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Enhancing effect of indirubin derivatives on 1,25-dihydroxyvitamin D_3- and all-*trans* retinoic acid-induced differentiation of HL-60 leukemia cells

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Abstract—The induction of differentiation represents a new and promising approach to cancer therapy, well illustrated by the treatment of acute promyelocytic leukemia (APL) with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid (ATRA). Using combinations of low, nontoxic concentrations of either 1,25-$(OH)_2D_3$ or ATRA and differentiation-enhancing chemicals, adverse effects such as hypercalcemic effects have been ameliorated, and long-term survival has been improved. Indirubin has been demonstrated to exert anti-leukemic effects in cases of chronic myelocytic leukemia. Previously, we synthesized a series of indirubin derivatives and evaluated their anti-proliferative properties against cancer cells. In this study, we determined the enhancing activities of these derivatives on 1,25-$(OH)_2D_3$- and ATRA-induced differentiation of human promyelocytic leukemia HL-60 cells. Importantly, some of these derivatives were found to synergistically enhance the differentiation of HL-60 cells in a concentration-dependent manner when coupled with low doses of either 1,25-$(OH)_2D_3$ or ATRA. The ability of indirubin derivatives to enhance the differentiation potential of 1,25-$(OH)_2D_3$ or ATRA may improve the ultimate outcomes of APL therapy.

1. Introduction

Leukemia may eventually prove treatable with agents that induce terminal differentiation, presumably with less morbidity than that associated with treatment with cytodestructive agents.[1] 1,25-$(OH)_2D_3$ and ATRA are both known to be able to induce terminal differentiation in leukemic cell lines, such as HL-60 and U937 cells, as well as in short-term cultured APL cells in humans.[2] Moreover, ATRA has been demonstrated to induce complete remission (CR) in almost all patients with APL, via the in vivo differentiation of APL blasts.[3] Although ATRA can bring about CR in cases of APL, treatment with ATRA alone is associated with certain severe side effects, including ATRA syndrome[4] and the induction of secondary ATRA resistance. Therefore, the current approach to solving this problem involves the introduction of a second chemical, which enhances the differentiation-inducing effects of 1,25-$(OH)_2D_3$ or ATRA at lower, nontoxic concentrations.

Indirubin, a 3,2′-bisindole, is an active ingredient of a traditional Chinese medical preparation, referred to as Danggui Longui Wan. Indirubin exhibits profound anti-leukemic effects against myelocytic leukemia.[5] Recently, both indirubin and indirubin derivatives have been shown to function as potent inhibitors of cyclin-dependent kinases (CDKs), evidencing growth inhibitory effects in human tumor cells.[6] Indirubin inhibits the proliferation of a variety of cell types via the arrest of the G1/S or G2/M phase of the cell cycle.[7]

In this report, we have assessed the enhancing effects of novel indirubin derivatives on the cellular differentiation of human promyelocytic leukemia HL-60 cells, in combination with low doses of 1,25-$(OH)_2D_3$ or ATRA. The human promyelocytic leukemia HL-60 cell culture has frequently been employed as an excellent model system

Keywords: Indirubin; Differentiation; Leukemia; 1,25-Dihydroxyvitamin D_3; All-*trans* retinoic acid.
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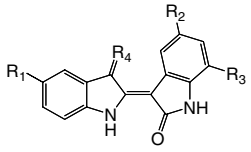
for the in vitro study of cellular differentiation. HL-60 cells differentiate into monocytic lineage when treated with 1,25-$(OH)_2D_3$, and granulocytic lineage when treated with ATRA.[2,8]

