Synthesis, characterization of strontium-bile acid salts and their bioactivity vs. the anti-osteoporosis drug strontium ranelate

Paola Bergamini\textsuperscript{a}, Elena Marchesi\textsuperscript{a}, Antonella Pagnoni\textsuperscript{a}, Elisabetta Lambertini\textsuperscript{b}, Tiziana Franceschetti\textsuperscript{b}, Letizia Penolazzi\textsuperscript{b}, Roberta Piva\textsuperscript{b,}\textsuperscript{*}

\textsuperscript{a}Dipartimento di Chimica, Universit\'a degli Studi di Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
\textsuperscript{b}Dipartimento di Biochimica e Biologia Molecolare, Sezione di Biologia Molecolare, Universit\'a degli Studi di Ferrara, Via Fossato di Mortara 74, 44100 Ferrara, Italy

\textbf{A R T I C L E  I N F O}

Article history:
Received 14 October 2008
Received in revised form 10 March 2009
Accepted 16 March 2009
Available online 25 March 2009

Keywords:
Strontium-bile acid salts
Osteoblasts
Osteoclasts
Strontium ranelate

\textbf{A B S T R A C T}

The strontium salts Sr(cholate)\textsubscript{2}, (Compound 1), Sr(dehydrocholate)\textsubscript{2}, (Compound 2) and Sr\textsubscript{3}(3-dehydrocholanoloidien-\textsuperscript{r}-tartrate)\textsubscript{3}, (Compound 3) have been prepared and characterized. The potential anti-osteoporotic activity of these compounds was tested on human primary osteoblasts (hOBs) and human primary osteoclasts (hOCs) in comparison with the bioactivity of strontium ranelate, previously registered as drug in the treatment of post-menopausal osteoporosis. Our results led to the hypothesis that the tested compounds, particularly Compound 2, may have requirements for modulating skeletal tissue regeneration or at least down regulating the loss of bone mass. In fact, all tested compounds have been shown to induce maturation in human primary osteoblasts (hOBs) and apoptosis of human primary osteoclasts (hOCs) at the same time.

\textcopyright 2009 Elsevier Inc. All rights reserved.

1. Introduction

Evidence is growing that strontium (Sr) influences bone cells and bone metabolism in vitro and in vivo. Many studies have shown that Sr\textsuperscript{2+} can stimulate bone formation and inhibit bone resorption both in vitro and in vivo [1,2]. Recently strontium ranelate (SrR), a compound containing two ions of stable bivalent strontium combined with ranelic acid, which acts as a carrier, is registered as drug in the treatment of post-menopausal osteoporosis [3–5].

SrR is hypothesized to be a dual-acting agent with both anti-resorptive and anabolic skeletal benefits [6].

The precise molecular mechanism responsible for SrR effects is not clear and needs to be investigated. Functional analyses that have been performed suggest that different signaling pathways may be involved in the osteoblastic and osteoclastic responses to SrR [7–9]. Although, SrR is approved in several countries for the treatment of post-menopausal osteoporosis, it is a relatively new drug and its long-term safety still needs to be documented for the ideal management of osteopenic diseases.

Although, SrR is now being administered to women for osteoporosis, it could have potentially therapeutic value in different osteopenic disorders, including Paget’s disease and cancer with bone metastases. To avoid possible adverse reactions in the greater population, it may be important to design analogous compounds that are better tolerated.

\textsuperscript{*} Corresponding author. Tel.: +39 0532 974405; fax: +39 0532 974484.
E-mail address: piv@unife.it (R. Piva).

Since it is evident that the therapeutic action of SrR is due exclusively to Sr\textsuperscript{2+}, we reasoned that the performance of strontium-based drugs could be improved by modification of the carrier anion. Ranelate bears four carboxylic groups and allows the transport of a large amount of Sr\textsuperscript{2+} metal (two cations per tetra-charged anion, 34.1% in mass), but it showed a few drawbacks. First, it is known that a large proportion of Sr\textsuperscript{2+} administrated as ranelate is eliminated via gastrointestinal secretion [10,11]; therefore SrR needs to be administrated as a daily dose of 2 g, resulting in a low patient compliance. Second, synthesis of ranelic acid requires a long, multistep chemical process which contributes to the high price of SrR [12]. Therefore, it would be of first interest to couple the active Sr\textsuperscript{2+} cation to a readily available natural non-toxic anion that can increase the intestinal absorption of the metal.

