

Liposomal Muramyl Tripeptide Phosphatidylethanolamine: Targeting and Activating Macrophages for Adjuvant Treatment of Osteosarcoma

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Abstract: About one third of osteosarcoma patients develop lung metastasis refractory to chemotherapy. Recent studies indicate that biological response modifiers activating the patient's immune system may help controlling minimal residual disease *via* pathways distinct from those used by cytotoxic drugs, and therefore prove effective against tumor resistance. Muramyl tripeptide phosphatidylethanolamine (MTP-PE) is a synthetic lipophilic glycopeptide capable of activating monocytes and macrophages to a tumoricidal state. When intercalated in multilamellar liposomes (L-MTP-PE) and injected intravenously, it targets lung, liver, and spleen macrophages. Therapeutic activity of L-MTP-PE was demonstrated in several preclinical models of experimental lung metastasis and in clinical trials in dogs with osteosarcoma. Although macrophage activation was shown to be directly involved in the *in vivo* anti-metastatic activity of this molecule, cytokine and chemokine secretion by activated macrophages could induce recruitment and stimulation of other immune cells, which may in turn indirectly contribute to the anti-tumor effect. L-MTP-PE has undergone clinical development in humans. In early trials, most side effects of L-MTP-PE were minimal. L-MTP-PE showed signs of efficacy in treatment of patients with recurrent osteosarcoma and the encouraging results from phase II studies led to a phase III trial conducted by the Children's Oncology Group in patients with newly diagnosed high-grade osteosarcoma. Patients were treated with or without L-MTP-PE in combination with multi-drug chemotherapy in adjuvant setting; significantly higher overall survival and disease-free survival were observed in the group receiving L-MTP-PE.

Keywords: Osteosarcoma, macrophages, innate immunity, muramyl peptides, liposomes, chemotherapy.

INTRODUCTION

Osteosarcoma is the most common form of bone cancer [1-3]. It primarily occurs in children and adolescents. The incidence is very low: there are approximately 900 new cases of osteosarcoma per year in the United States and around the same number in Europe. The standard treatment consists in surgical resection of the primary tumor, associated with chemotherapy in neoadjuvant and adjuvant settings. With this treatment, long-term survival is achieved in about 70% of patients. The remaining patients usually develop pulmonary metastasis, and represent an unmet therapeutic need.

In recent years, results from animal models and early clinical trials have indicated that biological response modifiers activating the patient's immune system may help controlling minimal residual disease *via* pathways either complementary or synergistic to those used by cytotoxic drugs, and may therefore prove effective in preventing the development of tumor resistance. Toll-like receptor (TLR) ligands as CpG oligonucleotides or lipid A derivatives activate adaptive and/or innate immune responses against tumors [1-3]. Muramyl tripeptide phosphatidylethanolamine (MTP-PE), a synthetic molecule derived from muramyl dipeptide (MDP), is also a stimulator of innate immunity. Formulated in multilamellar liposomes, it has been developed for the treatment of osteosarcoma.

In this review we will present an update of the preclinical and clinical studies conducted with liposomal MTP-PE (L-MTP-PE), and we will discuss the mechanisms of action *in vivo*.

THE MOLECULE AND ITS FORMULATION

MTP-PE is a synthetic molecule derived from MDP, the minimal peptidoglycan motif common to both Gram-positive and Gram-negative bacteria. The covalent addition of an alanine and of dipalmitoylphosphatidylethanolamine to MDP results in MTP-PE, a more lipophilic molecule due to the presence of two lipid acid tails. The chemical structure is shown in Fig. (1A).

Like MDP, MTP-PE activates *in vitro* monocytes and macrophages. This activation is measured by increased tumoricidal activity and secretion of cytokines and pro-inflammatory factors, including TNF- α , IL-6, IL-8, IL-1b, nitric oxide, PGE₂ [4-9]. Fogler and Fidler demonstrated a superiority of MTP-PE over MDP in the activation of human monocytes [10]. This was ascribed to the lipophilic properties of MTP-PE, resulting in higher cell uptake *via* passive transfer through the cytoplasmic membrane, with increased availability for an intracellular receptor. In addition, the lipophilic MTP-PE could be efficiently incorporated in the lipid bilayer of liposomal structures. Fidler and colleagues developed a liposomal formulation that, when injected intravenously, distributes primarily in the liver, spleen, and lungs, where it is phagocytosed by monocytes and macrophages [10-12]. These liposomes are concentric multilamellar vesicles (MLV) of approximately 2-3 μ m. They are constituted by the two synthetic

