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THE ANTITUMOR ACTIVITIES OF GNIDIMACRIN ISOLATED FROM STELLERA CHAMAEJASME L.

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Gnidimacrin, a diterpene compound, isolated from the methanol extract of *Stellera chamaejasme* L., showed significant antitumor activities against mouse leukemia P-388 and L-1210 *in vivo*, such as Lewis lung carcinoma, B-16 melanoma and Colon cancer 26. It showed ILSs of 40%, 49% and 41% at the dosages of 0.01-0.02 mg/kg ip, respectively. Gnidimacrin strongly inhibited cell proliferation of human cancer cell lines such as leukemia K562, stomach cancers Kato-III, MKN-28, MKN-45, and mouse leukemia L-1210 by the MTT assay and colony forming assay *in vitro*. The IC_{50} of gnidimacrin was 0.007 - 0.00012 μg/ml. It is concluded that gnidimacrin is the principal antitumor element in *Stellera chemaejasme* L. with strong antitumor activities.

Key words: Gnidimacrin antitumor drug, Plant *Stellera chamaejasme* L.

In the course of our studies on screening for chinese medicinal herbs, we found that a methanol extract of the root of *Stellera chamaejasme* L. (Rui Xiang Lang Du in Chinese) showed significant antitumor activities.[1] It have been reported that *Stellera chamaejasme* L. may be useful in treatment of malignant disease,[2] and it's crude extracts had effects on management of the lung cancer and the liver cancer by a clinical report.[3] In order to clarify the antitumor consituents of the plant and also to find out novel antitumor agent, we attempted to isolate the root of *Stellera chamaejasme* L. and purify it's active constituents using an antitumor bioassay to against mouse leukemia P388 as a active index. As the results, six antitumor diterpenes include gnidimacrin that showed significant antitumor effects were isolated.[4] We report herein the antitumor activities of gnidimacrin against animal tumors *in vivo* and human cancer cell lines *in vitro*.

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MATERIALS AND METHODS

Materials

Drugs and chemicals:

(1) Gnidimacrin: Dried root or *Stellera chamaejasme* L. collected in Tibet was extracted with methanol. By further extraction with petroleum ether, followed by purification of column chromatography using silica gel and HPLC using ODS column, gnidimacrin, a white pure powder, was isolated from the plant. the chemical structure of gnidimacrin was shown in Figure 1. Gnidimacrin was dissolved in physiological saline containing 1% DMSO to use for ip injection *in vivo* or dissolved in RPIM-1640 medium (Immunobiological Institute, Japan) supplemented by 10% FCS to use for cell culture *in*

vitro. (2) MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma Chemicals Co. Ltd., USA] was dissolved in PBS at a concentration of 2% (2 mg/ml). (3) Enriched McCoy's 5A was supplied by Dr. Reiko tokuzen, chemotherapy Division, National Cancer Center, Tokyo Japan.

Animals

Five weeks old female BDF1, C57BL/6 and BALB/c mouse supplies by Japan SLC Co. Ltd. were used.

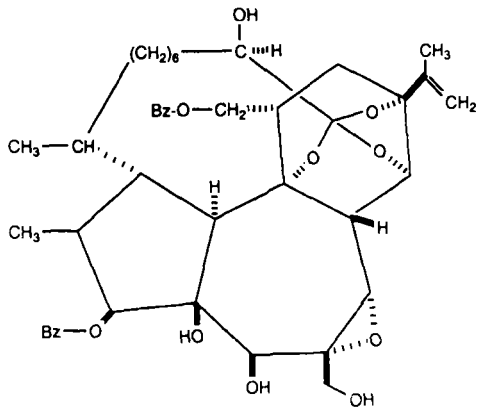

Fig 1. The chemical structure of gnidimacrin

Tumors

Mouse leukemia P388 and l1210 were inoculated ip and solid Lewis lung carcinoma, colon 26 adenocarcinoma and B-16 melanoma were implanted s.c. *in vivo*. The human cancer cell lines leukemia K562, stomach cancer Kato-III, MKN-28 and MKN-45, mouse leukemia L1210 were used *in vitro*. All of the tumors were maintained in National Cancer Center Institute, Tokyo, Japan.

