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Research Article

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Synthesis and Antileukemic Activity of Novel 4-(3-(Piperidin-4-yl) Propyl)Piperidine Derivatives

Kambappa Vinaya[1,2], Chandagirikoppal V. Kavitha[3,4], Siddappa Chandrappa[1], Doddakunche S. Prasanna[1], Sathees C. Raghavan[3] and Kanchugarakoppal S. Rangappa[1,*]

[1]Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore-570 006, India
[2]Department of Chemistry, Government First Grade College, Kadur-577 548, India
[3]Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India
[4]Department of Chemistry, Government College for Women, Mandya-571 401, India
**Corresponding author: Kanchugarakoppal S. Rangappa, rangappaks@chemistry.uni-mysore.ac.in; rangappaks@gmail.com*

To explore the anticancer effect associated with the piperidine framework, several (substituted phenyl) {4-[3-(piperidin-4-yl)propyl]piperidin-1-yl} methanone derivatives 3(a–i) were synthesized. Variation in the functional group at *N*-terminal of the piperidine led to a set of compounds bearing amide moiety. Their chemical structures were confirmed by ^{1}H NMR, IR and mass spectra analysis. Among these, compounds 3a, 3d and 3e were endowed with antiproliferative activity. The most active compound among this series was 3a with nitro and fluoro substitution on the phenyl ring of aryl carboxamide moiety, which inhibited the growth of human leukemia cells (K562 and Reh) at low concentration. Comparison with other derivative (3h) results shown by LDH assay, cell cycle analysis and DNA fragmentation suggested that 3a is more potent to induce apoptosis.

Key words: 4-amino benzoic acid, anticancer agents, apoptosis, cell death, DNA damage, leukemia

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Cancer is a serious disease with a complex pathogenesis that threatens human life greatly. Currently, much efforts have been put to the identification of novel anticancer targets and the discovery of anticancer drugs. In recent years, great progress has been made in the treatment for cancer, and so, the life expectation of cancer patients has been improving remarkably. Furthermore, during the past 5 years, the global effort for sequencing human genome has provided us with an enormous number of potential targets associated with cancer. The drugs designed are expected to have high affinity with the novel targets. They not only inhibit the proliferation but also differentiation of tumor cells and speed up their death. Cancer chemotherapy is now well established and is considered as a highly specialized field.

Among the modifications effected to the family of antitumor compounds, heterocyclic organic compounds have been extensively tried by many groups especially to modify the reactivity profile. Piperidine is a new class of heterocycle that showed interesting cytotoxicity profiles.

The piperidine nucleus can be found in numerous naturally occurring alkaloid and synthetic compounds with interesting biological and pharmacological properties (1–3). Piperidine moiety-containing molecules play an important role in the field of medicinal chemistry. Several derivatives of this class have found such useful biological applications as fungicidal, bactericidal, anti-inflammatory, antihistaminic, wound healing, anticancer, CNS stimulant, and depressant activities (4–10). Compounds containing amide bond substitution can alter the chemical properties, disposition, and biological activities of drugs (11). Various amides are currently used in the treatment for several diseases, and these include antidepressants, anti-inflammatory agents, antimalarial drugs, antipsychotic, antiviral agents, steroids, and general anesthetics (12).

Literature has several instances, wherein incorporation of two of the structural features required for activity in a single molecule has to significantly enhance the activity (13). Because of broad spectrum of activities reported in the literature so far, we attempted to synthesize a system that combines two biolabile components together targeting for potent molecules possessing antileukemic activity. In continuation of previous research on synthesis and anticancer studies of bioactive heterocycles (14–17), herein, we tried to investigate the antiproliferative activity and apoptosis induced by the newly synthesized piperidine analogs against human leukemia cells.

Materials and Methods

Infrared (IR) spectra were recorded using a Jasco FTIR-4100 spectrometer in the wave number range of 4000–400 /cm. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AM 400 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane as an internal standard. The chemical shifts are

expressed in δ, and the following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). The purity of the compounds was checked by thin layer chromatography (TLC). Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates. All the reagents and chemicals used were from Sigma Aldrich Chemicals Pvt Ltd.

Chemistry

For the synthesis of the novel compounds **3**(**a**–**i**), the reaction sequences outlined in Scheme 1 were followed. The coupling reaction of 4-[3-(piperidin-4-yl)propyl]piperidine (**1**) with different substituted aromatic/heterocyclic acids **2**(**a**–**i**) were carried out in the presence of base *N*-methyl morpholine, isobutyl chloroformate, and tetrahydrofuran (THF) as solvent with the yield ranging from 75% to 90%. Synthesized molecules **3**(**a**–**i**) were structurally characterized by ^{1}H NMR, mass and IR spectroscopic analyses. Compounds **3**(**a**–**i**) were confirmed by IR data, which showed disappearance of stretching frequencies of –COOH at 2995 /cm. From ^{1}H NMR spectra, this showed disappearance of –COOH at 12.03 ppm. The chemical structures and yield of the synthesized compounds are given in Table 1.

General procedure for the synthesis of 4-(3-(piperidin-4-yl)propyl) piperidine derivatives 3(a–i)

A solution of different substituted aromatic/heterocyclic acids **2**(**a**–**i**) (1.0 eq) in dry THF was taken and cooled to 0–5 °C. Then, isobutyl chloroformate (1.3 eq) and *N*-methyl morpholine (3.0 eq) were added to the cold reaction mixture. The reaction mixture was stirred for 15 min at same temperature, then added 4-[3-(piperidin-4-yl)propyl]piperidine **1** (1.0 eq) to the reaction mixture, and allowed the reaction mixture at room temperature for 4–5 h with stirring. The progress of the reaction was monitored by TLC. After completion of the reaction, water was added and the reaction mixture was filtered, washed with ether, and dried under vacuum.

