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

I. In vitro cytotoxicity

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Summary. *A new synthetic tripeptide (p-F-Phe-m-bis-(2-chloroethyl)amino-Phe-Met ethoxy HCl), PTT.119, was demonstrated to have significant cancericidal activity against seven in vitro tumor cell lines of different origins and etiologies and against primary human AMML, ALL, and hairy cell leukemias. Viabilities of each murine tumor and rabbit, marmoset, and human leukemia and lymphoma line were significantly reduced by treatment with 1–50 μg PTT.119 in media containing serum. Continuous 24-h exposure or pulse treatment as short as 15 and 30 min with the tripeptide resulted in irreversible damage to the tumor cells. Under identical treatment conditions, freshly isolated human leukemic cells, particularly ALL lymphoblasts, were even more susceptible to PTT.119 than any of the tested tumor cell models.*

Examination of the parameters of PTT.119 activity revealed that reductions of tumor cell survival were dependent on the concentration of the tripeptide. Prolongation of PTT.119 exposure from 15 min to 24 h increased the rates of tumor cell death but did not proportionally reduce the numbers of surviving cells. Assessment of tumor cell viabilities for 5 consecutive days following pulse exposure to PTT.119 demonstrated increasing reductions in tumor cell survival, which were greatest 5 days after treatment with the tripeptide.

The cancericidal activity of PTT.119 was compared with its three parental components either as individual agents or as a mixture. Both the alkylator moiety, m-sarcolysin (m.L.SL) alone or together with p-fluoro-phenylalanine and L-methionine ethoxy HCl, and L-PAM (L-phenylalanine mustard), the p-isomer of m.L.SL, were 1.5- to 3-fold less cytotoxic to L1210 leukemia and MJY-alpha mammary tumor cells than PTT.119. Covalent linkage of the amino acid residues to m.L.SL yielded a molecule with greatly augmented cancericidal activity capable of acting against a broad spectrum of tumor cells.

Introduction

Bergel and Stock [4] and Larionov et al. [17] attempted to enhance the anticancer potential of nitrogen mustard (mechlorethamine) by synthesizing the bis-(2-chloroethyl)amino-DL-phenylalanine derivative which they called merphalan and *p*-sarcolysin, respectively. Larionov et al. [17] reported an increase in the specificity of sarcolysin for certain tumor cells resulting from substitution of the methyl group of mechlorethamine with phenylalanine. Nyhan [19] subsequently hypothesized that *p*-sarcolysin possessed dual alkylator-antimetabolite activities, since the compound specifically inhibited incorporation of amino acid precursors in tumor cells. Further, examination of the racemic mixture of *p*-sarcolysin revealed that the L form (melphalan or L-PAM) was the active isomer [23].

These findings stimulated development of chemotherapeutic agents composed of synthetic peptides with cytotoxic activity due to the alkylating group and selective carrier function towards specific target cells resulting from linkage of various amino acid residues [8, 9]. Comparison of three structural isomers of bis-(2-chloroethyl)amino-L-phenylalanine revealed that the *m*-isomer (m.L.SL) was more active than the *p*-isomer (L-PAM) and less toxic than the *o*-isomer. Over 300 peptides were synthesized by adding various amino acid sequences in the L configuration at the amino and carboxyl groups of the phenylalanine residue of m.L.SL. Screening of these compounds demonstrated the central role of the peptide moieties in determining cytolytic intensity and selectivity of the synthesized derivatives. Only 14 derivatives proved to be as active or better than the parent compound. De Barbieri et al. [9] tested the chemotherapeutic efficacy of these peptides by combining the six most active molecules, consisting of four tripeptides, one tetrapeptide, and one pentapeptide. As anticipated, this combination was effective against several types of cancers. Reductions of growth of established tumors were observed without adverse physiologic, histologic, or enzymatic alterations in principal organs and tissues of the hosts [9, 16]. Efficacy of these synthetic peptides was established in phase-I and phase-II clinical trials; remissions were induced in patients with neuroblastoma, and bladder, cervical and laryngeal cancers, and also in patients with CML blastic crises [2, 9, 13, 15, 21].

