ISSN: 2249 –4820

Chemistry & Biology Interface, 2011, 1, 1, 59-67

Chemistry & Biology Interface

An official Journal of ISCB, Journal homepage; www.cbijournal.com

Research Article

Synthesis and antiproliferative activity of novel homopiperazine derivatives in leukemia cells

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Received 16 July 2011; Accepted 2 August 2011

Keywords: Homopiperazine, carboxamide, Cytotoxicity, Leukemia

Abstract: A series of novel homopiperazine derivatives were synthesized and characterized using 1H NMR, LC MS, IR and elemental analysis data. These novel molecules were evaluated for their antiproliferative activity against Reh, leukemia cells using trypan blue and MTT assays. All the molecules showed cytotoxicity with IC_{50} values between 50-100 µM as calculated by trypan blue assay and greater than 100 µM as calculated by MTT assay. Compound **6b** with 3,5-dinitro substituents on phenyl ring of the aryl carboxamide moiety attached to homopiperazine ring showed good activity with IC_{50} value of 41 µM.

Introduction

Cancer remains the leading cause of death in the World and as a result there is a pressing need for novel and effective treatments. One of the characteristic of cancer cells, that differs from their normal counterparts in a number of biochemical processes, particularly during the control of cell growth and division. Among different types of cancers, leukemia is one of the major causes of cancer related deaths [1,2].

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Hence, the identification of novel, efficient and less toxic anticancer agents remains an important and challenging task in cancer biology.

Synthetic heterocyclic compounds have been used extensively for drug development and the treatment of diseases including cancer [3]. Piperazines have been widely used in biological screening resulting In numerous applications [4] and constitutes an attractive pharmacological scaffold present in several drugs. This small and rigid heterocyclic backbone could act on various pharmacological targets. Especially, piperazine nucleus could be found in a broad range of biological active compounds displaying anticancer [5], antibacterial [6], antifungal [7], antimalarial, antipsychotic agents [8],

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HIV protease inhibitors [9], antidepressants [10] and anti-inflammatory activities [11]. Homopiperazines having similar structure as piperazine with one extra carbon atom in the ring have also shown various biological activities [12-15]. Earlier we reported the anticancer activity of benzhydryl piperazine derivatives against human cancer cell lines [16]. In continuation of our work, herein we report the synthesis and anticancer activity of benzhydryl homopiperazine derivatives.

Materials and Methods

Chemistry: Infrared (IR) spectra were recorded using a Jasco FTIR-4100 spectrometer in the wave number range of 4000-400 cm^{-1}. Nuclear magnetic resonance (^1H 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). Mass and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were obtained on Vario EL III Elementar. 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 were from Sigma Aldrich Chemicals Pvt Ltd.

Synthesis of 1-benzhydryl-4-(substituted phenylcarbonyl)-1,4-diazepane derivatives 6(a-f)

1-Benzhydryl-1,4-diazepane derivatives **6(a-f)** were synthesised by the method summarized in **Scheme 1**. Initially the compound **2**, benzhydrol was synthesised by reduction of benzophenone **1** using sodium borohydride and achieved 90% yield. Compound **2**was subsequently treated with thionyl chloride to give benzhydryl chloride **3**, which was directly treated with homopiperazine **4** and anhydrous potassium carbonate using dimethyl formamide as a solvent at 80 ^oC to give the target key intermediates 1-benzhydryl-1,4-diazepane **5**. Nucleophilic substitution reaction of compound 5 with different substituted benzoyl chlorides yielded the target compounds **6(a-f)**.

Synthesis of benzhydrol (2)

A solution of substituted benzophenone (20.0 g, 109 mmol) in methanol was taken and cooled to 0-5 ^oC. Sodium borohydride (8.28 g, 219.5 mmol) was added to the solution and stirred for 5 hr at room temperature. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. Finally water wash was given to the organic layer and dried with anhydrous sodium sulphate. The solvent was evaporated to get benzhydrol.

Synthesis of benzhydryl chloride (3)

A solution of substituted benzhydrol (16.0 g, 86.8 mmol) in dry dichloromethane was taken and cooled to 0-5 ^oC. Thionyl chloride (30.7 g, 258 mmol) was added to the solution and stirred for 4-5 hr at 0-10 ^oC. Upon completion, the solvent was removed under reduced pressure and residue was taken in dichloromethane for the removal of excess thionyl chloride to get benzhydryl chloride.

