The anti-neoplastic effect of Doxycycline in Osteosarcoma as a Metalloproteinase (MMP)-inhibitor. A systematic review

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Abstract

ABSTRACT Background Osteosarcoma is as a very aggressive primary bone tumor, affecting mainly young populations. Most cases diagnosed have distant macro and micro-metastases at the time of diagnosis. Surgical resection with neoadjuvant and adjuvant therapies improve overall and disease-free survival of patients. Doxycycline, a synthetic tetracycline has been found to act either as an antibiotic drug as well as a chemotherapeutic agent. Its anti-neoplastic role has been proven to be extremely significant in various types of cancer like prostate, intestinal, central neural system cancers and osteosarcoma. Inhibition of metalloproteinases (MMPs) in different stages of tumor expansion is the most well understood mechanism. MMPs are secreted molecules from various normal cells like fibroblasts, leucocytes and vascular smooth muscles but also from cells with high proliferative potential like tumor cells. In osteosarcoma, MMPs have been found to be overexpressed. MMPs help osteosarcoma cells to survive, grow and give metastases in distant sites, mainly in lungs. Doxycycline, blocks extracellular matrix and basic membranes degradation by suppressing MMPs function. As a consequence, osteosarcoma cells lose their ability to invade and give metastases. Additionally, doxycycline eliminates the secretion of vascular endothelial growth factor (VEGF) and deprives the supply of circulating nutritious components, by its anti-angiogenesis action. The aim of this review is to evaluate doxycycline's action against osteosarcoma cells as an MMP-inhibitor and interpret its usage as a chemotherapeutic agent. Methods We checked PubMed and Google Scholar for recent published data, on the tumor-supportive role of MMPs and VEGF in osteosarcoma cells. We further studied published experimental trials, on the role of doxycycline as a tumor-suppressive agent via MMPs and VEGF inhibition. Results MMPs and VEGF have been found to play a fundamental role in osteosarcoma cells survival and its high aggressiveness by in vitro, in vivo and clinical trials. Nevertheless, Doxycycline proved its tumor-suppressive effect, by in vivo experimental trials in various cancers, but not yet in osteosarcoma. Conclusion Doxycycline remains a promising chemotherapeutic agent against osteosarcoma, via MMPs inhibition, showing of the need, for further in vivo and clinical trials to carried out in the future. Key Words: Osteosarcoma, Metalloproteinase, VEGF, Doxycycline, Metastasis

Background

Osteosarcoma, also known as osteogenic sarcoma, is the most common primary bone tumor with highly metastatic and lethal behavior[1]. In the United States of America about 800-900 new cases of osteosarcoma are diagnosed every year[1]. The age distribution of osteosarcoma is bimodal, as the first age peak is recorded between first and third decade and the second smaller peak (10% of them) in the sixth decade[1]. Osteosarcoma counts 2% of all cancers in childhood and remains a challenging disease to prevent and treat[1]. For localized and resectable osteosarcomas the 5-year survival varies from 60% to 80% but for metastatic lung disease the survival rate diminishes to 40%[1]. Lung is the most common site for initial metastasis, as approximately 10% of osteosarcoma patients, have pulmonary nodules at the time of diagnosis[2].

Proliferation of tumor cells, increasing tumor size and local invasion combined with new vessels formation for necessary nutritious and oxygen intake are basic steps for osteosarcoma cells to expand and give distant metastases[3]. Osteosarcoma progression is strictly associated with extracellular matrix (ECM)-degrading matrix metalloproteinases (MMPs), which play a fundamental role in cancer survival and invasion, as well as with the development of a neoplastic vascular network[4]. MMPs are zinc-dependent endopeptidases, secreted by fibroblasts, leucocytes, vascular smooth muscles and rapidly proliferative tumor cells[5]. The biological attributes of MMPs appear in various physiological and pathological processes such as collagen and elastin degradation, endothelial cell formation during angiogenesis, migration of vascular smooth muscles and proliferation along with migration of tumor cells[5]. Currently, in humans there are 23 well- identified MMPs that stimulate cancer survival and expansion, being a target group for anticancer drugs[6].

Doxycycline, a chemically modified tetracycline, is a cheap drug with quite safe profile. It's the only MMP-inhibitor in a concentration dependent-manner, approved by the U.S. Food and Drug Administration for the treatment of periodontal disease[7]. It is composed of a four- ring core with an attached dimethyl amino group at the C4 carbon on the upper site of the molecule. At the lower oxygen-rich part of doxycycline, the formation of chelation bonds with Zn^{2+} and Ca^{2+} ions within MMPs can occur[8]. Despite of its antimicrobial usage, recent observations showed that doxycycline has a cytotoxic effect on tumor cells. Inhibition of MMPs secreted by osteosarcoma cells, can reduce neo-angiogenesis and tumor invasiveness by suppressing ECM destruction and preventing the formation of micro-metastasis at the very early stages of cancer expansion. As a prophylactic chemotherapeutic agent, doxycycline could improve the overall survival rate in patients suffering from osteosarcoma.

