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SYNTHESIS OF 4-ARYLPIPERIDIN-4-OL DERIVATIVES OF LOPERAMIDE AS AGENTS WITH POTENT ANTIPROLIFERATIVE EFFECTS AGAINST HCT-116 AND HL-60 CELLS

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This paper is dedicated to Professor Victor Snieckus for his 77th birthday.

Abstract – The structure of 4-arylpiperidin-4-ol, a constituent of the antidiarrheal loperamide, is key to μ opioid receptor activation. Some opioid derivatives were recently reported to induce tumor cell death, but the chemical structures responsible for their antitumor activity remain unclear. We synthesized loperamide analogs and tested their antiproliferative activity against HCT-116 colon tumor cells and HL-60 leukemia cells. The *N*-substituents on 4-arylpiperidin-4-ol units were found to play an important role in their antiproliferative activity, and the *N*-diphenylpropanol analogs exhibited the most potent antiproliferative activity. Furthermore, the *N*-diphenylpropanol analog activated caspase-3, as was found previously for opioids that exhibited antitumor effects.

Opioid analgesics, such as morphine, are widely used to treat severe pain, such as postoperative pain and cancer pain. Three opioid receptor subtypes were cloned in the early 1990s and were classified as G protein-coupled receptors.¹⁻⁴ Opioid receptors not only inhibit adenylyl cyclase through Gi protein, but also activate mitogen-activated protein kinases⁵ and phosphatidylinositol 3-kinases.⁶ These activation of these kinases by opioid receptors leads to cell proliferation and survival.⁷ The administration of high doses of morphine was recently reported to extend the life span of advanced cancer patients,⁸ suggesting that high doses or chronic opiate treatment might have anticancer activity.⁹ Morphine (**1**) displays anticancer activity against BALB/3T3 and KATO III tumor cells, and the inhibition of protein kinase C activation is considered to be the mechanism responsible for this effect.^{8,10} In human lung carcinoma A549 cells, buprenorphine (**2**) and loperamide (**3**) inhibit cell growth and induce DNA fragmentation. Buprenorphine also activates caspase-3 in A549 cells, which is suggestive of apoptosis induction.¹¹ These findings indicate that opiate analogs induce tumor cell apoptosis, but the molecular mechanism responsible for these effects remain unclear because a very high dose of opioids is required for the antitumor activity.

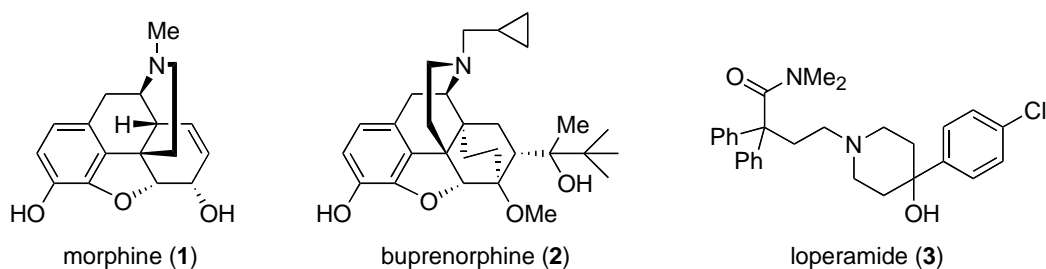
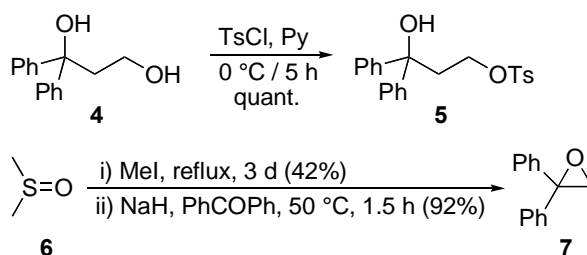


Figure 1. Structures of morphine (**1**) and its antitumor analogs (**2** and **3**)

