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PRECLINICAL STUDIES

Synthesis and identification of a new class of *(S)*-2,6-diamino-4,5,6,7-tetrahydrobenzo[d]thiazole derivatives as potent antileukemic agents

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Summary Benzothiazoles are multitarget agents with broad spectrum of biological activity. Among the antitumor agents discovered in recent years, the identification of various 2-(4-aminophenyl) benzothiazoles as potent and selective antitumor drugs against different cancer cell lines has stimulated remarkable interest. Some of the benzothiazoles are known to induce cell cycle arrest, activation of caspases and interaction with DNA molecule. Based on these interesting properties of benzothiazoles and to obtain new biologically active agents, a series of novel 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i) were synthesized and evaluated for their efficacy as antileukemic agents in human leukemia cells (K562 and Reh). The chemical structures of the synthesized compounds were confirmed by ^{1}H NMR, LCMS and IR analysis. The cytotoxicity of these compounds were determined using trypan blue exclusion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. Results showed that, these compounds mediate a significant cytotoxic response to cancer cell lines tested. We found that the compounds having electron withdrawing groups at different positions of the phenyl ring of the thiourea moiety displayed significant cytotoxic effect with IC_{50} value less than 60 μM. To rationalize the role of electron withdrawing group in the induction of cytotoxicity, we have chosen molecule 5g (IC_{50} ~15 μM) which is having chloro substitution at ortho and para positions. Flow cytometric analysis of annexin V-FITC/ propidium iodide (PI) double staining and DNA fragmentation suggest that 5g can induce apoptosis.

Keywords Tetrahydrobenzo[d]thiazole · Cytotoxicity · Apoptosis · DNA fragmentation · Cancer therapeutics · Leukemia

Introduction

Cancer chemotherapy targeting tumor progression represents one of the most relevant challenges of chemists and oncologist. Leukemia is one of the major types of cancer affecting a significant segment of the population, especially children. The American Cancer Society (ACS) estimated that 44,790 new cases of leukemia would be diagnosed in the United States in 2009, whereas about 21,870 adults and children would die of leukemia during 2009 [1]. In order to gain new insight into the complexity of the disease, robust screening methods for evaluating different natural or synthetic drugs have been carried out by the scientific community. Although the incidence rate of this disease remains almost unchanged, some success has been reported in its treatment. But even if the success of clinical trials in identifying new agents and treatment modalities has been significant, current treatments have many limitations related to their side effects and the development of acquired drug resistance [2].

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Thiazole is an important heterocyclic ring widely used in anticancer drug research area. The interest in the antineoplastic activity of thiazoles increased due to the presence of thiazole moiety in natural chemotherapeutic agents like tiazofurin [3, 4], distamycin [5], bleomycin [6, 7], netropsin and thianetropsin [8]. Benzothiazole derivatives have been studied extensively followed by the use of riluzole (6-trifluoromethoxy-2-benzothiazolamine) [9], which was found to interfere with glutamate neurotransmission. Some of the 2-aminobenzothiazole derivatives possess cytotoxicity on cancer cells which is comparable to that of cisplatin [10]. In this respect, the benzothiazole constitutes an important scaffold of drugs, possessing several pharmacological functions. The interesting anticancer properties of these compounds take the initiative of synthesis and anticancer studies of many new benzothiazole derivatives [11-15]. In addition, these derivatives are used as neurotransmission blocker [16-18], calmodulin (CaM) antagonists [19] and neuroprotective agent [20, 21]. Cantharidin containing benzothiazoles possess antitumor properties in hepatocellular carcinoma, breast cancer, acute myelogenous leukemia and non-small cell lung carcinoma. It is reported that compounds having substituted amine groups at position 2 and 6 of benzothiazole moiety show good antitumor activity [22]. Tetrahydrobenzothiazoles which are similar in structure to benzothiazoles also claim various biological activities [23-29]. Pramipexol, a 2,6-diamino-4,5,6,7-tetrahydrobenzo[d]thiazole derivative with (*S*) configuration at position 6 is being used as a drug for schizophrenia and Parkinson's disease.

Based on the results reported for the benzothiazole derivatives, biological importance of the 4,5,6,7-tetrahydrobenzo[d]thiazole moiety with substitution at position 6 and also from our own experience in the synthesis and evaluation of anticancer property of substituted heterocyclic compounds [30-37], we designed and synthesised a new series of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives with arylthiourea substitution at position 6. To the best of our knowledge, all 6-amine substituted derivatives of *(S)*-4,5,6,7-tetrahydrobenzo[d]thiazole-2,6-diamine are novel, and here we report the synthesis and cytotoxic activities of novel *(S)*-4,5,6,7-tetrahydrobenzo[d]thiazole-2,6-diamine derivatives on human leukemia cells.

Experimental

IR spectra were recorded using a Jasco FTIR-2008 series. ^{1}H NMR spectra were recorded on Shimadzu AMX 400-Bruker, 400 MHz spectrometer using DMSO as a solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Mass and purity were recorded on a LC–MSD-Trap-XCT. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.

General procedure for the synthesis 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazol-6-yl)-3-(substituted phenyl)thiourea derivatives 5(a–i)

To the solution of intermediate compound 4 (1 eq) in dichloromethane, triethylamine (3 eq) was added and cooled to 0 °C, respective aryl isothiocyanate (1 eq) were added at cold condition and stirred at room temperature for 6–7 h. Progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was concentrated, water was added and extracted thrice using ethyl acetate. The combined ethyl acetate layer was washed with brine solution and dried over anhydrous sodium sulphate. Ethyl acetate was evaporated under reduced pressure and the crude product obtained was purified by silica gel (60–120 mesh) column. The compounds 5(a–i) were eluted at 70–80% ethyl acetate in hexane.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazol-6-yl)-3-(4-methoxy phenyl)thiourea (5a)

The product obtained (0.33 g, 83%) by the reaction of 4 (0.2 g, 1.2 mmol) with 4-methoxy phenyl isothiocyanate (0.195 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.20–8.16 (dd, 1H, -NH), 8.06 (s, 1H, -NH), 7.31–7.29 (d, 2H, Ar-H), 6.85 (d, 2H, Ar-H), 6.70 (s, 2H, $-NH_2$), 4.04–4.01 (m, 1H, -CH-), 3.70 (s, 3H, $-OCH_3$), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, $-CH_2$), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, $-CH_2$), 1.79–1.72 (m, 1H, $-CH_2$). MS (ESI + ion): m/z = 335.57. IR (KBr, cm^{-1}): 3411, 3207, 1371, 1106.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazol-6-yl)-3-(4-fluoro phenyl)thiourea (5b)

