Antitumour Potential of Pollen Extract on Lewis Lung Carcinoma Implanted Intraperitoneally in Syngeneic Mice

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A defined pollen extract of selected plants has been reported to possess some pharmacological activities on chronic prostatitis or benign prostatic hyperplasia. This paper describes the antitumour potential of the water-soluble fraction (Cernitin T60) of the pollen extract against Lewis lung carcinoma implanted intraperitoneally in syngeneic mice. Cernitin T60 was not cytotoxic in cell cultures at concentrations up to 2.5 mg/mL, while it is significantly prolonged the life-span of mice carrying the tumour without any apparent side effects at 0.5 g/kg. In addition, Cernitin T60 demonstrated beneficial therapeutic effects in an additive fashion on the life-span of mice when it was combined with standard cytotoxic antitumour drugs such as adriamycin, cisplatin, vincristine, methotrexate, fluorouracil, or thioguanine. The antitumour potential of Cernitin T60 was completely abolished by treatment with inhibitors of macrophage functions (2-chloroadenosine or carrageenan), but not with the T-cell inhibitor (cyclosporin A). Cernitin T60 appears to be a potent immunostimulator of macrophages.

Keywords: antitumour; pollen extract; murine long tumour; immunomodulator

INTRODUCTION

Pollen extract of selected plants has been widely used for nearly 30 years as a phytotherapeutic agent to chronic prostatis or bening prostatic hyperplasia (Ebeling, 1986; Buck *et al.,* 1989, 1990) based on its α-adrengic blockage (Kimura *et al.,* 1986) antiinflammatory properities (Ito *et al.,* 1984; Loschen and Ebeling, 1992) and inhibitory potential on hormone-dependent growth of tissues of benign prostatic hyperplasia in nude mice (Wagner *et al.,* 1992) or cultivated prostate cells *in virto* (Habib, 1992). Pollen extract also has been reported to reduce hepatotoxicity of paracetamol (Juzwiak *et al.,* 1992) and to prevent poisoning caused by organic solvents (Ceglecka *et al.,* 1992). Numerous substances have been isolated from pollen extract; amino acids, carbohydrates, deoxyribosides, enzymes, coenzymes, vitamins, minerals, trace elements, sterols (Kvanta, 1986; Nielsen *et al.,* 1957; Nielsen and Holmström, 1957) and polyunsaturated fatty acids (Seppänen *et al.,* 1989). Cernitin T60 contains water-soluble α-amino acids (6.0% - 9.2%) and other water-soluble substances of pollen extract.

We now report the antitumour potential of a pollen extract on a system of Lewis lung carcinoma implanted intraperitoneally (i.p) in syngeneic mice (Furusawa and Furusawa, 1985, 1989, 1990), and the beneficial effects in combination with standard chemotherapeutic drugs.

MATERIALS AND METHODS

KB cell cultures for cytotoxicity test. KB cell line, a human pharyngeal carcinoma, adopted as a standard cell line for screening cytotoxic agents in the National Cancer Institute (Goldin *et al.,* 1977) was used in tests. The procedure has been described previously (Furusawa and

Table 1. Cytotoxicity of Cernitin T60 on KB cell cultures: comparison with standard cytotoxic drugs

Cultivation period	Degree of cytotoxicity (0 to 4+) and dose/mL of culture medium									
	Cernitin T60				ADR	DDP	VCR	MTX	5-FU	DACT
	10 mg	5 mg	2.5 mg	1.25 mg	0.2 μg	2 μg	0.01 μg	0.05 μg	1 μg	0.002 μg
Day 1	2+	0	0	0	2+	2+	2+	2+	2+	2+
Day 2	3+	1+	0	0	3+	3+	3+	3+	3+	3+
Day 3	4+	1+	0	0	4+	4+	4+	4+	4+	4+
Day 4		2+	0	0						
Day 5		3+	0	0						
Day 6		3+	0	0						

Graded doses of Cernitin T60 or 2 Minimum Toxic Dose of cytotoxic drugs in 0.1 mL of MEM medium was added into the tube cultures (1 mL of culture medium) of human KB cells immediately after seeding of ca. 2 × 10 000 cells/mL, and incubated for 6 days. One seeding was done on day 3 (4 times dilution of cell numbers) in the presence of T60. Degree of cytotoxic effect (0 to 4+) was determined by the morphological criteria, density of cells, degrees of acid production (pH changes of medium) in comparison with the control cultures. ADR: Adriamycin, DDP: Cisplatin, VCR: Vincristine, MTX: Methotrexate, 5-FU: 5-Fluorouracil, DACT: Actinomycin-D. μg = 0.001 mg

Furusawa, 1985) and a brief description is included in Table 1. The culture medium was composed of Eagle's minimum essential medium (MEM) with 5% fetal calf serum and gentamycin.

