NVP-BHG712 against postmenopausal osteoporosis in mice by targeting Cathepsin K

Yueran Zhao[1], Xin Liu[1], Shuang Liu[1], Huanxin Sun[1], Jing Zhang[1], Ming Li[1], Wendi Chen[1], Yuxuan Shi[1], Yongzhi Cao[1], Gang Lu[2], and Yunna Ning[1]

[1]Shandong University
[2]The Chinese University of Hong Kong School of Biomedical Sciences

November 21, 2022

Abstract

Background and Purpose Recent evidence suggests that postmenopausal osteoporosis is associated with increased bone resorption function in osteoclasts, of which Cathepsin K is a key molecule. In this study, we aimed to screen small molecule compounds targeting Cathepsin K and evaluate whether they affect osteoclast bone resorption function. Experimental Approach We screened out the small-molecule compound NVP-BHG712 targeting CTSK by molecular docking, and studied its pharmacological effect on bone resorption function of osteoclasts. To this end, we evaluated bone mass changes in postmenopausal mice by μCT, ELISA, and H&E staining. In addition, we also investigated the effects of NVP-BHG712 on osteoclast differentiation, bone resorption function and expression of osteoclast differentiation related factors in vitro. Key Results Surprisingly, we found that oral NVP-BHG712 treatment significantly reduced bone loss in postmenopausal mice. In vitro osteoclast culture, it was found that this effect was achieved by inhibiting osteoclast differentiation and bone resorption. Meanwhile, NVP-BHG712 significantly decreased the expression of genes related to osteoclast differentiation, including CTSK, MMP9, CTR, IP3R1, IP3R3, and OC-STAMP. Conclusion and Implications The present findings suggest that NVP-BHG712 reduces bone resorption function by inhibiting osteoclast differentiation, and is a potential drug for preventing and treating postmenopausal osteoporosis and other diseases.

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Running title

NVP-BHG712 against osteoporosis

Authors

Xin Liu[1,2,3], Shuang Liu[1,2,3], Huanxin Sun[1,2,3], Jing Zhang[1,2,3], Ming Li[1,2,3], Wendi Chen[1,2,3], Yuxuan Shi[1,2,3], Yongzhi Cao[1,2,3], Gang Lu[4], Yunna Ning[1,2,3,*], Yueran Zhao[1,2,3,*]

[1]Center for Reproductive Medicine, Shandong University, Jinan, Shandong, 250012, China

[2]Key laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, Shandong, 250012, China

[3]National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, Shandong, 250012, China

[4]CUHK-SDU Joint Laboratory on Reproductive Genetics, School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China

[*]Address correspondence to: Yunna Ning, Dr, Center for Reproductive Medicine, Shandong University, Jinan, Shandong, 250012, China. Email: yunna_ning@163.com

Yueran Zhao, PhD, Center for Reproductive Medicine, Shandong University, Jinan, Shandong, 250012, China. Email: *yrzhao@sdu.edu.cn*

Word count

4737.

Acknowledgements

Authors received funding for this work from The National Key Research and Development Program of China (No. 2017YFC1001002). All of these authors have no disclosures in relations to this manuscript. We acknowledge the Center for Reproductive Medicine for supporting the experiment.

The research reported here was not pre-registered in an independent, institutional registry.

Data Availability

All data presented in this article will be shared upon request.

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

Abstract

Background and Purpose

Recent evidence suggests that postmenopausal osteoporosis is associated with increased bone resorption function in osteoclasts, of which Cathepsin K is a key molecule. In this study, we aimed to screen small molecule compounds targeting Cathepsin K and evaluate whether they affect osteoclast bone resorption function.

Experimental Approach

We screened out the small-molecule compound NVP-BHG712 targeting CTSK by molecular docking, and studied its pharmacological effect on bone resorption function of osteoclasts. To this end, we evaluated bone mass changes in postmenopausal mice by μCT, ELISA, and H&E staining. In addition, we also investigated the effects of NVP-BHG712 on osteoclast differentiation, bone resorption function and expression of osteoclast differentiation related factors in vitro.

Key Results

Surprisingly, we found that oral NVP-BHG712 treatment significantly reduced bone loss in postmenopausal mice. In vitro osteoclast culture, it was found that this effect was achieved by inhibiting osteoclast differentiation and bone resorption. Meanwhile, NVP-BHG712 significantly decreased the expression of genes related to osteoclast differentiation, including CTSK, MMP9, CTR, IP3R1, IP3R3, and OC-STAMP.

Conclusion and Implications

The present findings suggest that NVP-BHG712 reduces bone resorption function by inhibiting osteoclast differentiation, and is a potential drug for preventing and treating postmenopausal osteoporosis and other diseases.

