Anticancer Activity of Narcissus Extracts in Mice[1] (37247)

E. FURUSAWA, N. SUZUKI, S. TANI, S. FURUSAWA, G. Y. ISHIOKA,[2] AND J. MOTOBU[2]
Department of Pharmacology, School of Medicine, University of Hawaii, Honolulu, Hawaii 96816

Through our antiviral screening work with 400 medicinal plants of the Pacific area, we have found one agent (narcissus alkaloid) which showed remarkable antiviral activity against several neurotropic RNA virus infections in mice (1, 2). Recently, this alkaloid has been found to increase the life span of mice made leukemic by infection with Rauscher virus in comparison with several standard antileukemic drugs (3, 4). There are two papers reporting as active (5) or negative (6) the anticancer activity of this alkaloid against transplantable tumors in mice. We now report that one of the neutral fractions (nonalkaloidal) of the narcissus bulbs demonstrates anticancer activity against Ehrlich ascites tumor cells and # $6C_3H$-ED lymphosarcoma (solid type) in mice. Also, we have confirmed that the narcissus alkaloid has moderate anticancer activity against these two tumors as well as MCDV-12 leukemia cells.

Materials and Methods. Tumor cell systems for chemotherapy. Ehrlich ascites tumor cells have been passaged intraperitoneally (ip) in a random strain of Swiss albino mice for many years. The # $6C_3H$-ED lymphosarcoma cells have been passaged subcutaneously (sc) for one year in a stable hybrid strain (black hair), originated from C_3H male (brown) and Swiss albino female mice. The transplantable ascites line of MCDV-12 leukemia cells was originally isolated from a Rauscher virus-inoculated BALB/c male mouse at NIH, and was supplied by Dr. M. A. Chirigos, NIH, and carried in BALB/c inbred mice for 2 years by Dr. P. Yoder, University of Hawaii. At present, no infective Rauscher virus can be detected in the cells.

Preparation of narcissus alkaloid. The method of extraction of the total alkaloidal fraction from the bulbs of *Narcissus tazetta* L. has been described (7). A yellow crystalline alkaloid (named 2-X) was isolated from the final chloroform fraction (which contained total alkaloids) after standing at 4° for a month. This was identified by Bristol Laboratories, NY and later in this laboratory as pseudolycorine. After removing the 2-X crystals, the chloroform fraction was shaken with water, and the water-soluble fraction (named Residual Alkaloid) was collected and concentrated. The 2-X was dissolved in water (pH 6) for use.

Preparation of neutral fraction of narcissus bulbs. Narcissus bulbs were extracted in 95% ethanol. The concentrated ethanol extracts were acidified with 2 *N* HCl and shaken with chloroform. The neutral and acidic substances were moved to chloroform portion. Then the portion was washed with 2 *N* potassium carbonate to remove the acidic substances (A). Most of the substance (99%) remaining in the chloroform portion was the neutral fraction (N) of the narcissus extracts. The concentrated neutral fraction was further separated into 7 portions by passing through an aluminum oxide column by running chloroform (1st to 4th portions), chloroform with 3% methanol and 3% acetone (5th portion), chloroform with 20% methanol (6th), and finally methanol with 30% chloroform (7th). Each fraction was flash dried then suspended in water (pH 8) at 20 mg solid/ml for use.

Results. Effect of narcissus agents on survival of mice inoculated with Ehrlich ascites tumor cells. Alkaloidal and nonalkaloidal fractions of the narcissus extracts were tested against Ehrlich ascites cells in adult Swiss albino mice (av. 20 g). The single and

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TABLE I. Effect of Narcissus Agents on Survival of Mice Inoculated with Ehrlich Ascites Tumor Cells.

