PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 186-191 (1976)

Therapeutic Activity of Pretazettine, a Narcissus Alkaloid on Rauscher Leukemia: Comparison with Tazettine and Streptonigrin[1] (39357)

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One of the narcissus alkaloids (residual alkaloid A-2) has been shown to be therapeutically effective against advanced Rauscher leukemia in mice (1-3). The alkaloid inhibits the growth of Rauscher virus (2), a viral reverse transcriptase (4), and cellular protein synthesis (5). The alkaloid was identified as pretazettine, an unstable free base (3). We have now succeeded to isolate enough amounts of pretazettine hydrochloride via the picrate (6). We now report the anti-Rauscher leukemic activity of pretazettine HCl (PTZ) in comparison with tazettine (TZ) and streptonigrin (SN), one of the few inhibitors of reverse transcriptase, effective *in vivo* against Rauscher leukemia in mice (7).

Materials and methods. Preparation of agents. The methods of isolation of PTZ and TZ from the bulbs of *Narcissus tazetta L* have been described (6). The antibiotic SN was obtained from Dr. Chirigos of NIH. PTZ and SN were dissolved in distilled water and TZ was dissolved in water slightly acidified with HCl (pH 5) before use.

Cell cultures. NIH/3T3 and BALB/3T3 mouse fibroblast cell lines, obtained from Dr. Aaronson of NIH and cultured in MEM medium with 10% fetal calf serum for several months in this laboratory, were used for the *in vitro* antiviral testing of the agents. XC cells, supplied by Dr. Hackett of Naval Biomed. Res. Lab., Oakland, Calif., and kept in MEM medium with 5% fetal calf serum, were used as detectors of Rauscher virus.

System of Rauscher viral leukemia and chemotherapy. An inoculum (0.2 ml) of a 1:40 dilution of leukemic plasma was injected intraperitoneally (ip) into 8-10-week-old BALB/c inbred mice, either male or female. Treatment with optimal dose of the agents was started subcutaneously (sc) 2 to 3 weeks after infection when 70 to 100% of the mice showed clearly palpable spleens. In some experiments, the treatment was started earlier. Agents were injected every other day for several weeks. The dose and the periods were chosen to avoid any apparent toxicity, as indicated by general weakness, ruffled hair, weak motions, or marked loss of body weight.

In vitro test for antiviral activity. NIH/3T3 or BALB/3T3 cells, freshly trypsinized and suspended in 5 ml of culture medium (ca. 2×10^4 cells/ml) in Falcon plastic flasks (25-cm growth area) were inoculated with 0.5 ml of 1:10 dilution of leukemic plasma (ca. 10^5 XC syncytial forming units). The cells were inoculated at 37° for 3 days with daily medium changes; then various doses of the agents in 0.1 ml of distilled water were added (on Day 3) and incubated for 2 more days. The culture medium was then collected (on Day 5) for the titration of virus yield and the cultures were overlaid with 5×10^5 XC cells on 5 ml of MEM medium with 5% calf serum. The number of syncytial giant cells per microscopic field under 40× magnification one day later (on Day 6) was counted. For the titration of the virus yield in the harvests, NIH/3T3 cells were used. The cells (10^4) freshly suspended in 0.5 ml of medium were inoculated with 0.1 ml of a 10-fold dilution of the harvests and incubated at 37° for 9 days with medium changes of every other day, then 5×10^4 XC cells in 1 ml were overlaid and incubated 1 more day. The syncytial cells were detected by the modified method of XC assay of Rowe *et al.* (8). Three tubes were used for each dilution. The virus titer was expressed by a median tissue culture infectious dose ($TCID_{50}$)/ml.

