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Original Article

L-MTP-PE and zoledronic acid combination in osteosarcoma: preclinical evidence of positive therapeutic combination for clinical transfer

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Abstract: Osteosarcoma, the most frequent malignant primary bone tumor in pediatric patients is characterized by osteolysis promoting tumor growth. Lung metastasis is the major bad prognosis factor of this disease. Zoledronic Acid (ZA), a potent inhibitor of bone resorption is currently evaluated in phase III randomized studies in Europe for the treatment of osteosarcoma and Ewing sarcoma. The beneficial effect of the liposomal form of Muramyl-TriPeptide-Phosphatidyl Ethanolamine (L-mifamurtide, MEPACT®), an activator of macrophage populations has been demonstrated to eradicate lung metastatic foci in osteosarcoma. The objective of this study was to evaluate the potential therapeutic benefit and the safety of the ZA and L-mifamurtide combination in preclinical models of osteosarcoma, as a prerequisite before translation to patients. The effects of ZA (100 µg/kg) and L-mifamurtide (1 mg/kg) were investigated *in vivo* in xenogeneic and syngeneic mice models of osteosarcoma, at clinical (tumor proliferation, spontaneous lung metastases development), radiological (bone microarchitecture by microCT analysis), biological and histological levels. No interference between the two drugs could be observed on ZA-induced bone protection and on L-mifamurtide-induced inhibition of lung metastasis development. Unexpectedly, ZA and L-mifamurtide association induced an additional and in some cases synergistic inhibition of primary tumor progression. L-mifamurtide has no effect on tumor proliferation *in vitro* or *in vivo,* and macrophage population was not affected at the tumor site whatever the treatment. This study evidenced for the first time a significant inhibition of primary osteosarcoma progression when both drugs are combined. This result constitutes a first proof-of-principle for clinical application in osteosarcoma patients.

Keywords: Osteosarcoma, liposomal mifamurtide, zoledronic acid, inhibition of lung metastases development, bone protection

Introduction

Osteosarcoma is the most frequent malignant primary bone tumor in young people with around 1000 new cases per year in Europe [1, 2]. It arises mainly in adolescents and young adults (median age 18 years), with a preferential location in the metaphysis of long bones. The standard treatment of osteosarcoma consists in multi-drug chemotherapy as neo-adjuvant and adjuvant settings associated with surgical local control [3, 4]. Despite recent advances in limb-salvage surgery and poly-chemotherapy combinations, event-free survival and overall survival have only slightly improved during the last decades remaining around 65-70% at 5 years for localized forms and only 25% for patients who present pulmonary metastases at diagnosis. The current drug regimens used for osteosarcoma pediatric patients associate MTX-VP-IFO (High dose methotrexate, Vincristin, platinium and ifosfamide) or API-AI for adults (adriamycin-platinium-ifosfamide). However, patients who do not respond to chemotherapy have an overall survival of only 20-25% at 5 years [5, 6]. Therefore, new therapeutic targets are urgently needed for these patients at high risk.

Recently, L-mifamurtide (Liposomal-Muramyl TriPeptide-PhosphatidylEthanolamine: L-MTP-PE) has been proposed as adjuvant therapy for osteosarcoma patients in the United States. It is a synthetic analog of the muramyl dipeptide (MDP), resulting from the covalent addition of alanin and dipalmitoylphosphatidyl ethanolamine to MDP, a peptidoglycan found in bacterial cell wall [7]. L-mifamurtide acts as a nonspecific immunomodulator by activating macrophages and monocytes related to the upregulation of tumoricidal activity and secretion of pro-inflammatory cytokines including tumor necrosis factor (TNF)-a, interleukin (IL)-1, IL-6, IL-8, IL-12, nitric oxide (NO), prostaglandin E2 (PGE2) and PGD2 [8-10]. The lipid composition of mifamurtide has been developed to target delivery of the drug selectively to monocytes and macrophages in liver, spleen and lungs [7, 11, 12]. It also facilitates signaling via phosphatidyl serine recognition by macrophages [13]. Moreover, the liposomal formulation can improve the safety profile of several drugs by modifying parent drug or solubilization agent toxicity [14]. In 2011, Buddingh and *al.* reported that tumor-associated-macrophages (TAMs) are associated with good prognosis in high grade osteosarcoma [15]. In contrast to most other tumor types, TAMs are associated with reduced metastasis and improved survival for those patients. A phase III randomized clinical trial was conducted by the Children's oncology group (COG) from 1993 to 1997 [16]. Significant improvement in EFS and overall survival were observed in patients randomized to receive L-mifamurtide [7, 17, 18]. In the European Union, except in France, L-mifamurtide is indicated in patients aged between 2 and 30 years with high-grade, resectable, nonmetastatic osteosarcoma after the complete surgical wide resection.

In France, OS2006 is the current clinical protocol for pediatric and adult osteosarcoma patients, a phase III randomized study associating Zometa® (ZA, zoledronic acid) with conventional poly-chemotherapy and surgery. This protocol is based on preclinical studies from our group demonstrating the advantage of combination of antitumor therapy with drugs that target the bone microenvironment [19, 20]. Indeed, interactions between tumor cells and bone cells are closely regulated in the so called "vicious cycle" as initially proposed by Paget [21]. Tumor cells proliferating in bone produce osteoclast activating factors such as PTH-rP, IL-11, IL-6 or the main regulator of osteoclast function: Receptor Activator of NF-κB Ligand (RANKL). When activated, osteoclasts are able to degrade bone, and allow the release of growth factors stored in the bone matrix such as Transforming Growth Factor-b, Insulin-like Growth Factor-1, Fibroblast Growth Factor... that in turn activate tumor cell proliferation [22].

