RESEARCH ARTICLE

2-Methoxyestradiol analogue ENMD-1198 reduces breast cancer induced osteolysis and tumor burden both *in vitro* and *in vivo*.

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ABSTRACT

It has been estimated that 70% of advanced breast cancer patients will face the complication of bone metastases. Three processes are pivotal during bone metastatic growth of breast cancer namely, tumor cell proliferation, angiogenesis and osteolysis. During tumor-induced osteolysis, a number of cytokines and growth factors are released from the degraded bone matrix. These factors stimulate further tumor growth, tumor angiogenesis and tumor-induced osteolysis. New therapies should target all relevant processes to halt this powerful feedback loop. Here, we characterized the new 2-methoxyestradiol analogue ENMD-1198 and showed that ENMD-1198 is cytotoxic to tumor cells. Moreover, it showed both anti-angiogenic and vascular disruptive properties and was capable of protecting the bone against tumor-induced osteolysis. We confirmed the *in vitro* data with a series of *in vivo* experiments showing the beneficial effects of ENMD-1198 and ENMD-1198-based combination treatments of metastatic breast cancer in bone both on tumor progression and on survival with long term ENMD-1198 treatment. We confirmed the *in vivo* relevance of the ENMD-1198 protective effect on bone both with X-ray radiographs and micro-CT. In addition, we combined ENMD-1198 treatment with low dose “metronomic” cyclophosphamide and the bisphosphonate, risedronic acid, leading to a mild increase in treatment efficacy.

INTRODUCTION

The preference of breast cancer to metastasize to bone followed by growth and bone destruction involves specific tumor-host interactions. It has been estimated that 70% of advanced breast cancer patients will face the complication of bone metastases. Complications of bone metastasis include osteolytic lesions resulting in severe bone pain, fracture, hypercalcaemia and nerve compression [1,2,3].

Several processes are crucial during bone metastatic growth. These processes include tumor growth and tumor – stroma interactions such as local pro-angiogenic signaling, local activation of the innate immune system and local suppression of the adaptive immune system [4,5,3,6]. Regulatory T (Treg) cells are capable of blocking local immune responses. A local increase in Treg cells is a major factor in the tolerance and immune avoidance of tumors [7]. Pro-angiogenic signaling and the local alterations of the immune system both have a positive feedback on tumor growth.

In addition, breast cancer cells are capable of releasing parathyroid hormone-related protein (PTHrP) which activates receptor activator for nuclear factor κ B ligand (RANKL) on stromal cells and osteoblasts. RANK-RANKL signaling not only leads to an increase in osteoclastogenesis but also prolongs the lifetime of osteoclasts, resulting in increased bone resorption. During bone resorption, several growth factors and cytokines are released from the bone matrix. These include transforming growth factor β (TGF-β), insulin like growth factors (IGFs), fibroblast growth factors (FGF-1 and -2), platelet derived growth factor (PDGF) and bone morphogenic proteins (BMPs). The released growth factors stimulate tumor cell proliferation and survival. TGF-β induces the upregulation in vascular endothelial growth factor (VEGF) expression by tumor cells leading to an increase in tumor angiogenesis [8]. Moreover, TGF-β stimulates the production and release of PTHrP by the tumor cells resulting in a self sustaining feedback loop known as the vicious cycle of bone metastases [9,10,11,12].

Due to the complex nature of bone metastasis new treatments should simultaneously target tumor cell proliferation, angiogenesis, immunological alterations and halt the cycle of bone metastases. In this study we describe the *in vitro* and *in vivo* efficacy of a combination therapy targeting these major processes involved in metastatic growth in bone. To do so we combined low dose “metronomic” cyclophosphamide (CTX), 2-methoxyestradiol (2ME2, Panzem) derivate ENMD-1198 and the bisphosphonate risedronic acid.

Previous research indicated that the therapeutic effect of metronomic chemo therapy is mainly due to a reduction of tumor angiogenesis rather than direct cytotoxicity [13,14]. In addition, metronomic cyclophosphamide selectively depletes Treg cells resulting in an enhanced tumor immune response [15,16].

The naturally occurring estrogen metabolite 2ME2 (Figure 1a) has been shown to have anti-tumor, anti-angiogenic and anti-resorptive effects [17,18]. *In vivo* therapeutic effects of 2ME2 are moderate due to the rapid inactivation via conjugation (positions 3&17) and oxidation (position 17). ENMD-1198 is a 2ME2 derivate with reduced metabolic liabilities (Figure 1b) [19]. ENMD-1198 displays anti-proliferative effects on tumor cells and neo-angiogenesis very similar to 2ME2. Itt inhibits pro-angiogenic signaling through downregulating HIF-1α and STAT3 and it has vascular disruptive effects [20,21,22]. To date, no studies on treatment of bone metastases or bone specific effects with ENMD-1198 have been published. ENMD-1198 is currently in clinical trial for treatment of primary breast cancer [23].

Bisphosphonate treatment blocks osteoclast function, thereby halting the cycle of bone metastatic growth [24,25,26]. It has been shown *in vivo* that bisphosphonate treatment mainly prevents the development of new metastases whilst exhibiting a transient effect on already established metastases [27]. Also, bisphosphonates reduce skeletal complications and morbidity in patients with bone metastases [28].

The combination treatment of metronomic cyclophosphamide, bisphosphonates and ENMD-1198 targets all of the aforementioned major processes involved in osteolytic bone metastasis of breast cancer, namely tumor cell proliferation, angiogenesis, local immune suppression and osteolysis. By targeting the tumor stroma, in addition to the cancerous cells, less drug resistance is to be expected over time. The aim of this study was to assess the efficacy of ENMD-1198 treatment on tumor growth and bone destruction in a murine model for osteolytic breast cancer metastases and to evaluate a possible synergy between ENMD-1198, metronomic cyclophosphamide and bisphosphonates.

MATERIALS AND METHODS

Animals

Pregnant Swiss Albino and female athymic mice (BALB/c *nu/nu*, 4-6 weeks old) mice were acquired from Charles River (Charles River, L'Arbresle, France), housed in individually ventilated cages whilst food and water were provided ad libitum. All surgical and analytical procedures were performed under isoflurane gas anesthesia. Tumor diameters in the animals were measured routinely. Animals were sacrificed by cervical dislocation at the end of the experimental period or when the tumor diameter exceeded 1 cm in concordance with local guidelines for the use of animals in cancer research. Animal experiments were approved by the local committee for animal health, ethics and research of Leiden University Medical Center.

Cell lines and culture conditions

The cell lines MDA-231-B/Luc+, (hereafter MDA-BO2) a bone-seeking and luciferase-expressing subclone from the human breast cancer MDA-MB-231 [29,30] cell line and RAW264.7, a murine osteoclast precursor cell line (ATCC, Manassas, VA USA) were cultured in DMEM (Invitrogen, Carlsbad, CA USA) containing 4.5g glucose/l supplemented with 10% fetal calf serum (FCS) (Lonza, Basel, Switzerland), 100 units/ml penicillin, 50µg/mL streptomycin (Invitrogen) and 800 µg/ml geneticin/G418 (Invitrogen).