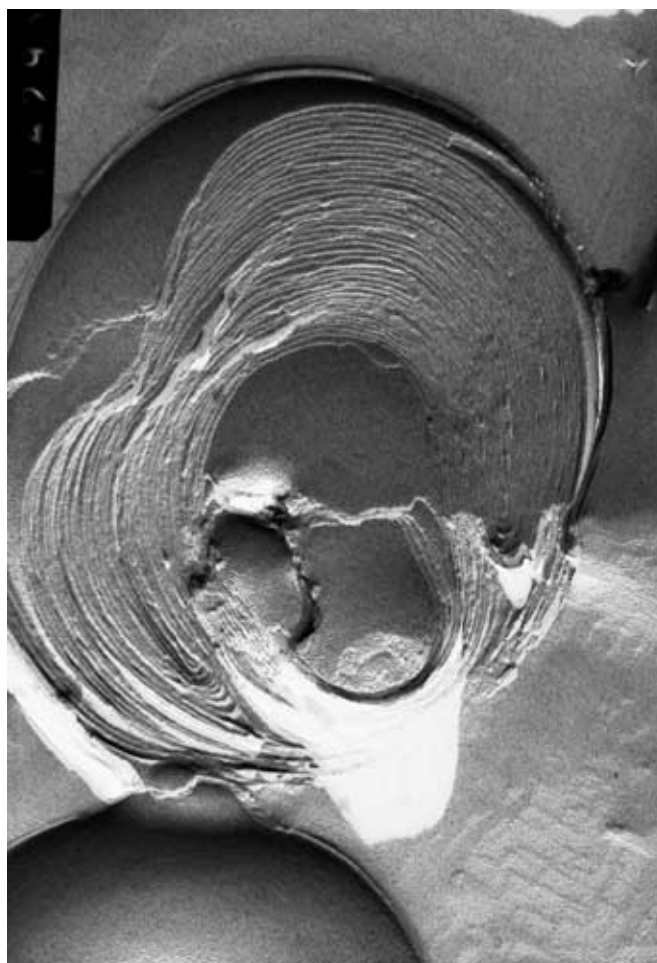
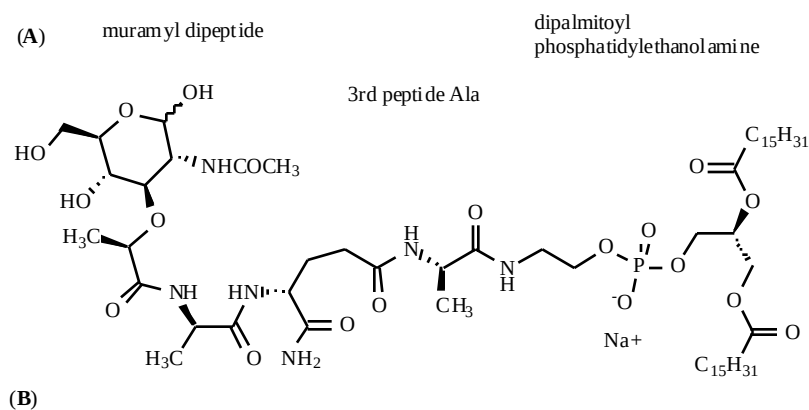
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phospholipids phosphatidylcholine and phosphatidylserine at a 7:3 w/w ratio. MTP-PE is intercalated within the vesicles at a 1:250 w/w ratio. A freeze-fracture electron microscopy photo of a multilamellar liposome is shown in Fig. **(1B)**. Formulation of MTP-PE into these phospholipids vesicles improves activation of macrophage and monocyte tumoricidal properties *in vitro*, and prolongs its presence in the lungs [10, 12, 13]. Compared to the free lipopeptide, the MLV formulation undergoes very rapid clearance from the circulation, due to drug targeting to the phagocytic cells of the reticuloendothelial system [12, 14]. Pharmacokinetics studies in dogs showed that, 5 minutes

after i.v. injection of L-MTP-PE, only 0.5% was found in plasma, compared to 93% when administrated as free lipopeptide [14]. Possibly because of this fast clearance, MTP-PE formulated in liposomes showed a no-adverse-event level ten times higher than free MTP-PE in rabbit and dog.

PRECLINICAL STUDIES AND ANIMAL TRIALS

In vitro, both free and liposomal MTP-PE are potent activators of monocytes and macrophages. Increased tumoricidal activity on melanoma and sarcoma cell lines has



been measured with murine alveolar macrophages (AM), Kupffer cells, or peritoneal macrophages after treatment with this molecule [6, 15, 16]. Interestingly, tumor cells surviving six sequential exposures to activated peritoneal macrophages did not show any reduction in susceptibility to macrophage killing compared to the parent cell line: thus, tumor cell destruction by activated macrophages was nonselective and did not lead to the development of resistant cells [6]. Tumor killing by L-MTP-PE-treated rat liver macrophages was reduced by about 75% with anti-TNF- α antibodies, indicating that TNF- α may be involved in anti-tumor activity [17].

In vitro-treated human monocytes killed allogeneic and autologous tumors (including melanoma, ovarian, colon, and renal carcinoma [7, 18-20]), but had no by-stander toxicity towards normal cells, even when mixed with the tumor targets [4, 21]. Importantly, several of these studies showed that monocytes from cancer patients could be activated to similar levels of tumoricidal activity as cells from healthy donors [7, 18-20].