Tumour-animal system for testing activities of agents. Lewis lung carcinoma was obtained from the National Cancer Institute and was maintained in syngeneic C57BL/6 mice in our laboratory. The tumour mass (2—3 g, 2—4 weeks old) was minced in 10 mL of MEM and forced through an 80-mesh screen with a 20-gauge needle. An aliquot (0.2 mL) of tumour homogenate containing 2—4 x 10^5 live tumour cells was injected intraperitoneally (i.p.) into young adult (18—20 g) C57BL/6 mice. They died of cancerous panperitonitis accompanied by disseminated solid tumour masses and massive ascitic bleeding within 10-20 days.

Preparation of agents. The water-soluble fraction (Cernitin T60) of defined pollen extract (product of SAPEC S.A., Barbengo, Switzerland) was dissolved in MEM culture medium for cytotoxicity test or in distilled water for i.p administration (0.1 mL/mouse). Adriamycin, cisplatin, vincristine, methotrexate, 5-fluorouracil, 6-thioguanine, 2-chloroadenosine, carrageen (Type III, kappa) and cyclosporine (cyclosporine A) were obtained from commercial sources. Drugs were freshly dissolved or suspended in distilled water before use or frozen until used.

Statistical analysis. The statistical significance of differences was analysed by Student's *t*-test for mean survival time (MST) and Fisher's exact test for ranking of survivors.

RESULTS

Cytotoxicity test of Cernitin T60 in KB cell cultures

To determine the degree of cytotoxicity, the graded doses of Cernitin T60, dissolved in MEM culture medium, were added to KB cell cultures. The results are shown in Table 1. It was found that Cernitin T60 was not cytotoxic at a high concentration of 2.5 mg/mL, although it became toxic at 5-10 mg/mL. Routinely, we designate a chemical or an extract of crude natural product origin as nontoxic when it was not cytotoxic at a concentration of 0.1-0.2 mg/mL, respectively. The nontoxicity of Cernitin T60 was also confirmed in murine cell line, BALB/3T3, NIH/3T3, and XC cells (data not listed)

Effect of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice

Preliminary toxicity test of Cernitin T60 in normal mice demonstrated that a single i.p. injection of 80 mg (4—4.4 g/kg) was lethal and 5 multiple (once/every other day) injections of 30 mg dose (1.5—1.7 g/kg) were barely tolerable with marked loss of body weight, while the same multiple i.p. injections of 10 mg dose were apparently not toxic and did not affect the natural increase of body weight. We also administered the graded doses in the nontoxic range (10—0.04 mg/mouse), i.p., every-other-day schedule for a total of 5 injections, starting 1 day or 2 days after tumour implantation. The results are shown in Table 2. It was found that the therapeutic administration of Cernitin T60 significantly prolonged the survival time of mice at the broad range of dosage (0.2—10 mg), although total cure was not observed.

Effect of Cernitin T60 in combination with standard cytotoxic drugs on i.p. implanted Lewis lung carcinoma

