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# Increased cancericidal activity of PTT.119; a new synthetic bis-(2-chloroethyl)amino-L-phenylalanine derivative with carrier amino acids

## II. In vivo bioassay

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***Summary.*** *The cancericidal efficacy of a new synthetic tripeptide was demonstrated using both in vitro cultures and in vivo tumorigenic assays. The antitumor agent PTT.119 (p-F-Phe-m-bis-(2-chloroethyl)amino-Phe-Met ethoxy HCl) was highly effective against three virulent murine tumor models: the L1210 leukemia, MJY-alpha mammary tumor and B16 melanoma. Treatment of tumor cells for periods as short as 15 min to 4 h with concentrations of 1–50 μg PTT.119/ml irreversibly reduced tumor cell viability, as evidenced by vital dye exclusion and abrogation of tumor formation and prolongation of host survival. Examination of the sensitivity of mice to PTT.119 revealed that the in vitro antitumor activity of the synthetic tripeptide was exerted at concentrations easily attainable and well tolerated in vivo.*

### Introduction

Derivatives of mechlorethamine (nitrogen mustard) comprise a family of chemotherapeutic agents developed from the basic concept of targeted delivery of cytolytic molecules to selected neoplastic cells. Numerous compounds containing the bis-(2-chloroethyl)amino bifunctional alkylating group coupled to peptides (di-, tri-, tetra-, penta-), polymers, and copolymers of amino acids have been synthesized [8, 26]. Several of these dervatives, including L-phenylalanine mustard (L-PAM) and a synthetic mixture of six (tri-, tetra-, and penta-) peptides each containing the meta structural isomer of L-phenylalanine mustard, *m*-sarcolysin (m.L.SL), have shown therapeutic activity against a variety of human and experimental cancers [1, 8, 14, 21].

Recently we reported the cancericidal activity of a new synthetic tripeptide (*p*-fluoro-phenylalanyl-*m*-bis-(2-chloroethyl)amino-L-phenylalanyl-methionineethoxyhydrochloride), PTT.119, against a broad spectrum of virulent leukemia, lymphoma, mammary tumor, and melanoma cells [31]. Our in vitro systems demonstrated the ability of PTT.119 to induce a dose-dependent, irreversible loss of murine, primate, and human cancer cell survival. We have continued our evaluation of the therapeutic potential of this synthetic tripeptide by assessing the tumorigenicitiy of L1210 leukemia, MJY-alpha mammary tumor, and B16 melanoma cells exposed to PTT.119. These studies, which are reported here, demonstrate that treatment with physiological dosages of PTT.119 effectively reduce or completely eliminate tumorigenicity of the tumor cells.

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### Materials and methods

*Cell cultures.* The epithelial BALB/cfC3H mammary tumor cell line, MJY-alpha, was utilized between the 25th and 65th in vitro subcultures [30]. The growth medium used was RPMI-1640 supplemented with 18% fetal bovine serum, 10 μ*M* bovine insulin, and antibiotics [30].

B16 melanoma cultures were established from transplantable tumors maintained in syngeneic C57BL/6Jx mice. Melanomas from three to five donors were cultured as explants using minimal essential medium containing D-valine and Earle's salts supplemented with 20% calf serum and antibiotics. Confluent cultures were subsequently passaged as single-cell suspensions using saline-trypsin-versene (STV) [30]. Two 4- to 12-month-old B16 cell cultures were used between their 13th and 45th in vitro passages.

Primary L1210 leukemia cell suspensions were initiated from ascitic DBA/2- Ha mice or their $f_1$ hybrid, $BDF_1$ (BALB/c × DBA/2) [3]. Leukemic cells separated on Ficoll-Hypaque gradients were > 98% viable by trypan blue exclusion. Suspension cultures were maintained in RPMI-1630 containing 10% fetal bovine serum and antibiotics.

Cultures were incubated at 37° C in a humidified atmosphere of 5% $CO_2$ in air and were routinely checked for contamination [30].