Morphine and loperamide as μ opioid receptor agonists, and buprenorphine as a partial μ opioid receptor agonist and κ opioid receptor antagonist, all of them have induced apoptosis in tumor cells, however, the other specific agonists or antagonists for opioid receptor subtypes did not inhibit the tumor cell viability.¹¹ Furthermore, some tumor cells that do not express opioid receptors exhibited morphine-mediated apoptosis. These results suggest that the chemical structures required for the antitumor activity of opioid analogs might be quite different from the reported pharmacophore to each opioid receptor subtype.¹² Loperamide (**3**) consists of a 4-aryl piperidin-4-ol unit and a *N*-substituent unit on piperidine backbone. In this study, we synthesized loperamide analogs, in which the 4-aryl group and *N*-substituent were altered, and assessed their antiproliferative effects against human HCT-116 colon tumor and human HL-60 leukemia-derived HL-60 cells.

To synthesize loperamide derivatives, we planned to alkylate the amino groups on piperidine molecules using various reagents. The preparations of alkylating reagents **5** and **7** were outlined in Scheme 1.

The primary alcohol on the diol **4**, which was synthesized according to the reported procedure,^{13,14} was selectively converted to the corresponding tosylate **5** in quantitative yield.¹⁵ The structure of oxirane **7**, which was synthesized from dimethylsulfoxide (**6**),^{16,17} was confirmed by ¹H-NMR without purification, and **7** was immediately reacted with arylpiperidine due to its instability.



Scheme 1. Synthesis of alkylating reagents **5** and **7**

Table 1. Synthesis of the loperamide derivatives

alkylating reagent	+ piperidine	conditions CH ₃ CN	loperamide derivative
Alkylating reagent	Piperidine	Conditions	Product
5		Na ₂ CO ₃ (2.0 eq.) reflux (16 h)	 10a (84%)
5		Na ₂ CO ₃ (2.0 eq.) reflux (16 h)	 10b (90%)
5		Na ₂ CO ₃ (2.0 eq.) reflux (16 h)	 10c (82%)
5		Na ₂ CO ₃ (2.0 eq.) reflux (16 h)	 10d (89%)
7		LiClO ₄ (2.0 eq.) reflux (10 h)	 11a (81%)
		Na ₂ CO ₃ (2.0 eq.) rt (3 h)	 12a (49%)

The alkylation of the amino groups on the piperidine molecules was shown in Table 1. Using tosylate **5** as the alkylating reagent, 1,1-diphenylpropanol derivatives **10a-d** were afforded in yields greater than 80%. The 1,1-diphenylethanol derivative **11a** was synthesized in 81% yield from oxirane **7** and 4-(*p*-chlorophenyl)piperidin-4-ol (**9a**) in the presence of lithium perchlorate.¹⁸ The use of propyl bromide (**8**) as an alkylating reagent resulted in a low yield of *N*-propyl derivative **12a** due to the release of dipropylated ammonium as a byproduct of the reaction.

Opioid analgesics, including loperamide (**3**), are mainly comprised of two chemical units: 4-arylpiperidine as a core receptor-activating unit and an *N*-substituent as a modulating unit for receptor binding.¹² The addition of lipophilic moieties, such as phenyl or diphenyl group attached to 2 or 3 carbons, to the nitrogen atom of 4-arylpiperidine has been reported to significantly increase the affinity of these molecules for μ opioid receptor; i.e., *N*-phenethyl and phenylpropyl analogs exhibited increased binding activity.¹⁹ On the other hand, the non-opioid clofedanol, which possesses a ω -amino-diphenylpropyl unit like loperamide, but not a 4-arylpiperidine unit, inhibits the proliferation of A549 human lung carcinoma cells.¹¹ These findings suggested that the diphenylpropanol derivative **10a**, in which the dimethylamide moiety on loperamide was replaced with a hydroxyl group, might display increased antiproliferative activity. In fact, **10a** significantly inhibited HCT-116 human colon tumor cell

