

**Investigating the *in vitro* and in vivo anti-resorptive effects of herbal-  
and TCM-based extracts on Cathepsin K activity**

by

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B.Sc., The University of British Columbia, 2018

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Biochemistry and Molecular Biology )

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2021

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Investigating the *in vitro* and in vivo anti-resorptive effects of herbal-and TCM-based extracts on Cathepsin K activity

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the degree of Master of Science

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## Abstract

Osteoporosis is a chronic skeletal disease primarily affecting post-menopausal women and men over the age of 50. Current pharmacological interventions, such as bisphosphonates (BP) and denosumab, target the bone resorption process and have limitations in efficacy due to the disruption of important bone remodeling mechanisms and thus reducing bone formation and quality. Cathepsin K (CatK) is the major enzyme that is responsible for the breakdown of type I collagen in the bone matrix, therefore inhibitors of CatK have been a new approach to the treatment of osteoporosis without decreasing bone formation. Several active site CatK inhibitors such as odanacatib (ODN) have shown high efficacy in clinical trials, but were not approved due to risks of adverse effects. Adverse effects were postulated to be due to inhibition of CatK normal physiological as well as pathophysiological functions such as its part in the catabolism of essential growth hormones. Exosite inhibitors extracted from red sage plants, such as tanshinone IIA sulfonic sodium (T06) and dihydrotanshinone-1 (DHT-1) have so far shown a potent anti-resorptive effect while demonstrating an increased specificity in targeting the disease-related collagenase activity of CatK. These inhibitors are able to bind at a site on CatK to prevent collagenase activity while leaving other enzymatic activities unaltered. The major aim of my thesis will be to elucidate the *in vitro* and *in vivo* efficacy of herbal-based CatK inhibitors in the treatment of osteoporosis. The *in vitro* and *in vivo* efficacy of a pan-tanshinone-containing extracts of *Salvia Milthoriza*

(SM) , and a formulation used in Traditional Chinese medicine where SM is part of a multi-herbal combination (XLGB Pills), which will be compared against a standard-of-care bisphosphonate through the analysis of bone microstructural as well as CatK activity subsequent to these treatments.

## **Lay Summary**

Osteoporosis is a chronic skeletal disease primarily affecting post-menopausal women and men over the age of 50. Current pharmacological interventions, such as bisphosphonates and denosumab, target the bone resorption process and have limitations in efficacy due to the disruption of important bone remodeling mechanisms. Cathepsin K (CatK) is an enzyme that is capable of breaking down collagen in the bone matrix, therefore inhibitors of CatK have been a new approach to the treatment of osteoporosis without affecting bone formation. Previously, compounds derived from *Salvia miltiorrhiza* (SM), a herb used in traditional Chinese medicine (TCM) for the treatment of cardiovascular and bone related disease have been shown to inhibit CatK activity. The major aim of this project will be to compare the efficacy of herbal formulations with SM and current Western interventions through in vitro and in vivo experiments.

## **Preface**

This research project was conducted under the supervision of Dr. Dieter Brömme in his research lab. I was responsible for the conducting the research as well as the statistical analysis of the experiments described in this thesis. Experimental planning as well as the CTx-1 ELISA was carried out and performed by Dr. Preety Panwar and myself together. *In vivo* Mice work for this project was conducted under the approval of Animal Care Committee (protocol number A16-0085) and my animal ethics certification number (20180820-02ABC).

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## Abbreviations

BP: Bisphosphonates

BMD : Bone Mineral density

BV/TV: Bone volume/tissue volume

CatK: Cathepsin K

C4-S: Chondroitin sulfate

CTX-1: C-terminal telopeptide 1

DHT-1: Dihydrotanshinone-1

DMSO: Dimethyl sulfoxide

DP: Dripping pill

IC<sub>50</sub> : Half-maximal inhibitory concentration

M-CSF: Macrophage colony-stimulating factor

NTx-1: N-terminal telopeptide 1

ODN: Odanacatib

OVX: Ovariectomized

RANK: Receptor activator of NF-κB

RANKL: Receptor activator of NF-κB ligand

SM: *Salvia miltiorrhiza*

SEM: Scanning electron microscopy

T06: Tanshinone IIA sulfonate sodium

TRAP: Tartrate-resistant acid phosphatase

TCM: Traditional Chinese Medicine

Tb.N: Trabecular numbers

Tb.Sp: Trabecular separation

Tb.Th: Trabecular thickness

XLGB pills: XianLingGuBao pills

ZOL: Zoledronate

Z-FR-MCA: Benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin

$\mu$ CT: Micro-computed tomography

## **Acknowledgements**

I would like to offer my gratitude to all the members of the Bromme lab that have provided me with tremendous support both towards my research and also emotionally through this journey. I would like to especially acknowledge Dr. Dieter Bromme, who has been of great guidance and a very supportive mentor who has motivated and guided me through my project.

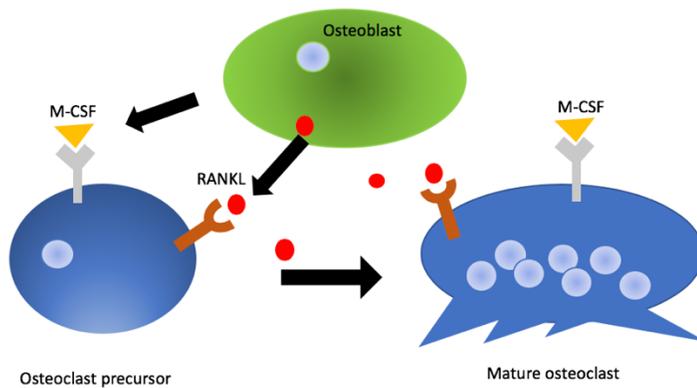
I would also like to thank Dr. Preety Panwar for her endless support in this project as well as her patient and clear explanations to my endless questions. I truly appreciate her guiding through me on various project techniques and providing constructive and encouraging feedback.

Lastly, I would like to address my appreciation for my parents for unconditionally supporting me through my education both financially and emotionally.

## **Chapter 1. Introduction**

### **1.1 Osteoporosis and current treatment options on the market**

Bone growth and health is maintained by the constant state of bone remodeling. Bone remodeling is determined by two processes: bone resorption and bone formation, which are mediated by osteoclasts and osteoblasts, respectively [1]. Initiation of the bone remodeling process starts with the generation of resorption pits by multinucleated osteoclasts, followed by the lay-down of type I collagen at the resorption site by osteoblasts [2]. Prior to the resorption process, mononucleated osteoclast precursor cells fuse into multinucleated osteoclasts regulated by cytokines and growth factors such as macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- $\kappa$ B ligand (RANKL) stimulation (Figure 1); mature osteoclasts form a sealing zone, which enables these cells to attach to the surface of the bone prior to acidifying the resorption compartment through the later established ruffled border membrane ( $\text{pH}<5$ ) [3]. The acidic pH exposes type I collagen in the organic bone matrix by dissolving the embedded minerals, which is then degraded by the osteoclast-secreted CatK [4]. The other part of bone remodeling, bone formation, is mediated by osteoblast cells which line the surface of the bones and are responsible for the synthesis and mineralization of the bone matrix [1]. In the case of an imbalance in the two processes, such as an increase in bone resorption due to increased osteoclast activity or reduced bone formation, a lower bone mass and bone mineral density (BMD) would result leading to the onset pathophysiological conditions such as osteoporosis [5].



**Figure 1. Osteoclast maturation and differentiation.** Schematic displaying of osteoblast and osteoclast cross-talk and osteoclast maturation into a multinuclear osteoclast through RANKL and M-CSF stimulation

Osteoporosis is a common chronic metabolic bone disorders and affects approximately 10 million people in the United States [6]. It affects both men and women, with increasing prevalence in post-menopausal women and elders over the age of 50 [6]. The pathophysiology of osteoporosis is defined by the imbalance in bone homeostasis, with increased bone resorption that ultimately leads to the destruction of the microarchitecture of the bone matrix, higher risk of bone fragility, fractures and subsequent hospitalization incidents [5].

It is estimated that the current economic burden of osteoporosis in Canada is \$ 4.6 billion, twice the amount of the previous estimation of \$2.3 billion in 2010 of the annual Canadian Health care expenditure spent on treatment and care for osteoporosis and related fractures [7], [8] With anticipated costs progressively increasing each year due to the globally aging population, osteoporosis is becoming one of the major health concerns nationwide and worldwide [7].

Bone mineral density (BMD) is an important parameter for the diagnosis of osteoporosis where the expected BMD for each age group and sex is expressed as a Z-score [6]. The criteria for assessing bone quality and risk of fractures are defined by the T-scores and Z-scores, which can be positive or negative depending on whether the individual falls above or below the mean of the adult group; additionally, the standard deviations away from the mean is noted by the numerical component of the score [6]. T-scores and Z-scores are important in assessing an individual's bone density different from the norm. Osteoporosis is defined by the World Health Organization (WHO) as a condition on the basis of a T-score, which compares an individual's bone density with a healthy 30-year-old of the same sex, with a threshold of -2.5 qualifying for osteoporosis diagnosis [9]. There are two main methods to obtain a T-score based on the BMD of different areas such as the lumbar spine, proximal femur, or peripheral sites such as the wrist and heel bone using quantitative computed tomography (qCT) and dual-energy x-ray absorptiometry (DXA). Currently, the standard method for the diagnosis of osteoporosis is DXA at central sites (spine or hips), reporting the BMD as a T-score from this analysis [10].

The application of bisphosphonates (BP) is currently the standard treatment for osteoporosis [11]. BP provide anti-resorptive effects through inducing osteoclast inactivation and apoptosis, leading to increased bone density and mass [12]. Other antiresorptive treatments such as estrogen supplement therapy [13] and denosumab have been shown to effectively decrease bone loss and increase bone density [11],[14].

Anabolic treatments such as parathyroid hormone therapy (PTH) and a recently developed sclerostin inhibitor, Romosozumab [15] are also used and show an increase in bone formation, with the latter drug exhibiting no additional resorption activation as seen with PTH analogues [16]. Osteoclast differentiation and proliferation requires the binding of RANKL to receptor activator of NF- $\kappa$ B (RANK) on osteoclasts [17]. Denosumab is a monoclonal antibody that targets this pathway by binding to RANKL and preventing the subsequent signaling and formation of osteoclasts [14]. BPs exert antiresorptive actions by inducing apoptosis of osteoclast cells [14]. However, side effects of these drugs such as decreased bone formation and bone quality have been attributed to the modulation of osteoclastogenesis and subsequent disruption of osteoblast and osteoclast coupling, thus making it less ideal for the use of continuous long term therapy [18], [19]. Additional long term adverse effects of BP and Denosumab have been shown to increase the risk of bone necrosis [20], while other interventions such as estrogen hormone replacement therapy have been reported to increase risks of breast cancer as well as clot formation leading to cardiac complications [21], [22]. In contrast to current interventions, CatK inhibitors do not affect osteoclast survival rates and osteoclastogenesis, which enables the cross-talk between osteoblasts and osteoclasts and therefore was considered as an alternative method to achieve anti-resorptive effects in the treatment of osteoporosis without negatively affecting bone formation [23].

## 1.2 TCM in the treatment of Osteoporosis

Traditional Chinese Medicine (TCM) has been used in the treatment of osteoporosis for extended periods of time in China. TCM is made from a combination of various medicinal plants (and sometimes animal products), either in the form of raw plants, or extracts of plants; the formulation is either packaged as a capsule/tablet, or given to patients in the raw plant form to be extracted in boiling water. Since western anti-osteoporotic treatments have presented with adverse effects in long term use, many laboratories in China continue to study the anti-osteoporotic effects of medicinal herbs and some standard antiosteoporosis drugs such as calcitriol and hormone replacement therapy treatment on bone mineral density (BMD) changes to minimize treatment adverse effects [24]. Systematic reviews displayed no significant difference in the therapeutic effects depicted by the changes in BMD of the standard anti-osteoporosis drug and the TCM prescriptions that were reviewed [24].