The third generation nitrogen-containing Bisphosphonates (N-BPs) and among them zoledronic acid are potent inhibitors of osteoclast functions resulting in inhibition of bone resorption [23, 24]. In addition, N-BPs slow down tumor growth in bone, both indirectly by inhibiting osteoclast activation and directly by inhibition of tumor cell proliferation, angiogenesis, migration and activation of γδT lymphocytes [25, 26]. Therefore, these drugs are currently used as therapeutic agents in pathologies associated with bone degradation from tumor origin or not (osteoporosis, multiple myeloma, bone metastases and primary bone tumors) [27, 28].

Considering that osteolysis promoting tumor growth and lung metastasis dissemination are the two key points in osteosarcoma progression, it is tempting to propose ZA and L-mifamurtide combination in osteosarcoma treatment, each of these drugs already proving their efficacy. Before clinical application, preclinical studies are needed to see whether L-mifamurtide could interfere with ZA induced prevention of associated bone lesions, and conversely whether ZA may interfere with L-mifamurtide induced inhibition of lung metastasis dissemination.

Material & methods

Cell culture

Human KHOS and murine K7M2 osteosarcoma cell lines were purchased from the American Type Culture Collection (ATCC, LGC Promochem, Molsheim, France) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Switzerland). Murine MOS-J (Jackson Laboratory) osteosarcoma cell line was cultured in Roosevelt Park Memorial Institute (RPMI) medium (Lonza). Media were supplemented with 5% fetal calf serum (Hyclone, USA). All cell lines were cultured in a humidified 5% CO_2/air atmosphere at 37°C.

Table 1. Primer sequences used in quantitative real-time PCR analysis

Gene	Forward primer	Reverse primer
hGAPDH	TGGGTGTGAACCATGAGATATG	GGTGCAGGAGGCATTGCT
mHPRT	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
mF4/80	TCCTCCTTGCCTGGACACT	GCCTTGAAGGTCAGCAACC
mFas	TGAATGGGGGTACACCAACC	TGCTCTTCATCGCAGAGTGT
hFas	GAAGGGAAGGAGTACACAGACA	TGGTATTCTGGGTCCGGGTG
CD64	AGGGCGGAAAGAGAAGATGC	ATGACTGGGGACCAAGCACT

Drugs

L-mifamurtide was provided by TAKEDA (IDM PHARMA SAS). Each vial containing 4 mg of mifamurtide powder in 1 g excipient was divided into 8 tubes and stored at 4°C. Powder was reconstituted extemporaneously in sodium chloride 0.9% and used within 6 hrs. ZA, 1-hydroxy-2-(1H-imidazole-1-yl) ethylidene-bisphosphonic acid supplied as the disodium salt by Novartis Pharma AG (Basel, Switzerland), was dissolved in PBS as 10 mM stock solution and stored at -20°C. Liposome-PBS (clodronateliposome.com, Netherlands) was used as control.

Cell proliferation

Osteosarcoma cell lines were plated in respective media and treated with L-mifamurtide or zoledronic acid at indicated concentration for 48 hours, cell growth being measured using crystal violet assay as described previously [29].

Quantitative reverse transcription-PCR

Total RNA was extracted from mice osteosarcoma biopsies (24 h after the last drug injection) using Direct-zol RNA MiniPrep (Zymo Research) with DNase treatment to remove residual genomic DNA. RNA quantity and quality was evaluated by determining A260/A280 ratio using NanoDrop. Complementary DNA was synthesized from isolated RNA using ThermoScript real-time polymerase chain reaction (RT-PCR) System (Invitrogen, Carlsbad, CA, USA). Quantitative-PCR (qPCR) was performed using primers (**Table 1**) on Chromo4 instrument (Biorad, Richmond, CA, USA) using SYBR Green Supermix reagents (Biorad). Target gene expression was normalized to GAPDH levels in respective samples as an internal standard, and the comparative cycle threshold (Ct) method was used to calculate relative quantification of target mRNAs. Each assay was performed in triplicate.

Animal models of osteosarcoma

Five week-old female Rj: NMRI nude mice for xenogeneic models or C57BL/6, BALB/c mice for syngeneic models were purchased from Janvier Breedings (Le Genest Saint Isle, France). Mice were anesthetized by inhalation of an isoflurane/air mixture (2%, 1 L/min). Primitive osteosarcoma was induced by intramuscular paratibial injection of 10^6 human KHOS osteosarcoma cells (xenogeneic model) or 3.10^6 MOS-J or K7M2 mice osteosarcoma cells respectively in C57BL/6 and BALB/c mice (syngeneic models). Tumor development is associated to osteolytic lesions and with dissemination of spontaneous lung metastasis mimicking the human pathology. The day after tumor cell injection, mice were randomly assigned (n=8-10) to vehicle (NaCl 0.9%), ZA, L-mifamurtide or ZA+L-mifamurtide (bitherapy) groups. ZA (100 µg/kg; s.c.) or L-mifamurtide (1 mg/kg; i.v.) was injected twice per week. Tumor volume was measured three times weekly (length*width*depth*0.5). Data points were expressed as mean tumor volume ± SEM.

Mice were sacrificed when the tumor volume reached 10% of body weight for ethical reasons. Primary bone tumor was harvested for immunohistochemistry analysis, lung metastases were macroscopically counted under a binocular loupe. Animal care and experimental protocols were approved by the French Ministry of Agriculture (agreement n°D-44015 for the Experimental Therapy Unit located at the Medical School of Nantes) and were realized in accordance with the institutional guidelines of the regional ethical committee (CREEA Pays de la Loire, PdL06, France), with the protocol agreement n°1280.01 and 1281.01, and under the supervision of authorized investigators.