Keywords

Osteoporosis; Osteoclast differentiation; Cathepsin K; NVP-BHG712

Introduction

The dynamic balance between bone formation and resorption is necessary to maintain bone homeostasis[Chen, Wang, et al., 2018); Matsuoka, Park, Ito, Ikeda, and Takeshita (2014); (Zaidi, 2007]. Due to internal and external factors, the disturbance of this dynamic balance leads to an increase in bone resorption activity and the occurrence of compensatory bone diseases, such as postmenopausal osteoporosis (PMPO) and rheumatoid arthritis (RA)[Eastell et al., 2016]. Therefore, inhibition of osteoclast resorption activity may be an effective method for treating pathological bone loss in these diseases[Chen, Zhi, Cao, et al., 2017; Chen, Zhi, Pan, et al., 2017; Chen, Zhi, et al., 2018; Zhi et al., 2018]. Osteoclasts are multinucleated giant cells that differentiate from mononuclear macrophages; osteoclasts are located on the surface of bone tissue and perform bone resorption functions by synthesizing and secreting a variety of organic acids and proteases to dissolve bone salts and degrade organic matter[Boissy, Saltel, Bouniol, Jurdic, & Machuca-Gayet, 2002]. In the process of osteoclast differentiation, they are stimulated by two essential cytokines, namely, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL)[Fumoto, Takeshita, Ito, & Ikeda, 2014; Park, Lee, & Lee, 2017]. The binding of the cytokine M-CSF to the receptor c-Fms maintains the survival and proliferation as well as the differentiation of BMMs into osteoclast precursor cells. The cytokine RANKL binds to the receptor RANK to promote the differentiation of osteoclast precursor cells into mature osteoclasts[Fumoto et al., 2014], and this interaction induces osteoclast differentiation and the expression of function-related genes, including CTSK, MMP9 and CTR.

CTSK is an important osteoclast enzyme that degrades type I collagen, and CTSK inhibitors have been used in the treatment of osteoporosis for many years[Ho et al., 2009; Hou et al., 1999; Reiser, Adair, & Reinheckel, 2010; Schilling et al., 2007]. Odanacatib, which is a drug that inhibits CTSK function in osteoclasts, has been abandoned by the Desert East Company because it increases the occurrence of cardiovascular events[McClung et al., 2019; Mullard, 2016; Saag et al., 2021]. A large amount of evidence has shown that CTSK inhibitors can effectively inhibit bone resorption by osteoclasts[Lotinun et al., 2013; Mukherjee & Chattopadhyay, 2016]. Therefore, the identification of potential CTSK inhibitors will facilitate the development of treatments for bone metabolic diseases.

NVP-BHG712, which is as a specific EphB4 inhibitor, has attracted much attention because of its effects in inhibiting tumor metastasis[Becerikli et al., 2015; Li et al., 2021; Troster et al., 2018]. Angiogenesis plays a key role in bone formation and osteoporosis[Filipowska, Tomaszewski, Niedźwiedzki, Walocha, & Niedźwiedzki, 2017; Kusumbe, Ramasamy, & Adams, 2014; Ramasamy, Kusumbe, Wang, & Adam
A recent study reported that NVP-BHG712 inhibited angiogenesis[You et al., 2017]. However, the role of NVP-BHG712 in osteoclast formation remains unclear. In our study, the results suggested that NVP-BHG712 may act as a potent inhibitor of osteoclast formation to improve bone loss in ovariectomized mice.

Methods

Molecular docking . To screen CTSK-targeting compounds, we downloaded crystal conformations from the Protein Data Bank database and screened CTSK-targeting compounds from more than 9,000 Selleckchem compounds. The binding pocket of CTSK (Fig. 1A) to which the compounds bind was identified according to the resolution, and the conformation of CTSK while bound to the known ligands 5TDI, 4DMY, 3KWZ, and 4X6H was selected according to the binding strength. According to the physical characteristics of the compounds, the compound NVP-BHG712, which binds to the CTSK molecule, was selected.

Animals . All animals care, ethical principles and experimental procedures were approved by the Cheeloo College of Medicine of Shandong University (Shandong, China). Animal studies are reported in compliance with the ARRIVE guidelines and with the recommendations made by the*British Journal of Pharmacology* . C57BL/6JN mice (6-8 weeks of age) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). To evaluate the effect of NVP-BHG712 on bone mass in OVX females, OVX

females underwent ovariectomy at 8 weeks of age. The mice were randomly divided into 6 groups (5 mice in each group): the Sham group, OVX group, and 5 mg kg^{-1}, 10 mg kg^{-1}, 20 mg kg^{-1}, and 40 mg kg^{-1} NVP-BHG712 groups. In the Sham group, the ovaries of the mice were only exposed to the surrounding adipose tissue, while in the OVX group and different NVP-BHG712 groups, both ovaries were removed from the mice. After one week of postoperative recovery, the Sham and OVX groups were treated with 0.5% CMC-Na, and the NVP-BHG712 group was treated with NVP-BHG712 dissolved in 0.5% CMC-Na. After 6 weeks, bilateral femurs and blood were collected from the mice for analyses.

Cell culture . The RAW264.7 cell line was purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China, RRID: CVCL_0493) and authenticated by STR profiling. The cells were cultured in DMEM medium (Gibco, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Solarbio, Beijing, China) at 37°C in a humidified atmosphere with 5% CO_2.

