

Targeting aggressive osteosarcoma with a peptidase-enhanced cytotoxic melphalan flufenamide

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Abstract

Background: Low survival rates in metastatic high-grade osteosarcoma (HGOS) have remained stagnant for the last three decades. This study aims to investigate the role of aminopeptidase N (ANPEP) in HGOS progression and its targeting with a novel lipophilic peptidase-enhanced cytotoxic compound melphalan flufenamide (melflufen) in HGOS.

Methods: Meta-analysis of publicly available gene expression datasets was performed to determine the impact of ANPEP gene expression on metastasis-free survival of HGOS patients. The efficacy of standard-of-care anti-neoplastic drugs and a lipophilic peptidase-enhanced cytotoxic conjugate melflufen was investigated in patient-derived HGOS *ex vivo* models and cell lines. The kinetics of apoptosis and necrosis induced by melflufen and doxorubicin were compared. Anti-neoplastic effects of melflufen were investigated *in vivo*.

Results: Elevated ANPEP expression in diagnostic biopsies of HGOS patients was found to significantly reduce metastasis-free survival. In drug sensitivity assays, melflufen has shown an anti-proliferative effect in HGOS *ex vivo* samples and cell lines, including those resistant to methotrexate, etoposide, doxorubicin, and PARP inhibitors. Further, HGOS cells treated with melflufen displayed a rapid induction of apoptosis and this sensitivity correlated with high expression of ANPEP. In combination treatments, melflufen demonstrated synergy with doxorubicin in killing HGOS cells. Finally, Melflufen displayed anti-tumor growth and anti-metastatic effects *in vivo*.

Conclusion: This study may pave the way for use of melflufen as an adjuvant to doxorubicin in improving the therapeutic efficacy for the treatment of metastatic HGOS.

Keywords: aminopeptidase, chemotherapy, melflufen, metastasis, osteosarcoma

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Introduction

Osteosarcoma is a tumor histologically characterized by the presence of mesenchymal cells and production of bone stroma.¹ It is the most common bone tumor, predominantly occurring in children, adolescents and young adults.² One important determinant for the outcome in patients with HGOS is the response to chemotherapy,^{3,4} with up to 50% of patients displaying a poor response to conventional chemotherapy.

The last significant improvement in the management of HGOS patients was achieved in the 1980s when combined therapy, including surgery and multi-agent chemotherapy consisting of methotrexate, adriamycin/doxorubicin, and cisplatin (MAP), was introduced.⁵ Thereafter, over the past three decades the survival rates remained stagnant and unsatisfactory, especially in patients with metastatic and relapsed disease.^{6,7} In addition, osteosarcoma has a high invasive and

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metastatic potential with pulmonary spread, which is fatal in the majority of patients.^{8,9} A recently completed randomized clinical trial from the European American Osteosarcoma Study Group (EURAMOS-1 trial) failed to improve outcomes in HGOS patients with a poor response to MAP therapy.⁴ In this investigation, the addition of etoposide and ifosfamide on top of the standard MAP protocol did not improve clinical outcomes. Thus, novel approaches for HGOS treatment are urgently needed.¹⁰

Aminopeptidases, like aminopeptidase N (ANPEP/CD13), have been implicated in the pathogenesis and metastasis of several cancer types through enhancing invasion, motility, and angiogenesis.^{11–13} In osteosarcoma, ANPEP has been identified as a driver of cell migration and invasion.^{14–16} The aminopeptidase activity of ANPEP has been associated with the activation of PI3K and MAPK pathways,^{14,15} known contributors to HGOS pathogenesis and metastasis.^{17,18} High-ANPEP expression was linked to poor survival in osteosarcoma patients.¹⁹ Thus, eradicating osteosarcoma cells with a high expression of ANPEP/CD13 may offer a unique therapeutic option, given the introduction of novel aminopeptidase-enhanced cytotoxic compounds, such as melflufen.^{11,20}

Melflufen is an aminopeptidase substrate carrying a nitrogen based alkylating moiety bis(2-chloroethyl)amine, also present in cyclophosphamide, bendamustine, and melphalan. Melflufen has demonstrated high anti-neoplastic efficacy in several pre-clinical models as well as clinical trials,^{20–24} but has not been evaluated in osteosarcoma. In this study we evaluated the role of aminopeptidase expression in metastatic HGOS and assessed the ability of melflufen to eradicate aggressive osteosarcoma cells. Our data demonstrate that elevated levels of ANPEP expression are linked to a higher metastatic potential of osteosarcoma. Importantly, we could show that these aggressive malignant cells can be targeted with a peptidase-enhanced cytotoxic compound melflufen. In addition, melflufen's anti-neoplastic effect is potentiated by doxorubicin, a long-term first-line agent in osteosarcoma treatment. Combined with the favorable toxicity profile of melflufen shown in this study using an *in vivo* model, this study suggests that melflufen may be an effective adjunct to the treatment of HGOS patients.

Materials and methods

Analysis of gene expression and statistical methods

Gene expression datasets (GSE3362, GSE14827, GSE21257, GSE32981, GSE43281, GSE74230) were downloaded from GEO Omnibus. The values for gene expression measured by microarray were extracted using GEO2R software. As for RNA-seq analysis, the count tables supplied by the providers were processed using DeSeq2. The metastasis-free survival was assessed by Cox regression analysis. The statistical significance between different samples was evaluated using Mann–Whitney *U* test for unpaired samples and Wilcoxon test for the paired samples. Spearman correlation analysis was employed for the correlation analysis. All statistical analyses were performed using GraphPad prism.

Ethical aspects

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Tumor sampling and data collection was performed following informed consent, and the study was approved by the Regional Ethical Committee in Uppsala (Dnr 2007/237). *In vivo* experiments were performed using chick embryos. This model is recognized as an alternative to mouse xenografts for *in vivo* experiments by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R, UK). Experiments with chick embryos do not require administrative procedures for obtaining ethics committee approval for animal experimentation (European Directive 2010/63/EU). It has been confirmed by the regional Ethical Committee in Grenoble (Inovotion-JV-01).

Cell lines and media

Pilot experiments were with osteosarcoma cell lines available in house. For main experiments, osteosarcoma cell lines U2OS, SaOS-2, CAL-72, MG-63, HOS, 143B/HOS, and MNNG/HOS were purchased from the American Type Culture Collection (ATCC) and immediately put in culture for conducting the experiments. Cells were maintained at a humidified cell-culture incubator at 37°C and 5%

CO₂. Primary osteosarcoma cell lines STA-OS-1, -2, -3, and -5 were previously established at St. Anna Children's Cancer Research Institute and characterized by single nucleotide polymorphism arrays.²⁵ Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat inactivated fetal calf serum, 1% Pen-Strep, 1 mM sodium pyruvate (all ThermoFisher Scientific). Regular mycoplasma testing was performed using MycoAlert assay (Lonza).

