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In Vitro Topoisomerase II Inhibitory and Apoptotic Activities of Novel 3,5 Disubstituted Thiophene-2-carboxylates

Ananda Hanumappa[1], Kothanahally S. Sharath Kumar[1], Mahesh Hegde[1], Kereyagalahally H.Narasimhamurthy[1], Sathees C. Raghavan[2] and Kanchugarakoppal S Rangappa[1]*

[1]*Department of Studies in Chemistry, University of Mysore, India*

[2]*Department of Biochemistry, Indian Institute of Science, India*

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***Corresponding author:** Kanchugarakoppal S Rangappa, Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore, 570006, India, Tel: +91-821-2419661; Email: rangappaks@gmail.com

Abstract

Topoisomerases (topoII) are crucial enzymes involved during DNA replication, repair and transcription. Recent studies have shown topo II as an interesting target for cancer therapy. In the current study we have synthesized and characterized few thiophene derivatives and determined their antiproliferative activity and apoptosis studies in cancer cell lines. Biochemical analysis showed that thiophene derivatives inhibit the alpha isoforms of topoisomerase II and hence these molecules can be considered as a potential anticancer drug.

Keywords: Thiophene-2-carboxylates; Aldol condensation; Topoisomerase II; Nalm6 cells; Cytotoxicity

Introduction

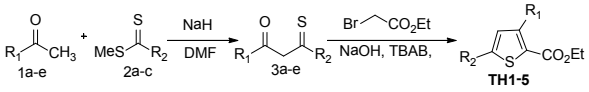
Thiophene derivatives represent an important class of sulfur containing heterocycles that is present in numerous bioactive natural products and pharmaceutically active agents [1,2]. Thiophene derivatives are well explored as cytotoxic agents and recognized as a promising topoisomearse I inhibitor [3,4]. From the literature survey, one may presume that thiophene derivatives can bind to various DNA structures and therefore may interfere with DNA associated enzymes. Topoisomerase are considered as one of the important targets for thiophene derivatives. Cancer cells are highly metabolically active due to their proliferative nature. Each time when a cell reolicate first replication of DNA takesplace and for which role of topoisomerases is very important [5].In eukaryotes, Topoisomerase II enzyme (Topo II) is highly over expressed in robustly dividing tumor cells, and plays an important role in DNA replication, nuclear genome maintenance and transcription [6-8]. Hence, this nuclear enzyme is an attractive target for cancer chemotherapy. Nearly 50% of all cancer therapeutics contains at least one entity targeted to htopo II [9-11]. All the topoisomerase II (topo II) -targeted anticancer drugs are clinically used for their antitumor properties. Broad range of topoII inhibitors are normally classified as topo II poisons and catalytic inhibitors [12]. Topoisomerase inhibitors have played a significant role in chemotherapy. The most extensively used topoisomerase II inhibitors include etoposide, doxorubicin, daunorubicin, mitoxantrone, teniposide and amsacrine. However, it is observed that unfortunately, treatment with some of the topo II inhibitors failed after early effective therapy. Therefore, there is need for the development of novel topoisomerase II targeted drugs, with the goal of disabling current boundaries.

Results and Discussion

Chemistry

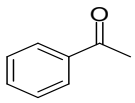
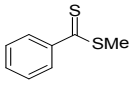
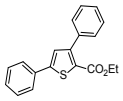
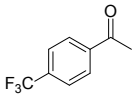
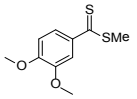
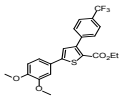
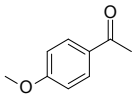
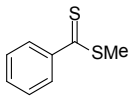
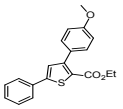
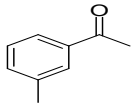
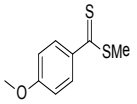
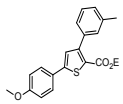
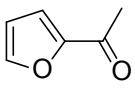
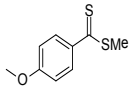
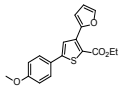
One of the important synthetic approaches for the preparation of substituted thiophene derivatives is from using functionalized polarized ketene dithioacetals as precursors [13]. However, most of the reported methods suffer from one or the other disadvantages, mainly formation of mixed alkylation products during one pot sequential alkylation with different alkyl halides. Later, selective sequential alkylation has been achieved by Asokan et al. [14], but this method is limited to introduce only alkyl thio group in the 2-position of the thiophene derivatives. Further, Ila et al. [15] have developed sequential one-pot three component route to tri- & tetra substituted/annulated thiophenes, this method produced comparatively low yields of thiophenes. In continuation of our synthetic efforts [16], we set out to identify the possible mild conditions under which, the intermediate β-alkyl thioenone would undergo intramolecular aldol condensation with synthetically useful rate. Initially we have started the synthesis of ethyl 3,5-disubstituted thiophene-2-carboxylate derivatives starting directly from 1,3-monothio-

