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Synthesis and anticancer activity of benzyloxybenzaldehyde derivatives against HL-60 cells

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Abstract—A series of benzyloxybenzaldehyde derivatives were prepared and tested against the HL-60 cell line for anticancer activity. Preliminary structure–activity relationships were established. It was discovered that 2-(benzyloxy)benzaldehyde (**17**), 2-(benzyloxy)-4-methoxybenzaldehyde (**26**), 2-(benzyloxy)-5-methoxybenzaldehyde (**27**), 2-(benzyloxy)-5-chlorobenzaldehyde (**28**), 2-[(3-methoxybenzyl)oxy]benzaldehyde (**29**), 2-[(2-chlorobenzyl)oxy]benzaldehyde (**30**), and 2-[(4-chlorobenzyl)oxy]benzaldehyde (**31**) exhibited significant activity at 1–10 μM. Among them, compound **29** was the most potent one. The morphological assessment and DNA fragmentation analysis indicated that these compounds arrested cell cycle progression at G2/M phase and induced cell apoptosis. They resulted in the loss of mitochondrial membrane potential after 12 h of treatment.
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1. Introduction

Apoptosis is essential for normal development, tissue homeostasis, and immune function. Apoptotic cell death is accompanied by the condensation and fragmentation of nuclei, apoptotic body formation, and chromosomal DNA fragmentation into about 180 bp oligomers.[1,2] Abnormal inhibition of apoptosis is a hallmark of cancer and autoimmune disease.[3,4] Inducing apoptosis either directly or after terminal differentiation or after cell cycle arrest has been important approach for cancer prevention or therapy.[5–7]

In our previous work, we have synthesized several benzyloxybenzaldehyde analogues as novel adenyl cyclase activator,[8] and have studied the action mechanism of the representing 2-(benzyloxy)benzaldehyde (**17**;

CCY1a).[9] In addition, we noticed that compound **17** exhibited anti-proliferation effect on the action of serum via the inhibition of the Ras/MAPK signal pathway and its downstream effectors.[10] To pursue our interest further into the anti-proliferation of compound **17** toward cancer cells, in the present work, we synthesized a series of benzyloxybenzaldehyde derivatives (Table 1) and performed pharmacological evaluation of these compounds in terms of their anti-proliferative activity in cancer cells and effects on progression of cell cycle.
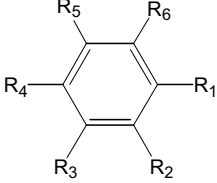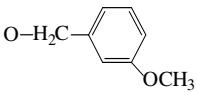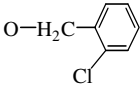
2. Results and discussion
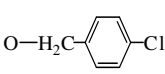
2.1. Chemistry

2.1.1. Preparation of substituted benzyloxybenzenes (17–34). As shown in Scheme 1, the starting substituted phenols (**1–12**) were subjected to *O*-benzylation by reacting with substituted benzyl chloride in THF, in the presence of K_2CO_3, to yield the corresponding substituted benzyloxybenzene derivatives (**17–31**). Substituted benzyloxybenzaldehydes **17–19** were allowed to react with hydroxylamine to afford corresponding substituted benzyloxybenzaldehyde oximes **32–34**.

Table 1. The viability of benzyloxybenzaldehyde derivatives on HL-60 cells

Compound	R_1	R_2	R_3	R_4	R_5	R_6	Viability[a] (%)
17 (CCY1a)	CHO	OCH$_2$Ph	H	H	H	H	32.36 ± 2.25
18	CHO	H	OCH$_2$Ph	H	H	H	86.11 ± 4.75
19	CHO	H	H	OCH$_2$Ph	H	H	97.04 ± 6.85
20	COCH$_3$	OCH$_2$Ph	H	H	H	H	92.62 ± 8.07
21	COCH$_3$	H	OCH$_2$Ph	H	H	H	71.36 ± 6.16
22	COCH$_3$	H	H	OCH$_2$Ph	H	H	102.99 ± 7.06
23	COOCH$_3$	OCH$_2$Ph	H	H	H	H	96.46 ± 6.23
24	CONH$_2$	OCH$_2$Ph	H	H	H	H	100.20 ± 6.28
25	CHO	OCH$_2$Ph	OCH$_3$	H	H	H	98.87 ± 3.04
26	CHO	OCH$_2$Ph	H	OCH$_3$	H	H	40.81 ± 3.00
27	CHO	OCH$_2$Ph	H	H	OCH$_3$	H	51.44 ± 3.06
28	CHO	OCH$_2$Ph	H	H	Cl	H	45.47 ± 5.40
29	CHO	O–H$_2$C–⟨⟩–OCH$_3$	H	H	H	H	24.32 ± 4.98
30	CHO	O–H$_2$C–⟨⟩–Cl	H	H	H	H	25.02 ± 3.55
31	CHO	O–H$_2$C–⟨⟩–Cl	H	H	H	H	35.32 ± 3.25
32	CH=NOH	OCH$_2$Ph	H	H	H	H	101.00 ± 4.31
33	CH=NOH	H	OCH$_2$Ph	H	H	H	90.42 ± 4.29
34	CH=NOH	H	H	OCH$_2$Ph	H	H	97.46 ± 4.51

