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Synthesis and characterisation of strontium and calcium folates with potential osteogenic activity

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Abstract

Compounds having the general formula $MFO \cdot 4H_2O$ where M = Ca or Sr and FO = folate anion were prepared and their structure and physical-chemical properties were determined by elemental and thermal TGA, DSC analysis, FTIR, and EDAX spectroscopies and DRX. The results indicate that the two compounds form stable structures where folic acid acts as self-bridging ligand via two bidentate carboxylate groups. Moreover the two compounds showed low toxicity in-vitro response as h-osteoblast cell viability was not negatively affected by the presence of the folate derivatives within the range of 0.063 – 0.5 mg/ml. Results also indicate that forming folate derivatives overcome the toxic effects related to free Sr^{2+} ions. The range of maximum cell viability corresponding with a concentration of SrFO falls within the *in vitro* physiologically active range for strontium while within the same range the

strontium derivative showed a potential osteogenic activity as indicated by the overexpression of ALP activity.

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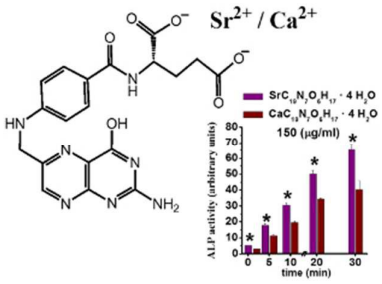

New strontium derivatives based on folic acid resulted in the formation of biocompatible SrFO compounds with osteogenic activity

Introduction

Different approaches in osteoporosis treatments include the use of strontium ranelate as bone promoter, especially indicated in patients unable to tolerate other anti-osteoporotic drugs such as bisphosphonates. However, this treatment presents some long-term safety concerns associated with the risk of blood clots and other secondary effects including, serious skin reactions, disturbances in consciousness, seizures, liver inflammation and reduced number of blood cells[1] leading to severe restriction of the use of this drug by the European Medicines Agency's Pharmacovigilance Risk Assessment Committee.[2] Taking these facts in consideration there is a major clinical need to develop new osteogenic drugs that provide a safer vehicle of strontium ions with low pharmacological side effects of the contra-ion. Since it is evident that the therapeutic action of strontium ranelate is dissociated from the ranelate moiety we present an alternative compound based on a folate carrier in which the capability of skeletal tissue regeneration and performance of the strontium based drug is improved.[3] The mechanism by which

strontium derivatives play a key role in preventing bone loss is still a matter of debate, however it has been demonstrated that the strontium cation is responsible for stimulating osteoblasts both in vivo and in vitro promoting the osteogenesis and preventing osteoclasts from resorbing bone .[4, 5] It is accepted that the anabolic effect of this cation involves calcium- sensing receptors that enhance preosteoblast replication, osteoblast differentiation, collagen type I synthesis, and bone matrix mineralization.[6] Meanwhile it has been hypothesised that strontium cation also plays a role in the inhibition of osteoclast differentiation and activity is mediated by the increase in osteoprotegerin markers and decrease in RANK ligand.[7]

In addition to the approved ranelate based carrier for strontium cations, only a few organic anions have been evaluated in order to enhance the strontium bioavailability avoiding some of the drawbacks associated with the ranelic moiety.[8] Folic acid derivative anion (folate) constitutes a very promising alternative carrier for Sr^{2+} that has not been explored yet and indeed the incorporation of B-group vitamins (folates) can significantly contribute to enhance cell metabolism reducing the risk of abnormal gene expression and DNA disorders, within cell replication and differentiation processes playing an additional beneficial role in bone regeneration for the treatment of osteoporosis and other bone diseases.[9-12]

This work describes the synthesis of two folate compound derivatives of divalent calcium and strontium cations and their characterization in order to elucidate their composition and physico-chemical characteristics and explore their potential as bioactive compounds for the treatment of osteoporosis.

Materials and Methods

Materials Folic acid, strontium chloride, calcium chloride, phosphate-buffered solution (PBS) pH 7.4, aqueous solutions of Triton X-100, tissue culture media,

additives, trypsin, and 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were all purchased form Sigma Aldrich and used without further purification. Thermanox (TMX) control disks were supplied by Labclinics S. L. Solvents used were HPLC grade and all other reagents were analytical grade purchased form Sigma-Aldrich.

