

Investigating the Underlying Molecular Mechanisms of Yunke on Bone Metastases from Prostate Cancer

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Objective: To explore analgesic effect and bone repair mechanism of non-radioactive technetium-99 conjugated with methylene diphosphonate (⁹⁹Tc-MDP, brand name, Yunke) on bone metastases (BM).

Procedures: In vivo experiment, mouse BM models of prostate cancer RM-1 cell were constructed and divided into Control, Yunke, ⁹⁹Tc+SnCl₂ and MDP groups based on medicine composition. Tumor specimens were inspected for size, X-ray, microCT and histopathology. In vitro experiment, with Cell Counting Kit-8 (CCK8), scratch, clone, apoptosis, Polymerase Chain Reaction (PCR) and Western Blot experiments, effects of Yunke on RM-1 cells and osteoclast-related cells were observed.

Results: In vivo experiment, there was no difference in tumor size between Yunke and control group. Contrasted with control group, in Yunke group, trabecular spacing (Tb.Sp) of tumor bone was lower, bone volume/total volume (BV/TV) on marrow cavity and bone cortex were higher. Tunnel staining showed that positive rate of apoptosis in Yunke group was higher than that in control group. Ki67 staining showed that Yunke could not inhibit proliferation of tumor cells. In vitro experiment, CCK8 and scratch experiments showed that Yunke neither can inhibit proliferation nor can inhibit migration of RM-1 cells. High concentration of Yunke promoted late apoptosis of RM-1 cells. Yunke could inhibit BMM cell proliferation, differentiation of osteoclasts, and osteoclast-related transcription factors. Yunke displayed different degrees of inhibitory effects on MAPKs signaling pathway during osteoclast differentiation. It had obvious inhibitory effects on osteoclast-related transcription factors, such as cFOS, NFATC1, ACP-5, CTSK, D2 and MMP-9, the strongest inhibitory effects were observed with ACP-5, CTSK and D2. Yunke also displayed different degrees of inhibitory effects on protein activities of JNK, pERK, ERK and pP38.

Conclusion: Yunke cannot inhibit the proliferation and migration of RM-1 cells, so we think it is not recommended for the treatment of primary tumors and prevention of occurrence of tumors metastatic to bones. The mechanism of therapeutic effect of Yunke on BM by inhibiting proliferation of BMM, inhibiting MAPKs signal transduction and activation of transcription factors during differentiation process of BMM-derived osteoclasts, inhibiting number and size of osteoclasts, inhibiting bone resorption and protecting bone destruction through enhancing bone hardness and bone mass. Thereby, we believe that Yunke is more suitable for promoting the repair induced by BMs, delaying its progression and reducing the occurrence of SREs.

Keywords: ⁹⁹Tc-MDP, bone metastases, molecular mechanisms, RM-1, prostate cancer

Introduction

The BM are common in prostate cancer (PCa) patients with an incidence rate up to 70%,¹ which mainly involve the axial and proximal appendicular skeleton, and a 5-year survival rate less than 15%. BM can cause skeletal-related events (SREs) such as pain and pathological fractures, seriously affecting the quality of life and survival rate of patients. Therefore, early diagnosis of BM is critically important. At present, the most commonly used method for diagnosing BM is radionuclide bone scan with (99m) technetium-methylene diphosphonate (99mTc-MDP) as an imaging agent, which can target the bone lesion. MDP is one kind of bisphosphonates, and bisphosphonate has been effective in prevention and treatment of SREs on various late malignant tumors

since its Introduction. It can bind to the sites with metabolically active and inhibit the activity of osteoclasts to protect the bone. Currently, the third-generation bisphosphonates mainly include zoledronic acid and ibandronate sodium. Finianos² summarized the therapeutic effect of zoledronic acid and pointed out that its use had the benefit of delaying SREs, but did not benefit the overall survival rate of PCa patients, and was also prone to cause some serious side effects such as osteonecrosis and nephrotoxicity.³ Another commonly used RANKL monoclonal antibody, denosumab, similarly to zoledronic acid, reduces SREs associated with bone lesions or metastases in patients with advanced solid tumors. However, it also has a similar worse treatment emergent adverse events,⁴ with a nephrotoxicity rate of 17%, neutropenia of 15%, and jaw osteonecrosis of 4%. Therefore, some patients cannot benefit from the above treatment. In clinical practice, we have found that non-radioactive ⁹⁹Tc-MDP, technetium-99 conjugated with methylene diphosphonate (trade name, Yunke, a new drug patented in China, Patent No. ZL94113006.1; [Supplementary Figure 7](#)), can target BM and show good analgesic effects and some bone repair in treatment of BM in PCa, breast cancer and lung cancer.⁵⁻⁷ ([Supplementary Figure 1](#)), and its effect is relatively mild and a few serious adverse events are observed.⁸

However, the mechanism of Yunke toward BM is not well defined. The existing studies believe that the mechanism is that the main active components of Yunke are technetium (⁹⁹Tc) and MDP. Sha Liu⁹ proposed in a study of 64 cases of rheumatoid arthritis treated by Yunke that the changes in the valence state of the technetium can remove free radicals in the body, protect the active of superoxide dismutase (SOD), inhibit the production of pathological complexes, prevent free radicals from destroying tissues, and inhibit the production of inflammatory mediators and immune factors interleukins, inhibit the production and release of prostaglandins E and histamin, and play an anti-inflammatory and immune-regulating roles. MDP, like other bisphosphonates, can inhibit osteoclast activity and interfere with tumor metastasis.^{5,10} Starting from clinical practice, the aim of this paper is to explore the mechanism of Yunke on tumor by constructing BM animal models of PCa in mice and in vitro cell experiments.

Materials and Methods

The research was divided into in vivo and in vitro experiments. In vivo experiment, mouse BM models of prostate cancer RM-1 cell were constructed and divided into four groups, including Control, Yunke, ⁹⁹Tc+SnCl₂ and MDP based on the medicine composition. The tumor specimens were inspected for size, X-ray, microCT and histopathology. In vitro experiment, we performed CCK8, scratch, clone, apoptosis, PCR and Western Blot experiments to observe the effects of Yunke and its components on the proliferation, migration and apoptosis of RM-1 cells, and the effects on BMM proliferation, osteoclast differentiation, expression of related transcription factors, and protein activity on the MAPK signaling pathway in the process of osteoclast differentiation.

The details of whole experimental materials and process of experiments were stated in [supplementary material and methods](#).