Synthesis of (4-fluoro-3-nitrophenyl) {4-[3-(piperidin-4-yl)propyl]piperidin-1-yl} methanone 3a

The product obtained was yellow oily from 4-fluoro-3-nitro benzoic acid **2a** (0.25 g, 1.35 mmol), isobutyl chloroformate (0.239 g, 1.79 mmol), *N*-methyl morpholine (0.409 g, 4.05 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.28 g, 1.35 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.81 (s, 1H, Ar–H), 8.28 (d, 1H, Ar–H), 7.56 (d, 1H, Ar–H), 2.93–3.08 (m, 4H), 2.45–2.60 (m, 4H), 2.04 (s, 1H, –NH), 1.92 (bs, 2H), 1.60 (m, 2H), 1.09–1.31 (m, 12H). MS (ESI) *m/z*: 378.21 (M+H$^+$). IR (KBr, /cm): 3343, 3092, 1671, 1517, 1335, 1256, 1176, 612.

Synthesis of (2-amino-1,2,3,4-tetrahydronaphthalen-2-yl) {4-[3-(piperidin-4-yl) propyl]piperidin-1-yl}methanone 3b

The product obtained was white solid from 2-amino-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid **2b** (0.25 g, 1.30 mmol), isobutyl chloroformate (0.23 g, 1.69 mmol), *N*-methyl morpholine (0.39 g, 3.9 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.27 g, 1.30 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.12 (dd, 2H, Ar–H), 6.90 (d, 2H, Ar–H), 3.90 (s, 2H, –NH_2), 3.43 (m, 2H, –CH_2), 2.95–2.98 (m, 8H), 2.91 (t, 2H, –CH_2), 2.81 (bs, 2H), 2.18 (t, 2H, –CH_2), 2.04 (s, 1H, –NH), 1.74 (m, 2H), 1.09–1.26 (m, 12H). MS (ESI) *m/z*: 384.3 (M+H$^+$). IR (KBr, /cm): 3329, 3074, 1656, 1615, 1525, 1169.

Synthesis of 2-amino-3-(2′-ethyl-4′-methoxybiphenyl-4-yl)-1-{4-[3-(piperidin-4-yl) propyl]piperidin-1-yl}propan-1-one 3c

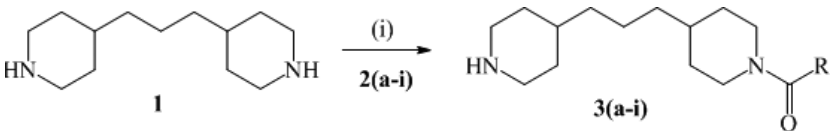
The product obtained was white amorphous solid from 2-amino-3-(2′-ethyl-4′-methoxylbiphenyl-4-yl)propanoic acid **2c** (0.25 g, 0.83 mmol), isobutyl chloroformate (0.147 g, 1.08 mmol), *N*-methyl morpholine (0.25 g, 2.49 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.174 g, 0.83 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.47 (d, 2H, Ar–H), 7.35 (d, 1H, Ar–H), 7.21 (d, 2H, Ar–H), 6.71 (s, 1H, Ar–H), 6.62 (d, 1H, Ar–H), 3.95 (m, 1H, –CH), 3.92 (s, 2H, –NH_2), 3.87 (s, 3H, –OCH_3), 3.23 (d, 2H, –CH_2), 2.89–2.98 (m, 8H), 2.81 (bs, 2H), 2.62 (m, 2H, –CH_2), 2.08 (s, 1H, –NH), 1.74 (m, 2H), 1.23 (t, 3H, –CH_3), 1.09–1.98 (m, 12H). MS (ESI) *m/z*: 492.35 (M+H$^+$). IR (KBr, /cm): 3315, 3052, 1668, 1610, 1538, 1180.

Synthesis of [3-(cyclopentyloxy)-4-methoxyphenyl] {4-[3-(piperidin-4-yl)propyl] piperidin-1-yl}methanone 3d

The product obtained was pale yellow liquid from 3-(cyclopentyloxy)-4-methoxybenzoic acid **2d** (0.25 g, 1.05 mmol), isobutyl chloroformate (0.18 g, 1.37 mmol), *N*-methyl morpholine (0.32 g, 3.17 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.22 g, 1.05 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.45 (d, 1H, Ar–H), 7.32 (s, 1H, Ar–H), 6.87 (d, 1H, Ar–H), 3.79 (s, 3H, –OCH_3), 3.72 (m, 1H, –CH), 2.88–2.93 (m, 8H), 2.83 (bs, 2H), 2.12 (m, 4H, –CH_2), 2.10 (s, 1H, –NH), 1.72 (m, 2H), 1.59 (m, 4H, –CH_2), 1.10–1.23 (m, 12H). MS (ESI) *m/z*: 429.31 (M+H$^+$). IR (KBr, /cm): 3430, 3042, 1641, 1603, 1366, 1190, 1057.

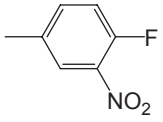
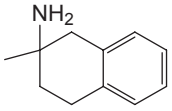
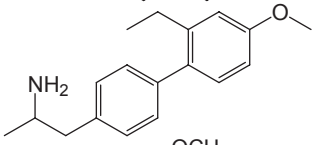
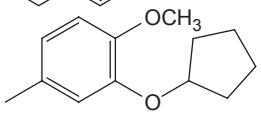
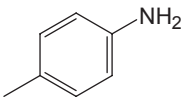
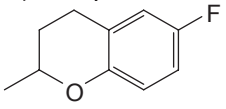
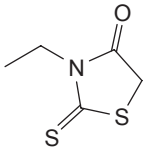
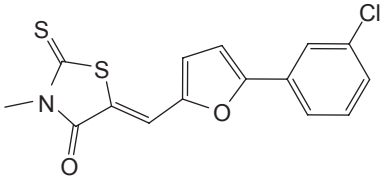
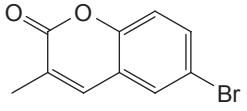
Synthesis of (4-aminophenyl){4-[3-(piperidin-4-yl)propyl]piperidin-1-yl}methanone 3e

The product obtained was pale yellow liquid from 4-aminobenzoic acid **2e** (0.25 g, 1.82 mmol), isobutyl chloroformate (0.32 g, 2.37 mmol), *N*-methyl morpholine (0.55 g, 5.46 mmol), and



Scheme 1: Synthesis of 4-[3-(piperidin-4-yl)propyl]piperidine derivatives. Reagents and conditions: (i) Aromatic/heterocyclic acids **2**(**a**–**i**), isobutyl chloroformate, *N*-methylmorpholine, tetrahydrofuran, 5–6 h.