Analysis of the characteristics of bile acids indicates that their anions may be promising alternative carriers for Sr\textsuperscript{2+}. Bile acids comprise a large group of natural or semi-synthetic molecules that are readily available at low cost; they have been used for pharmaceutical purposes for a long time, because of their known value as drug carriers [13]. Bile acid transport in the gastrointestinal tract is recognized as being an efficient high capacity system because of its involvement in reabsorption of bile salts following fat digestion. Bile acid anions are of therapeutic value because they are known to form complexes that increase the absorption of metal ions, like calcium and iron [14–16]. On this basis, we hypothesized that Sr\textsuperscript{2+} absorption also could be enhanced by the use of bile acids. To verify this hypothesis, and to ascertain if these observations can be...
exploited for the development of new strontium-based anti-osteoporosis drugs, it was essential to document that strontium bile salts are at least as active as SrR and are not cytotoxic. This was the aim of the present preliminary study.

2. Experimental

2.1. General

3-Dehydrocholanoyliden-l-tartaric acid was prepared as reported [17]. All the other chemicals and solvents were used as purchased (reagent grade). Elemental analysis (C, H, N) was performed using a Carlo Erba instrument, model EA1110.

The amount of strontium in each sample was determined by atomic absorption spectroscopy using 460.7 nm resonance line in an air-acetylene flame. Analysis was performed using a Perkin-Elmer atomic absorption spectrometer (Analyst 800); a strontium hollow cathode cave lamp was used (Slit width 0.2H, signal type AA time average, reading time 3 s, oxidant flow (air) 20 L/min, acetylene flow 2.5 L/min).

Samples and standards were added to 0.1% KCl solution, the 0.1% KCl solution being used as a calibration blank. Because considerable ionization of strontium occurs in an air-acetylene flame, it was controlled by the addition of an alkali salt. A calibration curve was made after reading four standard strontium solutions (1, 3, 5, 10 mg/L) prepared by diluting with deionized (18 MΩ cm) water (Milli-Q system, Waters Corp., Milford, MA). An atomic spectroscopy standard solution (1 l g/mL) of Strontium (Sr(NO₃)₂ in a HNO₃ 2% matrix, Perkin-Elmer Pure) and a blank solution were also read. For each concentration level 3 readings were taken; three samples, marked with numbers 1, 2 and 3 were read and their concentrations found to be in the calibration range. To check the accuracy of the method, a recovery test was performed adding these to those of known Sr concentration. Recoveries were found to be between 102% and 104%. The precision was tested taking triplicate readings, and the relative standard deviation (RSD%) was found to be less than 1%. FT-IR spectra were recorded on a Bruker Vertex 70 FT-IR instrument (4000–300 cm⁻¹/C). ¹H NMR spectra were recorded on a Bruker 200 AM. The splitting of proton resonances in the ¹H NMR spectra are defined as s = singlet, d = doublet, and m = multiplet. ¹³C NMR spectra were recorded on a Varian 400 NMR spectrometer (¹³C at 100.57). Peak positions shown are relative to tetramethylsilane.

2.2. Synthesis

2.2.1. Sr(cholate)₂ (1)

A solution of strontium hydroxide, Sr(OH)₂·8H₂O (0.5 g, 1.8 × 10⁻³ mol) in 75 mL of water was added to a suspension of cholic acid (1.5 g, 3.7 × 10⁻³ mol) in water (30 mL), which became immediately clear. The pH increased from 5.5 to 8.0. Occasional solid residues were eliminated by filtration. After stirring for 30 min at room temperature, the solution was evaporated to dryness giving a white residue that was washed with acetone and then dried at 60 °C for 4 h (Sr(cholate)₂·2H₂O, 1.5 g, 1.6 × 10⁻³ mol, yield 89%). The solubility in water of 1 is 0.02 g/mL at 25 °C.