Synthesis of 1-benzhydryl-1,4-diazepane (5)

To a solution of homopiperazine **4** (5.0 g, 49.9 mmol) in dimethyl formamide, anhydrous potassium carbonate (20.7 g, 149.7 mmol) was added followed by the addition benzhydryl chloride (9.1 g, 44.9 mmol) and the reaction mixture was heated to 80 ^oC for 8 hr. Completion of the reaction was monitored by TLC. After

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completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. Finally, water wash was given to the organic layer and dried with anhydrous sodium sulphate. The solvent was evaporated to get crude product, which was purified by column chromatography over silica gel (60-120 mesh) using chloroform: methanol (9:1) as an eluent.

General procedure for synthesis of 1-benzhydryl-4-(substituted phenylcarbonyl)-1,4-diazepane derivatives 6(a-f)

A solution of 1-benzhydryl-1,4-diazepane (**5**) (1.0 eq) in dry dichloromethane was mixed. Triethylamine (3.0 eq) was added to the reaction mixture and stirred for 10 min, and then different benzoyl chlorides (1.0 eq) were added. The reaction mixture was stirred for 5-6 hr at room temperature, and monitored by TLC. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. The organic layer was washed with 10% ammonium chloride solution and finally water wash was given to the organic layer and dried with anhydrous sodium sulphate. The solvent was evaporated to get crude product, which was purified by column chromatography over silica gel (60-120 mesh) using hexane:ethyl acetate (8:2) as an eluent.

Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(3-methoxyphenyl)methanone (6a)

The product **6a** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 3-methoxybenzoyl chloride (0.32 g, 1.88 mmol) and triethylamine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.92 (t, 1H, Ar-H), 7.83 (d, 2H, Ar-H), 7.48 (t, 4H, Ar-H), 7.31 (m, 2H, Ar-H), 7.18 (m, 4H, Ar-H), 6.98 (s, 1H, Ar-H), 4.74 (s, 1H, -CH-), 3.89 (s, 3H, -OCH_3), 2.93 (m, 4H, -CH_2-), 2.78 (d, 4H, -CH_2-), 1.52-1.63 (m, 2H, -CH_2-). MS (ESI, + ion): m/z = 401.3. IR (KBr, cm^{-1}): 1681, 1372, 1164. Elemental Analysis: Found: C, 78.07; H, 6.97; N, 7.07; Calculated for $C_{26}H_{28}N_2O_2$: C, 77.97; H, 7.05; N, 6.99.

Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(3,5-dinitrophenyl)methanone (6b)

The product **6b** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 3,5-dinitrobenzoyl chloride (0.43 g, 1.88 mmol) and triethylamine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.42 (s, 1H, Ar-H), 8.19 (s, 2H, Ar-H), 7.75 (t, 2H, Ar-H), 7.41 (m, 4H, Ar-H), 7.29 (m, 4H, Ar-H), 7.17 (m, 4H, Ar-H), 4.86 (s, 1H, -CH-), 2.65 (t, 4H, -CH_2-), 2.53-2.58 (m, 4H, -CH_2-), 1.62 (m, 2H, -CH_2-). MS (ESI, + ion): m/z =462.2. IR (KBr, cm^{-1}): 1686, 1368, 1165. Elemental Analysis: Found: C, 65.29; H, 5.37; N, 12.24; Calculated for $C_{25}H_{24}N_4O_5$: C, 65.21; H, 5.25; N, 12.17.

Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(2,6-difluorophenyl)methanone (6c)

The product **6c** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 2,6-difluorobenzoyl chloride (0.33 g, 1.88 mmol) and triethyl-amine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.57 (t, 1H, Ar-H), 7.45 (t, 4H, Ar-H), 7.32 (m, 2H, Ar-H), 7.21 (t, 4H, Ar-H), 7.16 (t, 2H, Ar-H), 4.72 (s, 1H, -CH-), 2.66 (t, 4H, -CH_2-), 2.53 (m, 4H, -CH_2-), 1.52-1.6 (m, 2H, -CH_2-). MS (ESI, + ion): m/z =407.2. IR (KBr, cm^{-1}): 1681, 1361, 1162. Elemental Analysis: Found: C, 74.01; H, 6.07; N, 6.97; Calculated for $C_{25}H_{24}F_2N_2O$: C, 73.87; H, 5.95; N, 6.89.