Table 1: Chemical structures and yield of the synthesized compounds **3**(**a**–**i**)

Compound	R	Yield (%)
3a		
3b		79
3c		75
3d		80
3e		89
3f		78
3g		90
3h		85
3i		81

4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.38 g, 1.82 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.78 (d, 2H, Ar–H), 6.75 (d, 2H, Ar–H), 4.1 (s, 1H, –NH_2), 2.90–2.96 (m, 8H), 2.79 (bs, 2H), 2.10 (s, 1H, –NH), 1.72 (m, 2H), 1.15–1.24 (m, 12H). MS (ESI) *m/z*: 330.25 ($M+H^+$). IR (KBr, /cm): 3748, 3425, 3062, 1630, 1603, 1342, 1177, 1086.

Synthesis of (6-fluoro-3,4-dihydro-2H-chromen-2-yl){4-[3-(piperidin-4-yl)propyl] piperidin-1-yl}methanone 3f

The product obtained was pale yellow liquid from 6-fluoro-3,4-dihydro-2H-chromene-2-carboxylic acid **2f** (0.25 g, 1.27 mmol), isobutyl chloroformate (0.22 g, 1.65 mmol), *N*-methyl morpholine (0.38 g, 3.82 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.26 g, 1.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 6.71 (m, 3H, Ar–H), 4.56 (t, 1H, –CH), 2.71 (t, 2H, –CH_2), 2.53 (t, 2H, –CH_2), 2.88–2.94 (m, 8H), 2.80 (bs, 2H), 2.12 (s, 1H, –NH), 1.72 (m, 2H), 1.12–1.24 (m, 12H). MS (ESI) *m/z*: 389.26 ($M+H^+$). IR (KBr, /cm): 3352, 3073, 1680, 1340, 1256, 1165, 618.

Synthesis of 3-(2-oxo-2-{4-[3-(piperidin-4-yl)propyl]piperidin-1-yl}ethyl)-2-thioxo thiazolidin-4-one 3g

The product obtained was brown solid from 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid **2g** (0.25 g, 1.30 mmol), isobutyl chloroformate (0.23 g, 1.7 mmol), *N*-methyl morpholine (0.39 g, 3.92 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.275 g, 1.3 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.15 (s, 2H, –CH_2), 3.83 (s, 2H, –CH_2-S), 2.90–2.96 (m, 8H), 2.83 (bs, 2H), 2.06 (s, 1H, –NH), 1.76 (m, 2H), 1.16–1.23 (m, 12H). MS (ESI) *m/z*: 384.17 ($M+H^+$). IR (KBr, /cm): 3440, 3087, 1668, 1346, 1251, 1172, 702.

Synthesis of 5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-3-(2-oxo-2-{4-[3-(piperidin-4-yl)propyl]piperidin-1-yl}ethyl)-2-thioxothiazolidin-4-one 3h

The product obtained was pink solid from 5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidine-3-carboxylic acid **2h** (0.15 g, 0.39 mmol), isobutyl chloroformate (0.07 g, 0.51 mmol), *N*-methyl morpholine (0.12 g, 1.18 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine **1** (0.083 g, 0.39 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.88 (d, 1H, Ar–H), 7.76 (t, 1H, Ar–H), 7.65 (d, 1H, Ar–H), 7.47 (d, 2H, Ar–H), 7.31 (d, 1H, Ar–H), 4.44 (s, 2H, –CH_2), 2.89–2.98 (m, 8H), 2.81 (bs, 2H), 2.05 (s, 1H, –NH), 1.74 (m, 2H), 1.09–1.26 (m, 12H). MS (ESI) *m/z*: 559.15 ($M+H^+$). IR (KBr, /cm): 3454, 3096, 1650, 1368, 1215, 1217, 769.

Synthesis of 6-bromo-3-(4-[3-(piperidin-4-yl)propyl]piperidine-1-carbonyl)-2H-chromen-2-one 3i

The product obtained was pale yellow solid from 6-bromo-2-oxo-2H-chromene-3-carboxylic acid **2i** (0.10 g, 0.37 mmol), isobutyl chloroformate (0.066 g, 0.484 mmol), *N*-methyl morpholine (0.112 g, 1.11 mmol), and 4-[3-(piperidin-4-yl)propyl]piperidine (**1**) (0.078 g, 0.37 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.90 (s, 1H, –CH=C), 7.72 (s, 1H, Ar–H), 7.27 (d, 2H, Ar–H), 2.88–2.95 (m, 8H), 2.83 (bs, 2H), 2.11 (s, 1H, –NH), 1.78 (m, 2H), 1.14–1.23 (m, 12H). MS (ESI) *m/z*: 462.13 ($M+H^+$). IR (KBr, /cm): 3432, 3063, 1609, 1378, 1274, 1172, 576.

Synthesis of 2-(5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidin-3-yl) acetic acid 2h

A mixture of 3-rhodanine-3-acetic acid **1** (1.0 g, 0.01 mmol), 5-(3-Chlorophenyl)-furfural **2** (1.08 g, 0.01 mmol), and anhydrous sodium acetate (1.3 g, 0.03 mmol) were taken in of glacial acetic acid 10 mL. The reaction mixture was heated to 120 °C in an oil bath for 10 h. Then, the reaction mixture was cooled, filtered, and

washed with ether. Finally, a reddish solid compound (1.75 g, 75%) was obtained. The schematic representation of the synthesized compound is shown in Scheme 2. ^{1}H NMR (DMSO-d_6, 400 MHz)δ: 10.5 (s, 1H, –COOH), 7.9 (d, 1H, Ar–H), 7.6 (t, 1H, Ar–H), 7.5 (d, 1H, Ar–H), 7.4 (d, 2H, Ar–H), 7.1 (d, 1H, Ar–H), 4.5 (s, 2H, $-CH_2$). MS: 379.97.