Drugs

Melflufen was obtained from Oncopeptides AB. The other drugs mentioned throughout the study were obtained from SelleckChem. All the drugs were dissolved in dimethyl sulfoxide (DMSO) as 10 mM or 100 mM (where solubility is appropriate) stock solutions.

Survival Assays

Serial drug dilutions (0.01–100 μM range) were prepared in phenol red-free cell-culture medium. Cells were seeded in 96-well plates at 5×10^5 cells/ml density and incubated with the drugs for 72 h at 37°C and 5% CO₂. Cell viability was assessed using the Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to a protocol provided by the manufacturer. The read out was done on EnSpire Multimode plate reader (PerkinElmer). To estimate the half maximal inhibitory concentration (IC₅₀) smoothed dose-response curves were fitted using GraphPad Prism (GraphPad Software). The IC₅₀ values were calculated by determining the mean of three independent experiments. Synergy scoring was done using SynergyFinder platform.²⁶

Ex vivo chemosensitivity in primary osteosarcoma cells was assayed using the fluorometric microculture cytotoxicity assay.²⁷ Briefly, tumor cells from the patient surgical specimens were prepared by enzyme treatment of minced samples, followed by density gradient separation to enrich the tumor cells (>70% for cytological approval). The cells from cryopreserved samples were seeded in 384-well microtiter plates (in 45 μl culture medium). Drugs were added immediately after cell seeding using acoustic droplet ejection with an Echo® 550 (Labcyte Inc.). The culture plates were incubated for 72 h at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. After incubation, culture medium was washed away and fluorescein

diacetate added to all wells. After 30 min of incubation, fluorescence was measured. The response was quantified using the Survival Index% (SI%) as $SI\% = 100\% (F_{EXP} - F_{BLANK}) / (F_{CONTROL} - F_{BLANK})$ where F_{EXP} , F_{BLANK} and $F_{CONTROL}$ which corresponds to the fluorescence reading of treated, blank, and untreated control wells, respectively.

Flow cytometry

Flow cytometry analysis was performed on an LSRFortessa (BD, USA). Cells were grown in the medium indicated previously, harvested by using Stem Cell Pro Accutase (Invitrogen). Phycoerythrin (PE)-conjugated monoclonal antibodies WM15, IM7 (both Invitrogen), CC2C6, MIH42, MIH2, JS11, SKI1.4, P1E6-C5, GoH3, and SHM16 (all Biolegend) directed against CD13, CD44, CD47, CD276/B7-H3, CD274/PD-L1, CD55, CD155, CD49b, CD49f, and EPHA2 were used for detection of osteosarcoma surface markers. PE-conjugated MOPC-21 (Biolegend) were used as isogenic control. A minimum of 30,000 events were recorded. Quantification was performed using BD Quantibrite™ PE-conjugated beads (BD Biosciences) according to the manufacturer's protocol. Quantitative data is provided (Supplemental Figure 4).

Cell death analysis

Apoptosis kinetics was assessed using Real Time-Glo Annexin V Apoptosis and Necrosis assay (Promega, USA) according to the manufacturer's protocol. The readout was made using the aforementioned EnSpire Multimode plate reader (PerkinElmer). The experiment was repeated three times.

Western blot analysis

Osteosarcoma cells were lysed using high-salt buffer (20 mM Tris*HCl, 400 mM NaCl, 0.5% NP40, 0.3% Triton X100) with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The protein content was assessed using the Bio-Rad Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). After denaturing the samples in NuPAGE® LDS Sample Buffer (Thermo Fisher Scientific), 25 μg proteins were separated by electrophoresis using NuPAGE® 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific), transferred onto Immobilon-FL PVDF membranes (Merck) with the XCell IITM

Blot Module (Thermo Fisher Scientific), and treated with Odyssey Blocking Buffer TBS (LI-COR). Antibodies directed against the following targets were used to probe the membranes: phospho-histone H2A.X (Ser139) (D7T2V) mouse monoclonal antibodies (Cell Signaling Technology) and GAPDH (Abcam), dilutions 1:1000 and 1:2500 respectively. The DyLight™ conjugated antibodies (Cell Signaling Technology) were used diluted 1:20,000 for visualization of specific bands, which was made on the Odyssey Imaging System (Li-COR). Quantification was performed in Image Studio Lite 5.2 (Li-COR).

In vivo studies

Fertilized White Leghorn eggs were incubated at 37.5°C with 50% relative humidity for 9 days (Inovation INC, France). On day E9, the upper chorioallantoic membrane (CAM) was dropped down by drilling a small hole through the eggshell into the air sac, and a 1 cm² window was cut in the eggshell above the CAM. Osteosarcoma cells 143B/HOS were detached with trypsin, washed with complete medium and suspended in graft medium. An inoculum of 500K cells was added onto the CAM of each egg. Eggs were then randomized into eight groups. On day E10, tumors became detectable and were treated with either vehicle [1% DMSO in 1 × phosphate-buffered saline (PBS)], doxorubicin at 110 nM, or melflufen at 440 nM. For all conditions, the injection volume of 100 µl/egg, was dropped onto the tumor. The concentration of melflufen chosen is based on the achievable safe plasma concentration. On day E18, the upper portion of the CAM containing tumor was removed, washed in 1 × PBS, and then directly transferred in paraformaldehyde and fixed for 48 h. The tumor was then washed, carefully cut away from normal CAM tissue, and weighed. To estimate toxicity, eggs were checked at least every 2 days for viability and visible macroscopic abnormalities. The number of dead embryos counted on day E18, combined with reported abnormalities, was used to evaluate total toxicity.

Metastasis

Analysis of metastasis was done in parallel. Briefly, a 1 cm² portion of the lower CAM was collected to evaluate the number of metastatic cells which have invaded the embryo from upper

CAM to the lower CAM. The presence of human DNA in the lower CAM was used as a measure of tumor invasion since it only can originate from human tumor cells in this model. Genomic DNA was extracted from the lower CAM and analyzed by quantitative polymerase chain reaction with primers specific for human *Alu* sequences.²⁸ Calculation of cycle quantitation value (Cq) for each sample, mean Cq, and relative amount of metastasis for each group was performed with the Bio-Rad® CFX Maestro® software.