Results

Effects of Yunke on C57 Mouse BM Model of PCa RM-1 Cell

Twenty-eight C57 mice, weighing 21.56±1.07g before drug intervention, weighed 22.64±1.22g after administration for four times and before the establishment of BM model, and were administrated continuously for six times. Mice were sacrificed when the long diameter of the tumor grew to about 1cm, and the weight was 24.40±1.49g. Body weights of mice increased steadily throughout the course of the experiment ($F=35.35$, $P=0.00$). The long diameter of the right tibial tumors were 11.79±1.75mm in Yunke group, 11.15±2.01mm in ⁹⁹Tc+SnCl₂ group, 11.61±1.02mm in MDP group, and 13.04±1.26mm in control group. Although from the data, tumor size of control group was slightly larger than that of drug intervention groups, but there was no statistically significant difference between them ($F=1.89$, $P=0.16$). This result preliminarily indicated that Yunke and its ingredients could not inhibit the growth of tumors on the gross specimens.

Subsequently, the tibial tumor specimens were scanned with microCT. Quantitative analysis of trabecular number (Tb.N), trabecular spacing (Tb.Sp), trabecular thickness (Tb.Th), bone volume/total volume (BV/TV) of marrow and BV/TV of cortical bone in each group was determined by CT Analyser (Version: 1.14.4.1). The Results showed ([Figure 1](#)) the Tb.Sp in Yunke (478.40±51.58um) and MDP (461.63±49.72um) groups were lower than that in control group (574.20±107.70um) ($P=0.04$, $P=0.02$). Additionally, BV/TV of marrow (3.25±0.67%) and bone cortex (39.22±6.29%) in Yunke group were higher

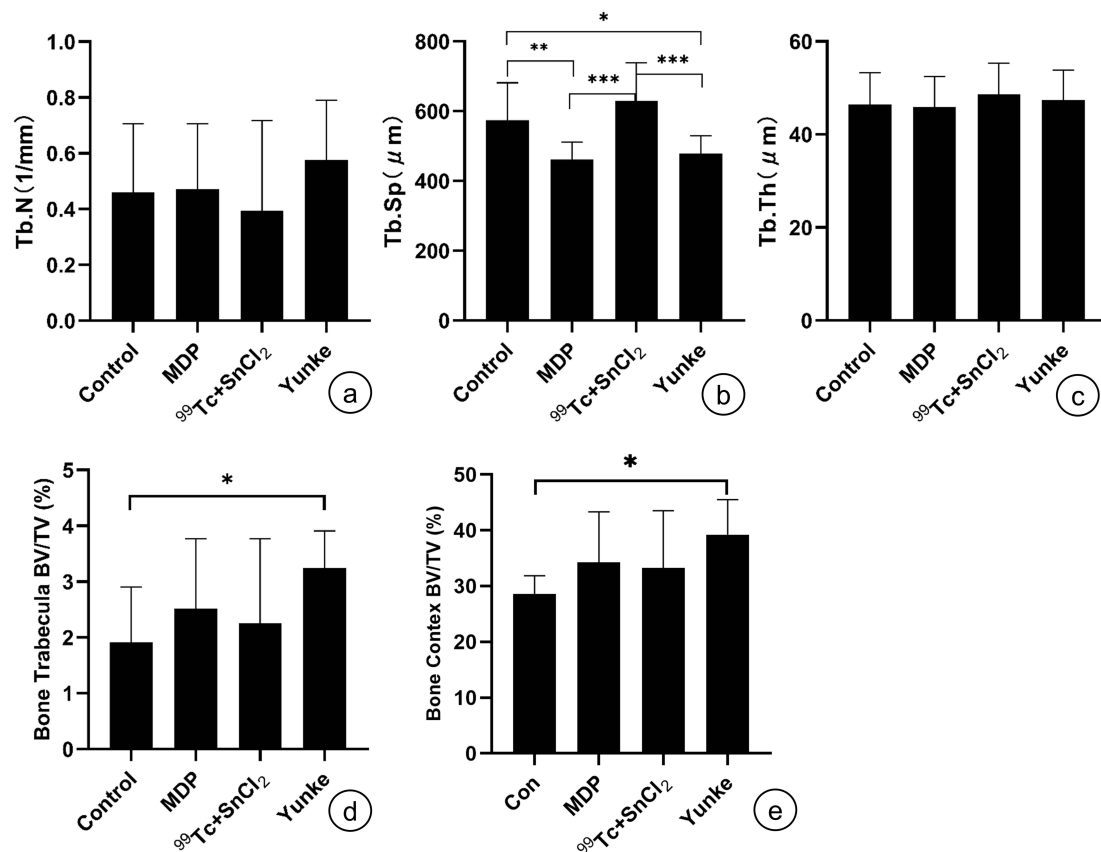


Figure 1 The bone morphology and densitometric quantitative analysis indicators of prostate cancer RM-1 cell tibial bone metastases. The Tb.Sp in Yunke and MDP groups were lower than that in control group (b). BV/TV of marrow (d) and bone cortex (e) in Yunke group were higher than in control group. However, the Tb.N (a) and Tb.Th (c) in four groups have no differences. *indicates that the P value is less than or equal to 0.05, which means there is a significant difference between the two sets of data; **indicates that the P value is less than or equal to 0.01, that is, the difference between the two sets of data is very significant; ***indicates that the P value is less than or equal to 0.001, indicating that the difference between the two sets of data is extremely significant.

than in control group (bone marrow, $1.91 \pm 0.99\%$; bone cortex, $28.57 \pm 3.27\%$) ($P=0.04$, $P=0.04$). The above results indicated that although Yunke and MDP could not inhibit the proliferation of tumor, they could inhibit bone resorption and had a certain protective effect on bone destruction.

All specimens were routinely pathologically sectioned, and stained by TRAP (Figure 2) to count osteoclast cells, stained by Ki67 and TUNEL (Figure 3) to observe proliferation and apoptosis of tumor cells. The results of osteoclast count showed that, compared with control group (106.43 ± 11.34), Yunke (32.43 ± 5.09) and MDP (65.29 ± 9.73) had a certain inhibitory effect on the proliferation of osteoclasts in bone marrow cavity and cortex ($F=220.35$, $P=0.00$). The pathological sections (Figure 2c) demonstrated that although $^{99}\text{Tc}+\text{SnCl}_2$ (3.14 ± 1.07) group had the most prominent inhibitory effect on osteoclasts, the X-ray still showed bone destruction of the mouse tibia, and there was some contradiction between them. TUNEL staining revealed the apoptosis rates in Yunke ($79.00 \pm 9.56\%$), MDP ($30.57 \pm 12.45\%$) and $^{99}\text{Tc}+\text{SnCl}_2$ ($74.14 \pm 15.40\%$) groups were higher than those in control group ($15.43 \pm 5.13\%$) ($F=54.87$, $P=0.00$), indicating that all of them promoted tumor cell apoptosis, the effect of MDP was weaker than that of other two groups. Ki67 staining revealed Yunke and MDP could not inhibit tumor cell proliferation ($P=0.98$, 0.87), while $^{99}\text{Tc}+\text{SnCl}_2$ had a significant inhibitory effect ($P<0.0001$).