BMMs were obtained as described previously[(Gooi, Chia, Vrahnas, & Sims, 2019; Maridas, Rendina-Ruedy, Le, & Rosen, 2018; Stern et al., 2012] and were cultured in α-MEM (Gibco, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Solarbio, Beijing, China) at 37°C in a humidified atmosphere with 5% CO_2.

CCK-8 . According to the reagent standard, BMMs were incubated in 96 well plates ($1x10^4$ cells per well) for 24 hours. BMMs were cultured in induction medium containing M-CSF (30 ng/ml) (R&D systems, Mn, USA) and RANKL (50 ng/ml) (R&D systems). Then, different concentrations of NVP-BHG712 (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 μM) were added and incubated for 72 hours. Then, CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well and incubated for another 2 h before the absorbance was measured.

TRAP staining . TRAP staining for the detection of mature osteoclasts was performed using a tartrate resistant acid phosphatase kit (Servicebio) according to the manufacturer's instructions. The number of TRAP+ cells per mm^2 was counted.

F-actin ring staining . BMMs were fixed in 4% paraformaldehyde and stained with F-actin (Beyotime Biotechnology). DAPI was used to stain the nuclei. Images were acquired using light and fluorescence microscopes.

Bone resorption . BMMs were seeded on bone plates (Corning, NYC, USA) and treated with M-CSF, RANKL and NVP-BHG712. The cells were removed from the bone plates on day 7. Images were acquired using light and fluorescence microscopes.

Western blotting . BMMs were harvested and subjected to Western blotting assays, which were performed as described previously[(Hu et al., 2019; Sun et al., 2019)], and the following antibodies were used for immunoblotting: anti-CTSK (Abcam, ab19027, RRID: AB_2261274), anti-MMP9 (Abcam, ab76003, RRID: AB_1310463), anti-CTR (Abcam, ab11042, RRID: AB_297696), anti-β-actin (Proteintech, 66009-1-Ig, RRID: AB_2687938), anti-rabbit IgG (Cell Signaling Technology (CST), #7074, RRID: AB_2099233), and anti-mouse IgG (CST, #7076, RRID:AB_330924).

RNA isolation and qPCR . BMMs were harvested, and total RNA was isolated using a RNeasy kit (Spark Jade). An aliquot of 1 μg of total RNA was subjected to reverse transcription with a SPARK script II reverse transcription PCR kit (Spark Jade) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green Master Mix (Roche). mRNA expression levels were calculated by the method using GAPDH as an internal control and further normalized to the mean expression level of the control group. The primers were as follows: mouse-MMP9: forward, 5'-CGTCGTGATCCCCACTTACT-3', and reverse, 5'-AACACACAGGGTTTGCCTTC-3'; mouse-CTR: forward, 5'-CCTGCAGA TGCCCAGTGAAG-3', and reverse, 5'-TGTGTAGTAGCCCTGCTCCC-3'; mouse-IP3R1: forward, 5'-AACTGTGGGACCTTCACCAG-3', and reverse, 5'-AACTCTCGCCAGTTTCTGG-3'; mouse-IP3R2: forward, 5'-GTTACAGGATGTCGTGGCCT-3', and reverse, 5'-ATTCGCCGTAATGTGCTACC-3'; mouse-IP3R3: forward, 5'-CAATGAGCACCACGAGAAGA-3', and reverse, 5'-AACTTGACAGGGGTCACCAG-3'; mouse-NFATc1: forward, 5'-GGGTCAGTGTGACCGAAGAT-

3', and reverse, 5'-GGAAGTCAGAAGTGGGTGGA-3'; mouse-OC-STAMP: forward, 5'-ATGAGGACCATCAGGGCAGCCACG-3', and reverse, 5'-GGAGAAGCTGGGTCAGTAGTTCGT-3'; mouse-DC-STAMP: forward, 5'-AAAACCCTTGGGCTGTTCTT-3', and reverse, 5'-GGCTGCTTTGATCGTTTCTC-3'; mouse-Atp6v1c1: forward, 5'-CACGTTGGCTGTCTTGGTTG-3', and reverse, 5'-CTTCGCGTTAGCAAACTTCC-3'; mouse-αv-integrin: forward, 5'-ACAAGCTCACTCCCATCACC-3', and reverse, 5'-ATATGAGCCTGCCGACTGAC-3'; mouse-GAPDH: forward, 5'-TCAAGAAGGTGGTGAAGCAG-3', and reverse, 5'-AGTGGGAGTTGCTGTTGAAGT-3'.

ELISA and microenzyme labeling method . Mouse serum was collected, and the tartrate-resistant acid phosphatase 5b (TRACP-5b) level was measured with a TRACP-5b ELISA Kit (Elabscience, Wuhan, China). Alkaline phosphatase (ALP) activity was measured with an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Microcomputed tomography . Bone density and bone volume of the right femur were measured using a Micro-CT SkyScan 1176 (Bruker, Germany). Briefly, samples were fixed in 4% paraformaldehyde and incubated in 70% ethanol. Scanning was performed using a voxel size of 9 mm, X-ray tube potential of 58 kV and X-ray intensity of 431 μA. For specimen scanning, the volumes of interest were evaluated using CT Analyzer software. Representative 3D images created using NRecon software.