Increased tumoricidal activity by isolated murine Kupffer cells and AM [15, 22, 23], dog monocytes and AM [5, 24] was also measured *ex vivo*, that is after treatment of the

animals with L-MTP-PE and isolation of cells. Dog AM cytotoxicity was even greater if the animals were treated with both doxorubicin and L-MTP-PE [5].

Cell activation was associated with production of cytokines and other inflammatory factors, including TNF- α , IL-6, IL-8, IL-1, nitric oxide, PGE₂ [4-9, 17, 25]. Fig. (2A) shows that human macrophages stimulated *in vitro* with MTP-PE secrete TNF- α , IL-1b, IL-6 and increased IL-8. No IL-12p70 and IL-10 were however detected. Asano and colleagues reported that the inflammatory chemokine CCL2 was also induced [26]. On human monocytes, L-MTP-PE stimulated up-regulation of the adhesion molecules LFA-1 and ICAM-1, which could be important for interaction with tumor cells; indeed, anti-LFA-1 antibody inhibited killing of melanoma cells [27].

Several studies demonstrated that IFN- γ synergizes with MTP-PE to increase tumoricidal activity and to induce secretion of cytokines [20, 23, 28-31]. We found that MTP-PE synergizes with IFN- γ in inducing tumor killing by human macrophages, and augmenting secretion of TNF- α and IL-1b (Fig. (2)). The mechanism for this potentiation is not known, but a 2-fold increase in liposome phagocytosis was observed after treatment of human monocytes with IFN- γ [29].

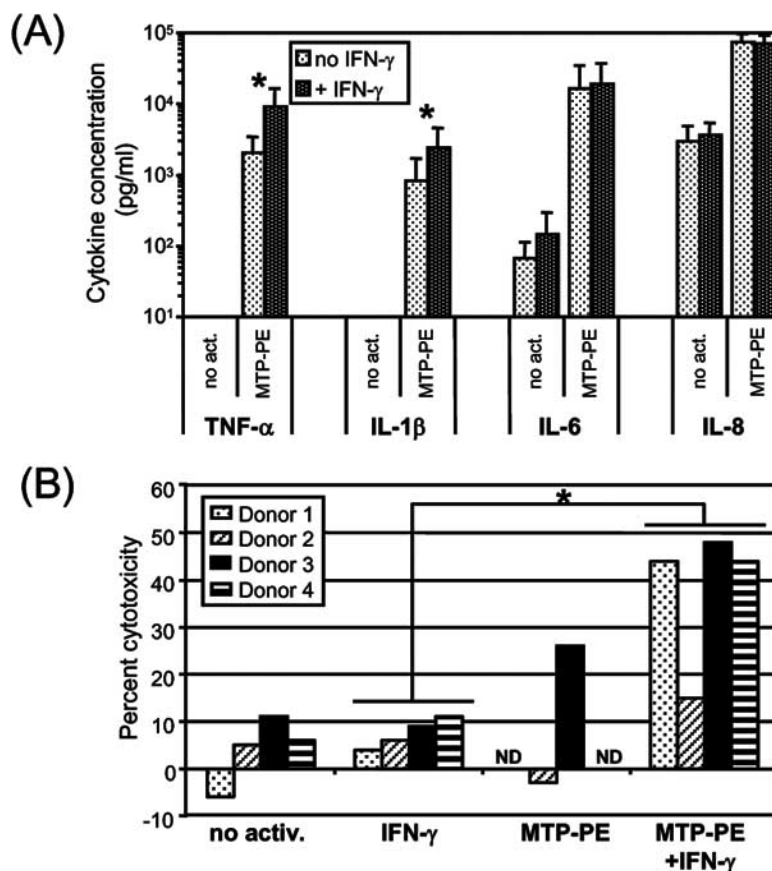


Fig. (2). MTP-PE, in synergy with IFN- γ , activates tumoricidal properties and secretion of TNF- α , IL-1b, IL-6, IL-8 in human macrophages. Macrophages were differentiated from monocytes by a 6-day incubation of total apheresis in the presence of GM-CSF [83]. The cells were then treated for 18 h with 1 μ g/ml MTP-PE +/- 166 U/ml of IFN- γ . **A.** Cytokine concentration (mean and SD from 4 independent experiments) was measured in culture supernatants harvested 18 h after cell activation. No IL-12p70 or IL-10 were detected. **B.** Activated and non-activated macrophages were purified and their tumoricidal activity against the human breast cancer cell line SK-BR-3 was evaluated in a 48-h cytotoxicity assay, at effector:target ratio of 20:1. Data from 4 independent experiments are shown. * $p < 0.05$ by paired one-tailed Student's t test. ND, not determined.

