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PTT.119, *p*-F-Phe-*m*-BIS-(2-CHLOROETHYL)AMINO-L-Phe-Met ETHOXY HCl, A NEW CHEMOTHERAPEUTIC AGENT ACTIVE AGAINST DRUG-RESISTANT TUMOR CELL LINES

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Abstract—PTT.119 [*p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met ethoxy HCl], a new synthetic tripeptide, was highly effective against the L-phenylalanine mustard (L-PAM) resistant (L1210/L-PAM and P388/L-PAM) tumor lines, as well as the sensitive L1210 leukemia. Cytolytic activity of PTT.119 against all three leukemias was significantly greater than equimolar doses of L-PAM. These *in vitro* results paralleled the significant increases in mean survival times of hosts and, in some cases, abrogations of tumor formation observed in the *in vivo* bioassays of PTT.119-treated L1210 and L1210/L-PAM cells. Dose–response studies failed to demonstrate cross-resistance to the tripeptide by L-PAM resistant cells. Doses of PTT.119 required to reduce the viable fraction by 50% (tissue culture dose 50, TCD_{50}) or 100% (TCD_{100}) were 1.3- to 3-fold lower for the L-PAM resistant cells than for the L1210 leukemia. In comparison, L-PAM was unable to completely eliminate cell survival; 0.2 to 3% of the cells in all three leukemias remained viable even at doses of 75 and 163 μM. In similar studies, L1210 leukemia cells made resistant to methotrexate (L1210 MTX) and cisplatin (L1210DDP) were also completely susceptible to PTT.119; TCD_{50} values of the two resistant lines were 1.94 μM for L1210 MTX and 0.525 μM for L1210DDP compared to 2.38 μM for the susceptible parent L1210S leukemia. Continuous low-dose PTT.119 treatment of MJY-alpha mammary tumor cells for 8 months and exposure of L1210 leukemia to escalating levels of tripeptide for over 100 passages failed to select or induce drug-resistant phenotypes in either cell line. PTT.119 appears to be a poor mutagen and is unlikely to readily increase the probability of drug-resistant mutants in the tumor cell populations.

A significant problem in cancer chemotherapy is the appearance of drug-resistant phenotypes in a tumor cell population which was sensitive at the onset of treatment [1]. Consequently, the efficacy of a chemotherapeutic drug is measured not only by its cytolytic capabilities against susceptible cells but also by its activity against tumor cell populations resistant to other agents, and by its ability to induce drug-resistant phenotypes [1, 2]. We are currently evaluating the chemotherapeutic potential of PTT.119 [*p*-F-Phe-*m*-bis-(2-chloroethyl)amino-L-Phe-Met ethoxy HCl], a synthetic derivative [3]. This new agent containing the bis-(2-chloroethyl)amino bifunctional alkylating group coupled to three amino acid residues was shown to be effective against human, primate and rodent leukemias and lymphomas and also reduces survival of mammary tumor and melanoma cells [4, 5]. The cytopathology, degree of susceptibility, and kinetics of tumor cell cytolysis depend on the dose and vary greatly with tumor cell type [6]. The data suggested that there were inherent differences in the biochemical interactions of PTT.119 and/or its alkylating intermediates with cellular components.

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The investigations presented here assess the efficacy of PTT.119 against leukemia cells resistant to L-phenylalanine mustard, methotrexate and cisplatin. Cross-resistance to PTT.119 was not observed and, in fact, three of the cell lines were collaterally sensitive to the tripeptide [7]. The mutagenic potential of the tripeptide was also determined by continuous low-dose exposure of tumor cells to PTT.119. Treatment for 8–12 months of both mammary tumor and leukemia cells failed to select for or induce PTT.119 resistant phenotypes. The results indicate that PTT.119, in addition to being highly active against both sensitive and resistant cells, is also a poor mutagen.

MATERIALS AND METHODS

Cell cultures. Three ascitic leukemia cell lines maintained in BDf_1 (C57BL/6 × DBA/2) mice were used in this study: L1210 leukemia and two L-PAM resistant lines, L1210/L-PAM and P388/L-PAM leukemia [8]. Both resistant lines were made available for these studies by the late Dr. Frank M. Schabel, Jr., Southern Research Institute, Birmingham, AL. The resistant lines were generated *in vivo* by intraperitoneal injections of L-PAM (5–10 mg/kg) 2 days following serial intraperitoneal passage. Repetitive transplantation and L-PAM treatment yielded cells with stable resistance to L-PAM within 10–15 passages. Leukemia cells were harvested and separated