H&E staining . Tissues were collected, cleaned to remove excess tissue, fixed in 4% paraformaldehyde and demineralized in 10% EDTA for 10–14 days before being embedded in paraffin wax. Sections were cut to 5 μm thicknesses using a Leica RM2165 and subsequently stained with H&E or TRAP.

Statistical Design . SPSS version 26.0 (IL, USA) and GraphPad Prism 9 (CA, USA) were used for statistical analysis. Two-tailed unpaired Student's t tests were used for comparisons of two groups, and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used for comparisons of more than two groups. P values less than 0.05 were considered statistically significant.

Results

3.1 NVP-BHG712 is a small molecule compound that targets CTSK.

We used a novel strategy to identify potential active small molecule compounds that target CTSK, and we ultimately identified NVP-BHG712 as a potential bioactive compound from the Selleckchem compound library.

As mentioned above in the Methods, the molecular docking method was used to search for small molecule compounds that target CTSK. First, we downloaded crystal conformations of human CTSK from the PDB database. According to the binding of the CTSK active pocket with known ligands, the ligands 7AS and OLC bind to CTSK in an L-shape, with part in the pocket and part extending upward, and the binding of the ligands KWZ and I37 to CTSK occurs in both upward and downward conformations (Fig. 1A, B). Analysis showed that I37 had the weakest binding strength among the four ligands because the molecule was small and the interaction with CTSK was weak, but its binding strength could reach an order of nM. Therefore, we selected these four molecular conformations of CTSK for molecular docking to search for new small molecule compounds that bind to CTSK. According to the results of molecular docking analysis with molDockTools software, a small molecule compound was identified to bind to the four conformations of CTSK (Fig. 1C), and the compound was NVP-BHG712 (Fig. 1D). Moreover, through a literature review, we did not find any reports of an effect of NVP-BHG712 on CTSK and osteoclast formation. Therefore, we next focused on the effects of NVP-BHG712 on CTSK and osteoclast formation.

3.2 NVP-BHG712 inhibited RANKL-induced osteoclastogenesis and bone resorption in vitro.

To investigate the cytotoxicity of NVP-BHG712, we observed the effects of NVP-BHG712 at 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μM on the proliferation of BMMs and RAW264.7 cells. After 72 hours of treatment with 0˜0.4 μM NVP-BHG712, the ratio of BMMs and RAW264.7 cells was 138% and 154%, respectively.

When the concentration was 0.8 to 1.6 μM, NVP-BHG712 promoted cell proliferation. However, when the concentration was increased to 3.2 μm, NVP-BHG712 significantly inhibited the proliferation of both cell lines and caused obvious cytotoxicity. The results indicated that there was no significant cytotoxicity when the concentration of NVP-BHG712 was lower than 1.6 μM (Fig. 2A). To further assess the effects of NVP-BHG712 on osteoclastogenesis, a RANKL-induced osteoclastogenesis assay was carried out in BMMs that were exposed to various concentrations of NVP-BHG712 (0, 0.1, 0.2, 0.4, 0.8, and 1.6 μM). The numbers of TRAP-positive multinucleated cells were counted to assess RANKL-induced osteoclast differentiation. As the results showed, the number of TRAP-positive multinucleated cells was increased when treated with NVP-BHG712 at 0˜0.2 μM and dramatically decreased when treated with NVP-BHG712 at 0.4˜1.6 μM (Fig. 2B, C).

Furthermore, a pit-formation assay was carried out to determine whether osteoclast functions were impaired. Bone resorption pits on bone slices are the direct result of bone resorption and are also the most reliable indicator of bone resorption function in vitro. BMMs exposed to RANKL were treated with different concentrations of NVP-BHG712 (0, 0.1, 0.2, or 0.4 μM), and then, the area of bone lacunae absorption was observed. The results showed that bone resorption activity was attenuated by NVP-BHG712 (Fig. 2D, E).

3.3 NVP-BHG712 inhibited osteoclastogenesis and F-actin ring formation in mature osteoclasts at the early phase in vitro.

To further identify at which specific phase NVP-BHG712 exerts its inhibitory effect on osteoclastogenesis, RANKL-treated BMMs were incubated with NVP-BHG712 (0.4 μM) at different time points and for different durations (days 1-3, days 3-5, days 5-6, and days 1-6). TRAP staining illustrated that osteoclast differentiation was more strongly inhibited by NVP-BHG712 treatments in the first several days than in the later days, suggesting that NVP-BHG712 mainly suppressed osteoclast differentiation in the early phase (Fig. 3A, B). F-actin rings are considered the characteristic structure of mature osteoclasts. To further determine the inhibitory effect of NVP-BHG712 on osteoclast function, immunofluorescence staining of the F-actin ring was performed in BMMs that were exposed to NVP-BHG712 treatments. Similar to the prior inhibitory effects, NVP-BHG712 more significantly inhibited the formation of F-actin ring structures by mature osteoclasts in the first several days than in the later days, as demonstrated by a decrease in F-actin numbers compared to total osteoclast numbers (Fig. 3C, D). In conclusion, the results suggest that osteoclast function is mainly inhibited by NVP-BHG712 in the early phase.