Immunohistochemistry

Immunohistochemical staining of tumors obtained from the chick embryos was performed using Bond Autostainer Kit (Leica) according to the manufacturer's protocol. Initially, 4 µm-thick sections were cut from formalin-fixed paraffin-embedded tissue blocks and were dried in a 37°C oven overnight. The sections were baked and dewaxed; antigens were retrieved and rehydrated using the deparaffinization protocol on the Leica Bond autostainer. This involved the incubation of the sections through several pre-programmed cycles at temperatures of up to 96°C using ER1 epitope retrieval buffer at pH 6.0. The sections were then automatically rinsed with Bond wash buffer. A Bond polymer refine peroxidase block was applied for 5 min then automatically rinsed with Bond wash buffer. Dako serum-free protein block was applied for 10 min without washing the slides, primary antibodies [anti-phospho-H2AX rabbit monoclonal antibodies EP854(2)Y (Abcam) at 0.025 µg/ml] and anti-cleaved caspase 3 rabbit monoclonal antibodies D3E9 (Cell Signaling Technology) at 0.275 µg/ml were applied for 30 min at ambient temperature, after which sections were rinsed with Bond wash buffer. Polymer refine (anti-rabbit horseradish peroxidase [HRP]) was subsequently applied to the slides for 15 min. All sections were then rinsed with bond wash buffer. Polymer refine 3,3'-diaminobenzidine (DAB) was then applied for 10 min. Following chromogenesis, the sections were washed with water and counterstained using the automated counterstaining protocol on the Bond; this involved incubating the sections with hematoxylin for 5 min followed by bluing with water. Once this automated program had finished, the slides were removed from the autostainer then dehydrated in an ascending series of ethanol (90–99%), cleared in three changes of xylene, and cover slipped under DePeX.

Results

ANPEP expression is elevated in aggressive osteosarcoma

To better understand the impact of ANPEP expression in osteosarcoma, publicly available data on gene expression in aggressive HGOS including xenograft²⁹ and orthotopic³⁰ mouse models as well as patient samples³¹ were analyzed. First, a cohort of HGOS patients ($n = 34$) from GSE21257,³² the only publicly available gene expression dataset, where corresponding clinical data on the time to the first detection of metastasis was available, was subjected to a Cox regression survival analysis. A significant decrease ($p = 0.0374$) of metastasis-free survival in patients with high ANPEP expression (top 30%) was found (Figure 1a). Moreover, the analysis of gene expression in the same cohort of patients³¹ indicated significantly higher ANPEP mRNA expression in patients that poorly responded to chemotherapy (Figure 1b) or developed metastasis (Figure 1c, d). Furthermore, in orthotopic murine osteosarcoma models³⁰ murine ANPEP mRNA was up-regulated in those primary tumors that produced metastasis in comparison with primary tumors lacking metastatic potential (Figure 1e). Also, ANPEP mRNA expression was higher in the pulmonary metastasis when compared with the corresponding primary tumor (Figure 1f). In addition, analysis of RNA-sequencing data²⁹ showed that ANPEP mRNA expression was up-regulated in highly invasive osteosarcoma cell lines 143B and MG63.3, metastatic derivatives of the well-known HOS and MG63 cell lines^{33,34} (Supplemental Figure 1). Moreover, samples of pulmonary metastasis isolated from murine xenografts of human osteosarcoma cell lines MG63, MG63.3, and 143B²⁹ also showed elevated expression of ANPEP mRNA (Supplemental Figure 1). Thus, elevated levels of ANPEP are indicative of aggressive osteosarcoma tumors with high invasive potential.

The aminopeptidase-enhanced cytotoxic agent melflufen is more effective against osteosarcoma cells than other alkylating agents in vitro and ex vivo

Elevated ANPEP expression in aggressive osteosarcoma prompted us to evaluate the potency of a novel lipophilic peptidase-enhanced cytotoxic agent melflufen which has shown potency in cancer models with high aminopeptidase expression.²⁰

Thus, we assessed the anti-proliferative potency of melflufen in a panel of osteosarcoma cell lines including low passaged patient derived neoplastic cells.²⁵ In addition, the potency of melflufen was compared with a panel of commonly used alkylating agents such as melphalan, cyclophosphamide, ifosfamide, busulfan and bendamustine, as well as standard of care protocol agents (doxorubicin, methotrexate, cytarabine), agents tested in the recent EURAMOS-1 studies (etoposide and ifosfamide)⁴ or drugs with proven pre-clinical efficacy (PARP inhibitors, MEK inhibitor).^{35,36} In concordance with the NCI sarcoma database³⁷ (Supplemental Figure 2), our results showed that common alkylating agents, including ifosfamide, demonstrated little efficacy against osteosarcoma cell lines within a physiologically relevant dose range (Figure 2a). Among the standard chemotherapy drugs, only doxorubicin was universally active in the panel of osteosarcoma cell lines we have employed, whereas methotrexate and etoposide did not show potency in several cell lines. Besides doxorubicin, melflufen was the only drug that was able to induce cytotoxic effect within a physiologically achievable dose range in all osteosarcoma cell lines tested (Figure 2a). Remarkably, melflufen was also able to induce cytotoxicity in a primary cell line STA-OS-3 resistant to both methotrexate and etoposide (Figure 2c, Supplemental Figure 3). Noteworthy, STA-OS-3 was established from a patient that poorly responded to the standard chemotherapy and died soon after the diagnosis.²⁵ Three other etoposide-resistant cell lines, SaOS-2, U2OS and STA-OS-1 also displayed sensitivity to melflufen (Figure 2a). Melflufen also showed better anti-proliferative activity in primary *ex vivo* cultures of patient derived osteosarcoma cells as compared with melphalan (Figure 2d–f), including a specimen resistant to doxorubicin (Figure 2d). Melflufen has demonstrated high *in vitro* efficacy in patient-derived HGOS cell lines (IC₅₀ 50–900 nM). Thus, melflufen has demonstrated a broad anti-proliferative effect against osteosarcoma cells even if they are resistant to commonly used chemotherapeutic drugs such as doxorubicin, etoposide, and methotrexate.

