

Synthesis and Antileukemic Activity of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)-3-(substituted phenyl)urea Derivatives

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Heterocyclic urea derivatives play an important role as anticancer agents because of their good inhibitory activity against receptor tyrosine kinases (RTKs), raf kinases, protein tyrosine kinases (PTKs), and NADH oxidase, which play critical roles in many aspects of tumorigenesis. Benzothiazole moiety constitutes an important scaffold of drugs, possessing several pharmacological functions, mainly the anticancer activity. Based on these interesting properties of benzothiazoles and urea moiety to obtain new biologically active agents, we synthesized a series of novel 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)-3-(substituted phenyl)urea derivatives and evaluated for their efficacy as antileukemic agents against two human leukemic cell lines (K562 and Reh). These compounds showed good and moderate cytotoxic effect to cancer cell lines tested. Compounds with electron-withdrawing chloro and fluoro substituents on phenyl ring showed good activity and compounds with electron-donating methoxy group showed moderate activity. Compound with electron-withdrawing dichloro substitution on phenyl ring of aryl urea showed good activity. Further, lactate dehydrogenase (LDH) assay, flow cytometric analysis of annexin V-FITC/propidium iodide (PI) double staining and DNA fragmentation studies showed that compound with dichloro substitution on phenyl ring of aryl urea can induce apoptosis.

Cancer is a group of disease having the hallmark of uncontrolled cell proliferation leading to total collapse of cellular homeostasis. Among different cancers, leukemia is one of the major causes of cancer related deaths. The leukemias account for the largest number of cases of childhood cancer and are the primary cause of cancer related mortality of children. Although tremendous improvement has occurred in the area of cancer diagnostics and therapeutics in the past one decade, still long way to go to cope with this deadly disease, especially when the average life span of human being is increasing day by day. The need of more targeted and relatively non toxic chemotherapeutics is quite urgent.

Within the past ten years, a huge volume of research on the synthesis, structure–activity relationships (SAR), and anticancer activities of the urea derivatives was reported. Heterocyclic urea derivatives play an important role in anticancer agents because of their good inhibitory activity against receptor tyrosine kinases (RTKs),[1–4] raf kinases,[5,6] protein tyrosine kinases (PTKs),[7] and NADH oxidase,[8] which play critical roles in many aspects of tumorigenesis. Many aromatic urea derivatives such as *N*-phenyl-*N*′-(2-chloroethyl)ureas and benzoylureas show good anticancer activity, and these compounds have mainly been proved to be tubulin ligands that inhibit the polymerization of tubulin.[9,10]

Currently, a benzothiazole derivative, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole, acting through a novel mechanism, has entered the clinical trial phase I in U.K. It shows high sensitivity toward the tumor cells by acting through aryl hydrocarbon receptor (AhR) signaling pathway. The activation of AhR pathway in sensitive tumor cells leads to induction of the microsomal enzyme CYP1A1. This converts the drug into highly reactive metabolites that form adducts with DNA causing cell death.[11] Further, the combinations of urea and thiourea derivatives with benzothiazoles have produced DNA topoisomerase[12,13] or HIV reverse transcriptase inhibitors.[14,15]

In continuation of our research on heterocyclic antileukemic agents[16–19] and with the goal of discovering new antileukemic agents, we synthesized a series of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)-3-(substituted phenyl)urea derivatives **2a**–**2i** and studied the effect of the novel derivatives for their antileukemic activity.

Chemistry. With the aim of obtaining new anticancer agents and to study the cytotoxic effect of urea derivatives on leukemia cells, we synthesized a series of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)-3-(substituted phenyl)-urea derivatives **2a**–**2i** and evaluated the antileukemic activity of these derivatives. Synthesis of **2a**–**2i** was done as outlined in Scheme 1. The key intermediate (*S*)-2,6-diamino-4,5,6,7-tetrahydrobenzo[*d*]thiazole (**1**) was synthesized using the earlier reported method.[20] Compound **1** on treatment with different aryl-substituted isocyanates in presence of triethylamine using dichloromethane as solvent gave compounds **2a**–**2i**. The synthesized novel compounds were characterized using the melting point, optical rotation, IR, ^{1}H NMR, ^{13}C NMR, LCMS, and elemental analysis data. The absence of –NH_2 peak around δ 2.5 and the presence of a peak around δ 8.5 corresponding to amide proton in the ^{1}H NMR spectrum of the final compounds and a strong absorption at 3395–3435 cm^{-1} for –NH and 1670–

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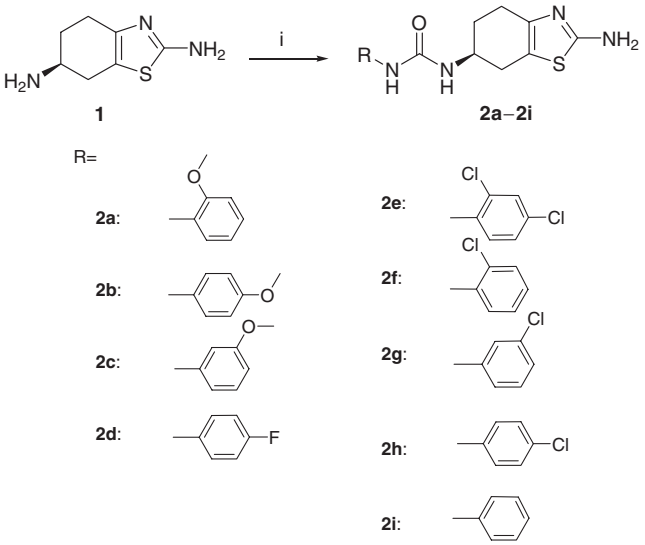

Scheme 1. Synthesis of compound **2**. Reagents and conditions: i) aryl isocyanates, triethylamine, dichloromethane, rt, 6–7 h.

$1690\,cm^{-1}$ for C=O in the IR spectrum of the compounds along with the LCMS and elemental analysis data confirmed the structure of the novel compounds **2a**–**2i**.

Biological Study. The human chronic myelogenous leukemia (CML) K562 and pre B cell line Reh were selected for the purpose of preliminary anticancer screening of newly synthesized compounds. To assess the cytotoxicity, we employed 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 **2a**–**2i**. In addition to this we also performed DNA fragmentation assay which is an indicator of apoptosis. To quantify the apoptotic cells formed upon treatment with **2e**, we performed Annexin V-FITC/PI double staining assay. Assays were carried out in duplicate in at least two independent experiments.