β-diketones in one pot operation. The monothio-β-diketone 3a was selected as a model substrate for optimizing the reaction condition for the formation of thiophene TH1 in the presence of various bases. Sodium hydroxide in benzene: water (2:1) system with TBAB as a phase transfer catalyst at room temperature was found to be better reaction condition for the synthesis of TH1 with 92% yield. With the optimized protocol in hand we went on to synthesize different thiophene derivatives (TH2-TH5). Thus the 1,3-monothio-β-diketones bearing electron donating and electron withdrawing substituents have also gave the considerable product yield. The probable mechanism for the formation of thiophene looks to be simple, treatment of 1,3-monothio-β-diketones with ethyl bromo acetate in-situ generates β-alkyl thioenone intermediate which underwent in-situ intramolecular aldol condensation to afford ethyl 3,5-disubstituted thiophene-2-carboxylate derivatives in good yield (Table 1) (Scheme 1).



Scheme 1: Synthetic route to tri substituted thiophene derivatives.

Table 1: Step-wise synthesis of Ethyl 3,5-disubstituted thiophene-2-carboxylates TH1-5.

Entry	Acetophenone	Dithioester	Product	Yield (%)
1	 1a	 2a	 TH1	92
	 1b	 2b	 TH2	86
3	 1c	 2c	 TH3	89
4	 1d	 2d	 TH4	90
5	 1e	 2e	 TH5	85

Biological studies

Effect of TH1 on various human cancer cell lines: The activity of newly synthesized thiophene compounds were checked in various human cancer cell lines such as Molt4, Nalm6, K562 and HEK 293T for their cytotoxic activity. The MTT assay results confirmed that the tested compounds inhibit the cell proliferation after 48h at different concentrations. Importantly, TH1 inhibited the growth of Molt4, Nalm6 and K562 cells in dose dependent manner with IC_{50} value of 68.9, 69.2 and 82.1μM respectively. Furthermore TH1 didn't inhibit the growth of normal cells such as Human epithelial kidney (HEK293T) cells with IC50value of 141.2μM. These results suggested that TH1 inhibits the growth of human cancer cells.

TH1 causes the accumulation of the cells in the SubG1 phase of the cell cycle in human leukemic cells: In order to assess the influence of TH1 on cell cycle, Nalm6 cells were treated with TH1 and examined the effect after 48h treatment of TH1 (25, 50, 75 & 100 μM) onNalm6 cells induced concentration dependent increase in SubG1 population of cell cycle and it was prominent at 100μM. This data indicated that TH1 could promote cell death through apoptosis without affecting to cell cycle arrest. The bar graph representing different stages of cell population (Figure 1A&1B) of TH1 treated cells appeared to induce cell death in concentration dependent manner in Nalm6 cells.

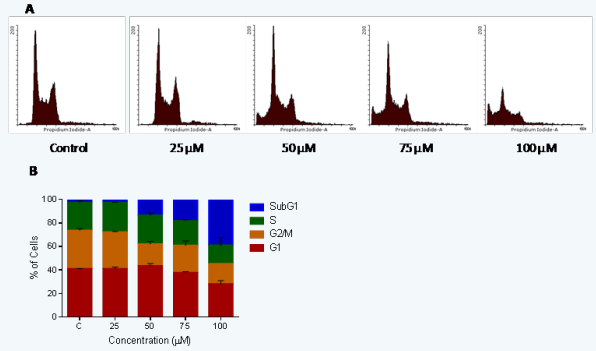


Figure 1: TH1 induces SubG1 phase arrest in Nalm6 cells.
Figure 1A: Cells were treated with different concentrations of TH1 (25, 50, 75 and 100 μ M) for 48 h and cell cycle progression was examined by flow cytometer.
Figure 2B: The bar diagram shows the percentage cell populations in G1, S, G2/M & SubG1 phases of TH1 treated Nalm6.

