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The skeletal impact of the chemotherapeutic agent etoposide

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Abstract

Summary—Effects of the chemotherapeutic agent etoposide on the skeleton were determined in mice. Numbers of bone marrow cells were reduced and myeloid cells were increased. Bone volume was significantly decreased with signs of inhibition of bone formation. Etoposide after pre-treatment with zoledronic acid still reduced bone but overall bone volume was higher than with etoposide alone.

Introduction—Chemotherapeutics target rapidly dividing tumor cells yet also impact hematopoietic and immune cells in an off target manner. A wide array of therapies have negative side effects on the skeleton rendering patients osteopenic and prone to fracture. This study focused on the pro-apoptotic chemotherapeutic agent etoposide and its short- and long-term treatment effects in the bone marrow and skeleton.

Methods—Six- to 16-week-old mice were treated with etoposide (20–25 mg/kg) or vehicle control in short-term (daily for 5–9 days) or long-term (3×/week for 17 days or 6 weeks) regimens. Bone marrow cell populations and their phagocytic/efferocytic functions were analyzed by flow cytometry. Blood cell populations were assessed by CBC analysis. Bone volume and area compartments and osteoclast numbers were measured by microCT, histomorphometry, and TRAP staining. Biomarkers of bone formation (P1NP) and resorption (TRAcP5b) were assayed from serum. Gene expression in bone marrow was assessed using qPCR.

Results—Flow cytometric analysis of the bone marrow revealed short-term etoposide reduced overall cell numbers and B220⁺ cells, with increased marrow apoptotic (AnnexinV⁺PI[−]) cells, mesenchymal stem-like cells, and CD68⁺, CD45⁺, and CD11b⁺ monocyte/myeloid cells (as a percent of the total marrow). After 6 weeks, the CD68⁺, Gr1⁺, CD11b⁺, and CD45⁺ cell populations were still relatively increased in etoposide-treated bone marrow. Skeletal phenotyping revealed etoposide decreased bone volume, trabecular thickness, and cortical bone volume. Gene expression in the marrow for the leptin receptor and CXCL12 were reduced with short-term

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etoposide, and an increased ratio of RANKL/OPG mRNA was observed. In whole bone, Runx2 and osteocalcin gene expressions were reduced, and in serum, P1NP was significantly reduced with etoposide. Treatment with the antiresorptive agent zoledronic acid prior to etoposide increased bone volume and improved the etoposide-induced decrease in skeletal parameters.

Conclusions—These data suggest that etoposide induces apoptosis in the bone marrow and significantly reduces parameters of bone formation with rapid reduction in bone volume. Pre-treatment with an antiresorptive agent results in a preservation of bone mass. Preventive approaches to preserving the skeleton should be considered in human clinical studies.

Keywords

Bisphosphonate; Bone; Chemotherapy; Efferocytosis; Etoposide; Macrophage

Introduction

Traditional cancer therapies act by targeting rapidly growing cells. The major goal is to induce cell death in proliferating cancer cells, hence eliminating or reducing the growth of the tumor [1–3]. In addition to the positive effects of reducing tumor cell burden, many chemotherapeutic drugs have potentially deleterious effects systemically as well as in the microenvironment surrounding the tumor.

Etoposide is a cytotoxic cancer drug approved by the FDA in 1983. It is used to treat a wide variety of cancers including Kaposi's sarcoma, Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, nonlymphocytic leukemia, glioblastoma, and others [4]. Etoposide is also used as a conditioning regimen prior to bone marrow or blood stem cell transplant [5]. For the treatment of cancer, it is often combined with other antineoplastic drugs. Etoposide belongs to a class of topoisomerase inhibitors and prevents religation of the DNA strands resulting in DNA strand breaks and apoptosis [6, 7]. Rapidly dividing cancer cells are heavy topoisomerase users and hence are particularly prone to apoptosis with etoposide.

Patients on chemotherapeutic treatments for cancer frequently present with osteopenia and increased fracture risk, but little has been done to identify underlying mechanisms or address prevention [8, 9]. One report indicates that any cancer treatment modality increases the incidence of osteoporosis to 10% in childhood survivors of cancer with a median age of 32 years [10]. Patients undergoing allogeneic stem cell transplantation with prior immunosuppression treatment have an increased risk of osteoporotic fracture [11], and Schimmer et al., report that within 2 months of bone marrow transplantation, 50% of patients have a diagnosis of osteopenia or osteoporosis mainly due to previously administered cancer treatments [12].

When such cancers are treated with anticancer agents, bone loss may be exacerbated and can be deleterious in the surviving patient. Bisphosphonates have been shown to be effective in preventing early bone loss in patients undergoing allogeneic stem cell transplantation [13], which suggests that increased osteoclastic activity is a central component to the negative skeletal impact of chemotherapeutics. Still, relatively little is known of the impact of

chemotherapeutic regimes and how to minimize potentially deleterious effects on the skeleton.

A better understanding of the impact of such agents will set the foundation to garner the benefits of the chemotherapeutic while minimizing adverse effects. The purpose of this study was to determine the impact of etoposide in the bone marrow microenvironment and skeletal homeostasis and evaluate the effectiveness of a potential intervention to prevent bone loss.

