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Synthesis and Anti-osteoporosis Potential of Two New Indirubin-3'-oxime Derivatives

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Two new indirubin-3'-oxime derivatives, indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime] (2) and indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime] (3) were synthesized. Their structures were confirmed by ESI-MS and NMR spectroscopic method. Both of them (5 μg/mL) significantly caused a elevation of cell growth, alkaline phosphate activity, and mineralization in osteoblastic MC3T3-E1 cells ($p<0.05$).

Key words: anti-osteoporosis, indirubin-3'-oxime, semi-synthesis

Indirubin-3'-oxime is a derivative of indirubin, an active ingredient of a traditional Chinese medicinal recipe (Danggui Longghui Wan), which is used to treat various diseases including chronic myelocytic leukemia. Indirubin and its analogs were early discovered as the cyclin-dependent kinases (CDKs) inhibitors [Hoessel *et al.*, 1999]. CDKs exert essential function in regulating the cell division cycle and are involved in cancers. They play important effects in the nervous system and are involved in various neurodegenerative diseases such as Alzheimer's, Parkinson's, Nieman-Pick's and ischemia diseases [Ribas *et al.*, 2006]. They are also involved in inflammation and insulin secretion by pancreatic cells. Apart from activity towards CDKs, there is evidence suggesting that indirubins inhibit glycogen synthase-3β, glycogen phosphorylase and c-Src kinase, c-Jun NH_2-terminal kinase, and also activate aryl hydrocarbon receptor, a co-transcriptional factor which mediates the effects of many xenobiotics such as dioxin [Damiens *et al.*, 2001]. It was reported that indirubin could suppress the nuclear factor-kappa B activation and lead to the tumor necrosis factor-induced apoptosis [Sethi *et al.*, 2006]. More recently, indirubin-3'-oxime has been found to inhibit auto-phosphorylation of fibroblast growth factor receptor-1, which stimulates extracellular signal-regulated kinase1/2 activity through long-term p38 mitogen-activated protein kinase activation [Olivier *et al.*, 2008]. However, there is no investigation on antio-steoporosis potential of this type of compounds.

The process of bone remodeling (resorted and rebuilt) is controlled by a balance of bone formation and bone resorption. Excessive bone resorption that overcomes bone formation results in bone abnormalities such as osteoporosis, which is characterized by a reduction in bone mass and a higher incidence of bone fractures [Weinreb *et al.*, 1989]. MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain their capacity to differentiate into osteoblasts, and may provide very useful information about the effect of phytochemicals on the differentiation of osteoblasts. During differentiation, osteoblast exhibits various characteristics in time-dependent manner: increase in alkaline phosphate (ALP) activity followed by extracellular matrix (ECM synthesis and result in mineralization. Therefore, ALP activity, collagen synthesis and mineralization are major osteoblast differentiation markers. And *in vitro* studies, when a phytochemical shown increase the growth of MC3T3-E1 cell and also significantly increase ALP activity, collagen synthesis and mineralization that would be concluded it stimulates proliferation and differentiation of osteoblast MC3T3-E1 cell, and hence increase bone formation.

The first synthesis of indirubin-3'-oxime was accomplished in 1913 by Farbwerke using condensation reaction between indirubin and hydroxyl amine in pyridine solution [Eisenbrand, 2006]. In one of previous papers, we reported the synthesis of indirubin-3'-oxime

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and acetylated derivatives of indirubin, the red pigment produced from *Strobilanthes cusia* leaves [Cuong *et al.*, 2010]. In this paper we report the semi-synthesis of two other new indirubin-3'-oxime derivatives and their effects of on the development of bone cells.

Materials and Methods

General experimental procedures. NMR spectroscopy was recorded by AVANCE 500 MHz (Bruker, Karlsruhe, Germany) instrument. Electrospray ionization mass spectrometry (ESI-MS) was measured by Agilent 1100 Series LC/MSD Trap SL (Agilent Technologies, Waldbronn, Germany). Indirubin-3'-oxime was synthesized from indirubin which was obtained from *Strobilanthes cusia* leaves [Cuong *et al.*, 2010].