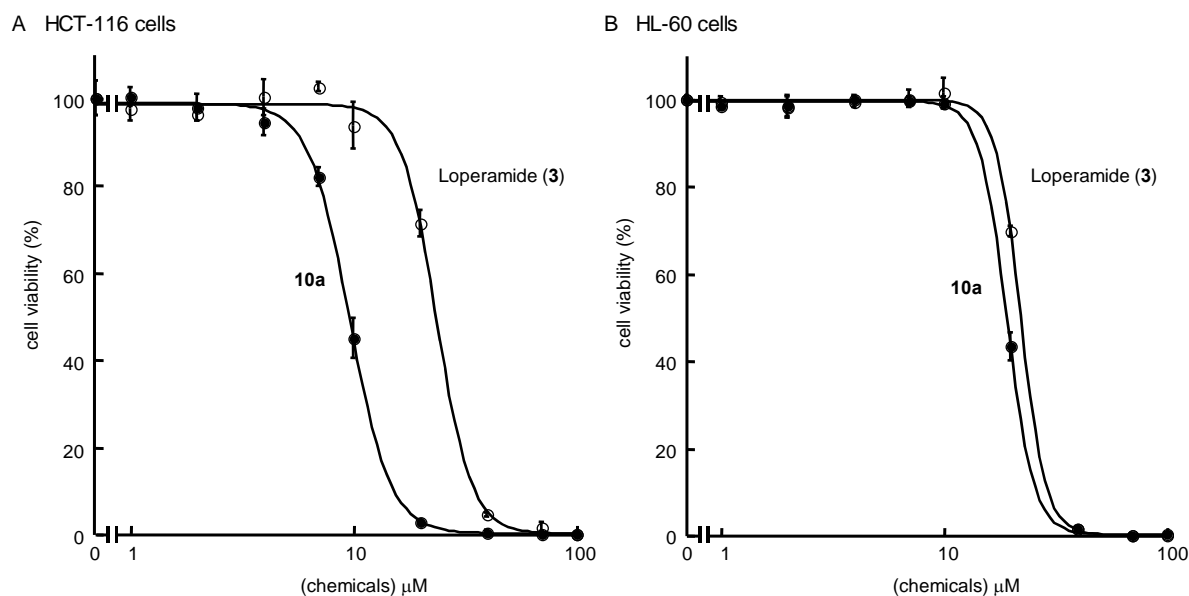
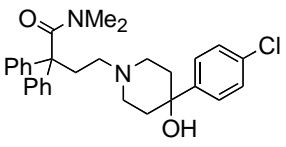
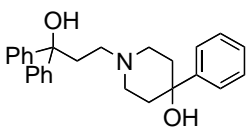
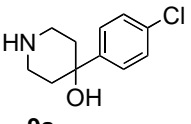
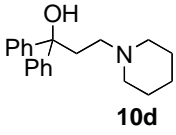
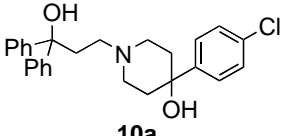
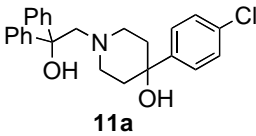
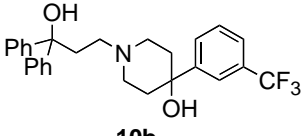
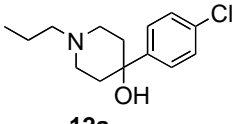


Figure 2. Dose-dependent effect of the loperamide derivative **10a** on tumor cell viability. HCT-116 cells (A) and HL-60 cells (B) were treated for 24 h with increasing concentrations of loperamide (open circles) or the *N*-diphenylpropanol derivative **10a** (filled circles), and then their tumor cell viability was analyzed using the MTT or WST-1 method. Data shown are mean \pm SEM values ($n = 3$).

growth and HL-60 human promyelocytic leukemia cell growth in a dose-dependent manner (Figure 2) and exhibited 2.5- and 1.2-fold increased activity against HCT-116 and HL-60 cells, respectively, compared with the LC₅₀ values of loperamide (Table 2).