Methods

Search Strategy

We searched for published articles from PubMed and Google Scholar. Our first aim, was to mention preclinical osteosarcoma trials, in order to link the important role of several subtypes of MMPs, with the level of invasiveness and metastatic attitude of osteosarcoma cells. Secondly, we checked bibliography for the effect of MMPs and VEGF in clinical trials of osteosarcoma, to guarantee that their neoplastic behavior has been proven not only in experimental models, but in real osteosarcoma human specimens too. After we recognized the tumorigenic action of MMPs, we focused our search on the anti-neoplastic effect of doxycycline, in various types of cancer to ensure that acts not only as an antibiotic drug, but as a tumor-suppressive agent via MMP-inhibition, too. Additionally, we searched for the indirect antineoplastic effect of doxycycline, by its VEGF-suppressing action that has been observed in malignant cells. Finally, we studied all published preclinical trials interpreting the antineoplastic effect of doxycycline in osteosarcoma experimental models, in order to identify its action on osteosarcoma cells.

Selection Criteria

We preferred to search most recent published articles. We mainly focused in bibliography from 2013 to 2019. Our helpful 5 key words in searching tools of PubMed and Google Scholar were Osteosarcoma, Metalloproteinase, VEGF, Doxycycline and Metastasis. We studied published articles referring to the neoplastic effect of MMPs only in osteosarcoma preclinical trials, excluding articles that mention their action in other types of cancer. We excluded published data on the anti-MMPs action of doxycycline in other types of cancers as well. Our first aim, was to focus our attention on the role of MMPs and doxycycline only in osteosarcoma cells.

Results

Thirty over forty-two (71.4%) cited articles were published first time from 2013 until 2019. From 1999 to 2013 interval, we observed a lack of experimental trials and published data referring to the antineoplastic effect of doxycycline in osteosarcoma.

Studies Content

We found only three in vitro preclinical trials, interpreting the antimetastatic behavior of doxycycline and no in vivo experimental trials on osteosarcoma animal models, that could examine the blockage of metastases in distant organ, from 1997 to 2019.

The metastatic role of MMPs in Osteosarcoma in preclinical trials

MMP-1 (also known as collagenase-1), has been found to be remarkably upregulated after intratibial injection of 143-B highly metastatic human osteosarcoma cells into SCID mice compared with injection of non-metastatic HOS cell line[9]. MMP-1 plays an important role in lung formation of micro and macro metastases in patients diagnosed with osteosarcoma[9]. The expression of MMP-2 (also known as gelatinase-A) is elevated in osteosarcoma patients, especially in those with pulmonary metastases and could be an independent prognostic marker for the total survival time after initial diagnosis of the primary tumor[10]. Additionally, circulating MMP-2 seems to alter sensitiveness of osteosarcoma cells in chemotherapeutic drugs as a shift from MMP-9 to MMP-2 secretion is correlated with poor response to them[11].The malignant phenotype in osteosarcoma cells is obviously promoted by another metalloproteinase, the MMP-3(also known as stromelysin-1)[12]. The migration and invasion properties of MMP-3 knockdown, MG-63 and TE85 highly metastatic osteosarcoma cells, found to be extraordinarily deteriorated[12]. MMP-9(also known as gelatinase-B) is another important biomarker which has been found to be involved in osteosarcoma cells invasion, with highly predictive role on development of lung metastases, especially when coexpressed with chemokine CXCR4[13,14,15]. Inhibition of MMP-11(also known as stromelysin-3) in MG-63 and U2OS osteosarcoma cells by upregulating the micro RNA-125a-5p, has proved the important function of another MMP during migration and invasion process[16]. The MMP-13(also known as collagenase-3) secreted by osteosarcoma cells has been found to be another important

molecule for the ability of tumor cells to invade the extracellular matrix and give lung metastases[17,18]. The downregulation of MMP-13 by injected MicroRNA-143 in highly metastatic human osteosarcoma cells, 143B, significantly suppress lung metastases, but proliferation potential remains the same[17].Conversely, upregulation of MMP-13 and stimulation of AKT-pathway mediated from MMP-13, by Interleukin-32, enhances the motility and invasion of osteosarcoma cells, confirming the key role of this metalloproteinase in lung metastases[18,19].