Consistent with the *in vitro* findings, intravenous injection of L-MTP-PE showed therapeutic activity in several models of experimental metastasis or neoplasia. In the B16 melanoma model developed by Fidler and colleagues, spontaneous lung and lymph node metastasis occur subsequent to the growth of the primary tumor implanted in the footpad of mice. Injection of L-MTP-PE after surgical removal of the primary tumor increased survival from 10% (control animals receiving placebo liposomes) to 70% [32]. Regression of established metastasis was associated with tumoricidal macrophages residing within the metastasis. Efficacy was dependent on the regimen and the tumor burden at the moment of treatment: best results were obtained starting the treatment 3 days after tumor removal, twice weekly for 4 weeks [33]. When the beginning of the treatment was delayed, the efficacy decreased, probably due to large metastatic lesions. Importantly, lung cells from surviving mice injected s.c. into syngeneic hosts did not develop tumors, indicating complete eradication by the treatment. Synergy with IFN- γ was also observed in this *in vivo* model, provided that IFN- γ was present in the same liposomes as MTP-PE [23].

In addition, i.v. L-MTP-PE delayed tumor growth and increased animal survival in mice with UV-induced skin tumors [34, 35], and in rats with liver metastasis [36, 37].

Clinical trials were conducted at the University of Wisconsin in dogs and cats with various malignancies: osteosarcoma, splenic hemangiosarcoma, melanoma or mammary adenocarcinoma. Results from these studies are particularly relevant because spontaneous tumors in these animals have similar characteristics and undergo similar treatment interventions as in humans. L-MTP-PE was administered as adjuvant treatment after resection of primary tumor, at a dose of 2 mg/m² twice weekly for 8 weeks, combined or not with chemotherapy (doxorubicin and cyclophosphamide, or cisplatin). MacEwen and colleagues observed significantly longer disease-free survival (DFS) and overall survival (OS) in dogs with osteosarcoma, splenic hemangiosarcoma or early-stage melanoma treated with L-MTP-PE [38-42]. Some of the data from these trials are summarized in Table 1. In contrast, L-MTP-PE had no effect in cats and dogs with mammary tumors after mastectomy [43, 44]. This lack of efficacy might have been due to a suboptimal activation of local macrophages: while some pulmonary recurrences were seen in the placebo group, recurrences in the treated group occurred only locally,

possibly as a consequence of the selective activation of lung macrophages [44].

Routes of administration other than i.v. were tested in some pre-clinical models. Given orally to mice, free or liposomal MTP-PE (phosphatidylcholine liposomes) activated both alveolar and peritoneal macrophages, and inhibition of lung and lymph node metastasis was observed [45, 46]. Intranasal or intratracheal MTP-PE stimulated macrophages and granulocytes in the lungs of rat, and increased mice survival in the B16 lung metastasis model [47, 48]. Intraperitoneal treatment enhanced survival of mice with i.p. syngeneic fibrosarcoma or human ovarian xenografts [49, 50]. Intravesical treatment with L-MTP-PE reduced tumor incidence in nude mice grafted orthotopically with human bladder carcinoma; activated macrophages were found in the bladder of treated mice [31]. Thus, MTP-PE may be active in several indications, provided that the drug is efficiently delivered to local macrophages.

INSIGHTS ON THE MECHANISMS OF ACTION

The outcome of L-MTP-PE administration is likely to be the result of

- 1- the cellular targets of the liposomes
- 2- the cellular receptor expression
- 3- additional indirect effects secondary to activation of the primary cellular targets.

I.v.-injected liposomes are taken-up by macrophages adjacent to sinusoids in liver and spleen. They also accumulate in lung capillaries, from which they cannot however spontaneously extravasate [51]. Circulating monocytes, rapidly activated by L-MTP-PE in the lung capillaries, can however cross the endothelium, be recruited to the alveoli as activated AM, and act at the level of the micrometastasis. One day after L-MTP-PE administration to mice, 15% of AM presented internalized liposomes [32].

Although both MTP-PE and lipopolysaccharide, a common monocyte/macrophage activator binding to TLR4, have been shown to activate the MAP kinases ERK-1/2 and the transcription factors NF- κ B and AP-1, the signaling pathways of the two molecules are clearly distinct [9, 17]. Fogler and Fidler found that MTP-PE is taken up by monocytes in a non-saturable, receptor-independent fashion, and therefore suggested that its receptor may be intracellular

Table 1. Randomized Clinical Trials in Dogs: Median Survival Times after Treatment with L-MTP-PE or Placebo Liposomes in Adjuvant Setting

Indication	Design	Median survival time	p-value	Ref.
Osteosarcoma	L-MTP-PE or placebo immediately after surgery	L-MTP-PE: 222 d Placebo: 77 d	<0.002	[38]
Osteosarcoma	After cisplatin (non-metastatic dogs)	L-MTP-PE: 14.4 mo Placebo: 9.8 mo	<0.01	[39]
Osteosarcoma	Together with cisplatin	L-MTP-PE: 10.3 mo Placebo: 7.6 mo	Not significant	[39]
Hemangiosarcoma	Together with doxorubicin and cyclophosphamide	L-MTP-PE: 277 d Placebo: 143 d	0.029	[40]

