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Synthesis of furopyrazole analogs of 1-benzyl-3-(5-hydroxymethyl-2-furyl)indazole (YC-1) as novel anti-leukemia agents

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Abstract—As part of our continuing search for potential anticancer drug candidates in **YC-1** analogs, several 1-benzyl-3-(substituted aryl)-5-methylfuro[3,2-*c*]pyrazoles were synthesized and evaluated for their cytotoxicity against HL-60 cell line. Among these compounds, 1-benzyl-3-(5-hydroxymethyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (**1**) showed more potency than **YC-1**. Through investigation of action mechanism, it was found that compound **1** induced terminal differentiation of HL-60 cells toward granulocyte lineage and promoted HL-60 cell differentiation by regulation of Bcl-2 and c-Myc proteins. Meanwhile, compound **1** also demonstrated apoptosis-inducing effect. Such anti-leukemia mechanism of action is apparently different from that of **YC-1** which mainly works by inducing apoptosis, but not cell differentiation. Therefore, compound **1** is identified here as a new lead compound of cell differentiating agent and apoptosis inducer for further development of new anti-leukemia agents.
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1. Introduction

Previously, we have synthesized a series of 1-arylmethyl-3-arylimidazole derivatives (**A**) and found that 1-benzyl-3-(5-hydroxymethyl-2-furyl)indazole (**YC-1**) was the most promising anti-platelet agent.[1,2] Subsequent investigation of its action mechanism revealed that **YC-1** is a unique NO-independent, and NO-enhancing, activator of soluble guaunylyl cyclase (sGC).[3,4] Due to such unique action mechanism of **YC-1**,[5–8] and the fact that sGC is associated with a lot of physiological functions, more than 200 papers deal with the biological functions and pharmacological action of **YC-1**, and related indazole derivatives, have been published during last decade. In some of these literatures, **YC-1** was reported to exhibit excellent anti-cancer activity. The anti-cancer effect of **YC-1** seemed to result from its multiple action, including apoptosis induction,[9–11] anti-angiogenesis,[12,13] anti-inflammation,[14] and inhibition of matrix metalloproteinases (MMPs).[15] Animal studies indicated that an oral dose of **YC-1** suppressed tumor growth and prolonged the medium survival time in xenograft animal models carrying various human cancers, including the non-small cell lung cancer,[13] hepatoma,[9] and prostate cancer.[11]

We recently found that **YC-1** acted against U937 leukemia cell in vitro via an intrinsic mitochondrial-dependent apoptosis pathway, and that **YC-1** induced apoptosis in a cGMP-independent pathway. The in vivo anti-leukemia effects of **YC-1**, were evaluated in BALB/c mice inoculated with WEHI-3B cells. The result indicated that **YC-1** enhanced survival rate and prevented the body weight loss in mice tested. This suggested that **YC-1** is an excellent lead compound for further development of anti-leukemia agents.[16]

In the course of our search for novel anti-leukemia drug candidates, we tried to replace the indazole skeleton of **YC-1** with a bioisosteric furo[3,2-*c*]pyrazole chromophore to give 1-benzyl-3-(5-hydroxymethyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (**1**) which was further derived into a series of furo[3,2-*c*]pyrazoles that were tested against HL-60 cells for anti-leukemia activity. It was found that the anti-leukemia mechanism of compound

Keywords: Furopyrazole; **YC-1**; Cell differentiation; Anti-leukemia agent.
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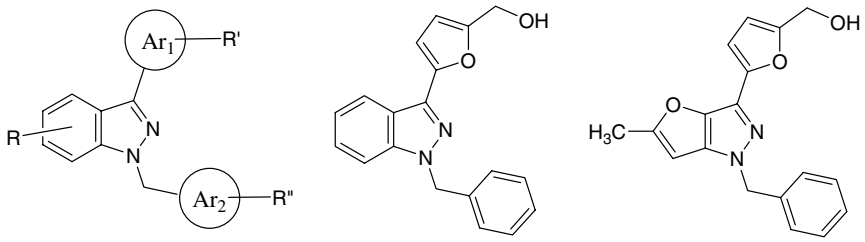


(A) YC-1 (1)

Ar_1, Ar_2 = benzene, thiophene, furan

1, comprising of inducing both cell differentiation and apoptosis, differed from that of **YC-1**. Therefore, the synthesis and anti-leukemia activities of these novel furo[3,2-*c*]pyrazoles are reported here.

2. Results and discussion

2.1. Synthesis of 1,3-disubstituted-5-methylfuro[3,2-*c*]pyrazole (1, 14–24)

Target compounds **1** and **14–24** were synthesized following similar procedure for the synthesis of **YC-1** as shown in Scheme 1.[2] The starting diaryl ketones (**6** and **7**) were prepared by reacting 5-methyl furan-2-carbonyl chloride (**2**), or 4-methoxycarbonyl benzoyl chloride (**5**), with substituted furans (**3** and **4**), respectively, according to Friedel–Craft's acylation condition. The resulted diaryl ketones (**6** and **7**) were then treated with hydrazines (**8** and **9**), respectively, to yield the corresponding hydrazones (**10–13**) which were subsequently treated with mixed reagents of $Pb(OAc)_4$ and $BF_3{\cdot}Et_2O$ to undergo oxidation and cyclization to give the desired 1,3-disubstituted furo[3,2-*c*]pyrazoles (**14–17**). The ester forms of compounds **14–17** were either hydrolyzed into their corresponding carboxylic acids (**18–21**) or reduced with $Ca(BH_4)_2$ to afford their corresponding carbinol derivatives (**1**, **22–24**).

