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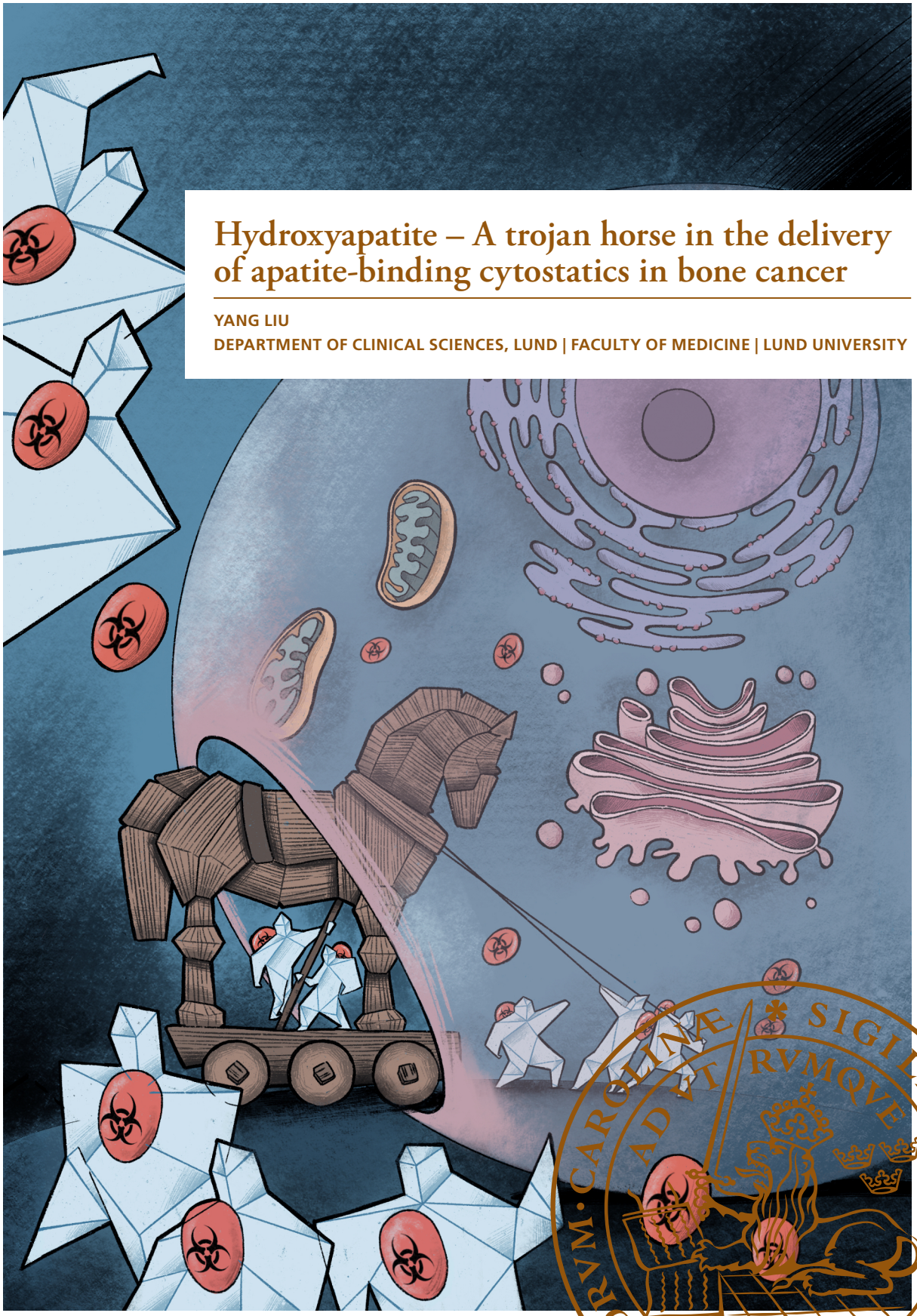

Hydroxyapatite

A trojan horse in the delivery of apatite-binding cytostatics in bone cancer

Yang Liu

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title and subtitle: Hydroxyapatite – A trojan horse in the delivery of apatite-binding cytostatics in bone cancer

Abstract

Osteosarcoma is a malignant cancer of the bone mainly affecting adolescents. Despite progress, the clinical management of osteosarcoma is still challenging. With the current chemotherapy protocol being used for more than 30 years, the number of poor responders is increasing. Although new treatments have been explored since then, no improved tumor eradication effect have been found. In the present thesis, we have developed a new treatment method for osteosarcoma, using hydroxyapatite (HA) based materials as a platform for local delivery of cytostatics. Doxorubicin (DOX), a cornerstone osteosarcoma drug, was chosen as a drug candidate, due to its binding capacity to HA. Different types of HA-based biomaterials were tested for local or targeted delivery of DOX. The efficacy of the developed system was evaluated in-vitro, in osteosarcoma cells as well as in-vivo, in mice bearing an aggressive osteosarcoma.

In Study 1, a clinically approved calcium sulphate (CaS)/HA biomaterial achieved a sustained and controlled release of DOX up to 28 days, both in-vitro and in-vivo. Compared to no treatment or the clinical standard with systemic DOX administration, the local delivery of DOX using a CaS/HA biomaterial significantly hindered tumor progression by inhibiting angiogenesis and cell proliferation.

In Study 2, we investigated the physicochemical interactions between DOX and different sizes of HA particles, both in-vitro and in-vivo. When delivered by HA nanoparticles, DOX is routed to the mitochondria causing insufficient ATP synthesis, less cell migration and cell apoptosis. This leads to stronger in-vivo tumor eradication compared to systemic administration of DOX. Furthermore, nHA mediated delivery of DOX may prevent further metastases in-vivo, which was indirectly verified by PET/CT data.

In Study 3, HA particles (nHA, mHA or n/mHA) were labelled with carbon 14 (^{14}C) to detect particle migration in-vivo. During the observational time of 28 days, the majority (>99.9%) of implanted HA particles, irrespective of the size, stayed in the implantation site (proximal tibia), without migrating to other vital organs. No pathological changes were detected in the vital organs.

In summary, we describe a new and efficient method to supplement osteosarcoma treatment, with a possible rapid translational potential, using clinically approved constituents. By using a hydroxyapatite-based biomaterial, DOX could be routed to the tumor site, more efficiently and with less side effects compared to systemic administration. The chemical interaction between DOX and HA lead to a sustained and controlled DOX release which further improved its tumor eradication effect. When using HA nanoparticles, DOX could be directed to the mitochondria causing tumor cell starvation, reduced migration and apoptosis, jointly leading to improved tumor eradication. The local administration of HA particles, irrespective of size, was confirmed as safe without damage to vital organs. In the future, chemotherapeutics with multi-release profile potentially could be applied by using a combination of nHA and mHA.

Key words: Biomaterials, Hydroxyapatite, Osteosarcoma, Doxorubicin, Local delivery

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To My Family and My Teachers....

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Popular Science

Osteosarcoma is a highly malignant tumor of the bone. The treatment is based on a combination of pre-operative drug treatment (chemotherapy) followed by surgery and post-operative chemotherapy. Advances in imaging and surgical techniques have helped to save limbs for these patients, but limb-preservation comes with a risk of local recurrence due to persisting cancer cells. An increasing proportion of patients respond poorly to chemotherapy due to drug resistance, which remains a challenge. For osteosarcoma, the drugs used as standard chemotherapy are high-dose methotrexate, doxorubicin (Adriamycin), and cisplatin (MAP). Even though chemotherapy has dramatically improved the prognosis for osteosarcoma patients, the options for the poor responders are few. The patients with a poor response to chemotherapeutics have a 5-year survival between 45 and 55%. Adding more drugs, like using MAP plus ifosfamide and etoposide, has been tested but did not improve the efficacy. In fact, less than 5-10% of the total administered drug reach the primary tumor site in the skeleton, and the rest may cause severe side effects and organ toxicity.

The aim of this thesis was to explore the possibility of using hydroxyapatite, a native bone mineral, as a biomaterial for controlled delivery of cytostatics. When placed inside the tumor, it shows similarities to the legendary Trojan horse in Homer´s Iliad. Local drug delivery comes with the advantage of achieving extremely high drug concentration inside the tumor, and thereby better drug efficacy. In addition, by using hydroxyapatite binding drugs, the surplus drug binds to the local hydroxyapatite in the surrounding bone, and less systemic distribution and less accumulation in vital organs would hypothetically cause fewer side effects.

In this thesis, firstly a calcium sulphate/hydroxyapatite (CaS/HA) biomaterial was shown to be an efficient carrier for immediate local delivery of doxorubicin (DOX) and more efficient than systemic DOX. Secondly, the chemical interaction between DOX and HA was validated as an electrostatic interaction. After binding to HA nanoparticles, DOX was redirected to the mitochondria instead of the cell nucleus. The efficacy of mitochondria targeted delivery of DOX was verified in-vivo and the tumor growth was significantly hindered, compared to using intravenously administered DOX. Last but not the least, the safety of HA nanoparticles (nHA) combined with microparticles (mHA) was confirmed by showing that particles, after being locally implanted in the tibia, did not migrate to the vital organs.

Abstract

Osteosarcoma is a malignant cancer of the bone mainly affecting adolescents. Despite progress, the clinical management of osteosarcoma is still challenging. With the current chemotherapy protocol being used for more than 30 years, the number of poor responders is increasing. Although new treatments have been explored since then, no improved tumor eradication effect have been found. In the present thesis, we have developed a new treatment method for osteosarcoma, using hydroxyapatite (HA) based materials as a platform for local delivery of cytostatics. Doxorubicin (DOX), a cornerstone osteosarcoma drug, was chosen as a drug candidate, due to its binding capacity to HA. Different types of HA-based biomaterials were tested for local or targeted delivery of DOX. The efficacy of the developed system was evaluated in-vitro, in osteosarcoma cells as well as in-vivo, in mice bearing an aggressive osteosarcoma.

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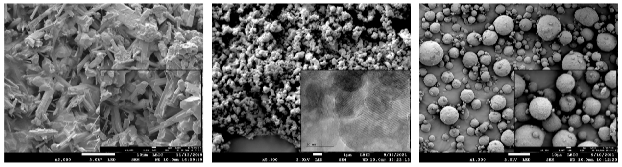
Thesis at a glance

Biomaterials

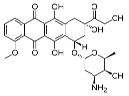


CaS/HA+DOX (Study 1)

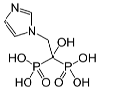
Nano-HA (Study 2 and 3)

Micro-HA (Study 2 and 3)

Drugs



Doxorubicin
A first-line osteosarcoma drug
(Study 1 and 2)

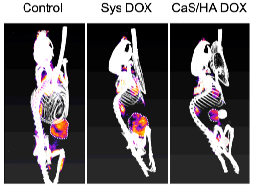


Zoledronic acid
Bisphosphonate with high affinity to hydroxyapatite
(Study 3)

Study 1

Aim: The efficacy of CaS/HA in locally delivering DOX.

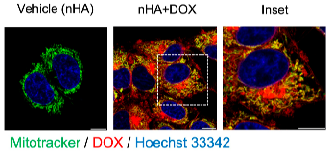
Conclusion: Local delivery of DOX by CaS/HA is superior to systemic administration.



Study 2

Aim: Investigate the efficacy and target organelles for intracellular DOX delivery using nano-HA.

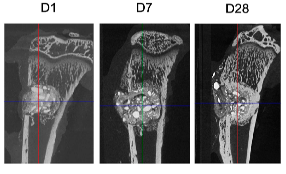
Conclusion: DOX can be delivered intracellularly to mitochondria with improved tumor eradication.



Study 3

Aim: Detect the biodistribution of HA particles in-vivo.

Conclusion: Majority of implanted HA particles (nHA or n/mHA) stayed locally without migrating to vital organs.



CaS/HA: Calcium sulfate/hydroxyapatite; DOX: Doxorubicin; Nano-HA, nHA: Hydroxyapatite nanoparticles; Micro-HA, mHA: Hydroxyapatite microparticles. n/mHA: a combination of nHA and mHA (50/50 w/w%).

List of papers

Paper 1: Sustained and controlled delivery of doxorubicin from an in-situ setting biphasic hydroxyapatite carrier for local treatment of a highly proliferative human osteosarcoma.

Liu Y, Raina DB, Sebastian S, Nagesh H, Isaksson H, Engellau J, Lidgren L, Tägil M.

Acta Biomaterialia. 2021 Sep 1;131:555-571.

Paper 2: Bone mineral: A trojan horse for bone cancers. Efficient mitochondria targeted delivery and tumor eradication with nano hydroxyapatite containing doxorubicin.

Liu Y, Nadeem A, Sebastian S, Olsson MA, Wai SN, Styring E, Engellau J, Isaksson H, Tägil M, Lidgren L, Raina DB.

Materials Today Bio. 2022 Feb 26;14:100227.

Paper 3: Long-term in-vivo biodistribution of nano and micro sized hydroxyapatite particles implanted in a bone defect

Liu Y, Sebastian S, Huang JT, Corbascio T, Engellau J, Lidgren L, Tägil M, Raina DB.

Submitted. 2022

Papers not included in the thesis

Paper 1: Synthetic hydroxyapatite: a recruiting platform for biologically active molecules.

Raina DB, **Liu Y**, Isaksson H, Tägil M, Lidgren L.

Acta Orthop. 2020 Apr;91(2):126-132.

Paper 2: Bone mineral as a drug-seeking moiety and a waste dump.

Raina DB, **Liu Y**, Jacobson OLP, Tanner KE, Tägil M, Lidgren L.

Bone Joint Res. 2020 Oct;9(10):709-718.

Paper 3: Spatio-temporal Delivery of a Heterodimer Bone Morphogenic Protein-2/7 via a Collagen Hydroxyapatite Scaffold Accelerates Critical Femoral Defect Healing at ultra-low Doses.

Liu Y, Puthia M, Sheehy EJ, Ambite I, Petrlova J, Prithviraj S, Oxborg MW, Sebastian S, Vater C, Zwingenberger S, Struglics A, Bourgine PE, O'Brien FJ, Raina DB.

Under Review. 2022

Paper 4: Bioactive hydroxyapatite nanomaterial functionalized by doxorubicin and zoledronic acid exerts improved tumor eradication and bone restoration on an orthotopic xenograft

Liu Y, Corbascio T, Sebastian S, Huang JT, Engellau J, Lidgren L, Tägil M, Raina DB.

In Manuscript.

