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Natural inhibitors targeting osteoclast-mediated bone resorption

Guang-Zhi Zeng,[a,b] Ning-Hua Tan,[a,*] Xiao-Jiang Hao,[a] Quan-Zhang Mu[a] and Rong-Tao Li[a]

[a]*State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

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Abstract—Human cathepsin K, matrix metalloproteinase 9, and $\alpha_V\beta_3$ integrin are the key regulators in osteoclast-mediated bone resorption. In this paper, we found natural inhibitors **1**–**10** for them by enzyme inhibition assays. Inhibitors **1**–**7**, **8**–**9**, and **10** are novel inhibitors of human cathepsin K, matrix metalloproteinase 9, and $\alpha_V\beta_3$, respectively.

Bone resorption is necessary in many physiological processes of the skeleton, and the physiological remodeling of bone in adults is strictly dependent on it.[1] Bone homeostasis is maintained by the balance between bone-resorbing osteoclasts and bone-forming osteoblasts. When the balance is upset and bone resorption exceeds bone formation, metabolic bone diseases will occur, like osteoporosis, which is an important disease commonly found among elderly populations, especially postmenopausal women.[1,2]

Osteoclast-mediated bone resorption is a multistep process initiated by the attachment of osteoclasts to the bone surface. Integrin $\alpha_V\beta_3$ is highly expressed on osteoclasts, and it can bind to the RGD tripeptide sequence found in extracellular matrix expressed on the bone surface, which facilitates the attachment of osteoclasts to the bone. It also can regulate osteoclast migration along the bone surface.[3] After attaching to the bone surface, a tightly sealed resorption lacuna is created. Then some main proteolytic enzymes expressed in osteoclasts, such as cathepsin K (CatK) and matrix metalloproteinase 9 (MMP-9), are secreted into the lacuna for the removal of bone mineral and the degradation of organic matrix proteins.[1,2,4,5] Furthermore, research also indicated that MMP-9 plays a role in osteoclastic bone resorption by facilitating migration of osteoclastic cells to the bone matrix, and MMP-9 deficiency or blocking MMP-9 activity slowed down the migration of osteoclasts dramatically.[6] So, $\alpha_V\beta_3$, CatK, and MMP-9 are the key regulators in osteoclast-mediated bone resorption, and their inhibitors may be as effective anti-resorption agents in osteoporosis therapy.

In order to search for new natural inhibitors against CatK, MMP-9, and $\alpha_V\beta_3$, we performed the inhibition assays of CatK, MMP-9, and $\alpha_V\beta_3$ on 96-well microtiter plates. With random screening, results indicated that 7 inhibitors (**1**–**7**) for cathepsin K, 2 inhibitors (**8**–**9**) for MMP-9, and 1 inhibitor (**10**) for integrin $\alpha_V\beta_3$ (Fig. 1) were found with IC_{50}s below 10 μg/ml. They are new natural inhibitors from plants, which are different from the inhibitors found for these enzymes before.

Natural compounds **1**–**10** were isolated and identified by us with their purities >95%. Detailed purifications and identifications of these inhibitors were described before.[7–12] Enzyme inhibition data were expressed as IC_{50} values, which were calculated by dose-response curves with at least four concentrations (dilution ratio = 1/2), and the highest tested concentrations are 2.5, 10, and 10 μg/ml for CatK, MMP-9, and $\alpha_V\beta_3$, respectively. Results are expressed as mean IC_{50} values ± standard deviation. Leupeptin (Sigma,

Abbreviations: Z-, carbobenzyloxy-; AMC, [7-amino-4-methyl] coumarin; Nma, *N*-methyl-anthranilic acid; Dnp, 2,4-dinitrophenyl; Leupeptin, acetyl-leucyl-leucyl-arginal.

Keywords: Cathepsin K; Matrix metalloproteinase 9; $\alpha_V\beta_3$; Natural inhibitor; Bone resorption.

