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Synthesis, characterization and biological activity of hydroxyl-bisphosphonic analogs of bile acids

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ABSTRACT

Bisphosphonates (BPs) are now the most widely used drugs for diseases associated with increased bone resorption, such as osteoporosis, and tumor bone diseases. A significant drawback of the BPs is their poor oral absorption that is enhanced by the presence of bile acid substituents in the bisphosphonate framework, with no toxic effects. A straightforward synthesis of bile acid-containing hydroxy-bisphosphonates and a full characterization of these pharmaceutically important molecules, including an evaluation of affinity and the mechanism of binding to hydroxyapatite, is presented. The biological activity of bile acid-containing bisphosphonate salts was determined using the neutral-red assay on the L929 cell line and primary cultures of osteoclasts. The bioactivity of the new compounds was found superior than bisphosphonates of established activity.

1. Introduction

Geminal bisphosphonates (BPs) are metabolically stable analogs of the naturally occurring inorganic pyrophosphate, which have shown to impair the formation and dissolution of calcium phosphate crystals [1]. Like pyrophosphate, bisphosphonates have high affinity for bone mineral hydroxyapatite (HAP) and, at high doses, can modulate calcification both in vitro and in vivo. There is no doubt, however, that the action in vivo is mediated through mechanisms other than the physico-chemical inhibition of crystal dissolution. BPs are internalized by osteoclasts and interfere with the action of the bone resorbing through specific biochemical processes. They can affect osteoclast-mediated bone resorption in a variety of ways that include osteoclast recruitment, differentiation, resorptive activity and apoptosis [2–4]. Therefore, a significant number of these compounds are currently being used for the treatment of several bone disorders such as osteoporosis, myeloma, hypercalcemia and Paget's disease [1b,c]. Chemically, the bisphosphonates used in humans are all characterized by two phosphonate groups bridged through a $-CR^1R^2-$ moiety, Fig. 1.

It has been proved that R^1 substituents with additional capability to coordinate the calcium sites as hydroxyl or amino groups display an enhanced attitude to bind to the mineral, in virtue of the additional hook. On the other hand, varying the R^2 substituents may results in differences in the antiresorptive potency. To date, most of the highly potent BP drugs include a R^2 moiety that contains a *N*-heterocycle, that makes these cyclic nitrogen-containing derivatives up to 10000-fold more active than the first generation bisphosphonates, as zoledronic and risedronic acids [5]. These enhancements in antiresorptive potency, resulting from differences in the R^2 groups, appear to be linked to the biochemical activities of these drugs, particularly inhibition of the farnesyldiphosphate synthase enzyme (FPPS) within the mevalonic acid pathway in osteoclasts [6–8]. Structure–activity studies have actually indicated that bioactivity and bioavailability of BPs is highly dependent on the nature of substituents linked to the bisphosphonic skeleton [9]. Bioavailability is a critical feature of these derivatives since bisphosphonates are very hydrophilic and consequently poorly absorbed from the gastrointestinal tract after oral administration [10]. The strategies for improving the bioavailability of poorly absorbed drugs are usually based on the use of enhancing agents as surfactants, bile acids and chelating agents [3,11] or, more recently, by employing liposome encapsulation [12] or nanoparticles [13]. Bile acids are cholesterol-derived facial amphiphiles responsible for the solubilization of cholesterol

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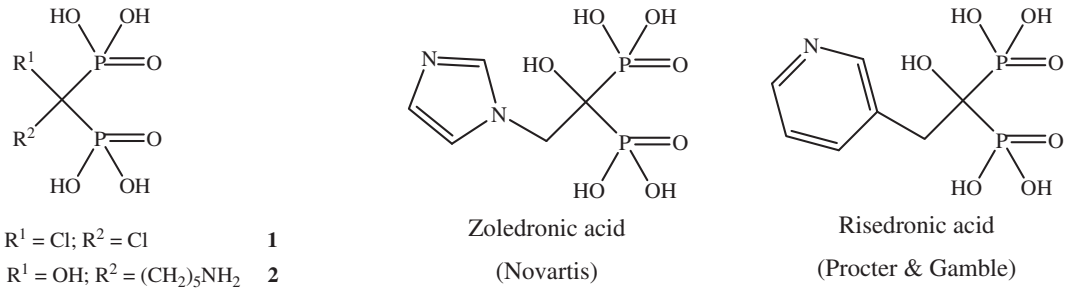


Fig. 1. Generic bisphosphonate structure, clodronate **1**, neridronate **2** and highly potent bisphosphonates zoledronic and risedronic acids.

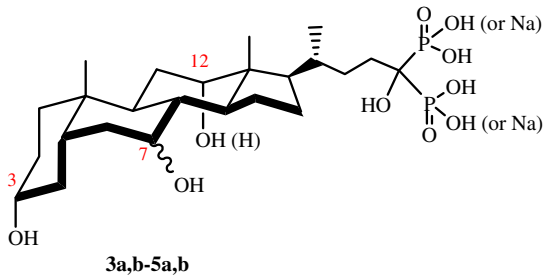
and fat through mixed micelle formation with phospholipids [14]. Their unique molecular properties provide a wealth of possibilities for applications in medicinal chemistry, that includes the improved absorption of the combination bile acid-drug [14b]. Accordingly we thought of interest the synthesis of a new class of bisphosphonates bearing in geminal position, together with an hydroxyl function, a bile acid-like R^2 substituent and we have selected for the scope the two human primary bile acids (cholic and chenodeoxycholic acids), the ursodeoxycholic acid, largely used as enterohepatic drug [15,16], and a couple of bile acids of non-human origin [17].

In this paper we report a straightforward synthesis of bile acid-containing hydroxyl-bisphosphonates and a study of the dual characteristic of this class of molecules that display both, a facial amphiphilic nature, and the capacity to bind to hydroxyapatite. In particular the coexisting presence of a hydrophobic side and a polar hydrophilic side is expected to raise the gastrointestinal absorption, thus increasing the amount of drug delivered to bone surface, with reduced toxic effects. Furthermore, the biological activity of the new compounds has been evaluated in two steps. Firstly, a cytotoxicity screening has been performed in order to define the concentration of compounds which passed to the following step. The toxicity of compounds was measured by using the neutral-red assay on the L929 cell line exposed to a wide range of drug concentrations [18]. Next, primary cultures of osteoclasts have been used to test the bioactivity of the new compounds in comparison with bisphosphonates of established activity [5,19,20].