The prognostic value of MMPs and VEGF expression in clinical trials

The detection of osteosarcoma, by using MMP-9 as a biomarker has been found to be extremely accurate by a metanalysis of Wang J. et al, in which a total number of 892 patients in different clinical stages were included[20]. Foukas A. et al, attempted in 2002, to interpret the prognostic metastatic value of MMP-9 in 55 patients with stage-IIB osteosarcoma around the knee. Immunohistochemical studies in resection specimens of these patients, showed that overexpression of MMP-9 in osteosarcoma cells, is significantly associated with metastases and poor overall survival[21]. However, the prognostic value for metastases of serum MMP-9 levels in patients suffering from osteosarcoma was found to be statistically non-significant[21]. A study by Han J. et al, among 177 cases diagnosed with osteosarcoma, revealed correlation between high levels of serum alkaline phosphatase (ALP) and MMP-9 for the prediction of metastatic disease and poor prognosis[22]. On the other hand, a metanalysis by Wen, X. et al, among five cohort studies revealed that expression of MMP-2 has a strong value for the prognosis of metastases and increased mortality of patients suffering from osteosarcoma[23].

Elevated levels of various MMPs can increase the expression of vascular endothelial growth factor (VEGF) and vise versa[24]. VEGF being, the most important molecule to establish a neoplastic network of new blood vessels, it conserves the ability of malignant cells to survive and spread in distant organs. Liu Y et al, found among 84 osteosarcoma samples that high expression of VEGF is significantly correlated with high possibility of metastases and poor prognosis in patients suffering from osteosarcoma[25]. A systematic and metanalysis by Chen D et al, in 2013, among 12 studies with a total of 559 patients suffering from osteosarcoma, revealed that VEGF expression is obviously associated with lower overall survival due to higher incidence of metastases[26].

The strict correlation between osteosarcoma lung metastases and poor survival, along with MMPs and VEGF overexpression, motivated research on inhibiting agents. One of them is probably doxycycline.

The anti-neoplastic role of Doxycycline as an MMP-inhibitor in various cancers

Doxycycline as an MMP inhibitor, mediates extracellular matrix degradation and acts as an anti-proliferative, anti-invasive and anti-angiogenic agent deteriorating the expanding and invasive potential of tumor cells. The molecular mechanism that supports MMPs inhibition by doxycycline has different

assumptions: i) MMP-mRNA becomes unstable, ii) inactivation of MMPs via Zn^{2+}chelates, iii) elimination of reactive oxygen species secreted by ECM cells that activate pro-MMPs, iv) blockage of MMPs activation via MT1-MMP pathway[27]. Inactivation of MMPs secreted by tumor cells, deprives the formation of new capillary vessels which could support survival of malignant cells and give a potential frame for invasion. As a consequence, doxycycline, acts as an indirect cytotoxic drug and bans the formation of pulmonary micro metastases. Tumor cells already settled in the lungs, require the construction of a supportive angiogenic environment to grow into clinically detectable metastases. The anti-metastatic action of doxycycline has been proven in different types of cancer.

A doxycycline treated group and a control group of engrafted human MHCC97H cells of hepatocellular carcinoma into BALB/c mice showed that doxycycline acted as an MMP-2 and MMP-9 inhibitor. Doxycycline, suppressed the growth of tumor cells and prolonged mice survival. The mechanism of vasculogenic mimicry, in which the formation of mosaic vessels from endothelium and tumor vessels along with bridging channels in tumors cells has been found to be occluded via doxycycline administration, losing the potential of malignant cells to migrate in distant organs[28].

The suppressive effect of doxycycline against oral squamous-cell carcinoma has been associated with MMPs inhibition. The over production of MMPs in this type of cancer leads to lymph node and distant organ metastases. The administration of doxycycline, can decrease tumor invasiveness in vitro SCC-15 cell line and growth volume of tumor in vivo in xenografted nude mice. After 24h treatment of SCC-15 cells, MMP-9-mRNA levels have been found to be significantly reduced while MMP-2 secretion deteriorated at post transcriptional stages, via Zn^{2+}chelates with doxycycline's molecule. It is obvious, to our knowledge, that doxycycline can eliminate MMPs either in pre transcriptional or post transcriptional level acting as an adjuvant, anti-invasive chemotherapeutic drug[29].

Inhibitory effects of doxycycline on the expression of MMPs in prostate cancer, consists another example of its antimetastatic usage. NF-kB signaling has been found to regulate the expression of MMPs in the nuclei of lipopolysaccharide (LPS)-induced PC3 human prostate cancer cells[30,31]. Doxycycline treatment downregulated the levels of MMP-2, MMP-8, MMP-9 and NF-kB in dose-dependent manner[30,31]. Consisting another important molecular pattern for restriction of tumor cell proliferation, invasion and angiogenesis.