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最后感谢自己不负时光。

List of abbreviations

MAP	Methotrexate, doxorubicin, cisplatin
MAPIE	Methotrexate, doxorubicin, cisplatin, ifosfamide, and etoposide
CaS/HA	Calcium sulphate/hydroxyapatite
DOX	Doxorubicin
HA	Hydroxyapatite
nHA	HA nanoparticles
mHA	HA microparticles
n/mHA	A combination of nHA and mHA (50:50 w/w%)
^{14}C	Carbon 14
WHO	World Health Organization
MSCs	Mesenchymal stem cells
BM-MSCs	Bone marrow-derived mesenchymal stem cells
ASCs	Adipose-derived mesenchymal stem cells
BMP2	Bone morphogenic protein 2
ECM	Extracellular matrix
MMPs	Metalloproteinases
SDF-1	Stromal derived factor 1
IL-6	Interleukin 6
VEGF	Vascular endothelial growth factor
MCP-1	Monocyte chemoattractant protein-1
GRO-α	Growth-regulated oncogene-α
EVs	Extracellular vesicles
HUVECs	Human umbilical vein endothelial cells
TGF-β	Transforming growth factor-β

FGF	Fibroblast growth factors
RANKL	Receptor activator of nuclear factor kappa B ligand
IGF1	Insulin-like growth factor 1
ZA	Zoledronic acid
HIF	Hypoxia-induced factor
EPCs	Endothelial progenitor cells
TAMs	Tumor-associated macrophages
APCs	Antigen presenting cells
DCs	Dendritic cells
HDMTX	High-dose methotrexate
NCI	National Cancer Institute
CTCAE	Common Toxicity Criteria for Adverse Events
AKI	Acute kidney injury
CIPN	Cisplatin-induced peripheral neuropathy
LDDS	Local drug delivery system
FA	Folic acid
FAR	Folate receptor
EPR	Enhanced permeability retention
FAP	Fluorapatite
CAP	Chlorapatite
ZA	Zoledronic acid
^{14}C-ZA	^{14}C labelled zoledronic acid
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
XRD	X-ray diffraction
FTIR	Fourier-transform infrared spectroscopy
IVIS	In-Vivo Imaging System Spectrum CT
PET-CT	Positron emission tomography–computed tomography
NIM	Nanoparticle-in-microparticle system

1. Introduction

1.1 Understanding osteosarcoma

1.1.1 The epidemiology of osteosarcoma

Osteosarcoma is the most common primary bone cancer, accounting for nearly two-thirds of all cases [1]. It was first described in 1805 and termed osteosarcoma by a French surgeon Alexis Boyer. Nowadays, the incidence is reported to be higher in adolescence, and peaks at 8–11 per million between age 15–19 years in various countries [2-4]. The second peak is in the elderly, with an incidence in the range of 2.5–5 per million in men and 1.5–4 in women. In Australia and Canada, the incidence is even higher for men aged 75 years and older compared to other countries [5]. The bimodal age incidence distribution of osteosarcoma has been observed worldwide.

Osteosarcoma usually occurs primarily at the metaphysis where bone growth takes place [6]. Approximately 80% of osteosarcomas occur in the extremities, primarily in the proximal tibia, distal femur and proximal humerus [7]. To confirm the diagnosis of osteosarcoma, the suspected tissue is usually biopsied for histopathological assessment with the most important characteristic of an osteoid within the tumor. Currently, World Health Organization (WHO) divided conventional osteosarcoma into three major subtypes: osteoblastic, chondroblastic and fibroblastic, based on the predominant cell type within the tumor. In addition, there are also other histological variants, like telangiectatic osteosarcoma, small cell osteosarcoma, parosteal and periosteal osteosarcomas, as well as low grade central and high-grade surface osteosarcomas [8]. The conventional osteosarcoma is the most common type (80%) with no significant differences in clinical outcomes among the sub-categories. The other types like telangiectatic, small-cell and low-grade osteosarcoma accounts for 4%, 1-2% and 1-2%, respectively [9].

1.1.2 The pathogenesis of osteosarcoma

Many studies have explored the pathogenesis of osteosarcoma within the last decades. Although the mechanism of its pathogenesis is still under exploration, various types of cells were reported to be involved in osteosarcoma-genesis.

1.1.2.1 Cell-of-origin for osteosarcoma

The cell-of-origin concept is that a normal cell type acquires the first cancer-promoting mutations and thereby initiates tumor formation [10]. For osteosarcoma, various studies have considered mesenchymal stem cells (MSCs) as the most likely cell-of-origin [11]. It has been reported that the orthotopic inoculation of $P53^{-/-}$ and $P53^{-/-}$+$RB^{-/-}$ bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived MSC (ASCs), consistently generated osteoblastic osteosarcoma reflecting human osteosarcoma characteristics. However, the osteosarcoma-related histological pattern was missing in the tumor where is away from the inoculation site, suggesting that bone microenvironment is important in osteosarcomagenesis. In addition, inoculation of $P53^{-/-}$+$RB^{-/-}$ MSC together with hydroxyapatite/tricalcium phosphate biomaterial in an ectopic location also demonstrated that bone microenvironmental factors, like bone morphogenic protein 2 (BMP2) and calcified substrates, were involved in the progress of osteosarcoma [12].

1.1.2.2 MSCs in osteosarcoma microenvironment

For osteosarcoma, the crosstalk between the osteosarcoma cells and the non-tumor cells plays a vital role for tumor progression and metastasis [13]. MSCs could react to the microenvironment due to the receptors on the cell membrane for multiple growth factors and chemokines. They could also modulate their microenvironment by secreting extracellular matrix (ECM) and various growth factors, cytokines, chemokines, and metalloproteinases (MMPs) [14]. Thus, MSCs have both autocrine and paracrine trophic properties, regulating cell metabolism of MSCs, osteoblasts, and endothelial cells [15]. Furthermore, MSCs could also secrete stromal derived factor 1 (SDF-1), interleukin 6 (IL-6) and growth factor vascular endothelial growth factor (VEGF) to promote osteosarcoma growth, metastasis and angiogenesis [16]. When co-injection of osteosarcoma-associated stromal cells with human osteosarcoma MNNG/HOS cells, the tumor induced by MNNG-HOS cells appeared as bone spines and an abundant osteoid matrix, which is similar to the microstructure of the osteosarcoma in patients, which was not found in the MNNG/HOS mouse model. In addition, a huge infiltration of peripheral blood mononuclear cells in vessel walls were found surrounded osteosarcoma metastases in the lungs [17]. This indicated that MSCs could affect osteosarcoma cells and MSC-induced osteosarcoma cells may in turn promote more osteoid matrix formation and higher immune infiltration. On the other side, Pietrovito et al. showed that MSCs, activated by osteosarcoma cells, could secrete more monocyte chemoattractant protein (MCP-1) (alias CCL2), growth-regulated oncogene (GRO-α) (also known as CXCL1), and IL-6 and -8, which in turn promote osteosarcoma cell motility, invasiveness, and trans-endothelial migration [13].

Extracellular vesicles (EVs) from MSCs were found to contain tumor supportive microRNAs, proteins and metabolites such as lactate and glutamate [18]. In

osteosarcoma, MSC-secreted EVs increased tumor cell survival and migration under serum starvation condition [19]. It was found also to be involved in revascularization. EVs, from MSCs under hypoxia, increased angiogenesis by activating the protein kinase A in human umbilical vein endothelial cells (HUVECs) [20]. MSC-secreted EVs have been also reported to activate angiogenic signals on endothelial cells [21], especially in hypoxic and ischemic conditions [22].

1.1.2.3 Osteoblasts and bone formation

One of the main histological features of osteosarcoma is the formation of osteoid matrix within the tumor [23]. This could induce the formation of Codman's triangles or bone spines presented as the sunburst periosteal reaction. Osteoblastic progenitors are MSCs, which mainly present in the bone marrow and differentiate into osteoblasts, chondroblasts, myoblasts and adipocytes under different specific transcription factors. Most of these cytokines or growth factors are involved in the development of osteosarcoma. For example, transforming growth factor beta (TGF-β) is involved in tumor growth and metastasis [24]. Blocking TGF-β activity in osteosarcoma cells inhibited tumor growth by regulating the cell communication between tumor cells and non-tumor cells [25]. Weekes et al. reported that using AZD4547 to block fibroblast growth factors (FGF) receptor signaling decreased lung metastases in mice [26]. Dickkopf-1, a monoclonal antibody against the WNT signaling, has been found to inhibit metastasis in a preclinical setting [27]. Thus, the evidence that osteoblasts play a vital role in tumor progression is emerging. Osteoblasts could regulate bone matrix synthesis directly and bone resorption indirectly through the release of Receptor Activator of Nuclear Factor kappa B Ligand (RANKL), which binds its receptor RANK on osteoclast precursors as well as MSCs. Navet et al. explored the role of RANK overexpression in osteosarcoma cell lines and tumor progression in-vivo [28]. It was found that activating RANKL–RANK pathway in osteosarcoma cells had no effect on cell proliferation and cell migration as well as tumor growth, suggesting that RANK overexpression is not directly involved in tumor progression. However, RANK-overexpressing osteosarcoma cells increased lung metastasis, which could be prevented by a RANKL antibody. The whole-body deletion of RANKL inhibited tumor progression and lung metastasis in genetically modified mice [29]. Thus, RANKL–RANK pathway activation seems to be indirectly involved in tumor progression.

1.1.2.4 Osteoclasts and osteolysis

Osteolysis is common in osteosarcoma development, causing frequent pain in tumor-bearing patients. The aggressiveness of osteosarcoma has been found related to osteolysis markers in patients [30]. Notably, the binding of RANKL (produced by osteoblasts and osteocytes [31]) to its receptor (RANK) (expressed on the cell surface of osteoclast precursors [32]), mainly regulates osteolysis through paracrine regulation. In osteosarcoma, osteoclast activity leads to a vicious cycle between osteosarcoma cell proliferation and bone degradation, leading to the release of pro-

tumor factors such as insulin-like growth factor 1 (IGF-1) or TGF-β from the bone matrix [33, 34]. The relationship between osteolysis and tumor progression were validated in preclinical studies by using chemical inhibitors (mainly zoledronic acid, ZA) [35], RANKL receptor competitors [29], and RANKL silencing [36]. Thus, inhibiting osteolysis became a promising therapeutic target in addition to the standard chemotherapy. However, no clear benefit but slightly worse therapeutic results were seen from a Phase III clinical trial by using ZA in addition to chemotherapy plus surgery [25]. Even though ZA has an inhibitory effect on osteosarcoma cells, its efficacy in hindering tumor progression remains controversial [37]. Denosumab, an antibody directed against human RANKL, is currently used for bone metastasis or giant cell tumors to efficiently inhibit osteoclast activity and prevent bone loss. However, no clinical evidence supported its usage in osteosarcoma [38].

1.1.2.5 Vascular microenvironment in osteosarcoma biology

Vascularization plays a vital role in tumor growth, providing oxygen and nutrients and supporting tumor cell migration or invasion. A tumor vasculature is one of the main features for cancer [39]. It is formed mainly through the neo-angiogenesis sprouted from existing vessels. Additional mechanisms such as vascular mimicry are also involved in the expansion of tumor vasculature [40].

Environmental stresses like hypoxia could initiate angiogenesis by promoting the secretome of pro-angiogenic factors such as hypoxia-induced factor (HIF) and VEGF in the tumor microenvironment. Osteosarcoma are highly vascularized bone tumors with a hypoxic and acidic microenvironment, which appear mainly in the metaphysis where type-H endothelial cells with ability to promote angiogenesis are located [41]. This suggests a contribution of endothelial cells to neo-angiogenesis. In tumors, endothelial progenitor cells (EPCs) can be recruited from their initial location to the tumor site, where tumor cells or stromal cells can regulate EPCs through differentiation paracrine signals. In osteosarcoma, the role EPCs in the development of neo-angiogenesis was recently highlighted in a rat model, where co-inoculation of osteosarcoma cells and EPCs increased vascularization [42]. It was found that osteosarcoma cells promoted EPCs migration and its angiogenesis through angiogenesis-related factors including VEGF and TGF-β1. The pro-angiogenic role of EVs from osteosarcoma cells was also verified based on angiocrines and angiogenesis-related miRNAs [43, 44].

The expression of neo-vascularization markers in samples collected from patients were investigated by several cohorts. Upregulated genes in the VEGF pathway, especially VEGF-A, were found in osteosarcoma patients, as well as at the protein level [45]. VEGF overexpression is positively related to tumor progression and metastasis [46]. Indeed, other studies indicated that VEGF overexpression in biopsies was related to worse disease-free survival and lower overall survival [46] [47]. A systematic review and meta-analysis including 559 patients suggested

VEGF expression as an promising biomarker for osteosarcoma prognosis [48]. Beside VEGF, its receptor VEGFR-2 is upregulated in osteosarcoma compared to healthy bone tissue, and its overexpression is related to poor prognosis [49]. It was also found that serum concentration of VEGF was higher in bone sarcoma (Ewing sarcoma, osteosarcoma, chondrosarcoma) compared to healthy samples [50]. Its prognostic value was addressed by showing significantly higher serum levels of VEGF in patients with metastasis at diagnosis than non-metastatic patients [51]. However, using circulating VEGFs as objective prognostic factors needs to be explored further in the future.

1.1.2.6 Immune cells in osteosarcoma microenvironment

The immune cells, involved in the osteosarcoma development, are mainly tumor-associated macrophages (TAMs), dendritic cells (DCs), lymphoid cells and myeloid cells [52]. The infiltration of TAMs could reduce metastasis and improve survival in high-grade osteosarcoma [53]. It is still unclear what role TAMs play inhibiting metastasis in osteosarcoma but infiltration of pro-inflammatory M1 and anti-inflammatory M2 was found in osteosarcoma. The presence of M2 macrophages might exert an anti-metastatic rather than a pro-metastatic effect [53]. The infiltration of antigen presenting cells (APCs), including CD1a DCs and CD68 macrophages, has been correlated with poor prognosis [54]. Furthermore, myeloid and lymphoid cells were both detected in osteosarcoma [52, 55]. From the biopsies, cytotoxic $CD8^+$ T lymphocytes were not as much as myeloid cells, indicating that osteosarcoma microenvironment was in lack of cytotoxic lymphocytes. In recent studies, $CD8^+$ T lymphocytes were detected from 50% osteosarcoma patient, who had a significantly lower risk of metastasis [56].