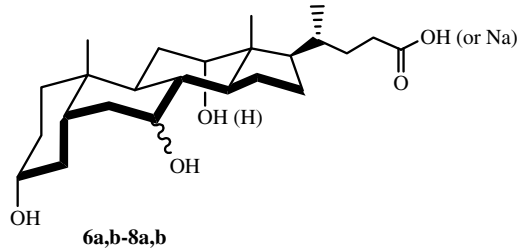
2. Chemistry, characterization and hydroxyapatite affinity

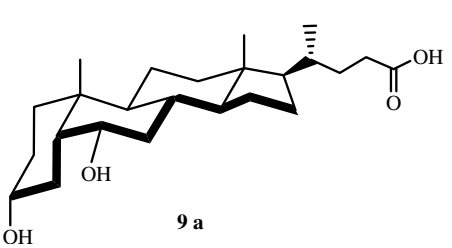
The one pot synthesis of bile acid-containing hydroxyl-bisphosphonates **3a**–**5a** shown in Fig. 2, and that of the corresponding sodium salts **3b**–**5b**, is general, simple and straightforward and has been accomplished according to Scheme 1.

Cholic **6a**, 3α,7α,12α-dihydroxy-5β-cholan-24-oic acid, chenodeoxycholic **7a** and ursodeoxycholic **8a** acids were first converted into their corresponding formyl derivatives **6c**–**8c** by heating with



	7	12
3a, **6a** (sodium salt **3b**, **6b**)	α–OH	α–OH
4a, **7a** (sodium salt **4b**, **7b**)	α–OH	--
5a, **8a** (sodium salt **5b**, **8b**)	β–OH	--





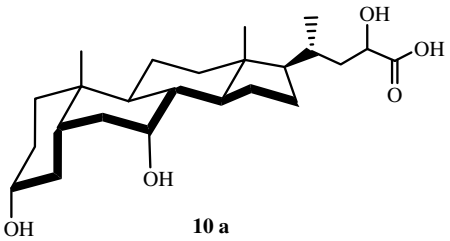


Fig. 2. Bile acid-derived hydroxyl-bisphosphonates used in this study **3a**–**5a** and related disodium salts **3b**–**5b**. Bile acids used as precursors for the synthesis **6a**–**8a** and related sodium salts **6b**–**8b**, utilized for control experiments in the evaluation of the biological activity.

formic acid. The formyl protected bile acids were treated with thionyl chloride for 5 h at 60 °C in benzene quantitatively affording, after evaporation under reduced pressure, the corresponding acyl chlorides that were used without further purification for the silylation step. Accordingly, 2.1 equivalents of tris(trimethylsilyl) phosphite were added to the bile acid acyl chlorides dissolved in THF [21]. After evaporation of the volatile fractions, the crude products were directly submitted to methanolysis with formation of bile acid-derived hydroxyl-bisphosphonates **3a**–**5a**, characterized by ^{1}H, ^{13}C, ^{31}P NMR, IR and ESI-HRMS analysis. In particular all ^{31}P NMR spectra showed signals between 20 and 22 ppm, characteristic of 1-hydroxymethylene-1,1-bisphosphonic acids whereas no typical signal of α-ketophophonates was observed around 0 ppm and in the ^{13}C NMR spectra no signals was observed in the carbonyl region. Conversion to the corresponding disodium salts **3b**–**5b** has been obtained by reaction with two equivalents of aqueous NaOH. The general feasibility of this reaction sequence has been applied to two additional bile acids of non-human origin as hyodeoxycholic acid **9a**, also known as 3α,6α-dihydroxy-5β-cholan-24-oic acid, present in mammalian species in different proportions and main constituent of hog bile and phocaecholic acid **10a**, a natural bile acid isolated from the bile of snakes, seals and other marine mammals [17]. The details of the characterization of the bisphosphonate derivatives of **9a** and **10a** are reported in the Experimental section.

Several groups have investigated the affinity and the mechanism of association of BPs to HAP based matrixes, using ^{31}P or ^{1}H NMR techniques [22,23], chromatographic [24] and computational methods [25], since this property would be particular helpful in predicting how BPs distribute and diffuse through the bone. Also in the present case the experiments were based on ^{31}P NMR spectra, using H_3PO_4 external standard as reference compound, and clodronate **1** and neridronate **2** as BP models. The chemisorption of **3b**–**5b** onto HAP was tested by dissolving equal amounts of **1**, **2** and the selected bile acid-containing bisphosphonate salt in water, pH 7.4. HAP was progressively added, increasing the amount from 0 mg/mL to 10 mg/mL. One mL of this suspension was take-up at 0, 3, 24, 48, 72 and 96 h. These samples were centrifuged (5000 rpm for 5 min), transferred into a 5 mm NMR tube containing the same sealed capillary filled with a solution of H_3PO_4 in D_2O (secondary standard) [22a] and analyzed via ^{31}P NMR. The unadsorbed amount at various times were determined comparing the integral of the specific bisphosphonate with the integral of the secondary standard and the pertinent results are collected in Table 1 for **4b**.

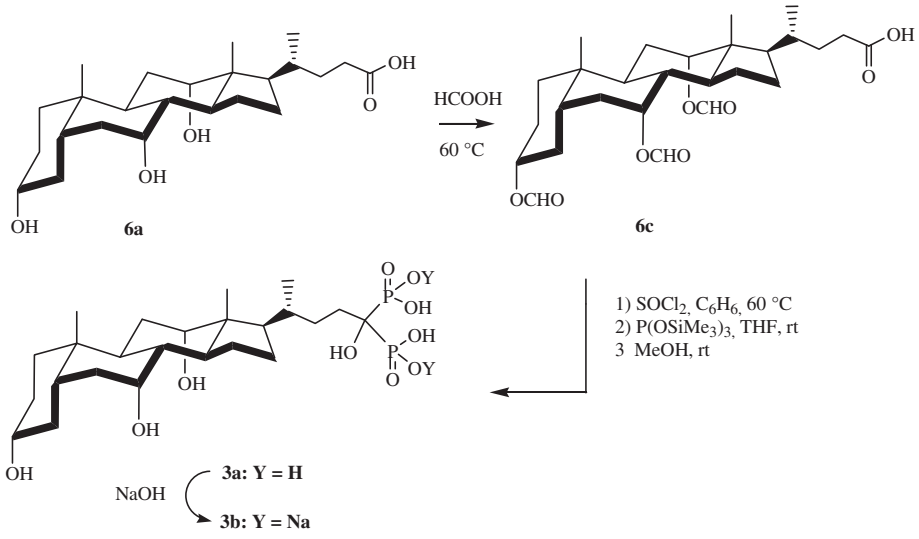
Table 1
Percentage of bisphosphonate unadsorbed as a function of time (h).[a]

Time h (mg/mL of HAP)	1	2	4b
0 (0)	100	100	100
3 (2.5)	100	98	91
24 (2.5)	100	96	64
48 (5.0)	100	85	32
72 (7.5)	96	82	12
96 (10.0)	93	67	3

[a] Determined by comparing the integral of the specific bisphosphonate to the integral of the H_3PO_4 as secondary standard.

