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Evaluation of *p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met-ethoxy HCl against transplantable and spontaneous murine neoplasia*

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Summary. The therapeutic efficacy of PTT.119, *p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met-ethoxy HCl, was evaluated using the transplantable L1210 leukemia and Ridgway osteogenic sarcoma tumor lines and the spontaneous C3H/StRos mammary tumor and AKR leukemia tumor models. Given in a single i.p. dose at 5–10 mg/kg on day 2 or in two injections of 5–7 mg each on days 2 and 9 to BDf_1 mice with peritoneal L1210 leukemia grafts, PTT.119 increased the life spans (ILS) of the population dying of tumor by 94%–313%. In addition, 10% of the mice receiving 7 mg PTT.119 on days 2 and 9 were free of L1210 leukemic grafts when autopsied at the end of the 70-day observation period. The average life span of AKR mice with Ridgway osteogenic sarcoma grafts was significantly increased from 36–40 days to >79 days following one or two s.c. injections of 5, 7, or 12.5 mg/kg PTT.119. Administration of PTT.119 at 14 or 14 and 21 days after tumor graft not only induced regression of palpable tumors but resulted in the absence of grafts in 60%–70% of the mice in several of the treated groups on autopsy at 180 days. In contrast, spontaneous mammary tumors were less susceptible to PTT.119; an ILS of only 15%–38% was observed in C3H/StRos mice, which eventually succumbed to tumor. Nevertheless, the total regression of initial tumors and the absence of further tumor incidence (>180 days) was confirmed by autopsy in 5%–10% of the C3H/StRos mice receiving multiple i.p. injections of 5 or 7.5 mg/kg PTT.119. The drug was highly effective against spontaneous AKR leukemia; multiple s.c. or i.p. injections for a total of 15–40 mg/kg PTT.119 increased the average 25-day life span up to 723% and sustained remission in 9%–40% of the animals for >6 months.

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Abbreviations: PTT.119, *p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met-ethoxy HCl; L-PAM, L-phenylalanine mustard

Introduction

The tripeptide, 3-(*p*-fluorophenyl)-L-alanyl-3-[*m*-bis-(2-chloroethyl)amino-phenyl-L-alanyl-L-methionine ethyl ester HCl, PTT.119, was synthesized in an attempt to enhance the selective uptake of the cytolytic bis-(2-chloroethyl)amino alkylating moiety by inclusion of carrier amino acids. Previous studies have shown that cellular uptake of PTT.119 occurs by at least two amino acid transport carriers that are quantitatively and qualitatively different from those involved in L-phenylanine mustard (L-PAM) entry [27, 28]. Unlike that of L-PAM, the cytolytic activity of PTT.119 was not inhibited by exogenous amino acids [24]. In addition, the tripeptide remained completely effective against multiple-drug-resistant cells possessing altered transport carriers [10, 11, 19, 24]. PTT.119 induced dose-dependent, irreversible cytostatic/cytolytic decreases in tumor cell populations in a variety of rodent, primate, and human leukemia cells derived from spontaneous disease and neoplasias induced by chemical carcinogens or by RNA and DNA viruses [22, 23]. The cytopathology, degree of susceptibility, and kinetics of cell cytolysis varied with the tumor cell type, suggesting inherent differences in the biochemical interactions of cellular components with PTT.119 and/or its alkylating intermediates [21].

The present investigations were undertaken to evaluate the in vivo efficacy of PTT.119 as a single therapeutic agent. The results indicate that the tripeptide not only could increase the life span of tumor-bearing mice but could also abrogate the growth of transplantable L1210 leukemia and Ridgway osteogenic sarcoma as well as rendering mammary-tumor-bearing C3H/StRos mice and AKR mice with spontaneous leukemia free of disease for as long as 6 months.