Both cell lines were grown in a humidified incubator at 37°C and 5% CO_2. All cell lines were monthly checked for mycoplasma infection by PCR. Cells are checked routinely for morphological changes, no other authentication tests have been done on these cell lines since they were acquired in the lab.

In vitro viability assays

Both MDA-BO2 and RAW264.7 were plated in a 96 wells plate (Costar, Cambridge, MA USA) in 100 µl medium at a density of 10.000 cells per well and left over night to adhere. The next day the medium was replaced with medium containing the experimental compounds, 6 wells per condition.

After 24, 48 or 144 hours, cell viability was measured using a non-radioactive colorimetric MTS viability assay (Promega Benelux, Leiden, The Netherlands) according to the manufacturer's protocol. Optical absorption was measured at 490nm using a Versamax absorbance microplate reader (Molecular Devices, Sunnyvale, CA USA).

In vitro vascularization models

In vitro angiogenesis was measured as outgrowth of endothelial capillary structures from cultures of 17-day old fetal mouse metatarsal bone explants, as described previously [31]. In short, pregnant Swiss Albino mice were sacrificed by cervical dislocation at day 17 of gestation and isolated foetal metatarsals were cultured in α-MEM medium containing 10% FCS (Lonza), penicillin (100 units/mL) and streptomycin (50µg/mL,Invitrogen) and left for 48 hours to adhere. Thereafter, the feed was replaced with medium containing VEGF (50ng/ml) (rhVEGF-A, Oncogene Science, Sanbio, Uden, The Netherlands). After 10 days of culture, the explants were fixed and stained with ER-MP12 antibody directed against murine PECAM-1 (CD31) (kind gift of Dr. P. Leenen, Erasmus University, Rotterdam, The Netherlands).

Images were obtained using a digital camera and the area of PECAM-1-positive tubular structures was quantified by image analysis using ImageJ 1.43r (Wayne Rasband, National Institute of Health, USA).

In vitro effects on newly established vasculature were examined as described previously [32]. In short, isolated 17-day old fetal metatarsals were left to adhere and cultured for 10 days in the presence of VEGF (50ng/ml) to stimulate capillary network formation. Thereafter, the feed, was replaced by medium containing the test substances. After 24 hours, bone explants were fixed and stained for PECAM-1 for further analysis as described before.

In vitro bone resorption assay

Bone resorption was assessed as described earlier [33]. In short, pregnant Swiss Albino mice were injected with 30µCi ^{45}Ca (1 Ci/mmol; PerkinElmer, Waltham, MA USA) on day 16 of gestational age. The animals were sacrificed on day 17 and the ^{45}Ca pre-labeled fetal metatarsals were isolated and pre-cultured in 1 mL of αMEM (Invitrogen) supplemented with 0.1% BSA in six well plates (Costar) for 24h. This allowed ^{45}Ca exchange in the bones with the culture medium. The metatarsals were then cultured in 250 µL of (αMEM, 0.1% BSA) in 24-well plates for 10 days in the presence of 100nM PTHrP (1-34) (Bachem, Bubendorf, Switzerland) with or without additives (n=6 per condition). Finally, medium was removed and residual ^{45}Ca was extracted from the bones in 5% trichloroacetic acid (TCA) for 24h. The amount of ^{45}Ca in both the culture media and the decalcification fluid was determined by liquid scintillation using a β-counter (Packard 1600 TR, Groningen, The Netherlands). Resorption was expressed as percentage of ^{45}Ca from the pre-labeled explants that is released in the medium during culture (%^{45}Ca release = ((^{45}Ca release into medium [cpm]) / (total^{45}Ca incorporated in metatarsals [cpm])) × 100%).

In vivo treatment of bone metastases

MDA-BO2 cells were injected into the right tibiae as described previously [30]. In brief, two holes were drilled through the bone cortex of the upper right tibia with a 25-gauge needle (25G 5/8, BD Micro-Fine, Becton Dickinson, Franklin Lakes, NJ USA) and bone marrow was flushed out. Subsequently, 250,000 MDA-BO2 cells/10µl PBS was injected into the right tibiae of 6-week old nude mice. Three days after intra-osseous inoculation of MDA-BO2 cells the animals were randomly divided in groups (n=10).

In a first experiment, 4 groups of mice (n=10) received either ENMD-1198 (200mg/kg/day, per oral gavage) or CTX (30mg/kg/day, through the drinking water as described previously [14]) or a combination of ENMD-1198 and CTX or vehicle control. Treatment started at day 7 after inoculation and was continued throughout the experiment. After 6 weeks the animals were sacrificed by cervical dislocation hind-limbs were fixed and kept for *ex vivo* X-ray analysis.

In a second experiment, 4 groups of mice (n=10) received ENMD-1198 (200mg/kg/day, per oral gavage) or ENMD-1198 + CTX (30mg/kg/day, through the drinking water) or ENMD-1198 + CTX + Risedronic Acid (Procter & Gamble Pharmaceuticals, Norwich, NY USA) (1.6µmol/kg/day, by sub cutaneous injection).

Bioluminescence Imaging

The progression of cancer cell growth was monitored weekly by bioluminescent imaging (BLI) using the IVIS100 Imaging System (Caliper Life Sciences, Hopkinton, MA USA).

X-ray radiographs and µCT analysis

At the experimental endpoints, mice were sacrificed and the tumor bearing hind- limbs were removed and assessed for osteolytic lesions by using a Faxitron 43805 (Hewlett Packard). X-ray radiographs were scanned and subsequently analyzed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

µCT Scans were made using a SkyScan 1076 µCT scanner (SkyScan, Kontich, Belgium) using a source voltage set to 40kV and a source current set to 250µA, with a step size of 0.9° over a trajectory of 180°. Images were taken with a frame average of 3 to reduce noise and an image pixel size of 9.03µm. Reconstructions were made using nRecon software (SkyScan) with a beam hardening correction set to 20% and a ring artefact correction set to 5. Volume measurements and visualizations were performed using MeVisLab (MeVis Medical Solutions AG, Bremen, Germany).

Statistics

Results are depicted as mean value +/- standard error of the mean (SEM). Differences between groups were determined by one-way analysis of variance for multiple comparisons followed by student’s t-test for differences between two specific groups.

RESULTS

ENMD-1198 reduces MDA-BO2 cell viability *in vitro*

The direct effect of the proposed treatment on tumor cells was assessed *in vitro.* In order to do this, the effect of ENMD-1198 on cell viability was investigated using an MTS colorimetric cell viability assay and with drug concentrations ranging from 0.1μM to 3.2μM. The MTS assay was performed after 48 hours exposure to ENMD-1198. ENMD-1198 inhibited MDA-BO2 cancer cell viability with an IC_{50} of approximately 0.8μM (Fig 2a).