[a] HL-60 cells were treated with each compound at concentration of 40 μM for 24 h. Viable cells were determined by propidium iodide exclusion method. Percentage of viability was calculated as the ratio of viable cell number in each group to that in ethanol-vehicle control group. The experiments were performed at least three times with similar results. Data are expressed as the means ± SD.
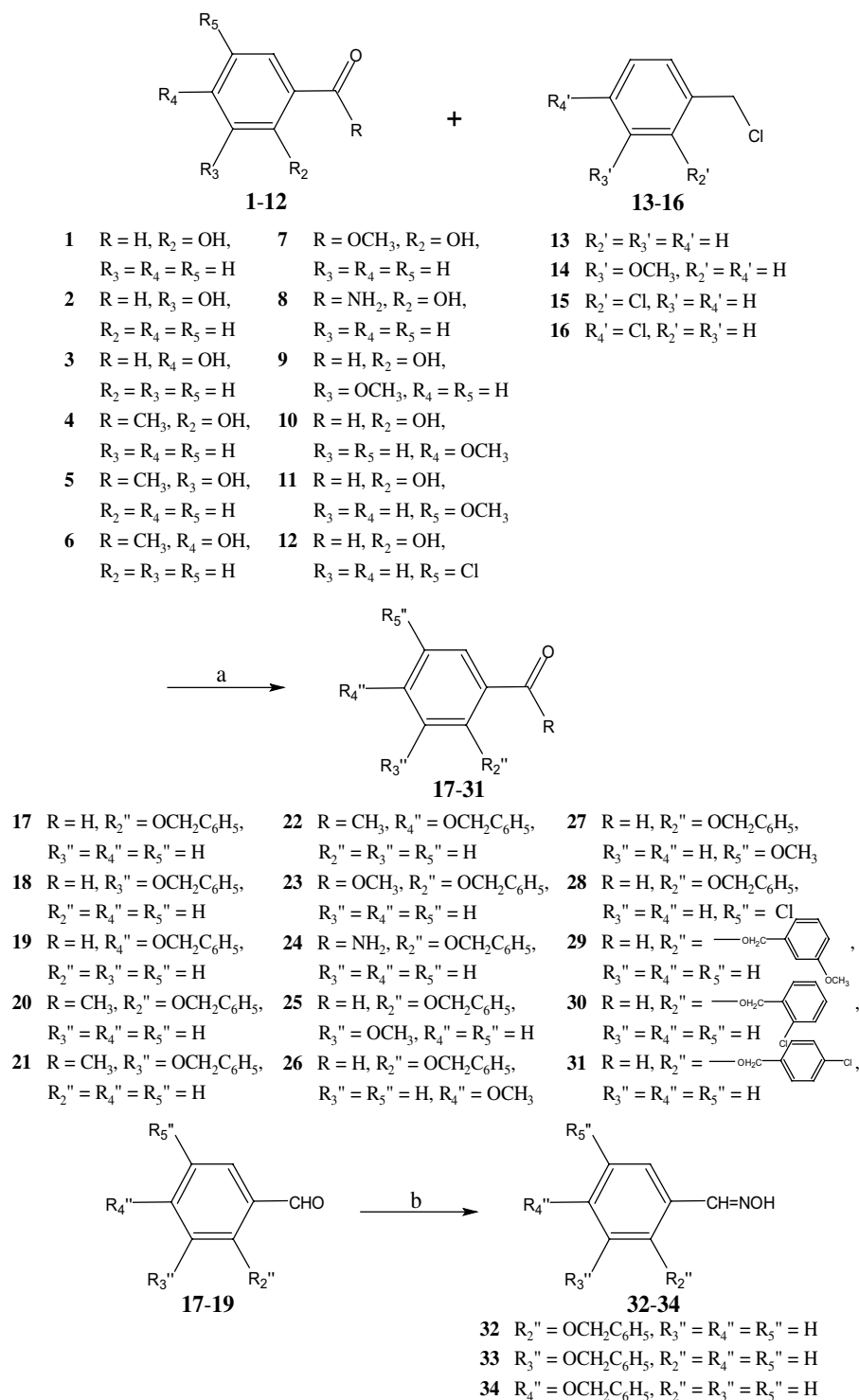
2.2. Cytotoxic effects on HL-60 cells

To evaluate the cytotoxicity of these compounds, we performed the propidium iodide (PI)-exclusion assay. Quercetin and vinblastine were included as positive controls for cytotoxicity assay because they have been demonstrated to inhibit HL-60 growth. The approximate IC$_{50}$s for quercetin and vinblastine on HL-60 cells were 30 and 0.5 μM, respectively. As shown in Table 1, compound **17** demonstrated significant activity, but the structural modification approach to shift its –OCH$_2$Ph group to *meta*-position (**18**) or *para*-position (**19**) resulted in considerably reduced activity. Meanwhile, the attempt to replace the CHO of compound **17** with COCH$_3$ (**20**), COOCH$_3$ (**23**), CONH$_2$ (**24**), or CH=NOH (**32**) also caused significant loss of activity. This seems to suggest that the CHO group is an important contributor for the potency of the compound **17**. In another trial, the introduction of OCH$_3$ into *meta*-position of compound **17** (**25**) also impaired its activity. It appears that most of the 2-(benzyloxy)benzaldehyde derivatives (**26–31**) displaying outstanding cytotoxicity have structure very similar to compound **17**. Among them, 2-[(3-methoxybenzyl)oxy]benzaldehyde (**29**) demonstrated the greatest activity. After treatment for 24 h, **17** and **26–31** compounds demonstrated potent anti-proliferation in a concentration-dependent manner (Fig. 1).

On the contrary, no significant cytotoxicity was observed in peripheral blood mononuclear cell (PBMC) exposed to these compounds at 20 μM for 24 h (data not shown). The above result clearly shows that these active 2-(benzyloxy)benzaldehyde derivatives (**17**, **26–31**) were less toxic for PBMC than for HL-60 cells.

2.3. Induction of apoptosis

To characterize the growth inhibition induced by 2-(benzyloxy)benzaldehyde derivatives, we observed the morphological change under phase-contrast microscope. We also examined nuclear morphology of dying cells stained with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent DNA-binding agent. After treatment with 10 or 20 μM of **17**, **26**, **28**, **29**, **30**, and **31** for 12 h, cells displayed typical morphological features of apoptotic cells, with condensed and fragmented nuclei (Fig. 2A). Furthermore, we performed DNA fragmentation assay to analyze the integrity of chromosomal DNA. DNA from HL-60 cells treated with 20 μM of **17**, **26**, **28**, **29**, **30**, and **31** for 24 h were fragmented as evidenced by the formation of DNA ladder on agarose gels (Fig. 2B). Results from both morphological assessment and DNA fragmentation analysis indicated that these 2-(benzyloxy)benzaldehyde derivatives induce HL-60 cells apoptosis.