Results. Effect of pretazettine HCl, tazettine, and streptonigrin on survival of mice

[1] Supported by Research Grants from National Cancer Institute (CA 12733) and American Cancer Society (CH-45).

with established Rauscher leukemia. Three initial experiments with the purified residual alkaloid, PTZ, have been performed to confirm our previous experiments (1–3) with the crude alkaloid fractions. Figure 1 or 2 shows the dynamic course of the leukemic disease which the treatment was started on the early (Day 7) or on the advanced (on Day 25) stage after the ip inoculation of Rauscher virus. The median survival time (MST) of the PTZ-treated group was 81 days (controls: 41 days) in Fig. 1, or 72 days (controls: 47 days) in Fig. 2. That is, the continuous administration of PTZ significantly increased ($P < 0.01$) the life span of the leukemic mice. As shown in Fig. 3, PTZ was still effective against the leukemia which was initiated by the intracerebral inoculation of the virus. While pretazettine (Fig. 4) is stable in acidic pH, it is unstable in basic pH and gradually rearranged to tazettine (Fig. 4) (9, 10) on standing. Since it is likely that PTZ will be converted to TZ in the body, it is of interest to know whether TZ has the biological potency. It is also of interest to compare PTZ with streptonigrin, an antibiotic which is one of the few inhibitors of viral reverse transcriptase, possessing therapeutic activity against Rauscher viral leukemia (7). Table I shows the results of six comparative experiments with TZ and three with SN. It was found that TZ was not significantly active (the mean prolongation effect of MST = 22%; $P > 0.05$), while SN demonstrated almost the same activity as that of PTZ. Although it is active, SN

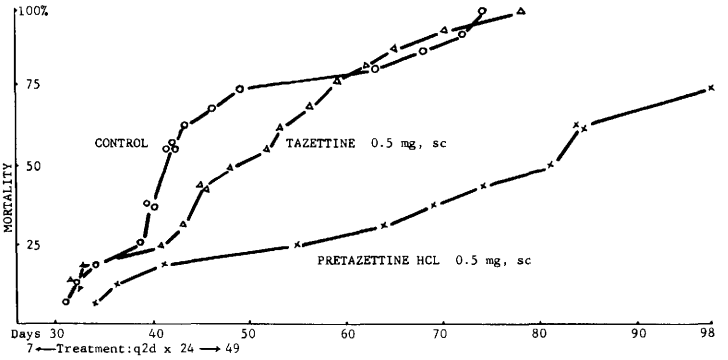

FIG. 1. Effect of pretazettine HCl on life span of established Rauscher leukemia: comparison with tazettine. Treatment was started on Day 7 postinfection, every other day, continued for 49 days (MST of control: 41 days; PTZ: 81 days; TZ: 49 days). Each 16 female mice used.

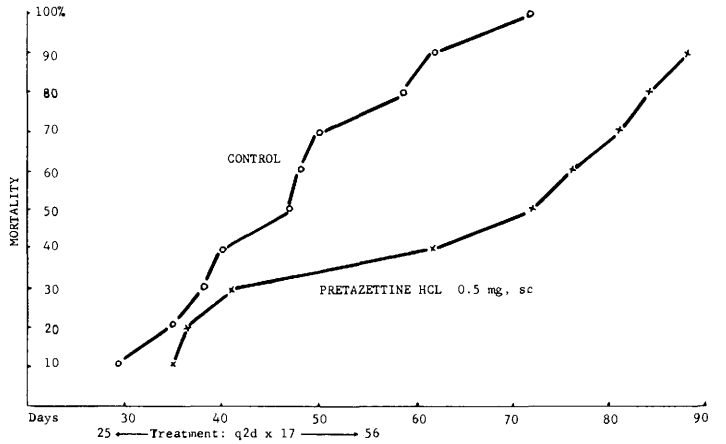

FIG. 2. Effect of pretazettine HCl on life span of advanced Rauscher leukemia. Treatment was started on Day 25 postinfection, every other day, for 56 days (MST of control: 47 days; PTZ: 72 days; prolongation of MST over control: 53%). Each 10 male mice used.