Pharmacology

To assess the antiproliferative activity, leukemic cells growing in log phase were treated with 10, 100 and 250 μM concentrations of 4-[3-(piperidin-4-yl)propyl]piperidine derivatives **3**(**a**–**i**). Because the compounds were dissolved in DMSO, it was used as vehicle control. The amount of DMSO used was equal to the DMSO in highest concentration of compound tested. We conducted trypan blue dye exclusion assay, MTT assay, LDH assay, and FACS analysis to evaluate the cytotoxicity of these derivatives. In addition, we have also performed DNA fragmentation assay, which is an indicator of apoptosis. Each experiment was repeated a minimum of two times (15).

Cell lines and culture

Human cell line, K562 (chronic myelogenous leukemia), was purchased from National Center for Cell Science, Pune, India, and Reh (B-cell leukemia) cell line was a kind gift from Prof. Michael Lieber, University of Southern California, USA. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 μg of streptomycin/mL and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Trypan blue exclusion assay

To determine the effect of 4-[3-(piperidin-4-yl)propyl]piperidine analogs **3**(**a**–**i**) on viability of K562 or Reh cells, approximately 7.5×10^4 cells/mL were seeded in a 6-well tissue culture plate for 24 h and compounds **3**(**a**–**i**) were added at a concentration of 10, 100, and 250 μM. Cells were collected at intervals of 24 h and resuspended in 0.4% trypan blue (viable-unstained and non-viable blue). The number of viable cells was counted using hemocytometer.

MTT assay

Cell survival was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the ability of viable cells to metabolize yellow tetrazolium salt to violet formazan. Exponentially growing K562 or Reh cells (1×10^4 cells/well) were plated in triplicates and incubated with 10, 100, and 250 μM of **3**(**a**–**i**). Cells were harvested after 48 and 72 h of treatment and incubated with MTT (5 mg/mL) at 37 °C. The blue MTT formazan precipitate was then solubilized in detergent (50% final concentration of *N,N*-dimethylformamide and 10% of sodium dodecyl sulfate). Absorbance was measured at 570 nm using ELISA plate reader. The mean absorbance of culture medium was used as the blank and was subtracted. IC_{50} values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h of compound treatment using Origin software. The absorbance of untreated (medium only) cells was taken as 100% viability, and the values of treated cells were calculated as percentage of control and presented as histograms (Figure 2).

LDH release assay

Release of lactate dehydrogenase (LDH) is an indicator of membrane integrity and hence cell injury. LDH assay was performed as per standard protocols to assess the LDH release in the culture media after treatment with **3a** or **3h** (10, 50 and 100 μM) on K562 cells for 24 and 48 h. The intracellular LDH was determined after lysing the cells by freezing at −70 °C and rapid thawing. The LDH release was measured at an absorbance of 490 nm. The LDH percent release was calculated as follows:

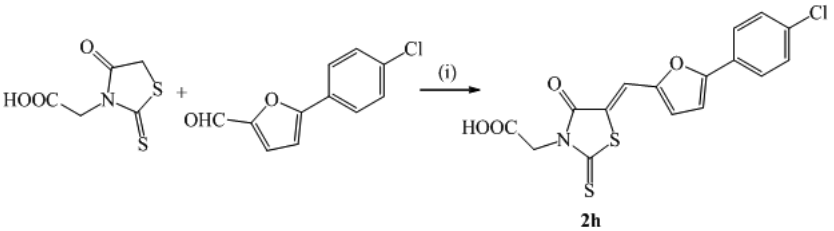
LDH release (%) = (LDH activity in media)/(LDH activity in media + LDH activity in total cells) × 100%.

Cell cycle analysis

Cellular DNA content was measured by flow cytometry. Flow cytometry was performed on FACScan (Becton Dickinson, San Jose, CA, USA), using CellQuest (Becton Dickinson) software for acquisition and WinList (Verity, Topsham, ME, USA) software for analysis. Approximately 7.5×10^4 cells/mL were cultured and treated with 10, 50, and 100 μM concentrations of **3a** or **3h**. Cells were harvested after 48 h of treatment, washed, fixed in 70% ethanol, and incubated with RNase A (Sigma-Aldrich, St Louis, MO, USA). Propidium iodide (PI, 50 μg/mL; Sigma-Aldrich) was added half an hour before acquiring the flow cytometric reading (FACScan; BD Biosciences, New Bedford MA, USA). A minimum of 10 000 cells were acquired per sample.

DNA fragmentation assay

To elucidate the mode of action of **3a** and **3h**, especially with respect to induction of oligonucleosomal DNA fragmentation, this



2h

Scheme 2: Synthesis of 2-(5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidin-3-yl) acetic acid. Reagents and conditions: (i) Gla AcOH/CH_3COONa, 120 °C, 10 h.

assay was performed. Briefly, K562 cells were cultured in the presence of **3a** at 5, 10, and 20 μM or **3h** at 10, 50, and 100 μM for 72 h. Cells were harvested, and genomic DNA was extracted using standard protocol. DNA was resuspended in 250 μL of TE buffer. To ensure the equal loading of DNA, pilot gel was run by loading equal volume of DNA into each well, and then, based on the intensity of bands, concentration of DNA was adjusted. Finally, the DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed.

