Effect of Long-Term Administration of Narcissus Alkaloid on Rauscher Leukemia and Combinations with Standard Drugs[1] (36606)

E. Furusawa, N. Suzuki, S. Ramanathan, S. Furusawa, and W. Cutting
Department of Pharmacology, School of Medicine, University of Hawaii, Honolulu, Hawaii 96816

An alkaloid from narscissus bulbs emerged as an antiviral agent from a screening program of medicinal plants of the Pacific area (1, 2). It showed a superior prolongation effect on the life span of established Rauscher leukemic mice in comparison with standard antileukemic drugs (3). The therapeutic activity of the alkaloid has now been further evaluated on long-term treatment, and in combination with standard drugs, and interferon inducers. Also, the effects of the long-term treatment on the mechanisms of antibody production and interferon induction in the leukemic mice have been studied.

Materials and Methods. 1. *Preparation of narcissus alkaloid and other drugs.* The methods of extraction of the total alkaloidal fraction from the bulbs of *Narcissus tazetta L.* have been described (4). A yellow crystalline alkaloid (referred to as 2-X) was isolated from the total alkaloidal fraction. This was tentatively identified as *pseudolycorine* by Drs. M. H. Pindell, I. R. Hooper and A. L. Vulcano of Bristol Laboratories. After removing 2-X crystals, the water-soluble portion (referred to as residual alkaloid) was collected. Polyriboinosinic–polyribocytidylic acid complex (poly I:C; 1 mg/ml solution) was purchased from Microbiological Associates; tilorone HCl was supplied by the Wm. S. Merrell Company, and cyclophosphamide (Cytoxan) was supplied by Dr. P. A. Tavormina of Mead Johnson Research Center. The 2-X was dissolved in acidic water (pH 6), mercaptopurine (6-MP) was dissolved in alkaline water (pH 8), and others in distilled water for injection.

2. *System of Rauscher's viral leukemia and chemotherapy.* Rauscher leukemogenic virus was supplied by Dr. M. A. Chirigos of NIH and passaged for 5 yr 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 6–8 weeks old inbred BALB/c mice (18–20 g), either male or female. Clear palpable splenomegaly (+2 degree: about 1 g wt) appeared in 70-100% of mice 12–15 days (female) or 18–20 days (male) after infection. Treatment with one maximum nontoxic dose (MNTD) of drug was started subcutaneously (sc) when 70–100% of the mice showed clearly palpable splenomegaly, and was continued once daily for 6–10 weeks. The dose was the maximum amount which normal and leukemic BALB/c could tolerate for 10 weeks continuous administration without apparent intoxication as indicated by general weakness, ruffled hair, weak motions, marked loss of body weight or marked decrease of intake of food and water.

Results. 1. *Effect of long-term treatment with narcissus alkaloid on survival of leukemic mice in comparison with standard drugs.* By preliminary experiments, the MNTD of each drug was determined as shown in Table I. The MNTD for 2 weeks schedule was the same used in our previous experiments (3). The MNTD for the 6–10 weeks schedule were used in the experiments reported in this paper. Table II shows the results of eight experiments. The average prolongation of median survival time (MST) over the control was 94% for 2-X alkaloid, 147% for the residual alkaloid, 64% for cyclophosphamide, 33% for vincristine, and 40% for 6-MP. Doubling the dosage of cyclophosphamide, vincristine or 6-MP in three experiments (E59, E60 and E67) made the life span short-

[1] Supported by Research Grants from Nat. Cancer Inst. (CA 12733-01) and from American Cancer Society (#IC-29).

TABLE I. Maximum Nontoxic Dose (MNTD)[a] of Drugs in Adult BALB/c Inbred Mice.

	Daily sc administration for about		
	2 wk	4 wk	6–10 wk
Narcissus residual alkaloid	50 mg/kg	25 mg/kg	25 mg/kg
2-X alkaloid (pseudolycorine)	10	10	10
cyclophosphamide (Cytoxan)	10	10	5
6-mercaptopurine (6-MP)	30	5	2.5
Vincristine (Oncovin)	0.15	0.04	0.02

[a] MNTD is the maximum tolerated dose at which mice show no visible toxic signs such as ruffled hair, weak motions, marked loss of body weight and marked decrease of intake of food and water.

er than that of controls, probably due to toxicity.