More recently we have successfully synthesized a new series of di-, tri-, tetra-, and penta-peptides in the L configuration with the bifunctional alkylating group in the meta position of the benzene ring of phenylalanine. The results reported here demonstrate the effectiveness of one of the tripeptides, *p*-fluoro-L-phenylalanyl-*m*-bis-(2-chloroethyl)-amino-L-phenylalanyl-methionine ethylester hydrochloride, designated PTT.119 (Fig. 1), against several tumor cell lines of murine, rabbit, primate, and human origin. Comparison of PTT.119 with m.L.SL and L-PAM revealed that equimolar doses of PTT.119 had superior cytolytic activity against tumor cells.

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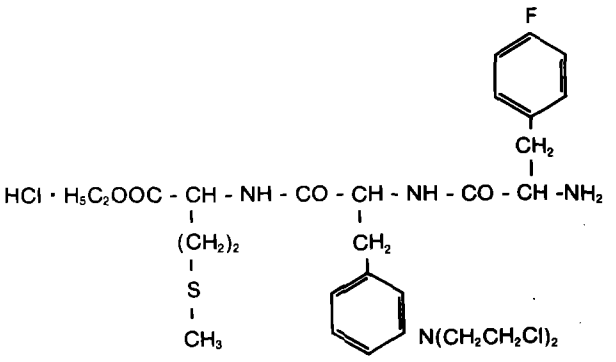

p-Fluoro-L-Phenylalanyl-*m*-*Bis*-(2-Chloroethyl) amino-L Phenylalanyl-Methionine Ethylester hydrochloride

Fig. 1. Molecular structure of the synthetic tripeptide, PTT.119

Materials and methods

Cell cultures. The murine mammary tumor cell line, MJY-alpha, was derived from adenocarcinomas induced in BALB/cfC3H females as a result of infection and oncogenesis by the mouse mammary tumor virus (MMTV) [27]. The epithelioid cell line was tumorigenic in syngeneic BALB/c hosts and was used between the 25th and 65th in vitro subcultures. The growth medium used was RPMI-1640 supplemented with 18% fetal bovine serum (FBS), 10 μ*M* bovine insulin, penicillin (250 U/ml), and streptomycin (100 μg/ml) [27].

B16 melanoma cell cultures were established from the transplantable B16 tumor line passaged SC in male, C57BL/6Jx mice. Melanomas from three to five mice were finely minced, washed three times with serum-free growth medium, and cultured as explants using minimal essential medium containing D-valine and Earle's salts supplemented with 20% calf serum and antibiotics. Confluent primary explant cultures were subsequently passaged weekly as single-cell suspensions using saline-trypsin-versene (STV) [27]. Two 4- to 12-month-old B16 cell cultures were used between their 13th and 45th in vitro passages. Both in vitro lines of B16 cells contained cells pigmented with melanin, and were tumorigenic in syngeneic mice.

Primary L1210 leukemia suspension cultures were initiated from the virulent, in vivo ascites line maintained in DBA/2 Ha mice or their f_1 hybrid, BDF_1 (BALB/c × DBA/2) [3]. L1210 cells were harvested by washing the peritoneal cavity of tumor-bearing mice with physiological saline (0.85% NaCl, pH 7.2) containing 5% heparin (v/v) without preservatives. Leukemic cells were separated on Ficoll-Hypaque gradients followed by several washes in the growth medium (RPMI-1630 with 10% FBS and antibiotics). Tumor cell preparations were consistently free of erythrocytes, and 98% of the L1210 cell population excluded trypan blue.

Human EBV-infected, B-cell lymphoma lines, Raji, and P_3HR-1 [14, 20, 22] were cultured as suspensions in RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin (250 U/ml), and streptomycin (250 μg/ml). Culture densities were adjusted to $5-10 \times 10^5$ cells/ml and media were replenished twice a week.

T-cell lymphoma, 5−78, derived from AACRB rabbits [7], and the EBV-infected, B9508, B-cell lymphoma from marmosets [18] were cultured as suspensions using growth conditions similar to those used for culture of the human lymphoma cell lines.

Human acute myelomonocytic leukemia (AMML), acute lymphocytic leukemia (ALL), and hairy cell leukemia were obtained from untreated patients undergoing leukophoresis prior to chemotherapy. Leukemic cells were separated on Ficoll-Hypaque gradients and processed as outlined for L1210 cells. Tumor cell preparations containd 99% viable cells.

All cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. Media, culture fluids, and cell suspension were routinely checked for bacterial, fungal, and mycoplasma contaminations [24].