3.4 NVP-BHG712 inhibited the RANKL-induced expression of osteoclast differentiation-related genes.

CTSK is a key enzyme in the process of bone resorption by osteoclasts. Under acidic conditions, activated CTSK participates in the degradation of collagen type I, osteopontin and other bone matrix proteins in the bone matrix, and it promotes the bone resorption function of osteoclasts. To observe whether the small molecule compound NVP-BHG712, which targets CTSK, can inhibit the protein expression of CTSK, Western blotting was used to measure the expression of CTSK in RANKL-treated BMMs-derived osteoclasts. The results showed that 0.4 μM NVP-BHG712 could inhibit CTSK expression in RANKL-treated BMMs-derived osteoclasts. (Fig. 4A, B). To investigate the effect of NVP-BHG712 on osteoclast differentiation-related genes, Western blotting was employed to measure the proteins levels of MMP9 and CTR. The results showed that the 0.4 μM concentration of NVP-BHG712 could significantly inhibit the RANKL-induced proteins expression of MMP9 and CTR in osteoclasts (Fig. 4A, C, D). Next, we measured the mRNA expression of MMP9 and CTR, the results showed that the mRNA expression of MMP9 and CTR was significantly inhibited by NVP-BHG712 (Fig. 4E, F).

Next, we measured the RANKL-induced protein expression of CTSK in BMMs-derived osteoclasts after adding 0, 0.1, 0.2, and 0.4 μM NVP-BHG712 to determine the effect of the different concentrations of NVP-BHG712 on the protein expression of CTSK in osteoclasts. The protein expression of CTSK in BMMs-derived osteoclasts treated with RANKL was measured after incubation with 0.4 μM VP-BHG712 for 1, 2, 3, and 4

days to determine the effect of different treatment times on the protein expression of CTSK in osteoclasts. The results revealed that the protein expression of CTSK was significantly decreased when the concentrations of 0.1, 0.2, and 0.4 μM NVP-BHG712 were added to the BMMs culture system (Fig. 4G, H). The expression of CTSK protein was not significantly changed after the addition of 0.4 μM VMP-BHG712 to the osteoclast differentiation and maturation culture system for 1 and 2 days; however, the protein expression of CTSK decreased significantly after 3 days and 4 days of culture (Fig. 4I, J). The mRNA expression levels of IP3R1, IP3R2, IP3R3, NFATc1, OC-STAMP, DC-STAMP, αV-integrin and Atp6v1c1 were measured by qPCR. We found that the mRNA expression of MMP9, CTR, IP3R1, IP3R3 and OC-STAMP was significantly inhibited by NVP-BHG712 (Fig. 5A-H). Therefore, NVP-BHG712 could inhibit the RANKL-induced expression of osteoclast differentiation-related genes in BMMs.

3.5 NVP-BHG712 did not affect the expression of osteoclast differentiation-related receptors.

To further investigate whether NVP-BHG712 affected the expression of receptors related to osteoclast differentiation and thus affected the downstream signal in the cells, we observed the expression of the c-Fms receptor and RANK receptor in BMMs-derived osteoclasts by immunofluorescence assay. The immunofluorescence of the c-Fms receptor did not change significantly after treatment of osteoclasts with NVP-BHG712 at a concentration of 0.4 μM (Fig. 5I, J). In addition, the immunofluorescence results showed that NVP-BHG712 had no significant effect on the expression of the RANK receptor, which is related to osteoclast differentiation (Fig. 5K, L).

3.6 NVP-BHG712 attenuated bone loss in ovariectomized mice.

The decrease in estrogen during postmenopausal osteoporosis leads to an increase in osteoclast activity, bone resorption rates become greater than bone formation rates, and the bone turnover rate increases, leading to bone loss related to high turnover. Ovariectomized female mice are widely used as a postmenopausal osteoporosis model in research[C. Y. Chen et al., 2019; K. Chen et al., 2019]. Thus, we established OVX models in 8-week-old female mice and tested whether the intragastric administration of NVP-BHG712 every 3 days for five weeks could attenuate bone mass loss in these OVX female mice. (Fig. 6A). The effect of NVP-BHG712 on bone mass in ovariectomized mice was confirmed by measuring the serum TRACP-5b levels by ELISA and the serum ALP levels with a microplate reader. The results of TRACP-5b measurement showed that the serum TRACP-5b levels in the mice in the OVX group were significantly increased compared with those in the Sham group (Fig. 6B), which indicated that the model of postmenopausal osteoporosis was successfully established. Similar results were observed in the measurement of ALP activity, which was lower in the OVX group than in Sham group (Fig. 6C). Both of these results proved the success of the operation. After administration of NVP-BHG712 at a concentration of 10-40 mg kg^{-1}, the serum TRACP-5b levels were significantly decreased in the mice. The serum ALP levels of the mice were significantly increased after the administration of NVP-BHG712 at a concentration of 5˜40 mg kg^{-1}. Together, these results suggest that gavage of NVP-BHG712 can be used in the treatment of high turnover osteoporosis.

