European Journal of Medicinal Chemistry 44 (2009) 3621–3626

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and anti-osteoporotic evaluation of certain 3-amino-2-hydroxypropoxyisoflavone derivatives

Chih-Hua Tseng [a], Yeh-Long Chen [a], Chih-Ming Lu [a], Chih-Kuang Wang [a], Yin-Tung Tsai [a], Ru-Wei Lin [b], Chain-Fu Chen [b], Yu-Fang Chang [b], Gwo-Jaw Wang [b,c], Mei-Ling Ho [b,d,**], Cherng-Chyi Tzeng [a,*]

[a] Faculty of Medicinal and Applied Chemistry, College of Life Science, Kaohsiung Medical University, Kaohsiung City 807, Taiwan
[b] Orthopaedic Research Center, Kaohsiung Medical University, Kaohsiung City 807, Taiwan
[c] Department of Orthopaedics, School of Medicine, Kaohsiung Medical University, Kaohsiung City 807, Taiwan
[d] Department of Physiology, School of Medicine, Kaohsiung Medical University, Kaohsiung City 807, Taiwan

ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form
3 December 2008
Accepted 19 February 2009
Available online 4 March 2009

Keywords:
Isoflavone
3-Amino-2-hydroxypropoxyisoflavone
Anti-osteoporotic activity

ABSTRACT

We report herein the synthesis and anti-osteoporotic evaluation of certain 3-amino-2-hydroxypropoxyisoflavone derivatives. The results indicated that 3-(3,4-dimethoxyphenyl)-7-(oxiran-2-ylmethoxy)-4*H*-chromen-4-one (**4**) and 3-{4-[3-(cyclohexylamino)-2-hydroxypropoxy]phenyl}-7-methoxy-4*H*-chromen-4-one (**5a**) exhibited significant inhibitory effects on osteoclast activity (TRAP activity in RAW 264.7 with an ED_{50} of 0.56 and 2.28 μM respectively). Both compounds have also exhibited very strong osteogenic effects, approximately a 10-fold effect of Ipriflavone on mineralization of osteoblasts (MC3T3E1 cells, a preosteoblast cell line derived from calvaria of C57BL/6 mice). Results indicated the potency on enhancing mineralization in D1 cells (a bone marrow mesenchymal cell line derived from *BALB/c* mice) decreased in an order **4** > Ipriflavone > **5a**. However, the potency on enhancing mineralization in human adipose tissue derived stem cells (hADSCs) decreased in an order **5a** > **4** > Raloxifene > Ipriflavone. Compound **5a** has been found to be non-cytotoxic and especially active in the enhancement of mineralization in human adipose tissue derived stem cells. Therefore, **5a** was selected as a potential lead for further structural optimization.

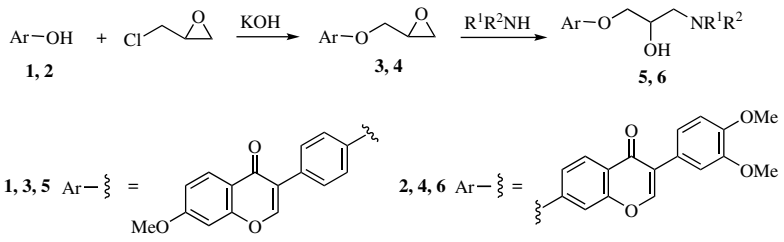
1. Introduction

Bone is subject to continual remodeling, renewed through a process of resorption of old bone by osteoclasts and formation of new bone by osteoblasts. An imbalance of the bone resorption over the bone formation leads to osteoporosis which is a major public health problem in the elderly population especially in women after menopause [1]. Hormone-replacement therapy (HRT) is effective in the prevention of bone loss in early menopause, but is also accompanied by several side effects such as uttering bleeding and carcinogenesis [2]. Isoflavonoids, a subclass of flavonoids which have structures similar to that of estrogen, have received considerable attention as alternatives to HRT for the prevention of postmenopausal osteoporosis and their actions suggested to be the inhibition of osteoclast differentiation [3–5].

Isoflavonoids present in large quantities in soybeans and soy products. These natural products along with their synthetic analogues possess a wide variety of biological activities including antiparasitic, antiproliferative, antifungal, antiviral, anti-inflammatory, antioxidant, and cardiovascular effects [6–13]. Genistein, a major isoflavone phytochemical in some plants, is known as a phytoestrogen that is capable of binding to the estrogen receptor. Much attention has been focus on the role of genistein in preventing bone loss resulted at least in part from the estrogen deficiency [14–17]. Ipriflavone (7-isopropoxyisoflavone) [18–22], an isoflavone synthesized from the soy isoflavone daidzein, has been approved for the treatment of involutional osteoporosis in some European countries and in Japan. However, only few reports have been dedicated to the improvement of the selective estrogen receptor modulator (SERM) activity and the anti-osteoporotic activity of Ipriflavone [23–25]. Delcanale et al. have synthesized certain aminoalkoxy analogues of Ipriflavone and evaluated for their capacity to inhibit bone resorption. Among them, 7-(aminoalkoxy)isoflavone analogues proved to be the most active [24]. The present report describes further the substitution of a hydroxyl group on the aminoalkoxy side chain of isoflavone derivatives and