To note that the direct measurement of the affinity in the presence of an excess of HAP resulted difficult to obtain due to the fast chemisorptions of **4b**. After 15 min, in fact the signal of **4b** was reduced to 22% and after 70 min the signal was almost gone. From the data of Table 1 we may assess that chenodeoxycholic-derived hydroxyl-bisphosphonate salt **4b** exhibited an affinity to HAP much higher than neridronate or clodronate. After 48 h, in fact the amount of **4b** chemisorbed is ca. 70% and it is almost completed within 3 days. A similar trend was observed using **3b** and **5b**, always in a comparison with **1** and **2**, although solubility problems interfere in the measurements pertaining to **5b**. The rank order of affinity for hydroxyapatite, extended also to experiments conducted on zoledronic acid disodium salt (ZOL), may be estimated as (high to low) **4b** > **3b** > **5b** > **2** > ZOL > **1**, being the latter part of the sequence in very good agreement with literature data [26]. This sequence was further validated with a second set of experiments in which a direct comparison between two bile acid-derived bisphosphonates was investigated. Table 2 reports the results found by comparing the affinity of **3b** and **4b**, present at the same time in solution.

Both bile acid-derived hydroxyl-bisphosphonates bind to hydoxyapatite with a concomitant release of phosphate PO_4^{3-} anions, according with a displacement mechanism based on a PO_3 for PO_4 exchange [22b,c]. This release may be followed by the increase of the ^{31}P NMR signal at ca. 1.75 ppm pertaining to $H_2PO_4^-$, see supplementary data for details. Chenodeoxycholic-derived bisphosphonate salt **4b** showed a higher affinity than **3b** (or **5b**) to hydroxyapatite that lets to hypothesize a superior biological activity, as discussed in next paragraphs.



Scheme 1. Synthetic sequence, shown for cholic acid **6a**, for the preparation of bile acid-containing hydroxyl-bisphosphonate disodium salts **3b**–**5b**.

Table 2
Ratio of unadsorbed **4b** with respect to **3b** and of released $H_2PO_4^-$ with respect to the external standard.

Time h (mg/mL of HAP)	Total[a] BPs/H_3PO_4	**4b**/**3b**[b]	NaH_2PO_4/H_3PO_4[b]
0 (0)	1.30	1.17	0
3 (2.5)	1.19	1.16	0.11
24 (2.5)	1.02	1.08	0.19
96 (7.5)	0.72	0.97	0.44

[a] Determined by comparing the integral of the specific bisphosphonate to the integral of the H_3PO_4 as secondary standard.
[b] Determined by comparing the integral of $H_2PO_4^-$ to the integral of the H_3PO_4 as secondary standard.

The observation that bile acid-containing hydroxyl-bisphosphonates quantitatively chemisorbed over HAP prompted us to prepare and characterize BP-HAP composites with the help of attenuated total reflection (ATR), thermogravimetry (TG) and X-ray powder diffraction (XRPD) spectra. In particular the ATR spectrum of chemisorbed **4b** showed the C—H stretching signals pertaining to the bile acid moiety, whereas the powder X-ray diffraction patterns of **4b**-HAP composite displayed the reflections characteristic of crystalline HAP with a modest amorphous contribution in the $3 < 2\theta < 12^\circ$ broad zone, Fig. 3(c).

Finally, the content of the organic component within the **4b**-HAP composite i.e. the bile acid moiety, has been calculated from thermogravimetry experiments to be ca. 10 wt %, Fig. 4, in very good agreement with the amount of **4b** used to prepare the composite, thus confirming that all the chenodeoxycholic hydroxyl-bisphosphonate salt is chemisorbed over hydroxyapatite.

Lipophilicity is an additional physico-chemical characteristics that control bioavailability and pharmacokinetics of drugs. This property is usually estimated by measuring the octanol-water partition coefficient (clog P), of the free acids, however for substances which contain hydrogen-like functions the distribution coefficient (log D) is used as a function of a given pH. Log D is usually obtained by calculation approaches [27]. Table 3 collects the partition coefficients calculated both at physiological pH (7.4) and in acid conditions. Bile acid-containing bisphosphonates are more liphophilic than clodronate or neridronate, i.e. less negative value of log D at physiological pH, and, according to other studies, are expected to exhibit cellular efficacy at lower concentration [28].

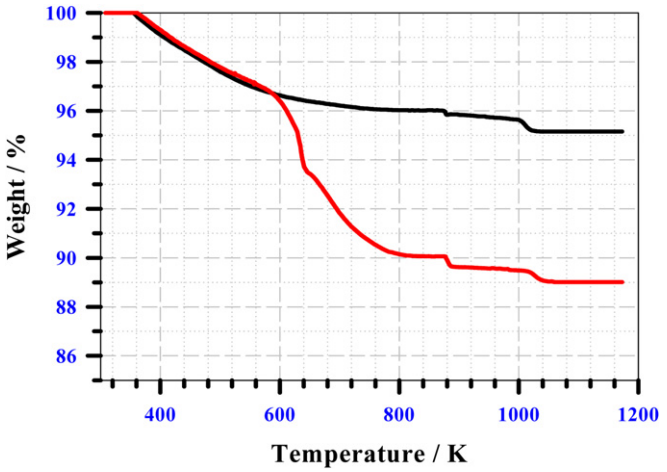


Fig. 4. Thermogravimetric analysis (TGA) of hydroxyapatite (black line) and of **4b** chemisorbed of hydroxyapatite (red line). For **4b** content calculations see Supplementary data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Biological evaluation

The cytotoxicity screening allowed to establish which compounds and concentrations are suitable for the biological activity evaluation. The neutral-red (NR) assay is a colorimetric test for the quantification of the membrane permeability and lysosomal activity of cells in response to drugs, chemical and environmental compounds, and nutrients [29]. The assay is based on the ability of viable cells to incorporate and bind neutral-red within lysosomes. NR is a weak cationic dye that readily penetrates the cell membrane, binds with anionic sites to the lysosomal matrix, and accumulates intracellularly in lysosomes. Xenobiotics may induce changes of the cell membrane which gradually become irreversible and lead to lysosomal fragility, with the inevitable consequence of a decreased uptake and binding of NR. Therefore, the quantity of dye incorporated into cells is directly proportional to the number of viable cells with an intact membrane. The concentrations to be used for cytotoxicity testing were chosen on the basis of