For analyzed the effect of *N*-substituents in loperamide on antiproliferative activity, the activities mediated by 4-(*p*-chlorophenyl)piperidin-4-ol derivatives were assessed (Table 2. loperamide (**3**), **9a**, **10a**, **11a**, and **12a**). The diphenylethanol derivative **11a**, in which the distance between its diphenylcarbinol and arylpiperidine units was shorter than the equivalent distance in **10a**, displayed decreased antiproliferative activity compared with **10a**. The removal of the diphenylcarbinol unit led to the loss of antiproliferative activity against both types of tumor cells (Table 2. **9a** and **12a**). These findings indicated that diphenylcarbinols, especially diphenylpropanol, as an *N*-substituents on piperidine have important effects on the antiproliferative activity. For analyzed the effect of arylpiperidine unit in **10a** on antiproliferative activity, the activities mediated by diphenylpropanol derivatives were assessed (Table 2. **10a**, **10b**, **10c**, and **10d**). The 4-(*m*-trifluorophenyl)piperidin-4-ol derivative **10b** exhibited slightly increased the activity compared with 4-(*p*-chlorophenyl)piperidin-4-ol derivative **10a**, and displayed the most potent activity of all of the analogs synthesized in the present study against both tumor cell lines. The 4-phenylpiperidin-4-ol derivative **10c** and piperidine derivative **10d** showed significantly reduced or absent activity; thus, the substituent on the phenyl group in arylpiperidin-4-ol could be crucial for antiproliferative activity.

Table 2. Effects of loperamide derivatives on tumor cell viability

Compound	LC ₅₀ values (μM) ^a		Compound	LC ₅₀ values (μM) ^a	
	HCT-116 cells	HL-60 cells		HCT-116 cells	HL-60 cells
 loperamide (3)	23.72 ± 0.71	22.29 ± 0.41	 10c	48.74 ± 0.66	50.00 ± 0.89
 9a	> 100	> 100	 10d	> 100	> 100
 10a	9.66 ± 0.11	19.29 ± 0.16	 11a	16.95 ± 0.47	33.62 ± 0.36
 10b	6.47 ± 0.10	18.14 ± 0.41	 12a	> 100	> 100

^a Data shown are mean ± SEM values.

Apoptosis is the major form of cell death, and plays an important role in chemically-induced tumor cell death. Caspase-3, which is a cysteine protease, plays a key role during the early stage of apoptosis and is activated during buprenorphine-induced A549 tumor cell apoptosis.^{11,20} Therefore we investigated its activity in HL-60 cells that had been treated with the loperamide derivative **10b**. The caspase-3 in HL-60 cells was activated by treatment with the loperamide derivative **10b** (Figure 3).

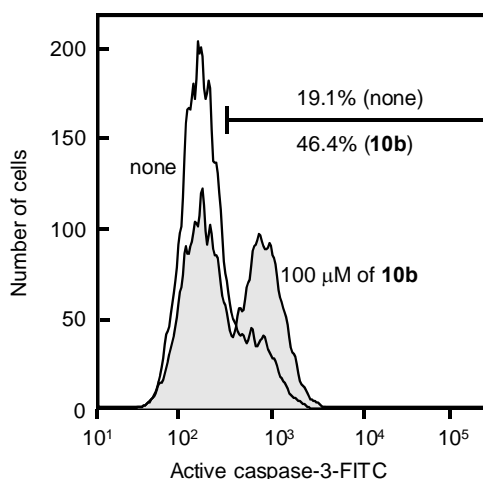


Figure 3. Caspase-3 activation by the loperamide derivative **10b** in HL-60 cells. HL-60 cells were treated for 4 h with (gray area) or without (clear area) 100 μ M of the loperamide derivative **10b**, and then the activated caspase-3 was stained with a fluorescein isothiocyanate-conjugated monoclonal antibody. Fluorescence was analyzed by flow cytometry. The percentage of active caspase-3 positive cells is shown.