Strontium folate salt was obtained from folic acid and strontium chloride solutions. Briefly, 25 mL of folic acid aqueous solution (0.4M) was prepared and pH adjusted to 7.4 with NaOH (1M), the solution then was refluxed at 50 °C for 2 hours. Subsequently, 25 ml of a $SrCl_2$ solution (1.2M) in ethanol/H_2O (50% v/v) were added and the reaction mixture was stirred for a further period of 1 h. The reaction was quenched by cooling down the mixture in an ice bath and the precipitate formed was collected by filtration. The dark orange solid product was recrystallized twice in hot water, milled and dried at 50 °C under vacuum until constant weight in the form of fine orange crystals. A similar procedure was also carried out for the preparation of the calcium folate complex that yielded fine dark orange crystals.

Characterization techniques

Elemental analysis. C, H and N were determined with an elemental analyser (LECO-932) equipped with an infrared and thermal conductivity detector. Strontium and Calcium were quantified by Inducted Coupled Plasma analysis using an inductively coupled plasma (ICP) optical emission spectrometer (Perkin–Elmer 430DV). A given weight of the complex was dissolved in HCl (2% p/v) and the solution made to volume in a measuring flask. Concentration of calcium and strontium were determined from a calibration curve constructed from absorbance of solution series prepared form standard stock solutions of chlorine salts in HCl (2% p/v).

X-ray analysis of the salt was carried out by Energy-dispersive X-ray spectroscopy (EDAX), recorded on a Phillips XL30, and X-ray diffraction (XRD) diffractograms were recorded respectively on a Bruker D8 Advance instrument that works with CuKα radiation at 0.02 step size and 0.5 seconds per step .

UV spectra were recorded on a Perkin Elmer Lambda 16 instrument while ATR-FTIR spectra were recorded on a Perkin–Elmer Spectrum One spectrophotometer with an ATR attachment.

Mass spectra were obtained with an electrospray ionization mass spectrometer (Agilent Series 1100) using 1mg/ml solutions in H_2O/methanol/acetonitrile (49.5:49.5:1 %v/v) as mobile phase.

Melting point (Tm) of the folate derivatives were measured by differential scanning calorimetry (DSC, Perkin–Elmer DSC 8500). Samples (10 – 15 mg) were placed in aluminium pans and heated under nitrogen flow (20ml · min^{-1}) from - 20 °C to 190 °C at constant rate of 10 °C · min^{-1}. Tm was taken as the onset of the heat capacity transition observed. Thermogravimetric diagrams were obtained using a thermogravimetric analyser TGA Q500 (TA instruments) apparatus, under dynamic nitrogen at a heating rate of 5 °C · min^{-1} in a range of 40-600 °C.

Cell culture studies were performed using human osteoblasts cells (Hob, European Collection of Cell Cultures, ECACC). The culture medium was Dulbeco's modified Eagle's Medium)/HAM F12 enriched with 110 mg·ml^{-1} of sodium pyruvate (DMEM,Sigma) and supplemented with 10 vol% of foetal bovine serum (FBS, Gibco), 200mM L-glutamine, 100 units·ml^{-1} penicillin and 100 mg·ml^{-1} streptomycin (Sigma, Spain). Cultures were maintained at 37 °C in humidified air with 5 vol% CO_2,

and the culture medium was carefully replaced at selected time intervals under sterile conditions.

The cytotoxicity of CaFO and SrFO compounds were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolimbromide) (Sigma) assay, and compared with folic acid, $SrCl_2$, $CaCl_2$ and culture medium. 0.5mg·mL^{-1} solutions of the respective sample were prepared in water and successively diluted to a concentration range of 1.0 –1·10^{-3} mg·ml^{-}). Cells were seeded in a sterile 96-well culture plate at a density of 5·10^4 cells ·ml^{-1} in complete medium and incubated to confluence for 24 h. Subsequently 50 ml of each sample dilution were added (n=8) and incubated for 24, 48, and 72 h. After each time point, the medium was removed and 100 mL of MTT solution prepared in warm PBS (0.5 mg·ml^{-1}) were added to each well and the plates incubated at 37 °C for 4 h. The excess medium and MTT were removed and 100 ml of DMSO was added to each well in order to dissolve the formazan crystals produced by viable cells. The absorbance was measured using a test wavelength of 570nm (Biotek Synergy HT).