Anticancer Tests

In vivo test against mouse tumors

P388 (10^6 cells) and L1210(10^5 cells) were implanted i.p. respectively. From 24 hr after implantation the mice were given ip injection of gnidimacrin 0.1 ml/mouse once a day for 9 days. Lewis lung carcinoma (10^6 cells), colon 26 (5×10^5 cells) and B-16 (20% homogenate 0.25 ml) were implanted sc respectively. From 24 hr after implantation, the mice were given ip injection of gnidimacrin 0.1 ml/mouse once daily for 10 days. the control group was injected 1% DMSO-saline ip 0.1 ml. there are 6 mice in each group and the experiments were repeated 3 times. survival days were investigated and the results were determined by Increase in Life span (ILS):

$$ILS=\left(\frac{\text{mean survival days of treatment group}}{\text{mean survival days of control group}}-1\right)\times 100\%$$

Cytotoxic activity test in vitro

(1) MTT assay: The cells (10^4/ml) were plated in a 96-well microplate in a volume of 180 μl and incubated at 37 ℃ in an atmosphere of 5% CO_2-incubator. 20 μl of gnidimacrin in different concentration or medium was added into each well 2 hr later, one concentration contain 4 wells. after the incubation for 96 hr, 50 μl of MTT was added to each well and further reincubate for 4 hr. after centrifugation (2000 rpm, 15 minutes), the ksupernatant was aspirated and formazan crystals formed were dissolved in 200 μl of 100% DMSO and 25 μl of glycine buffer (0.1M. pH 10.5). The absorbency was measured in a microplate-reader at 540 nm. the experiment was repeat 3 times. Result are expressed in terms of the drug concentration required to inhibit 50% of the cell-growth (IC_{50}). It is estimated as the absorbency value equal to 50% of that cells in the control wells. (2) Colony forming assay: Double layer assay was used in this study. Under layers were consisted of 3.5% agar and Enriched McCoy's 5A in a proportion of 1:5, then 1 ml of gnidimacrin in vary concentrations or medium only as control, 0.7 ml of RPMI 1640 medium contains 10% FCS, 0.1 ml of cancer cells (10^4/ml) and 0.1 ml of 3.5% agar. After incubation at 37 ℃ in a 5%CO_2 for 7 days, the numbers of colonies (≥40 cells) of each well were determined by a automatic colony counter. Each concentration of the drug formed 3 wells and the experiment repeated 3 times. result are expressed in terms of the drug concentration required to inhibit

50% of the colony forming (IC_{50}). It is estimated as the colony numbers equal to 50% of that in the control wells.

RESULTS

Antitumor Activity against Murine Tumors *in vivo*

Gnidimacrin showed significant antitumor activity against murine leukemia (P388 and L1210) increasing the life span of tumor-implanted mice 70% and 80% ($P<0.001$) at the dosages of 0.02-0.03 mg/kg by ip. In addition, the drug also significantly prolonged life span of the mouse implanted solid tumor, such as Lewis, B-16 and colon 26, the ILS were 40%, 49% and 41% respectively. the results were shown in Table 1. In this experiments, a few mice died because of the toxicity, when the dose of gnidimacrin ip was more than 0.05 mg/kg.

Table 1. Antitumor activities of gnidimacrin invivo

Tumor	Route	Dose	Survival days	ILS	*P*
P388	ip-ip	0. 00	8.1 ± 0.8		
		0. 02	13.8 ± 1.5	70	< 0. 001
L1210	ip-ip	0. 00	7.4 ± 0.5		
		0. 03	13.3 ± 3.7	80	< 0. 001
Lewis	sc-ip	0. 00	16.7 ± 1.4		
lung cancer		0. 02	23.3 ± 1.6	40	< 0. 05
B-16	sc- ip	0. 00	22.7 ± 3.1		
melanoma		0.02	33.8 ± 13.7	49	< 0. 05
colon	sc- ip	0.00	16.8 ± 1.6		
cancer 26		0.01	23.7 ± 11.5	41	< 0. 05

*: (1) 6 mice in each group. (2) Data is average ± standard division in 3 different tests.