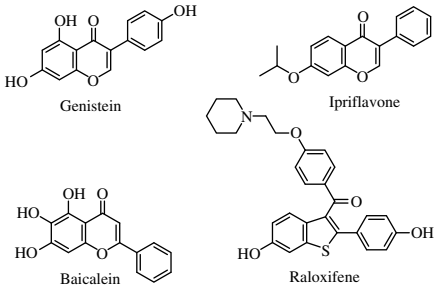
* Corresponding author. Tel.: +886 7 3121101x2123; fax: +886 7 3125339.
** Corresponding author. Orthopaedic Research Center, Kaohsiung Medical University, Kaohsiung City 807, Taiwan. Tel.: +886 7 3121101x2140; fax: +886 7 3234687.
E-mail addresses: homelin@kmu.edu.tw (M.-L. Ho), tzengch@kmu.edu.tw (C.-C. Tzeng).

0223-5234/$ – see front matter © 2009 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2009.02.025



Scheme 1.

evaluated for their anti-osteoporotic activities, *i.e.* the inhibition of osteoclasts and the promotion of osteoblasts. This type of 3-amino-2-hydroxypropoxy side chain is in common of certain clinically used cardiovascular drugs such as isoproterenol, salbutamol, and procaterol.



2. Chemistry

3-Amino-2-hydroxypropoxyisoflavone derivatives have been prepared as described in Scheme 1. Treatment of 3-(4-hydroxyphenyl)-7-methoxy-4*H*-chromen-4-one (**1**) [26] with epichlorohydrin gave 7-methoxy-3-[4-(oxiran-2-ylmethoxy)phenyl]-4*H*-chromen-4-one (**3**) which was treated with substituted amines respectively to afford the respective 3-amino-2-hydroxypropoxyisoflavone derivatives **5a**–**5c** in a good overall yield. Accordingly, compounds **6a** and **6b** have been prepared by amination of 3-(3,4-dimethoxyphenyl)-7-(oxiran-2-ylmethoxy)-4*H*-chromen-4-one (**4**) which in turn was obtained by the reaction of commercially available 7-hydroxy-3′,4′-dimethoxyisoflavone (**2**) and epichlorohydrin.

3. Pharmacological results and discussion

All the synthesized 3-amino-2-hydroxypropoxyisoflavones and their epoxide precursors were evaluated the tartrate resistant acid phosphatase (TRAP) activity [27] of RAW 264.7 cells, the precursor cells of osteoclasts, after RANKL induction for osteoclastogenesis, and the results are summarized in Table 1. Among these compounds, 3-{4-[3-(cyclohexylamino)-2-hydroxypropoxy]phenyl}-7-methoxy-4*H*-chromen-4-one (**5a**) exhibited significant inhibition of osteoclast activity. The inhibition of TRAP activity in RAW 264.7 with an IC_{50} of 2.28 μM is approximately 2-fold more active than its cyclopropyl counterpart **5c**, while the morpholinyl isomer **5b** was not effective on TRAP activity. Peripheral substitution of isoflavone core plays an important role in the inhibition of osteoclast activity, in which the inhibitory effect on TRAP activity was in an order of cyclohexyl > cyclopropyl > morpholinyl. Thus, 7-[3-(cyclohexylamino)-2-hydroxypropoxy]-3-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one (**6a**) inhibited the TRAP activity in RAW 264.7 with an IC_{50} of 4.17 μM while its morpholinyl counterpart **6b** was not effective on TRAP activity. Both epoxide precursors **3** and **4** have also been found to possess significant inhibitory effect on osteoclast inhibitory activity with IC_{50} of 6.39 and 0.56 μM respectively. With exception of **5b** and **6b**, all the isoflavone derivatives are more effective than the positive baicalein which exhibited an IC_{50} of 13.01 μM [28].

