J. Toxicol. Pub. Health
Vol. 16, No. 4, pp. 263-267, 2000

Antitumor Activity of 23, 24-dihydrocucurbitacin D Isolated from *Bryonia alba* L.

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(Received October 23, 2000)
(Accepted November 16, 2000)

ABSTRACT : *The cytotoxic and antitumor activity of 23,24-dihydrocucurbitacin D (dhc D) isolated from Bryonia alba L. was examined. Our results showed that dhc D strongly inhibited the viability of the cultured cancer cells, A-549, COLO 205, SK-MEL-2, and L1210. It also exhibited effective antitumor activity in ICR mice, which had been intraperitoneally implanted with sarcoma 180 ascites tumor cells. The dhc D also strongly inhibited the viability of immortalized macrophages RAW 264.7, but not normal peritoneal macrophages. These results indicate that dhc D has antiproliferative effect on cancer cells and it may more sensitive on immortalized cells than normal cells.*

Key Words : *23,24-Dihydrocucurbitacin D (dhc D), Bryonia alba L., Cytotoxicity, Macrophages, Antitumor activity*

I. INTRODUCTION

Bryonia alba L. (Cucurbitaceae) has been used for thousands years as a traditional medicine for the prevention or treatment of many diseases (Sepetchian, 1948). Chemical constituents from the roots of this plant have been previously identified (Konopa *et al.*, 1974a). Curcubitacin B, D, E, and I have been isolated from roots and their cytotoxic activities were demonstrated in various cancer cell lines (Konopa *et al.*, 1974b). Cucurbitacins found in other plants, such as *Bryonia dioica* (Hylands and Magd, 1986) and *Trichosanthes kirilowii* (Ryu *et al.*, 1994) were also shown to have cytotoxic activities against cancer cells. However, these compounds have not been developed as a therapeutic agent for cancer patients because of their high cytotoxicity *in vivo*. Furthermore, the mechanism responsible for the cytotoxic activities of cucurbitacins is still unknown.

Previously, we isolated a new cucurbitacin, 23,24-dihydocucurbitacin D (19-norlanost-5-ene-3,11,22-trione-2β, 16α, 20β, 25-tetrahydroxy-9-methyl) from the methanol extract of the *Bryonia alba* L. root collected in Armenia, and its cytotoxic activity was demonstrated against several cancer cell line (Baek *et al* ., 1995). These results led us to investigate its *in vivo* antitumor activity and the possible mechanism underlying this activity. Therefore, in this study, we investigated the antitumor activity of dhc D in mice bearing sarcoma 180 ascites tumor cells. Its *in vitro* cytotoxic effects on normal peritoneal macrophages and immortalized macrophages were also examined.

II. MATERIALS AND METHODS

1. Preparation of dhc D

Bryonia alba L. root was collected in the east area of Yerevan, Armenia, in September, 1993. The roots were sliced and dried at room temperature in open air to be constant weight. The dhc D was isolated from the root and identified from physical and spectroscopic data (m.p., IR, UV, ^{1}H-NMR, ^{13}C-NMR, MS)

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as described previously (Baek *et al.*, 1995). To determine *in vitro* cytotoxic activity and *in vivo* antitumor activity, dhc D was dissolved in a small amount of ethanol, then suspended in saline. The final concentration of ethanol did not exceed 0.2%. To study of the effect of dhc D on the viability of macrophages, it was dissolved in dimethylsulfoxide, then diluted with the culture medium. The final concentration of dimethylsulfoxide was less than 0.1%.

2. *In vitro* cytotoxicity test

We used four types of cancer cell lines to determine cytotoxic effect of dhc D. A549 (lung carcinoma, human), COLO 205 (colon, adenocarcinoma, human), SK-MEL-2 (malignant melanoma, metastasis to the skin of thighs, human) and L1210 (leukemia, murine) cells were cultured in an RPMI 1640 medium supplemented with glutamine, sodium bicarbonate, and 5% fetal bovine serum. For the growth inhibition studies, A549, COLO 205, and SK-MEL-2 cells were seeded in 24 well plates (1×10^5 cells/ml, 1 ml/well), and preincubated for 24 hr at 37°C under 5% CO_2, followed by treatment with dhc D for 48 hr. For L1210, cells were seeded in 24 well plates (5×10^4 cells/ml, 1 ml/well) and treated with the dhc D for 48 hr without the 24 hr preincubation. The cultured cells were collected by centrifugation at 1,200 × g for 10 min according to the method of Skehan *et al.* (1990). The cytotoxicity of dhc D at various concentration was evaluated as the net growth inhibition (%) of cells compared with the control group treated with 0.2% ethanol in saline. The concentration of dhc D, which inhibited cell viability by 50% (ED_{50} value) was determined graphically from a dose-response curve.