Materials and methods

Animal care and experiments

All animal experiments were performed with the approval of the University of Michigan Committee for the Use and Care of Animals. Female C57Bl/6J mice were obtained from Jackson Labs (Bar Harbor, ME). Etoposide (20–25 mg/kg; Sigma, St. Louis, MO) was administered i.p. daily for 5 days, then three times per week. Treatments were administered to 16-week-old adult mice for 6 weeks, and mice were sacrificed at 22 weeks of age. Early etoposide effects were investigated in 6- to 16-week-old mice after treatment ranging from 5 to 9 days. A subset of mice was treated subcutaneously with zoledronic acid (50 µg/kg), Sagent Pharmaceuticals, Schaumburg, IL) 3 days prior to administration of etoposide, then twice per week. In these mice, etoposide (or vehicle) was administered 10 times over 14 days for a total treatment regime of 17 days. Treatment dosage for etoposide (20 mg/kg) was established as the human equivalent dose of 1.6 mg/kg which is ~40% less than the 2.7 mg/kg/day used to treat testicular cancer. The dose for zoledronic acid was based on previous investigation [14].

Flow cytometric analysis

Murine long bone marrows were flushed with flow cytometry staining buffer (FSB) (2% FBS, 2 mM EDTA in 1× PBS) immediately after sacrifice and 1×10^6 cells were incubated with fluorescent-conjugated antibodies (PE 5 µl; FITC 2 µl; APC 4 µl) in 100 µl of FACS buffer for 20–30 min at 4 °C. Intracellular staining of CD68 utilized antibody specific reagents for a 15-min fixation followed by a 30 min incubation with the CD68 antibody in permeabilization buffer. Cells from all stains were then washed, and analyzed on a FACS Calibur or a FACS Aria IIu (BD Biosciences, San Jose, CA).

Antibodies for flow cytometric analyses included the following: Antimouse CD45 (30-F11), Lineage cocktail, sca1 (E13-161.7), CD29 (HM β1-1), and the Annexin PI kit were obtained from BD Biosciences (San Jose, CA). Antimouse CD11b (M1/70, APC) was obtained from eBioscience (San Diego, CA). Antimouse CD68 (FA-11) antibody and respective fixation and permeabilization reagents were purchased from AbD Serotec (Alexa Fluor 647; Leucoperm Reagents; Raleigh, NC) or Biolegend (FITC; Fixation Buffer/Permeabilization Buffer; San Diego, CA). B220 (RA3-6B2) was obtained from Biolegend.

MicroCT

Three-dimensional analyses of long bones were performed by microCT as previously described [15]. Briefly, formalin-fixed tibiae or femurs were embedded in 1% agarose and placed in a 19-mm-diameter tube and scanned over their entire length using a microCT system (μ CT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were as follows: 12 μ m voxel size, medium resolution, 70 kVp, 114 μ A, 0.5-mm AL filter, and an integration time of 500 ms. Trabecular bone parameters were measured over a 0.6 mm region using a 180 mg/cm³ hydroxyapatite (HA) threshold beginning 0.18 mm distal to the growth plate; cortical bone parameters were measured over 0.36 mm beginning 3 mm proximal to the tibia-fibular joint using a 280 mg/cm³ HA threshold.

Histology and osteoclast staining

Formalin-fixed tibiae were decalcified in 14% EDTA pH 7.2 for 14 days and processed for histology. Paraffin-embedded tibias were cut (5 μ m), stained with H&E and histomorphometric analyses performed using Osteomeasure (OsteoMetrics Inc., Atlanta, GA). Osteoclast staining on bone sections was performed using a tartrate resistant acid phosphatase (TRAP) staining system (Sigma). Manufacturer's instructions were followed and sections were counterstained with Gill's Hematoxylin and mounted with Aquamount (Lerner Laboratories, Kalamazoo, MI). Stained sections were visualized with a Nikon Microscope and analyzed using ImageJ software. Very little trabecular bone was observed more than 200 μ m distal to the proximal growth plate in 6-week etoposide-treated (22-week-old) mice. Therefore, analysis was performed in the proximal tibiae starting where the growth plate ends (osteoclasts directly on growth plate and cortical bone excluded) and encompassing 200 μ m distally. In 6- to 10-week-old mice, analysis was performed in the proximal tibiae beginning 150 μ m from the growth plate and extending 900 μ m distally, up to but excluding cortical bone. All analyses were performed in a blinded manner by investigators.

Engulfment assays

To measure phagocytosis, Dragon Green fluorescent carboxylated beads (Bang's Laboratories, Fishers, IN) were used as bait. Bone marrow was harvested from mice treated for 7 days, and one million cells were incubated with 700,000 beads for 2 h at 37 °C. Cells were then fixed, permeabilized, and stained with CD68 (FA-11; AlexaFluor647; AbD Serotec) for 30 min, washed, and flow cytometric analyses performed for dual CD68 and green fluorescence positivity.

To measure efferocytosis, bone marrow stromal cells (BMSCs) were used as the apoptotic cell bait. BMSCs were harvested from 4- to 6-week-old C57B6/J mice. Briefly, hind limb bone marrow was flushed with α -MEM (Life Technologies) supplemented with 20% FBS (Gemini Bio-Products, West Sacramento, CA), 1% PSG, and 10⁻⁸ M dexamethasone (Sigma). Cells were expanded over one passage, and just prior to confluence stained with Deep Red Cell Tracker (1:1000 in PBS, Invitrogen) for 30 min, recovered in complete medium for 30 min, and then induced to undergo apoptosis by UV treatment in PBS for 30 min. Cells were again allowed to recover in the PBS for 2 h, counted (cells were >85% trypan blue positive), and used for the efferocytosis assays. Whole bone marrow was

harvested from mice treated with etoposide for 9 days and incubated with stained apoptotic BMSCs at a 1:1 ratio for 5 h, fixed, permeabilized, and stained with CD68 (FA-11; FITC; Biolegend) for 30 min. Cells were washed and flow cytometric analysis performed for cells dually positive for CD68 and Red Cell Tracker.