Compounds **4** and **5a** are non-cytotoxic on RAW 264.7 and D1 cells as shown in Fig. 1A, and C respectively. Both compounds have also exhibited very strong osteogenic effects, approximately a 10-fold effect of Ipriflavone on mineralization of osteoblasts (MC3T3E1 cells, a preosteoblast cell line derived from calvaria of C57BL/6 mice), as shown in Fig. 1B. Results from Fig. 1D indicated the potency on enhancing mineralization in D1 cells (a bone marrow mesenchymal cell line derived from *BALB/c* mice) decreased in an order **4** > Ipriflavone > **5a**. However, cell viability of **4** decreased to 60–70% to that of control while **5a**, Raloxifene, and Ipriflavone respectively were non-cytotoxic in a 3-day treatment of human adipose tissue derived stem cells (hADSCs) as shown in Fig. 1E. Fig. 1F indicates the potency on the enhancement of mineralization in hADSCs decreased in an order of **5a** > **4** > Raloxifene > Ipriflavone. Raloxifene is a nonsteroidal benzothiophene and classified as a selective estrogen receptor modulator (SERM) on the basis it produced both estrogen-agonistic effects on bone and estrogen-antagonistic effects on uterine endometrium and breast tissue [29–32]. Compound **5a** has been found to be non-cytotoxic and especially active in the enhancement of mineralization in human adipose tissue derived stem cells and therefore, was selected as a potential lead for the discovery of anti-osteoporotic drug candidates. Further structural optimization and mechanism studies are on-going.

4. Conclusion

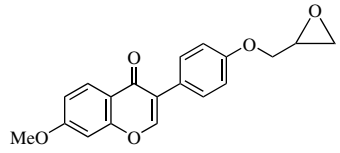
Peripheral substitution of isoflavone core plays an important role in the inhibition of osteoclast activity in which the TRAP activity decreased in an order of cyclohexyl > cyclopropyl > morpholinyl. Among these synthesized isoflavone derivatives, **4** and **5a** have been proved to possess dual actions, inhibition of osteoclast activity (TRAP activity in RAW 264.7) and enhancement of osteoblast activity (mineralization in MC3T3E1 cells, D1 cells, and hADSCs). The potency on enhancing mineralization in human adipose tissue derived stem cells (hADSCs) decreased in an order of **5a** > **4** > Raloxifene > Ipriflavone. Compound **5a** has been found to be non-cytotoxic and therefore, was selected as a potential lead for further structural optimization.

5. Experimental

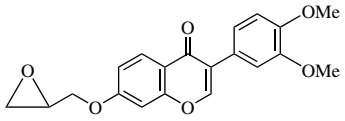
5.1. General

Melting points were determined on an Electrothermal IA9100 melting point apparatus and are uncorrected. Nuclear magnetic resonance (^1H and ^{13}C) spectra were recorded on a Varian Gemini 200 spectrometer or Varian-Unity-400 spectrometer. Chemical

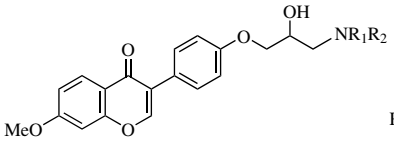
Table 1
Tartrate resistant acid phosphatase (TRAP) assay for testing effects of isoflavone derivatives on osteoclastic activity.



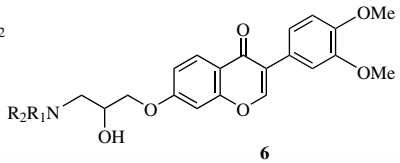
3



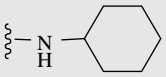
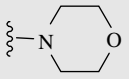
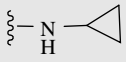
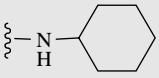
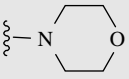
4



5



6

Compd.	$-NR_1R_2$	% Inhibition at 10 μM	IC_{50}
3	–	107	6.39 ± 1.09
4	–	119	0.56 ± 0.05
5a		119	2.28 ± 0.34
5b		0	ND
5c		103	4.85 ± 0.86
6a		120	4.17 ± 0.58
6b		0	ND
Baicalein		–	13.01 ± 0.06
Ipriflavone		31	ND

shifts were expressed in parts per million (δ) with tetramethylsilane (TMS) as an internal standard. Thin-layer chromatography was performed on silica gel 60 F-254 plates purchased from E. Merck and Co. The elemental analyses were performed in the Instrument Center of National Science Council at National Cheng-Kung University and National Chung-Hsing University using Heraeus CHN-O Rapid EA, and all values are within ±0.4% of the theoretical compositions.

