Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 1125–1132
Bioorganic & Medicinal Chemistry

Indeno[1,2-*c*]isoquinolines as enhancing agents on all-*trans* retinoic acid-mediated differentiation of human myeloid leukemia cells

Seung Hyun Kim,[a] Sang Mi Oh,[b] Ju Han Song,[a] Daeho Cho,[c] Quynh Manh Le,[b] Suh–Hee Lee,[b] Won-Jea Cho[b] and Tae Sung Kim[a,*]

[a]*School of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea*
[b]*College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju, Republic of Korea*
[c]*Department of Life Sciences, Sookmyung Women's University, Seoul, Republic of Korea*

Received 11 September 2007; revised 25 October 2007; accepted 26 October 2007
Available online 30 October 2007

Abstract—Induction of differentiation is a new and promising approach to cancer therapy, well illustrated by the treatment of acute myeloid leukemia with all-*trans* retinoic acid (ATRA). Using combination of ATRA and chemotherapy, adverse effects such as retinoic acid syndrome have decreased, and long-term survival has improved. In this study, we demonstrated that the indeno[1,2-*c*]isoquinolines markedly enhanced differentiation of human myeloid leukemia HL-60 and NB4 cells when simultaneously combined with a low dose of ATRA. Of the tested compounds, 6-(4-methoxybenzyl)-2,11-dimethyl-6*H*,11*H*-indeno[1,2-*c*]isoquinolin-5-one (IIQ-16), an indeno[1,2-*c*]isoquinoline derivative, showed the highest differentiation-enhancing activity via a pathway involved with protein kinase C, extracellular signal-regulated kinase, and c-Jun N-terminal kinase. The ability to enhance the differentiation potential of ATRA by IIQ-16 may improve outcomes in the therapy of acute promyelocytic leukemia.

1. Introduction

Leukemia can eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cyto-destructive agents.[1] ATRA is able to induce a terminal differentiation of leukemic cell lines, such as HL-60, NB4, and U937 cells, and of short-term culture APL cells from humans.[2] Moreover, ATRA is able to induce complete remission (CR) in almost all patients with APL through in vivo differentiation of APL blasts.[3] Although ATRA can bring about CR of APL, treatment with ATRA alone showed severe side effects, including ATRA syndrome and the induction of a secondary resistance to ATRA.[4] ATRA syndrome combines fever, respiratory distress, weight gain, pulmonary infiltrates, pericardial effusions, and hypotension. Therefore, current attempts to overcome this problem focus on the combination therapy with non-toxic concentrations of ATRA and compounds that have different mechanisms of action, such as paclitaxel, curcumin, and silibinin.[5–7]

The indenoisoquinolines are a class of cytotoxic molecules that have been demonstrated to inhibit topoisomerase I enzyme by intercalating between DNA bases at the enzyme's cleavage site. This mechanism of action is identical to the natural product camptothecin[8] and its clinically useful derivative topotecan.[9] Various topoisomerase I inhibitors such as 10-hydroxycamptothecin and camptothecin are known to induce and/or enhance the differentiation of leukemia cells.[10,11] Previously we synthesized a series of indeno[1,2-*c*]isoquinoline derivatives, which were demonstrated to exert inhibitory effects on topoisomerase I activity.[12]

In this report, we investigated enhancing effects of the indeno[1,2-*c*]isoquinoline derivatives on cellular differentiation of human myeloid leukemia HL-60 cells, in combinations of a low dose of ATRA. Human myeloid leukemia HL-60 cell culture has been employed as an excellent model system for studying cellular differentiation in vitro. HL-60 cells are differentiated into a granulocytic lineage when treated with ATRA.[2,13]

Keywords: Indenoisoquinoline; Differentiation; Leukemia; All-*trans* retinoic acid.
* Corresponding author. Tel.: +82 2 3290 3416; fax: +82 2 3290 3921; e-mail: tskim@korea.ac.kr

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.10.086

2. Results

In order to determine whether the indeno[1,2-*c*]isoquinoline derivatives exerted any effects on the differentiation of leukemia cells, HL-60 cells were seeded at a density of 2×10^5 cells/ml and treated with either medium alone, or treated for 72 h with 200 nM of each of the indeno[1,2-*c*]isoquinoline derivatives, in the absence or presence of a low (nontoxic) dose of ATRA (50 nM). As shown in Table 1, treatment with the indenoisoquinoline derivatives induced little increase in the differentiation of the HL-60 cells, by approximately 0.83–1.75%. Importantly, some of the indeno[1,2-*c*]isoquinoline derivatives significantly enhanced cell differentiation when combined with 50 nM ATRA, which by itself caused a relatively low level of differentiation. Among the tested derivatives, 6-(4-methoxybenzyl)-2,11-dimethyl-6*H*,11*H*-indeno[1,2-*c*]isoquinolin-5-one (IIQ-16, Fig. 1B) profoundly potentiated cell differentiation in a concentration-dependent manner (Fig. 2A). Treatment of the cells with more than 100 nM of IIQ-16 also inhibited cell proliferation in a dose-dependent manner (Fig. 2B). IIQ-16 also enhanced cell differentiation and inhibited cell proliferation of human myeloid NB4 cells in a dose-dependent manner, when combined with 50 nM ATRA (Fig. 2). For all treatments, cell viability was in excess of 98% throughout the incubation period, as shown by the results of the Trypan blue exclusion assay (data not shown).

To further determine the cell differentiation enhanced by the indeno[1,2-*c*]isoquinoline derivatives, the morphologic phenotypes and the expression of cell surface antigens on HL-60 cells were analyzed. As shown in Figure 3A, Giemsa-stained undifferentiated control HL-60 cells were predominantly myelocytes with round and regular cell margins, and large nuclei, suggesting that the cells were highly active in DNA synthesis and were rapidly proliferating. The cells treated with 250 nM IIQ-16 or 50 nM ATRA alone exhibited relatively small changes in cell morphology such as irregular cell margins. Combined treatment of HL-60 cells with 50 nM ATRA plus 250 nM IIQ-16 resulted in significantly decreased cell size, denser chromatin, and an increased cytoplasm to nuclear ratio, which suggested less DNA synthesis. As shown in Figure 3A, some cells showed a multilobed nucleus, which is a sign of cell differentiation into a granulocytic lineage.

Cytofluorometric analysis was also performed to determine the expression of specific surface antigens on HL-60 cells. CD11b (Mac-1) is expressed on activated monocytes, granulocytes, lymphocytes, and a subset of NK cells. HL-60 leukemia cells express a cell surface marker, CD11b, when differentiated into granulocytes by high concentrations of ATRA.[14] As shown in Figure 3B. indenoisoquinoline IIQ-16 significantly increased the number of CD11b-positive cells when combined with 50 nM ATRA, confirming that the indenoisoquinoline potentiated ATRA-induced HL-60 cell differentiation.

To determine an action mechanism by which the indenoisoquinoline derivative potentiates ATRA-induced HL-60 cell differentiation, HL-60 cells were treated with specific inhibitors for extracellular signal-regulated kinase (ERK) (PD 98059), c-Jun N-terminal kinase (JNK) (SP 600125), PKC (protein kinase C) (GF 109203X, chelerythrine), and phosphatidylinositol 3-kinase (PI3-K) (Wortmannin, LY 294002), in the presence of indenoisoquinoline IIQ-16 alone or in combination with 50 nM ATRA. Afterward, the degree of cellular differentiation was assessed by nitroblue tetrazolium reduction assay. Activation of PI3-K, PKC, and MAPK has been known to be involved in the differentiation of leukemia cells.[15] As shown in Figure 4, inhibitors for ERK, JNK, and PKC significantly suppressed HL-60 cell differentiation treated with IIQ-16 in combination with ATRA in a dose-dependent manner. In contrast, both PI3-K inhibitors had no effects on HL-60 cell differentiation enhanced by IIQ-16 in combination with ATRA.

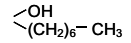
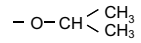
To further characterize involvement of PKC, ERK, and JNK in the cell differentiation induced by ATRA and IIQ-16, the protein levels of total PKC, pERK, and pJNK were determined by Western blot analysis. As shown in Figure 5, the levels of total PKC, pERK, and pJNK were increased in the cells treated with IIQ-16 and ATRA, compared with the levels in those treated with single treatments of either IIQ-16 or ATRA. These results demonstrate that indeno[1,2-*c*]isoquinolines potentiate ATRA-induced HL-60 cell differentiation via a PKC/ERK/JNK pathway.

3. Discussion

In this study, we demonstrated that indeno[1,2-*c*]isoquinoline derivatives can potentiate ATRA-induced differentiation in HL-60 promyelocytic leukemia cells, which are widely used as a model system for studies of differentiation. HL-60 cells were found to synergistically differentiate into granulocytes when treated with the indeno[1,2-*c*]isoquinoline derivative IIQ-16 in combination with ATRA. Many previous studies have uncovered some chemical combinations which exert either an additive or a synergistic effect on the differentiation of HL-60 cells. These combinations include retinoic acid with sodium butyrate, dimethylsulfoxide, hexamethylene bisacetamide, or thalidomide.[16]

The mechanism by which the indeno[1,2-*c*]isoquinoline derivative IIQ-16 potentiates ATRA-induced HL-60 cell differentiation remains to be adequately clarified. ATRA is believed to mediate biological responses, including cell differentiation, as a consequence of their interaction with nuclear receptors to regulate gene transcription and with a putative cell membrane receptor to generate rapid non-genomic effects including the opening of voltage-gated calcium and chloride channels,[17] and the activation of PI3-K, PKC, and MAPK.[15] In our study, inhibitors of PKC, ERK, and JNK significantly inhibited the enhancing effects on HL-60 cell differentiation induced by IIQ-16 in combination with ATRA. This finding strongly suggests that the potentiation of cell differentiation by the indenoisoquinoline derivative in combination with ATRA may occur via a PKC/ERK/JNK-mediated signaling pathway.

Table 1. Effects of the indeno[1,2-*c*]isoquinoline derivatives on HL-60 cell differentiation

	R1	R2	R3	Differentiation (%)	
				IIQ alone	ATRA + IIQ
Medium alone				0.83 ± 0.38	—
ATRA alone				—	27.00 ± 2.75
IIQ-1	–H	–H	–Me	1.50 ± 0.87	28.33 ± 13.99
IIQ-2	–H	–H		1.33 ± 1.23	30.67 ± 5.39
IIQ-3	–H	–Me	–Me	1.25 ± 0.66	46.75 ± 6.74**
IIQ-4	–H	–Me		1.42 ± 0.29	38.58 ± 0.88**
IIQ-5	–H	–OH	–Me	1.08 ± 0.52	23.42 ± 3.40
IIQ-6	–H	–OH		0.83 ± 1.01	29.42 ± 6.03
IIQ-7	–H	–OMe		0.92 ± 0.14	38.92 ± 9.00
IIQ-8	–H			1.17 ± 0.14	37.83 ± 3.06
IIQ-9	–H	–OEt	–Me	1.75 ± 0.43	33.08 ± 6.03
IIQ-10	–H	–$OCOCH_3$		1.58 ± 0.80	38.83 ± 6.03*
IIQ-11	–H			1.42 ± 0.29	36.50 ± 3.28*
IIQ-12	–H	–$(CH_2)_6$–CH_3		0.83 ± 0.14	40.75 ± 6.19*
IIQ-13	–Me	–H	–Me	1.25 ± 0.66	24.08 ± 5.92
IIQ-14	–Me	–H		1.25 ± 0.25	36.33 ± 0.76*
IIQ-15	–Me	–Me	–Me	1.75 ± 0.50	58.75 ± 6.95
IIQ-16	–Me	–Me		1.25 ± 1.00	76.08 ± 10.16**
IIQ-17	–Me	–OH	–Me	0.83 ± 0.29	28.50 ± 2.29
IIQ-18	–Me	–OH		1.33 ± 0.14	28.75 ± 1.15
IIQ-19	–Me	–OMe	–Me	1.17 ± 0.72	28.92 ± 11.00
IIQ-20	–Me	–OMe		1.42 ± 0.14	32.25 ± 6.67
IIQ-21	–Me	–OEt	–Me	1.42 ± 0.38	32.75 ± 6.43
IIQ-22	–Me	–OEt		0.92 ± 0.58	31.42 ± 7.52
IIQ-23	–Me	–OPr	–Me	1.33 ± 0.63	34.75 ± 8.89
IIQ-24	–Me	–OPr		1.25 ± 0.43	35.33 ± 8.71
IIQ-25	–Me			1.00 ± 0.25	35.92 ± 10.37
IIQ-26	–Me	–OBu	–Me	1.92 ± 0.29	34.58 ± 5.63
IIQ-27	–Me	–OBu		0.92 ± 0.14	30.00 ± 8.23
IIQ-28	–Me	–$O(CH_2)_4CH_3$	–Me	1.50 ± 0.66	28.42 ± 7.73

(*continued on next page*)

Table 1 (*continued*)

	R1	R2	R3	Differentiation (%)	
				IIQ alone	ATRA + IIQ
IIQ-29	–Me	$-O(CH_2)_4CH_3$		1.00 ± 0.25	35.08 ± 3.79*
IIQ-30	–Me		–Me	1.50 ± 0.87	33.00 ± 5.17
IIQ-31	–Me			1.17 ± 0.52	32.17 ± 6.33

HL-60 cells were treated for 72 h with medium or 50 nM ATRA alone, or with 50 nM ATRA in combination with 200 nM of the indeno[1,2-*c*]isoquinoline derivatives. The cell differentiation was assessed by the NBT assay. Each value represents the mean ± SE mean ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$, relative to a group treated with ATRA alone.

In addition, the methyl substitution at the R_1 and R_2 positions of indeno[1,2-*c*]isoquinoline (IIQ-15, -16) afforded higher enhancing effect on HL-60 cell differentiation, and additional methoxybenzyl substitution at the R_3 position (IIQ-16) exhibited the most profound synergistic effects on HL-60 cell differentiation when combined with ATRA. This indicates that the substitution of indeno[1,2-*c*]isoquinoline at R_1, R_2, and R_3 positions might affect the activity of signaling molecule(s) involved in the enhancement of HL-60 cell differentiation. Previously we reported that indeno[1,2-*c*]isoquinoline and its analogs were demonstrated to inhibit topoisomerase I activity.[12] Among the synthesized compounds, IIQ-22, IIQ-24, and IIQ-27 showed potent topoisomerase I inhibition activity. In contrast, IIQ-16 exhibited weak inhibitory activity on topoisomerase I, suggesting that the enhancing effects of the indeno[1,2-*c*]isoquinoline derivatives on HL-60 cell differentiation observed in this study were not correlated with the inhibitory effects on topoisomerase I activity.

In conclusion, the indeno[1,2-*c*]isoquinoline derivative IIQ-16 potentiates ATRA-induced HL-60 cell differentiation via a PKC/ERK/JNK signaling pathway. These findings imply that the indeno[1,2-*c*]isoquinoline derivatives may prove useful in the treatment of leukemic diseases.

4. Experimental

4.1. Materials

All-*trans* retinoic acid, phorbol 12-myristate 13-acetate (PMA), 2-[4-morpholinyl]-8phenyl-1[4*H*]-benzopyran-4-one (LY 294002), and 3-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (Wortmannin), ethanol, Giemsa staining solution, methanol-free paraformaldehyde, and all other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Chelerythrine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from the Tocris Cookson Ltd. (UK). SP 600125 was purchased from Calbiochem (San Diego, CA, USA). A stock solution of 1 mM ATRA was dissolved in dimethylsulfoxide. The indenoisoquinoline derivatives were dissolved in dimethylsulfoxide to make a stock solution of 20 mM. The solutions were diluted at least 1000-fold in the growth medium such that the final concentration of ethanol or dimethylsulfoxide had no effect on the differentiation and proliferation of HL-60 cells. All manipulations were performed in subdued light. The cell lines HL-60 and NB4 were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA).

4.2. Synthesis of indeno[1,2-*c*]isoquinoline derivatives

A number of indeno[1,2-*c*]isoquinolines with different substituted groups at three positions (Fig. 1A) were synthesized as previously described.[12] The four compounds **2**, **IIQ-8**, **IIQ-11**, and **IIQ-12** were newly prepared as shown in Scheme 1. The commercially available starting lactone **1** was treated with $PMBNH_2$ to give the indenoisoquinoline **2** in 95% yield. Indenoisoquinoline **2** was reacted with MeMgBr or *n*-hexylMgBr to afford the corresponding alcohols **IIQ-8** and **IIQ-11**, respectively, in good yield. Dehydroxylation of **IIQ-11** was performed with TMSCl, NaI, and MeCN to provide the desired **IIQ-12** in 84% yield.

4.3. General remarks

Melting points were determined by using the capillary method on Electrothermal IA9200 digital melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) data for ^1H NMR were taken on Bruker AMX-R300 and are reported in ppm, downfield from the peak of tetramethylsilane as an internal standard. The data are reported as follows: chemical shift, number of proton, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet). IR spectra were recorded on Nicolet 520P using KBr pellets. Mass spectra were obtained on Platform II of Micromas applying the electron-impact (EI) method. Column chromatography was performed on Merck silica gel 60 (70–230 mesh). TLC was carried out using plates coated with silica gel 60 F254 purchased from Merck. Chemical reagents were purchased from Aldrich Chemical Co. and used without further purification. Solvents were distilled prior to use: THF was distilled from sodium/benzophenone.

4.3.1. 6-(4–Methoxybenzyl)-6*H*-indeno[1,2-*c*]isoquinolin-5,11-dione (2). *p*-Methoxybenzyl amine (69 mg, 0.5 mmol) was added to a stirred solution of benz[*d*]indeno[1,2-*b*] pyran-5,11-dione (100 mg, 0.4 mmol) in CH_2Cl_2 (10 mL) at room temperature. The bright orange mixture

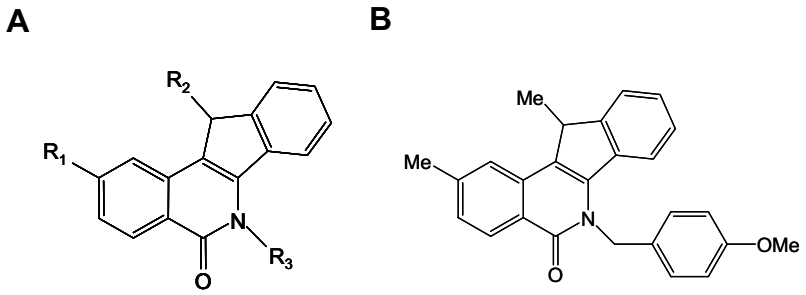


Figure 1. The structures of indeno[1,2-*c*]isoquinolines (A) and IIQ-16 (B).

was stirred overnight and the solvent was removed in vacuo to give the residue which was purified by column chromatography on silica gel with Hexane/Ethyl acetate (4:1) to afford the compound **2** (140 mg, 95%) as a reddish solid. Mp 213–214 °C. IR (cm^{-1}): 1700 (C═O), 1660 (amide). ^1H NMR ($CDCl_3$): δ 8.76 (d, J = 8.3 Hz, 1H), 8.38 (d, J = 9.0 Hz, 1H), 7.79–6.86 (m, 10H), 5.74 (s, 2H), 3.77 (s, 3H). EIMS *m/z* (%): 367 (M^+, 85%).

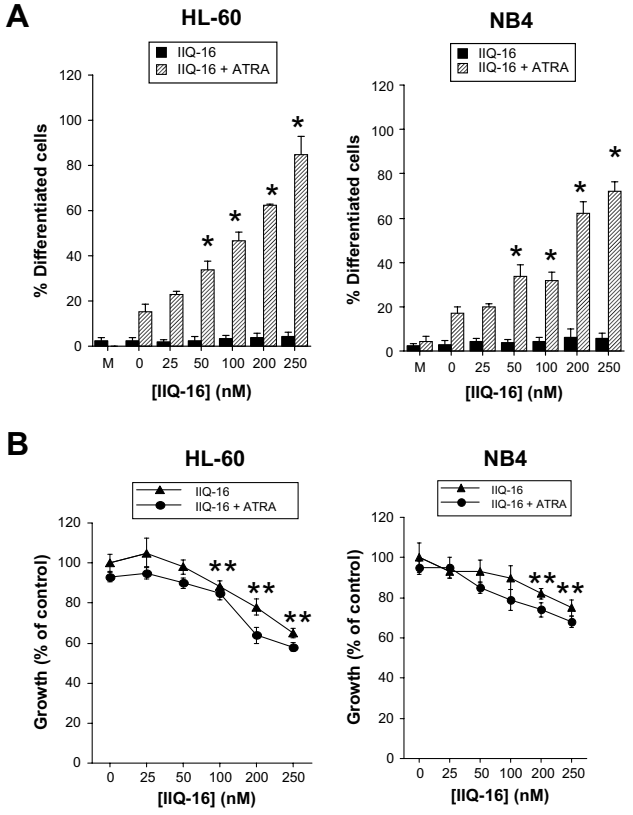


Figure 2. Effects of an indenoisoquinoline IIQ-16 on cell proliferation and differentiation of human myeloid leukemia cells. Human myeloid leukemia HL-60 and NB4 cells were, respectively, treated for 72 h with ATRA (50 nM) or IIQ-16 (0–250 nM) alone, or with ATRA in combination with various concentrations of IIQ-16 (0–250 nM). The cellular differentiation was assessed by the NBT assay (A) and the cell proliferation was determined by the MTT assay (B). Each value represents the mean ± SE mean (n = 3). $^*P < 0.001$, relative to an untreated group. $^{**}P < 0.001$, relative to a group treated with ATRA alone.

4.3.2. 11–Hydroxy-6-(4-methoxybenzyl)-11-methyl-6*H*, 11*H*-indeno[1,2-*c*]isoquinolin-5-one (IIQ-8). To a stirred solution of 6-(*4*-methoxybenzyl)-6*H*-indeno[1,2-*c*] isoquinolin-5,11-dione **2** (100 mg, 0.27 mmol) in THF (10 mL) at 0 °C, CH_3MgBr 1 M in butyl ether (0.6 mL, 0.54 mmol) was added and the reaction mixture was stirred while the temperature was increased to room temperature. After additional stirring for 12 h, the mixture was quenched by water and the reaction mixture was filtered through Celite. The CH_2Cl_2 layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness. The residue was purified by column chromatography on silica gel with *n*-hexane/ethyl acetate (2:1) to yield **IIQ-8** (82 mg, 79%) as a yellow solid. Mp 172–177 °C. IR (cm^{-1}): 3360 (OH), 1630 (amide). ^1H NMR ($CDCl_3$): δ 8.22–6.75 (m, 12H), 5.55 (d, J = 16.4 Hz, 1H), 5.25 (d, J = 16.4 Hz, 1H), 3.71 (s, 3H), 1.84 (s, 3H). EIMS *m/z* (%): 383 (M^+, 60%).

4.3.3. 11–Hexyl-11-hydroxy-6-(4-methoxybenzyl)-6*H*, 11*H*-indeno[1,2-*c*] isoquinolin-5-one (IIQ-11). The same procedure as described in the preparation of **IIQ-8** was used to give **IIQ-11** (98%) as a yellow solid. Mp 145–146 °C. IR (cm^{-1}): 3360 (OH), 1638 (amide). ^1H NMR ($CDCl_3$): δ 8.20 (d, J = 8.0 Hz, 1H), 7.72–6.67 (m, 11H), 5.54 (d, J = 16.5 Hz, 1H), 4.69 (m, 1H), 4.10 (m, 1H), 3.66 (s, 3H), 2.44–2.33 (m, 1H), 2.31–2.03 (m, 1H), 1.03–0.97 (m, 6H), 0.69 (t, J = 6.9 Hz, 3H), 0.57–0.46 (m, 2H). EIMS *m/z* (%): 453 (M^+, 72%).

4.3.4. 11–Hexyl-6-(4-methoxybenzyl)-6*H*,11*H*-indeno[1,2-*c*]isoquinolin-5-one (IIQ-12). To a mixture of Me_3SiCl (145 mg, 1.34 mmol), NaI (200 mg, 1.34 mmol) and CH_3CN (55 mg) was added a solution of 11–hexyl-11-hydroxy-6-(4-methoxybenzyl)-6*H*,11*H*-indeno[1,2-*c*]isoquinolin-5-one **IIQ-11** (101 mg, 0.22 mmol) in CH_2Cl_2 (2 mL). The mixture was stirred at room temperature for overnight. The reaction mixture was diluted with water and extracted with CH_2Cl_2. The organic layer was washed with water, brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel with hexane/ethyl acetate (4:1) to give the compound **IIQ-12** (81 mg, 84%) as a yellow solid. Mp 156–159 °C. IR (cm^{-1}): 1642 (amide). ^1H NMR ($CDCl_3$): δ 8.54 (d, J = 8.0 Hz, 1H), 7.76–6.80 (m,

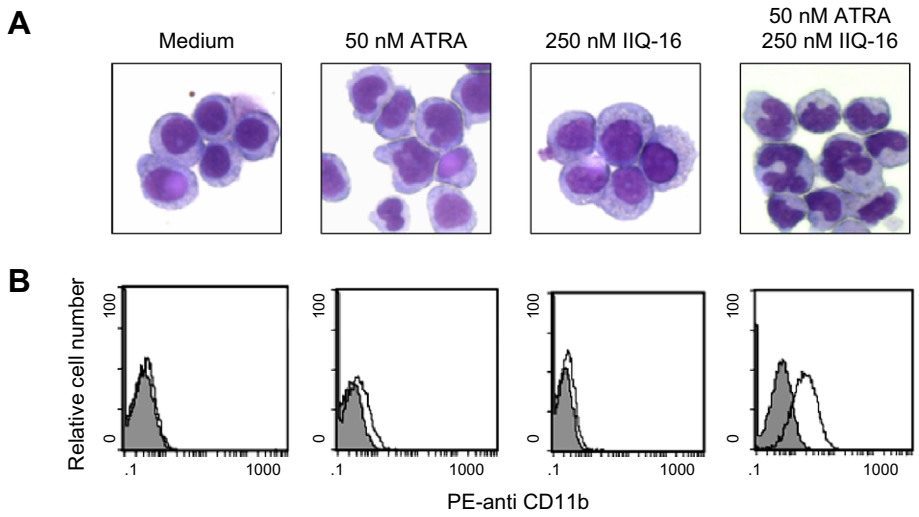


Figure 3. Morphologic and cytofluorometric analysis of HL-60 leukemia cells treated with ATRA alone or in combination with IIQ-16. (A) HL-60 cells were treated for 72 h with 50 nM ATRA or 250 nM IIQ-16, or with ATRA plus IIQ-16. The cells were assessed by morphologic analysis using Giemsa stain. (B) The cells were assessed by cytofluorometric analysis using PE-conjugated anti-CD11 mAb (*unshaded area*), or isotype control mAb (*shaded area*). The data are representative of three separate experiments.

11H), 5.81 (s, 2H), 4.13–4.10 (m, 1H), 3.73 (s, 3H), 2.28–2.22 (m, 1H), 2.18–2.13 (m, 1H), 1.16–1.05 (m, 6H), 0.98–0.82 (m, 2H), 0.77 (t, $J = 6.80$ Hz, 3H). EIMS *m/z* (%): 437 (M^+, 92%).

4.4. Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay as previously described.[18] Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. After incubation for 4 h at 37 °C, the crystals of viable cells were dissolved with 100 μl of 0.04 N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

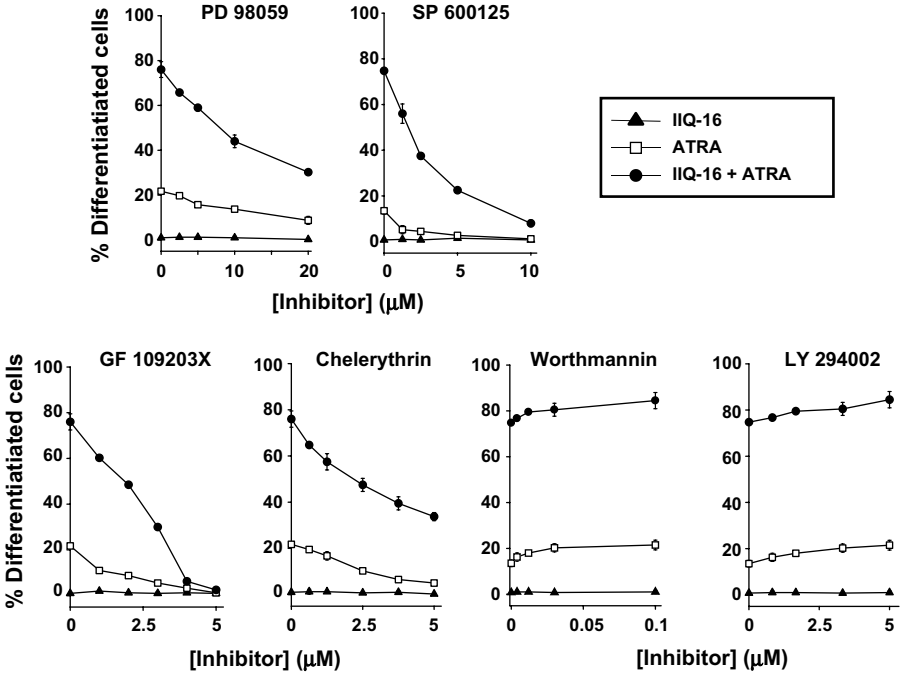


Figure 4. Effects of specific kinase inhibitors on HL-60 cell differentiation induced by 50 nM ATRA in combination with IIQ-16. HL-60 cells were treated for 40 min with various concentrations of ERK inhibitor (PD 98059), JNK inhibitor (SP 600125), PKC inhibitors (GF 109203X, chelerythrine), and PI3-K inhibitors (Wortmannin, LY 294002), followed by incubation with 50 nM ATRA or 250 nM IIQ-16, or with 50 nM ATRA plus 250 nM IIQ-16. The cellular differentiation was assessed by the NBT reduction assay. Data are presented as percentage of differentiated cells with means ± SE mean ($n = 3$).

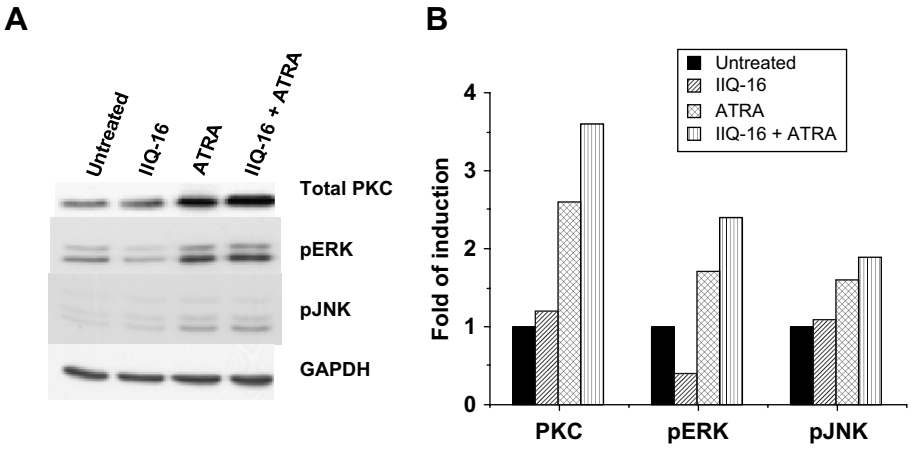
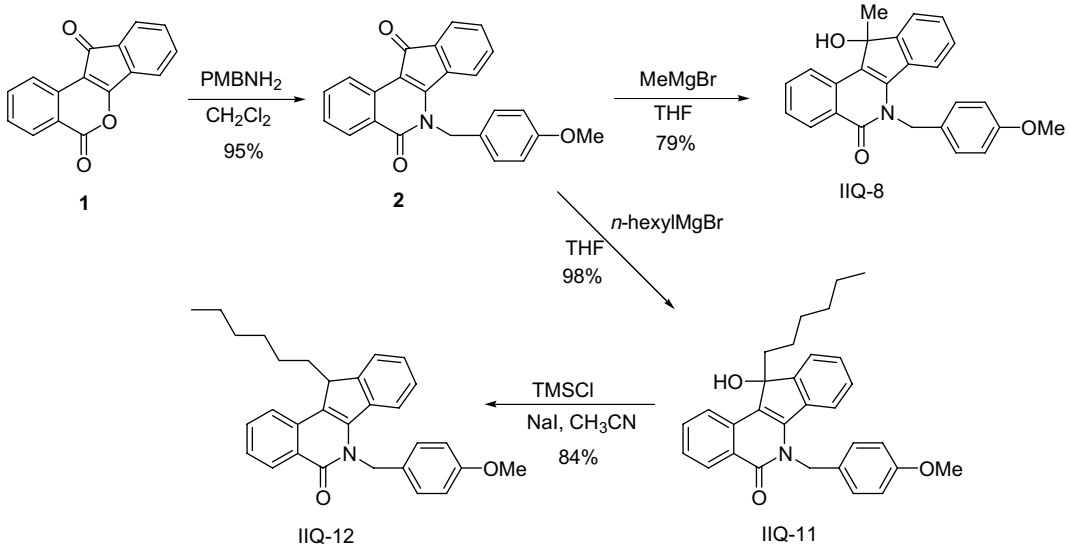


Figure 5. Involvement of PKC, JNK, and ERK in HL-60 cell differentiation induced by ATRA and IIQ-16. HL-60 cells were incubated for 24 h with 50 nM ATRA or 250 nM IIQ-16, or with ATRA plus IIQ-16, or untreated. The protein levels were determined by Western blot analysis. The band intensity of each of the treatment groups was determined via densitometric analysis and was expressed as fold of induction over untreated cells. The experiment was repeated at least twice with similar results.



Scheme 1. Synthesis of indeno[1,2-*c*]isoquinoline derivatives.

4.5. Determination of cell differentiation

Cell differentiation was assessed by the nitroblue tetrazolium reduction assay, as previously described.[19] This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in the dark. Cytospin slides were prepared and were examined for blue-black nitroblue diformazan deposits, indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

4.6. Morphologic studies

Single-cell suspensions were prepared and 2×10^5 cells were loaded into a cyto-funnel and spun at 500 rpm in a cytospin centrifuge. The slides were fixed with methanol and dried. The slides were stained with Giemsa staining solution for 20 min and rinsed in deionized water, air-dried, and observed under a microscope with a camera. The stained cells were assessed for size, regularity of the cell margin, and morphological characteristics of the nuclei.

4.7. Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic XL flow cytofluorograph equipped with a multi-parameter data acquisition and display system. Briefly, a single-cell suspension was collected from the various cultures and washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4). Afterward, phytoerythrin (PE)-conjugated anti-human CD11b monoclonal antibodies (Becton-Dickinson, San Jose,

CA, USA) were added, followed by incubation at 4 °C for 1 h. After incubation, the cells were washed with PBS and were fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was performed. Background staining was determined by staining the cells with PE-conjugated isotype control monoclonal antibodies. One-parameter fluorescence histograms were generated by analyzing at least 1×10^4 cells.