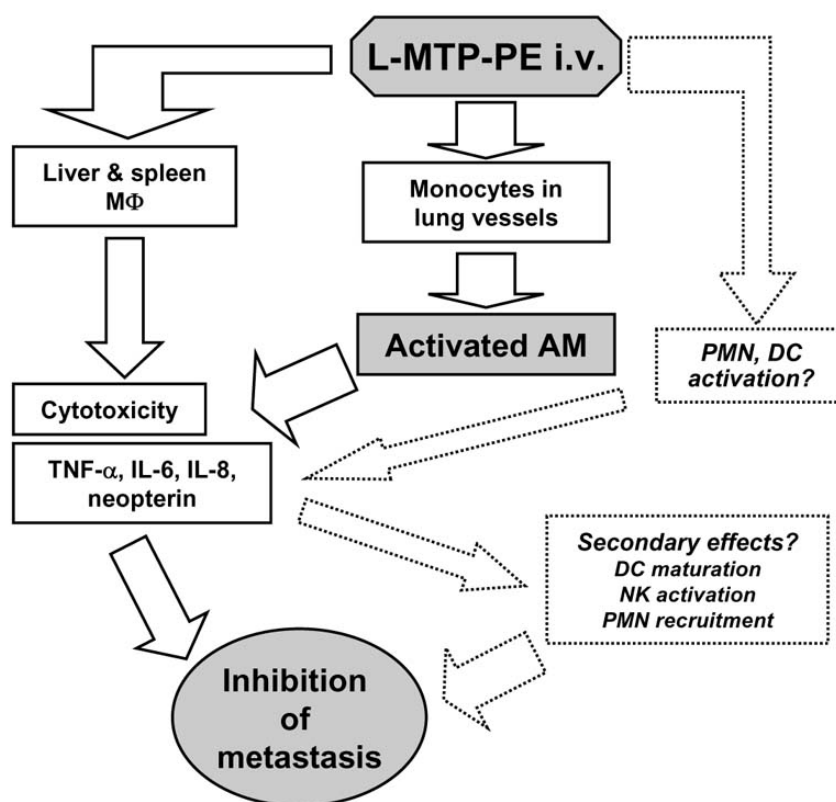


Fig. (3). Direct and indirect cellular targets of intravenous administration of L-MTP-PE. Tumoricidal macrophages are elicited in lungs, liver and spleen. They secrete cytokines and chemokines that may directly affect the micrometastasis, and increase the recruitment and activation of other cells, including dendritic cells, granulocytes, NK cells.

[10]. Bacterial-derived structures are often recognized by members of the Toll-like receptor family. In particular, the TLR2/TLR6 heterodimer is known to bind biacetylated lipopeptides like MALP-2. As MTP-PE is itself biacetylated, and TLR2 can be found intracellularly, in the phagosomal compartment [52], an interaction between the two molecules was conceivable. On the other hand, recent evidence indicates that MDP, the natural precursor of MTP-PE, signals through Nod2, an intracellular protein that induces NF- κ B activation and is implicated in innate immune defense against bacteria [53]. Nod2 is predominantly expressed in cells of the myeloid lineage, including monocyte/macrophages, myeloid dendritic cells, and granulocytes [54]. D. Philpott and colleagues recently found that MTP-PE is a Nod2, but not TLR2, agonist (J. Fritz *et al.* personal communication).

Thus, the cellular targets of the liposomes seem to be the same cells that also express the receptor for MTP-PE. *In vivo*, phagocytic cells of the myeloid lineage take up the liposomes containing MTP-PE and slowly degrade the multilamellar vesicles in the endo-lysosomal compartment [10]. MTP-PE is released into the cytosol where it interacts with Nod2, and the cells are consequently activated. Although the anti-tumoral activity of activated macrophages is far from being fully understood, direct tumor killing through TNF- α or other cytokines is a possible mechanism [17] (Fig. (3)).

There is direct evidence that the anti-metastatic activity of L-MTP-PE is due to macrophages. In the murine model

of melanoma lung metastasis, Fidler and colleagues showed that when MDP liposomes were not retained in the lung vessels (a particular type of liposomes entirely composed of phosphatidylcholine), they failed to activate AM or inhibit tumor growth [55]. More importantly, treatment with agents selectively impairing macrophage function (silica, carrageenan, hyperchlorinated drinking water) also resulted in lack of metastasis eradication by liposomal MDP, while depletion of NK or T cells had no effect. Finally, the authors demonstrated reduction of lung metastasis after adoptive transfer of syngeneic macrophages activated *in vitro*.