Melflufen induces rapid apoptosis in osteosarcoma cells

Having established the anti-proliferative efficacy of melflufen in survival assays, we analyzed whether melflufen can induce apoptosis and

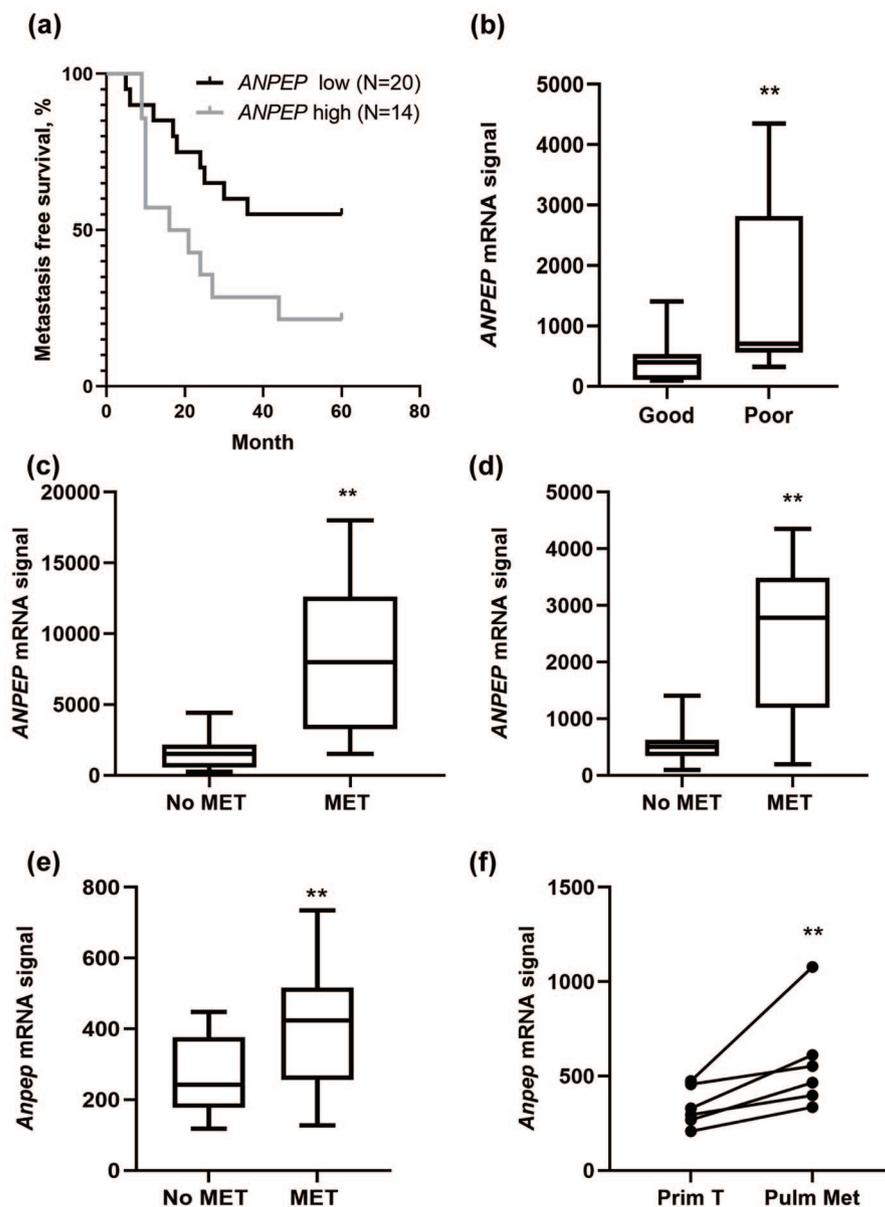


Figure 1. Analysis of ANPEP mRNA expression in murine and human osteosarcoma samples. (a) HGOS patients with higher ANPEP expression (grey) have significantly lower metastasis-free survival when compared with the HGOS patients with lower ANPEP expression. Mantel-Cox regression analysis, hazard ratio 2.749, 95% confidence interval 1.061–7.127, $p=0.0374$. (b) ANPEP mRNA is higher expressed in tumor samples of osteosarcoma patient with poor response to chemotherapy. GSE14827, $**p < 0.001$ Mann-Whitney U test. (c) ANPEP mRNA is up-regulated in osteosarcoma tumors produced pulmonary metastasis (MET) within 5 years upon initial diagnosis when compared with the samples from patients where no metastasis was detected 5 years upon initial diagnosis (No MET), GSE14827 $**p < 0.001$. (d) ANPEP mRNA is higher expressed in tumor samples of osteosarcoma patient with confirmed occurrence of pulmonary metastasis within 5 years upon diagnosis. GSE32981, $**p < 0.001$ Mann-Whitney U test. (e) Murine ANPEP mRNA is up-regulated in murine osteosarcoma tumors with metastatic (MET) potential when compared with the tumors which did not produce pulmonary metastasis (No MET), GSE43281, $**p < 0.001$ Mann-Whitney U test. (f) The tumors capable of producing pulmonary metastasis in a p53 mutated murine model display higher ANPEP mRNA expression in the metastasis (Pulm Met) when compared with the primary bone tumor (Prim T), GSE43281, $**p < 0.001$ Wilcoxon test. HGOS, high-grade osteosarcoma.

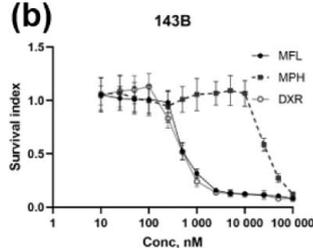
necrosis in HGOS cells. In real time experiments, melflufen was able to induce rapid apoptotic response in osteosarcoma cells with a maximum at 5–10h after treatment start, followed by the

accumulation of late apoptotic/necrotic cells (Figure 3a–d). The apoptosis induction was associated with a dose-dependent DNA damage as γ H2AX signal were detected by western blot

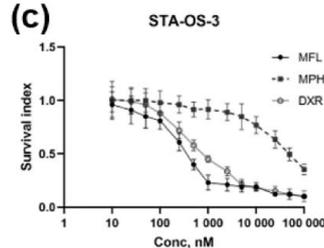
(a)