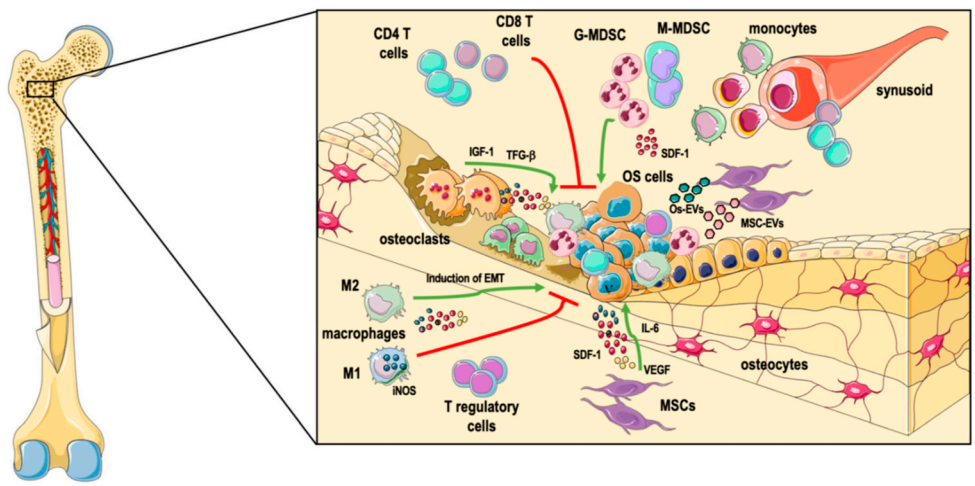

Figure 1. The complex osteosarcoma micro-environment in the bone tissue (adapted from Cascini C et al. 2021).

1.2 A brief history of osteosarcoma treatment

Prior to the 1970s, amputation of the effected limb was the treatment for osteosarcoma. With this approach, more than 80% of patients diagnosed as localized osteosarcoma died within 2 years due to pulmonary metastasis. In the early 1970s, clinicians started testing various chemotherapy agents for recurrent osteosarcoma. To confirm the benefit of chemotherapy, a multi-center randomized study was started in 1982 to explore the efficacy of multi-agent chemotherapy including high-dose methotrexate, Adriamycin, and cisplatin (MAP) on patients with non-metastatic osteosarcoma. The study was presented in 1986 demonstrating that at 2-year survival of 66% for patients treated with multi-agent chemotherapy compared to only 17% for patients on the control observation arm [57]. This multi-agent chemotherapy (MAP protocol) became standard practice for osteosarcoma patients, which is still widely used nowadays.

Methotrexate was discovered by Farber et al. in the 1940s, which was initially a drug for leukemia and lymphoma [58]. Later, high-dose methotrexate (HDMTX) was introduced for the treatment of osteosarcoma. It could deprive folates from the cell, which is necessary for DNA formation. The efficacy of methotrexate on osteosarcoma was confirmed in a randomized clinical trial called MIOS [59]. It was aimed to compare surgical ablation plus chemotherapy with only surgical ablation. For chemotherapy, methotrexate combined with other agents were used. Surgical resection plus chemotherapy showed a survival of 65% compared to only 5% for the control arm (surgery alone). The result was re-confirmed in another clinical study by Eilber et al. [60]. Numerous publications later strengthened its efficacy as chemotherapy in osteosarcoma.

Doxorubicin (DOX) was shown to be effective in osteosarcoma in the 1960s [61]. It could intercalate into DNA inducing DNA damage. It produced responses in 30-40% patients for different cancers with its single usage or combined with other agents. It also increases the sensitivity of radiation therapy. Accumulation of DOX locally may cause severe tissue necrosis and ulceration. However, the major severe side effect is cardiotoxicity. To avoid cardiotoxicity, the total cumulative dose is suggested under 300 mg/m^2 in children (< 6 years) and 450 mg/m^2 in adults. It is a vital part of the standard chemotherapy in pre- and postoperative regimens for osteosarcoma.

Cisplatin was introduced to treat osteosarcoma in the 1970s. It exerts its cytotoxic effect by platination, the formation of inter-strand DNA cross-links. It has been administered both intravenously and intra-arterially. Intravenously, it produced a 30-60% response in patients with metastatic disease, which is 60-90% intra-arterially [62, 63]. Indeed, the intra-arterial route could enhance the drug efficacy, especially by its effect on tumor neovascularity [64]. Unfortunately, its usage is limited by the labor insensitivity and requirements for conscious sedation or general

anesthesia. It was also suggested for pathological fractures with a rapid response. But similar responses were achieved by administrating cisplatin intravenously and hence administration of cisplatin intra-arterially has been replaced.

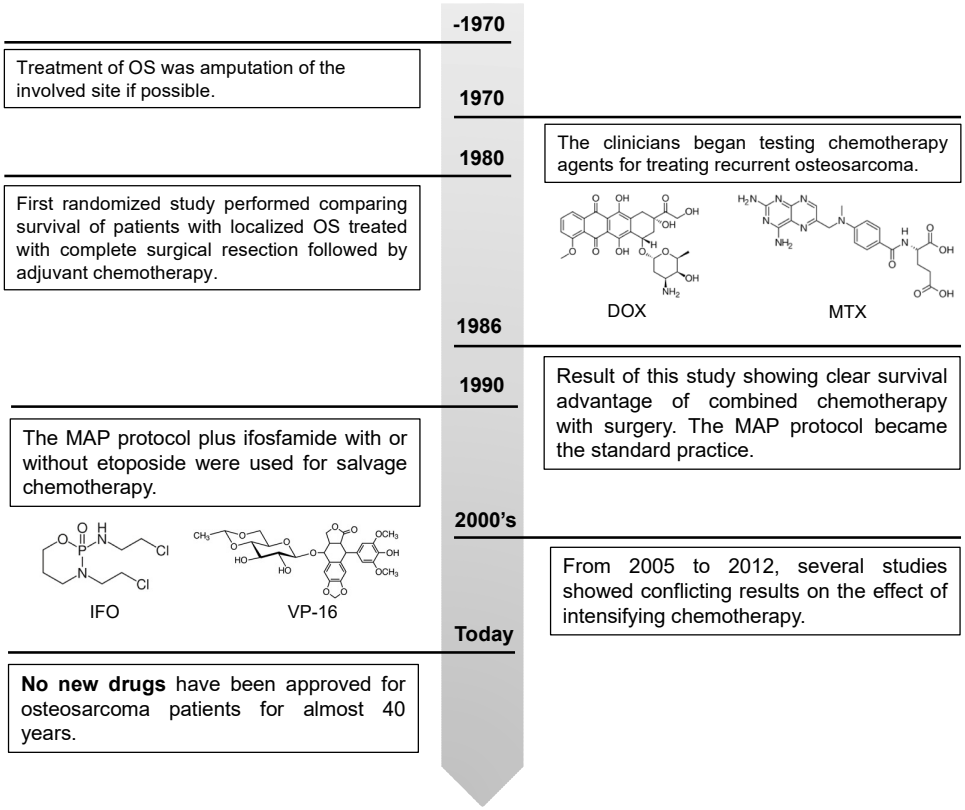

Figure 2. A brief history of the osteosarcoma treatments.

1.3 Current challenges of osteosarcoma treatment

Currently, a major challenge is the fact that no improved 5-year survival rate has been achieved during the last decades by using the current chemotherapy. The MAP protocol is still the backbone of standard treatment protocol for osteosarcoma and has significantly improved the prognosis for the affected individuals. However, very little progress has been achieved for the survival of osteosarcoma patients since the mid-1980s. This is already reported by National Cancer Institute (NCI) of US, in which the 5-year event-free survival of patients (15 to 19 years) was 61% (1987-1990), 68% (1991-1994), 62% (1995-1998), and 66.4% (1999-2002) [65]. In Europe, it was similar for patients (15 to 19 years) treated between 1988 and 1997,

with the 5-year survival of 51% [66]. Due to the continuation of MAP usage, patients have started to respond poorly to the current chemotherapy and approximately 40% of osteosarcoma patients exhibited a poor histological response [67]. For patients with poor histologic response (< 90% necrosis), the 5-year survival was only 45% to 55% [68, 69]. Intensified chemotherapy, adding more drugs like ifosfamide and etoposide into MAP protocol, has been tried to achieve better tumor eradication but without any improved outcome [70, 71].

The second challenge is that the continuation of current anti-tumor drugs has induced drug resistance. Drug resistance was first found in bacteria, which got resistant to the antibiotic. Then similar phenomenon was found on cancer and several other diseases [72]. For example, drug resistance in cancer was first noted in the 1940s [73], and over the years, it has become one of the significant concerns of cancer management. The initial solution to avoid drug resistance was to use poly-chemotherapy instead of single-agent chemotherapy, which means combining agents with non-overlapping mechanisms of action [74]. This approach worked remarkably well in various types of cancers [75, 76]. Multi-agent chemotherapy thus became a new trend for cancer therapy which led to multiple complex regimens for different cancers. Except that, different methods of drug administration [77], including shorter-interval administrations [78] or higher doses administrations [79], resulted in improved inhibition of early tumor growth. However, the successes achieved with poly-chemotherapy had largely plateaued since its introduction. Surgery and poly-chemotherapy were clearly not enough to cure osteosarcomas.

The third challenge is the serious side effects caused by highly toxic cytostatics. The administration of chemotherapy usually comes with severe side effects, including immediate signs of toxicity and late signs of chronic toxicity [80]. It can be divided into mild (grade 1), moderate (grade 2), severe (grade 3), life-threatening consequences (grade 4), or death (grade 5), according to the Common Toxicity Criteria for Adverse Events in cancer therapy (CTCAE) [81]. Immediate effects can be observed on various locations such as skin and hair, bone marrow and gastrointestinal tract. All organs of the body can be affected, including essential organs like the heart, lung and brain [82]. In addition, the chronic effects could induce drug resistance, carcinogenicity and infertility. High-dose methotrexate (> 500 mg/m^2) is used in multiple adult and childhood cancers. Although HDMTX is safe for most patients, it can still cause significant nephrotoxicity inducing acute kidney injury (AKI), which was found in 2-12% of patients [83]. AKI and other toxicities caused by HDMTX can lead to morbidity, treatment delays and impaired renal function [84]. DOX is known for its cardiac toxicity, especially with a cumulative dose over 550 mg/m^2 [85]. It might lead to life-threatening cardiomyopathy and congestive heart failure [86]. Cisplatin, as the first metal based anticancer drug, was associated with neurotoxicity, cardiovascular toxicity, renal and pulmonary toxicities [87]. Cisplatin-induced peripheral neuropathy (CIPN) is one of the most common side effects due to the increasing cumulative dose of

cisplatin [88]. The CIPN is usually seen with a cumulative dose over 300 mg/m^2. It will be gradually alleviated after drug discontinuation; however, it can still be durable in 20–40% of patients [89, 90]. In osteosarcoma, the overall treatment time of MAP protocol is long, usually 6–8 months. When administered systemically, the risk of off-target side-effects increases, primarily bone-marrow toxicity, damage to mucosal tissue, alopecia and heart failure, which may lead to multi-organ failure [91].

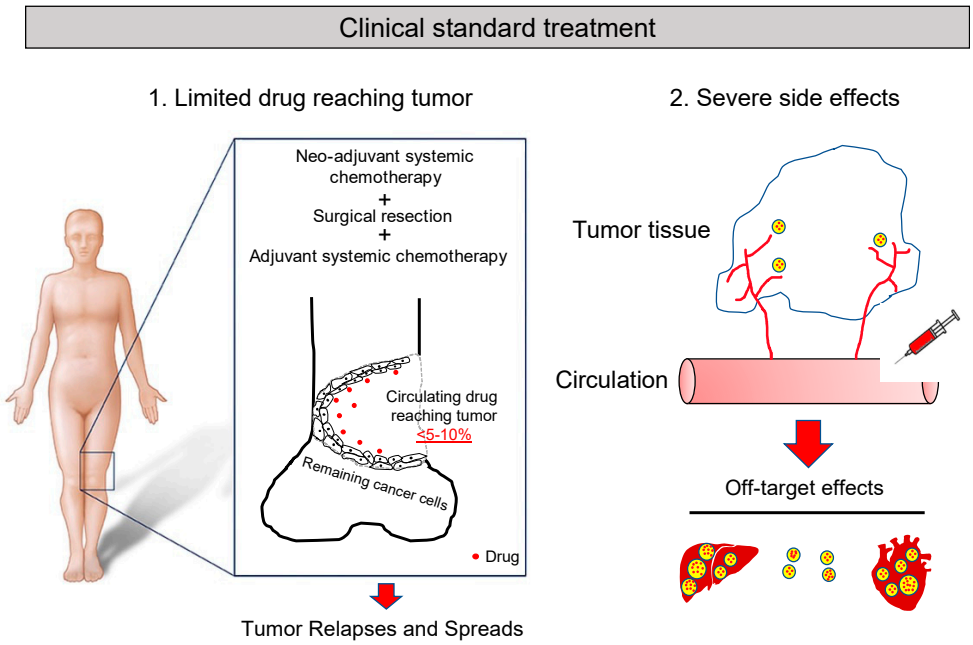

Figure 3. The disadvantages of the current osteosarcoma treatments.

1.4 Potential solutions from pre-clinical research

In the last decades, progress on the investigation of tumor microenvironment and fabrication of various biocompatible materials [92-94], have led to the development of drug carriers that can deliver cytostatics to tumor sites more efficiently achieving improved tumor eradication. Among all the delivery system, local drug delivery system (LDDS) has become a promising approach to deliver anti-tumor agents facilely and intensively in situ. It has several advantages especially for the chemotherapy postoperatively. (1) Biomaterials are mostly biodegradable with no need for surgical removal and biocompatible circumventing the chronic foreign-body immune response. (2) High local concentration is achieved by controlled and

sustained drug release. (3) Side effects on the adjacent healthy tissues and organs are avoided [95, 96]. For example, Cinar et al. showed that local controlled release of DOX by peptide amphiphiles nanogel is more efficient to inhibit tumor growth compared to local injection of free DOX, with 40% tumor reduction [97]. Co-delivery of doxorubicin, cisplatin and methotrexate locally by thermosensitive hydrogels induced a 60% tumor reduction compared to free drug administration [98]. Thus, improved tumor eradication could be well achieved by local delivery. Recent studies have also verified that no obvious damage to the surrounding tissues has been induced by local delivery with drug released in a controlled pattern [99].