2. Results

We have synthesized a series of indirubin derivatives,[9] which were then demonstrated to exert inhibitory effects on CDKs. In order to determine whether these indirubin 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 solvent (DMSO) alone, or treated for 72 h with 1 μM of each of the indirubin derivatives, in the absence or presence of low (non-toxic) doses of 1,25-$(OH)_2D_3$ (5 nM) or ATRA (50 nM). As shown in Table 1, treatment with the indirubin derivatives induced a relatively small increase in the differentiation of the HL-60 cells, by approximately 3.1–30.5%. Importantly, among the tested derivatives, 5-fluoro-indirubin (compound **6**) profoundly potentiated cell differentiation when combined with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA. As seen in Figure 1C, the addition of 5-fluoro-indirubin to cultures exposed to 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA, which when administered without the derivative induced relatively low levels of differentiation (19% and 29%, respectively) resulted in marked increases in cell differentiation. The cell growth and viability were determined in each of the treatment groups. As shown in Figure 1B, treatment with 10 μM 5-fluoro-indirubin inhibited cell growth to less than 10% of the control levels, according to the results of the MTT assay. 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).

In order to further characterize the enhancement of cell differentiation affected by 5-fluoro-indirubin, we conducted an analysis of morphologic phenotypes and cell surface antigen expression in the HL-60 cells. By Giemsa staining HL-60 cells had blastic and round nuclei in their centers, and a high nucleus/cytoplasm ratio (Fig. 2A). Cells treated with 1,25-$(OH)_2D_3$, ATRA or 5-fluoro-indirubin evidenced relatively minor changes in cell morphology, such as irregular cell margins (Fig. 2B–D). Cells treated with 1,25-$(OH)_2D_3$ or ATRA in combination with 5-fluoro-indirubin were larger than usual, and had a low nucleous/cytoplasm ratio; their

Table 1. Effects of the indirubin derivatives on HL-60 cell differentiation



Compound (1 μM)	R_1	R_2	R_3	R_4	% differentiated cells		
					Media	1,25-$(OH)_2D_3$ (5nM)	ATRA (50 nM)
1	H	H	H	O	7.76 ± 3.42	28.36 ± 0.15	39.61 ± 1.25
2	H	H	H	NOH	11.12 ± 0.31	24.52 ± 2.67	37.78 ± 1.06
3	H	CF_3O	H	O	6.27 ± 1.76	22.04 ± 0.38	41.01 ± 1.26
4	H	CF_3O	H	NOH	22.81 ± 2.38	28.36 ± 3.05	28.68 ± 0.23
5	H	NO_2	H	O	19.94 ± 2.02	48.51 ± 3.49	59.32 ± 1.54
6	H	F	H	O	7.53 ± 1.02	78.44 ± 6.88	75.08 ± 6.80
7	H	F	H	NOH	23.71 ± 1.73	24.29 ± 1.34	54.81 ± 1.65
8	H	CH_3	H	NOH	28.22 ± 2.04	35.49 ± 5.45	70.54 ± 2.17
9	H	Cl	H	NOH	20.97 ± 1.19	21.72 ± 1.22	72.40 ± 3.05
10	H	I	H	NOH	30.50 ± 4.97	33.27 ± 0.75	67.41 ± 1.36
11	H	CH_3	CH_3	NOH	3.07 ± 1.69	27.24 ± 1.32	33.99 ± 0.47
12	H	Cl	CH_3	NOH	8.37 ± 0.45	22.10 ± 0.90	43.17 ± 1.56
13	Br	H	H	NOH	8.69 ± 4.95	36.52 ± 1.27	23.13 ± 0.71
14	H	$Na^+SO_3^-$	H	O	9.36 ± 0.13	27.66 ± 1.28	51.49 ± 0.13
15	H	$Na^+SO_3^-$	H	NOH	11.34 ± 2.32	23.34 ± 0.65	34.50 ± 2.91
16	Br	H	H	O	11.63 ± 3.48	24.64 ± 0.65	28.23 ± 0.02
17	Br	NO_2	H	O	18.35 ± 0.02	48.28 ± 0.36	51.20 ± 3.70
18	H	CH_3	H	O	24.89 ± 6.05	41.66 ± 0.40	51.01 ± 0.88
19	H	Cl	H	O	26.18 ± 0.59	49.41 ± 0.08	48.39 ± 1.48
20	H	I	H	O	29.84 ± 1.12	44.43 ± 0.09	60.33 ± 0.51
21	H	CH_3	H	O	9.45 ± 0.92	21.74 ± 1.90	43.19 ± 1.73
22	H	Cl	H	O	8.85 ± 2.20	35.22 ± 0.55	55.57 ± 1.11
DMSO (negative control)					3.08 ± 1.29	19.21 ± 1.23	28.75 ± 2.65
100nM 1,25-$(OH)_2D_3$ (positive control)					75.32 ± 2.20		
1 μM ATRA (positive control)					80.08 ± 4.47		

