Therapeutic Activity of Narcissus Alkaloid on Rauscher Leukemia and Comparison with Standard Drugs[1] (35452)

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In an antiviral screening program with 400 Chinese, Japanese, and Hawaiian medicinal plants, it was found that narcissus alkaloid showed marked activity against neurotropic RNA virus infections in mice with EMC, JBE, and LCM viruses (1, 2). Recently, the alkaloid has been found to have a remarkable prolongation effect on the life span of mice made leukemic by infection with Rauscher virus (3). We now report further studies of the therapeutic activity of narcissus alkaloid, and comparison with cyclophosphamide (Cytoxan), vincristine sulfate (Oncovin) and mercaptopurine (6-MP;Purinethol) against mice in an advanced stage of Rauscher leukemia, especially in view of possible evaluation for human use.

Materials and Methods. 1. Preparation of narcissus alkaloid. The method of extraction of the total alkaloidal fraction from the bulbs of *Narcissus tazetta L.* has been described (4). A yellow crystalline alkaloid (named 2-X) has been isolated from the final concentrated chloroform fraction which contained total alkaloids by standing at 4° for a month. After removing 2-X crystals by paper-filtration, the chloroform fraction was shaken with water, and the water-soluble fraction (named residual alkaloid) was collected and concentrated. Recently, the 2-X alkaloid has been tentatively identified as Pseudolycorine (Fig. 1) by Drs. M. H. Pindell, I. R. Hooper, and A. L. Vulcano (personal communication from Bristol Laboratories). The 2-X was dissolved in acidic water for use.

2. System of Rauscher's viral leukemia and chemotherapy. Rauscher leukemogenic virus was supplied from Dr. M. A. Chirigos of NIH and passaged for 4 years in BALB/c inbred mice in this department. An inoculum of 0.2 ml of 1:10 dilution (about 1000 ID_{100}) of leukemic plasma was injected intraperitoneally (ip) into 5- to 6-week-old inbred BALB/c mice (18 to 20 g), either male or female. Clearly palpable splenomegaly (+2 degree:about 1 g wt) appeared in 70 to 100% of mice 12 to 15 days (female) or 18 to 20 days (male) after infection. Treatment with one maximum nontoxic dose (MNTD) of drug was started subcutaneously (sc) to the mice showing clearly palpable splenomegaly and continued once daily for various days. The dose was the maximum amount which the leukemic mice could tolerate against the 2 weeks continuous administration without apparent intoxication as indicated by general weakness, ruffled hair, and remarkable loss of body weight. Double this dose (2 MNTD) kills the mice unless the daily administration is stopped within 10 days. Administration of 1 MNTD of narcissus alkaloid or cyclophosphamide for 30 days showed no apparent toxicity, while 1 MNTD of vincristine or 6-MP showed intolerable toxicity, in that time, but ½ MNTD was tolerable. The 6-MP was dissolved in diluted

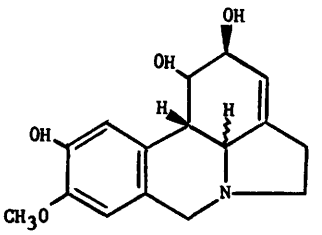

FIG. 1. Pseudolycorine (2-X alkaloid).

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alkaline (NaOH) solution and the others in distilled water for injection.

3. Titration of Rauscher virus in plasma. Each 10-fold dilution of leukemic plasma was inoculated into 3 female BALB/c inbred mice ip, allowing calculation of ID_{50} by the presence of splenomegaly (over 0.5 g) on the day 35.

Results. 1. Effect of narcissus alkaloid on survival time of established leukemic mice in comparison with standard drugs. Seven experiments were performed to confirm our initial work (3) which demonstrated the therapeutic effect of narcissus alkaloid on life span of the established leukemic mice with comparison of cyclophosphamide and vincristine.