Blood and bone marrow serum biochemical assays

Serum markers of bone resorption (tartrate-resistant acid phosphatase; TRAcP5b) and bone formation (procollagen type 1 N-terminal propeptide; P1NP) were measured using immunoassay analytics obtained from ImmunoDiagnostic Systems (Scottsdale, AZ) and performed according to manufacturer's instructions.

Real time RT-PCR gene expression assays

RNA was extracted from both bone marrow isolates (flushed) and whole bone (marrow + bone tissue). For bone marrow RNA, femurs were flushed with Trizol reagent (Invitrogen) and total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturers' protocols. For whole bone RNA, femurs were flash frozen in liquid nitrogen at sacrifice, then crushed with mortar and pestle (while still frozen), resuspended in Trizol, and then sheared using an 18G needle. As mentioned above, RNA was extracted, purified, and processed using the RNeasy Mini Kit. RNA was quantified using a Nano-Drop, and double-stranded cDNA was synthesized from 0.5 µg RNA using the TaqMan RT-PCR Kit (Applied Biosystems, Branchburg, NJ). Genes of interest were amplified using TaqMan PCR Master Mix and TaqMan primer/probe sets, including receptor activator of nuclear factor-κB ligand (*Rankl*; *Tnfrsf11*; Mm00441906_m1), osteoprotegerin (*Opg*; *Tnfrsf11*; Mm00435452_m1), leptin receptor (*Lepr*; Mm00440181_m1), C-X-C motif chemokine 12 (*Cxcl12*; Mm00445553_m1), Runt-related transcription factor 2 (*Runx2*; Mm00501584_m1), and osteocalcin (*Ocn*; *Bglap3*; Mm03413826_mH) (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer/probe set (Mm99999915_m1) served as an endogenous control. PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Samples were run in duplicate, and Ct values were calculated as an average of each duplicate per sample. Ct values of the genes of interest for each sample were normalized to the corresponding Ct value of GAPDH ($-Ct$).

Statistical analyses

Statistical analyses were performed by Student's *t* test or two-way ANOVA using the GraphPad InStat (San Diego, CA) statistical program or the Wilcoxon rank sum test using GraphPad Prism 7, with significance at $p < 0.05$. Data are presented as mean $\pm$ SEM.

Results

Peripheral blood analyses

The short-term effects of etoposide on peripheral blood cell populations were analyzed in 16-week-old C57Bl/6J female mice treated for 5 days with etoposide (Table 1). White blood cells, neutrophils, lymphocytes, and monocytes were all significantly reduced. In the red blood cell compartment, red blood cells, hemoglobin, hematocrit, and red cell distribution

width were significantly reduced whereas platelets were significantly increased with etoposide treatment.

Short-term effects of etoposide in the bone marrow

To investigate the effect of short-term etoposide treatment on the bone marrow, 16-week-old C57B6/J female mice were administered etoposide daily for 5 days. Total numbers of cells were reduced (vehicle $9.06 \times 10^6 \pm 0.52$ vs. etoposide $6.87 \times 10^6 \pm 0.63$; $*p < 0.05$). Etoposide treatment increased early apoptotic cells in the bone marrow revealed by an increase in AnnexinV⁺PI⁻ (Fig. 1a), confirming that etoposide-induced apoptosis in the bone marrow. CD68⁺ phagocytic cells and CD68⁺ cells express the Mer receptor (MER), an indication that apoptotic cell clearance was also increased (Fig. 1b, c). CD68, also known as microsialin, is considered to be a marker specific to professional phagocytic capacity [16, 17]. When bone marrow cells were induced to undergo apoptosis, macrophages and, in particular, phagocytic cells were likely preserved or upregulated to meet the demands of clearing the dying bone marrow cells. Six-week-old C57B6/J female mice were administered etoposide daily for 7 days, and flow cytometric analysis was performed to characterize bone marrow changes. Mesenchymal stem-like cell (MSC) markers Lin⁻CD29⁺sca1⁺ [18] (Fig. 1d) and the leukocyte marker CD45 (Fig. 1e) were significantly increased. As these are markers for less differentiated cells, their increase suggests that the bone marrow was attempting to replenish cells that had undergone apoptosis. Cells positive for either monocyte/macrophage marker CD11b (Fig. 1f) or phagocytic marker CD68 (Fig. 1g) were increased as a percent of the total marrow cells with etoposide treatment. Conversely, the marker for B cells (%B220) was decreased with etoposide treatment (Fig. 1h). When whole bone marrow from 8-week-old etoposide-treated (7–9 days) mice was evaluated for phagocytosis, there was an increase in CD68⁺ cells that engulfed apoptotic mimicry beads (Fig. 1i). When bone marrow from 8-week-old etoposide-treated mice was evaluated for efferocytosis, there was an increase in the CD68⁺ cells that engulfed apoptotic bone marrow stromal cells (BMSCs) (Fig. 1j).