* Corresponding author. Tel./fax: +86 871 5223800; e-mail: nhtan@mail.kib.ac.cn

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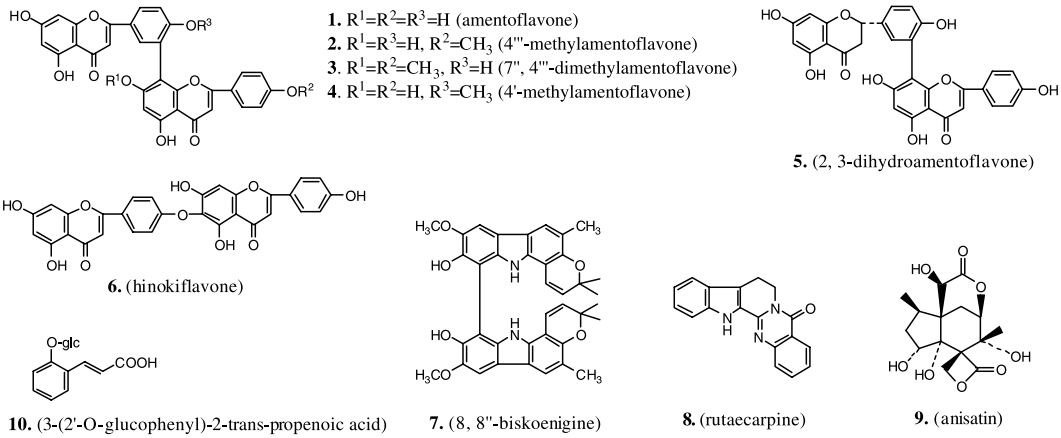

Figure 1. Chemical structures of inhibitors **1**–**10**.

Table 1. Activities of inhibitors **1**–**10** against human CatK, MMP-9, and $\alpha_v\beta_3$

Compound	Plant resources	IC_{50} (μg/ml)		
		Cathepsin K	Matrix metalloproteinase 9	$\alpha_V\beta_3$
1	*Taxodium mucronatum*	1.01 ± 0.20	—	—
2	*Taxodium mucronatum*	1.39 ± 0.15	—	—
3	*Taxodium mucronatum*	0.89 ± 0.23	—	—
4	*Cycas guizhouensis*	0.84 ± 0.14	—	—
5	*Cycas guizhouensis*	0.77 ± 0.08	—	—
6	*Cycas guizhouensis*	0.83 ± 0.20	—	—
7	*Murraya koenigii*	1.18 ± 0.04	—	—
8	*Winchia calophylla*	—	7.95 ± 0.43	
9	*Illicium simonsii*	—	5.80 ± 0.63	—
10	*Eupatorium adenophorum*	—	—	5.97 ± 1.65
RC-1	—	0.018 ± 0.006	—	—
RC-2	—	—	0.0028 ± 0.00054	—
RC-3	—	—	—	0.00028 ± 0.000032

RC-1, 2, and 3 represent leupeptin, (2*R*)-[(4-biphenylylsulfonyl) amino]-*N*-hydroxy-3-phenylpropionamide and Cyclo(Arg-Gly-Asp-D-Phe-Val), respectively.

Averages were calculated from at least two independent experimental data.

L9783), (2*R*)-[(4-biphenylylsulfonyl) amino]-*N*-hydroxy-3-phenylpropionamide[13] (Calbiochem, 444249), and Cyclo(Arg-Gly-Asp-D-Phe-Val)[14] (Calbiochem, 182015) were used as reference compounds for CatK, MMP-9, and $\alpha_v\beta_3$, respectively.

Compound inhibitory activity against CatK (Calbiochem, 219461) was referenced by what Aibe and Barrett et al. did before.[15,16] Test compounds were diluted with the reaction buffer (pH 5.0, 100 mM sodium acetate, 20mM L-cysteine, and 2 mM EDTA), and mixed with the enzyme solution for 5–10 min at 25 °C, then the 75 μM fluorescence substrate Z-GPR-AMC (Bachem, I-1150) was added to start the reaction. The final concentration of CatK in the assay mixture was 4.63 nM. After incubation for 120 min at 37 °C, the fluorescence was monitored at 460/40 nm after excitation at 360/40 nm in a cytofluor II fluorescence plate reader (PerSeptive Biosystems).

Compounds were tested for their ability to inhibit human MMP-9 (Calbiochem, PF024) using the quenched fluorescence assay.[17] Test compounds were diluted with the reaction buffer (pH 7.4, 50 mM Tris, 100 mM sodium chloride, 10 mM calcium chloride, and 0.01% Brij35), and were mixed with the enzyme solution for 120 min at 37 °C, then the 10 μM fluorescence substrate, Dnp-PChaGC(Me)HK(Nma)-NH_2 (Bachem, M-2055) was added to start the reaction. The final concentration of MMP-9 in the assay mixture was 0.58 nM. After incubation for 60 min at 37 °C, the fluorescence was detected at 460/40 nm after excitation at 360/40 nm in a cytofluor II fluorescent plate reader (PerSeptive Biosystems).