Another alternative promising delivery method is the targeted delivery system, which can be achieved by two approaches: the passive targeting and the active targeting. The passive targeting works mainly depending on carrier characteristics and tumor microenvironment [100]. For example, the marketed Doxil© and Caelyx©, both approved for clinical usage, serve as a golden standard for the application of passive tumor-targeted systems [101]. The active targeting is theoretically able to increase the quantity of drug delivered to the target cell compared to free drug or passive targeting. The mechanism of active targeting is to modify the surface of a drug carrier with a functional group that can bind to target cell receptors. For example, folic acid (FA) has been widely used to label the nanocarriers which would specifically bind to the folate receptor (FAR) presented in tumor microenvironment [102]. For both targeting systems, enhanced permeability retention (EPR) effect is the basic mechanism, by which molecules or drug carriers of certain sizes tend to accumulate more in tumor tissue compared to normal tissues [103]. However, not more than 5-10% of the systemically injected drug has been delivered at the targeted tumor site even with the most advanced afore-mentioned drug delivery methods [104].

1.5 Hydroxyapatite-based biomaterial being used in bone diseases

Hydroxy(l)apatite (HA-$Ca_5(PO_4)_3(OH)$), often stated as $Ca_{10}(PO_4)_6(OH)_2$ due to its hexagonal crystallographic symmetry, is the stoichiometric form of the inorganic mineral phase in bone and teeth. Stoichiometric HA is rare in nature; geologically fluorapatite (FAP, $Ca_5(PO_4)_3F$), chlorapatite (CAP, $Ca_5(PO_4)_3Cl$), and multiple substituted apatites are found in rocks and corals [105, 106]. More than 300 apatite-type minerals exist, with elements from the entire periodic table replacing Ca, P, and OH in the fundamental apatite crystal structure [107]. In mammalian bone and teeth, substitutions are also found, including both A and B position carbonate substituted apatites ($Ca_{10}[(PO_4)_{6-y}(CO_3)_y][(OH)_{2-2x}(CO_3)_x]$) [108, 109]. Based on its crystal structure and chemical structure, HA has been reported to bind various

molecules. In 1567, Leminus described that the root of the madder plant made bone red [110], which is the first report of a substance binding to bone. Later, Belchier in 1736 noticed that the pigs fed with mashed madder root had the red bone [110], which was the first report of a systemically administered agent traced in bone. John Hunter studied bone growth by intermittent feeding of pigs with madder root in the late 1700s [111] and found white and red bands in the growth zones of the pig mandible. More recently, Hoyte, in the 1950s, used alizarin (1,2-dihydroxyanthraquinone) extracted from the madder root for bone staining [112]. Nowadays alizarin is still used for dynamic histomorphometric studies of mineral apposition [112, 113]. Several other substances have been reported with affinity to apatite. In 1935, Georg de Hevesy, a Hungarian chemist, published the first study describing the radioactive bone mineral tracers ^{32}P sodium phosphate [114], which indicated continuous uptake of the phosphorus atoms in the bone. In 1950, ^{45}Ca, a bone mineral-seeking radioactive tracer, was systemically administered to study bone accretion and metabolism [115, 116]. Others bone-seeking radioactive isotopes such as ^{85}Sr was also used to identify bone turnover. This eventually paved the way for ^{99}Tc scintigraphy, which is now commonly used for detection of osseous infections, tumors, or other metabolic disorders. Further, with the advancement in chemistry/radiosynthesis techniques and modern imaging equipment, the same tracer ^{99}Tc has been coupled with hydroxy diphosphonate (^{99}Tc-HDP) or methylene diphosphonate (^{99}Tc-MDP) to study mineralization of human stem cells in vitro [117] and bone turnover in vivo in humans [118].

Nowadays, synthetic HA has been extensively investigated for various clinical applications [119]. For example, it has been well reported to use CaS/HA biomaterial to deliver antibiotics locally in bone and joint infections [120, 121]. HA can also be synthesized and grinded into nano sized particles. Nanoparticles in a defined volume have relatively larger surface area compared to microparticles, which dramatically improves the loading characteristics of a drug and consequently increasing drug delivery capacity [122]. It has been shown that HA nanoparticles exert anti-tumor effects on several types of cancer cells [123, 124]. However, the pristine nHA seems to be insufficient to eradicate an established tumor in-vivo, and therefore it needs modification to enhance its cytotoxicity. Using chitosan or bovine serum albumin as an intermediator has been explored to successfully incorporate anti-tumor drugs on the surface of nHA for local delivery [125]. But the burst drug release due to rapid degradation of the coating materials compromises the efficacy. Instead, chemical bonding as an alternative for drug loading has been explored in polymer-based nanomaterials [126]. However, it has rarely been investigated on HA due to the difficulty of modifying the surface [127]. HA, as the hydroxyl endmember of the complex apatite group in a hexagonal crystal system, has fundamental adsorption capacity due to its positively charged surface (Ca^{2+}) attracting anion pairing interactions with deprotonated carboxyl groups ($–CO_2^-$), and negatively charged groups, PO_4^{3-}, which promote interactions with protonated amines (–NH3+) [128]. It has been shown that various growth factors can be loaded on HA surface

due to its physicochemical structure without impairing its bioactivity [129, 130]. Furthermore, this molecule-HA interaction has also been verified in-vivo in bone regeneration and infections [130]. Tetracycline was first found accumulated in the bone after systemic administration in 1956 [131]. And it was confirmed and validated by other studies showing that tetracycline binds to HA through a chemical interaction [132, 133]. Our previous study showed that the serum concentrations of tetracycline can be recruited by the synthetic HA particles placed in targeted site and exert an adequate antibacterial effect [134]. Another example is zoledronic acid (ZA), a third-generation nitrogen-containing bisphosphonate. It has been reported to accumulate both in human bones and pre-implanted in synthetic HA in a rat muscle pouch [135, 136]. After being recruited by pre-implanted HA particles, ZA could modulate and activate HA, promoting bone formation around the implant and thereby enhancing bone anchorage [137]. Thus, synthetic HA materials could be promising carriers for local and targeted delivery of various drugs for bone disorders, enhancing the efficacy and preventing side effects.

2. Research questions

In this thesis, the aims are to answer the following questions:

1. Can a clinically approved CaS/HA biomaterial be used for local delivery of cytostatics like doxorubicin, and how does local DOX delivery perform in terms of tumor eradication compared to systemic administration? This was addressed in **Study 1**.

2. Is there a chemical interaction between DOX and HA, which could facilitate drug loading and controlled delivery of DOX? This was addressed in **Study 2**.

3. Compared to micro-HA, can nano-HA deliver DOX intracellularly? Does intracellular DOX delivery improve tumor eradication? This was addressed in **Study 2**.

4. What is the in-vivo biodistribution of HA nanoparticles when implanted alone or in combination with HA microparticles in a bone void? Do the particles migrate or do they stay at the implantation site? This was addressed in **Study 3**.

3. Experimental design

3.1 Biomaterial preparation

During this thesis work, three different HA-based biomaterials was evaluated for their potential to deliver DOX.

1. The first biomaterial is a commercially available calcium sulphate/ hydroxyapatite (CaS/HA) biomaterial (purchased from Bonesupport AB, Sweden), clinically used in several thousand patients. The material is injectable and consists of 60% alfa hemihydrate calcium sulphate and 40% hydroxyapatite. Importantly, the sulphate, embeds millions of hydroxyapatite microparticles and sets into a solid mass in-situ at low temperatures. Briefly, ceramic powder, 1 g (Calcium Sulphate-60%, Hydroxyapatite-40% by weight) and 430 µL of iodine-based contrasting agent (Iohexol, C-TRU, Bonesupport, Sweden) containing different amounts of DOX, were mixed in a 24-well plate and the paste was transferred into a 1 mL syringe. Uniform cylindrical discs were casted for in-vitro and in-vivo experiments in **Study 1** and characterized by SEM (Fig.4A).
2. 2. To explore whether the particle size plays a role in DOX loading and delivery, HA particles, both nano- and micro-sized HA, were purchased from the same company FLUIDINOVA, Portugal. The materials have been characterized by SEM, TEM and XRD. In **Study 2**, DOX solution was reacted with HA particles in a 2 mL Eppendorf tube to explore the in-vitro binding. For in-vivo binding, HA particles were implanted into muscle pouches of Sprague-Dawley rats under anaesthesia, followed by systemic injection of DOX. For cell experiments and its efficacy in-vivo, HA particles were pre-functionalized with DOX and used accordingly (Fig.4B-C).
3. The same HA particles described in Study 2 were used in **Study 3**. To track HA particles, ^{14}C-ZA solution was used to label HA particles with radioactivity, which would allow detecting its in-vivo biodistribution by using scintillation counting (Fig.4B-C).

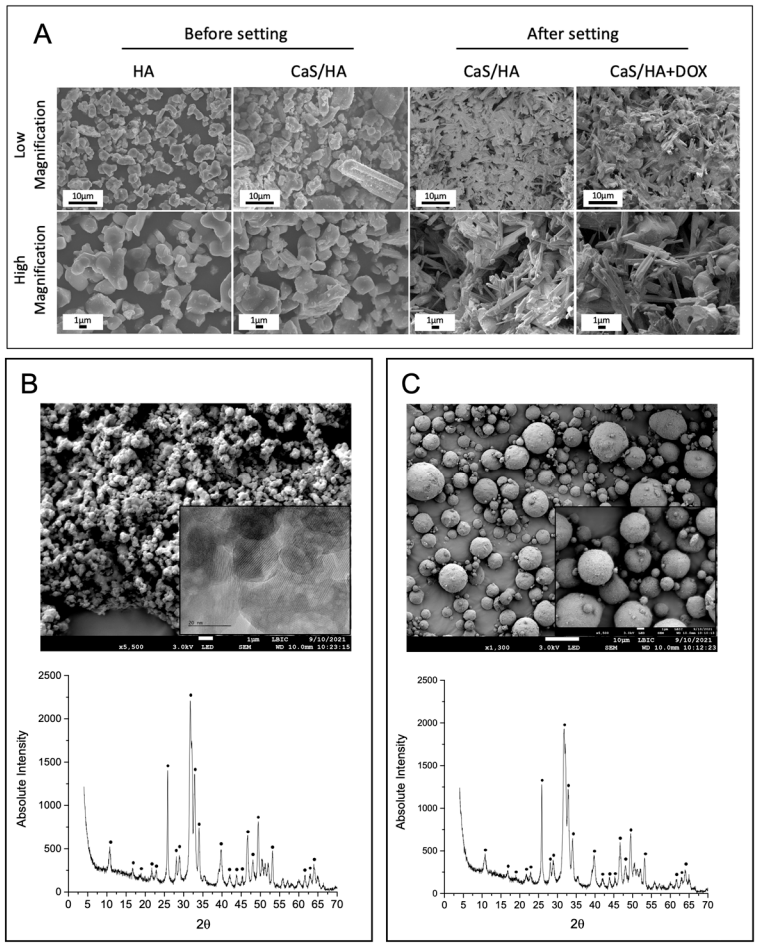

Figure 4. Biomaterials used in this thesis. (A) Microstructure of CaS/HA biomaterial before and after setting in presence of DOX, which was used in **Study 1**. (B-C) Shape, size and quality of pristine nano and micro-HA particles used in **Study 2 and 3**.

3.2 Animal models

During this thesis work, four different animal models were used to test the hypotheses.

1. An ectopic abdominal muscle pouch model in SD rats [138]: Briefly, the abdominal muscle was incised by a surgical knife to form a pouch in between the muscle layers, where the biomaterial could be placed. In **Study 1**, this model was used to detect the DOX release from CaS/HA in-vivo. In **Study 2**, it was used to explore the in-vivo accretion of DOX to pre-implanted HA particles.
2. A subcutaneous xenograft model (without tumor resection) on athymic nude mice, which mimic the pre-operative chemotherapy situation [139]: Briefly, the tumor-bearing mice were prepared by subcutaneously injecting the right flank of the animals with 0.1 mL of cell suspension containing 4×10^6 human 143B osteosarcoma cells in PBS. The interventions started 7 days later when the tumor had formed. It was used in **Study 1** to explore the efficacy of delivering DOX by CaS/HA compared to no treatment, CaS/HA alone and systemic administration of DOX (Fig.5A).
3. A subcutaneous xenograft model (with tumor resection) on athymic nude mice, which mimics the post-operative chemotherapy situation: Briefly, the subcutaneous cell injection was performed with a total of 2×10^6 human 143B osteosarcoma cells. One week after cell inoculation, when the tumor was palpable, the tumor core was resected using a biopsy punch (⌀=2 mm), leaving the tumor margins untouched. Then the biomaterials were implanted inside the defect from tumor resection. It was used in **Study 1** to explore the efficacy of delivering DOX by CaS/HA compared to no treatment and 3 times higher dose of DOX administrated systemically and in **Study 2** to explore the efficacy of DOX delivered by different size of HA particles compared to no treatment and systemic administration of DOX (Fig.5B).
4. A tibia defect model in rats [140]: The proximal tibia was drilled with a hand-held drill to create a defect (Ø = 3 mm). In **Study 3**, pellets (Ø = 3 mm) of radioactively labelled HA particles (nHA, mHA and n/mHA) were implanted into the defect and covered with a collagen membrane (Ø = 4 mm). The radioactivity in the tibia and vital organs were measured to evaluate the particle migration (Fig.5C).