Live dead assay for TH1 on Nalm6 cells: We carried out live dead cell assay using calcein and propidium iodide staining to confirm the cell death induced by TH1 on Nalm6 cells. Calcein stains live cells and propidium iodide stains only dead cells because of the damaged cell membrane. The 48 h treatment of TH1 showed increase in propidium iodide stained (single and double positive) cells in a dose dependent manner (50, 75 and 100 μM) and decreased number of calcein-AM stained cells

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which further confirming the cytotoxic effect caused by TH1 on Nalm6 cells (Figure 2A & 2B).

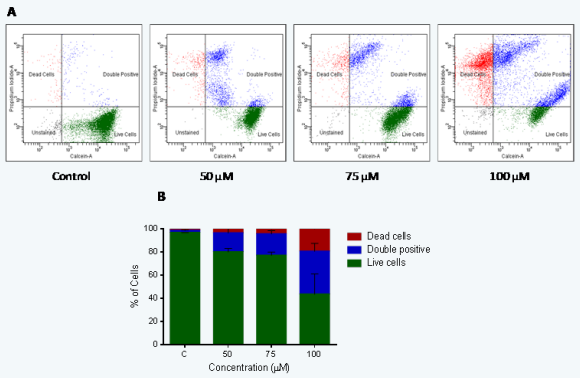

Figure 2: FACS analysis of Nalm6 cells were treated with TH1 by Live dead assay stain.
Figure 2A: Dot plots showing live dead analysis.
Figure 2B: Bar graph shows the percentage of cells in the live, double positive and dead cells. The data presented is derived from two independent experiments and error bars indicated.

Study of mitochondrial membrane potential assay: Study of mitochondrial membrane potential (Δψm) is an important parameter in anti-cancer drug discovery pipeline. We evaluated the effect of TH1 on mitochondrial membrane potential in Nalm6 cells using JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol -carbocyanineiodide) dye. In normal mitochondria JC-1 forms aggregate and emits red fluorescence, on the other hand the dead population with low mitochondrial membrane potential dye remains in monomeric form which emits green fluorescence. Interestingly, 48h of TH1 treatment (75 and 100 µM) lead decrease in the mitochondrial membrane potential in Nalm6 cells which was evident in the increased green fluorescence (Figure 3A&3B). This suggested that participation of mitochondrial apoptotic mechanism in induction of cell death upon treatment with TH1 in Nalm6 cells.

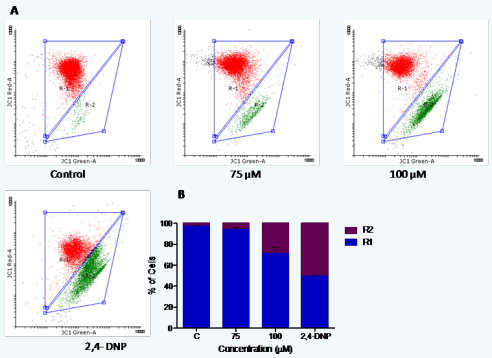

Figure 3 Detection of loss of mitochondrial membrane potential followed by TH1 treatment.
Figure 3A: Nalm6 cells treated with TH1 (75 and 100 µM) for 48 h, harvested and stained with JC-1 dye and analysed by flow cytometer. 2, 4- DNP treated Nalm6 cells were served as positive control. Density plot representing JC-1 stained cells at different concentration of TH1.
Figure 3B: Bar diagram showing ratio of red versus green fluorescent cells.