HL-60 cells were treated for 72 h with 1 μM of each of the indirubin derivatives alone, or in combination with 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA. The cell differentiation was assessed by the NBT assay.
Each value represents means ± SEM ($n = 3$).

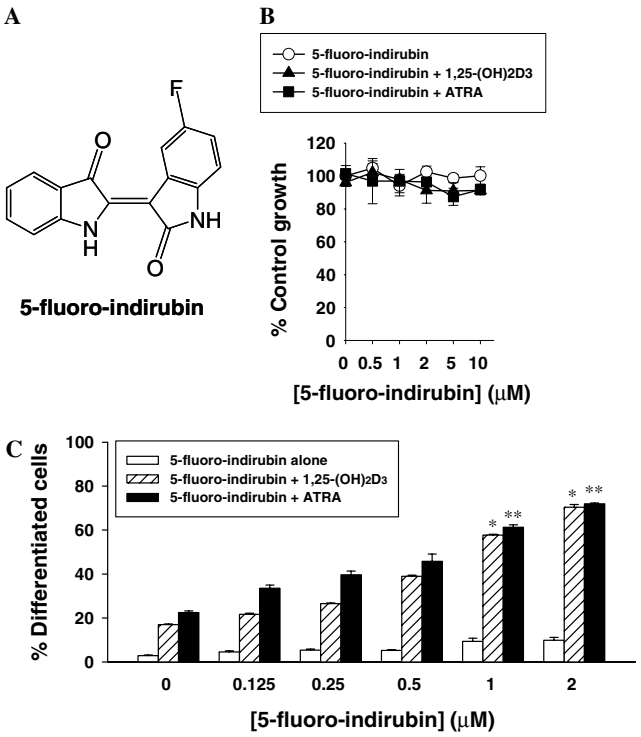


Figure 1. Effects of the indirubin derivative, 5-fluoro-indirubin, on HL-60 leukemia cell proliferation and differentiation. HL-60 leukemia cells were treated with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, or in combination with various concentrations of 5-fluoro-indirubin (chemical structure, A) for 72 h. Afterwards, cellular proliferation and differentiation were evaluated via MTT assay (B) and NBT reduction assay (C). Data are expressed as means ± standard deviations of triplicate determinations from one representative experiment. The differentiation experiment was repeated more than three times with similar results. *$P < 0.01$, relative to a group treated with 5 nM 1,25-$(OH)_2D_3$ alone; **$P < 0.01$, relative to a group treated with 50 nM ATRA alone.

nuclei were banded and segmented (Fig. 2E and F). As shown in Figure 2, some of the cells evidenced horseshoe-shaped nuclei, which is a sign of cell differentiation into a monocytic lineage (Fig. 2E). Some cells also evidenced multilobed nuclei, which is indicative of cell differentiation into a granulocytic lineage (Fig. 2F). Cytofluorometric analysis was also conducted in order to determine the expression of specific surface antigens on the HL-60 cells. HL-60 leukemia cells express a cell surface marker, CD11b, when differentiated into monocytes or granulocytes.[10] As shown in Figure 3A, 5-fluoro-indirubin treatment resulted in an increase in the number of CD11b-positive cells when administered in combination with 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA, thereby confirming that 5-fluoro-indirubin potentiated both 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation.