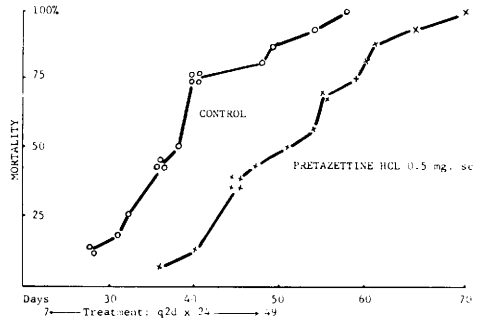

FIG. 3. Effect of pretazettine HCl on life span of established Rauscher leukemia by ic route-infection. Treatment was started 7 days after intracerebral inoculation of virus, every other day, continued for 49 days, total 24 injections (MST of control: 38 days; PTZ: 51 days; prolongation of MST over control: 34%, $P < 0.05$). Each 16 female mice used.

showed unfavorable delayed side effects such as local skin-muscle necrosis, ruffled hair, weak motions, and loss of body weight within 2 to 3 weeks, so further administration was not possible, while PTZ administration could be continued for over 100 days (E15) without apparent toxicity. In E16, the mice were inoculated with a washed leu-

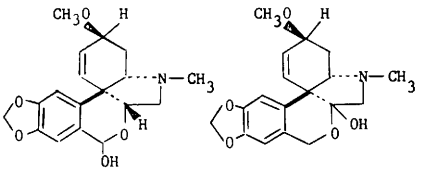

FIGURE 4

TABLE I. EFFECT OF PRETAZETTINE HCl ON SURVIVAL OF MICE WITH ESTABLISHED RAUSCHER LEUKEMIA: COMPARISON WITH TAZETTINE AND STREPTONIGRIN.

Expt. no.	Agent	Dose (mg/kg)	Times and period of treatment (days)	Median survival time (MST) (day and range)	Prolong. of MST over control (%)	P
E11	Control (each 20 female)			29 (24-46)[b]		
	Pretazettine	25	10 (18-37)[a]	45 (31-78)	55	<0.01
	Tazettine	25	10 (18-37)	37 (23-49)	28	<0.05
E12	Control (each 20 male)			53 (27-74)	55	<0.01
	Pretazettine	25	10 (23-42)	82 (32-107)	19	>0.05
	Tazettine	25	10 (23-42)	63 (30-78)		
E13	Control (each 20 female)			44 (28-50)		
	Pretazettine	25	17 (17-39)	109 (32-141)	148	<0.01
	Tazettine	25	17 (17-39)	53 (28-62)	25	<0.05
E14	Control (each 20 male)			43 (29-55)		
	Pretazettine	25	21 (19-56)	76 (32-85)	77	<0.01
	Tazettine	25	21 (19-56)	58 (31-62)	35	<0.05
E15	Control (each 20 female)			40 (19-62)		
	Pretazettine	25	56 (5-108)	98 (40-135)	145	<0.01
	Tazettine	25	56 (5-108)	49 (19-68)	23	>0.05
E16	Control (each 20 female)			29 (23-31)		
	Pretazettine	25	20 (12-53)	74 (30-77)	155	<0.01
	Tazettine	25	8 (12-27)	27 (23-34)	0	
E17	Control (each 10 female)			40 (21-49)		
	Pretazettine	25	7 (16-30)	61 (27-76)	53	<0.01
	Streptonigrin	0.05	7 (16-30)	63 (30-68)	58	<0.01
	Streptonigrin	0.025	12 (16-40)	48 (24-55)	20	>0.05
E18	Control (each 10 female)			44 (20-63)		
	Pretazettine	25	10 (18-37)	69 (33-89)	57	<0.01
	Streptonigrin	0.05	10 (18-37)	67 (35-81)	52	<0.01
	Streptonigrin	0.025	14 (18-45)	53 (32-64)	18	>0.05
E19	Control (each 20 male)			48 (21-78)		
	Pretazettine	25	10 (18-37)	79 (25-92)	65	<0.01
	Streptonigrin	0.05	10 (18-37)	85 (31-89)	77	<0.01
	Streptonigrin	0.025	14 (18-45)	60 (27-75)	25	<0.05

[a] Treatment with the optimal dose of each agent was started on Day 18 and continued every other day until Day 37, total 10 injections. Only in E13, five daily injections per week were done.

[b] Day of the first death to day of the last death during the observation periods of 100-150 days.

kemic whole blood (0.2 ml of 1:20 dilution, ip), freshly harvested, which contained at least 10^5 levels of leukemic cells. PTZ was also therapeutically effective against Rauscher leukemia established by transplanted leukemia cells.