1 R = H, R$_2$ = OH, R$_3$ = R$_4$ = R$_5$ = H
2 R = H, R$_3$ = OH, R$_2$ = R$_4$ = R$_5$ = H
3 R = H, R$_4$ = OH, R$_2$ = R$_3$ = R$_5$ = H
4 R = CH$_3$, R$_2$ = OH, R$_3$ = R$_4$ = R$_5$ = H
5 R = CH$_3$, R$_3$ = OH, R$_2$ = R$_4$ = R$_5$ = H
6 R = CH$_3$, R$_4$ = OH, R$_2$ = R$_3$ = R$_5$ = H

7 R = OCH$_3$, R$_2$ = OH, R$_3$ = R$_4$ = R$_5$ = H
8 R = NH$_2$, R$_2$ = OH, R$_3$ = R$_4$ = R$_5$ = H
9 R = H, R$_2$ = OH, R$_3$ = OCH$_3$, R$_4$ = R$_5$ = H
10 R = H, R$_2$ = OH, R$_3$ = R$_5$ = H, R$_4$ = OCH$_3$
11 R = H, R$_2$ = OH, R$_3$ = R$_4$ = H, R$_5$ = OCH$_3$
12 R = H, R$_2$ = OH, R$_3$ = R$_4$ = H, R$_5$ = Cl

13 R$_2$' = R$_3$' = R$_4$' = H
14 R$_3$' = OCH$_3$, R$_2$' = R$_4$' = H
15 R$_2$' = Cl, R$_3$' = R$_4$' = H
16 R$_4$' = Cl, R$_2$' = R$_3$' = H

17 R = H, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = R$_5$" = H
18 R = H, R$_3$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_4$" = R$_5$" = H
19 R = H, R$_4$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_3$" = R$_5$" = H
20 R = CH$_3$, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = R$_5$" = H
21 R = CH$_3$, R$_3$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_4$" = R$_5$" = H

22 R = CH$_3$, R$_4$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_3$" = R$_5$" = H
23 R = OCH$_3$, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = R$_5$" = H
24 R = NH$_2$, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = R$_5$" = H
25 R = H, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = OCH$_3$, R$_4$" = R$_5$" = H
26 R = H, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_5$" = H, R$_4$" = OCH$_3$

27 R = H, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = H, R$_5$" = OCH$_3$
28 R = H, R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = H, R$_5$" = Cl
29 R = H, R$_2$" = —OH$_2$C—(2-OCH$_3$-C$_6$H$_4$), R$_3$" = R$_4$" = R$_5$" = H
30 R = H, R$_2$" = —OH$_2$C—(2-Cl-C$_6$H$_4$), R$_3$" = R$_4$" = R$_5$" = H
31 R = H, R$_2$" = —OH$_2$C—(4-Cl-C$_6$H$_4$), R$_3$" = R$_4$" = R$_5$" = H

32 R$_2$" = OCH$_2$C$_6$H$_5$, R$_3$" = R$_4$" = R$_5$" = H
33 R$_3$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_4$" = R$_5$" = H
34 R$_4$" = OCH$_2$C$_6$H$_5$, R$_2$" = R$_3$" = R$_5$" = H

Scheme 1. Reagents and conditions: (a) K$_2$CO$_3$, KI, reflux, 6 h; (b) NH$_2$OH, EtOH, reflux, 3 h.

2.4. Loss of mitochondrial membrane potential

Mitochondria plays a crucial role in apoptosis.[11] One of the major parameters of mitochondrial dysfunction is the loss of mitochondrial membrane potential ($\Delta\Psi_m$).[12] We studied whether induction of apoptosis by 2-(benzyloxy)benzaldehyde derivatives involves alteration of mitochondrial membrane potential. As shown in Figure 3, 20 µM of **17**, **26**, **28**, and **29** obviously induced loss of mitochondrial membrane potential in HL-60 cells treated for 12 h. However, **32**, less cytotoxic to HL-60 cells, at a concentration of 50 µM did not exhibit significant effect on mitochondrial membrane potential. These results showed that mitochondria-mediated processes may be involved in the induction of apoptosis by 2-(benzyloxy)benzaldehyde derivatives.
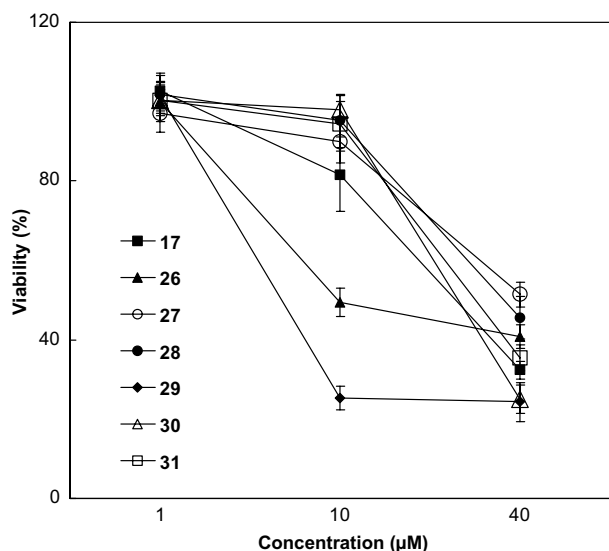
Figure 1. Concentration–response effects of 2-(benzyloxy)benzaldehyde derivatives on cell viability. The HL-60 cells were treated with 2-(benzyloxy)benzaldehyde derivatives for 24 h. The viable cells were measured by PI exclusion and immediately analyzed by flow cytometry. Percentage of viability was calculated as the ratio of viable cell number in each group to that in ethanol-vehicle control group.