	143B	MNNG	U2OS	SaOS-2	CAL-72	MG-63	STA-OS-1	STA-OS-2	STA-OS-3	STA-OS-5
MFL	6.31	5.74	5.72	6.05	5.82	6.52	6.84	6.39	6.52	6.05
MPH	4.58	4.31	4.03	4.57	4.68	4.09	4.81	5.02	4.27	4.61
BDM	4.82	4.58	4.31	4.92	4.85	5.02	5.21	5.03	4.58	4.87
IFO	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
CPH	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
BUS	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
DXR	6.28	6.02	5.74	6.34	6.00	7.04	6.28	7.05	6.28	6.29
MTX	7.49	8.00	7.58	6.30	6.64	7.27	7.31	6.63	4.00	7.02
CDDP	4.81	5.30	5.18	4.60	4.80	4.98	5.04	4.18	4.28	5.68
ETO	6.12	5.57	5.00	5.30	6.02	5.97	4.00	6.02	4.00	4.99
ARA-C	6.30	6.30	4.82	5.32	6.00	5.98	4.81	4.92	5.03	4.92
TAZP	6.25	4.57	4.00	4.58	5.33	6.30	4.20	6.57	4.35	4.98
OLAP	4.00	4.00	4.00	4.32	4.00	5.05	4.04	4.95	4.00	4.00
NIRA	5.57	5.00	4.57	5.00	4.60	6.00	4.60	5.60	5.00	4.60
PAMI	4.29	4.31	4.22	4.30	4.33	4.58	4.31	4.29	4.05	4.00
DAS	5.00	5.31	4.98	4.89	5.02	5.32	5.25	4.99	4.92	5.02
TRAM	8.03	7.33	7.31	4.29	6.28	4.71	4.49	7.71	4.96	4.82

(b)

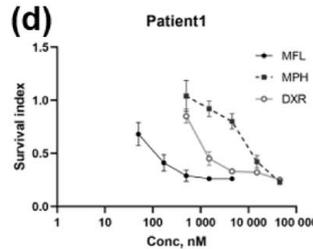


(c)

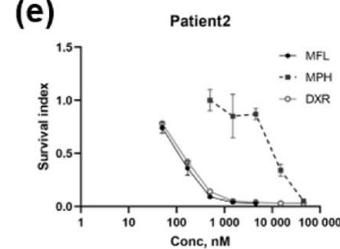
4,00 $-\log_{10}(\text{IC}_{50})$ 8,00100 μM

10 nM

(d)



(e)



(f)

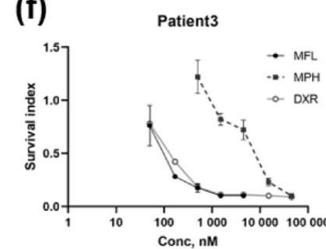


Figure 2. Drug sensitivity profile of osteosarcoma cell lines.

(a) Drug sensitivity profile of osteosarcoma cell lines in a panel of drugs used or proposed for osteosarcoma treatment. Panel of drugs include melflufen (MFL), melphalan (MPH), bendamustine (BDM), ifosfamide (IFO), cyclophosphamide (CPH), busulfan (BUS), doxorubicin (DXR), methotrexate (MTX), etoposide (ETO), cis-platin (CDDP), cytarabine (ARA-C), talazoparib (TAZP), olaparib (OLAP), niraparib (NIRA), pamiparib (PAMI), dasatinib (DASA), and trametinib (TRAM). The numbers are pIC_{50} [$-\log_{10}(\text{IC}_{50})$], thus larger values represent higher drug sensitivity, which is additionally reflected by more intense red color. (b, c) Representative pictures of growth inhibition of 143B (b) and STA-OS-3 (c) by melflufen (MFL), melphalan (MPH) and doxorubicin (DXR). (d–f) Growth inhibition of *ex vivo* primary osteosarcoma cells isolated from cryo-preserved patient samples, including doxorubicin-resistant sample (d) with demonstrated sensitivity to melflufen. IC_{50} , half maximal inhibitory concentration.

analysis of drug-treated osteosarcoma cells after 6 h (Figure 3e).

The anti-proliferative activity of melflufen on HGOS is dependent on aminopeptidase activity

It has been previously reported that in other tumor types melflufen's efficacy is dependent on the

activity of aminopeptidases.²⁰ To assess, whether aminopeptidase activity influences melflufen's cytotoxicity in osteosarcoma cells, an aminopeptidase inhibitor, bestatin, was employed.^{16,38,39} Pre-treatment of STA-OS-3 and 143B osteosarcoma cells with increasing concentrations of bestatin reduced the anti-proliferative effect of melflufen in a survival assays (Figure 4a–b), suggesting similar