*Salvia miltiorrhiza* (SM), along with *Epimedium brevicornum* are some of the main medicinal plants used historically in the treatment of osteoporosis and various skeletal diseases [25]. SM has been classified as a herb that is conventionally used in the treatment of osteoporosis as well as cardiovascular disease. Studies have shown that the SM treatment was previously able to improve BMD and trabecular mass in ovariectomized (OVX) rat models [26]. Dripping pills (DP) are a commercially available combination tablet containing approximately 85% SM, used conventionally for the treatment of cardiovascular diseases [27]; its effect on skeletal diseases such as

osteoporosis or osteoarthritis is still unclear. Similarly, *Epimedium brevicornum* is a widely known bone anabolic medicinal herb; its main bioactive constituent icariin has been studied extensively as the treatment for osteoporosis in the recent years [28].

Xianlinggubao (XLGB) is one of the most often used prescription drugs for the prevent treatment of osteoporosis in China. XLGB tablets contains 70% *Epimedium brevicornum* and has been shown to increase bone parameters in OVX rat models [29]; although studies have shown that no significant toxicity has been observed in a 26-week treatment period on OVX rat models [29], increasing reports of adverse toxicity effects on the liver in human patients has led to concerns of the safety of the long term use of XLGB.

However, the discussion around TCM has always been that there is a lack of a defined mechanism of action for the therapeutic effects that are observed; in addition, the varying climates as well as physical location and harvesting times all are contributing factors to variations of the same herbs [30], and thus increasing the difficulty for TCM quality standardization. In the recent years, many of these osteoporosis-targeting TCMs such as XLGB have been extensively investigated for their bioactive ingredients to further understand the therapeutic targets of the herbal formulation; currently there have been 15 categories of bioactive compounds found in TCM: some examples are flavones, glycosides and phytochromes [30]. As previously mentioned, SM being one of the major constituents of the dripping pills its major components are hydrophilic compounds such as salvianolic acids as well as flavonoids, while the hydrophobic component mainly consists of tanshinone analogues [25], [31]. Tanshinone analogues such as tanshinone IIA

sulfonate (T06) and dihydrotanshinone (DHT-1) have been shown to exhibit selective inhibition of CatK's collagenase activity [32], and therefore these analogues may serve as the foundation for future osteoporotic research. XLGB pills also contain SM (5%) but with the majority being comprised of *Epimedium brevicornum* (Herba Epimedii 70%) [28], icariin is the main bioactive component of these pills which have been found to be pharmacologically active in inhibiting CatK activity and thus osteoclast resorption [28]. Therefore recent research on TCM osteoporotic therapy has been looking at these bioactive compounds within these herbs in inhibiting CatK activity to achieve a targeted and selective inhibition of the collagenase activity of this enzyme.

### **1.3 CatK as a target for the treatment of Osteoporosis**

CatK is a lysosomal cysteine protease and belongs to the papain-like protease family [33]. It is encoded by the *CTSK* gene located on human chromosome 1 and contains eight exons and seven introns [34]. CatK is expressed in several cells but has its highest expression levels in osteoclasts [35]. Here, the expression of CatK is stimulated by the interaction of RANKL with the promoter complex of the *CTSK* gene [36]. It is also regulated by estrogen where increased estrogen levels reduce CatK mRNA and protein expression [36]. CatK mRNA and protein expression has been found to be present in various tissues non-exclusively to bone such as the lung, small intestines, heart, ovary and skin [37]. Typical cells besides osteoclast which express CatK are breast cancer cells, macrophages, epithelial cells [38], [39]. For example, the increased expression of CatK in

breast cancer cells has also been reported while further indicating a possible role of CatK relating to bone metastasis [40], [41].

Currently, the majority of osteoporotic treatments are aimed to maintain a balanced bone turnover activity through modulating the bone resorption process. With CatK being at the final steps of the resorption mechanism, it has been one of the more promising targets for the discovery of new osteoporotic treatments with minimal adverse effects [42]. During bone resorption, CatK is the main cathepsin secreted by osteoclasts [37]. CatK is expressed in its inactive zymogen form (38kDa) within the osteoclasts which then undergo autoactivation and cleavage to the mature active form (23.5kDa) in the lysosomal compartment of the osteoclasts [43]. The secretion of CatK into the resorption lacunae allows it to access and degrade type I collagen exposed from the bone matrix by the preceding acidic demineralization step. CatK does not only degrade collagen: it also is capable to degrade elastin, gelatin, various cytokines and growth factors, which may account for some side effects observed with CatK active site inhibitors such as skin fibrosis and cardiovascular side effects [44], [45]

#### **1.4 CatK as a type I collagen degrading protease**

Type I collagen makes up approximately 90% of organic bone matrix and is essential for providing tensile and shear strength to the bone [46]. It is composed of three intertwined  $\alpha$  chains ( $2\alpha_1$  and  $1\alpha_2$ ), forming the collagen triple helical structure (Fig 2a) [47]. Chondroitin 4-sulfate (C4-S) is an important sulfated glycosaminoglycan composed

of long chains of sugar moieties of repeating disaccharide units: N-acetylgalactosamine and hexuronic acids present in the bone and cartilage. It is present in the extra cellular matrix (ECM) of various connective tissues and is responsible for stabilizing collagen within the bone matrix [48], [49]. Previously, studies have found the oligomer formation between CatK and C4-S and other glycosaminoglycans such as keratan sulfate at a 2:1 molar ratio is essential for the collagenolytic activity of CatK [50]–[52]. The CatK-C-4S complex is thought to partially unwind the collagen triple helices, and allowing the cleavage of the protein at multiple sites within the triple helical region [52]. A CatK specific cleavage product is the C-terminal telopeptide I (CTx-I) which is used for diagnostic purposes (Fig 2a).

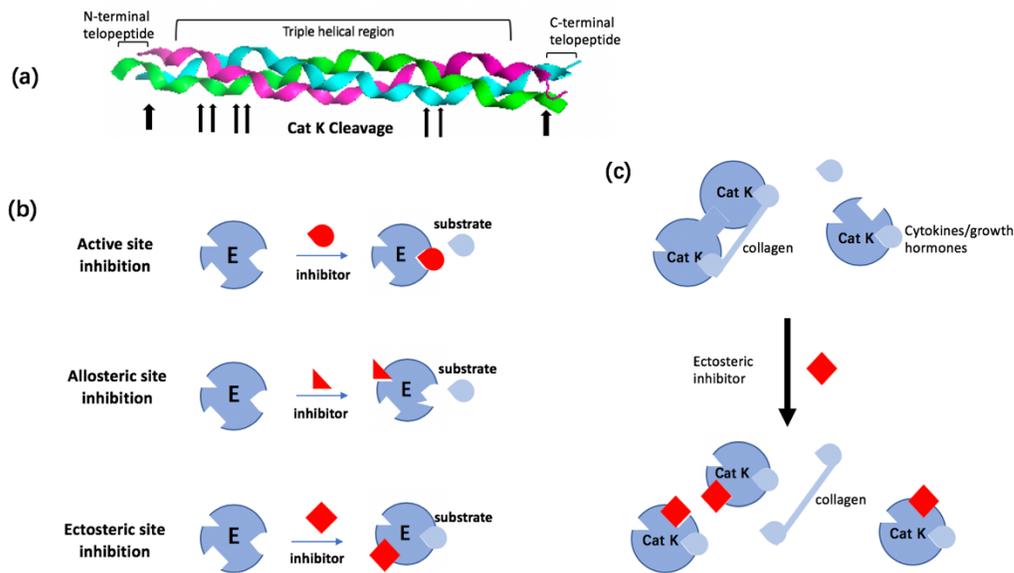
### **1.5 CatK inhibitor mechanisms: active site and exosite-targeting inhibitors**

CatK inhibitors have been seen as a potential therapeutic intervention in drug discovery for the treatment of osteoporosis due to its anti-collagenolytic actions and thus preventing the degradation of the organic bone matrix.

There are various approaches to inhibit the activity of an enzyme, such as active site and exosite inhibition. The first CatK inhibitors were designed to target the active site of the enzyme. These inhibitors bind to CatK at the active site, competitively inhibiting the degradation of all substrates (Fig 2b). Exosite inhibitors bind remote from the active site and include allosteric and ectosteric inhibitors. Upon binding at an allosteric site of CatK, a conformational change of the active site takes place, thus inhibiting or reducing the

degradation of substrates (Fig. 2b) [53]. Due to the multi-functional nature of CatK, both allosteric and active site-directed inhibition would inhibit the degradation of all substrates including those of important cytokines and growth factors along with collagen.

An alternative inhibition mechanism known as ectosteric inhibition was recently introduced on the basis of searching for compounds specifically inhibiting the CatK collagen-degrading activity while not affecting its other functions [32], [54]. Ectosteric inhibitors are a type of exosite inhibitor as they also bind at a site remote from the active site. In contrast to allosteric inhibitors, ectosteric inhibitors do not cause a change of the active site conformation of the enzyme (Fig. 2b) [54]. In the context of CatK, the ectosteric site that was seen as a new potential therapeutic target for further research was the complexation site between C4-S and CatK and protein-protein interaction sites in the CatK oligomers [54]. The oligomer formation between C4-S and CatK is essential to allow collagen degradation [52], [54], while other substrates such as growth factors do not depend on the oligomeric form of CatK for degradation (Fig. 2c) [54], [55]. Inhibiting the C4-S and interaction or protein-protein interactions in the complex is suggested to selectively inhibit only the collagen degradation activity of CatK and thus can be seen as a novel therapeutic approach for osteoporosis.



**Figure 2. CatK collagen degradation and inhibitor mechanisms.** (a) Multiple CatK cleavage sites on triple helical type I collagen (PDB ID: 1CAG). (b) Schematic outlining of the different enzyme-inhibition mechanisms. (c) Graphical representation of the concept of ectosteric inhibitor in the context of CatK. Abbreviations: E, Enzyme; CatK, Cathepsin K.

## 1.6 CatK inhibitors in research and clinical trials

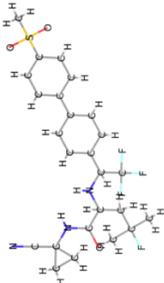
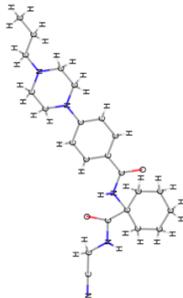
As CatK inhibitors became of interest in the past decade, several reversible CatK inhibitors have been developed and studied (Table 1). In preclinical studies, ovariectomized (OVX) rodents are often used for *in vivo* investigation of these inhibitors. Estrogen is an important hormone of the maintenance for proper bone health and formation [56]. OVX models mimic the physiological conditions of post-menopausal women by modelling osteoporosis caused by a deficiency in estrogen.

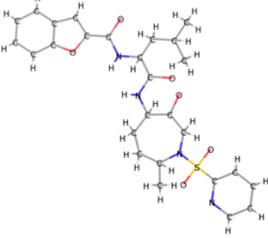
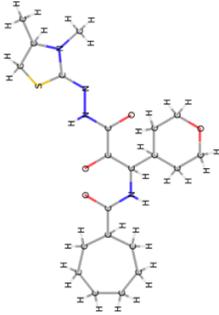
One of the most potent and selective of all the investigational CatK inhibitor was Odanacatib (ODN) [45]. ODN is an active site-directed CatK inhibitor developed by

Merck & Co. In preclinical studies, ODN was able to show 60-70% reduction on bone resorption through bone resorption markers such as CTx1 and NTx1. In contrast to BP and denosumab, ODN showed slight increases in bone formation markers while providing similar anti-resorptive effects [45]. Using OVX monkey models, ODN showed an increase in overall cortical bone mass and volume compared to the controls [45]. Entering clinical trials with an enrollment of 16,000 post-menopausal patients, ODN was able to show a reduction in osteoporotic fracture incidents as well as an increase in BMD [45]; however, the drug was withdrawn from phase III trial due to cardiovascular events [57]. Similarly, Balicatib, an active site directed CatK inhibitor developed by Novartis was dropped during Phase II clinical trials in 2006 due to dermatological side effects [44].

The side effects of active site-directed inhibitors of CatK were postulated due to off-target effects of CatK inhibition in other tissues as well as a complete inhibition of CatK activity due to active site inhibition [23]. However, the inhibition of the degradation of various growth factors such as TGF-  $\beta$ 1 and cytokines may have contributed to adverse effects of the active site-directed CatK inhibitors in an on-target fashion [55], [58].

