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Synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid derivatives and evaluation of their cytotoxicity and induction of apoptosis in human leukemia cells

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ABSTRACT

In order to explore the anticancer effect associated with the thiazolidinone framework, several 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid derivatives **5(a–l)** were synthesized. Variation in the functional group at C-terminal of the thiazolidinone led to set of compounds bearing amide moiety. Their chemical structures were confirmed by ^{1}H NMR, IR and Mass Spectra analysis. These thiazolidinone compounds containing furan moiety exhibits moderate to strong antiproliferative activity in a cell cycle stage-dependent and dose dependent manner in two different human leukemia cell lines. The importance of the electron donating groups on thiazolidinone moiety was confirmed by MTT and Trypan blue assays and it was concluded that the 4th position of the substituted aryl ring plays a dominant role for its anticancer property. Among the synthesized compounds, **5e** and **5f** have shown potent anticancer activity on both the cell lines tested. To rationalize the role of electron donating group in the induction of cytotoxicity we have chosen two molecules (**5e** and **5k**) having different electron donating group at different positions. LDH assay, Flow cytometric analysis and DNA fragmentation suggest that **5e** is more cytotoxic and able to induce the apoptosis.

1. Introduction

Among diseases cancer is considered as the deadliest and a substantial number of new antineoplastic agents have been discovered. Considerable insight has been gained into the mechanisms by which many of these compounds affect cellular growth and this knowledge has been used in the designing of new chemotherapeutic drugs.[1] Chemotherapy combined with surgery and/or radiotherapy still has an important role to play in the therapeutic approach of cancer.

Cancer cells differ from their normal counterparts in a number of biochemical processes, particularly during the control of cell growth and division. One characteristic of cancer cells, that distinguishes them from most normal cells, is their high proliferative index. As a result, targeting of proliferative pathways resulting in cell death via apoptosis or prevention of cell division via cell cycle arrest, are considered effective strategies for fighting this disease.

Leukemia is one of the major types of cancer affecting a significant segment of the population, especially children. Although development of better chemotherapy regimens has improved remission induction and overall survival in leukemia, relapse remains a common problem.[2,3] Development of innovative therapies and identification of more effective drugs, therefore, remain high priorities for leukemia research. Currently, combined anticancer therapies or multi-acting drugs are clinically preferred to traditional cytotoxic treatment, with the aim of overcoming resistance and toxicity drawbacks. These events often prevent successful treatment and are responsible for reduced survival times.[4,5] Hence in the field of chemotherapeutic drugs, the search for new, more active, more selective and less toxic compounds is still very intense, and new promising anticancer approaches are being tested.[6,7]

In discovering small anticancer molecules, a notable role is played by heterocyclic structures, and among these, a growing attention focuses on the synthesis and study of the biological properties of compounds containing various combinations of thiazolidinone or furfural moieties.[8] An important application of small molecule libraries is the preparation of a directed or focused combinatorial library for assay against a specific biological target. 4-thiazolidinones substituted in the 2 position were proven to be biologically very potent and selective.[9–11] A wide spectrum of

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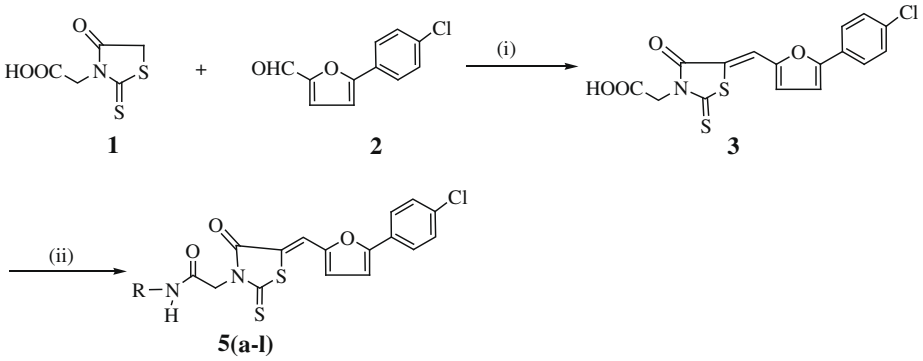


Scheme 1. Reagents and conditions: (i) CH_3COONa, CH_3COOH, 120 °C, 12 h; (ii) isobutyl chloroformate, triethyl amine, THF, R-NH_2 **4**(**a**–**l**), rt, 4–5 h. Where **4a** = 4-trifluoro methyl benzyl amine; **4b** = 4-cyanophenyl amine; **4c** = 4-fluorobenzyl amine; **4d** = 5-amino-2-(trifluoromethyl)benzonitrile; **4e** = 4-methoxy benzyl amine; **4f** = 4-methyl benzyl amine; **4g** = 4-bromo benzyl amine; **4h** = 2-chloro benzyl amine; **4i** = 1*H*-tetrazol-5-amine; **4j** = hexan-1-amine; **4k** = 3- methyl benzyl amine; **4l** = butan-1-amine.

pharmacological activities has been reported for these compounds. These include anticancer,[12–14] peptic ulcer,[15,16] antimicrobial,[17] antiviral,[18] anticonvulsant,[19] antitubercular,[20] antifungal,[21] anti-inflammatory,[22] analgesic[23] and antiproliferative[24] activities. Thiazolidinone derivatives also possess wide range of pharmacological action, especially on anti-HIV agents[25,26] and ability to inhibit the bacterial enzyme MurB.[27] It is also reported that, groups like furan and aryl furans contribute to the biological activities[28] and these would result in highly potent and selective anticancer agents.[29] The design, synthesis and biological study of new anticancer compounds to obtain compounds with enhanced activity are an ongoing research project in our group. We have recently reported the synthesis of novel derivatives of diazaspiro bicyclo hydantoin and induction of apoptosis in leukemia cells.[30,31] We also developed a series of thiazolidinone derivatives and investigated their anticancer properties against Ehrlich ascites tumor cells.[32–34] In continuation of our research in this field we designed and synthesized novel thiazolidonones having furan moiety.