*5.1.1. 7-Methoxy-3-(4-(oxiran-2-ylmethoxy)phenyl)-4H-chromen-4-one (**3**)*

A mixture of **1** (0.27 g, 1 mmol), K_2CO_3 (0.41 g, 3 mol) and epichlorohydrin (0.3 g, 3 mol) in MeCN (30 mL) was refluxed for 4 h (TLC monitoring). The mixture was then cooled and evaporated in vacuo to give a residue which was treated with H_2O (50 mL). The resulting precipitate was collected, washed with H_2O, and purified by column chromatography (MeOH:CH_2Cl_2 1/50) to give **2** (0.14 g, 43%). Mp: 153–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.72 (dd, 1H, J = 4.8, 2.8 Hz, 3′-H), 2.85 (dd, 1H, J = 4.8, 4.4 Hz, 3′-H), 3.34 (m, 1H, 2′-H), 3.85 (dd, 1H, J = 11.2, 6.4 Hz, 1′-H), 3.90 (s, 3H, OMe), 4.36 (dd, 1H, J = 11.2, 2.4 Hz, 1′-H), 7.01 (m, 2H, Ar–H), 7.08 (dd, 1H, J = 8.8, 2.4 Hz, 6-H), 7.15 (d, 1H, J = 2.4 Hz, 8-H), 7.52 (m, 2H, Ar–H), 8.02 (d, 1H, J = 8.8 Hz, 5-H), 8.42 (s, 1H, 2-H). ^{13}C NMR (100 MHz, DMSO-d_6): 43.78, 49.74, 56.12, 68.99, 100.56, 114.22 (2C), 114.81, 117.58, 123.29, 124.45, 126.95, 130.11 (2C), 153.56, 157.46, 157.99, 163.73, 174.63. Anal. calcd for $C_{19}H_{16}O_5$: C 70.36, H 4.97; found: C 70.46, H 4.95.

*5.1.2. 3-(3,4-Dimethoxyphenyl)-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (**4**)*

The title compound was obtained by the treatment of **2** with epichlorohydrin as described for **3**. Yield: 70%. Mp: 161–162 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 2.80 (dd, 1H, J = 4.8, 2.4 Hz, 3″-H), 2.95 (dd, 1H, J = 4.8, 4.0 Hz, 3″-H), 3.42 (m, 1H, 2″-H), 3.92 and 3.93 (two s, 6H, 3′- and 4′-OMe), 4.03 (dd, 1H, J = 11.2, 6.0 Hz, 1″-H), 4.38 (dd, 1H, J = 11.2, 2.8 Hz, 1″-H), 6.90 (d, 1H, J = 2.4 Hz, 8-H), 6.92 (d, 1H, J = 8.0 Hz, 5′-H), 7.02–7.06 (m, 2H, 6- and 6′-H), 7.20 (d, 1H, J = 1.6 Hz, 2′-H), 7.95 (s, 1H, 2-H), 8.21 (d, 1H, J = 8.8 Hz, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): 44.53, 49.76, 55.92, 55.94, 69.28, 101.04, 111.12, 112.45, 114.73, 118.75, 121.00, 124.53, 124.96, 127.90, 148.74, 149.08, 152.29, 157.73, 162.69, 175.83. Anal. calcd for $C_{20}H_{18}O_6 \cdot 1.0H_2O \cdot 0.8HCl$: C 59.81, H 5.23; found: C 59.90, H 5.46.