on Ficoll–Hypaque gradients as previously described [4]. Leukemia cell viability was greater than 98% as determined by trypan blue exclusion. Growth medium for primary suspension cultures was RPMI-1630 with 10% fetal bovine serum (FBS)† and antibiotics [4]. Long-term L1210 cultures were grown in RPML-1640 with 18% FBS and antibiotics. Cell culture-adapted lines of L1210 leukemia (the L1210S, L1210DDP and L1210 MTX) were maintained in logarithmic growth phase in Fischer's medium with 10% horse serum [9]. The resistant L1210DDP and L1210 MTX lines were developed by continuous exposure to weekly increases of drug [10, 11]. The resultant cell lines were then cloned in soft agar with their respective drug. L1210DDP cells were 20-fold more resistant to cisplatin than the susceptible L1210S in clonogenic assays. Likewise, L1210 MTX cells were 100 times more resistant to methotrexate. Resistance exhibited by both cell lines was stable, and each resistant cell line was grown in the absence of the drug. The epithelial BALB/cfC3H mammary tumor cell line, MJY-alpha, was grown in RPMI-1640 with 18% FBS, 10 μM bovine insulin and antibiotics [12]. All cell cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air.

Chemotherapeutic agents. The tripeptide, PTT.119 (*p*-F-phenylalanyl-*m*-bis-(2-chloroethyl)amino-L-phenylalanyl-methionine ethoxy hydrochloride) was provided by Proter S.p.A. (patented) [3]. PTT.119 was dissolved at 15 mM in *N*,*N*-dimethylacetamide, Tween 80 and propylene glycol (1:1:2). Stock solutions of 0.15 or 1.5 mM were made by dilution in aqueous 50% propylene glycol prior to use. L-PAM (Alkeran; Burroughs-Wellcome) was dissolved at 1.5 mM in the same solvent system prior to use. Stock solutions of the drugs were added directly to culture media containing serum to obtain final concentrations of 15 nM to 75 μM. Control cultures received identical volumes of solvent without drugs.

Treatment of leukemia cell suspensions. PTT.119 and L-PAM treatments were carried out at 37° in the appropriate complete growth media containing 10% or 18% sera as previously described [4]. Briefly, suspensions of 10^6 L1210, L1210/L-PAM and P388/L-PAM cells were exposed for 15, 30 or 60 min to 3.75, 7.5 and 15 μM PTT.119 or L-PAM. Cells were then pelleted at 200 *g* for 9 min at 5°, washed twice, and resuspended in RPMI-1630 medium with 10% FBS for *in vitro* cultures. Cells used for the *in vivo* bioassays were resuspended in serum-free RPMI-1630 after the final wash. Cells (10^6/ml) were also treated with 1.5 to 163 μM PTT.119 or L-PAM for 24 hr. All culture media were changed daily by gently pelleting the cells and exchanging 70% of the spent supernatant fraction.

In vitro *tumor cell survival*. Leukemia cell viability was ascertained immediately following and daily for 7 days after pulse exposure to PTT.119 or L-PAM [4]. The viable fraction of cells exposed to drugs for 24 hr was determined once at the end of treatment. Numbers of viable cells excluding trypan blue were enumerated by counting all fields in a haemocytometer. Means from twenty to forty evaluations were obtained for each time point, and the percent viable cells in treated cultures was determined by direct comparison with parallel untreated or solvent-treated cells. Since there were no significant differences in the viability of cells treated with solvent or media with no additives, these two experimental controls were pooled.

Bioassay of tumorigenicity. Bioassays were performed as described previously on suspensions of PTT.119 or L-PAM treated and control leukemia cells [5]. Hybrid female BDf_1 (C57BL/6 × DBA/2) mice weighing 20–26 g received 10^6 cells by intraperitoneal injections. Mice were monitored daily throughout the 45-day examination period and were autopsied at the time of death.

Development of cells resistant to PTT.119. Long-term cultures of L1210 leukemia and MJY-alpha mammary tumor cells were continuously exposed to low doses of PTT.119. L1210 cells passaged at 10^5 cells/ml every 3–14 days received 15–750 nM PTT.119 at the time of seeding. MJY-alpha mammary tumor cells were treated with 0.15 to 15 μM PTT.119 when subcultured every 7–21 days, and daily when the medium was changed.

Cells grown in the presence of PTT.119 were periodically tested for susceptibility to 1.5 to 75 μM tripeptide. L1210 cell suspensions were treated for 24 hr as previously described and viable cells enumerated using trypan blue. MJY-alpha cells from treated and parallel untreated control cultures were seeded at 2×10^5 cells/2 cm^2 well using media with the appropriate PTT.119 concentration. One day later confluent layers were tested for resistance to five elevated doses of tripeptide. After 24 hr, cells were released from substrate with 0.5 ml saline-trypsin-versene [12], and diluted 2- to 10-fold with trypan blue for enumeration.