Agent	Maximum nontoxic dose/kg (mg)	Treatment schedule (ip)	No. died/total		Median survival time (MST) (days)		Prolong. of MST over control (%)	Survivals without ascites[b] (%)
			Treated	Control	Treated	Control		
2-X alkaloid	200	single[a]	16/16	16/16	22	22	0	0
	100	7/2d[a]	18/20	20/20	33.5	21.5	56	10
	50	6/d[a]	20/20	20/20	31	21	48	0
Residual alk.	400	single	16/16	16/16	22	22	0	0
	200	7/2d	10/20	20/20	>50	21.5	>100	50
	100	6/d	20/20	20/20	29	20	45	0
Neutrals (N)	1600	single	20/47	47/47	>40	19.1	>100	58
Acids (A)	1000	single	16/20	20/20	26.3	19	39	20
Cytoxan	100	single	12/20	20/20	30.5	19	61	40
	50	6/2d	20/20	20/20	35	17	>100	0
½ dose	25	6/2d	20/20	20/20	22	17	29	0
¼ dose	12.5	6/2d	10/10	10/10	18	17	6	0

[a] Single = Only one ip injection on Day 1 (one day post tumor inoculation ip with 10^5 cells). 7/2d = Seven injections (one injection every other day, 7 times) started Day 1. 6/d = Six daily injections started Day 1.

[b] Survivals of treated group without appearance of ascites on Day 40 or 50 when observation was stopped.

TABLE II. Effect of Narcissus Agents on Growth of Solid Tumors of $\#6C_3H$-ED Lymphosarcoma and Ehrlich Carcinoma in Mice.[a]

Tumor and agent	Dose/kg (mg)	Treatment schedule	Day tumor collected	No. of mice with tumor: Treated	No. of mice with tumor: Control	Av wt of tumor/total (g): Treated	Av wt of tumor/total (g): Control	Reduction of tumor weight (%)
$\#6C_3H$-ED lymphosarcoma (10,000 cells, sc)								
Residual alk.	200	7/2d[a]	Day 15[a]	3/3	6/6	0.80	1.58	49.4
	200	7/3d	17	5/7	7/7	1.10	2.93	62.5
Neutrals (N)	800	7/2d	Day 15	4/6	6/6	0.32	1.58	79.7
	400	7/2d	15	6/6	6/6	1.53	1.58	3.2
Ehrlich carcinoma (100,000 cells, sc)								
Residual alk.	200	10/2d	Day 21	10/10	10/10	2.15	8.85	72.0
Neutrals (N)	1600	2/7d	Day 15	4/4	4/4	0.85	2.65	67.9
	800	10/2d	21	9/10	10/10	3.60	8.85	67.6

[a] The 10^4 cells of lymphosarcoma or 10^5 cells of Ehrlich ascites cells were inoculated sc at back side near neck. Seven injections (one inject every other day) started Day 1. On Day 15, the solid tumor mass was taken out and weighed.

multiple ip injections of agents were started one day after ip inoculation of 10^5 Ehrlich cells. For comparison, cyclophosphamide (cytoxan) was used as a standard drug. Table I shows the results. It was found that a single injection of neutral fraction (N) was remarkably effective, prolonging the median survival time (MST) more than 100% over the controls with 58% of the mice surviving without ascites (cure state at the time experiments stopped). Narcissus alkaloids were not active by a single administration but were active with repeated administration. The activity of the residual alkaloid was higher than that of the 2-X crystal alkaloid. The former prolonged the life span more than 100% over the controls, with 50% of the mice surviving. The activity of cytoxan was also demonstrated by single and multiple administrations. The anticancer activity of the narcissus neutral fraction appears to be the same as that of cytoxan when administered by the intraperitoneal route.

Effect of narcissus agents on growth of solid tumor in mice. For practical reasons, it is important to know if the systemic administration of the narcissus agents is inhibitory for solid tumor growth. A syngeneic solid type of lymphosarcoma ($\#6C_3H$-ED), which had been passaged sc in C_3H mice for years, has now been found to grow progressively in a stable hybrid strain (black hair) originated in this laboratory from C_3H male and Swiss albino female mice. Unlike the lymphosarcoma, the growth of Ehrlich solid tumor was not always progressive and sometimes regressed. Narcissus agents were administered ip one day after the sc inoculation of 10^4 cells of $\#6C_3H$-ED lymphosarcoma or 10^5 cells of Ehrlich ascites tumor in the hybrid black mice or Swiss mice. When the tumor size became almost maximum, Days 15–21, and before necrosis or regression occurred, the tumor mass was excised and weighed. Table II shows the results. Systemic administration of both narcissus alkaloidal and neutral fractions suppressed significantly ($p < 0.05$) the growth of these solid tumors.