Elemental analyses: see Table 1.

IR (KBr, cm⁻¹) selected peaks: 3700–3100 (broad, OH), 1544 (COO⁻), 1421.

¹H NMR (D₂O, ppm) 0.65 (3H, s, CH₃-19), 0.84 (3H, s, CH₃-18), 0.95 (3H, d, CH₂-21), 1.0–2.3 (24H, m, CH), 3.43 (1H, m, CH-3), 3.83 (1H, m, CH-7), 4.00 (1H, m, CH-12).

¹³C NMR (D₂O, ppm,) selected peaks: 184.28 (C-24), 72.99 (C-12), 71.46 (C-3), 68.12 (C-7).

2.2.2. Sr(dehydrocholate)₂ (2)

The same procedure as above, using dehydrocholic acid suspended in water, gave Sr(dehydrocholate)₂·H₂O, (1.5 g, 1.65 × 10⁻³ mol, yield 91.7%).

The solubility in water of 2 is 0.01 g/mL at 25 °C.

Elemental analyses: see Table 1.

IR (KBr, cm⁻¹) selected peaks: 3800–3000 (broad, H₂O), 1714 (ν C=O), 1557 (COO⁻), 1430.

¹H NMR (D₂O, ppm): 0.71 (3H, d, CH₃-21), 1.08 (3H, s, CH₃-18), 1.26 (3H, m), 1.36 (3H, s, CH₃-19), 1.5–2.2 (12H, m, CH, CH₂), 2.37 (2H, m, CH₂-11), 2.97–3.29 (7H, m, CH₂-2, CH₂-4, CH₂-6, CH₂-8).

¹³C NMR (D₂O, ppm,) selected peaks: 219.80 (C-12), 216.91 (C-7), 215.89 (C-3), 184.32 (C-24).

2.2.3. Sr₃(3-dehydrocholanoyliden-l-tartrate)₂ (3)

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>C%</th>
<th>H%</th>
<th>Sr%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Calc.</td>
<td>Found</td>
<td>Calc.</td>
</tr>
<tr>
<td>Sr(cholate)₂ (1)</td>
<td>C₄₈H₇₈O₁₀Sr₂H₂O</td>
<td>61.28</td>
<td>61.45</td>
<td>8.55</td>
</tr>
<tr>
<td>Sr(dehydrocholate)₂ (2)</td>
<td>C₄₈H₆₆O₁₀Sr₂H₂O</td>
<td>63.22</td>
<td>63.54</td>
<td>7.05</td>
</tr>
<tr>
<td>Sr₃(3-dehydrocholanoyliden-l-tartrate)₂ (3)</td>
<td>C₅₆H₇₀O₂₀Sr₃H₂O</td>
<td>49.15</td>
<td>49.40</td>
<td>5.35</td>
</tr>
</tbody>
</table>
The reaction of 3-dehydrocholanooyliden-\(\eta\)-tartaric acid and strontium hydroxide was carried out in water as above in a 1:1.5 molar ratio (250 mg, 4.7 \times 10^{-4} \text{ mol}) of 3-dehydrocholanooyliden-\(\eta\)-tartaric acid for 186.6 mg, 7 \times 10^{-4} \text{ mol} of Sr(OH)\(_2\) \cdot 8\text{H}_2\text{O}) giving Sr\(_2\)(3-dehydrocholanooyliden-\(\eta\)-tartarate)\(_2\) as an off-white solid (240 mg, 1.8 \times 10^{-4} \text{ mol}, 76.6% yield).

The solubility in water of 3 is 0.05 g/mL at 25 °C.

Elemental analyses: see Table 1.

IR (KBr, cm\(^{-1}\)) selected peaks: 3800–3000 (broad, H\(_2\text{O}\)), 1704 (\(\nu\) C=O), 1593 (COO\(^{-}\)), 1432.

