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Evaluation of 3-(*p*-fluorophenyl)-L-alanyl-3-[*m*-bis-(2-chloroethyl)aminophenyl]-L-alanyl-L-methionine ethyl ester HCl (PTT.119) against xenografts of human rhabdomyosarcoma*

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Summary. PTT.119 [*p*-*F*-phe-*m*-bis(2-chloroethyl)amino-L-phe-met ethoxy HCl], a synthetic tripeptide mustard, was evaluated for therapeutic efficacy against a spectrum of childhood rhabdomyosarcomas (RMS) maintained as xenografts in immune-deprived mice. These xenografts were established from previously untreated tumors, and sublines were selected in mice for resistance to L-phenylalanine mustard (L-PAM). PTT.119 caused regression of four of six RMS lines established from untreated tumors, and demonstrated activity similar to that of L-PAM in this model. Against tumors Rh18/L-PAM and Rh28/L-PAM, selected in situ for L-PAM resistance, PTT.119 had no significant activity. Rh28/L-PAM was cross-resistant also to oxazophosphorine mustards (ifosfamide, cyclophosphamide), and both tumors were cross-resistant to adriamycin and vincristine. PTT.119 caused hematologic toxicity similar to that of L-PAM, characterized by a marked decrease in white blood cells and thrombocytopenia.

Introduction

The identification of new agents effective in the treatment of childhood malignancies presents problems not often associated with more frequently occurring tumors in adults. Solid tumors in children are rare, hence preventing large, well-controlled, randomized trials, and in most instances new agents are studied in heavily pretreated patients, whose tumors may be resistant to multiple agents. Further, in the case of many childhood tumors, effective curative therapy is available for some patients, thus precluding the evaluation of new agents in previously untreated patients. What is needed, therefore, is the ability to provide data that would support evaluation of a new agent against previously untreated patients with poor prognosis, particularly, when that agent had failed to demonstrate significant activity in phase II evaluation against patients relapsing on conventional therapy.

We have therefore approached this problem by developing a series of tumor models, each specific for a particular histiotype, by heterografting human tumors into immune-deprived mice [9, 10]. Such xenografts appear to parallel the sensitivity of the clinical disease, in retrospective studies [11], and in prospective studies [12]. For example, the models of childhood rhabdomyosarcoma (RMS) used in the current study, identify each of the clinically effective agents. Used prospectively, they have identified L-phenylalanine mustard (melphalan, L-PAM) with very significant activity against a high proportion of independently derived lines [12]. The efficacy of L-PAM given at conventional dose levels (45 mg/m^2) has been demonstrated against previously untreated, advanced RMS [6, 7], the response rate being over 80%. These results indicate that xenograft models, used appropriately, may be of value in prioritizing drug evaluation in the less frequently occurring tumors. Despite the efficacy of L-PAM, however, its use is limited by severe myelosuppression and the development of resistance. We were therefore interested in evaluating the synthetic peptide PTT.119, which had demonstrated significant activity against multiple tumor lines in vitro [16, 19] and was active against L1210 leukemic cells selected for resistance to L-PAM [17].

Materials and methods

Immune deprivation of mice. Female CBA/CaJ mice (Jackson Lab, Bar Harbor, Me), 4 weeks of age, were immune-deprived by thymectomy, followed 3 weeks later by i.p. administration of 1-β-D-arabinofuranosylcytosine (200 mg/kg) 48 h prior to receiving whole-body irradiation (950 cGy) using a ^{137}Cs source [12].

Tumor lines. Six independently derived lines from previously untreated RMS have been described previously [6, 12]. Each tumor grows routinely in over 90% of recipient mice, and all are human as determined by karyotype and species-specific isoenzyme patterns [5, 10]. The chemosensitivity of these lines has been reported previously for conventional agents in the therapy of RMS [11] and for L-PAM [12].

Sublines selected in situ for resistance to L-PAM have been described elsewhere [8]. Briefly, for Rh18 and Rh28 tumors, mice were treated with a single administration of L-PAM (13 mg/kg) for 13 and 7 passages, respectively.

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Abbreviations: PTT.119, 3-(p-fluorophenyl)-L-alanyl-3-[m-bis-(2-chloroethyl)-aminophenyl]-L-alanyl-L-methionine ethyl ester HCl; L-PAM, L-phenylalanine mustard; VCR, vincristine; ADR, adriamycin; CTX, cyclophosphamide; Ifos, ifosfamide

For an equivalent response in the L-PAM resistant sublines a two- to threefold increase in dose was required. Resistance was stable in the absence of drug selection pressure.