Synthesis of indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime]. A mixture of indirubin-3'-oxime (83 mg, 0.3 mmol), anhydrous N,N'-dimethylformamide (DMF, 6 mL), 1,3-dibromopropane (120 μL, 0.9 mmol) and triethyl amine as catalyst (120 μL, 0.9 mmol) was stirred at room temperature for 48 h. Then the solvent was removed under low pressure and the organic products were washed with distilled water and dried to yield a crude product. The crude product was chromatographed on silica gel, eluting with CH_2Cl_2:MeOH (150:1, v/v) to give pure product **2** (74 mg, 62%) as red solid.

(*2'Z,3'E*)-Indirubin-3'-oxime (1): Red solid, $C_{16}H_{11}N_3O_2$; ESI-MS: 278 $[M+H]^+$, 276 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): 13.46 (1H, s, OH), 11.73 (1H, s, H-1'), 10.70 (1H, s, H-1), 8.65 (1H, d, *J*=7.5 Hz, H-4), 8.24 (1H, d, *J*=8.0 Hz, H-4'), 7.39 (1H, m, H-6'), 7.39 (1H, m, H-7'), 7.13 (1H, dt, *J*=1.0; 7.5 Hz, H-6), 7.03 (1H, m, H-5), 6.95 (1H, dt, *J*=1.0; 7.5 Hz, H-5), 6.90 (1H, d, *J*=8.0 Hz, H-7); ^{13}C-NMR (125MHz, DMSO-d_6): 170.91 (C-2), 151.28 (C-3'), 145.22 (C-2'), 144.82 (C-7'a), 138.29 (C-7a), 131.97 (C-6'), 127.92 (C-4'), 125.86 (C-6), 122.94 (C-4), 122.63 (C-3a), 121.39 (C-5'), 120.31 (C-5), 116.48 (C-3'a), 111.45 (C-7'), 108.78 (C-7), 98.88 (C-3).

(*2'Z,3'E*)-Indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime] (2): Yield 62%, red solid, $C_{19}H_{16}N_3O_2Br$; ESI-MS: 398 $[M+H]^+$ (^{79}Br), 400 $[M+2+H]^+$ (^{81}Br); ^{1}H-NMR (500 MHz, DMSO-d_6): 11.67 (1H, s, H-1'), 10.74 (1H, s, H-1), 8.59 (1H, d, *J*=7.8 Hz, H-4), 8.11 (1H, d, *J*=7.5 Hz, H-4'), 7.44 (1H, dt, *J*=7.5; 1.0 Hz, H-6'), 7.39 (1H, d, *J*=7.5 Hz, H-7'), 7.14 (1H, dt, *J*=7.5, 1.0 Hz, H-6), 7.00 (2H, m, H-5, H-5'), 6.89 (1H, d, *J*=7.5 Hz, H-7), 4.71 (2H, t, *J*=6.5 Hz, O$\underline{CH_2}$), 3.70 (2H, t, *J*=6.5 Hz, $\underline{CH_2}$Br), 2.44 (2H, qui, *J*=6.5 Hz, $CH_2\underline{CH_2}CH_2$); ^{13}C-NMR (125 MHz, DMSO-d_6): 170.84 (C-2), 151.56 (C-3'), 145.53 (C-2'), 143.77 (C-7'a), 138.69 (C-7a), 132.89 (C-6'), 128.30 (C-4'), 126.43 (C-6), 123.24 (C-4), 122.25 (C-3a), 121.46 (C-5'), 120.64 (C-5), 116.10 (C-3'a), 111.73 (C-7'), 108.97 (C-7), 100.28 (C-3), 74.23 (O$\underline{CH_2}$), 31.81 ($CH_2\underline{CH_2}CH_2$), 30.96 ($\underline{CH_2}$Br).

