]

[a] Mean $IC_{50} \pm s$ of 2-6 replicate assays except 3AO;
[b] Mean IC_{50} of leukemia cells;
[c] Mean IC_{50} of each histological type of solid tumor cells;
[d] Mean IC_{50} of whole solid tumor cells.

Time-response relationship Generally, the growth inhibition of K562 cells was increased along with prolonged exposure to salvicine at 10 $\mu mol \cdot L^{-1}$. This effect presented a linear relationship during the period of 1-34 h exposure, and reached maximum at 34 h exposure (Fig 2).

Perturbation of cell cycle progression The cell cycle phase distribution of untreated (control) K562 cells at indicating time points was shown. Generally, the proportion of cells in S phase was more than that in G_0-G_1 phase, although exhibited some fluctuations (Fig 3).

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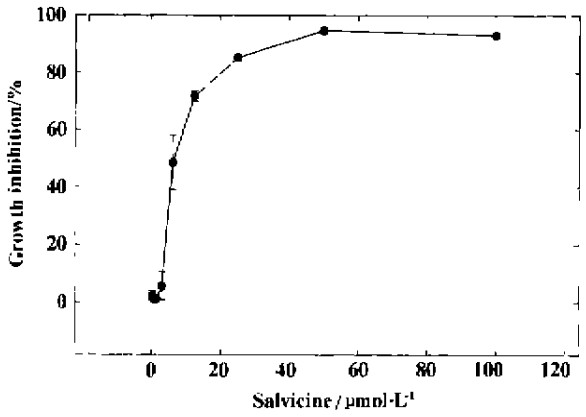

Fig 1. Concentration-dependent effects of salvicine on the inhibition of K562 cell proliferation (exposure: 24 h). $n = 2$ seperate experiments. $\bar{x} \pm s$.

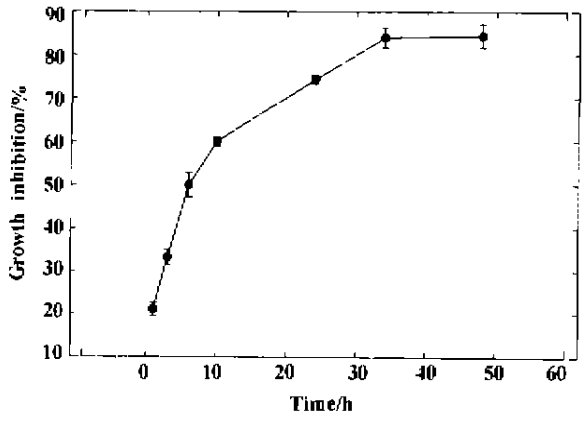

Fig 2. Time-dependent effects of salvicine on the inhibition of K562 cell proliferation (salvicine: 10 μmol·L⁻¹). $n = 2$ seperate experiments. $x \pm s$.

Cell cycle exhibited uncertain changes when concentration of salvicine below 12.5 μmol · L⁻¹. When the concentration exceeded 12.5 μmol · L⁻¹, salvicine caused an increase in the proportion of cells in G_1 phase, from 44.8 % (12.5 μmol · L⁻¹) to 69.29 % (50 μmol·L⁻¹) accompanied by a decrease in S phase, from 45.73 % to 14.22 %, and just a slight change was observed in G_2-M phase at the same time (Fig 4,5).

Exposing K562 cells to salvicine 25 μmol·L⁻¹ for different periods, no obvious changes were observed before 24 h exposure. Between 24 and 34 h, salvicine led to an increase of cells in G_1 phase, from 43.79 % (24 h) to 57.3 % (34 h) and a decrease of cells in G_2-M phase from 21.41 % to 0.39 %, and a slight

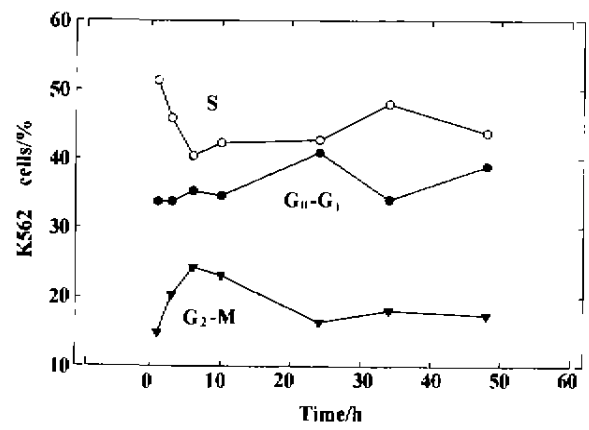

Fig 3. The cell cycle phase distribution of untreated (control) K562 cells.