Materials and methods

Transplantable tumor systems. L1210 leukemia was maintained in BDf_1 (C57BL/6 × DBA/2; Charles River) mice [12, 14] by biweekly i.p. passage of 10^6 cells [1, 2, 23]. The efficacy of PTT.119 was determined

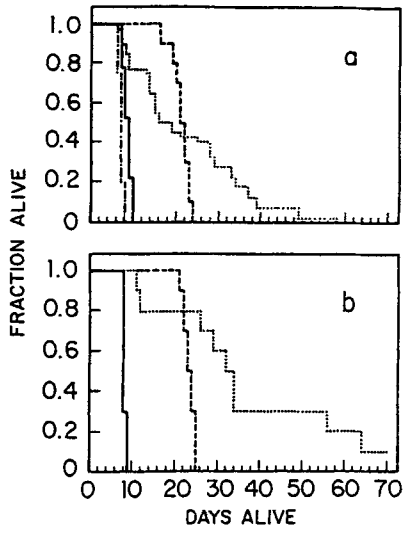

Fig. 1. BDf_1 mice grafted with L1210 leukemia on day 0 were given i.p. injections of PTT.119. **a** A single injection on day 2 of diluent ($n = 40$) (——), 5 mg/kg ($n = 10$) (– – –), 10 mg/kg ($n = 40$) (. . .), or 15 mg/kg ($n = 20$) (– · –). **b** Two courses of PTT.119 given on days 2 and 9 of diluent ($n = 10$) (——), 5 mg/kg ($n = 10$) (– – –), or 7 mg/kg ($n = 10$) (. . .)

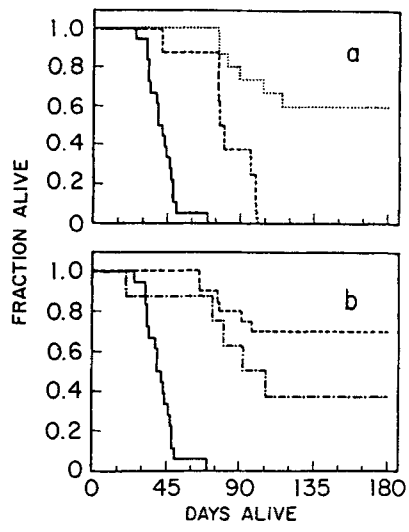

Fig. 2. Young AKR mice grafted with Ridgway osteogenic sarcoma on day 0 were given s.c. injections of PTT.119. **a** Mice were treated on days 14 and 21 with 5 mg/kg ($n = 10$) (– – –) or 7 mg/kg ($n = 15$) (. . .). **b** PTT.119 was also given at 12.5 mg/kg on day 14 ($n = 10$) (– · –) or 12.5 mg/kg on day 14 followed by 7 mg/kg on day 40 ($n = 10$) (– – –). All control animals received diluent on day 14 or days 14 and 21 or 14 and 40 (n = 30) (——)

using BDf_1 mice implanted i.p. with 10^6 L1210 cells on day 0. Cells for implants were harvested from donor mice by peritoneal lavage, and red blood cells were lysed by osmotic shock for 5 s. Viable L1210 cells enumerated by counting in a hemocytometer using trypan blue were then resuspended in 0.01 *M* phosphate-buffered saline (PBS, pH 7.4) at 10^7 cells/ml. Ridgway osteogenic sarcoma (ROS) was maintained by subcutaneous trocar grafts in young AKR/J mice (Jackson Laboratories) 8–10 weeks of age. Tumor passage and experimental chemotherapeutic regimens were completed before the mice could begin to develop spontaneous leukemia. Mice treated with chemotherapy received 1- to 2-mm grafts of ROS by trocar on day 0.

Spontaneous tumor models. The incidence of spontaneous mammary tumors in multiparous C3H/StRos mice (West Seneca Laboratory of Roswell Park Memorial Institute) is >90% by the age of 6–8 months. Mice that had 1–2 mammary tumors with dimensions of 8–11 mm were randomized and entered into treatment protocols on day 1. Retired AKR/J (Jackson Laboratories) female breeders were obtained at an age of 4–6 months and maintained until the development of spontaneous leukemia [4]. Clinical diagnosis of leukemia was made with 95% accuracy; animals were diagnosed as being leukemic when their leukocyte counts were >17,000/mm^3 and splenic and lymph node enlargement occurred. At this time the average splenic weight was 460 mg and that of the thymus, 640 mg, and the leukocyte count was 28,000/mm^3. Mice were randomized and entered into treatment protocols on day 1.

Chemotherapeutic compound. PTT.119 [*p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met-ethoxy HCl] was provided by Proter S. p. A. [6]. The drug was initially dissolved at 10 mg/ml in *N,N*-dimethyl-acetamide, Tween 80, and propylene glycol (1 : 1 : 2 by vol.) and was diluted in sterile saline containing 5% glucose immediately before administration. Control animals received injections of drug-free solvent diluted at similar ratios.