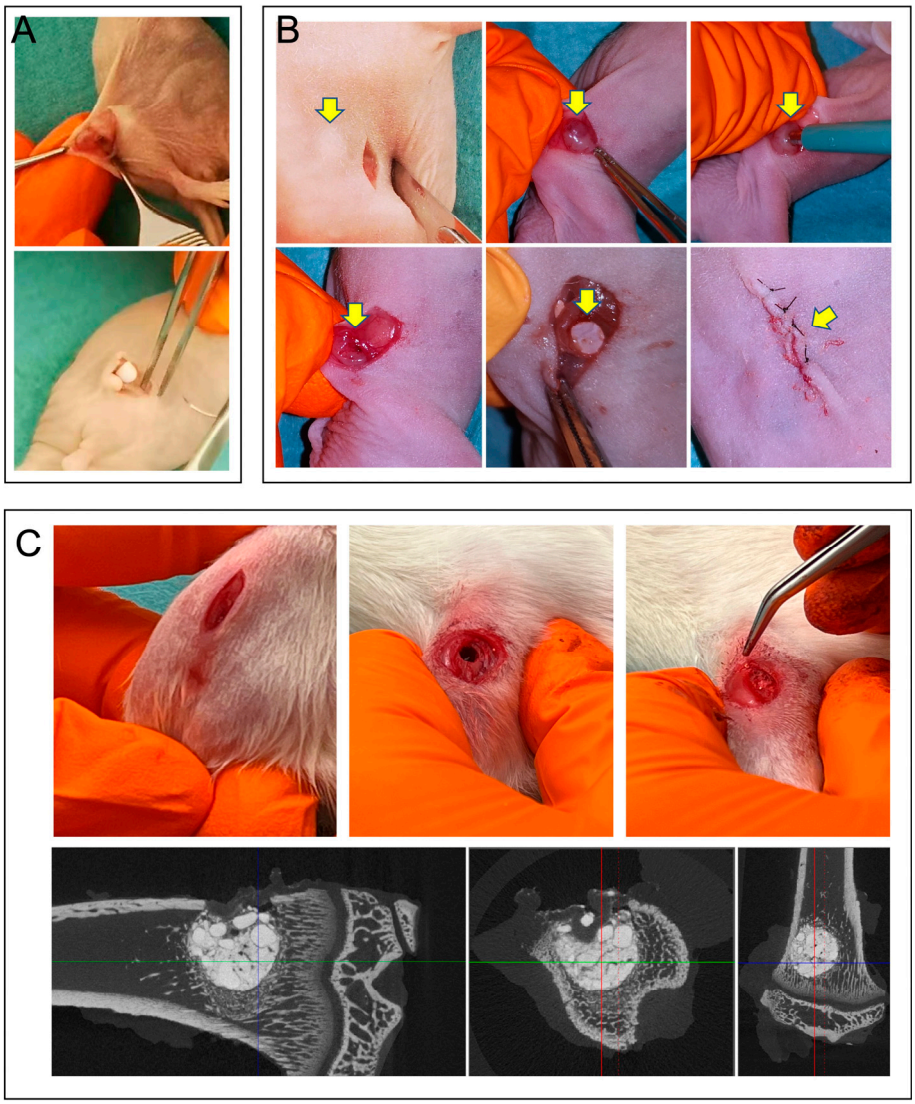

Figure 5. Animal models used in this thesis. (A) Subcutaneous xenograft without tumor resection mimicking the preoperative chemotherapy situation, used in **Study 1**. (B) Subcutaneous xenograft with tumor resection mimicking the postoperative chemotherapy situation, used in **Study 1 and 2**. (C) Tibia defect model for the implantation of different HA particles, used in **Study 3**. A and B show images from a mouse model while the images in C are from a rat model.

3.3 Experimental techniques

3.3.1 Cell culture

Cell culture techniques were used mainly in **Study 1** and **2**.

In **Study 1**, two human osteosarcoma cells (MG-63 and 143B) were used to investigate the efficacy of released DOX from CaS/HA biomaterial. The released fractions were collected from CaS/HA pellets containing DOX and stored at -20 ℃ before usage. MG-63 and 143B cells were cultured on 8-well chamber slides and treated with released DOX fractions. DOX has auto-fluorescence which could be detected at excitation 485 nm and emission 580 nm. It usually interacts with the cellular DNA in the nuclei by acting on the topoisomerase inducing breaks in the DNA causing apoptosis. After being stained with DAPI, confocal microscopy was used to detect the cellular distribution of the cells treated with released DOX as well as cell death on both MG-63 and 143B. Furthermore, both cell types were also seeded in 96-well plate. Cell viability after being treated with released DOX fractions was assessed using the MTT assay. To construct the subcutaneous xenograft, 143B cells, as one of the highly aggressive osteosarcoma cells, were injected under the skin of right flank on athymic nude mice to form a mature tumor.

In **Study 2**, 143B cells were used to investigate the intracellular delivery of DOX by HA nanoparticles and its effect on cell metabolism. The cell uptake of HA nanoparticles containing DOX was verified by confocal microscopy and flow cytometry with various endocytosis/pinocytosis inhibitors. To track the cellular distribution of HA nanoparticles containing DOX, 143B cells were grown on coverslip-bottom 8-well chamber slide, transfected with GFP-LAMP1 (lysosomal marker) to detect its co-localization with lysosome at 4 h and stained with Mitotracker and Tom20 (mitochondria marker) to detect its co-localization with mitochondria at 4 h and 24 h. The cells were stained with DAPI to visualize the nuclei. Leica SP8 inverted confocal system (Leica Microsystems) was used to capture the images. The co-localization findings from the above fluorescent images were also verified by TEM. To investigate the treatment effect on cell metabolism, luminescent ATP detection assay kit (ab113849) was used to measure ATP produced by human osteosarcoma cells treated with various interventions. Cell migration of 143B cells treated with HA nanoparticles containing DOX was detected using a standard wound closure experiment. Cell viability of treated cells was measured using MTT assay. To construct the subcutaneous xenograft, 143B cells, as one of the highly aggressive osteosarcoma cells, were injected under the skin of right flank on athymic nude mice as described above.

3.3.2 Material characterization

Biomaterial characterization was carried out in **Study 1-3**. The common techniques and the rationale of using those are provided below:

Scanning electron microscope (SEM) enables imaging of a sample by scanning the surface with a focused beam of electrons. In **Study 1**, SEM was used to visualize the size distribution of the hydroxyapatite microparticles used in the ceramic and the microstructure of CaS/HA before and after setting. The microstructure of CaS/HA containing DOX was also detected after setting. In **Study 2 and 3**, SEM was used to characterize the shape and size of the HA micro- and nanosized particles. All the materials were characterized at a resolution between 1 and 10 μm.

Fourier-transform infrared spectroscopy (FTIR) enables defining a compositional spectrum of absorption or emission of a solid, liquid, or gas. In **Study 1**, FTIR was used to validate that DOX has been successfully loaded on CaS/HA biomaterial. All the samples were prepared in powder form before testing.

X-ray diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material. It is widely used in ceramic chemistry, especially to detect the purity of a hydroxyapatite crystal after different chemical and physical treatments. XRD was used in **Study 1 and 2** to check the property and purity of hydroxyapatite. All the samples were prepared in powder form before testing.

Transmission Electron Microscopy (TEM) enables transmittance of an electron beam through a specimen to form an image. TEM was used in **Study 2** to check the crystal structure of HA nanoparticles and their intracellular distribution at a resolution between 20-1000 nm.

3.3.3 Cellular distribution of HA nanoparticles

Confocal microscopy, as one of the most important tools for fluorescence imaging, was used to detect the cellular uptake of HA nanoparticles containing DOX. DOX has its auto-fluorescence which could be detected at excitation 485 nm and emission 580 nm. To recognize the cells, DAPI was stained to show the nuclei. The images were taken on the treated 143B cells under confocal microscope to confirm the endocytosis. To further validate it, flow cytometry, as a technique used to detect and measure physical and chemical characteristics of a population of cells, was used for cells treated by HA particles containing DOX together with different endocytosis inhibitors. Live cell confocal microscopy was then used to track the HA nanoparticles intracellularly, especially its co-localization with lysosome and mitochondria. Transmission Electron Microscopy and energy dispersive x-ray spectroscopy (TEM EDS) is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image, and at the same time measure

chemical composition. To support the data from fluorescent images, the property of the intracellular HA nanoparticles was checked by TEM EDS.

3.3.4 Design of animal studies

Animal studies have been performed in all 3 studies entailed in this thesis. Groups for comparison were selected as summarized in Table 1 below.

Table 1. Overview of the animal studies in this thesis including biomaterial used, main aim, animal model, treatment groups and sample size.

Study	Biomaterial Used	Main Aim	Model	Groups for Comparisons	Sample Size/Group
1	CaS/HA	The efficacy of CaS/HA in local delivery of DOX compared to systemic administration	Subcutaneous xenograft with or without resection	No resection 1. No treatment 2. CaS/HA 3. Systemic DOX 4. CaS/HA DOX Resection 5. No treatment 6. Systemic DOX 7. CaS/HA DOX	G1-4 (n=8) G5-7 (n=7)
2	nHA mHA n/mHA	The efficacy of nHA or n/mHA in intracellular delivery of DOX	Subcutaneous xenograft with resection	1. No treatment 2. Systemic DOX 3. nHA+DOX 4. mHA+DOX 5. n/mHA+DOX	G1, G3 (n=8) G2, G4, G5 (n=9)
3	nHA mHA n/mHA	The migration of HA particles to vital organs after implantation with a follow up of 28 days	Tibia defect	1. ^{14}C-ZA labelled nHA 2. ^{14}C-ZA labelled mHA 3. ^{14}C-ZA labelled n/mHA	G1-3 (n=5/time point)

3.3.5 Imaging modalities used for in-vivo experiments

In-Vivo Imaging System Spectrum CT (IVIS) is an integrative platform that combines the full suite of IVIS optical features including Spectral Unmixing, 2D and 3D quantitative bioluminescence and fluorescence with fast and low dose CT imaging. In this thesis, it was used to detect the amount of DOX from the ex-vivo tissue samples based on its self-fluorescence (Excitation 487 nm and Emission 580 nm). In **Study 1**, it was used to detect in-vivo DOX release from CaS/HA pellets in a rat abdominal muscle pouch with a period of 28 days. In **Study 2**, it was used to validate in-vivo DOX accretion to HA particles which were pre-implanted in the abdominal muscle pouch. All the measurements were done within a sensitive detection range of the machine.

Positron emission tomography–computed tomography (PET-CT) is a nuclear medicine technique which combines PET and CT in a single gantry to acquire

sequential images from both devices in the same session. PET captured functional images presenting the spatial distribution of metabolic or biochemical activity in the body meanwhile CT captured the anatomic images which could be precisely aligned with PET images. In **Study 1 and 2**, it was used to monitor the tumor progression under different interventions. ^{18}F-FDG was chosen as the tracer based on the histopathology of the xenograft model.

3.3.6 Methodological considerations: Animal models and ethics

In this thesis, the tumor eradication efficacy of all the interventions were conducted on athymic nude mice because human osteosarcoma cells were used to create the tumor xenograft. Since DOX is a common first line anti-tumor drug, it can be predictable that this local delivery system might also work in other tumor models accordingly. To create the subcutaneous xenograft mimicking the clinical scenario, 143B, one of the most aggressive human osteosarcoma cells, were chosen for cell inoculation. 143B cells have been confirmed to have a strong ability of proliferation, migration, invasion, colony forming and tumorigenicity. Another reason to choose the aggressive 143B cells is that if the intervention could hinder the tumor progress formed by 143B it will be highly effective for other less aggressive cells. For the mice species, athymic nude mice is one of the most common species for tumor models. It is hairless, lacks a normal thymus gland, and has a defective immune system because of a genetic mutation. This makes it possible to use human cancer cells to form a mature tumor on animal without the risk of immune rejection. The hairlessness makes it easier to follow the growth of subcutaneous tumor.

In **Study 1**, the abdominal muscle pouch model on SD rats was used to detect the in-vivo DOX release from CaS/HA. This model has been widely used in bone formation as well as in-vivo drug release from biomaterials [138]. The muscle pouch could act as a closed system that keeps biomaterials locally releasing the drugs. It is also easy to collect the biomaterials from the pouch without disturbing it at different time points. The size of the muscle pouch would allow us to place a cylinder disc (Ø=5 mm) containing DOX, which stayed locally in a relatively uniform shape during the follow-up (up to 28 days). It gave solid evidence for the in-vivo DOX release. In **Study 2**, the abdominal muscle pouch model was used to explore the in-vivo DOX binding to HA. The pouch was used to keep the HA particles locally and followed by systemic injection of DOX 7 days after HA implantation. Theoretically, the circulation would have formed around the HA particles in a week. Then the injected DOX could reach HA particles through the newly formed circulation around HA particles. The whole system could strongly support the in-vivo binding between DOX and HA. In **Study 3**, a tibia defect model has been used to detect the in-vivo biodistribution of HA particles after implantation. It is commonly used to test bone regeneration for various interventions. The aim is to use HA nanoparticles to deliver apatite-binding cytostatics for bone cancers, but there are some concerns

about the safety of using HA nanoparticles in-vivo due to the risk of particle migration. Thus, HA particles (nHA, mHA and n/mHA) were implanted in the defect and detected its in-vivo biodistribution. In this defect, it will also have a bone microenvironment and the implanted HA particles are exposed to the circulation through the vasculature in the bone marrow cavity.

All three studies included in this thesis work have complied with the 3R's principle. Exclusion of unnecessary experimental groups in Study 2 has been done based the findings from Study 1. Replacement of animal models was not possible due to the multi-dimensional nature of the osteosarcoma progress. Power calculations and group sizes were based on the previous studies conducted and published by the group [141, 142]. All animal studies have been approved by the Swedish board of agriculture (Jordbruksverket, permit numbers 5.8.18-01018/2020-**Study 1, 2 and 3**, 5.8.18–15288/2019-**Study 1, 2 and 3**). All necessary steps to reduce animal suffering were followed and all animals had free access to regular food pellets and water throughout the experimental period. Animals were housed two per cage with hygienic conditions and 12 h long day/night light cycles.

3.3.7 Statistical analysis

Data are presented as Mean ± SD and checked for normality before statistical analysis. A student t-test was used to detect the difference between two unpaired groups. Paired t-test was used to compare difference between two paired groups. One-way ANOVA and Tukey's multiple comparisons test, Kruskal-Wallis test with Dunn's multiple comparisons test and One-way ANOVA with Dunnett's multiple comparisons were used to detect the difference among three or more independent groups. Repeated Measures ANOVA and Dunn's multiple comparisons test was used to compare three or more dependent groups. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. Data were tested using Prism 8 (MacOS, GraphPad Software Inc. CA, USA).

4. Results

4.1 Study 1 - Efficacy of local and controlled DOX delivery by CaS/HA compared to systemic administration

- A sustained and controlled delivery of DOX could be achieved up to 28 days, both in-vitro and in-vivo, with 28% and 63% pre-loaded DOX released respectively.
- Human osteosarcoma cells MG-63 and 143B treated with released DOX from the CaS/HA biomaterial showed DOX accumulation in the nucleus. This confirmed that DOX cytotoxicity remained unchanged after being incorporated in the CaS/HA material.
- Local delivery of DOX using CaS/HA biomaterial was superior to systemic DOX injection at clinical doses (3 mg/kg for human), as seen in-vivo in a subcutaneous xenograft model.
- After tumor resection, mimicking post-operative chemotherapy, a 3 times lower concentration of DOX delivered locally by CaS/HA biomaterial was still more efficient for tumor eradication than systemic injection.
- No obvious pathological changes of the surrounding healthy tissues were induced by locally delivered DOX leaking from the CaS/HA material.