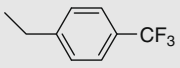
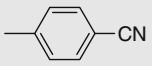
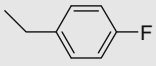
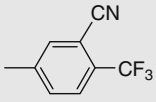
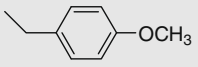
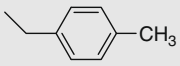
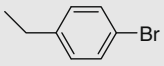
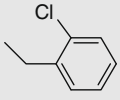
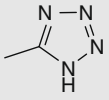
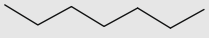
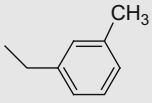
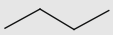
In this study, we investigated the antiproliferative activity and apoptosis induced by the newly synthesized thiazolidinone analogs against human leukemia cells. Our data indicate that compounds with more electron donating groups induce significant apoptosis rather than electron withdrawing groups.

2. Results and discussion

2.1. Chemical synthesis

Synthesis of the key intermediate 2-(5-((5-(4-chlorophenyl) furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid **3** is outlined in Scheme 1. Thiazolidine-2,4-dione **1** was treated with 5-(3-chlorophenyl)- furfural **2**, this undergoes aldol condensation to produce adduct **3**. This reaction requires use of sodium acetate as a base and acetic acid as solvent. The reaction of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid **3** with different aromatic amines (R-NH_2) were carried out in the presence of base triethyl amine, isobutyl chloroformate and tetrahydrofuran (THF) as solvent with a good yield ranging from 76% to 90%. Synthesized molecules **5(a–l)** were structurally characterized by ^{1}H NMR, IR analysis and Mass Spectra (MS). The coupling of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid **3** with different aromatic amines was confirmed by IR and ^{1}H NMR data. Compounds **5(a–l)** were confirmed by IR data, which showed disappearance of stretching frequencies of COOH at 2995 cm^{-1}. From ^{1}H NMR spectra, this showed appearance of –CONH at 12.03 ppm. The chemical structures, physical data and yield of all the synthesized compounds are given in Table 1.

Table 1
Chemical structure, yield and melting point of the synthesized compounds **5**(**a**–**l**)

Compound	R	Yield (%)	Mp (°C)
5a		76	182–184
5b		82	185–187
5c		79	192–194
5d		85	190–192
5e		88	179–181
5f		82	188–190
5g		80	176–178
5h		79	186–188
5i		85	195–197
5j		90	187–189
5k		84	185–187
5l		88	182–184

2.2. Thiazolidinones 5(a–l) induces cytotoxicity on leukemic cells

Induction of cell death or inhibition of cell proliferation is an important property for chemotherapeutic agents. In the present study, we have used trypan blue and MTT assay to investigate the effect of thiazolidinones **5(a–l)** on cell viability of different leukemic cell lines (K562 and Reh). The cells were counted at intervals of 24 h till the control cells attained stationary phase. Results showed that addition of compounds **5(a–l)** affected the viability of the cells in most cases in both the leukemic cell lines tested (Fig. 1 and data not shown). The cytotoxicity induced by the thiazolidinones **5(a–l)** was in a dose- and time-dependent manner. In both the cell lines tested, the lowest concentration (10 μM) of thiazolidinones **5(a–l)** was least effective (Fig. 1). However, an increase in the concentration of thiazolidinones **5(a–l)** to 100 or 250 μM affected the cell viability within 48–72 h (Fig. 1B, C and E–L) except in **5(a, d)** (Fig. 1A and D). It was found that the effect was maintained even after prolonged incubation periods. Interestingly, the DMSO control, corresponding to the highest concentrations of compounds tested did not show any significant toxic effect. Based on these results we identify that compounds **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **5k**, and **5l** induce cytotoxicity in the tested leukemic cells. Among the compounds identified, **5e** displayed the most prominent cytotoxicity which could be attributed to its electron donating methoxy group.

The cytotoxicity induced by thiazolidinones **5(a–l)** was further verified using MTT assay. In order to perform MTT assay, we incubated K562 or Reh cells with 10, 100 and 250 μM of the compounds and cells were harvested at 48 and 72 h. Results showed that **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **5k**, and **5l** were more cytotoxic and effected in dramatic reduction in cell proliferation at 100 μM or higher concentration after 48–72 h of treatment (Fig. 2 and data not shown). Based on these studies the IC_{50} value for thiazolidinones **5(a–l)** were calculated for 72 h (Table 2). Thus our results show that compounds having electron donating groups have more potent activity. Hence, we decided to compare the results within the different electron donating groups present at different positions. For that we have chosen compounds, **5e** and **5k** bearing methoxy (*para* position) and methyl group (*meta* position), respectively.