The effect of CTX on cell viability was investigated using an MTS colorimetric cell viability assay and with drug concentrations of the active CTX metabolite 4-hydroperoxycyclophosphamide (4-HC), ranging from 1nM to 10μM. The low 4-HC concentrations up to 0.1μM were similar to concentrations measured *in vivo* during metronomic CTX treatment [34]. Cell viability was measured after both 24 and 144 hours exposure to 4-HC to assess the effects of direct treatment and prolonged exposure, respectively. Neither short or long term effects of 4-HC treatment could be observed using drug concentrations up to 0.1μM (Fig 2b).

ENMD-1198 has both an anti-angiogenic effect as well as a vascular disruptive effect *in vitro*.

The vascular effect of ENMD-1198 was assessed *in vitro*. The anti-angiogenic effect of ENMD-1198 treatment was investigated using an *in vitro* fetal metatarsal outgrowth angiogenesis assay. The fetal metatarsal explants contain a multitude of stromal and vascular/endothelial precursor cells. This cellular complexity closely mimics the *in vivo* situation during angiogenesis [31]. Low concentrations of ENMD-1998 slightly enhanced the vascular outgrowth suggesting a possible biphasic effect of ENMD-1198 on angiogenesis. However, this enhancement was not statistically significant. Higher doses of ENMD-1198 had profound dose dependent effects on VEGF stimulated angiogenesis (Fig 3a-b).

Next, the effect of ENMD-1198 treatment on previously established vasculature was assessed using metatarsal explants and an optimized vascular disruption protocol as previously described

[32]. Like the metatarsal angiogenesis assay, this assay contains a multiple cell complexity making it a relevant assay mimicking the *in vivo* situation. The newly established vascular bed in this assay, like tumor vasculature, is immature and does not contain pericytes. Comparable to the angiogenesis assay, low doses of ENMD-1198 ENMD-1998 slightly enhanced the vascular outgrowth without statistical significance. Higher doses of ENMD-1198 disrupted newly established vasculature with an IC_{50} of approximately 0.8μM (Fig 3c-d).

ENMD-1198 reduces the viability of RAW264.7 osteoclast precursor cells and inhibits PTHrP stimulated bone resorption.

The bone effect of ENMD-1198 was assessed *in vitro*. The effects of ENMD-1198 and 2ME2 on the viability of RAW264.7 osteoclast precursor cells were investigated using an MTS colorimetric cell viability assay and with drug concentrations ranging from 0.1μM to 12.8μM. The MTS assay was performed after 48 hours of ENMD-1198 or 2ME2 exposure. Both ENMD-1198 and 2ME2 reduced RAW264.7 cell viability. With an IC_{50} of approximately 0.4μM, ENMD-1198 was four times more potent than 2ME2 (IC_{50} ~1.6 μM) (Fig 4a).

After investigating the effects of ENMD-1198 on osteoclast precursor cells, a ^{45}Ca release assay was performed to investigate whether ENMD-1198 could effectively block PTHrP stimulated bone resorption. The assay was conducted using ^{45}Ca-labeled fetal mouse metatarsals in serum free medium with 0.5% BSA. Bone resorption was stimulated by addition of 100nM PTHrP to the culture medium. This culture condition is especially suitable to study effects on PTHrP induced bone resorption [33]. ENMD-1198 effectively blocks PTHrP stimulated ^{45}Ca release in a dose dependent manner (Fig 4b).

ENMD-1198 based combination treatments inhibit tumor growth *in vivo*.

Following the *in vitro* characterization of ENMD-1198 on the three major processes involved in skeletal metastatic growth of breast cancer, the ENMD-1198 based combination treatment effects were assessed *in vivo*. To investigate the effect on tumor burden, mice were treated either with ENMD-1198, CTX or ENMD-1198 + CTX. Tumor growth was followed using BLI. In all treated groups the tumor burden was decreased significantly compared to the untreated controls by day 27. There was a trend that ENMD-1198 treatment was more effective than CTX alone. A difference between ENMD-1198 and the ENMD-1198 + CTX combination could not be observed (Fig 5a).

Next we wanted to investigate whether adding a bisphosphonate (risedronic acid) to the ENMD-1198 + CTX combination would improve the treatment outcome. The ENMD-1198 + CTX treated group had a significantly reduced tumor burden compared to the control by day 21. There was a trend that the ENMD-1198 and the ENMD-1198 + CTX + risedronic acid treated groups had a reduced tumor burden as well. The animals in the control group were sacrificed before these differences could reach significance because the tumor size in the control group exceeded 1cm in diameter, a defined endpoint stated in the Dutch guidelines for animal experiments in cancer research. However, differences in growth kinetics between the treated groups and the untreated group can still be appreciated. The tumor development in this experiment was more rapid than in the previous experiment. In conclusion, adding risedronic acid to the treatment did not improve treatment outcome (Fig 5b).

ENMD-1198 based combination treatments are capable to control tumor growth, but are unable to eradicate the tumor.

After investigating the treatment effects on tumor burden, the efficacy of long term treatment with ENMD-1198 or ENMD-1198 based combinations was assessed. Seven days after intra-osseous inoculation with MDA-BO2 cells, mice were treated with either ENMD-1198, ENMD-1198 + CTX, ENMD-1198 + risedronic acid, ENMD-1198 + CTX + risedronic acid or vehicle control. The treatment continued for a maximum of ten weeks. In accordance to the Dutch guidelines for animal experiments in cancer research, mice were sacrificed when the tumor diameter exceeded 1cm. All three ENMD-1198 based combination treatments were able to control tumor growth over a prolonged period of time. The animals receiving the combination of ENMD-1198 and risedronic acid appeared less healthy with a thinner skin and aberrant behavior, suggesting possible adverse effects of long term treatment with this combination of drugs. The tumors grew exponentially as soon as the treatment was stopped after ten weeks. The addition of low-dose CTX and/or risedronic acid did not improve treatment outcome (Fig 5 c-f).

ENMD-1198 protects the bone against tumor-induced osteolysis *in vivo*.

Finally, the effect of ENDM-1198 treatment on tumor induced osteolysis was assessed using both X-ray radiographs and high resolution µCT scans made *ex vivo* after sacrificing the mice. These include untreated controls and groups treated with ENMD-1198 or CTX or a combination of ENMD-1198 + CTX. The corresponding BLI data of these animals are depicted in Fig 5a. In the X-ray radiographs, the surface area of osteolytic lesions was measured after manually drawing a region of interest around the lesion. The osteolytic lesions were significantly smaller in groups which received ENMD-1198 than the groups which did not receive ENMD-1198 (Fig 6a).

Because the radiograph method is based on a manual definition of the osteolytic lesion we wanted to confirm these results in using a method that is less susceptible to bias. To do so, the bone volumes of the upper half of the tibiae of these mice were measured in µCT scans. The groups which received ENMD-1198 had a significantly larger bone volume than the groups which did not receive ENMD-1198, confirming the X-ray radiograph data (Fig 6b).