The alkaline phosphatase (ALP) activity was evaluated in confluent Hob cells cultured in the presence of SrFO and CaFO complexes. ALP catalyses the hydrolysis of ρ-Nitrophenyl phosphate (pNPP) to ρ-Nitrophenol. It has a strong absorbance at 405 nm. The rate of increased absorbance at 405 nm is proportional to the enzyme activity. Cell-free specimens were run alongside to correct for nonspecific background activity.

Results and discussion

Pure calcium and strontium based folate compounds were obtained in high yield. The synthetic procedure for producing the folate complexes from chlorine salts are expected to provide fewer impurities than hydroxide or carbonate derivatives and no other co-precipitates were obtained. Figure 1 displays the synthetic procedure applied in the preparation of strontium folate and analogue mechanism was followed for calcium folate. Table 1 shows the results obtained from elemental analysis and the formula deduced with yield calculated for both CaFO and SrFO products indicating that the compounds have a 1:1 stoichiometry.

Figure 2 illustrates the weight loss recorded for folic acid and folate derivatives during heating treatment under inert atmosphere. Below 200 °C the weight loss observed corresponded to four molecules of water within the proposed molecular formula displayed in Table 1. Above 200 °C the degradation profile of the folate complexes reveals a significant increase in thermal stability in comparison to the folic acid salt as observed by the shift onset of the second degradation step from 194 °C (folic acid) to 294 °C (SrFO and CaFO), and temperature for 50% weight loss from 433 °C (folic acid) to 663 °C (SrFO and CaFO). In the three cases, onset of degradation starts to occur after the melting points observed at 220 °C (folic acid) and 350 °C (SrFO and CaFO) as determined by DSC (Figure 3). This behaviour is in concordance with folic acid thermal stability and other folate-metal complexes described by different authors and reveals the formation of proposed compounds.[13-16]

Coordination between carboxylic group of folate derivatives and different cations have been described in the literature indicating the possibility of chelating mode through monodentate or bidentate bridging as shown in Figure 4. Nakamoto and McCarthy have established the methodology to elucidate between these two

modes by taking in consideration the direction of the frequency shift of the ν_{asy} COO^- and ν_s COO^- vibration bands with respect to those of the free ion.[17] Infrared spectra of folic acid and folate derivatives are shown in Figure 4a and the main vibrational bands summarized in Table 2. The main relevant range between 1700 and 1300 cm^{-1} is presented in Figure 4b in order to assign coordination modes of folate compounds. The strong vibration band observed at 1690 cm^{-1} for folic acid corresponds to free carboxylic group, which does not appear in the spectra of the SrFO and CaFO salts as expected. Meanwhile there are two bands at 1604 - 1518 cm^{-1} corresponding to ν_{asy} COO^- and other one centred at 1412 cm^{-1} assigned to ν_s COO^- of the folate derivatives. It can be seen that both the ν_{asy} COO^- and ν_s COO^- frequencies shifted to lower frequencies to the same extent, implying that the compounds prepared were bridging bidentate.

Energy dispersive X-ray analysis confirm the presence of expected elements with no significant impurities as shown in Figure 6 while X-ray diffraction powder patterns illustrated in Figure 7 reveals a change in the crystallinity and structure between the folic acid and the calcium and strontium folates. The diffractograms of CaFO and SrFO reveal the stacking of folate tetramers, with d spacing of 3.31 Å (2θ = 26.91°) for CaFO and 3.33 (2θ = 26.79º). These values are consistent with previously published data[18] and indicates the formation of a 2D hexagonal lattice. There are also well resolved reflections at d spacing of 8.26-8.74 for CaFO and 8.74 for SrFO that correlates well with a repeat distance between tetramer stacks. Another peak is clearly observed at d spacing 32.99 (2θ = 2.68°) for SrFO that agree with the hexagonal lattice. The reflections at 2θ around 5 and 10 cannot be indexed

in base of the hexagonal cell unit but the fully structural characterization is out of the scope of this paper and it will be reported later.