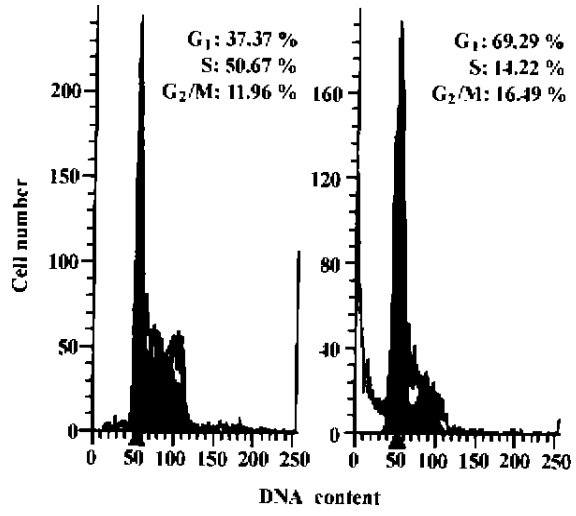

Fig 4. Cell cycle distribution of K562 cells treated with salvicine 50 μmol·L⁻¹ for 24 h.

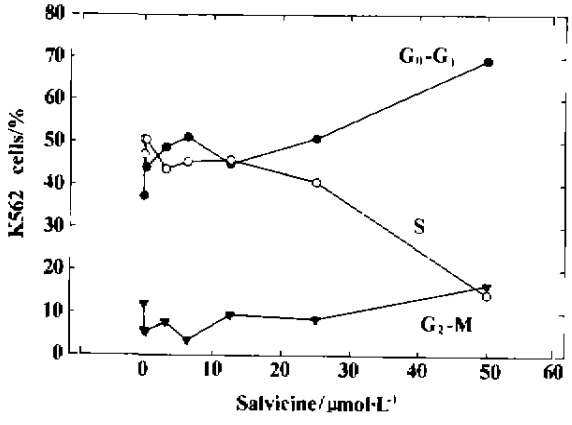

Fig 5. The cell cycle phase distribution of K562 cells after treatment with salvicine for 24 h.

increase of cells in S phase. When exposure excessed 34 h, salvicine caused a marked increase of cells in G_1 phase, from 57.3 % (34 h) to 86.95 % (48 h) and a decrease of cells in S phase, from 42.31 % to 8.99 %, meanwhile, the percentage of control cells in G_0-G_1 phase were 34.01 % and 38.91 %, and that in S phase was 47.90 % and 43.71 % (Fig 6).

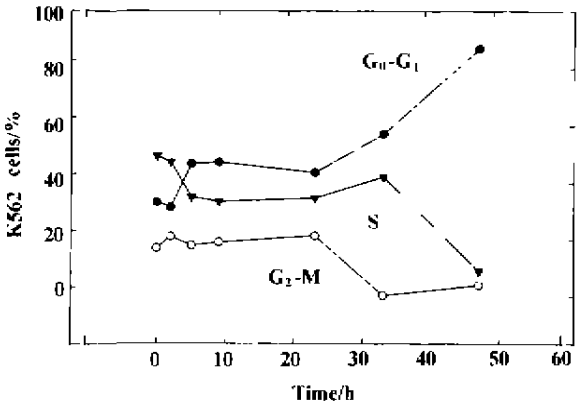

Fig 6. The cell cycle phase distribution of K562 cells treated with slavicine 25 μmol · L^{-1} for various exposure periods.

DISCUSSION

Salvicine, a novel compound of chemical modification from a naturally occurring diterpenoid quinone in a chinese medicinal plant *Salvia prionitis* Hance (labiatae), was selected for further study due to its potent antitumor activities in initial experiments *in vitro* and *in vivo* (previously described). In current study, we determined the cytotoxic activities of salvicine on 15 human tumor cell lines including leukemia, lung, gastric, ovarian, hepatic, colonic, and cervical carcinomas and its influence on cell cycle progression of K562 leukemia cells. Our results showed that salvicine had obvious cytotoxicity against various human tumor cell lines. The activity of salvicine was more potent than reference drugs VCR and VP-16 on 12 human solid tumor cells, and on an average, it was over 5.41- and 4.15-fold more cytotoxic than those of VCR and VP-16, respectively. Considering the limited number of cell lines per tissue, it was difficult to draw conclusion on the specificity of salvicine for a given histological type. However, results presented in Tab 2 showed that a better activity was obtained in lung and gastric cancer cell lines than in hepatic and ovarian cancer cell lines. Succedent experiment *in vivo* got a significant suppression of lung adenocarcinoma A-549 and LAX-83 xenograft growth in salvicine treated nude mice (data not shown). These results suggested that salvicine might possess a relative specificity to certain histological types of solid tumors, for example lung carcinomas. Unlike the results obtained from solid tumor cells, salvicine exhibited moderate cytotoxic activities against 3 leukemia cell lines, weaker than that of VCR and as the same as that of VP-16. On the contrary, VCR did not show any observable activities against solid tumor cell lines in this study. This is consistent with clinic that VCR is one of the main chemotherapeutic drugs for the treatment of leukemia, but it is rarely used alone for treatment of malignant solid tumors. In addition, the growth inhibitory effect of salvicine on K562 cells was concentration- and time-dependent.