2.2. Cytotoxic effects of compounds 1, 14–24 and YC-1 on HL-60 cells

All of the synthesized furo[3,2-*c*]pyrazoles (**1**, **14–24**) and positive control, **YC-1** were evaluated for their cytotoxic activities against HL-60 cell line by using propidium iodide (PI)-exclusion assay. As shown in Table 1, compound **1** was the only one exhibiting considerable cytotoxicity, whereas **YC-1** and all other furopyrazoles (**14–24**) resulted in ED_{50} values close to or greater than 20 μM. Further investigation of the concentration- and time-dependent viability of HL-60 cells exposed to compound **1** was conducted. As indicated by the result in Figure 1, compound **1** inhibited HL-60 cell proliferation by a dose- and time-dependent manner. To reconfirm the inhibitory effect in compound **1**-treated HL-60 cell, the mitochondrial reduction activity of compound **1** was measured by MTT assay, and the result agreed nicely with that of PI-exclusion method.

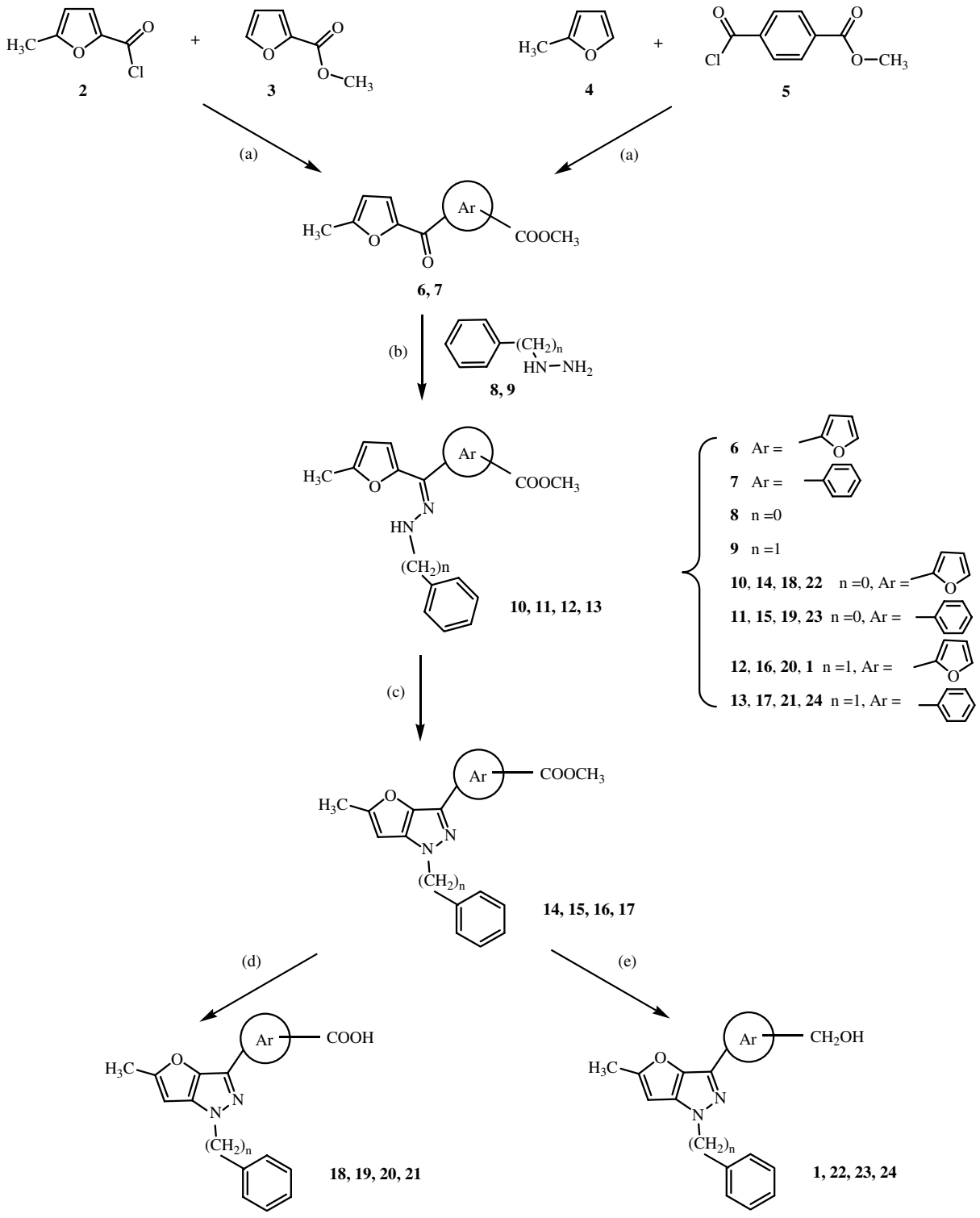
2.3. Compound 1 induced G0/G1 and sub G1 arrest in HL-60 cells

The cell cycle analysis was performed to determine whether compound **1** could influence the progression of cell cycle. As shown in Figure 2, the exposure of HL-60 cells to compound **1** for the duration of 24, 48, and 72 h resulted in significant increase in the percentage of cells accumulated in G0/G1 phase, accompanied by the slow increase in the percentage of cells in the sub G1 nuclei population. This finding suggested that compound **1** is effective in inducing differentiation and apoptosis of HL-60 cells. Similar phenomenon, however, was not observed when compound **1** was tested in other leukemia cell lines (U937 and K562).

2.4. Compound 1 induces differentiation of HL-60 cells toward granulocytic lineage

HL-60 cells are said to have dual potential because they are competent to differentiate into monocytes, in response to vitamin D_3 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or differentiate into granulocytes, in response to retinoic acid or DMSO.[17] To explore the ability of compound **1** in inducing differentiation of HL-60, Nitroblue Tetrazolium (NBT) reduction activity was assessed in HL-60 cells treated with 5 μM dose of compound **1** for 72 h.

Compared with the control cells, the results from NBT-reduction assay indicated that the number of differentiated cells increased significantly after 72 h of cultivation with compound **1** (Fig. 3A). On the contrary, the percentages of NBT-positive cells were 3.56% after treatment with **YC-1** (26 μM) (Fig. 3A), which indicated that **YC-1** can not induce differentiation in HL-60 cells. Meanwhile, after treatment with compound **1**, the flow cytometric diagrams exhibited significant elevation of surface CD11b expression (Fig. 3C), while the presence of monocyte-specific CD14 surface antigen is almost negligible if compared with that of CD11b. These findings together demonstrated that compound **1** is an effective inducer for differentiation of HL-60 cells in vitro, and is able to commit leukemia cells toward terminal maturation. Not only could compound **1** induce the differentiation of HL-60 cells into granulocyte-like cells, it also enhances the all-*trans* retinoic acid (ATRA)-induced differentiation (Figs. 3B and C). However, we



Scheme 1. Reagents and conditions: (a) Lewis acid/CH_2Cl_2; (b) AcOH/toluene; (c) $Pb(OAc)_4/BF_3{\cdot}Et_2O$; (d) $NaOH/H_3O^+$; (e) $Ca(BH_4)_2$/THF.

Table 1. Cytotoxicity data for compounds **1**, **14**–**24** and **YC-1** against HL-60 cell line

Cell line	Cytotoxicity ED_{50} (μM)												
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**1**	**24**	**YC-1**
HL-60	>20	>20	>20	19.36	17.22	>20	>20	>20	18.25	>20	5.12	19.25	25.27

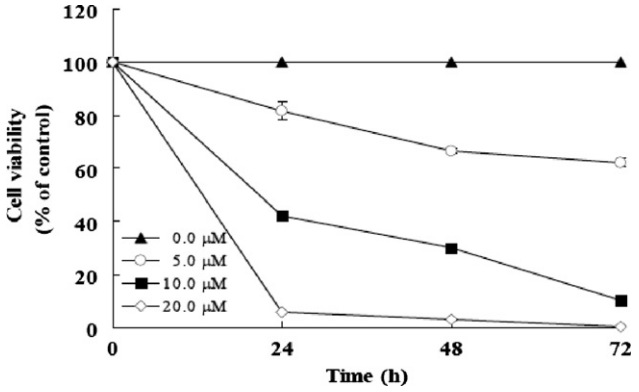


Figure 1. Effects of compound **1** on viability of HL-60 cells. Cells were treated with various concentrations of compound **1** for indicated duration. Viable cells were measured by PI exclusion and immediately analyzed by flow cytometry. The percentage of cell viability was calculated as a ratio between drug-treated cells and control cells. Each value represents mean ± SD from three independent experiments.

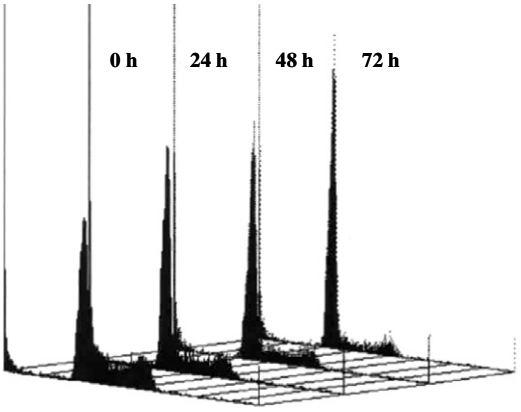
did not detect significant NBT-reduction in compound **1**-treated U937 and K562 cells (Table 2).

2.5. Compound 1 promotes HL-60 cell differentiation by regulation of Bcl-2 and cMyc proteins

Regulation of the relative levels of Bcl-2 protein may play an important role in modulating the susceptibility of cells to differentiation.[18] Intriguingly, this raises the possibility that c-Myc may be a key contributor to disease by deregulating cell proliferation, and by virtue of its opposing role in engendering apoptosis. Downregulation of c-Myc could inhibit the proliferation of normal and leukemia myeloid cells.[19] The expression levels of these Bcl-2 family proteins and transcription factor c-Myc were examined by Western blotting. The result shown in Figure 4 indicated that Bcl-2 and c-Myc protein levels decreased simultaneously during the differentiation of HL-60 cells into granulocytes that subsequently undergo compound **1**-induced apoptosis for 72–96 h. Our results indicated compound **1**-induced HL-60 cell differentiation may be associated with Bcl-2 and c-Myc protein levels.

2.6. Compound 1 and YC-1 induce apoptosis of HL-60 cells

The morphology of cells treated with compound **1** was studied to verify whether cell death was a result of apoptosis in HL-60 cells. The nuclear morphological changes of the cells were assessed by staining the cell with 4′-6-diamidino-2-phenylindole (DAPI). After 72-h culture with compound **1**, the cells exhibited nuclear shrinkage and chromatin condensation (Fig. 5B). Such morphological changes were not apparent in the control cells (Fig. 5A). Our results indicated that compound **1** induced HL-60 cell apoptosis. As was mentioned briefly in the introduction, we have previously observed **YC-1** inducing apoptosis of U937 cells. In this study, DAPI staining was assessed in HL-60 cells treated with **YC-1** (26 μM). After 48-h cultivation with **YC-1**, the cells exhibited nuclear shrinkage and chromatin condensation (Fig. 5C). This result indicated that **YC-1** induced HL-60 cell apoptosis at high dose.



Compound **1** (5 μM)

Time	Sub-G1	G0/G1	S	G2/M
0 h	3.61 ± 0.45	50.28 ± 0.41	39.24 ± 0.58	10.48 ± 0.19
24 h	11.11 ± 0.68*	68.07 ± 1.40*	23.84 ± 1.61	8.09 ± 0.22
48 h	12.62 ± 0.30*	69.94 ± 0.76*	22.89 ± 1.03	7.17 ± 0.30
72 h	16.43 ± 0.19*	79.93 ± 0.68*	13.78 ± 0.87	6.29 ± 0.51

Figure 2. Cell cycle progression of HL-60 cells after treatment with compound **1**. HL-60 cells were treated with compound **1** for the indicated incubation times, then stained for DNA with PI, and analyzed for cell cycle progression or apoptosis by flow cytometry. Cell cycle analysis showed that compound **1** induced a prominent G0/G1 population arrest in HL-60 cells. The percentage of positive cells was analyzed using a flow cytometer, and presented as mean ± SD. Three separate experiments were each tested in duplicate. Values significantly different from the 0 h-treated group are marked by $*P < 0.01$.

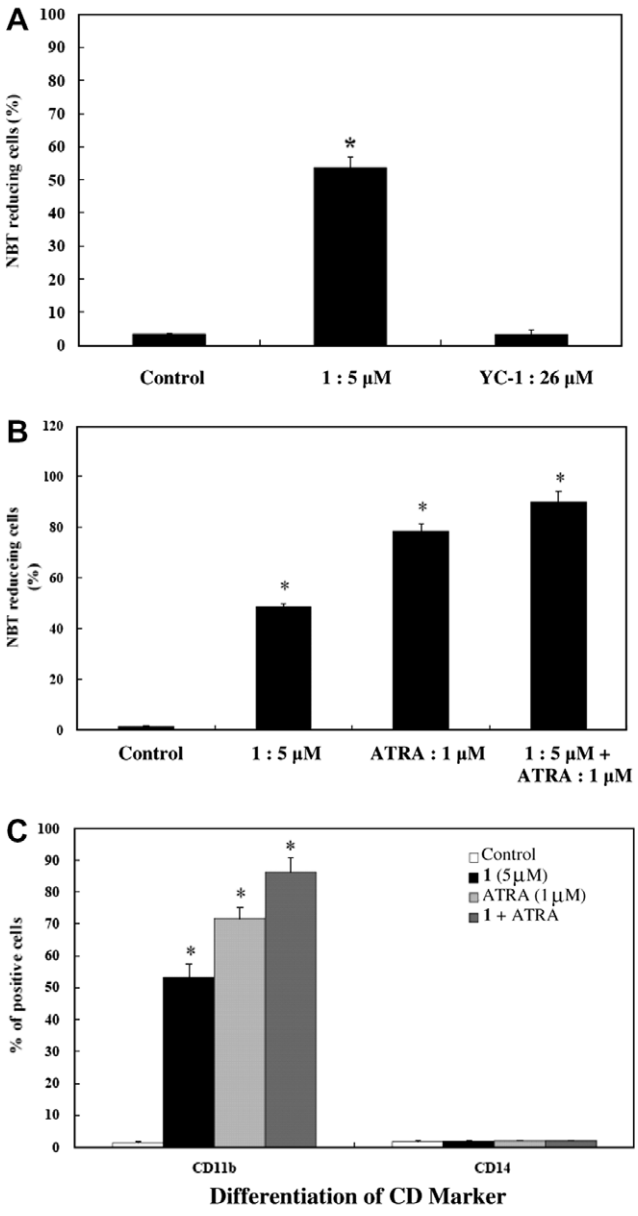


Figure 3. Compound **1**-induced HL-60 cell's differentiation presented by (A and B) NBT reduction and (C) CD11b and CD14 differentiation marker expression. NBT reduction was assay in HL-60 cells after 72-;h treatment with compound **1** (5 μM), **YC-1** (26 μM), and ATRA (1 μM). The percentage of formazan-containing NBT reduction cells was assessed microscopically. Expression of CD11b and CD14 in compound **1**- and/or ATRA-treated HL-60 cells. After 72 h-treatment of compound **1** and/or ATRA, HL-60 cells were stained with FITC-conjugated anti-CD11b and anti-CD14 antibodies. The percentage of positive cells was analyzed using a flow cytometer and presented as mean ± SD. Three separate experiments were each tested in duplicate. Values significantly different from the control group are marked by $^{*}P < 0.01$.

Table 2. NBT reduction in compound **1**-treated human leukemia cells

Compound **1**	NBT (%)		
	HL-60	U937	K562
0 μM	3.77 ± 0.25	3.01 ± 0.92	2.57 ± 1.38
5 μM	53.41 ± 2.38***	4.57 ± 0.39	3.11 ± 1.64

*** $P < 0.01$.

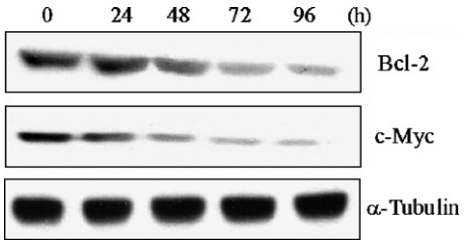


Figure 4. Compound **1**-induced Bcl-2, c-Myc, and α-tubulin protein levels' change in HL-60 cells assayed by Western blot analysis. Cells were treated with compound **1** for the indicated incubation times. Total protein extracts were analyzed by immunoblotting with antibodies specific to Bcl-2, c-Myc and α-tubulin.

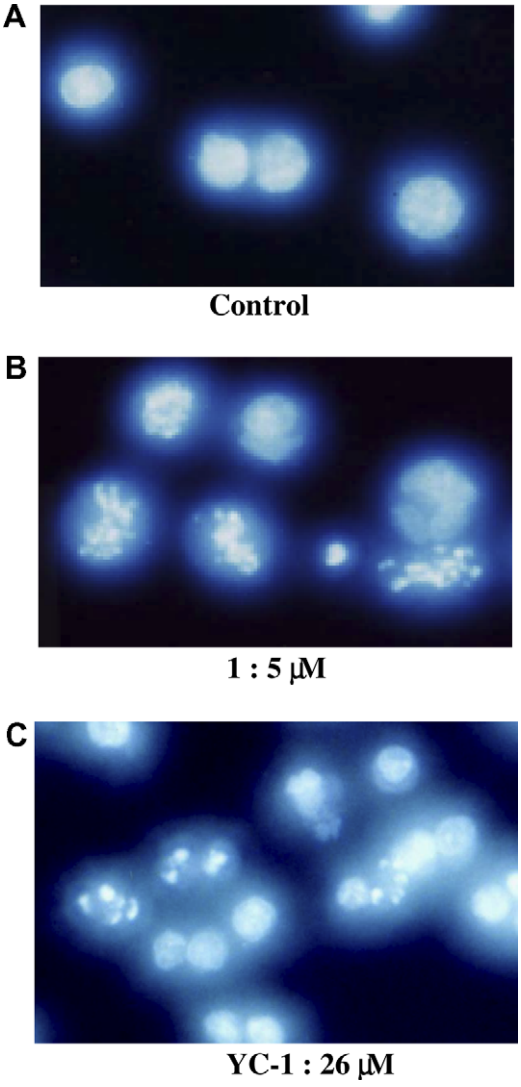


Figure 5. Compound **1**-induced apoptosis in HL-60 cells measured by DAPI staining (400×) followed by microscopic analysis. (A) 0.1% (v/v) DMSO-treated cells (control). (B) Compound **1**-treated cells. (C) **YC-1**-treated cells.

3. Conclusions

We have synthesized a series of furo[3,2-*c*]pyrazole analogs of **YC-1**. Through anti-leukemia activity evaluation, it was found that compound **1** demonstrated an

anti-leukemia mechanism of action different from that of **YC-1** which mainly works by inducing apoptosis. Compound **1** induced differentiation of HL-60 cells toward granulocyte lineage and promoted differentiation of HL-60 cells by regulation of the dual effects of Bcl-2 and c-Myc proteins. Meanwhile, compound **1** was also observed to induce apoptosis of HL-60 cells. Since cell differentiation and apoptosis are both considered important action mechanism for anti-leukemia therapy, the development of novel cell differentiation agents that also induce apoptosis would be the keystone for successful development of anti-leukemia agent. Thus, compound **1** is identified here as a new lead compound of cell differentiation agent and apoptosis inducer that will be further optimized for development of new anti-leukemia drug candidate.

4. Experimental

4.1. Chemistry

4.1.1. General methods. All of the solvents and reagents were obtained commercially and used without further purification. Reactions were monitored by thin-layer chromatography, using Merck plates with fluorescent indicator. Column chromatography was performed on silica gel. Melting points were determined with a Yanaco MP-500D melting point apparatus and are uncorrected. IR spectra were recorded on Shimadzu IRPrestige-21 spectrophotometers as KBr pellets. NMR spectra were obtained on a Bruker Avance DPX-200 FT-NMR spectrometer in $CDCl_3$ or DMSO. The following abbreviations are used: s, singlet; d, doublet; t, triplet; dd, double doublet; and m, multiplet. MS spectra were measured with an HP 5995 GC–MS instrument. Elemental analyses (C, H, and N) were performed on a Perkin-Elmer 2400 Series II CHNS/O analyzer and the results were within ±0.4% of the calculated values.

4.1.2. Synthesis of substituted furo[3,2-*c*]pyrazoles (1, 14–24)

4.1.2.1. 5-Methoxycarbonyl-2-furyl 5-methyl-2-furyl ketone (6). A mixture of 5-methylfuran-2-carboxylic acid (7.56 g, 0.06 mol) in 50 mL of dry CH_2Cl_2 and $SOCl_2$ (8.33 g, 0.07 mol) was heated under refluxing for 4–5 h. After evaporation, the crude product 5-methylfuran-2-carbonyl chloride (**2**) was obtained. Into the crude product **2** was added dry CH_2Cl_2 (50 mL), then methyl furan-2-carboxylate (**3**) (7.56 g, 0.06 mol) and anhydrous ferric chloride (9.75 g, 0.06 mol) were added. The reaction mixture was then heated under refluxing for 30 min, cooled and quenched with ice water. The CH_2Cl_2 layer was sequentially washed with water, 5% $NaHCO_3$ solution, and water, until neutral, then dried over $MgSO_4$ and filtered. The solvent of the filtrate was evaporated under reduced pressure, the residue was recrystallized from *n*-hexane to afford compound **6** (8.70 g, 0.037 mol). Yield: 62%; mp 122–125 °C; MS (EI, 70 eV): *m*/*z* 234 (M^+); found: C, 61.53; H, 4.29. $C_{12}H_{10}O_5$ requires: C, 61.54; H, 4.30; IR (KBr): 1635, 1728 (C=O) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.47 (s, 3H), 3.96 (s, 3H), 6.28–6.30 (m, 1H), 7.26–7.28 (m, 1H), 7.44 (d, 1H, $J = 3.7$ Hz); 7.74 (d, 1H, $J = 3.5$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 13.82, 100.13, 108.73, 121.43, 141.95, 158.32, 163.13.

4.1.2.2. *p*-Methoxycarbonylphenyl 5-methyl-2-furyl ketone (7). Anhydrous $AlCl_3$ (26.70 g, 0.20 mol) and 4-methoxycarbonyl benzoyl chloride (**5**) (9.93 g, 0.10 mol) were dissolved in CH_2Cl_2 (100 mL), then 2-methylfuran (**4**) (8.21 g, 0.10 mol) was added dropwise. The reaction mixture was then heated under refluxing for 30 min, cooled, and quenched with ice water. The CH_2Cl_2 layer was sequentially washed with water, 5% $NaHCO_3$ solution, and water, until neutral, then dried over $MgSO_4$ and filtered. The solvent of the filtrate was evaporated under reduced pressure, and the residue was recrystallized from *n*-hexane to afford compound **7** (14.20 g, 0.058 mol). Yield: 58.2%; mp 93–96 °C; MS (EI, 70 eV): *m*/*z* 244 (M^+); found: C, 68.87; H, 4.94. $C_{14}H_{12}O_4$ requires: C, 68.85; H, 4.95; IR (KBr): 1623, 1723 (C=O) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.45 (s, 3H), 3.95 (s, 3H), 6.22–6.24 (m, 1H), 7.12 (d, 1H, $J = 3.5$ Hz), 7.92–7.97 (m, 2H), 8.11–8.15 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 13.94, 52.17, 109.11, 123.14, 128.72, 129.30, 132.87, 141.07, 150.49, 159.06, 166.06, 181.05.

4.1.2.3. 1-Phenyl-3-(5-methoxycarbonyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (14). Into the solution of compound **6** (4.68 g, 0.02 mol) in toluene (100 mL) were added phenylhydrazine (4.36 g, 0.04 mol), and acetic acid (1.5 mL). The mixture was heated under refluxing for 3 h. After cooling, the solvent was evaporated and the residue was purified by column chromatography (silica gel, toluene) to give 5-methoxycarbonyl-2-furyl 5-methyl-2-furyl phenylhydrazone (**10**). The crude product **10** was dissolved in CH_2Cl_2 (50 mL), then $Pb(OAc)_4$ (26.60 g, 0.06 mol), followed with $BF_3{\cdot}Et_2O$ (98% in ether, 100 mL). After shaking for 10 min, the reaction mixture was poured into ice water (200 mL) and allowed to stand until two layers formed. The organic layer was washed with water, then 10% NaOH solution until neutral, then dried over $MgSO_4$ and filtered. The solvent of the filtrate was evaporated and the residue was purified by column chromatography (silica gel, $CHCl_3$) to afford compound **14** (1.81 g, 0.0056 mol). Yield: 28.1%; mp 130–131 °C; MS (EI, 70 eV): *m*/*z* 322 (M^+); found: C, 67.05; H, 4.39; N, 8.68. $C_{18}H_{14}N_2O_4$ requires: C, 67.07; H, 4.38; N, 8.69; IR (KBr): 1711 (C=O) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.53 (d, 3H, $J = 0.8$ Hz), 3.95 (s, 3H), 6.49 (d, 1H, $J = 0.9$ Hz), 6.96 (d, 1H, $J = 3.6$ Hz), 7.27–7.34 (m, 2H), 7.49 (t, 2H, $J = 8.3$ Hz), 7.77–7.81 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.92, 51.71, 96.04, 109.01, 117.75, 119.61, 124.21, 125.47, 129.21, 136.85, 139.84, 143.67, 149.81, 158.87, 162.00.

4.1.2.4. 1-Phenyl-3-(*p*-methoxycarbonylphenyl)-5-methylfuro[3,2-*c*]pyrazole (15). Compound **7** (4.48 g, 0.02 mol), phenylhydrazine (4.36 g, 0.04 mol), and acetic acid (1.5 mL) were allowed to react as in the preparation of compound **14** to afford **15** (2.13 g, 0.0064 mol). Yield: 32.1%; mp 156–159 °C; MS (EI, 70 eV): *m*/*z* 332 (M^+);

found: C, 72.31; H, 4.86; N, 8.42. $C_{20}H_{16}N_2O_3$ requires: C, 72.28; H, 4.85; N, 8.43; IR (KBr): 1713 (C=O) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.53 (d, 3H, $J = 0.9$ Hz), 3.95 (s, 3H), 6.48 (d, 1H, $J = 1.0$ Hz), 7.25–7.27 (m, 1H), 7.49 (t, 2H, $J = 8.4$ Hz), 7.78–7.82 (m, 2H), 8.14 (s, 4H); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.91, 51.88, 96.05, 117.52, 125.18, 125.64, 128.96, 129.21, 129.83, 131.66, 135.44, 137.01, 140.09, 145.35, 161.47, 166.77.

4.1.2.5. 1-Benzyl-3-(5-methoxycarbonyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (16). Compound **6** (4.68 g, 0.02 mol), benzylhydrazine (4.88 g, 0.04 mol), and acetic acid (1.5 mL) were allowed to react as in the preparation of compound **14** to afford **16** (1.76 g, 0.0052 mol). Yield: 26.2%; mp 114–117 °C; MS (EI, 70 eV): *m*/*z* 336 (M^+); found: C, 67.87; H, 4.78; N, 8.34. $C_{19}H_{16}N_2O_4$ requires: C, 67.85; H, 4.79; N, 8.33; IR (KBr): 1724 (C=O) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.38 (d, 3H, $J = 0.4$ Hz), 3.83 (s, 3H), 5.41 (s, 2H), 6.28 (d, 1H, $J = 0.9$ Hz), 6.85 (d, 1H, $J = 3.6$ Hz), 7.28–7.36 (m, 5H), 7.40 (d, 1H, $J = 3.7$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 14.89, 52.04, 54.84, 95.41, 108.55, 120.45, 121.55, 128.18, 128.91, 136.63, 139.35, 142.32, 142.92, 150.23, 158.45, 161.94.

4.1.2.6. 1-Benzyl-3-(*p*-methoxycarbonylphenyl)-5-methylfuro[3,2-*c*]pyrazole (17). Compound **7** (4.88 g, 0.02 mol), benzylhydrazine (4.88 g, 0.04 mol), and acetic acid (1.5 mL) were allowed to react as in the preparation of compound **14** to afford **17** (2.22 g, 0.0064 mol). Yield: 32.1%; mp 122–125 °C; MS (EI, 70 eV): *m*/*z* 346 (M^+); found: C, 72.85; H, 5.21; N, 8.11. $C_{21}H_{18}N_2O_3$ requires: C, 72.82; H, 5.24; N, 8.09; IR (KBr): 1712 (C=O) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.38 (d, 3H, $J = 0.9$ Hz), 3.84 (s, 3H), 5.40 (s, 2H), 6.25 (d, 1H, $J = 1.1$ Hz), 7.32–7.34 (m, 5H), 7.90–8.06 (m, 4H); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 14.90, 52.29, 54.82, 95.37, 125.28, 128.16, 128.39, 128.87, 130.17, 136.01, 136.80, 139.60, 143.76, 161.57, 166.18.

4.1.2.7. 1-Phenyl-3-(5-hydroxycarbonyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (18). Compound **14** (644 mg, 2.0 mmol) in 20 mL of 10% NaOH solution was heated under refluxing for 2 h, cooled and acidified with dilute HCl. The precipitates were collected, then recrystallized from ethanol to afford compound **18** (492 mg, 1.6 mmol). Yield: 79.9%; mp 247–250 °C; MS (EI, 70 eV): *m*/*z* 308 (M^+); found: C, 66.13; H, 4.01; N, 9.11. $C_{17}H_{12}N_2O_4$ requires: C, 66.23; H, 3.92; N, 9.09; IR (KBr): 1700 (C=O), 2500–3200 (OH) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.49 (s, 3H), 7.00 (d, 1H, $J = 3.6$ Hz), 7.04 (s, 1H), 7.27–7.37 (m, 2H), 7.53 (t, 2H, $J = 8.0$ Hz), 7.82 (d, 2H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 15.02, 97.21, 109.87, 117.58, 119.69, 123.98, 126.08, 130.00, 137.16, 139.49, 143.32, 144.90, 148.86, 159.41, 162.88.