Chemotherapeutic agents. The tripeptide, PTT.119 (*p*-fluoro-L-phenylalanyl-*m*-bis-(2-chloroethyl)amino-L-phenylalanyl-methionine ethylester hydrochloride) was provided by Proter S.p.A. (patent pending). PTT.119 was initially dissolved at 10 mg/ml in *N,N* dimethyl acetamide, absolute ethanol, and propylene glycol (1 : 1 : 2). Stock solutions of 1 or 2 mg/ml were obtained by dilution in aqueous 50% propylene glycol prior to use. L-PAM (Alkeran; Burroughs-Wellcome) was dissolved at 1 mg/ml in the same solvent system immediately prior to use. Aliquots of all stock solutions were added directly to culture media containing serum to obtain the final concentrations of 0.1−50 μg/ml. Control cultures received identical volumes of solvent without drugs.

Treatment of MJY-alpha and B16 cell layers. MJY-alpha mammary tumor cell layers were initiated at 2×10^5 cells/2-cm^2 well (Nunclon Multidish 4, Vangard International, Neptune, NJ) using spontaneously released cells from confluent, 6- to 8-day-old cultures. B16 melanoma cells obtained from 4-day-old confluent layers using STV were plated at 2.2×10^5 cells/2-cm^2 well. One day after subculture confluent cell layers were rinsed with medium and treated with medium, medium-solvent or medium-solvent with drugs at concentrations from 1 to 50 μg/ml. Treatment periods ranged from 15 min to 24 h at 37° C. Following treatment, layers were rinsed twice and reincubated with appropriate growth media. Media were changed daily until the cultures were terminated.

Treatment of lymphoid cell lines and human leukemias. All rabbit, marmoset, and human lymphoid in vitro cell lines, murine L1210 leukemia, and primary human leukemias were treated at cell concentrations of $6-10 \times 10^5$ cells/ml. Cultures were exposed at 37° C to 0.1−50 μg drug/ml or equivalent volumes of solvent. Following treatment for 15 min to 24 h cells were pelleted at 200 *g* for 9 min at 5° C, washed, and resuspended in fresh growth media. Culture media were changed daily by gently pelleting cells and exchanging 60%−70% of the spent supernatants for fresh media.

Tumor cell survival. Viability and outgrowth of tumor cell layers or suspensions following PTT.119 treatment were ascertained using 0.5% trypan blue in 0.1 *M* phosphate-buffered saline, pH 7.25. Cell layers were released from the substrate with 0.5 ml STV and diluted 2- to 10-fold with the vital stain; tumor cells in suspension cultures were directly mixed 1 : 1 with trypan blue. Viable tumor cells excluding trypan blue were enumerated in each sample by counting every field of the hemocytometer. Values from duplicate or triplicate, treated and parallel control cultures were averaged for each time point. Percentages of viable cells from treated cultures were determined by direct comparison of the numbers

of these cells excluding trypan blue to those in untreated or solvent-treated parallel cultures. There were no significant differences in viability between cells treated with solvent and untreated cells. Therefore, numbers of control cells for all dosages were averages of the two experimental controls.

Cell culture reagents and chemicals. All cell culture media, sera, and components for STV were obtained from Grand Island Biological Company (GIBCO), 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 cell separation was obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

Results

Comparison of the cancericidal activity of PTT.119 and parental components

The ability of this new synthetic tripeptide, PTT.119, to inhibit the growth and survival of neoplastic cells was first assessed using in vitro cultures of the MJY-alpha mammary tumor cell line and the transplantable L1210 leukemia. Numbers of viable cells in MJY-alpha cell layers and L1210 cell suspensions were enumerated following continuous 24-h exposure to 0.1–50 μg PTT.119/ml. The percentage of viable cells at each dosage was determined by comparison with parallel control cultures. Repetitive assessment using 25–40 evaluations revealed reproducible and significant decreases in tumor cell survival (Table 1). The viability of mammary tumor cells decreased by 47%–88% when treated with 1–50 μg PTT.119/ml. A greater reduction of 27%–97% of viable L1210 cells was observed following exposure to 0.1–25 μg PTT.119/ml. This cancericidal activity was pronounced despite inclusion of growth media containing 10%–18% FBS during treatment, indicating that amino acids and other serum components were not inhibitory [5, 25, 26]. The data also revealed that tumor cell cytolysis was the result of direct cellular interaction with PTT.119 and that PTT.119 did not require in vivo metabolic conversion for activation.