2.5. Arrest of cell cycle progression at G2/M phase

In parallel with the apoptosis studies described above, cell cycle analysis was performed to determine whether 2-(benzyloxy)benzaldehyde derivatives influence the progression of cell cycle. Exposure of HL-60 cells to **17**, **26**, **28**, **29**, **30**, and **31** for 12 or 24 h resulted in a significant increase in percentage of cells in G2/M phase, accompanied by the decrease in percentage of cells in G1 phase (Fig. 4). These results demonstrate that 2-(benzyloxy)benzaldehyde derivatives arrested cell cycle progression at G2/M phase after 12 h of treatment.

We also compared the growth inhibitory effects of 2-(benzyloxy)benzaldehyde derivatives on other human leukemia, lung cancer, and liver cancer cell lines. However, there were no significant effects of growth inhibition and cell cycle progression both on Hep G2 and Hep 3B (hepatocellular carcinoma cell lines) at 30 μM concentration for 48 h of treatment (data not shown). Moreover, based on cell morphology and NBT reduction assay, these compounds neither induce HL-60 cells differentiation alone nor promote all-*trans* retinoic acid-induced differentiation (data not shown).

3. Conclusion

In summary, we report here that 2-(benzyloxy)benzaldehyde derivatives exert anti-proliferation activities on HL-60 cells and that 2-[(3-methoxybenzyl)oxy]benzaldehyde (**29**) is the most potent compound. We found that the induction of cell cycle arrest at G2/M phase and apoptosis are the main inhibitory effects of
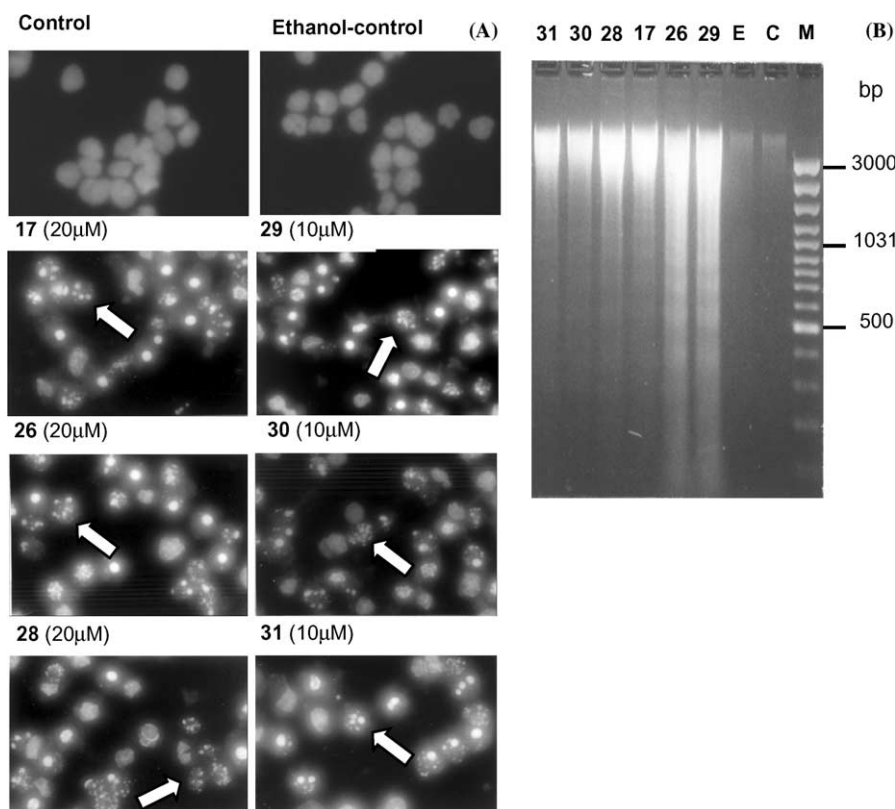
Figure 2. Induction of apoptosis by 2-(benzyloxy)benzaldehyde derivatives in HL-60 cell. (A) Nuclear morphology of HL-60 cells stained with 4′,6-diamidino-2-phenylindole (DAPI) after 12 h of treatment with 2-(benzyloxy)benzaldehyde derivatives. (B) Analysis of DNA fragmentation after 24 h of treatment with 20 μM 2-(benzyloxy)benzaldehyde derivatives. DNA extracts were electrophoresed in 1.0% agarose gel. M, DNA ladder marker; C, control; E, ethanol-control.
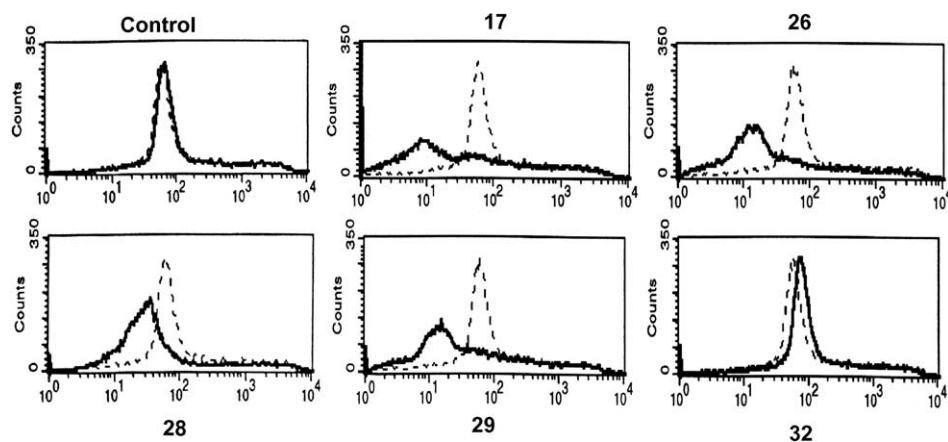
Figure 3. Inducing loss of mitochondrial membrane potential by 2-(benzyloxy)benzaldehyde derivatives. After 12 h of treatment with 20 μM 2-(benzyloxy)benzaldehyde derivatives (except 50 μM **32**), HL-60 cells were directly stained with 40 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)). Fluorescence intensity of cells was measured by flow cytometry. Dotted lines: vehicle control. Solid lines: compounds.
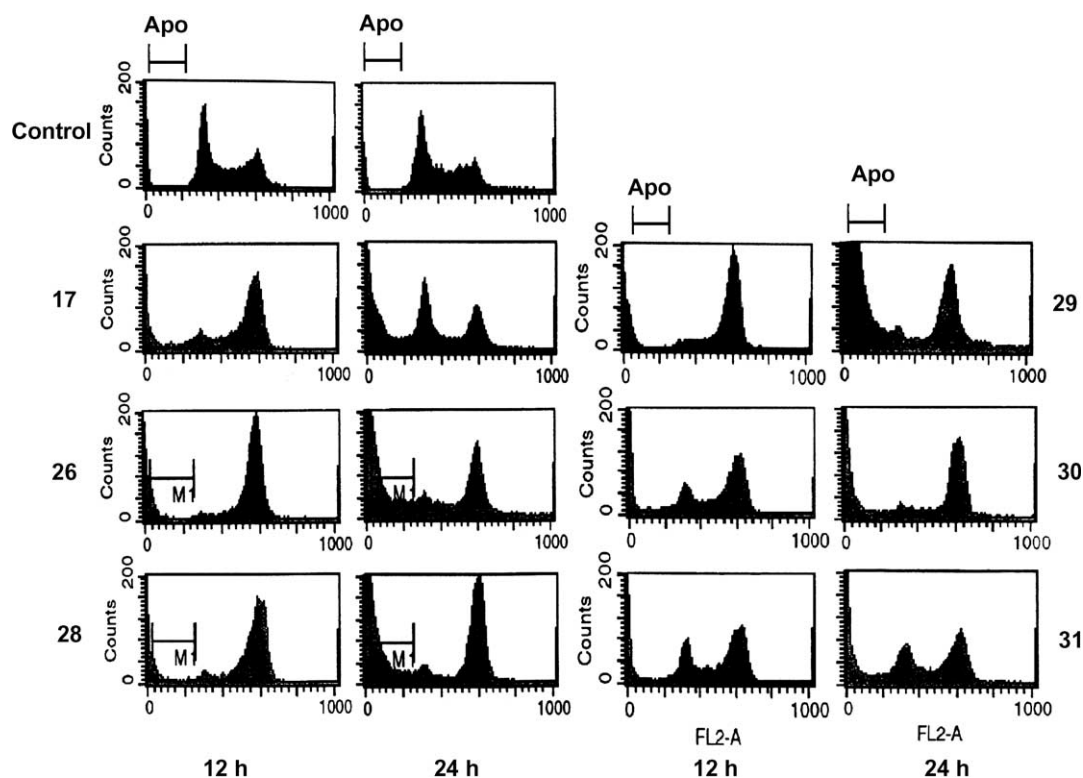
Figure 4. Effects of 2-(benzyloxy)benzaldehyde derivatives on cell cycle progression. HL-60 cells were treated with 10 μM 2-(benzyloxy)benzaldehyde derivatives for 12 or 24 h followed by incubation with hypotonic PI solution. DNA content was immediately analyzed by flow cytometry. [*]Apo indicated the Sub-G1 nuclei.