Effect of subfractions of the neutral fraction on Ehrlich ascites cells in mice. The neutral fraction (N) of narcissus bulbs has

TABLE III. Effect of Neutral Fractions of Narcissus Bulbs on Survival of Mice Inoculated with Ehrlich Ascites Tumor Cells (ip).[a]

Agent	Dose/kg (mg)	Treatment schedule (ip)	No. died/total		Median survival time (MST) (days)		Prolong. of MST over control (%)	Survivals without ascites (%)
			Treated	Control	Treated	Control		
Neutrals (N)	1600	single	8/20	20/20	>40	19	>100	60
Neutral N-1	150	single	10/10	10/10	19.5	14	39	0
N-2	350	single	8/10	10/10	24	19	26	20
N-3	200	single	10/10	10/10	24	20.5	17	0
	200	2/5d	6/8	8/8	24.5	15	63	25
N-4	200	single	7/10	10/10	22.5	17.5	28	30
N-5	200	single	15/40	40/40	>35.8	19.1	>88	63
N-6	200	single	8/10	10/10	25.8	18.8	39	20
N-7	200	single	8/8	8/8	21	15	40	0

[a] The 10^5 cells were inoculated ip. Single = Only one ip injection on Day 1. 2/5d = Two injections (on Day 1 and Day 6). The amount of N-1 to N-7 fractions was one-half of maximum non-toxic dose (½ MNTD). The amount of N was one MNTD. N was fractionated to 7 portions (N-1 to N-7) by passing aluminum oxide column.

been separated into seven parts by passing through an aluminum oxide column. Each subfraction was tested against Ehrlich ascites cells in mice by using the same method as indicated in Table I. See Table III. Fraction N-5 demonstrates a remarkable activity comparable to the crude original neutral fraction (N), although the other fractions also showed moderate activity. Fraction N-3 has been isolated as white crystals, while N-5 has not been purified.

Effect of narcissus agents on survival of mice inoculated with MCDV-12 leukemia cells. The syngeneic leukemia cells are more malignant than Ehrlich cells and kill BALB/c mice in a shorter time by both ip and sc routes. The range of survival time of mice is fairly proportional to the inoculum size of the cells by ip route. Preliminary experiments showed that the ip inoculation of 10^5 cells killed mice 8–9 days later, 10^4 cells killed 9–10 days later, 10^3 cells 10–12 days, and 10^2 cells still killed 100% of the mice 12–17 days later. The single or multiple administration of narcissus agents was started ip one day after the ip inoculation of 10^4 cells in BALB/c inbred mice. As shown in Table IV, only residual alkaloid was moderately active and the 2-X alkaloid and neutral fraction N-5 were not effective. Residual alkaloid increased the survival time to 11.7 days (26% over the control: $p < 0.05$); this is similar to a 90% reduction in tumor inoculum to 10^3 cells. It was of interest that neutral fraction N-5 remarkably enhanced the activity of cyclophosphamide (cytoxan) when mixed together. The subtoxic dose of 200 mg/kg of cytoxan, which the mice could barely tolerate showing marked loss of body weight and slower movement, increased the survival time to 18 days but could not protect most of the mice (7/8) from the final leukemic death. The combined treatment with cytoxan and N-5 fraction could completely eradicate the tumor cells in all the mice without apparent increase of toxicity (60 days observation). The optimal dose of cytoxan (100 mg/kg) without marked toxicity increased survival time by 63% with eventual death, but when combined with N-5 fraction, which did not increase toxicity, pro-

TABLE IV. Effect of Narcissus Agents on Survival of Mice Inoculated with MCDV-12 Leukemia Cells (ip).[a]