Efficacy of m.L.SL, *p*F-Phe, and L-Met, which make up PTT.119, was determined with the agents used under identical conditions either individually or as a mixture. The dosages were equivalent to their presence in the tripeptide where 1 g PTT.119 is composed of 46% m.L.SL, 24% *p*F-Phe, and 30% L-Met. Continuous exposure of L1210 and MJY-alpha cells to either *p*F-Phe or L-Met had no effect on cellular viability. The parent molecule, m.L.SL, which contained the bifunctional alkylator moiety did reduce the fraction of viable tumor cells (Table 1). Addition of *p*F-Phe and L-Met to m.L.SL did not affect the cytotoxic activity of m.L.SL. Both m.L.SL and L-PAM, as well as the mixture of all three compounds, were significantly less cytotoxic at all concentrations to L1210 leukemia and MJY-alpha mammary tumor cells than was PTT.119. Increased cancericidal efficacy of the tripeptide thus appears to be associated with the covalent attachment of *p*F-Phe and L-Met to the bifunctional alkylator.

Cytotoxic activity of PTT.119 against murine, rabbit, primate, and human cell lines

Assessment of the spectrum of the cancericidal activity of PTT.119 against various cancer cell types was initiated using in vitro cultures of T-cell leukemias and of melanoma and mammary tumor cells subjected to continuous PTT.119 treatment (Table 2). Decreases in tumor cell viabilities after a 24-h exposure were reproducibly observed in the 20–40 repetitive samplings of each of the tumor cell lines at all doses of PTT.119. The sensitivity of each tumor cell line was dose-dependent between the concentrations of 1 and 50 μg PTT.119/ml. Comparison of the three cancer cell types revealed that they were differentially susceptible to PTT.119-induced cytotoxicity at any given concentration of the tripeptide; leukemia cells were usually more susceptible to the cancericidal action of PTT.119.

Cancericidal activity of PTT.119 against human leukemias

Exposure of in vitro cell lines to PTT.119 demonstrated the activity of this tripeptide against a variety of cancer cell types from four species. To ascertain whether this cytolytic activity of PTT.119 was representative of the chemotherapeutic potential of the synthetic tripeptide, leukemic cells obtained by leukophoresis from untreated patients with hairy cell leukemia, AMML, and ALL were treated with 1–50 μg PTT.119/ml (Table 3). Lymphoblasts from a patient diagnosed to have acute lymphatic leukemia were particularly sensitive to PTT.119; cell viability was reduced by 75% following treatment with 1 μg PTT.119. AMML and hairy cell leukemias were also sensitive to all concentrations of PTT.119; numbers of

Table 1. Cytotoxic efficacy of PTT.119 and parental components following 24 h continuous treatment

Compound	Percent viable tumor cells[a]									
	L1210 Leukemia μg PTT.119 or equivalent/ml[b]					MJY-alpha mammary tumor μg PTT.119 or equivalent/ml[b]				
	0.1	1	5	10	25	1	5	10	25	50
PTT.119	73 ± 8	73 ± 6	63 ± 6	9 ± 9	3 ± 5	53 ± 12	39 ± 7	34 ± 6	25 ± 3	12 ± 3
*p*F-Phe	92 ± 3	102 ± 12	114 ± 2	104 ± 2	96 ± 14	100 ± 4	112 ± 8	116 ± 3	90 ± 6	102 ± 9
L-Met	100 ± 1	104 ± 8	99 ± 1	110 ± 11	100 ± 15	104 ± 2	87 ± 6	115 ± 10	109 ± 2	116 ± 1
m.L.SL	91 ± 13	100 ± 8	86 ± 1	75 ± 1	52 ± 5	62 ± 5	46 ± 11	52 ± 5	35 ± 11	32 ± 2
*p*F-Phe + L-Met + m.L.SL	115 ± 4	82 ± 3	73 ± 2	78 ± 10	49 ± 4	64 ± 6	55 ± 7	51 ± 8	50 ± 10	36 ± 14
L-PAM		104 ± 16	96 ± 2	73 ± 9	27 ± 10	65 ± 9	45 ± 11	43 ± 8	37 ± 6	29 ± 5

[a] Values are given ± SD and represent the means of 25–40 determinations. Viabilities of untreated and solvent-treated tumor cells were 98%–100% throughout the experimental periods

