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Synthesis and biological evaluation of indolyl bisphosphonates as anti-bone resorptive and anti-leishmanial agents ☆

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ABSTRACT

A series of indole conjugated bisphosphonate derivatives have been synthesized and evaluated for their in vitro anti-bone resorptive activity using bone marrow osteoclast culture. Two bisphosphonates **23** and **24** significantly inhibited osteoclastogenesis, **23** showed inhibition at 10 and 100 pM which was lower than the concentration of standard drug alendronate, and **24** inhibited osteoclastogenesis at 100 nM which was comparable to alendronate. Two other compounds **13** and **14** also showed inhibition comparable to alendronate, but were cytotoxic in the osteoblast cells. The two active bisphosphonates **23** and **24** induced significant osteoclast apoptosis at concentrations 100 nM for compound **24** and at 10 pM for compound **23** compared to alendronate. In vivo effect of active bisphosphonates **23** and **24** resulted in osteoclastogenesis of bone marrow cells (BMCs) to almost 40–50% (**23** showing 8.4% decrease and **24** showing 9.0%) compared to 16.5% of the ovariectomized group. Further, screening of anti-leishmanial activity, four compounds **24**–**25** and **27**–**28** showed more than 80% inhibition against both the promastigote and amastigote stages of the *Leishmania* parasite.

1. Introduction

Osteoporosis is a metabolic bone disorder, which affects 40–50% of the elderly female and 10–15% of the elderly male population. It is characterized by fragile bones, compromised bone strength leading to an increased risk of both vertebral and non-vertebral fractures, and has a devastating effect on the lives of postmenopausal women, leading to a worsening of the quality of life, substantial morbidity, and mortality.[1] The prevention and the treatment of this disease is therefore of paramount importance.

Nitrogen-containing bisphosphonates are the mainstay of anti-resorptive therapy for osteoporosis.[2,3] They inhibit bone resorption by reducing osteoclastic activity and induce osteoclast apoptosis.[4] Over the past 2–3 decades, many bisphosphonate derivatives have been developed and used in the treatment of many skeletal disorders, such as osteoporosis, Paget disease, bone pain,[5–7] and are effective in reducing fracture risks in women with postmenopausal osteoporosis. Bisphosphonates have also been found to be effective in vitro growth inhibitors of parasitic protozoa such as *Trypanosoma cruzi, Trypanosoma brucei rhodesiense*, *Leishmania donovani*, and *Leishmania mexicana*,[8–10] *Plasmodium falciparum, and Toxoplasma gondii*, which are the causative agents of Chagas's disease, visceral leishmaniasis, malaria, cryptosporidiosis, respectively. Residronate and pamidronate are active against experimental infections of both cutaneous and visceral leishmaniasis. Recent reports also show use of bisphosphonates in the management of cancer, particularly in cases of bone metastases in breast or prostate cancers.[11–13]

Bisphosphonates work through the binding of the phosphonate groups to hydroxyapatite. The presence of two phosphonate groups allows the molecules to act as a 'bone hook' essential for targeting to bone and for the molecular mechanism of action of the compounds.[14] There are two side groups in bisphosphonates: an R^1 and R^2 attached to the carbon bearing phosphonate groups. R^1 is generally a hydroxyl group that enables enhanced binding to bone and R^2 signifies the variable group.[15,16] The structure of the R^2 accounts for the significant differences in the biological potency between the various bisphosphonates and plays a potential role in modulating binding to bone mineral, and for differences in cellular and molecular effects on osteoclasts.[17] The presence and position of nitrogen within the structure of bisphosphonates is highly significant and is shown to influence the relative potency of various bisphosphonates.[18]

Most of the highly potent third-generation bisphosphonates contain heterocyclic rings with one or more nitrogen atom, for

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example, risedronic and zoledronic acids.[19] Among different heterocycles, indole nucleus is a fundamental constituent of a large number of natural and synthetic compounds. It is a well-established pharmacophore present in many drug molecules with remarkable biological activities.[20] The 2-phenyl indole nucleus has been explored as a possible estrogen mimic due to its structural similarity to estradiol. Recently, some tetracyclic indoles being rigid molecules have shown resemblance to natural estrogens with respect to binding affinity to the estrogen receptor and mixed estrogen agonist/antagonist activities.[21] Some tetracyclic N-substituted dihydrobenzothiepino and dihydrobenzoxepino indole derivatives synthesized in our group as selective estrogen receptor modulators have also shown substantial inhibition on bone resorption.[22] To extend our work further on development of anti-osteoporotic agents, we decided to synthesize new bisphosphonates having phosphonate groups conjugated to tetracyclic indoles through spacer carbon chain. In this paper, we report the synthesis and biological potency of these novel indolyl bisphosphonates.

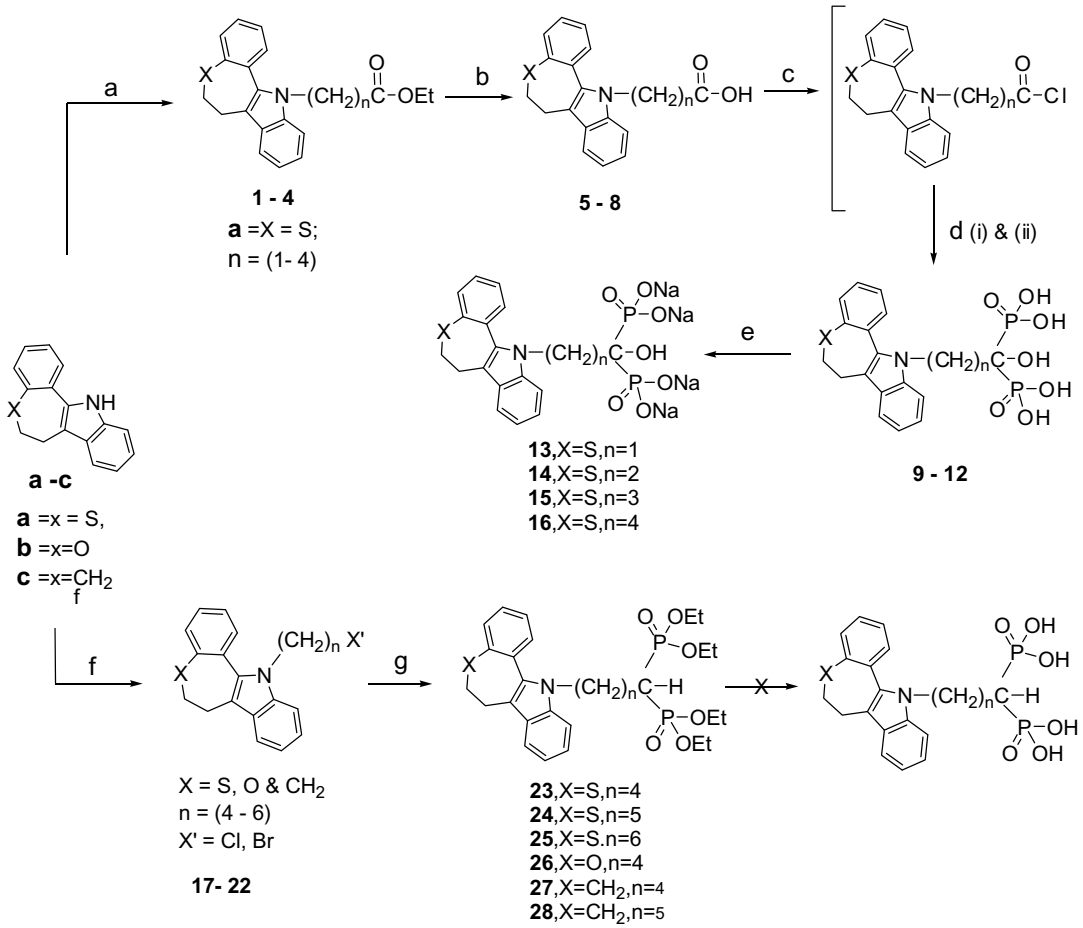
2. Results and discussion

2.1. Chemistry

Two different types of indolyl bisphosphonates were prepared, with and without hydroxyl group on 1-carbon atom bearing the N-alkyl chain linked to the tetracyclic indoles and the two phosphonate groups.