In order to determine the differentiation pathway exploited by the HL-60 cells upon treatment with 5-fluoro-indirubin and 1,25-$(OH)_2D_3$ or with 5-fluoro-indirubin and ATRA, the HL-60 cells were treated either with 5-fluoro-indirubin alone or in combination with either 1,25-$(OH)_2D_3$ or ATRA, after which cytofluorometric analyses were conducted, using mAb for the CD14 monocytic surface antigen. The CD14 antigen is exclusively expressed when cells differentiate into monocytes.[11] As shown in Figure 3B, the HL-60 cells treated with a combination of 5-fluoro-indirubin and 1,25-$(OH)_2D_3$ stained very strongly with anti-CD14 mAb. The cells treated with 1,25-$(OH)_2D_3$ alone also stained with anti-CD14 mAb, but to a lesser extent than did the cells treated with a mixture of 5-fluoro-indirubin and 1,25-$(OH)_2D_3$. These results indicate that 5-fluoro-indirubin stimulated 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation along the monocytic pathway. By way of contrast, the HL-60 cells treated with a mixture of

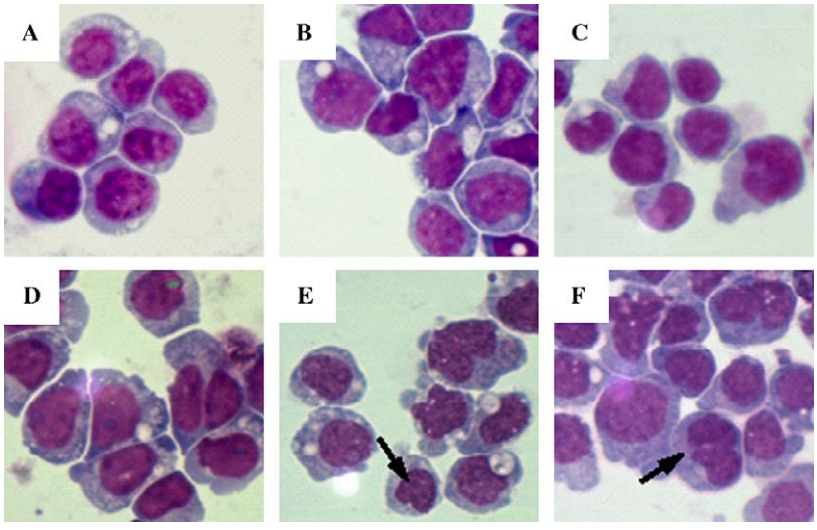


Figure 2. Morphologic analysis of HL-60 cells treated with 5-fluoro-indirubin alone, or in combination with either 1,25-$(OH)_2D_3$ or ATRA. HL-60 cells were treated for 72 h with media alone (A), 5 nM 1,25-$(OH)_2D_3$ (B) or 50 nM ATRA alone (C), 1 μM 5-fluoro-indirubin alone (D), 1 μM 5-fluoro-indirubin plus 5 nM 1,25-$(OH)_2D_3$ (E), or 1 μM 5-fluoro-indirubin plus 50 nM ATRA (F). Cytospin slides were prepared from HL-60 cells (2×10^5 cells/ml) and stained with Giemsa staining solution. The arrows represent horseshoe-shaped nuclei and multilobed nuclei, respectively. The data are representative of three separate experiments.