2. *Effects of combination of narcissus alkaloid and standard drugs on survival of leukemic mice.* In view of the possible use of narcissus alkaloid in man, it was of interest to know whether the alkaloid could demonstrate additive or synergetic effects on the viral leukemia when it was combined with the standard drugs. Mixtures of narcissus alkaloid with each of the other drugs (one MNTD of each) were injected sc into leukemic mice having clear splenomegaly. Treatment with the combination continued daily for 4–10 weeks. Table III shows the results of five experiments. The administration of narcissus alkaloid mixed with cyclophosphamide or 6–MP prolonged the life span of the leukemic mice an average of 210% for the former, and 160% for the latter, over the controls, in comparison with 114% ($p < 0.01$) for narcissus alkaloid alone, 58% ($p < 0.05$) for cyclophosphamide, and 42% ($p < 0.1$) for 6-MP (average of E46, E72, E82 and E84). In other words, the combination therapy of narcissus alkaloid and cyclophosphamide or 6-MP prolonged the life span 84% or 40% respectively over that of the single administration of narcissus alkaloid. In experiment E102, a significantly ($p < 0.01$) better prolongation effect was demonstrated even when the initial treatment with cyclophosphamide or 6-MP was changed after 28 days to narcissus alkaloid for the additional 17 days, in comparison with the effects of single drug administration of cyclophosphamide or 6-MP for the whole 45 days. The combination of narcissus alkaloid and vincristine showed no prolongation effect on the life span (E46). Throughout the long-term treatment with the mixtures of narcissus alkaloid and cyclophosphamide or 6-MP, each at one MNTD, the leukemic mice did not show any apparent intoxication but finally died of the leukemia, often accompanied by rupture of an enlarged spleen and epistaxis.

3. *Effects of the combination of narcissus alkaloid and interferon inducers on survival of leukemic mice.* Several papers have reported some effects, beneficial and adverse, of interferon and interferon inducers such as statolon or poly I:C on the course of established Friend viral leukemia in mice (5, 6). Therefore, it was also of interest to know whether combination treatment of narcissus alkaloid with poly I:C or tilorone HCl was more effective or not. One of three ip injections of the interferon inducers combined with the customary 20–50 daily sc injections of narcissus alkaloid were given to established Rauscher leukemic mice having splenomegaly. Table IV shows the results. It was found that a single administration of the interferon inducers did not increase the life span beyond that of the controls. Combinations with narcissus alkaloid were not more effective than narcissus alkaloid alone. It is suggested that these interferon inducers are not therapeutically effective on Rauscher leukemia.

4. *Effect of narcissus alkaloid on antibody production against sheep red cells in leuke-*

TABLE II. Effect of Narcissus Alkaloid on Survival of Mice with Established Rauscher Leukemia: Long-term Treatment and Comparison with Standard Drugs.