Cell culture reagents and chemicals. Cell culture media were purchased from the Grand Island Biological Co., Grand Island, NY. FBS was obtained from the Armour Pharmaceutical Co., Tarrytown, NY. Bovine insulin was purchased from the California Biochemical Co., La Jolla, CA, and penicillin and streptomycin were from Eli Lilly & Co., Indianapolis, IN. Ficol-Paque for cell separation was obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

RESULTS

PTT.119 activity against L-PAM resistant cell lines. Exposure of primary cultures of two L-PAM resistant lines, L1210/L-PAM and P388/L-PAM, and the sensitive L1210 leukemia to PTT.119 significantly reduced the fractions of surviving cells. Treatment with 3.75 μM PTT.119 for as short a time as 30 or 60 min reduced the percentages of viable L1210 and L1210/L-PAM cells to 40–50% and the resistant P388/L-PAM leukemia cells to 0–1% within 4–5 days after exposure. In contrast, 80–90% of the L1210, L1210/L-PAM and P388/L-PAM cells remained viable following either a 30- or 60-min exposure to 3.75 μM L-PAM. Both resistant lines were markedly more susceptible to 7.5 μM PTT.119 than the L1210

† Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); FBS, fetal bovine serum; LD_{100}, lethal dose 100; L-PAM, L-phenylalanine mustard; m.L.SL, *m*-L-sarcolysin; MST, mean survival time; MTX, methotrexate; TCD_{50}, tissue culture dose 50; and TCD_{100}, tissue culture dose 100.

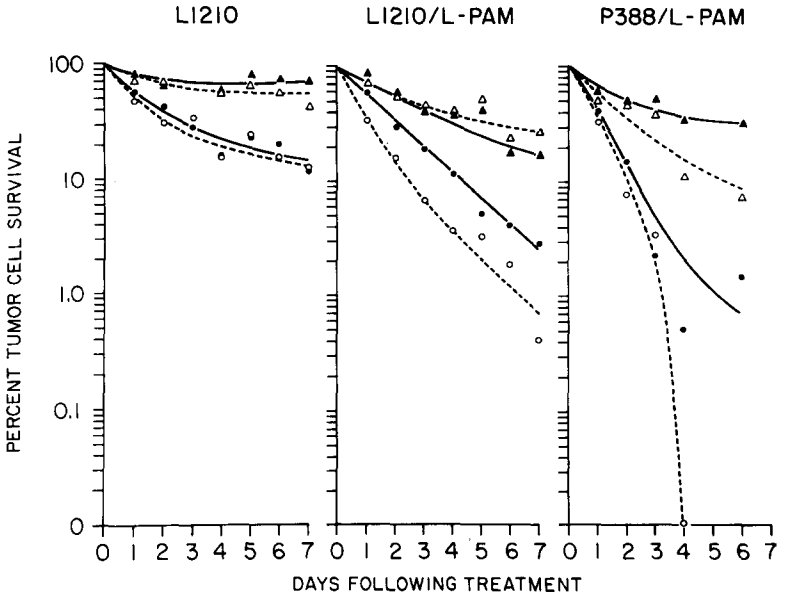

Fig. 1. Survival of L1210, L1210 L-PAM and P388/L-PAM leukemia suspensions following exposure to 7.5 μM PTT.119 for 30 (●——●) or 60 (○- - -○) min, or L-PAM for 30 (▲——▲) or 60 (△- - -△) min. Viability data are the means of twenty evaluations with standard deviations of less than 10%, and are representative of values obtained from two to four other experiments.

leukemia (Fig. 1). The increased cytotoxicity was observed when drug exposure was for either 30 or 60 min and was demonstrable throughout the 6- to 7-day period following PTT.119 treatment. At the end of the examination period, the surviving fractions of L1210/L-PAM and 388/L-PAM cells were reduced to 0.8 to 3% and 0 to 0.5%, respectively, compared to the 15% for the L1210 cells.

For comparison, the three leukemia lines were treated in parallel with an equimolar dose of L-PAM. Data depicted in Fig. 1 show that PTT.119 was always more effective; L-PAM was only able to reduce the surviving cell populations of L1210 to 60–80%, L1210/L-PAM to 3–20% and P388/L-PAM cells to 7–40%. Despite *in vivo* resistance of L1210/L-PAM and P388/L-PAM tumors to L-PAM, a sizable portion of the tumor cell population remained sensitive to the alkylating agent.