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Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(2-fluorophenyl)methanone (6d)

The product **6d** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 2-fluorobenzoyl chloride (0.30 g, 1.88 mmol) and triethylamine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.01 (t, 1H, Ar-H), 7.92 (d, 1H, Ar-H), 7.85 (d, 2H, Ar-H), 7.42 (m, 4H, Ar-H), 7.21-7.29 (t, 4H, Ar-H), 7.1-7.17 (t, 2H, Ar-H), 4.74 (s, 1H, -CH-), 2.73 (t, 4H, -CH_2-), 2.62 (m, 4H, -CH_2-), 1.65 (m, 2H, -CH_2-). MS (ESI, + ion): m/z =389.2. IR (KBr, cm^{-1}): 1678, 1360, 1160. Elemental Analysis: Found: C, 77.42; H, 6.57; N, 7.27; Calculated for $C_{25}H_{25}FN_2O$: C, 77.29; H, 6.49; N, 7.21.

Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(3-bromophenyl)methanone (6e)

The product **6e** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 3-bromobenzoyl chloride (0.41 g, 1.88 mmol) and triethylamine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.15 (s, 1H, Ar-H), 7.92 (d, 1H, Ar-H), 7.85 (d, 1H, Ar-H), 7.72 (t, 1H, Ar-H), 7.42 (m, 4H, Ar-H), 7.2-7.28 (d, 4H, Ar-H), 7.13 (m, 2H, Ar-H), 4.83(s, 1H, -CH-), 2.72 (t, 4H, -CH_2-), 2.31 (m, 4H, -CH_2-), 1.74 (m, 2H, -CH_2-). MS (ESI, + ion): m/z =450.2. IR (KBr, cm^{-1}): 1678, 1369, 1158. Elemental Analysis: Found: C, 66.92; H, 5.49; N, 6.32; Calculated for $C_{25}H_{25}BrN_2O$: C, 66.82; H, 5.61; N, 6.23.

Synthesis of (4-benzhydryl-1,4-diazepan-1-yl)(phenyl)methanone (6f)

The product **6f** was obtained by reaction of 1-benzhydryl-1,4-diazepane **(5)** (0.5 g, 1.88 mmol), 3-nitrobenzene sulfonyl chloride (0.26 g, 1.88 mmol) and triethylamine (0.57 g, 5.64 mmol) in dichloromethane using the general experimental procedure as described. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.96 (d, 2H, Ar-H), 7.77 (m, 2H, Ar-H), 7.52 (t, 1H, Ar-H), 7.48 (d, 4H, Ar-H), 7.27 (t, 4H, Ar-H), 7.14 (m, 2H, Ar-H), 4.72 (s, 1H, -CH-), 2.62 (t, 4H, -CH_2-), 2.44 (m, 4H, -CH_2-), 1.64 (m, 2H, -CH_2-). MS (ESI, + ion): m/z =371.1. IR (KBr, cm^{-1}): 1682, 1367, 1161. Elemental Analysis: Found: C, 80.94; H, 6.98; N, 7.67.; Calculated for $C_{25}H_{26}N_2O$: C, 81.05; H, 7.07; N, 7.56.

Biology:

Chemicals and reagents

All the chemicals used were of analytical grade and purchased from Sigma–Aldrich, USA or from SRL, India.

Cell lines and culture conditions

Reh, a B-cell leukemia cell line, a kind gift from Dr. M.R. Lieber, USA, was cultured in RPMI 1640 (Sera Lab, UK) containing 10% FBS (Gibco BRL, USA), 100 U of Penicillin G/ml and 100 µg/ml streptomycin (Sigma–Aldrich, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Trypan blue dye exclusion assay

The effect of compounds 6 (a-f) on cell viability was tested by trypan blue exclusion assay [17, 18]. Reh cells were cultured at a density of 1×10^5 cells/ml and the compounds were added at concentrations of 10, 50 and 100 µM after 24 h. Cells were harvested at an interval of 24 h and resuspended in 0.4% trypan blue (Sigma–Aldrich, USA). The live cells were counted and IC_{50} values (50% inhibition of cell growth) were estimated for 72 h of treatment with the compounds (Table 1). DMSO treated cells were used as vehicle control. Experiments were