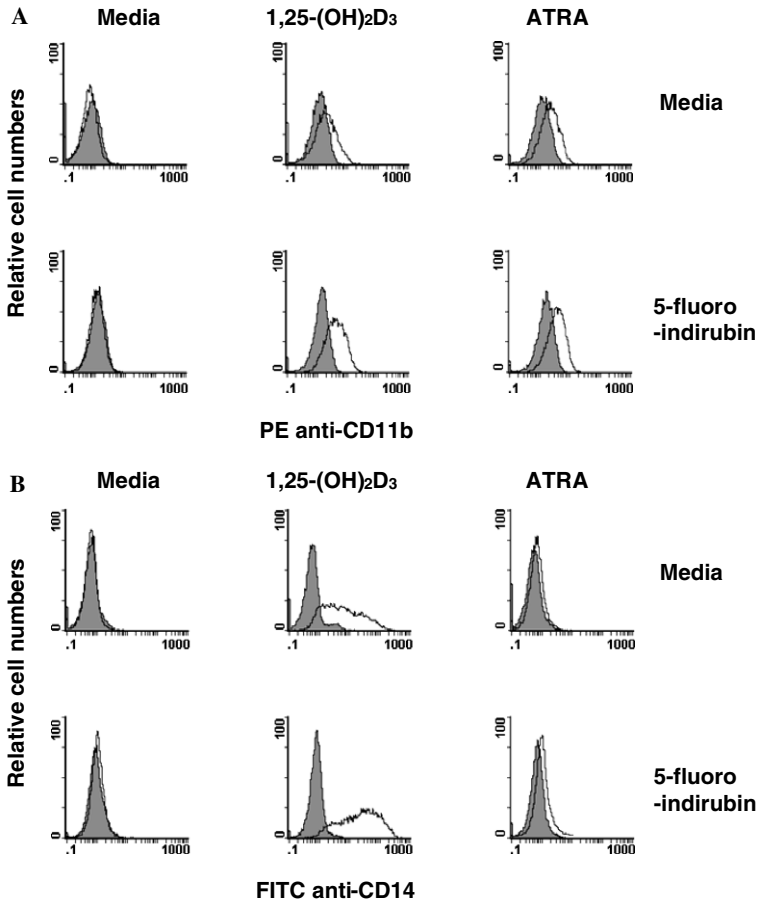


Figure 3. Cytofluorometric analysis of 5-fluoro-indirubin-enhanced HL-60 cell differentiation using mAbs, for the CD11b differentiation marker and for the CD14 monocytic cell surface marker. HL-60 cells were treated for 72 h with media alone, 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, 1 μM 5-fluoro-indirubin alone, 1 μM 5-fluoro-indirubin plus 5 nM 1,25-$(OH)_2D_3$, or 1 μM 5-fluoro-indirubin plus 50 nM ATRA. The cells were assessed via cytofluorometric analysis using PE-conjugated anti-CD11b mAb (A) or FITC-conjugated anti-CD14 (B) (unshaded area), or with the PE- or FITC-conjugated isotype control mAb (shaded area). Data are representative of three independent experiments.

5-fluoro-indirubin and ATRA exhibited only minor staining with anti-CD14 mAb (Fig. 3B), although the synergistic induction of cell differentiation was observed in an NBT reduction assay (Fig. 1). In addition, the HL-60 cells treated with a mixture of 5-fluoro-indirubin and ATRA stained strongly with mAb against the HL-60 cell differentiation marker, CD11b (Fig. 3A), which indicates that 5-fluoro-indirubin stimulated ATRA-induced HL-60 cell differentiation along the granulocytic pathway.

In order to determine the action mechanism by which the indirubin derivative, 5-fluoro-indirubin, potentiates the 1,25-$(OH)_2D_3$- and ATRA-induced differentiation of HL-60 cells, HL-60 cells were treated with enzyme inhibitors specific for: phosphatidylinositol 3-kinase (PI3-K) (wortmannin or LY 294002), protein kinase C (PKC) (H7, chelerythrine or GF 109203X), c-Jun N-terminal kinase (JNK) (SP 600125), extracellular signal-regulated kinase (ERK) (PD 98059), or phospholipase C (PLC) (U 73122), in the presence of 5-fluoro-indirubin, either alone or in combination with 1,25-$(OH)_2D_3$ or ATRA. The degree of cellular differentiation in these cells was subsequently assessed via NBT reduction assays. Activations of PI3-K, PKC, and MAPK are known to be involved in the differentiation of leukemia cells.[12] As shown in Figure 4, the inhibitors for PI3-K, PKC, JNK, and ERK significantly inhibited the HL-60 cell differentiation-enhancing effects associated with 5-fluoro-indirubin in combination with 1,25-$(OH)_2D_3$ or ATRA, and this inhibition occurred in a concentration-dependent manner. The PLC inhibitor evidenced no such effects. Therefore, the indirubin derivative, 5-fluoro-indirubin, potentiates HL-60 cell differentiation via PI3-K, PKC, JNK, and ERK when administered in combination with nontoxic concentrations of 1,25-$(OH)_2D_3$ or ATRA.

3. Discussion

In this study, we demonstrated that indirubin derivatives can potentiate 1,25-$(OH)_2D_3$- and 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 either monocytes or granulocytes when treated with the indirubin derivative, 5-fluoro-indirubin, in combination with either 1,25-$(OH)_2D_3$ or ATRA. Many previous studies have uncovered some chemical

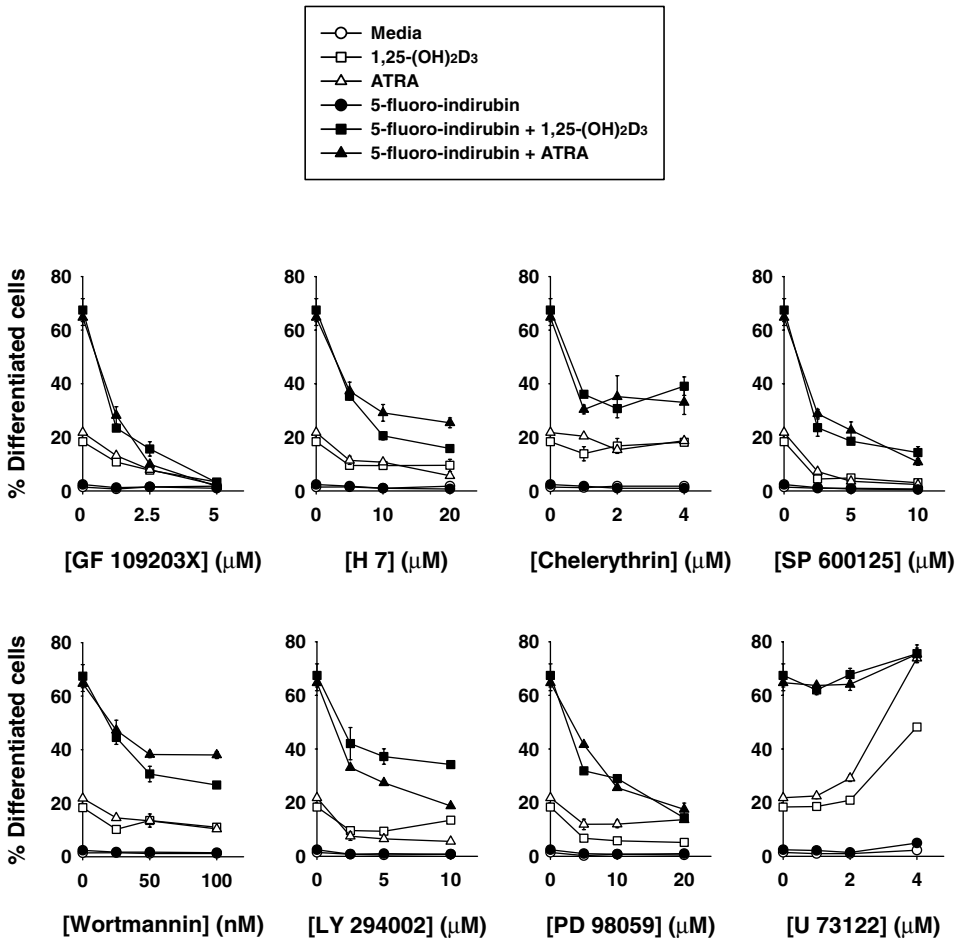


Figure 4. Effects of specific inhibitors for signaling molecules on HL-60 cell differentiation induced by either 1,25-$(OH)_2D_3$ or ATRA in combination with 5-fluoro-indirubin. HL-60 cells were treated for 40 min with various concentrations of PKC inhibitors (GF 109203X, H7, chelerythrine), JNK, inhibitor (SP 600125), PI3-K inhibitors (wortmannin, LY 294002), ERK inhibitor (PD 98059) or PLC inhibitor (U 73122), followed by incubation with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM ATRA alone, or in combination with 1 μM 5-fluoro-indirubin. Cellular differentiation was assessed via NBT reduction assay. The data are expressed as a percentage of differentiated cells with the means ± SEM ($n = 3$).