Expt. no.	Agent	Maximum nontoxic dose/kg	No. and sex of mice	Period of treatment once/day (days)	Median survival time (MST) (days)	Prolong. of MST over control (%)
E48	Control		10 f		49.0	
	2-X alkaloid	10 mg	10 f	(+15 to +58)	105.0	114
	cyclophosph.	5 mg	10 f	(+15 to +58)	90.0	84
	Vincristine	0.02 mg	10 f	(+15 to +58)	61.0	25
	6-MP	2.5 mg	10 f	(+15 to +58)	54.0	10
E51	Control		10 m		57.0	
	Residual alk.	25 mg	10 m	(+17 to +56)	96.0	69
	cyclophosph.	5 mg	10 m	(+17 to +56)	93.0	63
	6-MP	2.5 mg	10 m	(+17 to +56)	73.0	28
E56	Control		10 m		40.0	
	Residual alk.	25 mg	10 m	(+17 to +96)	128.0	220
	cyclophosph.	5 mg	10 m	(+17 to +96)	58.0	45
	Vincristine	0.02 mg	10 m	(+17 to +96)	56.0	40
	6-MP	2.5 mg	10 m	(+17 to +96)	73.0	82
E59	Control		10 m		45.5	
	Residual alk.	25 mg	10 m	(+19 to +84)	98.5	117
	cyclophosph.	10 mg[a]	10 m	(+19 to +84)	29.0	0
	Vincristine	0.04 mg[a]	10 m	(+19 to +84)	38.0	0
	6-MP	5 mg[a]	10 m	(+19 to +84)	31.5	0
E60	Control		10 f		36.5	
	Residual alk.	25 mg	10 f	(+18 to +77)	93.5	156
	cyclophosph.	10 mg[a]	10 f	(+18 to +77)	31.0	0
	Vincristine	0.04 mg[a]	10 f	(+18 to +77)	28.0	0
	6-MP	5 mg[a]	10 f	(+18 to +77)	28.0	0
E61	Control		10 m		50.0	
	Residual alk.	25 mg	10 m	(+13 to +80)	94.0	88
	cyclophosph.	10 mg[a]	10 m	(+13 to +80)	91.0	82
	Vincristine	0.04 mg[a]	10 m	(+13 to +80)	82.0	64
	6-MP	5 mg[a]	10 m	(+13 to +80)	58.0	16
E62	Control		10 f		30.0	
	Residual alk.	25 mg	10 f	(+21 to +60)	95.0	216
	cyclophosph.	10 mg[a]	10 f	(+21 to +60)	69.0	130
E67	Control		10 m		58.0	
	2-X alkaloid	10 mg	10 m	(+22 to +111)	100.0	73
	Residual alk.	25 mg	10 m	(+22 to +111)	151.0	160
	cyclophosph.	10 mg[a]	10 m	(+22 to +111)	54.0	0
	6-MP	5 mg[a]	10 m	(+22 to +111)	52.0	0

[a] Two maximum nontoxic doses (2 MNTD) for this schedule of treatment.

mic mice. There is well-known evidence that long-term administration of anticancer drugs has increased the risk and severity of bacterial or viral infections in patients as the result of the immunosuppressive side effect (7). Also, mice carrying viral leukemia, even without drug treatment, are in an immunologically deficient condition (8). Therefore, it was of importance to know whether the long-term administration of narcissus alkaloid injured the immune mechanisms of leukemic mice. Three leukemic mice with 2+ to 3+

TABLE III. Effect of Combinations of Narcissus Alkaloid and Standard Drugs on Survival of Mice with Rauscher Leukemia.

Expt. no.	Agent	Maximum nontoxic dose (MNTD)/kg (mg)	Period of treatment once/day (days)	Median survival time (MST) (days)	Prolong. of MST over control (%)
E46	Control (each 10 female mice)			31.5	
	Residual alkaloid	25	(+14 to +45)	57.5	82
	cyclophosphamide	10	(+14 to +45)	41.0	30
	Vincristine	0.04	(+14 to +45)	38.5	22
	6-MP	5	(+14 to +45)	49.0	56
	Mixture of residual alk. and cyclophosphamide	12.5 5	(+14 to +45)	83.0	163
	Mixture of residual alk. and vincristine	12.5 0.02	(+14 to +45)	48.0	52
	Mixture of residual alk. and 6-MP	12.5 2.5	(+14 to +45)	93.0	195
E72	Control (each 10 female)			33.5	
	2-X alkaloid	10	(+18 to +98)	99.0	196
	cyclophosphamide	5	(+18 to +98)	65.0	94
	Mixture of 2-X alkaloid and cyclophosphamide	10 5	(+18 to +98)	143.0	327
E82	Control (each 10 male)			51.5	
	2-X alkaloid	10	(+24 to +79)	103.0	100
	cyclophosphamide	5	(+24 to +79)	91.0	77
	6-MP	2.5	(+24 to +79)	69.0	34
	Mixture of 2-X alkaloid and cyclophosphamide	10 5	(+24 to +79)	113.5	124
	Mixture of 2-X alkaloid and 6-MP	10 2.5	(+24 to +79)	119.0	131
E84	Control (each 10 female)			35.5	
	2-X alkaloid	10	(+15 to +42)	63.0	78
	cyclophosphamide	5	(+15 to +42)	46.0	29
	6-MP	2.5	(+15 to +42)	48.5	37
	Mixture of 2-X alkaloid and cyclophosphamide	10 5	(+15 to +42)	115.5	225
	Mixture of 2-X alkaloid and 6-MP	10 2.5	(+15 to +42)	90.0	154
E102	Control (each 10 male)			41.0	
	2-X alkaloid	10	(+23 to +67)	70.0	71
	cyclophosphamide	5	(+23 to +67)	59.0	44
	[a]cyclophosphamide	5	(+23 to +50)	69.0	68
	then 2-X alkaloid	10	(+51 to +67)		
	6-MP	2.5	(+23 to +67)	61.0	49
	[a]6-MP	2.5	(+23 to +50)	75.0	83
	then 2-X alkaloid	10	(+51 to +67)		