*Chemotherapeutic compound.* PTT.119 or *p*-L-fluoro-phenylalanyl-*m*-bis-(2-chloroethyl)amino-L-phenylalanyl-L-methionine ethoxy hydrochloride [31] (molec. wt 666.1 g) was provided by Proter S.p.A. Research Division, Via Lambro 38, Opera Italy (patent pending). PTT.119 was dissolved in *N,N* dimethyl acetamide, absolute ethanol, and propylene glycol (1 : 1 : 2). Prior to use, the tripeptide was diluted to 2 mg/ml with 50% aqueous propylene glycol, and further dilutions of PTT.119 for in vitro treatment of tumor cells were made in the appropriate cell culture media containing serum. Control cultures were treated with equivalent volumes of solvent. Dilutions of PTT.119 for the in vivo studies were made with sterile saline containing 5% glucose.

*Treatment of tumor cell suspensions.* Single-cell suspensions of MJY-alpha mammary tumor cells and B16 melanomas were obtained by trypsinization of confluent cell layers using STV. Trypsinized tumor cells and L1210 leukemia cells were pelleted, resuspended, and incubated in their appropriate growth media for 30 min at 37° C prior to treatment. Concentrations of all tumor cell suspensions were adjusted to

$1 \times 10^6$ cells/ml. Cells were exposed to 1–50 μg PTT.119/ml for 0.25, 0.5, 1.2 and 4 h, after which treatment media were removed by centrifugation at 200 *g* for 9 min at 5° C. Cells were gently washed twice in cold media, and divided into two aliquots for resuspension in serum-free media for the in vivo bioassay or in complete growth media for in vitro cultures.

*In vitro tumor cell survival.* In vitro cultures of treated MJY-alpha and B16 tumor cells were accomplished in four-well Nuclon Multidishes using a seeding density of $2-2.2 \times 10^5$ cells/2-cm$^2$ well based on the initial tumor cell concentration before PTT.119 treatment. L1210 leukemia cells were maintained as suspensions at a concentration of $1 \times 10^6$ cells/ml. Media of all cultures were changed daily.

Tumor cell viabilities were determined immediately after PTT.119 treatment and daily over the following 7 days using trypan blue exclusion [11, 20]. MJY-alpha mammary tumor cells and B16 melanoma cells were released from the substrate with 0.5 ml STV and diluted 2- to 10-fold with the vital stain. L1210 cell suspensions were directly mixed (1 : 1) with the trypan blue dye and counted. Viable tumor cells excluding trypan blue were enumerated in each sample by counting all fields of the hemocytometer, and the values from replicate cultures were averaged. Percentages of viable cells from PTT.119-treated cultures were determined by direct comparison of the numbers of these cells excluding trypan blue with those in untreated or solvent-treated parallel cultures. Viabilities were similar in cells treated with varying concentrations of solvent and untreated cells, and consequently the numbers of control cells represent averages of the two experimental controls.

*Bioassay of tumorigenicity.* Suspensions of PTT.119-treated and untreated MJY-alpha mammary tumor, B16 melanoma, and L1210 leukemia cells were adjusted to yield $1 \times 10^7$ cells/ml based on tumor cell concentrations prior to PTT.119 exposure. Tumor cells were inoculated into syngeneic hosts at a concentration of $1 \times 10^6$ cells in 0.1 ml. Female BALB/c/Crgl recipients of MJY-alpha mammary tumor cells and male C57BL/6/Jx hosts receiving B16 melanoma cells were inoculated SC; $BDf_1$ mice were given IP injections of L1210 cells. All SC tumors were measured along their long and short axes every 1–3 days; tumor size is reported as the product of the two measurements (mm$^2$). The examination periods for mice receiving L1210, MJY-alpha, and B16 tumor grafts were 45, 80, and 85 days, respectively. All mice were autopsied at the time of death or sarcrifice.

*Maximum tolerable dosage of PTT.119.* In vivo effects of PTT.119 were assessed in male $BDf_1$ and female AKR/Jx mice. Groups of 10–25 animals received an IP injection of solvent, or of PTT.119 at dosages of 10, 12.5, 15, 17.5, 20, 25, and 30 mg/kg. These doses are equivalent to 35–105 mg/m$^2$, calculated using the formula (dose in mg/m$^2$) = $(km)_i \times$ (dose in mg/kg), where $(km)_i$ is the conversion factor based on the body weight of the mouse [12, 16, 23]. Mice were weighed every 1–3 days, and toxic effects and mortality monitored up to 1 month after treatment to determine the $LD_{10}$ and $LD_{50}$ [26].