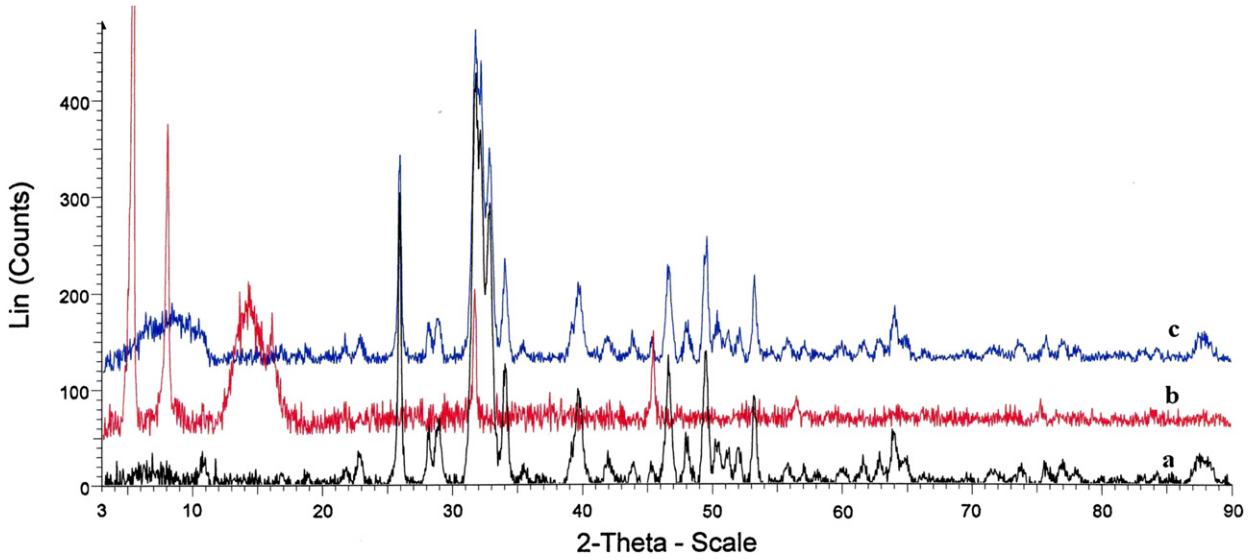


Fig. 3. X-ray powder diffraction analyses (XRPD) of (a) hydroxyapatite; (b) chenodeoxycholic acid-derives hydroxyl-bisphosphonate salt **4b**; (c) **4b** chemisorbed on hydroxyapatite.

Table 3
Distribution coefficients of bisphosphonates at various pH values.

Bisphosphonate	Log D (pH 7.4)	Log D (pH 1.5)
3a	−4.53	−0.76
4a	−3.34	0.44
5a	−3.34	0.44
Clodronate **1**	−5.04	−1.32
Neridronate **2**	−8.02	−8.01

therapeutic doses of neridronate which is given usually at 25–100 mg. The systemic administration of neridronate **2** allows the complete adsorption of the drug which is fivefold diluted in the bloodstream thus reaching the concentration ranging from 1 to 6 10^{-5} M [30]. Taking into account that the skeletal retention of bisphosphonates may be highly variable, the cytotoxic potential of the new compounds has been assessed by using a large range of concentrations (10^{-4}–10^{-11} M) [31]. We observed that two compounds, namely **3b** and **5b**, were poorly soluble in the culture medium. The low solubility is a major limitation for assessing the drug bioactivity on bone-resorbing cells, since pre-osteoclasts are engulfing cells and the presence of aggregates could stimulate phagocytosis, thus inhibiting the osteoclast-differentiation. For this reason, **3b** and **5b** were considered not suitable for the in vitro testing and they did not pass to the subsequent steps. On the contrary, **4b** did not show the above limitation. Fig. 5 shows the proportion of viable cells after 72 h of culture which is expressed as ratio between the neutral-red captured by L929 exposed to compounds (**4b** and **2**) and the negative control.

We observed that both compounds affect cell viability following a dose–response relationship, but at the major concentration (10^{-4} and 10^{-5} M) the chenodeoxycholic-containing hydroxyl-bisphosphonate salt is significantly less cytotoxic than neridronate ($p < 0.0001$). The biological activity of the compounds was tested by evaluating their capability to inhibit the osteoclastogenesis and to induce apoptosis. A more restricted range of concentrations was employed. As mentioned above, 10^{-5} M is considered as the therapeutic dose of neridronate [32]. Since the bile acid hydroxyl-bisphosphonate salts are intended for oral administration, 10^{-7} M was included because it corresponds to the 1% of the therapeutic dose which is calculated to be the absorption rate of conventional bisphosphonates [2]. In addition, 10^{-9} M was chosen because the culture system is a close environment where the bioactive agent is not excreted and the biological effect may be exacerbated.

In spite of the lower cytotoxicity, the chenodeoxycholic acid-derived hydroxyl-bisphosphonate **4b** was not less active than neridronate in inhibiting the osteoclastogenesis. As shown in Figs. 6 and 7 the new compound significantly decreased the formation of human osteoclasts which were obtained by cultivating peripheral blood mononuclear cells with proper differentiating agents.

At all the tested concentrations, **4b** was more active than neridronate even though the difference was not statistically significant. By itself, the chenodeoxycholic acid **7a** induced a slight decrease of the osteoclast formation, and the greater activity of **4b** seems to be the sum the biological effects of both the chenodeoxycholic-derived hydroxy-bisphosphonate salt and the chenodeoxycholic acid. In order to highlight the capability of active compounds in promoting the apoptosis of new-formed osteoclasts, mononuclear cells were cultured for seven days with differentiating agents and then added with the drugs. As shown in Fig. 8, the percentage of apoptotic osteoclasts was very low in control cultures (less than 5%), but increased significantly when cells were exposed to the highest concentration of neridronate and chenodeoxycholic-containing hydroxyl-bisphosphonate salt (10^{-5} M).