LDH release assay was performed to test the cell damage induced by the compounds **5e** and **5k**. For this, K562 cells were cultured with 10, 50 and 100 μM of **5e** or **5k** for 24 and 48 h. The release of LDH (an indicator of membrane integrity) was measured as mentioned in 'Materials and methods'. Results showed a dose-

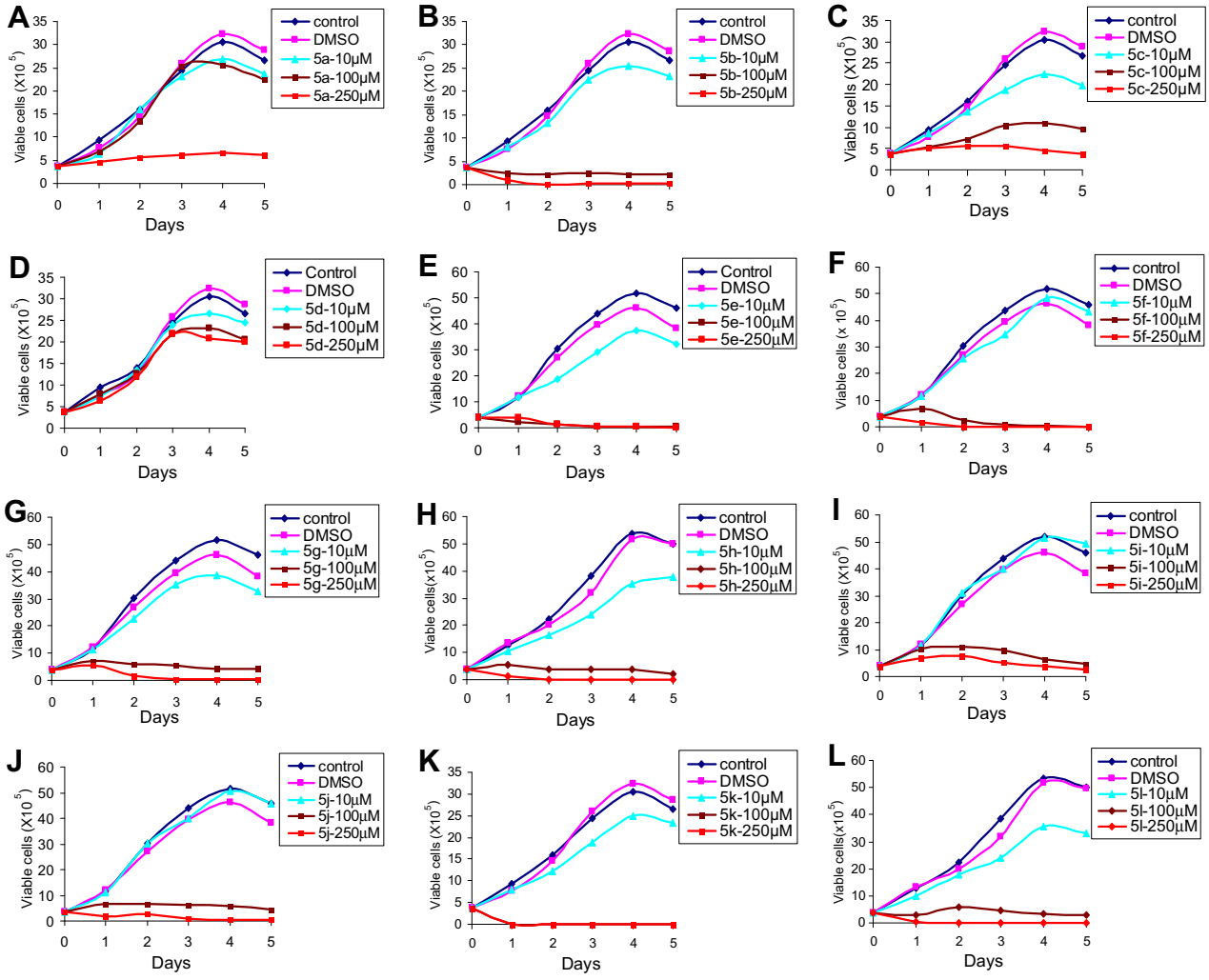


Figure 1. Dose-and time-dependent effect of thiazolidinones **5**(**a–l**) on K562 cell survival. Approximately 0.75 × 10^5 cells/ml were cultured followed by the treatment of compound **5(a–l)** after 24 h at a concentration of 10, 100 and 250 μM. Besides untreated cells, DMSO treated cells were used 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 was represented as a graph. Graphs A–L represents the treatment of compound **5(a–l),** respectively against K562 cells.