[b] Doses of parental components and L-PAM were equivalent to their weight in PTT.119: 1 μg PTT.119 contains approximately 0.46 μg m.L.SL, 0.24 μg *p*-F Phe and 0.3 μg L-Met; L-PAM was used at the same concentrations as m.L.SL

Table 2. Susceptibility of tumor cell lines to continuous exposure to PTT.119

Cell line	Cell type	Virus[a]		PTT.119 (μg/ml)	% Viable tumor cells with exposure for (h)[b]			
		Type	Production		1	4	8	24
Mouse								
L1210	Leukemia	MuLV	+	1	90	–	89	73
				5	99	88	–	63
				10	95	89	–	9
				25	50	39	–	3
B16	Melanoma	–	–	5	98	98	109	81
				10	88	94	65	64
				25	99	83	91	68
				50	81	97	58	33
MJY-alpha	Mammary tumor	MMTV	++	5	104	97	91	39
				10	110	113	107	34
				25	92	101	99	25
				50	98	87	92	12
Rabbit								
5–78	T lymphoma	HVA	+	5	104	88	–	66
				10	89	61	–	49
				25	75	32	14	6
				50	57	4	–	2
Marmoset								
B95–8	B lymphoma	EBV	+	10	100	102	–	40
				25	99	86	49	20
				50	77	42	26	6
Human								
Raji	B lymphoma	EBV	–	10	98	98	–	82
				25	100	97	97	35
				50	94	90	76	20
P_3HR-1	B lymphoma	EBV	+	10	96	91	–	75
				25	92	86	–	69
				50	93	75	–	50

[a] MuLV, murine leukemia virus; MMTV, mouse mammary tumor virus; HVA, herpesvirus ateles; EBV, Epstein-Barr virus

[b] Viability was determined immediately following exposure to PTT.119 for the prescribed time. Viabilities of parallel untreated and solvent-treated cultures for each cell line were 98%–100% throughout the assay periods. Results are the means of 20–40 evaluations

Table 3. Efficacy of PTT.119 against human cells

	Concentration of PTT.119 (μg/ml)	% Viable tumor cells with exposure for (h)[a]		
		1	4	24
Hairy cell leukemia	5	106	80	34
	10	91	71	31
	25	83	71	12
	50	38	21	7
Acute myelomonocytic leukemia	1	95	85	66
	5	50	52	66
	10	60	41	21
	25	20	15	14
	50	4	3	4
Acute lymphocytic leukemia	1	98	76	25
	5	46	29	7
	10	32	15	0
	25	15	15	0
	50	0	0	0

[a] Percentage viability at each dosage and exposure period of 1, 4, or 24 h was determined on duplicate samples by direct comparison to untreated and solvent-treated cultures. Viability of control cultures remained at 97%–100% throughout the experimental period

viable cells after 24 h treatment ranged between 4% and 66%. It is noteworthy that cytotoxicity was detected in the fresh leukemia preparations within the 1st h of PTT.119 treatment; the surviving fractions varied from 0% to 100% in a dose-dependent manner. The data indicate that the sensitivity of uncultured, human tumor cells to PTT.119 is significantly greater than that of culture-adapted human tumor cell lines.

Effects of treatment duration on PTT.119 cytotoxicity

Several of the tumor cell models and the primary human leukemias displayed PTT.119-induced cytotoxicity within 1–4 h of exposure to the tripeptide, indicating that continuous 24-h treatment may not be required for reduction of the tumor cell populations. To delineate the minimum concentrations and durations of PTT.119 exposure to induce cytolysis, MJY-alpha and B16 melanoma cell layers and L1210 leukemia cell suspensions were exposed to 0.5–50 μg PTT.119/ml for 0.25–4 h. PTT.119 was then removed, and the cells washed and reincubated in growth media. Tumor cell viabilities were determined 24 h after pulse PTT.119 exposure and compared with parallel control cultures. Cell viabilities of all three tumor cell lines were reduced by exposure to PTT.119 for as little as