Effects of Yunke on PCa RM-1 Cell

Effect of Yunke on the Proliferation of RM-1 Cells

The results of in vivo experiments showed that Yunke and MDP could not inhibit the proliferation of tumor. This conclusion was further verified by in vitro experiments in this study. Yunke and MDP with different concentration gradients from 25 to

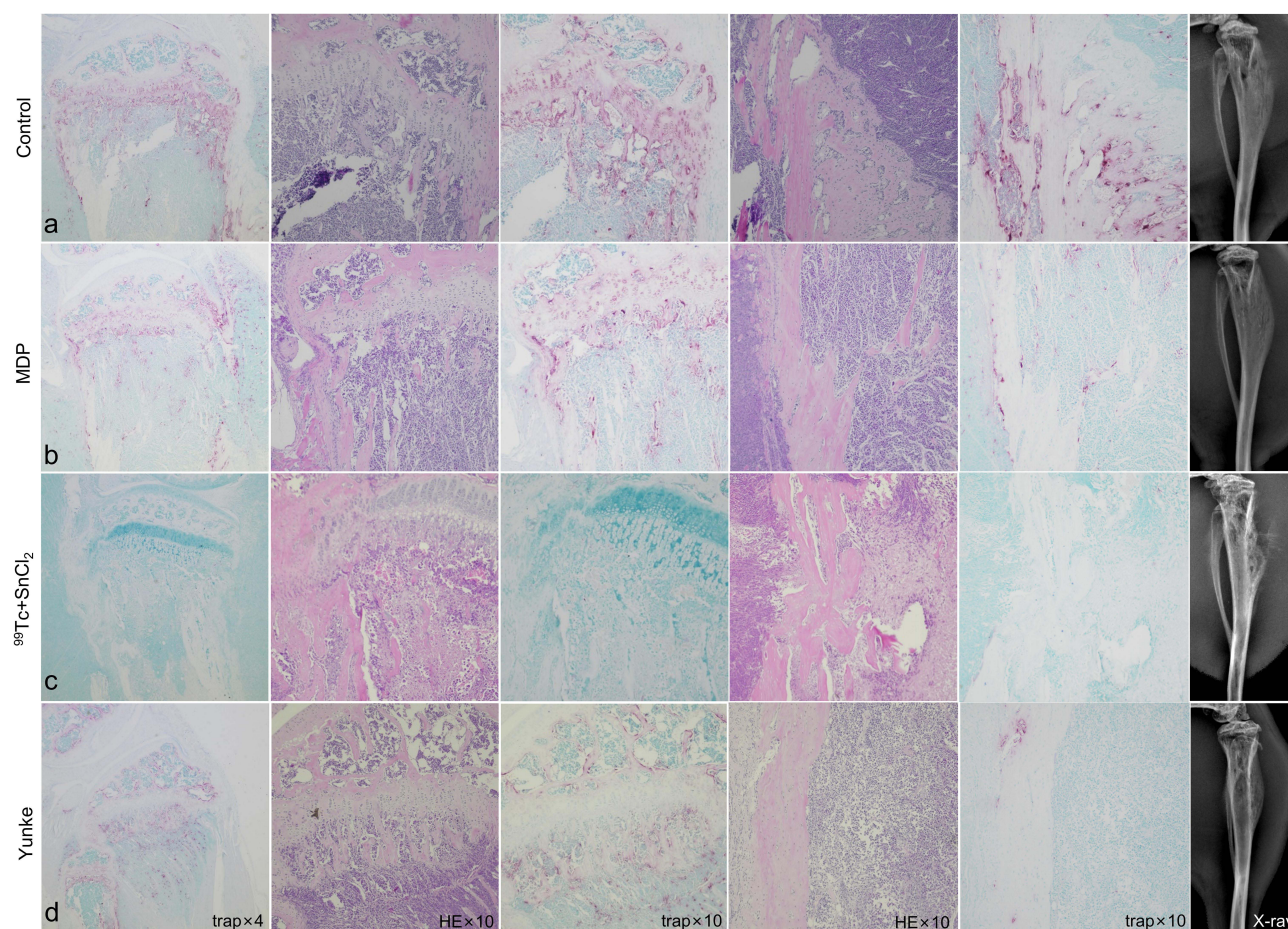


Figure 2 Osteoclast cells were counted in the TRAP-stained sections. Compared with control group (a), MDP (b) and Yunke (d) had a certain inhibitory effect on the proliferation of osteoclasts in bone marrow cavity and cortex; although $^{99}\text{Tc}+\text{SnCl}_2$ (c) group had the most prominent inhibitory effect on osteoclasts, the X-ray still showed bone destruction of the mouse tibia.

800umol/L could not inhibit the proliferation of RM-1 cells, and there was no statistical difference compared with control group ($P>0.05$) (Supplementary Figure 2). While the inhibition of $^{99}\text{Tc}+\text{SnCl}_2$ against the proliferation of RM-1 cells displayed a time- and concentration-dependent behavior, which was remarkably increased with the extension of time and enhancement of drug concentration (100, 200, 400 and 800umol/L), and the proliferation inhibition rates were 35.86%, 33.08%, 45.51% and 89.09%, which were obviously higher than that of control group (all $P<0.05$). Meanwhile, the cell cloning experiment (Supplementary Figure 3) showed that 400umol/L Yunke (179.00 ± 18.24) (Supplementary Figure 3e), 200umol/L ^{99}Tc (103.67 ± 14.50) (Supplementary Figure 3f), 200 (21.33 ± 5.51) and 400umol/L (2.33 ± 0.58) $^{99}\text{Tc}+\text{SnCl}_2$ (Supplementary Figure 3h and i), 200 (12.00 ± 1.73) and 400umol/L (2.33 ± 0.52) SnCl_2 (Supplementary Figure 3j and k), all had a certain inhibitory effect on RM-1 cell clones, and CFEs (Supplementary Figure 3l) were 35.83%, 37.03%, 4.29%, 0.83%, 7.62% and 0.83% respectively. Compared with control group (214.17 ± 20.18) (Supplementary Figure 3a), there were significant statistical differences ($F=76.39$, $P<0.05$). Similar to the results of CCK8, metal ions, especially SnCl_2 , had a significant inhibitory effect on the clonal proliferation of RM-1 cells. Although compared with control group, MDP (200umol/L, 160.67 ± 24.03 ; 400umol/L, 179.00 ± 18.25) (Supplementary Figure 3b and c), 200umol/L Yunke (113.00 ± 31.58) (Supplementary Figure 3d), and 400umol/L ^{99}Tc (130.67 ± 14.98) (Supplementary Figure 3g) had no significant statistical difference in the inhibition of tumor cell clone ($P>0.05$), but their tumor colonies were remarkably smaller than in control group. Therefore, Yunke still has a slightly inhibitory effect on the clonal proliferation of RM-1 cells.