Results and Discussion

All compounds were evaluated for their cytotoxic activity *in vitro* against human leukemia cells, K562 (chronic myeologenous leukemia) and Reh (pre-B cell). Preliminarily, we have used trypan blue and MTT assays to assess the effect of 4-[3-(piperidin-4-yl)propyl]piperidine derivatives **3**(**a**–**i**) on cell viability. For performing the antiproliferative assay, the compounds were dissolved in DMSO. To ensure that the solvent *per se* had no effect on the cell growth, negative control tests were performed using DMSO at the same concentration as used for highest concentration of the compound tested. In trypan blue assay, the cells were counted every day till the control cells reached stationary phase. Results are represented as graphs (Figure 1), which Results shows that the addition of **3**(**a**–**i**) affected the viability of the cells in both the cell lines investigated (Figure 1 and data not shown). The cytotoxicity induced by the 4-[3-(piperidin-4-yl)propyl]piperidine s **3**(**a**–**i**) was dose and time dependent. It was found that the effect was maintained even after prolonged incubation periods.

The cytotoxicity was further verified using MTT assay (Figure 2). The effect of the compounds was expressed as percentage of cell proliferation. IC_{50} value was determined after 72 h, and the results summarized in Table 2 with SD values show that most of the compounds inhibited the growth of both K562 and Reh cells in the micromolar range. Initially, structure–activity relationship of this series of compounds against K562 cell line was investigated. Different substituents on the *N*-terminal of the piperidine exhibited different levels of antitumor activity. On the basis of the data obtained, the following conclusion may be drawn. In general, the nature of the substituent at position *N*-amide of piperidine is crucial in influencing cytotoxicity. Thus, it was found that incorporation of fluoronitrobenzene (**3a**) resulted in more potent molecule with IC_{50} value of

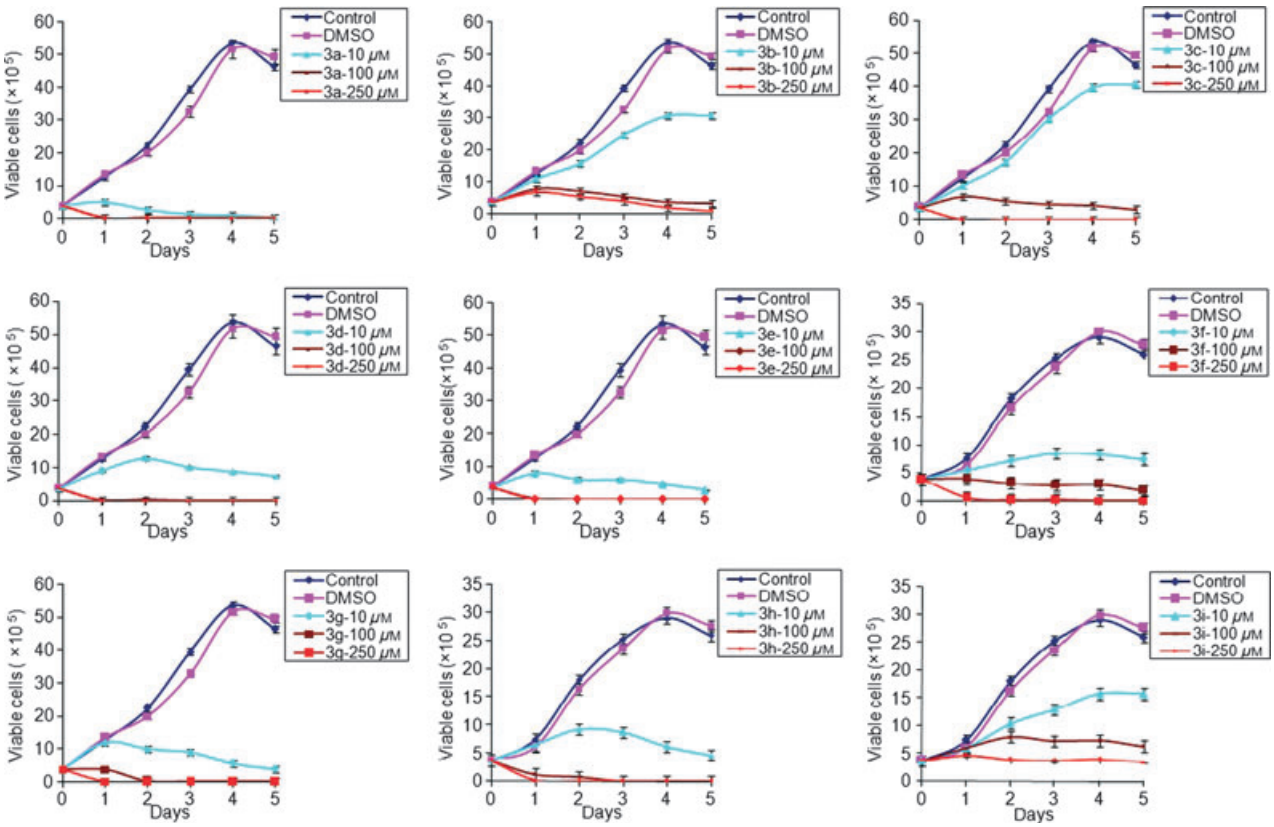


Figure 1: Dose- and time-dependent effect of 4-[3-(piperidin-4-yl)propyl]piperidines **3**(**a**–**i**) on K562 cell survival. Approximately 0.75×10^5 cells/mL were cultured followed by treatment of **3**(**a**–**i**) after 24 h at 10, 100, and 250 μM. Besides untreated cells, DMSO-treated cells were used as vehicle control. After every 24 h from the time of addition of compounds, cell viability was determined by trypan blue exclusion assay and data were represented as a graph. Mean ± SD error bars are represented in the figure. Graphs represent the treatment of compound **3**(**a**–**i**), respectively, against K562 cells.

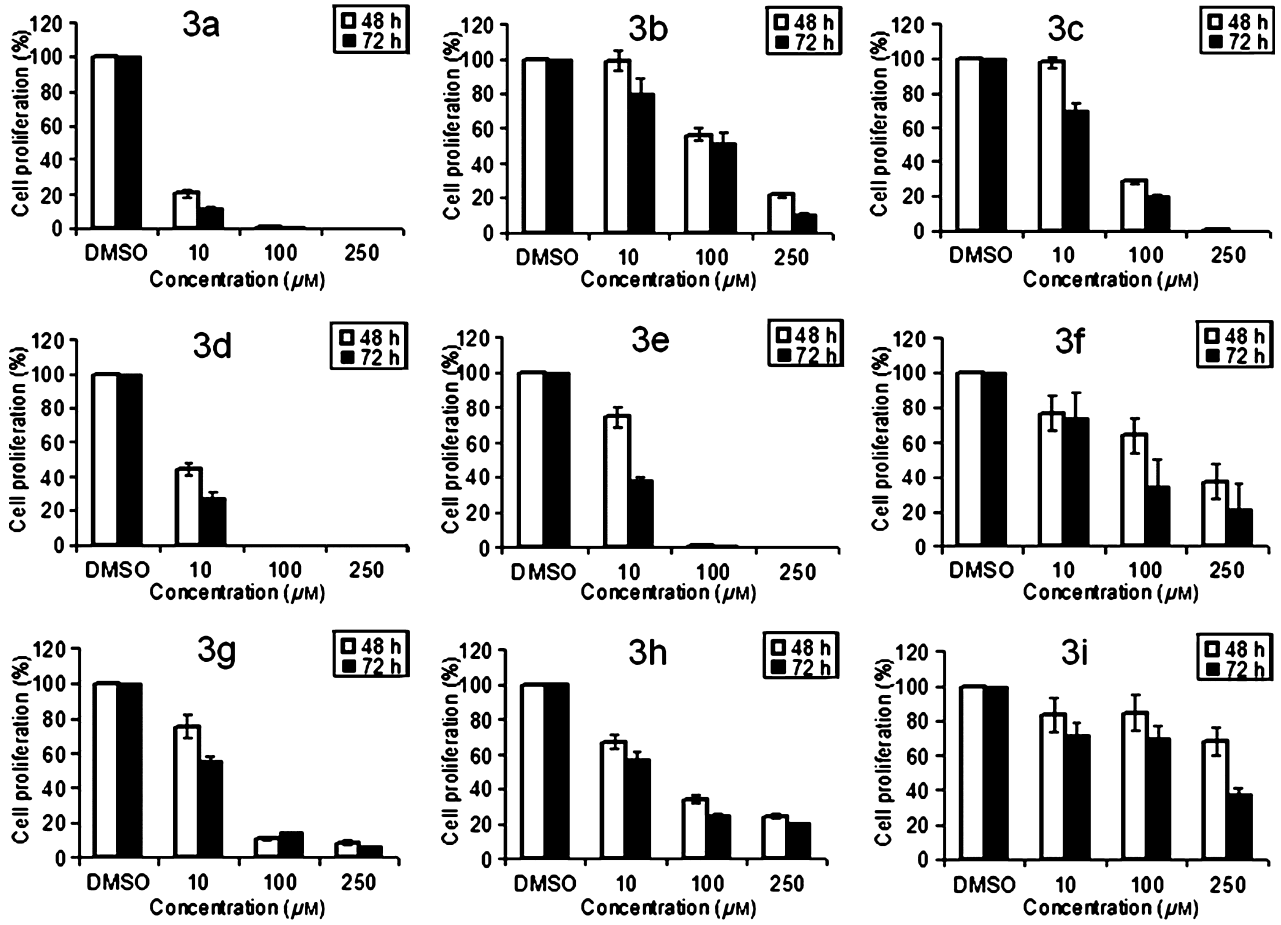


Figure 2: Determination of the effect of 4-[3-(piperidin-4-yl)propyl]piperidines **3**(**a**–**i**) on cell proliferation by MTT assay. After 48 and 72 h of exposure, K562 cells with **3**(**a**–**i**) (10, 100 and 250 μM) were incubated with MTT (5 mg/mL) in duplicates and resulting blue formazan precipitate was dissolved in detergent, and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation (the cell viability of vehicle cells was considered as 100%). Mean ± SD error bars are represented in the figure. Graphs represent the treatment of compound **3**(**a**–**i**), respectively, against K562 cells.

Table 2: IC_{50} with mean ± SD values (μM) of the synthesized compounds **3**(**a**–**i**) based on MTT assay

Compound	K562	Reh
3a	2.0 ± 1.3	2.3 ± 1.5
3b	100.2 ± 9.2	107.3 ± 9.8
3c	50.1 ± 7.5	48.0 ± 5.2
3d	5.1 ± 1.4	6 .0 ± 1.6
3e	5.5 ± 1.5	6.0 ± 1.8
3f	50.2 ± 6.5	45.0 ± 5.8
3g	20.5 ± 4.4	18.1 ± 4.1
3h	15.0 ± 1.6	17.2 ± 1.9
3i	125.1 ± 10.3	120.8 ± 8.6

2 μM. The inhibition by compound **3a** could be attributed to electron withdrawing fluoro and nitro groups present on the substituted benzamide at para and ortho position.

However, the replacement of nitro with cyclopentyloxy and fluoro with methoxy group in **3d** and removal of nitro and replacement of fluoro with amine group in **3e** reduced the potency with IC_{50} values of 5.1 and 5.5 μM, respectively. This suggests that the anticancer activity is dependent not only on the nature of the substituent but also on the nature of the groups attached to the phenyl ring of that substituent. Similarly, when we compare the potency of compounds **3b** (R = amino-1,2,3,4-tetrahydronaphthalene, IC_{50} = 100.2 μM), **3c** (R = amino-1,2,3,4-tetrahydronaphthalene, IC_{50} = 50.1 μM), and **3f** (R = fluoro-3,4-dihydro-2H-chromene, IC_{50} = 50.2 μM), 50% of activity was reduced in case of **3b**. To enhance the inhibitory activity, we replaced fluoro-3,4-dihydro-2H-chromene (**3f**, IC_{50} = 50.2 μM) with bromo-2-oxo-2H-chromene (**3i**, IC_{50} = 125.1 μM). But the potency to inhibit cell growth got reduced.