MTT cell viability and alkaline phosphatase activity assays were carried out in order to determine the effect of the two folate salts, SrFO and CaFO, using human osteoblast cells. Results of both analyses are shown in Figures 8 and 9, respectively. MTT assay shows that the cell viability was not negatively affected by the presence of the folate derivatives in the range studied and even showed a significant increase of cell viability for both SrFO and CaFO compounds within the range of 0.063 – 0.5 mg/ml when compared with non-treated cells (0 mg/mL) or treated with folic acid. Results also indicate that the formation of the folate derivatives avoid the toxic effects related to the presence free Sr^{2+} and Ca^{2+} ions in the culture media as it can be seen by the significant reduction on cell viability shown for $SrCl_2$ and $CaCl_2$ groups. Although a slight increase in cell viability is observed for CaFO with respect to SrFO these values were not statistically significant, howeverdifferences between the control group (Figure 8, cell viability over 100%) indicate that the folic acid derivative enhance cell metabolism and replication processes that can be used as non-cytotoxic contra-ion for strontium carriers. The range of maximum cell viability corresponding with a concentration of SrFO between 0.06 and 0.6 mg/ml is of great practical interest as it falls within the physiologically active range for strontium of 0.009 – 0.09 mg/ml (0.1 – 1 mM)[19] in which strontium induces osteoblast activity in the early stages of *in vitro* bone development and therefore overexpression of relatively early skeletal markers such as ALP is induced.[20, 21] This effect is clearly shown in Figure 9 where the increase in p-nitrophenol concentration is related with the increased ALP activity of cells cultured in the presence of both SrFO and CaFO. SrFO exhibits significantly higher and faster ALP activity in the groups treated with

SrFO in comparison with the CaFO, indicating the key role played by strontium in early stage bone development and therefore showing the potential of the new SrFO acting as new bioactive compound in the treatment of bone loss diseases such as osteoporosis.

Conclusions

The synthesis of calcium and strontium folate derivatives resulted in the formation of (1:1) metal:folic acid molar ratio $SrFO{\cdot}4H_2O$ and $CaFO{\cdot}4H_2O$ compounds where folic acid acts as a self-bridging ligand via two bidentate carboxylate groups as elucidated from thermal and elemental analysis, infrared, energy dispersive and diffraction X-ray spectroscopies. Moreover, the two compounds show a low toxic response against in vitro human osteoblast like cell cultures due to the presence of the folate moiety while the strontium derivative showed potential osteogenic activity as indicated by the overexpression ALP activity.

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Table 1. Molecular empiric formula, yield, elemental analysis and coordination water of the folate (FO) compounds

Molecular formula (Complex formula)	Yield[a]	Metal[b]		C[c]		N[c]		H[c]		Coordination water [d]	
		Cal %	Found %	Cal %	Found %	Cal %	Found %	Cal %	Found %	Cal %	Found %
$SrC_{19}N_7O_{10}H_{25}$ ($SrFO{\cdot}4H_2O$)	95	14.6	15.2	38.1	35.6	16.4	16.1	4.2	4.2	12.0	12.0
$CaC_{19}N_7O_{10}H_{25}$	96	15.9	16.9	41.4	36.2	17.8	16.3	4.5	4.5	13.6	13.0

($CaFO{\cdot}4H_2O$)

Determined by gravimetric analysis (a), ICP spectroscopy (b), elemental analysis (c) and TGA (d)

Table 2. ATR-FTIR main frequencies assignment (cm^{-1}) of folic acid and CaFO and SrFO compounds

Assignments	Folic acid	CaFO	SrFO
ν (OH); H2O	3545, 3417	3540	3545
ν (NH)	3322	3324	3324
νas(CH)	3109	3126	3130
νs(CH)	2975, 2923	2986, 2904	2981,2904
ν (COOH)	1690	-	-
νas (COO-)	1604, 1518	1508	1510
νs (COO-)	1412	1405	1405
ν (CN)	1182	1182	1182
δ (CC)	762	762	762

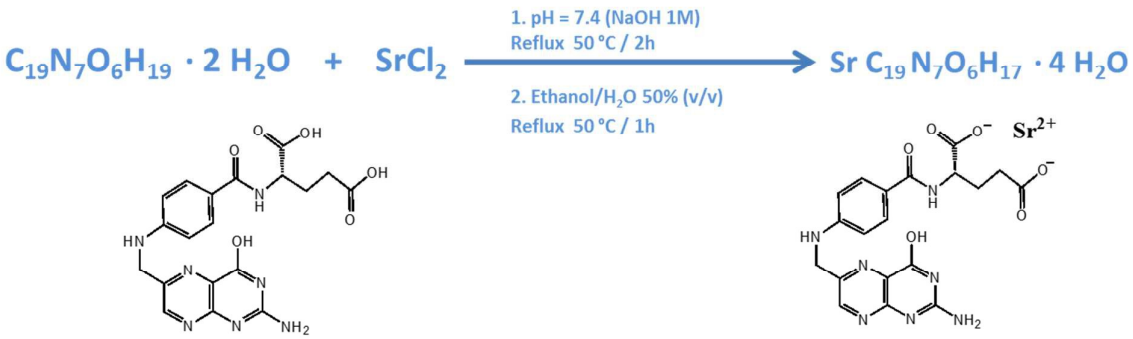


Figure 1. Synthesis of the Strontium folate complex.