*Cell culture reagents and chemicals.* Cell culture media, sera, and components for STV were obtained from Grand Island Biological Company, Grand Island, NY. Bovine insulin was purchased from California Biochemical Co., La Jolla, CA, and penicillin and streptomycin from Eli Lilly and Co., Indianapolis, IN. Ficoll-Paque for L1210 cell separation was obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

## Results

*In vivo bioassays*

The cancericidal efficacy of PTT.119 was determined by assessing the in vivo tumorigenicity of cells treated in vitro with the new synthetic tripeptide. Single-cell suspensions of the mouse mammary tumor virus-induced MJY-alpha mammary tumor, the spontaneously arising B16 melanoma, and the MCA-induced L1210 leukemia were exposed to 1–50 μg PTT.119 for 0.25–4 h. Cells were then washed and either injected into recipient syngeneic mice or maintained in in vitro cultures. Immediately following PTT.119 treatment no significant decreases in MJY-alpha and B16 tumor cell viabilities were observed; compared with untreated controls or cells treated with solvent, 90%–100% of the cells from every treatment schedule excluded trypan blue. L1210 leukemia cells were more susceptible to PTT.119, and tumor cell viabilities declined appreciably after exposure to tripeptide concentrations of 25 μg/ml for longer than 1 h, or 10 μg/ml for at least 4 h.

Bioassays of PTT.119-treated tumor cells demonstrated that exposures as short as 15 min caused reductions in the tumorigenicity of the cells ranging from inhibition to complete elimination of tumor growth. The data presented in Fig. 1 show that the L1210 leukemia cells were the most susceptible to the cancericidal activity of the tripeptide. Concentrations as low as 5 and 10 μg PTT.119/ml effectively abrogated the leukemogenicity of the L1210 cells, and all recipient mice receiving L1210 cells treated with 5 μg/ml for 2 h, or 10 μg/ml for 30 min or longer, remained free of tumor. The synthetic tripeptide effectively reduced the population of L1210 cells capable of proliferation to zero, since in our laboratory the

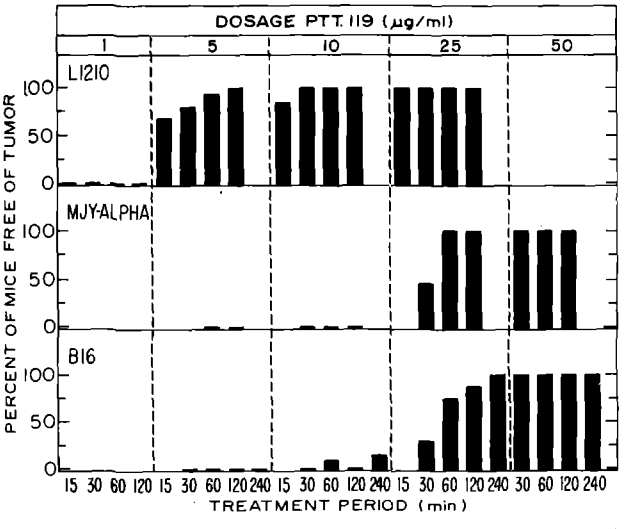


**Fig. 1.** In vivo tumorigenicity of L1210 leukemia, MJY-alpha mammary tumor, and B16 melanoma treated with 1–50 μg PTT.119/ml. Tumor cells were treated for 15 min to 4 h, washed, and $1 \times 10^6$ grafted into syngeneic hosts. Percentage of tumor-free recipients was determined on days 45 (L1210), 80 (MJY-alpha), and 85 (B16) after implantation. Values represents average of two experiments each performed with groups of 10–15 mice

**Table 1.** Mean survival time (MST) of syngeneic recipients of PTT.119-treated tumor cells

| Tumor cell[a] | PTT.119 (μg/ml) | MST (days) | | | | |
|---|---|---|---|---|---|---|
| | | Treatment period (min) | | | | |
| | | 15 | 30 | 60 | 120 | 240 |
| L1210 leukemia | 0 | 10 | 10 | 10 | 10 | – |
| | 1 | 10 | 10 | 11 | 13 | – |
| | 5 | 13† | 21†* | 32†* | TF[b] | – |
| | 10 | 8† | TF | TF | TF | – |
| | 25 | TF | TF | TF | TF | – |
| MJY-alpha mammary tumor | 0 | – | 42 | 42 | 42 | – |
| | 5 | – | – | 52* | 43 | – |
| | 10 | – | 44 | 46 | 61* | – |
| | 25 | – | 56†* | TF | TF | – |
| | 50 | – | TF | TF | TF | – |
| B-16 melanoma | 0 | – | 28 | 28 | 28 | 28 |
| | 5 | – | – | 31 | 33* | 41* |
| | 10 | – | 35* | 35†* | 35* | 45†* |
| | 25 | – | 52†* | 68†* | 65†* | TF |
| | 50 | – | TF | TF | TF | TF |