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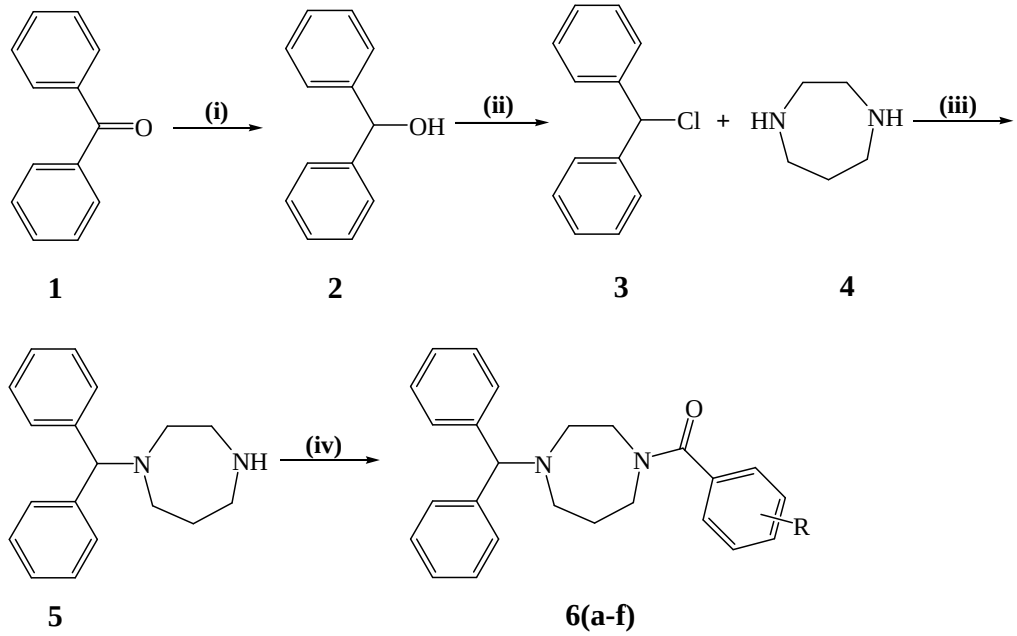
repeated at least 2 independent times and the values obtained were plotted as a graph



Scheme 1

Reagents and conditions: (i). $NaBH_4$, Methanol, r.t., 5 hr. (ii). Thionyl chloride, MDC, 0-5 ^oC, 4 hr. (iii). K_2CO_3, DMF, 80 ^oC, 8 hr. (iv). substituted benzoyl chloride, MDC, TEA, r.t., 5-6 hr.

MTT assay

The compounds, **6(a-f)** were tested for their effect on cell proliferation by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [19, 20]. Reh cells were cultured (duplicates) in 96-well plates and compounds were added after 24 h, at a concentration of 10, 50 and 100 μM of **6(a-f).** The cells were collected after 24, 48 and 72 h of incubation and used for the MTT assay. MTT reagent (5 mg/ml, Sigma–Aldrich, USA) was added to the cells and incubated for 4 h, resulting in formation of an insoluble purple colour formazan product. This product was then solubilized using a detergent containing 50% N,N-dimethylformamide (Sigma–Aldrich, USA) and 10% of sodium dodecyl sulphate (Amresco, USA) and incubated for another 2 h. The absorbance was measured at 570 nm using a multiwell ELISA plate reader (Molecular Devices, USA) scanning spectrophotometer. Cells treated with DMSO were used as vehicle control. The values obtained were plotted as % cell proliferation (The percentage of cell proliferation was calculated as: ratio of OD value of sample/OD value of control x 100). Each experiment was performed a minimum of two independent times and the values were plotted as a bar diagram with error bars.