Some of the opioid analogs synthesized in the present study might be applicable as novel anticancer analgesics for clinical use, however, the development of more potent analogs is required because the opioids that are currently used in clinical practice have to be administered at high doses to have antitumor effects.¹¹ In this paper, we synthesized loperamide analogs that exhibited potent antiproliferative activity against HCT-116 and HL-60 cells.

EXPERIMENTAL

General

Melting points were obtained with a Yanagimoto micro-melting-point apparatus and are uncorrected. IR spectra of solids (KBr) and liquids (NaCl) were recorded on a JASCO FT/IR-230 spectrophotometer. ^1H NMR spectra were recorded on a JEOL EX-400 (400 MHz) spectrometer or Bruker AVANCE500 (500 MHz) spectrometer with tetramethylsilane as an internal standard. ^{13}C NMR spectra were obtained on a JEOL EX-400 spectrometer or Bruker AVANCE500 spectrometer with CDCl_3 as an internal standard ($\delta_{\text{CDCl}_3} = 77.0$). EI- and FAB-MS were recorded on a JEOL JMS-SX102A spectrometer and ESI-MS were

recorded on a JEOL JMS-T100LP mass spectrometer. Elemental analyses of new compounds were performed by Yanaco CHN Corder MT-5. All chromatographic isolations were accomplished with BW-350 (Fuji Silysia) for column chromatography or with Kieselgel 60 PF₂₅₄ containing gypsum (Merck) for preparative TLC. CH₂Cl₂ was washed with water, dried over CaCl₂, and freshly distilled from P₄O₁₀. The recycling preparative HPLC was performed by LC-918 liquid chromatography (Japan Analytical Industry Co., Ltd.) equipped with JAIGEL-1H and -2H columns (polystyrene gels).

(3-Hydroxy-3,3-diphenyl)propyl *p*-toluenesulfonate (5). The *p*-toluenesulfonyl chloride (1.041 g, 5.46 mmol) was added to the solution of the diol **4** (831 mg, 3.64 mmol) in dry pyridine (3.64 mL) at 0 °C under atmosphere of argon, and the mixture was stirred at 0 °C for 5 h. The reaction quenched with water and extracted with Et₂O. The extracts were washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The tosylate **7** was isolated as a white solid in quantitative yield, mp 59-62 °C (dec.). IR (KBr): ν = 3503 (OH), 1355 (SO₃), 1170 (SO₃); ¹H NMR (400 MHz, CDCl₃): δ = 2.37 (1H, s), 2.43 (3H, s), 2.70 (2H, t, *J* = 7.2 Hz), 4.09 (2H, t, *J* = 7.2 Hz), 7.20-7.23 (2H, m), 7.27-7.32 (10H, m), 7.67 (2H, d, *J* = 8.2 Hz); ¹³C NMR (100 MHz, CDCl₃): δ = 21.6, 40.6, 87.6, 76.9, 125.6, 127.3, 127.9, 128.4, 129.8, 132.9, 144.7, 145.7; MS (ESI) *m/z* 405 ([M+Na]⁺); HRMS (ESI) *m/z* 405.1130 (calcd for C₂₂H₂₂NaO₄S, 405.1137).