Long-term effects of etoposide in the bone marrow

Longer term effects of etoposide were determined using 16-week-old female C57B6/J mice administered with etoposide daily for 5 days, then three times per week for 6 weeks. Body weights were recorded for mice treated with etoposide for 6 weeks, mice were sacrificed, and serum, long bones, and spleens were collected. There were no differences in body weight or spleen weights with etoposide vs. controls (data not shown). There was an overall reduction in cellularity in the bone marrow (vehicle $7.79 \times 10^6 \pm 1.42 \times 10^6$ vs. etoposide $5.86 \times 10^6 \pm 1.95 \times 10^6$ per tibia; $p = 0.05$). Flow cytometry was employed to characterize bone marrow populations. After 6 weeks of etoposide treatment, Lin⁻CD29⁺sca1⁺ MSCs as a percent of the total population were increased in the marrow (Fig. 1k). Etoposide treatment significantly increased the percent of CD45⁺ monocytes/neutrophils/granulocytes (Fig. 1l), the CD11b⁺ (Fig. 1m), and the CD68⁺ population in the bone marrow (Fig. 1n). Taken together, etoposide altered bone marrow cell populations with preference to MSCs and phagocytic cells.

Effects of etoposide on bone

Six weeks of etoposide treatment (age 16–22 weeks) significantly reduced microCT measures of trabecular bone volume (BV/TV) in proximal tibiae (Fig. 2a). Etoposide changes in BV/TV were due to significantly decreased trabecular thickness, increased trabecular spacing, with no significant effect on trabecular number (Fig. 2b–d). In diaphyseal tibia, etoposide significantly reduced cortical bone volume vs. vehicle (Fig. 2e). Histomorphometric analyses showed a similar and significant reduction in bone area (Fig. 2f). Of note, the decrease in tibial bone area (Fig. 2g) and femoral bone volume (Fig. 2h), was significant in as little as 9 days of etoposide treatment in 8 week old mice and 7 days of etoposide treatment in 6 week old mice (Fig. 2i).

In order to discern the early impact of etoposide that led to the long-term bone effects, 6-week-old mice were treated with etoposide for 7 days. The messenger RNA (mRNA) expression of the leptin receptor (*Lepr*) and *Cxcl12* were decreased in the bone marrow (Fig. 3a, b). *Lepr* and *Cxcl12* gene expressions are associated with mesenchymal cells responsible for bone formation and stem cell engraftment [19, 20]. There was a reduction in osteoprotegerin (*Opg*; *Tnfrsf11b*) but no difference in *Rankl* (*Tnfrsf11*) gene expression, resulting in an increase in the *Rankl/Opg* (*Tnfrsf11/Tnfrsf11b*) ratio (Fig. 3c) suggesting that at 1-week etoposide was tending to increase osteoclastogenesis at the local level. The mRNA levels for markers of osteoblastic activity *Runx2* and *Ocn* (*Bglap3*) were significantly and severely reduced in whole bone (marrow and bone tissue) (Fig. 3d, e) suggesting a robust negative impact of etoposide on bone formation. Significantly reduced serum PINP levels correlated with the decreased osteoblastic gene expression (Fig. 3f). In concert with the osteoclastic gene expression, histological assessment of TRAP stained sections revealed that osteoclast numbers per millimeter of linear bone were increased (Fig. 3g). However, serum TRAcP5b levels were significantly reduced (Fig. 3h), suggesting a disconnect at the local vs. systemic level that may be attributed to an overall reduction of bone resulting in less serum TRAcP5b reaching the circulation.

After 6 weeks of treatment, there were residual signs of reduced bone formation in etoposide-treated mice as evidenced by a slight but not statistically significant ($p = 0.15$) decrease in total procollagen type 1 N-terminal propeptide (PINP) (Fig. 3i). In contrast, serum TRAcP5b, a marker for bone resorption, was now significantly increased (Fig. 3j). Histological sections of the tibiae were stained for TRAP to visualize and enumerate osteoclast numbers. As trabecular bone was severely decreased with etoposide treatment, a region of interest close to the growth plate was used to analyze osteoclast numbers. While osteoclast numbers were marginally increased per linear bone ($p = 0.06$) with etoposide treatment (Fig. 3k), they were not significantly different, suggesting a normalizing effect.

Antiresorptive treatment effects on etoposide-induced bone loss

Since etoposide treatment resulted in a significant bone loss, the effects of an antiresorptive agent, zoledronic acid, as a preventive agent on etoposide-induced bone loss was evaluated (Fig. 4a). Mice were pre-treated with zoledronic acid (3 days) and then both zoledronic acid and etoposide administered for 14 days (total treatment regimen of 17 days). Complete blood counts revealed similar trends in the effects of etoposide as were seen after 9 days

(Table 2). Zoledronic acid alone revealed reductions in red cell distribution width, platelet numbers, and mean platelet volume relative to vehicle control; otherwise, there were no significant alterations in blood cell parameters. In general, the etoposide effects on blood counts were unchanged with zoledronic acid treatment with the exception of neutrophils. The etoposide associated reduction in neutrophils was inhibited when zoledronic acid was administered in combination with etoposide.