Action of TH1 leads to induction of apoptosis: The increase in subG1 population and cell death which were evident in cell cycle analysis and live dead assay induced by TH1 on Nalm6 cells lead us to detect the mode of the cell death induced by TH1. Hence we stained the Nalm6 cells which are treated with TH1 (48 h, 75 µM) with annexin-FITC and propidium iodide to study the different types of apoptotic cell populations. The externalization of phosphatidylserine (PS) in living cells is a hallmark of apoptosis. Soon after apoptosis is induced, PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet. We observed increase of early, late apoptotic cells (8.1%, 24.7%) at 75µM compared to DMSO treated control (5.7%, 3.6%) respectively (Figure 4A&4B). Results revealed that the Compound TH1 induced cell death in Nalm6 cells by inducing apoptosis.

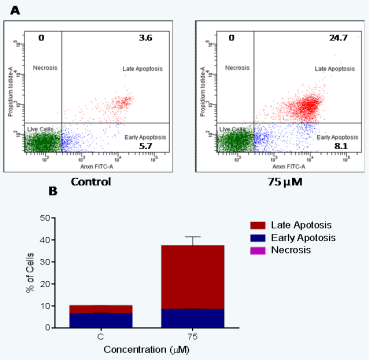

Figure 4: Flow cytometer analysis of apoptosis induction in Nalm6 cells treated with TH1.
Figure 4A: Dot plot represents Nalm6 cells were stained by Annexin V-FITC are cells in early apoptosis (bottom right quadrant), while cells stained with both Annexin V-FITC and PI (top right quadrant) are cells undergoing late apoptosis.
Figure 4B: Histogram showing the percentage of cells in early and late apoptosis phase.

Inhibition of human DNA topoisomerase II: It was evident that TH1 is the active potent compound in tested panel in ex vivo studies. While additional studies are necessary to develop a further understanding on mechanism of action of the TH1 compound. Majority of pharmacological agents bind to DNA and exhibit their effect through interference with the activity of topoisomerase [17,18]. The interaction with cellular macromolecules is the basis for the selective anticancer activity for any class of heterocyclic compounds. Thiophene derivatives are one among them which demonstrated drugs selective cytotoxicity and caused severe disturbance of the cell cycle and inhibited topoisomerases which related to DNA binding activity. Hence, we performed topoisomerase assay using human topoisomerase II. Furthermore we analyzed the effect of TH1 on human topoisomerase II alpha mediated relaxation assay. TH1 compound showed significant inhibition of topoisomerase II alpha activity from 50 µM onwards. VP16 (Topo II inhibitor) were used as positive control (Figure 5). Our results showed that inhibitory activities of topoisomerase II enzyme.

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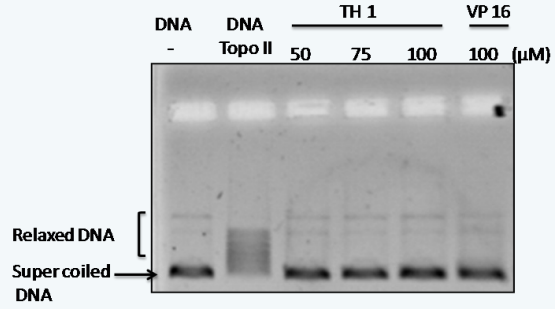

Figure 5: DNA relaxation assay for topoisomerase IIα. Effect of TH1on DNA relaxation catalyzed by Topo II at concentration of 50, 75 and 100 μM. VP16 (100 μM) was used as a positive control. Supercoiled DNA and converted relaxed DNA were marked.

Conclusion

In summary, we report step-wise synthesis and biological evaluation of new thiophene derivatives. Among synthesized molecules TH1 showed enhanced cytotoxicity in leukemic cell lines. Preliminary results about mechanism of action of compound suggested that it is having better capacity to induce apoptosis and substantial effect on topoisomerase II activity. These data revealed that significant correlation in the cytotoxicity. All of these above mentioned considerations tempted us to identify the capability of thiophene-2-carboxylates of generating DNA interactive species that lead to their anticancer activity.

Experimental Section

Reactions were monitored by TLC using precoated sheets of silica gel G/UV-254 of 0.25 mm thickness (Merck 60F254) using UV light for visualization. The melting points were determined on Selaco melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on an NMR spectrometer operating at 400 and 100 MHz, respectively, using the residual solvent peaks as reference relative to $SiMe_4$. Mass spectra were recorded using high resolution mass spectrometry. Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrophotometer.