Immunohistochemistry

Immunohistochemical stainings were performed on formalin-fixed and paraffin-embedded

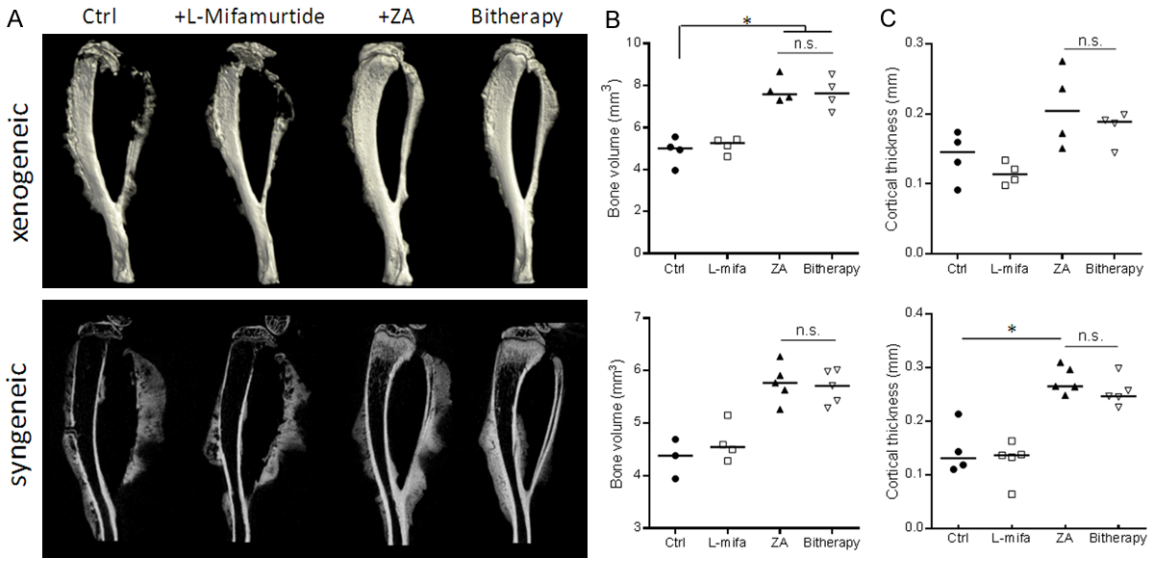

Figure 1. L-mifamurtide does not interfere with ZA-induced bone protection. A. Representative microCT and 3D reconstruction of the tumor-bearing tibia taken *ex-vivo*, from KHOS xenogeneic *(upper panel)* and MOS-J syngeneic *(lower panel)* models treated or not (Ctrl) with L-mifamurtide (1 mg/kg), ZA (100 µg/kg), or both (bitherapy) at day 1, twice a week, until week 4-5. B. MicroCT quantification of tibia bone volume (BV, mm^3) and C. Tibia cortical thickness (Co.Th, mm) were calculated on the tibia of tumor bearing mice of the different groups. Median were represented for each group (n=4). *p<0.05.

3 µm sections of tumor samples using adequate primary antibody. Immunodetection performed using secondary biotinylated antibodies and streptavidin HRP-complex was revealed with 3,3'-diaminobenyidine (DAB-Dako) followed by counterstaining with hematoxylin. Negative control was analyzed using a similar procedure without primary antibody. Same slides were used to estimate the percentage of necrosis area among the total tumor.

Micro-CT

Analyses of the bone microarchitecture were performed using a SkyScan 1076 *in vivo* micro-CT scanner (SkyScan, Kontich, Belgium). Tests were performed at early time (d21, tumor volume around 500 mm^3) or at necropsy (tumor volume around 2000 mm^3). All tibiae/fibulae were scanned using the same parameters (pixel size 18 µm, 50 kV, 0.5-mm Al filter and 0.7 degree of rotation step). Three-dimensional reconstructions and analysis of bone parameters were performed using NRecon and CTan softwares (SkyScan). Calculation of cortical bone volume (BV) following 3D morphometric parameters (bone ASBMR nomenclature) was performed on 5.5-7.2 mm of tibia length (depending on mice model) from the fibula insertion. This area corresponds to bone in close contact with osteosarcoma and excludes trabecular bone. Cortical thickness (Ct.Th) was defined as the mean cortical volume divided by the outer bone surface as previously described [30]. Trabecular bone parameters were also analyzed.

Statistical analysis

GraphPad InStat v3.02 software (La Jolla, CA, USA) was used. In vivo experimentation results were analyzed with the unpaired nonparametric method and Dunn's multiple comparisons following the Kruskal-Wallis test. A *p* value of less than 0.05 was considered statistically significant.

Results

L-mifamurtide does not interfere with ZA-induced bone protection

The first objective of the study was to investigate the potential interference of L-mifamurtide treatment with the protective zoledronic acid effects on bone during osteosarcoma progression. Two syngeneic and one xenogeneic models of osteosarcoma were used: respectively K7M2 and MOS-J induced in the BALB/c and C57BL/6 mouse strains, and KHOS induced in NMRI-nude mice. The day after tumor cell injec-