2-(benzyloxy)benzaldehyde derivatives on HL-60 cells. The mechanism of action exerted by **29** is under investigation and will be reported separately.

4. Experimental

All of the materials were obtained commercially (guaranteed reagent grade) 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 on a Yanaco MP-500D melting point apparatus and are uncorrected. IR spectra were recorded on Shimadzu IR-440 and Nicolet Impact 400 FT-IR spectrophotometers as KBr pellets. NMR spectra were obtained on a Bruker Avance DPX-200 FT-NMR spectrometer in CDCl$_3$. The following abbreviations are used: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; m, multiplet; and br,

broad. MS spectra were measured with an HP 5995 GC–MS instrument. The UV spectra were recorded on a Shimadzu UV-160A UV–vis recording spectrophotometer as methanolic solutions. Elemental analyses (C, H, 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. Preparation of substituted benzyloxybenzenes (17–31)

Substituted phenols (1–12) (0.1 mol) was dissolved in THF (100 mL). To the solution was added substituted benzyl chlorides (13–16) (0.13 mol), K_2CO_3 (20 g), and KI (26 g). The resulting mixture was stirred under reflux for 6 h. The solvent was removed in vacuo. Water (100 mL) was added and the mixture was extracted with $CHCl_3$, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography over silica gel, eluting with n-hexane/EtOAc to yield the corresponding substituted benzyloxybenzenes (17–31) (Table 1).

4.1.1. 2-(Benzyloxy)benzaldehyde (17).[8] Yield, 87%; mp 48–49 °C; MS (EI, 70 eV): m/z 212 (M$^+$); Found: C, 79.17; H, 5.54. $C_{14}H_{12}O_2$ requires: C, 79.25; H, 5.66; UV λ_{max} (log ε): 215.4 (4.46), 253.6 (4.00), 318.2 (3.89); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.20 (s, 2H), 7.01–7.09 (m, 2H), 7.35–7.48 (m, 5H), 7.54 (ddd, 1H, J = 8.2, 7.6, 1.9 Hz), 7.87 (dd, 1H, J = 8.0, 2.0 Hz), 10.58 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 70.24, 112.82, 120.80, 124.96, 127.08, 128.06, 128.23, 128.52, 135.70, 135.86, 160.84, 189.54.