\(^1\)H NMR (D\(_2\text{O}, \text{ppm}\)) : 0.71 (3H, d, CH\(_3\)-21), 1.05 (3H, s, CH\(_3\)-18), 1.28 (6H, m, CH\(_3\)-19 + CH) 1.30–2.30 (18H, m, CH, CH\(_2\)), 3.05 (3H, m, CH-11, CH-6, CH-8), 4.38 (2H, s, CH-25, CH-26).

\(^{13}\)C NMR (D\(_2\text{O}, \text{ppm}\)) selected peaks: 220.35 (C-12), 216.96 (C-1), 176.97 (C-27), 184.39 (C-24), 176.97 (C-27, C-28), 111.93 (C-3), 79.06, 78.71 (C-25, C-26).

2.3. Human primary bone cells

Human primary osteoblasts (hOBs) were obtained and cultured as previously described [18]. Recruitment of osteoblasts donors was in accordance with approved procedures and informed consent was obtained from each patient. Samples were collected during oral surgery and bone was cultured as previously described [18]. The bone was cut into small pieces which were rinsed and then cultured in Eagle's MEM supplemented with 20% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin, 100 \(\mu\text{g/mL}\) streptomycin, and 50 \(\mu\text{g/mL}\) ascorbate at 37 °C in a humidified atmosphere of 5% CO\(_2\). After 5–7 days of culture, outgrowth of bone cells from the bone chips was evident; confluence in 9 cm\(^2\) dishes was usually reached after 4–6 weeks. For the studies here presented, only first passage cells were used.

Human primary osteoclasts (hOCs) were prepared as reported by Matsuzaki et al. [19] with slight modification. Briefly, peripheral blood (PB) was collected from healthy adult volunteers after informed consent. PB mononuclear cells (PBMCs) were prepared from diluted PB (1:2 in Hanks Balanced Salt Solution) which was layered over Histopaque 1077 solution (Sigma Aldrich, St. Louis, MO, USA). PB mononuclear cells were prepared by centrifuging (400 \times g) and resuspended in alpha-minimum essential medium (MEM), (Euroclone, S.p.A., Milano, Italy)/10% FBS (Euroclone, S.p.A., Milano, Italy). PBMCs (3 \times 10^5 \text{cells/cm}^2) were plated in 24-well plates, or in chamber slides, and allowed to settle for 2 h; wells were then rinsed to remove non-adherent cells. Monocytes were then cultured in Dulbecco's MEM supplemented with 10% FCS, 100 U/mL penicillin and 10 U/mL streptomycin for 14 days in the presence of human M-CSF (25 ng/mL) and RANKL (30 ng/mL). Culture media were changed with fresh media every 3–4 days. Cells were used when mature multinuclear cells were predominant in the cultures [20].

2.4. Analysis of osteoblast activity

The alkaline phosphatase (ALP) activity was evaluated in confluent hOBs by the hydrolysis of \(p\)-nitrophenylphosphate (PNPP), according to Ibbotson [21]. Enzyme activity was expressed as Units (U)/mg of protein and normalized for protein level. One unit (U) was defined as the amount of enzyme that hydrolyzed 1 mmol/ PNPP per min.

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma) in the cells cultured for up to 35 days in osteogenic medium consisting in DMEM, high-glucose, supplemented with 10% FBS, 10 mM \(\beta\)-glycerophosphate, 0.1 mM dexamethasone and 50 mM ascorbate. In the committed cells, the osteogenic medium was changed every 3 days. The cells were then fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM AR-S (pH 4.2) for 10 min. at room temperature, washed five times with deionized water and incubated in PBS for 15 min. to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope. Matrix mineralization was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer (“Quantity one” software, Biorad).