For the preparation of 1-hydroxy-1,1-indolyl bisphosphonates (**13–16**), tetracyclic 6,7-dihydro-12*H*-benzothiepino [5,4-*b*] indole(**a**) was prepared by Fisher indole synthesis.[23] Alkylation with bromoesters of variable chain length formed N-alkylated esters (**1–4**) which on alkaline hydrolysis gave acids (**5–8**) in good yields. Acids, on reaction with oxalyl chloride converted into the corresponding acid chlorides, but were found to be unstable and decomposed on purification by silica gel chromatography. However, the in-situ formation of acid chlorides and subsequent reaction with two equivalents of tris(trimethylsilyl)phosphite (Arbuzov reaction)[24] at room temperature formed the silyl esters, which on methanolysis gave the desired 1-hydroxymethylene-1,1-indolyl bisphosphonic acids (**9–12**) as viscous oils in satisfactory yields. They were converted into their sodium salt by dissolving the liquids in methanol and treating with 2 N NaOH solution. On adjusting the pH of the solutions between 6 and 7, bisphosphonates precipitated as yellow colored crystalline solids (**13–16**) which were stable and could be stored for long periods. All the compounds were characterized by spectral analyses.

The second type of bisphosphonates (**23**–**28**), in which the 1-hydroxyl functionality is replaced by a hydrogen atom, were prepared as follows: alkylation of tetraethyl methylene bisphosphonate with different indolyl *N*-alkyl halides (**17–22**, prepared by the N-alkylation of tetracyclic indoles with ω-dihaloalkanes) in sodium hydride and dry THF gave tetraesters (**23–28**) in good yields. Acidic hydrolysis with hydrobromic–acetic acid solution to form bisphosphonic acids was not successful as the pure acids could not be isolated by column chromatography, hence the compounds



Scheme 1. Reagents: (a) bromoester/NaH/dry DMF; (b) 10% NaOH/dioxane/rt; (c) oxalyl chloride/stir/rt; (d) i—$P(OSiMe_3)_3$/dry THF/rt; ii—MeOH; (e) 2 N NaOH/MeOH; (f) ω-dihaloalkane/NaH/dry DMF; (g) $CH_2[P(O)(OCH_2CH_3)_2]_2$/NaH/dry THF.

were easily purified as tetraesters and identified by spectral analyses (Scheme 1).

2.2. Biological activity

Reduced osteoblast survival along with increased osteoclast functions are the two key cellular mechanisms observed in postmenopausal osteoporosis. Hence, while trying to inhibit osteoclast function or induce osteoclast cell death, ensuring osteoblast survival becomes essential. Therefore, all the synthesized bisphosphonates (**13–16** and **23–28**) were first tested in primary osteoblast cells for their effect on cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at concentrations ranging from 10 pM to 1 μM. The results showed that bisphosphonates **16** and **23–27** had no effect on osteoblast viability, while compounds **13–15** and **26** exhibited cytotoxic effect on osteoblast (Table 1).

The non-cytotoxic bisphosphonates **16** and **23–27** were then studied for their effect on osteoclastogenesis using murine bone marrow cultures. Alendronate was used as standard and 100 nM of alendronate maximally inhibited osteoclastogenesis (Fig. 1A) which was determined by the number of TRAP (tartarate resistant acid phosphatase) positive cells. Biphasic anti-osteoclastic action of alendronate has been reported in multiple myeloma bone disease.[25] Therefore, the effects of various bisphosphonates were compared at 100 nM concentration along with alendronate and it was observed that bisphosphonates **16**, **25**, and **27** inhibited osteoclastogenesis, whereas for **28**, osteoclastogenesis was reduced but was not found to be statistically significant. This activity was not comparable to that of alendronate (Fig. 1B). Activity of only bisphosphonate **24** in inhibiting osteoclastogenesis was found to be comparable with that of alendronate, however, it is remarkable that bisphosphonate **23**, which had no effect at 100 nM, inhibited osteoclastogenesis at 100 pM with comparable efficacy to that of alendronate (Fig. 2A). Osteoclastogenesis data for the two active compounds **23** and **24** at all the concentrations ranging from 10 pM to 1 μM have also been represented in Figure 2A and B, respectively.

Since the two bisphosphonates **23** and **24** had comparable effects to that of alendronate in inhibiting osteoclastogenesis, we next assessed their role in osteoclast apoptosis. Bone marrow cells were first differentiated to osteoclast following the protocol mentioned in Section 4. Differentiated osteoclasts were serum deprived for 4 h to stimulate apoptosis. Hoechst 33258 staining was performed to determine the characteristic chromatin condensation of apoptotic cells. Increased apoptosis was observed in the osteoclast cells treated with the bisphosphonates **23** and **24** (Fig. 3A). Further, whereas alendronate induced osteoclast apoptosis at 100 μM concentration, bisphosphonates **23** and **24** were able to do so at much lower concentrations of 10 pM and 100 nM, respectively (Fig. 3B). Similar to our results, other bisphosphonates, for example, risedronate, pamidronate, and clodronate have also been shown to promote apoptosis in osteoclasts in vitro and in vivo.[26]

Table 1
In vitro cell viability assay (MTT) in the osteoblast cells

Bisphosphonates	Cell proliferation assay in osteoblast cells (MTT)
13	Cytotoxic
14	Cytotoxic
15	Cytotoxic
16	Non-cytotoxic
23	Non-cytotoxic
24	Non-cytotoxic
25	Non-cytotoxic
26	Cytotoxic
27	Non-cytotoxic
28	Non-cytotoxic

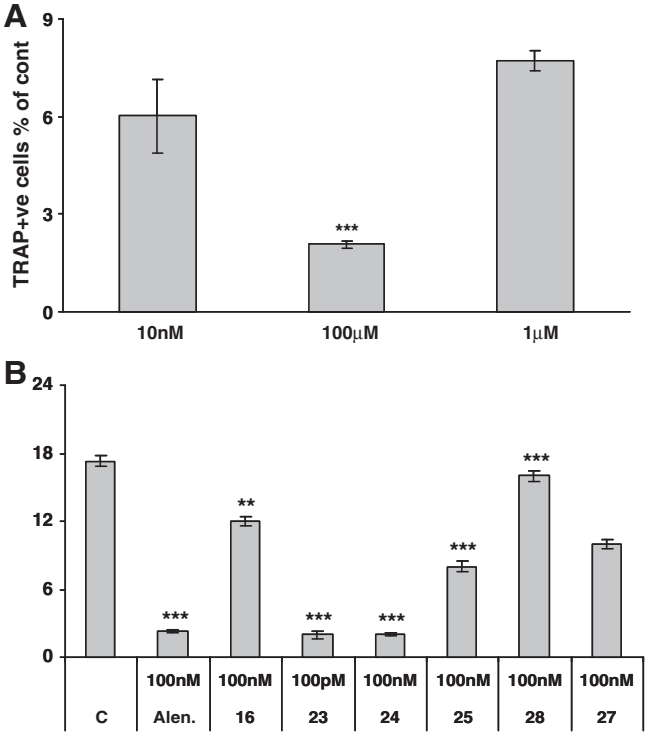


Figure 1. Effect of Alendronate (Alen.) (A) and synthesized compounds (B) on osteoclastogenesis in mouse bone marrow cultures. Bone marrow cells (3×10^5) were plated in the presence of MCSF (30 ng/mL) and RANKL (50 ng/mL) in 8-well chamber slides for a period of 7 days. The cultures were continued in the presence or in the absence of the synthesized compounds and most effective concentration of alendronate (100 nM) as control. Medium was changed after 3 days. At the end of experiment, cells were fixed in 4% paraformaldehyde and then proceeded for TRAP staining. Graph is plated to the % of TRAP + cells at various concentrations. Values represent mean SD of three ($n = 3$) independent experiments $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group.