Results and Discussion

Newly synthesized urea derivatives **2a**–**2i** containing 4,5,6,7-tetrahydrobenzo[*d*]thiazole pharmacophore were assessed for cytotoxicity against two human leukemia cell lines

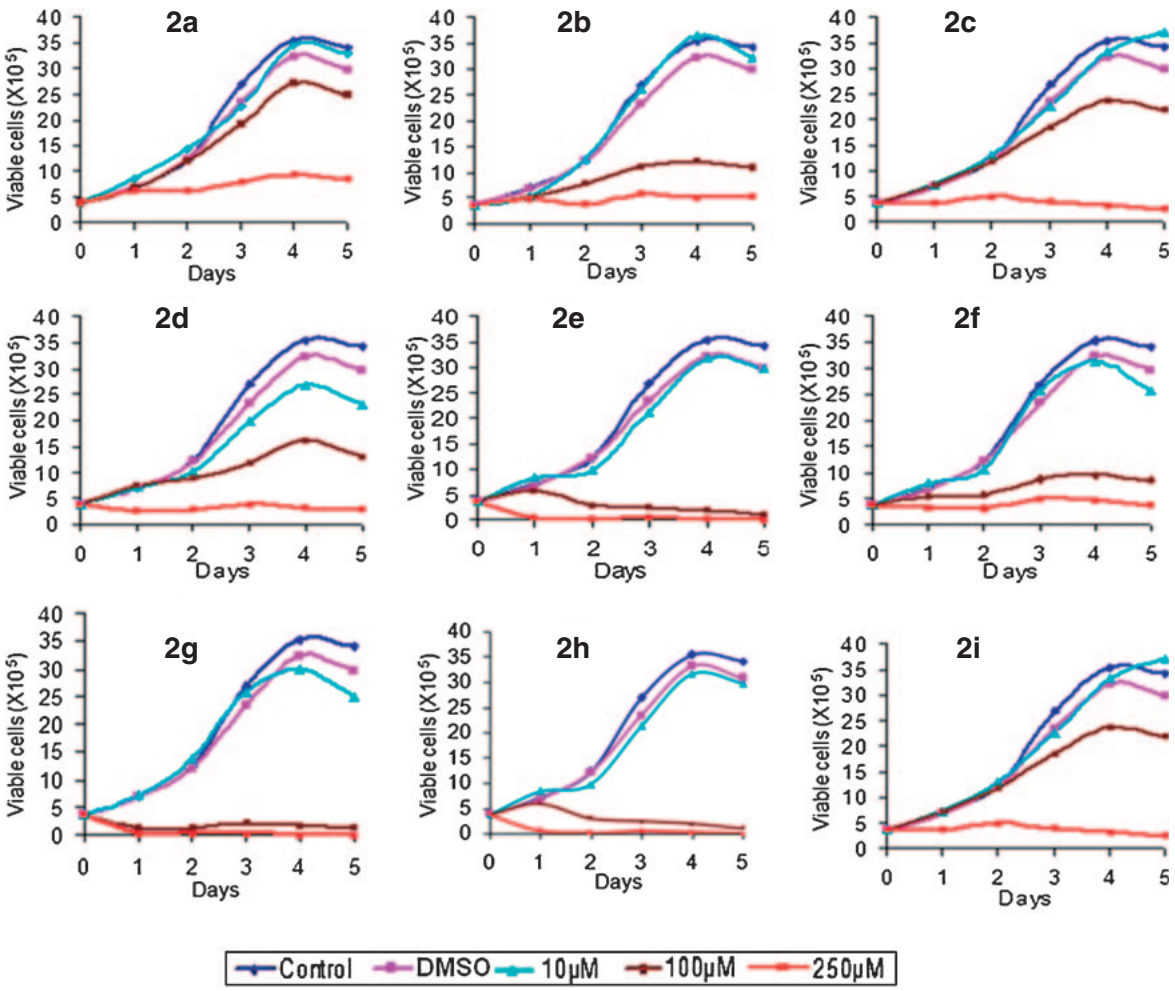

Figure 1. Dose- and time-dependent effect of 4,5,6,7-tetrahydrobenzo[*d*]thiazole derivatives **2a**–**2i** on K562 cell survival. Approximately 0.75×10^5 cells/mL were cultured and treated with **2a**–**2i** at a concentration of 10, 100, and 250 μM. In addition to control cells (without compound), DMSO was also used as vehicle control. 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.