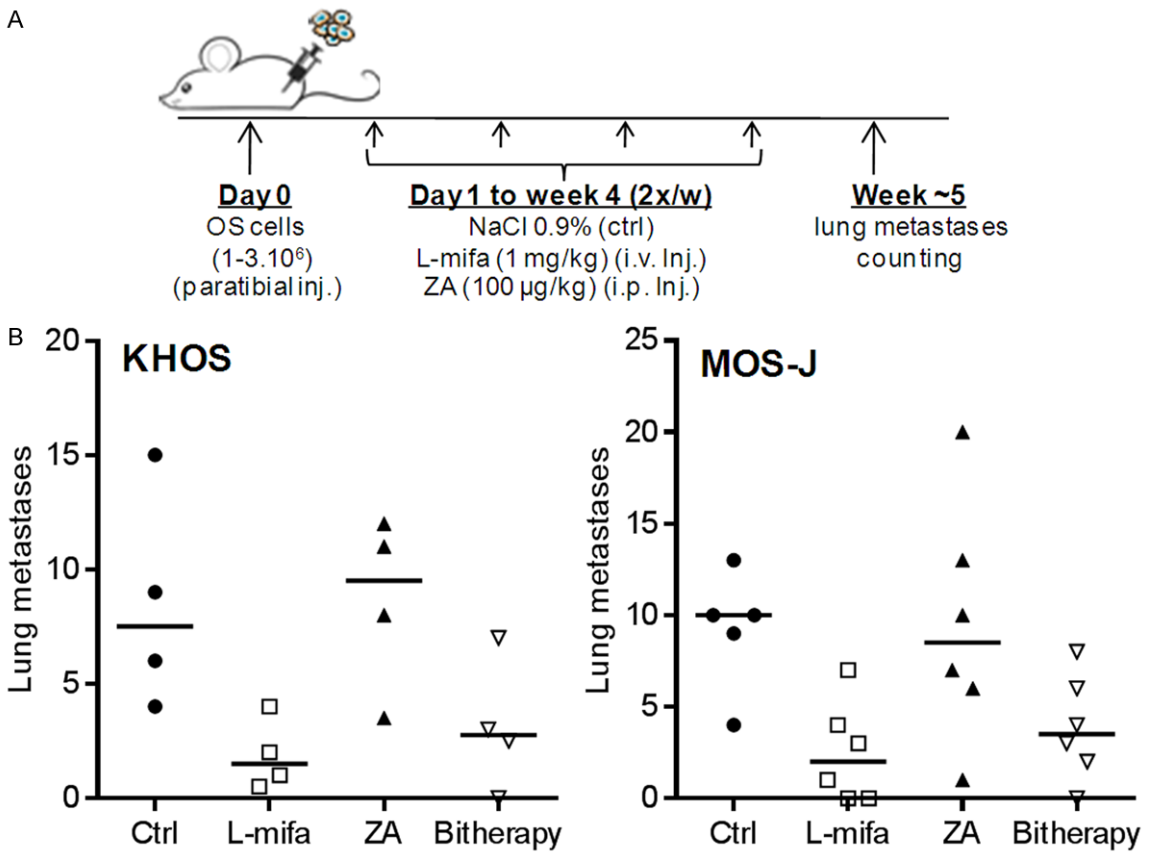

Figure 2. Treatment of osteosarcoma bearing mice with L-Mifamurtide alone or in association with ZA inhibits lung metastases development in both xenogeneic (KHOS) and syngeneic (MOS-J) models. A. NMRI-nude or C57BL/6 mice were injected respectively with KHOS (xenogeneic) or MOS-J (syngeneic) osteosarcoma cells and treated with vehicle (Ctrl), L-mifamurtide, ZA or both (bitherapy) as described in **Figure 1**. B. Graphs indicate individual (dots) and median (lines) numbers of lung metastases macroscopically counted in mice lungs from each group at equivalent primary bone tumor volume (2000 mm^3).

tion, mice were treated with ZA (100 µg/kg) and/or L-mifamurtide (1 mg/kg) twice a week. The bone microarchitecture parameters of the tumor-bearing tibia have been measured in all models and treatment conditions using X-ray, micro-CT and 3D reconstruction analysis (**Figure 1A**). Data revealed a decrease of the tumor-associated osteolysis in the ZA-treated mice as compared to the untreated control group. Addition of L-mifamurtide did not modulate ZA induced bone protection in all models tested (**Figure 1**). An extensive analysis of multiple bone parameters revealed an increase of tumor-associated bone quality in the ZA treated groups as compared to control group. In the xenogeneic KHOS mouse model, ZA treatment alone and combined with L-mifamurtide increased the cortical bone volume (BV) (from 4.88 to 7.79 and 7.62 mm^3 respectively as compared to control group, $p<0.05$; **Figure 1B**), the cortical thickness (Co.Th) (from 0.14 to 0.21 and 0.18 mm; **Figure 1C**), and trabecular parameters (not shown). Similar observations were made with the syngeneic mouse model of osteosarcoma MOS-J (**Figure 1A-C** lower panel), in which L-mifamurtide treatment did not affect the tumor-associated bone preservation as compared to control group.

Zoledronic acid does not interfere with L-mifamurtide-induced inhibition of lung metastasis dissemination and development

The second objective was to investigate the effect of L-mifamurtide and ZA on spontaneous metastasis dissemination from primary bone tumors induced in xenogeneic and syngeneic osteosarcoma models. Mice were treated by ZA and L-mifamurtide during ~5 weeks (**Figure 2A**) and metastases were counted at equivalent