DISCUSSION

This study was the first to assess the treatment efficacy of the 2ME2 derived compound ENMD-1198 on osteolytic bone metastasis of breast cancer. Our results indicate that ENMD-1198 interferes with all the crucial processes of bone metastatic growth of breast cancer namely, tumor cell proliferation, angiogenesis and bone resorption. We confirmed our *in vitro* data with a series of *in vivo* experiments showing the beneficial effects of ENMD-1198 and ENMD-1198 based combination treatments of metastatic breast cancer in bone both on tumor progression and survival with long term treatment over a period of 10 weeks. Furthermore, we showed the *in vivo* relevance of the ENMD-1198 protective effect on bone.

The observed *in vitro* effects of ENMD-1198 on cell viability were in line with earlier findings [19]. ENMD-1198 and 2ME2 are microtubule disrupting agents. Similar to other microtubule disrupting agents, they interfere with cell mitosis and thus have profound anti-proliferative effects on tumor cells and during neo-angiogenesis while resting cells are unaffected. Moreover, 2ME2 causes the phosphorylation of Bcl-2 and Bcl-xL leading to an upregulation of the intrinsic and extrinsic apoptotic pathways, a mechanism shared with paclitaxel [35].

ENMD-1198 and 2ME2 reduce angiogenesis by inhibiting the expression and nuclear accumulation of hypoxia-inducible factor (HIF)-1α. This results in a down regulation of VEGF expression and local pro-angiogenic signaling [36,37,21,22]. This impaired hypoxia sensing increases local concentrations of radical oxygen spiecies enhancing the pro-apoptotic effects of these compounds [21,22]. In addition, ENMD-1198 interferes with endothelial cell motility, chemotaxis and morphogenesis into capillary-like structures [22]. The results of our *in vitro* angiogenesis assay and vascular disruption assay confirmed these findings.

Metronomic CTX inhibits angiogenesis via three different mechanisms; by direct induction of apoptosis of proliferating tumor endothelial cells, by blocking or reducing the viability of circulating endothelial progenitor cells (EPCs) and by elevating levels of cellular and circulating thrombospondin-1 (TSP-1). These anti-angiogenic effects are enhanced in combination with

VEGF inhibitors [13,14]. Our finding that *in vitro* treatment with 4-HC is not cytotoxic to breast cancer cells were in line with earlier experiments conducted by Bocci *et al.* [13].

We expected to find a strong synergy between the anti-angiogenic effects of ENMD-1198 and metronomic CTX treatments *in vivo* since both treatments have been reported to inhibit angiogenesis via different mechanisms. The CTX + ENMD-1198 combination resulted in a better treatment outcome than CTX treatment alone, but this difference was not profound enough to suggest any synergy between CTX and ENMD-1198. Moreover, adding CTX to ENMD-1198 treatment did improve the overall outcome compared to ENMD-1198 alone in one *in vivo* experiment, but did not in the other.

ENMD-1198 reduced osteoclast precursor cell viability and was able to inhibit PTHrP stimulated bone resorption *in vitro*. When comparing the CTX treated group and the ENMD-1198 treated groups, there was a significant 60% reduction in osteolytic lesion size in the ENMD-1198 treated groups. The differences in tumor burden were much smaller and not significant between all groups. CTX treatment did not improve the osteolytic phenotype compared to the control group even though the tumor burden was significantly lower. Taken together, we conclude a strong bone specific effect of ENMD-1198 treatment.

To date, this is the first study showing these bone-specific effects of ENMD-1198. It has previously been shown that 2ME2 inhibits osteoclast differentiation and bone resorption and is cytotoxic to osteoclasts [38]. Interestingly, a 2ME2-mediated upregulation of osteoprotegerin has been described in osteosarcoma cells. This induction of osteoprotegerin can contribute to the anti-resorptive functions of 2ME2 in osteolytic metastatic lesions [39,40]. Induction of osteoprotegerin might be part of the mechanism in which ENMD-1198 protects the bone matrix from degeneration. The addition of risedronic acid to the combination treatment did not have an effect on tumor growth nor did it prolong the survival of the animals.

In conclusion, ENMD-1198 treatment of osteolytic bone metastases has profound *in vivo* effects on both tumor growth and osteolysis. ENMD-1198 based combination treatments can control tumor growth, however the tumor continues growing after treatment is stopped indicating a strong cytostatic effect. Due to the potent cytostatic and anti-angiogenic effects of ENMD-1198, only a weak additional value of having CTX treatment could be observed. However, any additional value of CTX treatment might be stronger in an immune competent model due to a selective depletion of Treg cells [41]. ENMD-1198 has strong anti-resorptive properties and the addition of risedronic acid to the treatment did not improve the overall treatment outcome. ENMD-1198 is a promising new compound in the field of metastatic bone disease affecting all processes that are crucial during development and growth of bone metastases.

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TITLES AND LEGENDS TO FIGURES

Figure 1

2ME2 and ENMD-1198

(a) Structural formula of the estrogen metabolite 2-methoxyestradiol (2ME2). Rapid inactivation of 2ME2 occurs *in vivo* via oxidation (at position 17) or conjugation (positions 3 and 17, both underlined).

(b) The 2ME2 derivate ENMD-1198 has been altered at positions 3 and 17 resulting in slower inactivation and increased plasma levels of the active compound *in vivo*. Both compounds have very low affinity for the estrogen receptor.

Figure 2

ENMD-1198 treatment reduces viability of MDA-BO2 cells *in vitro*, low dose cyclophosphamide does not.

(a) ENMD-1198 is reduces MDA-BO2 cell viability. Cell viability was measured after 48 hours of ENMD-1198 treatment.

(b) Low doses of 4-hydroperoxycyclophosphamide (4-HC) do not reduce MDA-BO2 cell viability. Cell viability was measured after 24 hours (direct effect) and 144 hours (prolonged metronomic treatment effect) of 4-HC treatment. 4-HC is the main active metabolite of cyclophosphamide.

Figure 3

ENMD-1198 has both an anti-angiogenic effect as well as a vascular disruptive effect *in vitro*.

(a) ENMD-1198 treatment inhibits angiogenesis in a mouse fetal metatarsal explants angiogenesis assay. Metatarsals were cultured for 10 days in the in the presence of VEGF and ENMD-1198 as indicated in the graph. After CD31 staining, the cultures were photographed and the surface area covered by the vascular outgrowth was quantified using ImageJ. Indicated statistical significant differences are in comparison with the VEGF treated control (* $p<0.05$; *** $p<0.001$). All differences in vascular outgrowth between the three ENMD-1198 concentrations are statistically significant.

(b) Representative images of the mouse metatarsal angiogenesis assay showing the metatarsal bone surrounded by vascular outgrowth.

(c) ENMD-1198 treatment disrupts pre-existing vasculature in a mouse fetal metatarsal explants vascular disruption assay. The fetal explants were treated with ENMD-1198 for 24 hours as depicted in the graph after 10 days of culture in the presence of VEGF (50ng/ml). After CD31 staining, the cultures were photographed and the surface area covered by the vasculature was quantified using ImageJ. Indicated statistical significant differences are in comparison with the untreated control (** $p<0.01$; *** $p<0.001$).