The product obtained (0.32 g, 84%) by the reaction of 4 (0.2 g, 1.2 mmol) with 4-fluoro phenyl isothiocyanate (0.181 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.16–8.12 (dd, 1H, -NH), 8.01 (s, 1H, -NH), 7.41–7.40 (d, 2H, Ar-H), 7.10–7.12 (d, 2H, Ar-H), 6.65 (s, 2H, $-NH_2$), 4.0–3.95 (m, 1H, -CH-), 2.77–2.73 (dd, 1H, -CH), 2.45–2.39 (m, 2H, $-CH_2$), 2.29–2.23 (dd, 1H, -CH), 1.80–1.72 (m, 1H, $-CH_2$), 1.77–1.71 (m, 1H, $-CH_2$). MS (ESI + ion): m/z = 323.52. IR (KBr, cm^{-1}): 3407, 3224, 1369, 1118.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazol-6-yl)-3-(3-chloro phenyl)thiourea (5c)

The product obtained (0.325 g, 81%) by the reaction of 4 (0.2 g, 1.2 mmol) with 3-chloro phenyl isothiocyanate

(0.2 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.26–8.23 (dd, 1H, -NH), 8.10 (s, 1H, -NH), 7.41 (s, 1H, Ar-H), 7.10–7.12 (m, 3H, Ar-H), 6.77 (s, 2H, -NH_2), 3.94–3.91 (m, 1H, -CH-), 2.94–2.89 (dd, 1H, -CH), 2.56–2.49 (m, 2H, -CH_2), 2.4–2.37 (dd, 1H, -CH), 1.79–1.73 (m, 1H, -CH_2), 1.69–1.62 (m, 1H, -CH_2). MS (ESI + ion): m/z = 339.94. IR (KBr, cm^{-1}): 3423, 3233, 1376, 1096.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-(3-methoxy phenyl)thiourea (5d)

The product obtained (0.33 g, 83%) by the reaction of 4 (0.2 g, 1.2 mmol) with 3-methoxyphenyl isothiocyanate (0.195 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.18–8.16 (dd, 1H, -NH), 8.06 (s, 1H, -NH), 8.02 (s, 1H, Ar-H), 7.35–7.21 (m, 1H, Ar-H), 6.95–6.91 (d, 2H, Ar-H), 6.70 (s, 2H, -NH_2), 4.04–4.01 (m, 1H, -CH-), 3.70 (s, 3H, -OCH_3), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, -CH_2), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, -CH_2), 1.79–1.72 (m, 1H, -CH_2). MS (ESI + ion): m/z = 335.40. IR (KBr, cm^{-1}): 3442, 3237, 1379, 1132.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-(2-fluoro phenyl)thiourea (5e)

The product obtained (0.32 g, 84%) by the reaction of 4 (0.2 g, 1.2 mmol) with 2-fluorophenyl isothiocyanate (0.181 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.20–8.18 (dd, 1H, -NH), 8.07 (s, 1H, -NH), 7.40–7.37 (d, 1H, Ar-H), 7.20–7.16 (m, 2H, Ar-H), 6.90–6.85 (m, 1H, Ar-H), 6.75 (s, 2H, -NH_2), 4.09–4.05 (m, 1H, -CH-), 2.89–2.74 (dd, 1H, -CH), 2.55–2.49 (m, 2H, -CH_2), 2.40–2.35 (dd, 1H, -CH), 1.90–1.84 (m, 1H, -CH_2), 1.77–