**Table 1. Selective active site Cathepsin K inhibitors in clinical trials**

Drug Name (Company)	Structure	Activity against CatK	Phase of Clinical Development	Clinical efficacy
<b>Odanacatib</b> <b>(Merck&amp;Co.)</b>		IC <sub>50</sub> = 0.2 nM <i>in vitro</i>	Withdrawn in Phase III trials	Increase in bone formation markers and providing anti- resorptive effects; increase in BMD but with cardiovascular side effects [57]
<b>Balicatib</b> <b>(Novartis)</b>		IC <sub>50</sub> = 1.4nM <i>in vitro</i>	Discontinued in Phase II trials	Reduction of bone resorption biochemical markers without affecting bone formation; increase in BMD but with dermatological side effects (rashes) [44]

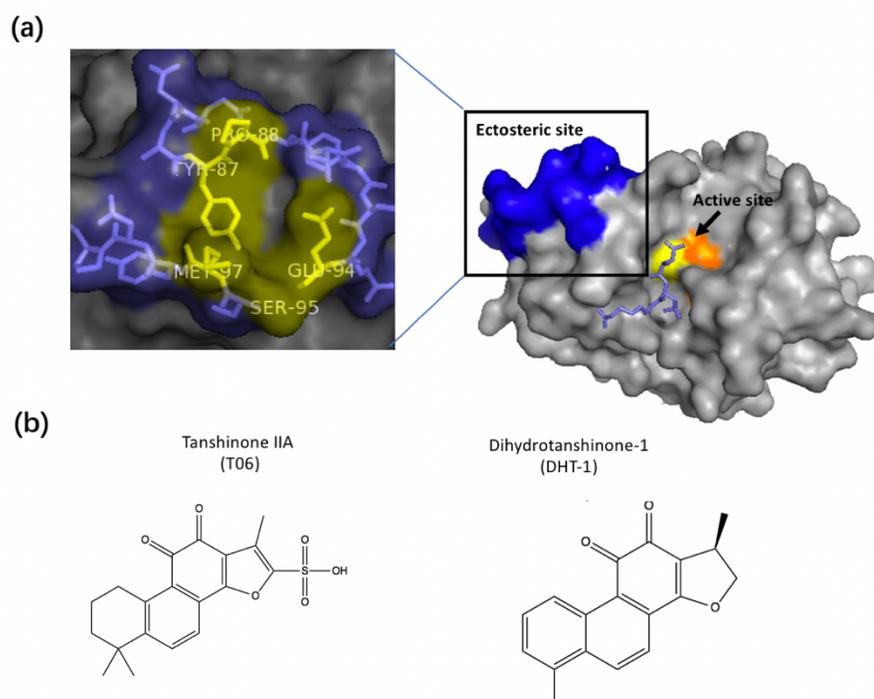
<b>Relacatib</b> (GlaxoSmithKline)		$K_{i, app}=41$ pM	Discontinued in Phase I trials (reason)	Reduction of bone resorption and high oral bioavailability [59]
<b>ONO-5334</b> (Ono Pharmaceuticals)		$K_{i, app}=0.1$ nM	Discontinued in Phase II trials (reason)	Reduction of bone resorption markers with little suppression of bone formation markers [60]
<b>MIV-711</b> (Medivir)	Not available	$K_i= 0.98$ nM	In Phase II trial	Reduction of bone resorption markers and preventing loss of cartilage [61]

### 1.7 Ectosteric inhibitors of CatK derived from Traditional Chinese Medicine

Previous research in our laboratory has been able to discover possible inhibitors that selectively target the collagenase activity of CatK. Recently, ectosteric site inhibitors that bind to sites of CatK oligomerization remote from the active site were introduced (Fig. 3a) [62]. These compounds are tanshinone-based and extracted from the roots of *Salvia miltiorrhiza* (SM), also known as Red Sage [54]. SM is a herb in traditional Chinese

medicine (TCM) used conventionally for the treatment of cardiovascular diseases [63].

Two tanshinone based molecules: Tanshinone IIA sulfonate sodium (T06) and Dihydrotanshinone-1 (DHT-1), were found to be the most potent ectosteric site inhibitors of CatK among numerous naturally occurring tanshinones found in the lipophilic component of SM (Fig. 3b) [54]. Molecular docking of T06 to the ectosteric site had revealed important residues such as Tyr87, Pro88, Met97, Glu94, and Ser95 for the compound protein binding interaction site (Fig. 3a) [64]. In contrast to active site inhibitors such as ODN, T06, and DHT-1 were reported to not affect the degradation of TGF- $\beta$ 1 and gelatin while exhibiting potent anti-collagenase activity [54], [55]. Both inhibitors were also reported to exhibit comparable activities in osteoclast-resorption assays to 50 nM ODN with 10  $\mu$ M DHT-1 exhibiting maximal reduction of approximately 60% CTx1 release compared to 80% in ODN [54].



**Figure 3. Molecular overview of CatK ectosteric site and inhibitors**

**a)** Molecular model of CatK bound to a pan-specific cysteine protease inhibitor E-64 (PDB ID: 1ATK) shown in surface form. Active site residues are shown in orange and yellow (His162 and Cys25). An ectosteric site is shown in blue with the 5 potentially interacting residues highlighted in yellow. **(b)** Structure of the two most potent ectosteric inhibitors discovered.

The major aim of my thesis will be to elucidate the *in vitro* and *in vivo* efficacy of herbal-based CatK inhibitors in the treatment of osteoporosis. The efficacy of alcoholic and water extracts of SM, and a formulation used in TCM where the aqueous extract of SM is part of a multi-herbal combination (XLGB Pills) and dripping pill (DP) will be first compared in *in vitro* CatK activity assay. XLGB Pills is a SM containing (5%) standard prescription TCM used for the treatment of osteoporosis; this capsule is selected to provide a comparison between the western and TCM approach to osteoporosis

treatment as well as to compare its anti-resorptive effects against the ethanol extract of SM. The second aim is to determine the *in vivo* efficacy of the alcoholic and water extracts of SM, and XLGB pills compared with a standard-of-care BP (Zoledronate) in an osteoporosis mouse model.

Tanshinones are the major hydrophobic components in SM and have been demonstrated to exhibit inhibitory effects on CatK in previous studies. My hypothesis is that the ethanol extract which contains the highest amount of tanshinones will be a more effective *in vitro* CatK collagenase assays as well as *in vivo* studies compared to other herbal formulations.

## **Chapter 2. Materials and Methods**

### **2.1 Preparation of SM crude extracts**

Crude SM roots were purchased from TongrengTang (Richmond, BC). SM ethanol extract was prepared through incubation of 500 g pulverized SM with 1.5 L of 100% ethanol over the course of two days in a shaker at room temperature; the process was repeated 3 times and the extract mixture was subsequently filtered with a filter paper to remove the insoluble content. The samples were then put on the rotary evaporator and transferred into a speed vacuum dryer to obtain a powdered form. SM water extract was prepared using 100 g of SM refluxed with 300 mL of water at 100°C for 4 hours. The mixture was then filtered using a filter paper and then lyophilized to powder form. The two commercial TCM-based medications XianLingGuBao (XLGB) (TongjiTang GuiZhou Pharmaceutical CO., China) and Dripping pills (Tong Ren Tang, China) containing approximately 5% and 83% SM, respectively, were pulverized, and then incubated with water on a shaker for 2 days. The samples were then centrifuged for 5 minutes at 13,000 rpm; the supernatant was collected and subsequently lyophilized to powder form.

### **2.2 Collagen degradation assay with soluble type I collagen**

C4-S was purchased from Sigma-Aldrich (St.Louis, MO, USA) and was used to optimize CatK collagen degradation ability. All reactions were prepared in 50  $\mu$ L reaction volume with 0.6 mg/mL of soluble bovine type I collagen, 400 nM of human recombinant wild-

type CatK, increasing concentrations of inhibitors and 200 nM of C4-S in sodium acetate buffer, pH 5.5 containing 2.5 mM dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA). Each reaction tube was incubated for 4 hours at 28°C and subsequently stopped with 2 µL of 100 µM of E-64 inhibitor, a pan-cysteine protease inhibitor. 8 µL of each reaction tube was taken and run on 8% SDS-polyacrylamide gels. SDS-polyacrylamide gels were then stained with Coomassie blue (BioRad, CA, US) for 4 hours and then de-stained overnight on a shaker. The intensity of the collagen  $\alpha$ 1 band on SDS-polyacrylamide gels was densitometrically quantified using ImageJ (NIH, Rasband, U.S.) to characterize the extent of collagen degradation. Dose response curves were constructed for each inhibitor sample using GraphPad PRISM 6 program (GraphPad , San Diego, C.A.) and the  $IC_{50}$  values for the pills and herbal extracts were determined. Three experimental replicates were obtained.

### **2.3 Gelatinase assay**

To evaluate potential active site inhibition, gelatinase assays were performed. Gelatin was prepared by heating soluble bovine type I collagen for 30 minutes at 95 °C. 0.6 mg/mL of gelatin was incubated with 5 nM of human wild-type CatK with the maximal concentration of each inhibitor previously used in the collagenase assays in sodium acetate buffer at 28 °C for 1 hour; 1 µL of 100 µM E-64 was subsequently added to adequately terminate the reaction. Samples were then loaded onto 8% SDS gels and the

$\alpha$ 1 band intensities were quantified for each condition. Three experimental replicates were obtained in the gelatin assay.

#### **2.4 Z-FR-MCA assay**

To further identify the potential active site inhibition of the compounds, a fluorogenic CatK active site substrate benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin (Z-FR-MCA) (Bachem Americas, Inc, Torrance, California, USA) was used. All reactions were pre-incubated for 5 minutes at room temperature with or without the presence of inhibitors and 5 nM CatK in sodium acetate buffer. Assays were performed by adding 5  $\mu$ M of Z-FR-MCA to the reaction after pre-incubation and monitoring the release of the fluorogenic group (MCA) on a Perkin Elmer Luminescence Spectrometer with an excitation and emission wavelength of 380 nm and 450 nm, respectively. The rate of release of the fluorogenic group, MCA, is indicative of the enzymatic activity of CatK. Three separate experimental repeats were performed to obtain the results.

#### **2.5 Insoluble collagen degradation analysis using hydroxyproline assay**

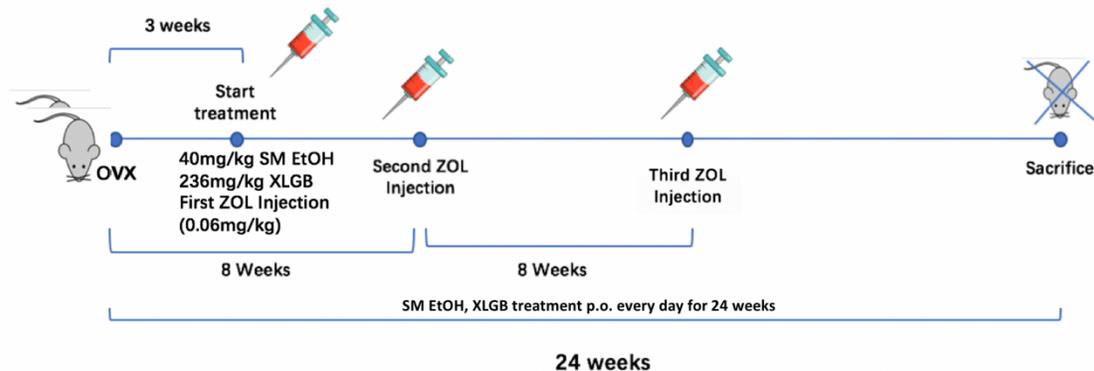
Collagen fibers were isolated from C57BL/6J mice tails and washed in 70% ethanol. 1 mg of collagen fibers was incubated with 2  $\mu$ M of CatK in 50  $\mu$ L reactions and varying concentrations of the inhibitors in sodium acetate buffer overnight at 28 °C. 2  $\mu$ L of 100  $\mu$ M E-64 was subsequently added to terminate the reaction. The reaction mixture was centrifuged for 5 minutes and the supernatant was obtained for hydroxyproline

quantification. A standard curve was prepared from serial dilutions of the hydroxyproline reference standard (Sigma-Aldrich). 50  $\mu$ L were taken from each reaction tube and hydrolyzed with concentrated HCl (12 M) for 3 hours. 50  $\mu$ L of the total reaction volume was then taken and transferred into 96 well plates and dried at 60 °C. Each dried reaction well was incubated with 100  $\mu$ L of chloramine T/ oxidation buffer at room temperature for 20 minutes and subsequently incubated with 100  $\mu$ L dimethylaminobenzaldehyde (1M DMAB) at 60 °C for 20 minutes. Reactions were quenched on ice and the absorbance values were taken at 560 nm with a microplate reader (Synergy BioTek, VT, USA). Three experimental repeats were performed.