Treatment. BDf_1 and AKR mice were randomly placed into control and treatment groups following transplantation with L1210 leukemia or ROS tumors, respectively. C3H/StRos and AKR mice with spontaneous disease were randomized following diagnosis. Animals were individually weighed and given the appropriate i.p. or s.c. dose of PTT.119 or solvent as described in the text [23].

Mice were weighed every 1–3 days and monitored for general health. Tumor width and length (mm) were obtained 2–3 times per week from C3H/StRos mice with spontaneous mammary tumors and AKR/J mice with ROS tumor grafts to determine tumor weight (mg; [(length) × (width)^2]/2). The examination periods for mice receiving L1210 and ROS grafts were 70 and 180 days, respectively. C3H/StRos and AKR mice with spontaneous disease were observed for 180 days following entry into the treatment groups. All mice were autopsied at the time of death or euthanasia.

Treatment regimens were assessed by two or more separate trials. Survival data of individual trials of PTT.119-treated animals and parallel, solvent-treated mice were combined and are presented as Kaplan-Meier estimates of survival for specific regimens, with significance being determined by the Wilcoxon test and the Mantel-Haenszel log-rank test. The percentage of increase in life span (ILS) was determined by the equation [100 × (T-C)/C], where T and C represent the median survival in days of treated and control mice, respectively. Significance was evaluated using Student's *t*-test. In all tumor systems examined, only neglible differences were observed among the various groups of control mice receiving solvent; the route, number, and day of injection(s) did not alter the duration of survival, nor were there significant changes ($P > 0.99$) in the average or median survival of the groups. Graphic representation of Kaplan-Meier estimates of individual control groups yielded indistinguishable, overlapping curves. Consequently, for graphic presentation only, control groups depicted in the figures represent the cumulative survival of control animals used in parallel for all PTT.119-treated groups shown.

Results

L1210 leukemia

BDf_1 mice given i.p. grafts of 10^6 L1210 leukemia cells on day 0 and treated with diluent on day 2 or days 2 and 9 had a median life span of 8.5 days, and all succumbed to tumor by days 9–10 (Fig. 1). A single i.p. injection of 5 or

Table 1. C3H/StRos mice with spontaneous mammary adenocarcinomas treated with PTT.119

Treatment[a]	Mice (*n*)	Life span in days		% ILS	%Free of tumor at 180 days
		Average	(Range)		
Days 1, 7, 13, 27:					
Diluent	19	51.1	(33– 72)	-	0
5 mg/kg PTT.119	20	66.3	(25–180)	38 (P <0.03)	10
Days 1, 11, 22:					
Diluent	23	49.6	(28– 89)	-	0
7.5 mg/kg PTT.119	20	55.3	(15–180)	15 (P <0.3)	5

[a] Mice received i.p. injections

Table 2. AKR mice with spontaneous leukemia treated with PTT.119

PTT.119 dose (mg/kg)[a]	[N]	Life span in days		% ILS
		Average	(Range)	
i.p. injections:				
0	10	11.7	(4– 27)	-
10	10	53.6	(24– 60)	471 (P <0.000013)
12.5	20	32.5	(13–104)	210 (P <0.000021)
20	17	29.5	(4– 83)	178 (P <0.09)
s.c. injections:				
0	20	11.9	(4– 21)	-
10	15	40.8	(16– 80)	257 (P <0.00002)
12.5	35	40	(3–180)[b]	321 (P <0.031)
20	11	16	(4– 63)	68 (P <0.48)

[a] Mice received a single injection of PTT.119 on day 1
[b] Two animals (5.7%) on this protocol were free of tumor after 180 days

10 mg/kg PTT.119 at 2 days after tumor graft increased the life spans of the mice to 21.5 and 17.5 days, respectively (Fig. 1a). Although none of the animals were cured of leukemia, the 94%–138% %ILS obtained by the treated animals in both protocols were significant (P <0.0001) according to the Wilcoxon log-rank test. Mice treated with 15 mg/kg PTT.119 received no benefit due to drug toxicity and their survival was similar to control values.

Administration of 5 mg/kg PTT.119 (i.p.) on days 2 and 9 resulted in a 194% ILS (P <0.0001) but was no more effective than a single dose (Fig. 1a, b). However, 10% of the mice receiving two courses of 7 mg/kg were alive at 70 days and were free of L1210 leukemia when autopsied (Fig. 1b). The median life span of all animals dying of leukemia in this group was 33 days vs 8.5 days for the diluent control, resulting in a 300% ILS (P <0.0001).