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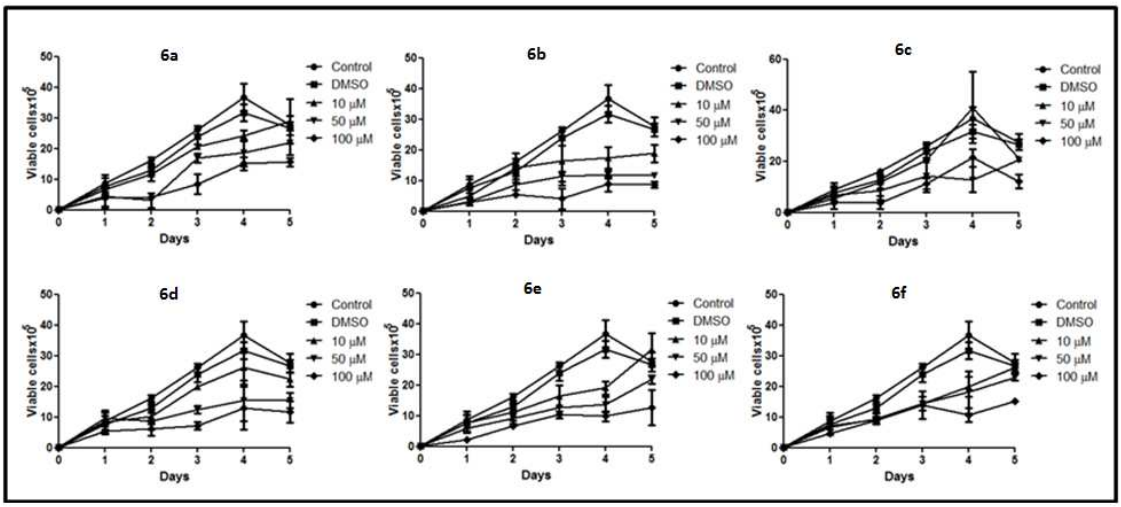

Figure 1. Dose- and time-dependent effect of 2, 3 series of compounds on cell viability of B-cell leukemic cell line, Reh. A. Approximately 1 x 10^5 cells/ml were cultured and 2 series compounds **6(a-f)** were added after 24 h at a concentration of 10, 50 and 100 µM. Cells alone and cells treated with DMSO were used as control and vehicle control, respectively. After addition of compounds, live cells were counted following staining with trypan blue at an interval of 24 h, till the cells reached stationary phase. Data obtained were plotted as a graph and error bars are indicated.

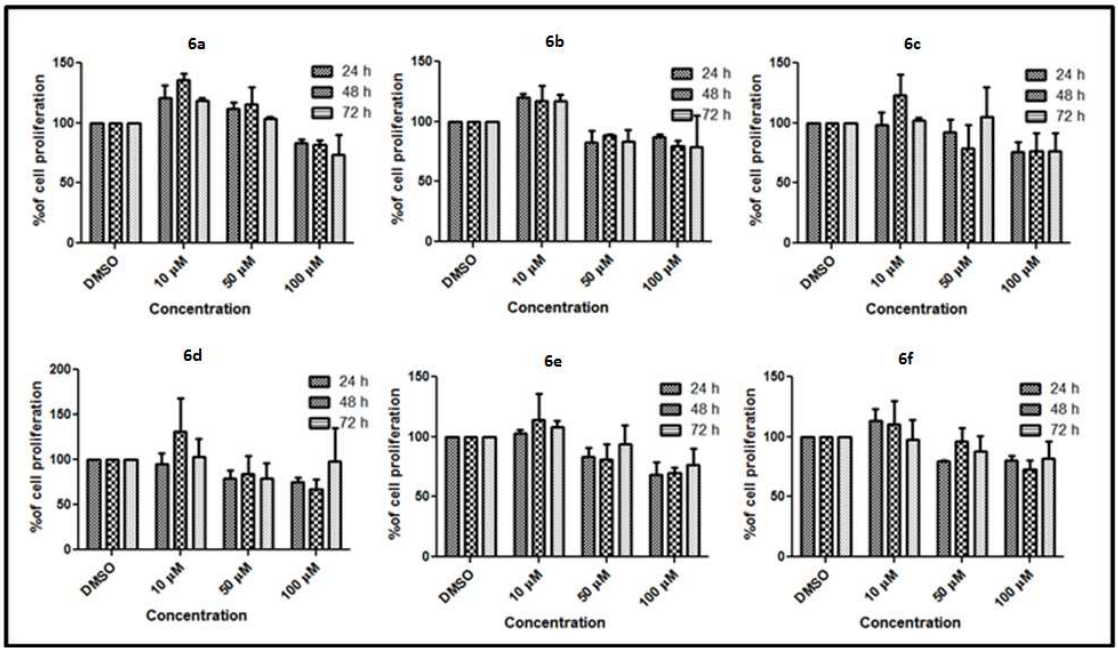

Figure 2. Determination of effect of compounds 6(a-f) on Reh cells by MTT assay. A. Reh cells, grown for 24 h, were treated with **6(a-f)** at a concentration of 10, 50 and 100 µM. The cells were collected following 24, 48 and 72 h of treatment and subjected to MTT assay as specified in methods. The percentage of DMSO treated (vehicle control) Reh cells were considered as 100% and the relative inhibition following treatment with the compounds is shown as a bar diagram.