Cytotoxicity test of agents against NIH/3T3 cells. Before testing the antiviral activity of the agents, the maximum nontoxic doses (MNTD) for the long-term and short-term cultivations of the cells were determined. It was found that the cells could be continuously passaged at a slightly reduced rate of growth in the presence of PTZ at the maximum concentration of 50 ng/ml, TZ at 5 μg (5000 ng)/ml, and SN at 0.5 ng/ml of medium. Doses higher than these were inhibitory to the growth of cells and decreased the cell populations gradually, and finally could not maintain the cultures. When the dose of PTZ or TZ was increased up to 5 or 100 μg/ml, respectively, the cells which had been making confluent or subconfluent monolayer became static within 2 days of incubation, recognized by reduced acid production, skinny fibroblastic forms, but still firmly attached to the wall. The majority of the cells quickly recovered when the agents were removed from the medium 2 days later. When the dose of SN was increased up to 100 ng/ml, the cells became apparently static within 2 days. The cells were affected by the agent at the range of 5 to 100 ng/ml without morphological changes, but many cells gradually detached from the wall during the further incubation, after removing the agent from the medium. Thus, the static status of the cells induced by PTZ (0.2 to 5 μg/ml) or TZ (20 to 100 μg/ml) was reversible while the same status by SN (5 to 100 ng/ml) was irreversible. Any higher levels of the agents (PTZ 10 μg, TZ 200 μg, and SN 200 ng/ml) destroyed the monolayers of the cells and over 50% of the cells were detached from the wall within 2 days. After checking their cytotoxic doses, the antiviral activity of these agents were tested at various doses up to MNTD for 2 days incubation.

Effect of PTZ, TZ, and SN on Rauscher virus growth in 3T3 cells. Table II shows the results using NIH/3T3 cells infected with the virus 3 days prior to the 2 day-treatment with the agents. The virus growth in the cells was directly estimated by XC cells overlaid on the cultures on Day 5. The three agents showed clear inhibitory effect on the growth of virus at the cell-static doses. PTZ possessed a wider effective range (0.5 to 5 μg/ml) compared with TZ (25 to 100 μg) and SN (50 to 100 ng). Table III shows the antiviral activity of these agents at the optimal dose both in NIH and BALB/3T3 cells, estimated by the virus titration of the medium in addition to the direct XC overlay. PTZ suppressed the virus growth 2 log below the level of controls, while TZ and SN one log.

Discussion. The free base pretazettine (residual alkaloid A-3, amorphous powder) was reported to be unstable in water above pH 7 and gradually rearranged to the stable isomer TZ even on standing at room temperature (9, 10). The hydrochloride of the pretazettine (PTZ), a stable form in water, is now quantitatively prepared by a modi-

TABLE II. EFFECT OF PTZ, TZ, AND SN ON RAUSCHER VIRUS GROWTH IN NIH/3T3 CELLS.[a]

Agent and dose/ml	Number of syncitial cells			
	Expt. 1	Expt. 2	Expt. 3	Mean (%)
Control (no agent)	65	63	20	100
Pretazettine HCl (μg)				
5	0	0		0
2.5	0	0	2	1
1.0	2	5		6
0.5	2	4	6	8
0.25	7	9		13
0.1	25		9	40
0.05	58	51	22	102
Tazettine (μg)				
100	3		0	2
50	6	8	5	13
25	28	35		49
10	62	70	18	101
5	72	68		110
Streptonigrin (ng)				
100	10	2	1	9
50	30	15		35
20	68	70	19	107
10	62	59		94
5	65		25	106

[a] Treatment with various nontoxic dose of each agent was started 3 days after the virus infection and continued for 2 days, then XC cells were overlaid. One day later, the number of syncitial giant cells per a microscopic field under 40× magnification was counted. The number is the average of 5 fields counted.