TABLE I. Effect of Narcissus Alkaloids on Survival of Mice with Established Rauscher Leukemia: Comparison with Standard Drugs.[a]

Expt. no.	Agent	Maximum nontoxic (dose/kg) (MNTD) (mg)	Mice		Period of treatment once/day (days)	Median survival time (MST) (days)	Prolong. of MST over control (%)	% of death when control reached 100% death
			No.	Sex				
E7	Control					22.5		
	2-X Alkaloid	10	10	f	(+13 to +22)	51.0	127	30
	Cyclophosphamide	10	10	f	(+13 to +22)	35.0	51	60
E12	Control		10	f		21.0		
	2-X Alkaloid	10	10	f	(+15 to +22)	38.5	83	10
	Cyclophosphamide	10	10	f	(+15 to +22)	30.5	45	50
	Vincristine	0.15	10	f	(+15 to +22)	29.0	38	60
E16	Control		10	f		26.5		
	2-X Alkaloid	10	10	f	(+18 to +31)	47.0	77	20
	Cyclophosphamide	10	10	f	(+18 to +31)	35.0	32	60
E22	Control		10	f		20.0		
	2-X Alkaloid	10	10	f	(+13 to +26)	43.5	118	10
	Vincristine	0.15	10	f	(+13 to +26)	35.5	75	50
E23	Control		10	m		44.0		
	Residual alk.	50	10	m	(+13 to +26)	105.0	138	30
	Cyclophosphamide	10	10	m	(+13 to +26)	73.0	67	60
	6-MP	30	10	m	(+13 to +26)	79.0	79	50
E24	Control		10	f		25.0		
	Residual alk.	50	10	f	(+12 to +25)	63.0	152	20
	Cyclophosphamide	10	10	f	(+12 to +25)	47.5	90	50
	6-MP	30	10	f	(+12 to +25)	45.5	82	50
E25	Control		10	m		41.0		
	2-X Alkaloid	10	10	m	(+19 to +32)	85.0	107	30
	Residual alk.	50	10	m	(+19 to +32)	97.0	136	20
	Vincristine	0.15	10	m	(+19 to +32)	60.0	46	60
	6-MP	30	10	m	(+19 to +32)	71.0	73	50
Mean values (E2 to E25)								
	2-X Alkaloid						102	28
	Residual alk.						132	23
	Cyclophosphamide						57	56
	Vincristine						53	57
	6-MP						78	50

[a] Residual alk. = residual alkaloid after removed 2-X from total alk. fraction of narcissus bulbs; 6-MP = 6-mercaptopurine; +13, etc. = the 13th day postinfection.

Table I shows the results. The administration of the drugs with one MNTD was started when the splenomegaly was clearly palpable in 70 to 100% of mice infected with about 1000 ID_{100} of leukemic plasma 12 to 19 days ago, and continued once daily for 8 to 14 days. The mean of the 7 expts. demonstrated that 2-X alkaloid prolonged the life span 102% over the control, the residual narcissus alkaloid prolonged it 132%, cyclophosphamide 57%, vincristine 53%, and 6-MP 78%. About 70% of mice treated with narcissus alkaloid survived, while none survived in the controls. The dynamic course of leukemic deaths in Expt. Nos. 24 and 25 in Table I is shown in Figs. 2 and 3. Narcissus alkaloid, as well as other drugs, definitely prolonged the survival time, which has become the most important criterion of drug evaluation, because progressive disease is not cured. Through the seven experiments, it was suggested that the prolongation effect of narcissus alkaloid on the life span of the established leukemic mice was superior to other standard antileukemic drugs at the same maximum tolerable dose (one MNTD).

2. Effects of narcissus alkaloid (2-X) on splenomegaly, number of nucleated blood cells, and amount of virus in plasma in established leukemic mice. The main characteris-

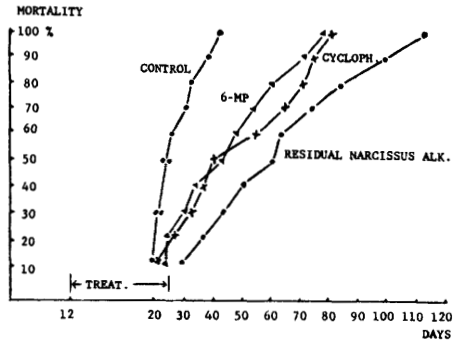

FIG. 2. Effect of narcissus alkaloid on survival of mice with established Rauscher leukemia: Comparison with cyclophosphamide and 6-MP (Expt. 24, Table I). Treatment was started on day 12 postinfection (80% mice with palpable splenomegaly), once daily, continued for 14 days. The narcissus residual alkaloid prolonged the life span 152% over the control, in comparison with 90% of prolongation for cyclophosphamide and 82% for 6-MP.

