Inhibitory Effects of Doxycycline on Tumor Progression *In vitro* and on Metastases in Early-Stage Osteosarcoma Xenografts Mice.

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Research

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Abstract

Background

Osteosarcoma (OS) is the commonest primary osseous malignant tumor with a high propensity to metastasize in lungs. Pulmonary widespread micrometastatic lesions are present in up to 80% of patients at initial diagnosis and they are associated with significantly worse prognosis. Doxycycline (Dox) is a synthetic tetracycline that has been shown to have anti-cancer properties *in vitro* and *in vivo*, and inhibit angiogenesis, effects that may prove beneficial for several types of cancer. The aim of the present work was to study how Dox affects OS cells' growth *in vitro* and *in vivo* and OS-driven pulmonary metastasis *in vivo*.

Methods

In vitro, the effect of Dox was measured in MG-63 and 143B human OS cells' viability, apoptosis, and migration. *In vivo*, highly metastatic143B cells were orthotopically implanted into the tibia of SCID mice and tumor growth as well as pulmonary metastases between Dox treated and untreated, non-amputated and early amputated xenografts were examined.

Results

Dox decreased the viability, inhibited the migration, and induced the apoptosis of OS cells *in vitro*. *In vivo*, Dox significantly enhanced tumor necrosis at primary OS sites, similarly to its *in vitro* effect. It also decreased the expression of Ki67, metalloproteinases 2 and 9 (MMP2 and MMP9), vascular endothelial growth factor A (VEGFA) and Ezrin in primary tumors. It also decreased the circulating VEGFA and MMP9 protein levels, in line with the decreased metastatic burden in Dox-treated mice in both non-amputated and early amputated xenografts.

Conclusions

Our results suggest that adjuvant administration of Dox may decrease OS growth and development of pulmonary metastases. Administration of Dox in combination with surgical resection and standard chemotherapeutic protocols in the early-stages of OS treatment is also supported. Moreover, Dox administration prior to the development of clinically detectable pulmonary macrometastases, is associated with enhanced clinically benefits from its anti-metastatic effect.

1. Background

Osteosarcoma (OS) is the most common primary malignant bone tumor with an estimated incidence of 1.7 - 4.4 per million people being diagnosed every year [1]. While OS frequently affects people between the age of 5 and young adulthood, it can also occur in people over 65 [2]. The distal femoral metaphysis and the proximal tibia are the most usual sites for primary OS to develop [3]. Usually, OS presents as a high-grade intramedullary bone lesion with malignant mesenchymal cells, which invade surrounding

tissues and produce immature bone, known as osteoid [3]. Approximately 80% of newly diagnosed OS cases have subclinical pulmonary micro metastases without radiologic evidence [4], leading to a significant decrease in the overall 5-year survival compared to the metastases-free OS patients (overall 5-year survival rate 30% compared to 70% for those free of distant metastases) [5]. Currently, the treatment protocol for OS includes neo-adjuvant chemotherapy (cisplatin, doxorubicin, ifosfamide, methotrexate) followed by wide surgical resection of the primary tumor and adjuvant chemotherapy [6]. Although the suggested OS treatment protocol presents the practice of choice for the treatment of OS, it does not seem to affect the metastatic rate of this very aggressive disease [7].

Dox is a synthetic third-generation tetracycline with a broad antibiotic spectrum. Its anti-cancer effects in several types of cancer have been previously examined with promising results [8, 9, 10, 11]. There is also limited *in vitro* evidence obtained from OS cell lines suggesting that Dox may be a potential OS anticancer agent [12, 13, 14]. One of the mechanisms through which Dox may exert its anticancer effects has been suggested to be its ability to inhibit matrix metalloproteinases (MMPs) due to calcium chelation [15]. MMPs are calcium-dependent, zinc-containing endopeptidases secreted by tumor cells with fundamental involvement in the degradation of the extracellular matrix (ECM), cancer cell invasion, metastasis and angiogenesis [16, 17]. MMPs may be also important for OS progression and targeting MMPs has been suggested as a potential therapeutic approach [18]. Experimental trials have suggested that MMPs interfere with the secretion of vascular endothelial growth factor A (VEGFA) from cancer cells [19]. MMPs and VEGFA help OS micro metastatic disease to stimulate local angiogenesis and remodel the microenvironment to support tumor neovascularization. In end-stage disease, micro metastases expand to become clinically detectable and give rise to lethal macro metastases [20].

The aim of the present study was to determine the *in vitro* and *in vivo* antitumor effect of Dox in OS and its potential for the prevention or limitation of pulmonary metastases in early-stage OS xenografts.

2. Materials And Methods

2.1 Cell cultures

143B (ATCC® CRL-8303) and MG-63 (ATCC® CRL-1427) human OS cell lines were purchased from the American Type Culture Collection [ATCC; (www.lgcstandards-atcc.org)]. OS cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 100 u/ml penicillin - 100 mg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2.

2.2 Cell viability Assay (Trypan blue exclusion)

OS cells were plated at a cell density of 3.5×10^4 cells/well in 6-well plates in EMEM supplemented with 10% FBS. Twenty-four and 48 h after seeding cells, trypan blue exclusion assay was used to determine the number of living MG-63 and 143B cells exposed to 0, 5, 10 and 20 μg/ml of Dox [21].

2.3 Cell migration assay (Wound healing assay)

OS cells cultured in EMEM supplemented with 10% FBS were seeded into 24-well tissue culture plate wells at a density of 2×10^6 cells/well so that after 24 h of growth, they should reach 90-95% confluency as a monolayer. The monolayers were scratched vertically with a sterilized 200 μl pipette tip across the center of the well. After scratching, the wells were washed twice with 1x phosphate-buffered saline (PBS) to remove the detached cells and replenished with fresh medium containing Dox (20 μg/ml) or PBS (control). Cells were photographed at 0, 8, 16, and 24 h after scratching, using an Olympus Bx40 microscope. TScratch software version 7.8 (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, Zurich, Switzerland) was then used to perform image analysis and measure the gap areas [21, 22].

2.4 Apoptosis Assay (Fluorescence-activated cell sorting/FACS)

The effect of Dox in OS cell lines apoptosis was carried out using flow cytometry. Briefly, 143B and MG-63 cells were fixed overnight at 4^0 C in 70% ethanol. The fixed cells were stained with RNase-containing propidium iodide (PI) and Annexin V FITC solution (TACS Annexin V FITC, Apoptosis Detection Kit, Gaithersburg, MD, USA). Cells were then separated as early apoptotic (Annexin V FITC-stained), late apoptotic (Annexin V FITC and PI-stained), necrotic (PI-stained) or non-apoptotic/live cells (no staining). DNA content was analyzed using a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA) and MoD Fit software (Verity Software House, Topsham, ME) [23].

2.5 In vivo orthotopic implantation of 143B human OS cells in mice xenografts

143B is a highly tumorigenic OS cell line with high metastatic rate [24, 25, 26]. The injection of 143B cells in mice tibias generates either spontaneous (tumor cells interact with their native microenvironment, invade local vessels, and move to distant sites) or experimental (direct seeding of tumor cells in lungs during the injection procedure) pulmonary metastases [27, 28, 29].