General Procedures for synthesis loperamide derivative (10a) using the tosylate as the alkylating reagent. Sodium carbonate (95.4 mg, 0.9 mmol) was added to the solution of (3-hydroxy-3,3-diphenyl)propyl *p*-toluenesulfonate **5** (114.7 mg, 0.3 mmol) and 4-(4-chlorophenyl)-4-piperidinol (63.5 mg, 0.3 mmol) in dry MeCN (1.0 mL) at room temperature under atmosphere of Argon, and the mixture was refluxed for 16 h. The reaction quenched with water and extracted with CHCl₃. The extracts were washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by column chromatography with CHCl₃/MeOH (30:1) to give 1,1-diphenyl-3-[4-hydroxy-4-(4-chlorophenyl)piperidin-1-yl]-1-propanol **10a** as a white solid in 84% yield. IR (KBr): ν = 3384 (OH), 1095 (Ar-Cl); ¹H NMR (400 MHz, CDCl₃): δ = 1.59 (1H, s), 1.73 (2H, brd, *J* = 12.2 Hz), 2.09-2.16 (2H, m), 2.40-2.53 (6H, m), 2.82 (2H, brd, *J* = 10.7 Hz), 7.16-7.21 (2H, m), 7.28-7.33 (6H, m), 7.40-7.43 (2H, m), 7.45-7.48 (4H, m), 8.28 (1H, brs); ¹³C NMR (100 MHz, CDCl₃): δ = 35.3, 38.5, 49.2, 54.9, 70.8, 79.1, 125.8, 126.0, 126.4, 128.1, 128.5, 133.0, 146.4, 147.8; MS (EI) *m/z* 421 ([M]⁺). The hydrochloride salts as white powders, mp 220 °C (dec.); Anal. Calcd for C₂₆H₂₈NO₂Cl·HCl: C, 68.12, H, 6.38, N, 3.06. Found: C, 68.11, H, 6.32, N, 2.99.

1,1-Diphenyl-3-[4-hydroxy-4-(3-trifluoromethylphenyl)piperidin-1-yl]-1-propanol (10b). Yield 90%, colorless oil; IR (KBr): ν = 3399 (OH), 1330 (CF₃); ¹H NMR (400 MHz, CDCl₃): δ = 1.74 (2H, brd, *J* = 14.6 Hz), 2.13-2.19 (2H, m), 2.42-2.54 (6H, m), 2.85 (2H, brd, *J* = 10.7 Hz), 7.19 (2H, t, *J* = 7.3 Hz), 7.30 (4H, t, *J* = 7.8 Hz), 7.44-7.48 (5H, m), 7.53 (1H, brd, *J* = 7.8 Hz), 7.66 (1H, brd, *J* = 7.8 Hz), 7.78

(1H, s), 8.30 (1H, brs); ^{13}C NMR (100 MHz, CDCl_3): δ = 35.2, 38.4, 49.1, 54.9, 71.0, 79.1, 121.5, 122.8, 124.0, 125.5, 125.8, 126.4, 128.1, 128.8, 130.6, 130.9, 131.2, 147.8, 148.9; MS (EI) m/z 455 ($[\text{M}]^+$). The hydrochloride salts as colorless prisms, mp 209 °C (dec.); Anal. Calcd for $\text{C}_{27}\text{H}_{28}\text{NO}_2\text{F}_3\cdot\text{HCl}$: C, 65.92, H, 5.94, N, 2.85. Found: C, 65.90, H, 5.88, N, 2.88.

1,1-Diphenyl-3-(4-hydroxy-4-phenylpiperidin-1-yl)-1-propanol (10c). Yield 82%, white solid; IR (KBr): ν = 3461 (OH); ^1H NMR (400 MHz, CDCl_3): δ = 1.76 (2H, brd, J = 12.6 Hz), 2.14-2.21 (2H, m), 2.43-2.53 (6H, m), 2.82 (2H, brd, J = 10.6 Hz), 7.18 (2H, brt, J = 7.5 Hz), 7.24-7.37 (7H, m), 7.46-7.49 (6H, m); ^{13}C NMR (100 MHz, CDCl_3): δ = 35.3, 38.5, 49.3, 54.9, 71.0, 79.0, 124.4, 125.8, 126.4, 127.2, 128.1, 128.4, 147.8, 147.9; MS (EI) m/z 387 ($[\text{M}]^+$). The hydrochloride salts as colorless needles, mp 232 °C (dec.); Anal. Calcd for $\text{C}_{26}\text{H}_{29}\text{NO}_2\cdot\text{HCl}$: C, 73.65, H, 7.13, N, 3.30. Found: C, 73.62, H, 7.02, N, 3.34.

