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Synthesis and biological evaluation of novel bisphosphonates with dual activities on bone in vitro

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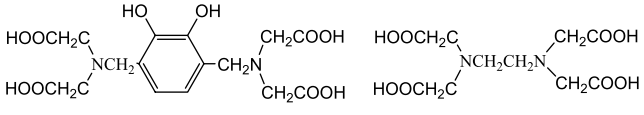
Abstract—In an effort to generate novel anti-osteoporosis agents, new bisphosphonates with various benzoyl groups on side chains have been synthesized and evaluated for activities on both bone resorption and bone formation in vitro. Several candidates showed distinct dual activities: promoting bone formation and inhibiting bone resorption.

Osteoporosis is a common skeletal disease characterized by gradual loss of bone mass and disrupted bone architecture as a result of an imbalance between the bone resorption activity of osteoclasts and the bone formation activity of osteoblasts.[1] Bisphosphonates, stable bone-targeted mimics of inorganic pyrophosphate, are used principally to inhibit osteoclastic bone resorption for the treatment of osteoporosis.[2] Since changes of bone mass can be induced pharmacologically by regulating either bone resorption or bone formation, or ideally both, there has been great interest in the discovery of new bisphosphonates causing anabolic effects on bone in addition to their anti-resorptive effects.[3] Our group and others' attempts[4,5] to achieve this goal by conjugating bisphosphonates with bone formation stimulants have not resulted in significant success so far, presumably because these conjugates, while inactive themselves, are difficult to be hydrolyzed to release active moieties in the bone. We now report the synthesis and biological evaluation of novel dually active bisphosphonates, able to both inhibit bone resorption and stimulate bone formation in cell cultures.

In the development of chelating agents for removing plutonium from bone, we serendipitously observed that CBMIDA, an analog of EDTA (Fig. 1), markedly increased osteoid volume in Beagle dogs.[6] Further studies showed that CBMIDA and other aromatic mimics of EDTA could induce proliferation of rat calvarial-derived osteoblasts in vitro whereas EDTA could not. We reason that incorporation of aromatic groups with simple bisphosphonates might also lead to an anabolic effect on bone. On the other hand, structure–activity relationship (SAR) studies of bisphosphonates have demonstrated that nitrogen-containing bisphosphonates are more potent inhibitors of bone resorption, and furthermore, introduction of a cyclic group on the side chain may increase the anti-resorptive potency.[7] Therefore, we designed and synthesized a new type of bisphosphonates with various aromatic rings on the side chains linked by amide bonds (Fig. 2). A hydroxyl group attached to the geminal carbon atom of the P–C–P



CBMIDA EDTA

Figure 1.

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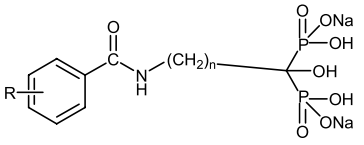


n = 2,3 and 5;
R = alkyloxyl, chloride, nitro etc as shown in Table 1

Figure 2. The structure of the target compound.

group supposedly increases their affinity for bone mineral because bisphosphonates chelate calcium ions more effectively by tridentate rather than bidentate.[2] In addition, the presence of a lipophilic aromatic group may also be favorable for oral bioavailability,[7] which is the major disadvantage of the clinically utilized bisphosphonates.

The synthesis of target compound **3** is shown in Scheme 1. Starting compound **1** was prepared according to Kieczykowski's method.[8] Aromatic acid chloride **2** was prepared from the corresponding benzoic acid. A freshly prepared benzoyl chloride (2 equiv) in THF was added to the solution of tetrasodium salt of aminoalkylenebisphosphonic acid in 2 N NaOH at room temperature. After completion of reaction, the solution was acidified to pH 3–4 with 2 N HCl and the disodium salts of compound **3** were obtained as white crystals. The hygroscopic free bisphosphonic acids can be obtained from ion-exchange chromatography.

Twelve desired compounds **3** have been synthesized in which benzoyl rings can have one or more various substitutes such as methyloxy groups, halogen, nitro, etc., and the length of the methylene chain can vary[9] (Table 1). Their biological activities were evaluated for both the inhibition of bone resorption and promotion of bone formation.

The effects of compound **3** and alendronate (one of most potent bone resorption inhibitors), as reference compound on bone resorption, were examined in bone marrow culture system.[10]

Their inhibitory potency varied according to the side chain length and the benzoyl ring substitution in a wide

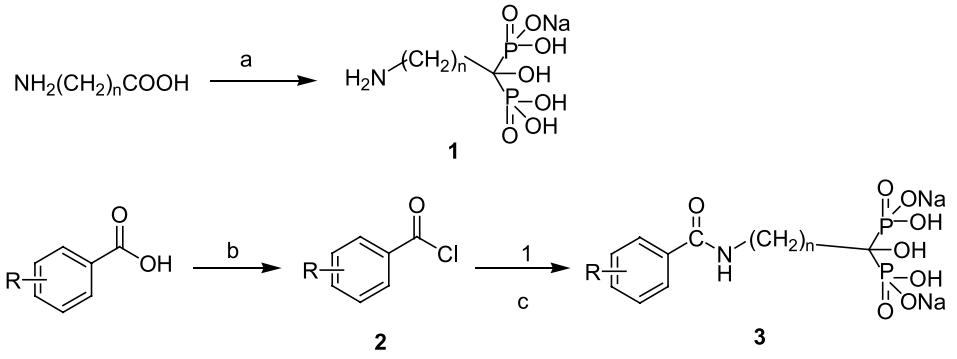
Table 1. Structure of compound **3** and their osteoclast inhibitory activity