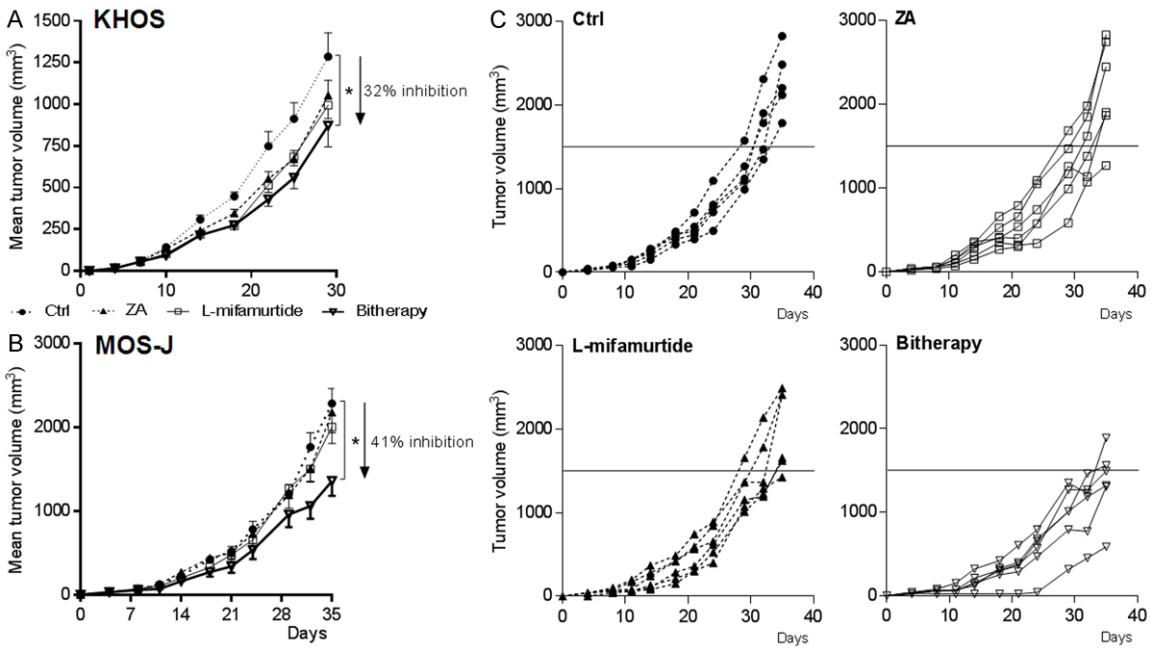

Figure 3. L-mifamurtide acts with ZA to reduce tumor growth both in syngeneic (MOS-J) and xenogeneic (KHOS) mouse models of osteosarcoma. Mice were treated with L-mifamurtide (1 mg/kg) and ZA (100 μg/kg) or both starting at day 1 after tumor cell injection as described in **Figure 1**. The mean tumor volume from (A) xenogeneic KHOS and (B) syngeneic MOS-J mice models, and individual tumor volumes (C) from xenogeneic KHOS model were compared between the 4 groups ± SEM (n=8). *p<0.001.

tumor volume. Whatever the model, a trend of diminished spontaneous lung metastasis dissemination was observed in L-mifamurtide treated groups (**Figure 2B**), and still when animals were treated with ZA combined with L-mifamurtide. No significant differences could be observed probably because of the high variability and the small animal number in each group. No difference was observed between control and zoledronic acid treated groups.

The combination of ZA and L-mifamurtide shows no interference on ZA induced bone protection effects and L-mifamurtide induced inhibition of lung metastasis development both in syngeneic and xenogeneic models of osteosarcoma in mice. ZA is known to have direct anticancer effects *in vitro* and *in vivo* in primary and secondary bone tumors [20]. To go further, we investigated whether L-mifamurtide would affect the antitumor effect of ZA on osteosarcoma primary bone tumor.

In vivo significant inhibitory effect of L-mifamurtide associated with zoledronic acid on primary bone tumor progression in syngeneic and xenogeneic models of osteosarcoma

The effect of L-mifamurtide and ZA therapeutic association was analyzed on primary tumor growth in syngeneic (MOS-J) and xenogeneic (KHOS) osteosarcoma models. In both cases, the tumor cells were injected in close contact to the tibia, after periosteum activation. The tumors were apparent within 10 days, and develop for 4-5 weeks. In each model, L-mifamurtide or ZA alone did not induce any significant effect on primary tumor development (**Figure 3A**, **B**). However, the combination of both drugs significantly inhibited tumor growth in xenogeneic and syngeneic models. The mean tumor size at 22 days for the xenogeneic KHOS model was 1285 ± 143 mm^3 in the control group, compared to 872 ± 128 mm^3 in mice treated with bitherapy (means ± SEM, p<0.001; **Figure 3A**). The mean tumor size at 35 days for the syngeneic MOS-J model was 2284 ± 176 mm^3 in the control group, compared to 1359 ± 177 mm^3 in the group treated with bitherapy (means ± SEM, p<0.001; **Figure 3B**). Similar results were obtained in the K7M2 syngeneic mice model (not shown). In summary, combinatory therapy induced 41% and 55% inhibition of mean tumor volume in syngeneic models (MOS-J and K7M2 respectively) and 37% in the xenogeneic model (KHOS) at final time point.