Table 4. Susceptibility of tumor cells to pulse treatment with PTT.119

Cell line	PTT.119 (μg/ml)	% Viable tumor cells at 24 h with treatment period of (h)[a]				
		0.25	0.5	1	2	4
MJY-Alpha	5	–	89 ± 7	80 ± 11	77 ± 4	76 ± 1
	10	69 ± 3	67 ± 5	51 ± 13	63 ± 9	48 ± 4
	25	61 ± 1	40 ± 6	34 ± 11	46 ± 9	44 ± 7
	50	45 ± 3	29 ± 8	23 ± 11	–	–
B-16 Melanoma	5	–	102 ± 8	91 ± 5	71 ± 5	86 ± 8
	10	–	91 ± 10	80 ± 12	76 ± 5	74 ± 13
	25	90 ± 10	77 ± 3	73 ± 3	59 ± 12	56 ± 13
	50	73 ± 10	68 ± 12	51 ± 12	–	–
L1210 Leukemia	0.5	96 ± 6	99 ± 9	73 ± 4	75 ± 8	34 ± 5
	1	88 ± 7	84 ± 11	77 ± 9	87 ± 6	48 ± 7
	5	64 ± 9	57 ± 10	49 ± 8	49 ± 10	37 ± 10
	10	43 ± 9	37 ± 11	35 ± 10	23 ± 9	7 ± 7
	25	7 ± 11	2 ± 4	1 ± 2	3 ± 5	0
	50	0	0	0	–	–

[a] Tumor cells were treated with PTT.119 for the prescribed period, then washed, and viability determined after 24 h of culture. Viabilities of untreated cultures and cells receiving solvent were 98%–100% throughout the experimental periods. Values are given ± SD and are the means of 20–40 evaluations

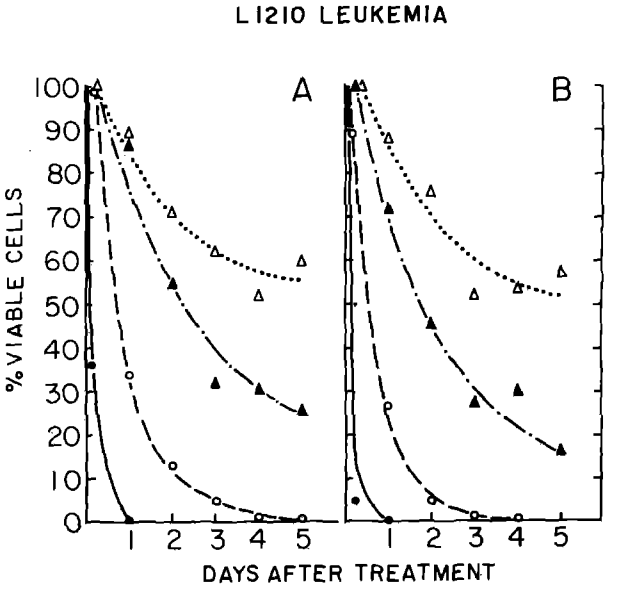

Fig. 2A and B. Survival of L1210 leukemia suspensions following a 30-min **(A)** or 1-h **(B)** pulse exposure to 1 (△ · · · · △), 5 (▲– · –▲), 10 (○– – –○), and 25 (●———●) μg PTT.119/ml. L1210 cell suspensions contained 6 × 10^5 cells/ml at the time of treatment and the range of viable control cells during the 5-day observation period was 6.5–1 × 10^5 cells/ml. Data are the means of 20 evaluations and are representative of values obtained from three other experiments