Compound	R	*n*	Osteoclast inhibitory ratio mean ± SE
3a	2,3-2OCH_3	2	68.1 ± 5.8
3b	3,4-CH_2O_2	2	60.2 ± 8.4
3c	4-CH_3	2	30.4 ± 4.6
3d	2,3-2OCH_3, 5-NO_2	2	64.4 ± 7.3
3e	3-OCH_3	2	26.9 ± 9.5
3f	H	2	9.5 ± 2.9
3g	3-OCH_3	3	34.2 ± 13
3h	H	3	35.6 ± 7.1
3i	2-CH_3	3	30.3 ± 17.7
3j	3-Cl	5	15.9 ± 5.3
3k	2-CH_3	5	9.4 ± 19.9
3l	4-OCH_3	5	39.0 ± 15.0
Aln			47.7 ± 10.3

The osteoclast precursor cells were plated at a density 5×10^4 cells/well in a 96-well plate cultured for 8 days with or without the presence of 10^{-7} M of bisphosphonates. After culturing, the cells were stained with tartrate-resistant acid phosphonates (TRAP) to count the number of positive cells containing three or more nuclei. The data were inhibitory ratio mean ± SE, $n = 5$. The inhibitory ratio (%) = (number of control cells − number of treated cells)/number of control cells × 100.

range from 9.18% to 68.08%. The overall trend can be seen that electron-donating substituents and short side chains ($n = 2$) provide more effective inhibitors. Three compounds (**3a**,**b**,**d**) with similar structures ($n = 2$, two methyloxy groups or a methylenedioxy group on the ring) displayed stronger activities (68.08%, 60.18%, 64.43%, respectively) than the reference agent, alendronate (47.74%). This result suggests that alkoxy substituents might enhance the anti-resorptive activity.

Compounds **3** were also tested for the proliferative activity in cultures of SD rat calvarial osteoblasts.[10] Results showed that five compounds (**3a**,**b**,**d**,**e**,**g**) stimulated cellular proliferation and this effect was strongly dependent upon the dose and peaked at 72 h after treatment (Table 2). In particular, **3a** markedly increased the cell number over controls throughout the entire concentration range tested, with the peak effect, a 74% increase at 10^{-7} M. Interestingly, there is some overlap in SAR between the proliferative effect on bone formation and the inhibitory effects on bone resorption. Alkylphenolic moieties and short side chains ($n = 2$) not only resulted



Scheme 1. Reagents: (a) H_3PO_3, PCl_3; (b) $SOCl_2$; (c) NaOH.

Table 2. Effects of bisphosphonates on rat calvaria osteoblast proliferation at different concentrations

Compound	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
3a	35.2 ± 8.7	47.4 ± 6.0	74.4 ± 5.1	26.6 ± 3.8
3b	9.3 ± 5.7	−8.3 ± 4.8	−7.3 ± 8.6	−0.2 ± 3.3
3d	17.7 ± 15.5	−1.5 ± 3.6	21.4 ± 9.8	−0.8 ± 1.4
3e	8.9 ± 6.9	7.1 ± 1.6	−5.5 ± 2.6	0.6 ± 3.5
3g	0.7 ± 2.4	−5.5 ± 1.9	−12.4 ± 2.4	3.7 ± 6.7

The cells were plated at a density of 2×10^4 cells/well in 24-well plates and treated with various concentrations of bisphosphonates for 72 h. Proliferation was determined using MTT colorimetric assay. The data were proliferative ratio mean ± SE, $n = 3$. The proliferative ratio (%) = (number of treated cells − number of control cells)/number of control cells × 100.

in more potent inhibitors of osteoclasts, but also are essential for the proliferative effect on osteoblasts. Moreover, compound **3a**, the most potent promoter and inhibitor, shares the same catechol-like scaffold with CBMID, the original lead compound (Fig. 2). Given that similar structural features frequently appear in natural anti-osteoporosis compounds such as estrogen (Fig. 3), we believe that phenolic or alkylphenolic moieties might provide biological benefits to anti-osteoporosis agents.

Intrigued by the extraordinary proliferative effect of compound **3a**, we further investigated its effect on the maturation and differentiation of osteoblasts with bioassay of the activity of alkaline phosphatase (ALP) and the synthesis of Collagen type I carboxyterminal propeptide (CICP) and osteocalcin (OC).[10] In our study (Table 3), alkaline phosphatase levels increased 61% over controls within 72 h after the treatment of **3a** at 10^{-6} M, indicating enhanced differentiation of the existing cell population. While not significantly affecting the synthesis of Type I collagen, compound **3a** remarkably enhanced osteocalcin expression up to fourfold compared to controls in a dosage-dependent manner. These results indicate that compound **3a** is also a promoter of osteoblast maturation.