## **2.6 Animal experiment protocol**

Animal experiments were conducted in accordance to guidelines outlined by the Canadian Council in Animal Care (CCAC). 2-months old female C57BL/6 mice with a starting weight of ~ 20 g were used to conduct this study. All mice were ovariectomized with the exception of the sham group and were given a 3-weeks period to develop the onset of osteoporosis. For the following 6 months, drugs other than Zoledronate (ZOL) were mixed into the chow with each mice given 3 g of chow + treatment diet to control drug uptake; animal weight was monitored 2-3-times per week for any fluctuations that may have an effect on the uptake of the amount of food containing the drug; 60  $\mu$ g/kg ZOL was administered through tail vein injection (i.v) every two months (Figure 4). The groups were: sham+ vehicle (chow) (n=12); OVX+ vehicle (n=10); OVX+ 40 mg/kg/d

SM Ethanol extract (SM EtOH) (n=10); OVX+ 236 mg/kg/d XLGB (n=10); OVX+ 0.06 mg/kg ZOL (n=10). At the time of autopsy, left femur bones were isolated and stored in 70% ethanol for micro-computed tomography ( $\mu$ CT) scanning, the right femur was collected and fixed in 10% formaldehyde for 48 hours and subsequently decalcified in 14% EDTA solution for histology. Lungs, liver, heart, skin, uterus, kidneys, and spleen were also preserved in 10 % formaldehyde for 24 hours prior to long term storage in 70% ethanol at 4 °C for future studies. Blood was collected by heart puncture, centrifuged and collected for plasma for later determination of plasma CTx-1 levels by ELISA. Uterine weights were taken immediately post-sacrifice to analyze for possible pseudo-estrogen effects of the treatments.



**Figure 4. Experimental design of animal study.** Studies were conducted for 24 weeks (n=10 for each treatment group and 12 animals for the control group). Injections of ZOL were given at 3 time points with 8 weeks gap in between each injection. TCM-related drugs were given daily by food.

## **2.7 Micro-CT analysis**

Left femur bones were scanned using a Scanco Micro CT 100 (Scanco Medical, Bruttisellen, Switzerland). The bone samples were scanned with a nominal resolution of 7.4  $\mu\text{m}$  with 0.5 mm Aluminum filter in batches of four. 3-D reconstruction and bone microstructure analysis was achieved by using a Feldkamp, David, Kress (FDK) algorithm. The femur trabecular regions were selected by contours. 150 continuous bone slices were analyzed for the trabecular parameters starting 10-15 slices distal of the growth plate using the merging point of the growth plate as reference point. Trabecular parameters such as trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular space (Tb.Sp), bone volume/tissue volume (BV/TV) and connectivity (Conn.D) were analyzed. 3-D reconstruction was also carried out to display the trabecular microstructure.

## **2.8 Uterus weight measurement and ELISA of mice plasma for CTx-1**

Blood was collected post-sacrifice of mice and centrifuged for 10 minutes at 2000 rpm to obtain the plasma and stored at -80 °C until use. Samples were thawed and aliquoted to small tubes on ice. The blood serum was analyzed for CTx-1, a type-1 collagen degradation product used as a bone resorption marker using the CTx-1 ELISA kit (Cloud-Clone Corp.) following the kit protocol. This assay kit employs a competitive inhibition ELISA, therefore concentration calculation of CTx-1 was determined with inverse correlation calculation on a semi-log graph. For determination of possible estrogenic

effects of treatments, uterine weight was recorded. Uterus was removed carefully from the mice immediately subsequent to sacrifice and weighed; weight was recorded for each mouse and compared with each treatment group.

## **2.9 Histological analysis and staining of femoral bone**

Right femoral bones were collected at the time of sacrifice and were fixed in 10% formalin for 48 hours and were subsequently submerged in 14% EDTA solution for 2-3 weeks. To ensure proper decalcification, femoral heads were confirmed to be soft prior to embedding. All decalcified femoral heads were stored in 70% ethanol until processing. Femoral bones samples were all processed for paraffin embedding by dehydrating in ascending concentration of ethanol (70%, 80%, 90%, and 100%); samples were then cleared in xylene and paraffin. Samples were all embedded in paraffin. 4  $\mu$ m bone sections were cut using the Leica RM 2265 microtome and dried overnight at room temperature; 25 sections were obtained from each sample and 5 sections were stained with hematoxylin and eosin (H&E) and 5 other were stained for tartrate-resistant acid phosphatase (TRAP) for the presence of osteoclasts. All paraffin sections prior to staining were melted 60°C in an electric oven. For H&E staining, slides were subsequently cleared in two washes with xylene for 10 minutes each and subsequently dehydrated in descending grade of ethanol incubations (100%, 95%, 90%, 80%) and washed in distilled water. Sections were then incubated with Mayer's hematoxylin to stain for cell nucleus for 5 minutes and kept under running tap water until stain develops from purple to blue.

Sections were then counterstained with Eosin to stain the cytoplasm of the cell and quickly dehydrated again in ascending grades of ethanol (80%, 90%, 100%); prior to mounting, sections were processed in 50%/50% ethanol/xylene and 100% xylene. Prior to TRAP staining, the stain was mixed fresh, filtered, heated and kept in a 37°C oven prior to use. Sections were also placed in the electric oven (60°C) for an hour followed by two xylene incubations (10 minutes/each) for paraffin removal. Samples were then dehydrated in descending grades of ethanol (100%, 95%, 90% and 80%) and submerged in distilled water. TRAP stain was pipetted onto the bone sections and incubated in the electric oven at 37°C for 1.5 hours and subsequently washed in distilled water for 5 minutes and counterstained with hematoxylin (2 minutes). Slides were then washed in running tap water (10 minutes) and processed for mounting (95%, 100% ethanol and cleared in xylene). Representative images were obtained with 10x, 20x and 40x objectives (Nikon Eclipse Ci microscope).

### **2.9.1 Histological analysis for TRAP**

Data points for the analysis of the number of osteoclasts/bone parameter were collected by taking the average number of osteoclasts found in the 10 ROIs (250  $\mu\text{m}$  x 250  $\mu\text{m}$ )/bone slice along the growth plate of the femoral bone head and divided over the length of the bone surface ( $\mu\text{m}$ ) within the defined ROIs. 5 bone slices from each of the 5 treatment groups and 10 ROIs were obtained for each bone slice. The analyses of bone slices as well as the counting of osteoclasts were obtained under 20x magnification (Nikon Eclipse

Ci microscope). Focus of the microscope was constantly adjusted to obtain an accurate number of osteoclasts near the growth plate within the set ROIs.

## **2.10 Statistical analysis**

Dose response curves were constructed on a semi-log scale and fitted on a sigmoidal curve; the average and standard deviation of three replicates of each concentration on the dose response curve were taken and plotted on the dose-response curve. GraphPad Prism version 6.01 was used to conduct these analyses. All error bars indicate the standard of deviation (SD).

We have used an ordinary-one way ANOVA multiple comparisons for the statistical analyses of the trabecular bone parameters obtained by  $\mu$ CT in Figure 12 between each treatment condition and the OVX group. Statistical analysis of ELISA CTx-1 concentrations between treatment conditions compared to the OVX group was also obtained using ANOVA for multiple comparisons. Similarly, for the analysis of the difference between mean uterine weights in Figure 10, an unpaired parametric t-test was used with the Welch's correction applied. GraphPad Prism version 6.01 was used to conduct these statistical analyses; results are expressed as  $\pm$  SD with P-value  $< 0.05$  considered to be significant.

## Chapter 3. Results

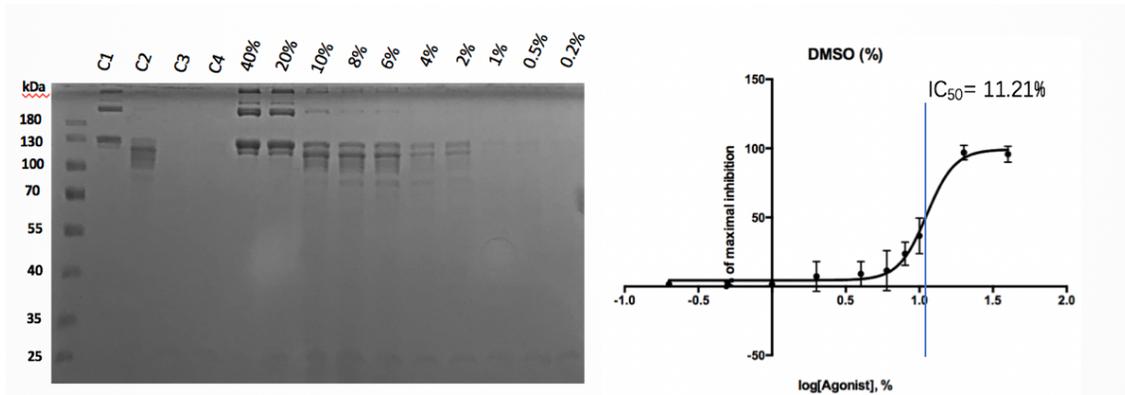
### 3.1 Inhibition of CatK-mediated soluble collagen degradation

Soluble bovine collagen was used to determine the inhibition of the CatK collagenase activity. The efficacies ( $IC_{50}$ -values) of the inhibitors (DP, XLGB, SM Ethanol and SM water extracts) were analyzed using SDS-PAGE and subsequently quantified by the densitometric determination of the relative amount of remaining  $\alpha 1$  band. These data were used for  $IC_{50}$  determination through dose response curves (Figure 6). All compounds were dissolved in either water or 1% DMSO.

DMSO is known to reduce the CatK collagenase activity, therefore, the  $IC_{50}$  of DMSO on CatK collagenase activity was also evaluated (Figure 6). Results displayed a concentration dependent increase of DMSO inhibitory effects on CatK collagenase activity (Figure 6). The  $IC_{50}$  value of DMSO was determined to be  $11.21 \pm 0.04\%$ . Therefore, all samples with DMSO as a solvent had a final DMSO concentration of 1% to eliminate any substantial solvent effect.

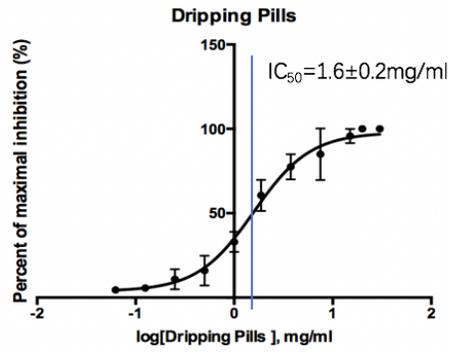
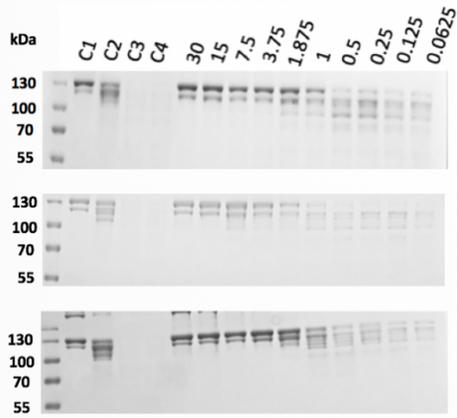
All four herb-based inhibitor samples showed a concentration-dependent increase in the inhibition of the collagenase activity for (Figure 6; A-D). Comparing the response between each of the four samples, DP, and XLGB pills displayed similar inhibition potencies, with  $IC_{50}$  values of  $1.6 \pm 0.2$  mg/mL and  $1.7 \pm 0.1$  mg/mL, respectively (Figure 6A and B); the SM water extract was shown to be 10x more potent than the commercial pills, with an  $IC_{50}$  value of  $0.15 \pm 0.02$  mg/mL (Figure 6D). Comparing the potencies of the four samples, SM ethanol extract displayed the greatest inhibition potency, with 3x

the potency of the corresponding SM water extract with an  $IC_{50}$  value of  $0.047 \pm 0.002$  mg/mL (Figure 6C). The results obtained were from densitometric analysis of SDS-PAGE gels in three independent replicates.