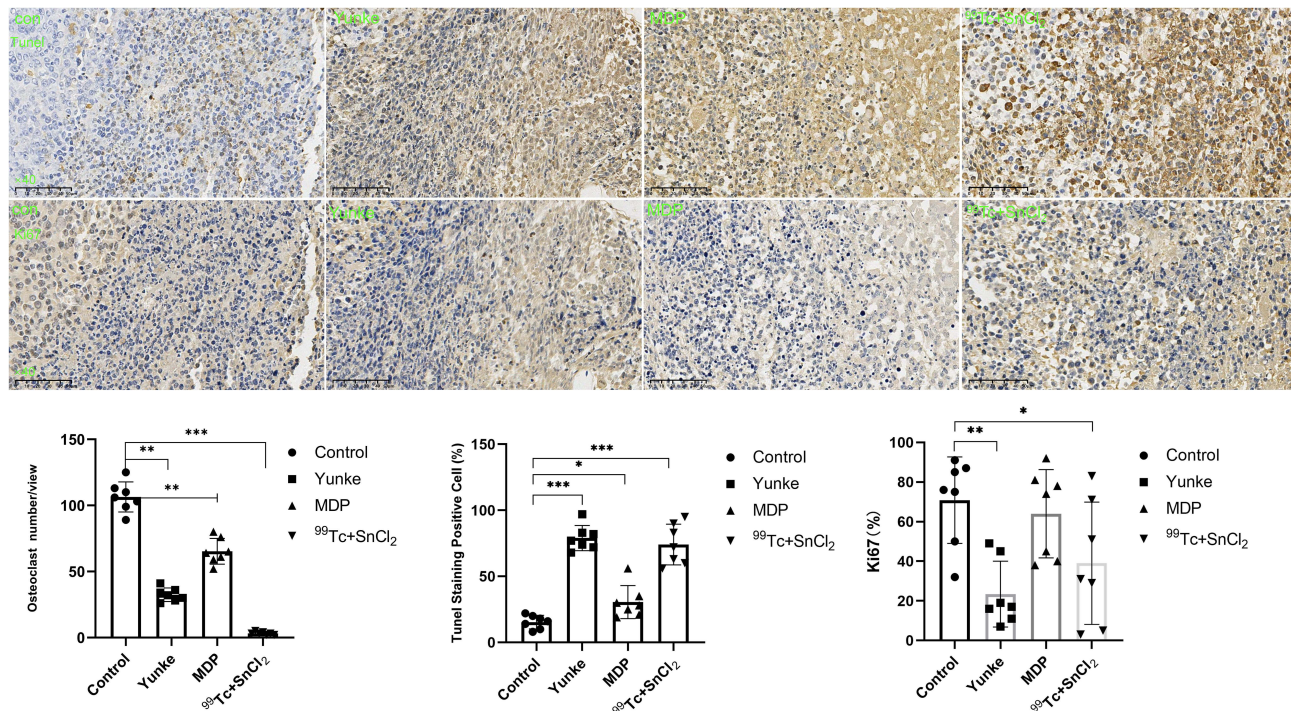


Figure 3 TUNEL (top row) and Ki67 (middle row) staining and quantification of TUNEL, Ki67-positive tumor cells. TUNEL staining revealed the apoptosis rates in Yunke, MDP and ⁹⁹Tc+SnCl₂ groups were higher than those in control group; Ki67 staining revealed Yunke and MDP could not inhibit tumor cell proliferation ($P=0.983, 0.872$), while ⁹⁹Tc+SnCl₂ had a significant inhibitory effect ($P<0.0001$). *indicates that the P value is less than or equal to 0.05, which means there is a significant difference between the two sets of data; **indicates that the P value is less than or equal to 0.01, that is, the difference between the two sets of data is very significant; ***indicates that the P value is less than or equal to 0.001, indicating that the difference between the two sets of data is extremely significant.

Effects of Yunke on the Migration Ability of RM-1 Cells

The results of 24h scratch test showed (Supplementary Figure 4), compared with control group, neither Yunke nor MDP could inhibit the migration of RM-1 cells ($P>0.05$), contrasting to the inhibitory of ⁹⁹Tc and ⁹⁹Tc+SnCl₂ to the migration of tumor cells. The highest inhibition was found with 400umol/L ⁹⁹Tc+SnCl₂.

Effects of Yunke on the Apoptosis of RM-1 Cells

Compared with control group (Supplementary Figure 5n), 400 (Supplementary Figure 5j), 200umol/L (Supplementary Figure 5k) Yunke, 100–800umol/L ⁹⁹Tc+SnCl₂ (Supplementary Figure 5e–h), and 50 (Supplementary Figure 5d), 200 (Supplementary Figure 5b), 400umol/L (Supplementary Figure 5a) MDP, all of them indicated various degrees of apoptosis in the tumor cells, especially the late apoptosis. Among them, 400 (Supplementary Figure 5f), 800umol/L (Supplementary Figure 5e) ⁹⁹Tc+SnCl₂ showed the strongest effect, Yunke at a concentration of 400umol/L also significantly promoted late apoptosis of RM-1 cells. However, 100umol/L MDP (Supplementary Figure 5c), 50umol/L ⁹⁹Tc+SnCl₂ (Supplementary Figure 5i), and low concentrations of Yunke (Supplementary Figure 5m and 5l) had no effect on the apoptosis of tumor cells.

In summary, similarly to the results of in vivo experiments, Yunke could not inhibit the proliferation and migration of RM-1 cells, while it could promote the late apoptosis of tumor cells. In view of this, our study further explored the effects of Yunke on osteoclasts.

Effects of Yunke on Osteoclasts

Effect of Yunke on Osteoclast Differentiation from BMM to Osteoclasts

Our study observed the effects of Yunke, MDP, SnCl₂ and ⁹⁹Tc with different concentration gradients from 0.1 to 12.5umol/L on osteoclast differentiation from BMM to osteoclasts. The results showed Yunke and its ingredients presented some inhibitory effects on osteoclast differentiation from BMM to osteoclasts with increasing drug concentrations (Figure 4). Compared with the control group, the osteoclast differentiation was inhibited at a concentration as small as 0.1umol/L.