Similarly to what observed in the inhibition of osteoclastogenesis, **4b** was more active than neridronate [2]. This is a remarkable finding since the capability to inhibit osteoclast formation is mainly related to nitrogen containing bisphosphonates which act on the mevalonate pathway and affect the post-translational modification (prenylation) of small GTPases which are important signaling proteins which regulate a variety of cell processes important for osteoclast function, including cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles and apoptosis [33]. However, other authors demonstrated that chenodeoxycholic acid is able to promote apoptosis of various cell types, and our findings suggest that this effect may potentiate the pharmacological activity of conventional bisphosphonate irrespective of the presence of amino groups.

4. Conclusions

In this paper we have reported a simple and efficient synthesis of bile acids-derived hydroxyl-bisphosphonates from the two human primary bile acids (cholic and chenodeoxycholic acids) and from ursodeoxycholic acid, compounds of well-established pharmacological activity. The relative affinity of these derivatives

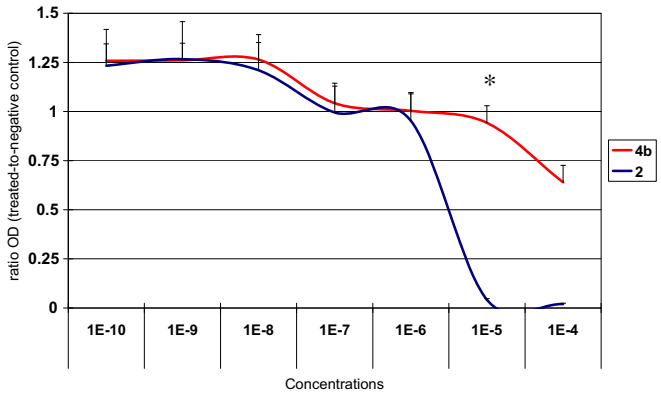


Fig. 5. Cytotoxicity of chenodeoxycholic acid-derived hydroxyl-bisphosphonate salt **4b** in comparison to a bisphosphonate of established activity **2**. The graph shows the ratio between the OD measured in L929 exposed to compounds and OD measured in negative control, after 72 h of culture. Mean ± SEM of 9 different experiments which were performed in triplicate wells. $^{*}p < 0.0001$.

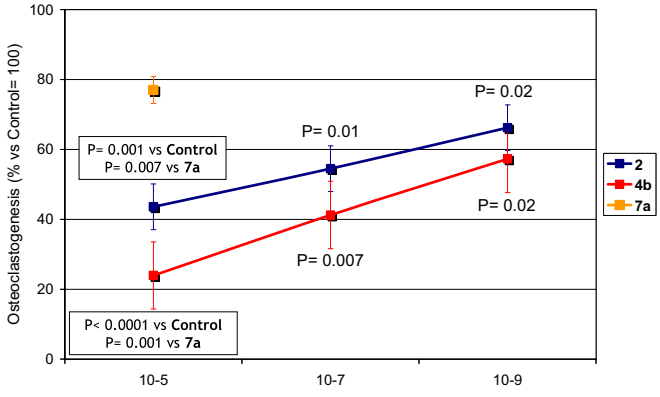


Fig. 6. Inhibition of osteoclastogenesis. The active compounds were added together with the osteoclast differentiating agents to highlight the ability of drugs in interfering the formation of new osteoclasts. The graph shows the percentage of osteoclasts counted after exposure to bisphosphonates and calculated in comparison to positive control. The number of osteoclast formed in positive control represented the maximum ability (100%) to generate multinucleated and TRAPC positive cells in presence of osteoclast differentiating agents. Mean ± SEM of 4 cultures which were obtained from two different donors. Neridronate **2**, chenodeoxycholic acid-derived hydroxyl-bisphosphonate salt **4b**, chenodeoxycholic acid **7a**.

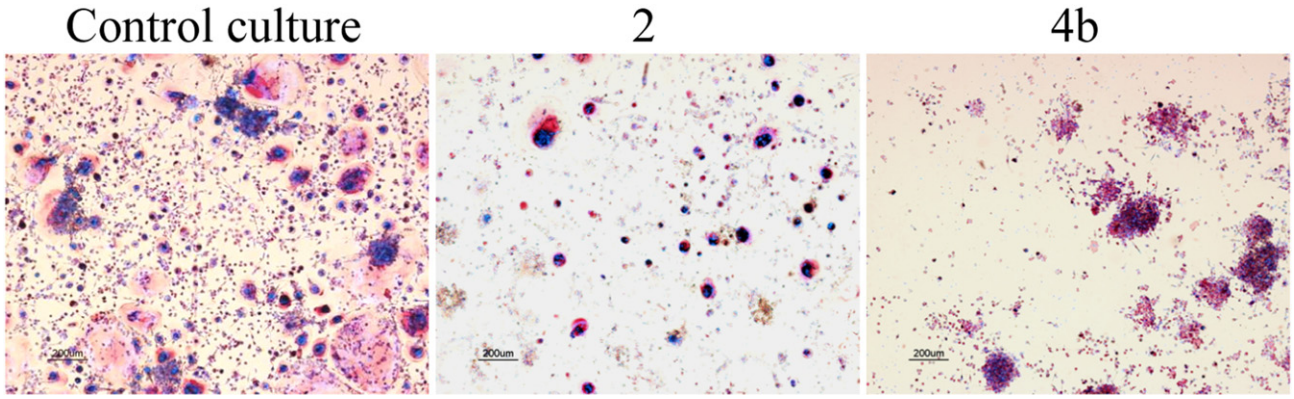


Fig. 7. The representative pictures show the morphological differences which were found in osteoclast cultures treated or not treated with bisphosphonates. The control culture was performed by using a cocktail of differentiating factors which induced the formation of osteoclasts which are multinucleated, TRACP positive and giant cells. Neridronate **2** decreases the number and the size of osteoclasts. Cell cultures treated with chenodeoxycholic acid-derived hydroxyl-bisphosphonate **4b** show many aggregates of mononuclear cells, but the typical osteoclast morphology is no longer recognizable.

toward hydroxyapatite has been estimated in a comparison with clodronate and neridronate drugs, using ^{31}P NMR experiments. The high affinity for HAP allowed the preparation of the composite **4b**-HAP and its characterization by means of attenuated total reflection (ATR), thermogravimetry (TG) and X-ray powder diffraction (XRPD) spectra. Chenodeoxycholic-containing hydroxyl-bisphosphonate salt demonstrated a higher activity than neridronate in inhibition of osteoclastognesis. This result is particularly significant since the capability to inhibit osteoclast formation is mainly related to nitrogen containing bisphosphonates. Even though **4b** seems to be very promising, at the moment we cannot demonstrate the higher bioavailability of the chenodeoxycholic-derived hydroxyl-bisphosphonate salt, in comparison with conventional drugs. In this regard, further experiments will be conducted to evaluate in vivo the pharmacokinetics and the pharmacodynamics of the new compound.