4.1.2. 3-(Benzyloxy)benzaldehyde (18).[8] Yield, 92%; mp 57–58 °C; MS (EI, 70 eV): m/z 212 (M$^+$); Found: C, 79.20; H, 5.59. $C_{14}H_{12}O_2$ requires: C, 79.25; H, 5.66; UV λ_{max} (log ε): 215.0 (4.57), 251.0 (4.46), 311.0 (3.92); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.13 (s, 2H), 7.23–7.32 (m, 1H), 7.34–7.49 (m, 8H), 9.98 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 0.01, 113.07, 121.97, 123.45, 127.32, 127.99, 128.46, 129.90, 136.09, 137.61, 159.10, 191.85.

4.1.3. 4-(Benzyloxy)benzaldehyde (19).[8] Yield, 92%; mp 67–68 °C; MS (EI, 70 eV): m/z 212 (M$^+$); Found: C, 79.13; H, 5.56. $C_{14}H_{12}O_2$ requires: C, 79.25, H, 5.66; UV λ_{max} (log ε): 214.0 (4.60), 252.0 (4.23), 315.0 (3.91); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.16 (s, 2H), 7.09 (d, 2H, J = 8.8 Hz), 7.35–7.47 (m, 5H), 7.85 (d, 2H, J = 8.8 Hz), 9.89 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 70.05, 114.93, 127.26, 128.11, 128.51, 129.91, 131.78, 135.73, 163.52, 190.57.

4.1.4. 1-[2-(Benzyloxy)phenyl]ethanone (20).[8] Yield, 70%; mp 41–42 °C; MS (EI, 70 eV): m/z 226 (M$^+$); Found: C, 79.58; H, 6.11. $C_{15}H_{14}O_2$ requires: C, 79.65; H, 6.19; UV λ_{max} (log ε): 211.0 (4.57), 246.0 (4.10), 305.0 (3.80); IR (KBr): 1665 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 2.63 (s, 3H), 5.17 (s, 2H), 7.02–7.06 (m, 2H), 7.39–7.50 (m, 6H), 7.78 (dd, 1H, J = 8.0, 2.0 Hz); ^{13}C NMR (50 MHz, CDCl$_3$): δ 31.98, 70.40, 112.58, 120.66, 127.38, 128.05, 128.42, 128.51, 130.27, 133.47, 135.98, 157.82, 199.76.

4.1.5. 1-[3-(Benzyloxy)phenyl]ethanone (21). Yield, 75%; MS (EI, 70 eV): m/z 226 (M$^+$); Found: C, 79.57; H, 6.14. $C_{15}H_{14}O_2$ requires: C, 79.65; H, 6.19; UV λ_{max} (log ε): 216.0 (4.60), 249.0 (4.12), 305.0 (3.62); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 2.60 (s, 3H), 5.12 (s, 2H), 7.19 (ddd, 1H, J = 8.2, 2.4, 1.1 Hz), 7.30–7.49 (m, 6H), 7.54–7.60 (m, 2H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 26.53, 69.95, 113.32, 120.07, 121.11, 127.36, 127.93, 128.44, 129.42, 136.27, 138.27, 158.74, 197.71.

4.1.6. 1-[4-(Benzyloxy)phenyl]ethanone (22). Yield, 93%; mp 89–90 °C; MS (EI, 70 eV): m/z 226 (M$^+$); Found: C, 79.62; H, 6.10. $C_{15}H_{14}O_3$ requires: C, 79.65; H, 6.19; UV λ_{max} (log ε): 211.0 (4.46), 269.0 (4.57); IR (KBr): 1678 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 2.56 (s, 3H), 5.14 (s, 2H), 7.02 (d, 2H, J = 8.9 Hz), 7.37–7.45 (m, 5H), 7.95 (d, 2H, J = 8.9 Hz); ^{13}C NMR (50 MHz, CDCl$_3$): δ 26.12, 69.90, 114.32, 127.24, 128.02, 128.47, 130.38, 130.48, 135.95, 162.39, 196.54.

4.1.7. Methyl 2-(benzyloxy)benzoate (23). Yield, 87%; MS (EI, 70 eV): m/z 242 (M$^+$); Found: C, 74.18; H, 5.74. $C_{15}H_{14}O_3$ requires: C, 74.30; H, 5.78; UV λ_{max} (log ε): 211.0 (4.60), 294.0 (4.60); IR (KBr): 1728 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 3.92 (s, 3H), 5.19 (s, 2H), 6.97–7.04 (m, 2H), 7.32–7.54 (m, 6H), 7.85 (dd, 1H, J = 8.0, 2.0 Hz); ^{13}C NMR (50 MHz, CDCl$_3$): δ 51.79, 70.35, 113.67, 120.36, 120.55, 126.59, 127.55, 128.32, 131.55, 133.19, 136.56, 156.90, 166.78.

4.1.8. 2-(Benzyloxy)benzamide (24).[8] Yield, 95%; mp 114–115 °C; MS (EI, 70 eV): m/z 227 (M$^+$); Found: C, 74.08; H, 5.65; N, 6.14. $C_{14}H_{13}NO_2$ requires: C, 74.00; H, 5.73; N, 6.17; UV λ_{max} (log ε): 210.0 (4.57), 290.0 (4.43); IR (KBr): 1647 (C=O) cm^{-1}; 3406 (NH$_2$); ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.19 (s, 2H), 6.08 (br, 1H), 7.05–7.14 (m, 2H), 7.38–7.52 (m, 6H), 7.75 (br, 1H), 8.25 (dd, 1H, J = 7.8, 1.9 Hz); ^{13}C NMR (50 MHz, CDCl$_3$): δ 71.06, 112.45, 120.92, 121.29, 127.64, 128.49, 128.75, 132.46, 133.12, 135.34, 156.90, 166.78.

4.1.9. 2-(Benzyloxy)-3-methoxybenzaldehyde (25). Yield, 89%; mp 58–59 °C; MS (EI, 70 eV): m/z 242 (M$^+$); Found: C, 74.21; H, 5.83. $C_{15}H_{14}O_3$ requires: C, 74.36; H, 5.82; UV λ_{max} (log ε): 207.0 (4.44), 259.6 (4.42); IR (KBr): 1693 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 3.94 (s, 3H), 5.18 (s, 2H), 7.10–7.20 (m, 2H), 7.33–7.40 (m, 6H), 10.23 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 56.01, 76.28, 117.98, 118.99, 124.26, 128.53, 128.59, 128.67, 130.29, 136.34, 151.05, 153.06, 190.32.