Effect of bisphosphonates **23** and **24** was also studied in vivo in ovariectomized (OVx) Balb/c mice (menopausal model of osteoporosis). Osteoclastogenesis in response to RANKL (receptor activator for nuclear factor κ B ligand) + MCSF(macrophage colony-stimulating factor) treatment for 7 days in bone marrow cells (BMCs) from various groups was compared. Figure 4 shows that OVx resulted in ~2.5-fold increase in osteoclastogenesis of BMCs compared with sham group. OVx mice treated with bisphosphonates **23** and **24** exhibited reduced osteoclastogenesis of BMCs compared with OVx mice treated with vehicle. In fact, treatment of OVx mice with bisphosphonates **23** and **24** resulted in osteoclastogenesis of BMCs comparable to that of sham group. Similar results have already been reported[27] with alendronate given to mice for periods up to 4 weeks which suppressed bone resorption.

From the results, it can be inferred that the reason for the activity of active compounds **23** and **24** includes inhibition of osteoclast formation from precursors and inhibitory or toxic effect on mature osteoclasts. Osteoclast apoptosis may therefore be a major mechanism whereby these bisphosphonates reduce osteoclast numbers and activity. Further, it was clearly observed that 1-hydroxy bisphosphonates showed greater toxicity as compared to tetraesters bearing no free hydroxyl functionality. The optimum carbon chain of 5 and 6 carbon atoms linking phosphonate groups to the tetracyclic indole was found to be the best (compounds **23** and **24**), further increase or decrease in the carbon atoms resulted in the loss of activity.

The efficacy of the synthesized bisphosphonates **13–16** and **23–28** was also explored on the inhibition of proliferation of promatigotes and amastigotes of parasite *L. donovini*, the causative agent of

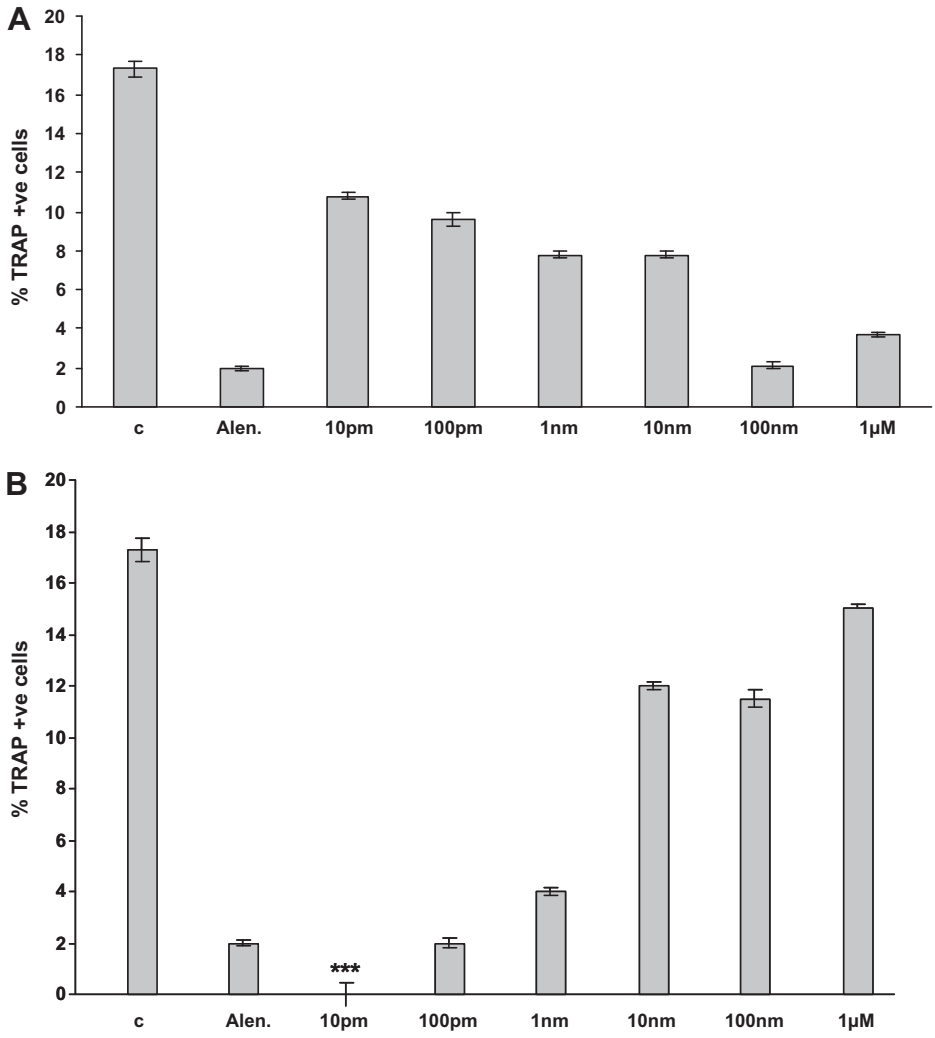


Figure 2. Effect of the two active compounds **23** (A) and **24** (B) on osteoclastogenesis. Various concentrations ranging from 10 pM to 1 μM, of the compounds were used to study osteoclastogenesis as per method given in Figure 1. Data show mean ±SD of three independent experiments. $^{*,**,***}p < 0.01$, $p < 0.005$, and $p < 0.001$, respectively, and have been compared to standard drug alendronate.

visceral leishmanasis. Cell viability was first determined using the MTT to study the cytotoxic effects expressed as 50% lethal dose. IC_{50} values were estimated through the preformed template. The in vitro screening on the viability of *Leishmania* promastigotes for anti-promastigote activity was assessed by monitoring the MTT metabolism after a 96 h culture period in the presence of the respective compounds in mouse macrophage cell line (J-774A1) infected with promastigotes in stationary culture stage. The compounds were also assessed for their anti-amastigote (semi-in vivo) activity against the amastigote stage of the parasite. Four compounds (**24, 25, 27**, and **28**) showed >80% inhibition against both the stages of the parasite. These compounds were then studied for IC_{50} evaluation against amastigotes and three compounds **24, 25, and 27** showed significant activity (IC_{50} = <5 μg/mL), whereas compound **28** was found toxic (Table 2). As in the case of anti-bone resorptive activity, here also tetraesters showed anti-leishmanial activity compared to 1-hydroxy bisphosphonates.

3. Conclusion

This study has shown that attachment of two phosphonate groups through linker carbon chain at the tetracyclic indole nitrogen formed novel bisphosphonates of which two bisphosphonates **23** and **24** showed significant bone inhibitory effects by inhibiting osteoclast functions which were similar to or better than alendronate. Presence of ester groups in these two compounds increases the lipophilic character which may be responsible for their better anti-osteoclastogenic activity despite lacking a hydroxyl group on C-1 atom. However, the cytotoxicity and lack of any significant anti-osteoclastogenic activity by 1-hydroxy 1,1-bisphosphonic acid sodium salts is surprising.

4. Experimental

4.1. General methods

Melting points were taken in open capillary tube on an electrically heated melting point apparatus Complab, and are uncorrected. IR spectra were recorded on Perkin-Elmer RX-1 spectrophotometer using KBr pellets. The FAB spectra were recorded using a beam of Argon (2–8 eV) on Joel SX 102/DA-6000 mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX-200 (at 200 MHz for ^{1}H and at 50 MHz for ^{13}C) or DRX-300 (at 300 MHz for ^{1}H and at 75 MHz for ^{13}C) spectrometers using $CDCl_3$ and TFA-d as solvent. Tetramethylsilane served as an internal standard in ^{1}H NMR and $CDCl_3$ in ^{13}C spectra. Silica gel (60–120 mesh) was used for column chromatography, while silica gel (230–400 mesh) was used for flash chromatography. TLC was