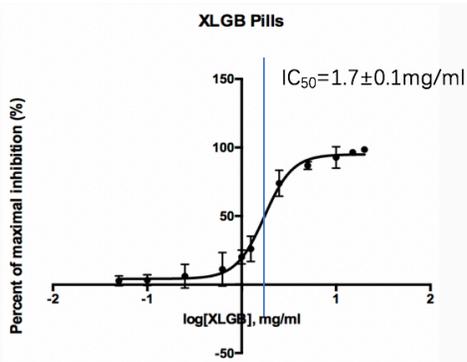
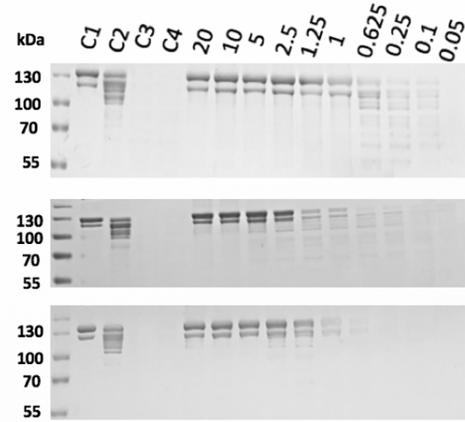


**Figure 5. Inhibition of CatK-mediated degradation of soluble collagen by DMSO**  
SDS-PAGE analyses and  $IC_{50}$  plot for type I collagen with varying concentrations of DMSO. Collagen control (C1), collagen with and without C4-S (C2, C3) and solvent control (C4) were used; CatK concentration was 400 nM, and digestion was performed at pH 5.5 and 28°C for 4 h ( $n = 4$ );  $IC_{50} = 11.21\%$ .

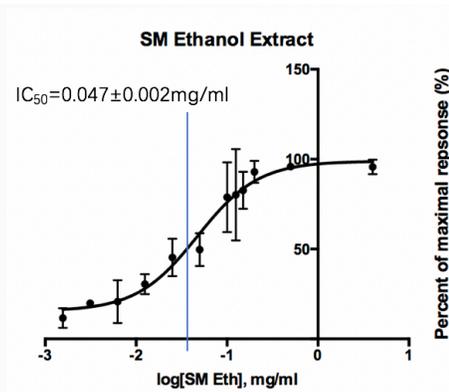
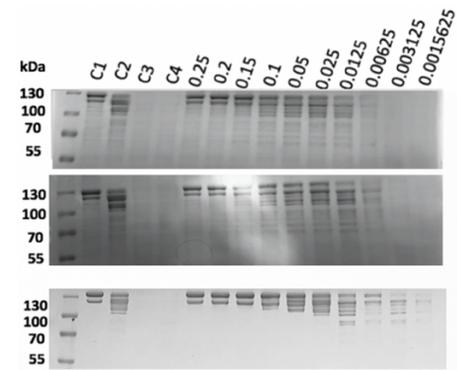
### A. Dripping Pills



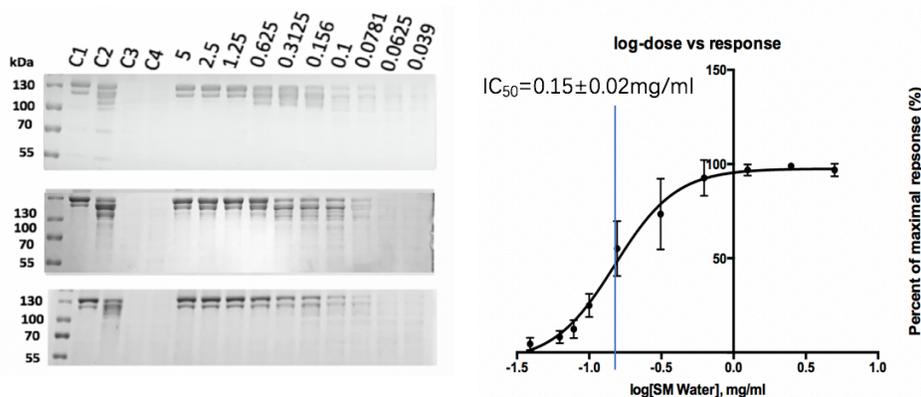
### B. XLGB Pills



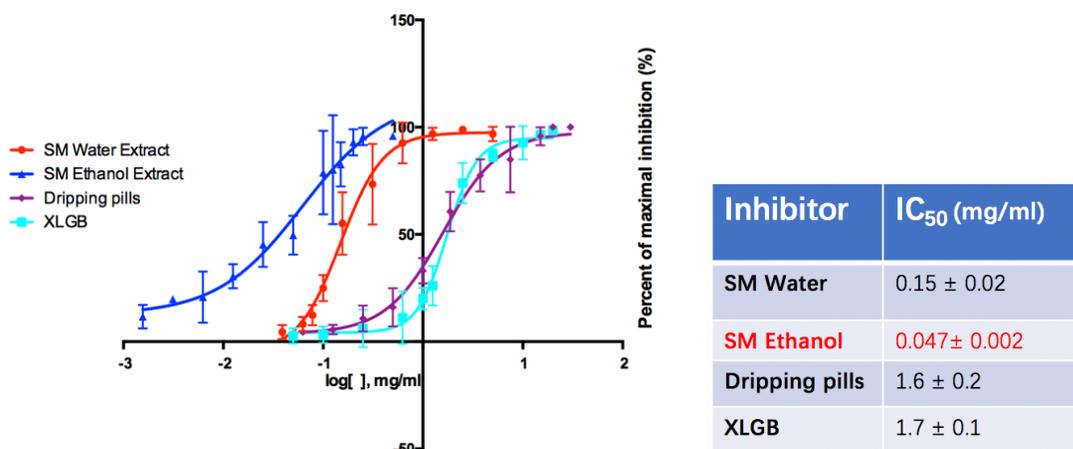
### C. SM Ethanol Extract



## D. SM Water Extract



## E. Summary

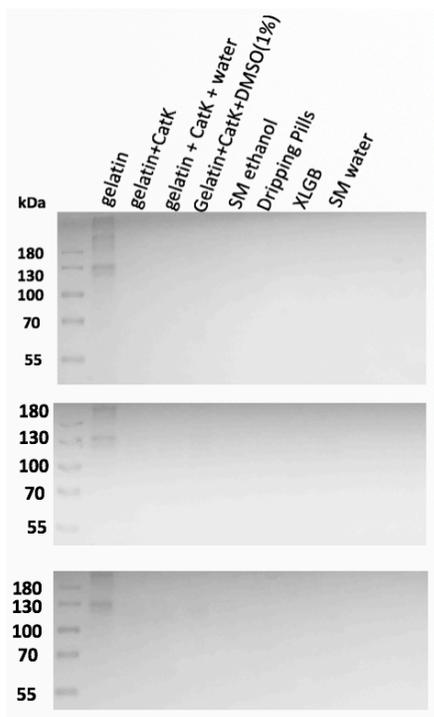


### Figure 6. Inhibition of the degradation of soluble collagen by SM water extract, ethanol extract, as well as Dripping Pill and XLGB Pill water extracts

SDS-PAGE analyses and  $IC_{50}$  plots for type I collagen with (A,B) commercial pills and (C,D) herbal extracts. (A-D) SDS-PAGE gels display bands indicative of increasing collagen degradation profile; collagen control (C1), collagen with and without C4-S (C2,C3) and solvent control (C4) were used. CatK concentration was 400 nM, and digestion was performed at pH 5.5 and 28°C for 4 h. The  $IC_{50}$  of Dripping Pills and XLGB were  $1.6 \pm 0.2$  mg/mL and  $1.7 \pm 0.1$  mg/mL, respectively (n=3); the  $IC_{50}$  of SM water and ethanol extracts were  $0.15 \pm 0.02$  mg/ml and  $0.047 \pm 0.002$  mg/mL (n=3). (E) Summary plot of the dose response curves of the four drugs.

### 3.2 Gelatinase activity

Gelatinase assays were used to evaluate potential active site inhibition of the four compounds that were contributing to the collagen degradation. The SDS-PAGE gels showed that there were no alpha 1 bands observed after 1 hour incubation with CatK and the compounds, suggesting that the compounds do not significantly inhibit the gelatinase activity of CatK (Figure 7). As the hydrolysis of gelatin does not require CatK oligomerization, we deduced the lack of CatK inhibition as proof that the extracts were not blocking the active site of the protease. The results were obtained by densiometric analysis and the experiment was conducted in three replicates.

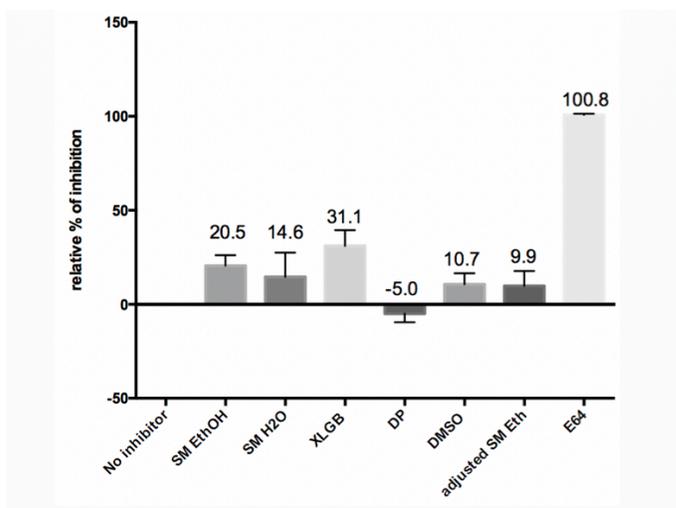


**Figure 7. Inhibition of gelatin degradation by inhibitors**

SDS-PAGE analyses for gelatin degradation with Dripping pills, XLGB pills, SM water, and ethanol extract. CatK concentration was 5nM, and digestion was performed at pH 5.5 and 28°C for 1 h (n = 3)

### **3.3 Z-FR-MCA activity**

To further evaluate a potential active site inhibition of the four extracts, the fluorogenic substrate Z-FR-MCA was used. Z-FR-MCA hydrolysis is not affected by C4-S. After a 5-minute pre-incubation with 5  $\mu$ M of CatK and extract (concentrations of extracts tested were the highest concentration used in the collagenase assays (DP: 30 mg/mL, XLGB: 20 mg/mL, SM water: 5 mg/mL, SM ethanol: 0.25mg/mL). results show that XLGB pills, SM ethanol, and water extracts all displayed varying degrees of active site inhibition (Figure 8). E-64 was used as a positive control and displayed maximal (100%) inhibition of CatK active site. SM ethanol displayed the lowest % of CatK active site inhibition (9.9%) compared to the no inhibitor control, while XLGB pills (31.1%) displayed the most CatK active site inhibition of the compounds tested. It is interesting to note that DP showed the opposite effect on CatK active site inhibition compared to the other three compounds by increasing CatK active site activity, showing a slightly increased rate of fluorescence release compared to the no inhibitor control (Figure 8). The measurements were calculated based on the release rate of the fluorogenic MCA-group and were repeated 3 times.



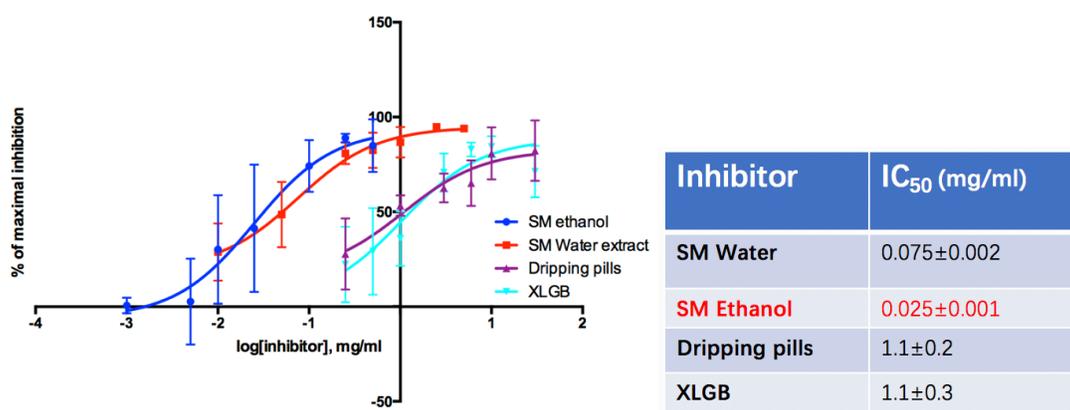
**Figure 8. Active site inhibition of Z-FR-MCA hydrolysis by SM ethanol extract, SM water extract, XLGB Pills and DP Pills.**

Z-FR-MCA analyses of the four inhibitors showing the relative % CatK active site inhibition (n=3). CatK concentration was 5nM, and reactions were pre-incubated with inhibitors for 5 minutes prior to the addition of Z-FR-MCA substrate. The concentration of the four inhibitors for DP, XLGB, SM ethanol extract and SM water extract were 30, 20, 5, 0.25 mg/mL, respectively).