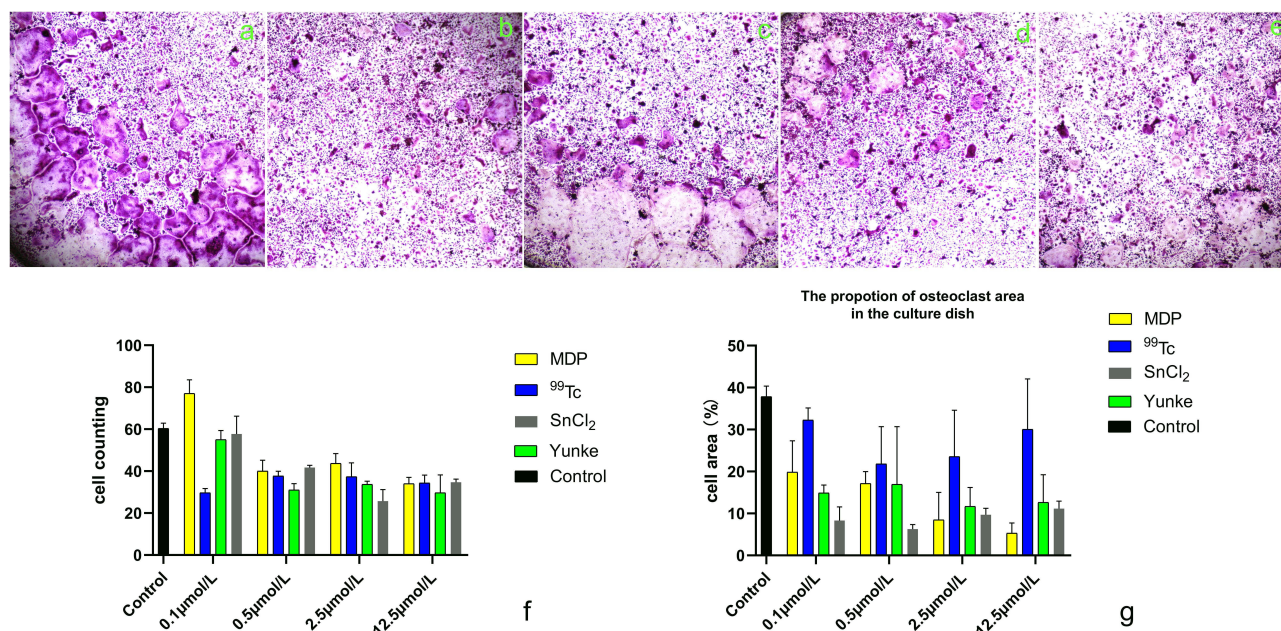


Figure 4 Compared with control group (a), the effects of MDP (b), ⁹⁹Tc (c), SnCl₂ (d), Yunke (e) with 12.5 μmol/L on inhibiting the number and size of osteoclast differentiation from BMM to osteoclasts. The lower panel (f and g) was with different concentrations.

However, compared with the control group, Yunke, MDP and SnCl₂ had no effect on the number of osteoclasts ($P=0.36$, 0.23 , 0.65), but the size of osteoclasts was significantly inhibited ($P=0.03$, 0.05 , 0.00). A 0.1 – 12.5 μmol/L ⁹⁹Tc had no significant inhibitory effect on the size of differentiated osteoclasts ($P>0.05$), but the number of osteoclasts was significantly less than that of control group ($P<0.05$). Drugs at concentrations of 0.5 and 2.5 μmol/L could effect the number of differentiated osteoclasts ($P=0.02$, 0.02), and inhibition by Yunke and metal ions were obvious ($P<0.05$). As showed in Figure 4f, excepting the lowest concentration of MDP, 0.5 – 12.5 μmol/L MDP presented some inhibition of the number of differentiated osteoclasts, and the inhibitory effect was also enhanced with increase of the concentration ($P=0.02$). Although there was no statistically significant difference in inhibition of the number of differentiated osteoclasts between 12.5 μmol/L of each group versus control ($P=0.13$), but this drug concentration showed an obvious inhibitory effect on the size of differentiated osteoclasts ($P=0.02$). The inhibitory effect of Yunke on the size of osteoclasts was not enhanced with the increase of concentration, but highly potent on the number of osteoclasts ($P=0.02$). The inhibitory effects of MDP on the number and size of osteoclasts were enhanced with the increase of drug concentration ($P=0.02$, 0.02). Overall, Yunke and its components played different roles in inhibiting osteoclast differentiation from BMM to osteoclasts.

Effect of Yunke on Cell Proliferation of BMM

The effects of Yunke, MDP, SnCl₂ and ⁹⁹Tc with different concentration of 5 – 40 μmol/L on cell proliferation of BMM were observed. The results showed (Supplementary Figure 6) that, compared with control group, 40 μmol/L MDP and Yunke presented obvious inhibition of cell proliferation of BMM, and the inhibitory effect was also enhanced with increase of the concentration, and the inhibition rates reached 78.41% and 78.73% . However, metal ions had no obvious inhibitory effect on BMM cells, which was basically consistent with the results of in vivo experiments. It was also confirmed from the cellular level that Yunke acted by inhibiting bone destruction through its major component MDP.

Effects of Yunke on the Express of Osteoclast-Related Transcription Factors

The results of BMM cells stimulated for five days (Figure 5) showed that, compared with Con+R group, excepting that ⁹⁹Tc+SnCl₂ had no obvious inhibitory effect on cFOS, 25 μmol/L Yunke and its components all exhibited obvious strong inhibition on cFOS, NFATC1, ACP-5, CTSK, D2, MMP-9 ($P<0.05$), and the strongest inhibitory effect was observed with ACP-5, CTSK and D2. This indicated that Yunke could effectively inhibit the express of osteoclast-related transcription factors.