In summary, we have identified a series of novel bisphosphonates that significantly promote osteoblastic bone formation, aside from their roles as potent inhibitors of osteoclastic bone resorption in cell cultures. This result suggests that these compounds, especially **3a**, might offer distinct advantages over currently available bisphosphonates, which selectively target osteoclasts. The further investigation of their bioavailabilities and activities in vivo is in progress.

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9. Analytic data for a representative compound **3a**: Anal. Calcd for $C_{12}H_{17}O_{10}NP_2Na_2{\cdot}4H_2O$: C, 27.96; H, 4.85; N,

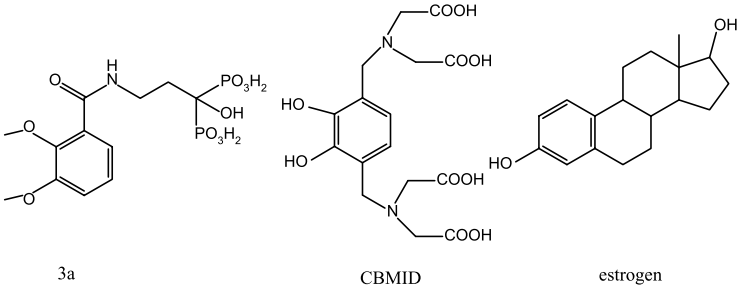

Figure 3. The structures of three anti-osteoporosis compounds.

Table 3. Effect of compound **3a** on the levels of alkaline phosphatase, osteocalcin and Type I collagen

Compound	Concentration (M)	ALP	OC	CICP
3a	10^{-6}	3.60 ± 0.84*	11.73 ± 1.42***	4.06 ± 0.04
	10^{-7}	2.15 ± 0.91	8.63 ± 0.17***	4.17 ± 0.09
	10^{-8}	0.75 ± 0.30	4.73 ± 0.01**	4.03 ± 0.02
	10^{-9}	0.56 ± 0.41	4.07 ± 0.16	4.27 ± 0.07
Control		2.23 ± 1.50*	3.92 ± 0.32***	4.07 ± 0.27

The cells were plated at a density of 5×10^3 cells/well in 96-well plates and treated with various concentrations of compound **3a** for 72 h. The alkaline phosphatase activity was determined by an established technique with *p*-nitrophenylphosphate as the substrate. Collagen type I carboxyterminal propeptide (CICP) and osteocalcin (OC) concentrations were measured from the cell lysates. All the markers were normalized to the relative number of viable cells as determined directly in the 96-well plates. Data are presented as mean ± SE ($n = 5$, *$0.1 > P > 0.05$; **$0.05 > P > 0.01$; ***$P < 0.01$).

2.72. Found: C, 27.97; H, 4.82; N, 2.81; ^{1}H NMR (D_2O) ppm: 2.35–2.50 (m, 2H, CH_2<); 3.80–3.89 (m, 2H, –$NHCH_2$–); 3.91 (s, 1H, –OCH_3); 3.98 (s, 1H, –OCH_3); 7.24–7.36 (m, 3H, ArH); ^{13}C NMR (D_2O) ppm: 35.10 (CH_2<); 38.15 (–$NHCH_2$–); 58.49 (–CH_3); 64.22 (–OCH_3); 75.04 (1-C); 118.21–154.59 (6C, Ar); 171.21 (C=O); ^{31}P NMR (D_2O) ppm: 19.49.

10. Materials and methods of bioactivity evaluation: Osteoclasts were prepared from thighbone, shoulder bone, and tibia of 24 h new born rat. The cells were stained with tartrate-resistant acid phosphonates (TRAP) to count the number of positive cells. Primary osteoblast-enriched cultures were prepared from newborn Sprague-Dawley rat calvaria at axenic circumstances. Parietal bones were dissected free from the sutures. The periosteal layers from both sides of the bones were removed carefully. The bones were cut into chips and digested with pancreas for 30 min. After centrifugation, the precipitate cells were diluted with Dulbecco's Modified Eagles Medium (DMEM). Then the obtained osteoblast-enriched cell suspension was seeded in culture flask and cultured at 37 °C in the presence of 5% CO_2. Medium was changed every 3 days. Cell viability was measured using a MTT Cell Proliferation Assay. Absorbance readings of the wells were taken of 492 nm. The alkaline phosphatase activity was determined by an established technique with *p*-nitrophenylphosphate as the substrate. T Collagen type I carboxyterminal propeptide (CICP) concentration was determined in cell culture supernatants with an ELISA. Osteocalcin was measured by the use of serum osteocalcium radioimmunity kit from the Radioimmunity Institute of the Liberation Army's General Hospital. All the makers of osteoblastic differentiation values were normalized to the relative number of viable cells as determined directly in the 96-well plates using the above-mentioned proliferation assay.