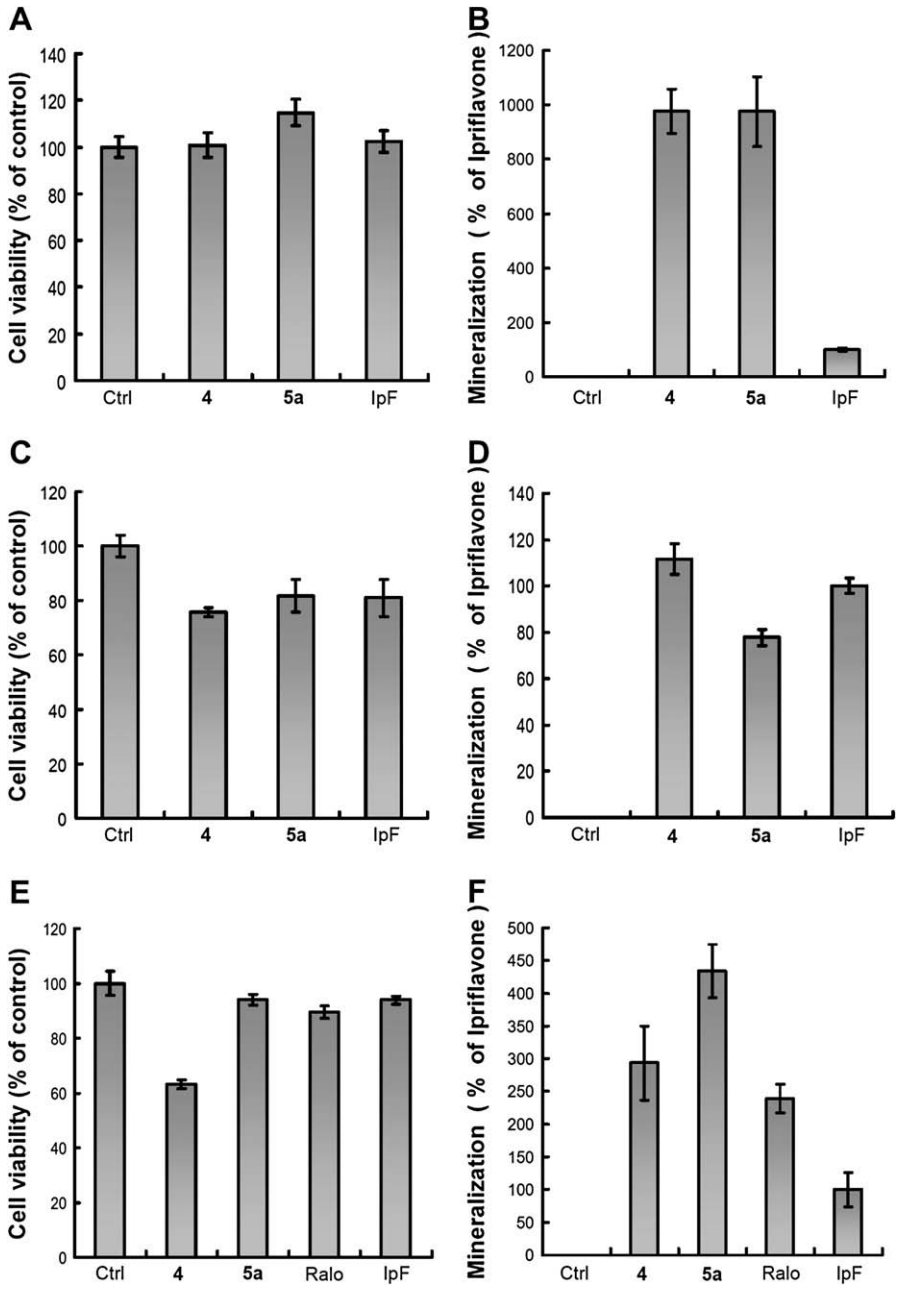


Fig. 1. Effects of compounds **4**, **5a**, Raloxifene (Ralo) and Ipriflavone (IpF) (10 μM) on (A) cell viability in RAW 264.7 cells (a 3-day treatment), (B) mineralization in MC3T3E1 cells (a 12-day treatment), (C) cell viability in D1 cells (a 3-day treatment), (D) mineralization in D1 cells (a 7-day treatment), (E) cell viability in hADSCs (a 3-day treatment), and (F) mineralization in hADSCs' cells (a 13-day treatment).

*5.1.3. 3-(4-(3-(Cyclohexylamino)-2-hydroxypropoxy)phenyl)-7-methoxy-4H-chromen-4-one (**5a**)*

A mixture of **3** (0.32 g, 1 mmol), cyclohexylamine (0.50 g, 5 mol) in EtOH (30 mL) was heated at reflux for 4 h (TLC monitoring) and then evaporated in vacuo to give a residue which was treated with H_2O (50 mL). The precipitating material was collected, washed with H_2O, and purified by column chromatography (MeOH:CH_2Cl_2 1/20) to give **5a** (0.27 g, 67%). Mp: 125–126 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 1.05–1.32 (m, 5H, cyclohexyl-H), 1.60–1.95 (m, 5H, cyclohexyl-H), 2.47 (m, 1H, cyclohexyl-H), 2.75 (dd, 1H, J = 12.4, 7.6 Hz, 3′-H), 2.93 (d, 1H, J = 12.4, 3.6 Hz, 3′-H), 3.92 (s, 3H, OMe), 3.97–4.08 (m, 3H, 1′- and 2′-H), 6.85 (d, 1H, J = 2.4 Hz, 8-H), 6.96–7.01 (m, 3H, Ar–H and 6-H), 7.48 (m, 2H, Ar–H), 7.92 (s, 1H, 2-H), 8.19 (d, 1H, J = 8.8 Hz, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): 24.97 (2C), 25.99, 33.44, 33.67, 48.74, 55.81, 56.82, 68.26, 70.52, 100.05, 114.54 (2C), 114.57, 118.37, 124.54, 124.78, 127.76, 130.13 (2C), 152.09, 157.93, 158.67, 163.95, 175.84. Anal. calcd for $C_{25}H_{29}NO_5 \cdot 0.1H_2O$: C 70.59, H 6.93, N 3.29; found: C 70.38, H 6.87, N 3.29.

*5.1.4. 3-(4-(2-Hydroxy-3-morpholinopropoxy)phenyl)-7-methoxy-4H-chromen-4-one (**5b**)*

The title compound was obtained by the treatment of **3** with morpholine as described for **5a**. Yield: 68%. Mp: 117–118 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 2.47–2.72 (m, 6H, morpholinyl-H and 3′-H), 3.70–3.79 (m, 4H, morpholinyl-H), 3.92 (s, 3H, OMe), 4.03 (m, 2H, 1′-H), 4.13 (m, 1H, 2′-H), 6.85 (d, 1H, J = 2.4 Hz, 8-H), 6.98 (m, 3H, Ar–H and 6-H), 7.49 (m, 2H, Ar–H), 7.92 (s, 1H, 2-H), 8.20 (d, 1H, J = 8.8 Hz, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): 53.75, 55.81, 61.02,

65.37, 66.97 (2C), 70.22, 94.38, 100.07, 114.55 (2C), 114.58, 118.38, 124.62, 124.78, 127.77, 130.15 (2C), 152.10, 157.94, 158.65, 163.97, 175.85. Anal. calcd for $C_{23}H_{25}NO_6$: C 66.40, H 6.19, N 3.37; found: C 66.09, H 6.17, N 3.34.