### 3.4 Inhibition of CatK-mediated degradation of insoluble collagen fibers

To further evaluate the CatK inhibition profile of the four extracts to a more physiological substrate, CatK collagenase activity was evaluated on type I insoluble collagen fibers that were isolated from mouse tails, which are more closely related to the bone forming collagen fibers. Results showed comparable results as obtained by the collagenase assay using soluble triple helical type I collagen; The ethanolic SM sample was also here the most potent extract of the four samples tested, showing a 3x greater potency compared to the water extract with an  $IC_{50}$  value of  $0.025 \pm 0.001$  mg/mL (Figure 9). Similarly, the water extract was found to be approximately 10x more potent than the

commercial pills, with an  $IC_{50}$  value of  $0.075 \pm 0.001$  mg/mL when compared to the DP and XLGB pills with  $IC_{50}$  values of  $1.1 \pm 0.2$  and  $1.1 \pm 0.3$  mg/mL, respectively (Figure 9). The results were obtained by hydroxyproline quantification and experiments were conducted in three replicates.



**Figure 9. Inhibition of CatK-mediated insoluble collagen fiber degradation by an ethanolic and aqueous SM extracts as well as Dripping Pill, and XLGB Pill extracts.**

CatK concentration in the assays was 2  $\mu$ M and the digestion was performed at pH 5.5 overnight at 28°C with 1mg of collagen fiber in each reaction (A) The % inhibition of collagenase activity of the inhibitors was quantified on the basis of maximum inhibition in the collagen control, and no inhibition in the CatK control (n=3). (B) The collagenase activity in the presence of extracts were quantified on the basis of hydroxyproline levels in the assay supernatants (n=3).

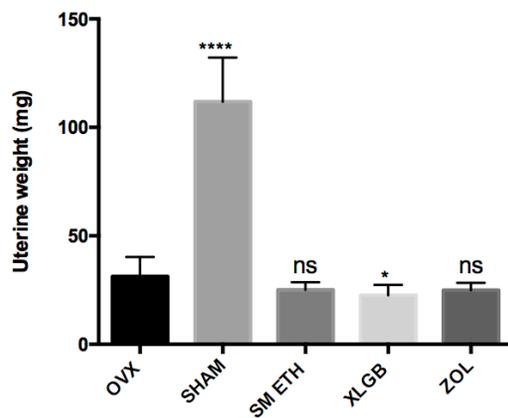
### 3.5. Analysis of anti-resorptive activities of TCM extracts and bisphosphonate in

#### OVX-mouse model of osteoporosis

##### 3.5.1 Analysis of estrogenic effects

Many medicinal herbs may show phytoestrogenic activities, which could cause a CatK-independent anti-resorptive effect. Therefore, the potential effect on the uterus in OVX mice was investigated. As the size and weight of the uterus is significantly reduced in

these mice and an estrogenic effect of the extracts would have been expected to increase the uterus weight, the weight of the organ was determined. The uteri were excised from mice and weighed after 6 months of treatment : SM Eth: 40mg/kg; XLGB: 236mg/kg, ZOL: 0.06mg/kg. OVX resulted in an 72% decrease in uterine weight (Figure 10) compared to the sham group ( $p < 0.001$ ); SM ethanol and ZOL treatment groups did not show a significant difference in uterine weight, suggesting an absence of estrogenic effects of the compounds; XLGB-treatment mice displayed a slight decrease in uterine weight (27.8%) compared to the OVX control group which would even more exclude an estrogenic effect of the herbal treatment (Figure 10).

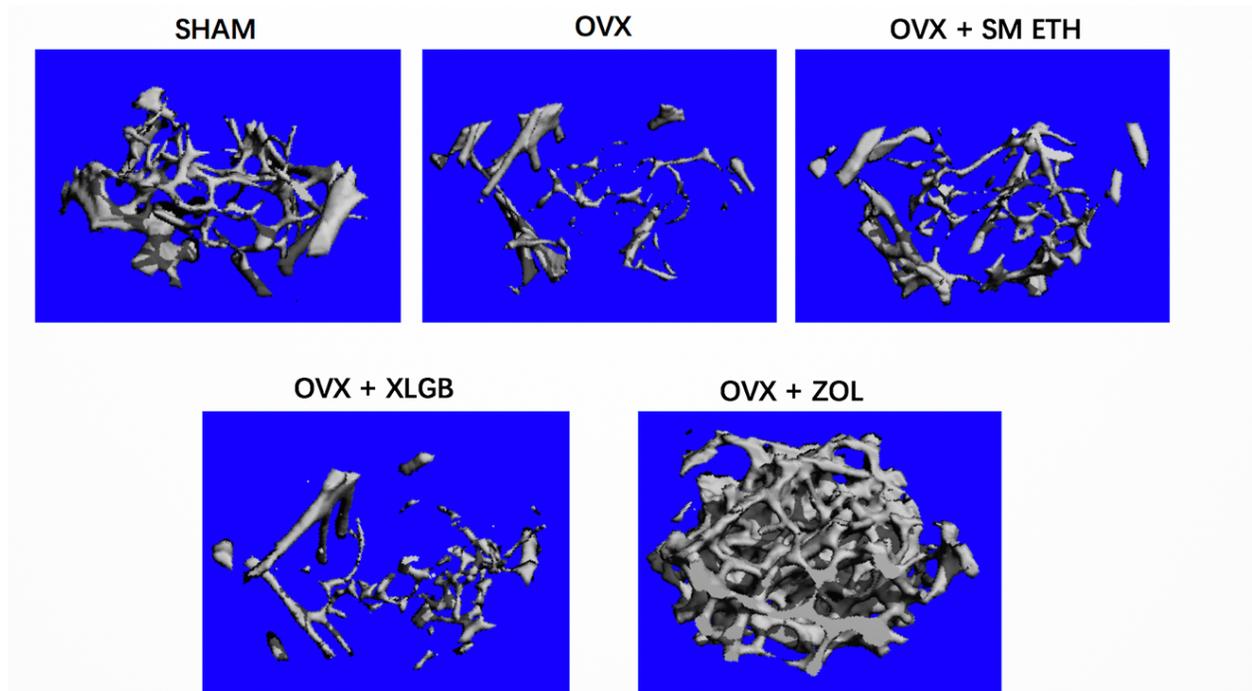


**Figure 10. Comparison of Uterine weight between different treatment groups.** Data represent mean  $\pm$  SD (n = 10). ns, not significant; \*  $p < 0.05$  and \*\*\*\* $p < 0.0005$  significantly different from OVX mice. The various treatments did not increase uterus weight.

### 3.5.2. $\mu$ CT analysis of trabecular femur bones

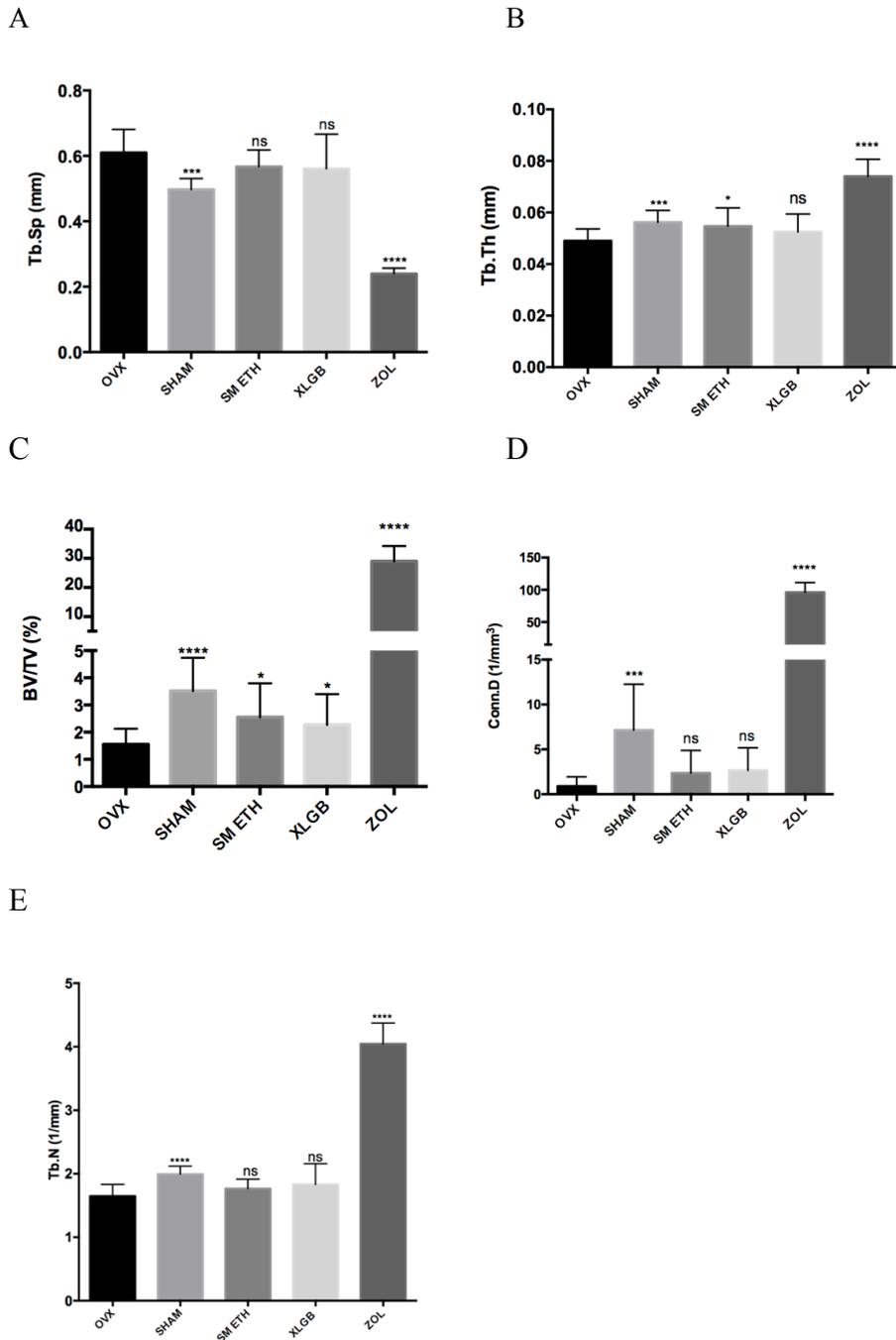
Left femur bones were dissected from all groups of mice and subjected to  $\mu$ CT scans. 3-D reconstruction of the  $\mu$ CT scans showed significant loss of trabecular bones in OVX mice compared to the sham group (Figure 11). The ZOL-treated group showed the

greatest change in bone microstructure compared to all the other treatments, with significantly reduced trabecular separation (Tb.Sp) (60%; Figure 12A) and increased trabecular thickness (Tb.Th) (51%; Figure 11B), BV/TV (Figure 12C), connectivity (Conn.D) (~100 fold; Figure 12D) and trabecular numbers (Tb.N) (~ 3.5 fold; Figure 12E) compared to the OVX group. Mice treated with SM Ethanol extract increased about 60% the BV/TV (Figure 12C) and about 12% in trabecular thickness when compared to the OVX group (Figure 12B). SM ethanol extract treatment group were able to display an upwards trend in connectivity and trabecular number compared to the OVX group (Figure 12 D,E). XLGB treatment group had similar effects on the trabecular parameters as SM ethanol extract by increasing BV/TV by 47% (Figure 12 C) while also increasing trabecular thickness by 7%. The difference between the effects of XLGB on femur bone microstructure compared to the SM ethanol treatment group were more subtle than the effect of ZOL (Figure 10, Figure 11A-E).