(d) Representative images of the mouse metatarsal vascular disruption assay. Note the remaining fragments of endothelial structures indicating areas where the vascular network has been disrupted.

Figure 4

ENMD-1198 has anti-resorptive effects *in vitro*.

(a) Both 2ME2 and ENMD-1198 reduce the viability of RAW264.7 cells, an osteoclast precursor cell line. ENMD-1198 had an IC_{50} of approximately 0.4μM and was shown to be four times more potent than 2ME2 (IC_{50} ~1.6μM). Cell viability was measured after 48 hours of 2ME2 or ENMD-1198 treatment. Indicated statistical significant differences are in between 2ME2 and ENMD-1198 (* $p<0.05$; *** $p<0.001$).

(b) ENMD-1198 treatment inhibits bone resorption in a mouse fetal metatarsal explants ^{45}Ca release assay. Radioactive ^{45}Ca-labeled murine fetal metatarsals were culture under the conditions depicted in the graph. The ratio of the amount of ^{45}Ca in the culture medium and the amount of ^{45}Ca in the bone was measured after 10 days of culture. Indicated statistical significant differences are in comparison with the untreated control, stimulated with PTHrP. Although the 5μM was not significantly different from the untreated control, it was different from the lower treatment concentrations ($p=0.006$ when compared to 0.1µM) (** $p<0.01$; *** $p<0.001$).

Figure 5

ENMD-1198 based combination treatments reduce tumor burden, but does not eradicate the tumor.

(a) Treatment started 7 days after inoculation (T0). All treated groups had a reduced tumor burden compared to the untreated control (* $p<0.05$). There was a trend that the ENMD-1198 treated group had a lower tumor burden compared to the CTX treated group ($p=0.063$).

(b) The ENMD-1198 + CTX combination group had a statistically significant decreased tumor burden compared to the control group at day 21 ($p=0.026$). There was a trend that the ENMD-1198 and the ENMD-1198 + CTX + risedronic acid treated groups had a reduced tumor burden compared to the untreated controls ($p=0.088$ and $p=0.086$ respectively). However, the control mice died before this difference could reach significance.

(c) Survival curves of mice treated with ENMD-1198 based combinations. Mice were inoculated intra-osseous with MDA-BO2 cells. Treatment started 7 days after inoculation (T0). Mice were sacrificed when the tumor exceeded 1cm diameter in accordance with local guidelines. Treatment was stopped after 10 weeks. One mouse in the ENMD-1198 treated group died in week 4 due to an accident with the oral gavage. The tumor size reached a steady state in the treated groups during the treatment period, but rapidly expanded after the treatment was stopped.

(d-f) Individual graphs for each of the combination treatments. ENMD-1198 versus control (d). ENMD-1198 + CTX versus control (e). ENMD-1198 + CTX + risedronic acid versus control (f).

Figure 6

ENMD-1198 treatment protects the bone against tumor-induced osteolysis *in vivo*.

(a) Quantification of osteolytic lesion size by surface measurement on X-ray radiographs. X-ray radiographs were made after termination of the experiment. The lesion surface was measured in number of pixels. Graphs show the average and standard error of the mean. Groups treated with ENMD-1198 have smaller lesions than the other groups. (** $p<0.01$; *** $p<0.001$)

(b) Quantification of osteolytic lesion size volume measurement in μCT scans. μCT scans were made after termination of the experiment. Bone volumes of the upper half of the tibia (region of interest is indicated in the inset), cut-off planes were always defined in a 90 degree angle to the bone. Graphs show the average and standard error of the mean. Groups treated with ENMD-1198 had a larger bone volume than the other groups confirming the radiograph data. (* $p<0.05$; ** $p<0.01$)

(c) Representative X-ray and μCT images of all the treatment groups. Both X-ray and μCT analysis show a reduction in osteolysis in ENMD-1198 treated groups.