Despite this compelling experimental evidence for the role of macrophages, it seems difficult to exclude that effects secondary to their activation may contribute to L-MTP-PE anti-tumor activity *in vivo*. Talmadge and co-workers isolated activated NK cells from lung and liver of mice treated with L-MTP-PE. They also demonstrated, by NK cell depletion, that the anti-metastatic activity of L-MTP-PE in a prophylactic model was completely resting on NK cells [22]. As NK cells were not activated *in vitro* by L-MTP-PE, it is likely that their activation was induced by factors produced *in vivo* by macrophages. Dendritic cells (DC) maturation could also be stimulated during L-MTP-PE treatment, either directly (particularly for myeloid DC, which express the Nod2 receptor) [56], or indirectly, following inflammatory cytokine production by monocyte/macrophages. If matured, pulmonary DC that had taken up tumor antigens could migrate to the draining lymph nodes and stimulate a T cell response to the tumor. Finally, granulocytes may take up the liposomes and be

directly stimulated by L-MTP-PE, while their recruitment to the lung could be enhanced by IL-8 produced by activated macrophages (Fig. (3)). The precise role of these other cell types in the *in vivo* anti-tumor response triggered by L-MTP-PE awaits further investigations.

CLINICAL DEVELOPMENT: FROM PHASE I TO PHASE III

Safety and Biological Activity

Exploratory phase I clinical studies were conducted in approximately 150 patients with various advanced malignancies (colorectal, melanoma, renal cell carcinoma, lung, breast, stomach and salivary gland cancers as well as sarcoma) to determine L-MTP-PE maximum tolerated dose (MTD) and optimal biological dose (OBD) (Table 2) [57-60]. MTD was found to be 4-6 mg/m² whereas the OBD, based on monocyte activation, was 0.5-2.0 mg/m² [57]. Most side effects of L-MTP-PE were minimal and included fever and rigors in the majority of patients, some tachycardia and nausea in approximately half the patients, as well as chills, myalgias, malaise and fatigue. Side effects decreased in intensity and frequency during the course of treatment [58, 59, 61]. They were believed to result directly from L-MTP-PE biological activity, particularly the secretion of

inflammatory cytokines by activated monocytes [62, 63]. Biological activity was consistently observed during the phase I and II trials, and included: increased monocyte tumoricidal activity and secretion of IL-1, as well as increased TNF- α , IL-6, C-reactive protein, neopterin in plasma (but no IFN- γ or IL-1) [57, 59, 61-65]. Chronic treatment resulted in monocyte tachyphylaxis for cytokine secretion but not tumor cytotoxicity, which remained elevated [63].

In later trials, including the phase III study, the dose of L-MTP-PE was escalated in each patient until occurrence of fever, chills or increase in the C-reactive protein: from a starting dose of 2 mg/m² to a maximal dose of 2 mg/m² + 2 mg [66]. In the phase III study, where L-MTP-PE was associated to chemotherapy (see below), there was no difference among the treatment arms for most of the toxicities reported [66].

As fever and chills, the major side effects following L-MTP-PE infusion, could be prevented by administration of anti-inflammatory drugs, a series of studies were conducted to determine whether COX inhibitors interfere with L-MTP-PE action. *In vitro*, only high doses of ibuprofen suppressed the generation of tumoricidal phenotype, as well as IL-1 and TNF- α production, in monocytes treated with L-MTP-PE [67]. Therefore, for the management of L-MTP-PE side

Table 2. Clinical Trials with L-MTP-PE

Indication	Phase	Design	Ref.
Various metastatic	I	28 patients Dose escalation, PK	[57, 58, 62]
Advanced malignancies	I	37 patients Dose escalation, biological activity	[60]
Advanced malignancies	I	27 patients Dose escalation, biological activity	[59]
Metastatic cancers	I	14 patients PK, biological activity	[61]
Advanced melanoma	I	14 patients Biological activity	[63]
Stage III and IV melanoma	I/II	18 patients Adjuvant or neo-adjuvant Biological activity	[65]
Stage III and IV melanoma	I/II	18 patients	[70]
Relapsed Osteosarcoma	II	28 patients, historical ctr Randomized; Dose escalation	[64, 71, 73]
Soft tissue sarcomas	II	20 patients	[69]
Relapsed Osteosarcoma	IIB	9 patients Adjuvant or neo-adjuvant Combination with ifosfamide	[79]
Osteosarcoma less than 30 days after diagnostic	III	677 patients adjuvant Combination with chemotherapy; Randomized to chemotherapy alone	[66]

effects, the authors suggested not to exceed blood ibuprofen levels of 10 mg/ml. On the other hand, the anti-tumor activity of diclofenac and L-MTP-PE in a murine fibrosarcoma model were lost when the two agents were used in combination [68], indicating that COX inhibitors can attenuate the anti-tumor effect of L-MTP-PE *in vivo*.

Indication

Phase II studies were performed in various indications including melanoma, non-small cell lung carcinoma, colorectal cancer and osteosarcoma (Table 2).