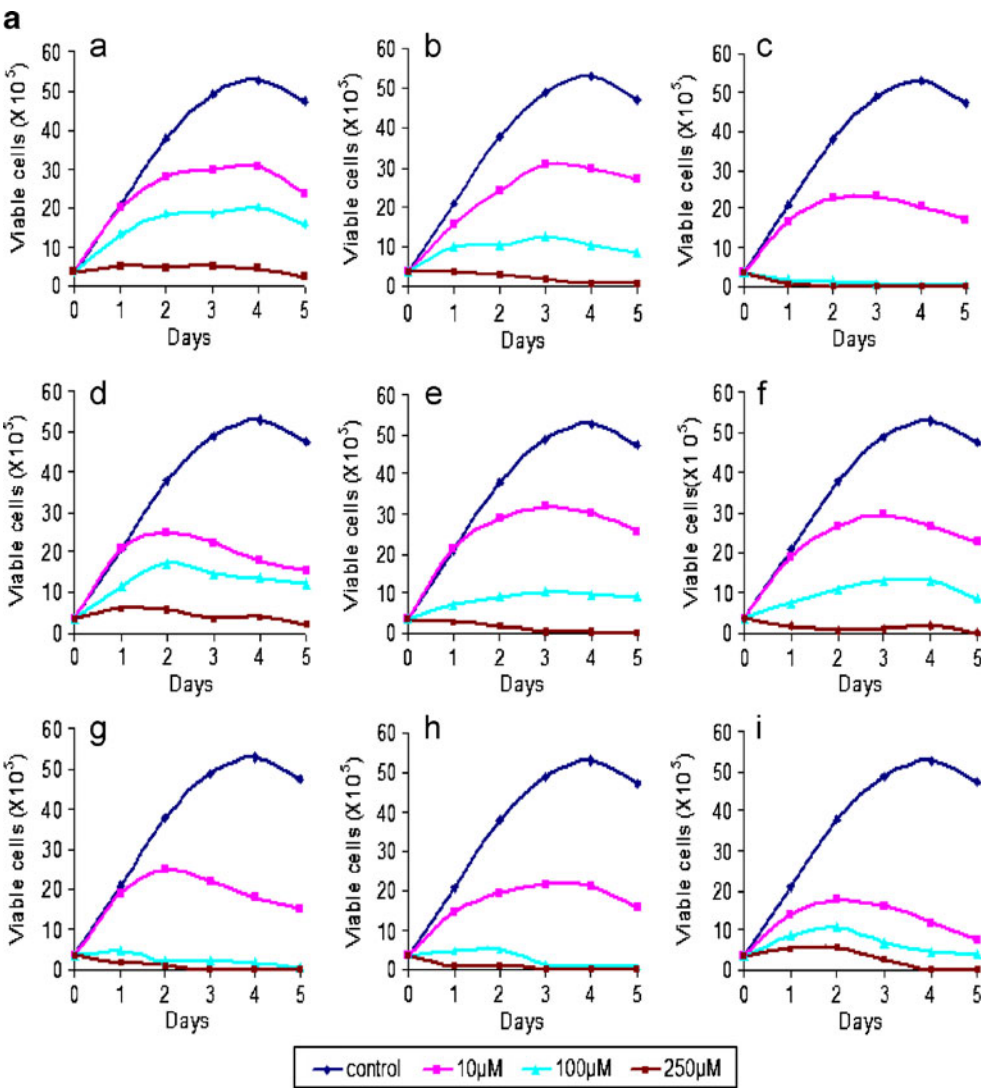


Fig. 1 Dose- and time-dependent effect of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i) on K562 (**a**) and Reh (**b**) cell survival. Approximately, 0.75×10^5 cells/ml were cultured and treated with 5 (a–i) at a concentration of 10, 100 and 250 μM. Cell viability was determined by trypan blue exclusion assay. Viable cells were counted everyday till the control cells reached stationary phase and the data was represented as a graph

1.70 (m, 1H, -CH_2). MS (ESI + ion): m/z = 323.61. IR (KBr, cm^{-1}): 3404, 3212 1381, 1116.

Synthesis of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-(2-chloro phenyl)thiourea (5f)

The product obtained (0.325 g, 81%) by the reaction of 4 (0.2 g, 1.2 mmol) with 2-chlorophenyl isothiocyanate (0.2 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.18–8.16 (dd, 1H, -NH), 8.06 (s, 1H, -NH), 7.38–7.35 (d, 1H, Ar-H), 7.23–7.19 (m, 2H, Ar-H), 6.94–6.89 (m, 1H, Ar-H), 6.70 (s, 2H, -NH_2), 4.04–4.01 (m, 1H, -CH-), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, -CH_2), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, -CH_2), 1.79–1.72 (m, 1H, -CH_2). MS (ESI + ion): m/z = 339.77. IR (KBr, cm^{-1}): 3397, 3186, 1361, 1092.

Synthesis of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-(2,4-dichloro phenyl)thiourea (5g)

The product obtained (0.345 g, 78%) by the reaction of 4 (0.2 g, 1.2 mmol) with 2,4-dichlorophenyl isothiocyanate (0.241 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.18–8.16 (dd, 1H, -NH), 8.07 (s, 1H, -NH), 7.38 (s, 1H, Ar-H), 7.23–7.19 (m, 2H, Ar-H), 6.70 (s, 2H, -NH_2), 4.04–4.01 (m, 1H, -CH-), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, -CH_2), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, -CH_2), 1.79–1.72 (m, 1H, -CH_2). MS (ESI + ion): m/z = 374.50. IR (KBr, cm^{-1}): 3452, 3246, 1392, 1133.

Synthesis of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-(4-chloro phenyl)thiourea (5h)

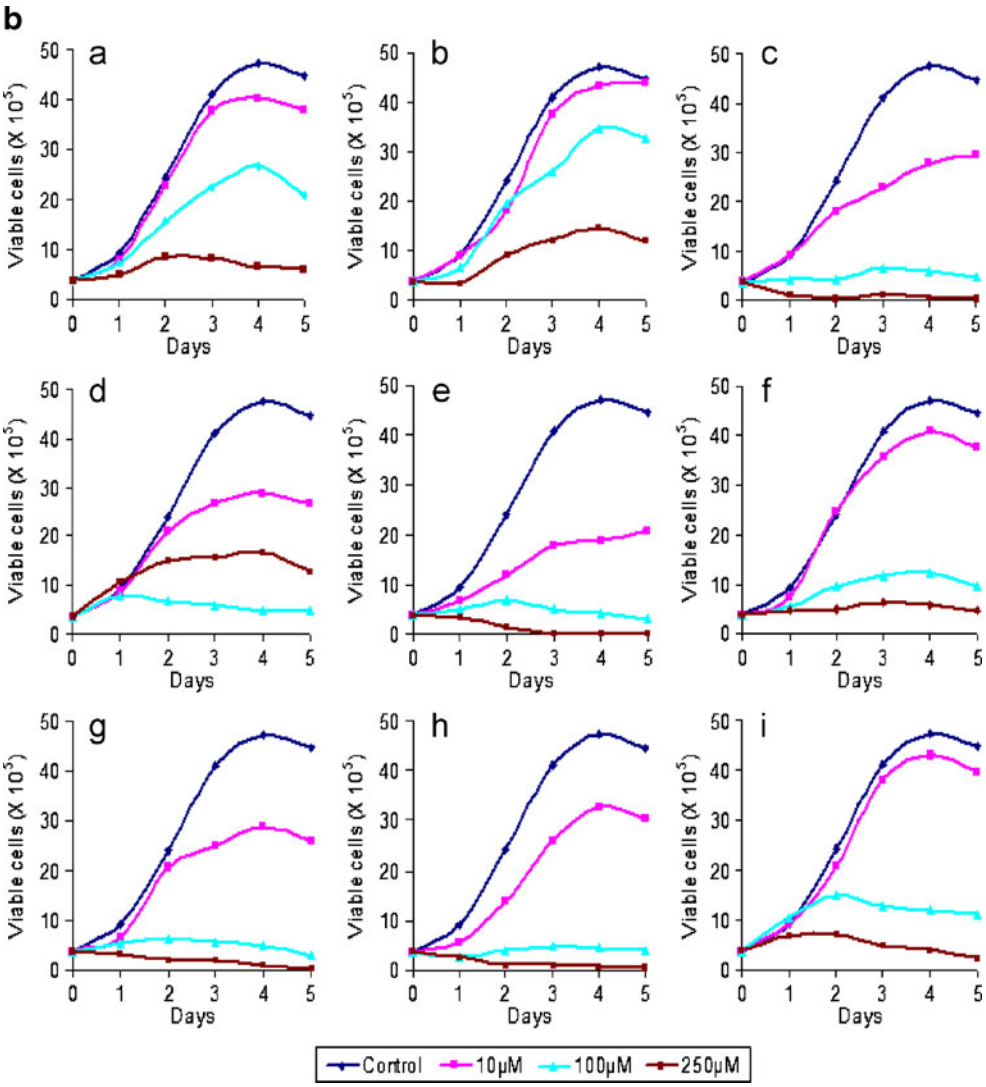


Fig. 1 (continued)

The product obtained (0.325 g, 81%) by the reaction of 4 (0.2 g, 1.2 mmol) with 4-chlorophenyl isothiocyanate (0.2 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.20–8.16 (dd, 1H, -NH), 8.06 (s, 1H, -NH), 7.31–7.29 (d, 2H, Ar-H), 6.86–6.84 (d, 2H, Ar-H), 6.70 (s, 2H, -NH_2), 4.04–4.01 (m, 1H, -CH-), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, -CH_2), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, -CH_2), 1.79–1.72 (m, 1H, -CH_2). MS (ESI + ion): m/z = 339.68. IR (KBr, cm^{-1}): 3433, 3225, 1377, 1123.

Synthesis of 1-(*(S)*-2-amino-4,5,6,7-tetrahydrobenzo[d] thiazol-6-yl)-3-phenyl) thiourea (5i)

The product obtained (0.31 g, 83%) by the reaction of 4 (0.2 g, 1.2 mmol) with phenyl isothiocyanate (0.160 g, 1.2 mmol) and triethylamine (0.36 g, 3.6 mmol) in dichloromethane (3 ml) using the general experimental procedure described was white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.22–8.19 (dd, 1H, -NH), 8.07 (s, 1H, -NH), 7.30 (m, 5H, Ar-H), 6.70 (s, 2H, -NH_2), 4.04–4.01 (m, 1H, -CH-), 2.84–2.79 (dd, 1H, -CH), 2.51–2.46 (m, 2H, -CH_2), 2.41–2.36 (dd, 1H, -CH), 1.89–1.82 (m, 1H, -CH_2), 1.79–1.72 (m, 1H, -CH_2). MS (ESI + ion): m/z = 305.63. IR (KBr, cm^{-1}): 3403, 3211, 1379, 1114.

Biology

Human leukemia cells K562 (chronic myelogenous leukemia) and Reh (B-cell leukemia) were selected for the purpose of preliminary anti-cancer screening of newly synthesized compounds. The cytotoxicity was assessed by

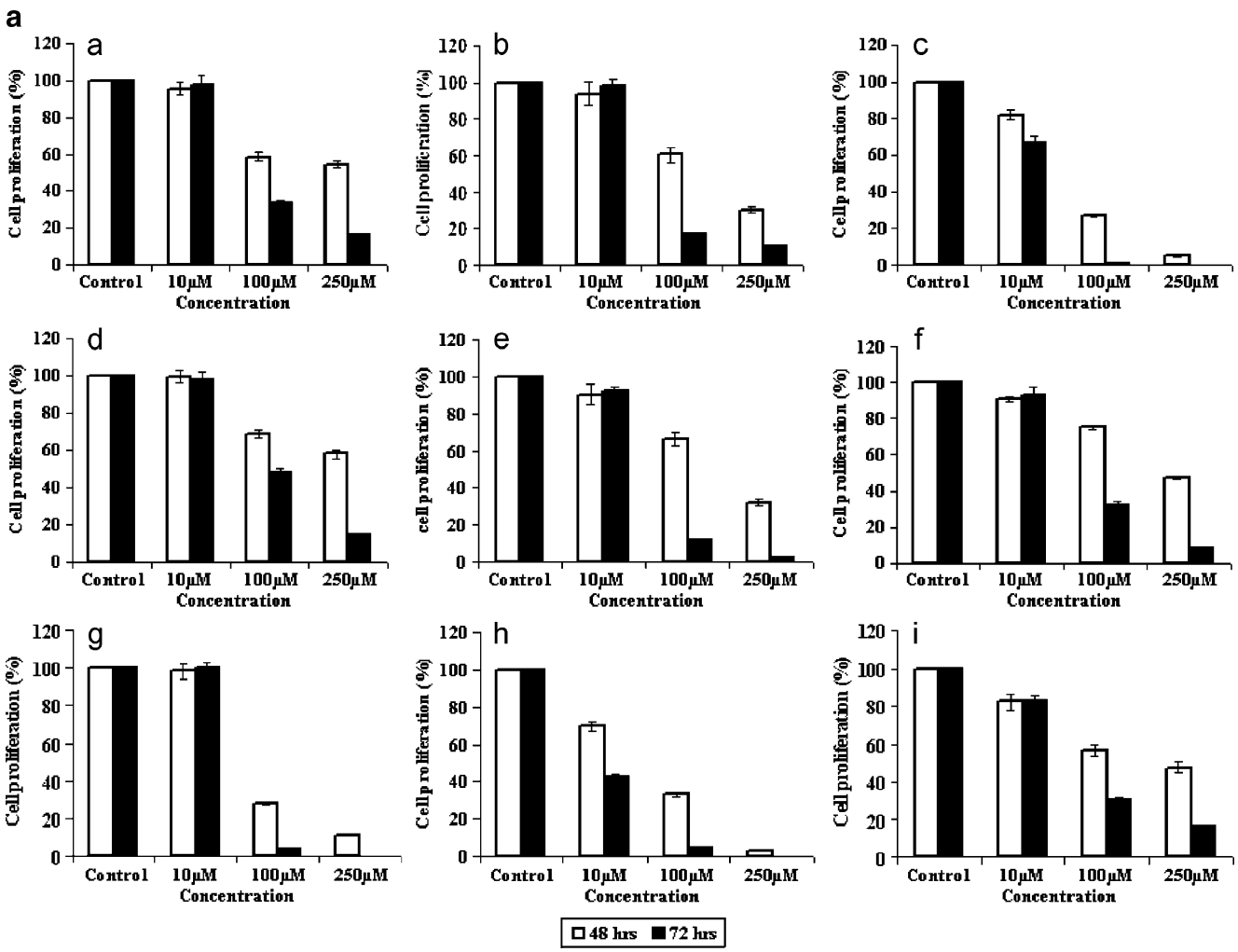


Fig. 2 Determination of cell proliferation by MTT assay. After 48 h and 72 h of exposure, K562 cells (**a**) or Reh cells (**b**) treated with 5(a–i) at different concentrations (10, 100 and 250 μM) were incubated with MTT (0.5 mg/ml) in duplicates and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation. Error bars are represented in the figure