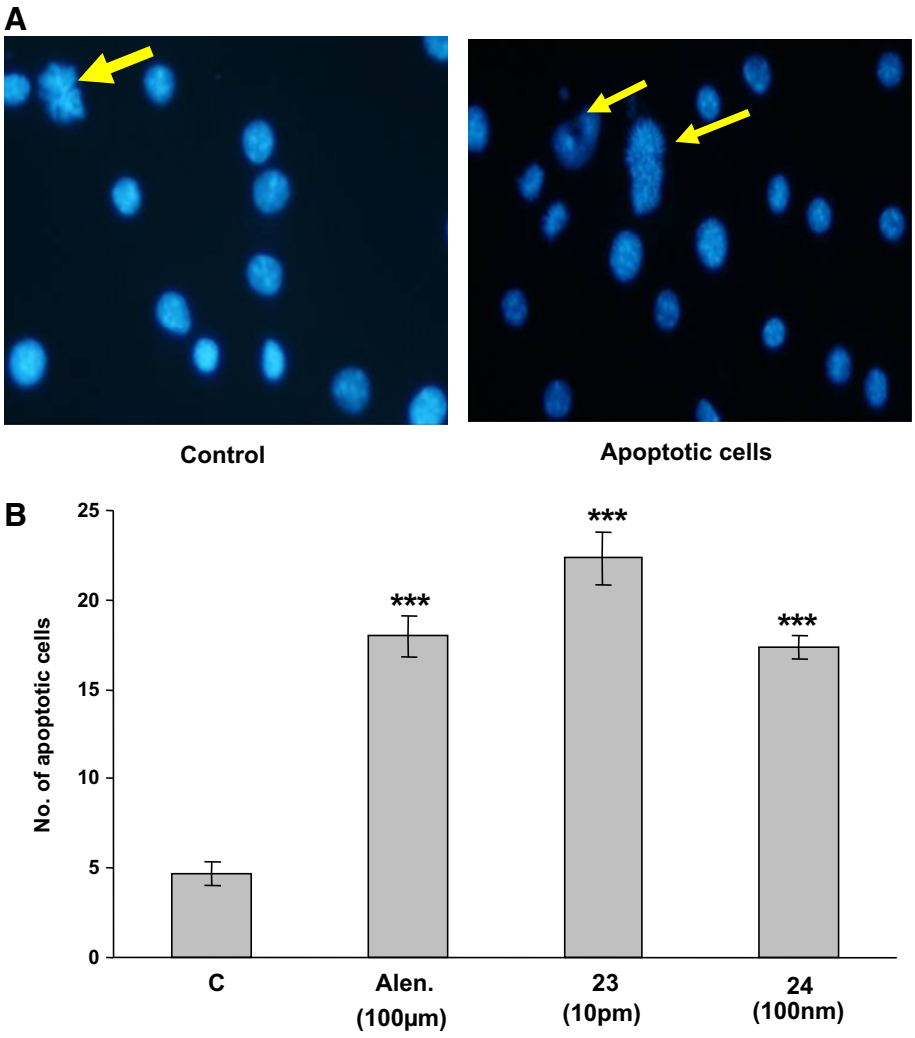


Figure 3. The figure shows the effect of two active compounds **23** and **24** on osteoclast apoptosis. Bone marrow cells (3×10^5) were plated in the presence of MCSF (30 ng/mL) and RANKL (50 ng/mL) in 8-well chamber slides for a period of 6 days and allowed them to form multinucleated osteoclasts. On day 6, cells were serum-deprived for 4 h and then compound treatment (**23** and **24**) was given to the cells for 48 h. Cells were then fixed and stained with Hoechst-33258 (0.2 mM) to confirm apoptosis. (A) Representative dark field photomicrograph of Hoechst-33258 stained cells. Non-apoptotic osteoclasts presented normal nuclei (left panel) and apoptotic osteoclasts presented condensation of chromatin (right panel) indicated by arrows. (B) Number of apoptotic cells were counted in each well (5 fields/well) of the chamber slide. Data have been expressed as the number of apoptotic cells for each compound and have been compared to control and alendronate. Values represent mean SD of three independent experiments ($n = 3$) $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control group.

run either on precoated silica gel 60 *F*254 and RP-18 F_{254} (Merck) or handmade plates. Detection of spots was done either by iodine vapors or by spraying with 1% ceric sulfate in 1 M H_2SO_4 followed by heating at 110 °C.

4.1.1. (6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-acetic acid ethyl ester (1)

To a suspension of NaH (60% suspension in oil, 60 mg, 1.5 mmol) in 5 mL dry DMF was added 6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (**a**, X = S) (251 mg, 1 mmol) dissolved in 5 mL dry DMF at 0 °C with stirring under nitrogen atmosphere. After 15 min, 2-bromoethyl acetate (250 mg, 1.5 mmol, dissolved in 10 mL DMF) was added dropwise and stirring was continued at room temperature for 2 h. The reaction mixture was poured into water and extracted with ethyl acetate (3× 10 mL), organic layer was washed with water (2× 10 mL), dried over sodium sulfate, and concentrated. The desired ester was purified by column chromatography and recrystallized from benzene/hexane to yield compound **1** as crystalline solid, 252 mg (75%), mp 92 °C; IR (KBr) ν_{max}: 1210, 1367, 1460, 1742, 2932 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.27 (t, J = 4.8 Hz, 3H), 3.07 (t, J = 6.6 Hz, 2H), 3.51 (t, J = 6.6 Hz, 2H), 4.26 (q, J = 4.8 Hz, 2H), 4.78 (s, 2H), 7.42–7.18 (m, 6H, ArH), 7.61 (d, J = 7.4 Hz, 1H, ArH), 7.76 (d, J = 7.5 Hz, 1H, ArH); FAB-MS (*m*/*z*): 337 $[M]^+$.

4.1.2. 3-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-propionic acid ethyl ester (2)

Yield 66%; mp 93 °C; IR (KBr) ν_{max}: 1222, 1360, 1464, 1746, 2933 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.08 (t, J = 7.1 Hz, 3H), 2.45 (t, J = 7.7 Hz, 2H), 3.14 (t, J = 6.4 Hz, 2H), 3.46 (t, J = 6.4 Hz, 2H), 3.95 (q, J = 7.1 Hz, 2H), 4.48 (t, J = 7.7 Hz, 2H), 7.43–7.06 (m, 6H, ArH), 7.53 (d, J = 7.7 Hz, 1H, ArH), 7.70 (d, J = 7.5 Hz, 1H, ArH); ESI-MS (*m*/*z*): 351 $[M+H]^+$.

4.1.3. 4-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-butyric acid ethyl ester (3)

Yield 90%; oil; IR (Neat) ν_{max}: 1219, 1357, 1466, 1748, 2931 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 (t, J = 7.0 Hz, 3H), 1.25–1.19 (m, 2H), 1.90 (t, J = 6.5 Hz, 2H), 2.04 (t, J = 5.8 Hz, 2H), 3.54 (t, J = 5.8 Hz, 2H), 3.92 (t, J = 6.5 Hz, 2H), 4.35 (q, J = 7.0 Hz, 2H), 7.75–7.16 (m, 8H, ArH); ESI-MS (*m*/*z*): 366 $[M+H]^+$.