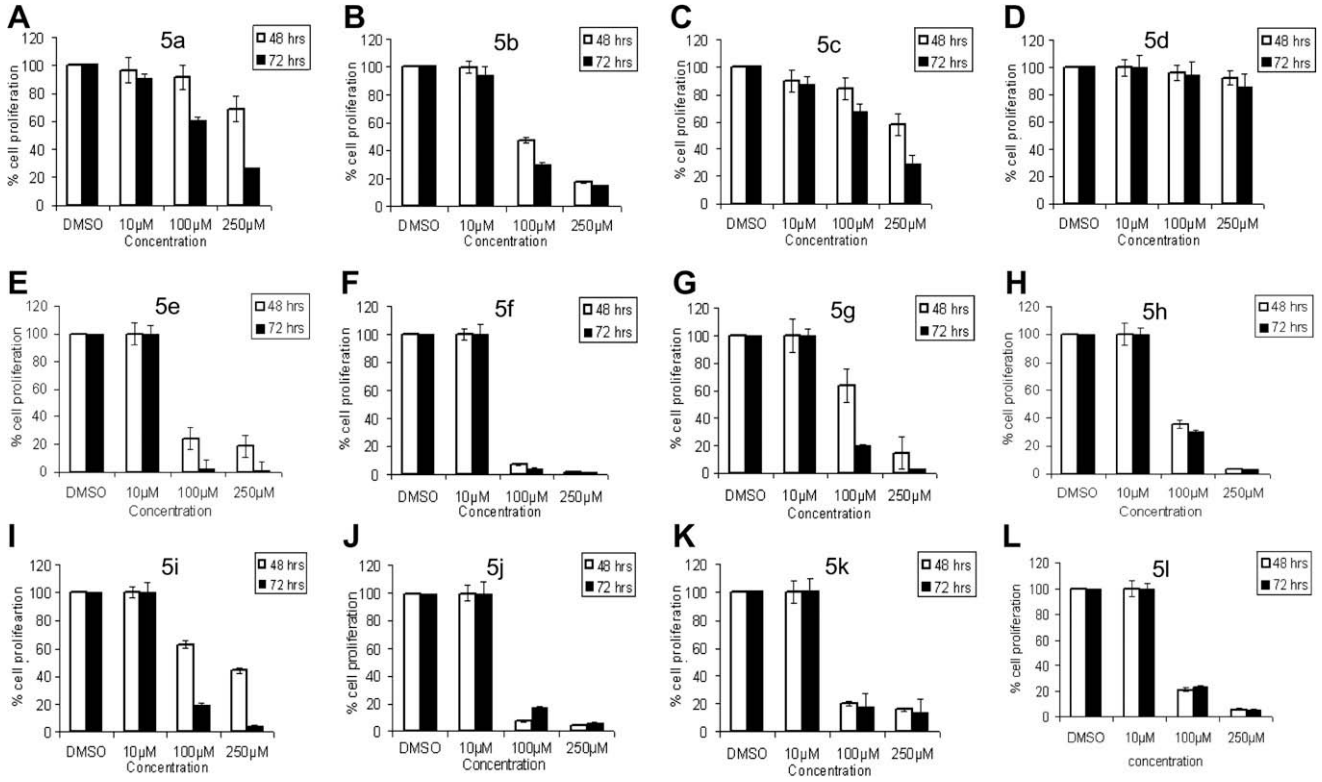


Figure 2. Determination of the effect of thiazolidinones **5**(**a**–**l**) on cell proliferation by MTT assay. After 48 and 72 h of exposure, K562 cells with **5(a–l)** (10, 100 and 250 μM) were incubated with MTT (5 mg/ml) in duplicates and resulting blue formazan precipitate was dissolved in detergent and absorbance was measured at 570 nm. Results are presented as percentage of cell proliferation (the cell viability of vehicle cells were considered as 100%). Error bars are represented in the figure. Graphs A–L represents the treatment of compound **5(a–l)**, respectively against K562 cells.

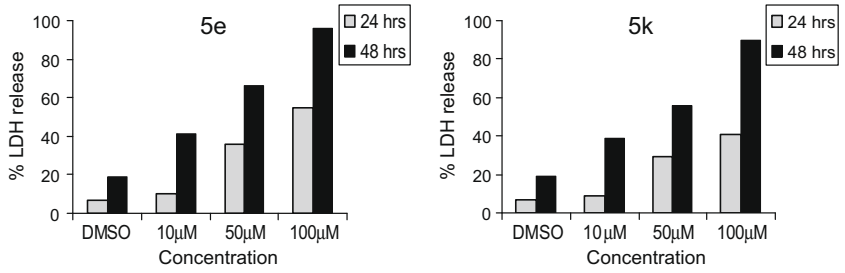


Figure 3. Time-and dose dependent LDH release in K562 cells treated with **5e** or **5k**. K562 cells were incubated for 24 and 48 h with different concentrations of **5e** or **5k**. Release of LDH in the medium was measured at 490 nm. Results are presented as percentage of LDH release.

and time-dependent increase in LDH release (Fig. 3), further confirming the above results. We observed that **5e**, which has a strong electron donating methoxy group at *para* position is more potent than **5k** with a less electron donating methyl group at *meta* position.

Table 2
IC_{50} values of thiazolidinones **5**(**a**–**l**) as determined based on MTT assay

Compound	IC_{50} (μM)	
	K562	Reh
5a	155 ± 7	170 ± 9
5b	85 ± 4	90 ± 5
5c	150 ± 6	165 ± 7
5d	>250 ± 15	>250 ± 14
5e	40 ± 2	44 ± 2
5f	45 ± 2	45 ± 3
5g	68 ± 4	75 ± 4
5h	64 ± 3	70 ± 3
5i	52 ± 3	50 ± 3
5j	50 ± 3	55 ± 5
5k	48 ± 3	53 ± 4
5l	50 ± 3	55 ± 4

2.3. Assessment of cell cycle profile upon treatment with 5e or 5k on K562 cells

Data from trypan blue exclusion assay and MTT assay showed that **5e** and **5k** induced reduction in the number of live cells. Based on this, we were interested in studying cell cycle distribution by fluorescence activated cell sorting (FACS). K562 cells were harvested after 48 h of compound treatment (10, 50 and 100 μM), PI stained and subjected to flow cytometry. The histogram of vehicle control (DMSO) treated cells showed a standard cell cycle pattern, which include G1 and G2 peaks separated by S phase peak (Fig. 4). SubG1 peak (mostly dead cells) was either absent or not prominent. Interestingly upon addition of **5e** or **5k** to K562 cells, a concentration dependent change was observed in the cell cycle pattern (Fig. 4A–D). However, such a change was less prominent

