Zoledronic Acid as a New Adjuvant Therapeutic Strategy for Ewing's Sarcoma Patients

Guillaume A. Odri1,2,3, Sophie Dumoucel1,2,4, Gaëlle Picarda1,2, Séverine Battaglia1,2, François Lamoureux1,2, Nadège Corradini5, Julie Rousseau1,2, Franck Tirode5, Karine Lourd5, Olivier Delattre5, François Gouin1,2,3, Dominique Heymann1,2, and Françoise Redini1,2

Abstract

Ewing’s sarcoma (ES) is the second most frequent pediatric bone tumor also arising in soft tissues (15% of cases). The prognosis of patients with clinically detectable metastases at diagnosis, not responding to therapy or with disease relapse, is still very poor. Among new therapeutic approaches, bisphosphonates represent promising adjuvant molecules to chemotherapy to limit the osteolytic component of bone tumors and to protect from bone metastases. The combined effects of zoledronic acid and mafosfamide were investigated on cell proliferation, viability, apoptosis, and cell cycle distribution of human ES cell lines differing in their p53 and p16/ink4 status. ES models were developed to reproduce both soft tissue and intraosseous tumor development. Mice were treated with 100 μg/kg zoledronic acid (two or four times per week) and/or ifosfamide (30 mg/kg, one to three cycles of three injections). ES cell lines showed different sensitivities to zoledronic acid and mafosfamide at the cell proliferation level, with no correlation with their molecular status. Both drugs induced cell cycle arrest, but in the S or G2M phase, respectively. In vivo, zoledronic acid had no effect on soft tissue tumor progression, although it dramatically inhibited ES development in bone. When combined with ifosfamide, zoledronic acid exerted synergistic effects in the soft tissue model: Its combination with one cycle of ifosfamide resulted in an inhibitory effect similar to three cycles of ifosfamide alone. This very promising result could allow clinicians to diminish the doses of chemotherapy.

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Introduction

Ewing’s sarcoma (ES) is the second most frequent primitive malignant bone tumor in children and adolescents after osteosarcoma. Its frequency is around three cases per million in people under 20 years old, with a peak incidence in the second decade of life (1). Current treatments developed in the Euro-EWING99 protocol consist of neoadjuvant chemotherapy, local surgical resection with limb salvage, followed by adjuvant chemotherapy with or without radiotherapy. These treatments lead to a 70% overall survival, but it is less than 40% in patients with metastasis and only 15% for bone metastasis. Radiographs show marked osteolysis with periosteal reaction and frequent soft tissue invasion around the primary tumor. ES are included in the primitive neuroectodermal tumors (PNET) sharing a chromosomal translocation involving the EWS gene on chromosome 22 with a gene of the erythroid transformation sequence (ETS) family (2). In 85% of cases, the translocation t(11;22)(q24;q12) occurs leading to the EWS-FLI1 fusion gene, which behaves as an oncogene.

Bisphosphonates are PPi derivatives that selectively concentrate at the bone resorption surface (3) and induce osteoclast apoptosis, resulting in inhibition of bone resorption (4). In addition, they inhibit tumor cell adhesion, invasion, and proliferation, and induce apoptosis in a variety of human tumor cell lines in vitro including osteosarcoma (5–10). Among all bisphosphonates tested, zoledronic acid, one of the third-generation nitrogen-containing bisphosphonates, shows the greatest inhibitory effects on both osteoclast activity and tumor cell proliferation (11). Quite appropriately, these agents are increasingly used alongside anticancer treatments to prevent skeletal complications and relieve bone pain. Concerning primary bone tumors, zoledronic acid has been recently combined with conventional chemotherapy and surgery in the French OS2006 phase III randomized clinical trial for osteosarcoma treatment, after promising preclinical results had been found on survival and tumor growth (10).

The rationale for the therapeutic use of zoledronic acid in bone tumors is linked to the existence of a stimulatory
feedback loop (the "vicious cycle") between tumor cell proliferation and bone resorption during tumor development in bone (12). Thus, zoledronic acid may have complementary actions on bone tumors: an indirect antitumor effect by inhibiting osteoclastogenesis and a direct action on tumor cells. To date, only one preclinical study reported the therapeutic benefit of using zoledronic acid in ES, showing an inhibitory effect on osteolysis; however, no study on survival and tumor volume has been done (13). In the present study, in vitro experiments were performed to evaluate the effect of zoledronic acid on eight human ES cell lines, all expressing the EWS-FLI1 fusion gene but differing in their molecular characteristics (p53 mutation, p16/ink4 deletion). Then, in vivo experiments were realized to study the effects on tumor progression and animal survival, as a single therapeutic agent or in combination with ifosfamide, a conventional drug used in ES clinical protocols. Two different animal models of ES were developed, reproducing both clinical behaviors of ES: soft tissue or bone development (15% and 85%, respectively, of total ES).