The cytotoxic effect of doxycycline via MMPs inhibition, also demonstrated with in vitro and in vivo preclinical trials of xenografted HuTu-80 human duodenal adenocarcinoma cells in mice[32]. Tumor growth was reduced in mice treated with doxycycline compared with the control group and survival rates were found significantly higher, as well[32]. The antineoplastic effect of doxycycline on intestinal neoplasias, has already been confirmed previously by Sagar et al, in colorectal cancer cells (HT29 cell line)[32].

Glioblastoma multiforme, the most common brain cancer found to be susceptible in doxycycline, after in vitro treatment of highly aggressive U251HF human cells[33]. Doxycycline found to decrease extracellular levels of MMP2 after the formation of chelation bonds, but it has not the ability to downregulate MMP2-

mRNA at pre-translational stages[33]. As a consequence, anti-proliferative effect and reduced cell invasiveness occur due to an MMP inhibition, by doxycycline.

Anti-angiogenetic effect of Doxycycline via MMP-inhibition

MMPs provoke angiogenesis which is crucial for tumor viability, cells proliferation and metastases in distant organs. MMPs contribute in angiogenesis by two distinct ways. Firstly, MMPs degrade basement membrane and extracellular matrix, and give the chance to endothelial cells of existing vessels to migrate and settle in new positions, creating a new supporting capillary network for tumor cells[34]. Secondly, the degradation of extracellular matrix releases angiogenic factors like vasculo-endothelial growth factor (VEGF), fibroblast growth factor (bFGF) and tumor growth factor (TGFβ), which trigger intracellular angiogenic pathways for new vessels formation. VEGF, also increases vessels permeability and allow tumor cells to enter blood circulation and move in distant sites. Additionally, these angiogenetic factors induce secretion of MMPs by endothelial cells, conserving high amount of local MMPs in the tumor area[34]. In vitro inhibition of VEGF expression in U2OS osteosarcoma human cells promoted tumor cells apoptosis and reduced cell proliferation through vascular insufficiency[35]. Chen et al, in a meta-analysis of 12 studies showed that secretion of VEGF is a powerful prognostic factor for the total survival rate of patients suffering from osteosarcoma. Among 559 patients, the overall disease- free survival found to be strictly associated with expression levels of VEGF[36].

According to Merentie M. et al, doxycycline reduced the expression of VEGF in endothelial cells in vitro with a dose dependent manner, but failed to downregulate its expression in transgenic mice in vivo[37]. In contrast, doxycycline has been found to eliminate angiogenesis indirectly as an MMP-9 inhibitor, via suppression of VEGF expression in cerebral matrix of a mouse model[38]. Additionally, doxycycline proved to increase tissue inhibitors of metalloproteinases-1 (TIMP-1), and stop human aortic smooth muscle cell (HASMCs) migration by inhibiting VEGF expression[39]. As an MMP-inhibitor doxycycline presented a promising anti-angiogenetic agent through its anti-VEGF action.

Inhibition of MMPs by Doxycycline in Preclinical Osteosarcoma models

In Vitro Studies

Fife R et al, were the first in 1997 to study the anti-proliferative and apoptotic effect of doxycycline in cultured human osteosarcoma cells in vitro[40]. Their hypothesis that doxycycline, a non-toxic antibiotic drug could act either as an MMP-2 inhibitor and as an anti-neoplastic chemotherapeutic agent in osteosarcoma cells proved realistic. Osteosarcoma cells from six patients and one human osteosarcoma cell line U2OS were cultured in the presence or absence of 5 mg/ml and 10 mg/ml of doxycycline. Their preclinical trial showed that doxycycline at a dose of 10 mg/ml suppressed cell proliferation three to seven-fold of osteosarcoma cultures. MMP-2 activity significantly inhibited only at a dose of 5mg/ml[40].

Cakir Y and Hahn KA, in 1999 described the suppressive effect of Doxycycline on MMP-1, beyond its antimicrobial usage. Their in vitro study on canine osteosarcoma cells, showed that 5mg/ml and 10mg/ml of Doxycycline, could minimize tumor volume 50% and 75%, respectively[41]. MMP-1 secretion, reduced 35% and 50% at Doxycycline's doses of 10mg/ml and 20mg/ml, respectively[34]. Their in vitro trial, proved the antiproliferative usage of a chemically modified tetracycline, against osteosarcoma cells via MMP-1 inhibition[41].