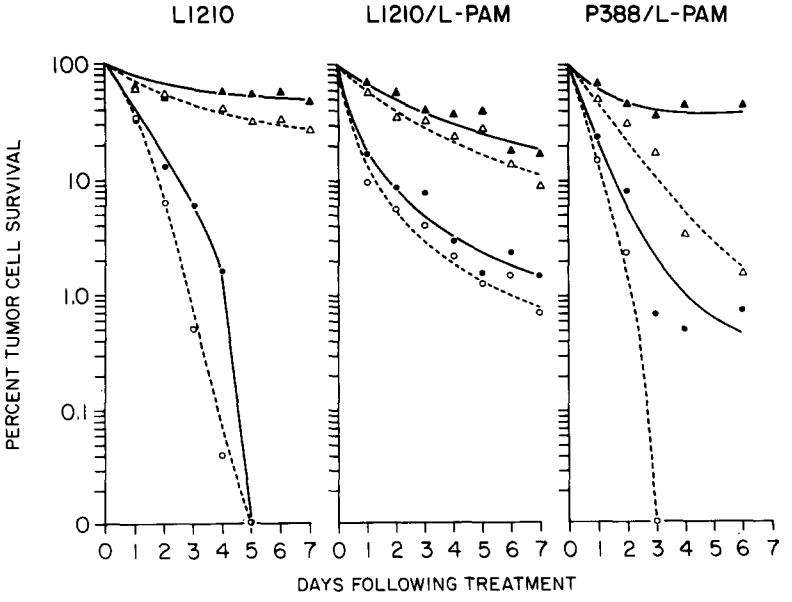

Fig. 2. Survival of L1210, L1210/L-PAM and P388/L-PAM leukemia suspensions following exposure to 15 μM PTT.119 for 30 (●——●) or 60 (○- - -○) min, or L-PAM for 30 (▲——▲) or 60 (△- - -△) min. Viability data are the means of twenty evaluations with standard deviations of less than 10%, and are representative of values obtained from two to four other experiments.

Increasing the dosage of PTT.119 to 15 μM resulted in further reductions in cell survivals and also increased the rate of cell cytolysis of all three leukemias (Fig. 2). The most significant change was observed with the sensitive L1210 leukemia; exposure to 15 μM PTT.119 for either 30 or 60 min completely eliminated viable cells within 5 days of treatment. Total elimination of viable P388/L-PAM cells was only accomplished by exposure to PTT.119 for 60 min. PTT.119 at 15 μM reduced the viable fraction of L1210/L-PAM to 0.8 to 1.5% with either a 30 or 60 min pulse but did not completely reduce the cell population to zero. These data indicate that, at this PTT.119 concentration, the L1210 cell line was more susceptible to the tripeptide cytotoxicity than the two L-PAM resistant lines.

Doubling the L-PAM concentration to 15 μM increased its cytolytic activity against the sensitive L1210 cells almost 2-fold (Figs. 1 and 2). Significant decreases in L1210/L-PAM and P388/L-PAM cell viabilities were also observed when treatment was carried out for 60 min although no changes in cell survival were observed between 7.5 and 15 μM L-PAM when treatment was for 30 min. In addition, L-PAM at any dosage was unable to eliminate all viable cells in any of the three leukemia cell lines.

In vivo *bioassays of PTT.119 and L-PAM treated cells*. The tumorigenicity of L1210 and L1210/L-PAM leukemia cells was determined following *in vitro* treatment with PTT.119. Significant increases in the mean survival times (MST) were observed in recipients which did succumb to PTT.119-treated L1210 or L1210/L-PAM tumor cells (Table 1). BDf_1 mice free of tumor were also present in every group receiving leukemia cells treated with PTT.119 but not with L-PAM. Reduction of tumorigenicity of L1210/L-PAM cells increased as the concentration of PTT.119 and the duration of exposure increased.

Table 1. Mean survival time (MST) of BDf_1 mice receiving leukemia cells treated with PTT.119 or L-PAM*

Tumor cell	Drug (μM)	MST in days: Treatment period (min) 15	30	60
L1210	0	10	10	10
	7.5 PTT.119		21†‡	32†‡
	15 PTT.119	8†	TF§	TF
L1210/L-PAM	0	13	13	13
	7.5 PTT.119		19†‡	TF
	15 PTT.119	16†‖	18†‖	TF
	7.5 L-PAM		15	15†
	15 L-PAM	14	16	TF

* Tumor cells (10^6/ml) were treated *in vitro*, washed, and implanted into syngeneic hosts. Numbers of surviving animals were determined daily. All values are MST of animals who die of tumor and represent the average of two experiments each containing groups of ten to fifteen mice.

† Group also contained tumor-free animals.

‡ $P < 0.001$.

§ Tumor free.

‖ $P < 0.01$.

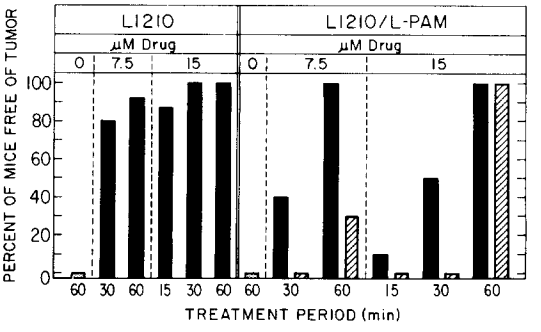

Fig. 3. *In vivo* tumorigenicity of L1210 and L1210/L-PAM leukemia treated with solvent (stippled bar), 7.5 or 15 μM PTT.119 (solid bar), or L-PAM (striped bar). Tumor cells were treated for 15, 30 or 60 min, washed, and 10^6 cells injected i.p. into BDf_1 hosts. The number of tumor-free mice was determined 45 days after implantation. Values represent averages of two experiments each containing groups of ten to fifteen mice.

Abrogation of L1210/L-PAM tumors was obtained by treatment with either a 7.5 or 15 μM concentration of tripeptide, and all animals receiving cells treated for 60 min were free of tumor at the end of the 45-day examination period. These results indicate that PTT.119 effectively reduced the population of treated leukemia cells capable of proliferation in the tumor-free mice to 0 since the lethal dose 100 (LD_{100}) is a single cell. In comparison, *in vitro* treatment of L1210/L-PAM leukemia with L-PAM resulted in diminution of tumorigenicity only when cells were treated with 7.5 or 15 μM L-PAM for 60 min; all mice receiving cells treated with either dose of L-PAM for 15 or 30 min died of tumor (Fig. 3) with no observable increase in their MST (Table 1). The overall efficacy of PTT.119 again appeared to be slightly greater against the susceptible L1210 leukemia than the L-PAM resistant, L1210/L-PAM. The number of mice free of tumor in every group of animals receiving L1210 cells treated with 7.5 or 15 μM PTT.119 for 15, 30, or 60 min was 80–100%. However, since PTT.119 was able to effectively abrogate L1210/L-PAM tumor growth at all doses, it is apparent that the possible resistance of these cells to the tripeptide was marginal.

Dose response of L-PAM resistant leukemia cells to PTT.119. Treatment of the L-PAM resistant L1210/L-PAM and P388/L-PAM leukemias with increasing doses of PTT.119 for 24 hr revealed that these two tumor lines were more susceptible to the tripeptide than the sensitive L1210 leukemia (Fig. 4). As summarized in Table 2, PTT.119 levels which decreased resistant cell survival by 50% (TCD_{50}) or totally eliminated viable cells (TCD_{100}) were markedly lower compared to those for L1210 cells.

Parallel treatment of the three tumor cell lines with L-PAM demonstrated significant differences between the efficacies of PTT.119 and L-PAM (Fig. 4). The cytolytic activity of PTT.119 was always greater than equimolar concentrations of L-PAM. In addition to this quantitative difference, a plateau was observed in the dose–response curves of L1210/L-PAM and P388/L-PAM leukemia treated with L-

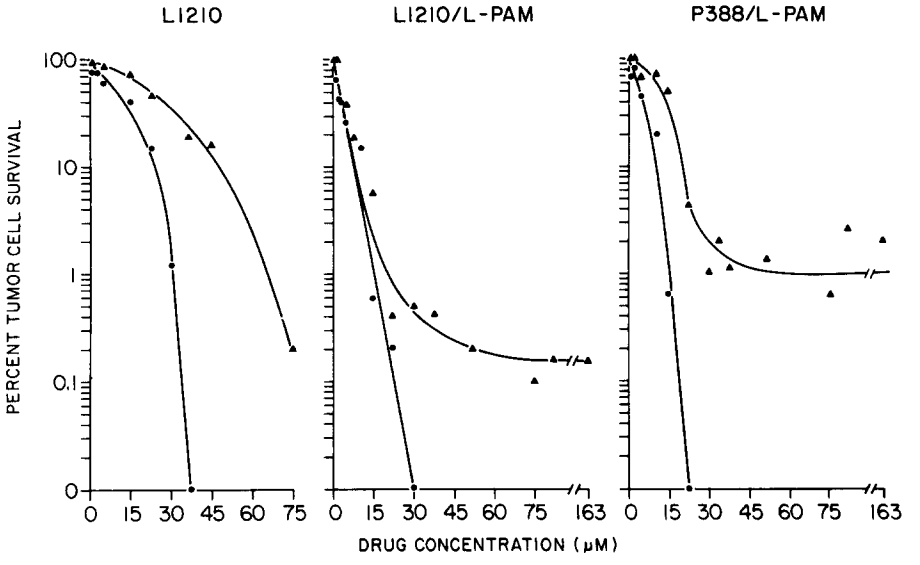

Fig. 4. Survival of L1210, L1210/L-PAM and P388/L-PAM leukemia suspensions following 24-hr exposure to PTT.119 (●——●) or L-PAM (▲—▲). Data are the means of thirty to forty evaluations obtained from three experiments.

PAM. A residual number of cells in each of the leukemia populations was unaffected by increasing the L-PAM concentration in excess of 30 μM. As a result, the survival of these L-PAM resistant lines could not be completely abrogated, and at 163 μM L-PAM approximately 0.2% of the L1210/L-PAM and 3% of the P388/L-PAM populations remained viable (Table 2). The results show that these tumor cell lines maintained in mice are a heterogeneous mixture of both L-PAM susceptible and resistant cells. However, the complete cytolysis of all L1210/L-PAM and P388/L-PAM cells by PTT.119 at a concentration similar to that used in the susceptible L1210 leukemia demonstrates the lack of any detectable cross-resistance to the tripeptide (Fig. 4; Table 2).

*Dose response of methotrexate and cisplatin resistant leukemia cells to PTT.*119. The cytolytic efficacy of PTT.119 against leukemic cell lines made resistant to the 2,4-diaminofolate antagonist methotrexate (L1210 MTX; TCD_{50} 0.5 μM MTX) and the alkylating agent *cis*-diamminedichloroplatinum (II) (L1210DDP; TCD_{50} 30 μM cisplatin) was examined and compared to the parental L1210S (TCD_{50} is 5 nM for MTX and 1.5 μM for cisplatin). Viability of both resistant lines was decreased in a dose-dependent manner following 24-hr exposure to PTT.119 (Fig. 5). The L1210 MTX line was more susceptible in comparison to the L1210S leukemia; the TCD_{50} and TCD_{100} of L1210 MTX were 1.94 and 3.75 μM, respectively, compared to 2.38 μM (TCD_{50}) and 7.5 μM (TCD_{100}) for the L1210S. The L1210DDP line was also completely susceptible to the tripeptide although the dose–response curve indicated the existence of at least two subpopulations of leukemic cells with different sensitivities to PTT.119. Approximately 60% of the L1210DDP leukemic cells were highly susceptible to PTT.119 doses of 1 μM (TCD_{50} 0.525 μM) with the remaining population becoming sensitive at 4–15 μM. The small plateau in cell viability was consistently observed in every trial with the tripeptide. The data suggest that the cloned population of L1210DDP which displays a homogeneous response to cisplatin treatment is heterogenous with respect to its sensitivities to another alkylating agent.

Table 2. Cytolytic efficacy of PTT.119 and L-PAM*

	TCD_{50} (μM)		TCD_{100} (μM)	
Cell line	PTT.119	L-PAM	PTT.119	L-PAM
L1210	9.0	22.5	37.5	75.0†
L1210/L-PAM	3.0	3.8	30.0	163.0†
P388/L-PAM	5.0	11.0	22.5	163.0†

* L1210, L1210/L-PAM and P388/L-PAM cell suspensions (10^6 cells/ml) were treated for 24 hr, and the percentage of viable cells was compared to untreated cells and control cells receiving diluent. TCD_{50} and TCD_{100} Values represent the means of twenty-five to forty determinations. Viabilities of untreated and solvent-treated tumor cells were 98–100%.

† Cultures contained 0.2 to 3% viable cells at 24 hr.

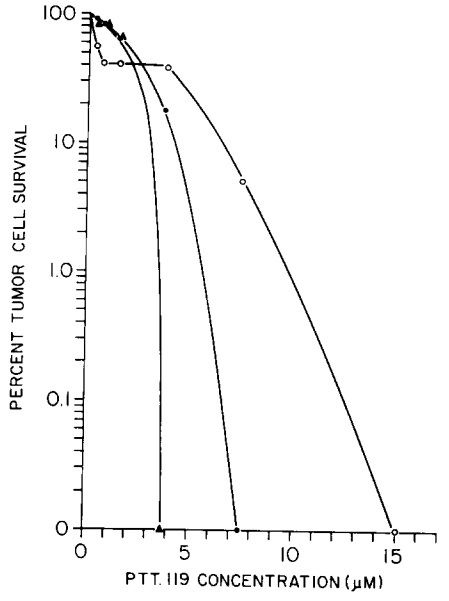

Fig. 5. Survival of L1210S (●——●), L1210DDP (○—○), and L1210 MTX (▲——▲) leukemia cell lines following 24-hr exposure to PTT.119. Data are the means of three experiments carried out in duplicate.

Development of PTT.119 resistant cell lines. The ability of PTT.119 to induce drug-resistant cells was examined by maintaining tumor cell lines in the presence of low concentrations of the tripeptide. The first series of these experiments involved continuous treatment of the susceptible MJY-alpha cells (TCD_{50} 3.75 μM) to 0.15, 1.5, 7.5 or 15 μM PTT.119. Monolayers grown in the presence of 15 μM PTT.119 did not survive beyond the second passage. MJY-alpha cells exposed to the three lower concentrations of PTT.119 were successfully maintained for 8 months. The growth rates of these treated cells (alpha-0.15, alpha-1.5, alpha-7.5) were diminished, resulting in their subcultures every 1.5 to 2 weeks compared to the weekly passage of the untreated parental cells. The susceptibility of alpha-0.15, alpha-1.5 and alpha-7.5 to PTT.119 was tested periodically by 24-hr exposure to six concentrations of the tripeptide (1.5, 3.75, 7.5, 15, 37.5 and 75 μM). No alterations in the dose–response curves of the three PTT.119-treated lines were observed compared to previously untreated MJY-alpha cells.

Long-term *in vitro* suspension cultures of L1210 leukemia cells have also been treated continuously with PTT.119 at 15, 37.5, 52.5, 75, 150, and 1500 nM. Cells initially treated with tripeptide doses of 37.5 nM or greater were all rendered nonviable within the first two or three subcultures. Two cell lines, L1210-T1 and L1210-T2, were maintained in 15 nM PTT.119 for 28 and 23 passages respectively. The dose response of L1210-T1 and L1210-T2 cells to 24-hr PTT.119 treatment was repetitively tested. An increase in resistance to PTT.119 at concentrations of 15 and 22.5 μM was observed in both lines between the 8th and 16th passages. At these two doses, PTT.119 cytotoxicity was found to be 20–30% lower compared to untreated L1210 cells. However, resistance was not stable and by the 20th passage the dose–response curves of L1210, L1210-T1 and L1210-T2 were identical.

Attempts have been made to induce an L1210 line resistant to PTT.119 by stepwise escalation of the tripeptide dosage. At the 29th passage of L1210-T1, the concentration of PTT.119 was increased to 37.5 nM. The dose response of L1210-T1 cells maintained at this higher PTT119 concentration for 50 passages was identical to the parental, sensitive L1210 line. Further escalation of the tripeptide concentration to 52.5, 75 and 150 nM has been successfully carried out over the past 12 months. L1210-T1 cells maintained in 150 nm PTT.119 still had a dose response to 1.5 to 75 μM tripeptide similar to that of the sensitive L1210 cells. After 100 passages in PTT.119, the L1210-T1 cells had a TCD_{50} of 8.3 μM and a TCD_{100} of 30 μM, which are slightly lower than the L1210 cells (Table 2). These studies with L1210 leukemia and MJY-alpha mammary tumor cells demonstrate the difficulty in inducing resistance to PTT.119 and strongly indicate that inclusion of carrier amino acids in the parental bis-(2-chloroethyl) amino bifunctional alkylating moiety rendered the tripeptide an extremely poor mutagen.

DISCUSSION

PTT.119, a new bifunctional alkylating agent, is highly cytotoxic against L1210, L1210/L-PAM and P388/L-PAM leukemia cell suspensions, and the L1210S, L1210DDP and L1210 MTX cell lines. We have hypothesized previously that the observed increase in PTT.119 cytolytic activity is related to the structural alteration of the parental *m*-L-sarcolysin (m.L.SL) molecule which contains the bis-(2-chloroethyl)amino bifunctional alkylating moiety [4]. The evidence suggests that linkage of Phe and Met in the L configuration of the amino- and carboxyl-groups of the Phe residue of m.L.SL alters and/or increases the transport of PTT.119 across the tumor cell membrane. This change in PTT.119 transport could account for the increased cytolytic efficacy of the tripeptide compared to m.L.SL or its structural isomer L-PAM observed in this investigation and in our previous study [4]. It has been shown that L-PAM-induced cytolysis is directly related to drug uptake which, in turn, is severely affected by the environmental mileu of the cells at the time of treatment [13–16]. The TCD_{100} of L1210 cells in Dulbecco's buffer was 7.0 μM L-PAM, whereas, in the presence of medium containing leucine and other amino acids, this concentration of L-PAM only reduced the viable population to 83%. We obtained similar levels of L1210 survival following exposure to medium containing 7.5 μM L-PAM; 75% of the L1210 cells excluded trypan blue 7 days after the 30-min treatment. In comparison, treatment of L1210 cells with 7.5 μM PTT.119 under identical conditions reduced the viable fraction to 15%. The data demonstrated that PTT.119 uptake and, hence, cytolytic activity were considerably less sensitive than L-PAM to the presence of exogenous amino acids transported by the two L-amino acid transport systems [13–17].

Evidence that PTT.119 and L-PAM enter tumor cells by different transport systems was obtained in experiments using the L-PAM resistant line, L1210/L-PAM. Redwood and Colvin [18] reported that L1210/L-PAM cell resistance was due to a specific mutation which decreased the affinity of the L1210 L-system carrier for L-PAM. As a consequence, both the kinetics of L-PAM uptake and the resultant intracellular concentration of the drug were reduced in the resistant compared to the sensitive L1210 cell line. If cellular entry of PTT.119 and L-PAM is mediated by the same amino acid carrier system, tripeptide uptake and subsequent cellular cytotoxicity should also be decreased in the L-PAM resistant line. The lack of diminution of PTT.119 cytolytic activity suggests that entry of the tripeptide is not via the L-system.

Other factors which have important roles in drug efficacy include the metabolic half-life of the compound, the susceptibility of intracellular macromolecular targets such as DNA to alkylation, and the ability of the target tumor cells to repair drug-induced damage [19–22]. Under physiological conditions the half-life of L-PAM has been reported to be 60–90 min [23]. In comparison, the biological activity of PTT.119 measured by cellular cytotoxicity indicated that the tripeptide has a half-life of approximately 5.5 hr. Thus, intracellular components of target cells are exposed longer to a higher concentration of active PTT.119 molecules.

PTT.119 and L-PAM could also have different preferred sites of intracellular action and/or vary in their reaction rates of drug-induced alterations [8]. Our previous study suggested that tripeptide cytolytic activity was due to several mechanisms since the cellular damage of tumor cells of different histopathologies was quantitatively and qualitatively unique following PTT.119 treatment [6]. The tripeptide not only induced significant nuclear disruption of varying cytopathologies, but also appeared to interfere with the directional protein initiating and synchronizing mitosis [6]. Similar intracellular activities have not been observed following exposure of tumor cells to L-PAM. PTT.119 and L-PAM reactions rates of alkylation of cellular components could also be different as has been observed for two similar bifunctional alkylators, L-PAM and HN_2 [21, 24]. These variances were observed for the rate of formation of monoadducts and their conversion to cross-linked moieties, as well as for their removal and repair by the cells [18, 22, 25].

The L-PAM resistant (L1210/L-PAM and P388/L-PAM) tumor lines and the cisplatin resistant (L1210DDP) and methotrexate resistant (L1210 MTX) cell lines were all totally susceptible to PTT.119. Comparison of the dose–response curves of the resistant lines to the sensitive parental cell lines, L1210 and L1210S, revealed that L1210/L-PAM, P388/L-PAM and L1210 MTX cells were collaterally sensitive to PTT.119 [7]. This increased sensitivity to the tripeptide by the resistant cell lines is not observed with other chemotherapeutic agents. Both the L1210/L-PAM and P388/L-PAM lines are cross resistant to other alkylating drugs; L1210/L-PAM has an incomplete but marked resistance to L-PAM and is moderately resistant to cyclophosphamide, chlorozotocin, dianhydrogalactitol, and thio-TEPA, and is completely cross-resistant to cisplatin. P388/L-PAM is totally resistant to both L-PAM and cisplatin although it is susceptible to other alkylating agents [2, 8, 26]. The *in vitro* data generated by this investigation indicate that PTT.119 could be a highly effective chemotherapeutic agent in situations where tumor cells have developed resistance to other drugs.

The rates of random spontaneous mutation of drug resistant phenotypes vary greatly and selection of cells resistant to alkylating agents usually occurs during chronic, low-dose drug exposure [1, 8, 27]. L1210 cells are highly responsive to low-dose selection and approximately 1 in 10^5 tumor cells will mutate in the continuous presence of a sublethal dose of drug [2, 8, 26]. A small number of resistant cells should be generated within several serial subcultures and overgrowth by drug-resistant cells could occur within 6–8 passages [2, 28]. Our attempts to generate an L1210 cell line resistant to PTT.119 have been unsuccessful. No decrease in PTT.119 sensitivity could be detected even after constant exposure of L1210 cells for over 100 passages using low and escalating doses of the tripeptide. Similar experiments attempting to select for PTT.119 resistance in the MJY-alpha mammary tumor cell line were also unsuccessful after constant low-dose exposure to the tripeptide for 8 months.

The inability of PTT.119 to induce cell lines resistant to the tripeptide demonstrates that this new synthetic bifunctional alkylating compound is neither a mutagen nor an inducer and indicates that administration of PTT.119 will not readily increase the probability of drug-resistant mutants in the tumor cell population. This further suggests that PTT.119 is not carcinogenic and has a low potential for causing cellular transformation in hosts exposed to the drug. These findings of low mutagenicity coupled with the high efficacy of the tripeptide against susceptible and drug-resistant tumor cells strongly demonstrates the potential benefits of PTT.119 as a chemotherapeutic agent.

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