PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 771–777 (1974)

Therapeutic Activity of Narcissus Alkaloids on Rauscher Leukemia: Antiviral Effect *in Vitro* and Rational Drug Combination *in Vivo*[1] (37892)

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One of the alkaloids from narcissus bulbs, pseudolycorine, has been shown to be remarkably effective against Rauscher leukemia in mice (1, 2). The residual alkaloid, which is a water-soluble portion of the narcissus alkaloids after the removal of pseudolycorine, also showed a considerable prolongation of the life span of established Rauscher leukemic mice in comparison with standard antileukemic drugs (cyclophosphamide, vincristine, and 6-mercaptopurine) (1, 2). The combination of these alkaloids with cyclophosphamide or 6-mercaptopurine was more effective than the administration of either drug singly. In addition, the narcissus alkaloids did not impair humoral antibody production against sheep red cells nor did they suppress interferon induction by polyriboinosinic–polyribocytidylic acid complex in leukemic mice (2). Recently, Papas *et al.* have demonstrated that the residual alkaloid strongly inhibits the activity of RNA-dependent DNA polymerase (reverse transcriptase) which is associated with various oncogenic RNA viruses. This alkaloid is peculiar in that it works by binding to the enzyme itself rather than interacting with nucleic acid template (3). We report now the results of our experiments on antiviral activity of the residual alkaloid in cell cultures and on its therapeutic effect *in vivo* compared with other inhibitors of reverse transcriptase and standard antileukemic agents using Rauscher leukemia virus.

Materials and Methods. Preparation of the narcissus alkaloids and other drugs. The methods of extraction of the total alkaloidal fraction and pseudolycorine from the bulbs of *Narcissus tazetta* L. have been described (1). After the removal of pseudolycorine from the total alkaloidal fraction, the crude residual alkaloid was obtained in a water-soluble fraction. Residual alkaloid A-2 is a partially purified grade, soluble even in cold water, and obtained by repeatedly washing and drying with water; residual alkaloid A-3 is a chromatographically pure alkaloid isolated from A-2 grade by passing through an aluminum oxide column with chloroform and then with the mixture of chloroform and methanol (9:1). Alkaloid A-3 is the main component (ca. 80%) of alkaloid A-2 and although the chemical structure is unknown, it is thought to be a phenolic precursor of tazettine.

6-Mercaptopurine (6-MP) and cyclophosphamide (CY) obtained from commercial sources were used as the standard antileukemic drugs. Inhibitors of reverse transcriptase, ethidium bromide (EB) (4) and rifamycin SV (5) were purchased from Calbiochem, CA, acetyl adriamycin (personal communication), and cinerubin A (6) were obtained from Dr. M. A. Apple of the Cancer Research Institute of the University of California.

Cell culture. Secondary tube cultures of BALB/c mouse embryo (ME) cells were used as amplifiers of Rauscher leukemia virus (RLV). ME cells were grown

[1] Supported by Research Grants from National Cancer Institute (CA 12733) and from American Cancer Society (IC-29).

in Eagle's minimum essential medium (EMEM) with 10% unheated fetal calf serum, 2 m*M* glutamine and antibiotics, and maintained in EMEM with 3% fetal calf serum after the cultures became subconfluent. XC cells were employed as detectors of RLV. This rat tumor cell line was supplied by Dr. A. Hackett of Naval Biomedical Research Laboratory, Oakland, CA and kept in EMEM with 10% fetal calf serum with frequent subpassages.

Virus. RLV was supplied by Dr. M. A. Chirigos of NIH and passaged for 6 yr in BALB/c inbred mice in this department. An inoculum of 0.2 ml of a 1:40 dilution of leukemic plasma was injected intraperitoneally (ip) into 6- to 8-week-old BALB/c mice. Infected mice were bled by cardiac puncture about 4 weeks postinfection when enlarged spleens were easily palpable. Pooled plasma was used as the RLV inoculum in the following experiments *in vivo* and *in vitro*.

System of Rauscher viral leukemia and chemotherapy. An inoculum of 0.2 ml of a 1:20 or 1:40 dilution of leukemic plasma was injected ip into 6- to 8-week-old BALB/c mice (18–20 g), either male or female. Treatment with one maximum nontoxic dose (MNTD) was started subcutaneously (sc) 16–18 days after infection when 70–100% of the mice showed clearly palpable spleens. All agents, singly or combined, were injected every other day. Pseudolycorine and its combinations with CY and/or 6-MP were administered for 30 days, and residual alkaloid and its combinations, for about 40 days. Rifamycin SV and EB were given for 23 and 17–53 days, respectively, and acetyl adriamycin and cinerubin A were administered for 9 days. These intervals were chosen to avoid any 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.

