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Enhancement of Antineoplastic Effects of Cisplatin by Calmodulin Antagonists in Nude Mice Bearing Human Ovarian Carcinoma[1]

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ABSTRACT

The present study was designed to potentiate the antineoplastic effects of cisplatin by combination with calmodulin antagonists [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5)] in nude mice bearing human ovarian carcinoma. Tumor growth of nude mice treated with W-7 or W-5 combined with cisplatin was significantly inhibited, compared to that of nude mice treated with W-7 alone, W-5 alone, or cisplatin alone. Although treatment with cisplatin alone markedly inhibited lytic activity of spleen cells from tumor-bearing nude mice against the tumor cells, the inhibitory effect was eliminated by combination with W-7 or W-5. There was no significant difference in survival time among untreated, cisplatin-treated, W-7-treated, and W-5-treated groups. Only when cisplatin was followed by W-7 or W-5 was a significant enhancement by W-7 or W-5 of the antitumor effect of cisplatin observed with respect to inhibition of tumor growth as well as prolongation of survival time.

INTRODUCTION

Since the discovery of the antineoplastic effects of cisplatin by Rosenberg *et al.* (1), cisplatin-based combination chemotherapy has been undertaken for ovarian cancer patients and improvement in the survival and response rates has been reported (2–4). However, the relatively marked side effects of cisplatin are one of dose-limiting factors. We have reported previously that combinations of calmodulin antagonists and anticancer drugs resulted in adjuvant effects with regard to the inhibition of tumor cell proliferation *in vitro* (5). In addition, we have demonstrated that in the *in vitro* study timing of administration of calmodulin antagonists is important to obtain optimum adjuvant effects to anticancer drugs (6). On the basis of these previous observations, we attempted to determine timing of administration of calmodulin antagonists (W-7[3] and W-5) and to enhance anticancer effects of cisplatin, using nude mice bearing human ovarian carcinoma.

MATERIALS AND METHODS

Agent. W-7 and W-5 were obtained from Rikaken Co., Ltd., Nagoya, Japan.

Cells. SN cells derived from a patient with clear cell carcinoma of the ovary were used exclusively in this study. The passage number is about 105. The tumorigenicity of the SN cells in the nude mice was 100%. Doubling time and plating efficiency were 26 h and 30%, respectively. The cells were cultured as described previously (7). Briefly, cells were incubated in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml; Grand Island Biological Co.) in a 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days, and the cells were passed when confluency was achieved.

Mice. Approximately 8-week-old female BALB/c nude mice were purchased from Japan Clea Laboratories, Tokyo, Japan, and maintained in a pathogen-free environment.

In Vivo Treatment. SN (5×10^5) cells were inoculated s.c. to right flank of nude mice. After 7 days of tumor inoculation, 2 mg/kg cisplatin and 15 mg/kg W-7 or W-5 were administered i.p. every week for 5 weeks. The mice were inspected daily, and tumor growth was determined by the measurement of diameters in two dimensions of the tumor nodule with a caliper once a week. Tumor volume (cm^3) was calculated according to the formula

$$\frac{4\pi/3 \times (r_1 + r_2)^3}{8}$$

where r_1 is the longitudinal radius and r_2 is the transverse radius. The values were presented as mean ± SD. Blood from tail vein was collected to hematocrit tubes every week and hematocrit values and body weight were measured for monitoring of side effects of drugs.

Measurement of Lytic Activity. Spleen cells of nude mice bearing SN cells or intact nude mice were used as effector cells. The spleen cells were prepared as described previously (8). SN cells were used as target cells. Aliquots containing 10^6 target cells were labeled with 100 μCi of sodium [^{51}Cr]chromate solution (New England Nuclear, Boston, MA) for 1 h in 1 ml of medium. After three washings, 10^4 cells in 0.1 ml of medium were pipetted into microtiter plates (Linbro Scientific, Inc., Hamden, CT). Various concentrations of effector cells in 0.1 ml of medium were added in triplicate to give effector:target cell ratios of 100:1 and 50:1, respectively. After incubation for 18 h at 37°C in a humidified atmosphere of 5% CO_2 in air, supernatants were collected with a Titertek collection system (Flow Laboratories, Inc., Rockville, MD) and counted in a gamma counter. The specific ^{51}Cr release was calculated as

$$\% \text{ of specific activity} = \frac{\text{cpm test release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100$$

Spontaneous and maximum releases are cpm releases from target cells incubated in medium and in medium to which 1 N HCl was added, respectively.