Thirty-two 6 to 8-week-old female severe combined immunodeficient (SCID) mice were obtained from the National Center for Scientific Research (Demokritos, Greece). Mice were acclimatized for 10 days without any interventions after transportation to the Laboratory for Experimental Surgery and Surgical Research "*N.S. Christeas*", Medical School, University of Athens, Greece, where the *in vivo* study was conducted. The animals were housed individually in clean metabolic cages placed in a well-ventilated house with optimum conditions (temperature 23 ± 1°C; photoperiod 12 h natural light and 12 h dark; humidity 45-50%) with access to food and water ad libitum during the entire study period. On day 0, the animals were anesthetized using a ketamine (100 mg/kg, Imalgene 1000; Merial, France) - xylazine (10 mg/kg Rompun; Bayer Animal Health GmbH, Germany) mixture injected intraperitoneally (IP)[30]. 143B OS cells (1×10^6 in 100 μl of PBS) were injected into the left proximal tibia of SCID mice using a 25-G needle [31]. All procedures were approved by the Regional Veterinary Service (no:366107/July 9, 2019) the Ethics Committee of the NKUA/Medical School (no:163/September 18, 2019) and were in accordance with National Legislation and European Directive 63/2010.

2.6 Study design

Mice were randomly divided into four groups. Mice in group A, Dox^{-}/Amp^{-} (n=7), comprised the untreated control group. Mice in group B, Dox^{+}/Amp^{-} (n=8), received Dox hyclate 50 mg/kg (D9891; Sigma Aldrich Chemical Co., St Louis, MO, USA) diluted in water and injected IP daily for 28 consecutive days [32]. Mice in group C, Dox^{-}/Amp^{+} (n=7), underwent transfemoral amputation using a standardized method (Day 5) [33]. Mice in group D, Dox^{+}/Amp^{+} (n=6), underwent transfemoral amputation (Day 5) and received Dox hyclate (50 mg/kg) IP daily for 28 consecutive days.

Four weeks after tumor inoculation (Day 28), all animals were anesthetized, blood was drawn from the orbital sinus and mice were euthanized via cervical dislocation [34, 35]. During the inoculation process 4/32 mice died after the acute onset of tachypnea, possibly due to pulmonary embolism. The left tibias (primary tumor sites) were evaluated with x-rays (Chirana type RK 75-10 EKv 2mm AL, Film Agfa 100NIF 25x30, Cassette Agfa CR MD 4.0 General) three weeks after engraftment of 143B cells to assess tumor formation at primary sites.

Resected xenograft tibias and tumor dimensions were measured using a digital caliper according to the formula: volume = (L + W) (L) (W) (0.2618). The value assigned as width (W) was the average between the anterior - posterior and medial - lateral planes of the proximal tibia. The value assigned as length (L) was the distance between the most proximal and the most distal tumor margin [28]. The lung volumes were calculated with a digital weight scale, and lung tissues were investigated for macroscopically detectable metastases using a stereoscope.

2.7 Surgical technique

Mice were anesthetized using a ketamine - xylazine mixture, as described above, injected IP [30]. A small animal heating pad was used during surgery to maintain normothermia. While under anesthesia, the left tibia of the mouse was shaved, cleaned with povidone iodine, and then rinsed with alcohol. The knees were flexed in 90^{0} and tibias were removed after midshaft femur cut to achieve tumor-free margins. The skin was closed with size 4-0 monofilament nylon sutures [33]. During this procedure, a mouse from group C died due to excessive bleeding (group C, new n=6). Buprenorphine (0.1 mg/kg, IP every 8 h; Bupaq® 0.3, Neocell Ltd.) was used for pain control over the first 24 h after amputation (Day 5) [36].

2.8 Quantitative measurement of blood biomarkers MMP9 and VEGFA

On day 28, blood samples were aspirated from mice under anesthesia with retro-orbital technique using fine-walled glass Pasteur pipettes (diameter:150mm). The blood samples were collected in heparin coated microhematocrit tubes and stored on crushed ice for no longer than 30 min before being centrifuged at 8000 rpm at 4°C for 10 min. Mouse serum was examined for MMP9 and VEGFA protein levels by ELISA (#ab100610 and #ab100662 respectively, Abcam, Cambridge, UK), according to manufacturer's instructions. The cell line we used to generate the *in vivo* OS model was human and we used human anti-VEGFA and anti-MMP9 ELISA kits to avoid cross-reaction with mouse VEGFA and MMP9 [37].

2.9 Histological Examination And Immunohistochemistry (Ihc)

Primary tumors and lungs were collected from mice, fixed in 4% paraformaldehyde in Tris-buffered saline (TBS) at 4°C for 18 h. Paraffin embedded tissue specimens were sectioned at a thickness of 3.0 μm, followed by deparaffinization in xylene and dehydration in a graded series of ethanol solutions

Histological examination

Each section was photographed in its entirety and subsequently, digitally processed utilizing the IpWin6 program. Tumorous areas were manually demarcated, and the program proceeded to assess the percentage of tumor areas to overall tissue surface. The same procedure was conducted to assess the percentage of necrotic tumor areas to the overall tumor surface in primary sites.

IHC: Antigen retrieval was performed by heating the samples for 20 min at 95°C in citrate buffer (pH 6.0), and endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature. Afterwards, the sections were incubated with anti-MMP9 antibody (rabbit polyclonal, #ab38898, Abcam), anti-MMP2 antibody (rabbit polyclonal, #ab97779, Abcam), anti-vimentin antibody (rabbit monoclonal [EPR3776], #ab92547, Abcam), anti-E-cadherin antibody (mouse monoclonal [4A2], #ab231303, Abcam), anti-Ki67 antibody (rabbit polyclonal, #ab15580, Abcam), anti-Ezrin antibody (mouse monoclonal, #ab4069, Abcam), anti-VEGFA antibody (rabbit polyclonal, cat. #ab46154; Abcam), all at a dilution of 1:300 at 4°C overnight. After washing with PBS, the sections were incubated with biotinylated secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG, cat. no. 20775; Millipore, Burlington, USA) for 10 min at room temperature (RT). They were then incubated with Streptavidin HRP (#20774, Millipore, Burlington, USA) for 10 min at RT, and the reaction was visualized using 3,3'-diaminobenzidine (#D12384, Sigma-Aldrich, Gillingham, UK). Eventually, the specimens were counterstained with Mayer’s hematoxylin at RT for 1 min. Images were photographed using a Nikon Eclipse 80i microscope equipped with a digital camera image system (Cellsens). The immunostaining of antibodies was scored on the scale of semi-quantitative assessment by evaluating the intensity and percentage of positively stained cells. The intensity of antibodies staining was scored as follows: 0, none; 1, weak; 2, moderate; and 3, strong. Samples were blindly inspected by two independent experienced pathologists [38].