[a] Tumor cells were treated with PTT.119 in vitro, washed and implanted into syngeneic hosts. Numbers of surviving animals were quantitated every 2 days. All values are MST of animals who died of tumor and each represents the average of two experiments each with groups of 10–15 mice

[b] TF, tumor-free

* $P < 0.001$

† Group also contained tumor-free animals

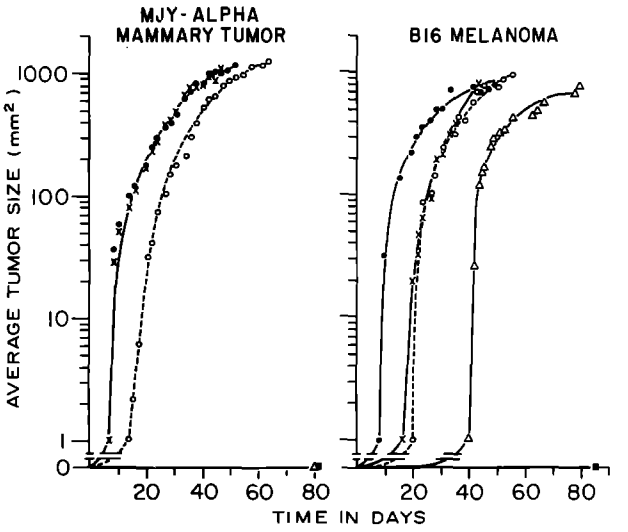


**Fig. 2.** Average tumor size of MJY-alpha mammary tumor cells or B16 melanoma implanted SC into BALB/c and C57BL/6 mice, respectively, as a function of time after implantation. Tumor cells ($10^6$) were treated for 2 h with 0 (●———●), 5 (×———×), 10 (○– – –○), 25 (△———△), and 50 (■– – –■) μg PTT.119/ml, washed, and grafted. Tumor areas were determined and the values given represent the means of tumor sizes in 25–40 animals per group

$LD_{100}$ of this tumor system is a single cell in $DBA/_2$ or $BDf_1$ hosts. To ascertain whether the recipients' immune responses were elicited against the tripeptide-treated leukemia cells, $BDf_1$ mice which had survived unsuccessful grafts of PTT.19-treated L1210 leukemia cells for 45 days were challenged with a second graft of 1,000 untreated L1210 cells. Compared with control hosts, there were no differences in the leukemogenesis of L1210 cells in these recipients. All mice died of tumor on days 12 and 13, demonstrating that implantation of L1210 cells treated with tripeptide did not protect the hosts against future grafts.

Loss of tumorigenicity of MJY-alpha mammary tumor and B16 melanoma tumor cells was also apparent following tripeptide treatment (Fig. 1). In both tumor systems, complete absence of tumor growth was observed in recipients of $10^6$ cells treated with PTT.119 concentrations of 25 μg/ml or higher for 30–240 min. Exposure to PTT.119 reduced the viable population of MJY-alpha and B16 tumor cells by at least 99.9%, since their $LD_{100}$ doses are 100 viable cells. At lower PTT.119 dosages where complete elimination of L1210 leukemia, MJY-alpha mammary tumor and B16 melanoma tumorigenicity were unattainable significant extensions of the mean survival time (MST) of the recipient mice were observed (Table 1). $BDf_1$ mice receiving L1210 leukemia cells treated with 5 μg PTT.119 for 30 or 60 min had an ILS of 110% and 220%, respectively. Similarly, the MSTs of BALB/c mice that received implants of $10^6$ MJY-alpha mammary tumor cells or C57BL/6 mice receiving $10^6$ B16 melanoma cells treated with 5–25 μg PTT.119/ml were extended significantly beyond the 42 or 28 days, respectively, observed in the control hosts.