combinations which exert either an additive or a synergistic effect on the differentiation of HL-60 cells. These combinations include 1,25-$(OH)_2D_3$ with silibinin, tumor necrosis factor-α, or tretinoin tocoferil,[13] as well as retinoic acid with sodium butyrate, dimethylsulfoxide, hexamethylene bisacetamide, or thalidomide.[14]

The mechanism by which 5-fluoro-indirubin potentiates 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation remains to be adequately clarified. 1,25-$(OH)_2D_3$ and ATRA are both 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,[15] and the activation of phosphatidylinositol 3-kinase (PI3-K), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK).[12] In our study, inhibitors of PI3-K, PKC, and ERK significantly inhibited the enhancing effects on HL-60 cell differentiation induced by 5-fluoro-indirubin administered in combination with 1,25-$(OH)_2D_3$ or ATRA. This finding strongly suggests that the potentiation of cell differentiation by 5-fluoro-indirubin in combination with 1,25-$(OH)_2D_3$ or ATRA may occur via a PI3-K/PKC/ERK-mediated signaling pathway. Approximately, 40% of the cells treated with 5-fluoro-indirubin plus 1,25-$(OH)_2D_3$ differentiated into granulocytes in the presence of the PI3-K inhibitors, indicating that PI3-K may be partially involved in the ATRA-induced HL-60 cell differentiation enhanced by 5-fluoro-indirubin. In contrast, ERK and PKC inhibitors completely inhibited the enhancing effects of 5-fluoro-indirubin on both 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation. This suggests that both PKC and ERK may be common signaling components involved in the 5-fluoro-indirubin-associated enhancement of HL-60 cell differentiation into granulocytic and monocytic lineages.

In addition, the fluoro substitution at the R_2 position of indirubin afforded the most profound synergistic effects on HL-60 cell differentiation when combined with 1,25-$(OH)_2D_3$ or ATRA. This indicates that the indirubin derivatives harboring lone pair electron-rich groups, such as halides, might interact preferentially with signaling molecule(s) involved in the enhancement of HL-60 cell differentiation. Indirubin and its analogs have been

shown to selectively inhibit cyclin-dependent kinases (CDKs), via competition with ATP for binding to the catalytic site of the kinase.[6] However, 5-fluoro-indirubin, which evidenced the most profound enhancing effects, had only minimal effects on cell proliferation at the concentrations employed in this study,[9] suggesting that the enhancing effects of the indirubin derivatives on HL-60 cell differentiation observed in this study were not correlated with inhibitory effects on CDK. We also previously reported that some indirubin-3′-oxime analogs have one of the anti-proliferative activities, such as inhibition of CDK2.[9] As shown in Table 1, 5-fluoro-3′-monoxime indirubin (compound **7**), an oxime analog of 5-flouro-indirubin, has no synergistic activities on 1,25-$(OH)_2D_3$- or ATRA-induced HL-60 cell differentiation even though the oxime analog has little effects on HL-60 cell differentiation by itself. These results suggest that the oxime analogs have no synergistic activities with 1,25-$(OH)_2D_3$ or ATRA on HL-60 cell differentiation.

1,25-$(OH)_2D_3$and some of its analogs are also utilized in treatments for psoriasis.[16] ATRA has been employed in the treatment of patients suffering from leukemia.[17] The results of this study suggest that treatment with combinations of 5-fluoro-indirubin and 1,25-$(OH)_2D_3$, or 5-fluoro-indirubin and ATRA, may yield a greater therapeutic response than treatment with 1,25-$(OH)_2D_3$- or ATRA alone, and these combination treatments may also prove less toxic.

In conclusion, the indirubin derivative, 5-fluoro-indirubin, potentiates 1,25-$(OH)_2D_3$- and ATRA-induced HL-60 cell differentiation via the PI3-K/PKC/ERK signaling pathway. These findings imply that the indirubin derivatives might prove useful in the treatment of leukemic diseases.