2.10 Statistical analysis

All statistical analysis was performed using SPSS version 26 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY). All data were tested for normality using a Kolmogorov–Smirnov test and displayed parametric or nonparametric distributions ($p < 0.05$). The measurements of blood biomarkers, number of lung metastases, lung weight and quantitative IHC in lungs were analyzed using one-way analysis of variance (ANOVA) (Kolmogorov–Smirnov significance $p > 0.05$) followed by a post-hoc Bonferroni test. The measurements for the tumor volume, tumor weight, tumor necrosis and quantitative IHC in primary

tumors were analyzed using an independent samples t test, as the data showed normality. P<0.05 was considered as a statistically significant difference.

3. Results

3.1 In vitro studies

3.1.1 Doxycycline inhibits viability and induces early and late apoptosis of MG-63 and 143B human OS cells

Exogenous administration of Dox decreased the number of both MG-63 and 143B viable cells in a concentration-dependent manner. The decrease was evident at 24 h with the higher used Dox concentrations and was significant even with the lower Dox concentrations at 48 h (Fig. 1A).

The apoptotic effect of Dox on the same cells was examined by Annexin V-FITC and PI staining following a 24 h exposure of cells to either 20 μg/ml Dox or solute (PBS). Dox significantly enhanced late apoptosis in both types of OS cells, but also induced necrosis of these cells (Fig. 1B), in line with its inhibitory effect on the number of viable cells shown in Fig. 1A.

3.1.2 Doxycycline inhibits migration of MG-63 and 143B human OS cells

The *in vitro* effect of Dox in the migration capacity of OS cells was examined by a wound healing assay. Both Dox treated OS cell lines (MG-63 and 143B) presented a significant reduction in their mobility even at 8 h after the onset of treatment ($p < 0.0001$ in both cases) (Fig. 2).

3.2 In vivo studies

3.2.1 Endpoint metrics in 143B OS xenografts

The volume (cm^3) and weight (g) of primary tumors in left tibias were measured in the Dox^-/Amp^- and Dox^+/Amp^- groups. No differences in primary tumor volume and weight were found between Dox treated and non-treated xenografts (Fig. 3A). The percentage of primary tumor necrosis in the Dox^+/Amp^- group was significantly increased ($P < 0.0001$) compared to the Dox^-/Amp^- group (Fig. 3B). Histopathologic evaluation of primary tumors with hematoxylin and eosin (H&E) staining revealed malignant tumor and stroma cells with destruction of normal bone (Fig. 3C).

3.2.2 Dox decreases the expression of the prognostic factors Ki67, VEGFA, MMP2, MMP9 and Ezrin in primary tumors

Based on IHC staining, the primary tumors of both Dox^-/Amp^- and Dox^+/Amp^- xenografts were vimentin positive and E-cadherin negative (Fig. 4A), verifying the mesenchymal nature of OS [39, 40, 41].

The Ki67 nuclear protein is a well-established prognostic and predictive indicator in OS biopsies [42, 43]. We found a statistically significant decrease (P <0.041) in the expression of Ki67 in primary tumors of the Dox^+/Amp^- group (20.62 ± 8.21%) compared to the Dox^-/Amp^- group (45.00 ± 21.90%) (Fig. 4A).

Using quantitative IHC analysis, we found that the expression of MMP2 in Dox^+/Amp^- group (7.50 ± 8.01%) was significantly decreased (P < 0.002) compared to the Dox^-/Amp^- group (22.85 ± 6.98%). Similarly, the expression of MMP9 in primary tumors of the Dox^+/Amp^- group (4.25 ± 4.68%) was significantly (P < 0.0001) decreased compared to the Dox^-/Amp^- group (25.71 ± 5.34%). The expression of VEGFA in Dox^+/Amp^- group (2.12 ± 7.03%) was also significantly decreased (P < 0.0001) compared with the Dox^-/Amp^- (72.85 ± 13.49%). Finally, based on the notion that Ezrin expression may be a marker and a target in OS [44], we looked for its expression in primary tumors and found that it was significantly decreased (P < 0.003) in the Dox^+/Amp^- group (8.12 ± 8.83%) compared to the Dox^-/Amp^- group (26.42 ± 10.29%) (Fig. 4).

3.2.3 Doxycycline prevents the formation of pulmonary macro metastatic disease

In all groups, abnormal lesions were recognized on the lung surfaces of numerous mice and they were recorded as metastatic sites. We used H&E staining (Fig. 5A) to evaluate the percent metastatic surface / total lung surface ratio for each xenograft from our groups. As shown in Fig. 5B, the number of metastases was significantly lower in the Dox^+/Amp^- compared to the Dox^-/Amp^- group. Similarly, the number of metastases was significantly lower in the Dox^-/Amp^+ compared to the Dox^-/Amp^- group. The decrease observed in the Dox^+/Amp^+ group compared to the Dox^-/Amp^+ group did not reach statistical significance. Early amputation also resulted in a significantly lower lung weight independently of Dox treatment (Fig. 3C).

3.2.4 The expression of markers in pulmonary macrometastatic lesions

The expression of Ki67, MMP2, MMP9, VEGFA and Ezrin in metastatic lesions was high in all xenografts and no evidence of statistically significant difference between the Dox-treated and untreated groups was found (Fig. 6 and Table 1). The metastatic lesions were vimentin positive and E-cadherin negative, similarly to the primary tumors shown in Fig. 4.

Table 1
The intensity of antibodies staining in pictures like those presented in Fig. 6 was scored as described in Methods and results are expressed as mean ± SD of the percentage of tumor cells which express the marker to the overall tumor surface. Statistical analysis was performed by one-way Anova.

	Dox+/Amp+ (n=6)	Dox-/Amp+ (n=6)	Dox+/Amp- (n=8)	Dox-/Amp- (n=6)	P value
MMP2	6.66 ± 12.11	2.50 ± 6.12	2.12 ± 4.01	5.83 ± 9.17	0.670
MMP-9	8.33 ± 13.29	8.33 ± 13.29	12.50 ± 14.63	18.33 ± 17.22	0.607
VEGFA	26.66 ± 41.31	30.00 ± 46.47	50.00 ± 41.74	55.00 ± 42.77	0.573
Ki-67	28.33 ± 44.00	29.16 ± 45.21	50.00 ± 41.74	58.33 ± 45.68	0.546
Ezrin	13.33 ± 32.65	13.33 ± 24.22	16.87 ± 27.11	16.66 ± 18.61	0.990

3.2.5 Blood levels of VEGFA and MMP9 correlate with the number of experimental metastases

The hypothesis that blood levels of VEGFA could have a predictive and prognostic value in OS patients has been previously assessed, showing that VEGF blood levels are significantly higher in OS patients compared with healthy counterparts and correlate with response to chemotherapy or metastasis [45]. The predictive role of MMP9 blood levels has been discussed for several types of tumors [46], but not yet in OS patients. In the present work, we evaluated the correlation between VEGFA and MMP9 blood levels and response to Dox. Both VEGFA and MMP9 blood levels were high in the non-treated Dox^-/Amp^- control group and they were significantly decreased in all other groups ($P < 0.0001$, one way ANOVA). The decrease observed in the Dox^+/Amp^+ group compared to the Dox^-/Amp^+ group did not reach statistical significance (Fig. 7), similarly to what has been observed in the formation of pulmonary metastases shown in Fig. 5B.