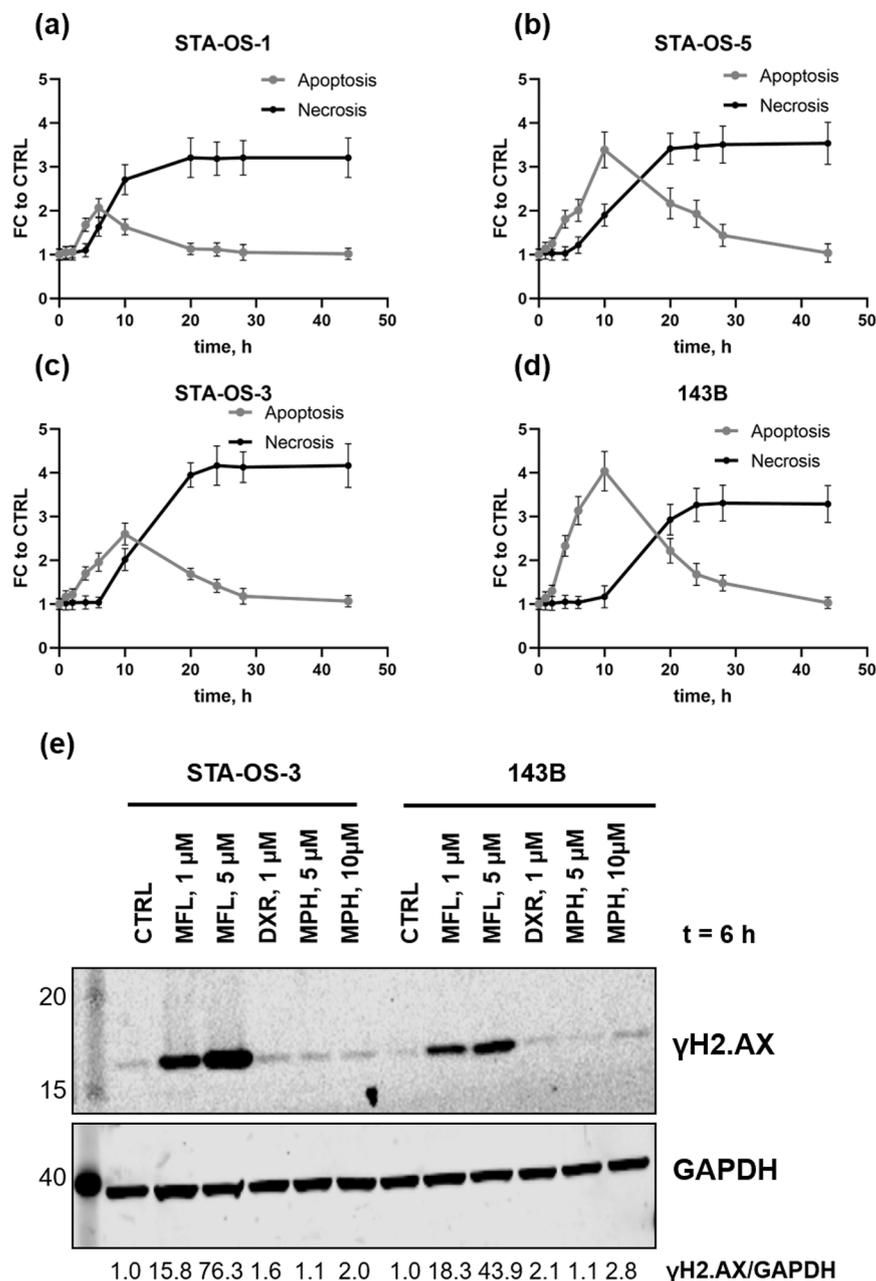


Figure 3. Effect of melflufen on apoptosis and DNA damage in osteosarcoma cells. (a–d) Indicated high-grade osteosarcoma (HGOS) cell lines were cultivated in the presence of 1 μ M melflufen and analyzed at 0, 1, 2, 4, 6, 10, 20, 24, 26, and 44 h for apoptosis and necrosis as described in the Materials and methods, $n=3$. (e) Western blot analysis of osteosarcoma cells 143B and STA-OS-3 treated with vehicle control (DMSO), melflufen (MFL), melphalan (MPH), and doxorubicin (DXR) at indicated concentrations. Quantification of the γ H2AX to GAPDH signal ratio is shown. DMSO, dimethyl sulfoxide.

aminopeptidase dependency as seen in other cancer types.^{20,40} In addition, ANPEP protein expression in the osteosarcoma cell lines used in this study was evaluated by quantitative flow cytometry analysis (Supplemental Figure 4) and was correlated with IC_{50} values determined for melflufen.

We found a positive correlation between sensitivity to melflufen (high pIC_{50}) and ANPEP/CD13 expression (Spearman $R = 0.72$, $p = 0.006$) in osteosarcoma cell lines (Figure 4c), suggesting that melflufen might be more effective in cell lines with high ANPEP levels.

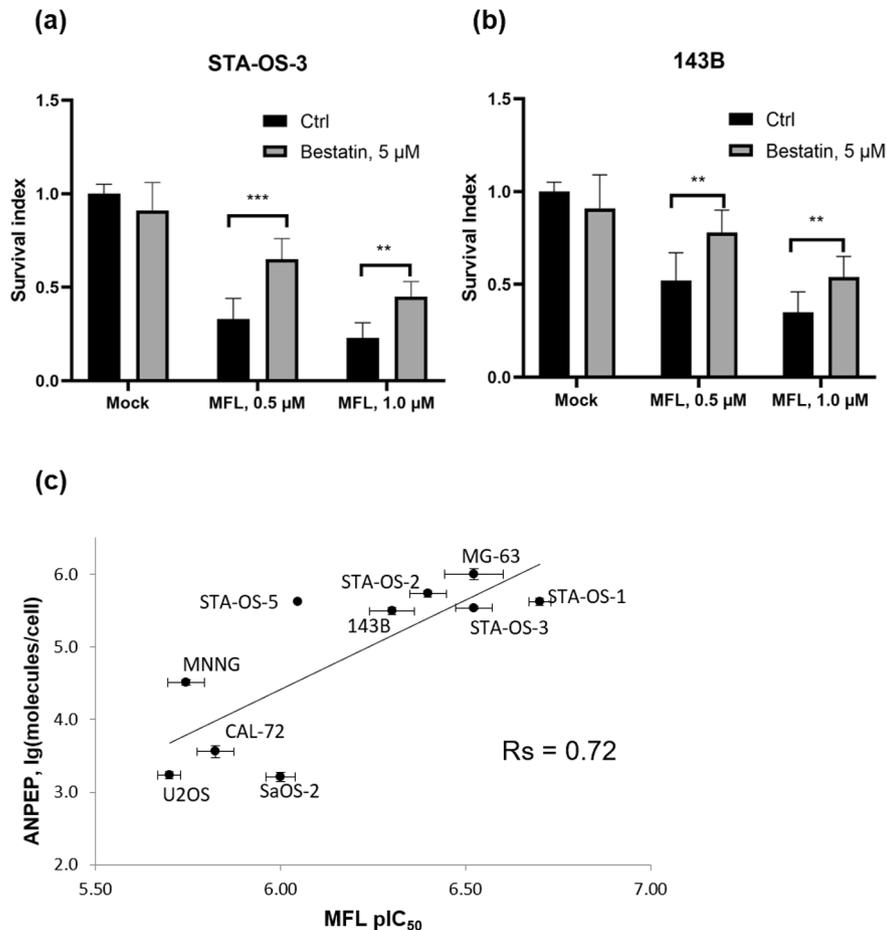


Figure 4. Melflufen's activity in relation to aminopeptidase activity and expression.

Effect of aminopeptidase inhibition by bestatin on melflufen's cytotoxicity in STA-OS-3 (a) and 143B (b) cells. (c) Correlation between melflufen pIC₅₀ and expression of ANPEP/CD13 (molecules per cell). The correlation analysis was done using the Spearman method. $R = 0.72$, $p = 0.006$

IC₅₀, half maximal inhibitory concentration. ** $p < 0.01$, *** $p < 0.001$.

Doxorubicin pre-treatment up-regulates ANPEP expression and synergizes with melflufen in killing osteosarcoma cells

It has been previously shown that elevated *ANPEP* transcript levels are detected in doxorubicin-induced cardiotoxicity in humans.⁴¹ We thus hypothesized that doxorubicin could also potentiate melflufen's cytotoxic effects in osteosarcoma cells with low *ANPEP/CD13* expression. Interestingly, our gene expression analysis of previously published data revealed that *ANPEP* mRNA is up-regulated upon doxorubicin treatment of osteosarcoma cells U2OS (Figure 5a).^{42,43} Moreover, *ANPEP* mRNA has been found up-regulated in the doxorubicin-resistant subline of highly aggressive osteosarcoma cell line 143B.⁴⁴ Thus, we first tested whether addition of doxorubicin leads to up-regulation of *ANPEP/CD13* expression in our panel of osteosarcoma cell lines.