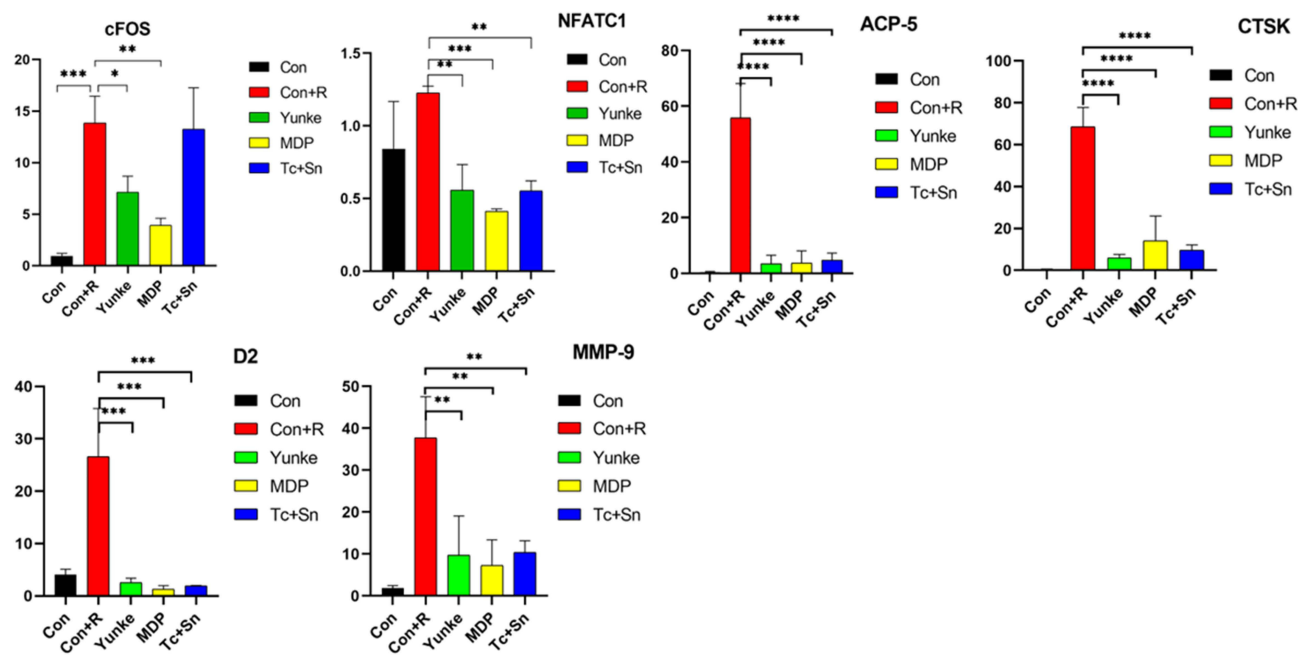


Figure 5 Yunke and its components all had obvious inhibitory on the express of osteoclast-related transcription factors, such as cFOS, NFATC1, ACP-5, CTSK, D2, MMP-9.

Effects of Yunke on the activity of ERK, JNK and P38 in the MAPK signaling pathway during osteoclast differentiation

The WB results of effects of Yunke and its components on the MARPs signals mediated by three different RANK receptors (ERK, JNK and P38) (Figure 6) showed that, compared with control group, excepting without inhibition on the activity of P38 and pJNK, Yunke presented different degrees of inhibition on JNK, pERK, ERK and pP38. The highest

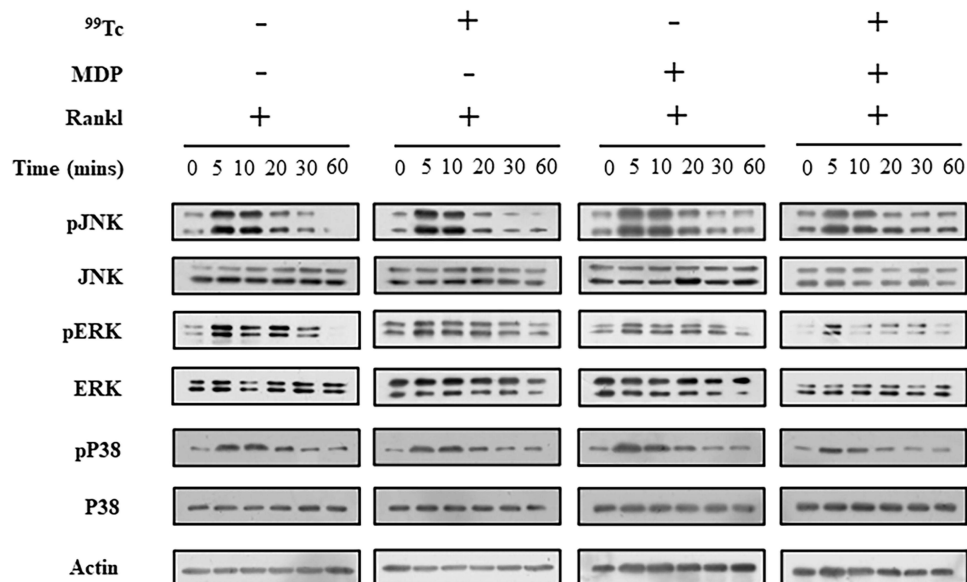


Figure 6 Effects of Yunke and its components on the activity of ERK, JNK and P38 in the MAPK signaling pathway during osteoclast differentiation. Compared with control group, the activity of JNK, pERK, ERK and pP38 exhibited different degrees of inhibition by Yunke. JNK and pERK only exhibited weak inhibition by MDP. There was no significant inhibit on the above proteins by ^{99}Tc . *indicates that the P value is less than or equal to 0.05, which means there is a significant difference between the two sets of data; **indicates that the P value is less than or equal to 0.01, that is, the difference between the two sets of data is very significant; ***indicates that the P value is less than or equal to 0.001, indicating that the difference between the two sets of data is extremely significant; ****indicates that the P value is less than 0.0001, which is very strong evidence that there is a significant difference between the two sets of data.

inhibitory effect was observed on pERK, exerting a certain inhibitory at 0 min, reaching to the peak at 10–30min, disappearing at about 60min. MDP only presented weak inhibitory effect on JNK and pERK, and the effect disappeared after 20min. ^{99}Tc had no significant inhibit on the above proteins.

Discussion

In a large amount of clinical practices, Yunke has been effectively used in the treatment of many autoimmune and orthopedic diseases, such as rheumatoid arthritis, hyperthyroidism with infiltrating exophthalmos, gout and other endocrine and metabolic diseases. It can inhibit BM and has an analgesic effect. It can also be used in preventing and treating osteoporosis, preventing fractures. Additionally, Yunke also has a therapeutic effect on aseptic osteonecrosis.^{8,11–14} It is currently thought that ^{99}Tc , the main component in Yunke, is the product in the decay of $^{99\text{m}}\text{Tc}$, has a valence of +4 with active chemical property, and easily gain and lose an electron to become +3 or +5. Gain or loss of electrons can remove free radicals in human body and enhance autoimmunity. MDP is one of bisphosphonates that targets osteoclasts, thus suppressing the function of osteoclasts, and thereby reducing bone resorption.¹⁵ Tassone et al¹⁶ indicated that zoledronic acid had effect on PCa cells by suppressing the proliferation and induction of apoptosis in vitro. Yunke is one kind of bisphosphonates but labeled with nuclide. The specific mechanism of its therapeutic effect on BM in clinical practices remains unclear. In this study, we investigated its specific mechanism both in vitro and in vivo.