4. Discussion

Concurring to the literature [12, 13, 14], our *in vitro* evidence suggests a dose-dependent effect of Dox in decreasing the number and the migration of OS cell lines. In addition, Dox effectively induced both apoptosis and necrosis of OS cell lines, in line with the decreased number of cells and the enhanced necrosis observed *in vivo*. The *in vitro* anti-tumor effect of Dox on MG-63 and 143B cells was studied at a dose between 5 to 20 μg/ml. To our knowledge, this *in vitro* treatment range has been proposed as the equivalent human dosage for Dox [11–13].

Since lung is the most common site of metastasis in OS patients and pulmonary metastasis is associated with a significant worse prognosis in OS patients, we also aimed to assess the use of Dox as a supportive therapy in OS patients for the prevention of pulmonary metastasis. In our *in vivo* human 143B OS xenograft model, doxycycline hyclate at 50 mg/kg/d was intraperitoneally administered for 28 days in dox-treated mice. A study by Luccheti J *et al*, revealed that the intraperitoneal injection of Dox in

C57BL76 mice at a dose of 10 - 100 mg/kg had led in a peak plasma concentration of 2-10 μg/mL which is superimposable to the established oral treatment of Dox at 100-200 mg per day in humans [47]. In addition, we designed our study in accordance with a previously conducted study which reported a great tolerance of Dox when administered at a dose of 50 mg/kg/d in breast BALB/c xenografts [32]. The only study which evaluated the suppressing effect of Dox in tumor progression of human OS- or rhabdomyosarcoma- implanted athymic mice had used Dox at ∼30 mg/kg/d in drinking water with promising results [48]. However, in our opinion the administration of Dox 'ad libitum' via drinking water is not well controlled and this consists a limitation of the experimental study by Dickens D *et al*. Other limitations of that study are that the effect of Dox in the prevention of pulmonary metastatic disease was not examined in lung tissue biopsies, and the design of that study had not mimicked the standard treatment strategy of human OS, which includes neoadjuvant and adjuvant administration of chemotherapeutic agents accompanied with surgical excision of primary site [6]. Our study is the first experimental trial that has mimicked the clinical therapeutic strategy as in human OS. We administered Dox preoperatively, as well as postoperatively, after wide resection of the primary tumor in one of our study groups (Dox^+/Amp^+) and the results were compared with control groups.

In our study, we observed that treatment with 50 mg/kg Dox resulted in a smaller number of metastases compared with the numerous metastatic lesions in the Dox^-/Amp^- control group. In addition, the mice treated with neo-adjuvant Dox, early amputation and adjuvant administration of Dox were almost free of metastases. Dox has been suggested to have anti-cancer properties through inhibition of MMPs, but the timing of an MMP inhibitor application in cancer is critical to achieve the desired therapeutic effect [49]. However, previously conducted phase I, II and III clinical trials that have evaluated the effect of MMP-inhibitors (e.g., the broad-spectrum MMP inhibitor Marimastat and the chemically modified tetracycline Col-3) as chemotherapeutic agents in advanced end-stage metastatic cancers (e.g., breast, pancreatic, gastric) [50, 51, 52, 53] were not encouraging. This may have resulted from the fact that these MMP inhibitors had not been used at early-stage cancers before the establishment of macro metastatic disease. A novel proposal of our *in vivo* study is the administration of Dox in early-stage OS that seems to prevent the progression of micro metastatic to macro metastatic lethal disease.

The present study demonstrated that circulating levels of human VEGFA and MMP9, secreted by the human OS cells, were decreased by Dox in line with the fewer macro metastases, resulting in better prognosis. The circulating levels of human VEGFA and MMP9 in the Dox^-/Amp^+ and Dox^+/Amp^+ groups were much lower compared to the groups that did not undergo early amputation. In these groups, both VEGFA and MMP9 were most likely secreted by the OS cells that formed pulmonary micro metastases and could be used as a biomarker even in cases when these metastases cannot be detected.

Similarly, to the *in vitro* model, the Dox-treated mice developed increased tumor necrosis in primary tumors, supporting the prognostic value of tumor necrosis in primary site for the development of systemic disease in OS [54]. Furthermore, Dox treated mice depicted fewer metastases compared to non-treated mice. On the contrary, the tumor size and the tumor volume were not affected by Dox administration and were not correlated with a higher or a lower metastatic surface / total lung surface

ratio. This finding is not in accordance with bibliography which tends to recognize the significance of primary tumor size as a prognostic factor for subsequent lung metastases in OS [55]. The reason for this discrepancy was that some of our non-amputated mice developed limping and skin ulcers on primary sites and had to be euthanized early from the 28th day respectful to their welfare. Consequently, a significant difference in tumor size between Dox-/Amp- and Dox+/Amp- groups could not be entirely assessed.

The effect of Dox on EMT reversal in our *in vivo* model is verified by the immunohistochemical evaluation of E-cadherin (epithelial marker) and Vimentin (mesenchymal marker) [56]. In our study, primary tumors as well as pulmonary metastatic lesions were Vimentin positive and E-Cadherin negative. No significant difference in the intensity of the signal was recognized among Dox-treated and non -treated xenografts. Subsequently, we concluded that Dox prevents metastatic spreading to the lungs in OS, but this effect is not related to an EMT process, which has been also described in other tumors, such as lung cancer [57].

In Dox-treated mice, the expression of VEGFA, MMP2, MMP9, Ki67 and Ezrin in primary tumors were all downregulated and were strictly associated with a better prognosis. The association between VEGFA, MMP2, MMP9, Ki67 and prognosis is well known for many types of cancer [58, 59]. Ezrin has been shown to positively regulate the expression of MMPs and VEGFA in tumor cells and promote the metastatic potential of tumor cells [60, 61]. In pulmonary metastases, the expression of these markers was decreased in the amputated compared to the non-amputated groups but were not affected by Dox treatment. This may be due to the well described differences between metastatic cell clones and primary tumor cells that include their chemosensitivity to anticancer agents [62, 63].

Despite our effort to provide a close approximation of human OS using a humanized mouse model and provide therapeutic outcomes of Dox with clinical relevance, our study has two main limitations. The first limitation is that orthotopic implantation of allogeneic OS cells in SCID mice do not fully recapitulate the human immune and stroma-tumor interactions occurring in *de novo* tumors [64]. Secondly, the administration of Dox in Dox^{+}/Amp^{-} and Dox^{+}/Amp^{+} mice had started the same day with 143B cells intramedullary engraftment. Certainly, in most human cases the diagnosis does not coincide with the onset of treatment. However, tumor progression could not have been accurately monitored in mice to start treatment immediately prior the development of poorly controlled numerous metastases and therefore, we have chosen to start the administration of Dox from a baseline time for all treated mice. Nevertheless, the achievement of experimental metastases from the start points (seeding of OS cells in lungs during their engraftment in tibias) remains a strong point of our study design, as it replicates the clinical scenario for 80% of humans who suffer from pulmonary OS micro metastases already from the day in which diagnosis is made and chemotherapy is given.

5. Conclusions

Our data encourage the design of further clinical studies with adjuvant administration of Dox in non-metastatic OS disease (IA-IIB), supporting the use of Dox in combination with surgical resection and

standard chemotherapeutic protocols in the early stages of OS treatment, prior to the development of clinically detectable pulmonary macro metastases.