2.5. Tartrate-resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining was performed as previously reported [20]. Cells were fixed in para-formaldehyde (3%) with cacodylate buffer, 0.1 M, pH 7.2 (0.1 M Sodium cacodilte, 0.0025% CaCl\(_2\)) for 15 min, extensively washed in the same buffer and stained for TRAP (Acid Phosphatase Kit n. 386—Sigma Aldrich, St. Louis, MO, USA). After distilled water washing and drying, TRAP-positive multinucleated cells containing more than three nuclei were counted as hOCs.

2.6. Immunocytochemistry

Immunocytochemistry analysis was performed employing the streptavidin–biotin method utilizing the Ultrastain Polyvalent-HRP Immunostaining Kit (Ylem—Avezzano (AQ), Italy).

Cells grown in chamber slides were fixed in cold 100% methanol, and permeabilized with 0.2% (v/v) Triton X-100 (Sigma Aldrich, Chemical Co. St. Louis, MO, USA) in TBS (Tris-buffered saline). Cells were incubated in 3% H\(_2\text{O}_2\); the endogenous peroxidase activity was blocked with Super Block reagent. After application of the different primary antibodies, cells were incubated at 4 °C overnight: polyclonal antibodies for human matrix metalloproteinase 9 (MMP-9) (1:500 dilution, Santa Cruz Biotec, USA) and human Runx2 (M-70; 1:2000 dilution) (Santa Cruz, Biotechnology, CA).

Cells were then incubated at room temperature with anti-polyvalent Biotinylated Antibody (Ultrastain Polyvalent-HRP Immunostaining Kit, Ylem—Avezzano (AQ), Italy). After rinsing in TBS, Streptavidin HRP (Ultrastain Polyvalent-HRP Immunostaining Kit, Ylem) was applied; then Substrate-chromogen mix (AEC Cromogeno kit, Ylem) was added. After washing, cells were mounted in glycerol/TBS 9:1 and observed using a Leitz microscope.

2.7. Measurement of apoptosis

At the end of appropriate days of treatment, cells were rinsed twice with PBS solution and fixed for 25 min in 4% para-formaldehyde at room temperature. Apoptotic cells were detected by the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Moreover all cells were subjected to hematoxylin solution, showing blue stained nuclei. For OC cells, apoptosis was calculated as a percentage of apoptotic nuclei (dark brown nuclei) versus total nuclei of multinucleated TRAP-positive cells, evaluated for three different experiments.

2.8. Cytotoxicity studies

Cytotoxicity was determined on in vitro cultured human hOBs. The cells were plated in 96-well plates. Determinations of viable cells were performed using a colorimetric assay with MTT (thiazolyl blue). The assay, based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells [22], provides a quantitative determination of viable cells.
After 72 h of treatments in triplicate, 25 μL of MTT were added to each well, and the plate was incubated for 2 h at 37 °C. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformamide. Spectrophotometric absorbance of each sample was then measured at 570 nm.

2.9. Statistical analysis

The data are expressed as mean ± SEM of at least three replicates from at least three independent experiments. Statistical analysis was carried out by using Student’s paired t-test. Values were considered statistically significant when P < 0.05.

3. Results and discussion

3.1. Synthesis and characterization of the compounds 1–3

Among the bile acids strontium salts prepared for this study, we excluded those less soluble in water (deoxycholate, ursodeoxycholate) and we chose for this study the Sr salt of natural cholic acid (Compound 1); the Sr salt of oxidized form dehydrocholic acid (Compound 2), which has already been successfully tested as carrier for platinum-based drugs [23,24]; and the Sr salt of the semi-synthetic dehydrocholanoyl tartaric acid (Compound 3), a tri-carboxylic acid, able to bind a larger amount of metal than monocarboxylic bile acids.

The elemental analysis of C and H and the determination of Sr content by atomic absorption supported the indicated composition of the three compounds (see Table 1). In the IR spectra, the vC=O of the carboxyl group was diagnostic, being found at 1544 cm⁻¹ in the salt of 1, and at 1714 cm⁻¹ in free cholic acid. Similarly, the appearance of a peak at 1558 cm⁻¹ in the IR of 2 and at 1593 for 3, due to the C=O stretch of COO⁻, allowed 2 and 3 to be distinguished from their parent free acids. The ¹H and ¹³C NMR signals in D₂O of the strontium salts were substantially unchanged with respect to their corresponding acids or sodium salts in the same solvent. The only appreciable difference was found in the ¹H NMR of 3, where the peak assigned to CH-25 and CH-26 was observable at 4.38 ppm, while it was seen at 4.66 ppm in the parent tri-acid in D₂O.