4.1.2.8. 1-Phenyl-3-(*p*-hydroxycarbonylphenyl)-5-methylfuro[3,2-*c*]pyrazole (19). Following the same synthetic procedure for compound **18**, compound **19** (548 mg, 1.7 mmol) was prepared by reacting compound **15** (664 mg, 2.0 mmol). Yield: 86.2%; mp 285–288 °C; MS (EI, 70 eV): *m*/*z* 318 (M^+); found: C, 71.66; H, 4.44; N, 8.81. $C_{19}H_{14}N_2O_3$ requires: C, 71.69; H, 4.43; N, 8.80; IR (KBr): 1691 (C=O), 2500–3200 (OH) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.50 (s, 3H), 7.06 (d, 1H, $J = 0.9$ Hz), 7.30 (t, 1H, $J = 7.3$ Hz), 7.54 (t, 2H, $J = 8.4$ Hz), 7.87 (d, 1H, $J = 7.8$ Hz), 8.02–8.12 (m, 4H); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 15.06, 97.26, 117.42, 125.74, 129.99, 130.42, 131.15, 134.75, 137.38, 139.69, 144.76, 162.57, 167.23.

4.1.2.9. 1-Benzyl-3-(5-hydroxycarbonyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (20). Following the same synthetic procedure for compound **18**, compound **20** (482 mg, 1.5 mmol) was prepared by reacting compound **16** (676 mg, 2.0 mmol). Yield: 74.8%; mp 231–234 °C; MS (EI, 70 eV): *m*/*z* 322 (M^+); found: C, 67.09; H, 4.40; N, 8.67. $C_{18}H_{14}N_2O_4$ requires: C, 67.07; H, 4.38; N, 8.69; IR (KBr): 1681 (C=O), 2500–3100 (OH) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.37 (s, 3H), 5.39 (s, 2H), 6.28 (s, 1H), 6.82 (d, 1H, $J = 3.6$ Hz), 7.32–7.38 (m, 6H); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 14.89, 54.84, 95.40, 108.39, 119.63, 121.82, 128.19, 128.91, 136.67, 139.33, 142.33, 144.33, 149.72, 159.45, 161.88.

4.1.2.10. 1-Benzyl-3-(*p*-hydroxycarbonylphenyl)-5-methylfuro[3,2-*c*]pyrazole (21). Following the same synthetic procedure for compound **18**, compound **21** (504 mg, 1.5 mmol) was prepared by reacting compound **17** (692 mg, 2.0 mmol). Yield: 75.9%; mp 211–214 °C; MS (EI, 70 eV): *m*/*z* 332 (M^+); found: C, 72.30; H, 4.84; N, 8.45. $C_{20}H_{16}N_2O_3$ requires: C, 72.28; H, 4.85; N, 8.43; IR (KBr): 1712 (C=O), 2500–3400 (OH) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.39 (s, 3H), 5.41 (s, 2H), 6.27 (s, 1H), 7.26–7.44 (m, 5H), 7.97 (dd, 4H, $J = 8.2$, 15.1 Hz); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 14.94, 54.78, 95.41, 125.14, 128.16, 128.90, 129.80, 130.34, 135.54, 136.86, 139.57, 143.72, 161.55, 167.30.

4.1.2.11. 1-Phenyl-3-(5-hydroxymethyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (22). Compound **14** (644 mg, 2.0 mmol) was dissolved in a homogeneous solution of THF (50 mL) dispersed with $CaBH_4$ (1.26 g, 0.018 mol). The mixture was heated under refluxing for 6 h and then filtered. The solvent was evaporated and the residue was recrystallized from *n*-hexane and then purified by column chromatography (silica gel, *n*-hexane–ethyl acetate) to afford compound **22** (512 mg, 1.7 mmol). Yield: 87.1%; mp 127–130 °C; MS (EI, 70 eV): *m*/*z* 294 (M^+); found: C, 69.35; H, 4.78; N, 9.50. $C_{17}H_{14}N_2O_3$ requires: C, 69.38; H, 4.79; N, 9.52; IR (KBr): 3200–3500 (OH) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.49 (d, 3H, $J = 0.9$ Hz), 4.73 (s, 2H), 6.43–6.45 (m, 2H), 6.82 (d, 1H, $J = 3.3$ Hz), 7.19–7.27 (m, 1H), 7.42–7.50 (m, 2H), 7.72–7.77 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.85, 57.34, 96.04, 108.84, 109.38, 117.59, 125.05, 125.31, 129.15, 136.61, 139.99, 143.59, 145.82, 154.04, 161.56.

4.1.2.12. 1-Phenyl-3-(*p*-hydroxymethylphenyl)-5-methylfuro[3,2-*c*]pyrazole (23). Following the same synthetic procedure for compound **22**, compound **23** (526 mg, 1.7 mmol) was prepared by reacting compound **15**

(664 mg, 2.0 mmol). Yield: 86.5%; mp 114–116 °C; MS (EI, 70 eV): *m/z* 304 (M^+); found: C, 74.95; H, 5.31; N, 9.18. $C_{19}H_{16}N_2O_2$ requires: C, 74.98; H, 5.30; N, 9.20; IR (KBr): 3100–3400 (OH) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.51 (s, 3H), 4.74 (s, 2H), 6.46 (d, 1H, $J = 0.9$ Hz), 7.20–7.27 (m, 1H), 7.44–7.52 (m, 4H), 7.77–7.81(m, 2H), 8.08 (d, 2H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.90, 64.99, 96.01, 117.38, 124.83, 126.10, 127.09, 129.15, 130.36, 132.51, 136.84, 140.21, 140.40, 145.14, 161.23.