Abbreviations

OS
Osteosarcoma, Dox:Doxycycline
VEGF
Vascular Endothelial Growth Factor
MMP
Metalloproteinase
SCID
Severe Combined Immunodeficient
Amp
Amputation
ECM
extracellular matrix
IHC
Immunohistochemistry
H&E
Hematoxylin and Eosin
IP
Intraperitonially
NKUA
National and Kapodistrian University of Athens

Declarations

Ethics approval: Our *in vivo* experimental trial in osteosarcoma xenograft mice followed the standard criteria of the 'NC3Rs primates' (replacement, reduction, refinement) guidelines. All procedures were approved by the Regional Veterinary Service (no:366107/July 9,2019), the Ethics Committee of the NKUA/Medical School (no:163/September 18,2019) and were in accordance with national legislation and European Directive 63/2010.

Consent for publication: Not applicable

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Figures

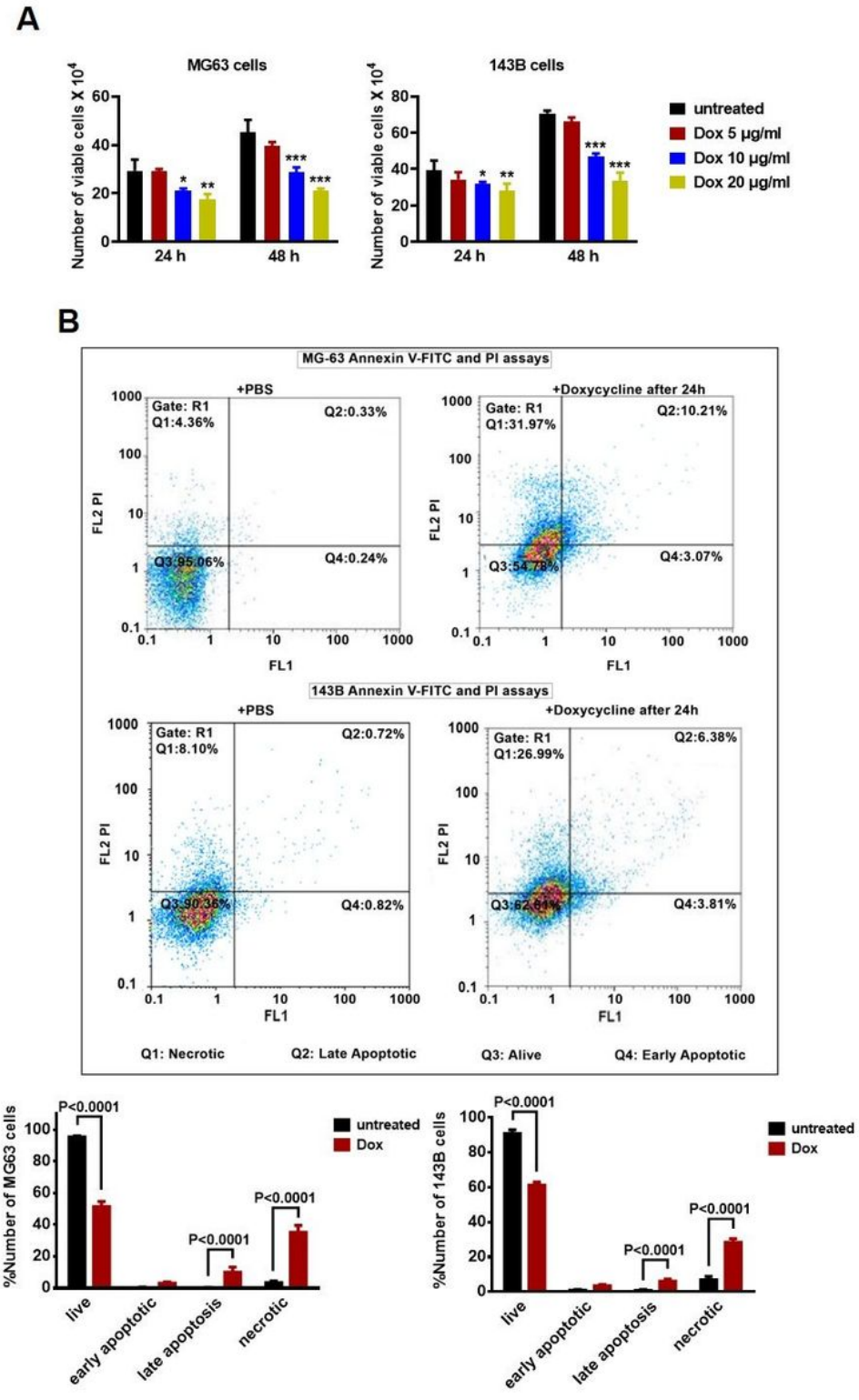

Figure 1

The effect of Dox in the viability and apoptosis of OS cell lines. (A) The number of viable MG63 and 143B cells was measured at 24 and 48 h after treatment with the shown Dox concentrations using the trypan blue exclusion assay. Data are expressed as mean ± SD of the number of viable cells from three independent experiments. (B) Representative pictures of FACS analysis of cells stained with annexin V and propidium iodide. Viable cells are in in the Q3 region, early apoptotic cells are those present in the Q4 region, late apoptotic cells are in the Q2 region and necrotic cells at the Q1 region.