[a] Treatment with cyclophosphamide (5 mg/kg) or 6-MP (2.5 mg/kg) was started on the 23rd day, continued to the 50th day, and then changed to 2-X alkaloid started on the 51st day, continued until the 67th day.

degrees of splenomegaly were selected from each group receiving the long-term treatment with drugs, and given sheep red cells ip. Then 5 or 6 days later, heart blood was

TABLE IV. Lack of Increased Survival of Leukemic Mice after Combined Treatment with Narcissus Alkaloid and Interferon Inducers.

Expt. no.	Agent	Maximum nontoxic dose (MNTD)/kg (mg)	Period of treatment once/day (days)	Median survival time (MST) (days)	Prolong. of MST over control (%)
E85	Control (each 10 male mice)			56.0	
	Residual alkaloid (sc)	25	(+28 to +78)	169.0	202
	Tilorone HCl (ip)	50	(+28, 35, +42)	59.0	6
	[a]Combination of residual alk.	25	(+28 to +78)	88.0	57
	(sc) and tilorone (ip)	50	(+28, 35, +42)		
E86	Control (each 10 female)			33.5	
	2-X alkaloid (sc)	10	(+15 to +64)	85.0	154
	Tilorone HCl (ip)	100	(+15 only)	39.5	18
	Combination of 2-X alk.	10	(+15 to +64)	76.0	127
	(sc) and tilorone (ip)	100	(+15 only)		
E88	Control (each 10 female)			49.0	
	Residual alkaloid (sc)	25	(+19 to +58)	102.0	108
	Tilorone HCl (ip)	50	(+19, +33)	43.0	0
	Combination of residual alk.	25	(+19 to +58)	93.0	90
	(sc) and tilorone (ip)	50	(+19, +33)		
E92	Control (each 10 male)			44.0	
	Residual alkaloid (sc)	25	(+19 to +69)	107.0	143
	Poly I:C (ip)	10	(+19 only)	39.0	0
	Combination of residual alk.	25	(+19 to +69)	88.5	101
	(sc) and poly I:C (ip)	10	(+19 only)		
E94	Control (each 10 female)			25.5	
	Residual alkaloid (sc)	25	(+17 to +37)	44.0	75
	Poly I:C (ip)	5	(+17, +24)	30.0	18
	Combination of residual alk.	25	(+17 to +37)	39.5	55
	(sc) and poly I:C (ip)	5	(+17, +24)		

[a] The group was given 25 mg/kg daily of residual alkaloid subcutaneously (sc) from the 28th to the 78th day, and also was given 50 mg/kg of tilorone HCl on the 28th, 35th and 42nd days intraperitoneally (ip).

collected for antibody titration. Table V shows the results. The three standard drugs significantly inhibited antibody production, especially the daily sc administration of cyclophosphamide (30–40 days) deeply suppressed the immunological reactions. On the other hand, the mice receiving the same long-term administration of narcissus alkaloid fully maintained this immune response.