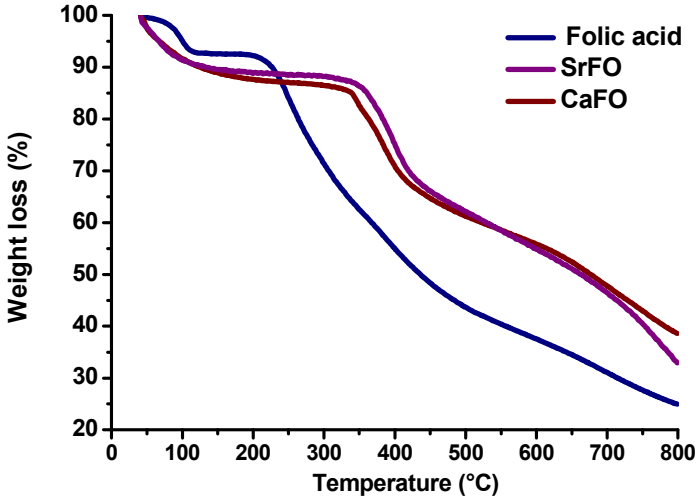


Figure 2. TGA thermograms of folic acid, strontium folate (SrFO) and calcium folate (CaFO) complexes.

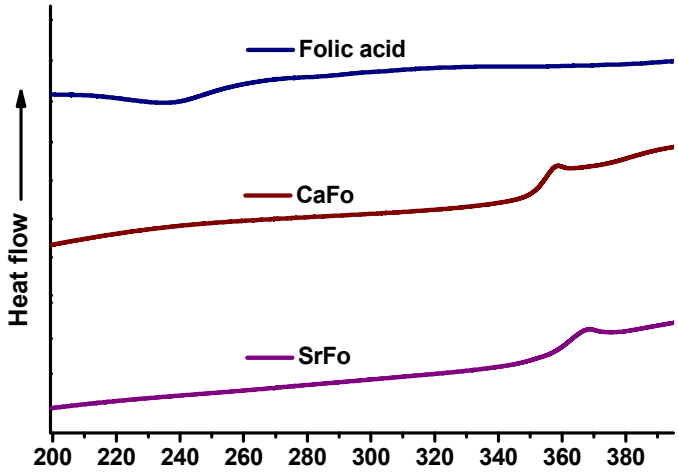


Figure 3. DSC thermograms of folic acid, strontium folate (SrFO) and calcium folate (CaFO) complexes.

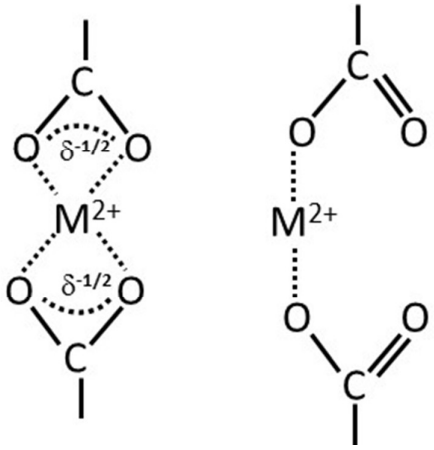
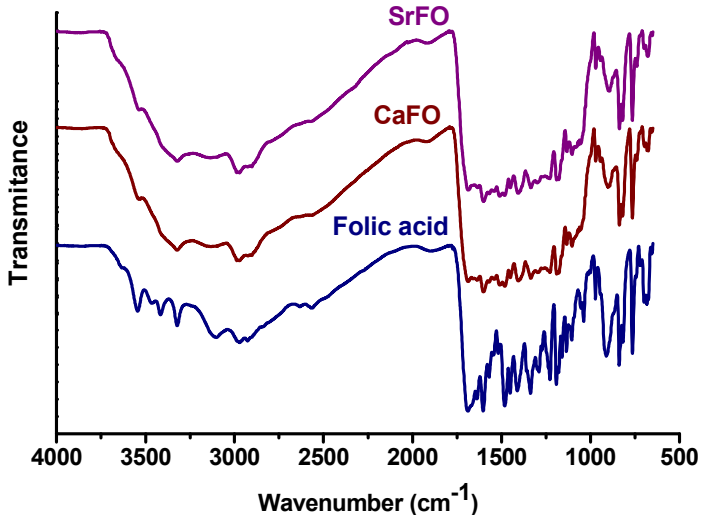