Growth inhibition studies. Mice bearing bilateral subcutaneous tumors each received a single i.p. administration of agent when tumors were 1 cm or more in diameter. Tumor response was determined at 7-day intervals using digital calipers (maxcal) interfaced through an RS 232 to a Suntronics AT microcomputer. Two perpendicular diameters were used to compute volumes [12]. Growth delay was calculated from the difference in days required for treated tumors to grow to four fold their volume at the time of treatment compared with vehicle-treated controls. For each treatment group, six or seven tumor-bearing mice were used. Relative tumor volumes were calculated from the formula RTV = (V_x/V_o), where V_x = volume on day x and V_o is the volume of tumor at time of treatment. To equate responses in tumor lines that demonstrate different rates of growth, inhibition was normalized by expressing this as a function of tumor volume-doubling time. Mean volume-doubling times during exponential growth for Rh12, Rh18, Rh28, Rh30, Rh35, and Rh39 tumors were 7.9, 6.3, 9.9, 9.3, 8.0, and 13.3 days, respectively. For L-PAM-resistant sublines of Rh18 and Rh28, doubling times were 4.2 and 10.1 days. Grading of tumor responses is given in Table 1; the definition of $\geq 50\%$ regression required that each tumor within a group, at some time point after treatment, demonstrated such a reduction in volume.

Hematologic toxicity. Immune-deprived, non-tumor-bearing mice each received a single dose of PTT.119 (12.5 or 4 mg/kg) or L-PAM (13 mg/kg or 4 mg/kg). Mice were bled from the retro-orbital sinus on alternate days. White blood cells and platelets were analyzed using a Sysmex 18A instrument.

Cytotoxic agents. L-PAM was purchased from Sigma Chemical Co. (St. Louis, Mo), PTT.119 was supplied by Proter S. p. A. and dissolved *N,N*-dimethylacetamide, absolute ethanol and propylene glycol (1:1:2), the final concentration of vehicle being $\leq 10\%$ v/v in saline. L-PAM was dissolved in 1 *M* HCl and back-titrated with NaOH as described elsewhere [12], after which it was made up to volume in saline. Vincristine was a gift from Eli Lilly Co. (Indianapolis, Ind), and ifosfamide was obtained from DCT, National Cancer Institute. Adriamycin was purchased through the pharmacy at SJCRH. Dilutions were made in saline. Each mouse received a single i.p. administration (0.1 ml per 10 g body weight) of each agent or the appropriate vehicle.

Results

Preliminary experiments using immune-deprived, non tumor-bearing mice showed that PTT.119 and L-PAM were equitoxic at 12.5 and 13 mg/kg, respectively (data not shown), and that these doses were the maximum tolerated as single administrations (LD_{5-10}).

Efficacy against RMS xenografts

PTT.119 (10 mg/kg or 12.5 mg/kg) demonstrated marked activity against all lines of RMS, causing complete regression of advanced tumor in Rh28, Rh30, Rh35 and Rh39. As shown for Rh35, the rate of tumor regression was rapid (12.5 mg/kg; Fig. 1), with no tumors regrowing during the period of observation. At 4 mg/kg PTT.119, there was marked activity against Rh35 and Rh30 xenografts. PTT.119 data are summarized in Table 1, and have been

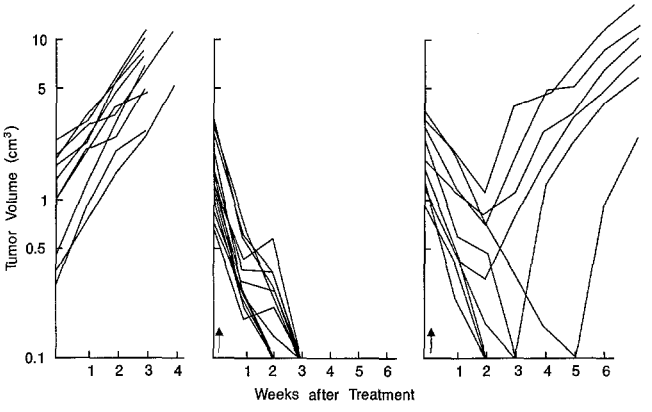

Fig. 1. Response of Rh35 xenografts in mice receiving a single administration of PTT.119. *Left:* growth of control (vehicle treated); *center:* PTT.119 12.5 mg/kg; *right:* 4 mg/kg. Each *curve* shows growth of an individual tumor

Table 1. Responses[a] of RMS xenografts to PTT.119 and L-PAM

Agent	Dose (mg/kg)	Tumor					
		Rh12	Rh18	Rh28	Rh30	Rh35	Rh39
PTT.119	12.5	ND[b]	+++	++++++	++++++	++++++	++++++
	10	+++	ND	ND	ND	ND	ND
	4	++	ND	+++	++++++	++++	ND
L-PAM[c]	13	++++++	+++	+++++	+++++	++++++	++++++
	10	+++++	+++	++++	+++++	+++++	+++++
	4	+	ND	−	++++	ND	++++