As shown in Fig. 5D-F, the NVP-BHG712-treated OVX mice had the lower trabecular bone mass phenotypes than the OVX mice, as revealed by the 2D and 3D μCT reconstructed images of distal femurs, the much higher BMD, BS/TV, BV/TV, Tb.N, Tb.Th values, and the lower BS/BV, Tb. Pf, Tb.Sp values. These results revealed that NVP-BHG712 increased the bone mass in OVX mice. The results of HE staining and quantitative analysis of the distal femur showed that NVP-BHG712 increased the density and number of trabecular bones in OVX mice (Fig. 6G, H). Together, these findings indicate that NVP-BHG712 exerts a protective effect against bone loss in ovariectomized mice, which is related to the decreased osteoclast resorption induced by NVP-BHG712.

After the operation, we recorded the weight of the mice before each feeding, and the results showed that the weight of the mice in the OVX group, different NVP-BHG712 treatment groups and Sham group increased gradually, and no significant weight loss was observed (Fig. 7A). The organ index of the mice was calculated as the ratio of the organ weight to the body weight of the mice. Compared with those of the Sham group, the heart index, spleen index, kidney index and tibia index of the OVX group did not change significantly,

and gavage with different doses of NVP-BHG712 did not cause significant differences compared with OVX alone (Fig. 7B-E). No differences in the heart weight/tibia length results were observed between the different NVP-BHG712 groups and the OVX group (Fig. 7F). Based on these results, we found that NVP-BHG712 did not cause toxicity in ovariectomized mice when administered by gavage at a dose of 40 mg kg^{-1}.

In conclusion, our findings identified a novel compound for the prevention of postmenopausal osteoporosis (Fig. 7). With skeletal aging and menopause, osteoclastic bone resorption activity increases relatively or absolutely. During osteoclast bone resorption, CTSK is released from the bone matrix into the bone marrow, where CTSK and other cytokines promote bone resorption. NVP-BHG712 inhibits osteoclast bone resorption by inhibiting the function of the CTSK protein, thereby slowing the loss of bone mass that is associated with aging or postmenopausal bone.

Discussion

Our investigation to understand the role of NVP-BHG712 in osteoclasts establishes that CTSK is the target of NVP-BHG712 and promotes RANKL-induced osteoclast differentiation and maturation. This assertion stems from the strong binding of NVP-BHG712 to each of the four molecular conformations of CTSK and its inhibitory effect on RANKL-induced osteoclast differentiation and maturation. When compared with other compounds that inhibit osteoclast differentiation and maturation, several striking features emerge in our results. In particular, to our knowledge, the magnitude with which NVP-BHG712 inhibits osteoclast differentiation and maturation is matched by OCN. Through a literature search, it was found that NVP-BHG712 had not been previously reported in studies on CTSK targets and osteoclasts. EphrinB2/EphB4 signaling pathway was activated to inhibit osteoclast differentiation, while decreased osteoclast maturation and differentiation was observed when NVP-BHG712, an EphB4-specific inhibitor, was added, suggesting that EphB4 inhibition was not the primary role of NVP-BHG712 in this process. Therefore, in this study, we did not continue to investigate the role of Ephrin signaling pathways. Therefore, we did not continue to investigate the role of Ephrin signaling pathways. We observed a decrease in osteoclast maturation and differentiation with the addition of NVP-BHG712, so we speculate that inhibition of EphB4 to promote osteoclast maturation is not the primary effect of NVP-BHG712 in this process. Therefore, we did not continue to investigate the role of Ephrin signaling pathways. Moreover, we found that in vitro osteoclast proliferation can be promoted by NVP-BHG712, but bone resorption can be inhibited by NVP-BHG712, indicating that NVP-BHG712 can inhibit osteoclast resorption. When we explored the time at which NVP-BHG712 affected osteoclasts, we found that NVP-BHG712 mainly inhibited osteoclast differentiation and maturation in the early stage. Additionally, our findings show that the formation of the F-actin loop, which is used to demonstrate osteoclast function, is mainly inhibited in the early stage after NVP-BHG712 treatment at different time periods. However, NVP-BHG712 could not reverse the differentiation and maturation of osteoclasts because mature osteoclasts were still formed when NVP-BHG712 was added at a later stage. We hypothesize that once CTSK-dependent osteoclast maturation is disrupted, the formation of the bone resorption microenvironment is blocked, and bone resorption function is lost (Fig. 6G).