3. *In vivo* antitumoric test

Sarcoma 180 ascites tumor cells were intraperitoneally transplanted in male ICR mice of an approximate 25 g body weight. They were housed under a 12 hr-light-dark cycle at 22±1°C and provided with free access to water and food. After 14 days, the cells were collected from the ascitic fluids of the mice and washed several times in ice-cold saline. These cells were diluted with saline to adjust the tumor cell number to 1×10^7 cells per ml. Then, 0.1 ml of the cell suspension was inoculated into the peritoneal cavity of healthy mice. The mice were randomly assigned into four experimental groups eg., normal (saline), control (sarcoma + saline), sarcoma + dhc D 5 mg/kg/day, and sarcoma + dhc D 10 mg/kg/day with 10 mice in each group. The dhc D dissolved in saline was injected into mice intraperitoneally 24 hr after the tumor implantation at a dose of either 5 or 10 mg/kg body weight in the two experimental groups, respectively. Administration of dhc D was repeated every 24 hrs for 10 consecutive days. The body weight of mice was recorded daily.

4. Effect on the viability of macrophages

Thioglycolate-elicited peritoneal macrophages were obtained from Balb/c mice as described previously (Ding *et al.*, 1988). Peritoneal macrophages were cultured in RPMI 1640 media containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were dispensed in 96 well plates (1×10^6/ml, 0.1 ml/well) and incubated for 2~3 hr to ensure the attachment, then treated with various concentrations of dhc D. Peritoneal macrophages were washed twice with culture media to remove nonattached cells before the dhc D treatment. RAW 264.7 cells as an immortalized cells were also cultured and treated with the same procedure. After incubating for 20 hr, the viabilities of both peritoneal macrophages and immortalized cells were determined by tetrazolium reduction using the MTT assay (Denizot and Lang, 1986).

III. RESULTS

1. Cytotoxicity of dhc D on cancer cells

The cytotoxic activity of dhc D on various cancer cells is summarized in Table 1. ED_{50} values of dhc D on A549, SK-MEL-2, COLO 205, and L1210 cells were 1.59, 0.87, 1.60, and 0.49 μg/ml, respectively. The cytotoxic effect of dhc D on the A549 cell line was slightly less than the activity of 5-fluorouracil (1.44 μg/ml) under the same conditions, which is known as exceptionally active cytotoxic substance. Although these ED_{50} values are lower than those of cucurbitacin B or D from *Trichosanthes Kirilowii* (Ryu *et al.*, 1994), the

Table 1. The cytotoxic activity of dhc D on cancer cells

Cell lines	ED_{50} values (μg/ml)*
A549	1.59
SK-MEL-2	0.87
COLO 205	1.60
L1210	0.49

The compound was examined in duplication at five different concentrations.
*ED_{50} value of 23,24-dihydrocucurbitacin D on each cancer cell line was defined as a concentration (μg/ml) that caused 50% inhibition of cell growth. The values are mean of at least four replications.

dhc D also showed a high cytotoxicity against these cells. The unusual potency of the cytotoxic effect of cucurbitacins on various cell lines *in vitro* indicates that they are promising antitumor agents.

2. Antitumor activity of dhc D against sarcoma 180 ascites tumor cells in mice

Antitumor effect of dhc D was evaluated *in vivo*. The *in vivo* results of dhc D's antitumor activity are presented in Fig. 1 and 2. As shown in Fig. 1, the body weight of control mice increased by approximately 86%, 20 days after implantation of sarcoma 180 tumor cells. However, the body weight increase of mice injected with either 5 or 10 mg/kg of dhc D per day was remarkably suppressed as only 42% and 33%, respectively. During the same period, more than half the mice in the treatment groups showed no increase in body weight. These results suggest that growth of sarcoma 180 ascites tumor cells was inhibited by the treatment of dhc D . Separately, when dhc D was injected alone intraperitoneally without sarcoma 180 implantation, mice grew with normal body weight gain and exhibited no discernible symptoms (Data not shown). The dhc D treatment was also shown to prolong the viability of mice implanted with sarcoma 180 tumor cells in a dose dependent manner (Fig. 2). All mice in the control group died within 21 days after the implantation of tumor cells. However, all mice in the treatment groups survived longer than in control group and several of them were healthy until 60 days after sarcoma implantation (3 among 10 mice treated with 5 mg/kg/day, and 6 among 10 mice treated with 10 mg/kg/day). These results show that the administration of dhc D remarkably increased the survival of tumor bearing mice.

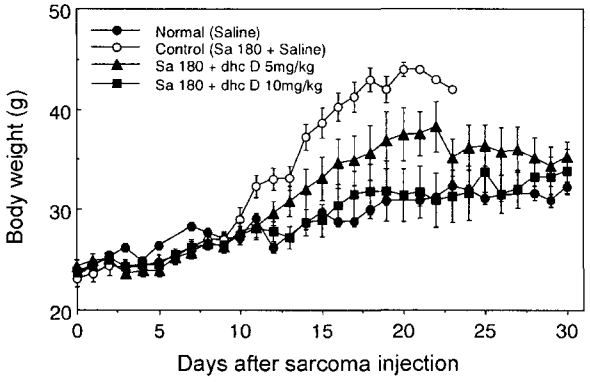

Fig. 1. Body weight changes of mice after implantation of sarcoma 180 tumor cells. Each point represents a mean± SEM for male mice (n = 1 to 10).