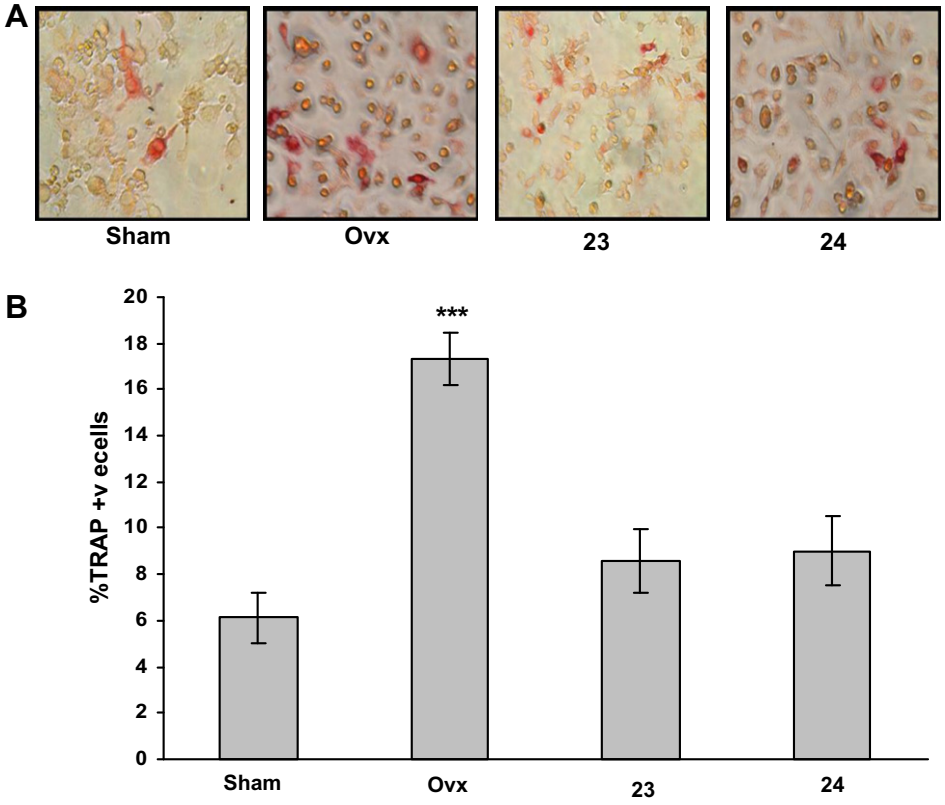


Figure 4. The figure shows the effect of bisphosphonates **23** and **24** in vivo in ovariectomized (OVx) Balb/c mice. Six- to 8-week old mice were ovariectomized and fed with compound **23** and **24** (10.0 mg/kg body weight in 20% ethanol) for 30 days. Sham and OVx controls were administered 20% ethanol (vehicle). At the end of treatment the mice were sacrificed and bone marrow flushed and bone marrow cells (5×10^5) were cultured in 48-well plate in the presence of MCSF (30ng/mL) and RANKL (50 ng/mL) for 7 days. Cells fixed on day 7 and stained for TRAP. (A) Representative photomicrograph of TRAP-stained cells that were counted. (B) Percentage of TRAP positive cells in sham + vehicle, Ovx + vehicle and Ovx + compounds **23** and **24**. Data have been expressed as the number of animals taken in each group ($n = 5$) $^{***}p < 0.001$ compared with sham group.

4.1.4. 5-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-pentanoic acid ethyl ester (4)

Yield 66%; oil; IR (Neat) ν_{max}: 1223, 1359, 1466, 1739, 2928 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.12 (t, $J = 6.9$ Hz, 3H), 1.34–1.22 (m, 2H), 2.05 (t, $J = 9.3$ Hz, 2H), 2.26 (t, $J = 7.2$ Hz, 2H), 3.34 (t, $J = 7.1$ Hz, 2H), 3.96 (t, $J = 9.3$ Hz, 2H), 4.06 (t, $J = 7.1$ Hz, 2H), 4.18 (q, $J = 6.9$ Hz, 2H), 7.39–7.08 (m, 6H, ArH), 7.53 (d, $J = 7.6$ Hz, 1H, ArH), 7.70 (d, $J = 7.6$ Hz, ArH); ESI-MS (m/z): 380 $[M+H]^+$.

4.1.5. (6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-acetic acid (5)

Compound **1** (1.0 g, 3 mmol) was dissolved in dioxane (20 mL), 10% aqueous solution of NaOH (5 mL, 7.7 mmol), was added and stirred the mixture was stirred for 3 h. The reaction mixture was neutralized by adding 20% aqueous HCl solution, and then extracted with ethyl acetate (3× 20 mL), the organic layer was washed with water (2× 10 mL), dried over Na_2SO_4, and concentrated at reduced pressure. The desired product was purified by crystallization in benzene/hexane to yield **5** as white solid, 800 mg (87%), mp 142 °C; IR (KBr) ν_{max}: 1278, 1352, 1461, 1699, 2370, 2923 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.09 (t, $J = 6.2$ Hz, 2H), 3.52 (t, $J = 6.2$ Hz, 2H), 5.65 (s, 2H), 7.44–7.17 (m, 6H, ArH), 7.62 (d, $J = 7.5$ Hz, 1H, ArH), 7.76 (d, $J = 6.9$ Hz, 1H, ArH); ESI-MS (m/z): 310 $[M+H]^+$.

4.1.6. 3-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-propionic acid (6)

Yield 78%; mp 132 °C; IR (KBr) ν_{max}: 1272, 1358, 1458, 1693, 2372, 2926 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.56 (t, $J = 7.9$ Hz, 2H), 3.00–2.71 (m, 2H), 3.53–3.47 (m, 2H), 4.54 (t, $J = 7.6$ Hz, 2H), 7.49–7.14 (m, 6H, ArH), 7.60 (d, $J = 7.7$ Hz, 1H, ArH), 7.75 (d, $J = 7.5$ Hz, 1H, ArH); ESI-MS (m/z): 324 $[M+H]^+$.

4.1.7. 4-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-butyric acid (7)

Yield 83%; mp 138 °C; IR (KBr) ν_{max}: 1277, 1354, 1465, 1695, 2329, 2927 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.82–1.78 (m, 2H), 2.01 (t, $J = 7.2$Hz, 2H), 2.87–2.79 (m, 2H), 3.62–3.59 (m, 2H), 4.28 (t, $J = 7.2$Hz, 2H), 7.38–7.06 (m, 6H, ArH), 7.54 (d, $J = 7.6$Hz, 1H, ArH), 7.68 (d, $J = 7.6$Hz, 1H, ArH); ESI-MS (m/z): 338 $[M+H]^+$.

4.1.8. 5-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-pentanoic acid (8)

Yield 76%; mp 142 °C; IR (KBr) ν_{max}: 1275, 1361, 1466, 1689, 2328, 2933 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.36–1.22 (m, 2H), 1.64–1.59 (m, 2H), 2.11 (t, $J = 7.1$Hz, 2H), 2.87–2.84 (m, 2H), 3.70–3.67 (m, 2H), 4.25 (t, $J = 6.9$ Hz, 2H), 7.48–7.11 (m, 6H, ArH), 7.60 (d, $J = 7.7$ Hz, 1H, ArH), 7.76 (d, $J = 7.5$ Hz, 1H, ArH); ESI-MS (m/z): 352 $[M+H]^+$

4.1.9. [2-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-1-hydroxy-1 phosphono ethyl]-phosphonic acid sodium salt (13)

(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-acetic acid, **5** (0.8 g, 2.4 mmol) was dissolved in oxalyl chloride (10 mL) and stirred overnight. Excess of oxalyl chloride was removed under reduced pressure to give (6,7-dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-acetyl chloride, which was immediately used without further purification. The crude 6,7-dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-acetyl chloride was dissolved in dry THF (10 mL) and added tris (trimethylsilyl) phosphite (2 mol) one mole each at

Table 2
In vitro anti-leishmanial activity of bisphosphonates against promastigotes and amastigotes of *L. donovini*

Compound	Anti-promastigotic activity		Anti-amastigotic activity (MQ/amast.model)		Cytotoxicity CC_{50} (μg/mL)
	Concn tried (μg/mL)	% Inhibition compared to control	Concn tried (μg/mL)	% Inhibition compared to control IC_{50} (μg/mL)	
13	10	NI	10	33.47	
14	10	23.48	10	NI	
15	10	NI	10	6.3	
16	10	NI	10	55.31	
23	10	27.78	10	9.45	
24	10	100	10	78.2	3.66
	6.25	99.62	5	58.75	
			2	16.75	
			1	4.2	
				IC_{50} = 1.69	
25	10	100	10	85.0	4.50
	6.25	90.13	5	67.21	
			2	40.05	
			1	25.5	
				IC_{50} = 2.56	
26	10	39.43	10	NI	
27	10	99.76	10	82.32	5.81
	6.25	86.36	5	78.30	
			2	80.56	
			1	3.0	
				IC_{50} = 24	
28	10	85.65	10	Toxic	
	6.25	26.07			
Sodium stibogluconate (reference drug)	3000	86.81	200	99.80	297.38
	2000	72.82	100	78.83	
	1000	55.53	50	48.35	
	500	29.1	25	11.45	
		IC_{50} = 940		IC_{50} = 53.62	

15 min interval was added. On completion, excess of solvent was removed under reduced pressure, 20 mL of methanol (for the hydrolysis of silyl ester) was added and the mixture was stirred for 2 h to give hydroxyl compound **9** as viscous solid. It was converted into sodium salt form by dissolving the viscous compound in methanol and by adding 2 N NaOH solution dropwise to the reaction mixture, when the sodium salt precipitated out. It was filtered, washed with methanol, and acetone to yield **13** as yellow solid, 550 mg (48%), mp > 250 °C; ^{1}H NMR (D_2O): δ 3.10 (t, J = 6.2 Hz, 2H), 3.51 (t, J = 6.2 Hz, 2H), 5.49 (s, 2H), 7.81–7.29 (m, 8H, ArH); ^{31}P NMR ($CDCl_3$): δ *+19.46;* ESI-MS (m/z): 544 $[M+H]^+$.