3.2. Effects on osteoblast cell viability

Two different preparations of hOBs (hOB1 and hOB2) obtained from bone specimens collected as described in Experimental section, were used for these experiments. All samples were character-

![Fig. 1](image)

Fig. 1. (a) Human primary osteoblasts (hOB1 and hOB2) cultures were characterized by alkaline phosphatase (ALP) and Runx2. Original magnification 20×. (b) The effect of different concentrations (0.1–0.001 mM) of Compounds 1, 2, 3 and SrR on cell survival of hOBs. Results are expressed as a percentage of surviving cells, compared to that of the untreated cells, which has been set as 100%.
ized for their osteoblastic phenotype by expression of specific markers, including ALP (alkaline phosphatase) activity and Runx2 (Fig. 1a).

To determine whether the newly synthesized compounds had any effect on these bone forming cells, the viability of these hOBs was examined by the colorimetric MTT assay. Studies were done using 0.1–0.001 mM concentrations of Compounds 1, 2, and 3. As shown in Fig. 1b, cells treated with these newly synthesized compounds displayed only weak dose-dependent inhibition of cell viability. Therefore, these compounds may be considered to be no more cytotoxic than SrR.

3.3. Effects on osteoblast function

hOBs cultured under appropriate conditions followed a proliferation–differentiation sequence that led to mature osteoblasts. In the experiment shown in Fig. 2, after 5 days of culture the two hOB samples (hOB1 and hOB2) exhibited different basal ALP activity levels, probably reflecting their different stages of maturation; as expected, this value increased on day 10. It is well known that ALP activity, an early osteoblastic marker, is associated with extracellular matrix maturation, and decreases with osteoblast differentiation [25]. Recently it was shown that SrR increased the level of osteoblast maturation and the prevalence of osteocytes in cultures of hOBs [8]. The effects of compounds 1, 2, and 3 (0.1 mM) on ALP activity were analysed in hOBs cultured for 5 or 10 days. As shown in Fig. 2, treatment with compounds 2 and 3 significantly decreased ALP activity on day 10 of culture, compared with the control. Treatment with compound 1 induced only a faint (non-significant) decrease of ALP activity. This effect differed from the other two compounds tested; this is probably due to the different basal ALP activity levels expressed. Together, these results suggest

![Fig. 2. ALP activity was evaluated after treatment of two hOBs (hOB1 and hOB2) with 0.1 mM of the tested compounds for 5 and 10 days. C = untreated cells. Data are the mean of three different experiments.](image)

![Fig. 3. Mineralization pattern of hOBs in response to the tested compounds. (a) Surface of mineralized nodules formed by the two samples analysed (hOB1 and hOB2) after incubation with 0.1 mM of the tested compounds for 35 days. The results are expressed as a percentage of the control; data are expressed as the mean ± SEM. (b) Representative images of the cells subjected to Alizarin red staining. C = untreated cells. Original magnification 20×.](image)
that the tested compounds may increase the extent of maturation in hOBs.

Subsequently, we determined whether these compounds may affect the formation of mineralized nodules in hOBs cultured in osteogenic medium for 35 days. As shown in Fig. 3 these hOBs, cultured in presence of ascorbate and β-glycerophosphate, formed mineralized nodular structures that were increased after treatment with SrR. Otherwise, there was a variable pattern of mineralization exhibited by the cells in response to the three new compounds. In particular, the amount of mineral formed upon treatment with compound 1 did not differ from that formed in untreated cells. Compound 3 slightly increased the mineralization process; however, compound 2 significantly increased mineralization, not only when compared to the control, but also when compared to the SrR-treated cells. These results suggest that significant anabolic properties can be obtained from Sr^{2+} by replacing ranelate with the dehydrocholate.