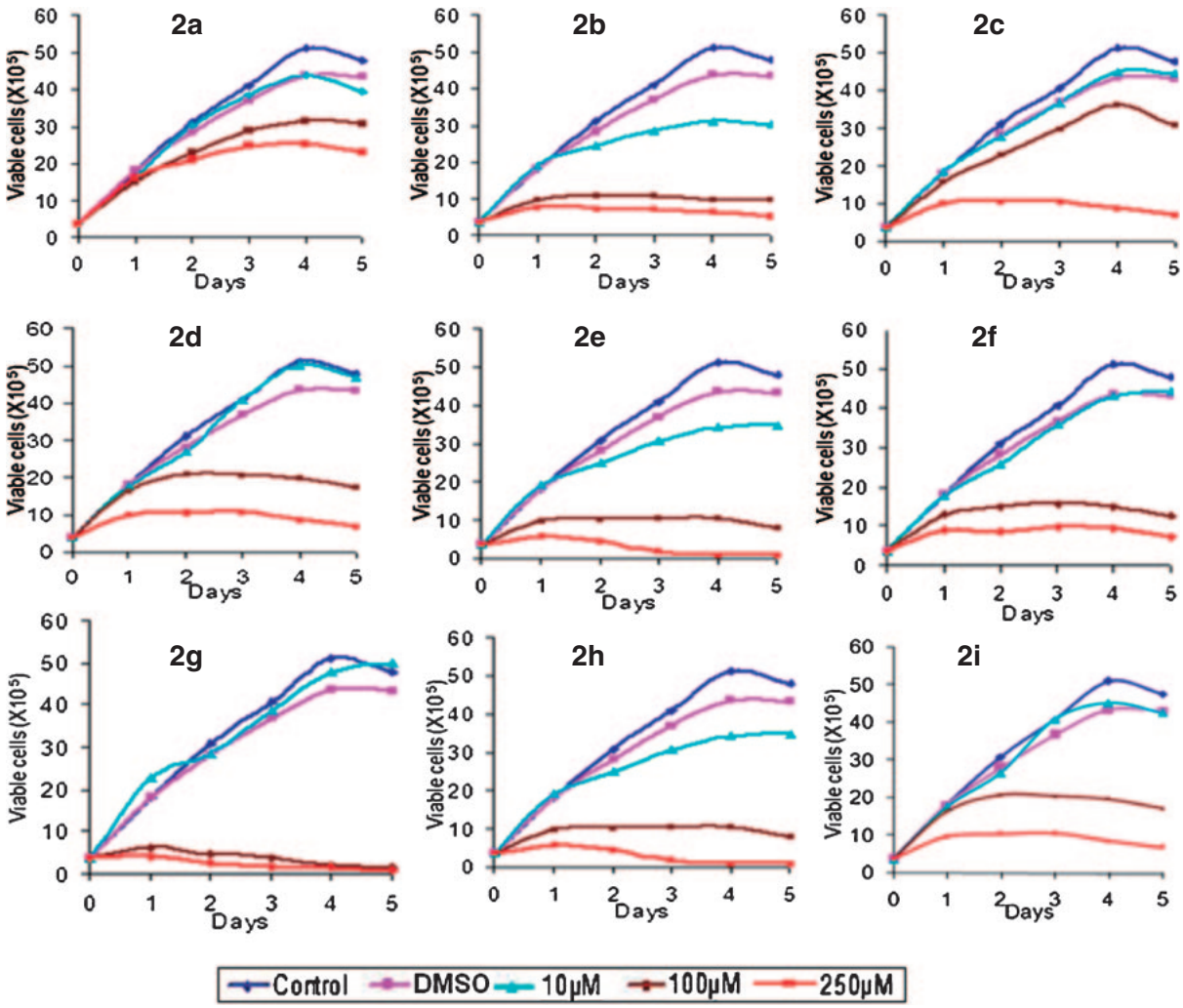

Figure 2. Dose- and time-dependent effect of 4,5,6,7-tetrahydrobenzo[*d*]thiazole derivatives **2a**–**2i** on Reh cell survival. Approximately 0.75×10^5 cells/mL were cultured and treated with **2a**–**2i** at a concentration of 10, 100, and 250 μM. In addition to control cells (without compound), DMSO was also used as vehicle control. 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.

(K562 and Reh). The effective concentrations of these 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 Figures 1 and 3 (K562 cells) and Figures 2 and 4 (Reh cells), the exposure of these derivatives for different time points 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 **2a**–**2i**, compounds **2d**, **2e**, **2f**, **2g**, and **2h** showed good inhibition against K562 cells with IC_{50} values of 70.11, 40.06, 80.26, 48.03, and 42.00 μM, respectively and for Reh cells the corresponding IC_{50} values were 90.32, 48.02, 90.21, 55.16, and 48.02 μM respectively (Table 1). The other compounds like **2a**, **2b**, **2c**, and **2i** exerted moderate inhibitory activity.

Comparing the IC_{50} values of the newly synthesized urea derivatives **2a**–**2i**, we were able to draw some of the SAR. In the first SAR, we observed that compounds **2d**, **2e**, **2f**, **2g**, and **2h** which showed good inhibition against both the cell lines are having electron-withdrawing chloro and fluoro groups at different positions of phenyl ring of aryl urea. Compounds **2a**, **2b**, and **2c** having electron-donating methoxy groups at different positions of phenyl ring of aryl urea and compound **2i** which is unsubstituted showed moderate inhibition. This shows that electron-withdrawing groups on phenyl ring increases the antiproliferative activity of compounds and electron-donating groups on phenyl ring of the aryl urea moiety decreases the activity. Second, we observed the effect among the compounds having chloro group at *ortho*-, *meta*-, and *para*-positions on the phenyl ring of the aryl urea moiety. Compound **2h** having chloro at *para*-position showed good inhibition with IC_{50} values of 42.00 and 48.02 μM against K562 and Reh cells respectively compared to compounds **2g** and **2f** having chloro at *meta*- and *ortho*-positions with IC_{50} values 48.03 and 80.26 μM respectively against K562 cells and 55.16 and 90.21 μM respectively against Reh cells.

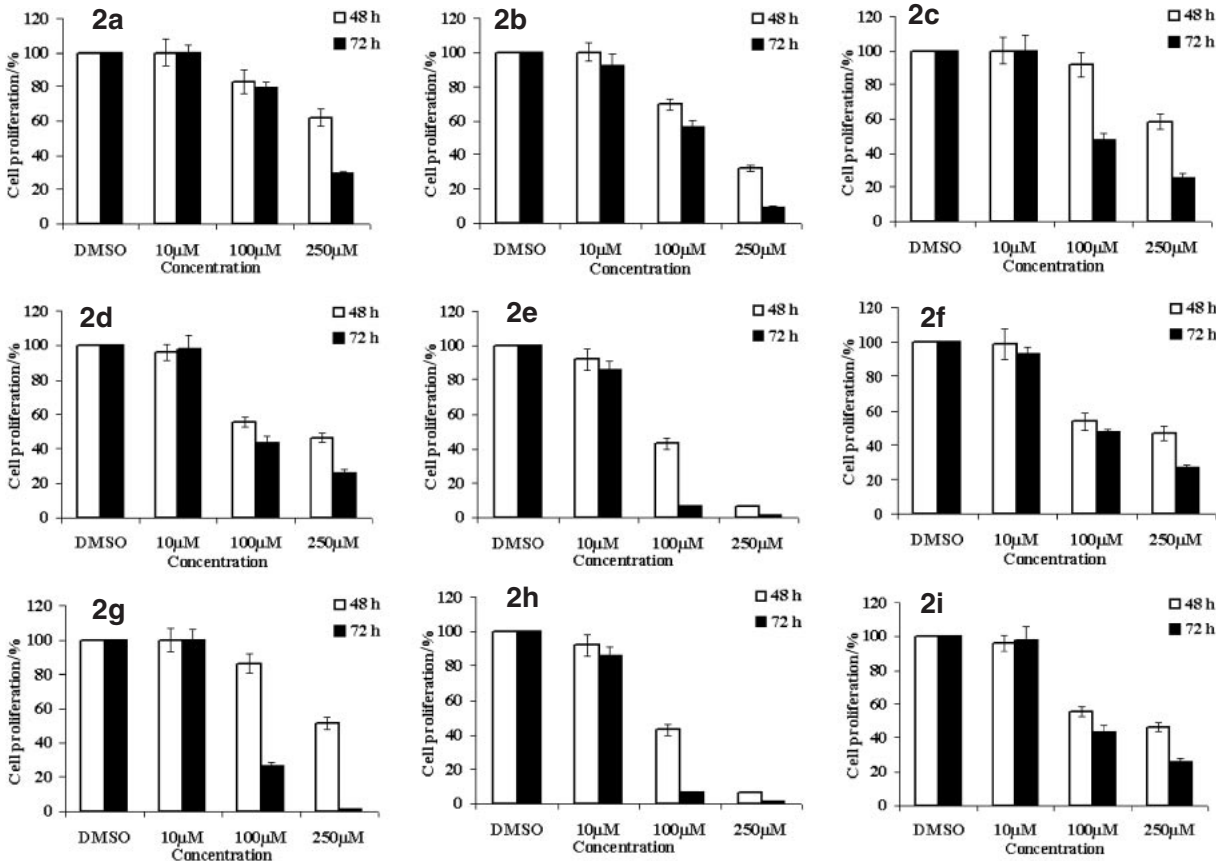

Figure 3. Determination of the effect of 4,5,6,7-tetrahydrobenzo[*d*]thiazole derivatives **2a**–**2i** on cell proliferation by MTT assay. After 48 and 72 h of exposure, K562 cells treated with **2a**–**2i** at different concentrations (10, 100, and 250 μM) were incubated with MTT (0.5 mg mL^{-1}) in duplicates and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation (the cell viability of vehicle cells were considered as 100%). Error bars are represented in the figure.

When we compare the activity of compound **2e** having a *ortho*- and *para*-dichloro substitution and compound **2h** having a *para*-chloro substitution on the phenyl ring of arylurea moiety with compounds **2f** and **2g** having monochloro substitution at *ortho*- and *meta*-positions respectively, we observed that compound **2e** and **2h** with chloro at *para*-position showed most significant activity against both K562 and Reh cells.

We chose the dichloro substitution containing derivative **2e** for further studies. We performed LDH release assay to assess the extent of cell damage induced by **2e**. For this, K562 cells were cultured with 10, 50, and 100 μM of **2e** 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 on treatment with **2e** (Figure 5).

Further, with an interest of quantifying the early and late apoptotic cells after treatment of cells with **2e**, we performed Annexin V-FITC/PI double staining assay. K562 cells were taken for this assay. K562 cells were harvested after 48 h of treatment with **2e** (10, 50, and 100 μM) and were used for double staining followed by FACS analysis. Results showed that apoptosis induced by **2e** is 3.5-fold higher than DMSO at 100 μM (Figure 6). These results suggest a total disruption of cell membrane and further damage to the chromosomal DNA upon treatment with compound **2e**. To validate this we carried out DNA fragmentation assay.

K562 cells treated with different concentrations of **2e** were harvested after 72 h, the chromosomal DNA was extracted and used for agarose gel electrophoresis as described earlier. The results showed observed smear in the lanes 3 and 4 (Figure 7) is due to the DNA breakage at multiple positions across the chromosomal DNA. The intensity of smear is maximum at 100 μM. These results in conjunction with annexin V-FITC/PI staining further suggest that compound **2e** induces fragmentation of chromosomal DNA leading to apoptosis.

Further, to check the cytotoxicity of **2e** on normal cells, MTT assay was performed using 293T cells (human embryonic kidney epithelial cells). Compound **2e** did not show any significant effect on proliferation of 293T cells (human embryonic kidney epithelial cells) (Figure 8).

Experimental

Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. 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-d_6 as a solvent and TMS as internal standard (chemical shift in δ). Spin multiplets are given as s (singlet), d (doublet), t (triplet), and m (multiplet). ^{13}C NMR spectra were recorded on Shimadzu AMX 400-Bruker, 100 MHz spectrometer using DMSO-d_6 as a solvent. Mass

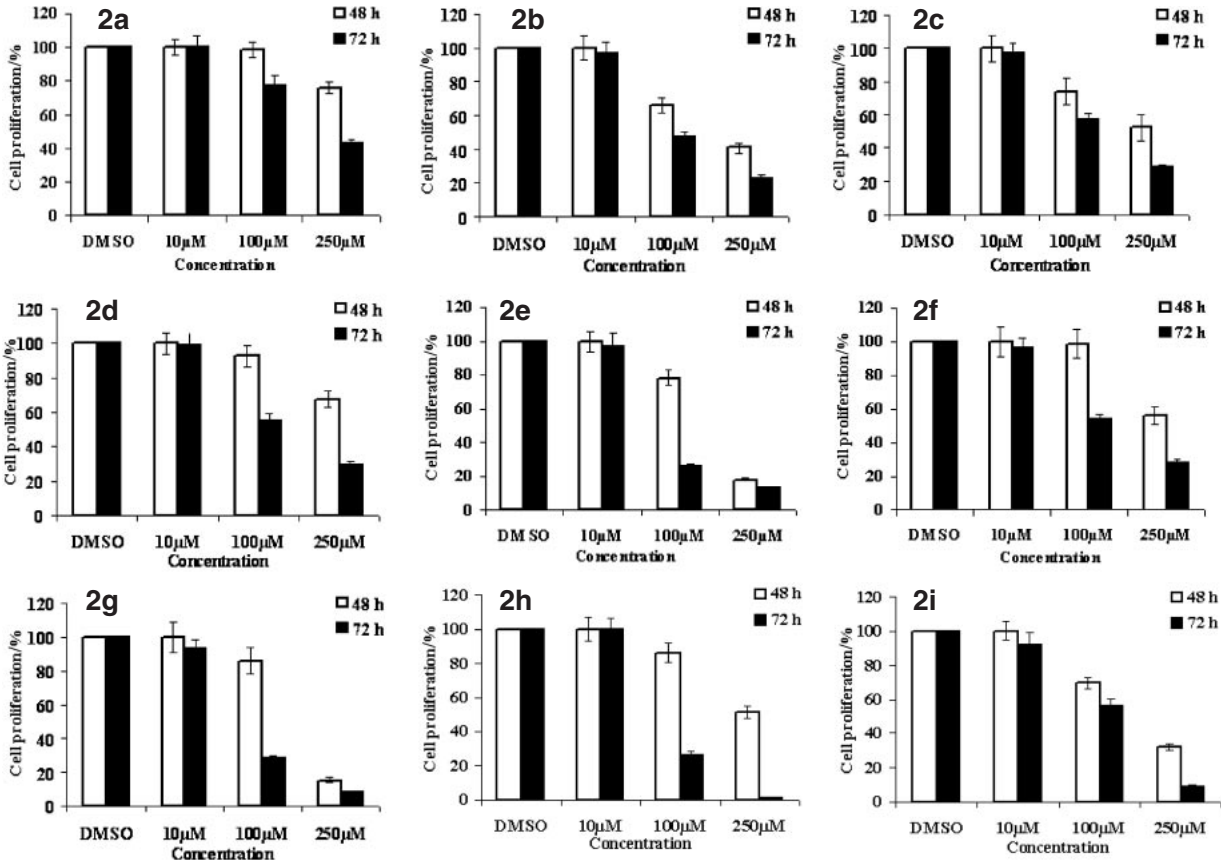

Figure 4. Determination of the effect of 4,5,6,7-tetrahydrobenzo[*d*]thiazole derivatives **2a**–**2i** on cell proliferation by MTT assay. After 48 and 72 h of exposure, Reh cells treated with **2a**–**2i** at different concentrations (10, 100, and 250 μM) were incubated with MTT (0.5 mg mL^{-1}) in duplicates and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation (the cell viability of vehicle cells were considered as 100%). Error bars are represented in the figure.

Table 1. IC_{50} Values of 4,5,6,7-Tetrahydrobenzo[*d*]thiazole Derivatives as Determined based on MTT Assay

Compound	IC_{50}	
	K562/μM	Reh/μM
2a	125.01 ± 6.82	215.03 ± 10.95
2b	110.03 ± 5.57	100.11 ± 5.16
2c	100.23 ± 5.21	125.24 ± 6.94
2d	70.11 ± 4.38	90.32 ± 5.26
2e	40.06 ± 2.95	48.02 ± 4.13
2f	80.26 ± 4.68	90.21 ± 4.98
2g	48.03 ± 3.47	55.16 ± 5.43
2h	42.00 ± 3.14	48.02 ± 3.56
2i	100.35 ± 5.20	110.63 ± 5.42

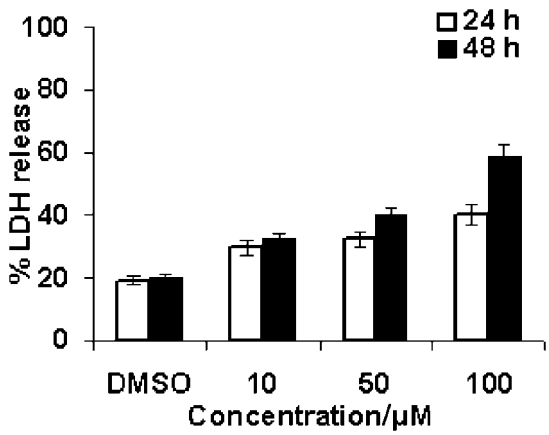

Figure 5. Time- and dose-dependent LDH release in K562 cells treated with **2e**. K562 cells were incubated for 24 and 48 h with different concentrations of **2e**. Release of LDH in the medium was measured at 490 nm. Results are presented as percentage of LDH release.

and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were obtained on Vario EL III Elementar. 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)urea Derivatives 2a–2i. To the solution of intermediate compound **1** (1.0 equiv) in dichloromethane, triethylamine (3.0 equiv) was added and cooled to 0 °C, respective aryl isocyanate (1.0 equiv) was 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 and water was added and extracted thrice using ethyl acetate. The combined ethyl acetate layer was washed with brine solution and dried over anhydrous sodium sulfate. Ethyl acetate was evaporated under reduced pressure and the crude