The most recent study on the inhibition effect of doxycycline, in pediatric human osteosarcoma cell line U2OS was held by M.W. Roomi et al, in 2013[42]. Osteosarcoma cells proved to be sensitive to doxycycline exposure, as MMP-2 and MMP-9 secretion were suppressed in a dose-dependent manner. Gelatinase zymography revealed total blockage of MMP-2 at 5 mg/ml, and blockage of MMP-9 at 10mg/ml of doxycycline[42]. As MMPs were recognized in several experimental trials as crucial molecules for invasive properties of tumor cells, doxycycline was interpreted as a potential adjuvant and neoadjuvant chemotherapeutic drug.

Discussion

Osteosarcoma aggression and metastases is strictly associated with the ability of tumor cells to secrete MMPs[3]. MMPs are secreted from normal tissues like smooth muscles and endothelial cells, supporting their ability to survive, expand and migrate participating in normal processes[5]. However, their presence in pathological tumorigenic processes is well identified. The expression of MMPs play a key role in tumor spreading as they can modify the surrounding microenvironment of osteosarcoma cells. The extracellular matrix and basic membrane degradation accompanied with new vessels formation under direct or indirect action of MMPs, make an ideal frame of osteosarcoma cells to survive, expand and transport in distant organs[3,4]. The supporting neo-angiogenesis promoted by MMPs conserves the survival of local tumor and the formation of lung-micro metastasis[5,34]. Additionally, distant osteosarcoma metastatic lesions continue to secrete MMPs in order to adhere in normal tissues and form mature secondary tumors[34]. Constructing the supportive surrounding environment with a new network of blood vessels.

It is well-established to our knowledge that doxycycline has an anti-MMP behavior. Due to this function it could be used not only as an antibiotic drug, but also as an adjuvant and neo-adjuvant chemotherapeutic agent. Doxycycline as an MMP-inhibitor blocks the potential growth and invasiveness of malignant cells as well as the formation of neoplastic new blood vessels, and promises improvement of total survival rates in patients suffering from osteosarcoma[5,34]. Doxycycline administration on early stages of osteosarcoma expansion could diminish the mortality and expand life expectancy of patients[27]. Due to the fundamental role of MMPs in the high metastatic attitude of malignant osteosarcoma cells, doxycycline showed a promising anti-cancer action by inhibiting MMPs. In vitro trials with osteosarcoma cells have revealed the tumor suppressing effect of doxycycline, as well as in vivo experimental trials in other types of cancer, but not in osteosarcoma yet[28 - 33].

Conclusions

Searching for published articles on preclinical experimental trials for the anti-metastatic effect of doxycycline as an MMP-inhibitor we concluded that bibliography lacks. We found only three preclinical in vitro trials, interpreting the tumor-suppressing effect of doxycycline via MMP-inhibition. Surprisingly, we found no available publications on this topic, during the period from 1999 to 2013. We conclude that further preclinical trials must be designed, and especially high quality in vivo experimental studies which have not been tried before. In our opinion the usage and safety of doxycycline as an anti-neoplastic agent must be further studied in order to be valued as a potential chemotherapeutic agent by FDA.

Abbreviations

Metalloproteinase **(MMP),** Vascular Endothelial Growth Factor **(VEGF),** Extracellular Matrix **(ECM),** Alkaline Phosphatase **(ALP),** Doxycycline **(DOX),** Fibroblast Growth Factor **(bFGF),** Tumor Growth Factor **(TGFβ).**

Declarations

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All authors who participated in the article's composition have a special interest in orthopedic oncology, especially in the treatment of patients suffering from osteosarcoma. Doxycycline as a Metalloproteinase (MMP)-inhibitor found to be a potential chemotherapeutic agent in osteosarcoma, motivating the authorship of this article. We used PubMed and Google Scholar, to export all data and study materials from all journals subscribed in our library of Medical School of National and Kapodistrian University of Athens. All figures attached, have been designed by the authors of this article. Being the first author of this article, I would like to thank the study participants Athanasios F. Foukas, Olga D. Savvidou, Andreas F. Mavrogenis, Amanda K. Psyrri, and Professor Panagiotis J. Papagelopoulos for their guidance. We also wish to thanks the editorial members of BMC cancer for their special help on publishing our research.

Competing interests

The authors declare that they have no competing interests.

Conflict of interest

None of the authors have any competing interests.

Authors' contributions

Argyris C. Hadjimichael and Athanasios F. Foukas contributed to the design of this study. Olga D. Savvidou, Andreas F. Mavrogenis, Amanda K. Psyrri and Panagiotis J. Papagelopoulos provided the

appropriate study database and guided the authorship of this study. All authors drafted, read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not Applicable

Consent to publish

Not Applicable

Availability of data and materials

All data generated for or analyzed in this study are included in this published article. We made references from published articles in PubMed and Google Scholar and all data supporting our findings can be found there. All figures included in this article were designed by its authors and they are attached in supplementary information files.