5. *Effect of narcissus alkaloid on interferon-inducing activity of poly I:C in leukemic mice.* In addition to immune reactions, interferon production is an important natural defense mechanism against exogenous viral infections. Therefore, it was also of importance to know whether the long-term administration of drugs affected the interferon system. Three leukemic mice selected from each group receiving narcissus alkaloid or cyclophosphamide for 60 days were given 20 μg of poly I:C ip. Twenty hr later, heart blood was collected and the amount of interferon in the individual serum titrated by using the L cell-VS virus system. Interferon titer was expressed by the reciprocal of the highest dilution of serum which inhibited 50% of CPE produced by 100 $TCID_{50}$ of VS virus inoculum. Table VI shows the results. Neither narcissus alkaloid nor cyclophosphamide was found to have any inhibitory effect on interferon-induction mechanisms stimulated by poly I:C. It is of interest that leukemic

TABLE V. Effect of Narcissus Alkaloid on Antibody Production Against Sheep Red Cells in Leukemic Mice: Comparison with Standard Drugs.

Days post infection: Treatment started / Red cells injected / Treatment stopped and blood taken		Exp. 1[a] Day 15 / Day 35 / Day 41		Exp. 2 Day 15 / Day 45 / Day 50	
Agent		HA titer	No. of leukocytes	HA titer	No. of leukocytes
Narcissus residual alkaloid	50 mg	64	10,900	32	9,800
	25	128	12,600	32	10,600
Cyclophosphamide	10 mg	<4	10,300	<4	10,100
Vincristine	0.04 mg	32	19,100		
	0.02			8	22,000
6-MP	5 mg	32	8,600		
	2.5			16	15,800
No drug (leukemic mice)		128	28,800	32	35,000
No drug (normal mice)		256	4,800	64	5,200

[a] Treatment with agents was started 15 days after Rauscher virus inoculation and continued once a day until the 41st day. On day 35, washed sheep red cells (12%, 0.2 ml) were injected ip. On day 41, heart blood was collected from 3 mice and mixed for hemagglutinin (HA) antibody titration and nucleated white cell counting.

mice receiving the cyclophosphamide-treatment continued to show effective interferon systems although their immune systems were severely damaged.

Discussion. In a previous paper (3), we showed that the short-term administration (2 weeks) of narcissus alkaloid produced a superior prolongation of the life span of established Rauscher leukemic mice in comparison with standard drugs. In this paper, long-term administration (6–10 weeks) was equally effective and allowed combinations with standard drugs. The narcissus alkaloid alone was superior to the standard drugs (cyclophosphamide, vincristine and 6-MP) but combinations with cyclophosphamide and 6-MP gave still better results. One of the characteristics of narcissus alkaloid is the low toxicity, allowing an effective dose to be administered for as long as 10 weeks without

TABLE VI. Amount of Serum–Interferon Induced by Poly I:C in Rauscher Leukemic Mice Receiving Long-Term Treatment.[a]

	Interferon titer per ml		
Serum	no. 1	no. 2	no. 3
Control leukemic mice with palpable splenomegaly	1,024	1,024	2,048
Leukemic mice treated with 2-X alkaloid (10 mg/kg) for 60 days	1,024	1,024	4,096
Leukemic mice treated with cyclophosphamide (5 mg/kg) for 60 days	1,024	1,024	2,048
Control nonleukemic mice	2,048	4,096	2,048

[a] Results in three separate sera. Poly I:C (20 μg) was injected ip 20 hr before blood collection. Serum–interferon was titrated in an L cell–vesicular stomatitis (VS) virus system by the criterion of 50% reduction of CPE from 100 $TCID_{50}$ inoculum.

damaging natural defense systems (immune and interferon) against infections. In contrast, vincristine had to be reduced to 1/8, and 6-MP to 1/12 the original dose (from the 2 weeks schedule) when they were administered for 10 weeks because of intolerable toxicity. Cyclophosphamide demonstrated remarkable immunosuppressive activity which would be a serious side effect in the use of the agent as an anticancer drug. Nevertheless, this agent did not affect interferon induction after stimulation by poly I:C in our experiments, although the agent has been reported to have an inhibitory effect on interferon production by Sendai virus (9). It is desirable to develop new drugs which have no immunosuppressive effect for the treatment of acute leukemia in children in which the main direct cause of death (70%) is at present exogenous infections (10).