Figure 4.- Bidentate (left) and monodentate (right) coordination modes of carboxylate group with divalent cations.



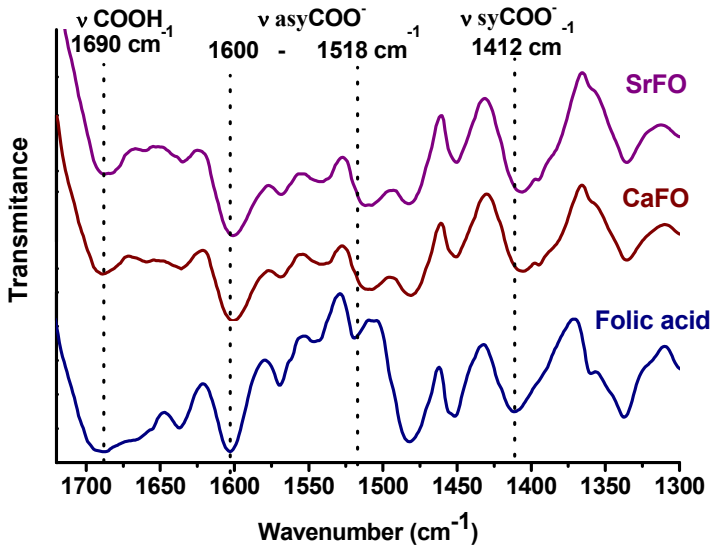
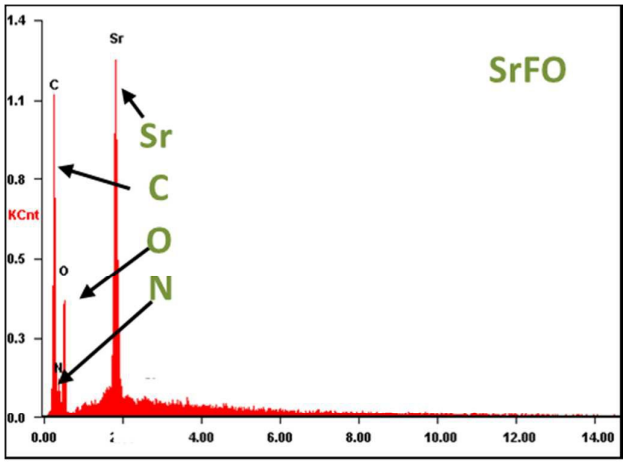


Figure 5. ATR-FTIR spectra (upper) and region of interest (lower) obtained for folic acid, strontium folate (SrFO) and calcium folate (CaFO) complexes.



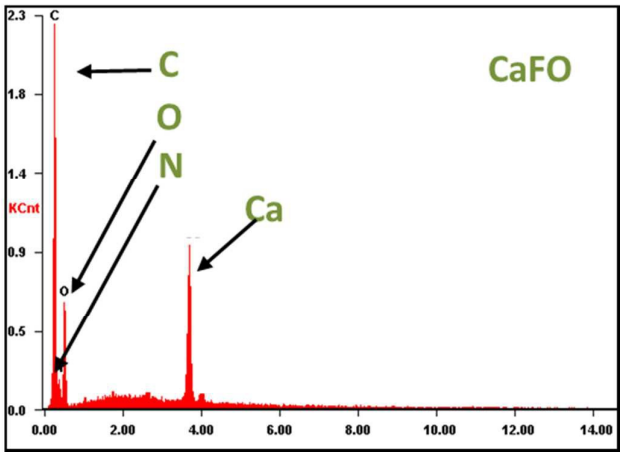


Figure 6. EDAX spectra of strontium folate (SrFO) and Calcium folate (CaFO) salts.