4.1.10. 2-(Benzyloxy)-4-methoxybenzaldehyde (26). Yield, 84%; mp 65–66 °C; MS (EI, 70 eV): m/z 242 (M$^+$); Found: C, 74.12; H, 5.81. $C_{15}H_{14}O_3$ requires: C, 74.36; H, 5.82; UV λ_{max} (log ε): 208.0 (4.65), 273.5 (4.56), 312.0 (4.41); IR (KBr): 1675 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 3.85 (s, 3H), 5.17 (s, 2H), 6.52 (d, 1H, J = 2.2 Hz), 6.57 (dd, 1H, J = 8.6, 0.6 Hz), 7.38–7.45 (m, 5H), 7.85 (d, 1H, J = 8.6 Hz), 10.39 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 55.40, 70.21,

99.02, 105.96, 119.10, 127.05, 128.07, 128.51, 130.27, 135.72, 162.54, 165.83, 188.05.

4.1.11. 2-(Benzyloxy)-5-methoxybenzaldehyde (27).
Yield, 80%; mp 47–48 °C; MS (EI, 70 eV): m/z 242 (M$^+$); Found: C, 74.29; H, 5.78. $C_{15}H_{14}O_3$ requires: C, 74.36; H, 5.82; UV λ_{max} (logε): 223.0 (4.54), 257.0 (4.11), 346.0 (3.89); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 3.81 (s, 3H), 5.16 (s, 2H), 7.01 (d, 1H, $J = 9.0$ Hz), 7.12 (dd, 1H, $J = 9.0, 3.2$ Hz), 7.34–7.44 (m, 6H), 10.52 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 116.02, 121.60, 125.83, 126.50, 126.96, 126.93, 128.16, 132.06, 135.40, 157.04, 188.06.

4.1.12. 2-(Benzyloxy)-5-chlorobenzaldehyde (28).
Yield, 85%; mp 78–79 °C; MS (EI, 70 eV): m/z 246 (M$^+$); Found: C, 68.20; H, 4.40. $C_{14}H_{12}O_2Cl$ requires: C, 68.29; H, 4.47; UV λ_{max} (logε): 219.0 (4.48), 251.0 (4.48), 330.0 (3.58); IR (KBr): 1682 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.19 (s, 2H), 7.01 (d, 1H, $J = 8.9$ Hz), 7.36–7.43 (m, 5H), 7.47 (dd, 1H, $J = 8.9$, 2.7 Hz), 7.81 (d, 1H, $J = 2.8$ Hz), 10.48 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 70.72, 114.53, 125.87, 126.51, 127.10, 127.80, 128.26, 128.59, 135.12, 135.35, 159.21, 188.16.

4.1.13. 2-[(3-Methoxybenzyl)oxy]benzaldehyde (29).
Yield, 89%; mp 73–74 °C; MS (EI, 70 eV): m/z 242 (M$^+$); Found: C, 74.32; H, 5.71. $C_{15}H_{14}O_3$ requires: C, 74.38; H, 5.79; UV λ_{max} (logε): 214.0 (4.57), 252.0 (4.11), 319.0 (3.79); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 3.83 (s, 3H), 5.18 (s, 2H), 6.90 (ddd, 1H, $J = 7.8, 2.4, 1.2$ Hz), 7.01 (d, 1H, $J = 9.0$ Hz), 7.00–7.09 (m, 4H), 7.31 (dd, 1H, $J = 8.1, 6.4$ Hz), 7.54 (ddd, 1H, $J = 7.9, 7.8, 1.9$ Hz), 7.87 (dd, 1H, $J = 7.9, 1.9$ Hz), 10.58 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 55.05, 70.09, 112.58, 112.80, 113.39, 119.16, 120.81, 124.95, 128.29, 129.59, 135.68, 137.44, 159.69, 160.77, 189.52.

4.1.14. 2-[(2-Chlorobenzyl)oxy]benzaldehyde (30).
Yield, 85%; mp 86–87 °C; MS (EI, 70 eV): m/z 246 (M$^+$); Found: C, 68.21; H, 4.40. $C_{14}H_{11}O_2Cl$ requires: C, 68.29; H, 4.47; UV λ_{max} (logε): 214.0 (4.67), 252.0 (4.37), 315.0 (4.04); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.29 (s, 2H), 7.04–7.11 (m, 2H), 7.27–7.46 (m, 3H), 7.52–7.60 (m, 2H), 7.88 (dd, 1H, $J = 7.9, 1.8$ Hz), 10.59 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 67.51, 112.82, 121.06, 125.05, 126.89, 128.47, 128.51, 129.17, 129.34, 132.46, 133.57, 135.72, 160.49, 189.38.

4.1.15. 2-[(4-Chlorobenzyl)oxy]benzaldehyde (31).
Yield, 85%; mp 80–81 °C; MS (EI, 70 eV): m/z 246 (M$^+$); Found: C, 68.27; H, 4.41. $C_{14}H_{11}O_2Cl$ requires: C, 68.29; H, 4.47; UV λ_{max} (logε): 218.0 (4.53), 253.0 (4.51), 318.0 (3.72); IR (KBr): 1685 (C=O) cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.16 (s, 2H), 7.00–7.10 (m, 2H), 7.38 (m, 4H), 7.54 (ddd, 1H, $J = 7.9, 7.8, 1.9$ Hz), 7.86 (dd, 1H, $J = 7.7, 1.8$ Hz), 10.54 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 69.47, 112.69, 121.05, 124.96, 128.40, 128.48, 128.72, 133.91, 134.33, 135.69, 160.50, 189.33.