Assessment of tumor growth rates of SC-implanted MJY-alpha mammary tumor and B16 melanoma cells revealed increases in the initial lag period between inoculation of tumor cells and presence of palpable tumors. In both solid tumor model systems the rate of tumor appearance was inversely related to the dosage of PTT.119 exposure (Fig. 2). However, the tripeptide did not alter tumor progression once the graft was established; tumor growth rates of untreated and PTT.119-treated MJY-alpha and B16 cells were identical, and the maximum size of the tumor at the time of death of the hosts remained unchanged. This indicated that the tripeptide did not alter the kinetics of tumor cell proliferation, nor did it appear to be selectively cytotoxic for one subpopulation of tumor cells.

*Sensitivitiy of mice to PTT.119*

To assess whether the effective cancericidal concentrations of PTT.119 found in the bioassays were attainable in vivo, mice were given a single IP inoculation of the tripeptide. Demonstrable PTT.119 toxicity required administration of high concentrations of the synthetic tripeptide. All mice tolerated doses of 52.5 mg/m$^2$ (15 mg/kg) with no observable signs of discomfort or pathologies. $BDF_1$ males which have been previously shown to be very sensitive to alkylating agents tolerated 61.25 mg PTT.119/m$^2$ (17.5 mg/kg; $LD_{10}$), whereas this dose increased to 74.6 mg/m$^2$ (21.3 mg/kg) in AKR females. The $LD_{50}$ for male $BDF_1$ mice was 81.6 mg/m$^2$ (23.3 mg/kg) and an $LD_{50}$ of 93.5 mg/m$^2$ (27 mg/kg) was recorded for female AKR mice.

*In vitro survival of PTT.119-treated tumor cells*

The reversibility of the cancericidal activity of PTT.119 on L1210 leukemia, MJY-alpha mammary tumor, and B16 melanoma cells was also monitored in the nonhostile tissue culture environment in parallel to the in vivo bioassays.

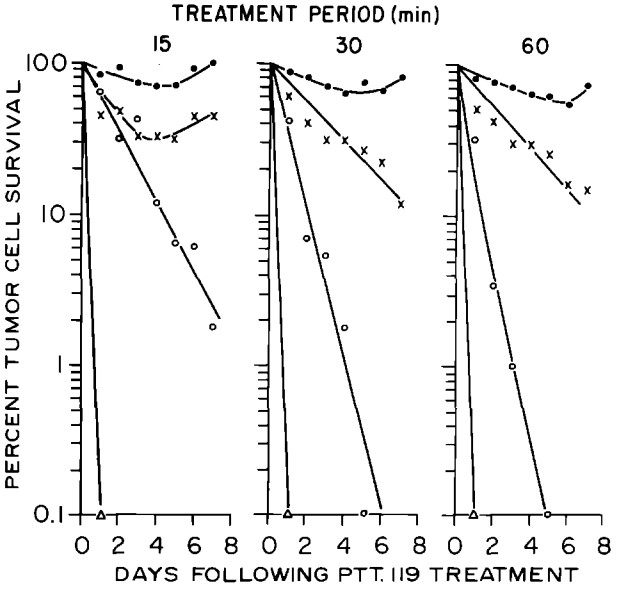


**Fig. 3.** In vitro viability of L1210 leukemia cells following 15, 30, and 60 min treatment with 1 (●———●), 5 (×———×), 10 (○———○), and 25 (△———△) μg PTT.119/ml. L1210 cell suspensions contained $1 \times 10^6$ cells at the time of treatment. Viability was determined for 7 consecutive days. Data are the means of at least 40 evaluations; standard deviations were less than 11%

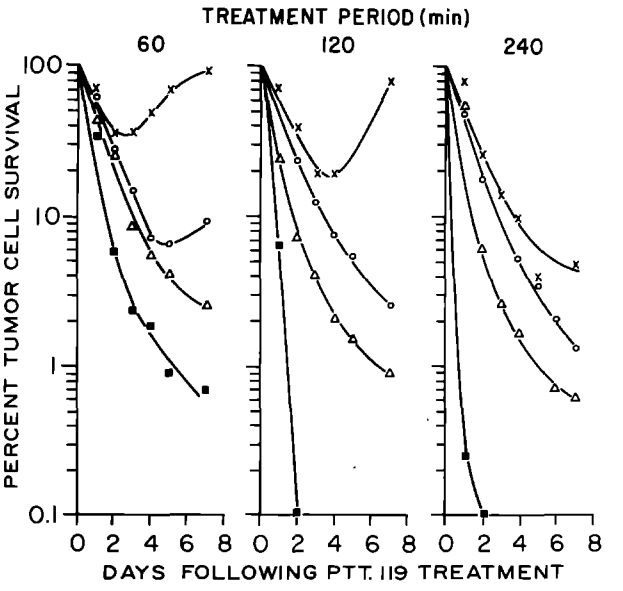


**Fig. 5.** In vitro viability of B16 melanoma cells following 60-, 120-, and 240-min exposure to 5 (×———×), 10 (○———○), 25 (△———△), and 50 (■———■) μg PTT.119/ml. B16 cells were treated at a concentration of $1 \times 10^6$, washed, and seeded at $2.2 \times 10^5$ cells/2 $cm^2$, and viability determined for 7 consecutive days. Data represent means of at least 20 evaluations; standard deviations were less than 13%

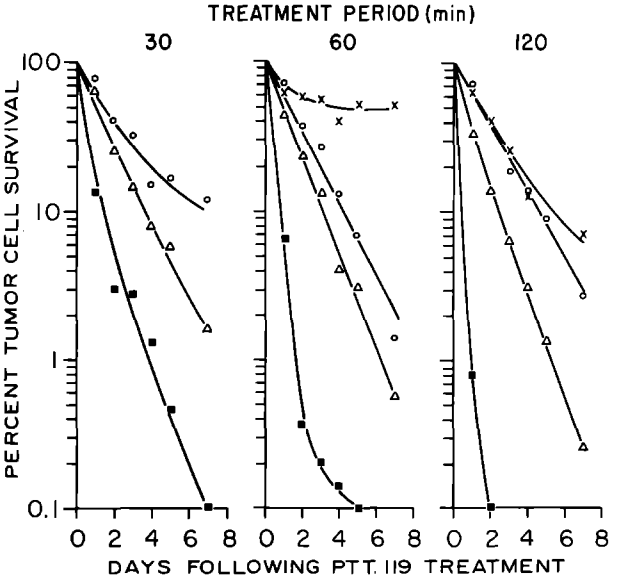


**Fig. 4.** In vitro viability of MJY-alpha mammary tumor cells following 30-, 60-, and 120-min exposure to PTT.119 at concentrations of 5 (×———×), 10 (○———○), 25 (△———△), and 50 (■———■) μg/ml. Cells were treated at $1 \times 10^6$ cells/ml and were then seeded at $2 \times 10^5$ cells/2 $cm^2$ following washing. Viability was determined daily for 7 consecutive days. Data represent means of at least 25 evaluations; standard deviations were less than 13%

Significant reductions in tumor cell survival were observed in vitro when the L1210 leukemia (Fig. 3), MJY-alpha mammary tumor (Fig. 4), and B16 melanoma (Fig. 5) tumor cells were observed 24 h following tripeptide exposure and for the next 6 days.

Longitudinal determination of tumor cell viabilities demonstrated that cytolysis continued to increase days after PTT.119 treatment and also revealed the repopulation of tumor cell cultures treated with low concentrations of the tripeptide. A step-wise gradation in cytolysis with increasing concentrations of PTT.119 was observed in the in vitro survival curves of the three tumor cell systems. At any dose of PTT.119, tumor cell survival also decreased when the treatment periods were lengthened, although the L1210, MJY-alpha, and B16 cells were refractory to these duration-related changes at several low concentrations of the tripeptide. These apparent refractory phases were observed at concentrations of 10 and 25 μg PTT.119/ml in MJY-alpha and B16 cells and at 1 and 5 μg tripeptide/ml in L1210 leukemia cultures.

## Discussion

Our in vitro and in vivo bioassays have demonstrated that the new synthetic tripeptide PTT.119, containing an amino acid analog, *p*-fluoro-L-phenylalanine at the amino-group and L-methionine ethylester at the carboxyl end of *m*-sarcolysin, has strong cancericidal potency against three virulent tumor models. Brief exposures as short as 15 min to 2 h to PTT.119 were sufficient to reduce the viable cell population of MJY-alpha mammary tumor, B16 melanoma and L1210 leukemia cells by 99.9%–100%. The in vitro doubling times of these tumor cells were approximately 16–21 h and our use of asynchronous cultures ensured that tumor cell populations would be comprised of cells in all phases of replication. As a consequence, th tripeptide-induced abrogation of tumor cell proliferation and tumorigenicity was independent of the replicative phase of the tumor cells at the time of exposure. PTT.119's lack of cycle specificity is characteristic of other alkylating compounds which exert their greatest effects as the

drug-containing cells enter into the S phase [6]. This suggests that when PTT.119 is administered in vivo all neoplasic cells exposed to the tripeptide would be at risk.

The cancericidal activity of PTT.119 was solely attributable to its ability to directly inhibit tumor cell proliferation. Reductions in the numbers of viable tumor cells resulted in lengthening of the lag phases between implantation of PTT.119-treated cells and the appearance of measurable tumors. The irreversible nature of the interaction between the synthetic tripeptide and tumor cells was manifested by the significant increases in mean survival times of the hosts and the complete prevention of tumor growth. The tripeptide did not appear to affect the rate of cellular proliferation or tumor progression. PTT.119 also did not alter the susceptibility of the host to the lethal tumor, nor did it appear to change the antigenicity of the treated tumor cells.

PTT.119 was more cancericidal to L1210 leukemia cells than to either of the two tumor cell lines derived from an adenocarcinoma and a melanoma. The enhanced susceptibility of leukemic cells was evident when tumor cell survival was determined either in vivo or by in vitro cultivation of PTT.119-treated cells. The differences were not a reflection of fluctuations of cellular proliferation since the generation times of the three tumor cell types were similar [10, 27]. Neoplastic cells of different histological origins have inherent degrees of sensitivity or resistance to chemotherapeutic agents, including bifunctional alkylators. Lethality of these latter class of agents is also related to general metabolic parameters of the cells, including uptake/transport [4, 5, 24, 28, 29], intracellular retention/efflux [2, 15, 19], and rate of repair of lesions resulting from alkylation of cellular components, particularly DNA [22, 25, 27, 28]. The role of these metabolic factors in PTT.119 cytolytic activity is curently under investigation.

In vitro determination of tumor cell viability following PTT.119 treatment was effective in predicting in vivo tumorigenicity of the cell population. The best correlation occurred when PTT.119 reduced tumor cell survival in vitro to less than 1% of control within the 7-day period; such tumor cells were unable to produce tumors in mice. Minimal decreases in tumor cell survival or repopulation of the in vitro cultures after PTT.119 exposure correlated with successful tumor grafts. No clear association between viability in vitro and tumorigenicity could be established between these two extremes.

The efficacy of this cancericidal compound, PTT.119, as a chemotherapeutic agent is currently under evaluation. Our studies of the sensitivity of mice to PTT.119 have indicated that the tripeptide has a lower toxicity than other alkylators containing a bis(2-chloroethyl)amino-L-phenylalanine moiety. The tripeptide had no observable toxic effects at doses of 52.5 mg/m$^2$ (78.8 $\mu M$/m$^2$), and the maximum tolerable doses of PTT.119 in male and female mice were 61.3–74.6 mg/m$^2$ (92–112 $\mu M$/m$^2$). These doses are higher than the historical values for L-phenylalanine mustard (L-PAM) in mice of the same strains; the $LD_{10}$ and $LD_{50}$ are 52.4 $\mu M$/m$^2$ and 73.4 $\mu M$/m$^2$, respectively [13, 18, 26]. This is particularly significant since our previous study [31] demonstrated that PTT.119 was 1.5- to 9-fold more cancericidal than L-PAM when tested against in vitro tumor systems, and suggests that the in vivo therapeutic index of the tripeptide would also be greater.

Calculation of the hypthetical in vivo doses of PTT.119 which would have to be administered to obtain the levels of tripeptide equivalent to those demonstrated to be effective in vitro suggest that concentrations of 5–25 μg PTT.119/ml could be achieved [9]. Assuming PTT.119 is uniformly distributed in the plasma fraction, as has been demonstrated for other bis-(2-chloroethyl)amine derivatives [8, 27], an adult mouse whose blood volume is approximately 280 ml/m$^2$ [7, 17] would require minimum dosages of 0.77–3.85 mg/m$^2$. Using the standard prediction that only 10% of the administered drug would reach the plasma, the doses needed to achieve plasma levels of 5–25 μg PTT.119/ml would be 7.7–38.5 mg/m$^2$ (11.6–57.8 $\mu M$/m$^2$). These levels are below the well tolerated dose observed and are substantially less than the maximum tolerable dose for mice. These doses are thus realistic in terms of the concentrations that should be attainable in vivo and indicate that PTT.119 should be an effective chemotherapeutic agent when administered to a tumor-bearing host.

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