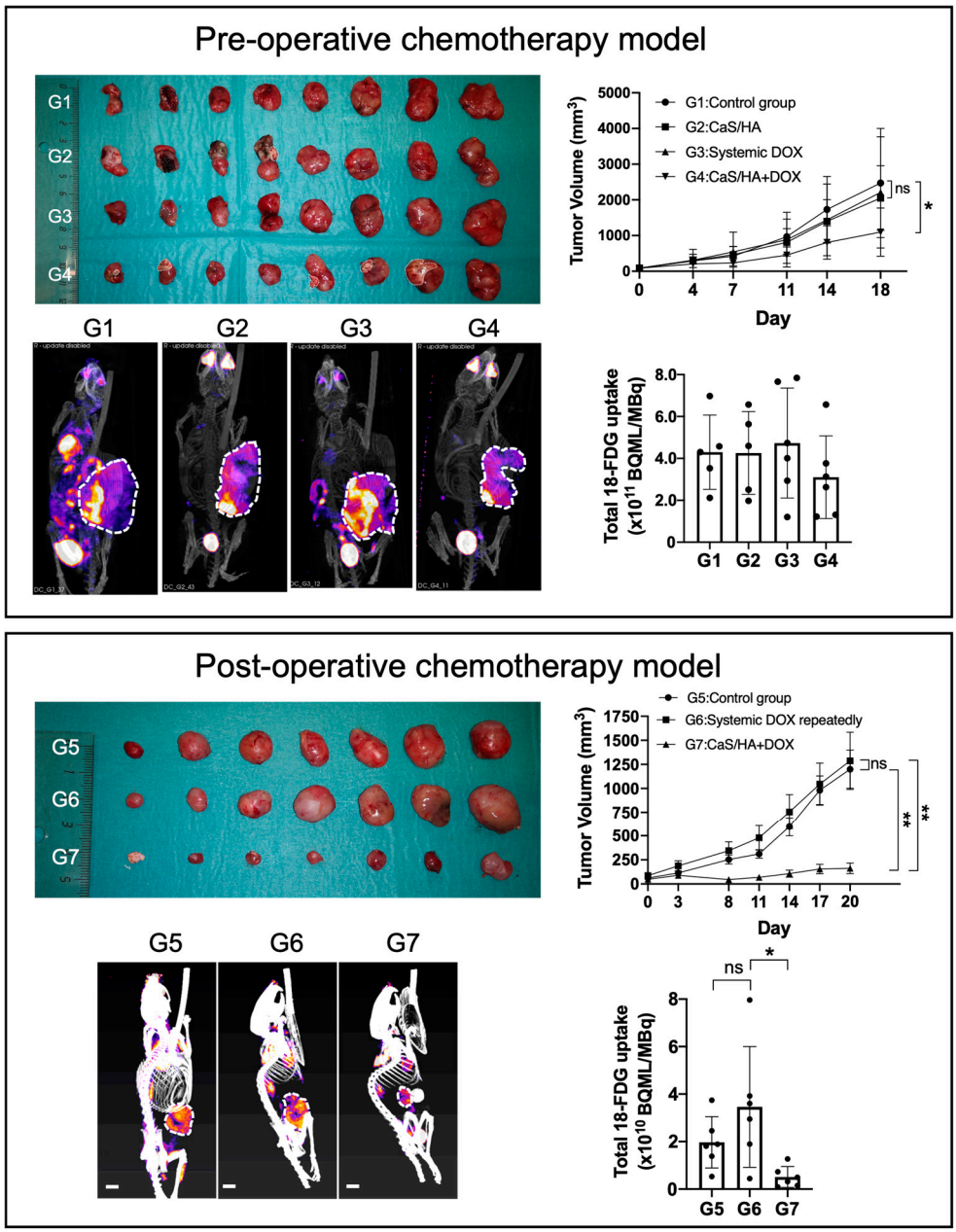

Figure 6. The efficacy of locally delivered DOX by CaS/HA biomaterial in pre-operative (top) and post-operative (bottom) chemotherapy models. The collected tumor tissue, tumor volume, representative PET-CT images and quantification of ^{18}F-FDG uptake are presented. * indicates $p<0.05$, ** indicates $p<0.01$. Figure has been reproduced from [139].

4.2 Study 2 - Intracellular delivery of DOX by HA nanoparticles is mitochondria-targeted

- DOX, containing free hydroxyl groups, has high affinity to different sizes of HA particles (both in-vitro and in-vivo). The DOX-HA interaction is reversible, which is beneficial for drug loading and controlled release.
- Pre-implanted HA particles in the abdominal muscle pouch recruit systemically administered DOX (DOX-HA interaction in-vivo). This indicates a novel potential targeted drug delivery method for cancer treatment, which might improve the drug concentration in the tumor, and alleviate the off-target side effects.
- HA nanoparticles, functionalized with DOX, are endocytosed and accumulated in endolysosomes. The acidic lysosomal pH induces a pH-dependent release of DOX.
- After being released from the lysosome, free DOX is routed to the mitochondria causing insufficient ATP synthesis, reduced cell migration and cell apoptosis. This has implications in preventing further metastases in-vivo.
- By using HA microparticles as a carrier for nanoparticles delivering DOX, the strong tumor killing effect of nano-HA DOX was not compromised and might provide a novel method for multiple-stage release profile of cytostatics. Micro-HA particles can exert extracellular drug release while nano-HA can act as a Trojan horse, entering the cancer cell, delivering the cargo and eventually efficiently killing the cancer cell.

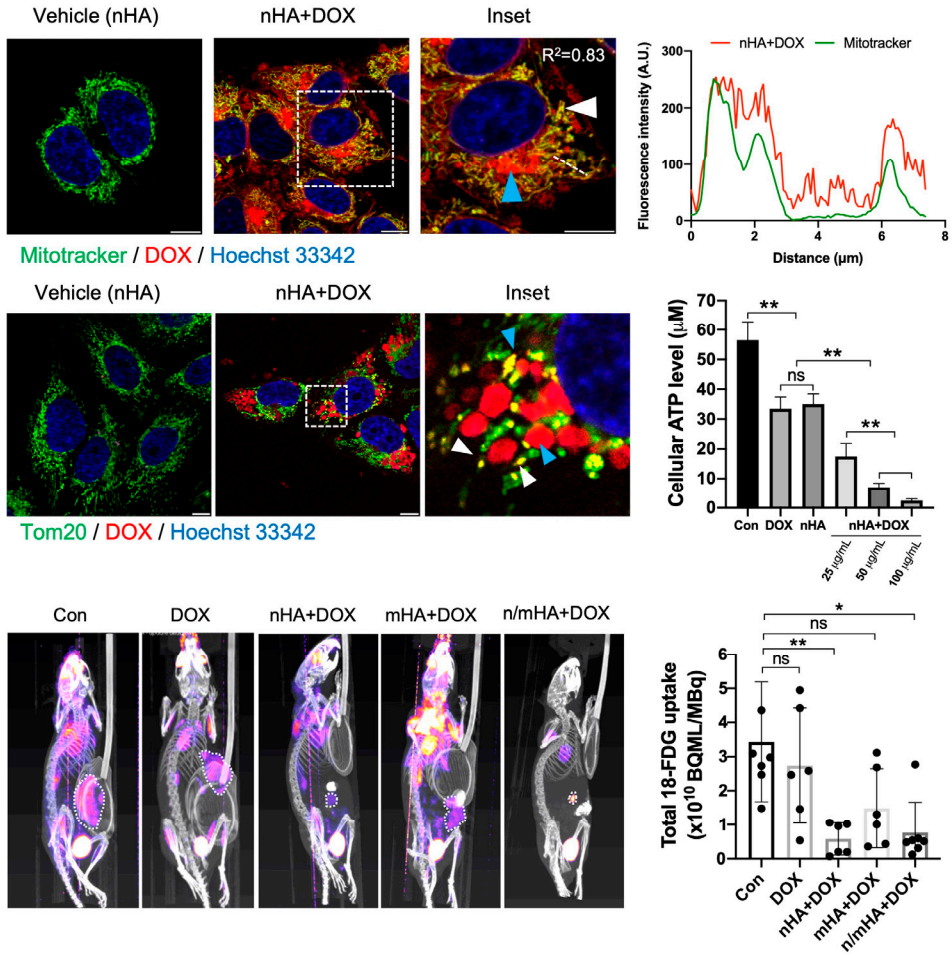

Figure 7. The intracellular delivery of DOX targeted to mitochondria by nHA and its in-vivo efficacy. The top and middle panel shows that nHA+DOX was co-localized with mitochondria, inducing cell starvation. The bottom panel shows its efficacy in-vivo by detecting the metabolically active tumor. ns indicates no significant difference. * indicates $p<0.05$, ** indicates $p<0.01$. Figure has been reproduced from [143].

4.3 Study 3 - In-vivo biodistribution of implanted HA particles

- The radioactive labelling of ZA by ^{14}C did not affect ZA affinity to HA particles. Irrespective of the HA particle size, >90% of ^{14}C-ZA was bound to the HA particles.
- Majority of HA nanoparticles stayed locally at the proximal tibia up to 28 days without migrating to other vital organs.
- Increasing HA particles or free ^{14}C-ZA were seen in the spleen and kidney at 28 days. Although the total radioactivity detected in spleen and kidney was < 0.1 % of the total implanted “radioactivity”, this should be explored further using a longer follow-up.
- No obvious pathological changes could be observed in vital organs.

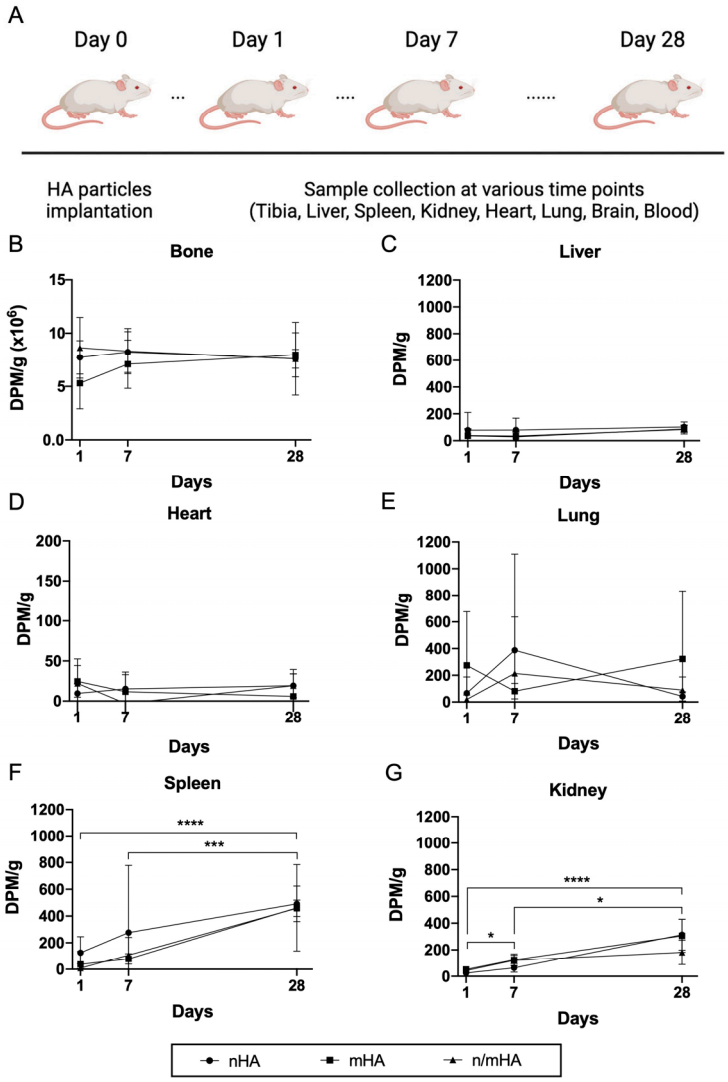

Figure 8. The radioactivity counts in the collected proximal tibia and vital organs including liver, heart, lung, spleen and kidney in the follow-up (up to 28 days).

5. Connections between the studies

All studies described in this thesis form a continuous pattern. Lessons learned from preceding studies were implemented in the following studies.

In **Study 1**, it was reported that CaS/HA could be used for local delivery of DOX in a sustained and controlled release pattern, achieving better tumor eradication compared to systemic administration. For the in-vivo DOX release, 37% of loaded DOX remained in the CaS/HA material, which matches the amount of HA (40% w/w) in this biomaterial. Is there any interaction between DOX and HA? Another point is HA microparticles were used in CaS/HA biomaterial. Can HA nanoparticles be more efficient in delivering DOX locally, especially for intracellular delivery?

In **Study 2**, it was reported that there is an electrostatic interaction between DOX and HA, which led to this slow release. Compared to microparticles, HA nanoparticles could deliver DOX intracellularly, first to the lysosome and then mitochondria, causing cell energy-starvation, reduced migration and apoptosis. The improved tumor eradication of nHA+DOX was also verified in-vivo. Then the important question that remains is whether it is safe to use HA nanoparticles in-vivo? Do the particles migrate and if so, does HA particle migration cause any damage to vital organs?

In **Study 3**, it was reported that majority of implanted HA particles, irrespective of the size, stayed locally at the proximal tibia site without migrating to vital organs. No obvious pathological damage could be detected. This indeed helped in establishing the safety of HA particle usage in-vivo.

6. Discussion

6.1 CaS/HA biomaterial for controlled delivery of DOX

The clinical management of osteosarcoma includes tumor resection combined with pre- and/or post-operative chemotherapy. However, this regimen is sometimes insufficient for local control to prevent tumor recurrence, thus affecting morbidity and long-term survival [144]. The ideal carrier would deliver a high local concentration of a cytostatic locally in a controlled release pattern, at the same time avoiding systemic side effects, which would radically improve the treatment of osteosarcoma. In **Study 1**, the efficacy of delivering DOX locally by a clinically approved CaS/HA biomaterial was verified by comparing to systemic administration. It could be a promising supplementary treatment to the standard chemotherapy with highly translational potential. Recent studies showed that controlled release of DOX (only up to a week) is more efficient than local injection of free DOX to inhibit tumor growth [97]. Thus, the first step in **Study 1** was to detect the release profile of DOX from CaS/HA. Ma et al. delivered DOX with thermosensitive PLGA−PEG−PLGA hydrogels, in which almost 75% of loaded DOX was released within 12 days [98]. Kamba et al., using calcium carbonate nanocrystal carrier, showed that more than 70% of the drug was released in 1 day [145]. In yet another study, 50% of DOX was released in 5 days from a calcium phosphate-phosphorylated adenosine material [146]. Compared to previous studies, a controlled and sustained release of DOX from a biphasic CaS/HA biomaterial in-vitro was demonstrated, where 28% and 36% of the loaded drug was released when dissolved in PBS at pH 7.4 and pH 5 over a 4-week period, respectively. The in-vivo release, using an IVIS imaging platform, has only been explored in few other studies [147, 148]. In **Study 1**, nearly 63% of the loaded DOX from CaS/HA was released in 28 days with another 37% remaining in the material, which was investigated in a rat abdominal muscle pouch model. Thus, this biphasic nature of the material leads to a sustained and controlled delivery of DOX, with a burst release pattern, early on due to the fast-resorbing CaS phase, and a more controlled release follows due to slowly resorbing HA particles. The bioactivity of released DOX was then tested on two human osteosarcoma cells, indicating no impairment to its bioactivity.