Synthesis of indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime]. A mixture of indirubin-3'-oxime (97 mg, 0.35 mmol), DMF (6 mL), methyl bromoacetate (90 μL, 19.75 mmol) and triethyl amine as catalyst (150 μL, 11.25 mmol) was stirred at room temperature for 2 h. Then water was added to the solution and filtered out to obtain a crude solid. The crude product was chromatographed on silica gel, eluting with dichloromethane to give pure product **3** (87 mg, 71%) as red solid.

(*2'Z,3'E*)-Indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime] (3): Yield 71%, red solid, $C_{19}H_{15}N_3O_4$; ESI-MS: 350 $[M+H]^+$, 348 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): 11.64 (1H, s, H-1'), 10.77 (1H, s, H-1), 8.41 (1H, d, *J*=7.5 Hz, H-4), 8.20 (1H, d, *J*=7.5 Hz, H-4'), 7.47 (1H, dt, *J*=7.5, 1.0 Hz, H-6'), 7.42 (1H, d, *J*=8.0 Hz, H-7'), 7.16 (1H, dt, *J*=7.5, 1.0 Hz, H-6), 7.06 (1H, dt, *J*=7.5 Hz, 1.0 Hz, H-5'), 6.93 (2H, m, H-5, H-7), 5.25 (2H, s, O$\underline{CH_2}$), 3.77 (3H, s, O$\underline{CH_3}$); ^{13}C-NMR (125 MHz, DMSO-d_6): 170.88 (C-2), 169.43 ($CH_2\underline{C}OO$), 152.45 (C-3'), 145.86 (C-2'), 143.42 (C-7'a), 138.94 (C-7a), 133.42 (C-6'), 128.89 (C-4'), 126.84 (C-6), 123.43 (C-4), 122.14 (C-3a), 121.60 (C-5'), 120.70 (C-5), 116.08 (C-3'a), 111.94 (C-7'), 109.18 (C-7), 100.99 (C-3), 72.46 (O$\underline{CH_2}$), 52.06 (O$\underline{CH_3}$).

Cell culture and materials. Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GibcoBRL, Grand Island, NY). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. When cells reached confluence, cells were subcultured using 0.02% EDTA-0.05% trypsin solution.

Cell viability. Cells were suspended in medium supplemented with 10% FBS, and cell suspension containing 5×10^3 cells was added to the individual wells of 48-well microplates. The plates were incubated at 37°C in a CO_2 incubator for 48 h. After discarding the culture medium and washing the cells with phosphate-buffered saline (PBS), serum-free medium containing 0.3% bovine serum albumin (BSA) and compounds at appropriate concentrations was added to the cell culture and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h. Surviving cells was counted by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. MTT 20 μL in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/mL), was added to each well, and

the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Alkaline phosphatase activity. The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid, to initiate differentiation. The medium was changed every 2-3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 days. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100, and the lysate centrifuged at 14,000×g for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Asan Co., Hwaseong, Korea).

Collagen content. The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. The medium was changed every 2-3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and compounds for 2 days. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Collagen content was quantified by Sirius Red-based colorimetric assay. Cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm.

Calcium deposition assay. The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. After 12 days, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 days. On harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinum chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.

Statistics. The results are expressed as the mean±SEM (n=5). Statistical analysis was performed using a one-way ANOVA (p<0.05) with the SAS statistical software.

Results and Discussion

The synthesis of two new indirubin-3'-oxime derivatives including indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime] (**2**) and indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime] (**3**) (Fig. 1) is carried out by a substitution reaction between indirubin-3'-oxime and different halogen derivatives in N,N'-dimethylformamide. The reaction was performed at room temperature for 48 hr in the presence of Et_3N as base catalyst. The products were then purified by column

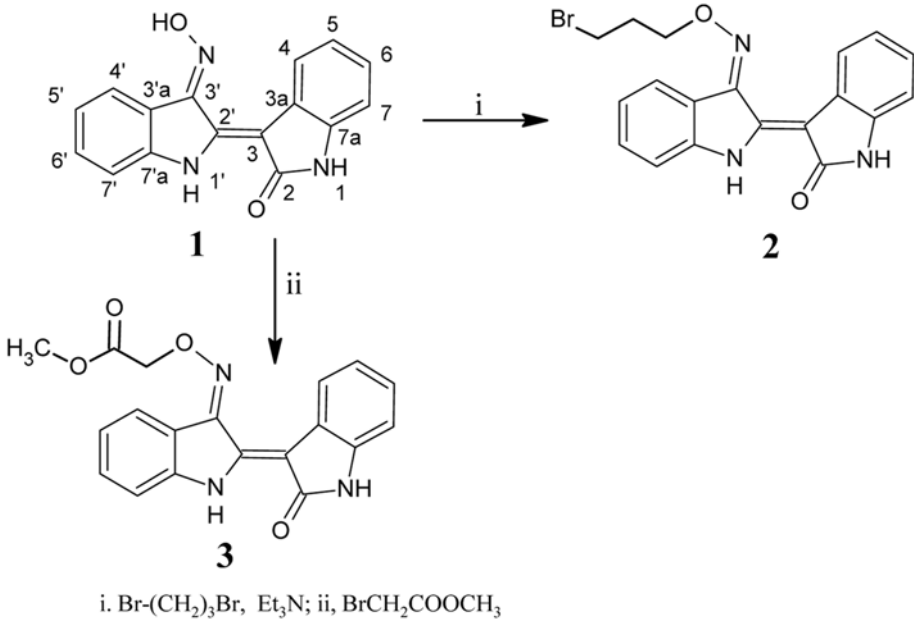

Fig. 1. Synthesis of indirubin-3'-oxime derivatives.

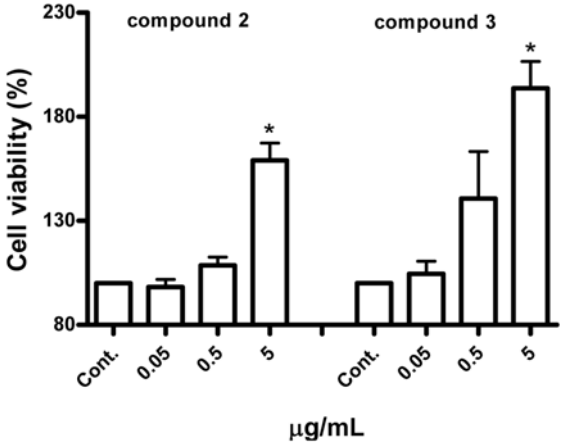

Fig. 2. Effect of compounds 2 and 3 on the viability of MC3T3-E1 cells.

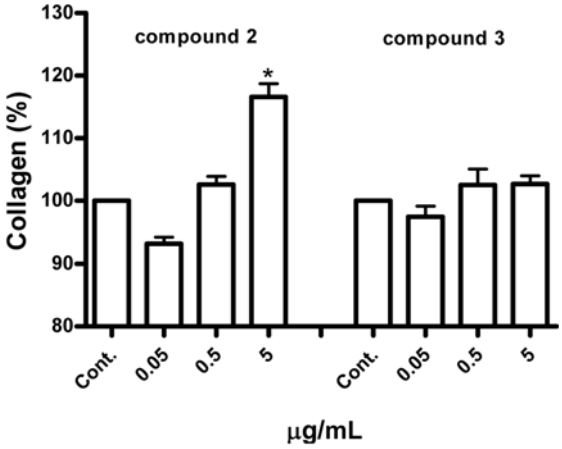

Fig. 3. Effect of compounds 2 and 3 on the collagen synthesis of MC3T3-E1 cells.