“Guidelines for Follow-up and Health Management of Breast Cancer in China (2022 Edition)”¹⁷ recommends that breast cancer patients with low bone mass, osteoporosis or high-risk factors who receive endocrine drug aromatase inhibitor therapy, ovarian castration or chemotherapy, in addition to improve the lifestyle, should accept appropriate drug management in time. These patients should take in vitamin D and calcium supplements, be treated with denosumab or bisphosphonate, of which bisphosphonate treatment can last for five years. Referring to this guideline, we administered Yunke for bone health management in advance before establishing the BM model in mice. The drugs were totally administrated for 10 times. The size of tibial BM was measured, and the results showed that there was no significant statistical difference in tumor size between the three drug-intervened groups and control group. This indicated that Yunke and its components could not inhibit tumor growth in vivo. Zhang JM¹⁸ used Yunke to observe its effect on the Walker 256 cancer bone invasion and osteolysis rat model, and reported that there was also no difference in tumor weight between control group and Yunke group. Next, we performed Ki67 staining on the pathological sections of tumor tissue. The results showed that Yunke and MDP could not effectively inhibit the proliferation of tumor cells, while $^{99}\text{Tc}+\text{SnCl}_2$ with significant inhibitory effect. We analyzed this may be associated with the formation of Yunke after the conjugation between ^{99}Tc and MDP, the toxic effect of individual metal ions on cells disappeared, so the inhibitory effect on tumor cell proliferation was significantly weakened. Therefore, from the in vivo experimental results, we believe that Yunke act as tumor suppressor not by inhibiting tumor cell proliferation. Meanwhile, our study also verified this corollary from in vitro experiments. The results of CCK8 proliferation experiment of RM-1 cells showed that, compared with control group, Yunke and MDP with different concentration gradients could not inhibit the proliferation of RM-1 cells. However, 400umol/L Yunke still presented some inhibitory effect on the clonal proliferation of tumor cells with the CFE of 35.83% in the cloning experiment. MDP has no obvious inhibitory effect, but the tumor cell colony size under MDP action was still smaller than that of control group from the visual view. $^{99}\text{Tc}+\text{SnCl}_2$ displayed a significant inhibitory effect and enhanced with the increase of time and concentration, and the proliferation inhibition rate reached 89.09% on the 4th day. The colony experiment also showed the same result with the CFE of 0.83%–7.62%, in which SnCl_2 played the main role. The results were consistent with that of in vivo experiments. We thought this was related to the cytotoxicity of metal ions.¹⁹ Wang YC²⁰ et al reported that the proliferation inhibition rate of ^{99}Tc -MDP at 50, 100 and 200umol/L on human breast cancer MDA-MB-231 cells were 36.00%, 37.00% and 57.10% respectively, which had an obvious inhibitory effect. Zhang JM¹⁸ used Yunke to observe its effect on the Walker 256 cancer bone invasion and osteolysis rat model, the results of flow cytometry showed Yunke induced apoptosis while inhibiting cell proliferation in transplanted tumor cells. However, our results were different from the above two. Only 400umol/L Yunke showed a weak inhibitory rate on the clonal proliferation of tumor cells. One of the reasons of different results may be related to the different cells types. Prostate cancer cells are more malignant than breast cancer cells and prone to BM. In addition, Wang YC only from in vitro experiments and Zhang JM only from in vivo experiments, and both only used one method (flow cytometry) to

indicate the inhibitory effect of Yunke on the proliferation of tumor cells, lacking of comprehensive researches. In our study, the effects of Yunke on tumor cells were repeatedly verified using pathological immunohistochemical staining, CCK8 and clonal proliferation experiments *in vivo* and *in vitro*. Compared with previous researches, our conclusions are more reliable. Additionally, the drug components of Yunke were studied separately. MDP, its main component, had a similar effect on proliferation of tumor cells as Yunke, which was consistent with previous reports that bisphosphate exerts therapeutic effects not by inhibiting the proliferation of tumor cells.²¹ In summary, we believe that Yunke cannot effectively inhibit the proliferation of PC RM-1 cells, so it is not recommended for the treatment of primary tumors.

One of the mechanisms of BM is the ability of malignant tumor cells to leave the primary site and migrate to bone tissue.^{22,23} We studied the effects of Yunke on the migration ability of RM-1 cells. The results of 24h scratch test showed that Yunke and MDP could not inhibit the migration of tumor cells, while ⁹⁹Tc and ⁹⁹Tc+SnCl₂ could. This may be related with the direct killing effect of metal ions.¹⁹ Therefore, we considered that the therapeutic effect of Yunke on BM does not act by inhibiting migration of tumor cells; therefore, it is also not recommended for prevention of BMs. Wang YC²⁰ proposed that ⁹⁹Tc-MDP could directly induce the apoptosis of human breast cancer MDA-MB-231 cell line with dose-dependent. The research of Zhang JM¹⁸ also found Yunke could cause DNA damage before Walker 256 cells enter the DNA synthesis phase, arrest cells at the G0/G1 phase, reduce S-phase entry and induce apoptosis during this phase. Our study also observed Yunke, ⁹⁹Tc+SnCl₂ and MDP could induce apoptosis of RM-1 cell, especially the late apoptosis, similarly with the dose-dependent manner. ⁹⁹Tc+SnCl₂ showed the strongest effect at the 400 and 800 μmol/L concentration followed by 400 μmol/L Yunke. At the same time, we performed the TUNEL staining on specimens to observe the apoptosis of tumor cells. The results were basically consistent with *in vitro* experiments, the positive rates of apoptosis in Yunke, ⁹⁹Tc+SnCl₂ and MDP group were higher than that in control group. This results indicated Yunke and its components differently promoted tumor cell apoptosis, in which Yunke with the strongest effect, MDP with the weakest effect.

In summary, the results of *in vivo* and *in vitro* experiments all indicated that Yunke could not inhibit the proliferation and migration of prostate cancer RM-1 cells but could induce the late apoptosis. Therefore, we considered that Yunke exert its therapeutic effect on BMs not by directly acting on tumor cells. Bone metastasis is a multistage process.^{22,23} The circulating tumor cells can settle at the normal vessels of bone marrow and the endosteal niches, remaining in a dormancy state. They may become active after several years, cause bone resorption with osteoclasts and bone formation with osteoblasts, finally promote bone destruction. Therefore, our study further explored the effect of Yunke and its components on osteoclasts.