A phase II study in 20 patients with metastatic soft tissue sarcoma failed to show any clinical efficacy [69].

A pilot study in melanoma patients at high risk of recurrence suggested a possible benefit in survival. Eighteen patients with either resectable stage III or resectable stage IV limited to lungs, lymph nodes and subcutaneous tissues received L-MTP-PE before (for 4 weeks) and after (for 20 weeks) resection. Mean survival was 80.5 months (range 69 to more than 91 months) with 4 patients remaining free of disease for more than 5 years after surgery [70].

The first signs of biological activity of L-MTP-PE at the level of lung metastasis were obtained in osteosarcoma patients who had recurrent lung nodules after treatment with L-MTP-PE; in these nodules, peripheral tumor fibrosis was frequently observed. This finding was clearly different from the central necrosis generally observed after chemotherapy and was therefore considered to result from a local effect of L-MTP-PE [71]. The incompleteness of the fibrosis encouraged the investigators to extend treatment duration in a second group of patients from 12 weeks to 24 weeks [72].

These phase II trials (described in more detail below) in patients with osteosarcoma provided the basis for the phase III trial design.

The choice of osteosarcoma as the indication was based on the aforementioned signs of biological activity, the availability of a clinically relevant model in dog, and the prominent medical need. It is however worth mentioning that Kleinerman *et al.* [73] suggested that other solid tumors in which lung metastases pose a treatment problem could benefit from further investigations with L-MTP-PE.

Combination with Chemotherapy

The probability of 5-year disease-free survival (DFS) for patients with osteosarcoma treated with surgery alone is less than 20% [74]. Incorporation of chemotherapy has significantly improved both DFS and overall survival (OS): patients presenting localized, primary extremity osteosarcoma and treated in a neo-adjuvant or adjuvant setting have now a 5-year event-free survival (EFS) above 70% [74]. Various chemotherapeutic agents and regimens have been tested, none of them showing any obvious superiority [75, 76]. For example, a study conducted by the European Osteosarcoma Intergroup failed to reveal any difference in survival of more than 400 patients randomized to receive a two-drug regimen (doxorubicin and cisplatin) or a complex multidrug regimen (vincristine, methotrexate, doxorubicin, bleomycin, cyclophosphamide, dactinomycin)

[77]. More recently, Meyers and colleagues showed that addition of ifosfamide to a three-drug regimen had no significant effect on DFS and OS [66].

Before testing the efficacy of combining chemotherapy and L-MTP-PE, it was first necessary to define whether cytotoxic agents interfere with monocyte activation by L-MTP-PE. Kleinerman and co-workers measured the tumoricidal activity of blood monocytes from osteosarcoma patients treated with cisplatin, high-dose methotrexate, cyclophosphamide or doxorubicin, following *in vitro* activation with L-MTP-PE. They found no difference with monocytes from normal donors, except when cyclophosphamide was used together with doxorubicin, a combination that inhibited monocyte activation [78].

Combination therapy was therefore evaluated during a phase IIb study (Table 2). Nine patients with pulmonary lesions that had developed during chemotherapy, or were present at diagnosis, persisted despite chemotherapy and recurred after surgical excision, were treated with ifosfamide and L-MTP-PE in either adjuvant or neo-adjuvant setting. No increase in ifosfamide toxicity was observed upon combination with L-MTP-PE. Serum cytokines and monocyte-mediated tumoricidal activity were not different from what had been reported following treatment with L-MTP-PE alone. Signs of tumor fibrosis and infiltration by inflammatory cells were observed in surgical specimens from patients in the neo-adjuvant group, strengthening the notion that chemotherapy does not mitigate L-MTP-PE activity and supporting the rationale for combination [79].

Clinical Efficacy

Another phase II trial (Table 2) was conducted in 28 osteosarcoma patients with lung metastases that developed during adjuvant therapy or that were present at diagnosis, persisted during therapy and recurred following surgical excision. After surgical resection, 12 patients received L-MTP-PE twice a week for 12 weeks (Group 1). Nodules that recurred after L-MTP-PE therapy were excised in 6 patients and compared with lesions that had been resected prior to study entry. Based on the type of the histologic changes observed in pulmonary lesions removed from patients in Group 1 (see above), the therapy was altered for the next 16 patients, who were treated for 24 weeks instead of 12. An historical control group (Group 3) included 21 patients who had been treated post-operatively with chemotherapy in the same institution. No significant improvement in progression-free survival was observed in the first group of patients compared to the historical controls. In the second group of patients, receiving L-MTP-PE for 24 weeks, progression-free survival was significantly longer than the historical control group: 9 months versus 4.5 months, respectively ($p < 0.03$) [73].