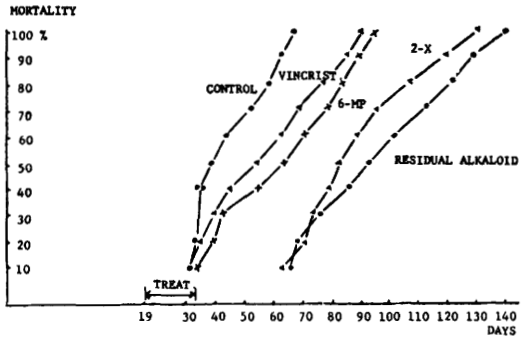

FIG. 3. Effect of narcissus alkaloid on survival of mice with established Rauscher leukemia: Comparison with vincristine and 6-MP (Expt. 25, Table I). Treatment was started on day 19 postinfection (80% palpable splenomegaly, male mice), continued once daily for 14 days. The 2-X crystal alkaloid prolonged the life span 107% over the control, the residual narcissus alkaloid 136%, vincristine 46%, and 6-MP 73%.

tics of Rauscher disease (splenomegaly, increase of nucleated blood cells and viremia) have been used as other criteria for evaluation of the narcissus agent. An experiment was carried out to determine whether the prolongation effect of narcissus agent on life span is due to the antitumor effect, the antiviral effect, or a combination of the two. Treatment with 2-X alkaloid at 2 MNTD (20 mg/kg:400 μg/mouse) was started on the 19th day postinfection in 6 mice having palpable (2+ degree) splenomegaly, and continued once daily for 5 days (3 mice), or for 10 days (3 mice). On days 24 and 29, the mice were sacrificed. The individual spleen weight and number of nucleated cells in cardiac blood were calculated, and the infectivity of mixed plasma was titrated. Figure 4 shows the results. It was found that narcissus alkaloid 2-X inhibited the enlargement of spleen, the increase of nucleated blood cells and virus increase in plasma.

The antileukemic activity of 2-X alkaloid was again compared with the standard drugs using the same criteria and methods mentioned above. Table II shows the results. All the 4 agents significantly decreased the spleen weight and number of nucleated blood cells, while only 2-X and vincristine decreased the amount of virus in plasma. Cyclophosphamide and 6-MP did not suppress

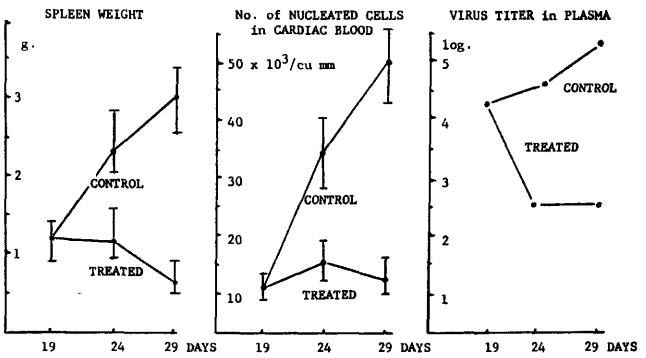

FIG. 4. Effects of narcissus alkaloid (2-X) on splenomegaly, nucleated cell number and virus amount in cardiac blood in mice with established Rauscher leukemia. Treatment (20 mg/kg:400 μg/mouse) was started on day 19 postinfection against 6 mice with palpable (2+ degree) splenomegaly, and continued once daily for 5 days (3 mice) or for 10 days (3 mice). On days 24 and 29, the individual spleen weight and number of nucleated cells in blood were calculated, and the infectivity of plasma mixed from 3 mice was titrated.

the virus titer in plasma, so they may act as antitumor agents only, not as antiviral agents. Vincristine appeared to exert antiviral activity, it produced a more pronounced effect in retarding the splenomegaly, although this may be due to toxicity since the mice on the last day (21) were extremely weak. Thus, in comparison with standard drugs, narcissus alkaloid has been confirmed to have definite antileukemic activity on Rauscher disease in mice, probably due to both an antitumor and antiviral effect.