Ethyl 3,5- disubstituted thiophene-2-carboxylates (TH1-5)

Monothio-β-diketones (3a-e) were synthesized by the reaction between different acetophenones (1a-e) with dithioesters (2a-c) [19,20]. To a stirred solution of monothio-β-diketones 3a-e (1.0 mmol), 10% aq. Na OH (2.0 m mol) and TBAB (catalytic) in benzene (10 volume), ethylbromoacetate (1.0 m mol) was added drop wise at 0oC. The reaction mixture was brought to room temperature and further stirred for 4 h. Reaction was monitored by TLC and after the completion of the reaction, mixture was diluted with water and extracted to ethylacetate. The organic layer was washed with brine solution and dried over anhydrous sodium sulphate, filtered and solvent was evaporated under reduced pressure to get a crude product which was purified by silica gel column chromatography using hexane ethylacetate as eluent to get pure product.

Ethyl 3,5-diphenylthiophene-2-carboxylate (TH1)

Off white solid (92%): mp 67-69 ^{0}C; Rf 0.5 (2:8 EtOAc : Hexane); IR (KBr, Cm^{-1}) 2976, 1668, 1602, 1462, 1435, 1365, 1269, 1131, 819, 764, 657, 501; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (d, J = 7.2 Hz, 2H, ArH), 7.51-7.48 (m, 2H, Ar H), 7.44-7.36 (m, 6H, ArH), 7.29 (s, 1H, C_4H), 4.27-4.22 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 1.27-1.23 (t, J = 7.2 Hz, 3H, OCH_2CH_3); 13C NMR (100 MHz, CDCl3) δ 161.0, 135.3, 135.2, 133.4, 131.0, 129.8, 129.0, 127.9, 127.3, 127.0, 124.7, 121.5, 119.9, 60.3, 14.1; HRMS (ESI) *m/z* Cal cd for $C_{19}H_{16}O_2S$ $[M + Na]^+$ 331.0871, found 331.0895.

Ethyl5-(3,4-dimethoxyphenyl)-3-(4-(trifluoromethyl) phenyl)thiophene-2-carboxylate(TH2)

Pale yellow solid (86%): mp 108-110 ^{0}C; Rf 0.35 (2:8 EtOAc : Hexane); IR (KBr, Cm^{-1}) 2988, 2849, 1678, 1627, 1461, 1299, 1152, 1098, 845, 739, 634, 538; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66-7.64 (d, J = 8.0 Hz, 2H, ArH), 7.60-7.58 (d, J = 8.0 Hz, 2H, ArH), 7.24 (s, 1H, C_4H), 7.114-7.110 (d, J = 1.6 Hz, 2H, ArH), 6.90-6.88 (d, J = 8.0 Hz, 1H, ArH), 4.24-4.22 (q, J = 8.0 Hz, 2H, OCH_2CH_3), 3.93 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 1.26-1.22 (t, J = 7.0 Hz, 3H, OCH_2CH_3); 13C NMR (100 MHz, $CDCl_3$) δ 160.2, 149.0, 132.8, 132.8, 129.0, 127.9, 127.8, 125.7, 125.19, 125.15, 125.13, 125.11, 122.1, 120.0, 119.7, 109.4, 109.2, 61.5, 55.99, 55.95, 14.0; HRMS (ESI) m/z Calcd for $C_{22}H_{19}F_3O_4S$ [M + Na]+ 459.444, found 459.0854.

Ethyl3-(4-methoxyphenyl)-5-phenylthiophene-2-carboxylate (TH3)

Off white solid (89%): mp 91-93^{0}C; Rf 0.4 (2:8 EtOAc : Hexane); IR (KBr, Cm^{-1}) 2978, 1661, 1603, 1462, 1437, 1365, 1268, 1134, 819, 764, 657, 505; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65-7.63 (d, J = 8.0 Hz, 2H, ArH), 7.45-7.32 (m, 5H, ArH), 7.25 (s, 1H, C_4H), 6.94-6.92 (d, J = 8.0 Hz, 2H, ArH), 4.27-4.21 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 3.83 (s, 3H, OCH_3), 1.28-1.23 (t, J = 7.2 Hz, 3H, OCH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.6, 145.3, 140.0, 133.4, 131.0, 129.5, 129.0, 127.9, 127.6, 127.0, 126.7, 121.5, 114.9, 60.8, 55.4, 14.1; HRMS (ESI) m/z Calcd for $C_{20}H_{18}O_3S$ [M + Na]+ 361.0977, found 361.0994.