The 17-day etoposide/zoledronic acid regimen resulted in a significant reduction in trabecular BV/TV (Fig. 4b, c) with a concurrent decrease in trabecular number with etoposide (Fig. 4d). Zoledronic acid significantly increased bone volume regardless of vehicle or etoposide treatment (Fig. 4c). However, the combination of zoledronic acid and etoposide resulted in a decrease relative to zoledronic acid alone. Trabecular number and thickness were increased and trabecular spacing decreased in the zoledronic acid-treated mice (Fig. 4d, e). Etoposide reduced total marrow cell numbers in both control and zoledronic acid-treated mice (Fig. 4g) and increased the relative percent of CD11b, CD45, and CD68 positive cells in the bone marrow whereas zoledronic acid alone, or in combination with etoposide, did not impact the percent of CD11b or CD68 positive cells (Fig. 4h–j). The 14-day treatment of etoposide alone reduced the serum PINP bone formation marker albeit to a lesser extent than seen after 7 days of administration. Zoledronic acid alone also reduced levels, and the combination of zoledronic acid and etoposide resulted in further significant reduction (Fig. 4k). The serum bone resorption marker, TRAcP5b, was decreased similarly and significantly with etoposide and zoledronic acid alone, with a further reduction with the combination treatment (Fig. 4l). Of note, and similar to Fig. 3f, mice treated with etoposide had increased osteoclast numbers per millimeter of bone, but this effect was abolished when combined with zoledronic acid treatment. Zoledronic acid, regardless of combination treatment, significantly decreased osteoclast numbers (Fig. 4m). Taken together, zoledronic acid administration increased bone mass such that the reduced bone mass evoked by etoposide treatment was mitigated.

Discussion

The list of medications that have been reported to be associated with low bone mass in humans is extensive including aluminum, anticonvulsants, anticoagulants, aromatase inhibitors, cancer chemotherapeutics, glucocorticoids, gonadotropin-releasing hormone agonists, heparin, immunosuppressants, lithium, progesterone, proton pump inhibitors, selective serotonin uptake inhibitors, tamoxifen (pre-menopausal use), and thiazolidinediones [21]. Many of these drugs, and in particular cancer chemotherapeutics, alter the hematopoietic and/or immune system, and hence, their impact on bone may be indirect through cells in the bone marrow that support skeletal homeostasis.

The bone marrow contains diverse cell populations which serve as a reservoir for the rest of the body. The cellular composition of the bone marrow microenvironment has important implications for bone. In the present study, etoposide, a potent chemotherapeutic drug, was found to have a dramatic effect on bone marrow cell populations as well as a negative impact on bone mass. Administration of a bisphosphonate prior to and during etoposide treatment increased the bone mass such that the etoposide demise was not as net negative.

Etoposide is a plant-derived topoisomerase II inhibitor with application in cancer due to the overexpression of topoisomerase II in tumor cells [22, 23]. Inhibition of topoisomerase II leads to failure to properly repair DNA damage and activates the caspase-mediated apoptosis program. Although tumor cells may be particularly prone to etoposide, normal cells are also impacted. In mice, etoposide has been reported to reduce peripheral monocytes, yet the etoposide impact in the bone marrow of murine models is unclear [24]. Human mesenchymal stem cells were found to be relatively resistant to etoposide, and their differentiation was preserved in vitro [25] which is similar to what the present study found in mice in vivo. In humans, etoposide is most frequently administered with other chemotherapeutic agents and hence, little is known about the specific effect of etoposide on cells in the bone marrow or bone. Experimental investigations suggest that the hematopoietic stem cell niche may be compromised by etoposide treatment since bone formation was impeded; osteoblastic cells were found to be damaged, and in vitro colony-forming potential was hindered [26–28]. The hematopoietic stem cell niche is integrally linked to skeletal homeostasis and bone formation suggesting that agents which negatively impact the niche may also negatively impact the skeleton [29, 30].

Macrophages are hematopoietic cells that comprise a significant proportion of the bone marrow, and a subpopulation of macrophages adjacent to the bone surface has been termed “osteal” macrophages or “osteomacs” [31, 32]. Osteal macrophages promote bone formation and support fracture healing [33]. One of the primary roles of macrophages is to phagocytose apoptotic cells, which has been associated with polarization of monocyte/macrophages to M2 macrophages [34]. Interestingly, efferocytic peritoneal M2 macrophages produce factors such as TGF β and IL-10 which facilitate wound healing and regeneration [35, 36]; however, little is known regarding how bone marrow efferocytosis affects its microenvironment. In the present study, when mice were challenged with etoposide, there was a preservation of macrophages and their efferocytic activities, which may alter the bone microenvironment; however, this did not appear to serve a protective function in bone formation since P1NP levels were not elevated and gene expression markers of bone formation were reduced.

In the present study, etoposide resulted in increased cell apoptosis in the bone marrow as evidenced by Annexin V⁺ cells, a reduction in monocytes in the peripheral blood, and an increase in monocytic cells in the bone marrow suggesting that macrophages were preferentially located in the marrow for corpse clearance (efferocytosis). The role of macrophage efferocytosis is well described in tissues such as the peritoneum, spleen, and liver, but little has been described in bone. After irradiation, widespread cell death occurs and is followed by increased macrophage numbers likely to provide clearance of dead cells [37]. A preservation of macrophages was also seen in bone marrow after increased apoptosis via etoposide treatment; however, in contrast, irradiation treatment did not result in a difference in bone area (whereas etoposide decreased). While the difference may have to do with the fact that these two cell death induction models were performed on different aged mice, another possible explanation could be that etoposide and irradiation effects have differential modes or targets in the induction of bone marrow cell apoptosis.