**Figure 11. Micro CT 3D reconstruction of trabecular bone**

Representative  $\mu$ CT images of the cross section of distal mice femoral bones showing a loss of trabecular bone in the OVX group and the varying effects of the treatments on trabecular bone microstructure.



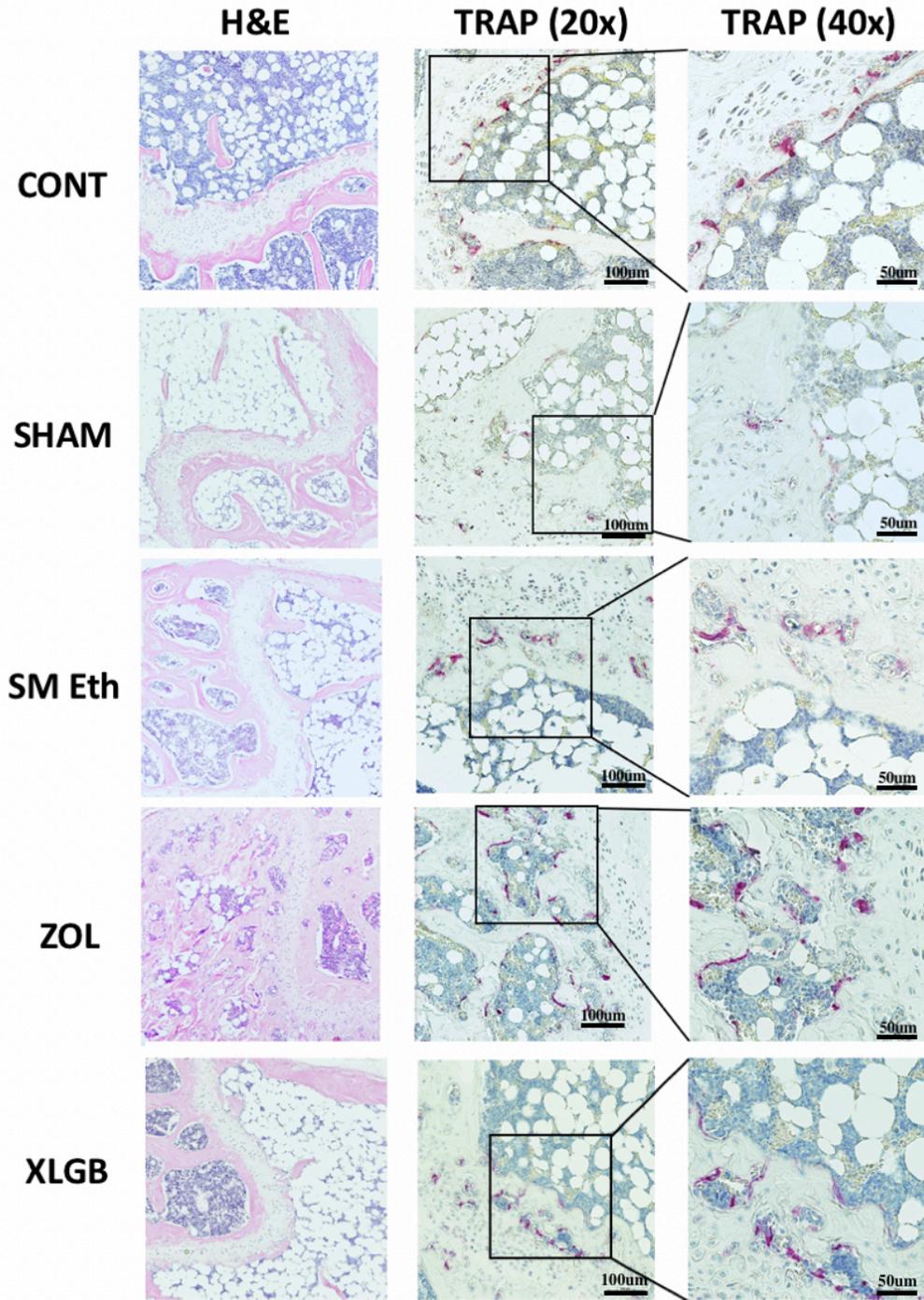
**Figure 12 . μCT parameters of mice femoral bone for different treatment groups (n=10)**  
 Graphs represent quantification of various parameters in different group. (A) trabecular space (Tb.Sp); (B) trabecular thickness (Tb.Th); (C) Bone volume over total volume (BV/TV); (D) Connectivity (Conn.D); (E) trabecular number (Tb.N). Data represent mean ± SD (n = 3). ns, not significant; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.005 and \*\*\*\*p < 0.0001 significantly different from OVX mice.

### **3.5.3 Effect of treatments on osteoclast numbers and bone microstructure**

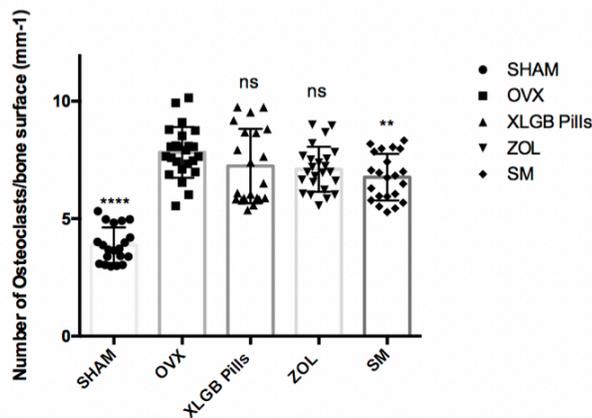
To further investigate the effects of treatments on bone microstructure and resorption, right femur bones were isolated and TRAP positive osteoclasts were identified on transverse sections of the femoral head. Representative H&E staining of the microstructure of the bones showed a loss of trabecular bone in the control (OVX), XLGB, and SM ethanolic extract-treated animals when compared to the SHAM group, with the latter showing a smaller loss compared to the OVX and XLGB group; a substantial increase in trabecular bone was observed in the ZOL-treated mice compared to the SHAM group (Figure 12A). From TRAP stained osteoclasts, not much differences were seen between the treatment groups compared to the OVX control group, whereas SHAM showed substantially lower number of osteoclasts stained compared to the OVX control group. Number of osteoclasts/ bone surface showed a non-significant decrease of 7.4 % and 9.2% decrease in the XLGB and ZOL treatment group compared to the OVX group, respectively (Figure 12B). It is noted from the raw analysis data that in the trabecular region, a higher number of TRAP stained osteoclasts in the ZOL-treated mice was found not to be attached to the surface of the bone compared to the other treatment (Figure 13A). A larger decrease in the number of osteoclast/bone surface was observed in the SM ethanolic extract treatment group to OVX compared to the other treatment groups, displaying a 13.5% ( $p < 0.01$ ) decrease (Figure 12B). Overall a slight decreasing trend in the number of osteoclasts/bone surface is observed in XLGB, ZOL, and SM

ethanolic extract treatment groups compared to OVX, with the latter displaying a significant decrease.

(A)



(B)

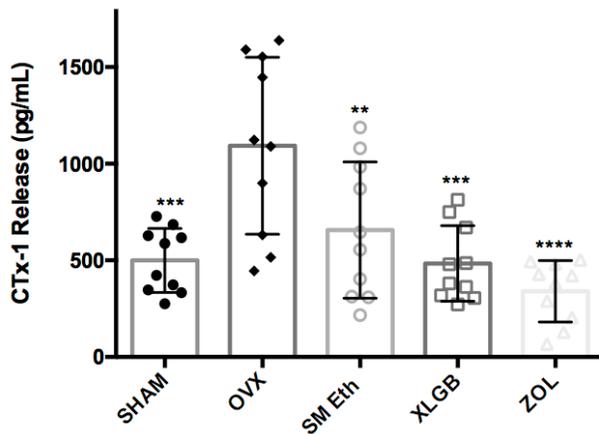


**Figure 13. *In vivo* effect of treatments on osteoclast numbers.** (A) Representative images from femoral head sections of CONT, SHAM, SM ethanol extract, ZOL, and XLGB group. Data shows HE staining (10x) and TRAP staining (20x and 40x) (B) Number of Osteoclasts/bone surface (mm), Data represents mean  $\pm$  SD (n=25 bone slices for each treatment group and control, 10 ROIs/ bone slice taken for analysis). \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  significant versus OVX.

### 3.5.4. Effects of treatments on bone turnover

CTx-1 is a CatK-specific marker for bone resorption and indicator of bone turnover; release of bone resorption marker CTx-1 in plasma was determined using ELISA technique. The ZOL treatment group showed the largest decrease in CTx-1 plasma levels (67.9%; Figure 14) when compared to the OVX group. CTx-1 plasma levels in mice treated with XLGB and SM ethanolic extract also displayed a decrease compared to OVX; XLGB treatment group displayed a larger decrease in CTx-1 plasma levels (54.4%; Figure 14) when compared to SM ethanolic extract treatment group (31.5%; Figure 14). Plasma CTx-1 levels in XLGB treated groups were similar to the plasma

CTX-1 level found in the SHAM group; compared to the SHAM group, SM ethanolic extract-treated mice had a slightly higher mean CTx-1 plasma level (31.1%; Figure 14) while mice treated with ZOL were seen with lower CTx-1 plasma level (32.0%; Figure 14). Overall, all treatment groups (SM ethanol extract, XLGB and ZOL) over a 6 month period were able to elicit a decrease in CTx-1 plasma levels compared to OVX mice.



**Figure 14. *In vivo* effect of treatments on CTx-1 release.** ELISA quantification of CTx-1 release in blood serum of SHAM, OVX, SM ethanol, XLGB and ZOL-treated mice, n=10. Data represents mean  $\pm$  SD, \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  significantly different from OVX treatment.

## Chapter 4. Discussion

CatK active site inhibitors have been widely studied and brought into clinical trials. With increasing interest and research in the use of herbal based medicine in the treatment and management of osteoporosis, our laboratory had previously discovered tanshinone-based ectosteric site inhibitors such as T06 to be potent CatK collagenase inhibitors [62]. Since tanshinones are mostly phenolic compounds in SM, I had performed an ethanol extract in attempt to obtain a crude extract of the various tanshinones. To compare the efficacy of this approach to other treatments available on the market, I compared one of the currently used standard osteoporotic treatments such as ZOL infusion and a prescription drug used in China, XLGB pills with the ethanolic SM extract in an OVX mouse model.

### 4.1 Effects of TCM extracts on the collagenase activity *in vitro*

Evaluating the potency of the four compounds in blocking collagenase activity of CatK, the soluble collagen degradation assays showed that out of the four samples tested, SM Ethanol had displayed the lowest  $IC_{50}$  values and thus the most potent CatK collagenase inhibition followed by the water extract. This was expected as the SM ethanol extract likely has the highest abundance of tanshinone-based compounds as they are hydrophobic and should be found present in the ethanol extract rather than the hydrophilic water extract. Previous studies have shown that natural tanshinone

compounds such as T06 and T12 have potent inhibitory effects on the CatK collagenase activity [32], therefore, it is likely that the potency of the SM ethanol extract is contributed to the tanshinone compounds. Although the SM water extract did not exhibit an as potent CatK collagenase inhibitory effect as the ethanol extract, it was still found to be 10x more potent than the commercial Dripping and XLGP pill water extracts. This was slightly surprising as DP is known to be 70-85% composed of water extracted SM, therefore we would expect it to exhibit similar potency as the SM water extract. The difference in the anti-collagenase activity seen between the DP and SM water extract are likely contributed to the nature of the herb itself. SM, like any herb, can widely differ in its contents depending on the origin of the plant that it was collected, conditions of growth, method of collection. The variations between the preparation of the TCM and SM water extract as well as the origin of the herb itself make it difficult to standardize the various compounds, as well as tanshinones or active compounds within each herbal extract and TCM formulation would likely explain the difference observed in the anti-collagenase activity between the two (Figure 5). An alternative explanation could possibly be that the water extraction of the SM to obtain the DP was performed differently than in our lab, resulting in varying extraction proportions of the hydrophobic and hydrophilic components of SM present in the extract. It could be that our water extract could likely contain more of the hydrophobic tanshinones compounds resulting in a 10x potency difference between the DP and SM water extract. Comparing XLGB and DP, they exhibited approximately similar anti-collagenase effects from the collagenase

assays (Figure 5E). XLGB is known to contain approximately 5-8% SM compared to DP which contains up to 85%; the comparable anti-collagenolytic effect between the two compounds would suggest that other compounds within XLGB may also be contributing to the collagenolytic effect. Previous studies have found that icariin, a main component of *Herba Epimedii*, is pharmacologically active and exhibits an anti-collagenase activity [65]. XLGB is known to be composed of 70% of *Herba Epimedii* in addition to *Radix Dipsaci* (10%), *Salvia miltiorrhiza* (5%) as well as other trace herbs; thus, the abundance of icariin in the XLGB compound is likely to contribute to its CatK collagenase inhibitory effects. The relative potencies of the 4 extracts were confirmed by the results obtained in the insoluble collagen fiber degradation assays (Figure 9), indicating that these compounds can also inhibit degradation of collagen fibers in addition to soluble collagen in a similar manner.