Some chemotherapeutic agents used in the clinic commonly act in cell cycle phase specific manner, eg antimetabolites methotrexate and nucleotide analogues are inhibitors of DNA synthesis and mainly kill the cells in S phase[11-12]. The *Vinca* alkaloids (vincristine and vinblastine) are inhibitors of mitosis and dominantly act to the cells in G_2-M phase[11-12]. These observations imply that these agents exert their antitumor efficiency through interfering specific cellular processes in proliferating cells. Our results obtained from proliferating human leukemia K562 cells demonstrated that salvicine regulated important cell cycle-related mechanism: the progression of proliferating cells through G_1 phase. Both 24-h exposure at concentrations above 12.5 μmol · L^{-1} or 25 μmol · L^{-1} for longer than 24-h exposure led to a dramatic accumulation of cells in G_1 phase and loss of cells in S phase, indicating cells continue mitosis but can not pass G_1 phase to enter S phase and fail to trigger DNA synthesis. Furthermore we also observed that a population of cells with decreased DNA content (below that of G_1 phase cells) became apparent at the same time of the loss of S phase cells from the culture (data not shown). So the loss of cells in S phase may be a result of specific degradation of DNA in these cells triggered by salvicine[13]. In addition, another experiment in our laboratory showed that salvicine was able to inhibit the activity of DNA topoisomerase II

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(data not shown). DNA topoisomerase Ⅱ inhibitor TN and m-AMSA triggered DNA degradation specifically in S phase of HL-60 and KG_1 cells and induced a cell population with diminished DNA stainability[12-13], which is in agreement with the results obtained in the present study. In summary, salvicine exhibited potent inhibitory effects on the growth of human tumor cell lines, especially solid tumor cells, arrest human leukemia K562 cells in G_1 phase and S phase may be the susceptible phase of salvicine action.

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REFERENCES

1 Yang BJ, Huang XL, Huang Y, Wang XM, Lin LZ. Study on the chemical constituents of *salvia prionitis*. Acta Botan Sin 1988; 30: 524-7.
2 Lin LZ, Wang XM, Huang XL, Huang Y, Yang BJ. A new diterpenoid quinone dehydromiltirone. Acta Pharm Sin 1988; 23: 273-5.
3 Lin LZ, Wang XM, Huang XL, Huang Y, Yang BJ. Diterpenoids from *salvia prionitis*. Planta Med 1988; 54: 443-5.
4 Lin LZ, Blasko G, Cordell GA. Diterpenes of *Salvia prionitis*. Phytochemistry 1989; 28: 177-81.
5 Lin LZ, Cordell GA. Salvonitin, a diterpene from *Salvia prionitis*. Phytochemistry 1989; 28: 2846-7.
6 Lin LZ, Wang XM, Huang XL, Huang Y. A new diterpenoid quinone sapriparaquinone. Acta Pharm Sin 1990; 25: 154-6.
7 Huang XL, Wang XM, Huang Y, Zhang JS, Lin LZ. Structural elucidation of salpriolactone. Acta Botan Sin 1990; 32: 490-1.
8 Zhang JS, Huang Y. Two new diterpenoids, prioketolactone and neoprionitone from *salvia prionitis*. Nat Prod Res Develop 1995; 7: 1-4.
9 Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, *et al*. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988; 48: 589-601.
10 Mosmman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983; 65: 55-63.
11 Chabner BA, Myers CE. Clinical pharmacology of cancer chemotherapy. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer principles and practice of oncology. Philadelphia: JB Lippincott Co; 1982. p157, 184.
12 Del Bino G, Darzynkiewicz Z. Camptothecin, teniposide, or 4′-(9-acridinylamino)-3-methanesulfon-m-anisidide, but not mitoxantrone or doxorubicin, induces degradation of nuclear DNA in the S phase of HL-60 cells. Cancer Res 1991; 51: 1165-9.
13 D'Arpa P, Beardmore G, Liu LF. Involvement of nucleic acid synthesis in cell killing mechanism of topoisomerase poisons. Cancer Res 1990; 50: 6919-24.

新二萜醌化合物杀尔威辛的体外细胞毒作用

R979-1

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杀尔威辛 抗肿瘤药 细胞毒性

关键词 醌类; 培养的肿瘤细胞; 四唑鎓盐类; 流动血细胞计数; 长春新碱; 去甲鬼臼甙

白血病

目的: 研究杀尔威辛体外抗肿瘤活性及对人肿瘤细胞周期分布的影响. **方法**: 四唑鎓盐还原法(MTT)测定体外抗肿瘤活性; 流式细胞术检测细胞周期时相分布. **结果**: 杀尔威辛对3株人白血病细胞的活性与鬼臼乙叉甙(VP-16)相同, 但弱于长春新碱(VCR). 对12株人实体瘤细胞的作用分别是VCR和VP-16的5.41和4.15倍以上, 对人胃癌和肺癌有相对高的活性, 分别是VCR的10.81和7.68倍, VP-16的9.03和3.24倍. 杀尔威辛阻滞增殖的K562细胞于G_1期, 细胞DNA断裂增加和S期细胞明显减少. **结论**: 杀尔威辛对人肿瘤细胞尤其是实体瘤细胞有较强的生长抑制效应, 阻滞增殖的人白血病K562细胞于G_1期, S期可能是其作用的敏感期.

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