Integrin $\alpha_V\beta_3$ ELISA was performed using a modification of the method of Charo and Smith et al.[18,19] Fibrinogen (Sigma, F3879) was dissolved in buffer A (pH 8.2, 100 mM $NaHCO_3$, 100 mM NaCl) with the concentration of 1 mg/ml. Sulfo-*N*-hydroxysuccinimido-Biotin (Pierce, 21217) was added as a solid (0.2 mg of biotin ester in 1 ml fibrinogen solution) and gently mixed for 30 min at room temperature. Unreacted biotin ester was removed by exhaustive dialysis against buffer B (pH 7.4, 50 mM Tris, 100 mM NaCl, and 0.05% NaN_3) at 4 °C, and the biotinylated-Fibrinogen (Fg-Biotin) was stored at −70 °C until used. Integrin $\alpha_V\beta_3$ (Chemicon, CC1019) was diluted with coating buffer (pH 7.5, 20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% NaN_3) with the

concentration of 250 ng/ml, and then immediately added to 96-well microtiter plates (Costar, 9018) at 100 μl/well and incubated overnight at 4 °C. Plates were blocked with 200 μl/well of 1% BSA in coating buffer for 3 h at 37 °C. Then 100 μl Fg-Biotin solution containing 2 μl sample solution was added to each well and incubated for 2 h at 30 °C. Hundred microliters of avidin-alkaline phosphatase (Sigma, A2527) solution was added to each well, and incubated for 15 min at room temperature, and then 100 μl *p*-NPP (Pierce, 34045) solution (10 mg/ml) was added and incubated for 120 min at 37 °C. Finally, OD values were read at 405 nM in a colorimetric plate reader (Molecular Devices, SpectraMax 190). Every time before different solution was added, washing plate with wash buffer (pH 7.4, 50 mM Tris, 100 mM NaCl, 0.1% BSA, and 0.05% Tween 20) was necessary.

Osteoporosis is a disease characterized by excessive bone resorption, which causes changes in the microstructure of the bone matrix and makes bone prone to fracture. Costs of osteoporosis are staggering and increasing every year. Antiresorptive therapies and medicines include the estrogen replacement therapies, selective estrogen receptor modulators, vitamin D, PTH analogues, and bisphosphonates, which show efficacy but reveal serious side effects. Thus, other nonhormonal and more specific therapies are needed.[5] $\alpha_V\beta_3$ integrin, CatK, and MMP-9, as novel and specific drug targets of anti-osteoporosis, have attracted much attention because inhibition of them will decrease activities of osteoclasts. Many inhibitors for these enzymes have been synthesized or isolated, such as E-64, ketoamide derivatives for cathepsin K;[20] TIMP and *N*-sulfonylamino acid derivatives for MMP-9;[13] echistatin and cyclic RGD peptides for $\alpha_V\beta_3$.[14,21] Some of them also displayed good bioavailability in vivo for bone resorption. As part of anti-osteoporosis research, our work was concentrated on osteoclasts. We hope to find novel and specific natural inhibitors for the key targets in osteoclats, and some work has been done before, such as assay of carbonic anhydrase II, another key target in osteoclasts.[22] In this research, compounds we discovered are novel natural inhibitors for cathepsin K, MMP-9, and $\alpha_V\beta_3$ with IC_{50}s below 10 μg/ml. They are nonpeptidic inhibitors against these three enzymes with natural chemotypes of biflavones, alkaloids, sesquiterpene lactones, and lignans (Fig. 1). As demonstrated in Table 1, these compounds exhibited somewhat different activities against CatK, MMP-9, and $\alpha_V\beta_3$, though their inhibitory activities were not as potent as the synthesized, natural or peptide-based ones (Table 1), they provided us new insights into the study of the potent inhibitors of CatK, MMP-9, and $\alpha_V\beta_3$. Some of these compounds showed cytotoxicity on cancer cell lines (data not published before, see footnote[†]).

[†] Compounds **1**–**10** were tested for their cytotoxicity on cancer cell lines. With the test concentration of 10 μg/ml, compounds **1**, **2**, **3**, **7**, and **10** showed weak cytotoxicity with IC_{50}s from 1.60–9.77 μg/ml on A549, BGC-823, SGC-7901, DU145, MDA-MB-231, HT-29, BEL-7402, MCF-7, and B16.

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