Etoposide increased numbers of osteoclasts in the bone in the short-term studies and increased serum TRAcP5b after 6 weeks. Interestingly, in the short-term studies, serum TRAcP5b levels were significantly decreased. Though this is perplexing, there is previous evidence of this opposite effect seen in ovariectomy models resulting in increased osteoclast numbers per linear bone and either no change or decrease in serum TRAcP5b [38, 39]. In the present study, with the administration of a potent agent that induces widespread cell death, cells of the myeloid lineage could be preferentially directed to the efferocytic macrophage lineage to enable the clearing of dead cells and debris. Osteoclasts themselves could be employed with cell clearance during early etoposide treatment (which may not elicit TRAcP5b secretion) since there is evidence that osteoclasts are capable of efferocytosis [40]. A likely explanation is that the magnitude of etoposide impact on osteoclastic activity is minimal as evidenced by the low TRAcP5b levels in the long-term study and secondary to the impact on bone formation. Systemic serum TRAcP5b significantly decreased with etoposide may reflect an overall reduction in the bone available that could be resorbed. Notably, overall serum TRAcP5b levels are higher in younger mice than older mice and are likely be impacted at a greater magnitude. Nevertheless, this data suggests that serum TRAcP5b markers with etoposide treatment should be interpreted with caution as they may differ from histologic assessment, and vary in a temporal and age-dependent manner.

The clinical implications of this work center on the ability of a potent antiresorptive to buffer against bone loss induced by the chemotherapeutic agent etoposide. Bone is commonly affected in cancer and at times in association with chemotherapeutic agents [41]. Quach et al. elegantly showed that chemotherapeutic treatments such as cyclophosphamide, irradiation with bone marrow transplant, and 5-fluorouracil (5-FU)-induced bone loss [42]. They also showed that zoledronic acid was able to prevent bone loss in irradiated and 5-FU models. The current study expanded these findings to include etoposide, a common chemotherapeutic used to treat many cancer types. In contrast to the Quach study, where all treatments were given as a single dose, the current study investigated multiple dosings of both zoledronic acid and etoposide. After a single chemotherapeutic dose, Quach et al. found temporal effects in osteoblast, osteoclast histological parameters (peaks found at 4–10 days), early upregulation in serum P1NP (days 4, 7) which then decreased (vs. control) 2 weeks post administration, and an increase in *Rankl/Opg* mRNA 1–2 weeks post treatment. Serum TRAcP5b was not measured. Due to differences in dosing regimens, feedback mechanisms and temporal changes would likely differ between the two studies. However, in agreement, the current study we also found an increase in resorption parameters such as local osteoclast number (after 7 and 17 days) and increased *Rankl/Opg* gene expression (after 7 days). We further showed a severe decrease in marrow *Lepr* and *Cxcl12* and osteoblastic whole bone mRNA *Runx2* and *Ocn*. Interestingly, decreases in serum P1NP seem to be in accordance with the gene expression data, while the decrease in serum TRAcP5b was less clear. After 7 days of etoposide treatment, TRAcP5b was only decreased by ~25% while P1NP was decreased by >50% suggesting significant compromise in early parameters of bone turnover, with a more severe effect on formation. With the resultant bone loss, the systemic TRAcP5b could be lowered due to the reduction in overall bone mass available for resorption. Indeed, our analysis of increased local osteoclast numbers per linear

bone is also explained by the decrease in overall bone with etoposide treatment since the analyses of numbers of osteoclasts per tissue area (N.Oc/TA) were not significantly different (24.75 ± 1.81 vehicle vs. 25.36 ± 2.52 etoposide; $p = 0.85$). Taken together, the data suggest a more prominent negative effect on bone formation vs. bone resorption.

In the 17-day study, zoledronic acid was administered 3 days prior to etoposide and twice per week until sacrifice which resulted in increased bone volume that could still be reduced with etoposide dosing over time. This is an important comparison of zoledronic acid alone to zoledronic acid with chemotherapeutic since although bone volume was preserved with zoledronic acid (plus etoposide), it is likely not solely due to a specific antiresorptive effect countering the etoposide. Previously, zoledronic acid was found to increase hematopoietic stem cells in the marrow osteoblastic niche [43]. Bone resorption has been implicated in facilitating tumor metastasis by providing growth factor release from the mineralized tissues [44]. As such, antiresorptives like zoledronic acid inhibit tumor development in states of increased resorption like ovariectomy animal models [45]. Hence, the addition of an antiresorptive agent might be a prudent strategy to increase bone mass that could minimize the negative long-term effects of etoposide on the skeleton while also limiting the bone resorptive release of potentially tumor favorable growth factors from the mineralized matrix.

Potentially, adverse side effects should be taken into account when considering the use of a bisphosphonate. Antiresorptive agents have been associated with adverse events including osteonecrosis of the jaw and atypical fractures [46]. These side effects are more likely to occur with prolonged and/or higher doses of bisphosphonates such as those used for the treatment of skeletal metastasis. In addition, use of bisphosphonates is generally avoided in children as they can delay skeletal growth and tooth eruption [47]. However, using a low-dose bisphosphonate in conjunction with the etoposide chemotherapeutic approach could serve to protect against its deleterious skeletal effects.

In summary, etoposide treatment was found to induce apoptosis in the bone marrow which stimulated a phagocytic macrophage response and associated increases in mesenchymal stem cell expansion. In concert with this, etoposide treatment dramatically reduced parameters of bone formation and to a lesser extent increased osteoclastogenesis corresponding with reduced bone mass. Intervention with the bisphosphonate zoledronic acid mitigated the bone volume reduction with little impact on the hematopoietic effects of etoposide.

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Fig. 1.