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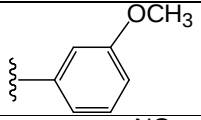
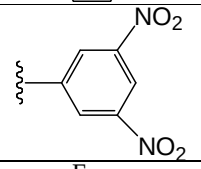
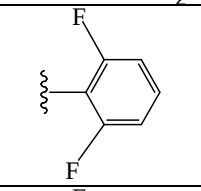
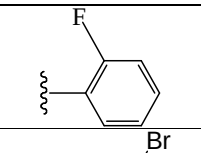
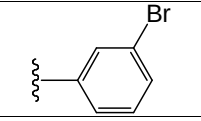
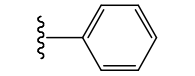
Table 1: Chemical structure and IC_{50} values of the synthesized compounds **6(a-f)** on Reh cells (as determined based on trypan blue assay).

Compound	R	IC_{50} in µM
6a		69
6b		41
6c		75
6d		50
6e		80
6f		>100

Results and discussion

In the present study, we have synthesised a series of novel aryl carboxamide and benzhydryl substituted homopiperazine derivatives. All the novel molecules were charactesised using 1H NMR, LC MS, IR and elemental analysis data. Then we have investigated the cytotoxic effect of **6(a-f)** compounds on Reh, a B-cell leukemia cell line. Concentrations of 10, 50 and 100 µM were used for testing. Cells alone and cells with DMSO were used as control and vehicle control, respectively. In order to determine the cell viability, trypan blue assay was performed. Results showed that the addition of **6(a-f)** compounds showed cytotoxicity at 100 µM concentration (Figure 1). All the compounds exhibited IC_{50} values in the range of 50-100µM in trypan blue assay (Table 1). These results suggest that compounds **6(a-f)** induced cytotoxicity at higher concentration and induced cell death on Reh cells in a time- and dose-dependent manner. Compound **6b** with 3,5-dinitro substituents on the phenyl ring of the aryl carboxamide moiety attached to the homopiperazine ring showed good activity compared to all the derivatives in the series with an IC_{50} value of 41µM. Compound **6f** without any substituents on the phenyl ring showed poor activity with IC50 value of around 100 µM.

The effect of compounds **6(a-f)** on cell proliferation was further tested by using

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MTT assay. Reh cells were treated with 10, 50 and 100 µM. The cells were harvested after 24, 48 and 72 h of treatment and subjected to MTT assay. The proliferation of Reh cells was determined by the reduction of MTT by the metabolic activity of viable cells. Results showed that addition of compounds **6(a-f)** affect the cell proliferation (Figure 2) at concentration of 100 µM. Based on MTT assay, the IC_{50} was calculated and; most of them showed IC_{50} values >100 µM. These results suggest that the activity depends on the substitution on the phenyl ring of aryl carboxamide moiety.

Conclusion

In conclusion, a series of novel homopiperazine carboxamide derivatives were synthesised and evaluated for their antiproliferative activity against Reh cells. Compound **6b** with 3,5-dintro substitution on the phenyl ring of the aryl carboxamide moiety attached to homopiperazine showed good activity. Further derivatisation of the key intermediate to increase the efficacy and to study the structure activity relationship associated with the nature and position of the substituents on the phenyl ring are underway. Further, the study of the mechanism of action of these novel molecules in inhibition of the proliferation of Reh cells is also under progress and the results will be reported in future.

Acknowledgements

We are grateful to CSIR and UGC, Govt. of India for financial support to KSR. We thank Mridula Nambiar for critical reading of the manuscript. This work was supported by Lady Tata Memorial Trust international award for leukemia research (London) for SCR. KPM is supported by IISc postdoctoral fellowship, India.

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