In both syngeneic models, the mean decrease in tumor volume of mice treated with ZA and

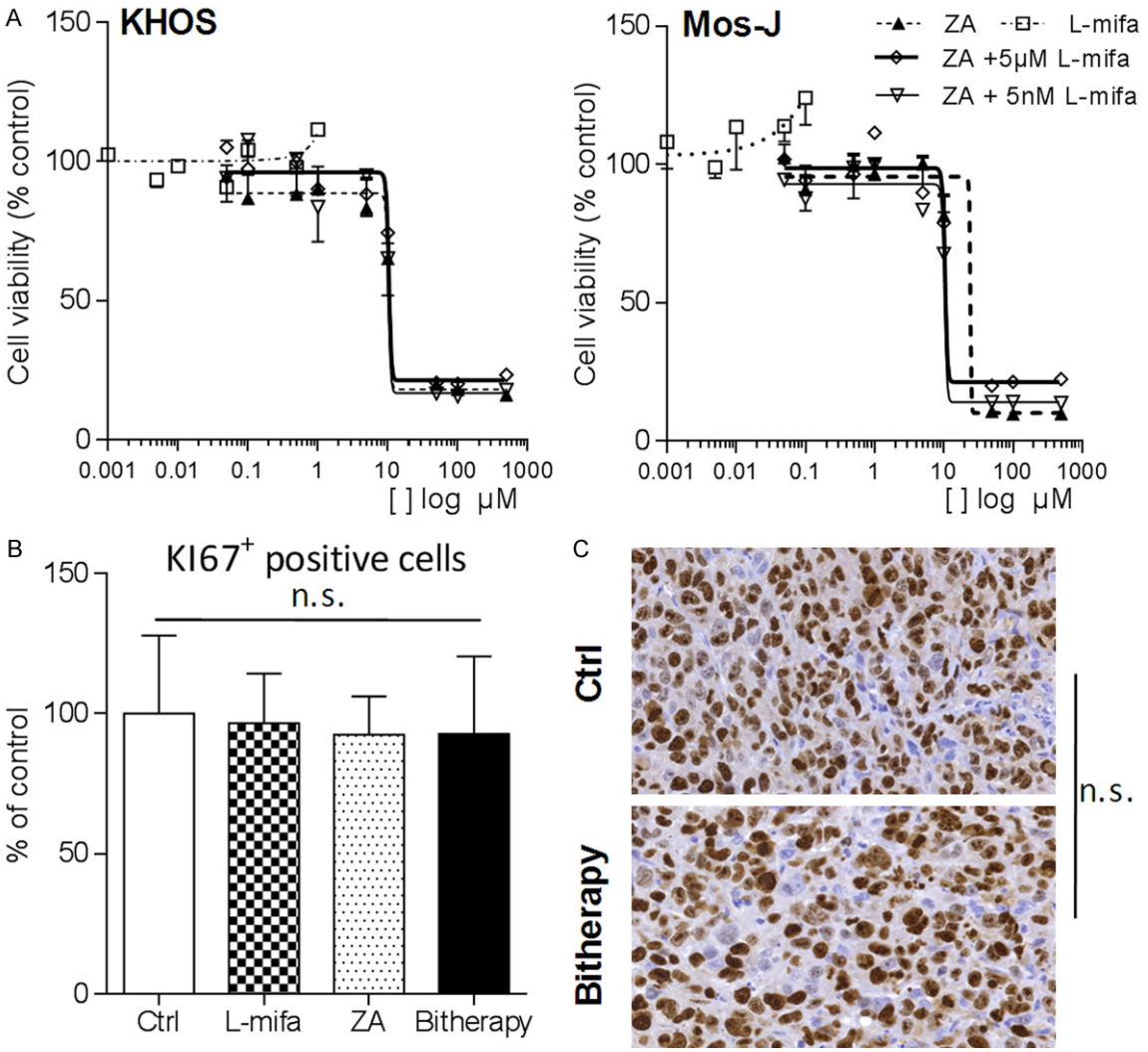

Figure 4. L-mifamurtide alone or associated with ZA does not directly affect tumor cell proliferation *in vivo* and *in vitro*. A. Osteosarcoma cells were treated with ZA, L-mifamurtide or both at indicated doses for 48 h. Cell growth was determined by crystal violet analysis and compared to control untreated cells. B. KHOS tumor biopsies were collected and Ki67 staining was evaluated by immunohistochemical analysis. Specimens were scored and estimated in percentage mean of $Ki67^+$-cells compare to the control group, which corresponds to the mice bearing tumor that did not receive any treatment but vehicle. C. Representative picture of Ki67 staining for control and bitherapy treated groups.

L-mifamurtide reflects a slowing tumor growth in some mice, and no response in others (**Figure 3C**). These unexpected results demonstrate that L-mifamurtide acts with ZA to reduce primary tumor growth *in vivo*.

L-mifamurtide does not affect osteosarcoma cell proliferation in vivo or in vitro

In order to analyze the mechanisms involved in the tumor growth inhibition observed *in vivo* with the L-mifamurtide + ZA therapeutic association, the direct effect of each drug alone and in combination was studied on osteosarcoma cell proliferation *in vitro,* and *in vivo* by Ki67 staining on tumor biopsies. Results show that L-mifamurtide alone has no direct effect on the proliferation rate of the two osteosarcoma cell lines MOS-J and KHOS *in vitro* (**Figure 4A**). In addition, L-mifamurtide does not significantly modify the ZA-induced inhibition of osteosarcoma cell proliferation (**Figure 4A**). Considering the *in vivo* mechanisms of action, no significant difference could be observed by Ki67 immunohistochemical (IHC) staining of KHOS osteosarcoma *in situ* cell proliferation between treated