RESULTS

Adjuvant Effects of Calmodulin Antagonists to Cisplatin on Tumor Growth. When 5×10^5 SN cells were inoculated s.c. to the right flank of nude mice, all mice developed palpable tumor on Day 21. Tumor volumes in a cisplatin-treated group were significantly smaller than that in an untreated group 42 and 49 days after tumor inoculation. Treatment with W-7 or W-5 alone did not result in inhibitory effects on tumor volumes. When treatment with W-7 or W-5 was followed by cisplatin, tumor growth was significantly inhibited 42 and 49 days after tumor inoculation, compared to that in untreated group but not in the cisplatin-treated group. If cisplatin was administered before injection of W-7 or W-5, the tumor volumes were significantly smaller on both Days 42 and 49 than those not only in the untreated group but also in the cisplatin-treated group, suggesting the importance of timing of W-7 or W-5 treatment to

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[3] The abbreviations used are: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide.

Table 1 *Adjuvant effects of W-7 or W-5 to cisplatin on tumor growth on SN cells grown in nude mice*

In cisplatin→W-7- or cisplatin→W-5-treated groups, cisplatin was administered 3 h before treatment with W-7 or W-5. In W-7→cisplatin- or W-5→cisplatin-treated groups, W-7 or W-5 was administered 3 h before treatment with cisplatin. Each experimental group consisted of 10 mice. Statistical analysis was by Mann-Whitney *U* test.

Days after inoculation	Tumor volume (cm^3)							
	Untreated group	Cisplatin-treated group	W-7-treated-group	W-7→cisplatin-treated group	Cisplatin→W-7-treated group	W-5-treated group	W-5→cisplatin-treated group	Cisplatin→W-5-treated group
14	Palpable	Palpable	Palpable	Palpable	Palpable	Palpable	Palpable	Not palpable
21	0.48 ± 0.52[a]	0.13 ± 0.15	0.38 ± 0.41	0.15 ± 0.11	0.04 ± 0.05	0.35 ± 0.32	0.13 ± 0.10	0.01 ± 0.01
28	1.36 ± 1.22	0.52 ± 0.35	1.06 ± 0.98	0.55 ± 0.37	0.34 ± 0.34	0.84 ± 0.91	0.45 ± 0.44	0.16 ± 0.21
35	3.21 ± 3.04	1.03 ± 0.88	2.52 ± 3.01	1.05 ± 0.79	0.28 ± 0.36	2.02 ± 1.99	0.92 ± 0.84	0.23 ± 0.26
42	6.56 ± 4.51	1.99 ± 0.85[b]	5.14 ± 4.02	1.33 ± 0.87[b]	0.27 ± 0.18[b, c]	4.56 ± 3.13	1.98 ± 0.96[b]	0.30 ± 0.09[b, d]
49	7.07 ± 5.73	3.85 ± 2.79[b]	6.24 ± 4.97	2.68 ± 1.26[b]	0.58 ± 0.37[b, c]	6.88 ± 4.72	3.17 ± 1.81[b]	0.51 ± 0.15[b, d]

[a] Mean ± SD.
[b] $P < 0.05$, compared to untreated group.
[c] $P < 0.05$, compared to cisplatin-treated group and W-7-treated group.
[d] $P < 0.05$, compared to cisplatin-treated group and W-5-treated group.

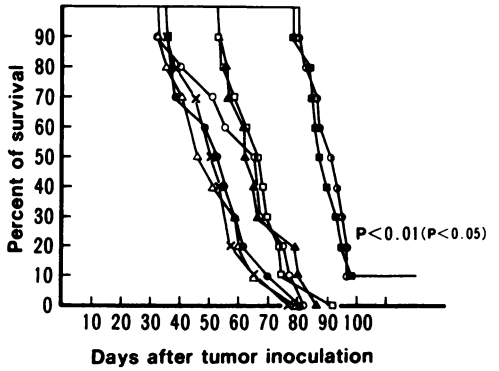

Fig. 1. Effect of combination of cisplatin and calmodulin antagonists (W-7 and W-5) on survival time of nude mice bearing human ovarian carcinoma. The statistical analysis was by Mann-Whitney *U* test. *P*, when compared to untreated control. *P* in parentheses, when compared to cisplatin alone-, W-7 alone-, and W-5 alone-treated groups. ×, untreated control; ○, cisplatin alone; ●, W-7 alone; △, W-5 alone; ▲, W-7 followed by cisplatin; □, W-5 followed by cisplatin; ■, cisplatin followed by W-7; ◑, cisplatin followed by W-5.

potentiate adjuvant effects of W-7 or W-5 on cisplatin (Table 1). As shown, in Fig. 1, there was no significant difference in the survival time among untreated, W-7 alone-, W-5 alone- and cisplatin alone-treated groups, although the survival times in W-7→cisplatin- and W-5→cisplatin-treated groups seemed to be marginally significantly prolonged only when compared to those in untreated group. On the other hand, those in the cisplatin→W-7- and cisplatin→W-5-treated groups were significantly longer than that not only in untreated, W-7, and W-5 alone-treated groups but also in cisplatin-treated groups (Fig. 1).