CTSK plays a role in bone resorption, the immune system, tumorigenesis and invasion, the circulatory system, the cardiovascular system and other systems and participates in extracellular matrix remodeling in different organs. Under acidic conditions, CTSK can bind to collagen to degrade it, leading to increased bone fragility(Gruber, 2015; Mujawar et al., 2009). Furthermore, because NVP-BHG712 is a CTSK-targeting compound that was identified by molecular docking, we hypothesized that NVP-BHG712 inhibits the function and expression of CTSK. Interestingly, Western blotting results showed that NVP-BHG712 inhibited the protein expression of CTSK and inhibited the protein expression of MMP9 and CTR, which are markers related to osteoclast differentiation. In addition, we also found an inhibitory effect of NVP-BHG712 on IP3R1, IP3R3 and OC-STAMP mRNA expression, among which IP3R1 and IP3R3 are related to intracellular calcium oscillation, and OC-STAMP is related to osteoclast maturation. c-Fms and RANK receptors play an important role in the proliferation and differentiation of osteoclast precursor cells. M-CSF participates in the proliferation of osteoclast precursor cells by binding to the c-Fms receptor, and RANKL participates in

the differentiation of osteoclast precursor cells by binding to the RANK receptor. Our immunofluorescence staining results showed that NVP-BHG712 had no inhibitory effect on the expression of c-Fms and the RANK receptor, which indicated that the regulation of osteoclast proliferation, differentiation and maturation by NVP-BHG712 did not occur through the M-CSF/c-Fms and RANKL/RANK signaling pathways.

The pathogenesis of PMOP involves bone remodeling caused by an imbalance in bone resorption and bone formation(Darby, 1981). Estrogen withdrawal leads to increased osteoclast production, enhanced bone resorption, and substantial bone loss(Kameda et al., 1997; Mundy, 2007). Therefore, targeting overactivated osteoclasts provides an effective therapeutic option for PMOP. Osteoclast hyperactivation promotes excessive bone loss in the pathophysiology of PMOP. We further confirmed our in vitro findings by modelling the pathological state of PMOP in an ovariectomized mouse model. Before the start of the experiment, we measured the bone mass of 8-week-old female mice at 3-6 weeks after ovariectomy by H&E staining of femur sections. It was found that the trabecular bone density of mice was significantly reduced 5 and 6 weeks after ovariectomy. Therefore, NVP-BHG712 administration for 5 weeks after the operation can be used to explore its effect in vivo. By measuring the serum levels of TRACP-5b and ALP, we found that NVP-BHG712 significantly inhibited osteoclast bone resorption activity. Using microcomputed tomography and H&E staining of the distal femur, we found that NVP-BHG712 significantly attenuated the loss of bone mass in mice after ovariectomy. In conclusion, NVP-BHG712 inhibited osteoclast maturation and bone resorption in vivo and prevented bone loss caused by ovariectomy. Additionally, it was observed that the body weights of the mice in the Sham group, OVX group and different NVP-BHG712 treatment groups were continuously increased and did not decrease. The cardiac index, spleen index, kidney index, tibial index, and heart weight to tibial length ratio were not significantly different in OVX mice treated with different concentrations of NVP-BHG712 (Fig. 6B-E), suggesting that 0˜40 mg kg^{-1} NVP-BHG712 was not toxic to mice in vivo. Our results suggest that NVP-BHG712 at 0˜40 mg kg^{-1} concentration has a protective effect on bone loss in ovaries excised mice, but the effect and toxicity of NVP-BHG712 at higher concentration need further investigation.

In summary, our work identifies a previously unknown small molecule compound with anti-osteoporosis effects, namely, NVP-BHG712, which inhibits osteoclast activation by inhibiting CTSK function. In the ovariectomized female mouse model, NVP-BHG712 slowed the loss of bone mass.Although the molecular mechanism of the effect of NVP-BHG712 on osteoclast differentiation needs further investigation, this study may provide new insights into the effect of NVP-BHG712 on bone metabolism in osteoporosis.

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Figure legends.

Figure 1. NVP-BHG712 is a small molecule compound that targets CTSK.

A, Chemical formulas of the ligands KWZ, OLC, I37 and 7AS for the molecular conformation of CTSK. B, Images of molecular conformations of CTSK (3KWZ, 4DMY, 4X6H and 5TDI) bound to ligands (KWZ, OLC, I37 and 7AS). C, Molecular docking diagram of the small molecule compound NVP-BHG712 with the four molecular conformations of CTSK. D, Chemical structure of NVP-BHG712.

Figure 2.NVP-BHG712 inhibited RANKL-induced osteoclastogenesis and bone resorption in vitro.

A, CCK-8 analysis of the cytotoxicity of NVP-BHG712 in RAW264.7 cells and BMMs. Experiments were repeated 3 times. B, C Formation of TRAP-positive cells from RANKL-treated BMMs treated with or without different concentrations of NVP-BHG712 (0, 0.1, 0.2, 0.4, 0.8, or 1.6 μM) for 7 days (B) and quantification of osteoclast numbers (C). D, E Representative images of pit formation by osteoclasts derived from RANKL-treated BMMs with or without different concentrations of NVP-BHG712 (0, 0.1, 0.2, or 0.4 μM) (D) and quantification of pit area (E). The data are presented as the mean ± SD (n=3) (***P <.001)

Figure 3.NVP-BHG712 inhibited osteoclastogenesis and F-actin ring formation in mature osteoclasts at the early phase in vitro.