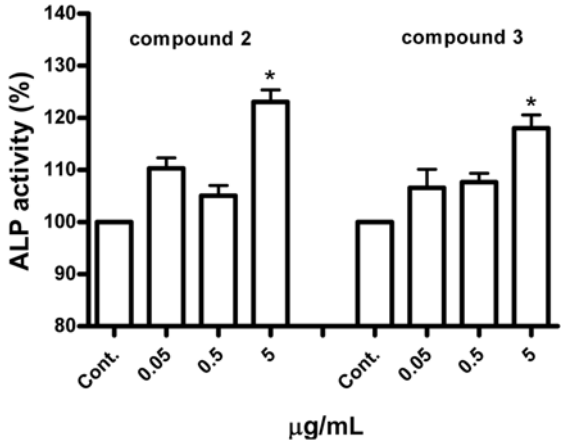

Fig. 4. Effect of compounds 2 and 3 on the alkaline phosphatase activity of MC3T3 E1 cells.

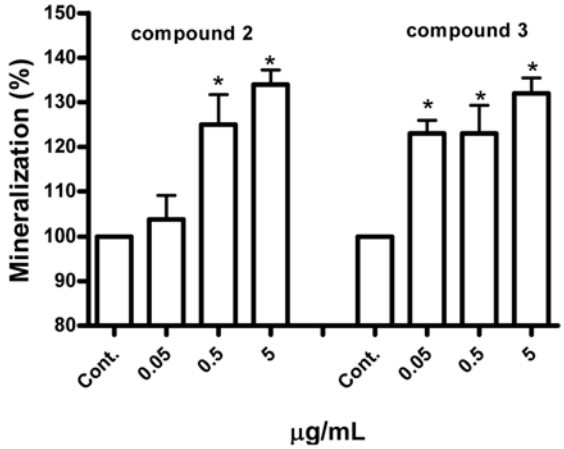

Fig. 5. Effect of compounds 2 and 3 on the mineralization of MC3T3-E1 cells.

chromatography and their structures were determined by NMR and ESI-MS. In the ^{1}H-NMR spectrum of compound **2**, there were three more signals in the up field (δ 4.71, 2H, triplet, J=6.5 Hz; δ 3.70, 2H, triplet, J=6.5 Hz and δ 2.44, 2H, quintet, J=6.5 Hz) as compared to those of the starting material, indirubin-3'-oxime, indicating that the reaction has occurred. The structure of derivatives **2** and **3** was determined and confirmed by the combination of NMR and ESI-MS methods.

The effect on the function of osteoblasts was investigated using pre-osteoblastic target cell line, MC3T3-E1, as an *in vitro* model for osteoblast differentiation. MC3T3-E1 cell growth was significantly (p<0.05) elevated by presence of **2** and **3** at concentration of 5 μg/mL (Fig. 2). Since both of these compounds significantly increased osteoblast growth, the effect of them on collagen synthesis was investigated using Sirius Red-based colorimetric assay. The experimental data showed that compound **2** significantly increased collagen synthesis at the concentration of 5 μg/mL while compound **3** had no effect on the collagen content of MC3T3-E1 cells (Fig. 3). The effects of compounds **2** and **3** on osteoblast differentiation was further assessed by measuring the ALP activity, which is one of the major osteoblast differentiation markers. Both compounds significantly increased the ALP activity of osteoblastic MC3T3-E1 cells at concentration of 5 μg/mL (Fig. 4). Next, the effects of compounds **2** and **3** on mineralization, another important process in differentiation, were evaluated by measuring the calcium deposition by Alizarin Red staining. In consistentcy with ALP activity, both compounds showed significantly stimulatory effect on mineralization (Fig. 5). Our results demonstrated that **2** and **3** increased the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

In conclusion, two new derivatives of indirubin-3'-oxime, indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime] and indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime] were

synthesized by substitution reaction with different halogen derivatives using triethylamine as a base catalyst. The effects of these products to bone cell development was investigated for the first time. Our data indicated that indirubin-3'-[*O*-(3-bromoprop-1-yl)-oxime] and indirubin-3'-[*O*-(methoxycarbonylmethyl)-oxime] possess stimulative effects on MC3T3-E1 cell growth and differentiation.

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