5.1.5. 3-(4-(3-(Cyclopropylamino)-2-hydroxypropoxy)phenyl)-7-methoxy-4H-chromen-4-one (**5c**)

The title compound was obtained by the treatment of **3** with cyclopropylamine as described for **5a**. Yield: 78%. Mp: 105–106 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 0.22 (m, 2H, cyclopropyl-H), 0.36 (m, 2H, cyclopropyl-H), 2.11 (m, 1H, cyclopropyl-H), 2.64–2.77 (m, 2H, 3′-H), 3.88-4.05 (m, 6H, OMe, 1′- and 2′-H), 4.97 (1H, NH), 6.99 (m, 2H, Ar–H), 7.08 (dd, 1H, $J = 8.8$, 2.0 Hz, 6-H), 7.17 (d, 1H, $J = 2.0$ Hz, 8-H), 7.51 (m, 2H, Ar–H), 8.03 (d, 1H, $J = 8.8$ Hz, 5-H), 8.43 (s, 1H, 2-H). ^{13}C NMR (100 MHz, DMSO-d_6): 6.17 (2C), 30.29, 52.21, 56.13, 67.95, 70.83, 100.07, 114.20 (2C), 114.80, 117.59, 123.38, 124.00, 126.95, 130.05 (2C), 153.48, 157.46, 158.52, 163.71, 174.65. Anal. calcd for $C_{22}H_{23}NO_5 \cdot 0.1H_2O$: C 68.94, H 6.11, N 3.66; found: C 68.69, H 6.06, N 3.46.

5.1.6. 7-(3-(Cyclohexylamino)-2-hydroxypropoxy)-3-(3,4-dimethoxyphenyl)-4H-chromen-4-one (**6a**)

The title compound was obtained by the treatment of **4** with cyclohexylamine as described for **5a**. Yield: 54%. Mp: 70–71 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 1.03–1.32 (m, 5H, cyclohexyl-H), 1.62–1.95 (m, 5H, cyclohexyl-H), 2.45 (m, 1H, cyclohexyl-H), 2.76 (dd, 1H, $J = 12.4$, 8.0 Hz, 3′-H), 2.96 (d, 1H, $J = 12.4$, 4.0 Hz, 3″-H), 3.92 and 3.93 (two s, 6H, 3′- and 4′-OMe), 3.99–4.09 (m, 3H, 1″- and 2″-H), 6.89 (d, 1H, $J = 2.4$ Hz, 8-H), 6.92 (d, 1H, $J = 8.4$ Hz, 5′-H), 7.01–7.06 (m, 3H, 6- and 6′-H), 7.21 (d, 1H, $J = 2.4$ Hz, 2′-H), 7.95 (s, 1H, 2-H), 8.21 (d, 1H, $J = 8.8$ Hz, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): 24.96 (2C), 25.98, 33.61, 33.91, 48.52, 55.90, 55.92, 56.75, 68.04, 71.04, 100.82, 111.09, 112.42, 114.82, 118.53, 120.99, 124.55, 124.90, 127.75, 148.71, 149.04, 152.26, 157.77, 163.05, 175.86. Anal. calcd for $C_{26}H_{31}NO_6 \cdot 0.2H_2O$: C 68.32, H 6.92, N 3.06; found: C 68.51, H 6.99, N 3.01.

5.1.7. 3-(3,4-Dimethoxyphenyl)-7-(2-hydroxy-3-morpholinopropoxy)-4H-chromen-4-one (**6b**)

The title compound was obtained by the treatment of **4** with morpholine as described for **5a**. Yield: 51%. Mp: 135–136 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 2.47–2.73 (m, 6H, morpholinyl-H and 3″-H), 3.71–3.80 (m, 4H, morpholinyl-H), 3.92 and 3.93 (two s, 6H, 3′- and 4′-OMe), 4.11 (m, 2H, 1″-H), 4.17 (m, 1H, 2″-H), 6.90 (d, 1H, $J = 2.4$ Hz, 8-H), 6.92 (d, 1H, $J = 8.4$ Hz, 5′-H), 7.02–7.06 (m, 2H, 6- and 6′-H), 7.20 (d, 1H, $J = 2.0$ Hz, 2′-H), 7.95 (s, 1H, 2-H), 8.21 (d, 1H, $J = 8.8$ Hz, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): 30.85, 53.69, 55.91, 55.93, 60.78, 65.08, 66.92 (2C), 70.67, 100.89, 111.12, 112.45, 114.77, 118.61, 121.00, 124.54, 124.93, 127.80, 148.73, 149.07, 152.26, 157.75, 162.99, 175.83. Anal. calcd for $C_{24}H_{27}NO_7$: C 65.29, H 6.16, N 3.17; found: C 65.20, H 6.19, N 3.14.