To assess if the inhibition CatK collagenase activity contributed to blocking the catalytic site or exosite, gelatinase and Z-FR-MCA assays were conducted. Although the gelatin assays showed that high concentrations of all four compounds did not inhibit the gelatinolytic activity of CatK, the Z-FR-MCA assay displayed some active site inhibition of three out of the four extracts, with the exception of DP (Figure 8). As noted, the gelatinase assay has a reaction time of 1 hour in comparison to the 5 minute incubation time with the Z-FR-MCA assay. It is possible that these compounds are reversible and have a relatively low affinity for the active site. Therefore with a longer incubation time with the enzyme, there would be increased chance for gelatin to get hydrolyzed by CatK.

Interestingly, DP was shown to even slightly increase active site activity of CatK rather than exhibiting inhibitory effects (Figure 8). As DP contains three other herbs other than SM, a plausible explanation could be that one of the compounds within the other herbs could act as a weak enzyme activator by binding to allosteric sites on the enzyme to increase the Z-FR-MCA activity of CatK. This slight increase in CatK active site activity by DP could also serve as an alternative explanation to the significantly lower potency of DP compared to SM water extract in blocking CatK collagenase activity. The possible presence of a weak enzyme activator in the other herbs present in DP may increase CatK active site activity, off-setting some of the inhibitory effects on CatK.

#### **4.2 Effects of TCM extracts on mice bone microstructure and bone resorption marker**

SM ethanol extract, XLGB pills were orally administered in chow-mix daily and ZOL was injected at three time points (every 8 weeks) over a 24-week treatment period. The efficacy of SM ethanol treatment was seen to be slightly higher than the XLGB treatment, with a significant increase in BV/TV and trabecular thickness compared to the OVX group (Figure 12C); this result aligns with the *in vitro* results obtained for XLGB and SM ethanol extract as the latter had a high anticollagenase activity. However, the effect of SM ethanol extract and the XLGB pills on improving bone microstructure is relatively modest when compared to the ZOL treatment group. In agreement with previous published data for BP on the inhibition of bone resorption [66], femur bones of

mice treated with ZOL were seen to cause a dramatic increase in trabecular numbers, thickness as well as exhibiting a 10-fold increase in BV/TV when compared to the OVX group (Figure 12C). Previous research showed that C57BL/6 (B6) mice are more sensitive than other mice strains to ZOL and other BPs, with response to ZOL in B6 mice being approximately two times shown in BV/TV% compared to A/J mice after 8 weeks treatment (one injection/4 weeks) [66], while ZOL was able to also elicit a 2x increase in BV/TV% compared to the vehicle baseline control. Thus, the dramatic increase in trabecular bone parameters observed in the ZOL treatment group (10x) compared to the SHAM group is likely due to the increased sensitivity for the class of drug for C57BL/6 mice; a longer treatment period in our study (6 months) was performed to further investigate the side effects of these treatments in future studies may also account for the substantial increase in response to ZOL compared to the previous literature of 8 weeks since the baseline bone parameters (SHAM and vehicle treated) would likely decrease with increase age. It should also be noted that SM ethanol extract is derived directly from the SM herb to extract tanshinones, while XLGB is a prescription drug used in China for the treatment of osteoporosis. Achieving comparable results *in vivo* between the two compounds suggests that the hydrophobic components do indeed contain pharmacologically active compounds, most likely tanshinones. When analyzing bone resorption changes between treatments through a histological approach, histological staining of sections was able to allow the visualization of the number of osteoclast at the femoral growth plate. Investigating the anti-resorption activity of these treatments, TRAP

staining results were able to show that compared to the OVX treatment, SM ethanol extract treatment showed a comparable decrease in the number of osteoclasts when compared to the XLGB treatment (Figure 12). This decrease in osteoclast numbers may be attributed to the tanshinone contents within the SM extract. Some studies have shown that some additional bioactive tanshinones to tanshinone IIA sulfonate and DHT-1 such as tanshinone VI have also been able to exhibit antiresorptive effects through directly inhibiting RANKL and induction of NFkB-pathway associated cytokines, thus preventing osteoclastogenesis [67]. Additional tanshinones such as tanshinone IIA, which represents one of the most abundant tanshinones in SM, approximately at 29% of all tanshinones content [68] but does not inhibit CatK collagenase activity [32], have also been shown to exhibit a wider range of anti-resorption activities through the expression of genes controlling osteoclast differentiation, RANKL and prevention of the formation of the sealing zone essential for osteoclast activity by preventing actin ring formation [69]. However, since tanshinones that exhibit anti-resorptive activities are not the major tanshinones found in SM extracts [70], the trace amount of anti-resorptive tanshinones in the crude extract could contribute to the slight decrease in osteoclast number compared to the OVX group.

However, when comparing the CTx-1 plasma content in SM ethanol extract and XLGB treated mice, both extracts revealed decreases in the type I collagen degradation marker CTx-1 when compared to the OVX controls and approach the levels of the SHAM control. The effect of XLGB was slightly stronger than that of SM Ethanol

extract (Figure 13). This suggests that XLGB may have additional effects on inhibiting osteoclast differentiation and thus the overall resorptive activity compared to the SM ethanol extract. The increased suppression of osteoclast activity per osteoclast by XLGB compared to SM could be due to the additional active compounds in XLGB. Herba Epimedii, which makes up 70% w/w of the XLGB capsule, contains the main active constituent icariin [28]. Icariin has been shown to increase expression of a core binding factor responsible for the regulation of osteoblastic activity [28] and thus likely contributing to the anti-osteoporotic effects observed. Additional research has suggested that asperosaponin VI, a main constituent of the Radix Dipsaci (10% w/w of XLGB) also induces osteoblast differentiation, which may also explain for the additional anti-osteoporotic effects seen in the capsule compared to the crude extract [71]. It is also important to note that the SM extract is a crude extract, whereas the XLGB pills are a commercially available TCM prescription on the market; thus additional optimizations in the extraction process of SM as well as chemical modifications of the purified active constituents in SM would likely increase the anti-osteoporotic effects to the same or higher effects as the XLGB pills.

Interestingly, BP treatment (ZOL) did not show a significant difference in osteoclast numbers compared to the OVX treatment group, however was able to substantially suppress osteoclast resorption activity shown through a decrease in CTx-1 ELISA (Figure 13). Previous studies have shown that nitrogen BPs (ZOL is also a nitrogen BP) such as ibandronate and alendronate under low dosing regimen showed increase in osteoclast

numbers compared to the vehicle administered control, likely due to increased RANKL concentration induced by these BPs [72], [73]; however in that study, it was also noted that ZOL and high dose ibandronate showed no difference in osteoclast numbers when compared to the saline-injected control [72], similar to what we observed in the ZOL treatment group (Figure 12B). This absence of an increase in osteoclast numbers seen in ZOL-treated mice seems to be due to a higher number of apoptotic osteoclasts observed in ZOL-treated mice [73], thus offsetting some of the effects of increased RANKL-associated osteoclastogenesis. It is known that nitrogen containing BPs mainly inhibit bone resorption activity through interfering with the mevalonate pathway and majorly disrupting osteoclast resorption function [74] and not through directly inducing osteoclast apoptosis which is a result from the accumulation of cytotoxic analogues of ATP within the osteoclast, thus explaining for the unchanged osteoclast numbers in ZOL-treated mice while still eliciting the highest anti-resorptive effects compared to all other treatment groups through a substantial decrease in CTx-1 levels (Figure 13). This overall decrease in osteoclast activity and bone resorption is visualized through the increase in bone mass observed from the  $\mu$ CT analysis as well as the histology sections. When analyzing the histology sections, it is interesting to note that in some bone sections of the ZOL treatment group, TRAP staining displayed some larger osteoclasts that were not attached to the bone surface; this osteoclast phenotype is likely due to possible early onset of osteopetrosis, a common side effect observed with chronic use of BPs [75] as a result of disrupting the osteoclast-osteoblast cross talk. Although BPs are a major standard care of

treatment for osteoporosis, the presence of side effect is the reason for our research in investigating CatK tanshinone-based inhibitors.

Previously, our lab had tested the pure synthetic tanshinone, T06, which was able to restore bone microstructure to a similar state as the SHAM treatment group. T06 is a hydrophilic derivative of tanshinone IIA in addition to having preferential binding to the ectosteric site, which would likely explain the difference in observed efficacies with a combined increased bioavailability of the derivative [76] as well as selective binding to CatK in preventing the collagenase activity of CatK. The presence of antiresorptive effects shown from the crude SM extract in the *in vivo* and *in vitro* results of this thesis along with our previously tested pure tanshinone compounds suggests that with chemical modifications to the structure of tanshinones, highly effective antiresorptive drugs can be generated with less adverse effects and without disrupting the osteoblast-osteoclast cross talk by targeting only the collagenase activity of CatK.

### **4.3 Possible estrogenic effects**

Some natural herbal medicine such as red clover, ginseng have been thought to help manage post-menopausal symptoms by exhibiting estrogen-like activities [77]. In the presence of a phytoestrogen, uterine weights would increase while a loss of estrogen would decrease the uterine weights as seen with the OVX group when compared to the SHAM group (Figure 10). Results indicated that uterine weights were not affected by the treatment groups administered at 40 mg/kg/d for SM ethanol and 236 mg/kg/d for XLGB

pills (Figure 10), showing no significant differences between the treatment groups and the OVX group. Thus at the concentration administered *in vivo*, the possible estrogenic effects of the compounds contributing to the improvement in bone trabecular parameters can likely be excluded. Additional ELISA for plasma estradiol concentrations and sex hormone binding protein, globulin, could be conducted to confirm this finding.

#### **4.4 Conclusion**

CatK has been an attractive drug target for the treatment of osteoporosis for the recent decade and various enzyme inhibitors have been tested in hope of delivering an anti-osteoporotic therapy with minimal side effects. Previously, TCM and herbal-based compounds such as some of the tanshinones found in SM roots have been able to exhibit anti-osteoporotic activity through an ectosteric site inhibition of the CatK enzyme. XLGB pills have also been used conventionally in China for the treatment of osteoporosis; therefore, the first part of the thesis through *in vitro* experiments serves as a preliminary comparison of the crude herbal ethanol and water extracts as well as the extracts of the XLGB pills to compare the anti-resorptive activities of these mixtures through inhibition of CatK activity. The second part of my thesis focuses on the *in vivo* experiments in OVX mice, serving as a further general comparison of the standard of care bisphosphonates used on the western market, the conventionally used XLGB pills in the TCM market and the crude ethanol SM extracts. The comparable anti-resorptive activities of XLGB pills

compared to the crude ethanol SM extracts in both the *in vitro* and *in vivo* experiments is encouraging for future tanshinone research for anti-osteoporotic treatments.

#### **4.5 Future work and direction**

This thesis has compared osteoporosis treatments on the Chinese (XLGB, Dripping pills) and western markets (BP) with a *Salvia miltiorrhiza* extracts. To further elucidate and better compare the effects of herbal-based XLGB pills, SM extracts, future studies need to be conducted to:

1. HPLC fingerprint the major known active compounds in these formulations (XLGB and SM extract) and perform a parallel comparison between these two compounds to further explain the relative potencies in inhibition osteoclastic resorption.
2. Use scanning electron microscopy (SEM) of the insoluble collagen fibers after treatments to further visualize and compare the inhibitory effects of these treatments on the morphological changes of collagen fibers

Additional chemical modifications to purified compounds such as T06 and other tanshinone derivatives to optimize the effect of the inhibitory effects on bone resorption would be the ultimate goal, in which the potency could be comparable to standard of care osteoporotic drugs such as bisphosphonates and active-site CatK inhibitors with less adverse effects.

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