employing trypan blue dye exclusion assay, MTT assay, LDH assay and FACS analysis. For this, cells growing in log phase were treated with different concentrations (10, 100 and 250 μM) of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i). Besides, DNA fragmentation assay was performed to assess whether the potent molecule 5g is able to damage DNA which is an indicator of apoptosis. Annexin V-FITC/ propidium iodide (PI) double staining assay was done to quantify the apoptotic cells formed upon treatment with 5g. All the assays were carried out in duplicate in two independent experiments.

Cell lines and culture

Human cell line, K562 was purchased from National Center for Cell Science, Pune, India and Reh 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, 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

Cell viability was monitored by the trypan blue exclusion assay. Cells (K562 or Reh) growing in exponential phase were seeded at a density of 0.75×10^5 cells/ml in a 6-well tissue culture plate for 24 h prior to the experiments and cells were exposed to 10, 100 and 250 μM concentrations of 5(a–i). Cells were collected at intervals of 24 h and resuspended in 0.4% trypan blue and further incubated for 5 min after which the number of viable cells was estimated in a haemocytometer chamber. The results are presented as graphs in Fig. 1.

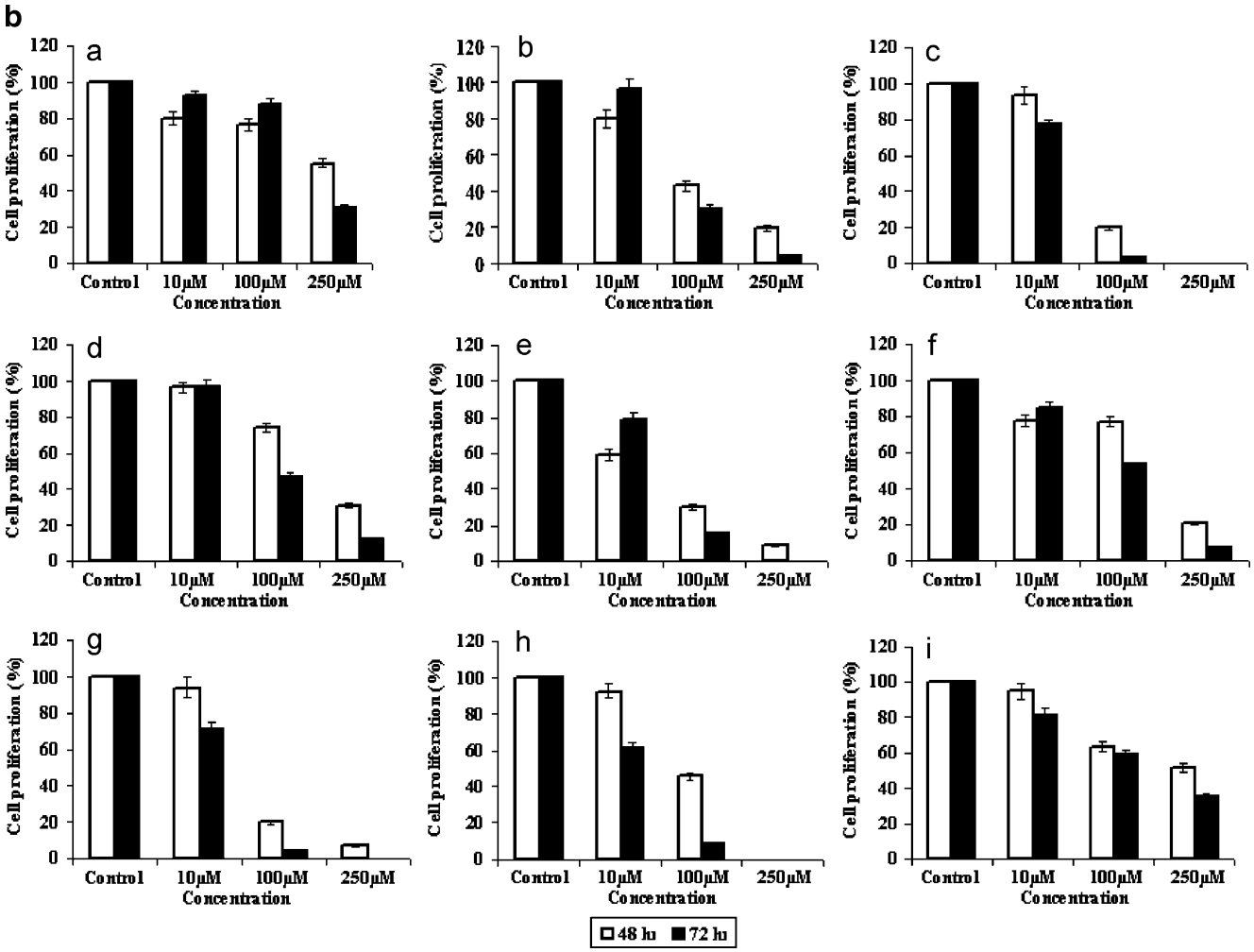


Fig. 2 (continued)