[a] Tumor response criteria: −, no growth inhibition; ±, transient response, inhibition $<Td_2$; +, growth inhibition $\geq Td_2$; ++, growth inhibition $\geq 2 \times Td_2$; +++, growth inhibition $\geq 3 \times Td_2$; ++++, growth inhibition $>3 \times Td_2$ plus volume regression $\geq 50\%$; +++++, complete regression with subsequent regrowth; ++++++, complete regression with no growth during the period of observation (≥ 84 days). Td_2, mean time for tumor volume to double

[b] ND, not determined

[c] Data from [12]

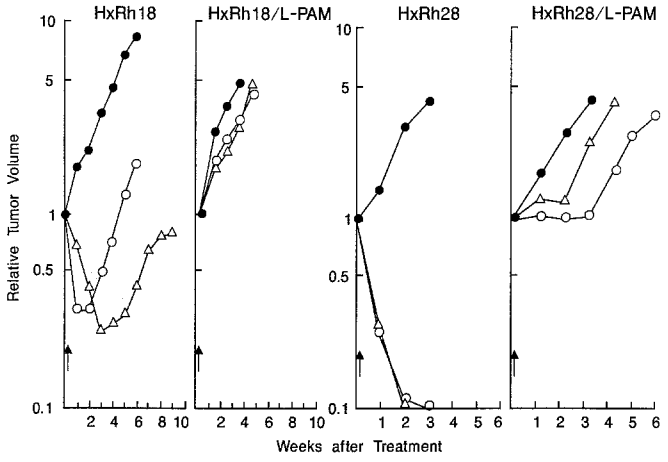

Fig. 2. Responses of Rh18 and Rh28 tumors and their sublines selected in situ for resistance to L-PAM. ●, Control; ○, L-PAM 13 mg/kg; △, PTT.119 12.5 mg/kg. Each *curve* represents the mean for 12 to 14 tumors, expressed as the tumor volume relative to its volume at time of treatment

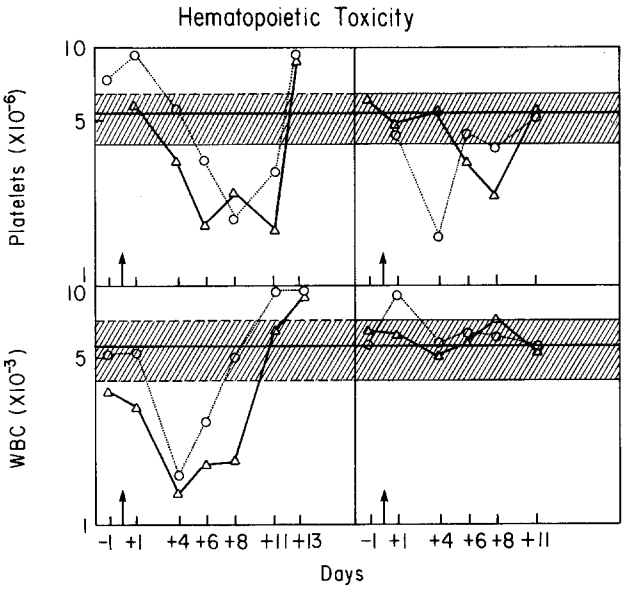

Fig. 3. Hematologic toxicity of PTT.119 and L-PAM in non-tumor-bearing immune-deprived mice. ○, L-PAM; △, PTT.119. *Left panel:* 13 mg/kg L-PAM, 12.5 mg/kg PTT.119; *right panel:* 4 mg/kg L-PAM, 4 mg/kg PTT.119. *Top panels* show changes in platelet and *lower panels* demonstrate changes associated with peripheral white blood cells (mm^3). Each *point* represents the mean for three mice

compared with the responsiveness of these tumors to L-PAM reported previously [12]. Against these tumors, both agents appeared to have similar therapeutic activity, PTT.119 being slightly more effective than L-PAM in Rh18 tumors when both agents were tested concomitantly (Fig. 2).

Response of L-PAM-resistant tumors

Two RMS lines selected in situ for resistance to L-PAM were derived (Rh18/L-PAM and Rh28/L-PAM). Both lines exhibited low-level, stable resistance to the selecting agent. The responses of Rh18, Rh28 and their L-PAM resistant lines to PTT.119 are presented in Fig. 2. Clearly there was cross-resistance between PTT.119 and L-PAM in both Rh18/L-PAM and Rh28/L-PAM.