Agent	Dose/kg (mg)	Treatment schedule (ip)	No. died/total		Median survival time (MST) (days)		Prolong. MST over control (%)	Survivals without ascites (%)	p
			Treated	Control	Treated	Control			
2-X alkaloid	100	4/2d[a]	16/16	16/16	10.5 (10–11)[b]	9.5 (9–10)	11	0	>0.05
Residual alk.	200	4/2d	24/24	24/24	11.7 (11–12)	9.3 (9–10)	26	0	<0.05
	100	7/d[a]	24/24	24/24	11.7 (11–12)	9.3 (9–10)	26	0	<0.05
Neutral N-5	200	single[a]	16/16	16/16	9.5 (9–10)	9.5 (9–10)	0	0	
	200	4/2d	16/16	16/16	9.5 (9–10)	9.5 (9–10)	0	0	
	400	single	8/8	8/8	9.5 (9–10)	9.0 (9–10)	6	0	>0.05
Cytoxan	200	single	7/8	8/8	18.0 (14–41)	10.0 (9–10)	80	13	<0.01
Cytoxan and N-5	200 / 200	single mixture	0/8	8/8	>60.0	10.0 (9–10)	>500	100	<0.01
Cytoxan	100	single	16/16	16/16	15.5 (14–25)	9.5 (9–10)	63	0	<0.01
Cytoxan and N-5	100 / 200	single[c] mixture	8/16	16/16	>50.0 (16–32)	9.5 (9–10)	>400	50	<0.01
Cytoxan	50	single	8/8	8/8	12.0 (10–13)	10.0 (9–10)	20	0	<0.05
Cytoxan and N-5	50 / 200	single mixture	8/8	8/8	13.0 (11–15)	10.0 (9–10)	30	0	<0.05

[a] The 10^4 cells were inoculated ip on Day 0. 4/2d = 4 injections every other day. 7/d = 7 daily injections. Single = only one injection on Day 1.

[b] Range of days (first and last deaths).

[c] Agents were mixed together just before the injection on Day 1 only.

tected 50% of the mice from leukemic death with over a 500% increase of MST. See Table IV.

Cytotoxicity of narcissus agents in KB cell cultures. It was previously reported that KB cells became static at the concentration of 1 μg/ml of 2-X alkaloid (2, 3). Now, it has been found that KB cells became static at the concentration of 5 μg solid of the residual alkaloid and 50 μg/ml of the neutral fraction N-5 after 2 days of incubation. The culture medium was composed of MEM with 4% fetal calf serum.

Discussion. Narcissus tazetta L. called *shui-hsien,* was first mentioned in an early Chinese herbal for use against tumors as a liniment (8), and has been listed as a medicinal herb in Kwangtung province where a paste of the bulb is applied topically to cancer of breast (9). Wu Te-Cheng *et al.* (5) reported that the total alkaloidal fraction of the bulbs possessed anticancer activity against Ehrlich ascites carcinoma in mice. However, Fitzgerald *et al.* (6) indicated that the anticancer activity against Sarcoma 37 was not associated with the alkaloidal fraction and suggested the possible presence of small amounts of an unknown highly active amorphous water-soluble substances in the crude alkaloidal extract. We have confirmed that narcissus alkaloids possess anticancer activity against Ehrlich ascites tumor in mice. The residual alkaloid (a water-soluble fraction of total alkaloids after removal of the 2-X crystal alkaloid) has demonstrated a superior activity to the pure 2-X alkaloid. Narcissus alkaloid was found to be inactive against L1210 leukemia cells in VDF_1 mice when the daily ip administration of drug was started on Day 3 (personal communication from Dr. R. J. Speer of Wadley Institutes).

The neutral fraction of narcissus extracts, after removing alkaloidal and acidic fractions, has remarkable activity against Ehrlich ascites tumor and #$6C_3$H-ED lymphosarcoma in mice. Also, the partly purified fraction N-5 enhanced the activity of cyclophosphamide against MCDV-12 leukemia cells without increasing the toxicity. The action mechanism is now obscure but of interest in view of the possible clinical application of N-5 by combination with cyclophosphamide or other established anticancer drugs. The 3rd subfraction of neutrals (N-3) is a pure substance having moderate activity, and one would expect to synthesize more active derivatives when the chemical structure is defined. Further purification of the most active N-5 fraction and the residual alkaloidal fraction is in progress.

Summary. Anticancer activity of the alkaloidal and non-alkaloidal neutral fractions of narcissus bulbs has been demonstrated against Ehrlich ascites tumor and #$6C_3$H-ED solid lymphosarcoma cells in mice. The residual alkaloid also showed moderate activity against syngeneic MCDV-12 leukemia cells in BALB/c mice and the 5th subfraction of neutrals (N-5) enhanced the activity of cyclophosphamide against the cells although N-5 itself was not active. The single ip administration of the neutral fraction was enough to eradicate Ehrlich ascites cells in about 50% of mice, while the alkaloidal fraction needed multiple administrations to suppress tumor growth. The cytotoxicity of the narcissus agents on KB cells was also investigated.

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