FIGURE 1

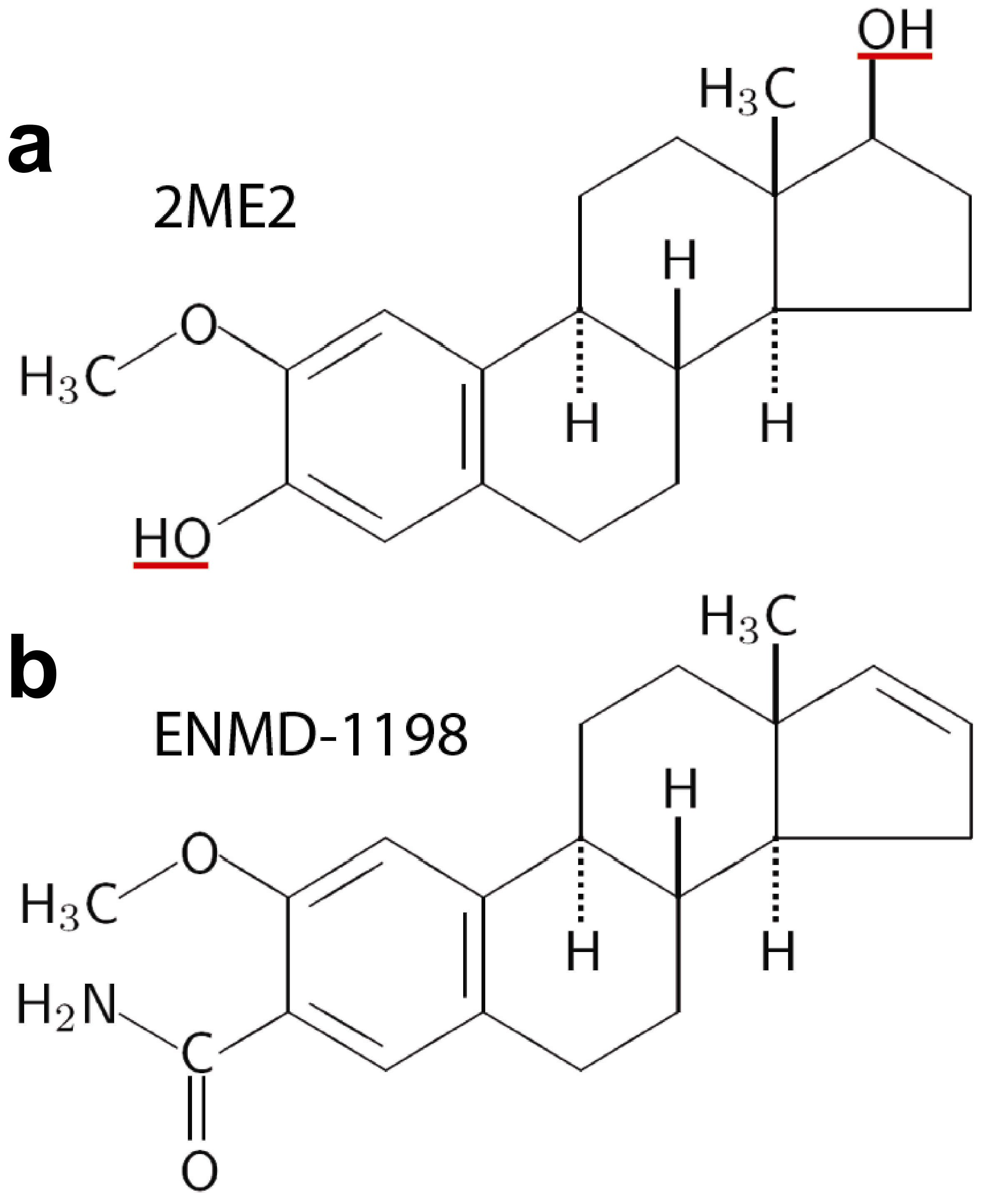


FIGURE 2

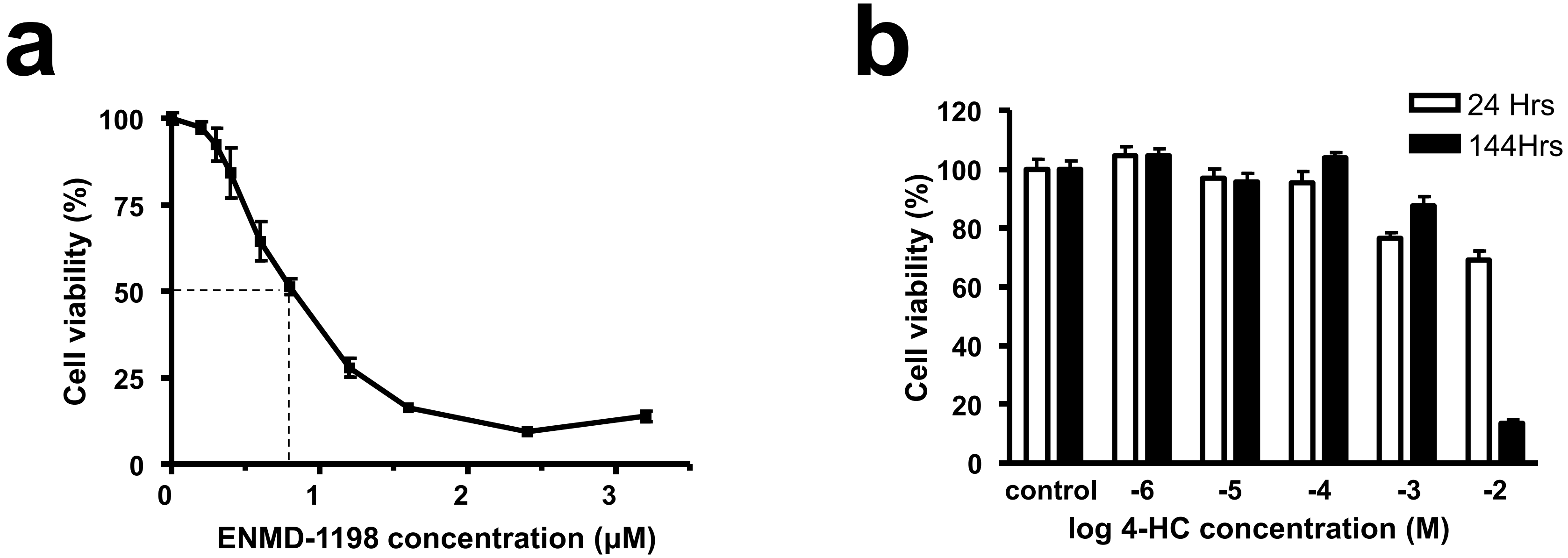

a
Cell viability (%)
100
75
50
25
0
0
1
2
3
ENMD-1198 concentration (µM)
b
□ 24 Hrs
■ 144Hrs
Cell viability (%)
120
100
80
60
40
20
0
control
-6
-5
-4
-3
-2
log 4-HC concentration (M)