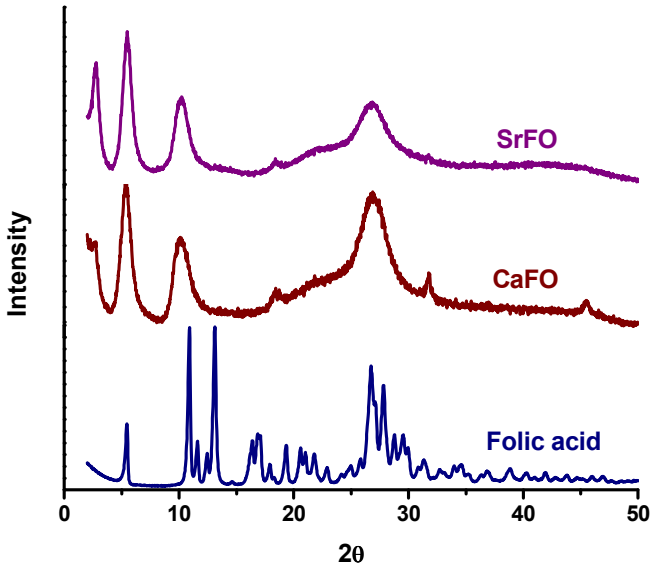


Figure 7. DRX obtained for folic acid, strontium folate (SrFO) and calcium folate (CaFO) complexes.

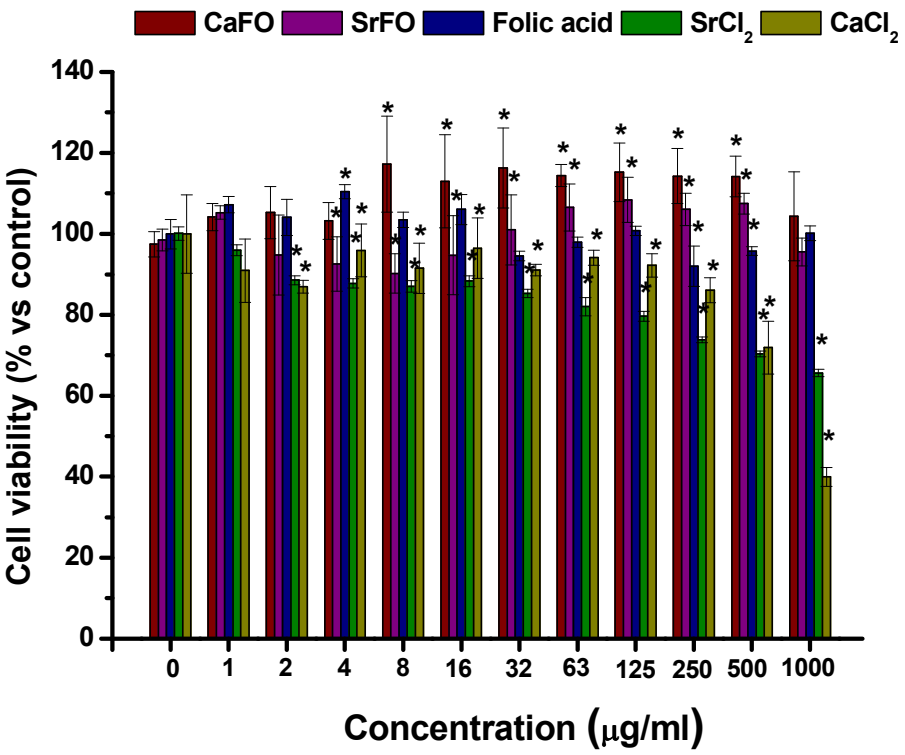


Figure 8. MTT cytotoxicity results for control (0 μg/ml), CaFO, SrFO, folic acid, $SrCl_2$ and $CaCl_2$ Results are mean with standard deviation. Statistical analysis (n= 8) of each compound was performed with respect to control at significance level of $p < 0.01$.