After microCT scans performed on tibial prostate cancer BM samples, we quantitatively analyzed the five main parameters of bone morphology and density. The results showed the Tb.Sps of Yunke and MDP group were lower than that of control group, but there was no significant difference between Yunke and MDP. Additionally, the BV/TVs of tumor bone trabecular and cortical bone in Yunke group were higher than that of control group. Thus, from the perspective of quantitative analysis, we considered Yunke and MDP inhibited bone resorption by enhancing bone hardness and increasing bone mass, and had protective effect on bone destruction with the effect of Yunke more evident. Soares et al²⁴ studied the long-term effects of tumor doses of zoledronic acid on jaws and femurs in young rat model. They found that compared with control group, the BV/TV and Tb.Th in treated group were increased, while the Tb.Sp decreased. Ozasa et al²⁵ applied ibandronate to treat the osteoporosis model of ovariectomized aged rats, and also found that bisphosphonates could increase bone BV/TV. These results were consistent with ours. In our study, we further stained osteoclasts for TRAP activity and counted the number in the pathological specimens, the results also showed the numbers of osteoclasts in bone marrow cavity and cortical area in Yunke and MDP groups were significantly lower than that in control group, which indicated that Yunke and MDP both have obvious inhibitory effects on osteoclasts, and the inhibitory effect of Yunke is higher than that of MDP. In summary, the *in vivo* experiments, no matter from the perspective of cell or bone quantitative analysis, suggested that Yunke inhibits bone resorption by inhibiting the production of osteoclasts, enhancing bone hardness and increasing bone mass, and has a protective effect on bone destruction.

Osteoclasts are multinucleated giant cells formed by the fusion of BMMs differentiated from myeloid progenitor cells in the bone marrow. The early immature proliferative BMM is called osteoclast precursor, which enters blood circulation under the action of chemical factors, and then enters the cavity of bone structure under the action of signaling factors released by the basal multicellular unit. Under the stimulation of signaling factors such as chemokines, transcription factors and cytokines,

they fuse into multinucleated cells and eventually are activated into osteoclasts.²⁶ Therefore, our study explored the effect of Yunke on the proliferation of BMM cells in vitro experiments. The results presented 40 $\mu\text{mol/L}$ MDP and Yunke had obvious inhibitory effect on the proliferation of BMM, and the inhibitory effect was more obvious with prolonged time. The inhibitory rate reached 78.41% and 78.73%, and there was no difference between the two. However, ^{99}Tc and SnCl_2 had no obvious inhibitory effect on BMM cells, which was basically consistent with the results of in vivo experiments. Additionally, Osteoclast differentiation is regulated by RANK, nuclear factor- κB (NF- κB), mitogen-activated protein kinase (MAPK) and other signal mediation.^{27,28} These pathways are involved in numerous cellular developmental processes, including cell growth, development, division and differentiation. Among these signaling pathways, various transcription factors such as cFOS, NFATC1, ACP-5, CTSK, D2, MMP-9 play a key role in regulating the differentiation of osteoclasts.^{29–31} Thus, in our study, we found that Yunke and its components can effectively inhibit the expression of the above-mentioned osteoclast-related transcription factors, with the strongest inhibitory effect observed with ACP-5, CTSK and D2, thereby inhibiting the differentiation of osteoclasts. Furthermore, we studied the effect of Yunke on three different RANK-mediated MAPKs signaling, namely receptors p38, JNK and ERK which translocate to the nucleus for phosphorylation the above transcription factors and promote osteoclasts differentiation,^{32,33} which 3 important parallel pathways in MAPK family. The results showed that Yunke inhibited the activity of JNK, ERK, pERK and pP38 to different extents, however, without inhibitory effect on pJNK and P38. The strongest inhibitory effect was found with pERK, reaching to the peak at 10–30min, and then gradually disappears with time. MDP only showed a weak inhibitory effect on JNK and pERK, and the effect disappears after 20min. This is compatible with the above results of the inhibitory effect of Yunke and MDP on transcription factors. This indicates Yunke the activation of MAPK signaling pathways inside or/and outside the cell nucleus, with the most obvious inhibitory effect observed on pERK in the nucleus, thereby inhibiting the differentiation and growth of osteoclasts. However, ^{99}Tc has no effect on the activities of the three upstream proteins, and only directly inhibit the transcription factors activated after the protein translocating to the nucleus. Gong W³⁴ studied the mechanism of 0.01 $\mu\text{g/mL}$ ^{99}Tc -MDP on RANKL-induced osteoclastogenesis, in vitro experiments found that ^{99}Tc -MDP could inhibit the expression of MMP-9, cFOS and CTSK, and also inhibit the activation of MAPK signaling in RAW264.7 cells after RANKL stimulation. Shen S³⁵ reported ^{99}Tc -MDP with 0.001–100 $\mu\text{g/mL}$ different concentrations all had inhibitory effects on RAW264.7 cell line, induce cell apoptosis and decreased the expression of RANK-related proteins. Compared with previous reports, our research is more comprehensive and meticulous to explore the effects of ^{99}Tc -MDP on the main signaling pathways and transcription factors related to osteoclasts differentiation. The final differentiated osteoclasts were also affected by Yunke. The results showed that Yunke and its components with 0.1–12.5 $\mu\text{mol/L}$ different concentrations presented different degrees of inhibition on osteoclast differentiation from BMM to osteoclasts, with the dose-dependent manner. Yunke, MDP and SnCl_2 can not only inhibit the number of generated osteoclasts but also make the size of osteoclasts significantly smaller than that of the control group. The inhibitory effect of SnCl_2 is relatively weak, while ^{99}Tc mainly inhibits the number of generated osteoclasts. In summary, at the cellular level, we believe that Yunke and its components play different roles in inhibiting the proliferation of BMM cells, the MAPKs signal transduction and the activation of transcription factors during the process of osteoclasts differentiation of BMM cells, and the number and/or size of generated osteoclasts.

In conclusion, this study comprehensively and systematically expounded the effect of Yunke on BMs of PCa from in vivo and in vitro experiments, from the perspectives of cell proliferation, cloning, migration, apoptosis, bone quantitative analysis of specimens and pathology. Yunke cannot effectively inhibit the proliferation and migration of tumor cells but can induce its late apoptosis. Therefore, the therapeutic effect of Yunke on BMs is not by directly killing tumor cells, so it is not recommended for the treatment of primary tumors and prevention of occurrence of tumors metastatic to bones. Our research shows that Yunke play a role of therapy by in inhibiting the proliferation of BMM cells, the MAPKs signal transduction and the activation of transcription factors during the process of osteoclasts differentiation of BMM cells, and the number and/or size of generated osteoclasts, by enhancing bone hardness and increasing bone mass, and having a protective effect on bone destruction. Thereby, we believe that Yunke is more suitable for promoting the repair induced by BMs, delaying its progression and reducing the occurrence of SREs. Our present study provided an important theoretical ground for Yunke to treat BMs of malignant tumors and is of great significance for its clinical application. There are some limitations in the study, such as only one cell line involved. It may be that some underlying mechanisms have not yet been explored. We are going to expand and improve our research.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable institutional and/or national guidelines for the care and use of animals were followed. Animal experiments were approved by the Ethics Committee of the Tenth People's Hospital of Shanghai (ID Number SHDSYY-2023-6462).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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