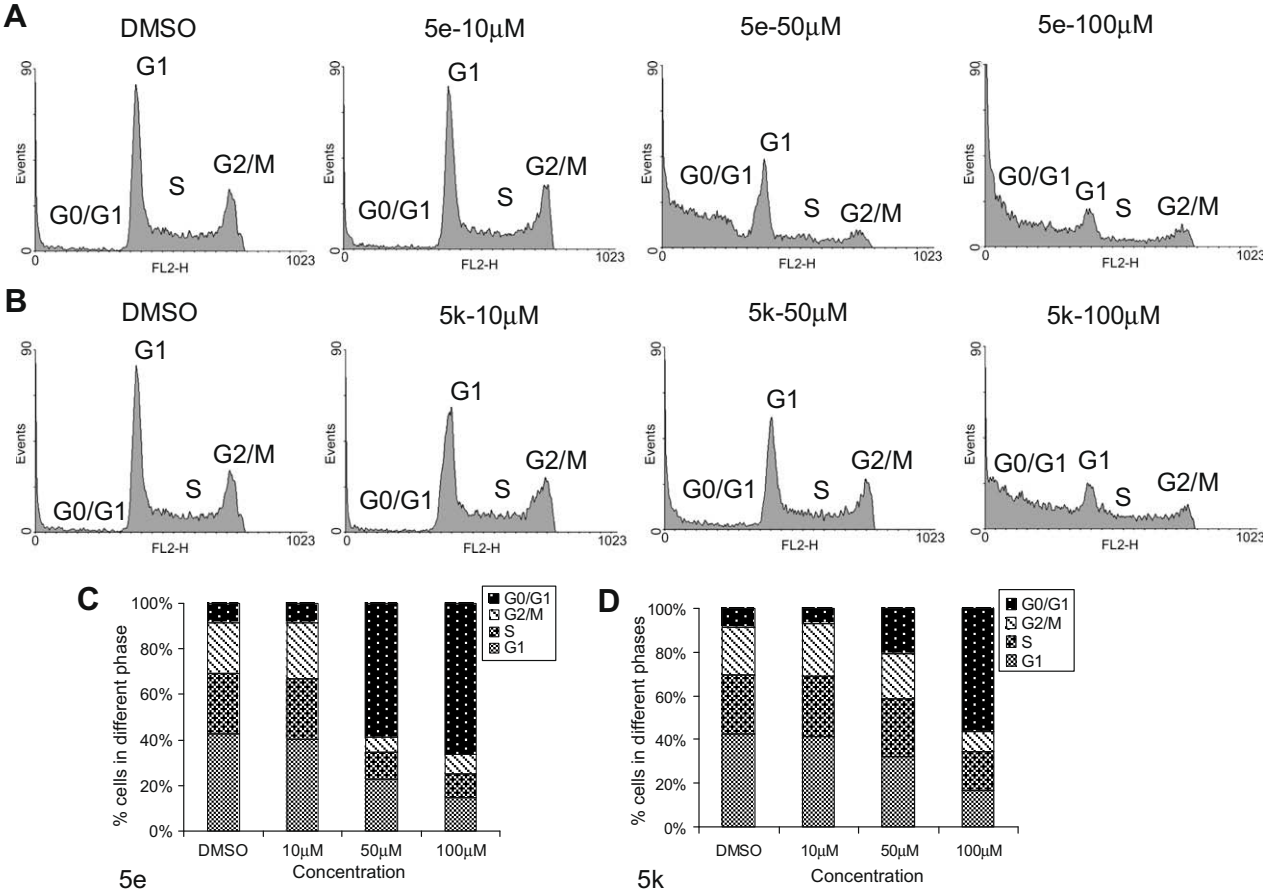


Figure 4. Cell cycle analysis of K562 cells treated with thiazolidinones **5e** or **5k**. K562 cells (0.75×10^5 cells/ml) were incubated at 37 °C with **5e** or **5k** (10, 50 and 100 μM). Following 48 h of incubation, cells were fixed and stained with propidium iodide and subjected to FACS analysis.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

when the cells were treated with **5k** at 50 μM when compared with **5e** at 50 μM. Although we were not able to visualize prominent arrest at any stage of the cell cycle, we noted that there was a remarkable accumulation of subploid cells, the subG1 phase (G0/G1), and decline of both G1 and G2/M (Fig. 4C and D of **5e** and **5k**, respectively) phases when compared with untreated cells. Therefore, our studies further confirm that growth inhibition could be due to apoptosis.

2.4. DNA fragmentation induced by 5e or 5k

The parameter which was considered to assess the DNA damage upon treatment with compounds was chromosomal DNA fragmentation. The chromosomal DNA extracted from the K562 cells treated with increasing concentrations (10, 50 and 100 μM) of **5e** or **5k** after 72 h, was used for agarose gel electrophoresis as described in 'Materials and methods'. The results showed fragmentation of the DNA leading to a smear in the lanes in which cells were treated with **5e** or **5k** (Fig. 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 case of **5e**, 50 μM showed moderate smearing and 100 μM showed maximum (Fig. 5A), however, it was limited to a lesser extent even in 100 μM concentration in case of **5k**. These results further suggest that **5e** having methoxy group induces fragmentation of chromosomal DNA leading to apoptosis more efficiently than **5k** having methyl group.

Thiazolidine is an important scaffold known to be associated with several biological activities.[35] Some of the derivatives of 2-aryl-4-oxo-thiazolidin-3-yl amides have the ability to inhibit the

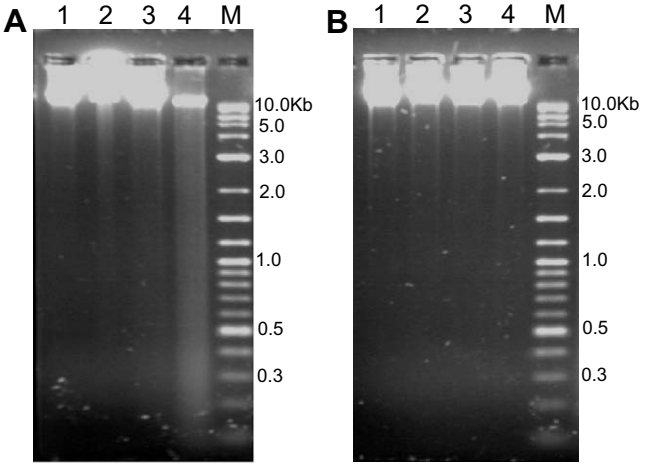


Figure 5. Detection of **5e** or **5k** induced DNA damage in K562 cells. The chromosomal DNA was extracted from K562 cells following treatment with different concentrations of **5e** (A) and **5k** (B). The purified DNA was then resolved on a 1% agarose gel at 30 V for 6 h. In both panels, Lane 1: DMSO;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