4.8. Preparation of cell lysates and Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml phenylmethanesulfonyl fluoride) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 rpm at 4 °C for 10 min. The proteins (15 μg) of the supernatants were separated using a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to the nitrocellulose membrane. The blots were probed with rabbit anti-human PKC, mouse anti-pJNK, and mouse anti-pERK, washed, and exposed to horseradish peroxidase-conjugated anti-mouse IgG2a or rabbit IgG antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

4.9. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between values for various experimental and control groups. A P value of <0.05 was considered as significant.

Acknowledgments

This study was supported by grants from the Korea Health 21 R&D Project, Ministry of Health and Welfare (01-PJ10-PG6-01GN16-0005) and the Seoul Research and Business Program (10582) to T.S. Kim, and a grant from the Korea Research Foundation (KRF-2004-C00325) to W.-J. Cho.

References and notes

1. Beere, H. M.; Hickman, J. A. *Anti-Cancer Drug Des.* **1993**, *8*, 299.
2. (a) Breitman, T. R.; Selonick, S. E.; Collins, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2936; (b) Chomienne, C.; Balitrand, N.; Cost, H.; Degos, L.; Abita, J. P. *Leuk Res.* **1986**, *10*, 1301.
3. Degos, L.; Wang, Z. Y. *Oncogene* **2001**, *20*, 7140.
4. Frankel, S. R.; Eardley, A.; Lauwers, G.; Weiss, M., ; Warrell, R. P., Jr. *Ann. Intern. Med.* **1992**, *17*, 292.
5. Hershberger, P. A.; Yu, W. D.; Modzelewski, R. A.; Rueger, R. M.; Johnson, C. S.; Trump, D. L. *Clin. Cancer Res.* **2001**, *7*, 1043.
6. Sokoloski, J. A.; Shyam, K.; Sartorelli, A. C. *Oncol. Res.* **1997**, *9*, 31.
7. Kang, S. N.; Lee, M. H.; Kim, K. M.; Cho, D.; Kim, T. S. *Biochem. Pharmacol.* **2001**, *61*, 1487.
8. Kohlhagen, G.; Paull, K.; Cshman, M.; Nagafuji, P.; Pommier, Y. *Mol. Pharmacol.* **1998**, *54*, 50.
9. Staker, B. L.; Hjerrild, K.; Feese, M. D.; Behnke, C. A.; Burgin, A. B., Jr.; Stewart, L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15387.
10. Ling, Y. H.; Tseng, M. T.; Nelson, J. A. *Differentiation* **1991**, *46*, 135.
11. Aller, P.; Rius, C.; Mata, F.; Zorrilla, A.; Cabanas, C.; Bellon, T.; Bernabeu, C. *Cancer Res.* **1992**, *52*, 1245.
12. (a) Cho, W.-J.; Le, Q. M.; Van, H. T. M.; Lee, K. Y.; Kang, B. Y.; Lee, E.-S.; Lee, S. K.; Kwon, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3531; (b) Van, H. T. M.; Le, Q. M.; Lee, E.-S.; Kwon, Y.; Kim, T. S.; Le, T. N.; Lee, S.-H.; Cho, W.-J. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5763.
13. Tanaka, H.; Abe, E.; Miyaura, C.; Shiina, Y.; Suda, T. *Biochem. Biophy. Res. Commun.* **1983**, *117*, 86.
14. Kansas, G. S.; Muirhead, M. J.; Dailey, M. O. *Blood* **1990**, *76*, 2483.
15. (a) Pan, Q.; Granger, J.; O'Connell, T. D.; Somerman, M. J.; Simpson, R. U. *Biochem. Pharmacol.* **1997**, *54*, 909; (b) Song, X.; Bishop, J. E.; Okamura, W. H.; Norman, A. W. *Endocrinology* **1998**, *139*, 457; (c) Marcinkowska, E. *Anticancer Res.* **2001**, *499*.
16. (a) Breitman, T. R.; He, R. Y. *Cancer Res.* **1990**, *50*, 6268; (b) Noguchi, T.; Shinji, C.; Kobayashi, H.; Makishima, M.; Miyachi, H.; Hashimoto, Y. *Biol. Pharm. Bull.* **2005**, *28*, 563.
17. (a) Norman, A. W.; Okamura, W. H.; Hammond, M. W.; Bishop, J. E.; Dormanen, M. C.; Bouillon, R.; van Baelen, H.; Ridall, A. L.; Daane, E.; Khoury, R.; Farach-Carson, M. C. *Mol. Endocrinol.* **1997**, *11*, 1518; (b) Zanello, L. P.; Norman, A. W. *J. Biol. Chem.* **1997**, *272*, 22617; (c) Haussler, M. R.; Whitfield, G. K.; Haussler, C. A.; Hsieh, J. C.; Thompson, P. D.; Selznick, S. H.; Dominguez, C. E.; Jurutka, P. W. *J. Bone Miner. Res.* **1998**, *13*, 325.
18. Kim, T. S.; Kang, B. Y.; Lee, M. H.; Choe, Y. K.; Hwang, S. Y. *Br. J. Pharmacol.* **2001**, *134*, 571.
19. Collins, S. J.; Ruscetti, F. W.; Gallagher, R. E.; Gallo, R. C. *J. Exp. Med.* **1979**, *149*, 969.