4.1.10. [3-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-1-hydroxy-1-phosphono-propyl]-phosphonic acid sodium salt (14)

Yield 51%; mp >250 °C; ^{1}H NMR (TFA-d): δ 3.13 (t, J = 7.9 Hz, 2H), 3.48 (t, J = 7.6 Hz, 2H), 3.81 (t, J = 7.6 Hz, 2H), 4.84 (t, J = 7.9 Hz, 2H), 8.13–7.64 (m, 8H, ArH); ^{31}P NMR ($CDCl_3$): δ *+23.98;* ESI-MS (m/z): 558 $[M+H]^+$.

4.1.11. [4-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-1-hydroxy-1 phosphono butyl]-phosphonic acid sodium salt (15)

Yield 51%; mp >250 °C; ^{1}H NMR (TFA-d): δ 2.64–2.49 (m, 2H), 2.91 (t, J = 7.2 Hz, 2H), 3.61 (t, J = 7.2 Hz, 2H), 3.84 (t, J = 7.0 Hz 2H), 4.96 (t, J = 7.2 Hz, 2H), 8.31–7.62 (m, 8H, ArH); ^{31}P NMR ($CDCl_3$): δ +23.80; ESI-MS (m/z): 572 $[M+H]^+$.

4.1.12. [5-(6,7-Dihydro-5-thia-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-1-hydroxy-1-phosphono-pentyl]-phosphonic acid sodium salt (16)

Yield 42%; mp >250 °C; ^{1}H NMR (TFA-d): δ 2.06–2.02 (m, 2H), 2.36–2.30 (m, 2H), 3.31 (t, J = 7.1 Hz, 2H), 3.99–3.91 (m, 4H), 4.53 (t, J = 7.1 Hz, 2H), 7.76–7.32 (m, 8H, ArH); ^{31}P NMR ($CDCl_3$): δ +23.90; ESI-MS (m/z): 586 $[M+H]^+$.

4.1.13. 12-(4-Chloro-butyl)-6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (17)

To a suspension of NaH (60% suspension in oil, 0.48 g, 11.9 mmol) in 10 mL dry DMF was added 6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (**a**) (2.0 g, 7.9 mmol, dissolved in 10 mL dry DMF) at 0 °C with stirring under nitrogen atmosphere. After 15 min, 1-bromo-3-chlorobutane (1.1 mL, 9.5 mmol) was added dropwise and stirring was continued at room temperature for 2 h. The reaction mixture was poured into water and extracted with ethyl acetate (3× 20 mL), organic layer was washed with water (2× 20 mL), dried over Na_2SO_4, and concentrated. The desired product was purified by column chromatography to yield compound **17** as a crystalline solid, 2.2 g (81%) mp 72 °C; ^{1}H NMR ($CDCl_3$): δ 1.55–1.46 (m, 2H), 1.77–1.73 (m, 2H), 3.10 (t, J = 6.2 Hz, 2H), 3.24 (t, J = 6.2 Hz, 2H), 3.55–3.51 (m, 2H), 4.30 (t, J = 6.2 Hz, 2H), 7.46–7.16 (m, 6H, ArH), 7.61 (d, J = 7.4 Hz, 1H, ArH), 7.78 (d, J = 7.6 Hz, 1H, ArH); ESI-MS (m/z): 342 $[M+H]^+$.

4.1.14. 12-(5-Bromopentyl)-6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (18)

Yield 66%; mp 123 °C; ^{1}H NMR ($CDCl_3$): δ 1.09–1.01 (m, 4H), 1.51–1.44 (m, 4H), 3.10 (t, J = 6.8 Hz, 2H), 3.48–3.44 (m, 2H), 4.18 (t, J = 7.0 Hz, 2H), 7.07–7.15 (m, 6H, ArH), 7.51 (d, J = 7.5 Hz, 1H, ArH), 7.69 (d, J = 7.5 Hz, 1H, ArH); ESI-MS (m/z): 401 $[M+H]^+$.

4.1.15. 12-(6-Bromohexyl)-6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (19)

Yield 68%; mp 131 °C; ^{1}H NMR ($CDCl_3$): δ 1.11–1.08 (m, 4H), 1.67–1.55 (m, 6H), 3.23 (t, J = 6.8 Hz, 2H), 3.56–3.52 (m, 2H), 4.25

(t, J = 7.0 Hz, 2H), 7.45–7.15 (m, 6H, ArH), 7.60 (d, J = 7.7 Hz, 1H, ArH), 7.77 (d, J = 7.5 Hz, 1H, ArH); ESI-MS (m/z): 414 $[M]^+$.

4.1.16. 12-(4-Chlorobutyl)-6,7-dihydro-12*H*-5-oxa-12-aza-dibenzo[*a*,*e*]azulene (20)

Yield 81%; oil; ^{1}H NMR ($CDCl_3$): δ 1.57–1.34 (m, 2H), 1.80–1.66 (m, 2H), 2.96 (t, J = 6.4 Hz, 2H), 3.30 (t, J = 6.4 Hz, 2H), 4.23 (t, J = 6.4 Hz, 2H), 4.51 (t, J = 6.4 Hz, 2H), 7.37–7.05 (m, 7H, ArH), 7.48 (d, J = 7.4 Hz, 1H, ArH); ESI-MS (m/z): 326 $[M+H]^+$

4.1.17. 12-(3-Chlorobutyl)-5,6,7,12-tetrahydro-benzo[6,7]cyclohepta[1,2-*b*]-indole (21)

Yield 83%; oil; ^{1}H NMR ($CDCl_3$): δ 2.03–1.96 (m, 4H), 2.23–2.17 (m, 2H), 2.50 (t, J = 6.5 Hz, 2H), 2.58 (t, J = 6.9 Hz, 2H), 3.20 (t, J = 6.2 Hz, 2H), 4.40 (t, J = 6.9 Hz, 2H), 7.38–7.04 (m, 7H, ArH), 7.53 (d, J = 7.5 Hz, 1H, ArH); ESI-MS (m/z): 324 $[M]^+$.

4.1.18. 12-(5-Bromopentyl)-5,6,7,12-tetrahydro-benzo[6,7]cyclohepta[1,2-b]-indole (22)

Yield 49%; oil; ^{1}H NMR ($CDCl_3$): δ 1.25–1.17 (m, 2H), 1.75–1.69 (m, 4H), 2.34–2.24 (m, 2H), 2.59 (t, J = 6.6 Hz, 2H), 2.66 (t, J = 7.1 Hz, 2H), 3.23 (t, J = 6.6 Hz, 2H), 4.30 (t, J = 7.1 Hz, 2H), 7.41–7.11 (m, 7H, ArH), 7.61 (d, J = 7.3 Hz, 1H, ArH); ESI-MS (m/z): 382 $[M]^+$.

4.1.19. [1-(Diethoxy-phosphoryl)-5-(6,7-dihydro-5-thia-12-aza-dibenzo[*a*,*e*] azulen-12-yl) pentyl]-phosphonic acid diethyl ester (23)

To a suspension of NaH (0.26 g, 6.5 mmol, 60% oil suspension) in dry THF (20 mL) was added tetraethylmethylene bisphosphonate (1.3 mL, 5.2 mmol) dropwise at 0 °C under nitrogen atmosphere with continuous stirring for 1.5 h. Thereafter, 12-(4-chloro-butyl)-6,7-dihydro-12*H*-5-thia-12-aza-dibenzo[*a*,*e*]azulene (**17**) (1.5 g, 4.3 mmol, dissolved in 15 mL of THF) was added dropwise and the solution was allowed to stand for 2 weeks. The reaction mixture was filtered. The filtrate obtained was concentrated under reduced pressure and the desired product was purified by column chromatography to yield compound **23** as an oil, 110 mg (45%), ^{1}H NMR ($CDCl_3$): δ 1.15–1.11 (m, 12H), 1.34–1.27 (m, 4H), 1.92–1.88 (m, 4H), 3.92–3.89 (m, 2H), 4.08–4.04 (m, 12H), 4.41 (t, J = 6.4 Hz, 2H), 7.81–7.14 (m, 7H, ArH), 8.06 (d, J = 7.4 Hz, 1H, ArH); ^{31}P NMR ($CDCl_3$): δ +19.46; ESI-MS (m/z): 594 $[M+H]^+$.