It is worthy to mention that previously reported 2-(5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidin-3-yl)acetic acid derivatives (18) exhibited weak antiproliferative activity when compared with **3h** (R = 5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidine IC_{50} = 15 μM) having almost same group as a substituent in the current series. This data clearly show that the enhanced activity exhibited by compound **3h** could be due to

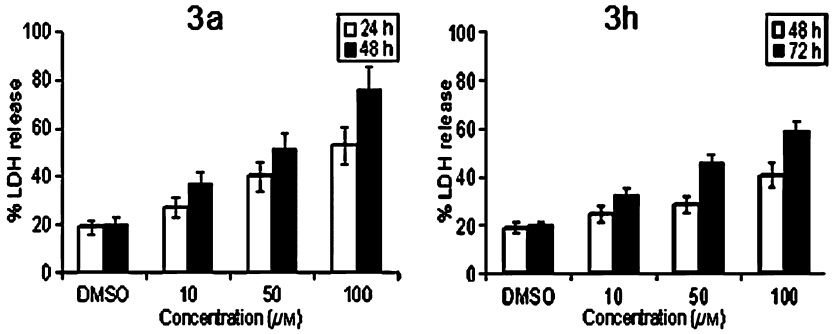


Figure 3: Time- and dose-dependent LDH release in K562 cells treated with **3a** or **3h**. K562 cells were incubated for 24 and 48 h with different concentrations of **3a** or **3h**. Release of LDH in the medium was measured at 490 nm. Results are presented as percentage of LDH release. Mean ± SD error bars are represented in the figure.

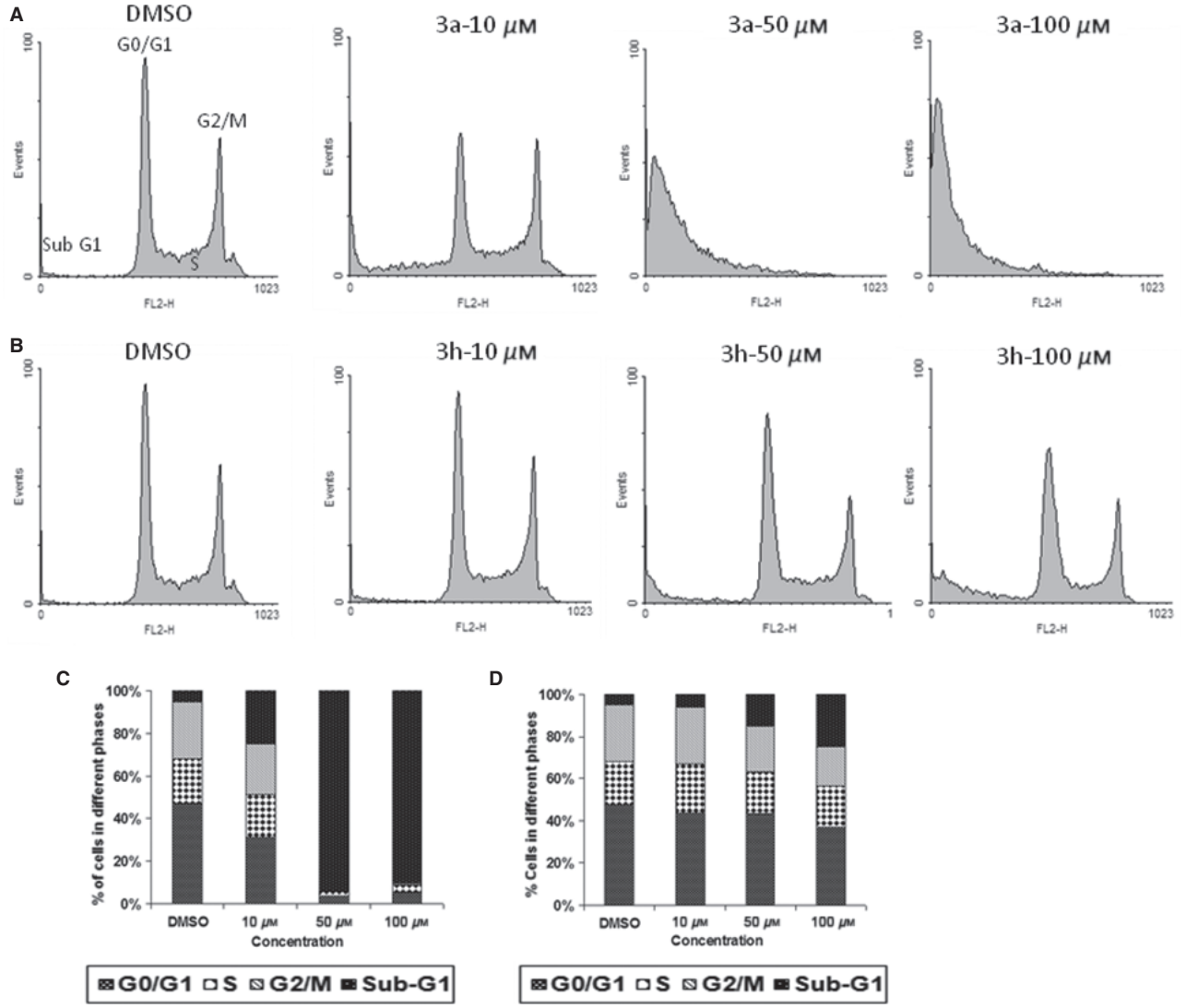


Figure 4: Cell cycle analysis of K562 cells treated with **3a** or **3h**. K562 cells (0.75×10^5 cells/mL) were incubated at 37 °C with **3a** or **3h** (10, 50 and 100 μM). Following 48 h of incubation, cells were fixed and stained with propidium iodide and subjected to FACS analysis. Panels A and B show histograms comparing the effect of **3a** and **3h** at specific cell cycle stages. In Panel A and B, the first histogram represents DMSO-treated cells. Panels C and D show the quantification of cells in different stages of cell cycle after treatment with **3a** and **3h**, respectively.

the presence of amide and the 4-[3-(piperidin-4-yl)propyl]piperidine moiety. The presence of amide group is necessary to lower systemic toxicity and to improve specificity in cancer treatment (11). In addition to this, compound **3g** having thiazolidinone group attached to the *N*-terminal of the piperidine also displayed good cytotoxic activity.