Cross-resistance patterns of L-PAM-resistant xenografts

To ascertain whether L-PAM resistance and cross-resistance to PTT.119 were related to an alteration in amino acid uptake and hence transport of these cytotoxic agents, the activity of oxazophosphorine mustards (cyclophosphamide, ifosfamide) was examined. Two unrelated agents (adriamycin and vincristine) were examined also, as we had noted previously that Rh28/L-PAM was cross-resistant to VCR [8]. The data are summarized in Table 2. Rhabdomyosarcoma lines selected for resistance to L-PAM exhibited cross-resistance to other classes of bifunctional alkylating agents and resistance to adriamycin and VCR.

Hematologic toxicity

In immune-deprived mice not transplanted with tumor, PTT.119 and L-PAM appeared to exert similar effects on the hematopoietic system (Fig. 3). The WBC nadir for each drug given at the MTD was on day 4 after administration. Both agents caused a significant and prolonged depression in platelets, which recovered to pretreatment levels by day 11. At the lower dose (4 mg/kg) both agents caused a decrease in platelets, but had little effect upon total WBC levels.

Discussion

The tripeptide PTT.119 has been shown to have marked cytotoxic activity in vitro [16, 19] against a number of different tumor lines. Further, no cross-resistance to this agent was determined in L1210/L-PAM [17]. We have shown previously that L-PAM has high therapeutic efficacy against human RMS xenografts, and this has been tested prospectively in children with this tumor [7, 12]. Data indicate a high response rate in this disease [7]. At this time it is not known why RMS is very sensitive to L-PAM, but one possibility is that there is selective uptake due to some increase or alteration in either the L-system or the ASC-

Table 2. Responses of RMS xenografts selected for resistance to L-PAM

	L-PAM	PTT.119	IFOS	CTX	VCR	ADR
Rh18	+++[a]	++++	ND	+++	+++	+++
Rh18/L-PAM	−	−	ND	ND	−	−
Rh28	++++++	++++++	+++	++++	+++++	++++[b]
Rh28/L-PAM	++	±	±	−	−	−

[a] Responses as for Table 1. Agents given in a single administration at the MTD
[b] Adriamycin 10 mg/kg every 7 days × 2

system for amino acid transport [15]. As PTT.119 and L-PAM share common transport mechanisms [15, 18] and PTT.119 was not cross-resistant in L1210/L-PAM, it was of interest to compare PTT.119 with L-PAM in a comprehensive model of childhood RMS.

At the MTD (12.5 mg/kg), PTT.119 caused complete regression of advanced tumors established from previously untreated xenografts (Rh28, Rh30, Rh35, Rh39). At the lower dose (4 mg/kg), PTT.119 was slightly more effective against Rh28 and Rh30 than was L-PAM at a similar dose. Overall, the efficacy of these two agents was similar, suggesting that PTT.119 may have clinical utility against childhood RMS.

For PTT.119 to have a significant advantage over L-PAM, it would be important to demonstrate activity against L-PAM-resistant sublines of responsive RMS. Two such lines were developed, Rh18/L-PAM and Rh28/L-PAM. Against these tumors PTT.119 was ineffective, however, indicating cross-resistance under these experimental conditions. In addition, cross-resistance to both cyclophosphamide and ifosfamide was determined in Rh28/L-PAM, suggesting that resistance was not a consequence of altered uptake by amino acid transport systems [8]. Further, both L-PAM-resistant lines were cross-resistant to adriamycin, possibly implicating glutathione metabolism in resistance [4]. Of note, also, was that Rh28/L-PAM [8] and Rh18/L-PAM (unpublished data) are cross-resistant to VCR. The mechanism for this cross-resistance is not understood, although murine leukemia P388/L-PAM is also cross-resistant to VCR [14].

Melphalan is limited clinically by causing severe myelosuppression, mainly leukopenia and thrombocytopenia, whereas other organ toxicities are not encountered until far higher dose levels such as are used for marrow transplantation protocols are reached [13]. It was thus of importance to determine whether PTT.119 and L-PAM caused similar hematopoietic toxicities in mice. Depression of WBC and platelets was similar for both agents both at the MTD and at 4 mg/kg. As the mouse is a good model for predicting toxicity in man [1–3] it would appear likely that PTT.119 would be limited by hematologic toxicity in man. Further studies comparing the effect of PTT.119 and L-PAM on specific hemopoietic precursor cells in vitro may, however, distinguish between these two agents.

In conclusion, PTT.119 is a potent agent with a high therapeutic efficacy against xenografts of childhood RMS. Its major toxicity in mice is hematologic, being equivalent to that of L-PAM. Against two L-PAM-resistant lines, cross-resistance to PTT.119 was determined. These data suggest that PTT.119 may be effective in the treatment of childhood RMS, but may not be superior to L-PAM.

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