4.1.20. [1-(Diethoxy-phosphoryl)-6-(6,7-dihydro-5-thia-12-aza-dibenzo[*a*,*e*]-azulen-12-yl)-hexyl]-phosphonic acid diethyl ester (24)

Yield 21%; oil; ^{1}H NMR ($CDCl_3$): δ 1.11–1.07 (m, 4H), 1.36–1.30 (m, 12H), 1.44–1.38 (m, 2H), 1.64–1.59 (m, 2H), 2.17 (t, J = 6 Hz, 2H), 3.54–3.49 (m, 2H), 4.13–4.07 (m, 8H), 4.23 (t, J = 6 Hz, 2H), 7.45–7.15 (m, 6H, ArH), 7.60 (d, J = 7.2 Hz, 1H, ArH), 7.77 (d, J = 7.5 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): δ 16.66 (CH_3), 16.74 (CH_3), 16.80 (CH_3), 16.84 (CH_3), 23.78 (CH_2), 25.78 (CH_2), 26.73 (CH_2), 28.99 (CH_2), 29.12 (CH_2), 34.37 (CH_2), 37.02 (CH_2), 44.38 (CH), 62.69 (CH_2), 62.83 (CH_2), 62.88 (CH_2), 62.98 (CH_2), 110.67 (CH), 116.17 (C), 118.37 (CH), 119.87 (CH), 122.43 (CH), 127.49 (C), 127.88 (CH) 128.37 (CH), 129.80 (CH), 135.50 (C), 136.14 (CH), 136.37 (C), 137.49 (C), 137.96 (C); ^{31}P NMR ($CDCl_3$) δ +3.98; ESI-MS (m/z): 608 $[M]^+$.

4.1.21. [1-(Diethoxy-phosphoryl)-7-(6,7-dihydro-5-thia-12-aza-dibenzo[*a*,*e*]-azulen-12-yl-heptyl]-phosphonic acid diethyl ester (25)

Yield 24%; oil; ^{1}H NMR ($CDCl_3$) δ 1.13–1.09 (m, 4H), 1.32–1.27 (m, 12H), 1.45–1.38 (m, 2H), 1.78–1.63 (m, 2H), 1.93–1.80 (m, 2H), 2.20 (t, J = 6.7 Hz, 2H), 3.55–3.51 (m, 2H), 4.15–4.07 (m, 8H), 4.23 (t, J = 6.7 Hz, 2H), 7.60–7.14 (m, 6H, ArH), 7.61 (d, J = 7.8 Hz, 1H, ArH), 7.78 (d, J = 7.5 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): δ 16.57 (CH_3), 16.76 (CH_3), 16.80 (CH_3), 16.88 (CH_3), 23.06 (CH_2), 25.80 (CH_2), 26.63 (CH_2), 29.26 (CH_2), 30.14 (CH_2), 34.43 (CH_2), 37.08 (CH_2), 39.73 (CH_2), 44.34 (CH), 62.71 (CH_2), 62.85 (CH_2), 63.00 (CH_2), 63.11 (CH_2), 110.69 (CH), 116.11 (C), 118.39 (CH), 119.85 (CH), 122.41 (CH), 127.50 (C), 127.90 (CH), 128.36 (CH), 129.84 (CH), 135.53 (C), 136.14 (CH), 136.41 (C), 137.46 (C), 137.96 (C); ^{31}P NMR ($CDCl_3$) δ +24.02; ESI-MS (m/z): 622 $[M+H]^+$.

4.1.22. [1-(Diethoxy-phosphoryl)-5-(6,7-dihydro-5-oxa-12-aza-dibenzo[*a*,*e*]azulen-12-yl)-pentyl]-phosphonic acid diethyl ester (26)

Yield 5.7%; oil; ^{1}H NMR ($CDCl_3$): δ 1.27–1.19 (m, 12H), 1.54–1.39 (m, 2H), 2.12–2.07 (m, 4H), 3.13–3.09 (m, 2H), 3.83–3.79 (m, 2H), 4.09 (t, J = 6 Hz, 2H), 4.13–4.06 (m, 8H), 4.55 (t, J = 6 Hz, 2H), 7.36–7.01 (m, 7H, ArH), 7.63 (d, J = 7.5 Hz, 1H, ArH); ^{31}P NMR ($CDCl_3$) δ +19.46; ESI-MS (m/z): 578 $[M+ H]^+$.

4.1.23. [1-(Diethoxy-phosphoryl)-4-(6,7-dihydro-5H-benzo[6,7]-cyclohepta[1,2-*b*]-indol-12-yl)-pentyl]-phosphonic acid diethyl ester (27)

Yield 5.5%; oil; ^{1}H NMR ($CDCl_3$): δ 1.18–1.09 (m, 12H), 1.24–1.20 (m, 2H), 1.31–1.27 (m, 4H), 2.17 (t, J = 6 Hz, 2H), 2.30 (t, J = 6 Hz, 2H), 2.62 (t, J = 6 Hz, 2H), 4.05–3.89 (m, 8H), 4.30 (t, J = 6 Hz, 2H), 7.37–7.13 (m, 7H, ArH), 7.38 (d, J = 6.7 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): δ 14.98 (CH_3), 14.99 (CH_3), 15.06 (CH_3), 15.16 (CH_3), 18.75 (CH_2), 27.96 (CH_2), 28.40 (CH_2), 31.54 (CH_2), 31.74 (CH_2)32.80 (CH_2), 35.25 (CH_2), 42.37 (CH), 61.24 (CH_2), 61.28 (CH_2), 61.32 (CH_2), 61.36 (CH_2), 108.76 (CH), 113.57 (C), 116.87 (CH), 117.97 (CH), 120.23 (CH), 124.77 (CH), 125.86 (CH), 126.45 (CH), 128.59 (CH), 131.40 (C), 132.81 (C), 134.74 (C), 135.86 (C), 141.00 (C); ^{31}P NMR ($CDCl_3$) δ +23.98; ESI-MS (m/z): 576 $[M+H]^+$.

4.1.24. [1-(Diethoxy-phosphoryl)-6-(6,7-dihydro-5*H*-benzo[6,7]-cyclohepta[1,2-*b*]-indol-1-yl)-hexyl]-phosphonic acid diethyl ester (28)

Yield 19%; oil; ^{1}H NMR ($CDCl_3$): δ 1.30–1.19 (m, 12H), 1.48–1.40 (m, 2H), 1.71–1.66 (m, 4H), 2.32–2.27 (m, 4H), 2.60 (t, 2H), 2.67 (t, J = 4.5 Hz, 2H), 4.15–4.09 (m, 8H), 4.28 (t, J = 5 Hz, 2H), 7.41–7.15 (m, 7H, ArH), 7.62 (d, J = 7.1 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): δ 16.34 (CH_3), 16.36 (CH_3), 16.42 (CH_3), 16.44 (CH_3), 19.98 (CH_2), 26.53 (CH_2), 28.67 (CH_2), 29.66 (CH_2), 32.81 (CH_2), 34.84 (CH_2), 36.61 (CH_2), 38.38 (CH_2), 43.87 (CH), 62.43 (CH_2), 62.52 (CH_2), 62.60 (CH_2), 62.69 (CH_2), 110.04 (CH), 116.17 (C), 118.15 (CH), 119.17 (CH), 121.40 (CH), 126.04 (CH), 127.14 (CH), 127.68 (CH), 129.86 (CH), 135.51 (C), 136.47 (C), 136.66 (C), 137.49 (C), 137.96 (C); ^{31}P NMR ($CDCl_3$) δ +23.98; ESI-MS (m/z): 590$[M]^+$.

4.2. Experimental protocols for anti-bone resorptive activity

For the screening of anti-bone resorptive activity, cell culture media, and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals and cytokines MCSF and RANKL were purchased from Sigma Aldrich (St. Louis, MO).