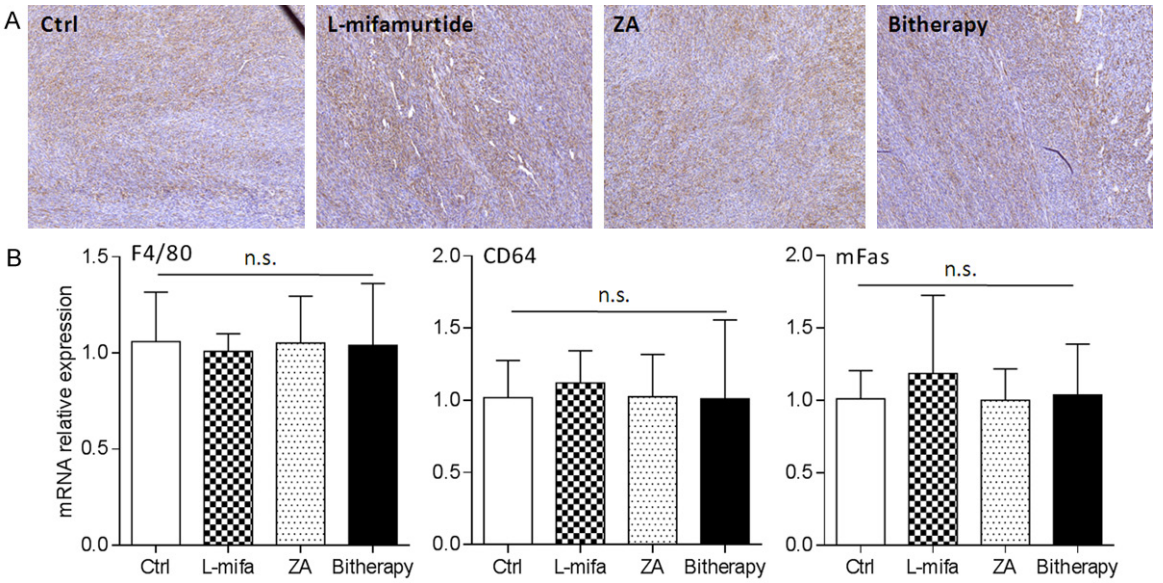

Figure 5. L-mifamurtide and/or zoledronic acid treatments did not affect monocyte/macrophage markers in osteosarcoma syngeneic tumor biopsies. A. Staining of osteosarcoma tissues from MOS-J syngeneic mouse model for F4/80 22 days after tumor induction. B. Expression of *F4/80*, *CD64*, *Fas* mRNA by MOS-J tumor biopsies. NS: no statistic differences between groups. Mean ± SD.

and untreated mice (**Figure 4B**, **4C**). Moreover, necrosis area percentage is not affected by any treatment and similar observations were obtained for xenogeneic models (not shown). It should be mentioned that osteosarcoma presents high heterogeneity inter- and intra-groups leading to difficult interpretation of IHC results at significant level.

Therefore, based on these *in vitro* results, the additive effect of both therapies cannot be explained by a direct anti-tumor effect of L-mifamurtide.

Mechanisms of action of L-mifamurtide and ZA combination in vivo

Because L-mifamurtide is known to activate macrophage- and monocyte-based immune response, the significant inhibitory effect of bitherapy that was observed on primary tumor progression may be linked to the modulation of these immune cell populations by the association of both drugs. Therefore, monocyte/macrophage populations were analyzed at primary site by the relative proportion of their markers at transcript (qPCR) and protein levels (IHC) on mouse osteosarcoma biopsies of each group.

IHC analyses showed no differences in the macrophages F4/80$^+$ infiltrates in primary bone tumors of the different groups in the syngeneic MOS-J mouse model (**Figure 5A**). QPCR analyses from tumor biopsies confirmed that any treatment affected the F4/80 (macrophages) or CD64 (monocytes/macrophages) mRNA expression (**Figure 5B**). Similar observations were made with the KHOS xenogeneic mouse model (not shown). Moreover, IHC scoring of iNOS (M1 macrophages) and Argl (M2 macrophages) did not show any differences between groups (not shown). Therefore, *Fas* mRNA expression was evaluated at the primary tumor site in osteosarcoma models under ZA and L-mifamurtide treatments. Results showed no significant modification of Fas mRNA expression in syngeneic MOS-J (**Figure 5B**) or xenogeneic KHOS (not shown) bone tumor biopsies.

In summary, neither ZA nor L-mifamurtide significantly alters the monocyte/macrophage populations in the osteosarcoma models used in this study. Furthermore, our results suggest that L-mifamurtide does not affect macrophage activation in the primary tumor site as analyzed by iNOS/Argl IHC staining.

Discussion

The absence of overall survival improvement for more than 40 years since the application of Rosen poly-chemotherapy protocols [31] is

associated with the lack of relevant therapeutic options for high-grade osteosarcoma patients (especially patients with lung metastases). In addition to conventional chemotherapy, multiple investigational agents have being studied. Among them, zoledronic acid has already demonstrated its therapeutic interest for bone malignancies including osteosarcoma [19, 20]. L-mifamurtide is also one of the more promising drugs for osteosarcoma acting as immune stimulatory agent. At clinical level, Meyer's study was designed to assess whether the addition of L-MTP-PE and/or ifosfamide to a standard chemotherapeutic regimen (doxorubicin-cisplatin-high dose methotrexate) would increase both EFS and overall survival in newly diagnosed patients with high-grade osteosarcoma. ZA is currently proposed in clinical trials for osteosarcoma (OS2006 - NCT00470223) and Ewing sarcoma (EWING2008 - NCT00987636, EuroEWING2012 - ISRCTN92192408) patients, and L-mifamurtide therapy has been conducted in phase II (MEMOS - ISRCTN82138287) and phase III trials in patients with newly diagnosed OS [18, 32]. Therefore, the combination of both drugs could represent a promising therapeutic option as both drugs target different complementary pathways: L-mifamurtide activates macrophages and monocytes which help to eradicate lung metastasis development, and ZA inhibits osteoclast function by inducing their apoptosis [33], and therefore indirectly induced tumor growth inhibition.