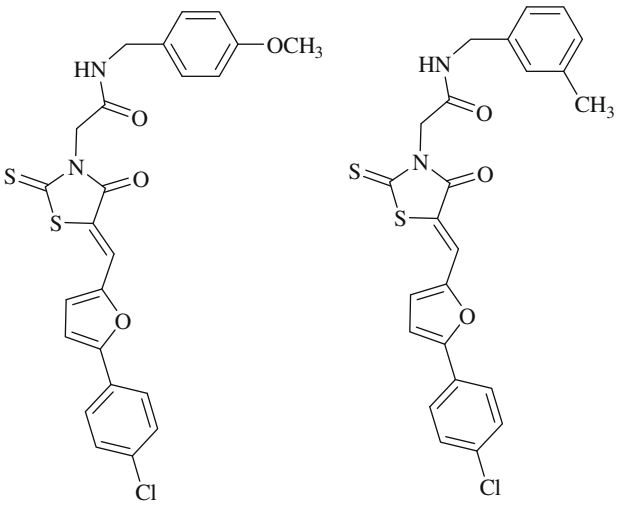


Figure 6. Structures of the potent molecules.

growth of different prostate cancer cells with improved selectivity compared to serine amide phosphates.[36] Some representative of 2-phenylimino-4-thiazolidinones have been investigated as potent inhibitors of the growth of human colon carcinoma cell lines with a different COX-2 expression.[37–41] Holla et al., reported the 2-(5-arylfurfurylidene/5-nitrofurfurylidene)-5-aryl-7-(2,4-dichloro-5-fluorophenyl)-5*H*-thiazolo[2,3-*b*]-pyrimidin-2(1*H*)-ones exhibited in vitro antitumour activity with moderate to excellent growth inhibition against a panel of 60 different cancer cell lines.[42] Thiazolidine-2,4-dione and 2,4-thione derivatives have been reported for inhibitors of translation initiation.[12] In view of the above findings, the anticancer activity was checked by carrying out the reactions of 2-(5-((5-(4-chlorophenyl) furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid with different amines containing substituted aromatic rings.

From the SAR studies, it reveals that the substitution at C-terminal of the phenyl ring play a key role in its biological activity. **5e** and **5k** having electron donating groups enhances the activity, whereas **5(a–d)** having electron withdrawing groups (CN, F, CF_3) decreases the activity. Interestingly, compounds **5g**, **5h** and **5i** having electron withdrawing bromo (*para*), chloro (*ortho*) and tetrazole groups, respectively showed good activity. This could be due to the less electron withdrawing effect of chloro, bromo and tetrazole group when compared to fluoro group **5c**. On the other hand, as the electron donating efficiency increases, the activity also increases. Introducing electron donating methoxy and methyl groups **(5e**, **5f)** on the C-terminal of the phenyl ring at 4th position resulted in increase in the activity, but the position of methyl group from 4th to 3rd position lead in the loss of activity and also in **5j** and **5l**, shows good activity due to the presence of electron donating alkyl chain. However, further studies are required to understand the exact mechanism of its action. The structures of the potent molecules are shown in Figure 6.

3. Conclusion

In the present study, we have shown that novel thiazolidinone derivatives **5(a–l)** showed moderate to strong antiproliferative activity against human leukemia cells. Interestingly from the cytotoxic assays we noted that the compounds with electron donating groups at C-terminal of the phenyl ring resulted in an increase in the activity by inducing cell death. FACS analysis of compounds **5e** and **5k** showed a remarkable accumulation of subploid cells, the subG1 phase (G0/G1) followed by the decline of both G1 and G2/M phases. Therefore, our studies further suggest that the observed growth inhibition could be due to apoptosis. In addition to that we have also observed the inter nucleosomal DNA fragmentation induced by the compound **5e**, which is a characteristic of apoptosis mediated cytotoxicity.

4. Experimental

4.1. Chemistry

Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded using a Jasco FTIR-4100 series. Nuclear magnetic resonance (^{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 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.

4.2. Synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4- oxo-2-thioxothiazolidin-3-yl)acetic acid (3)

A mixture of 3-rhodanine-3-acetic acid **1** (1.0 g, 0.01 mmol), 5-(3-chloro phenyl)-furfural **2** (1.08 g, 0.01 mmol) and anhydrous sodium acetate (1.3 g, 0.03 mmol) were taken in 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, was with ether. Finally get a reddish solid compound (1.75 g, 75%). ^{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.

4.3. General procedure for the synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid derivatives 5(a-l)

A solution of 2-(5-((5-(4-chlorophenyl) furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid **3** (1.0 equiv) in dry THF was taken cool to 0–5 °C. Then isobutyl chloroformate (1.5 equiv) and triethyl amine were added to the cold reaction mixture. The reaction mixture was stirred for 15 min at same temperature, and then adds appropriate amines (1.0 equiv) to the reaction mixture. Allow the reaction mixture to room temperature and stirred 4–5 h. The progress of the reaction was monitored by TLC. After completion of the reaction, water was added and the reaction mixture was filtered. Finally washed with ether and dried under vacuum.