Ridgway osteogenic sarcoma

AKR mice with s.c. ROS grafts all succumbed to tumor within 70 days with a median life span of 36–40 days (Fig. 2). Two courses of 5 mg/kg PTT.119 given s.c. on days 14 and 21 increased the median life span of the animals to 79 days. This 93% ILS (P <0.0003) correlated with a diminution in ROS tumor growth observed during the first 45 days in most of the PTT.119-treated animals. However, tumor regression was transient and regrowth of the grafts 24 days after PTT.119 treatment eventually resulted in the death of all mice by day 101 (Fig. 2a).

Increasing the dose of PTT.119 to 7 mg/kg extended the period of tumor regression to >60 days and increased the mean survival of those animals succumbing to ROS tumor to >90 days (Fig. 2a). In addition to an ILS of 110% (P <0.0002) in the uncured population, 60% of the mice receiving this treatment protocol were free of ROS tumor at the time of autopsy after 180 days (Fig. 2a).

Increasing the dose to 12.5 mg/kg resulted in a small number of deaths due to toxicity following the first injection 2 weeks after tumor implantation; consequently, animals did not receive a second dose on day 21. Survival of animals in this abbreviated treatment group was not as good as that in those receiving 14 mg/kg total in two doses, although the median life span of animals eventually dying from ROS grafts was increased by 98% (P <0.002; Fig. 2b). At the end of the 180-day experimental period, autopsy revealed that 37% of these mice were free of ROS tumor. In an attempt to inhibit the regrowth of ROS tumor

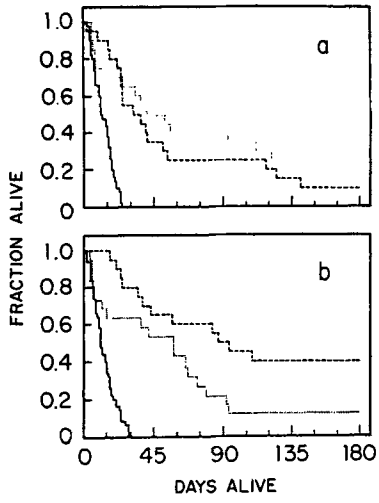

Fig. 3. AKR mice with spontaneous leukemia treated with PTT.119. a Mice received i.p. injections of either diluent on days 1, 8, 20, and 36 or 1 and 19 ($n = 20$) (——) or PTT.119 at 5 mg/kg on days 1, 8, 20, and 36 ($n = 20$) (– – –) or 10 mg/kg on day 1 followed by 5 mg/kg on day 19 ($n = 20$) (. . .). b AKR mice were also treated via the s.c. route with either diluent on days 1, 8, 20, and 41 or 1 and 19 ($n = 20$) (——) or PTT.119 at 10 mg/kg on days 1, 8, 20, and 41 ($n = 20$) (– – –) or 12.5 mg/kg on day 1 followed by 7.5 mg/kg on day 19 ($n = 20$) (. . .)

observed at 45–60 days, animals were treated with 12.5 mg/kg on day 14, followed by a second course of 7 mg/kg PTT.119 on day 40. The median life span of mice in this group that eventually died of tumor was increased to 76.5 days, and the proportion of mice at 180 days with no evidence of disease increased to 70%.

C3H/StRos spontaneous mammary adenocarcinoma

Survival of C3H/StRos breeding females possessing 1–2 spontaneous mammary lesions no larger than 8–11 mm in any dimension usually varies between 4 and 13 weeks, with an average life span of approximately 50 days (Table 1). Although the rate of growth and virulence of each lesion was unique, there were no significant differences among the control and various treatment groups in the numbers of lesions and total tumor burden of the mice at the initiation of the experiments and at the time of death of the host. PTT.119 given as four i.p. courses of 5 mg/kg over 4 weeks or as three courses of 7.5 mg/kg over 3 weeks increased the median duration of survival of those animals eventually dying of tumor by only 15%–38% (Table 1). However, multiple administration of the tripeptide did decrease the rate of tumor growth and induced complete regression of the initial tumors in a portion of these animals. In all, 25%–50% of the treated mice also remained free of further tumor incidence, and no spontaneous tumors were detected on autopsy after the 180-day period. Comparison of the inital tumor volumes and numbers of those animals achieving apparently complete regression with those data from animals with progressive disease revealed no correlation between initial tumor burden with tumor regression/progression and host survival.