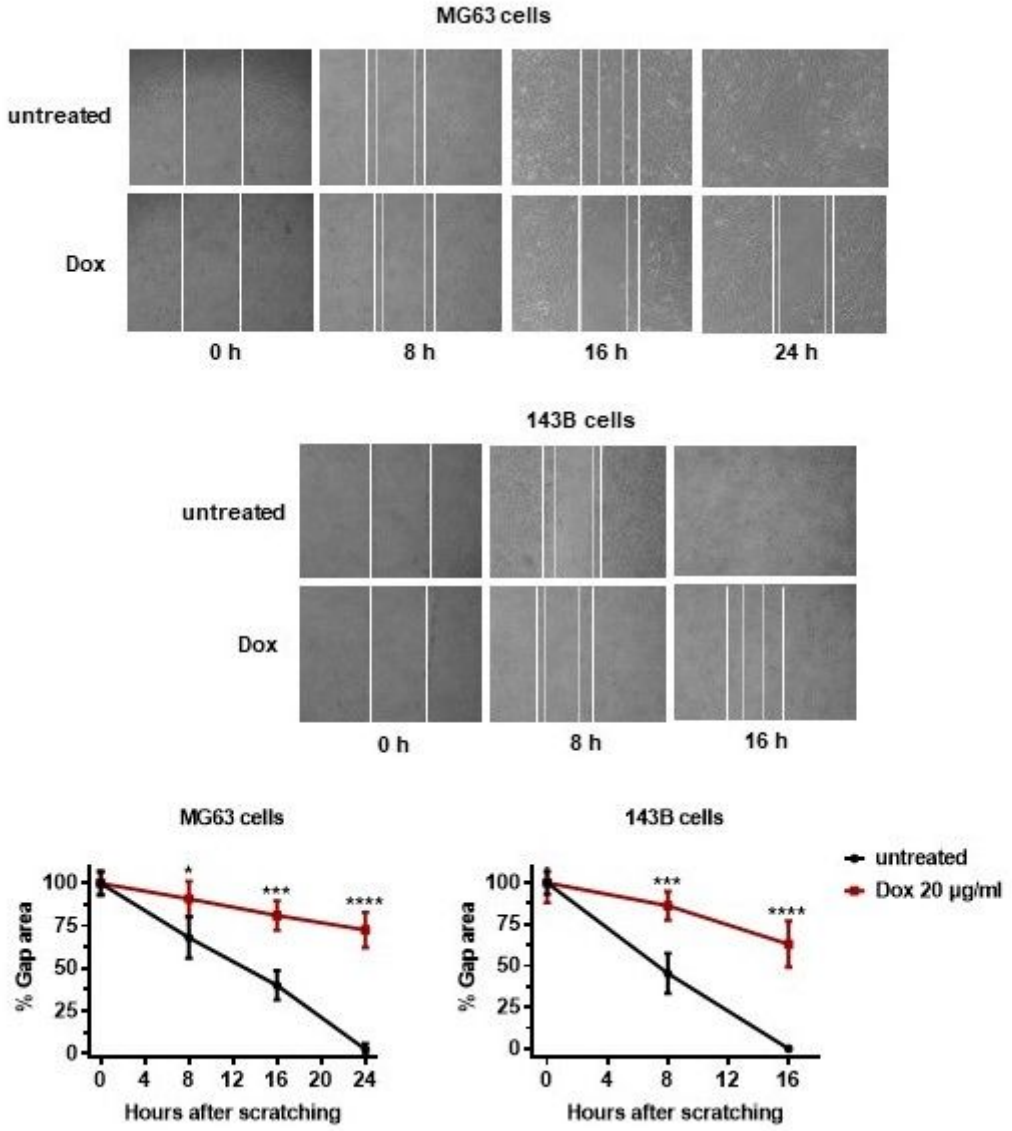

Figure 2

The effect of Dox in the migration of OS cell lines. Migration of MG63 and 143B cells was detected via wound healing assays (magnification X20). At different time points after scratch, the gap areas were quantified using proper software as described. Solid lines mark the initially scratched area and dotted lines show the area that was not covered with cells at the time points shown. When the whole area was covered with cells, no lines are present. The area that remained uncovered with cells was quantified at

each time point and expressed as percent remaining gap area compared to the initially scratched area at time=0 h. Results are expressed as mean ± SD from three independent experiments.

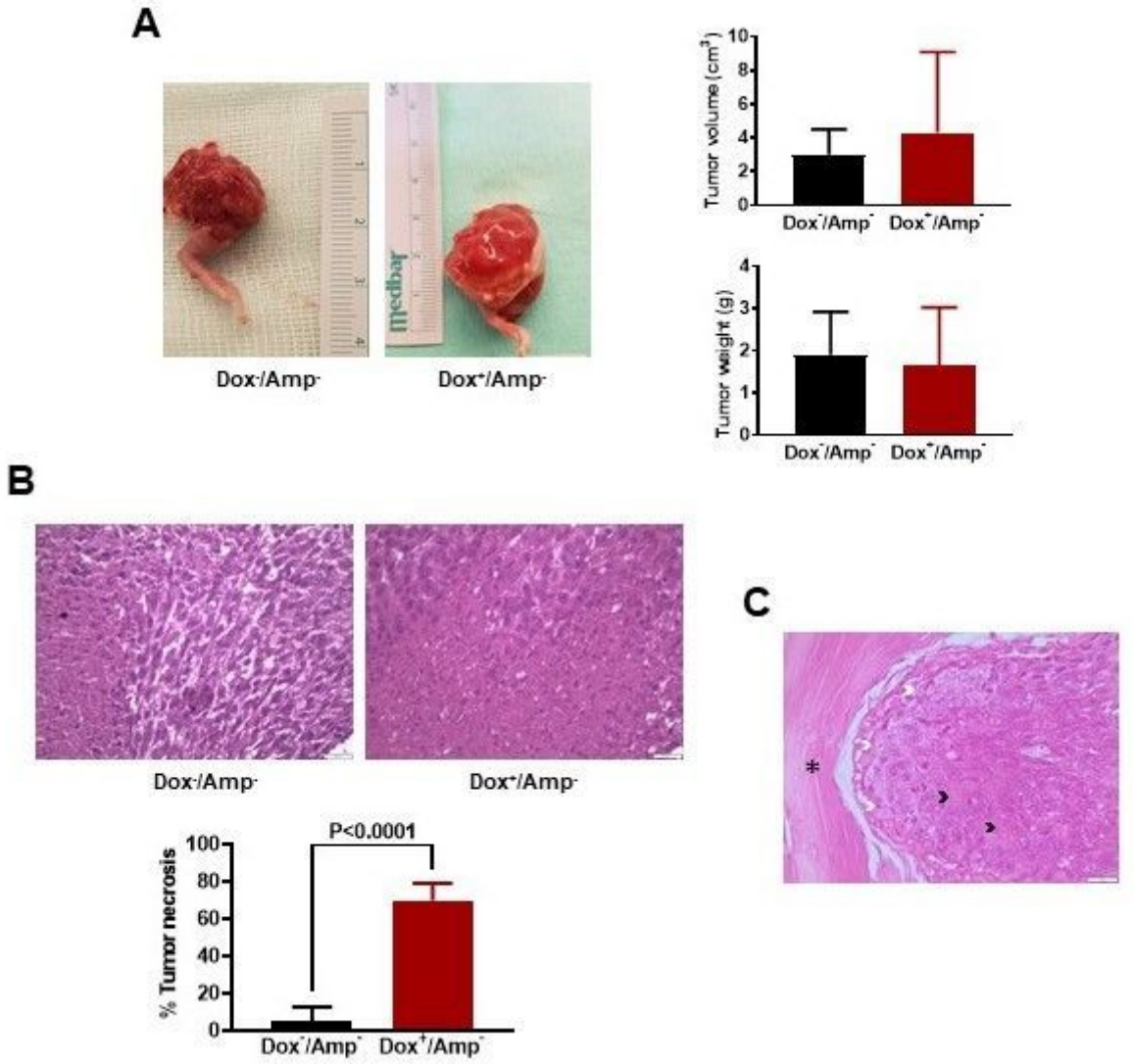

Figure 3

Dox does not affect the size of primary tumors but induces excessive primary tumor necrosis. (A) Representative pictures and the measured volume and weight (expressed as mean ± SD, Dox-/Amp- n=6; Dox+/Amp- n=8) of primary tumors are shown. (B) Representative photos of H&E-stained paraffin-embedded primary tumors are shown. Each section was photographed in its entirety and subsequently, digitally processed utilizing the IpWin6 program. Tumorous areas were manually demarcated, and the program proceeded to assess the percentage of necrotic tumor areas to the overall tumor surface. The percent primary tumor necrosis is expressed as mean ± SD (Dox-/Amp- n=6; Dox+/Amp- n=8). (C) Representative H&E-stained paraffin-embedded primary tumor showing malignant tumor cells (white arrows) with formation of interstitial osteoid (black arrows) and destruction of normal bone (asterisk).