To explore its tumor eradication effect in-vivo, a subcutaneous xenograft was constructed, followed by different interventions. By using CaS/HA biomaterial, a

high local DOX concentration was achieved, leading to a significant tumor size reduction by 50% in a period of 18 days in pre-operative chemotherapy model. The dose used in our study is a clinically relevant dose of 3 mg/kg [149]. Rahman et al. [150] showed that the terminal plasma half-life of doxorubicin was 17.3 h, which means that it is eliminated from the body within 2-3 days. This emphasizes the necessity of repeated infusions of DOX in clinical practice. However, by delivering DOX locally with a CaS/HA carrier, the drug can be retained within the tumor, with a prolonged and more effective duration. This was verified in the post-operative chemotherapy model, showing that even 3 times lower dose of DOX delivered locally by CaS/HA has a stronger tumor killing effect than systemic administration. Thus, a single local dose could be delivered by using CaS/HA carrier to achieve better efficacy compared to systemic injections [151, 152].

DOX, being used locally, has not been approved for clinical usage due to its high cytotoxicity. Thus, the safety concern of local DOX delivery was addressed. To explore the local inflammation caused by DOX, CaS/HA biomaterial containing DOX was implanted under the skin and compared to free DOX injection. No obvious local inflammatory reactions were induced by CaS/HA+DOX to the overlaying healthy tissue compared to free DOX.

6.2 DOX is a hydroxyapatite-binding cytostatic

It has been shown that various growth factors can be loaded onto the HA surface due to its physicochemical structure [129, 130]. In **Study 2**, HA particles, irrespective of the size, could easily adsorb to and form an electrostatic interaction with DOX in a physiologic buffer solution. nHA (<50 nm) had a stronger affinity for DOX compared to mHA (10 μm). HA has two binding sites, the C site which is rich in Ca^{2+} and the P site, which is rich in PO_4^{3-}. Both possess affinity towards biological macromolecules such as proteins [153]. DOX has abundant hydroxyl groups in its chemical structure, which makes this interaction with HA easier to form [154]. The molecular simulation studies indicate that there is a reversible electrostatic interaction between DOX and HA. This was experimentally validated in-vitro both on nHA+DOX and mHA+DOX composites, showing that more DOX was released at an acidic pH. The in-vitro DOX-HA interaction was also verified in in-vivo using a rat muscle pouch model, in which we confirmed that HA can be used as a moiety for attracting systemically circulating hydroxyapatite-binding cytostatics like DOX. Interestingly, significantly more DOX was found in the HA materials than the collagen scaffold or surrounding muscle. This recruitment of circulating DOX is a consequence of progressive vascularization at the tissue-implant interface between day 2 and 10 after implantation. Due to the high expression of vascular endothelial factor (VEGF), and an intricate vascular network forming around the material, circulating drugs with possible affinity to HA thereby

could reach the material [155]. After being injected systemically as per the clinical protocol, DOX would accumulate in various organs through the circulation, particularly in the liver, kidney, lung and heart [150]. By implanting HA particles before drug administration, more DOX can be recruited to the implanted site thereby reducing the exposure to other vital organs and potentially alleviating the off-target side effects. Other drugs have also been shown to be effective in animal models. For instance, zoledronic acid, when administrated systemically, could seek pre-implanted hydroxyapatite particles and induce more bone formation around an implant [136, 137]. Whether recruited DOX by HA will have a tumor inhibiting effect needs to be explored further in an in-vivo tumor model.

This interaction between DOX and HA might be associated with the structure and crystallinity of the material. The physico-chemical properties of HA such as the porosity or the degree of crystallinity can also provide further accessible surfaces for electrostatic interaction affecting the drug loading [156]. Since this binding is reversible and controllable, a "switch" for smart delivery of hydroxyapatite-binding drugs is created. There are several factors which could affect this reversible interaction and make DOX loaded HA a choice for controlled DOX release. For example, DOX-HA interaction is very sensitive to acidic microenvironment under which this interaction breaks and the drug will be released [157]. Previous studies have confirmed that the tumor microenvironment is acidic (pH 5.6 to 6.8) [158], which can lead to an increased drug release in the tumor compared to normal tissues. The release can also be affected by the ions around the HA [159], which gives the possibility to further control the drug delivery based on the ionic distribution around the HA-DOX complex [160]. Ultrasonic irradiation has also been shown to break this type of chemical interaction, which provides the possibility to control the drug release by providing external physical stimuli [161].

Similar interactions of other biomolecules, particularly the 3rd generation bisphosphonates like zoledronic acid, known to bind to HA, have been widely used systemically for bone tumor inhibition [162]. HA-binding antimicrobial peptides have been designed for bone infections [163]. Thus, the chemical structure of various drugs could be chemically modulated, based on the HA binding sites for targeted delivery in bone disorders.

6.3 Lysosomal pH-dependent release as a smart "switch" for intracellular controlled drug delivery

Cells of different tissue origins can endocytose nanoparticles fabricated from a variety of substrate materials in the nanometer range (<1000 nm) [164]. It has been confirmed that HA nanoparticles can penetrate cancer cell membranes via endocytosis, eventually inducing apoptosis [165]. Despite an inhibitory effect on

tumor cells, using pristine HA nanoparticles to eradicate tumors in-vivo has been unsuccessful due to its limited cytotoxicity on an established tumor [122]. To overcome this limitation, a few studies tried to couple HA particles with paclitaxel by chitosan or bovine serum albumin (BSA) coatings, but a rapid release of paclitaxel was observed within the first 3-5 days together with impaired drug bioactivity [125]. In this study, DOX was successfully delivered intracellularly by the nHA without impairing its biological activity. nHA+DOX which had been engulfed by the cells, were seen in the lysosome first and then moved to the mitochondria instead of the nucleus as is the case with free DOX [166]. Lysosome, which is the intrinsic digestive system of cells, receiving and sequestering cargoes from phagocytosis, endocytosis and auto phagocytosis engulfs the nHA+DOX composite during endocytosis [167]. For cancer cells, endocytosis is a fundamental biological process to internalize (bio)molecules and nanoparticles [168]. The size of the particles may affect the uptake efficiency and kinetics, the internalization mechanism and also the subcellular distribution [169]. A size-dependent uptake in different cell lines has been observed for various nanoparticles [170, 171] with the maximum cellular uptake in the range of 30-50 nm. In **Study 2**, nHA with less than 50 nm was only used for intracellular delivery of DOX. The size and shape of HA nanoparticles for optimal DOX loading and delivery needs to be explored in future studies. For lysosomes, an acidic environment (pH 4.5–5.5) promotes protein degradation during cellular metabolism, which in our case disturbs the DOX-HA interaction, resulting in pH-dependent drug release as verified in-vitro. Various studies have explored the pH-dependent controlled drug delivery from acidic tumor microenvironment and the lysosomes [172]. For example, when delivered by a calcium sulphate/micro hydroxyapatite biomaterial, DOX has been confirmed to have a higher in-vitro release in acidic pH, which mimics the tumor microenvironment [139]. However, few studies have paid attention to intracellular pH-dependent release of drugs, especially with nanomaterials [173].

6.4 Mitochondria-targeted delivery for osteosarcoma

Mitochondria are essential intracellular organelles that regulate energy metabolism, cell death, and signaling pathways that are important for cell proliferation and differentiation. Emerging evidence suggests that cancer is primarily a mitochondrial metabolic disease [174]. Mitochondrial function in cancer is fundamental for the dissemination of tumor cells to distant organs, or metastasis [175], which is responsible for over 90% of cancer deaths [176].

Various biomaterials have been explored to target mitochondria inducing damage and apoptosis [177, 178]. In **Study 2**, it was found that HA nanoparticles, when loaded with DOX, could enter the cancer cell and eventually accumulate in the mitochondria inducing mitochondrial dysfunction and collapse, eventually resulting

in less cell migration. Mitochondria are semi-autonomous organelles that perform essential functions in cellular metabolism and in the regulation of cell death [179]. DOX, delivered by nHA, could inhibit the production of ATP after accumulating in the mitochondria, which was significantly reduced compared to the free drug. As a result, the cancer cell migration was dramatically inhibited due to insufficient ATP synthesis. It even induced the collapse of mitochondria. A stronger cytotoxicity was achieved by mitochondria-targeted delivery of DOX instead of nuclear delivery. Except for ATP production, mitochondria also play a vital role in cancer through other energy and macromolecular synthesis, impacting metastasis [180]. As a prominent signal for cancer severity, mitochondrial dysfunction showed a significant correlation with poorer tumor progression and decreased metastasis [181]. In the nHA+DOX group, the tumors had the lowest ^{18}F-FDG uptake indicating a rather low "metabolic" tumor with lower risk for further metastasis.

6.5 Safety of HA nanoparticles alone or combined with microparticles in-vivo

The limitation of using HA nanoparticles encompasses the risk for migration and accumulation in vital organs like liver, kidney or even brain where a slow degradation could have detrimental effects [182, 183]. For instance, gold nanoparticles have been shown to interact with Mac-1 and induce inflammation by activating NF-κB signaling pathway [184]. The inflammation induced by gold nanoparticles was found in the lung with the presence of infiltrating lymphocytes and enhanced IL-1α expression in a rat study [185]. Likewise, titanium dioxide nanoparticles, were found to be deposited in the heart causing myocardial injury [186], in liver causing angiectasis and hyperaemia [187, 188] and in spleen causing lymphocyte infiltration and fatty degeneration [188].

HA based biomaterials have been widely used as a bone-void fillers in orthopedic surgery, for instance, after resection of diseased bone due to an infection or tumor. To mimic the clinical application of HA as a void filler, a bone defect in the proximal tibia in rats was created using a well-established animal model [137]. Proximal tibia in rats is highly vascularized and provides an environment that allows for systemic migration of implanted particles. Radioactively labelled HA particles were molded into pellets with a diameter of 3 mm using hyaluronic acid as a binder followed by implantation into the defect. The radioactivity of the collected tibia and vital organs was quantified as a surrogate marker for particle migration. It was found that the majority of implanted HA particles (>99.9%), irrespective of size (nHA or mHA) stayed locally within the defect up to 28 days, indicating the safety of HA particle usage at least in the bone. Hyaluronic acid, being used as a binder in this study, has been approved for clinical usage for years. It is an important component of the

extracellular matrix (ECM) found in the fluids in the eyes and joints, and has been used in scaffolds for bone disorders [189]. Dennis et al. have used hyaluronic acid as a binder for HA nanoparticles based on the colloidal networks for bone defect filling [190]. With its suitable in-vivo degradation, embedded HA particles will be free and exert their therapeutic effect [191]. Another advantage of using hyaluronic acid is its viscosity and the rheological properties of HA-hyaluronic acid paste allows for minimally invasive application of HA particles via an intra-tissue injection [192]. Other hydrogels based on chitosan or alginate as the liquid phase could also be interesting to be explored for HA implantation [193].

One of the strategies to retain nanoparticles locally is to embed them together with microparticles, which would stay at site for longer periods of time [194]. It has also been reported that the combination of nano- and micro-size HA shows less cytotoxic effects on healthy osteoblasts indicating better biocompatibility compared to only nanoparticles of HA [143]. In **Study 3**, nano-/micro-HA composite were implanted in the tibial defect and from the biosafety profile of HA established in this study, biphasic HA could be a potentially efficient material for drug delivery in bone disorders. Owing to their size, nHA loaded with drugs such as antibiotics or cytostatics could be delivered intracellularly to specific cellular compartments of cancer cells or sessile bacteria hiding within the cells [143, 195]. When used as a combination as nanoparticle-in-microparticle (NIM) system, multiple release profiles (burst release from outer particles and sustained release from internal components or intracellular release from nHA and extracellular release from mHA) can be envisaged. This phenomenon has been reported by Jelvehgari et al. who used theophylline loaded NIM to reduce the burst drug release of the outer microparticles [196]. Apatite-binding drugs like zoledronic acid, tetracycline or doxorubicin could be loaded on NIM system for both intra- and extracellular drug delivery with better efficacy for combatting bone diseases such as osteoporosis, tumors or infections [197, 198].

6.6 Future work

6.6.1 Deliver multiple drugs by CaS/HA

Even though some progress has been achieved in terms of anticancer therapeutics, increasing drug distribution to solid tumors with improved efficacy remains a challenge [199, 200]. For systemic administration, the amount of drugs reaching the tumor, relies on drug properties and physiological characteristics of the tumor microenvironment [201, 202]. Systemic chemotherapeutics often fail due to difficulties achieving sufficient therapeutic levels in the tumor. For example, less than 0.5% of the total dose of intravenously infused paclitaxel is locally available in

the tumor [96]. To improve drug efficacy, loco-regional drug delivery platforms have become an alternative strategy to supplement conventional chemotherapy. Further, it is logical that local drug delivery could be more cost-effective by reducing total administered doses or repeated hospital visits. In this thesis, CaS/HA was used for local delivery of DOX with improved tumor eradication compared to systemic administration. However, multiple cytostatics are usually needed to be combined to eradicate the tumor without recurrence in the clinical scenario due to the heterogeneity of cancer cells. In the future, other cytostatics like cisplatin combined with DOX delivered locally by CaS/HA should be explored for tumor eradication. It did also show high local concentrations of DOX did not induce any inflammation or even necrosis compared to free drug, which indeed makes the case favorable for delivering more cytostatics locally by CaS/HA. Based on its injectability and in-situ setting, it can also be used for unresectable tumors that are difficult to reach surgically.