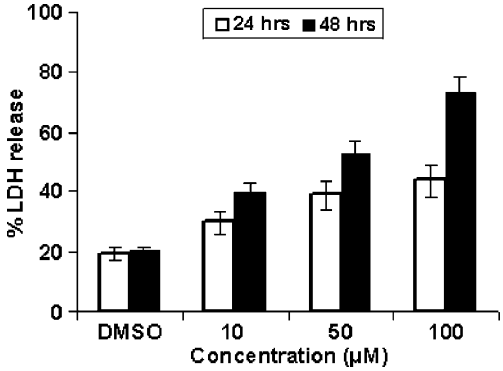


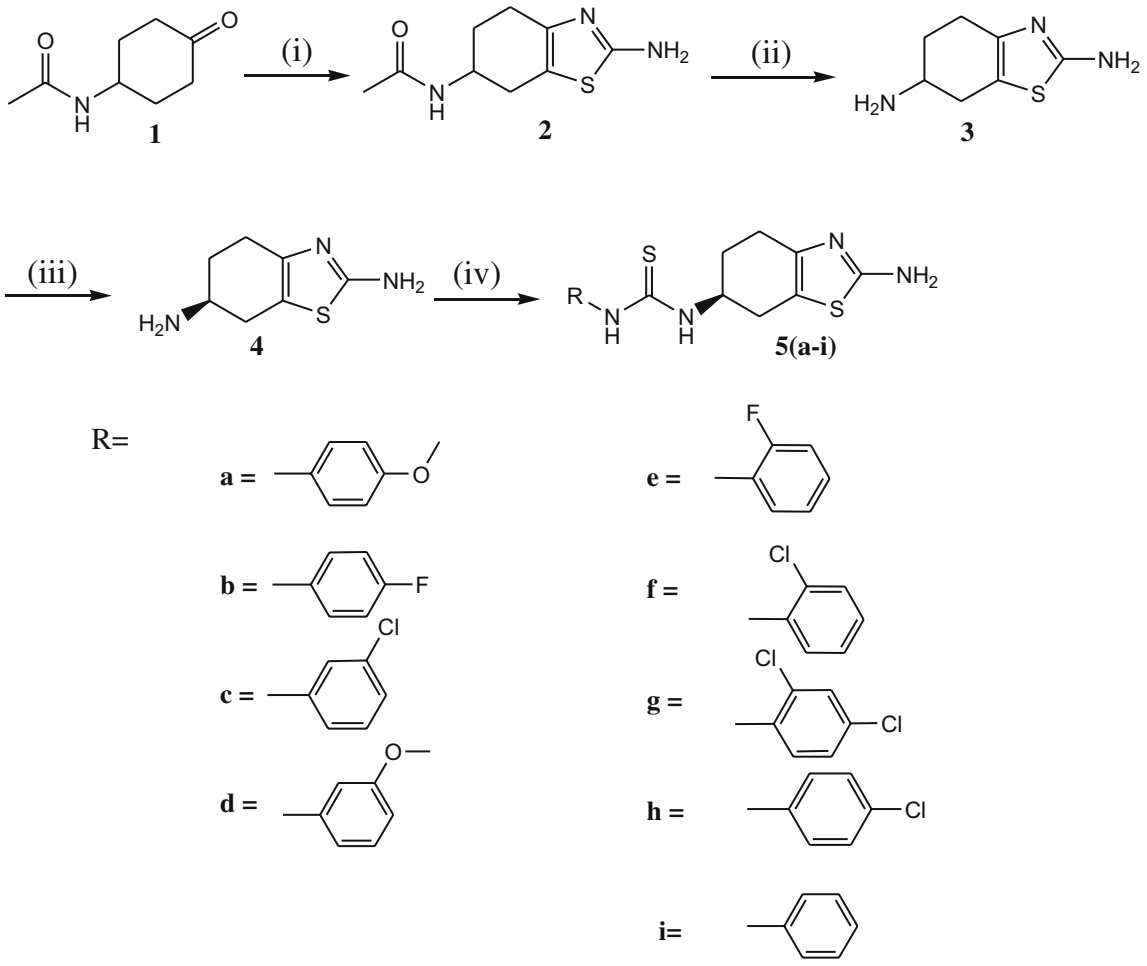
Fig. 3 Time- and dose-dependent LDH release in K562 cells treated with 5g. K562 cells were incubated for 24 h and 48 h with different concentrations of 5g. Release of LDH in the medium was measured at 490 nm. Results are presented as percentage of LDH release

Table 1 IC_{50} values of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i) as determined based on MTT assay

Compound	IC_{50}	
	K562 (in μM)	Reh (in μM)
5a	65±5.5	125±7.5
5b	42±3.5	45±3.5
5c	20±4.0	30±2.5
5d	95±5.1	90±6.2
5e	30±3.2	34±5.5
5f	45±3.5	55±4.3
5g	11±3.5	20±3.6
5h	30±6.0	30±3.5
5i	50±7.4	185±7.5

MTT assay

Cell proliferation was further assessed by MTT assay, which is based on the ability of viable cells to metabolize a yellow tetrazolium salt to violet formazan. Exponentially growing K562 or Reh cells (1×10^4 cells /well) were plated in duplicates and incubated with 10, 100 and 250 μM of 5 (a–i). Cells were harvested after 48 and 72 h of treatment



Scheme 1 Reagents and conditions: i) (**a**) Br_2, AcOH, 60 °C, 1 h; (**b**) thiourea, 1 h; ii) 47% aq. HBr; reflux, 15 h; iii) (**a**). L-(+)-tartaric acid, 75 °C; (**b**). KOH; iv) aryl isothiocyanates, triethyl amine, dichloromethane, rt, 6–7 h

and incubated with MTT (0.5 mg/ml) as described earlier [31]. The percentage cell proliferation was calculated and IC_{50} values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h of 5(a–i) treatment. The histograms are plotted as shown in Fig. 2.

LDH release assay

The cytotoxicity of 5g was further assessed by LDH release assay, which is an indicator of membrane integrity and hence cell injury. LDH assay was performed as per standard protocol to estimate the release of LDH in the culture media