The objectives were to determine the potential therapeutic efficacy of zoledronic acid alone or in combination with chemotherapy that could help reduce the chemotherapy dosing regimen in preclinical and then clinical protocols for ES.

Materials and Methods

Cell lines

Human ES cell lines kindly provided by Dr. S. Burchill (Children’s Hospital, Leeds, United Kingdom; A673, TC32, SKES1, SKNMC, and RDES cells) or by Dr. O. Delattre (Institut National de la Santé et de la Recherche Médicale U830, Paris, France; EW24, TC71, and EW7) were used. They all contain the EWS/FLI1 chromosomal translocation EWS/FLI1 but differ in their p53 and p16/ink4 status (Supplementary Data). A673, TC32, SKES1, and RDES cells were cultured in DMEM (BioWhittaker) with 10% fetal bovine serum. Cells were collected with the supernatant and centrifuged for 24 to 72 hours or with 1 μg/mL staurosporine for 6 hours added. The cells in each well were then manually counted to determine the ratio of dead cells to viable cells.

Apoptosis analysis

Caspase-3 activity was determined using the CaspACE assay system fluorometric kit (Promega). Fifteen thousand cells seeded in 24-well plates were treated as described above for 6 to 24 hours or with 1 μmol/L staurosporine for 6 hours as a positive control before lysis. Caspase-3 activity is reported per microgram protein as determined with copper sulfate diluted in bicinchonic acid (both from Sigma).

Cell cycle analysis

Two hundred thousand cells were seeded in 25-cm² flasks, then test compounds were added for 24 to 72 hours. Cells were collected with the supernatant and centrifuged at 800 × g for 5 minutes. The cells in the pellet were fixed in ethanol, centrifuged, and rinsed twice. The final pellet was resuspended in citrate phosphate buffer, incubated for 30 minutes at room temperature, and then centrifuged. The pellet was finally rinsed with PIB-RNase (DPBS+ Triton X-100 + 0.5 μL/L EDTA). After 30 minutes of incubation at 37°C, 50 μg of propidium iodine were added to each tube. After a final incubation at 4°C for 20 minutes, cell cycle analysis was performed using a FC500 flow cytometer.

Mouse models of ES

All procedures involving mice [their housing in the Experimental Therapeutic Unit at the Faculty of Medicine of Nantes (France) and care, the method by which they were anesthetized and killed, and all experimental protocols] were conducted in accordance with the institutional guidelines of the French Ethical Committee (CEEA.PdL.O6). Four-week-old male athymic mice were purchased from Harlan. The soft tissue ES model was induced by an i.m. injection of 2 × 10⁶ ES cells next to the tibia, leading to a rapidly growing tumor in soft tissue with secondary contiguous bone invasion. For the bone model, the same number of cells was injected in the medullar cavity of the tibia, leading to a slow-growing intraosseous tumor that progressively destroyed the cortical bone and invaded the surrounding soft tissues. Mice were anesthetized by inhalation of a combination of isoflurane/air (1.5%, 1 L/min) and buprenorphine (0.05 mg/kg; Temgésic, Schering-Plough). Mice were randomly assigned to treatment groups 1 day after tumor cell injection. The tumor volume was calculated by using the formula L × (L/2)^2, where L and l are the longest and the smallest perpendicular diameter, respectively. The mice died either spontaneously or were killed by CO₂ inhalation when the tumor volume exceeded 6,000 mm³. The tumor-bearing hind limb was dissected and kept in 10% paraformaldehyde for radiography, microcomputed tomography (micro-CT), and histologic analyses.