Localized immunotherapy was pioneered by William Coley in the late 1800s. He tried to simulate infection within the inoperable sarcoma on patients by injecting intra-tumorally a mixture of attenuated Streptococcus pyogenes and Serratia marcescens (known as “Coley's toxins”), leading to tumor regression in 50% patients [203]. It was validated later that immune infiltration is of significant prognostic and therapeutic importance which promoted the intra-tumoral administration of immunotherapy [204]. Intra-tumoral immunotherapy is aimed to trigger a local immune response that disseminates systemically to attack distant untreated tumors or micro metastases. The retention of immunomodulatory antibodies within tumor microenvironment is strategically important by promoting existing immune infiltrates meanwhile avoiding nonspecific immune activation, poor drug accumulation and systemic side effects. Moreover, in tumors with elevated lymphatic drainage, intra-tumoral administration could potentially target downstream lymphoid tissues, further improving the efficacy of immunomodulatory agents [205, 206]. However, bolus intra-tumoral injections are susceptible to rapid clearance due to the tumor microenvironment. In this scenario, repeated injections are needed to sustain a robust immune response, which could be cumbersome and traumatic for the tumors with difficulty to access. In our case, CaS/HA, composed of 60% alfa hemihydrate calcium sulphate and 40% hydroxyapatite, have a suitable degradation rate leading to sustained drug release pattern (up to 28 days) for various drugs [139, 207]. The material is injectable and sets in situ, which further confirms its potential in delivering immunotherapy locally. The efficacy of using CaS/HA to deliver immunotherapy alone or combined with cytostatics would be very interesting to be further explored.

6.6.2 Screen additional hydroxyapatite-binding cytostatics, similar to DOX, based on the chemical structure

In this thesis, it was found that DOX could bind to HA both in-vitro and in-vivo through an electrostatic interaction, irrespective of the size of the HA particles. Based on the DOX-HA interaction, this reversible electrostatic bonding could be applied in two different ways. First, it can be used for drug loading like functionalizing HA particles with DOX in **Study 2**. Since it is reversible especially in acidic microenvironment, this interaction could be disrupted specifically when it reached acidic pH like in the lysosome or within the tumor. And this could be a way to control the drug release at specific location. Second, the pre-implanted HA particles could be used to recruit the systemically administrated DOX, which was shown an in-vivo binding in **Study 2**. Our previous studies have shown that pre-implanted HA particles could be used to recruit bisphosphonate like ZA and exert the local effect [137]. Whether recruited cytostatics like DOX will have a local effect needs to be explored further. This could be a novel concept to attract more cytostatics to the tumor site with better efficacy and less side effects.

So far, we have only tested the affinity of DOX to HA since it is one of the first line anti-osteosarcoma drugs. It would be very interesting to screen other cytostatics with similar chemical structure as DOX to find additional drugs to be used in HA-based carrier systems.

6.6.3 Modifying the drug structure to increase affinity to HA

Our group have recognized various drugs with affinity to synthetic HA, including bisphosphonate (zoledronic acid), antibiotics (tetracycline and rifampicin) and cytostatics (doxorubicin) [136, 143]. More drugs are under evaluation. However, the mechanisms behind drug-HA interaction have not been thoroughly explored among available drugs. Part of the work has been done by our group using computer simulation, which needs further experimental validation. With the understanding of drug-HA interactions, we will be able to modify the chemical structure of current drugs with specific functional groups achieving higher affinity to HA. Then by using HA-based biomaterials, more drug categories can be sent to the target site as expected. A previous study has reported that 12b80 compound was fabricated by covalently conjugating antineoplastic compound doxorubicin to a bone targeting bisphosphonate (zoledronic acid), which has much lower toxicity on healthy cells compared to doxorubicin and stronger tumor inhibition in-vivo [208]. In this compound, zoledronic acid was used to target the bone (exposed hydroxyapatite), thus increasing the local accumulation of DOX in the tumor site. In the future, more drugs could be modified with higher affinity to HA for bone disorders.

6.6.4 Eradicating tumor, preventing infection and regenerating bone by the delivery of multiple drugs

In this thesis, it was proved that delivering DOX more efficiently improved tumor eradication. However, reconstruction of a bone defect and managing the risk of post-operative infection after resection of a malignant bone tumor is still a big challenge. Various methods like allografts, vascularized fibular grafts, autogenous extracorporeally devitalized tumor bearing bone graft have been used to regenerate the defect after resection, but with limitations [209]. The main problem is the recurrence due to the remaining cancer cells which are not able to be cleared during the surgery. By using hydroxyapatite-based biomaterials delivering cytostatics locally, the remaining cancer cells could be eradicated with high local concentrations of cytostatics. However, to repair the defect and to prevent infection, other bone active molecules need to be combined with cytostatics.

We have in a previous pre-clinical study shown that healing of cortical and cancellous bone requires different healing stimuli [142]. Healing of cortical compartment requires delivery of potent growth factors such as bone morphogenic proteins (BMPs), which are contraindicated in cancer patients. Cancellous bone on the other hand can be regenerated with the use of local and controlled delivery of bisphosphonates, such as zoledronic acid (ZA) [210]. ZA is also used as a cancer drug in patients with skeletal metastasis from breast and prostate cancer and in patients with giant cell tumors. Horstmann et al., used a CaS/HA material containing gentamycin for the local delivery of ZA in a metaphyseal void in rats mimicking a post-resection bone defect in patients. They demonstrated that the use of ZA alone without the addition of BMPs led to significant cancellous bone regeneration in the proximal tibia [210] and the simultaneous delivery of gentamycin did not have any negative consequences on bone formation. The pre-clinical study was then followed up by the same team in patients with benign and borderline bone lesions wherein the void formed after curettage was filled with CaS/HA or gentamycin loaded CaS/HA [211]. The authors concluded that the CaS/HA material resorbed within a year and remodeled into bone and restored the cortical bone thickness in the patients. Therefore, simultaneous co-delivery of chemotherapeutic agents, antibiotics and bone regenerating molecules could be a promising future strategy to address the current challenges associated with the management of bone defects after the resection of cancerous bone.

6.6.5 Develop a device which could be used for the mixing and injection of nHA+DOX compound in a closed system

With the ability to deliver DOX to the mitochondria, HA nanoparticles could improve the efficacy of DOX delivery. In **Study 2**, HA nanoparticles were mixed DOX solution in a 2 mL tube and then washed the unbound DOX from the surface

to get the functionalized HA nanoparticles. In the clinical scenario, a closed system would be preferred to mix HA nanoparticles with DOX to avoid staff exposure, which can incur high toxicity. The particles then need to be mixed with a binder to achieve correct rheological properties for the material to be injected into the target site.

The first step is to find suitable gels as carrier for the injection of HA nanoparticles. Various hydrogels have been studied for the injection of inorganic nanoparticles [212]. By using a gel matrix, inorganic nanoparticles often set in situ, reducing local reactions induced by nanoparticle [213]. With appropriate compositions, it can preserve the structural integrity and the functionalities of nanoparticles, as well as the possibility of additional engineering flexibility to improve the therapeutic efficacy. Thus, different materials could be interesting to explore for the acceptable injectability of HA nanoparticles. To ease its translational potential, the clinically approved gels like hyaluronic acid can be also used as an alternative. In the future, factors including physical factors, such as temperature, light and pressure, as well as chemical stimuli, such as changes in ionic strength or composition, molecule recognition, and covalent binding could also been taken into consideration to achieve additional therapeutic effect.

The second step is to develop the suitable mixing device. Hydroxyapatite based bone cement have been used in the clinic for many years. The mixing steps have changed from mixing the material powder and liquid in a bowl manually to using a closed system to mix powder and liquid without any leakage. This could be referred to as a closed mixing system for nHA+DOX. However, a novel device which could increase the wetting and binding of DOX to HA during the mixing should be explored to increase drug loading.

7. Conclusions

- The biphasic CaS/HA biomaterial could be a depot of cytostatics for local, controlled and high-dose intra-tumoral cytostatic delivery.
- The controlled and sustained release of DOX in the tumor site enhanced primary tumor response without incurring any obvious systemic toxicity.
- DOX, a cornerstone osteosarcoma drug, showed accretion to particulate HA particles both in-vitro and in-vivo, which could be reversible in an acidic microenvironment.
- Pre-implanted HA particles recruiting circulating DOX (DOX-HA interaction in-vivo) indicates a novel potential targeted drug delivery method for cancer treatment, which might improve the drug efficacy and alleviate the off-target side effects.
- The mitochondria-targeted delivery of DOX by nHA leads to reduced cell migration and increased apoptosis, which could potentially aid in preventing further metastasis in-vivo, indirectly verified by PET/CT data.
- By applying the nHA/mHA carrier system for DOX, a significant reduction in the tumor growth was seen compared to a systemic DOX treatment regimen. The approach of locally injecting nano-/micro-HA particles pre-functionalized with a HA binding cytostatic drug-DOX opens new avenues for complimentary treatment of localized solid tumors.
- By labelling HA with ^{14}C-ZA, nHA alone or in combination with mHA showed a very promising safety profile for local implantation in a bone defect model. Using nHA/mHA carrier system delivering other drugs like antibiotics needs to be explored further in the future.

8. Summary in English

The main treatment in osteosarcoma, a malignant cancer of the bone, has been chemotherapy using a standard MAP protocol (high dose methotrexate, doxorubicin and cisplatin) for more than 30 years. The current challenges are that 1) a limited dose of the anti-cancer drugs will reach the tumor site; 2) the majority of the systemically administrated chemotherapeutics accumulate in vital organs causing severe side effects; 3) an increasing number of patients respond poorly to the current chemotherapies and 4) for decades no new drugs have been developed for osteosarcoma treatment.

In this thesis work, it is shown that hydroxyapatite-based biomaterials are promising alternatives to deliver chemotherapeutics locally within the tumor. Local chemotherapeutic delivery can achieve high local concentrations and is envisaged to have less off-target side effects.

In the first study, it was found that sustained and controlled delivery of DOX by a clinically approved CaS/HA biomaterial could achieve improved tumor eradication compared to standard-of-care systemic chemotherapy. Surprisingly, this sustained release could even minimize the cytotoxicity of DOX to the healthy surrounding tissues. The controlled release mechanism of DOX via an HA based carrier was investigated further and, it was found that there is a reversible electrostatic interaction between DOX and HA, which makes HA a suitable candidate for DOX loading and consequently providing a controlled release of the drug over a long period of time.

Based on this drug-HA binding mechanism, both apatite-binding and non-binding anti-cancer drugs can be combined for local delivery by HA-based materials, which can achieve multiple release profiles. The efficacy of using pre-implanted HA materials to recruit HA seeking cytostatics to the tumor site is envisaged to be groundbreaking and should be explored further. The size of HA particles is key to achieve therapeutic efficacy. By coupling DOX to HA nanoparticles, it was shown that DOX could be delivered intracellularly to the mitochondria instead of the nucleus with improved tumor eradication. This targeted delivery approach opens new avenues for targeted intracellular delivery of hydroxyapatite-binding cytostatics.

After establishing that the nano sized HA particles were more efficient than micro-HA particles in DOX delivery, by entering the cell, the next step was to evaluate the

long-term safety of using nano HA particles in bone. This was studied by in-vivo tracking of radioactively labelled HA nanoparticles. The results showed that most implanted HA particles stayed locally without migrating to vital organs up to 28 days, which emphasizes the safety and the translational potential of using HA nanoparticles as a DOX carrier in the future. Finally, it is recommended that screening more hydroxyapatite-binding cytostatics and using HA nanoparticles for mitochondria-targeted delivery of apatite-binding cytostatics might be a good supplementary treatment for the treatment of bone cancer and the therapeutic potential of the developed drug delivery system could be extended to other soft tissue tumors in the future.

9. Sammanfattning på svenska

Osteosarkom är en aggressiv form av bencancer med hög risk för spridning. Vanligast är spridning till lungorna. Sjukdomen drabbar unga personer men också patienter över 75 år. Fram till 1960-talet var amputation det enda behandlingsalternativet och dödligheten var hög. Cellgiftsbehandling, bestående av en kombination av doxorubicin, metotrexat och cisplatin , introducerades på 70 talet och kombineras med kirurgisk excision av tumörvävnaden. Överlevnaden har ökat dramatiskt med denna kombinationsbehandling men samtidigt har även antalet patienter som svarar dåligt på behandlingen ökat. Några nya läkemedel har inte tagits fram under de tre senaste decennierna. Utmaningarna med nuvarande behandlingsmetoder är framförallt att 1) mängden cellgift som når tumörvävnaden är begränsad (i bästa fall 5% av den totala dosen givet läkemedlet), 2) allvarliga biverkningar uppstår på grund av oönskad ackumulering av cellgifter i friska organ, 3) ett ökat antal patienter svarar dåligt på behandlingen på grund av en resistensutveckling mot kemoterapin och 4) inga nya och effektiva läkemedel har utvecklats för behandling av osteosarkom.

I denna avhandling visas att hydroxyapatit (HA), ett naturligt förekommande benmineral, är ett lovande bärarmaterial för lokal frisättning av cytostatika i tumörvävnaden. Lokal tillförsel av cytostatika ger höga lokala koncentrationer i och omkring tumören och förväntas ge färre och mindre allvarliga systemiska biverkningar.

I den första studien fann vi en mer långvarig och kontrollerad frisättning av doxorubicin (DOX) då vi använde ett kliniskt godkänt HA-baserat biomaterial samt en förbättrad tumöreeliminering jämfört med tidigare systemisk kemoterapi. Vi fann också att denna fördröjda frisättning kunde minimera cytotoxiciteten av DOX i de friska omgivande vävnaderna.

Mekanismen för en kontrollerad frisättning av DOX via en HA-baserad bärare undersöktes ytterligare. Vi fann en reversibel elektrostatisk interaktion mellan DOX och HA, som gör HA till en lämplig kandidat för DOX-bindning som samtidigt tillåter en kontrollerad frisättning av läkemedlet över en längre tidsperiod. Storleken på HA-partiklarna verkar vara nyckeln till en god terapeutisk effekt. Genom att koppla DOX till HA-nanopartiklar kunde vi visa att DOX frisätts intracellulärt, och till mitokondrier i stället för till cellkärnan. Detta ger en förbättrad tumöreradikering

och öppnar för en möjlighet till riktad intracellulär distribution av hydroxyapatitbindande cytostatika.

Efter att ha fastställt att HA-partiklarna i nanostorlek var mer effektiva än HA-partiklar i mikrostorlek, både in vitro och in vivo, var nästa steg att utvärdera den säkerheten med nano-HA-partiklar i ben. Vi studerade radioaktivt märkta HA-nanopartiklar in vivo och visade att en majoritet av de implanterade HA-partiklarna kvarstannade lokalt i upp till 28 dagar utan att migrera till vitala organ. Vi tror att en utökad screening av andra typer av hydroxyapatitbindande cytostatika, liksom användning av HA-nanopartiklar med apatitbundna cytostatika som är målstyrda till mitokondrierna, i kombination med systemisk cellgiftsbehandling kan bli en kompletterande behandling för skelettcancer. Vi tror slutligen att den terapeutiska potentialen hos det utvecklade bärarsystemet i framtiden kan utvidgas till andra mjukdelstumörer.

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