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Figures

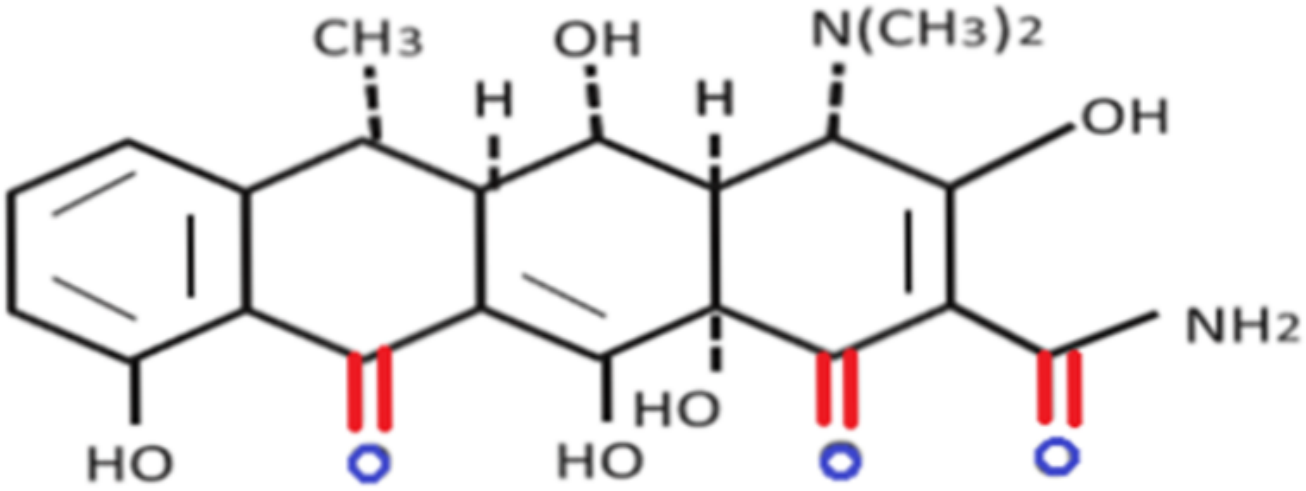

Figure 1

The chemical structure of Doxycycline. The lower oxygen-rich part is able to form chelation bonds with MMPs.