5. Experimental section

5.1. Chemistry

5.1.1. General materials and methods

Melting points are uncorrected and obtained on a 510 Büchi Meltig Point instrument. TLC was performed on pre-coated Silica Gel plates (thickness 0.25 mm, Merck), and phosphomolybdic acid

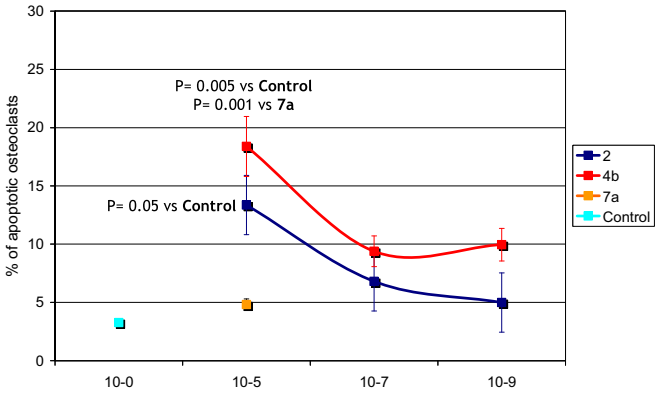


Fig. 8. Mononuclear cells were cultured for seven days with osteoclast differentiating agents and then added with the active compounds in order to highlight the pro-apoptotic activity of drugs. The graph shows the percentage of apoptotic osteoclasts calculated on the total number of multinucleated and TRACP positive cells. Mean ± SEM of 4 cultures obtained from two different donors.

solution was used as the spray reagent to visualize the steroids. The ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in $CDCl_3$ and/or CD_3OD and/or D_2O solution in 5 mm tubes, at room temperature, with a Varian Gemini 300 or a Varian Mercury Plus 400 spectrometers with TMS as internal standard for ^{1}H and ^{13}C spectra and with phosphoric acid as external standard for ^{31}P spectra. ESI-HRMS were acquired on an Agilent Dual ESI Q TOF 6520, in negative-ion mode, using mixtures of methanol-water. IR spectra were recorded on a Perkin–Elmer 1310 infrared spectrometer using nujol suspension technique. Theoretical calculations of the octanol/water distribution coefficient (log D) of bisphosphonates as a function of pH were calculated using MarvinSketch software ver. 5.4.1.1 (www.chemaxon.com). X-Ray powder diffraction data (Cu $K_{\alpha 1}$, $\lambda = 1.5406$ Å) were collected on a Bruker D8 advance diffractometer. The generator was operated at 40 kV and 40 mA. Receiving slit 0.2 mm. A long scan was performed within $3 < 2\theta < 50°$, 0.020°/2 s. The thermogravimetric analysis (TGA) was conducted with a Mettler Toledo TGA/SDTA 851 instrument. The instrument was calibrated using high purity indium and high purity aluminum standards. The experiment was conducted under nitrogen flow between 308 and 873 K and under air flow between 873 and 1173 K. The flow was maintained at 70 ml min^{-1}. The specimen, about 10 mg, was contained in a 70 mL alumina pan. It was carried from 308 to 1173 K at variable heating rate: the heating rate was 1 K min^{-1}, when the loss weight was higher than 2 mg s^{-1}, and 20 K min^{-1}, when the loss weight was less than 1 mg s^{-1}. The spectroscopic characterization of samples was carried out by Attenuated Total Reflectance (ATR) using a FT-IR spectrometer (Bruker mod. IFS88) equipped with: a Globar source in SiC, beamsplitter in KBr, Michelson Interferometer, ATR sampling accessory (Pike mod. MIRacle), Mercury Cadmium Telluride (MCT) detector. The spectra was scanned from 4000 to 650 cm^{-1} at 16 cm^{-1} of resolution. A thousand scans were monitored for each spectrum. The spectrometer was purged with dry air to reduce the atmospheric water vapor.

Cholic **6a**, chenodeoxycholic **7a**, ursodeoxycholic **8a,** hydeoxycholic **9a** and phocaecholic **10a** bile acids are commercially available compounds used without further purification. The formylated bile acids **6c**–**10c** were prepared according to the literature procedures [34].

5.1.2. General procedure for the synthesis of 24-hydroxy-24,24-bisphosphonobile acids ***3a*–*5a*, *11*, *12***

Freshly distilled thionyl chloride (0.5 mL, 6.85 mmol) was added dropwise to a solution of formylated bile acid **6c**–**10c** (4.95 mmol)

in 10 mL of dry benzene and a drop of DMF at 0 °C. The reaction mixture was stirred at 60 °C for 5 h and then evaporated to dryness under vacuum. Dry benzene (5 mL) was added and the syrup evaporated in twice to complete remove thionyl chloride. The bile acid chloride was dissolved in 10 mL of dry THF under nitrogen and tris(trimethylsilyl) phosphite (3.3 mL, 10 mmol) was added dropwise, maintaining the temperature 25–35 °C. The reaction mixture was then stirred at room temperature for 3 h. After evaporation of volatile fractions under vacuum, the crude product was hydrolyzed with methanol (20 mL) for 2 days at room temperature. After evaporation of solvent under vacuum, the obtained compound was washed with diethyl ether and a few drops of methanol and recovered by filtration to give bile acid-derived bisphosphonates **3a**–**5a**, **11** and **12** as white or pale yellow solids.

*5.1.2.1. 3α,7α,12α-Trihydroxy-5β-cholan-24-hydroxyl-24,24-bisphosphonic acid **3a**.* Yield 97%; mp 200–203 °C. IR: ν (cm^{-1}) 3300 (O–H), 2939 (C–H), 1189 (P=O), 1011 (P–O); ^{1}H NMR (300 MHz, CD_3OD) δ: 0.74 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.04 (d, J = 6.8 Hz, 3H, 21-CH_3), 1.15–2.35 (m, 28H), 3.39 (m, 1H, 3β-CH), 3.82 (br s, 1H, 7β-CH), 3.98 (br s, 1H, 12β-CH). ^{13}C NMR (75 MHz, CD_3OD) δ: 11.6, 16.5, 21.7, 22.8, 26.4, 27.2, 28.1, 28.8, 29.7, 30.3, 34.4, 34.5, 35.1, 36.3, 39.0, 39.6, 41.5, 41.7, 46.0, 46.8, 67.8, 71.5, 72.8, 73.3 (t, J = 108 Hz). ^{31}P NMR (121.5 MHz, CD_3OD) δ: 21.67. HRMS of $[M-H]^-$ ions: calculated for $C_{24}H_{43}O_{10}P_2$ 553.2336, found 553.2333.