In the present study, we described for the first time a strong trend for L-mifamurtide to inhibit lung metastasis dissemination in both syngeneic and xenogeneic osteosarcoma models in mice. Moreover, its combination with ZA showed no interference of both drugs on each other. Relevant complementary orthotopic models of osteosarcoma with spontaneous pulmonary metastasis development were used to better reproduce the human clinical features and to demonstrate the value of this therapeutic approach [34]. Preclinical studies using xenogeneic and syngeneic models of osteosarcoma demonstrated that ZA did not interfere with L-mifamurtide-inducing lung metastases reduction, and in parallel, L-mifamurtide did not modulate the ZA-induced bone protection. These results in murine models are in agreement with previous preclinical data reported by only one team who described the benefit of L-mifamurtide on lung metastasis formation in dog models of osteosarcoma [35].

Even if a strong trend (but not significant) toward an anti-metastatic activity of L-mifamurtide was shown in our mouse models, we could not observe any increase of the overall survival in these groups, as it has been previously described in dogs [35] and patients [18]. Overall, analyses of tumor behavior are rendered complicated due to the high tumor heterogeneity in osteosarcoma. The general source of heterogeneity is caused by specific tumor microenvironment, composed of stromal cells, the availability of vascular network, and the host's immune system [36]. Primary osteosarcoma progression was so rapid in our models that pulmonary metastasis dissemination was systematically observed, prompting us to sacrifice the mice early. In these cases, it could not be possible to observe any significant effect of L-mifamurtide, but only harm trends. The efficacy of the L-mifamurtide should be investigated in models with slower metastasis development.

In the second part of our study, we wanted to understand why the L-mifamurtide and ZA combination induced a synergistic and significant inhibition of primary osteosarcoma progression in our mice models whereas each drug showed no significant effect. This unexpected result was observed both in xenogeneic and syngeneic models with 32% and 41% inhibition of primary tumor growth respectively. Previous results from the literature showed a direct and indirect anti-tumor activity of L-mifamurtide on osteosarcoma cells *in vitro* or in metastases development after resection of the primary tumor [8, 37]. Moreover, this inhibitory effect on primary tumor growth is even more unexpected as ZA and L-mifamurtide were not associated with conventional chemotherapy as usually in clinics. Several hypotheses could be proposed to explain those results.

First, both drugs act synergistically by directly inhibiting tumor proliferation. However, when we studied the potential direct anti-tumor activity of L-mifamurtide alone and associated with ZA *in vitro,* no inhibition of osteosarcoma cell growth could be observed. In addition, tumor biopsies were collected in mice for histological analysis 24 hours after treatment at early (week 3) or end time point (week 5). We were unable to show a decreased proliferation rate as analyzed by Ki67 staining in the bitherapy group in our different models. However, we may

wonder whether the effects of ZA and L-mifamurtide are transient as a consequence of fever and pro-inflammatory cytokines up regulation for example. In that case, these parameters have to be analyzed earlier. In fact, the synergic interaction of ZA and L-mifamurtide on tumor progression may be due to a transient but sufficient effect on pro-inflammatory cytokine upregulation.

Various studies have reported the stimulation of macrophages by L-mifamurtide + IFN-γ *in vitro* [38, 39]. 'Priming' of macrophages by IFN-γ may enhance liposome up-take and improve the response to bacterial components such as MDP or presumably mifamurtide. We therefore hypothesized that ZA treatment may increase the IFN-γ level at plasma or local location high enough to 'prime' macrophages. It has been reported by clinicians that zoledronic acid therapy leads to fever symptoms in patients for 24-48 h, probably due to transient TNF-α and IL-6 increases, but not IFN-γ [40]. We indeed confirm these data, as we were unable to show a detectable IFN-γ plasma level in the mice 24 hours after zoledronic acid treatment (not shown). It is known that L-mifamurtide binds to the cytosolic NOD2 "receptor" to stimulate NF-κB signaling pathways, activating the expression of inflammatory cytokines including IL-12 [41]. It is admitted that plasma IL-12 increases after administration of L-mifamurtide, and that IL-12 upregulates FAS expression in human osteosarcoma lung metastases. It also known that Fas expression inversely correlates with metastatic activity in osteosarcoma [42]. However, we were unable to observe any enhancement of Fas mRNA expression (following L-mifamurtide treatment) in primary bone tumor in both xenogeneic and syngeneic mouse models of osteosarcoma (not shown).

Because L-mifamurtide acts as a potent stimulator of macrophage activity preferentially at lung site [43], the question raised about its action at the primary osteosarcoma site. In parallel, the role of ZA in tumor macrophages population is controversial. Because ZA induced osteoclast apoptosis (via the inhibition of the small G protein prenylation), it could be suggested that ZA also affects macrophages, as previously described in breast cancer [44], especially those present in the bone tumor microenvironment. We hypothesize that L-mifamurtide may counterbalance the potential side effect of ZA on TAMs. Different approaches by IHC, qPCR and flow cytometry were used to study macrophage sub-populations in mice tumor biopsies from xenogeneic and syngeneic osteosarcoma models. However, no differences in macrophage sub-populations infiltrating primary bone tumor could be observed whatever the methodological approaches used.

In conclusion, no interference between zoledronic acid and the liposomal form of mifamurtide was observed for the studied parameters. Combination of both drugs showed no significant difference on ZA induced bone protection effects and on L-mifamurtide induced inhibition of lung metastasis development. Unexpectedly, an additive and in some cases synergistic inhibitory effect was observed on primary osteosarcoma progression that could not be explained by a direct synergy between drugs on osteosarcoma cell proliferation, neither by a modification of TAMs population at the primary bone site. Even if the mechanism remains unclear up to now, those results constitutes a promising demonstration for clinical application in osteosarcoma patients.

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Declaration of conflict of interest

None.

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