Recently, Dr. M. A. Chirigos of NIH demonstrated that narcissus alkaloid strongly suppressed the plaque formation of several murine leukemia viruses (MLV, RLV and AKR) by using XC cell assay (personal communication).

One of the biggest problems in cancer chemotherapy is the serious side effects of anticancer drugs. These may be life threatening and require that administration be stopped. Therefore, many clinical attempts to reduce the side effects by the combination of several drugs as in the "POMP" program (Prednisolon, Oncovin, Methotrexate and Purinethol) (11) in acute leukemia have been reported (12, 13). Combinations of narcissus alkaloid with cyclophosphamide or 6-MP produced a clear additive effect, while combinations with vincristine or interferon inducers were not effective. In these combinations, the leukemic mice did not show any signs of intoxication. The narcissus alkaloid was still effective when given to leukemic mice that had already been given cyclophosphamide or 6-MP in an initial series of injections (E102, Tabel III). This activity of narcissus alkaloid as a sequential drug might have practical meaning in clinical use.

Summary. Long-term treatment with narcissus alkaloid (pseudolycorine or the residual alkaloid) at well tolerated doses was remarkably effective against established Rauscher leukemic mice, in comparison with standard antileukemic drugs. Combination of narcissus alkaloid with cyclophosphamide or 6-MP was more effective than the single administration of either drug, while combination with vincristine or interferon inducers (poly I:C or tilorone HCl) did not increase the effect. Narcissus alkaloid did not impair humoral antibody production in the leukemic mice, while the standard drugs were immunosuppressive during long-term administration. Also, narcissus alkaloid did not suppress interferon induction (by poly I:C) in leukemic mice.

We thank Dr. M. A. Chirigos, NIH, for supplying Rauscher virus, antiviral testing by XC cell assay, and helpful advice; and Dr. P. A. Tavormina, Mead Johnson Res. Center, for supplying cyclophosphamide (Cytoxan).

1. Cutting, W., Furusawa, E., Furusawa, S., and Wook, Y. K., Proc. Soc. Exp. Biol. Med. **120**, 330 (1965).

2. Furusawa, E., and Cutting, W., Ann. N.Y. Acad. Sci. **173**, 668 (1970).

3. Furusawa, E., Furusawa, S., Morimoto, S., and Cutting, W., Proc. Soc. Exp. Biol. Med. **136**, 1168 (1971).

4. Ramanathan, S., Furusawa, E., Kroposki, M., Furusawa, S., and Cutting, W., Chemotherapy **13**, 121 (1968).

5. Wheelock, E. F., and Larke, R. P. B., Proc. Soc. Exp. Biol. Med. **127**, 230 (1968).

6. Barker, A. D., Rheins, M. S., and Wilson, H. E., Proc. Soc. Exp. Biol. Med. **137**, 981 (1971).

7. Borella, L., and Webster, R. G., Cancer Res. **31**, 420 (1971).

8. Chakrabarty, A. K., Friedman, H., and Ceglowski, W. S., Cancer Res. **30**, 617 (1970).

9. Robinson, T. W. E., and Heath, R. B., Nature (London) **217**, 178 (1968).

10. Perry, S., Cancer Chemoth. Rep. Part 3, **2**, 99 (1971).

11. Carter, S. K., Cancer Chemother. Rep. Part 3, **2**, 81 (1971).

12. Saito, T., Ohira, S., Wakui, A., and Yokoyama, M., Antimicrob. Anticancer Chemother. **2**, 321 (1970).

13. Ota, K., Kurita, S., and Hoshino, A., Antimicrob. Anticancer Chemother. **2**, 317 (1970).

Received Mar. 8, 1972. P.S.E.B.M., 1972, Vol. 140.