*5.1.2.2. 3α,7α-Dihydroxy-5β-cholan-24-hydroxy-24, 24-bisphosphonic acid **4a**.* Yield 95%; mp 150–153 °C. IR: ν (cm^{-1}) 3400 (O–H), 2955 (C–H), 1255 (P=O), 1065 (P–O); ^{1}H NMR (300 MHz, CD_3OD) δ: 0.74 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.03 (d, J = 6.8 Hz, 3H, 21-CH_3), 1.1–2.38 (m, 28H), 3.38 (m, 1H, 3β-CH), 3.83 (br s, 1H, 7β-CH). ^{13}C NMR (75 MHz, CD_3OD) δ: 10.8, 14.0, 17.7, 20.4, 22.0, 23.2, 27.8, 28.8, 29.9, 30.4, 32.6, 34.5, 34.8, 35.1, 36.3, 39.0, 39.4, 39.7, 42.2, 50.1, 55.9, 67.7, 71.4, 73.1 (t, J = 110,2 Hz). ^{31}P NMR (121.5 MHz, D_2O) δ: 20.83. HRMS of $[M-H]^-$ ions: calculated for $C_{24}H_{43}O_9P_2$ 537.2387, found 537.2390.

*5.1.2.3. 3α,7β-Dihydroxy-5β-cholan-24-hydroxy-24, 24-bisphosphonic acid **5a**.* Yield 96%; mp 182–185 °C. IR: ν (cm^{-1}) 3400 (O–H), 2955 (C–H), 1255 (P=O), 1065 (P–O). ^{1}H NMR (300 MHz, CD_3OD) δ: 0.73 (s, 3H, 18-CH_3), 0.99 (s, 3H, 19-CH_3), 1.02 (d, J = 6.8 Hz, 3H, 21-CH_3), 1.1–2.25 (m, 28H), 3.50 (m, 2H, 3β-, 7α-CH). ^{13}C NMR (75 MHz, CD_3OD) δ: 11.3, 17.8, 21.0, 22.57, 26.6, 28.2, 28.8, 29.6, 30.3, 33.8, 34.7, 36.2, 36.6, 37.2, 39.3, 40.2, 42.6, 43.1, 43.3, 55.2, 56.2, 70.6, 70.7, 73.1 (t, J = 109.5 Hz). ^{31}P NMR (121.5 MHz, CD_3OD) δ: 21.56 (d, J = 41.6 Hz), 21.59 (d, J = 41.6 Hz). HRMS of $[M-H]^-$ ions: calculated for $C_{24}H_{43}O_9P_2$ 537.2387, found 537.2399.

*5.1.2.4. 3α,6α-Dihydroxy-5β-cholan-24-hydroxy-24, 24-bisphosphonic acid (bisphosphonate of hyodeoxycholic acid) **11**.* Yield 96%; mp 207–210 °C. IR: ν (cm^{-1}) 3400 (O–H), 2950 (C–H), 1255 (P=O), 1068 (P–O). ^{1}H NMR (300 MHz, CD_3OD) δ: 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 0.98 (d, J = 6.8 Hz, 3H, 21-CH_3), 1.1–2.25 (m, 28H), 3.51 (m, 1H, 3β-CH), 4.01 (m, 1H, 6β-CH). ^{13}C NMR (75 MHz, CD_3OD) δ: 12.5, 19.0, 21.9, 24.1, 25.3, 29.2, 29.9, 30.2, 31.1, 31.6, 35.5, 36.2, 36.8, 36.9, 37.6, 41.3, 41.4, 43.9, 49.8, 57.4, 57.68, 68.7, 72.3, 74.5 (t, J = 108.9 Hz). ^{31}P NMR (121.5 MHz, CD_3OD) δ: 21.40 (d, J = 40.6 Hz), 21.71 (d, J = 40.6 Hz). HRMS of $[M-H]^-$ ions: calculated for $C_{24}H_{43}O_9P_2$ 537.2387, found 537.2394.

*5.1.2.5. 3α,7α,23(R)-Trihydroxy-5β-cholan-24-hydroxy-24,24-bisphosphonic acid (bis- phosphonate of phocaecholic acid) **12**.* Yield 93%; mp 212–215 °C. IR: ν (cm^{-1}) 3410 (O–H), 2950 (C–H), 1255 (P=O), 1068 (P–O). ^{1}H NMR (300 MHz, CD_3OD) δ: 0.72 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.01 (d, J = 6.8 Hz, 3H, 21-CH_3), 1.02–2.30 (m, 27H), 3.36 (m, 1H, 3β-CH), 3.80 (br s, 1H, 7β-CH), 4.20 (m, 1H, 23-CH). ^{13}C NMR (75 MHz, CD_3OD) δ: 12.2, 18.7, 21.8, 23.4, 24.6, 29.4, 31.3, 33.8, 34.0, 35.8, 36.2, 36.5, 39.2, 40.4, 40.7, 41.1, 43.1, 43.8, 51.6, 58.2, 69.1, 72.3, 72.8, 77.2 (t, J = 108.3 Hz). ^{31}P NMR (121.5 MHz, CD_3OD) δ: 11.59. HRMS of $[M-H]^-$ ions: calculated for $C_{24}H_{43}O_{10}P_2$ 553.2336, found 553.2351.

*5.2. General procedure for the synthesis of 24-hydroxy-24,24-bisphosphonobile acid disodium salts **3b**–**5b** and bile acids disodium salts **6b**–**8b***

4 mmol of the bisphosphonobile acids **3a**–**5a**, dissolved in 20 mL of EtOH/H_2O (1:1 v/v), were treated with an aqueous solution of NaOH (8 mmol). The mixture was stirred for 1 h at r.t. The reaction mixture was lyophilized giving the expected disodium salts in almost quantitative yield. **3b**: ^{31}P NMR (121.5 MHz, D_2O) δ: 17.80 (d, J = 32.8 Hz), 18.20 (d, J = 32.8 Hz). **4b**: ^{31}P NMR (121.5 MHz, D_2O) δ: 19.58 (d, J = 31.5 Hz), 19.90 (d, J = 31.5 Hz). **5b**: ^{31}P NMR (161.9 MHz, D_2O) δ: 18.81 (d, J = 34 Hz), 18.46 (d, J = 34 Hz). An almost identical procedure was followed for the preparation of the simple bile acids sodium salts **6b**–**8b** used in biological investigations.