4.2.1. Culture of calvarial osteoblasts

Rat calvarial osteoblasts were obtained following our previously published protocol of sequential digestion.[28] Briefly, calvaria from ten to twelve 1- to 2-day-old Sprague–Dawley rats (both sexes) were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10–15 min) digestions at 37 °C in a solution containing 0.1% dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were collected,

centrifuged, resuspended, and plated in T-25 cm^2 flasks in α-MEM (modified Eagle's medium) containing 10% FCS (fetal calf serum) and 1% penicillin/streptomycin (complete growth medium).

4.2.2. Cell proliferation assay

Osteoblast cells were cultured in absence or in presence of various concentrations [C, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM] of compounds from series **13–16** and **23–28** for 48 h. After incubation, the cells were washed with phosphate buffer saline (PBS). The cells were then treated with MTT solution (5 mg/10 mL in DMEM devoid of (Phenol Red) for 4 h. Formazon crystals formed were dissolved in dimethyl sulfoxide and optical density (OD) was taken at 570 nm.[29]

4.2.3. Osteoclastogenesis

Mice were killed by cervical dislocation. Femur and tibia bones were isolated in a Petri dish in MEM without FCS (washing medium). Bone marrow was flushed with medium using a syringe. The cell suspension (stem cells) was centrifuged at 1500 rpm for 7 min. The pellet was resuspended in 1 mL of α-MEM containing FCS, and then transferred to T25 cm^2 culture flask in growth medium containing MCSF (30 ng/μl) and RANKL (50 ng/μl). The flask was kept in a 37 °C incubator with 5% carbon dioxide supply. After 24 h, the overlaying medium was discarded, cells were washed twice, and centrifuged at 1500 rpm for 7 min. Supernatant was discarded and pellet was resuspended in 1 mL of culture medium. Ten microliters of suspension was taken and cells were counted with hemocytometer. The cells were loaded in the 8-well chamber slides. The compound was added at various concentrations [C, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM] on the first day of culture. The medium was changed on alternate days. After 7 days of cell culture, cells were fixed in formaldehyde. The cells were TRAP (tartarate resistant acid phosphatase) stained. After staining, cells were observed, those cells which were pink were TRAP positive cells.

4.2.4. Apoptosis experiment

4.2.4.1. Hoechst-33258 staining. Bone marrow cells were differentiated into osteoclasts in the presence of cytokines for 6 days. On day 6, cells were serum-deprived for 4 h and then compound treatment (**23** and **24**) was given to the cells for 48 h. Cells were then fixed and stained with Hoechst-33258 to confirm apoptosis. Staining was performed in order to visualize nuclear morphology and nucleosomal DNA fragmentation in osteoclasts. The wells were washed with PBS, mounted, and analyzed under epifluorescence microscope. Cells with nuclei containing condensed chromatin or cells with fragmented nuclei were defined as apoptotic cells.

4.2.5. Ex-vivo experiments

The study was conducted in accordance with current legislation on animal experiments [Institutional Animal Ethical Committee (IAEC)] at C.D.R.I. Ten female mice weighing ~40–50 g were taken for the study (n = 10/group). Mice were ovariectomized (OVx). The other 10 rats were exposed to a sham surgical procedure. All mice were individually housed at 21 °C, in 12 h/12 h light–dark cycles. Normal chow diet and water were provided ad libitum. Compounds **23** and **24** were given at a dose of 10 mg/kg body weight (compounds dissolved in 20% ethanol) for one month to ovarictomized mice. Treated mice were sacrificed at the end of the month and their femoras and tibea were dissected to remove bone marrow cells (BMCs), which were then cultured in α-MEM plus ascorbate-2-phosphate (1.0 mM), β-glycerophosphate, and dexamethasone. Cells were plated for osteoclastogenesis in the presence of osteoclast differentiation medium containing MCSF and RANKL for 7 days. For osteoclastogenesis, the cells were plated in 48-well plate for a period of 7 days. At the end of the experiment, the cells were fixed in 4% paraformaldehyde and then stained for TRAP positive cells.

4.2.6. Statistical analysis

Results are presented as the means ± SEM of results from three or four cultures, and the significance of differences was analyzed by Student's t-test. Groups were analyzed via t-tests (two-sided) or ANOVA for experiments with more than two subgroups. Probability values of $p < 0.05$ were considered to be statistically significant. For osteoclastogenesis experiments, results are expressed as the % of TRAP positive cells for the various concentrations taken.

4.3. Experimental protocols for in vitro screening of anti-promastigote activity

Parasite: The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) obtained from Imperial College, London (UK) in 1979 has been maintained since then in this laboratory in vitro as promastigotes in NNN medium and as amastigotes in golden hamsters and was used for in vitro testings.

4.3.1. Experimental protocols for extra cellular anti-promastigotes activity

The effect of compounds on the viability of *Leishmania* promastigotes was assessed by monitoring the MTT metabolism (Sigma Chemical Co.) after a 96-h culture period in the presence of the respective compounds.[30] Parasites in stationary culture stage were seeded at 1×10^6/100 μl medium 199 per well in 96-well flat-bottomed microtitre plates (Cellstar). Further, 100 μl of medium 199 per well with different concentrations of test compounds or drug standard, dissolved in DMSO, was added in triplicate to achieve desired concentrations (5 and 10 μg mL^{-1}). Parallel dilutions of DMSO alone did not affect the parasite growth. The plates were incubated at 25 °C for 92 h prior to MTT (20 μl per well of a 5 mg mL^{-1} PBS stock) addition, and then for further 4–5 h. MTT processing was stopped and formazan crystals were solubilized by adding 50 μl per well acidified 20% SDS (Qualigens, India) and by incubating overnight at 37 °C. The relative amount of formazan per well produced by viable cells was measured photometrically at 570 nm. As a control, the activity of each compound was determined, and no substantial interaction was found. Sodium stibogluconate was used in each set of experiments as experimental control.

4.3.2. Intracellular anti-amastigote activity

For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J-774A-1) infected with promastigotes in stationary culture stage was used.[31] Cells were seeded in 16-well chamber slides (Nunc) (5×10^4cell/100 μl/well) in RPMI-1640 containing 10% fetal calf serum and the slides were incubated at 37 °C in a CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing stationary-phase promastigotes (2.5×10^5/100 μl/well). Promastigotes invade the macrophage and are transformed into amastigotes. At 24 h of internalization of promastigotes, test material in appropriate concentrations (1–10 μg/mL) in complete medium was added after replacing the previous medium, and the plates were incubated at 37 °C in a CO_2 incubator for 72 h. After incubation, the drug-containing medium was decanted and cells are fixed with methanol and stained with 5% Geimsa stain for 45 min, and at least 100 infected macrophages per sample were counted under optical microscope. Efficacy was expressed as percent inhibition of amastigote multiplication using formula given below. Sodium stibogluconate was used in each set of experiment as experimental control.

$$\text{Percentage Inhibition(PI)} = \frac{\text{AT} \times 100}{\text{AC}}$$

PI: Percent inhibition of amastigote multiplication.
AT: Average number of amastigotes/100 macrophage cells in treated groups.
AC: Average number of amastigotes/100 macrophage cells in control groups.

4.3.3. Cytotoxicity assay

The cell viability was determined using the MTT assay.[32] J774A-1 cell lines were maintained in RPMI medium (Sigma) supplemented with 10% fetal calf serum and 40 mg/mL gentamycin. Exponentially growing cells (1×10^4 cells/100 μl/well) were incubated with different drug concentrations for 72 h and were incubated at 37 °C in a humidified mixture of CO_2 and 95% air in an incubator. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μl of MTT reagent (5 mg/mL) in PBS medium, followed by syringe filtration was added to each well and incubated at 37 °C for 2 h. At the end of the incubation period, the supernatants were removed by tilting plate completely without disturbing cell layer and 150 μl of pure DMSO was added to each well. After 15 min of shaking, the readings were recorded as absorbance at 544 nm on a microplate reader. The cytotoxic effect was expressed as 50% lethal dose, that is, as the concentration of a compound which provoked a 50% reduction in cell viability compared to cells in culture medium alone. IC_{50} values were estimated through the preformed template as described.[33]

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