Bone marrow population analyses. Sixteen-week-old C57B6/J mice were administered etoposide (20 mg/kg) daily for 5 days. Bone marrow was flushed and stained via flow cytometry to determine the presence of **a** AnnexinV⁺PI⁻ early apoptotic cells, **b** phagocytic CD68⁺ cells, or **c** CD68⁺ cells also positive for MER (a tyrosine kinase receptor associated with phagocytosis of apoptotic cells), representative FAC image on the *right*. Six-week-old mice were administered etoposide (20 mg/kg) daily for 7 days. Bone marrow was flushed and analyzed for **d** Lin⁻sca1⁺CD29⁺ mesenchymal stem-like cells, **e** CD45⁺ leukocytes, **f**

CD11b⁺ myeloid cells, **g** CD68⁺ cells, and **h** B220⁺ cells. Eight-week-old C57B6/J mice were administered etoposide (25 mg/kg) daily for 7–9 days, bone marrow flushed and incubated ex vivo with dragon green carboxylated beads (2 h), or Deep Red-stained apoptotic BMSCs (5 h), fixed, stained with CD68, and then flow cytometry performed. **i** The percent of double positive CD68⁺ with beads⁺ (indicating phagocytosis) was increased with 7 days of etoposide treatment. **j** The percent of double positive CD68⁺ cells with apoptotic BMSCs (indicating efferocytosis) was increased in 9-day etoposide-treated bone marrow. Representative flow cytometry dot plots with *dashed boxes* indicate double positive populations. **k–n** Long-term etoposide effects. Sixteen-week-old female C57B6/J mice were injected with etoposide (20 mg/kg) daily for 5 days, then 3×/week for 6 weeks. Bone marrow was flushed, stained, and FAC analysis performed for Lin[−]sca1⁺CD29⁺ mesenchymal stem-like cells (**k**), CD45⁺ leukocytes (**l**), CD11b⁺ myeloid cells (**m**), and CD68⁺ cells (**n**). Data is mean ± SEM; $n = 5–11/\text{gp}$ (**a–j**); $n = 7–8/\text{gp}$ (**k–n**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. vehicle (VEH) controls

Fig. 2.

Bone microCT and histomorphometric analyses of etoposide treatment. **a–e** Sixteen-week-old female C57B6/J mice were administered etoposide (20 mg/kg) or vehicle daily for 5 days, then 3×/week for a total of 6 weeks. MicroCT analyses and representative images of tibiae revealed that etoposide **a** decreased bone volume/total volume, **b** did not alter trabecular number, **c** decreased trabecular thickness, **d** increased trabecular spacing, and **e** reduced cortical bone volume. **f** Histomorphometric analysis and representative H&E stained images of tibiae revealed a decrease in bone area with 6-week etoposide regimen. Eight-week-old female C57B6/J mice administered etoposide (25 mg/kg) or vehicle for 9 days had reduced **g** bone area and **h** bone volume. **i** Six-week-old female C57Bl/6 mice administered etoposide (20 mg/kg) or vehicle for 7 days had reduced bone area. Data are mean ± SEM; * $p < 0.05$, ** $p < 0.01$ vs. vehicle (VEH) controls; $n = 7–11$ /gp

Fig. 3.

Bone formation and resorption indices after etoposide treatment. **a–h** Six-week-old female C57B6/J mice were treated with etoposide (20 mg/kg) for 7 days. RNA was harvested from bone marrow (**a–c**) or whole bone (marrow + bone tissue) (**d, e**) and real-time qPCR performed. Data was analyzed using the delta delta CT method and the y-axis represents the relative change of leptin receptor (*Lepr*) (**a**), C-X-C motif chemokine ligand 12 (*Cxcl12*) (**b**), receptor activator of NFκB ligand (*Rank*; *Tnfrsf11*) and osteoprotegerin (*Opg*; *Tnfrsf11b*) (**c**), runt-related transcription factor 2 (*Runx2*) (**d**), and osteocalcin (*Ocn*; *Bglap3*) (**e**). The ratio

of RANKL/OPG (*Tnfsf11/Tnfrsf11b*) mRNA expression is also indicated in **c**. Serum analyses of P1NP (**f**). TRAP staining was performed on tibial sections and analyzed in the proximal tibiae beginning 150 μ m from the growth plate and extending 900 μ m distally, up to but excluding cortical to determine osteoclast numbers per linear bone (OC#/mm) (**g**). Serum analyses of TRAcP5b (**h**). Sixteen-week-old female C57B6/J mice were treated with etoposide (20 mg/kg) daily for 5 days, then 3 $\times$ /week for a total of 6 weeks. Serum analyses of P1NP (**i**) and TRAcP5b (**j**). TRAP staining was performed on tibial sections and analyzed immediately distal to the growth plate (200 μ m) to determine **k** osteoclast numbers per linear bone (OC#/mm). Data is mean $\pm$ SEM; $n = 7-11$ /gp; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. vehicle (VEH) controls

Fig. 4.

Zoledronic acid effects on etoposide-induced bone loss. **a** Experimental design. Ten-week-old C57B6/J female mice were treated with vehicle, zoledronic acid (ZA) (50 µg/kg), etoposide (25 mg/kg), or the combination over 17 days as indicated by arrows. **b** Representative images and **c-f** tibial microCT analyses of bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing (Tb.Sp). **g-j** Bone marrow cell analyses: **g** total bone marrow cell count; flow cytometric analyses from bone marrow flush for percent CD11b⁺ (**h**), CD45⁺ (**i**), and CD68⁺ (**j**) cells after etoposide and zoledronic acid treatment regimen. Serum was analyzed for **k** P1NP and **l** TRAcP5b levels.

m Osteoclast number per linear bone (OC#/mm) was enumerated after TRAP stain. Data is mean $\pm$ SEM; $n = 8-10/\text{gp}$ (**b-j**, **m**), $n = 15-16/\text{gp}$, two experiments combined (**k-l**); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. vehicle; # $p < 0.01$, ### $p < 0.0001$ vs. etoposide alone; ^T $p < 0.05$, ^{TT} $p < 0.01$, ^{TTT} $p < 0.0001$ vs. zoledronic acid alone