5.2. Pharmacological evaluation

5.2.1. Tartrate resistant acid phosphatase (TRAP) solution assay [28]

10^3 Raw 264.7 cells were cultured in 96-well plates with 100 ng/mL RANKL (R&D Systems, Minneapolis, MN). Cultures were incubated at 37 °C in 5% CO_2 for 5 days with addition of media containing fresh RANKL on day 3. In the TRAP solution assay, enzyme activity was examined by the conversion of α-naphthyl phosphate (4 mmol/L; Sigma Chemical Co.) to α-naphthol in the presence of 2 mol/L L-tartrate solution (Sigma Chemical Co.) in each well. Absorbance was measured at 405 nm using a microplate reader (model 550; Bio-Rad Labs.).

5.2.2. Cell culture and drug treatment

D1 cell, which is a mesenchymal stem cell line cloned from bone marrow cells of *BALB/c* mice, was purchased from American Type Culture Collection (Rockville, MD). D1 cells can be induced into osteoblasts, adipocytes and chondrocytes. D1 cells were maintained in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100 U/mL of penicillin and streptomycin. MC3T3E1 cell, which is a preosteoblast cell line derived from calvaria of C57BL/6 mice, was obtained from the American Type Culture Collection (Rockville, MD). MC3T3E1 cells were maintained in αMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100 U/mL of penicillin and streptomycin. They exhibit osteogenic properties in Dulbecco modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 50 μg/mL sodium ascorbate in a humidified atmosphere of 5% CO_2 at 37 °C, and the medium was changed every 2 days. New synthetic compounds were dissolved in DMSO to a final concentration of 10 mM and stored at −20 °C. The stock solution was freshly diluted with medium to a final concentration of 10 μM containing 0.1% of DMSO. Control cultures were treated with the same amount of DMSO as used in the corresponding experiments.

5.2.3. Cell viability by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

The MTT assay is a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals [33]. After compound treatments, 350 μL of MTT solution (0.5 μg/mL in PBS) was added to each well and incubated for 4 h. DMSO was then added for another 0.5 h to thoroughly dissolve the dark blue crystals. The absorbance at 570 nm was measured with an ELISA reader. Inhibition of mitochondrial metabolism was shown as relative activity (% of control).

5.2.4. Cytotoxicity assay by lactate dehydrogenase (LDH) leakage

Lactate dehydrogenase (LDH) leakage from cells was measured to quantify the cytotoxicity by using a cytotoxicity detection kit (Roche, Germany) [34]. Cells were previously seeded into 48-well plates (4×10^4 cells/well). After drug treatment, the supernatants and cell layers of the cultures were collected for assay. According to the manufacturer's guidelines for the detection kit, cell layers were lysed with 1% Triton X-100, and cell lysates and supernatants were assayed in a 96-well plate, respectively. Briefly, 100 μL of catalyst solution was added in each assay well for 20 min. Absorbance was measured with an ELISA reader with a 490 nm filter. LDH leakage was shown as relative activity (% of total cell toxicity).

5.2.5. Osteogenic differentiation and quantification of mineralization

Osteogenic differentiation was induced by culturing cells in osteo-induction medium (OIM, 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and L-Ascorbic 2 phosphate 100 μM in low glucose DMEM) for 7–14 days. The extracellular matrix calcification was estimated by using Alizarin red S stain [35]. The Alizarin red S-stained mineral was quantified by the osteogenesis quantification kit (CHEMICON®).

Acknowledgment

Financial support of this work by the Ministry of Economics (94-96-EC-17-A-17-S1-041, Taiwan, ROC) is gratefully acknowledged. We also thank the *National Center for High-Performance Computing* for providing computer resources and chemical database services.