3.4. Effects on osteoclast survival

In order to evaluate the influence of strontium ranelate on the behavior of osteoclasts, the primary bone-resorbing cells, we treated hOCs with all four compounds. On day 14 of culture, multinucleated TRAP-positive cells expressing the MMP-9 osteoclast-associated antigen were detected, confirming that this cellular phenotype was achieved at the molecular level (Fig. 4a). hOCs were treated for 72 h with the Compounds 1, 2, and 3 (0.1 mM); apoptosis levels were then measured using TUNEL staining and compared with those of untreated or SrR-treated cells. As evident in Fig. 4b, the three new compounds tested induced different levels of OCs apoptosis: compound 3 (71%) > compound 1 (46.5%) > compound 2 (20.5). These results indicate that compounds 1 and 2, even though showing a lower cytotoxicity to OCs than SrR, nevertheless exhibited a substantial ability to induce apoptosis.

4. Conclusions

This study describes the synthesis of three strontium-bile acid salts and their potential bioactivity as anti-osteoporosis drugs. To assess if they have the pre-requisites for modulating bone tissue regeneration, these strontium salts Sr(cholate)_2 (Compound 1), Sr(dehydrocholate)_2 (Compound 2) and Sr_3(3-dehydrocholanoyliden-L-tartrate)_2 (Compound 3) were tested on cultured human bone cells, showing effects that, on the whole, were anabolic on osteoblast differentiation and apoptotic on osteoclasts. Since there is increasing evidence that bone health depends on a delicate equilibrium between the activity of osteoblasts and osteoclasts, the dual effect of the tested compounds on these cells suggests that they may have potential therapeutic applications to certain osteopenic disorders.

In addition, considering that complexation with bile acid anions increases the absorption of metal ions, like calcium and iron, we suggest that Sr^{2+} absorption also may be enhanced by these bile acids. Considering the fact that results from animal and human studies have demonstrated that bone cells from different sources behave differently toward SrR treatment [7,8,26], it is important

![Fig. 4.](a) Human primary osteoclast cultures (hOCs) characterized by TRAP and MMP-9. (b) Detection of apoptosis by TUNEL assay. Cells were incubated either alone (C) or with 0.1 mM of the tested compounds for 72 h. The mean percentage ± SEM of the TUNEL-positive nuclei are reported in the graphs. Statistical significance was determined using the paired t-test (P < 0.05). The presence of apoptotic cells after treatment with the tested compounds, is also shown at the top of the panel. Brown colour reaction indicates cells that have undergone apoptosis. Original magnification 20×.)
to emphasize that the human primary cells we used to test strontium bile salts bioactivity may be considered a good experimental model for potential human treatment. Nevertheless, further work needs to be performed on the animal models in order to demonstrate the “in vivo” effects of these compounds. Of those tested, we consider that Compound 2 is the most promising. In as much as no one has yet achieved ideal management of osteopenic disorders in terms of adverse reactions to anti-osteoporotic drugs [1,27,28], further “in vivo” studies of our compounds are needed to demonstrate whether they possess better tolerability than those currently in use.

5. Abbreviations

hOBs human primary osteoblasts
hOCs human primary osteoclasts
SrR strontium ranelate
PBMCs peripheral blood mononuclear cells
FBS fetal bovine serum
HRP horseradish peroxidase
MEM minimum essential medium
ALP alkaline phosphatase
PNPP p-nitrophenylphosphate
TRAP tartrate-resistant acid phosphatase
TBS tris-buffered saline
PBS phosphate-buffered saline
MMP-9 matrix metalloproteinase 9
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SEM standard errors of the mean

Acknowledgments

E.M. thanks Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei sistemi Biologici (C.I.R.C.M.S.B.) for a research grant. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento. This work was supported by Regione Emilia Romagna, Programma di Ricerca Regione Universita’ 2007–2009.

References