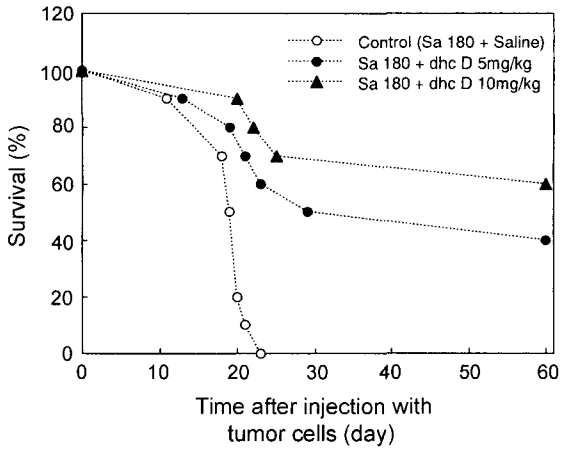

Fig. 2. Survival curve for mice of control and 23,24-dihydrocucurbitacin D treated ones. Each survival curve was generated from male 10 mice.

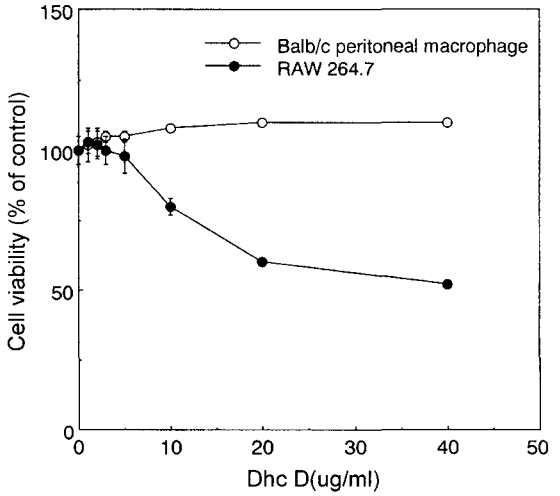

Fig. 3. Effect of 23,24-dihydrocucurbitacin D on the viability of macrophages.

3. Effect of dhc D on the viability of macrophage cells

Figure 3 shows the effect of dhc D on the viability of non-dividing peritoneal macrophages and immortalized dividing macrophage-like cell line RAW 264.7 cells. Dhc D suppressed the viability of RAW 264.7 cells in a dose dependent manner, but did not suppress that of peritoneal macrophages. This and *in vivo* study's results described above suggest that dhc D could work to inhibit the viability of only dividing cell.

IV. DISCUSSION

Cucurbitacins have been found mainly in *Cucurbitacea*, including *Bryonia alba* L., and isolated more than 8 homologues of cucurbitacins from *Cucurbitacea*. However, the compositions of cucurbitacins from the *Bryonia alba* L. root are inconsistent (Konopa *et al.*, 1974a; Baek *et al.*, 1995). Such differences in the composition of cucurbitacins in *Bryonia alba* L. root may be due to differences in collection times, sites, and plant ages, and may cause different cytotoxic potencies. Cucurbitacins are reported to have a wide range of biological activities, including a cytotoxic effect on various cancer cells *in vitro* and *in vivo* (Hylands and Magd, 1986; Ryu *et al.*, 1994; Kupchan *et al.*, 1972). Although some cucurbitacins have been exhibited a high cytotoxic activity, these compounds are not yet interested as antitumor drug (Konopa *et al.*, 1974b).

The present study demonstrated that dhc D isolated from the root of *Bryonia alba* L. exhibits a potent cytotoxic effect *in vitro* on various cancer cells. The dhc D exhibited a strong antitumor effect in mice implanted with sarcoma 180 ascites tumor cells. Furthermore, mice treated solely with dhc D did not show any discernible toxic signs during the experimental period. These results indicat that dhc D may not be toxic in mice. It is interesting that dhc D *in vivo* exhibits different antitumor effect from 23,24-cucurbitacin D in mice (Konopa *et al.*, 1974b). This fact suggests that the hydroxyl group at the side chain site on cucurbitacin D could play a pivotal role in its cytotoxicity.

In addition, dhc D did not exhibit a toxic effect in peritoneal macrophages, although it strongly suppressed the growth of RAW 264.7 cells. These results indicate that dhc D is cytotoxic only on dividing cells, such as RAW 264.7 and tumor cells, but not in normal cells. This compound preferentially increases the viability of peritoneal machrophages by 5%, although not considered significant.

The mechanism of this compounds cytotoxic activity is not yet clearly understood, although the binding of the cucurbitacins to glucocorticoid receptors has been suggested as a cytotoxic mechanism (Witkowski and Konopa, 1981). Macrophages play a central role in the inflammatory process and express high affinity receptors for glucocorticoids. In general, glucocorticoid suppresses the immune response at a high concentration (Witkowski and Konopa, 1981). Therefore, dhc D possibly acts directly on cancer cells with specific receptors for glucocorticoids. The antitumor activity of dhc D may be mediated, at least in part, by the binding with glucocorticoid receptors.

The results from our experiments imply that the dhc D has potent antitumor effect on tumor cells *in vitro* and *in vivo* without any specific toxicity in normal cells, and this effect may be due to its antiproliferative action.

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