Dosing regimens and experimental protocols

Zoledronic acid prepared in PBS was injected s.c. at 100 μg/kg, this dose being equivalent to the clinical dose of 4 mg every 3 to 4 weeks (14). Zoledronic acid treatment started at day 1 after tumor cell inoculation and was...
administered either twice a week with a 3-day interval or on 4 consecutive days each week. Ifosfamide (ASTA Medica) was injected i.p. at a final dose of 30 mg/kg. The first ifosfamide sequence, starting 10 days after tumor cell injection when the tumor was detectable, comprised a 3-day treatment with a 24-hour interval. A second and third cycle was administrated 1 and 2 weeks later depending on the protocols. The mice in the control group were injected with an equivalent volume of PBS. The protocol was determined in accordance with clinicians to start the treatment at a tumor stage comparable with that of patients at their first visit to an oncologist.

Radiographic analysis
Radiographs on anesthetized animals [xylazine (Rompun)-ketamine (Imalgène 500), 8% and 13%, respectively, in PBS; 100 μL/10 g] were taken every week and at necropsy with a PLANMED Sophie mammography apparatus (SN RAH 40710).

Micro-CT analysis
Micro-CT tomography was performed with a high-resolution X-ray micro-CT system for small-animal imaging SkyScan-1072. Three-dimensional reconstructions were built for each hind limb bone to quantify and compare the bone microarchitecture parameters between control and treated animals.

Bone and tumor histology
After sacrifice, the tibiae were conserved and fixed in 10% formalin, decalcified (PBS-EDTA), and embedded in paraffin for tartrate-resistant acid phosphatase (TRAP) staining: 4-μm sections were cut and stained for TRAP by incubating for 1 hour in a solution of 1 mg/mL naphthol-AS-TR-phosphate, 60 mmol/L N,N-dimethylformamide, 100 mmol/L sodium tartrate, and 1 mg/mL fast red TR salt solution (all from Sigma). A hematoxylin counterstain was performed. Apoptotic cells in primary bone tumor were determined using the in situ cell death detection kit (Roche Diagnostics), based on the terminal-deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method. TUNEL-positive cells were counted by microscopic examination on four to six histologic sections per animal.

Statistical analysis
Regression models were used to assess the effect of zoledronic acid and mafosfamide on cell proliferation of each cell line in vitro. Splines developed with generalized additive models were used to look for nonlinear effects of both chemotherapies on each cell line (15). In case of important departure from linearity, a log transformation was used and linearity was assessed again. When linearity was adequate, linear regression models were constructed to independently test the effect of each chemotherapy and search for an interaction. Nonparametric tests (Kruskal-Wallis test) with Dunn’s post hoc test were used for the other in vitro experiments. Tumor volume averages were also compared using the nonparametric Kruskal-Wallis test with Dunn’s post hoc test. Mice survival were estimated with the Kaplan-Meier estimator and compared with the log-rank test. The level of statistical significance was chosen at 0.05; all tests were bilateral. R software and Excel software were used for these analyses.

Results

*In vitro experiments*

**Cell viability.** Figure 1A summarizes the sensitivity to zoledronic acid and mafosfamide of eight human ES cell lines, but no correlation with p53 status could be evidenced. Results obtained with the A673 cell line are presented as an example in all the forthcoming figures. A linear effect of zoledronic acid and mafosfamide was found on A673 cell proliferation after log transformation of the data (Fig. 1B; \( P < 0.0001 \)), revealing that sensitivity of A673 cells to zoledronic acid and mafosfamide is concentration dependent with an IC\(_{50}\) of 3 μmol/L and 5 μg/mL, respectively. When these molecules were combined, no synergistic effect on ES cell proliferation could be shown by covariate analysis whatever the drug combination tested (Fig. 1C).

**Cell death—apoptosis.** Studies on cell viability were performed using trypan blue on cells treated by zoledronic acid and mafosfamide for 72 hours. Figure 2A shows that the proportion of dead cells increased with increasing zoledronic acid and mafosfamide concentration. To characterize the cell mechanisms involved in this process, apoptosis and cell cycle were analyzed. An increase in caspase-3 activation was observed in cells treated with both drugs but in a lower extent with zoledronic acid than mafosfamide (Fig. 2B). However, the use of the pangenomic caspase inhibitor Z-VAD-FMK did not abrogate the zoledronic acid–induced inhibition of cell proliferation (Supplementary Data). Cell cycle distribution analyzed by flow cytometry revealed that zoledronic acid treatment increases the proportion of A673 cells in the S phase (34% versus 18% in controls) whereas mafosfamide blocks cells in the G\(_2\)M phase (84% versus 33% in untreated cells; Fig. 2C). Therefore, this therapy combination may overcome the potential resistance to either drug.