4.2. Preparation of substituted benzyloxybenzaldehyde oximes (32–34)

To a solution of substituted benzyloxybenzaldehydes **17**–**19** (20 mmol) in EtOH (150 mL) was added hydroxylamine (2.8 g, 40 mmol) and then five drops of acetic acid were added. After reflux for 3 h, the reaction mixture was evaporated in vacuo. The residue was chromatographed through a silica gel column with *n*-hexane/EtOAc to give the corresponding substituted benzyloxybenzaldehyde oximes **32**–**34** (Table 1).

4.2.1. 2-(Benzyloxy)benzaldehyde oxime (32).
Yield, 83%; mp 62–63 °C; MS (EI, 70 eV): m/z 227 (M$^+$); Found: C, 74.05; H, 5.67; N, 6.19. $C_{14}H_{13}NO_2$ requires: C, 74.01; H, 5.73; N, 6.17; UV λ_{max} (logε): 212.0 (4.63), 253.5 (4.00), 304.0 (4.03); ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.13 (s, 2H), 6.95–7.02 (m, 2H), 7.30–7.46 (m, 6H), 7.76 (dd, 1H, $J = 8.0, 2.0$ Hz), 8.44 (s, 1H), 8.60 (s, 1H,); ^{13}C NMR (50 MHz, CDCl$_3$): δ 70.13, 112.37, 120.74, 120.89, 126.44, 127.11, 127.85, 128.42, 131.01, 136.31, 146.29, 156.51.

4.2.2. 3-(Benzyloxy)benzaldehyde oxime (33).
Yield, 89%; mp 81–82 °C; MS (EI, 70 eV): m/z 227 (M$^+$); Found: C, 74.03; H, 5.70; N, 6.21. $C_{14}H_{13}NO_2$ requires: C, 74.01; H, 5.73; N, 6.17; UV λ_{max} (logε): 216.0 (4.52), 256.0 (4.18), 298.0 (3.81); ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.09 (s, 2H), 7.03 (ddd, 1H, $J = 8.1, 2.6, 1.0$ Hz), 7.16 (d, 1H, $J = 7.6$ Hz), 7.25–7.47 (m, 7H), 8.14 (s, 1H), 8.34 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 69.84, 112.11, 116.94, 120.09, 127.32, 127.84, 128.39, 129.63, 133.08, 136.45, 150.08, 158.82.

4.2.3. 4-(Benzyloxy)benzaldehyde oxime (34).
Yield, 87%; mp 105–106 °C; MS (EI, 70 eV): m/z 227 (M$^+$); Found: C, 73.98; H, 5.71; N, 6.18. $C_{14}H_{13}NO_2$ requires: C, 74.01; H, 5.73; N, 6.17; UV λ_{max} (logε): 209.0 (4.39), 264.0 (4.41); ^{1}H NMR (200 MHz, CDCl$_3$): δ 5.10 (s, 2H), 7.00 (d, 2H, $J = 8.8, 1.0$ Hz), 7.34–7.46 (m, 5H), 7.53 (d, 2H, $J = 8.8$ Hz), 8.11 (s, 1H), 8.33 (s, 1H); ^{13}C NMR (50 MHz, CDCl$_3$): δ 69.84, 114.94, 124.63, 127.25, 127.89, 128.30, 128.43, 136.31, 149.67, 160.01.

4.3. Cell culture

The human HL-60 leukemia cells were obtained from the Culture Collection and Research Center (CCRC, Hsiu-Chu, Taiwan). Cells were cultured in RPMI-1640 (GIBCO BRL, Life Technologies, MD, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (HyClone, UT, USA) at 37 °C, 5% CO$_2$ in a humidified incubator. All the compounds were dissolved in ethanol. Control experiments were performed with ethanol (0.1%) as the vehicle.

4.4. Cell proliferation and viability assay[13]

HL-60 cells were resuspended at 2.5×10^5 cells/mL in RPMI-1640 medium and treated with different concentrations of **17**–**34**. At the indicated time of treatment,

cells were collected, resuspended in phosphate-buffered saline (PBS) containing 4 μg/mL PI. The number of viable cells was analyzed by flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA). The percentage of cell viability was calculated as a ratio between compound-treated cells and 0.1% vehicle-control cells.

4.5. Analysis of apoptotic cells by DAPI staining

Treated cells (2.5×10^5 cells/mL) were collected, resuspended, and fixed in 4% paraformaldehyde in PBS. After PBS washing, cells were stained with DAPI for 30 min followed by another PBS wash. The stained cells were examined under a fluorescent microscope (Zeiss, Axiovert 25, Germany).

4.6. Evaluation of apoptosis by DNA fragmentation[14]

After indicated treatment, cells were collected and lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.2% Triton X-100). The cell lysates were treated with 0.1 μg/mL proteinase K at 50 °C overnight, followed by 50 μg/mL RNase A digestion at 37 °C for 30 min. After precipitation, the DNA pellets were subjected to electrophoresis in a 1.0% agarose gel and ethidium bromide staining and visualized by UV exposure.

4.7. Measurement of mitochondrial transmembrane potential

Loss of mitochondrial transmembrane potential was monitored according to the method of Wang et al.[15] Briefly, HL-60 cells were exposed to 2-(benzyloxy)benzaldehyde derivatives and directly stained with 40 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (Molecular Probes, Eugene, Oregon, USA) for 15 min at 37 °C in the dark. Fluorescence intensity of stained cells was measured by flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA).

4.8. Cell cycle analysis[16]

After the indicated treatments, cells were harvested, washed twice with PBS, and resuspended at 1×10^6 cells/mL in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL PI. After 30 min of incubation, the fluorescence intensity of PI-stained nuclei in 10,000 cells was analyzed by flow

cytometry. The cell cycle phases and apoptotic nuclei were analyzed by ModFit software (Becton Dickinson). The ratio of apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated at the sub-G0/G1 peak.

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