4.1.2.13. 1-Benzyl-3-(5-hydroxymethyl-2-furyl)-5-methylfuro[3,2-*c*]pyrazole (1). Following the same synthetic procedure for compound **22**, compound **1** (480 mg, 1.6 mmol) was prepared by reacting compound **16** (676 mg, 2.0 mmol). Yield: 77.9%; mp 117–120 °C; MS (EI, 70 eV): *m/z* 308 (M^+); found: C, 70.10; H, 5.25; N, 9.10. $C_{18}H_{16}N_2O_3$ requires: C, 70.12; H, 5.23; N, 9.09; IR (KBr): 3200–3500 (OH) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6, δ): 2.36 (s, 3H), 4.43 (d, 2H, $J = 5.7$ Hz), 5.33 (t, 1H, $J = 5.8$ Hz), 5.35 (s, 2H), 6.25 (d, 1H, $J = 1.0$ Hz), 6.40 (d, 1H, $J = 3.3$ Hz), 6.60 (d, 1H, $J = 3.2$ Hz), 7.29–7.33 (m, 5H); ^{13}C NMR (50 MHz, DMSO-d_6, δ): 14.91, 54.55, 55.82, 95.30, 107.25, 109.04, 122.92, 128.06, 128.86, 137.03, 138.97, 141.89, 145.69, 155.31, 161.36.

4.1.2.14. 1-Benzyl-3-(*p*-hydroxymethylphenyl)-5-methylfuro[3,2-*c*]pyrazole (24). Following the same synthetic procedure for compound **22**, compound **24** (504 mg, 1.6 mmol) was prepared by reacting compound **17** (692 mg, 2.0 mmol). Yield: 79.2%; mp 118–121 °C; MS (EI, 70 eV): *m/z* 318 (M^+); found: C, 75.41; H, 5.71; N, 8.78. $C_{20}H_{18}N_2O_2$ requires: C, 75.45; H, 5.70; N, 8.80; IR (KBr): 3200–3500 (OH) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$, δ): 2.36 (s, 3H), 4.71 (s, 2H), 5.35 (s, 2H), 5.61 (d, 1H, $J = 0.9$ Hz), 7.26–7.38 (m, 5H), 7.42 (d, 2H, $J = 8.1$ Hz), 7.96 (d, 2H, $J = 8.2$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.78, 55.28, 64.99, 94.29, 125.73, 127.12, 127.92, 128.01, 128.52, 130.55, 130.83, 135.70, 138.83, 139.86, 144.05, 160.63.

4.2. Biological evaluation

4.2.1. Cell culture and viability assay. Leukemia cells were obtained from the Culture Collection and Research Center (CCRC, Taiwan, ROC), originally from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 cell culture medium (Gibco BRL, Life Technologies, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UT, USA), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL) (Gibco BRL), and incubated at 37 °C in humidified 5% CO_2 atmosphere. For cell viability and cell cycle analysis, cells [2.5×10^5 cells/mL] were seeded in a 24-well culture plate (Falcon, CA, USA). Compound was added to each well and the plates were incubated at 37 °C for 24, 48, and 72 h. Cell viability was estimated by propidium iodide (PI)-exclusion assay.

4.2.2. Cell cycle analysis. Cells were harvested together, washed twice in PBS, and fixed in 70% ethanol at 4 °C. After washing with PBS, low-molecular-weight fragments of DNA were extracted for 10 min in citrate buffer (Na_2HPO_4, $C_6H_3O_7$, pH 7.8), RNA was removed by ribonuclease A (5 mg/mL), and DNA was stained with propidium iodide (20 μg/mL PBS) for 30 min in the dark. Fluorescence was measured using a flow cytometer (FACSCalibur, Becton–Dickinson, San Jose, CA) equipped with an argon laser at 488 nm wavelength for excitation.

4.2.3. NBT assay. Cells were suspended in Hanks' balanced salt solution (HBSS) at a concentration of 1×10^6 cells/mL and then incubated for 30 min at 37 °C with an equal volume of NBT test stock solution (0.2 mL of HBSS containing 1 mg/ml NBT and 5 μg/mL TPA) (Sigma, MO, USA). After incubation, the positive cells reduced NBT to give intracellular black-blue formazan deposit. The percentage of differentiated cells was assessed microscopically by counting a minimum of 200 cells. Three separate experiments were each tested in duplicate.

4.2.4. Analysis of cellular surface antigen CD11b/CD14 expression. A direct immuno-fluorescence method was employed to detect the CD11b/CD14 expression of cellular surface antigens on HL-60 cells after differentiation induction.[20] Cells collected from 72 h cultures were treated with monoclonal antibodies anti-CD11b-FITC and anti-CD14-PE (Pharmingen, CA, USA) at 37 °C for 30 min, then washed with PBS. The percentage of positive cells was analyzed using flow cytometry (FACS Calibur, Becton–Dickinson, San Jose, CA).

4.2.5. Western blot analysis. Total protein extracts were prepared with RIPA protein lysis buffer (50 mM Tris (pH 6.8), 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 10 μM aqueous PMSF). The concentration of protein was determined by the Bradford method using the Bio-Rad protein assay dye reagent (Amresco, SO, USA).[21] The lysates containing 30 μg of protein were separated by SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Nonspecific binding sites were blocked with 5% non-fat milk in PBST buffer (0.05% Triton X-100 in PBS) for 1 h. The PVDF membrane was incubated overnight at 4 °C with specific primary antibodies against Bcl-2 (Upstate Biochemistry, NY, USA), c-Myc (Pharmingen, CA, USA), and α-Tubulin (Santa Cruz Biotechnology, CA, USA). After washing with PBST buffer, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). Immunoreactive proteins were detected by using Western Blotting Chemiluminescence Reagent *Plus* kit (NENTM Life Science, MA, USA) and exposed to X-ray films.

4.2.6. Statistical analysis. Results in this report are presented as means ± SD. Differences between the different treatment groups, which consisted of matched samples, were assessed by Student's *t* test. A confidence level of 1% (*P* value of less than 0.01) was considered to be significant.

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