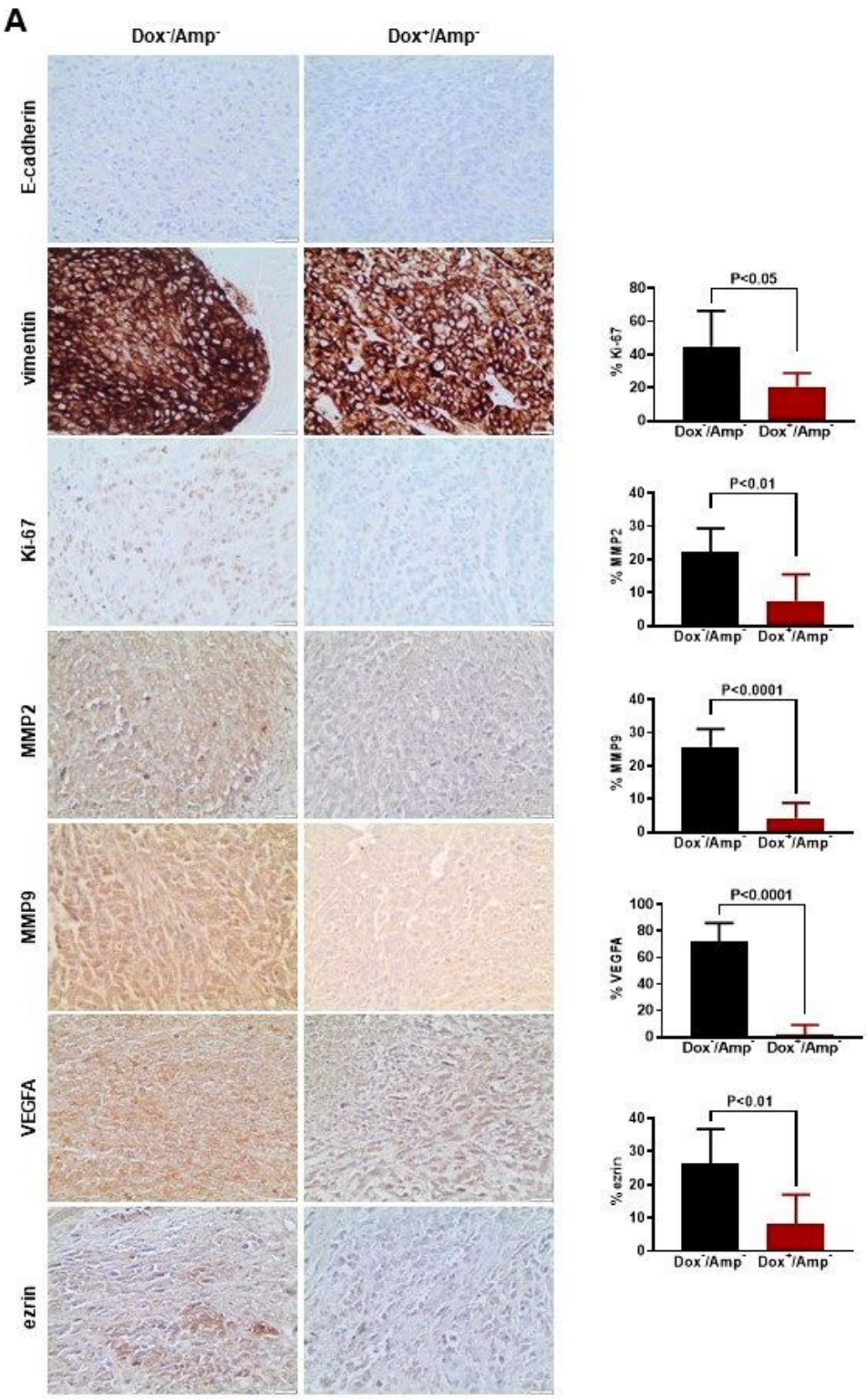

Figure 4

Dox suppresses the expression of Ki67, MMP2, MMP9, VEGF and Ezrin in primary tumors. (A) Paraffin-embedded primary tumor sections were stained using antibodies against vimentin and E-cadherin, Ki67, MMP2, MMP9, VEGFA and Ezrin. Positive staining is brown. Counterstain with hematoxylin is shown as blue. Representative figures are shown at X400 magnification. The intensity of antibodies staining was

scored as described in Methods and results are expressed as mean ± SD (Dox-/Amp- n=6; Dox+/Amp- n=8) of the percentage of tumor cells which express the marker to the overall tumor surface