AKR spontaneous leukemia

The life span of leukemic AKR mice diagnosed by splenic and lymph node palpation and leukocyte count in these experiments varied between 3 and 30 days from the day of diagnosis, with an overall median of 11 days (1.1 SE) for untreated mice ($n = 30$) and 11.5 days (0.91 SE) for animals ($n = 100$) injected either i.p. or s.c. with the various schedules of diluent. A single i.p. or s.c. injection of 10, 12.5, or 20 mg/kg PTT.119 on day 1 increased the average life span of the animals, resulting in significant increases in the median survival of mice treated on the six protocols (Table 2). Comparison of these three doses revealed that 10 mg/kg was the most effective concentration by either route and that i.p. injections were only slightly more efficacious ($P < 0.65$) than s.c. injections. In addition to the observed increases in survival, two animals receiving an s.c. injection of 12.5 mg/kg were alive at 180 days and had no detectable disease when autopsied. Toxicity was observed at higher drug concentrations (12.5, 20, and 22.5 mg/kg), contributing to the early deaths observed in these study groups.

PTT.119 was also given as a series of injections in an attempt to increase the benefit of tripeptide treatment. Two or three i.p. or s.c. injections of 5, 7, or 10 mg/kg given at 3- to 7-day intervals did not result in further increases in survival. However, mice treated with 5 mg/kg i.p. on days 1, 8, 20, and 36 had an average life span of 58.5 days (228% ILS, $P < 0.0000535$); Fig. 3a). In addition, 10% of these treated animals remained free of disease at 180 days after the initiation of PTT.119 treatment. The best results for multiple s.c. administration of a single concentration were obtained after the injection of 10 mg/kg on days 1, 8, 20, and 41 (Fig. 3b). This regimen increased the average life span of the animals to 103.7 days. This significant increase in survival of 762% ($P < 0.0000002$); was attributable to increases in remission duration and abrogation of spontaneous leukemia in 40% of the treated mice autopsied after 180 days.

Varying combinations of two concentrations of PTT.119 given in 2–5 injections between days 1 and 41 were also carried out. Of the 20 schedules examined, most did not result in significant increases in the average life span of the animals over that observed following a single or multiple injection(s) of a given concentration of PTT.119. Alteration of the dose and schedule to 10 mg/kg PTT.119 on day 1 followed by 5 mg/kg on day 19 resulted in an average life span of 75.7 days and an ILS of 304% ($P < 0.0037$). This regimen resulted in a sustained remission of 25% in the leukemia-positive AKR mice as determined by autopsy after 180 days (Fig. 3a). Administration of 12.5 mg/kg s.c. PTT.119 on day 1 followed by 7.5 mg/kg on day 19 yielded an average survival of 63.1 days, with a 452.3% ILS ($P < 0.01$). Complete remission was obtained in 15% of the animals that remained disease-free after 180 days (Fig. 3b).

Discussion

The synthetic tripeptide PTT.119 is a bifunctional alkylating agent of the nitrogen mustard family, consisting of methionine, *p*-F-phenylalanine, and phenylalanine with a bis-(2-chloroethyl)amino group in the meta position of the benzene ring. In addition to our initial findings that this tripeptide was effective against a variety of tumor cells in vitro, our previous investigations demonstrated that L1210 and P388 leukemia cells resistant to L-PAM, methotrexate, and *cis*-diamminedichloroplatinum(II) and cross-resistant to other alkylating drugs such as cyclophosphamide and thioTEPA were as sensitive to PTT.119 as were the susceptible parental leukemia strains [24]. These studies also revealed that exposure of L1210 leukemia to escalating levels of PTT.119 for over 100 passages could not select or induce drug-resistant phenotypes, indicating that the tripeptide was a poor mutagen. These in vitro results, coupled with our current findings that PTT.119 is therapeutically effective against spontaneous and transplantable murine tumors, demonstrate the potential benefit of PTT.119 as a chemotherapeutic agent.

The present studies, using the transplantable L1210 leukemia and Ridgway osteogenic sarcoma and the spontaneous AKR leukemia and C3H/StRos mammary tumor models, demonstrate that PTT.119 given as a single agent can reduce tumor progression and significantly prolong the survival of murine hosts with established tumors. PTT.119 given in one or two injections could significantly increase the survival of BDf_1 mice with L1210 leukemia grafts; in one treatment regimen, 10% of the hosts were alive at the end of the 70-day observation period, with no detectable disease. This indicated that the tripeptide effectively reduced to zero the population of L1210 leukemia cells capable of proliferation, since in our laboratory, as in others, the LD_{100} of this tumor system is a single cell in BDf_1 hosts within 24 days [1, 12, 14, 23].