In vitro test for antiviral activity. Immediately after treatment with DEAE–dextran (final concn: 20 μg/ml) at 37°C for 40 min, and subsequent washings, ME cultures were infected with 0.1 ml of a 1:2 or 1:4 dilution of the leukemic plasma. These were incubated at 37°C for 3 days with daily medium changes then one MNTD of each agent was added and incubated for another 3 days with daily medium changes, including one MNTD of the agent. The culture fluids were collected and kept frozen at −70°C for virus yield titration. In preliminary experiments, no syncytial giant cell formation was detected when culture fluids were collected 3 days after inoculation of the virus.

RLV yield titrations were performed by the method of XC cell assay developed by Klement *et al.* (7) and Rowe *et al.* (8) with a slight modification. Cultures were treated with DEAE–dextran in the same way as described above, inoculated with 0.1 ml of 10-fold diluted specimens from the nontreated controls and drug-treated tubes, and held at 37°C for 1.5 hr. Then 1 ml of medium was added and the culture tubes were kept at 37°C with daily medium changes. After 5–6 days, the cultures were overlayed with about 1.5×10^6 of freshly trypsinized XC cells. Syncytial giant cells were detected by a microscope under low magnification 1 or 2 days after the XC cell overlay. Two to three tubes were used for each dilution. RLV titers were calculated after Reed and Muench (9), and expressed by a median tissue culture infectious dose ($TCID_{50}$)/0.1 ml.

Results. Effects of narcissus alkaloids and other inhibitors of reverse transcriptase on Rauscher virus growth in ME cells. Pseudolycorine and narcissus residual alkaloid A-2 were tested for their inhibitory activity of the proliferation of RLV in ME culture cells in comparison with the standard inhibitors of reverse transcriptase, rifamycin SV and EB. Two tubes were used for each dilution in RLV yield titration tests. Table I shows the result from a representative experiment. RLV titers in the culture fluids from the tubes treated with pseudolycorine, narcissus residual alkaloid A-2, rifamycin SV, and EB were 0.5, 0.0, 2.5 and 3.5 log $TCID_{50}$/0.1 ml, respectively while nontreated control tubes gave a titer of 3.5 log $TCID_{50}$/0.1 ml. The narcissus residual alkaloid A-2 inhibited the virus growth almost completely and rifamycin SV was only

TABLE I. Effect of Narcissus Alkaloids and Other Standard Inhibitors of Reverse Transcriptase on Rauscher Virus Growth in ME Cells.

Agent[a]	Dose (μg/ml)	Virus yield[b] ($\log_{10}TCID_{50}$/0.1 ml)
Control (no drug)	—	3.5
Pseudolycorine	0.5	0.5
Narcissus alkaloid A-2	5.0	0.0
Rifamycin SV	12.5	2.5
Ethidium bromide	2.5	3.5

[a] Treatment with one MNTD of each agent was started 3 days after the virus infection and continued for 3 days.

[b] Titration of the virus amount per 0.1 ml of the culture fluids collected after the treatment was done by the method of XC cell assay. Any differences over 1 log between titers are significant ($P < 0.05$).

slightly inhibitory, while EB was not effective at all against RLV proliferation in ME cells.

By further purification of the residual alkaloid, a chromatographically pure alkaloid was recovered in the form of crystals, referred to as residual alkaloid A-3. In the following experiment, narcissus alkaloid A-3 and four other inhibitors of reverse transcriptase (rifamycin SV, EB, cinerubin A, and acetyl adriamycin) were tested for their inhibitory effects on the replication of the virus. The results from a representative experiment are summarized in Table II. In this case, the amount of EB added in the RLV-infected ME cells was reduced to 1.25 μg/ml because 2.5 μg/ml of this agent, the dose used in the previous experiment, was slightly toxic to ME cells. Each dilution of the virus titration was done in triplicate. The virus yield in the control tubes was 3.25 log $TCID_{50}$/0.1 ml, and no giant cells were detected in the tubes inoculated with the harvest from RLV-infected cultures treated with narcissus residual alkaloid A-3. Rifamycin SV and EB were only slightly inhibitory, and cinerubin A and acetyl adriamycin were significantly effective against RLV growth, but not as much as narcissus residual alkaloid A-3. In this experiment (Table II), in addition to the titrations of the virus yield, counting of the giant cell numbers was performed by overlaying the XC cells directly onto the ME cell cultures immediately after the removal of culture fluids. By this method, all the agents except EB were found to significantly suppress the giant cell formation, whereby the average number of giant cells in control tubes was 94. The inhibition tests using ME tissue cultures show the narcissus residual alkaloid to be the most potent inhibitor of RLV growth of all the agents tested; all of which have been shown to be inhibitors of reverse transcriptase.