Cytotoxic Activity Test in vitro

Gnidimacrin showed strong antitumor activities against human leukemia K562 and stomach cancer Kato-III, MKN-28, MKN-45, and also murine leukemia L1210. The 50% inhibitory concentration of cell growth (MTT assay) and colony forming (Colony forming assay) against those cancer were among 0.007 -- 0.00012 μg/ml. results were shown as Table 2

Cytotoxic activities of gnidimacrin in vitro

Cell lines	origin	IC_{50} (μg/ml)	
		MTT assay	Colony forming assay
K562	human leukemia	4.5×10^{-4}	6.0×10^{-4}
Kato-III	human stomach cancer	7.5×10^{-4}	2.0×10^{-3}
MKN-28	human stomach cancer	6.4×10^{-3}	5.0×10^{-3}
MKN-45	human stomach cancer	1.2×10^{-4}	7.0×10^{-4}
L1210	murine leukemia	7.0×10^{-3}	2.0×10^{-3}

*: Data is average ± standard division in 3 different tests.

DISCUSSION

There are two kinds of plants being used in clinical practices of traditional Chinese medicines as "Lang Du", these are *Stllera chemaejasme* L. (Rui Xiang Lang du in chinese, Thymelaeaceae) and *E. fischeriana* STEUD. (Bai Lang Du in chinese, Euphorbiaceae), in which *Stellera chemaejasme* L. was usually called the certified quality goods.[2] We have reported that the methanol extracts of Stellera chemaejasma L. were more active against cancer than other species of "Lang Du ", such as *E. fischeriana* STEUD. previously.[5] We also found the plant collected in Tibet showed higher activity than that of Inner Mongolia. In our antitumor experiment against P388, the methanol extract of *Stellera chemaejasme* L. showed ILS of 79% at a dosage of 10 mg/kg. Following further extract by petroleum ether, the ILS of the extracts was 57% at a dosage of 1 mg/kg by ip. Thus, it is suggested that the antitumor activity of the plant was enhanced significantly following the each extractions. the dosage of gnidimacrin agined similar antitumor effects with the crude methanol extract of the plant was only the one of five hundredth of the latter. This indicated that gnidimacrin is the principal antitumor element of *Stellera chemaejasme* L. Gnidimacrin also showed strong antitumor activities against mouse leukemia L1210 and solid tumors, such as Lewis lung cancer, B-16, colon cancer 26 those were tested regularly. It was demonstrated that gnidimacrin have wide antitumor spectrum. Gnidimacrin showed significant cell growth inhibitory effects and colony forming inhibitory effects in vitro. The antitumor activities against L1210 both *in vivo* and *in vitro* indicated the antitumor mechanism of gnidimacrin was a direct cytotoxicity. However, gnidimacrin showed toxicities while the ip dose over 0.05 mg/kg and the effective dosage is near with toxicitic dosage. Further studies of toxicology of gnidimacrin are necessary.

Gnidimacrin is a diterpene compound, it have similar chemical structure with Taxol, a antitumor diterpene alkaloid newly using in clinic. A bleb formation on the membrane of cells caused by gnidimacrin[6] and decrease of mitosis and some cell changes, such as increase of multiple and large nuclear cells, enlargement of cells and decrease of stainability of cells were found by preliminary morphological observation.[4]

Our study demonstrated that gnidimacrin, a diterpene compound, is the principal antitumor element in *Stellera chemaejasme* L. It have strong antitumor activities and it is necessary to do more studies on gnidimacrin.

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