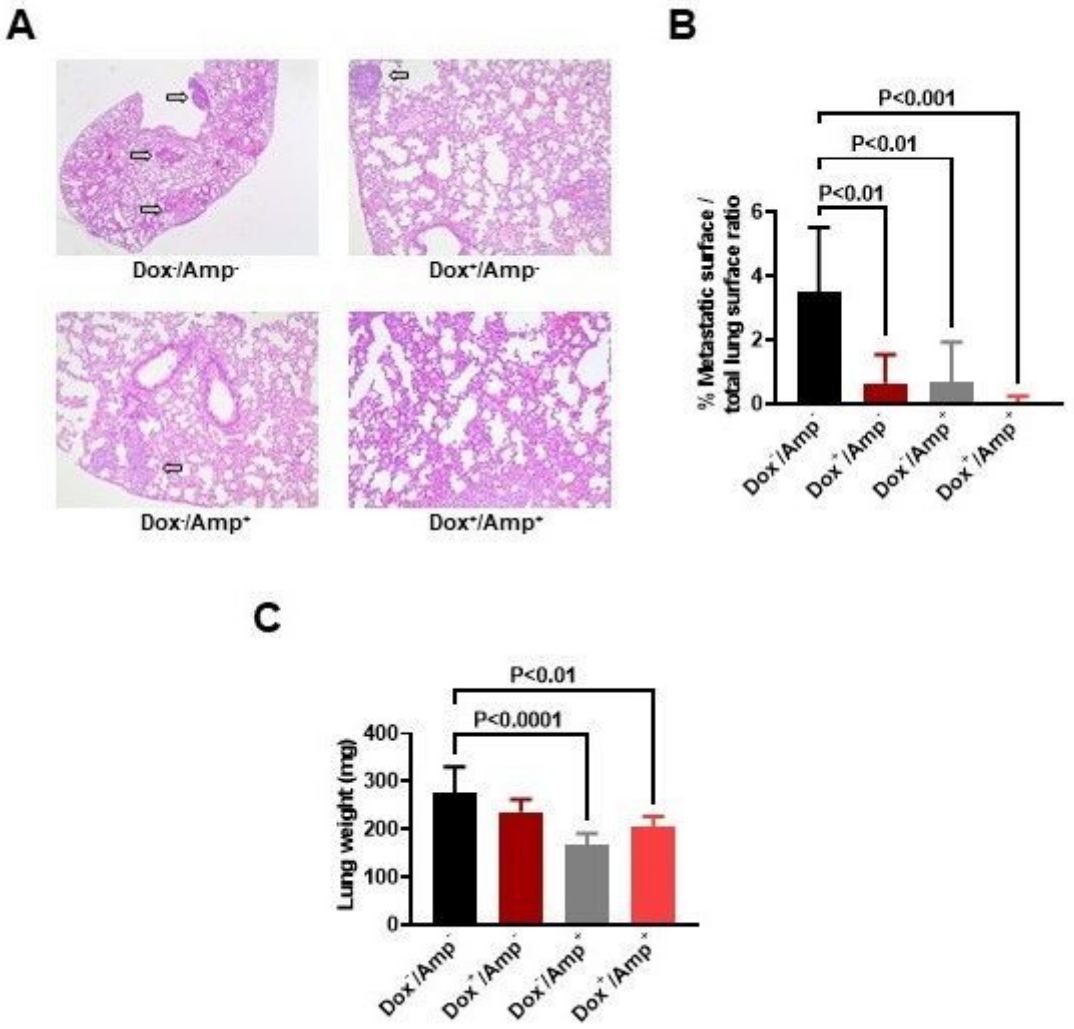

Figure 5

Dox prevents the formation of pulmonary macrometastases. (A) Representative H&E-stained paraffin-embedded lung sections. (B) Each section was photographed and digitally processed utilizing the IpWin6 program. Tumorous areas were manually demarcated, and the program proceeded to assess the percentage of tumor areas compared to overall tissue surface. Results are expressed as mean ± SD. (C) Weight of the lungs at the end of the experiment expressed as mean ± SD. In all groups n=6 except Dox+/Amp- in which n=8.

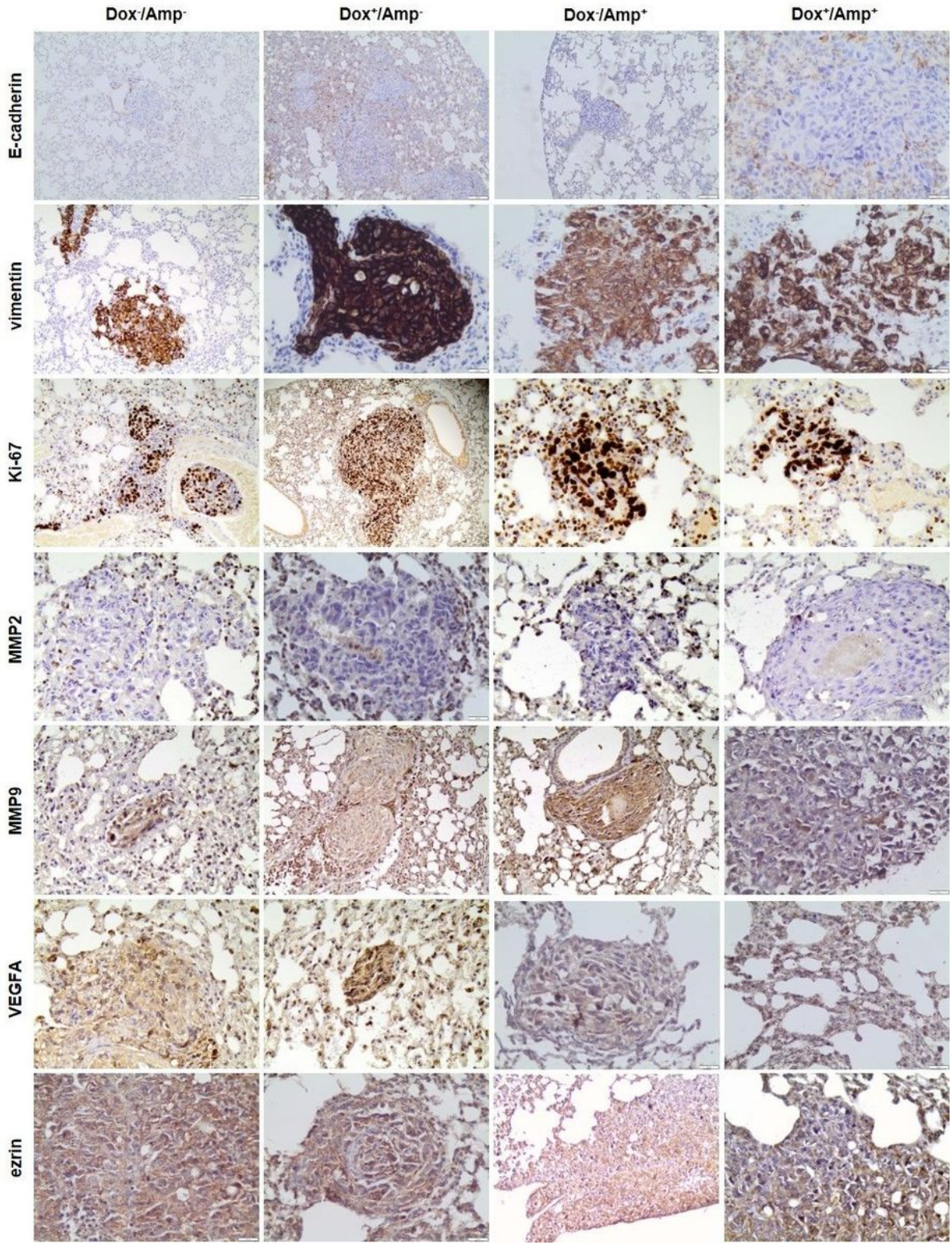

Figure 6

Expression of markers in pulmonary macrometastatic lesions. Representative paraffin-embedded mouse lung sections stained using antibodies against vimentin and E-cadherin, Ki67, MMP2, MMP9, VEGFA and Ezrin. Positive staining is brown. Counterstain with hematoxylin is shown as blue. Quantification is presented in Table 1.

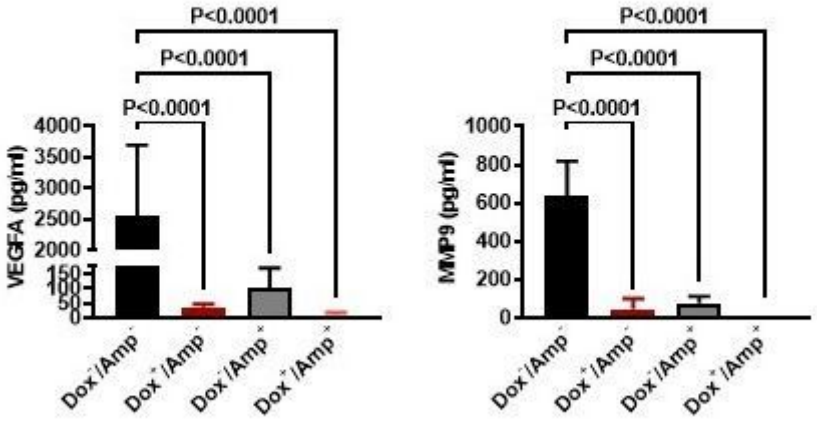

Figure 7

Blood levels of VEGFA and MMP9. The levels of VEGFA and MMP2 were measured in the blood of all mice from each of the studied groups and expressed as mean ± SD per group. In all groups n=6 except Dox+/Amp- in which n=8.