3. Effect of (2-X) alkaloid on encephalomyocarditis (EMC) virus propagation in KB cell cultures and comparison with cyclophosphamide, vincristine, and 6-MP. Narcissus alkaloid has been primarily screened as an antiviral agent (1–3). It reduced the mortality of mice infected with several typical neurotropic RNA viruses such as EMC, LCM, and Japanese B encephalitis (JBE) viruses. In tissue culture (KB cells), it was inhibitory to the growth of EMC, LCM, JBE, vesicular stomatitis, polio I, II, and vaccinia viruses, but was not active on adeno types 12 and 18 and reovirus type 2. So far as we know,

TABLE II. Effects of Narcissus Alkaloid (2-X) on Splenomegaly, Nucleated Cell Number, and Quantity of Virus in Blood in Established Leukemic Mice with Comparison of Standard Drugs.[a]

			Spleen wt (g)				No. of nucleated cells in cardiac blood/mm³ (× 1000)				
			Individual				Individual				Virus titer in mixed plasma from nos. 1, 2, 3 (10 log)
	(Day)	Dose/kg (2 MNTD) (mg)	No. 1	No. 2	No. 3	Mean	No. 1	No. 2	No. 3	Mean	
Control	15		1.15	0.93	1.24	1.11	10.5	12.3	9.4	10.7	4.2
	21		2.03	2.10	2.33	2.15	33.9	34.4	45.2	37.8	4.5
2-X alk.	21	20	0.43	0.67	0.95	0.68	4.7	5.5	7.3	5.8	2.8
Cyclophos.	21	25	0.63	0.50	0.55	0.56	5.1	6.0	5.8	5.6	4.5
Vincristine	21	0.25	0.42	0.22	0.28	0.31	3.6	4.2	4.0	3.9	2.5
6-MP	21	60	0.48	0.68	0.43	0.53	7.6	9.5	6.3	7.8	4.2

[a] The dose of which drug (2 MNTD) was not toxic for 5 days administration except vincristine which showed toxicity. Treatment was started on day 15 postinfection against 3 mice in each group with palpable (2+ degree) splenomegaly and continued once daily for 5 days. On day 21, mice were sacrificed. The individual spleen weight and nucleated cell number in blood were calculated, and the infectivity of the mixed plasma from 3 mice was titrated.

TABLE III. Effect of 2-X Alkaloid on EMC Virus Propagation in KB Cells in Comparison with Cyclophosphamide, Vincristine, and 6-MP.[a]

Agent	Maximum nontoxic dose for 1 day incubation (μg)	Results at 24 hr postinfection	
		CPE	Virus yield ($TCID_{100}$)
Control (no drug)		4+	10^7
2-X alkaloid	0.5	0	10^2
Cyclophosphamide	400	4+	10^7
Vincristine	0.01	4+	10^7
6-MP	400	4+	10^7

[a] Agents were added just before virus inoculation (100 $TCID_{50}$). Incubation was stopped 24 hr after infection. Noninfected KB cells became slightly static at the doses of the agents in 1 ml of medium 199 with 2% calf serum.

there is no chemically defined agent among the established anticancer drugs that shows marked antiviral activity against RNA virus infections in mice. In this experiment, three anticancer drugs (cyclophosphamide, vincristine, and 6-MP) having different action mechanisms were tested for antiviral activity against EMC virus infection in KB cells. Table III shows the results. These anticancer agents did not suppress the cytopathic effect (CPE) and the growth of EMC virus at a maximum nontoxic dose, which made the cells static but did not kill them, while the 2-X alkaloid showed strong antiviral activity. It is planned to perform antiviral testing against Rauscher leukemia virus in some adequate tissue culture systems.

Discussion. Our initial work on antileukemic activity of narcissus alkaloid was reported as a part of studies of antiviral activity (1–4). Further studies have been focused on the therapeutic activity against established viral leukemia because human acute leukemia can be diagnosed only after the latent period from a possible initial viral infection, at a time when treatment must be therapeutic rather than prophylactic. Through repeated experiments (Table I), it has been confirmed that one of the characteristics of the narcissus alkaloid is the better prolongation of survival time of leukemic mice in comparison with that produced by cyclophosphamide, vincristine, or 6-MP. Also, it should be emphasized that narcissus alkaloid, without remarkable toxicity, was definitely inhibitory to splenomegaly and the elevated number of nucleated blood cells and significantly dropped the virus titer in plasma. The mode of action *in vivo* seems to be, therefore, both antiviral and antitumor although confirmation of the antiRauscher virus activity in adequate tissue culture has not yet been demonstrated. Unlike other oncogenic virus–animal host systems, it is suggested that in murine leukemia, viral multiplication throughout the course of the disease is responsible, at least in part, for the progressive increase in the number of malignant cells.