1,1-Diphenyl-3-(piperidin-1-yl)-1-propanol (10d). Yield 89%, white solid, mp 120-121 °C; IR (KBr): ν = 3421 (OH); ^1H NMR (400 MHz, CDCl_3): δ = 1.58-1.63 (6H, m), 2.37-2.43 (7H, m), 7.15-7.19 (2H, m), 7.26-7.31 (4H, m), 7.44-7.47 (4H, m); ^{13}C NMR (100 MHz, CDCl_3): δ = 24.2, 26.1, 35.1, 54.4, 55.5, 78.9, 125.8, 126.3, 128.0, 148.1; MS (EI) m/z 295 ($[\text{M}]^+$); HRMS (EI) m/z 295.1943 (calcd for $\text{C}_{20}\text{H}_{25}\text{NO}$, 295.1936).

1,1-Diphenyl-2-[4-hydroxy-4-(4-chlorophenyl)piperidin-1-yl]-1-ethanol (11a). The solution of oxirane **7** (785 mg, 4.0 mmol), 4-(4-chlorophenyl)-4-piperidinol (846 mg, 4.0 mmol) lithium perchlorate (851 mg, 8.0 mmol) was refluxed for 16 h under atmosphere of Argon. The reaction quenched with water and extracted with CHCl_3 . The extracts were washed with brine, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by column chromatography with $\text{CHCl}_3/\text{MeOH}$ (30:1) to give **11a** as a white solid in 81% yield. IR (KBr): ν = 3420 (OH), 1094 (Ar-Cl); ^1H NMR (400 MHz, CDCl_3): δ = 1.47 (1H, s), 1.56-1.60 (2H, m), 1.97 (2H, dt, J = 3.9, 12.7 Hz), 2.43 (2H, brd, J = 11.2 Hz), 2.65-2.70 (2H, m), 3.33 (2H, s), 5.38 (1H, brs), 7.16-7.20 (2H, m), 7.27-7.31 (6H, m), 7.36-7.40 (2H, m), 7.50-7.53 (4H, m); ^{13}C NMR (100 MHz, CDCl_3): δ = 38.6, 50.6, 67.8, 70.4, 74.1, 125.6, 126.1, 126.6, 128.1, 128.4, 132.9, 146.5, 147.1; MS (FAB, glycerol) m/z 408 ($[\text{M}+\text{H}]^+$). The hydrochloride salts as colorless prisms, mp 195 °C (dec.); Anal. Calcd for $\text{C}_{25}\text{H}_{26}\text{NO}_2\text{Cl}\cdot\text{HCl}$: C, 67.57, H, 6.12, N, 3.10. Found: C, 67.56, H, 6.06, N, 3.17.

N-Propyl-4-(4-chlorophenyl)-4-piperidinol (12a). Sodium carbonate (95.4 mg, 0.9 mmol) was added to the solution of 1-bromopropane (43.0 mg, 0.6 mmol) and 4-(4-chlorophenyl)-4-piperidinol (63.5 mg, 0.3 mmol) in dry MeCN (1.0 mL) at room temperature under atmosphere of Argon, and the mixture was stirred for 3 h at room temperature. The reaction quenched with water and extracted with CHCl_3 . The extracts were washed with brine, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by column chromatography with $\text{CHCl}_3/\text{MeOH}$ (30:1) to give **12a** as a colorless

crystal in 49% yield, mp 99-100 °C. IR (KBr): ν = 3168 (OH), 1095 (Ar-Cl); ^1H NMR (400 MHz, CDCl_3): δ = 0.94 (3H, t, J = 7.3 Hz), 1.56-1.65 (2H, m), 1.70 (1H, brs), 1.74 (2H, dd, J = 2.1 and 14.3 Hz), 2.19-2.26 (2H, m), 2.42-2.53 (4H, m), 2.90 (2H, d, J = 10.4 Hz), 7.31 (2H, d, J = 8.5 Hz), 7.45 (2H, d, J = 8.5 Hz); ^{13}C NMR (100 MHz, CDCl_3): δ = 12.0, 20.0, 38.3, 49.4, 60.7, 71.0, 126.1, 128.4, 132.7, 146.7; MS (ESI) m/z 254 ($[\text{M}+\text{H}]^+$); HRMS (ESI) m/z 254.1301 (calcd for $\text{C}_{14}\text{H}_{21}\text{ClNO}$, 254.1312).