**Experimental in vivo protocols**

Two experimental models of ES were developed (in soft tissue and in bone) to reproduce as closely as possible the pathologic variants observed in patients. The in vivo experimental results obtained in the tumor model induced by A673 cells are presented in this article, but were confirmed in at least another ES model (Supplementary Data).

**Zoledronic acid and chemotherapy exert a synergistic effect in the soft tissue model of ES.** Zoledronic acid treatment of mice bearing ES, induced in soft tissue by using A673 or TC71 cells, did not inhibit tumor progression regardless of the dose used (10, 50, or 100 μg/kg; two or four times a week for 4 weeks, beginning at day 1; data not shown). Zoledronic acid was then combined with ifosfamide following two dosing regimens (Fig. 3A): zoledronic acid (100 μg/kg twice a week, beginning at day 1) and three courses of ifosfamide, each consisting of three injections of 30 mg/kg ifosfamide at 24-hour intervals beginning when the tumor was detectable (around day 10, then one course per week; left), and zoledronic acid (100 μg/kg twice a week from day 1) and one
course of ifosfamide (30 mg/kg beginning at day 10; right). In the first set of experiments, zoledronic acid alone had no effect on tumor development, ifosfamide alone decreased tumor progression by 27% at day 25 compared with control mice \((P < 0.05)\), and the combination of both drugs exerted an effect similar to that of ifosfamide alone (Fig. 3A, left). In the second set of experiments, zoledronic acid and ifosfamide alone had no significant effect on tumor progression,
but the combination reduced the mean tumor volume by 54% at day 25 compared with untreated animals (P < 0.01; Fig. 3A, right), a higher extent than three courses of ifosfamide alone (Fig. 3A, left). Taken together, both these experiments show that the combination of zoledronic acid with a single course of ifosfamide exerts a greater inhibitory effect on tumor progression than three courses of ifosfamide alone, suggesting that chemotherapy combined with bisphosphonate may allow the reduction of chemotherapy doses. Complementary radiography (Fig. 3B) and micro-CT (Fig. 3C) analyses showed a global reduction of bone mineralization, marked osteolysis with disruption of cortical continuity leading to multiple fractures in untreated mice bearing ES (Fig. 3B and C). The severity of these bone lesions was strongly diminished in zoledronic acid–treated mice (alone or combined with ifosfamide), indicating that zoledronic acid protects bone from tumor-associated osteolysis. Quantitation of specific bone volume [BV/total volume (TV)] confirmed these observations: BV/TV of tibia from zoledronic acid– and zoledronic acid + ifosfamide–treated mice is strongly increased (53.41% and 53.24%) compared with the ifosfamide and untreated groups (33% and 25%, respectively; Fig. 3C), being comparable with normal tibia (52.1%). Histology analysis confirmed the protective effect of zoledronic acid on bone, leading to the prevention of tumor cell invasion in the bone marrow (Fig. 3D). However, it cannot protect against tumor progression in soft tissue; thus, chemotherapy is needed in combination with zoledronic acid in this case.

Zoledronic acid alone prevents ES development in bone. This model closely reproduces ES development in patients, with a slower tumor growth than that observed in the previous model in soft tissue (42 and 27 days, respectively, to reach a tumor volume of 4,000 mm³). Figure 4A shows that zoledronic acid alone (100 μg/kg, four times a week for 9 weeks) strongly inhibits tumor development as shown by a 97% reduction in mean tumor volume 46 days after tumor cell inoculation. The corresponding radiography analyses showed that zoledronic acid protects bone from tumor cell invasion compared with untreated animals (Fig. 4B). This result was confirmed at the osteoclast level as revealed by TRAP staining (Fig. 4C): Osteoclasts were clearly evident at the tumor site in control animals but were absent in zoledronic acid–treated mice. The direct effect of zoledronic acid on ES cell apoptosis was confirmed in vivo by increased TUNEL staining in the tumors from the zoledronic acid–treated group compared with untreated animals (Fig. 4D).

Zoledronic acid combined with ifosfamide prevents tumor relapse in bone. The combination of zoledronic acid (100 μg/kg, twice a week) and ifosfamide (3 courses of 3 × 30 mg/kg) was tested for 4 weeks in the above-described bone model. Zoledronic acid alone exerted a stronger inhibitory effect on tumor progression (~65%, P < 0.01 at day 30 posttumor cell inoculation) than ifosfamide alone (~45% at the same day, P < 0.01; Fig. 5A). Moreover, ifosfamide efficacy declined over time, and relapse was observed from day 18. When zoledronic acid was combined with ifosfamide, the mean tumor volume curve could be superimposed on that of zoledronic acid alone, suggesting that zoledronic acid combined with ifosfamide prevents the early relapse that occurs with ifosfamide alone.