A, B Representative images of the formation of TRAP-positive cells treated with 0.4 μM NVP-BHG712 (Group 1: without NVP-BHG712) at different time points and four durations (Group 2-5: day 1-3, day 3-5, day 5-6, day 1-6, respectively) (A) and quantification of osteoclast numbers (B). C, D Representative immunofluorescence images of the F-actin ring structures of mature osteoclasts derived from RANKL-induced BMMs treated with 0.4 μM NVP-BHG712 (Group 1: without NVP-BHG712) at different time points and for durations (Group 2-5: day 1-3, day 3-5, day 5-6, day 1-6, respectively) (C) and quantification of F-actin ring formation of osteoclasts (D). Group C:M-CSF, Group R: M-CSF+RANKL, Group N: M-CSF+RANKL+NVP-BHG712. The data are presented as the mean ± SD (n=3) (*P <.05, **P <.01, ***P <.001).

Figure 4.NVP-BHG712 inhibited the RANKL-induced expression of osteoclast differentiation-related genes. A-D, Representative Western blots of the protein expression of CTSK, MMP9 and CTR in osteoclasts derived from RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 (A) and the quantification of CTSK (B), MMP9 (C) and CTR (D) expression. E-F, Representative quantification of the mRNA expression of MMP9 (E) and CTR (F) in osteoclasts derived from RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 by qPCR.

G, H Representative Western blots of the protein expression of CTSK in osteoclasts derived from RANKL-treated BMMs treated with or without different concentrations of NVP-BHG712 (0, 0.1, 0.2, or 0.4 μM) (G) and the quantification of CTSK (H) expression. I, J Representative Western blots of the protein expression of CTSK in osteoclasts derived from RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 for different time periods (days 1, 2, 3, and 4) (I) and the quantification of CTSK (J) expression. Group C:M-CSF, Group R: M-CSF+RANKL, Group N: M-CSF+RANKL+NVP-BHG712. The data are presented as the mean ± SD (n=3) (*P <.05, *P <.01, ***P <.001).

Figure 5. NVP-BHG712 did not affect the expression of osteoclast differentiation-related receptors.

A-H, Representative quantification of the mRNA expression of IP3R1 (A), IP3R2 (B), IP3R3 (C), NFATc1 (D), OC-STAMP (E), DC-STAMP (F), αV-integrin (G) and Atp6v1c1 (H) in osteoclasts derived from RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 by qPCR. I, J Representative immunofluorescence staining images of c-Fms in RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 (I) and the quantification of c-Fms (J) expression. K, L Representative immunofluorescence staining

images of RANK in RANKL-treated BMMs treated with or without 0.4 μM NVP-BHG712 (K) and the quantification of RANK (L) expression. The data are presented as the mean ± SD (n=3) (*P <.05, *P <.01, ***P <.001)

Figure6. NVP-BHG712 attenuated bone loss in ovariectomized mice.

C, Serum ALP (B) and TRACP-5b (C) levels in the Sham group, ovariectomized group and ovariectomized groups treated with different concentrations of NVP-BHG712 (5, 10, 20, or 40 mg kg^{-1}) for 5 weeks. C-E, Representative microcomputed tomography (μCT) 2D (C) and 3D (D) reconstruction images of distal femurs from the Sham group, ovariectomized group and ovariectomized groups treated with different concentrations of NVP-BHG712 (5, 10, 20, or 40 mg kg^{-1}) for 5 weeks. Quantitative morphometric properties of distal femurs showing bone mineral density (BMD), bone volume (BV), bone surface/volume ratio (BS/BV), bone surface density (BS/TV), fractional bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular pattern factor (Tb.Pf) (F). n=5. G, H H&E staining images (G) and statistical analysis (H) of mouse femur slices. Scale bars = 40 μm. All the experiments were performed with five biological replicates per group without independent repetition. The data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. (*P <.05, *P <.01, ***P <.001)

Figure 7. NVP-BHG712 was not toxic in ovariectomized mice.

A-F, Changes in the body weights of mice before each feeding (A), heart index (B), spleen index (C), kidney index (D), forelimb grip-strength index (E), and heart weight/tibial length (F) in the Sham group, ovariectomized group and ovariectomized groups treated with different concentrations of NVP-BHG712 (5, 10, 20, or 40 mg kg^{-1}) for 5 weeks. G, Schematic diagram of the mechanism by which NVP-BHG712 inhibits osteoclast function. With skeletal aging and menopause, osteoclastic bone resorption activity increases relatively or absolutely. During osteoclast bone resorption, CTSK is released (purple dots) from the bone matrix into the bone marrow, where CTSK and other cytokines promote bone resorption. NVP-BHG712 inhibits osteoclast bone resorption by inhibiting the function of the CTSK protein, thereby slowing the loss of bone mass associated with aging or postmenopausal bone. All the experiments were performed with five biological replicates per group without independent repetition. The data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA.