Novel cathepsin K inhibitors block osteoclasts \textit{in vitro} and increase spinal bone density in zebrafish†

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Cathepsin K (Cat K) is a predominant cysteine protease and highly potent collagenase expressed in osteoclasts. Cat K inhibitors are anti-resorptive agents to treat osteoporosis. A novel scaffold of cathepsin K inhibitors, exemplified by lead compound 1x, was used as the template for designing and synthesizing a total of 61 derivatives that have not been reported before. An exploratory structure–activity relationship analysis identified the potent Cat K inhibitor A22, which displayed an IC\textsubscript{50} value of 0.44 \mu M against Cat K. A22 was very specific for Cat K and caused a significantly higher \textit{in vitro} inhibition of the enzyme as compared to that of lead compound 1x. A surface plasmon resonance analysis confirmed \textit{in vitro} binding of A22 to Cat K. Molecular docking studies indicated several favourable interaction sites for A22 within the active pocket of Cat K. Furthermore, A22 also blocked active osteoclasts \textit{in vitro} and increased spinal bone density in zebrafish, in which it showed an activity that was higher than that of the marketed therapeutic bone metabolizer etidronate disodium. A22 represents a very promising lead compound for the development of novel antiresorptive agents functioning as orthosteric inhibitors of Cat K.

1. Introduction

Osteoporosis (OP) is a systemic disease associated with a declining rigidity and mechanical stability of the bones.\textsuperscript{1} Current estimates indicate that over 200 million people worldwide suffer from this disease, causing more than 8.9 million fractures annually. In fact, an osteoporotic fracture is estimated to occur every 3 s, and one out of every three women and one in five men over 50 years of age will have an OP-related fracture in their lifetime.\textsuperscript{2,3} A better knowledge about the progression of bone metabolic disorder and the development of effective treatments will have important socio-economic and medical consequences. The most commonly prescribed OP medications include nitrogen-containing bisphosphonates, denosumab, raloxifene, hormone replacement therapy, and calcitonin.\textsuperscript{4} Although these therapies improve the quality of life in patients with OP, they have many side effects.\textsuperscript{5-7} Thus, the discovery of novel OP medications is a priority that would provide new options for OP patients and represent a major advancement in the treatment of OP.

Bone health in adults depends on resorption that involves the continuous removal of old or damaged bone tissue by osteoclast (OC) cells, which is balanced by the deposition of new bone formed by osteoblast (OB) cells. As the skeleton ages, the action of the OCs outpaces that of the OBs, causing a shift toward excessive resorption and the development of chronic OP associated with reduced bone mineral density (BMD) and an increased risk of bone fractures.\textsuperscript{8-9} Cathepsin K (Cat K) is a lysosomal cysteine protease released by OCs, which cleaves the collagen matrix of bone tissue.\textsuperscript{10,11} Cat K knockout mice have been shown to develop osteopetrosis with elongated bones and vertebrae and abnormal joint morphology due to a deficit in matrix degradation,\textsuperscript{12} indicating the potential role of Cat K in OP and other bone diseases. In recent years, Cat K inhibitors have been intensively studied by pharmaceutical companies to find antiresorptive therapies for OP.\textsuperscript{13-15} The most advanced drug candidates include odanacatib,\textsuperscript{16,17} relacatib,\textsuperscript{18,19} balicatib\textsuperscript{20,21} and ONO-5334 (Fig. 1).\textsuperscript{22,23} Odanacatib and balicatib are

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Fig. 1 Structures of Cat K inhibitors.
reversible covalent Cat K inhibitors carrying a cyano group that can form a covalent bond with C25 of the active pocket in Cat K.

Odanacatib was once the most advanced drug candidate as a Cat K inhibitor that reduced fractures in postmenopausal women in a large multinational randomized, double-blinded phase III clinical study. However, odanacatib was also associated with an increased risk of cerebrovascular accidents and subsequently withdrawn from the regulatory approval process.

Thus, there is significant value in the study of Cat K because it is a major drug target for OP and related-bone disorders.

Our research team has been working on the development of anti-OP drugs for many years.24,25 Our recent efforts are focused on the discovery of Cat K inhibitors as potent antiresorptive agents. A virtual screen of our in-house compound library against the Cat K (PDB code: 4dmy, Fig. 2) active site identified compound 1q which was reported by Alexander G. Dossetter and his colleagues (Fig. 2a).26 The orientation of 1q also showed a high fitness comparable to that of 14 (Fig. 2b). The scaffold of 1q has not been reported by others as a Cat K inhibitor. A search of our in-house compound library identified several structural analogues of 1q that were all tested for in vitro Cat K inhibiting effects using an enzymatic assay. However, 1q was not active against Cat K at 100 μM. Interestingly, we identified compound 1x (Fig. 2) with a scaffold of benzimidazole-2-substituted pyridine propyl ketene that inhibited Cat K at a concentration below 100 μM. Two other compounds (Fig. 3, 1o and 1p) with a scaffold containing benzoazole or benzothiazole did not inhibit Cat K. We also found that the carbonyl group in 1x scaffold is probably essential for the Cat K inhibiting activity because the switch to a hydroxyl group (see 1s and 1t) abolished the activity.

The IC_{50} of compounds 1o, 1p, 1s, and 1t against Cat K were all above 100 μM.

In this study, we synthesized derivatives based on the structure of 1x with the benzimidazole scaffold and explored their antiresorptive potential. In total, 61 compounds containing either benzimidazole-2-substituted phenyl or pyridine propyl ketenes were synthesized. The compounds were evaluated for their potential to inhibit the kinase activity of Cat K. Many derivatives with benzimidazole scaffold showed inhibitory activity at less than 10 μM against Cat K in vitro. Then, we tested the selectivity of 1x, A20, A22, and A32 (Table 1) against several other cathepsins (L, S and B) and found that these compounds were highly selective for Cat K. Modelling studies identified several favourable interactions of A22 with the active pocket of Cat K. Surface plasmon resonance (SPR) analysis confirmed the high affinity of A22 to Cat K. Compounds A20, A22 and A32 showed inhibitory activity toward OCs and also inhibited bone resorption caused by C-telopeptide (CTX-I) decrease observed in vitro. Moreover, A22 also increased spinal bone density in zebrafish.

2. Results and discussion

2.1 Chemistry

The search for potential Cat K inhibitors led to the identification of compound 1x with a scaffold containing benzimidazole, a carbonyl group, and a double bond that is required for in vitro Cat K inhibition. The design and synthesis of a series of 1x derivatives were performed as follows. The synthesis route of benzimidazole-2-substituted phenyl or pyridine propyl ketenes is described in Scheme 1. In total, 61 derivatives have been synthesized. There are different substituents on the two aromatic rings. In this round of synthesis, we only implanted substituents with smaller size, such as methyl, halogen or cyano groups. The detailed structures of the compounds are reported in Table 1.

2.2 Inhibition of Cat K in vitro and preliminary structure–activity relationships

The inhibitory activities of our focused compound library against Cat K are reported in Table 1 including odanacatib, which was used as a positive control. Importantly, many derivatives of scaffold A showed Cat K inhibitory effects at concentrations below 100 μM, which confirmed that this novel scaffold has a high potential to serve as a template for antiresorptive agents.

The phenyl or pyridine substitution (such as A4 and A39) in the scaffold is favoured. The 2-substitution on the phenyl ring seems detrimental to Cat K inhibitory activities according to the IC_{50} data (>100 μM) of A11, A15, A27, and A30. The
5-halogen substitution on benzimidazole appears to be favourable for the activity. A20, A22, A32, and A41 showed inhibitory activities below 1 μM. Halogen and cyano group substitution on the terminal phenyl or pyridine ring may also contribute to the activity. The molecular docking calculations of A22 (Cat K $IC_{50} = 0.44 \mu M$, Fig. 4) in the Cat K active pocket also suggested that its imidazole H atom formed a hydrogen bond with the O atom of the backbone carbonyl group at N161. This could explain why compounds benzoazole 1o and benzothiazole 1p did not show any Cat K inhibiting effect. They lack this key hydrogen bond donor in their scaffold which appears to be a necessity for this activity. The phenyl ring of benzimidazole was coplanar with the glycine shelf (G23) and generated a hydrophobic interaction. The hydrophobic interaction between benzimidazole and C25 also enhanced the affinity to Cat K.

Thus, chlorine substitution on benzimidazole of A22 is beneficial to the affinity to Cat K.

2.3 Selectivity of the inhibition of cathepsins in vitro
We compared the inhibitory activities of compounds 1x, A20, A22, and A32 against Cat K, Cat L, Cat S, and Cat B (Table 2).
Compounds 1x, A20, A22, and A32 showed an inhibitory effects against the cathepsins, especially toward Cat K and Cat L. The order of the inhibitory activities of the four test compounds against the cathepsins were Cat K > Cat L > Cat S > Cat B. The selectivity of A22 for Cat K over Cat S and Cat B was 6.5 times for both Cat K and Cat L, as compared to the orientation of Y193 (purple) of Cat S, Y67 (green) of Cat K has much less clash with pyridine ring of A22. Compared to the orientation of Y193 (purple) of Cat S, Y67 (green) of Cat K has much less clash with pyridine ring of A22. As compared to the inhibitory activities of odanacatib, the positive control odanacatib had a higher activity against Cat K and Cat L, but a lower activity against Cat S and Cat B. The selectivity of odanacatib for Cat K over Cat S and Cat B was 6.5 times for both enzymes. The order of the inhibitory activity of odanacatib against the cathepsins was Cat K > Cat S = Cat B > Cat L. These results indicated that A22 and odanacatib differed in their selectivity toward cathepsin family proteins and, therefore, may differ in their activity and toxicity. Therefore, A22 has the potential to be novel antiresorptive agents.

| Compd. | Cat K (IC₅₀ (μM)) | Cat L | Cat S | Cat B |
|--------|-----------------|-------|-------|-------|
| 1x     | 10.04           | 15.82 | >100  | >100  |
| A20    | 0.60            | 1.64  | 24.73 | 47.73 |
| A22    | 0.44            | 2.62  | 46.72 | 73.85 |
| A32    | 0.88            | 1.79  | 25.34 | 191.76|
| Odanacatib | 0.002        | 8.46  | 0.013 | 0.013 |

### 2.4 Antiresorptive effects in vitro

Type I collagen comprises about 95% of the entire collagen content of bone. During skeletal renewal, type I collagen is degraded, releasing short peptide fragments into the bloodstream. The bone resorption process can be studied in vitro by culturing bone cells on bone sections or dentin sheets. During the process of bone resorption by OCs, the type I collagen C-terminal peptide (CTX-I) is degraded into fragments and released. In this experiment, the concentration of CTX-I released into the cell culture medium by bone degradation can be detected and quantified by ELISA. The in vitro antiresorptive effect of our compounds was tested in RANKL-induced RAW264.7 cells. Osteoclastic features can be induced in RAW264.7 by RANKL with high efficiency and reproducibility. We detected the CTX-I concentration released into the culture medium from RANKL-induced RAW264.7 cells by ELISA. Importantly, RAW264.7 cells released much less CTX-I in the presence of A20, A22, A32, 1x, and positive control odanacatib, as compared to that released by untreated RAW264.7 cells. The concentrations of all experimental compounds and positive control drugs were 5 μM. The antiresorptive effects of all the experimental compounds was similar to that of odanacatib, especially the activity of A20 was much stronger than that of the control drug. And also the efficacy of all the test compounds was significantly better than that of 1x on RAW264.7 cells (Fig. 5A).
2.6 Binding affinity to Cat K tested by SPR

To determine whether the active compounds can bind to Cat K, real-time binding analysis by SPR (Biacore 8K) was performed using Cat K and different dilutions of A20 and A22 (Fig. 6). The two compounds showed moderately strong affinities to Cat K. The calculated affinities (KD) of A20 and A22 to Cat K were 53.2 ± 43.7 μM and 17.2 ± 5.6 μM, respectively. Moderately strong affinities implied that the compounds may show antiresorptive efficacy in vivo via inhibition of Cat K.

2.7 OP therapeutic activity in zebrafish

To probe into the in vivo activities, we chose A22 as a pilot compound and evaluated its activity in zebrafish. Because A22 was the most potent Cat K inhibitor in vitro among our focused library compounds and showed high selectivity and affinity to Cat K. Etidronate disodium was used as a positive control drug. Etidronate disodium is a bone resorption inhibitor and now mainly used as an antiresorptive medication.

Its action mechanism involves the hydroxyapatite crystal growth inhibition according to the in vitro chemisorption onto the crystal surface. Prednisone is a glucocorticoid medication and often used to suppress the immune system and relieve inflammation. The use of prednisone can induce bone loss. In our study, we use prednisone to set up a zebrafish bone loss model.

Our dosing pilot study showed at the concentration of 1.00 μM, A22 didn’t show obvious toxicity to the 30 zebrafish in the group. We then tested OP therapeutic activity in zebrafish of A22 at concentrations of 0.11, 0.33, and 1.00 μM. Fluorescent chromophore calcine, which binds specifically to calcified skeletal structures, was used to indicate zebrafish spine bone density. Fluorescence intensities were enhanced as compared to that of the model group, indicating that A22 treatment improved bone density at tested concentrations. A22 also showed dose-response relationship in OP zebrafish. At the highest concentration group, A22 exhibited higher activity than etidronate disodium (ED) (Fig. 7). The OP therapeutic effect in zebrafish bone loss model showed that A22 is a promising anti-OP agent.

3. Experimental section

3.1 Chemistry

3.1.1 General information. All reagents and solvents were purchased and used without further purification unless specified. The 1H NMR and 13C NMR spectra were recorded with a Bruker BioSpin GmbH spectrometer (400 MHz) in d6-DMSO and the internal solvent residual peaks were used as references. The high-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL with an ESI mass selective detector. The melting points (mp) were determined using Mettler Toledo MP 90. Flash column chromatography using a silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd was performed on a Combibluff RF200.

3.1.2 Synthesis of A22. To a 250 mL three-necked round bottom bottle, 4-chlorobenzene-1,2-diamine (2, 7.13 g, 0.05 mol) and 85% 2-hydroxypropanoic acid (7.95 g, 0.075 mol) were added and mixed with 40 mL of 4 mol L⁻¹ HCl. The mixture was heated to 90 °C and stirred for 5 h. After the full conversion of the starting material (as monitored by TLC), the reaction was cooled to 30 °C. The pH of the mixture was adjusted to 8 using ammonia water. A white precipitate formed that was collected by filtration, washed with water, and dried under air in a culture dish followed by a drying step performed under vacuum in an oven. A product with the designation 1-(5-chloro-1H-benzo[d]imidazo[1-2-γ]ethan-1-ol was obtained as a white solid (3, yield 93%), MS (ESI) m/z: 197 [M + H]+

Fig. 7 Therapeutic effect of A22 in OP zebrafish. (A) Zebrafish spine fluorescence intensity data were collected within the white rectangle area. A22 was administered at a dose of 0.11 μM, 0.33 μM, 1.00 μM, and etidronate disodium (ED) was administered at a dose of 600 μM; (B) spine fluorescence intensity data were calculated based on values obtained from 30 fish each group. (C) A22 therapeutic effect on zebrafish OP. ***Differences from the model group with probability p < 0.001. The p value is calculated using Dunnett’s T test and differences with probability p < 0.05 are considered statistically significant.

Fig. 6 Binding curves of A20 and A22 dilutions. The affinities (KD) were calibrated with the steady state affinity 1:1 binding model using the evaluation software of Biacore 8K.
1-(5-Chloro-1H-benzo[d]imidazol-2-yl)ethan-1-ol (3, 1.97 g, 0.01 mol), TEMPO (0.016 g, 0.1 mmol) and KBr (0.12 g, 1 mmol) were added to a 100 mL three-necked round bottom bottle. Then, 20 mL of acetic acid and 20 mL of water were added. An 8% aqueous sodium hypochlorite (13.96 g, 0.015 mol) solution was added dropwise within 20 min while keeping the temperature of the mixture between 10 °C and 15 °C. The mixture was stirred for 30 min until full conversion (as monitored by TLC). The mixture was extracted by ethyl acetate (50 mL × 3). The organic phases were combined and washed with 60 mL of 10% sodium thiosulfate and 60 mL of brine. The organic layer was then separated, dried over anhydrous Na2SO4 and evaporated to recover 1-(5-chloro-1H-benzo[d]imidazol-2-yl)ethan-1-one (4, yield 71%), mp 156.1–157.4 °C; MS (ESI) m/z: 195 [M + H]+.

1-(5-Chloro-1H-benzo[d]imidazol-2-yl)ethan-1-one (4, 607.2 mg, 3.1 mmol) and ethanol (10 mL) were added to a 25 mL three-necked round bottom bottle. The resulting mixture was stirred until 4 was fully dissolved. NaOH (0.12 g, 3.12 mmol) was then added and then stirred for another 15 min. 6-Chloropicolinaldehyde (0.44 g, 3.12 mmol) was added. The reaction was stirred for 2 h until full conversion (as monitored by TLC). The reaction was quenched with 5 mL of water while stirring. A yellow solid precipitated from the solution. Filtration was performed to collect the solid and wash it with water. The solid was first dried under air in a culture dish and then separated by flash column chromatography (PE : EA = 10 : 1). 6(1H)-1-(5-chloro-1H-benzo[d]imidazol-2-yl)-3-(6-chloropyridin-2-yl)prop-2-en-1-one (A22, yield 54%) was obtained as yellow solid. mp 251.4–252.7 °C; 1H NMR (400 MHz, d6-DMSO) δ 13.68 (s, 1H), 8.39 (d, J = 15.7 Hz, 1H), 7.99–7.93 (m, 2H), 7.88 (d, J = 15.7 Hz, 1H), 7.84 (d, J = 7.5 Hz, 1H), 7.58 (d, J = 7.6 Hz, 2H), 7.43–7.34 (m, 1H); 13C NMR (101 MHz, d6-DMSO) δ 181.26, 153.43, 151.18, 141.46, 134.07, 130.75, 126.19, 125.80, 124.38, 123.28, 121.01, 114.84, 112.88; HRMS calcd for C15H10ON3Cl2 [M–C; MS (ESI) m/z: 7.6 Hz, 2H), 7.43–7.34 (m, 1H); 13C NMR (101 MHz, d6-DMSO) δ 181.26, 153.43, 151.18, 141.46, 134.07, 130.75, 126.19, 125.80, 124.38, 123.28, 121.01, 114.84, 112.88; HRMS calcd for C15H10ON3Cl2 [M+C; MS (ESI) m/z: 318.01954, found 251.4–252.7 °C; 1H NMR (400 MHz, d6-DMSO) δ 13.68 (s, 1H), 8.39 (d, J = 15.7 Hz, 1H), 7.99–7.93 (m, 2H), 7.88 (d, J = 15.7 Hz, 1H), 7.84 (d, J = 7.5 Hz, 1H), 7.58 (d, J = 7.6 Hz, 2H), 7.43–7.34 (m, 1H); 13C NMR (101 MHz, d6-DMSO) δ 181.26, 153.43, 151.18, 141.46, 134.07, 130.75, 126.19, 125.80, 124.38, 123.28, 121.01, 114.84, 112.88; HRMS calcd for C15H10ON3Cl2 [M+C; MS (ESI) m/z: 318.01942, found 318.01942.

3.1.3 Syntheses of A1–A21 and A23–A61. The syntheses of A1–A21 and A23–A61 used the protocol described for the synthesis of 22 and illustrated in Scheme 1. The starting materials differed. Specifically, the syntheses of A1–A13, A14–A17, A18–A33, A34, A35–A37, A38–A41, and A42–A61 were started from benzene-1,2-diamine, 4-methylbenzene-1,2-diamine, 4-chlorobenzene-1,2-diamine, 4-chlorobenzene-1,2-diamine, 4-chloro-6-nitroaniline, 5-chloro-2-nitroaniline, 4-bromobenzene-1,2-diamine, and 4-nitrobenzene-1,2-diamine, respectively. The data for the characterization of these compounds are provided in the ESI.†

3.2 Molecular docking calculations
CDOCKER of Discovery Studio 4.5 was used to carry out the docking simulation of the compounds to the protein. X-ray crystal structure of Cat K (PDB code 4dmy) was used for the docking studies. The ligand was extracted from the catalytic active site of Cat K, which was defined as the docking site for binding. Both the receptor and the compounds were employed using the CHARMM force field before docking. A higher CDOCKER score (i.e. –DOCKER _ENERGY) indicates a more favourable affinity. Using the docking calculations of CDOCKER, we also obtained the exact pose of the ligand in 4dmy.

3.3 Enzymatic activity assays in vitro
Quenched fluorescent resonance energy transfer (QFRET) technology was used to test the inhibitory activity of the compounds against Cat K (ENZO BML-SE535) catalysing the cleavage of the synthetic peptide Z-Phe-Arg-AMC. Compounds were tested at eight concentrations (diluted from 200 μg mL−1, three replicates per dilution). To each test sample well on a 96-well plate, 2 μL of test compound were mixed with 25 μL of Cat K (0.3 ng mL−1). In control wells, DMSO was added instead of the compounds. In blank wells, DMSO was added instead of compounds and buffer was added instead of Cat K solution. The plate was incubated for 15 min at 37 °C. The fluorescence (F1) was determined at a wavelength of 355 nm excitation and 460 nm emission. Then, 25 μL of 20 μM of Z-Phe-Arg-AMC was then added and the plate was incubated for 1 h at 37 °C. Then, the fluorescence (F2) was measured again at a wavelength of 355 nm excitation and 460 nm emission. The calculation was performed as follows: inhibition (%) = [control (F2–F1) − sample (F2–F1)]/[control (F2–F1) − blank (F2–F1)] × 100%. IC50 was calculated using SPSS.

The Cat L (Sigma, C6854), Cat S (Sigma, SRP6297), and Cat B (Sigma, C8571) inhibitory assays were carried out using a similar protocol, except that the substrates for Cat S and Cat B were replaced with Ac-Lys-Gln-Lys-Leu-Arg-AMC and Z-Arg-Arg-AMC, respectively.

3.4 The inhibition of TRAP in RANKL-induced OC
RAW264.7 cells were seeded into a sterile 96-well plate at a density of 1 × 102 cells per well. To the DMEM culture medium, 10 ng mL−1 RANKL was added to induce the differentiation in RAW264.7 cells. Test compounds were added at 20 μg mL−1; each compound was tested in triplicate. Wells without a compound were used as blanks. The cells were incubated for 72 h at 37 °C in 5% CO2. Then, the culture medium was removed. The residues in the wells were fixed with fixative solution (25.5% citrate solution + 66.3% aceton + 8.2% of 37% formaldehyde) for 30 s and washed with deionized water. Then mixing solution (naphthol AS-BI phosphate solution and tartrate solution) was added and the plate was incubated at 37 °C in the dark. After a final washing step with deionized water, the TRAP-positive cells were counted.

3.5 CTX-I concentration
RAW264.7 cells were seeded into a sterile 12-well plates with bovine femur slices at a density of 1 × 105 cells per well. To the DMEM culture medium, 50 ng mL−1 RANKL was added to induce the differentiation in RAW264.7 cells. Test compounds were added at a concentration of 5 μM. The cells were cultured for five days at 37 °C under 5% CO2. The culture supernatants were collected by centrifugation for analysing the CTX-I concentrations by ELISA.
3.6 Real-time binding to Cat K and analysed by SPR

SPR experiments were carried out using a Biacore 8K (GE Healthcare) at 25 °C. Cat K (ENZO BML-SE553) was preconditioned in pH 5.0 sodium acetate and then immobilized to a CM7 chip by amine coupling. Running buffer was 50 mM NaOAc (pH 5.5), 0.01% P20, 2 mM EDTA, 100 μM DTT. When the affinities of compounds were tested, 5% DMSO was added. Ten concentrations of each compound were used. A20 was diluted from 100 μM by two-fold serial dilution. A22 was diluted from 50 μM by two-fold serial dilution. Compounds were injected to the surface of the protein-coupled chip channels at the flow rate of 30 μL min⁻¹. The affinities (K_d) were calibrated with the steady state affinity 1:1 binding model with the evaluation software of Biacore 8K. The affinities of A20 and A22 were tested twice and five times, respectively.

3.7 OP therapeutic effects in zebrafish B

OP was induced in zebrafish at the age of 2 dpf (day-post-fertilization) by treating normal wild-type AB zebrafish with prednisone for 96 h. Zebrafish larvae were randomly added to wells in 6-well plates. There were 30 fish per well. Etidronate disodium and different concentrations of A22 were dissolved by water and then added to different wells to target concentration. The zebrafish larvae were incubated with the compounds for 96 h at 28 °C.

Zebrafish larvae aged at 7 dpf was dyed with 0.05% calcine solution for 15 min. Then it was washed with water for 15 min on the shaking table (repeated three times). The zebrafish larvae were fixed with methyl cellulose. The spine fluorescent intensity (SFI) images of the calcine-stained vertebra column were captured with AZ100 fluorescence microscopy (Nikon). The integral optical density of 1 to 4 segments of vertebra was measured by Image J analysis software. OP therapeutic effects (%) = (sample SFI - model SFI)/(normal SFI - model SFI) × 100%.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Institute of Medicinal Biotechnology, Chinese Academy of Medical Science and Peking Union Medical College and approved by the Animal Ethics Committee of Institute of Medicinal Biotechnology.

4. Conclusions

In this study, a total of 61 derivatives were designed and synthesized with a shared scaffold that has not been reported elsewhere as Cat K inhibitors and anti-osteoporosis agents. Several derivatives showed an IC₅₀ value below 10 μM against Cat K. We rigorously analysed the structure–activity relationship. A20, A22, and A32 had a significantly higher inhibitory activity against Cat K in vitro than that of the lead compound 1x. Real-time binding analysis by SPR and molecular docking calculations confirmed the binding of A22 to Cat K in vitro. A22 improved spinal bone density in zebrafish with induced OP at an efficacy that was higher than that of the marketed therapeutic bone metabolizer etidronate disodium. Hence, we believe that A22 represents a novel lead compound for antiresorptive drugs that will be examined for further improvements in structure-based drug design, therapeutic efficacy, and pharmacokinetics.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1 S. Tanaka, Am. J. Nephrol., 2007, 27, 466–478.
2 E. Hernlund, A. Svedbom, M. Ivergard, J. Compston, C. Cooper, J. Stenmark, E. V. McColleskey, B. Jönsson and J. A. Kanis, Arch. Osteoporos., 2013, 8, 1–115.
3 O. Ström, F. Borgström and J. A. Kanis, Arch. Osteoporos., 2011, 6, 59–155.
4 Z. Amso, J. Cornish and M. A. Brimble, Med. Res. Rev., 2016, 36, 579–640.
5 S. Fujiwara, E. Hamaya, M. Sato, P. Graham-Clarke, J. A. Flynn and R. Burge, Clin. Interventions Aging, 2014, 5, 1879–1893.
6 B. R. Bhavnani and F. Z. Stanczyk, J. Steroid Biochem. Mol. Biol., 2014, 142, 16–29.
7 S. Sanders and S. A. Geraci, South. Med. J., 2013, 106, 698–706.
8 G. Mazzotti, J. Bilezikian, E. Canalis, D. Cocchi and A. Giustina, Endocrine, 2012, 41, 58–69.
9 J. A. Kanis, E. V. McCloskey, H. Johansson, A. Oden, L. J. Melton and N. Khaltaev, Bone, 2008, 42, 467–475.
10 A. G. Costa, N. E. Cusano, B. C. Silva, S. Cremers and J. P. Bilezikian, Nat. Rev. Rheumatol., 2011, 7, 447–456.
11 M. Novinec and B. Lenarčič, Biol. Chem., 2013, 394, 1163–1179.
12 M. Gowen, F. Lazner, R. Dodds, R. Kapadia, J. Field, M. Tavaria, I. Bertoncello, F. Drake, S. Zavarselk, I. Tellis, P. Hertzog, C. Debouck and I. Kola, J. Bone Miner. Res., 1999, 14, 1654–1663.
13 M. T. Drake, B. L. Clarke, M. J. Oursler and S. Khosla, Endocr. Rev., 2017, 38, 325–350.
14 J. Wijkmans and J. Gossen, Expert Opin. Ther. Pat., 2011, 21, 1611–1629.
15 M. Frizler, M. Stirmberg, M. T. Sisay and M. Gütscshow, Curr. Top. Med. Chem., 2010, 10, 294–322.
16 J. Y. Gauthier, N. Chauret, W. Cromlish, S. Desmarais, L. T. Duong, J. P. Falgueyet, D. B. Kimmel, S. Lamontagne, S. Léger, T. LeRiche, C. S. Li, F. Massé, D. J. McKay, D. A. Nicoll-Griffith, R. M. Oballa, J. T. Palmer, M. D. Percival, D. Riedeau, J. Robichaud, G. A. Rodan, S. B. Rodan, C. Seto, M. Thérien,