Effects of narcissus residual alkaloid and other inhibitors of reverse transcriptase on the life span of Rauscher leukemic mice. To test the narcissus residual alkaloid and the other four inhibitors of reverse transcriptase for their therapeutic activities in established leukemic mice, treatment with single agents

TABLE II. Effects of Reverse Transcriptase Inhibitors on Rauscher Virus Growth.

Agent[a]	Dose (μg/ml)	Virus yield[b] ($\log_{10}TCID_{50}$/0.1 ml)	Inhibition of giant cell formation[c] (%)
Control (no drug)	—	3.25	—
Narcissus alkaloid A-3	2.5	0.0	100
Rifamycin SV	12.5	2.25	90
Ethidium bromide	1.25	2.50	0
Cinerubin A	0.05	1.50	100
Acetyl adriamycin	0.05	1.25	89

[a] Treatment with one MNTD of each agent was started 3 days after the virus infection and continued for 3 days.

[b] Titration of the virus amount per 0.1 ml of the culture fluids collected after the treatment was done by the method of XC cell assay.

[c] Any differences over 0.5 log between titers are significant ($P < 0.05$).

TABLE III. Effects of Narcissus Residual Alkaloid on Survival of Mice with Established Rauscher Leukemia: Comparison with Other Inhibitors of Reverse Transcriptase.

Expt no.	Agent	Maximum nontoxic dose/kg (mg)	Period of treatment (days)	Median survival time (MST) (days)	Prolongation[a] of MST over control (%)
E170	Control (each 20 female)			27.5	
	Residual alkaloid[b]	50	(+18 to +40)[c]	54.5	98
	Rifamycin SV	100	(+18 to +40)	34.5	26
E180	Control (each 20 female)			33.0	
	Residual alkaloid	50	(+16 to +32)	67.0	103
	Cyclophosphamide[d]	10	(+16 to +32)	47.0	43
	Ethidium bromide	25	(+16 to +32)	34.5	4
E195	Control (each 20 female)			40.0	
	Residual alkaloid	25	(+18 to +70)	72.0	80
	Ethidium bromide	5	(+18 to +70)	37.5	0
E207	Control (each 20 male)			52.5	
	Cinerubin A	2.5	(+18 to +26)	64.0	22
E209	Control (each 20 female)			58.5	
	Acetyl adriamycin	2.5	(+18 to +26)	82.0	42
E213	Control (each 20 male)			48.0	
	Cinerubin A	1.3	(+18 to +26)	46.0	0

[a] Any prolongation of MST over 25% is significant ($P < 0.05$).
[b] The crude fraction of residual alkaloid was used.
[c] Treatment with agent was started on the 18th day, continued every other day until the 40th day.
[d] Cytoxan was used as a standard antileukemic agent for comparison.

was performed. Each agent was injected sc every other day for the period indicated in Table III which is also a representative result. Prolongations of median survival time (MST) with narcissus residual alkaloid were 98%, 103%, and 80% (E170, E180, and E195) over the controls, which are consistent with the results of our previous experiments (2). Because of the treatment with 25 mg/kg of EB (E180) or 2.5 mg/kg of cinerubin A (E207) showed some toxicity (i.e., ruffled hair and loss of body weight) the doses of these two agents were reduced to 5 mg/kg for EB and 1.3 mg/kg for cinerubin A in the next experiments (E195, E213). However, no MST prolongation effects were demonstrated after this reduction in dosage. Rifamycin SV prolonged the MST only by 26% (E170), while acetyl adriamycin was moderately effective (42%, E209), comparable with CY (43%, E180) which was employed as a standard antileukemic drug.

Effect of combination treatment of narcissus alkaloids and standard antileukemic drugs on MST of Rauscher leukemic mice. The results obtained in the above experiments, both *in vitro* and *in vivo,* show the narcissus alkaloid to have the most potent inhibitory activity against RLV growth. Therefore, combination treatments using narcissus alkaloids (including pseudolycorine) with standard antileukemic drugs (CY and 6-MP) were attempted. One of the several results is shown in Table IV. The route and time intervals of injections were the same as in the preceding experiments. Treatment with a single agent of pseudolycorine or the residual alkaloid showed constantly significant therapeutic effects, giving about 80% prolongation of MST over the controls. Combination treatments of pseudolycorine with CY or 6-MP (E138) resulted in 178% or 93% MST prolongation, respectively, indicating a similar effect as in the previous experiments (2). When pseudolycorine was combined with CY and 6-MP, MST's were considerably prolonged (223%, E138 and 123%, E144).