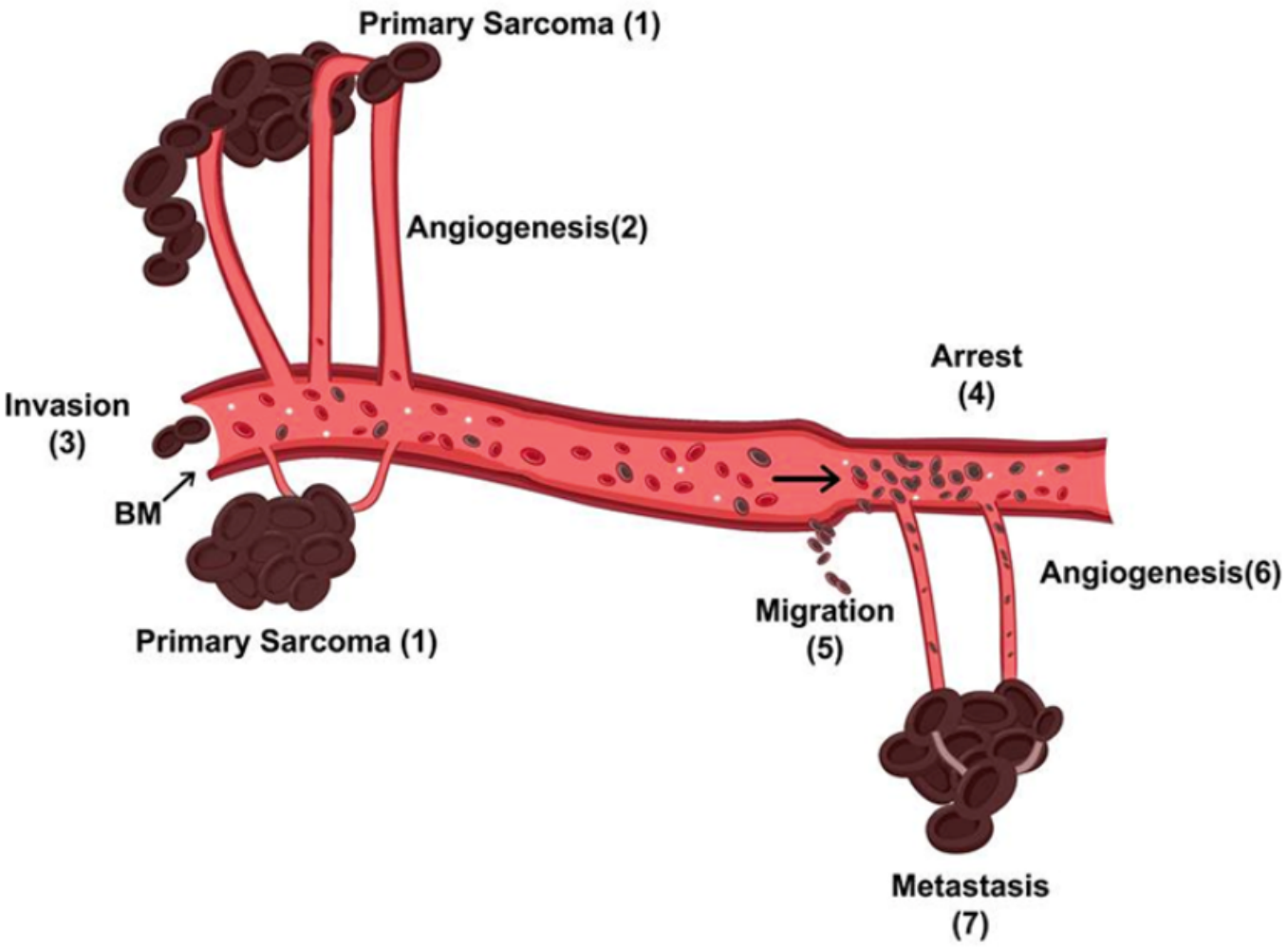

Figure 2

The role of neo-angiogenesis for tumor metastasis from local to distant sites. 1) The local proliferation and expansion of primary osteosarcoma cells. 2) MMPs cause overexpression of VEGF leading to new blood vessels formation. 3)Degradation of extracellular matrix and basic membranes by MMPs help malignant cells to invade in blood vessels. 4) Osteosarcoma cells arrested from endothelial cells of blood vessels in distant organs. 5)Tumor cells migrate in distant sites following blood circulation where they adhere. 6) Secondary tumors secrete MMPs in order to form a sufficient neoplastic blood vessels network. 7)Osteosarcoma cells in distant tissues create mature metastatic tumors.