The optimal dose of standard cytotoxic drugs for combination therapy in Lewis lung tumour system has already been established (Furusawa and Furusawa, 1990). Administration of cytotoxic drugs at the optimal single dose was done 1 or 2 days after tumour implantation, and treatment with Cernitin T60 at dose of 1.5.or 2.0 mg/mouse was started on day 1, 2 or 3 and continued every other day for a total of 4 or 5 injections. Table 3 shows the results. The combination therapy of Cernitin T60 with adriamycin (1μg: 1/100 of the maximum tolerable dose, MTD), cisplatin 10 μg: 1/15 MTD), vincristine (5μg: 1/4 MTD), 5-flurouracil (100μg: 1/10 MTD), methotrexate (200 μg: 1/2 MTD) or 6-thiogunine (150 μg: 1/2 MTD) significantly prolonged the survival time in an additive fashion, and some mice survived in a tumour-free state at the end of the observation period. These optimal doses of cytotoxic drugs, except methotrexate and 6-thioguanine, were relatively low and not produce any toxic signs even when combined with Cernitin T60. The optimal dose of methotrexate or 6-thioguanine was one-half of the MTD and produced some loss of body weight, but the combined regimen with Cernitin T60 did not show any further loss of body weight. This means that Cernitin T60 seems to be a safe supplemental agent in increasing the efficacy of chemotherapeutic drugs, without additional increase of drug toxicity in mice.

Effect of 2-chloroadenosine, carrageenan, or cyclosporine on the antitumour potential of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice

As mentioned before, Cernitin T60 is noncytotoxic in cell cultures at a high concentration of 2.5 mg/mL (Table 1), while multiple administration at a low dose of 0.2 mg/mouse/day was effective against tumour cells *in vivo* (Table 2). Therefore, it is unlikely that the agent suppresses the tumour cell growth by direct contact, but instead indirectly by enhancing the host immune system, such as macrophage and/or lymphocyte lineages. The potential of Cernitin T60 with concomitant treatment with 2-choloadenosine or carrageenan, both specific inhibitors of macrophage functions (Schultz and Altom, 1986; Schultz *et al.,* 1986; Rumjunek and Brent, 1978; Neveu and Thierry, 1982), or with cyclosporine A, a specific inhibitor of T lymphocyte functions (DiPadova, 1989) was examined. The multiple administration of 2-chloroadenosine (0.1 mg, i.p.), carregeenan (0.25 mg, i.p.) or cyclosporine (2 mg, s.c.) was started on day 1 and followed by multiple administration of Cerniltin T60. Table 4 shows the results. The antitumour potential of Cernitin T60 was completely abolished by treatment with 2-chloroadenosine or carrageenan, but not with cyclosporine.

Table 2. Effect of Cernitin T60 on intraperitoneally implanted Lewis lung carcinoma in syngeneic mice: comparison with standard cytotoxic drugs

Agent	Dose/mouse and schedule, i.p.	MST ± SD days	Number of mice survived/total	ILS %
Control (experiment 1)		10.5 ± 2.2	0/10	
Cernitin T60	10 mg, days 1, 2, 4, 6, 8	18.3 ± 6.0[a]	0/10	74
Control (experiment 2)		11.5 ± 2.4	0/6	
Cernitin T60	5 mg, days 2, 4, 6, 8, 10	23.6 ± 8.6[a]	1/5	105
	1 mg, days 2, 4, 6, 8, 10	21.8 ± 6.7[a]	0/5	90
	0.2 mg, days 2, 4, 6, 8, 10	22.8 ± 7.9[a]	0/5	98
Control (experiment 3)		12.3 ± 2.8	0/12	
Cernitin T60	2 mg, days 1, 3, 5, 7, 9	23.9 ± 7.8[a]	0/12	94
	0.2 mg, days 1, 3, 5, 7, 9	16.3 ± 4.6[b]	0/6	33
	0.04 mg, days 1, 3, 5, 7, 9	14.2 ± 3.5	0/6	15
Control (experiment 4)		13.6 + 5.4	0/5	
Vincristine	10 μg (1/2 MTD), day 1	26.4 ± 9.2[a]	0/5	94
Control (experiment 5)		14.9 ± 4.4	0/10	
Cisplatin	10 μg (1/15 MTD), day 1	25.3 ± 14.1[b]	0/8	70
Control (experiment 6)		13.9 ± 2.9	0/9	
Adriamycin	2 μg (1/50 MTD), day 1	24.2 ± 11.6[b]	0/9	74
Control (experiment 7)		12.6 + 2.4	0/10	
5-Fluoroucracil	0.5 mg (1/2 MTD), day 1	20.4 ± 13.9[b]	0/5	62
Control (experiment 8)		13.2 + 2.9	0/5	
Methotrexate	0.5 mg (1 MTD), day 1	19.4 ± 6.0[b]	0/5	47

[a] $p < 0.01$, [b] $p < 0.05$ compared with controls. MST, mean survival time; ILS, increase in life span; MTD, maximum tolerable dose.
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic C57BL/6 mice (18-20 g body weight). Treatment with Cernitin T60 was started i.p. on the next day (day 1) or day 2 and continued until 8, 9, or 10, a total of 5 injections. Treatment with cytotoxic drugs was done i.p. on day 1 only. Observation was done until day 50.