At the bone level, micro-CT analysis showed impressive bone degradation and disorganized bone remodeling in mice bearing ES (untreated) compared with naïve control animals (no tumor; Fig. 5B). Animals treated with ifosfamide showed the same extent of bone degradation. When mice were treated with zoledronic acid alone or combined with ifosfamide, a protection from bone lesions was observed with an absence of
Figure 3. Synergistic effect of zoledronic acid and ifosfamide (IFOS) on the development of ES in soft tissue. A, A673 ES cells (2 × 10⁶) were injected into the tibial muscle of immunodeficient mice. Animals were then divided into four groups (eight mice per group) designed as control, zoledronic acid (100 μg/kg, two times per week beginning at day 1 after tumor cell injection), ifosfamide [30 mg/kg, three cycles of three injections (left) or one cycle of injection (right)], and zoledronic acid + ifosfamide. The tumor volume was reported as mean tumor volume per group (*, P < 0.05; **, P < 0.01). B, radiography analysis of osteolytic lesions. C, micro-CT analysis of tibia from normal mice or mice bearing ES induced by A673 cell injection as described above, treated (ifosfamide, zoledronic acid, and ifosfamide + zoledronic acid) or untreated (UT). Specific bone volume is indicated in each case (BV/TV). D, histologic analysis of the cortical bone in mice treated with zoledronic acid (hematoxylin eosin staining, magnification ×100).
Figure 4. Monotherapy with zoledronic acid prevents ES development in bone. A, A673 ES cells (2 × 10⁶) were injected in the medullar cavity of the tibia of immunodeficient mice. Mean tumor volume of control and zoledronic acid (100 μg/kg, two times per week beginning at day 1 after tumor cell injection) groups (8–10 mice per group) is reported. B, radiography analysis of osteolytic lesions in one representative mouse of each group. C, TRAP staining of osteoclasts in mice treated with zoledronic acid compared with untreated mice bearing ES tumor and normal mice (no tumor). D, TUNEL staining was performed on 6-μm sections of A673 ES tumors treated or not with zoledronic acid (magnification ×100 and ×200).
cortical disruption, increased cortical bone density, and lack of fractures (Fig. 5B). In these cases, the bone architecture was conserved, even with extensive ectopic bone formation in the zoledronic acid + ifosfamide group. These results were confirmed by measuring TRACP5b levels in the serum of corresponding mice. TRACP5b levels were indeed significantly diminished in both the zoledronic acid–alone and zoledronic acid + ifosfamide–treated groups compared with untreated controls or ifosfamide-alone groups: 4 and 6 units/L versus 12.5 and 20.5 units/L (P < 0.001; Fig. 5C), respectively.

Discussion

Among the new investigational approaches to improve therapy in ES and OS, bone-specific agents may improve survival and/or quality of life on “continuation” therapy. This would include regimens with fewer short- and long-term side effects and better results for tumors in difficult locations and patients with recurrent disease.

Among primary bone tumors, giant cell tumors of bone are characterized by a major osteoclastic component that justifies the clinical use of bisphosphonates in this pathology (16). Because osteosarcoma and ES cells are metabolically active bone cells (17, 18), studies with bisphosphonates and more specifically nitrogen-containing bisphosphonates have shown selective uptake and “poisoning” of these cells (19–23). We have previously shown that zoledronic acid is able to limit tumor progression in a rat model of osteosarcoma, to prevent tumor relapse compared with chemotherapy alone and to prevent osteolytic lesions (10). Our results have provided the rationale for the French randomized clinical protocol OS2006, which combines zoledronic acid with conventional therapy for adult and pediatric patients. Since then, other fundamental or preclinical studies have confirmed the beneficial effect of bisphosphonates in osteosarcoma (20–25).