4.3.1. Synthesis of *N*-(4-(trifluoromethyl)benzyl)-5-((5-(4-chlorophenyl)furan-2-yl) methylene)-4-oxo-2-thioxothiazolidine-3-carboxamide (5a)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-trifluoro methyl benzyl amine **4a** (0.046 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.52 (s, 1H, –NH), 7.94 (s, 1H, =CH), 7.81 (d, 2H, Ar-H), 7.72 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.3 (d, 2H, Ar-H), 7.01 (d, 1H, Ar-H), 6.68 (d, 1H, Ar-H), 5.1 (s, 2H, $-CH_2$), 4.53 (s, 2H, $-CH_2$). MS: 536.5. IR (KBr, cm^{-1}): 3430, 1641, 1603, 1366, 1190, 1057, 812.

4.3.2. Synthesis of *N*-(4-(cyano)phenyl)-5-((5-(4-chlorophenyl) furan-2-yl) methylene)-4-oxo-2-thioxothiazolidine-3-carboxamide (5b)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-cyanophenyl amine **4b** (0.031 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.51 (s, 1H, –NH), 7.93 (s, 1H, =CH), 7.79 (d, 2H, Ar-H), 7.7 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.31 (d, 2H, Ar-H), 7.1 (d, 1H, Ar-H), 6.67 (d, 1H, Ar-H), 4.54 (s, 2H, –CH_2). MS: 479.8. IR (KBr, cm^{-1}): 3433, 2224, 1638, 1609, 1376, 1196, 1050, 803.

4.3.3. Synthesis of *N*-(4-(fluoro)benzyl)-5-((5-(4-chlorophenyl) furan-2-yl) methylene)-4-oxo-2-thioxothiazolidine-3-carboxamide (5c)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-fluorobenzyl amine **4c** (0.032 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.51 (s, 1H, –NH), 7.92 (s, 1H, =CH), 7.77 (d, 2H, Ar-H), 7.68 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.31 (d, 2H, Ar-H), 7.05 (d, 1H, Ar-H), 6.68 (d, 1H, Ar-H), 5.12 (s, 2H, –CH_2), 4.54 (s, 2H, –CH_2). MS: 486.9. IR (KBr, cm^{-1}): 3430, 1635, 1606, 1368, 1192, 1055, 816.

4.3.4. Synthesis of *N*-(3-cyano-4-(trifluoromethyl)benzyl)-5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidine-3-carboxamide (5d)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 5-amino-2-(trifluoromethyl)benzonitrile **4d** (0.048 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.53 (s, 1H, –NH), 7.96 (s, 1H, =CH), 7.85 (d, 1H, Ar-H), 7.79 (d, 2H, Ar-H), 7.49 (d, 2H, Ar-H), 7.35 (d, 2H, Ar-H), 7.1 (d, 1H, Ar-H), 6.7 (d, 1H, Ar-H), 4.56 (s, 2H, -CH_2). MS: 547.8. IR (KBr, cm^{-1}): 3444, 2225, 1642, 1616, 1367, 1193, 1056, 806, 725.

4.3.5. Synthesis of *N*-(4-methoxybenzyl)-2-(5-((5-(4-chloro phenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl) acetamide (5e)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-methoxy benzyl amine **4e** (0.035 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.5 (s, 1H, –NH), 7.9 (s, 1H, =CH), 7.76 (d, 2H, Ar-H), 7.65 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.3 (d, 2H, Ar-H), 7.02 (d, 1H, Ar-H), 6.67 (d, 1H, Ar-H), 5.1 (s, 2H, –CH_2), 4.54 (s, 2H, –CH_2), 3.91 (s, 3H, –OCH_3). MS: 498.8. IR (KBr, cm^{-1}): 3435, 1638, 1606, 1367, 1193, 1056, 805.

4.3.6. Synthesis of *N*-(4-methylbenzyl)-2-(5-((5-(4-chlorophenyl) furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide (5f)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-methyl benzyl amine **4f** (0.031 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.51 (s, 1H, –NH), 7.9 (s, 1H, =CH), 7.75 (d, 2H, Ar-H), 7.65 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.3 (d, 2H, Ar-H), 7.04 (d, 1H, Ar-H), 6.67 (d, 1H, Ar-H), 5.1 (s, 2H, –CH_2), 4.53 (s, 2H, –CH_2), 2.91 (s, 3H, –CH_3). MS: 482.9. IR (KBr, cm^{-1}): 3430, 1640, 1607, 1367, 1192, 1056, 805.

4.3.7. Synthesis of *N*-(4-bromobenzyl)-2-(5-((5-(4-chlorophenyl)-furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide (5g)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 4-bromo benzyl amine **4 g** (0.048 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.5 (s, 1H, –NH), 7.91 (s, 1H, =CH), 7.76 (d, 2H, Ar-H), 7.66 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.31 (d, 2H, Ar-H), 7.05 (d, 1H, Ar-H), 6.67 (d, 1H, Ar-H), 5.1 (s, 2H, –CH_2), 4.57 (s, 2H, –CH_2). MS: 547.6. IR (KBr, cm^{-1}): 3435, 1645, 1607, 1367, 1193, 1056, 804.

4.3.8. Synthesis of *N*-(2-chlorobenzyl)-2-(5-((5-(4-chloro- phenyl)-furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide (5h)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid **3** (0.1 g, 0.26 mmol), 2-chloro benzyl amine **4h** (0.037 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.51 (s, 1H, –NH), 7.93 (s, 1H, =CH), 7.82 (d, 2H, Ar-H), 7.7 (d, 2H, Ar-H), 7.65 (d, 2H, Ar-H), 7.35 (d, 2H, Ar-H), 7.13 (d, 1H, Ar-H), 6.7 (d, 1H, Ar-H), 5.13 (s, 2H, –CH_2), 4.56 (s, 2H, –CH_2). MS: 503.5. IR (KBr, cm^{-1}): 3436, 1644, 1607, 1369, 1193, 1056, 727.