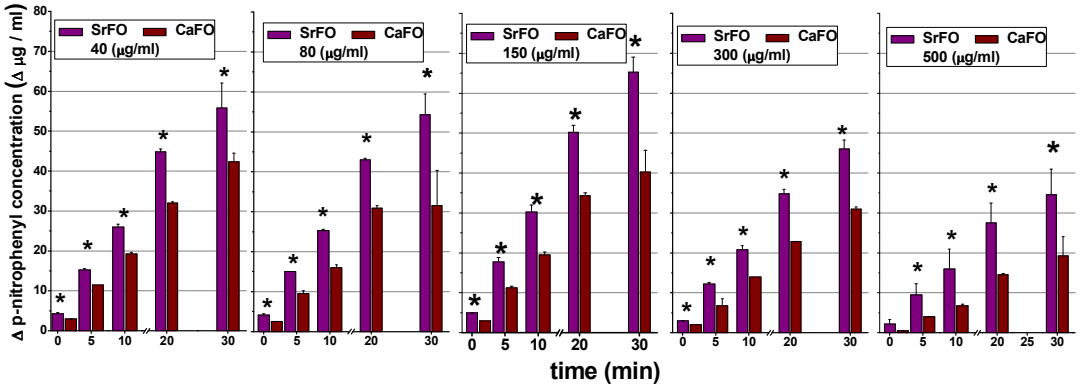


Figure 9. Variation of ALP activity expressed as variation of p-nitrophenol concentration liberated at different concentrations of SrFO and CaFO compounds in hOb cells cultures. Statistical analysis (n= 8) between groups was performed at significance level of $p < 0.01$.

1. S. Silverman and C. Christiansen, *Osteoporosis International*, 2012, 23, 797-809.
2. in *EMA/10206/2014*, European Medicines Agency, 2014.
3. I. Naharci, E. Bozoglu, N. Karadurmus, O. Emer, N. Kocak, S. Kilic, H. Doruk and M. Serdar, *Archives of Gerontology and Geriatrics*, 2012, 54, 469-472.
4. P. J. Marie, P. Ammann, G. Boivin and C. Rey, *Calcified Tissue International*, 2001, 69, 121-129.
5. S. Pasqualetti, G. Banfi and M. Mariotti, *Journal of Trace Elements in Medicine and Biology*, 2013, 27, 375-379.
6. Y. Zhang, L. Wei, J. Chang, R. J. Miron, B. Shi, S. Yi and C. Wu, *Journal of Materials Chemistry B*, 2013, 1, 5711-5722.
7. J. E. Fonseca, *Rheumatology*, 2008, 47, iv17-iv19.
8. P. Bergamini, E. Marchesi, A. Pagnoni, E. Lambertini, T. Franceschetti, L. Penolazzi and R. Piva, *Journal of Inorganic Biochemistry*, 2009, 103, 891-897.
9. I. P. Pogribny, A. G. Basnakian, B. J. Miller, N. G. Lopatina, L. A. Poirier and S. J. James, *Cancer Research*, 1995, 55, 1894-1901.
10. I. P. Pogribny, B. J. Miller and S. J. James, *Cancer Letters*, 1997, 115, 31-38.
11. E. Wainfan and L. A. Poirier, *Cancer Research*, 1992, 52, 2071s-2077s.
12. P. S. Low, W. A. Henne and D. D. Doorneweerd, *Accounts of Chemical Research*, 2008, 41, 120-129.
13. A. Vora, A. Riga, D. Dollimore and K. Alexander, *Journal of Thermal Analysis and Calorimetry*, 2004, 75, 709-717.
14. E. Hamed, M. S. Attia and K. Bassiouny, *Bioinorganic chemistry and applications*, 2009, 2009, 1.
15. M. G. A. El-Wahed, M. S. Refat and S. M. El-Megharbel, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2008, 70, 916-922.
16. P. R. Dametto, B. Ambrozini, F. J. Caires, V. P. Franzini and M. Ionashiro, *Journal of Thermal Analysis and Calorimetry*, 2014, 115, 161-166.
17. K. M. Nakamato, P.J. , *Spectroscopy and Structure of Metal Chelate Compounds*, John Wiley, New York, 1968.
18. R. Atluri, M. N. Iqbal, Z. Bacsik, N. Hedin, L. A. Villaescusa and A. E. Garcia-Bennett, *Langmuir*, 2013, 29, 12003-12012.
19. E. S. Place, L. Rojo, E. Gentleman, J. P. Sardinha and M. M. Stevens, *Tissue Engineering - Part A*, 2011, 17, 2713-2722.
20. E. Bonnelye, A. Chabadel, F. Saltel and P. Jurdic, *Bone*, 2008, 42, 129-138.
21. E. Gentleman, Y. C. Fredholm, G. Jell, N. Lotfibakhshaiesh, M. D. O'Donnell, R. G. Hill and M. M. Stevens, *Biomaterials*, 2010, 31, 3949-3956.