The mixture of the residual alkaloid with

TABLE IV. Effect of Combinations of Narcissus Alkaloids and Standard Drugs on Survival of Mice with Established Rauscher Leukemia.

Expt no.	Agent	Maximum nontoxic dose/kg (mg)	Period of treatment (days)	Median survival time (MST) (days)	Prolongation of MST over control (%)
E138	Control (each 20 female)			28.5	
	Pseudolycorine	20	(+15 to +57) [a]	50.5	77
	Mixture of pseudolycorine	20	(+15 to +57)	79.5	178
	and CY[b]	10			
	Mixture of pseudolycorine	20	(+15 to +57)	55.0	93
	and 6-MP[b]	5			
	Mixture of pseudolycorine,	20	(+15 to +57)	92.0	223
	CY	10			
	and 6-MP	5			
E144	Control (each 20 male)			43.5	
	Pseudolycorine	20	(+17 to +57)	78.0	79
	Mixture of CY	10	(+17 to +57)	75.0	72
	and 6-MP	5			
	Mixture of pseudolycorine,	20	(+17 to +57)	97.0	123
	CY	10			
	and 6-MP	5			
E150	Control (each 20 female)			30.5	
	Residual alkaloid[c]	50	(+19 to +49)	56.5	85
	Mixture of residual alkaloid	50	(+19 to +49)	87.5	187
	and CY	10			
	Mixture of residual alkaloid	50	(+19 to +49)	70.0	129
	and 6-MP	5			
	Mixture of residual alkaloid,	50	(+19 to +49)	154.0	405
	CY	10			
	and 6-MP	5			
E158	Control (each 20 male)			37.5	
	Residual alkaloid	50	(+17 to +47)	68.0	81
	Mixture of CY	10	(+17 to +47)	52.5	40
	and 6-MP	5			
	Mixture of residual alkaloid	50	(+17 to +47)	81.0	116
	and CY	10			
	Mixture of residual alkaloid	50	(+17 to +47)	85.0	127
	and 6-MP	5			
	Mixture of residual alkaloid,	50	(+17 to +47)	101.0	170
	CY	10			
	and 6-MP	5			

[a] Treatment with agent was started on Day 15, and continued every other day until Day 57.

[b] CY: cyclophosphamide (Cytoxan); 6-MP: 6-mercaptopurine (Purinethol) as the standard antileukemic agents.

[c] The crude fraction of residual alkaloid was used.

CY prolonged the MST by 187% (E150) and 116% (E158), and with 6-MP, about 128% (E150 and E158). Moreover, the triple combination of the residual alkaloid with CY and 6-MP was highly effective against Rauscher leukemia in mice, with 405% (E150) and 170% (E158) prolongation of MST over the controls. The mixture of the two standard antileukemic agents, CY and 6-MP, was not as effective as the combination of narcissus alkaloids with CY or 6-MP (E144 and E158), although no increase in toxicity was noticed in the mice. We found both pseudolycorine

and residual alkaloid, when mixed with CY and 6-MP, to be the most effective against Rauscher leukemia in mice without an increase in toxicity.

Discussion. Through our screening investigation for antiviral agents from the higher plants of the Pacific area (10, 11), narcissus alkaloids were found to be promising agents for possible application to chemotherapeutic treatment of human leukemia (2). Pseudolycorine, one of the narcissus alkaloids, has been shown to be highly effective against established Rauscher leukemia in mice (1, 2). We found also, that the residual alkaloid is more effective than pseudolycorine in the treatment of Rauscher leukemia (1, 2). In addition, the narcissus residual alkaloid has been found to be a potent inhibitor of reverse transcriptase (3), while pseudolycorine did not inhibit the activity of this enzyme (personal communication from Dr. M. A. Chirigos of NIH). This might be due to the relatively low solubility of pseudolycorine in water. Residual alkaloid and other inhibitors of reverse transcriptase (EB, rifamycin SV, acetyl adriamycin, and cinerubin A) were tested for their inhibitory effects on the replication of RLV in ME tissue culture cells and for their therapeutic activities in Rauscher leukemic mice. The results indicate that, while all drugs are claimed to have an inhibitory activity to reverse transcriptase in enzyme inhibition tests (4, 5, 7), only rifamycin SV and acetyl adriamycin significantly suppressed the virus replication in the culture cells (Tables I and II). However, these two agents could not prolong significantly the life span of the leukemic mice (Table III). The reason for this discrepancy in their effectiveness *in vitro* and *in vivo* might be that an effective dose could not be obtained *in vivo* because of their severe toxicity to mice. In contrast, effective doses of the narcissus residual alkaloid can be administered repeatedly over a long period of time (2). Thus, the narcissus residual alkaloid is one of the few inhibitors of reverse transcriptase which is effective both *in vivo* (mice) and *in vitro* (tissue cultures and enzyme inhibition tests).

For years, multiple drug combinations have been developed in the treatment of various neoplasias. As reported in our previous paper (2), the combination of the narcissus alkaloids with CY or 6-MP was a more effective treatment against Rauscher leukemia in mice than treatment with any one of these agents singly. Now we have results which indicate that the narcissus alkaloids in triple combination with CY and 6-MP are even more effective in the treatment of Rauscher leukemia (Table IV). This may be an example of a rational combination therapy involving agents of different action mechanisms; one being an antiviral agent possessing strong inhibitory activity against reverse transcriptase, and the others being classical cytotoxic agents. Moreover, the residual alkaloid, when given in repeated administrations, possesses moderate anticancer activity against Ehrlich ascites carcinoma in mice (12). Therefore, the narcissus residual alkaloid, with its inhibitory activity against reverse transcriptase, anticancer activity, relatively low toxicity, and without inhibitory activity to humoral antibody production in mice (tests of cell-mediated immunity are in progress), might be a potential agent for the treatment of human leukemia.

Summary. Narcissus alkaloid (pseudolycorine and the residual alkaloid) in comparison with standard inhibitors of reverse transcriptase (ethidium bromide, rifamycin SV, acetyl adriamycin, and cinerubin A) were tested for their antiviral activities against Rauscher leukemia virus in mouse embryo cell cultures. The narcissus alkaloid remarkably suppressed the virus replication in mouse embryo cells, while rifamycin SV, acetyl adriamycin and cinerubin A were moderately inhibitory, and ethidium bromide showed no effect.

In the treatment of Rauscher leukemia in mice, with single-drug administration, the narcissus alkaloid showed a significant prolongation of the life span of leukemic mice, while acetyl adriamycin and cinerubin A were only slightly effective, and rifamycin SV and ethidium bromide were found to be ineffective. The triple combination treatments using narcissus alkaloids with 6-mercaptopurine and cyclophosphamide showed

a superior prolongation effect on the survival time over two-combination treatments, which have been consistently demonstrated to be more effective than the administration of single agents.

We thank Dr. M. A. Chirigos of NIH for testing on reverse transcriptase; Dr. A. Hackett of Naval Biomedical Research Laboratory, Oakland, CA for supplying XC cells; and Dr. M. A. Apple of the Cancer Research Institute of the University of California for supplying acetyl adriamycin and cinerubin A. Appreciation is also expressed to Dr. P. Yoder of University of Hawaii for his criticisms and comments on the manuscript.

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

2. Furusawa, E., Suzuki, N., Ramanathan, S., Furusawa, S., and Cutting, W., Proc. Soc. Exp. Biol. Med. **140**, 1034 (1972).

3. Papas, T. S., Sandhaus, L., Chirigos, M. A., and Furusawa, E., Biochem. Biophys. Commun. **52**, 88 (1973).

4. Hirschman, S. Z., Science **173**, 441 (1971).

5. Gurgo, C., Ray, R. K., Thiry, L., and Green, M., Nature (London) New Biol. **229**, 111 (1971).

6. Apple, M. A., 5th International Congress on Pharmacology (San Francisco). Abstracts p. 8, 48 (1972).

7. Klement, V., Rowe, W. P., Hartley, J. W., and Pugh, W. E., Proc. Nat. Acad. Sci. USA **63**, 753 (1969).

8. Rowe, W. P., Pugh, W. E., and Hartley, J. W., Virology **42**, 1136 (1970).

9. Reed, L. J., and Muench, H. A., Amer. J. Hyg. **27**, 493 (1938).

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

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

12. Furusawa, E., Suzuki, N., Tani, S., Furusawa, S., Ishioka, G. Y., and Motobu, J., Proc. Soc. Exp. Biol. Med. **143**, 33 (1973).

Received Oct. 22, 1973. P.S.E.B.M., 1974, Vol. 145.