4.3.9. Synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-*N*-(1*H*-tetrazol-5-yl)acetamide (5i)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 1*H*-tetrazol-5-amine **4i** (0.026 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.45 (s, 1H, –NH), 7.91 (s, 1H, =CH), 7.82 (s, 1H, N–H), 7.7 (d, 2H, Ar-H), 7.65 (d, 2H, Ar-H), 7.35 (d, 1H, Ar-H), 6.7 (d, 1H, Ar-H), 4.5 (s, 2H, –CH_2). MS: 464.8. IR (KBr, cm^{-1}): 3433, 3306, 1645, 1609, 1365, 1194, 1056.

4.3.10. Synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)-methylene)-4-oxo-2-thioxo thiazolidin-3-yl)-*N*-hexylacetamide (5j)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), hexan-1-amine **4j** (0.026 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.1 (s, 1H, –NH), 7.87 (s, 1H, =CH), 7.7 (d, 2H, Ar-H), 7.65 (d, 2H, Ar-H), 7.35 (d, 1H, Ar-H), 6.7 (d, 1H, Ar-H), 4.45 (s, 2H, –CH_2), 3.1 (t, 2H, –NCH_2), 1.3–1.02 (m, 8H, –$(CH_2)_4$), 0.78 (t, 3H, –CH_3). MS: 462.9. IR (KBr, cm^{-1}): 3402, 2852, 1645, 1608, 1364, 1193, 1055.

4.3.11. Synthesis of *N*-(3-methylbenzyl)-2-(5-((5-(4-chloro-phenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide (5k)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), 3-methyl benzyl amine **4 k** (0.031 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6,

400 MHz) δ: 10.49 (s, 1H, –NH), 7.92 (s, 1H, =CH), 7.73 (d, 2H, Ar-H), 7.63 (d, 2H, Ar-H), 7.41 (d, 2H, Ar-H), 7.32 (d, 2H, Ar-H), 7.08 (d, 1H, Ar-H), 6.7 (d, 1H, Ar-H), 5.12 (s, 2H, $-CH_2$), 4.56 (s, 2H, $-CH_2$), 2.95 (s, 3H, $-CH_3$). MS: 483. IR (KBr, cm^{-1}): 3430, 1640, 1607, 1367, 1192, 1056, 805.

4.3.12. Synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl)-methylene)-4-oxo-2-thioxo thiazolidin-3-yl)-*N*-butylacetamide (5l)

The product obtained was reddish solid from 2-(5-((5-(4-chlorophenyl)furan-2-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)-acetic acid **3** (0.1 g, 0.26 mmol), butan-1-amine **4l** (0.019 g, 0.26 mmol), isobutyl chloroformate (0.053 g, 0.39 mmol) and triethyl amine (0.078 g, 0.78 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.13 (s, 1H, –NH), 7.85 (s, 1H, =CH), 7.72 (d, 2H, Ar-H), 7.63 (d, 2H, Ar-H), 7.30 (d, 1H, Ar-H), 6.71 (d, 1H, Ar-H), 4.51 (s, 2H, $-CH_2$), 3.15 (t, 2H, $-NCH_2$), 1.22–1.02 (m, 4H, $-(CH_2)_2$), 0.81 (t, 3H, $-CH_3$). MS: 434.8. IR (KBr, cm^{-1}): 3402, 2852, 1645, 1608, 1364, 1193, 1055.

4.4. Biology

To investigate the antiproliferative activity of thiazolidinones which is an important potency indicator for chemotherapeutic drugs, leukemic cells growing in log phase were treated with 10, 100 and 250 μM. Since the compounds were dissolved in DMSO, it was used as vehicle control. The amount of DMSO used was corresponding to the DMSO in highest concentration of compound tested, which did not show any significant effect on the cell lines tested. To assess the cytotoxicity of newly synthesized thiazolidinones, we employed trypan blue dye exclusion assay, MTT assay, LDH assay and FACS analysis. In addition to this we also performed DNA fragmentation assay which is an indicator of apoptosis. Each experiment was repeated a minimum of two times.

4.4.1. 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.

4.4.2. Trypan blue exclusion assay

To determine the effect of thiazolidinone analogs **5(a–l)** on viability of K562 or Reh cells, approximately 0.75×10^5 cells/ml were seeded in a 6-well tissue culture plate for 24 h and compounds **5(a–l)** 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 were counted using haemocytometer.

4.4.3. MTT assay

Cell survival was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay,[43] 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 triplicates and incubated with 10, 100 and 250 μM of **5(a–l)**. 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 sulphate). 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. The absorbance of vehicle cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control and presented as histograms (Fig. 2).

4.4.4. 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 following the treatment with **5e** or **5k** (10, 50 and 100 μM) on K562 cells for 24 and 48 h.[44] 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 percentage of LDH release was calculated as: (LDH activity in media)/(LDH activity in media + LDH activity in total cells) × 100%.

4.4.5. Cell cycle analysis

Cellular DNA content was measured by flow cytometry. Approximately 0.75×10^5 cells/ml were cultured and treated with 10, 50 and 100 μM concentrations of **5e** or **5k**. Cells were harvested after 48 h of treatment, washed, fixed in 70% ethanol and incubated with RNase A (Sigma–Aldrich, USA). Propidium iodide (PI, 50 μg/ml, Sigma–Aldrich, USA) was added half an hour before acquiring the flow cytometric reading (FACScan, BD Biosciences, USA). A minimum of 10,000 cells were acquired per sample and histograms were analyzed by using WinMDI 2.8 software.

4.4.6. DNA fragmentation assay

DNA fragmentation was performed for elucidating the mode of action of the investigated compounds, especially with respect to induction of oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis.[45,46] 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. Briefly, K562 cells were cultured in presence **5e** or **5k** 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. The DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed.

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