PTT.119 given to AKR mice with Ridgway osteogenic sarcoma also significantly increased host survival and rendered 37%–70% of the mice free of detectable ROS tumor after 180 days. Similar increases in life span and numbers of animals with complete elimination of ROS grafts have not been observed by other investigators following the administration of L-PAM or other similar compounds, using over 25 different treatment schedules for each agent [13, 17, 18]. For example, AKR mice with ROS treated with 16 μ*M*/kg L-PAM (days 14, 28, 42, and 56; i.p.) had an ILS of 130% and all animals eventually died of tumor. Increasing the dose of L-PAM to 26.2–29 μ*M*/kg further increased the ILS to 197%–207% and resulted in 10%–20% of the tumor hosts being free of disease [17, 18]. In comparison, administration of two i.p. injections of 10.5 μ *M*/kg PTT.119 (7 mg/kg; days 14 and 21) resulted in an ILS of 111%, with 60% of the AKR hosts being rendered free of ROS tumors (Fig. 2).

Administration of PTT.119 to C3H/StRos mice with spontaneous mammary tumor increased their life spans by 15%–38%. These increases are similar to the 29% ILS we previously obtained after mammary-tumor-bearing animals were given two courses of the combination of cytoxan (41 mg/kg, i.p.), 5-FU (34 mg/kg, i.v.) and Adriamycin (2.9 mg/kg, i.v.) on days 1 and 8 [26]. However, in contrast to this three-drug regimen, on which all mice succumbed to tumor within 100 days, PTT.119 could abrogate the initial mammary tumor growth, and 5%–10% of the mice remained free of any palpable mammary lesions for a period of >5 months.

PTT.119 was highly effective in increasing the life spans of AKR mice with spontaneous leukemia and rendered a significant proportion (10%–25%) of the animals disease-free for >180 days. As demonstrated by Skipper and co-workers [15], survival of leukemic AKR mice for as long as 90 days after therapy would not be expected if the animals had residual primary tumor, indicating that PTT.119 had completely eradicated the leukemic cells initially present. This apparent cure by PTT.119 treatment is significant, since AKR leukemia is highly refractile to cure by chemotherapeutic agents [16]. In numerous trials with single agents (e.g., cytoxan, L-PAM, nitrogen mustard, MeCCNU, BCNU, vincristine, actinomycin D), combinations of two or three of these compounds resulted in an ILS of up to 153%, 196%, or 246%, respectively, with 10%–13% of the animals surviving for >120 days on any protocol [16]. Our previous experience with chemotherapeutic treatment of leukemic AKR mice yielded similar results [3, 5]; i.p. administration of vincristine (0.75 mg/kg) on day 0, cytoxan (125 mg/kg) on day 3, and MeCCNU (25 mg/kg) on day 7 increased their average life expectancy to 37 days, resulting in a significant ILS of 103% ($P < 0.003$).

The reason(s) for the apparent extended protection rendered by PTT.119 in both retrovirus-infected, spontaneous leukemia and mammary tumor models is unknown. The tripeptide could exert an antiviral as well as a cytolytic effect, as suggested by our in vitro demonstration of a 31% reduction in the production of the B-type mouse mammary tumor retrovirus (MuMTV) within the first 24 h after exposure to PTT.119 [25]. Continued exposure of the productively infected MJY-alpha mammary tumor cells at noncytolytic drug levels for another 24 h further reduced RNA tumor virus production by 70%. The data indicated that PTT.119 interfered with late steps in MuMTV processing and maturation and that MuMTV synthesis was not restored immediately following removal of the tripeptide. Similar antiviral activities could depress MuMTV viremia in the C3H/StRos mice and result in reductions in the incidence of new virally induced mammary lesions. Likewise, PTT.119 given to AKR mice with leukemia/lymphoma may interfere with replication of the murine leukemia virus (MuLV), which induces leukemia in the AKR mice. In light of the increasing frequency of detection of human retroviruses and their proposed role in human leukemia [7–9, 20], this possible antiviral activity of PTT.119 could enhance its potential as a chemotherapeutic agent.

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