Table 3. Effect of Cernitin T60 in combination with standard cytotoxic drugs on i.p. implanted Lewis lung carcinoma in syngeneic mice

Agent	Dose/mouse and schedule, i.p.	MST ± SD	Number of mice survived/total	ILS %
Control (experiment 1)		11.5 ± 1.6	0/10	
Vincristine 2 or 5 μg, day 1		16.9 ± 5.4[a]	0/10	47
Cernitin T60	1.5 mg, days 1, 3, 5, 7 or 2 mg, days 2, 4, 6, 8, 10	20.7 ± 6.0[a]	0/10	80
Vincristine + T60	same dose	32.3 ± 13.1[b]	3/10	181
Control (experiment 2)		12.1 ± 2.2	0/10	
Cisplatin 5 or 10 μg, day 1		19.7 ± 5.6[a]	0/10	63
Cernitin T60	2 mg, days 2, 4, 6, 8 or 2 mg, days 3, 5, 7, 9	19.6 ± 6.5[a]	0/10	62
Cisplatin + T60	same dose	34.4 ± 11.3[a]	2/10	184
Control (experiment 3)		11.5 ± 2.1	0/10	
Adriamycin 1 μg, day 1 or 2		15.9 ± 3.5[a]	0/10	38
Cernitin T60	2 mg, days 2, 4, 6, 8, 10 or 2 mg, days 3, 5, 7, 9, 11	18.1 ± 5.5[a]	0/10	57
Adriamycin + T60	same dose	37.6 ± 17.6[a]	4/10[b]	227
Control (experiment 4)		11.3 ± 2.0	0/10	
Fluorouracil 100 μg, day 1		21.9 ± 8.1[a]	0/10	94
Cernitin T60	2 mg, days 2, 4, 6, 8, 10	20.2 ± 6.9[a]	0/10	79
Fluorouracil + T60	same dose	37.0 ± 15.4[a]	3/10	228
Control (experiment 5)		11.3 ± 2.5	0/10	
Methotrexate 200 μg, day 1		19.2 ± 6.0[a]	0/10	70
Cernitin T60 2 mg, days 3, 5, 7, 9		17.9 ± 6.6[a]	0/10	58
Methotrexate + T60 same dose		44.2 ± 18.1[a]	4/10[b]	291
Thioguanine 150 μg, day 1		20.0 ± 5.0[a]	0/10	77
Thioguanine + T60	same dose	32.3 ± 16.4[b]	3/10	186

[a] $p < 0.01$, [b] $p < 0.05$ compared with controls vs. single agent only (T60 or drug), or single agent only vs. the combination (T60 + drug).
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic C57BL/6 mice (18-20 g body weight). The i.p. administration of cytotoxic drugs on day 1 or 2 was followed by i.p. administration of T60 on day 1, 2, or 3 and continued every other day for a total of 4 or 5 injections. Observation was done until day 50.
Experiment 1, 2, or 3 is sum of each two experiments. For example, experiment 1 is composed of two experiments which were done with different dose and schedule: 2 μg of vincristine, day 1 and 1.5 mg of T60, days 1, 3, 5, 7; 5 μg of vincristine, day 1 and 2 mg of T60, days 2, 4, 6, 8, 10; and 5 mice used in each group.