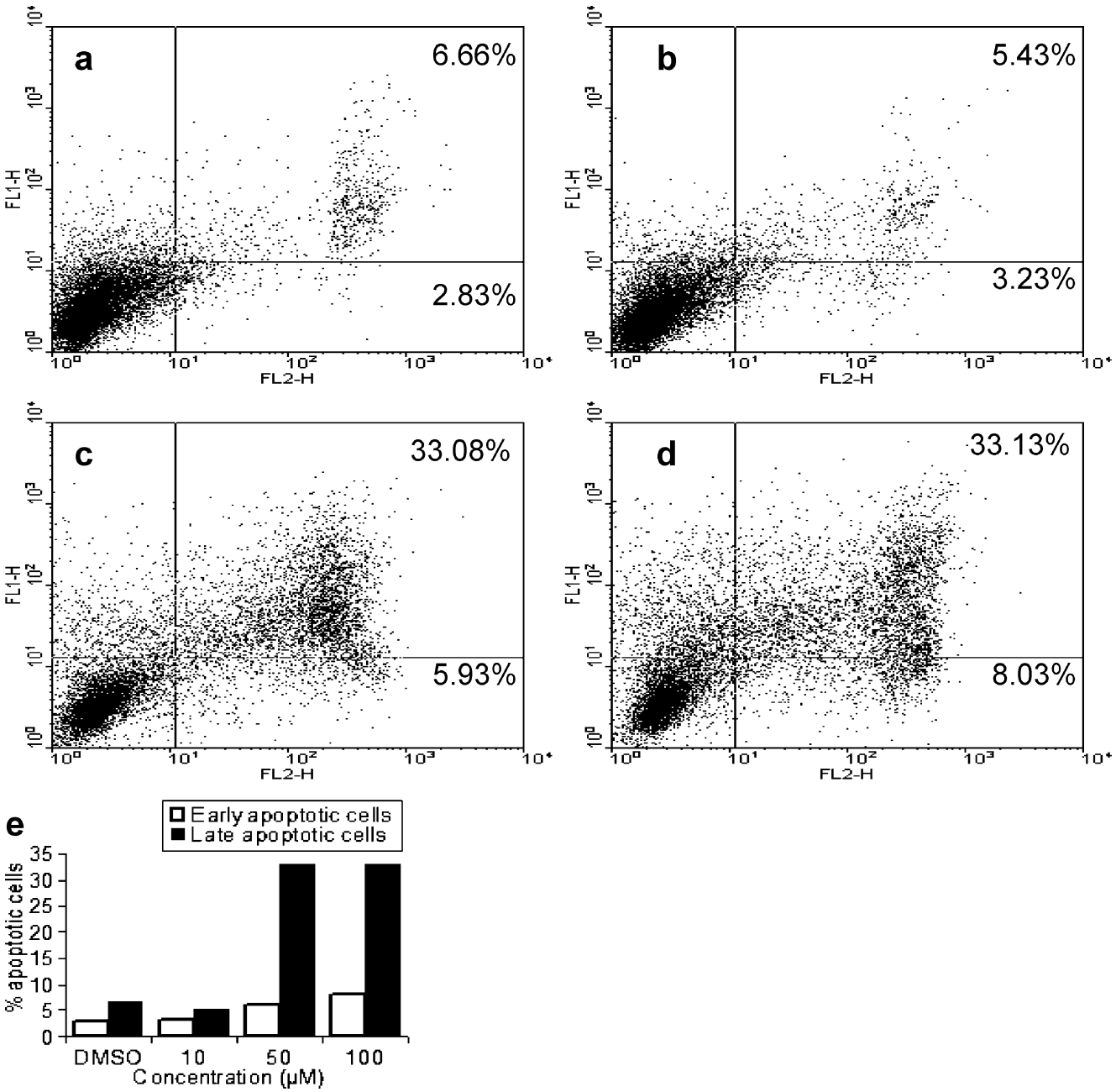


Fig. 4 Detection of apoptosis induced by 5g in K562 cells by flow cytometry using Annexin V-FITC/PI double staining. K562 cells (0.75×10^5 cells/ml) were incubated with 5g (10, 50 and 100 μM) for 48 h and processed for annexin V-FITC/PI double staining. The cells were then quantitatively monitored. In each panel, lower left quadrant shows cells which are negative for both annexin V-FITC and PI, lower right shows annexin V positive cells which are in the early stage of apoptosis, upper left shows only PI positive cells which are dead, and upper right shows both annexin V and PI positive, which are in the stage of late apoptosis or necrosis. Panels shown are annexin V-FITC/PI incubated with K562 cells, which are treated with DMSO (**a**), 10 μM (**b**), 50 μM (**c**) or 100 μM (**d**). **e** is the histogram showing comparison of early apoptotic and late apoptotic cells at different doses of 5g

upon treatment with 10, 50 and 100 μM of 5g in K562 cells. After 24 and 48 h of compound exposure, the LDH release was measured [31]. The percentage of LDH release was calculated and plotted as shown in Fig. 3.

Annexin V-FITC flow cytometric analysis

The translocation of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane is considered as one of the earliest events in apoptosis and can be measured by annexin V-FITC/PI double staining. In brief, after 48 h of treatment with 10, 50 and 100 μM of 5g, K562 cells were washed in PBS and resuspended in binding buffer (HEPES–NaOH 10 mM pH 7.4, 144 mM NaCl and 25 mM $CaCl_2$). Annexin V-FITC (0.2 mg/ml) and PI (0.05 mg/ml) were added and incubated in dark for 20 min. Cells were then subjected to FACS (FACScan, BD Biosciences, USA) analysis. At least 10,000 events were recorded and represented as dot plots.

DNA fragmentation assay

To determine the apoptotic response induced by 5g, we analyzed apoptotic DNA fragmentation which is considered as hallmark of apoptosis using agarose gel electrophoresis. Briefly, K562 cells were cultured in absence or presence of 5g at 10, 50 and 100 μM for 72 h. Cells were harvested and genomic DNA was extracted using standard protocol. The DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed.

Results and discussion

The chemistry of 4,5,6,7-tetrahydrobenzo[d]thiazole moiety has long attracted the interest of synthetic chemists due to its wide range of biological activity. Benzothiazole derivatives have been shown to be useful for treatment of various diseases including neurodegenerative disorders, local brain ischemia, Huntingtons's disease and cancer [38]. With the aim of obtaining new anticancer agents, a series of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5 (a–i) were synthesised. Synthesis of 5(a–i) was done as outlined in Scheme 1. The intermediate *(S)*-2,6-diamino-4,5,6,7-tetrahydrobenzo[d]thiazole was synthesized using the earlier reported method [39]. The bromination of 4-acetamidocyclohexanone 1 with bromine in hot acetic acid produced 2-bromo-4-acetamidocyclohexanone and cyclisation of bromo compound with thiourea gave 6-acetamido-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazole 2, which on hydrolysis with refluxing aqueous HBr yielded racemic 2,6-diamino-4,5,6,7-tetrahydrobenzo[d]thiazole 3. Optical resolution of 3 with L-(+)-tartaric acid afforded the *(S)* isomer 4. This *(S)* isomer when treated with different aryl substituted isothiocyanates gave compounds 5(a–i).