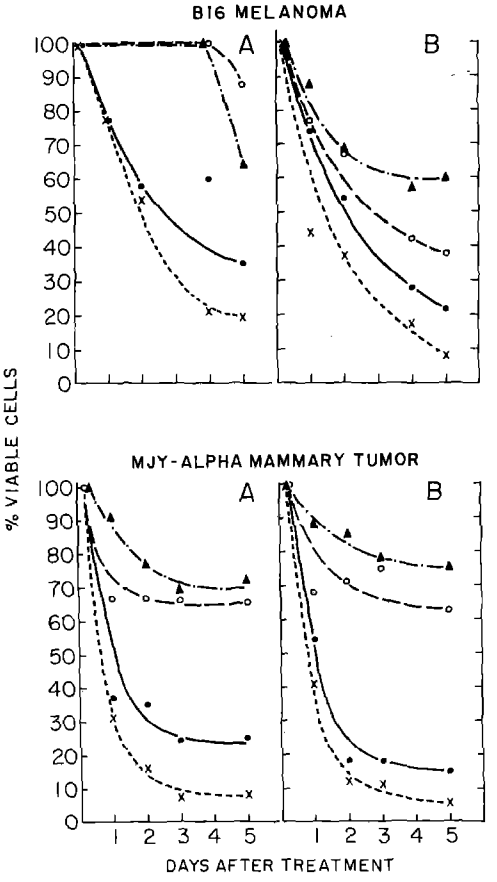

Fig. 3A and B. Survival of MJY-alpha mammary tumor and B16 melanoma cell layers following 30-min **(A)** or 1-h **(B)** pulse treatment with 5 (▲– · –▲), 10 (○– – –○), 25 (●———●), and 50 (×– – –×) μg PTT.119/ml. Numbers of cells in the 24-h-old layers at the time of treatment were 2.9–3.2 × 10^5 MJY-alpha cells and 2.7–3.0 × 10^5 B16 melanoma cells per well. The numbers of viable control cells during the 5-day observation period were 3.5–12 × 10^5 MJY-alpha cells and 3.5–6 × 10^5 B16 cells per well. Data are the means of 20 evaluations and are representative of values obtained from four other similar experiments

15 min (Table 4). At any given concentration of PTT.119, the degree of cytolysis generally increased with extension of the treatment periods from 15 min to 4 h. However, a concentration-dependent decrease in cell viability was observed in the three murine tumor models for each treatment period. Comparison of the data in Tables 2 and 4 reveals that cells expose to PTT.119 for 1 h were irreversibly damaged when assayed a day later, despite their apparent viability immediately after treatment. Of greater importance was the demonstration that 0.25- to 4-h exposures to any concentration of

PTT.119 were almost as effective as continuous 24-h treatment in reducing the numbers of viable tumor cells.

Effects of pulse PTT.119 treatment

Pulse exposures of mammary, melanotic, and leukemic tumor cells with PTT.119 were found to have reduced the numbers of viable cells on examination 24 h after exposure. To ascertain whether these remaining cells were viable and able to replicate and repopulate the culture vessels, longitudinal assessments of the surviving cell fractions were made. MJY-alpha mammary tumor and B16 melanoma cell layers and L1210 leukemia cell suspensions were treated with 1–50 μg PTT.119/ml for 30 min or 1 h. Cultures were washed and reincubated, and cell viabilities were determined over the following 5 days (Figs. 2 and 3). Daily assessment of the treated cultures revealed that in all three systems the numbers of viable cells continued to decline during the examination period. The observed decreases in viability, as determined by trypan blue exclusion, indicated that within the tumor cell population cytolysis occurred at varying rates. Consequently, the cumulative lethal effects of pulse PTT.119 exposure was revealed only by longitudinal examination of the treated cells. The degrees and rates of tumor cell susceptibility were dependent on the tumor cell system, as well as on the concentration and duration of PTT.119 exposure. Of the three tumor models, the L1210 leukemia was the most susceptible; at any given concentration of PTT.119, approximately five times more L1210 cells were destroyed than mammary tumor and melanoma cells.

Unlike either the L1210 leukemia and B16 melanoma cultures, which showed a gradual progression of cell cytotoxicity with increasing dosage, there was a distinct separation of cell viabilities between 10 and 25 μg/ml in MJY-alpha mammary tumor cell cultures (Fig. 3). Whether this reflected a critical threshold level of PTT.119 for this cell line or demarcated two populations of susceptible cells is not known. In all three tumor systems, extension of the treatment period from 0.5 to 1 h generally increased the rate of cell death but did not have an appreciable effect on the total numbers of viable cells remaining at the end of the 5-day assay period.

Discussion

The synthetic tripeptide PTT.119 is a new bifunctional alkylating agent of the nitrogen mustard group consisting of methionine, phenylalanine, and *p*-fluoro-phenylalanine in the L configuration with a bis-(2-chloroethyl)amino group in the meta position of the benzene ring of the phenylalanine residue. Our in vitro studies demonstrated the marked cytotoxic activity of this synthetic tripeptide against murine, rabbit, primate, and human tumor cell lines, and also against fresh AMML, ALL, and hairy cell leukemias. Degrees of cytotoxicity induced by continuous exposure to PTT.119 varied with tumor cell type and history. Among the examined cell lines, murine, and rabbit leukemias and EBV-transformed marmoset lymphoma were the most susceptible to PTT.119 when assessed by either concentration or duration of tripeptide treatment. The least susceptible to PTT.119 were the two EBV-transformed human B lymphomas. Whether these differences in cellular susceptibility were inherent in individual cell lines, types of tumor cells, or their metabolic activities is not yet known. In addition, the role of RNA or DNA oncogenic virus infection/transformation in the sensitivities of these tumor cells to PTT.119 remains to be examined.