Table 4. Abrogation of antitumour activity of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice by concomitant treatment with 2-chloroadenosine or carrageenan but not with cyclosporine

Agent	Dose/mouse and schedule, i.p.	MST ± SD	Number of mice survived/total	ILS %
Control (experiment 1)		10.1 ± 1.5	0/10	
Chloroadenosine 0.1 mg, i.p. days 1, 2, 3, 4, 6		11.5 ± 2.9	0/10	15
Cernitin T60	2 mg, i.p. days 2, 4, 6, 8	26.4 ± 16.0[a]	0/10	161
Chloroadeno. + T60	same dose	10.7 ± 1.5	0/10	6
Control (experiment 2)		11.2 ± 1.1	0/5	
Carrageenan 0.25 mg, i.p. days 1, 3, 5, 7		11.0 ± 0.7	0/5	−2
Cernitin T60 1.5 mg, i.p. days 1, 3, 5, 7		19.4 ± 7.3[a]	0/5	73
Carrageenan + T60	same dose	12.4 ± 3.2	0/5	11
Control (experiment 3)		13.7 ± 2.8	0/6	
Cyclosporine 2 mg, s.c., days 1, 2, 3, 4, 6		10.8 ± 0.5	0/5	−21
Cernitin T60 10 mg, i.p. days 1, 2, 3, 4, 6		30.0 ± 18.4	2/5	119
Cyclosporine + T60	same dose	31.4 ± 17.4[a]	2/5	129

[a] $p < 0.05$ compared with controls vs treated. s.c. subcutaneously.
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic mice (18–20 g). Each agent was administered i.p. or s.c. daily or every other day for a total of 4 or 5 injections. Chloroadenosine or carrageenan was preceded 1 h to T60 when administered on the same day.

DISCUSSION

The standardized pollen extract preparation of selected plants, recognized as a phytotherapeutic agent, has been shown to posses definite symptomatic efficacy on chronic prostatis or benign prostatic hyperplasia (Vahlensieck and Rutishauser, 1992). The pharmacological basis of action seems to be multiple, such as α-adrenergic blockage (Kimura *et al.,* 1986), antiinflammatory (Ito *et al.,* 1984; Loschen and Ebeling, 1986) and growth-inhibition on prostate cells (Habib, 1992) or tissues (Wager *et al.,* 1992). The pollen extract is also reported to reduce hepatotoxicty of drug (Juźwiak *et al.,* 1992) or poisoning of organic solvents (Ceglecka *et al.,* 1992).

We have demonstrated the antitumour potential of the water-soluble fraction (Cernitin T60) of pollen extract against Lewis lung carcinoma implanted i.p. in syngeneic mice. The system of Lewis lung carcinoma in syngeneic C57BL/6 semi-syngerenic BDF1 (C57BL/6 X DBA/2) mice was adopted for antitumour screening by the National Cancer Institute on 1975, and about 80% of the cytotoxic drugs that had been active on the Lewis lung tumour screen were reported to be active at a clinical level (Staquet *et al.,* 1983). In addition, we have found that Lewis lung carcinoma implanted i.p. in syngeneic mice is relatively sensitive to immunotherapy by exogenous immunostimulators of chemical or plant origin (Furusawa and Furusawa, 1989, 1990; Furusawa *et al.,* 1992). We have also found the Cernitin T60 possessed beneficial efficacy in an additive fashion on the survival time and the cure rate when combined with standard cytotoxic drugs. This suggests a possible clinical application of the pollen extract as an active supplemental agent in cancer chemotherapy without fear of additional toxicity. The optimal effective dose (2 mg/mouse = 100-111 mg/kg, every other day) which is 1/20 of the maximum tolerable dose (20 mg/mouse, daily) could be continuously administered without any side effects, even when combined with cytotoxic drugs. Cernitin T60 was not directly cytotoxic in cell cultures of human KB cells, murine BALB/3T3. NIH/3T3 or XC cells, but it seemed to kill tumour cells directly *in vivo* via activation of macrophage lineage of host immune system, which could be abrogated by the administration of 2-chloroadenosine or carrageenan, both specific inhibitors of macrophage function (Shultz *et al.,* 1986; Rumjanek and Brent, 1978). We did not observe a dependency of the antitumour action of Cernitin T60 upon T-cell activation which should have been abolished by

the concomitant administration of cyclosporine A, a potent immunosuppressor specific for T-cell lineage (diPadova, 1989). This is contrary to our previous finding that antitumor activity of polysaccharide of an edible mushroom origin was abolished by the pretreatment with cyclosporine in our Lewis lung tumour system (Furusawa *et al.,* 1992)

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