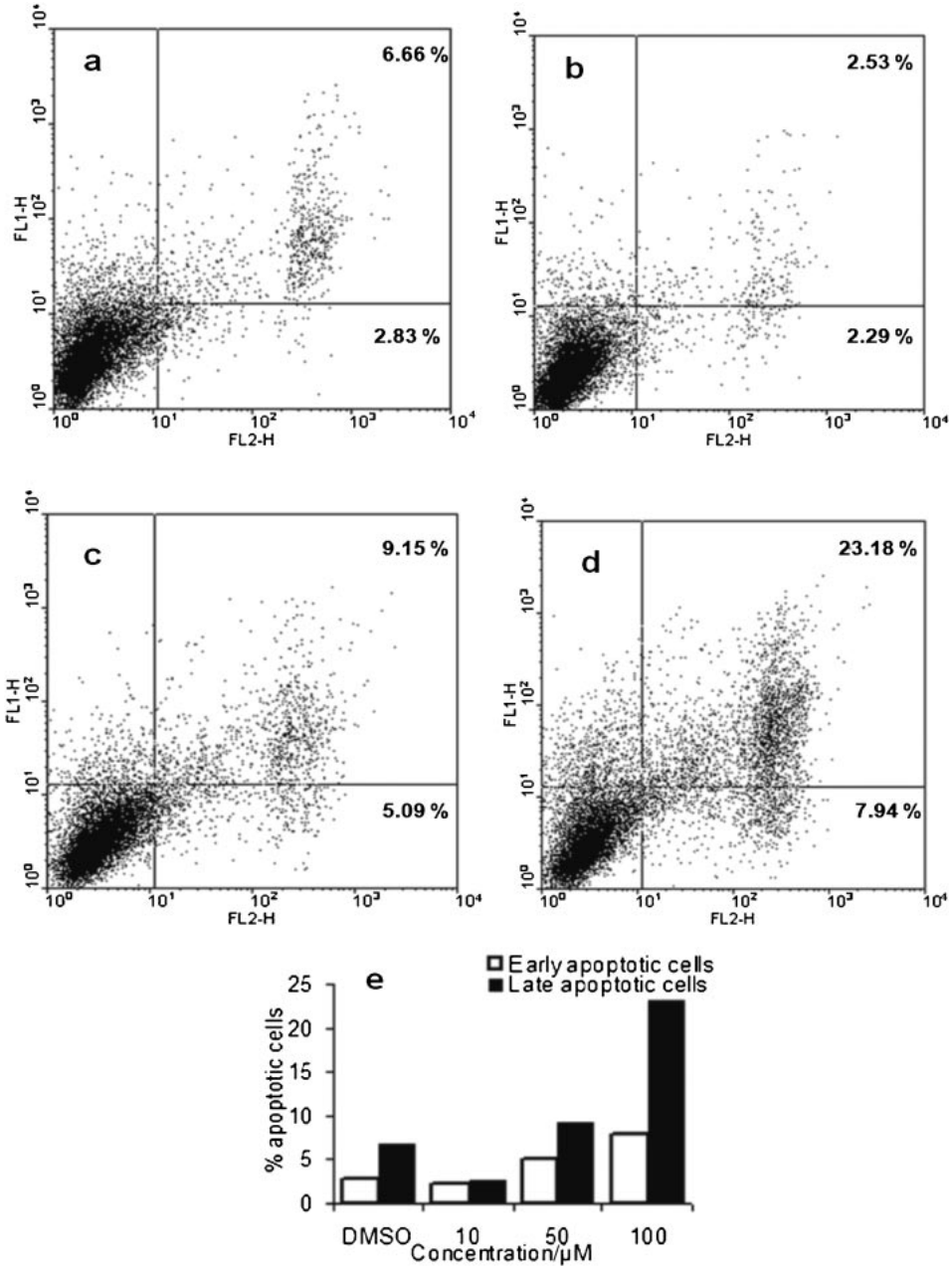

Figure 6. Detection of apoptosis induced by **2e** in K562 cells by flow cytometry using Annexin V-FITC/PI double staining. K562 cells (0.75×10^5 cells/mL) were incubated with **2e** (10, 50, and 100 μM) for 48 h and processed for annexin V-FITC/PI double staining. The cells were then quantitatively or qualitatively 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 incubated with K562 cells, which are treated with DMSO (a), 10 (b), 50 (c), or 100 μM (d). In both the panels (e) is histogram showing comparison of early apoptotic and late apoptotic cells at different doses of **2e**.

product obtained was purified by silica gel (60–120 mesh) column. The compounds **2a**–**2i** 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-(2-methoxyphenyl)urea (2a): The product obtained (0.320 g, 83%) by the reaction of **1** (0.200 g, 1.2 mmol) with 2-methoxyphenyl isocyanate (0.176 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a pale blue solid. Mp: 168–170 °C; $[\alpha]_D^{25}$ −67.3 (*c* 1, CH_3OH); ^1H NMR (DMSO-d_6, 400 MHz): δ 8.24 (s, 1H, –NH), 8.07 (s, 1H, –NH), 7.38 (d, 1H, $J = 10.62$ Hz, 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–), 3.64 (s, 3H, $-OCH_3$), 2.87 (dd, 1H, $J = 4.96$ Hz, $J = 4.88$ Hz, –CH), 2.55–2.49 (m, 2H, $-CH_2$), 2.38 (dd, 1H, $J = 5.92$ Hz, $J = 5.68$ Hz, –CH),

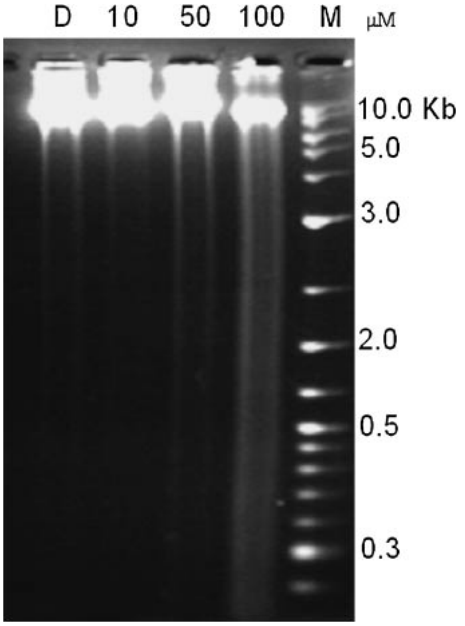

Figure 7. Detection of DNA damage induced by **2e** in K562 cells. The chromosomal DNA was extracted from K562 cells treated with different concentrations of **2e**. The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In both panels, Lane 1: DMSO; Lane 2–4: K562 cells treated with 10, 50, and 100 μM, respectively. M is Marker.

1.90–1.84 (m, 1H, –CH_2), 1.77–1.70 (m, 1H, –CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.5, 155.8, 154.2, 144.4, 130.6, 128.6, 127.3, 120.5, 114.5, 112.8, 55.6, 45.3, 30.0, 28.7, 24.3; MS (ESI + ion): $m/z = 319.4$; IR (KBr, cm^{-1}): 3403, 3211, 1679. Elemental analysis: Found: C, 56.66; H, 5.71; N, 17.58; S, 10.09%. Calculated for $C_{15}H_{18}N_4O_2S$: C, 56.58; H, 5.70; N, 17.60; S, 10.07%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(4-methoxyphenyl)urea (2b): The product obtained (0.315 g, 82%) by the reaction of **1** (0.200 g, 1.2 mmol) with 4-methoxyphenyl isocyanate (0.176 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a white solid. Mp: 190–192 °C; $[\alpha]_D^{25}$ −66.6 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.20 (s, 1H, –NH), 8.06 (s, 1H, –NH), 7.30 (d, $J = 9.24$ Hz, 2H, Ar–H), 6.85 (d, 2H, $J =$ 8.96 Hz, Ar–H), 6.70 (s, 2H, –NH_2), 4.04–4.01 (m, 1H, –CH–), 3.70 (s, 3H, –OCH_3), 2.82 (dd, 1H, $J = 4.92$ Hz, $J = 4.98$ Hz, –CH), 2.51–2.46 (m, 2H, –CH_2), 2.41–2.36 (dd, 1H, $J = 5.90$ Hz, $J = 5.62$ Hz, –CH), 1.89–1.82 (m, 1H, –CH_2), 1.79–1.72 (m, 1H, –CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz); δ 166.5, 155.3, 154.3, 144.5, 134.1, 119.6 (2C), 114.4 (2C), 112.9, 55.6, 45.2, 30.0, 28.7, 24.2; MS (ESI + ion): $m/z = 319.5$; IR (KBr, cm^{-1}): 3415, 3224, 1689; Elemental analysis: Found: C, 56.55; H, 5.64; N, 17.54; S, 10.00%. Calculated for $C_{15}H_{18}N_4O_2S$: C, 56.58; H, 5.70; N, 17.60; S, 10.07%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(3-methoxyphenyl)urea (2c): The product obtained (0.325 g, 85%) by the reaction of **1** (0.200 g, 1.2 mmol) with 3-methoxyphenyl isocyanate (0.176 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL)

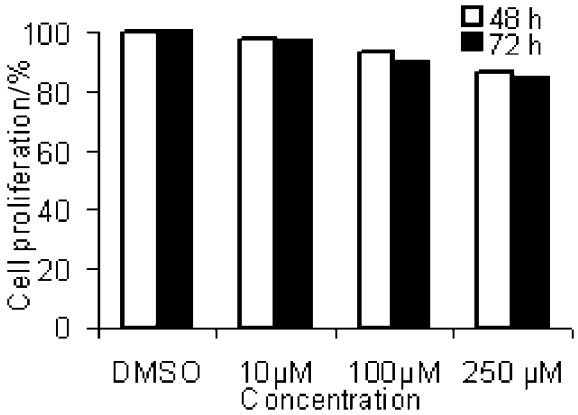

Figure 8. Effect of **2e** on the growth of normal cells. After 48 and 72 h of exposure, 293T cells (human embryonic kidney epithelial cells) treated with **2e** at 10, 100, and 250 μM concentrations were incubated with MTT (0.5 mg mL^{-1}) in duplicates and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation (the cell viability of vehicle cells were considered as 100%).