5.3. Hydroxyapatite affinity measurements

In a 15 mL cap vial, magnetically stirred at 25 °C, approximately 20 mg of clodronate **1**, 20 mg of neridronate **2** and 40 mg of chenodeoxycholic acid-containing hydroxyl-bisphosphonate sodium salt **4b** were dissolved in 10 mL of bidistilled water adjusting the pH to 7.4. HAP was added increasing the amount from 0 mg/mL to 10 mg/mL (Table 1). One mL of this suspension was take-up at 0, 3, 24, 48, 72 and 96 h. These samples were centrifuged (5000 rpm for 5′), transferred into a 5 mm NMR tube containing, every time, the same sealed capillary filled with a solution of H_3PO_4 in D_2O (secondary standard) [24a] and analyzed via ^{31}P NMR.

5.4. Cytotoxicity screening

5.4.1. Cell cultures

The L929 cells (mouse fibroblasts, ATCC CCLl, NCTC clone 929) were maintained in complete minimum essential medium (MEM) with Earle's salts, containing 10% fetal calf serum (FCS), 20 mM glutamine, 1% non-essential amino acids penicillin (100 U mL^{-1}) and streptomycin (l00 μg/mL^{-1}) at 37 °C in a humidified atmosphere of 5% CO_2 95% air. Confluent cells were detached by trypsin and 0.8 × 10^3 cells in 0.2 mL of culture medium were seeded in flat-bottomed 96 microplate wells (Costar, Cambridge, MA, USA). The cells were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 95% air in order to obtain a complete cell adhesion. Twenty-four hours after cell seeding, the medium was aspirated from the monolayer and replaced with 0.2 mL of complete MEM containing serial dilution (from 10^{-4} to 10^{-10} M) of the sodium salts of bile acid-derived hydroxyl-bisphosphonates (**3b**, **4b**, **5b**) and neridronate **2**. Stock solutions (10^{-2} M) were obtained dissolving compounds in phosphate buffer saline (PBS) at pH 7.4. As controls, cultures were added with 0.2 mL of complete MEM (negative control), and methanol fixed cells provided the positive controls. Sample and control cultures were tested in triplicate. The plates were incubated for 24, 48 and 72 h.

5.4.2. Neutral-red uptake assay

At the end points, the medium was aspirated from the monolayer and replaced with 0.1 mL per well of 0.4% neutral-red diluted 1:80 in complete MEM. After incubation for 2 h at 37 °C the dye-containing medium was discarded, the wells were washed twice with saline and 0.1 mL of extractant solution (50% ethanol in acetic acid, 1%) were

added. Microplates were shaken gently onto a microplate rotator for 2 min and the absorbance of the extracted dye was detected on a spectrophotometer microplate reader equipped with a 540 nm filter (Spectra SLT, Tecan, Crailsheim, Germany). Results from triplicate samples were recorded as optical density units (OD) and averaged after blank subtraction. Cytotoxicity data were expressed as ratio of the OD measured in sample exposed to compounds (**4b** and **2**) and OD measured in negative control.

5.5. Evaluation of the biological activity on osteoclast cultures

5.5.1. Osteoclast isolation

Osteoclast cultures were obtained from human peripheral blood mononuclear cells (PBMC) [35]. Fresh buffy coats were diluted with PBS (1:1 ratio), then PBMC were separated on Ficoll-Hystopaque gradient (Sigma–Aldrich) at 2000 rpm for 30 min and washed with PBS. The cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (Euroclone, Milan, Italy) added with 10% fetal bovine serum (FBS) (Euroclone), and seeded at the density of 3×10^6 cells/cm^2 in wells of 0.81 cm^2 surface. PBMC were incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO_2 95% air in order to obtain the monocyte adhesion. After 1 h, non-adherent cells were removed and the osteoclast-differentiation medium was added, consisting on complete DMEM containing 25% of supernatant of confluent SH-SY5Y cell line (human neuroblastoma, ATCC, CRL-2266 [36]).

5.5.2. Inhibition of osteoclast formation

The assay served to highlight the activity of the new compounds in inhibiting the osteoclastogenesis. For this purpose, three concentrations of **4b** and **2** (10^{-5}, 10^{-7}, 10^{-9} M) were added together with to the osteoclast-differentiation medium. As positive control of the osteoclastogenesis cells were maintained in osteoclast-differentiation medium. In order to control the biological activity of the bile acid component a unique concentration of the **7a** compound was evaluated (10^{-5}). Sample and control cultures were tested in duplicate. After seven days, the differentiation of PBMC into tartrate-resistant acid phosphatase (TRACP) positive multinucleated cells was evaluated. The cells were fixed with 3% paraformaldehyde and 2% sucrose for 30 min, permeabilized with Triton 0.5% in HEPES for 5 min at room temperature, and stained for TRACP using naphthol AS-BI phosphoric acid and tartrate solution (Acid Phosphatase kit, Sigma–Aldrich, USA) for 60 min at 37 °C.

5.5.3. Induction of osteoclast apoptosis

The assay tested the ability of the new compounds in inducing the apoptosis of new formed osteoclasts, thus reducing the number of active bone-resorbing cells. For this purpose, after seven days from seeding, the cultures of TRACP positive multinucleated cells were exposed to the above mentioned concentrations of compounds. After seven days from the incubation, the cells were fixed and permeabilized as described above. Then they were incubated for 30 min with phalloidin-TRITC 0.3 μg/mL (Sigma) and the nuclei were counterstained with 2.25 μg/mL of Hoechst 33258 (Sigma), for 30 min at room temperature. The multinucleated cells exhibiting F-actin ring and with apoptotic nuclear bodies were counted across the whole well surface (0.81 cm^2) at the magnification of 40×.

5.6. Statistical analysis

Statistical analysis was performed using StatView 5.01 for Windows (SAS Institute Inc, Cary, NC). Quantitative data were expressed as arithmetic mean plus or minus the standard error of the mean (SEM). We hypothesized that the biological activity of compounds could be influenced by multiple independent variables, i.e. the type of compound and the drug concentration. The analysis of variance (ANOVA) was applied to detect the effects of the independent variables on the quantitative results, and the *post hoc* Bonferroni–Dunn test was applied to highlight the differences among the groups. P values less than 0.05 were considered as statistically significant.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.03.020.

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