Ethyl5-(4-methoxyphenyl)-3-m-tolylthiophene-2-carboxylate (TH4)

Yellow solid (90%): mp 97-99^{0}C; Rf 0.35 (2:8 EtOAc : Hexane); IR (KBr, Cm^{-1}) 2999, 2843, 1674, 1479, 1448, 1297, 1256, 1242, 1019, 802, 729, 533; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71-7.69 (m, 2H, ArH), 7.61-7.59 (m, 2H, ArH), 7.56-7.54 (d, J = 8.0 Hz, 2H, ArH), 7.24-7.22 (d, J = 8.0 Hz, 2H, ArH), 7.21 (s, 1H, C_4H), 4.27-4.22 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 2.39 (s, 3H, Ar-CH_3), 1.28-1.24 (t, J = 7.2 Hz, 3H, OCH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.0,

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151.8, 137.4, 135.7, 134.6, 133.9, 130.2, 127.4, 127.5, 126.6, 126.3, 126.0, 121.5, 114.4, 60.2, 55.3, 21.4, 14.1; HRMS (ESI) m/z Calcd for $C_{21}H_{20}O_3S$ [M + Na]+ 375.1133, found 375.1156.

Ethyl3 - (furan-2-yl)-5-(4-methoxyphenyl) thiophene-2-carboxylate (TH5)

Pale yellow solid (85%): mp 101-103^{0}C; Rf 0.3 (2:8 EtOAc : Hexane); IR (KBr, Cm^{-1}) 2988, 2842, 1671, 1485, 1467, 1278, 1237, 1186, 1029, 828, 745, 521; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (br s, 1H, ArH), 7.60-7.58 (d, J = 8.0 Hz, 2H, ArH), 7.559-7.551 (d, J = 3.2 Hz, 1H, ArH), 7.24 (s, 1H, C_4H), 6.93-6.91(d, J = 8.0 Hz, 2H, ArH), 6.509-6.505 (d, J = 1.6 Hz, 1H, ArH), 4.36-4.31 (q, J = 8.0 Hz, 2H, OCH_2CH_3), 3.83 (s, 3H, OCH3), 1.39-1.35 (t, J = 8.0 Hz, 3H, OCH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.5, 159.1, 148.8, 141.0, 135.5, 126.3, 123.6, 122.9, 116.7, 114.4, 111.5, 109.3, 106.1, 60.5, 55.1, 14.0; HRMS (ESI) m/z Calcd for $C_{18}H_{16}O_4S$ [M + Na]+ 351.0769, found 351.0812.

Biology

All chemical reagents were obtained from SRL, India, and Sigma-Aldrich, USA. Culture medias were from Sera Laboratory International limited (West Sussex, UK), FBS and Pen Strep were (from Gibco BRL, USA). Topoisomerase IIα enzyme (Topo GEN Inc USA).

Cell lines and culture

Human cell lines, K562, HEK 293T were purchased from National Center for Cell Science, Pune, India. Cells were grown in RPMI 1640, MEM and DMEM 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. Nalm6 and Molt4 cells were cultured and maintained as described [21]. The cells were grown under the similar culture conditions as mentioned above.

MTT assay

The antiproliferative effect of thiophene derivatives against different cancer cell lines was determined by the MTT dye uptake method as mentioned previously [22,23]. Molt4, Reh, and Nalm6 cells (5 × 10^4 cells/ml) were treated with different concentration of thiophene derivatives (25, 50, 75 & 100 µM) for 48 h and 100 µl cell suspensions was taken duplicates in a 96 well plate. Thereafter, 20 µ l MTT (5 mg/ml in PBS) was added and incubated for 4h at 37°C and the resulting blue crystals were dissolved upon adding 69 µl of lysis buffer (50% final concentration of N, N-dimethylformamide and 10% of sodium dodecyl sulfate, 30 min at 37°C).and subsequently the optical density (OD) was measured at 570 nm using ELISA plate reader.