4. Experimental

4.1. Materials

The HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). All-*trans* retinoic acid, phorbol 12-myristate 13-acetate, 2-[4-morpholinyl]-8-phenyl-1[4*H*]-benzopyran-4-one (LY 294002), 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), wortmannin, anthrapyrazolone (SP 600125), Giemsa staining solution, methanol-free paraformaldehyde, and other reagents were purchased from 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 Tocris Cookson Ltd (UK). Stock solutions of 1 mM ATRA were dissolved in dimethylsulfoxide. The indirubin derivatives were dissolved in dimethylsulfoxide to generate a 20 mM stock solution. The solutions were diluted at least 1000-fold in the growth medium, such that the final dimethylsulfoxide concentration had no effect on the differentiation and proliferation behavior of the HL-60 cells. All manipulations were conducted under subdued light conditions.

4.2. Synthesis of indirubin and indirubin-oxime derivatives

The indirubin derivatives employed in these experiments were synthesized and characterized via high-resolution mass spectrometry and ^{1}H NMR in order to confirm their identity and purity.[9] In brief, to a solution of 176 mg (1 mmol) of indoxyl acetate and 1 mmol of isatin analogs in 5 ml of methanol was added 256 mg (2.5 mmol) of Na_2CO_3 under nitrogen atmosphere. The mixture was stirred for 2–3 h at room temperature. The dark violet precipitate was filtered and washed twice with methanol and several times with cold water and was dried under reduced pressure (yield 50–60%). To a solution of indirubin analogs (1 mmol) in 10 ml of pyridine was added 6 mmol of hydroxylamine hydrochloride. The mixture was refluxed for 2–3 h at 120 °C. After cooling, the product was neutralized with 1 N HCl and the precipitate was filtered and washed with water. Analytical data of compound **6** (5-fluoro-indirubin): mp >330 °C; MS (MALDI-TOF) 280 (M^+); Anal. Calcd for $C_{16}H_9FN_2O_2$: C, 68.57; H, 3.24; N, 10.00; O, 11.42. Found: C, 68.05; H, 2.97; N, 9.85. ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 11.1 (1H, s, N–H), 10.92 (1H, s, N–H), 8.57 (1H, dd, J = 2.7, 10.5 Hz), 7.67 (1H, d, J = 6.9 Hz), 7.43 (2H, m), 7.02 (3H, m).

4.3. Determination of cell viability and growth

Cell viability was evaluated via Trypan blue exclusion assay, as described previously.[18] Viability was calculated as the percentage of live cells in the total cell population. Cell growth was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each of the treatments, 10 μl MTT (5 mg/ml) was added to each well of the 96-well plates. After 4 h of incubation at 37 °C, the crystals of the 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.

4.4. Determination of cell differentiation

HL-60 cell differentiation was assessed via nitroblue tetrazolium reduction assay, as described previously.[19] This assay is based on the ability of phagocytic cells to generate superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested via 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 darkness. Cytospin slides were prepared and examined for blue-black nitroblue diformazan deposits, which are indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed in each experiment.

4.5. Morphologic studies

Single-cell suspensions were prepared and 2×10^5 cells were loaded into a cytofunnel and spun at 500 rpm in

a cytospin centrifuge. The slides were fixed with methanol and dried. The slides were then stained with Giemsa staining solution for 20 min, rinsed in deionized water, air-dried, and observed under a microscope equipped with a camera. The stained cells were evaluated with regard to size, cell margin regularity, and nuclear morphological characteristics.

4.6. Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were conducted using an Epic XL flow cytofluorograph equipped with a multiparameter data acquisition and display system, as described previously.[19] In brief, single-cell suspensions were collected from the various cultures and washed twice in ice-cold phosphate-buffered saline (PBS, pH 7.4). Afterwards, phycoerythrin (PE)-conjugated anti-human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) were added, followed by 1 h of incubation at 4 °C. After incubation, the cells were washed with PBS and fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was conducted. Background staining was performed via the staining of the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. One-parameter fluorescence histograms were generated via the analysis of at least 1×10^4 cells.

4.7. 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 the values of various experimental and control groups. A P value of <0.05 was considered to be significant.

Acknowledgments

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