Effects of Cisplatin and Calmodulin Antagonists on Lytic Activity to SN Cells of Spleen Cells in Nude Mice. In addition, we attempted to elucidate effects of combination of cisplatin and calmodulin antagonists (W-7 and W-5) on immune function in tumor-bearing nude mice. As shown in Table 2, spleen cells of nude mice before tumor inoculation did not have any lytic activity to SN target cells. At 3 weeks after inoculation, the spleen cells in untreated nude mice acquired significant lytic activities, while nude mice treated with cisplatin alone had the depressed lytic activities. The inhibitory effect of cisplatin was eliminated by combination with W-7 and W-5. Although the lytic activity in nude mice treated with cisplatin after injection of W-7 or W-5 decreased to about one-half of the level at 5 weeks after tumor inoculation compared to that at 3 weeks, the enhanced lytic activity in nude mice treated with cisplatin before injection of W-7 or W-5 remained unchanged even at 5 weeks. These results suggest that synergistic effects of W-7 or W-5 to cisplatin on inhibition of tumor growth and prolongation of

Table 2 *Effects of cisplatin and calmodulin antagonists (W-7 or W-5) on lytic activity to SN target cells of spleen cells in nude mice with SN cells*

Spleen cells from nude mice before, 3 weeks after, and 5 weeks after tumor inoculation were used as effector cells, respectively. Cisplatin, W-7, and W-5 were administered as described in Table 1. The results were analyzed using the Mann-Whitney *U* test.

Spleen cells	% of cytotoxicity	
	50:1[a]	100:1
Before inoculation	−3.7 ± 0.4[b]	−4.2 ± 0.5
3 wk after inoculation, untreated group	10.9 ± 0.3	19.1 ± 1.3
W-7 alone-treated group	12.1 ± 0.6	22.3 ± 1.4
W-5 alone-treated group	11.2 ± 0.7	21.8 ± 2.0
Cisplatin-treated group	2.2 ± 0.2[c]	4.9 ± 0.3[c]
Cisplatin→W-7-treated group	16.2 ± 2.2[d, e]	30.5 ± 3.1[d, e]
Cisplatin→W-5-treated group	18.1 ± 3.4[d, e]	31.2 ± 2.8[d, e]
W-7→cisplatin-treated group	12.9 ± 1.0[d]	23.1 ± 1.4[d]
W-5→cisplatin-treated group	13.2 ± 2.1[d]	23.4 ± 1.7[d]
5 wk after inoculation, untreated group	3.3 ± 1.1[f]	9.5 ± 0.6[f]
W-7 alone-treated group	6.1 ± 2.3	11.7 ± 1.7
W-5 alone-treated group	5.0 ± 1.4	10.3 ± 1.1
Cisplatin-treated group	0.8 ± 0.1[c]	2.6 ± 0.2[c]
Cisplatin→W-7-treated group	15.4 ± 1.3[d, e]	28.5 ± 1.7[d, e]
Cisplatin→W-5-treated group	9.7 ± 2.6[d, e]	19.9 ± 1.3[d, e]
W-7→cisplatin-treated group	6.8 ± 0.4[d]	8.0 ± 0.6[d]
W-5→cisplatin-treated group	4.9 ± 0.5[d]	10.0 ± 2.1[d]

[a] Effector:target cell ratio.
[b] Mean ± SD.
[c] $P < 0.01$, compared to untreated group.
[d] $P < 0.01$, compared to cisplatin-treated group.
[e] $P < 0.05$, compared to W-7→cisplatin- or W-5→cisplatin-treated group as well as untreated group.
[f] $P < 0.01$, compared to untreated group in 3 weeks.

survival time observed in this study may result in part from enhancement of the lytic activities in nude mice bearing SN cells. Next, we attempted to elucidate the direct effects of W-7 or W-5 on the lytic activity in spleen cells of nude mice before inoculation. The lytic activity of their spleen cells was defective. The defective lytic activity could be restored by preincubation of target cells with W-7 and W-5 (Table 3).