Furthermore, the potency of both **3a** and **3h** was verified by LDH assay, cell cycle analysis, and DNA fragmentation against K562 cells. The release of LDH is an indication of cell injury and hence cell death. Upon treatment with **3a** or **3h**, the LDH gets released into media, which was measured. Data show that the release of LDH was time and dose dependent (Figure 3). In case of **3a** treated with 100 μM, maximum release of LDH was observed at 48 h. This further confirmed that **3a** is more potent.

Flow cytometry was used to evaluate the effects of **3a** and **3h** on the growth and division of K562 cells, by measuring the DNA content of both treated and untreated cells stained by propidium iodide. Interestingly, upon addition of **3a** or **3h** to K562 cells, a concentration-dependent change was observed in the cell cycle pattern (Figure 4). As evident from Figure 4, that **3a** caused remarkable accumulation of sub-G1 cells (mostly dead cells) at concentration of 10 μM and higher with almost complete decline of both S and G2/M phase cells, which indicated the sign of apoptosis. However, such a drastic change was not observed when the cells were treated with compound **3h** at the same concentration levels. The histogram of vehicle control (DMSO treated) cells showed a standard cell cycle pattern, which include G0/G1 and G2/M peaks separated by S phase peak with almost no dead cells. Therefore, the studies further confirmed that remarkable growth inhibition caused by **3a** could be due to apoptosis.

Finally, we were interested to ascertain whether **3a** or **3h** caused DNA strand breaks or not, which is a characteristic feature of the programmed cell death or apoptosis. During the apoptotic process, activated nucleases degrade the higher-order chromatin structure of DNA into mono- and oligonucleosomal DNA fragments. Apoptotic degradation of DNA was analyzed by agarose gel electrophoresis. To verify this, chromosomal DNA was extracted after 72 h from the K562 cells treated with increasing concentrations of **3a** (5, 10, and 20 μM) or **3h** (10, 50, and 100 μM) as also from vehicle control-treated cells. This DNA was used for agarose gel electrophoresis. The results showed fragmentation of DNA leading to a smear in the lanes in which cells were treated with **3a** or **3h** (Figure 5). The observed smear is the result of DNA breakage at multiple positions across the chromosomal DNA. The intensity of smear increased with the dose in both **3a**- and **3h**-treated cells. In **3a**-treated cells, maximum smear was observed even at 10 μM. With respect to the DNA damage, we find that the extent of DNA strand breakage by **3a** is similar to that by **3h**. However, the concentrations required by **3a** to achieve this are much lesser compared with those by **3h**. These results further suggest that **3a** having fluoro and nitro phenyl ring induces fragmentation of chromosomal DNA leading to apoptosis more significantly than **3h** having 5-{[5-(4-chlorophenyl)furan-2-yl]methylene}-4-oxo-2-thioxothiazolidine group.

Conclusion

In summary, this study demonstrates that the 4-[3-(piperidin-4-yl)propyl]piperidine derivatives exhibit interesting antileukemia properties, depending on a delicate balance between the piperidine substitution pattern and nature of the acid moieties. They exhibited IC_{50} values of 2–125 μM. While **3a** was highly potent, a few others (**3d**, **3e**, **3g** and **3h**) exhibited moderate activity. The substituents at the *N*-amide of the pyridine ring were shown to be vital for potency. Work is in progress to optimize antileukemia activity by optimizing the *N*-substituted piperidine, to establish the mechanism of action.

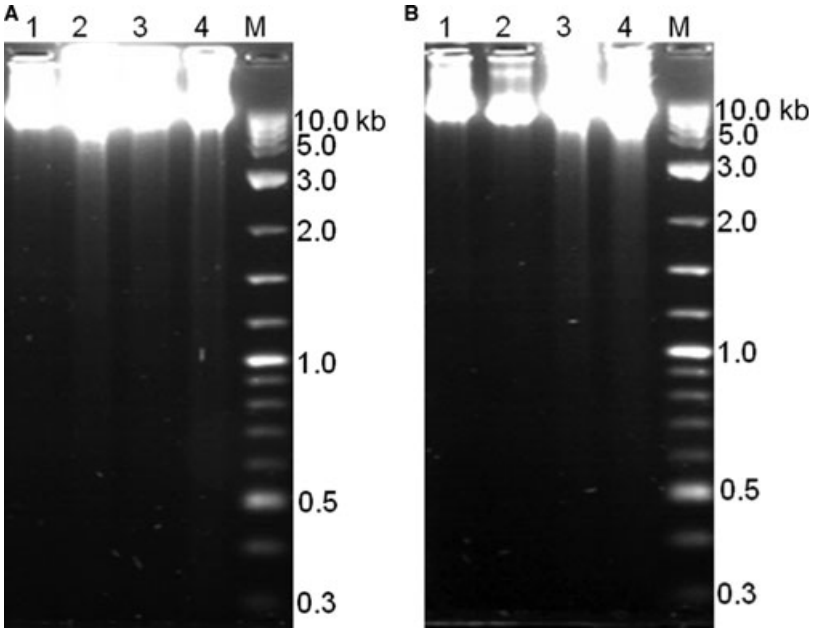


Figure 5: Detection of **3a** or **3h** induced DNA damage in K562 cells. The chromosomal DNA was extracted from K562 cells following treatment with different concentrations of **3a** (A) and **3h** (B). The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In both panels, Lane 1: DMSO; Lane 2, 3 and 4: K562 cells treated with 5, 10, and 20 μM of **3a**, and 10, 50 and 100 μM of **3h**, respectively. 'M' is Marker.

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