FIGURE 3

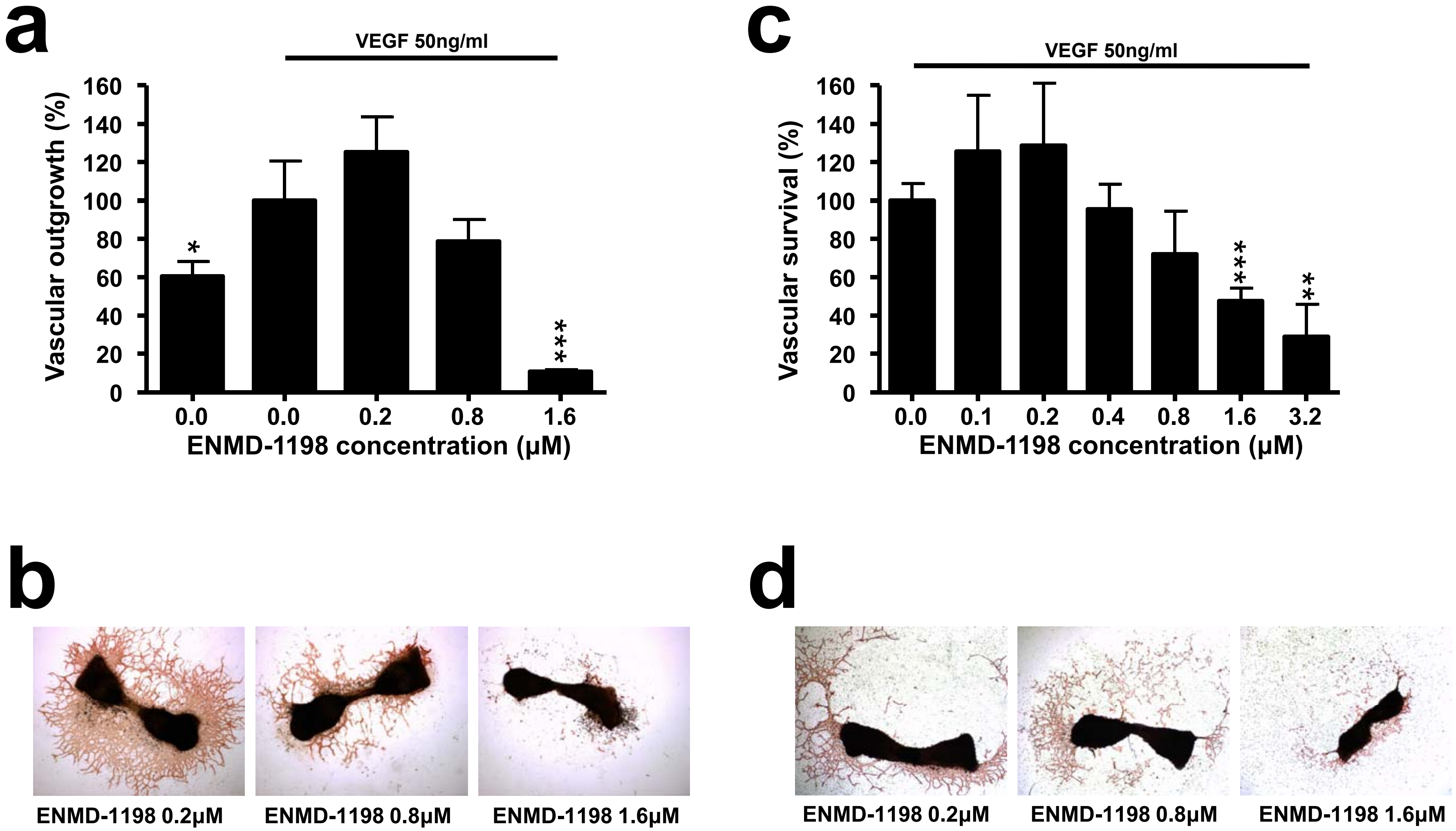

a
VEGF 50ng/ml
Vascular outgrowth (%)
160
140
120
100
80
60
40
20
0
*
***
0.0
0.0
0.2
0.8
1.6
ENMD-1198 concentration (µM)
c
VEGF 50ng/ml
Vascular survival (%)
160
140
120
100
80
60
40
20
0
***
**
0.0
0.1
0.2
0.4
0.8
1.6
3.2
ENMD-1198 concentration (µM)
b
ENMD-1198 0.2µM
ENMD-1198 0.8µM
ENMD-1198 1.6µM
d
ENMD-1198 0.2µM
ENMD-1198 0.8µM
ENMD-1198 1.6µM

FIGURE 4

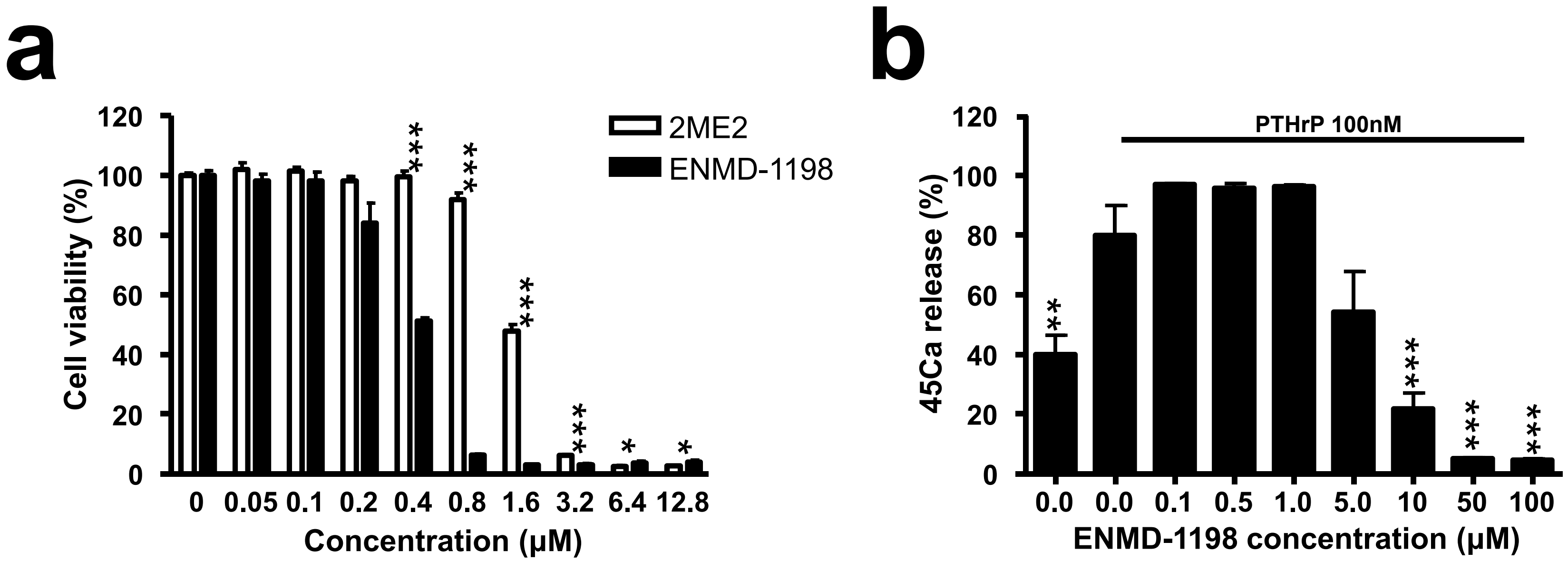

a
Cell viability (%)
120
100
80
60
40
20
0
2ME2
ENMD-1198
***
***
***
***
*
*
0
0.05
0.1
0.2
0.4
0.8
1.6
3.2
6.4
12.8
Concentration (µM)
b
45Ca release (%)
PTHrP 100nM
120
100
80
60
40
20
0
**
***
***
***
0.0
0.0
0.1
0.5
1.0
5.0
10
50
100
ENMD-1198 concentration (µM)