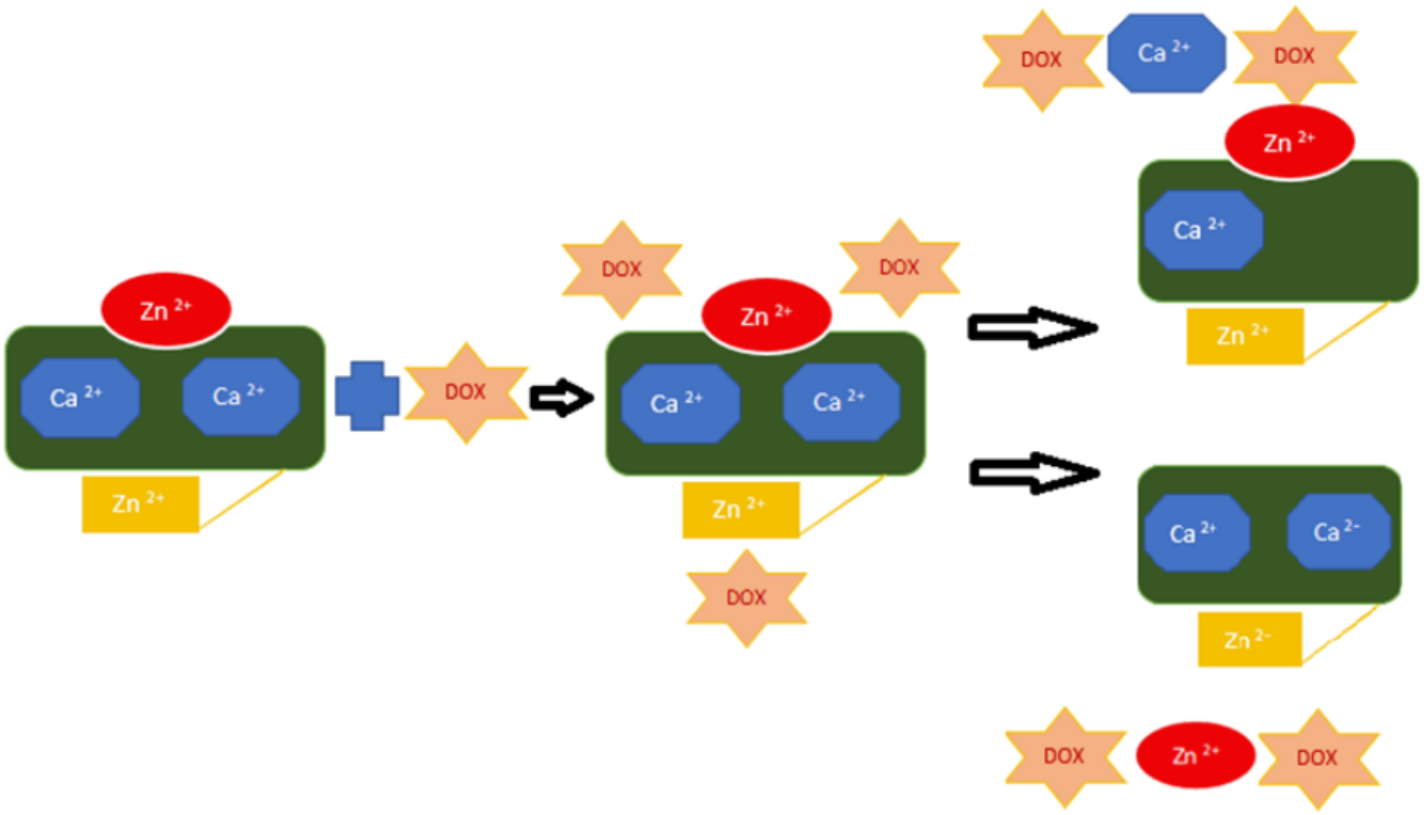

Figure 3

Mechanism of MMPs inactivation by Doxycycline. Osteosarcoma cells secrete MMPs which support survival and invasiveness of malignant cells. Doxycycline (DOX) inactivates MMPs by forming chelation bonds with their Zn2+ and Ca2+ ions.

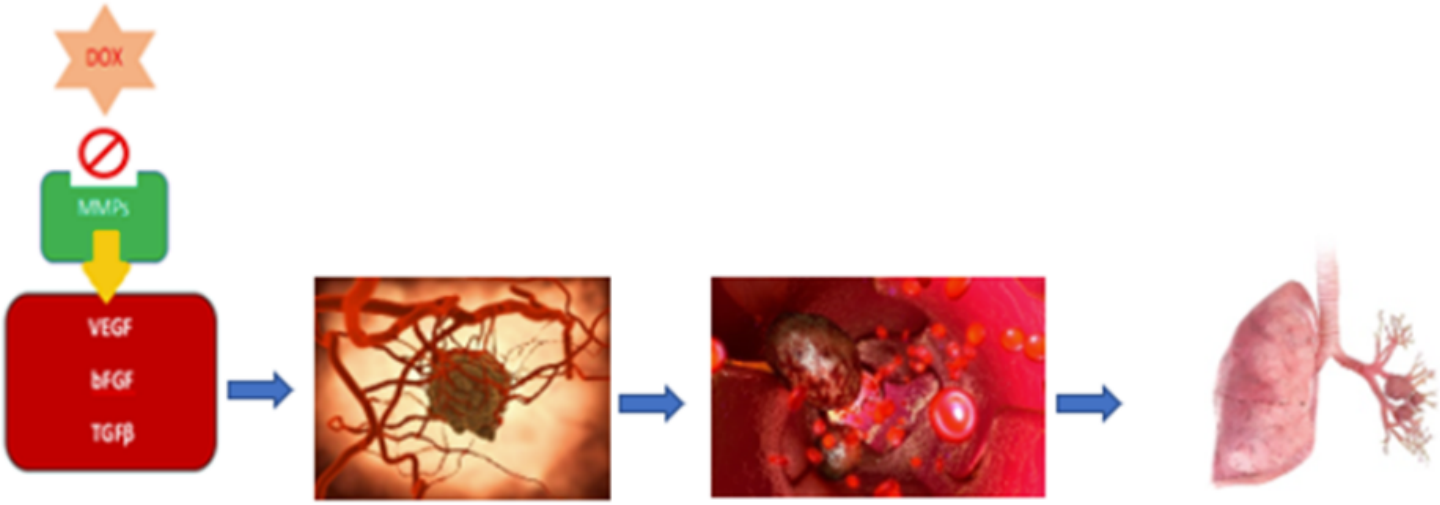

Figure 4

The indirect anti-angiogenesis effect of Doxycycline via MMPs inhibition. MMPs increase the secretion of proangiogenic factors like VEGF, bFGF and TGF promoting the formation of new network of blood

vessels. Adequate blood supply helps osteosarcoma cells to survive, proliferate and penetrate blood vessels to give metastases in distant organs, mainly in lungs. Doxycycline acts as a cytotoxic agent by inhibiting MMPs and depriving tumor cells from nutritional necessities.

Supplementary Files

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