Table 1

Complete blood counts after etoposide (5 days)

	Vehicle	Etoposide
WBC (K/ml)	2.83 ± 0.30	2.56 ± 0.18 **
NE (K/ml)	0.48 ± 0.04	0.25 ± 0.05 **
LY (K/ml)	2.23 ± 0.25	1.26 ± 0.14 **
MO (K/ml)	0.09 ± 0.02	0.04 ± 0.01 *
EO (K/ml)	0.021 ± 0.008	0.014 ± 0.007
BA (K/ml)	0.010 ± 0.002	0.003 ± 0.002
RBC (M/ml)	9.15 ± 0.10	7.45 ± 0.26 ***
Hb (g/dl)	12.61 ± 0.14	10.25 ± 0.23 ***
HCT (%)	39.06 ± 0.43	31.76 ± 0.91 ***
MCV (fl)	42.66 ± 0.17	42.71 ± 0.42
MCH (pg)	13.79 ± 0.23	13.83 ± 0.85
MCHC (g/dl)	32.37 ± 0.09	32.34 ± 0.44
RDW (%)	17.23 ± 0.25	16.26 ± 0.12 **
PLT (K/ml)	768.14 ± 17.90	1125.25 ± 65.10 ***
MPV (fl)	4.11 ± 0.08	4.16 ± 0.05

Data is mean ± SEM; $n = 5-6/\text{gp}$

WBC white blood cells, *NE* neutrophils, *LY* lymphocytes, *MO* monocytes, *EO* eosinophils, *BA* basophils, *RBC* red blood cells, *Hb* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red cell distribution width, *PLT* platelets, *MPV* mean platelet volume

*
 $p < 0.05$;

**
 $p < 0.01$;

 $p < 0.001$ vs. vehicle

Table 2

Complete blood counts after zoledronic acid (ZA) and etoposide (17 days)

	Vehicle	Etoposide	ZA	ZA + etoposide
WBC (K/ μ l)	2.74 $\pm$ 0.24	1.58 $\pm$ 0.21 ^{**}	3.20 $\pm$ 0.32	2.08 $\pm$ 0.18 ^T
NE (K/ μ l)	0.56 $\pm$ 0.05	0.29 $\pm$ 0.04 ^{**}	0.62 $\pm$ 0.10	0.51 $\pm$ 0.07 [#]
LY (K/ μ l)	2.08 $\pm$ 0.18	1.21 $\pm$ 0.16 ^{**}	2.42 $\pm$ 0.21	1.45 $\pm$ 0.13 ^T
MO (K/ μ l)	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	0.07 $\pm$ 0.02	0.06 $\pm$ 0.01
EO (K/ μ l)	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.07 $\pm$ 0.03	0.05 $\pm$ 0.01
BA (K/ μ l)	0.01 $\pm$ 0.003	0.01 $\pm$ 0.01	0.02 $\pm$ 0.01	0.01 $\pm$ 0.003
RBC (M/ μ l)	9.81 $\pm$ 0.10	7.32 $\pm$ 0.26 ^{***}	9.72 $\pm$ 0.15	7.05 $\pm$ 0.20 ^{TTT}
Hb (g/dl)	12.98 $\pm$ 0.17	9.56 $\pm$ 0.36 ^{***}	12.91 $\pm$ 0.16	9.33 $\pm$ 0.32 ^{TTT}
HCT (%)	44.66 $\pm$ 0.58	31.79 $\pm$ 1.07 ^{***}	44.00 $\pm$ 0.65	31.12 $\pm$ 1.08 ^{TTT}
MCV (fl)	45.50 $\pm$ 0.27	43.71 $\pm$ 0.43 ^{**}	45.31 $\pm$ 0.28	44.16 $\pm$ 0.25 ^T
MCH (pg)	13.24 $\pm$ 0.10	13.04 $\pm$ 0.18	13.28 $\pm$ 0.13	13.25 $\pm$ 0.12
MCHC (g/dl)	29.08 $\pm$ 0.26	30.06 $\pm$ 0.44	29.37 $\pm$ 0.29	30.02 $\pm$ 0.32
RDW (%)	16.44 $\pm$ 0.12	15.46 $\pm$ 0.19 ^{**}	17.27 $\pm$ 0.26 [*]	15.97 $\pm$ 0.22 ^T
PLT (K/ml)	618.80 $\pm$ 12.33	998.00 $\pm$ 60.40 ^{**}	552.80 $\pm$ 18.28 [*]	830.40 $\pm$ 48.42 ^{#TT}
MPV (fl)	3.79 $\pm$ 0.06	3.89 $\pm$ 0.04	3.97 $\pm$ 0.04 [*]	3.92 $\pm$ 0.06

Data is mean $\pm$ SEM; $n = 8-10$ /gp

WBC white blood cells, *NE* neutrophils, *LY* lymphocytes, *MO* monocytes, *EO* eosinophils, *BA* basophils, *RBC* red blood cells, *Hb* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red cell distribution width, *PLT* platelets, *MPV* mean platelet volume

^{*}
 $p < 0.05$;

^{**}
 $p < 0.01$;

^{***}
 $p < 0.0001$ vs. vehicle;

[#]
 $p < 0.05$ vs. etoposide;

^T
 $p < 0.05$;

^{TT}
 $p < 0.01$;

^{TTT}
 $p < 0.0001$ vs. ZA