The degree of PTT.119-induced cytotoxicity was dependent on the concentration of the tripeptide and duration of exposure. Comparison of MJY-alpha, B16, and L1210 tumor cell survivals demonstrated that prolongation of treatment from 0.25 to 4 h did not proportionally increase cellular cytolysis, nor could it necessarily compensate for reductions in PTT.119 concentrations. This suggests that each tumor cell must be exposed to a minimum concentration of PTT.119 for only a short period of time in order to be irreversibly damaged.

Longitudinal examination of tumor cells following pulse PTT.119 exposure demonstrated that the total cancericidal activity of PTT.119 was not discernable at the time of or within 24 h following tripeptide treatment. Tumor cell viability declined significantly with time and was most apparent 3–5 days following PTT.119 treatment. This irreversible but delayed cytotoxicity of the tripeptide was probably attributable to the bis-(2-chloroethyl)amino component of the molecule which is involved in strongly electrophilic reactions. Alkylation of guanine residues is a primary reaction, and this results in DNA miscoding, excision of altered residues, and possible cross-linking of two nucleic acid chains or nucleic acids to proteins. As a consequence, toxicity is usually expressed at the S and G_2 phases of the tumor cell cycle [6, 12]. It appeared that these cytotoxic effects of PTT.119 were not readily repaired by cellular mechanisms, since the viabilities of MJY-alpha, B16, and L1210 tumor cells continued to decrease throughout the observation period.

Comparison of the cytolytic effects of PTT.119 with those of the parental molecule, m.L.SL, or the *p*-isomer, L-PAM, of the bifunctional alkylator revealed that both isomers behaved similarly and that the tripeptide was significantly more cancericidal than either. The cytolytic activity of PTT.119 was 1- to 3-fold greater than the cumulative cytotoxicities of L-Met, m.L.SL, and *p*F-Phe. These results leave little doubt that the covalent linkage of *p*-fluoro-L-phenylalanine at the amino, and methionine ethylester at the carboxyl group of m.L.SL altered the capabilities of the individual components and yielded a molecule with significantly enhanced cancericidal activity. This increased cytotoxicity could be accounted for by a variety of mechanisms. It is possible that PTT.119 could exert antimetabolic activity and inhibit protein synthesis as observed following treatment with the closely related multipeptide complex, PTC [1, 9, 10]. Synthesis of the tripeptide could lengthen the half-life of the alkylating component by reducing the rate of intramolecular cyclization and the subsequent carbonium ion formation of the 2-chloroethyl side chains involved in alkylation. In addition to changes in the stability of the m.L.SL portion of the molecule, the linkage of amino acid residues could alter the cellular uptake and/or retention of PTT.119 compared with m.L.SL or L-PAM. It has been reported that L-PAM enters tumor cells via two leucine-preferring carrier transport systems [5, 25, 26]. Exogenous amino acids in the treatment medium effectively inhibited this carrier-dependent uptake, particularly in L1210 leukemia cells [25, 26]. Covalent attachment of the two other amino acids to m.L.SL to yield the tripeptide probably altered the requirements for its intercellular uptake, as reflected by the high sensitivity of L1210 leukemia cells to PTT.119 in the presence of exogenous amino acids. It is likely that this difference in PTT.119 metabolism played a role in the susceptibility of other tumor cell types.

The chemotherapeutic potential of this synthetic tripeptide is currently under investigation. Our in vitro results presented

here demonstrate that this new compound, PTT.119, exerts cancericidal action against a broad spectrum of neoplasms. The cytolytic activity of the tripeptide was 1- to 3-fold greater than equimolar concentrations of the parental m.L.SL molecule or its structural isomer, L-PAM. In addition, the cancericidal efficacy of PTT.119 was more pronounced against fresh, human ALL, AMML, and hairy cell leukemias than against established tumor cell lines of murine, rabbit, primate, and human origins. We have conducted bioassays designed to evaluate the tumorigenicity of PTT.119-treated cells in syngeneic hosts. The biosassays confirm our in vitro findings of the potent cancericidal activity of PTT.119 and are presented in detail in part II of this paper.

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