References

[1] W.J. Boyle, W.S. Simonet, D.L. Lacey, Nature 423 (2003) 337.
[2] H.K. Genant, D.J. Baylink, J.C. Gallagher, Am. J. Obstet. Gynecol. 161 (1989) 1842.
[3] N. Morabito, A. Crisafulli, C. Vergara, A. Gaudio, A. Lasco, N. Frisina, R. D'Anna, F. Corrado, M.A. Pizzoleo, M. Cincotta, D. Altavilla, R. Lentile, F. Sqadrito, J. Bone Miner. Res. 17 (2002) 1904.
[4] C.M. Rassi, M. Lieberherr, G. Chaumaz, A. Pointillart, G. Cournot, J. Bone Miner. Res. 17 (2002) 630.
[5] T. Yamagishi, E. Otsuka, H. Hagiwara, Endocrinology 142 (2001) 3632.
[6] Z.P. Xiao, D.H. Shi, H.Q. Li, L.N. Zhang, C. Xu, H.L. Zhu, Bioorg. Med. Chem. 15 (2007) 3703.
[7] A.V. Stachulski, N.G. Berry, A.C. Lilian Low, S.L. Moores, E. Row, D.C. Warhurst, I.S. Adagu, J.F. Rossignol, J. Med. Chem. 49 (2006) 1450.
[8] D.A. Vasselin, A.D. Westwell, C.S. Matthews, T.D. Bradshaw, M.F.G. Stevens, J. Med. Chem. 49 (2006) 3973.
[9] B.B. Aggarwal, S. Shishodia, Biochem. Pharmacol. 71 (2006) 1397.
[10] H. Kikuchi, T. Ohtsuki, T. Koyano, T. Kowithayakorn, T. Sakai, M. Ishibashi, J. Nat. Prod. 70 (2007) 1910.
[11] P. Laupattarakasem, P.J. Houghton, J.R.S. Hoult, Planta Med. 70 (2004) 496.
[12] W. Ren, Z. Qiao, H. Wang, L. Zhu, L. Zhang, Med. Res. Rev. 23 (2003) 519.
[13] T.C. Wang, I.L. Chen, P.J. Lu, C.H. Wong, C.H. Liao, K.C. Tsiao, K.M. Chang, Y.L. Chen, C.C. Tzeng, Bioorg. Med. Chem. 13 (2005) 6045.
[14] S.F. Wang, Q. Jiang, Y.H. Ye, Y. Lia, R.X. Tan, Bioorg. Med. Chem. 13 (2005) 4880.
[15] Y. Ishimi, M. Yoshida, S. Wakimoto, J. Wu, H. Chiba, X. Wang, K.I. Takeda, C. Miyaura, Bone 31 (2002) 180.
[16] J. Wu, X.X. Wang, M. Takasaki, A. Ohta, M. Higuchi, Y. Ishimi, J. Bone Miner. Res. 16 (2001) 1829.
[17] A. Bitto, B.P. Burnett, F. Polito, H. Marini, R.M. Levy, M.A. Armbruster, L. Minutoli, V. Di Stefano, N. Irrera, S. Antoci, R. Granese, F. Squadrito, D. Altavilla, Br. J. Pharmacol. 155 (2008) 896.
[18] K.A. Head, Altern. Med. Rev. 4 (1999) 10.
[19] K. Imamiya, H. Kawamoto, Patent No. JP 11012265, January 1999.
[20] I. Yamazaki, Y. Sawa, Patent No. EP-A2-136569, September 1984.
[21] M. Ferrari, Patent No. EP-A1-0941992, September 1999.
[22] M. Ferrari, Patent No. EP-B1-0941992, May 2002.
[23] P. Chiest, P. Ventura, V. Servadio, M. Delcanale, G. Amari, E. Armani, M. Civelli, M. Giossi, E. Galbiatti, PCT No. WO 98/29403, July 1998.
[24] M. Delcanale, G. Amari, E. Armani, M. Lipreri, M. Civelli, E. Galbiati, M. Giossi, P.L. Caruso, P. Crivori, P.A. Carrupt, B. Testa, Helv. Chim. Acta 84 (2001) 2417.
[25] G.E. Kelly, A. Heaton, J. Bremner, Patent No. US 0100238, May 2006.
[26] G.Y. Gao, D.J. Li, W.M. Keung, Bioorg. Med. Chem. 11 (2003) 4069.
[27] S. Bandyopadhyay, J.M. Lion, R. Mentaverri, D.A. Ricupero, S. Kamel, J.R. Romero, N. Chattopadhyay, Biochem. Pharmacol. 72 (2006) 184.
[28] M.H. Kim, S.Y. Ryu, M.A. Bae, J.S. Choi, Y.K. Min, S.H. Kim, Food Chem. Toxicol. 46 (2008) 3375.
[29] S. Morishima, M.A. Shibata, M. Ohmichi, Y. Otsuki, Med. Mol. Morphol. 41 (2008) 132.
[30] W.L. Lee, H.T. Chao, M.H. Cheng, P.H. Wang, Maturitas 60 (2008) 92.
[31] C. Jochems, M. Lagerquist, C. Haakansson, C. Ohlsson, H. Carlsten, Clin. Exp. Immunol. 152 (2008) 593.
[32] P.D. Delmas, S.R. Davis, J. Hensen, S. Adami, S. Os, E.A. Nijland, Osteoporosis Int. 19 (2008) 1153.
[33] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Cancer Res. 47 (1987) 936.
[34] J.G. Crowston, A.N. Akbar, P.H. Constable, N.L. Occleston, J.T. Daniels, P.T. Khaw, Invest. Ophthalmol. Vis. Sci. 39 (1998) 449.
[35] C.A. Gregory, W.G. Gunn, A. Peister, D.J. Prockop, Anal. Biochem. 329 (2004) 77.