TABLE III. EFFECT OF PTZ, TZ, AND SN ON RAUSCHER VIRUS GROWTH IN NIH AND BALB/3T3 CELLS.[a]

Agent and dose/ml	Virus yield[b] 10 log $TCID_{50}$/ml	Syncitial cell[c] formation (%)
NIH/3T3 cells		
Control	5.5	100
PTZ 1 μg	3.5	9
TZ 50 μg	4.2	15
SN 50 ng	4.2	32
BALB/3T3		
Control	4.5	100
PTZ 1 μg	2.2	1
TZ 50 μg	3.5	21
SN 50 ng	3.2	19

[a] Treatment with the optimal dose was started 3 days after infection and continued for 2 days, then the medium was harvested for the virus titration and XC cells were overlaid.

[b] The virus yield in the harvests were titrated using NIH/3T3 cells as described in Methods.

[c] The number of syncitial cells per a microscopic field (40×) was counted 1 day after XC overlay.

fied method of Wildman (6) in this laboratory. It was of interest to know whether TZ, a more stable isomer of pretazettine, is still active. It was found that TZ was not significantly effective against Rauscher leukemia in mice and it was needed at 50 to 100 times greater dose of PTZ to inhibit the virus growth *in vitro* (Tables II and III), while PTZ was therapeutically effective as same as residual alkaloid A-2 previously reported (3). At 60 μg/ml, PTZ was inhibitory (50%) to reverse transcriptase of avian myeloblastosis virus (communication from Dr. Chirigos of NIH) and we have confirmed the inhibitory activity on the reverse transcriptase of Rauscher virus at concentrations of 50 to 100 μg/ml (unpublished data), while TZ showed no activity on the enzymes of both viruses. Recently, at 10 μg/ml, PTZ has been found to inhibit the cellular protein synthesis without affecting the syntheses of DNA and RNA in KB, P388, and Ehrlich ascites cells in this laboratory (5), and in HeLa and Krebs II cells (communication from Dr. D. Vazques, Inst. Cellular Biology, Madrid, Spain) but TZ showed no inhibition. Such findings mentioned above suggest that the PTZ is the active form and TZ is an inert isomer. The MNTD of SN for the long-term cultivation of NIH/3T3 cells (0.5 ng/ml) was almost equivalent to the MNTD of the plating efficiency of cell colony formation methods reported by other laboratories: 0.16 to 0.33 ng/ml by Price *et al.* (11) and 0.5 ng/ml by Woods *et al.* (12). Although SN at such a low concentration which did not suppress the cell growth was reported to inhibit the *in vitro* transformation of high passage rat embryo cells by 3-methylcholanthrene (3CM) (11), it was not inhibitory to Rauscher virus growth in NIH/3T3 cells. In our system, at least 100 times more concentration (50 ng/ml) of SN was required to stop the on-going virus growth initiated 3 days previously, and such a high concentration of SN made the cells apparently static but actually irreversibly destroyed the growth viability. On the contrary, PTZ at a low concentration which was not inhibitory to cell growth (0.05 μg/ml) could not prevent the transformation of the rat cells by 3CM (communication from Dr. Price of Microbiological Associates, Maryland); however, at 10 times higher concentration (0.5 μg/ml) PTZ made the cells reversibly static and was inhibitory to the on-going growth of Rauscher virus in 3T3 cells.

Summary. The therapeutic activity of narcissus alkaloid pretazettine HCl (PTZ) on established Rauscher leukemia has been demonstrated and compared with the isomer tazettine (TZ) and an antibiotc, streptonigrin (SN). PTZ and SN showed remarkable prolongation effect on the life span of the leukemic mice and the antiviral activity has been confirmed in mouse 3T3 cells infected with Rauscher virus. TZ showed no significant activity in the leukemic mice and was inhibitory to the virus growth in the cells at much higher doses than PTZ. It is suggested that the stereochemical rearrangement from PTZ to TZ inactivates the biological activity of PTZ.

We thank Dr. M. A. Chirigos of NIH for supplying streptonigrin; Dr. S. A. Aaronson of NIH for supplying mouse 3T3 cells; and Dr. A. Hackett of Naval Biomed. Res. Lab., Calif., for supplying XC cells.

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Received January 19, 1976. P.S.E.B.M. 1976, Vol. 152.