The limits of ES therapy in the current European protocol EuroEWING99 mainly concern patients with bone or medullar metastases. Therefore, bisphosphonates may be
useful as adjuvant therapy to target osteoclasts and consequently to diminish the bone lesions associated with these tumors, or to prevent the development of bone metastases (26, 27). Very few studies specifically concern bisphosphonate action in ES. Two previous studies have shown cytotoxic and growth-inhibitory effects of bisphosphonates in ES/PNET cell lines and xenograft models (21, 25, 28). Another study has reported the inhibition of ES/PNET tumor growth by zoledronic acid, apoptosis induction, and alteration of the tumor lytic phenotype (13). However, all cell lines used to induce ES in these studies possess the p53 mutation and therefore are not representative of ES tumors in which p53 mutations are relatively uncommon (∼13% of patients; ref. 29).

We thus wanted to extend these studies by using eight human ES cell lines, all expressing the fusion gene EWS-FLI1 but differing in their p53 (mutation) and p16/ink4 (homozygous/hemizygous deletion) status. The results presented here, showing that all these ES cell lines were sensitive to zoledronic acid whatever their p53 mutation or p16 deletion status, are encouraging for treating a large cohort of ES patients. The data on ES cell growth inhibition can be compared with others obtained with different bisphosphonates: Sonnemann and colleagues’ study showed that 50 μmol/L pamidronate reduced cell number by up to 80% in eight ES cell lines, whereas clodronate had limited effects, reducing cell viability by maximally 40% at 1 mmol/L (28). To investigate the mechanism of action of zoledronic acid in ES, apoptosis and cell cycle analyses were performed. The results showed that zoledronic acid inhibits cell viability by blocking the cell cycle in S-G2M phase, as previously described for osteosarcoma cells (30). In addition, a low caspase-3 activation was shown in ES cells, more probably reflecting a postmitotic process linked to the cell cycle blockade rather than a caspase-dependent mechanism. Indeed, the use of the panpangenomic caspase inhibitor Z-VAD-FMK had no effect on zoledronic acid–induced ES cell proliferation.

A subsequent step was to confirm the inhibitory effects of zoledronic acid in vivo. In patients, ES tumors mainly develop in bone (85% of cases), but it can also occur as isolated soft tissue tumors. Because zoledronic acid targets not only osteoclasts but also the tumor cells themselves in vitro, this therapy was also tested in a soft tissue model. Interestingly, zoledronic acid strongly inhibited tumor progression in bone (∼97% after 9 weeks of treatment in the A673 model), this result being in accordance with the beneficial effects of bisphosphonate widely reported in several experimental models of bone lesions associated with primary or secondary tumors (10, 14, 31, 32). On the contrary, the same doses of zoledronic acid had no inhibitory effect on ES progression in soft tissue. This result is consistent with data from other models of soft tissue tumors or visceral metastases. Indeed, the inhibitory effect of bisphosphonate on tumor growth is still inconsistent for soft tumors (33–35). Even at the clinical level, few data have reported an antitumor effect of zoledronic acid against visceral metastases (36, 37), and conflicting results have been highly discussed (38). In our ES models, it is clear that the doses of zoledronic acid that prevented tumor development in bone were unable to significantly influence soft tissue tumor growth. The best efficacy of zoledronic acid in bone may be explained by the fact that bisphosphonates concentrate in bone due to their high tropism for the calcified bone matrix. The efficacy of zoledronic acid may be therefore potentiated in bone tissue by a dual mechanism: antitumoral and antibone resorption activities. Higher concentrations could be tested in soft tissue models, but would be irrelevant to clinical dosing regimens.

For many years, bisphosphonates have been used as an adjuvant to chemotherapy in several clinical protocols (39). In vitro and in vivo studies showed a synergistic interaction between bisphosphonates and chemotherapeutic agents, augmenting their efficacy (23, 25, 40, 41) and (re-)sensitizing to chemotherapy (22), to diminish side effects or to improve the function and quality of life (42). In our study, we showed that zoledronic acid exerts synergistic activity with low doses of ifosfamide in soft tissue ES, inducing the same extent of tumor growth inhibition as high doses of ifosfamide alone. This result suggests that chemotherapy dosing regimens could be diminished in the presence of adjuvant bisphosphonate.

Because ES is characterized by marked bone resorption, therapies that target osteoclasts such as bisphosphonates are promising. Our study shows, by complementary fundamental and preclinical analyses, that zoledronic acid represents a promising therapeutic agent for ES patients as a first-line therapy in combination with chemotherapy to limit bone lesions and to prevent bone tumor relapse, and also as an adjuvant therapy for the treatment of bone or medullary metastases. This is particularly important in the context of the EuroEWING09 protocol, which is nearing completion, and for the choice of new future strategies, especially for patients at high risk of relapse.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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