using the general experimental procedure as described was an off-white solid. Mp: 174–176 °C; $[\alpha]_D^{25}$ −64.7 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.18 (s, 1H, –NH), 8.05 (s, 1H, –NH), 7.82 (s, 1H, Ar–H), 7.35–7.21 (m, 1H, Ar–H), 6.90–6.86 (m, 2H, Ar–H), 6.70 (s, 2H, –NH_2), 4.05–4.02 (m, 1H, –CH–), 3.72 (s, 3H, –OCH_3), 2.82 (dd, 1H, $J = 4.90$ Hz, $J = 4.96$ Hz, –CH), 2.51–2.46 (m, 2H, –CH_2), 2.39 (dd, 1H, $J = 5.86$ Hz, $J = 5.60$ Hz, –CH), 1.88–1.81 (m, 1H, –CH_2), 1.78–1.71 (m, 1H, –CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.6, 158.8, 155.2, 144.5, 132.6, 122.6, 120.4, 116.9, 112.9, 108.8, 55.6, 45.2, 30.1, 28.7, 24.3; MS (ESI + ion): $m/z =$ 319.2; IR (KBr, cm^{-1}): 3396, 3197, 1674; Elemental analysis: Found: C, 56.64; H, 5.72; N, 17.64; S, 10.02%. Calculated for $C_{15}H_{18}N_4O_2S$: C, 56.58; H, 5.70; N, 17.60; S, 10.07%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(4-fluorophenyl)urea (2d): The product obtained (0.305 g, 83%) by the reaction of **1** (0.200 g, 1.2 mmol) with 4-fluorophenyl isocyanate (0.161 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a pale yellow solid. Mp: 185–187 °C; $[\alpha]_D^{25}$ −68.2 (*c* 1, CH_3OH). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.30 (s, 1H, –NH), 8.06 (s, 1H, –NH), 7.40 (d, 2H, $J = 8.12$ Hz, Ar–H), 7.11 (d, 2H, $J =$ 8.96 Hz, Ar–H), 6.70 (s, 2H, –NH_2), 4.04–4.01 (m, 1H, –CH–), 2.82 (dd, 1H, $J = 4.92$ Hz, $J = 4.96$ Hz, –CH), 2.51–2.46 (m, 2H, –CH_2), 2.39 (dd, 1H, $J = 5.86$ Hz, $J = 5.64$ Hz, –CH), 1.89–1.82 (m, 1H, –CH_2), 1.79–1.72 (m, 1H, –CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.6, 155.0, 144.6, 142.4, 138.6, 129.6 (2C), 115.6 (2C), 45.3, 29.9, 28.7, 24.3; MS (ESI + ion): $m/z = 307.5$; IR (KBr, cm^{-1}): 3415, 3220, 1682; Elemental analysis: Found: C, 54.98; H, 4.89; N, 18.32; S, 10.44%. Calculated for $C_{14}H_{15}FN_4OS$: C, 54.89; H, 4.94; N, 18.29; S, 10.47%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(2,4-dichlorophenyl)urea (2e): The product obtained (0.335 g, 78%) by the reaction of **1** (0.200 g, 1.2 mmol) with 2,4-dichlorophenyl isocyanate (0.22 g, 1.2 mmol)

and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a white solid. Mp: 180–182 °C; $[\alpha]_D^{25}$ −66.4 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.28 (s, 1H, –NH), 8.05 (s, 1H, –NH), 7.36 (s, 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–), 2.81 (dd, 1H, $J = 4.88$ Hz, $J = 4.96$ Hz, –CH), 2.51–2.46 (m, 2H, $-CH_2$), 2.39 (dd, 1H, $J = 5.94$ Hz, $J = 5.68$ Hz, –CH), 1.89–1.82 (m, 1H, $-CH_2$), 1.78–1.71 (m, 1H, $-CH_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.6, 154.5, 144.4, 136.5, 128.8, 128.0, 125.4, 121.9, 121.7, 112.7, 45.2, 29.8, 28.4, 23.9; MS (ESI + ion): $m/z = 358.1$; IR (KBr, cm^{-1}): 3423, 3216, 1683; Elemental analysis: Found: C, 47.18; H, 4.02; N, 15.60; S, 9.07%. Calculated for $C_{14}H_{14}Cl_2N_4OS$: C, 47.07; H, 3.95; N, 15.68; S, 8.98%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(2-chlorophenyl)urea (2f): The product obtained (0.325 g, 84%) by the reaction of **1** (0.200 g, 1.2 mmol) with 2-chlorophenyl isocyanate (0.181 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a pale pink solid. Mp: 176–178 °C; $[\alpha]_D^{25}$ −65.8 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.34 (s, 1H, –NH), 8.08 (s, 1H, –NH), 7.36 (d, 1H, $J = 10.06$ Hz, Ar–H), 7.23–7.19 (m, 2H, Ar–H), 6.94–6.89 (m, 1H, Ar–H), 6.72 (s, 2H, $-NH_2$), 4.06–4.03 (m, 1H, –CH–), 2.82 (dd, 1H, $J = 4.92$ Hz, $J = 4.96$ Hz, –CH), 2.51–2.46 (m, 2H, $-CH_2$), 2.38 (dd, 1H, $J = 5.88$ Hz, $J = 5.64$ Hz, –CH), 1.88–1.82 (m, 1H, $-CH_2$), 1.75–1.70 (m, 1H, $-CH_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.5, 154.7, 144.3, 132.5, 129.6, 127.2, 126.3, 122.1, 121.6, 113.0, 45.3, 29.8, 28.5, 24.1; MS (ESI + ion): $m/z = 323.7$; IR (KBr, cm^{-1}): 3425, 3194, 1671; Elemental analysis: Found: C, 52.18; H, 4.65; N, 17.41; S, 10.02%. Calculated for $C_{14}H_{15}ClN_4OS$: C, 52.09; H, 4.68; N, 17.36; S, 9.93%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(3-chlorophenyl)urea (2g): The product obtained (0.325 g, 84%) by the reaction of **1** (0.200 g, 1.2 mmol) with 3-chlorophenyl isocyanate (0.181 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was an off-white solid. Mp: 147–149 °C; $[\alpha]_D^{25}$ −64.9 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.32 (s, 1H, –NH), 8.06 (s, 1H, –NH), 7.41 (s, 1H, Ar–H), 7.11 (d, 2H, $J = 8.74$ Hz, Ar–H), 6.70 (s, 2H, $-NH_2$), 4.05–4.01 (m, 1H, –CH–), 2.83 (dd, 1H, $J = 4.96$ Hz, $J = 4.94$ Hz, –CH), 2.51–2.46 (m, 2H, $-CH_2$), 2.39 (dd, 1H, $J = 5.90$ Hz, $J = 5.68$ Hz, –CH), 1.87–1.80 (m, 1H, $-CH_2$), 1.75–1.69 (m, 1H, $-CH_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.5, 154.8, 144.4, 134.3, 131.8, 129.6, 125.4, 124.1, 122.6, 112.9, 45.3, 29.8, 28.4, 24.0; MS (ESI + ion): $m/z = 323.9$; IR (KBr, cm^{-1}): 3437, 3241, 1687; Elemental analysis: Found: C, 52.12; H, 4.75; N, 17.52; S, 9.99%. Calculated for $C_{14}H_{15}ClN_4OS$: C, 52.09; H, 4.68; N, 17.36; S, 9.93%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-(4-chlorophenyl)urea (2h): The product obtained (0.325 g, 84%) by the reaction of **1** (0.200 g, 1.2 mmol) with 4-chlorophenyl isocyanate (0.181 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was a white solid. Mp: 221–223 °C. $[\alpha]_D^{25}$ −66.9 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.24 (s, 1H, –NH), 8.12 (s, 1H, –NH), 7.29 (d, 2H, $J = 10.02$ Hz, Ar–H), 6.86 (d, 2H, $J = 9.74$ Hz, Ar–H), 6.72 (s, 2H, $-NH_2$), 4.06–4.02 (m, 1H, –CH–), 2.85 (dd, 1H, $J = 4.88$ Hz, $J = 4.90$ Hz, –CH), 2.53–2.49 (m, 2H, $-CH_2$), 2.40 (dd, 1H, $J = 5.92$ Hz, $J = 5.64$ Hz, –CH), 1.88–1.81 (m, 1H, $-CH_2$), 1.77–1.72 (m, 1H, $-CH_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.6, 155.0, 140.0, 129.0 (2C), 124.9, 119.4 (2C), 112.8, 45.2, 29.9, 28.6, 24.2: MS (ESI + ion): $m/z = 339.6$; IR (KBr, cm^{-1}): 3433, 3225, 1677, 1123; Elemental analysis: Found: C, 52.08; H, 4.70; N, 17.60; S, 9.97%. Calculated for $C_{14}H_{15}ClN_4OS$: C, 52.09; H, 4.68; N, 17.36; S, 9.93%.