Cytotoxic effect of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i) on human leukemia cells

The antiproliferative activity of all the newly synthesized 5 (a–i) compounds has been evaluated *in vitro* against human leukemia cells (K562 and Reh). The effective concentrations of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives required to inhibit K562 or Reh cell growth and survival were determined first by carrying out dose response experiments using trypan blue dye exclusion and MTT assays. The cells were counted at intervals of 24 h till the control cells attained stationary phase. As shown in Fig. 1a & 2a (K562 cells) and Fig. 1b & 2b (Reh cells), the exposure of these derivatives for different durations decreased the number of live cells in a time and concentration dependent manner. It was observed that, the effect was improved linearly when incubation time was prolonged. Among the compounds 5 (a–i), compounds 5b, 5c, 5e, 5f, 5g and 5h showed strong inhibition against K562 cells with IC_{50} values of 42 μM, 20 μM, 30 μM, 45 μM, 11 μM and 30 μM, respectively

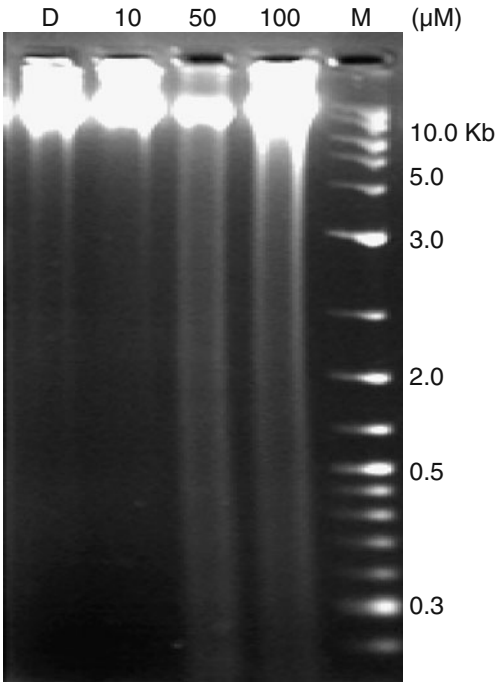


Fig. 5 Detection of DNA damage induced by 5g in K562 cells. The chromosomal DNA was extracted from K562 cells treated with different concentrations of 5g. The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In this panel, D represents DMSO; M represents Marker

and for Reh cells the corresponding IC_{50} values were 45 μM, 30 μM, 34 μM, 55 μM, 20 μM and 30 μM respectively (Table 1). The other compounds like 5a, 5d and 5i exhibited moderate inhibition. The inhibition shown by 5b, 5c, 5e, 5f, 5g, and 5h could be attributed to the presence of electron withdrawing halogen group on the phenyl ring. However, it is noteworthy to mention that *(S)*-6-(substituted arylthiourea)-2-amino-4,5,6,7-tetrahydrobenzo[d]thiazole molecules with chloro as substituent on the phenyl ring showed significant antiproliferation against leukemia cells. Similar results were shown in our previous reports where halogen substituted analogs of different heterocycles possessed potent antitumor activity [30, 31]. To confirm the above statement, molecule 5g with ortho, para dichloro substitution was selected for further studies.

LDH release assay was performed to test the cell damage induced by 5g. For this, K562 cells were cultured with 10, 50 and 100 μM of 5g for 24 and 48 h, the release of LDH (an indicator of membrane integrity) was measured. Results showed a dose- and time-dependent increase in LDH release (Fig. 3), which further confirmed our results.

From the SAR studies, it reveals that the substituents on phenyl ring of arylthiourea play a key role in the antiproliferative activity. Further, the number of halogen atoms also plays a role in the determination of cytotoxicity of these molecules. The investigations of these structural modifications and preliminary SAR would help to further design and develop more potent compounds.

Treatment of 5g in K562 cells led to translocation of phosphatidyl serine to the outer membrane

The annexin V has a strong affinity for phosphatidyl serine which is externalized in the membranes of apoptotic cells. Data from cytotoxic studies showed that 5g is more potent and able to induce apoptosis. Our interest was to quantify the different types of apoptotic cells induced by 5g. In order to detect and quantify the apoptosis induced by 5g, we used annexin V-FITC/PI double staining. K562 cells harvested after 48 h of treatment with 5g (10, 50 and 100 μM) were used for double staining followed by Fluorescence-activated cell sorting (FACS) analysis. Results showed that apoptosis induced by 5g treatment was remarkable at 50 μM compared to 10 μM and the effect was retained even at 100 μM, which is 5 fold higher than dimethyl sulfoxide (DMSO) treated cells (Fig. 4). These results suggest the total disruption of cell membrane and further damage to the chromosomal DNA upon treatment with 5g. This was further confirmed by DNA fragmentation assay.

DNA fragmentation and strand breakage induced by 5g in K562 cells

To elucidate the mode of action of the investigated compounds DNA fragmentation was performed, which is a characteristic feature of the programmed cell death or apoptosis [40, 41]. K562 cells treated with different concentrations of 5g were harvested after 72 h, the chromosomal DNA was extracted and used for agarose gel electrophoresis. The result showed fragmentation of DNA leading to a smear in the lanes in which cells were treated with 5g (Fig. 5). The observed smear was the result of DNA breakage at multiple positions across the chromosomal DNA. The intensity of smear was maximum at 50 and 100 μM. These results in conjunction with annexin V-FITC/PI staining further suggested that 5g induces fragmentation of chromosomal DNA leading to apoptosis.

Conclusion

In this study, we have described the synthesis of 4,5,6,7-tetrahydrobenzo[d]thiazole derivatives 5(a–i), which showed strong and moderate growth inhibitory activity against human leukemic cell lines, K562 and Reh. The derivatives 5b, 5c, 5e, 5f, 5g and 5h with chloro and fluoro substituents at different positions showed significant inhibitory effects whereas 5a, 5d and 5i showed moderate inhibitory activity. The best results were obtained for compound 5g having a dichloro group on the phenyl ring of arylthiourea at the 2nd and 4th position. Further investigations to understand the mechanism by which these molecules induce apoptosis is under progress in our laboratory.

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