Gressor *et al.* (5) reported that initiation of interferon treatment even 1 week after viral inoculation, at a time when splenic enlargement had already developed, still exerted a significant inhibitory effect on the further development of splenomegaly. Wheelock and Larke (6) also reported that interferon inducers (Sendai virus and statolon) had no inhibitory effect on established splenomegaly, but prolonged the life of Friend leukemia mice presumably by an antiviral action which suppressed continuous malignant transformation. On the other hand, the mode of action of chemically defined anticancer drugs on viral leukemia seems to be confusing at present, especially as to antiviral activity *in vivo*. Chirigos (7) reported that cyclophosphamide (70 mg/kg), vincristine (0.5 mg/kg), 6-MP (75 mg/kg) decreased Rauscher virus titer in plasma at least 2 logs when treatment was initiated 5 days after infection and continued for 4 days. His previous report (8) noted that cyclophosphamide, although effective against the virus-induced disease, did not exert any antiviral effect. Dawson *et al.* (9) reported that 6-MP (30 mg/kg) and triethylene melamine (TEM) did not decrease Friend virus titer in spleen, and their recent paper (10) again indicated that 6-MP (30 mg/kg) did not affect Friend virus replication in the spleen. Mirand *et al.* (11), however, reported that 6-MP (30 mg/kg) demonstrated antiviral activity both *in vivo* and *in vitro* (6-MP and virus mixtures without cells) although there is no clear indication in decrease of virus titer *in vivo*. Sidwell (12) reported that 6-MP (29

mg/kg) and 6-MP-ribonucleoside decreased Friend virus titer in plasma, while Toyoshima *et al.* (13) indicated that 100 mg/kg of 6-MP was effective but 60 mg/kg was not effective against established Rauscher leukemia. Toyoshima suggested that the primary effect of 6-MP is antiviral and a secondary effect is the inhibition of splenomegaly. Such discrepancies of interpretation of the mode of action of anticancer agents against viral leukemia probably come from the difficulty of separating antiviral action from antitumor activity, because virus propagation in leukemic cells will be greatly affected if the cells are severely damaged by anticancer activity of an agent without any direct antiviral activity. In our experiments (Table II), cyclophosphamide (25 mg/kg) and 6-MP (60 mg/kg), which showed definite inhibition of splenomegaly and elevation of nucleated blood cells, did not decrease the virus amount in plasma, so they seem to act only as antitumor, not as antiviral agents. Vincristine (0.25 mg/kg) demonstrated a strong effect in retarding splenomegaly, and a decrease of virus titer in plasma in mice which became weak, probably due to toxicity. The 2-X alkaloid (20 mg/kg) decreased the virus titer in plasma without remarkable toxicity in addition to an inhibitory effect on splenomegaly of the same degree as that of cyclophosphamide and 6-MP. It could be concluded that the superior prolongation effect of 2-X alkaloid on life span of leukemic mice might be due to the antiviral (suppression of Rauscher virus growth) activity because 2-X has only moderate effect on splenomegaly, comparable to cyclophosphamide and 6-MP at nontoxic doses. It was also suggested from regular antiviral (EMC virus suppression) activity, which could not be seen in the standard anticancer drugs with which it was compared.

Summary. An alkaloid (2-X), tentatively identified as pseudolycorine, has been isolated from *Narcissus tazetta L.* This alkaloid, primarily studied as a new antiviral agent derived from the screening of medicinal plants of the Pacific area, has been shown to exert a superior prolongation effect on the life span of established Rauscher leukemic mice having palpable splenomegaly, in comparison with standard antileukemic drugs. It was found that the alkaloid suppressed the development of splenomegaly and the increase in number of nucleated blood cells, and dropped the virus titer in plasma without apparent toxicity. A second alkaloidal complex, called residual alkaloid, also showed remarkable antileukemic activity.

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