From November 1993 through November 1997, the Children's Cancer Group and the Pediatric Oncology Group (now collectively known as Children's Oncology Group) conducted a phase III study to assess whether the addition of L-MTP-PE and/or ifosfamide to a standard 3-drug chemotherapeutic regimen (doxorubicin, cisplatin and high-dose methotrexate) would increase DFS in newly diagnosed patients with high-grade osteosarcoma. The trial design

Table 3. Phase III Clinical Trials with L-MTP-PE: Design and Results from Prospective Analysis. A Total of 664 Eligible Patients with Non-Metastatic, Resectable Disease were Randomized to 4 Regimens

Regimen A: standard chemotherapy	Regimen A + L-MTP-PE (A ⁺)				
Regimen B: standard chemotherapy + ifosfamide	Regimen B + L-MTP-PE (B ⁺)				

	# of patients	Relapses or deaths ^a	DFS p-value	Deaths ^a	OS p-value
No L-MTP-PE (A and B)	332	123	-	80	-
+ L-MTP-PE (A ⁺ and B ⁺)	332	99	0.030	61	0.039

^a at the end of the study, corresponding to a median follow-up of 4.8 years.

implied analysis in patients with non-metastatic disease that was considered resectable at diagnosis, though patients with metastatic or unresectable disease were also allowed to be enrolled at selected sites. A total of 664 eligible patients with non-metastatic disease amenable to resection were enrolled and IDM evaluated them for disease-free and overall survival. No significant improvement in DFS or OS was observed by the addition of ifosfamide in the dose and schedule used in this study ($p=0.934$ and 0.992 respectively). However, significant improvement in DFS and OS were observed in patients randomized to receive L-MTP-PE ($p=0.030$ and 0.039 respectively, Table 3). In this group of patients, the risk of recurrence and death were decreased by 25% and 30% respectively. The 6-year survival probability went from 67.5% in the absence of L-MTP-PE to 77.2% when it was added to the treatment.

This study was a factorial design with patients randomized to one of four arms (3 drugs $\pm$ L-MTP-PE and 4 drugs $\pm$ L-MTP-PE) but with the intent to analyze based on (i) 3 versus 4 drugs and (ii) $\pm$ L-MTP-PE. Because of a suggested interaction between L-MTP-PE and ifosfamide (which was however not significant in the prospectively defined analysis), Meyers and colleagues performed an analysis of individual arms [66]. This analysis failed to reveal any significant differences in DFS or OS, but the trial was not powered for such comparison.

One hundred and thirteen patients entered the study with metastatic or unresectable disease. We analyzed this population separately. The trends in this group were the same as in the larger group with non-metastatic resectable disease, though the number was too small to observe any significant differences. When all eligible patients ($n=777$) were analyzed together, a highly significant improvement in progression-free ($p=0.011$) and overall ($p=0.015$) survival was observed in patients in the L-MTP-PE arms, with the risk of recurrence/progression and death reduced by 26% and 30%, respectively.

In summary, although this study raised many additional questions, including the best way to combine chemotherapy and L-MTP-PE, it clearly demonstrated the clinical benefit associated with L-MTP-PE in the treatment of osteosarcoma.

CONCLUSION

Activated macrophages have been used in adoptive therapies against various cancers, including bladder and ovary cancers, mesothelioma and glioblastoma, and may be able to eradicate microscopic but not macroscopic tumors [80, 81]. The mechanism of *in vivo* anti-tumor activity of activated macrophages is not fully understood, but it probably implies pathways completely or partially distinct from those used by chemotherapeutic agents. Despite many attempts over the past decade to improve treatment of osteosarcoma by chemotherapy, varying drugs and regimens, it appears that about 30% of the patients present chemotherapy-resistant tumor cells at diagnosis [73]. MTP-PE-activated monocytes and macrophages may eliminate some metastases, provided they are small enough. It could be speculated that the 10% increase in OS (from 67.5% to 77.2% at 6 years) observed in the phase III trial is due to a L-MTP-PE beneficial effect on a subset of patients with micrometastasis. The limited efficacy of activated macrophages on large metastatic lesions [80] would argue in favor of L-MTP-PE addition as early as possible.

Combination therapy has always been a standard practice in medical oncology. In the recent years, combination of chemotherapy with immunotherapy is of increasing interest in various types of cancer [82]. The demonstration that L-MTP-PE and chemotherapy can be used together effectively in the treatment of osteosarcoma is encouraging and there is great interest in attempting to optimize this combination to take further advantage of any potential synergy.

ABBREVIATIONS

AM	=	Alveolar macrophages
DFS	=	Disease-free survival
EFS	=	Event-free survival
IFN	=	Interferon
L-MTP-PE	=	Liposomal MTP-PE
MDP	=	Muramyl dipeptide
MLV	=	Multilamellar vesicles

MTD	=	Maximum tolerated dose
MTP-PE	=	Muramyl tripeptide phosphatidylethanolamine
NK	=	Natural killer
OBD	=	Optimal biological dose
OS	=	Overall survival
PK	=	Pharmacokinetics
TNF	=	Tumor necrosis factor

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