Cell lines and cell cultures

For testing the antiproliferative cells activities, two types of cancer cell lines were used in this study: HCT-116 cells (human colon cancer) and HL-60 cells (human promyelocytic leukemia), which were purchased from the American Type Culture Collection (VA, USA). The HCT-116 cells were maintained in McCoy 5A medium with L-glutamine and 10% heat inactivated (55 °C for 30 min) fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2 . The HL-60 cells were cultured in RPMI-1640 medium with L-glutamine and 10% heat inactivated FBS at 37 °C in an atmosphere of 5% CO_2 .

Cell viability assays

The HCT-116 cells viability assay, by MTT method, it was carried out following the method described by Mosmann.²¹ Briefly, cells were placed in 96-well flat-bottomed tissue culture plates with 6.0×10^3 cells per well in 100 μL culture medium. This was followed by incubation at 37 °C in an atmosphere of 5% CO_2 for 24 h to allow cells attachment onto the wells. The cells were treated by the indicated concentrations of test agents in culture medium without FBS. Following a further 24 h incubation, 10 μL of MTT (5 mg/mL in PBS buffer) was added per well and the plate was incubated for 4 h to allow metabolism of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved by the addition of 100 μL of DMSO. The absorbance of purple formazan was read at 570 nm using a microplate reader. The results following test agents exposure were calculated as a percentage relative to untreated controls.

The HL-60 cells viability assay, by WST-1 method, it was carried out adopting the method described by Ishiyama.²² The cells were seeded in 96-well flat-bottomed tissue culture plates with 3.0×10^4 cells per well in 100 μL of the FBS contained culture medium with the indicated concentrations of test agents. Following a further 24 h incubation, 10 μL of a mixture of WST-1/1-methoxy PMS solution containing 5 mM WST-1 and 0.2 mM 1-methoxy PMS in 40 mM HEPES-NaOH (pH 7.4) was added per well and the plate was incubated for 3 h to allow metabolism of WST-1 by cellular mitochondrial dehydrogenases. The absorbance of yellow formazan was read at 415 nm using a microplate reader. The results following test agents exposure were calculated as a percentage relative to untreated controls.

FACS analysis for detection of apoptosis

HL-60 cell were incubated with test agents in Krebs-Ringer's HEPES buffer containing 15 mM HEPES-NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 0.7 mM MgSO₄, 1.2 mM CaCl₂ and 1.8 g/L glucose for 4 h at 37 °C. After washing the cells with ice-cold PBS buffer, the cells were fixed with Fixed Buffer I (BD Biosciences Pharmingen, NJ, USA) for 30 min on ice. The cells were then permeabilized and stained with anti-active caspase-3 monoclonal antibody (BD Biosciences Pharmingen, NJ, USA) in Perm/Wash Buffer I (BD Biosciences Pharmingen, NJ, USA) at 37 °C in the dark for 30 min. The cells were then washed and diluted with staining buffer (2% FBS in PBS buffer), and measured the fluorescence using by FACSCanto with BD Diva software (BD Biosciences).

Calculation

Concentration-cell viability relationships were fitted to a four-parameter logistic equation using a nonlinear curve-fitting program, which derived the LC₅₀ values (Kaleida-graph; Synergy Software, Reading, PA). Where appropriate, the results were expressed as means ± sem, with n = 3 or higher in one of at least three similar experiments.

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