Flow cytometry analysis demonstrated that indeed doxorubicin treatment promotes expression of *ANPEP* (Figure 5c–d). Second, we tested whether a 24h doxorubicin pre-treatment acts synergistically to enhance the cytotoxic effect of melflufen. Synergy scoring in two-dimensional survival assays and apoptosis analysis indicated that doxorubicin enhances the anti-neoplastic effect of melflufen and this was even more pronounced in cell lines with initially low *ANPEP/CD13* expression and low sensitivity to melflufen, such as U2OS, CAL-72, and MNNG (Figure 5b).

Melflufen shows high anti-neoplastic and anti-metastatic activity in osteosarcoma in vivo

To evaluate the anti-neoplastic activity of melflufen *in vivo*, we established a chicken CAM model of osteosarcoma. 143B cells were xeno-transplanted

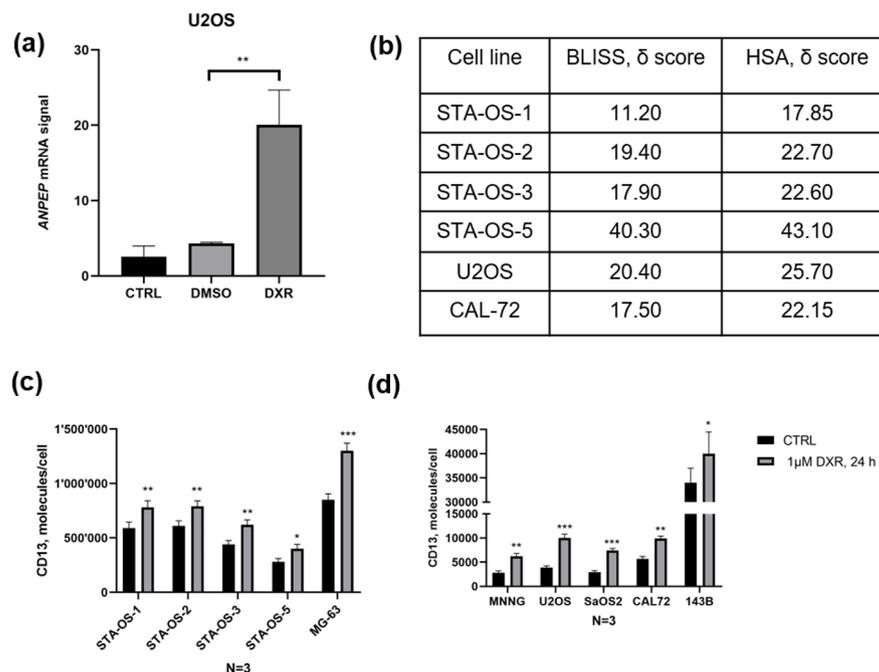


Figure 5. Effect of doxorubicin on aminopeptidase expression and melflufen's efficacy.

(a, b) The effect of doxorubicin on ANPEP/CD13 expression in osteosarcoma cells. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(c) The effect of doxorubicin on ANPEP expression in U2OS cell line. GSE46493, U test $p < 0.001$. (d) Synergy scores (Bliss, HSA and CI) for a combination doxorubicin melflufen in a panel of osteosarcoma cell lines. High δ scores (>20) correspond to synergistic effect, as do CI values below 0.9.

CI, combination index; HSA, Highest Single Agent.

onto the upper CAM of day E9 embryos (Figure 6a). Melflufen treatment was started on day E10 and doxorubicin was used as a positive control. The comparison of the number of dead chicken embryos in control, doxorubicin-treated, and melflufen-treated eggs indicated no noticeable toxicity of doxorubicin and melflufen in the chicken embryo (Figure 6b). Analysis of the tumors recovered at day E18 showed that melflufen significantly inhibited 143B tumor growth compared with the untreated control (Figure 6c). Thus, melflufen was well tolerated at a dose sufficient to induce an anti-osteosarcoma effect. Immunohistochemistry analysis of the tumor sections revealed increased cleaved caspase-3 signals in doxorubicin- and melflufen-treated samples (Figure 6e). Also, melflufen-treated samples showed an elevated level of γ H2AX, confirming our *in vitro* observations (Figure 3e). Importantly, analysis of the presence of 143B cells at the lower CAM allowed the accurate detection of osteosarcoma cell dissemination (Figure 6d). At the physiologically achievable and tolerable concentration of 440 nM, melflufen significantly reduced the metastasis ability of 143B cells compared with the untreated control, demonstrating

that melflufen inhibits both tumor growth and invasion of osteosarcoma cells *in vivo*.

Discussion

Overcoming stagnant survival rates in HGOS is a key objective in pediatric oncology.^{2,5,10,45} We provide evidence that melflufen is broadly effective at pharmacologically feasible doses against HGOS, employing cell lines, sensitive and insensitive to compounds, that are used as a standard of care or are in clinical development and patient-derived samples *in vitro*, as well as a CAM *in vivo* model. This anti-tumor activity is dependent on ANPEP activity and protein levels and can be enhanced by doxorubicin pre-treatment.

According to our meta-analysis, higher expression levels of the aminopeptidase ANPEP mRNA were attributable to osteosarcoma cells with higher metastatic potential. Moreover, pulmonary metastases displayed elevated expression of ANPEP transcripts as shown by gene expression analysis of several previously published datasets. In former studies, it has been shown that ANPEP/