Synthesis of 1-((*S*)-2-Amino-4,5,6,7-tetrahydrobenzo[*d*]-thiazol-6-yl)-3-phenyl)urea (2i): The product obtained (0.31 g, 81%) by the reaction of **1** (0.200 g, 1.2 mmol) with phenyl isocyanate (0.143 g, 1.2 mmol) and triethylamine (0.360 g, 3.6 mmol) in dichloromethane (3 mL) using the general experimental procedure as described was an off-white solid. Mp: 164–166 °C; $[\alpha]_D^{25}$ −67.9 (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.22 (s, 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–), 2.81 (dd, 1H, $J = 4.98$ Hz, $J = 4.96$ Hz, –CH), 2.51–2.46 (m, 2H, $-CH_2$), 2.39 (dd, 1H, $J = 5.86$ Hz, $J = 5.70$ Hz, –CH), 1.89–1.82 (m, 1H, $-CH_2$), 1.75–1.70 (m, 1H, $-CH_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.5, 155.2, 144.6, 132.6, 124.2 (2C), 121.6, 119.2 (2C), 112.8, 45.3, 30.1, 28.7, 24.1; MS (ESI + ion): $m/z = 305.6$; IR (KBr, cm^{-1}): 3403, 3211, 1679, 1114; Elemental analysis: Found: C, 58.58; H, 5.62; N, 19.61; S, 11.07%. Calculated for $C_{14}H_{16}N_4OS$: C, 58.31; H, 5.59; N, 19.43; S, 11.12%.

Antileukemic Activity. 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 (FBS), 100 U mL^{-1} 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 as reported earlier.[18] 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 and cells were exposed to different concentrations (10, 100, and 250 μM) of **2a**–**2i**. 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 hemocytometer chamber. The results of these are represented as graphs as shown in Figures 1 and 2.

MTT Assay: Cell proliferation was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,[18] 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 **2a**–**2i**. Cells were harvested after 48 and 72 h of treatment and incubated with MTT (0.5 mg mL^{-1}). 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 compound treatment. The histograms were plotted as shown in Figures 3 and 4.

LDH Release Assay: The cytotoxicity of the compound **2e** was further assessed by LDH release assay,[18] which is an indicator of membrane integrity and hence cell injury. LDH assay was performed as per standard protocols to estimate the LDH release in the culture media following the treatment of **2e** (10, 50, and 100 μM) on K562 cells for 24 and 48 h. The LDH release was measured. Percentage of LDH release was calculated and plotted as a graph as shown in Figure 5.

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 apoptosis detection kit (Santacruz, USA). In brief, after 48 h of treatment with **2e** (10, 50, and 100 μM), 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^{-1}) and PI (0.05 mg mL^{-1}) were added and incubated in dark for 20 min. Cells were then subjected to FACS (FACScan, BD Biosciences, USA) analysis. At least 10000 events were recorded and represented as dot plots.

DNA Fragmentation Assay: To determine whether an induction of apoptosis accompanied the growth inhibition by 4,5,6,7-tetrahydrobenzo[*d*]thiazole derivative **2e**, we analyzed apoptotic DNA fragmentation by agarose gel electrophoresis using the procedure as reported earlier.[18] For this assay, K562 cells were cultured in absence or presence of **2e** 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.

Conclusion

In summary, we have described the synthesis of 1-((*S*)-2-amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)-3-(substituted phenyl)urea derivatives **2a**–**2i**, which showed moderate and weak inhibitory activity toward human leukemic cells lines, K562 and Reh. The derivatives **2d**, **2e, 2f**, **2g**, and **2h** with electron-withdrawing chloro and fluoro substituents at different positions showed good inhibitory effects. Compounds **2a**, **2b**, **2c**, and **2i** showed moderate inhibitory activity against both the cell lines tested. Further investigations to understand the mechanism by which these molecules induce apoptosis and developing more potent molecules are under progress in our laboratory.

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