DISCUSSION

In the present study, we have demonstrated that a combination of cisplatin and calmodulin antagonists (W-7 and W-5) resulted in enhancement of its antitumor effects only when treatment with cisplatin was followed by treatments with calmodulin antagonists. We have already observed that in order to result in adjuvant effects of calmodulin antagonists to 5-fluorouracil on the inhibition of tumor cell proliferation *in vitro*, calmodulin antagonists should be administered after (not before) treatment with anticancer drugs (6). Calmodulin inhibitors have been reported to stabilize tumor cell membranes (9).

Table 3 *Effects of W-7 or W-5 on lytic activity of spleen cells in intact nude mice to SN cells*

Spleen cells from intact nude mice were used as effector cells. After effector or target cells were incubated in the absence or presence of 5 μM W-7 or 5 μM W-5 for 24 h, these cells were washed 3 times with fresh medium and used for ^{51}Cr release assay.

Effector cells	Untreated target cells	W-7-treated target cells	W-5-treated target cells
Untreated			
50:1[a]	−1.0 ± 1.2[b]	5.4 ± 1.0[c]	4.9 ± 1.2[c]
100:1	−1.0 ± 2.1	7.8 ± 1.1[c]	6.7 ± 1.4[c]
W-7-treated			
50:1	1.7 ± 1.3	3.6 ± 1.4	
100:1	5.8 ± 2.6[d]	6.8 ± 1.5	
W-5-treated			
50:1	1.2 ± 1.4		3.1 ± 1.0
100:1	2.1 ± 3.1		5.2 ± 1.5

[a] Effector:target cell ratio.
[b] Mean ± SD.
[c] $P < 0.01$, compared to untreated target cells.
[d] $P < 0.01$, compared to untreated effector cells.

Recently, it has been reported that calmodulin antagonists caused a marked inhibition in recovery from bleomycin-induced potentially lethal damage of cells by preventing the repair of damaged DNA (10). Similarly, it is possible that W-7 disturbs the repair process of DNA damaged by cisplatin. Treatment with cisplatin followed by W-7 or W-5 brought about a significant prolongation in survival time. The concentrations of W-7 or W-5 and cisplatin used in the present study did not result in any adverse side effects as confirmed by measurement of body weight and hematocrit values (data not shown). Recently, we reported that preincubation of target cells with calmodulin antagonists (W-7 or W-5) resulted in restoration of decreased lytic activity of autologous human lymphocytes (11). Accordingly, to determine whether adjuvant effects of W-7 or W-5 to cisplatin on tumor growth can be mediated through such immunological mechanisms, we measured lytic activities to tumor target cells of spleen cells in tumor-bearing nude mice receiving treatment with cisplatin and calmodulin antagonists or receiving no treatment. Calmodulin antagonists have been reported to be inhibitors of human natural killer activity (12). Since spleen cells in athymic nude mice do not contain normal T-cells (13), the results shown in the present study are considered to be different from previous observations obtained using human peripheral blood lymphocytes (12). Although nude mice have been shown to have T-cell precursors that can be differentiated further under the microenvironment of the thymus (14), such T-cell precursors in spleen cells of nude mice appear to differentiate to killer T-cells by exposure to calmodulin antagonists. Beattie *et al.* (15) demonstrated evidence of induction of a small population of functional T-cells *in vivo* following xenografts of human tumors. Similarly, we also confirmed that functional T-cells could be induced 3 weeks after xenograft of human ovarian carcinoma (SN cells). Cisplatin seems to damage not only tumor cells but also spleen cells, subsequently lowering cytotoxicity of spleen cells to the tumor cells. The lowering effect of cisplatin on cytotoxicity could be eliminated by subsequent treatment with W-7 or W-5. When treatment with W-7 or W-5 was followed by cisplatin, the enhancing effects of W-7 or W-5 on cytotoxicity were partly abrogated by subsequent treatment with cisplatin. The antitumor effect of calmodulin antagonists against experimental tumors in mice has been reported (16). However, in a concentration of calmodulin antagonists used in this study, neither W-7 nor W-5 had any antitumor effects. Virtually identical antitumor activity was observed when cisplatin was combined with W-7 or W-5, yet W-5 is a very weak calmodulin antagonist in comparison to W-7 (17). With respect to the adjuvant effects to cisplatin on tumor growth and the restorative effects of cisplatin-induced depressed immune function, W-5 had as much adjuvant and restorative effects as W-7. Recently, Onoda *et al.* (18) described the synergistic cytotoxicity of cisplatin combined with the calcium channel blocker, nifedipine. Therefore, it is possible that naphthalenesulfonamide potentiation of cisplatin activity is related more to calcium than to calmodulin. These observations provide a new strategy for enhancement of sensitivity of cancer cells to anticancer drugs and circumvention to their resistance.

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