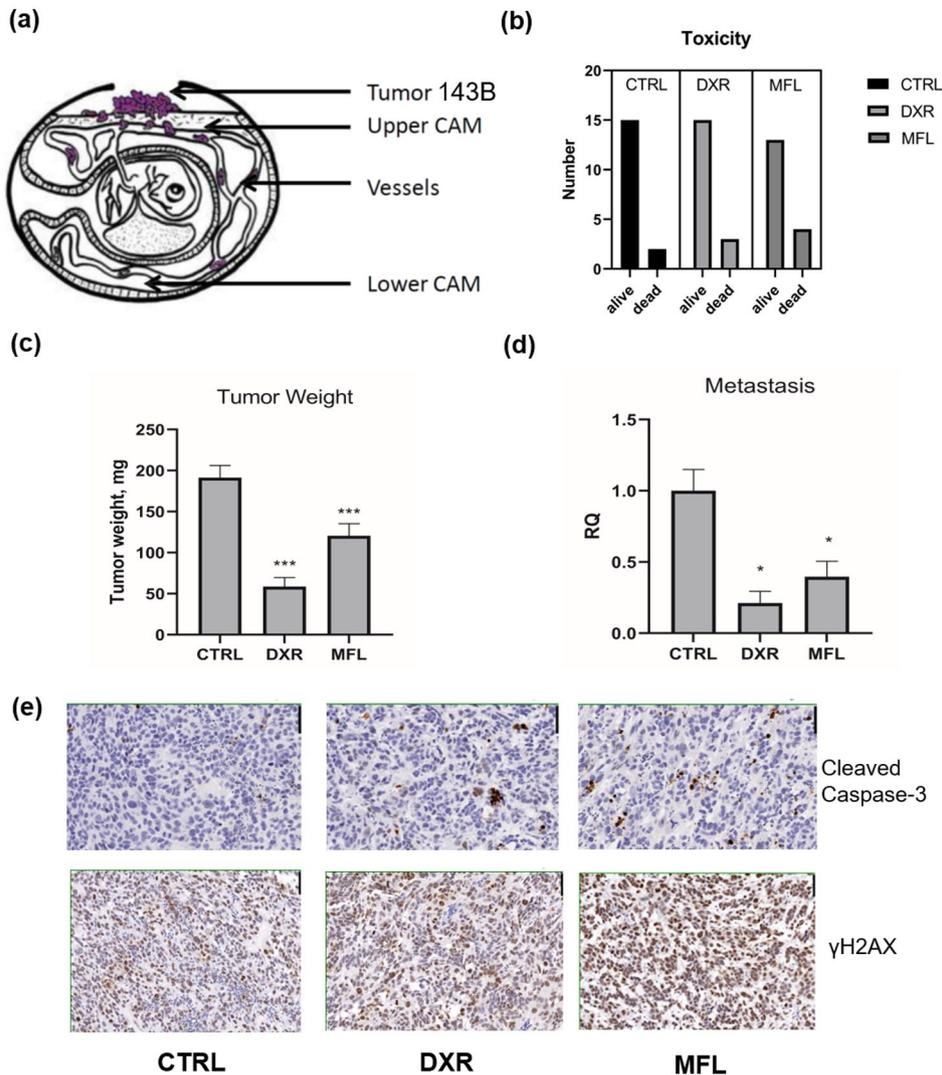


Figure 6. *In vivo* evaluation of anti-osteosarcoma activity of melflufen.

(a) Schematic representation of the chick embryo CAM model used in this study. 143B cells were transplanted onto the upper CAM of E9 chicken embryos. Treatment with 100 nM doxorubicin (DXR) or 440 nM melflufen (MFL) was started at E10. Controls were subjected to DMSO only. (b) Toxicity assessment: number of dead chick embryos upon drug application. (c) Average weight of tumors isolated at day E18 from the xenografts, analysis of variance test. * $p < 0.05$, *** $p < 0.001$. (d) Metastasis rate measured by the presence of tumor-derived markers and human *Alu* sequences by quantitative polymerase chain reaction in the lower CAM (e) Immunohistochemistry analysis of tumor sections stained for the apoptosis marker cleaved caspase-3 and a DNA damage marker γ H2AX. The scale bars (50 μ m) are at the upper right corners. DMSO, dimethyl sulfoxide.

CD13 enzymatic activity aids invasion of HGOS cells, a process contributing to the aggressiveness of the disease.^{11,14–16,46} These observations can be attributed to the pivotal role of ANPEP/CD13 in remodeling the extracellular matrix and promoting angiogenesis, two processes implicated in HGOS metastasis. Moreover, in our study, biopsy samples of poor responders showed higher levels of *ANPEP* mRNA expression in comparison with that of good responders.¹⁹ Thus, a promising clinical strategy is to eradicate HGOS cells with

high ANPEP expression. We here show that this is achieved using melflufen, a lipophilic peptide-conjugated alkylator potentiated by aminopeptidase activity.²⁰

Previous studies have shown an anti-neoplastic effect of melflufen in both hematological neoplasms^{21–24,47} and solid tumors including a pediatric malignancy neuroblastoma.³⁹ In this study, melflufen has shown a promising efficacy in HGOS. Our data demonstrate that melflufen is

the first-in-class alkylating agent, showing anti-neoplastic activity in osteosarcoma models *in vitro* and *in vivo*. Other alkylating agents used in the study including ifosfamide display poor cytotoxic activity against osteosarcoma cell lines, supporting previous observations made in a cell-culture system.^{37,48} On the other hand, application of melphalan by isolated lung perfusion in sarcoma patients suffering from pulmonary metastasis greatly increased the pulmonary progression-free survival from 33% to 63%.⁴⁹ It is tempting to speculate that melflufen might improve these results given its superior efficacy, provided an acceptable toxicity profile.

In our study, melflufen's cytotoxic activity against osteosarcoma cells positively correlated with ANPEP levels. Moreover, this effect was reduced by bestatin, a universal aminopeptidase inhibitor, indicating that aminopeptidase activity is essential for melflufen's anti-osteosarcoma effect. We also observed that melflufen demonstrated cytotoxic activity in osteosarcoma cells that failed to respond to methotrexate or etoposide, proving its potential in eradicating chemoresistant disease. Osteosarcoma cells with low ANPEP expression displayed lower sensitivity to melflufen. Interestingly, in this case melflufen's cytotoxic activity could be potentiated by doxorubicin pre-treatment since it results in up-regulation of ANPEP expression. Doxorubicin pre-treatment, however, was not able to increase the efficacy of melphalan or bendamustine in HGOS cell lines (data not shown), which further supports the rationale for a doxorubicin plus melflufen combination treatment. This combination has indeed shown synergistic effects in our panel of osteosarcoma cells, providing a strategy for targeting even osteosarcoma cells with low ANPEP expression that show low sensitivity to melflufen single treatment. Intriguingly, doxorubicin treatment of U2OS osteosarcoma cells led to the upregulation of additional aminopeptidases, such as *LAP3*, *RNPEP*, and *ERAP1/2*, as seen in the dataset GSE84863.⁴³ As doxorubicin is part of the HGOS standard of care treatment, its combination with aminopeptidase-enhanced cytotoxic agents may represent a promising strategy for eradicating metastatic osteosarcoma cells. Melflufen could also be combined with other novel approaches to eradicate chemo-resistant osteosarcoma.⁵⁰

Finally, *in vivo* studies have demonstrated anti-proliferative and anti-metastatic properties of melflufen in an animal model. Notably, melflufen

did not show any significant toxicities at the effective dose. Thus, melflufen represents a novel drug for the treatment of HGOS, given its favorable toxicity profile, and could complement the MAP protocol in future clinical trials.

Conflict of interest statement

KB, AS, FL are employed by Oncopeptides AB; FL and JG have equity in Oncopeptides AB; JG is a founder of Oncopeptides AB and currently provides consultancy to Oncopeptides AB.

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Supplemental material

Supplemental material for this article is available online.

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