FIGURE 5

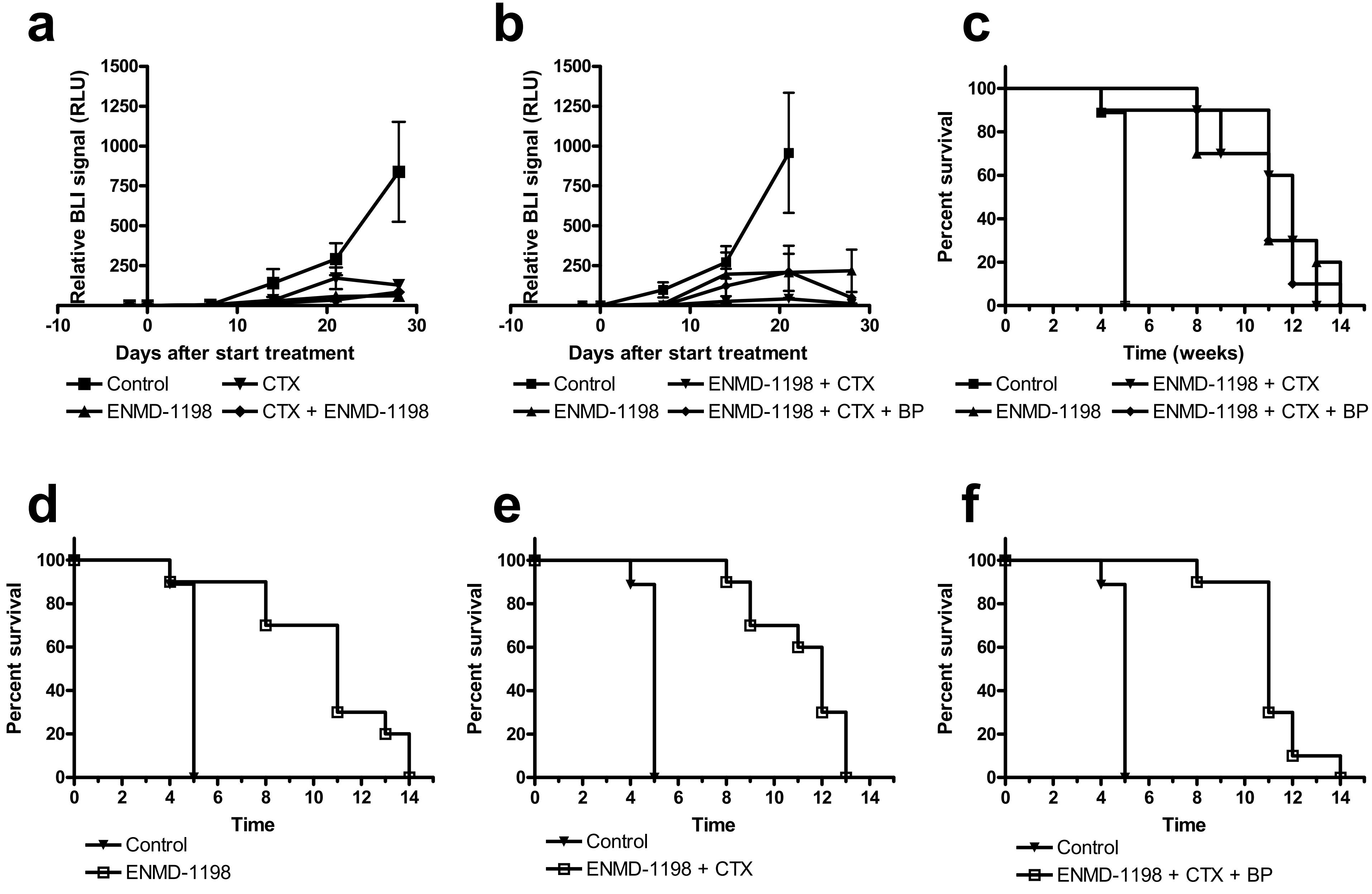
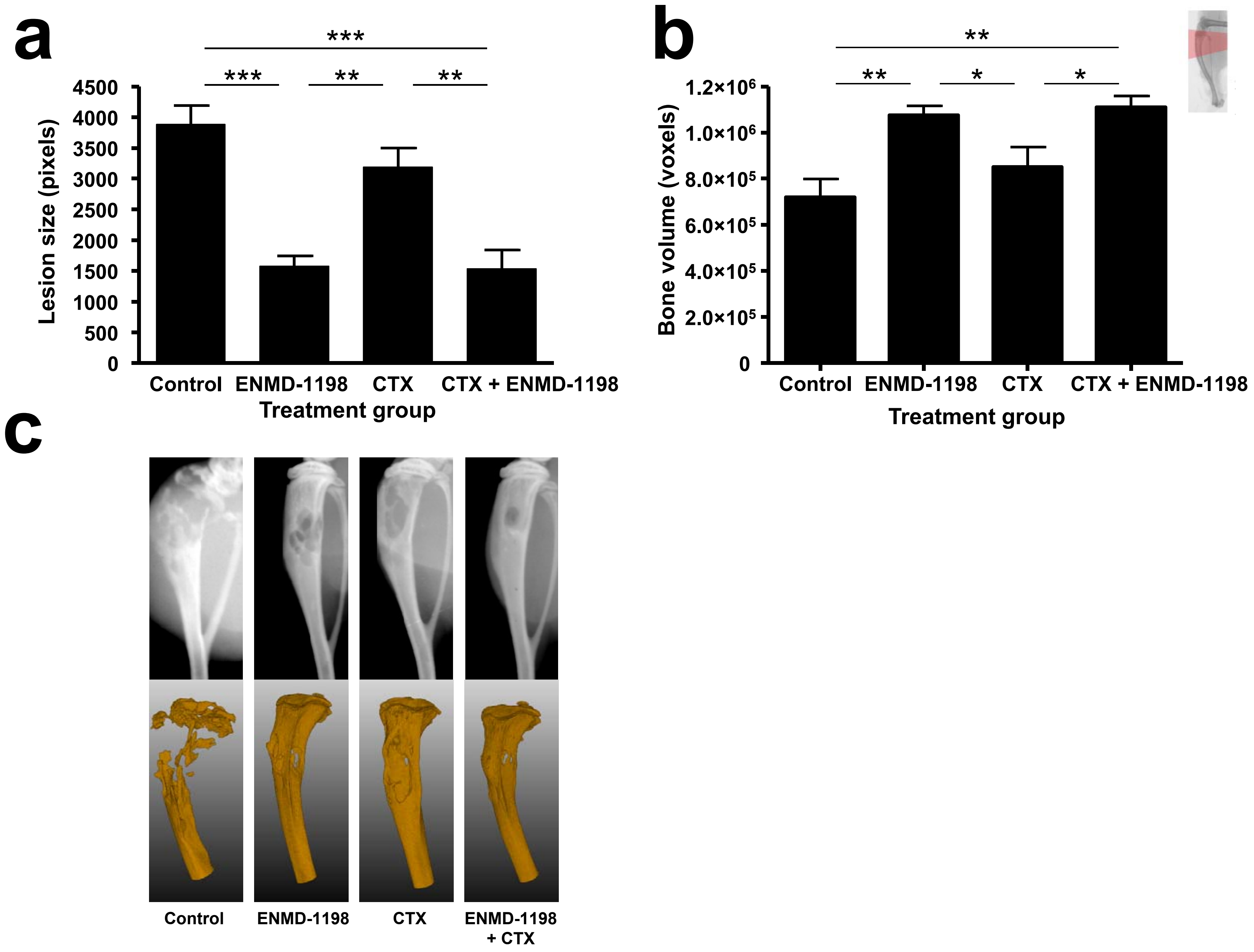

a
Relative BLI signal (RLU)
1500
1250
1000
750
500
250
-10
0
10
20
30
Days after start treatment
Control
CTX
ENMD-1198
CTX + ENMD-1198
b
Relative BLI signal (RLU)
1500
1250
1000
750
500
250
-10
0
10
20
30
Days after start treatment
Control
ENMD-1198 + CTX
ENMD-1198
ENMD-1198 + CTX + BP
c
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (weeks)
Control
ENMD-1198 + CTX
ENMD-1198
ENMD-1198 + CTX + BP
d
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time
Control
ENMD-1198
e
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time
Control
ENMD-1198 + CTX
f
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time
Control
ENMD-1198 + CTX + BP

FIGURE 6


a
***
***
**
**
Lesion size (pixels)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
ENMD-1198
CTX
CTX + ENMD-1198
Treatment group
b
**
**
*
*
Bone volume (voxels)
1.2×10⁶
1.0×10⁶
8.0×10⁵
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
Control
ENMD-1198
CTX
CTX + ENMD-1198
Treatment group
c
Control
ENMD-1198
CTX
ENMD-1198 + CTX