Cell cycle analysis

The study of DNA substance analysis in cells was performed as mentioned earlier [24]. Nalm6 cells were seeded (0.5x10^5 cells/ml) and treated with different concentrations of TH1 (25, 50, 75 100 µM) for 48 h. Cells were harvested, washed and fixed with 80% cold ethanol, and treated with RNase-A (50 µg/ml). Cells were stained with propidium iodide (10µg/ml) and cell cycle progression was monitored using flow cytometer (BD FACSVerse™). A minimum of 10000 cells were acquired. Results were analyzed using Flowing Software (Version 2.5) and plotted histograms.

Live dead cell assay

The live dead cell assay was performed to determine the viability of cells [25]. It is a two color fluorescence assay that simultaneously determines live and dead cells. Live cells have intracellular esterases that convert non fluorescent, cell permeable Calcein acetoxymethyl (Calcein-AM). Dead cells have damaged membranes, the propidium iodide (PI) enters damaged cells and is fluorescent when bound to nucleic acids, PI produces a bright red fluorescence in damaged or dead cells [26]. Cells were treated with different concentrations of TH1 (50, 75 and 100 µM for 48 h). Cells were harvested after processed and finally acquired for live cells the excitation (max) and emission (max) are 480nm and 527/32 nm, respectively and dead cells, the excitation (max) and emission (max) are 480 nm and 586/42 nm, respectively were used (FACS Verse™, BD Biosciences, USA). Results were analyzed in FACSDIVA software and presented as dot plot. Quantification of live and dead cells was shown as bar diagram based on two independent experiments.

JC-1 Assay

The study of mitochondrial permeability conversion is an important hallmark for study of apoptosis [27]. To assess the mitochondrial membrane potential, Nalm6 cells were seeded in 12 well plates (0.5 x 10^5 cells per ml), treated with TH1 at different concentration (75 and 100 µM). After incubation at 37°C for 48 h, cells were harvested, washed with 1x PBS and stained with (0.5 µg/ml) JC-1 dye for 30 min at 37°C. After incubation, cells were washed and re-suspended in 0.3 ml of 1x PBS and acquired in to flow cytometer (FACSVerse™, BD Biosciences, USA) using Cell Quest Pro Software. Minimum of 10,000 cells were acquired per sample and 2, 4-Dinitrophenol (2, 4-DNP) was used as positive control. Data were analyzed using Flowing Software (Version 2.5).

Detection of apoptosis by Annexin V/PI staining

Quantification of apoptotic cells at the single cell level was performed by Annexin V FITC/PI (Santacruz, USA) staining. Cells were seeded in 6 well plates (0.75x10^5 cells/ml) and treated 75 µM TH1 for 48 h Cells were harvested and processed according to the manufactures instructions [28]. Briefly, cells were washed with 1x PBS, resuspended in binding buffer (10 m M HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) containing annexin V-FITC (0.2 mg/ml) and propidium iodide (0.05 mg/ml), incubated at room temperature for 15 min and analyzed immediately in flow

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cytometer (FACSVerse™, BD Biosciences, USA). A minimum of 10000 cells were acquired and analyzed the data in FACSDIVA software. Control cells were treated with equal volume of DMSO. Cells in various stages of early, late and necrotic population were plotted in dot plot and quantification was presented in bar diagram.

Topoisomerase II DNA relaxation assay

The topoisomerase II assay was performed in a reaction mixture containing pBS-SK+ plasmid (Sigma Aldrich, USA), two units of recombinant human DNA topoisomerase II (Topo GENInc, USA) along with the various concentrations of test compounds [29]. Reaction was carried out at 37°C for 30 min in a relaxation buffer 1x topo II buffer (50 mMTris-Cl (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM dithiothreitol and 30 µgs BSA/ml) and reactions were terminated by adding 5x stop buffer containing 5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol. The DNA samples were electrophoresed on 1% agarose gel at 40 volts for 4 h with 0.5x TBE (Tris-borate-EDTA). The gels were stained for 30 min in milli Q water containing ethidium bromide (0.5 mg/ml) followed by distaining for 30 min in milli Q water and images were taken.

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