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Original article

Synthesis and biological evaluation of novel 2-aralkyl-5-substituted-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivatives as potent anticancer agents

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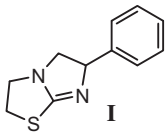
ABSTRACT

Levamisole, the imidazo[2,1-b]thiazole derivative has been reported as a potential antitumor agent. In the present study, we synthesized, characterized and evaluated biological activity of its novel analogues with substitution in the aralkyl group and on imidazothiadiazole molecules with same chemical backbone but different side chains namely 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazoles (**SCR1**), 2-aralkyl-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR2**), 2-aralkyl-5-formyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR3**) and 2-aralkyl-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR4**) on leukemia cells. The cytotoxic studies showed that **3a**, **4a**, and **4c** exhibited strong cytotoxicity while others had moderate cytotoxicity. Among these we chose **4a** (IC_{50}, 8 μM) for understanding its mechanism of cytotoxicity. FACS analysis in conjunction with mitochondrial membrane potential and DNA fragmentation studies indicated that **4a** induced apoptosis without cell cycle arrest suggesting that it could be used as a potential chemotherapeutic agent.

1. Introduction

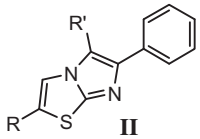
Development of anticancer drugs with fewer or no side effects is important for the treatment of cancer. The search for such potential anticancer drugs have led to the discovery of synthetic small molecules with anti-carcinogenic activity and limited harmful side effects particularly with respect to the immune system. Alternatively, stimulation of the body's immune system could provide a valuable support in cancer treatment, since it is capable of eradicating the neoplastic cells completely. Research in this area is expanding rapidly and some promising leads have emerged.

Levamisole (I) appears to be the most effective in patients with small tumor burdens and it acts by stimulating the responsiveness of lymphocytes to tumor antigens [1]. In addition, the imidazo[2,1-b]thiazole derivatives of Levamisole have been reported as potential antitumor agents (II) [2]. Later, antitumor activity of 5-formyl-6-arylimidazo-[2,1-b][1,3,4]thiadiazole sulfonamides (III) were also reported [3]. The promising results obtained in that study prompted us to prepare a new series of analogues including fluorine at position 4 of 6-phenyl in imidazo-[2,1-b]-1,3,4-thiadiazole (IV).



I

Levamisole



II

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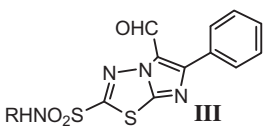
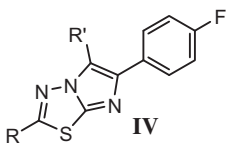


III

5-formyl-6-aryl-imidazo[2,1-b][1,3,4]thiadiazole sulfonamide



IV

6-(4'-fluorophenyl) imidazo[2,1-b][1,3,4]thiadiazoles (SCR1-SCR4)

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Previously, we have reported purification and synthesis of many natural and synthetic compounds, respectively. These were also studied for their biological activity and were shown to possess different levels of cytotoxicity on cancer cells [4–17]. In the present study, we synthesized, characterized and evaluated biological activity of new analogues of levamisole (I) with substitution in the aralkyl group and on fused imidazo[2,1-b][1,3,4]thiadiazole ring with same chemical backbone but different side chains namely 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazoles (**SCR1**), 2-aralkyl-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR2**), 2-aralkyl-5-formyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR3**) and 2-aralkyl-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]-thiadiazoles (**SCR4**). Based on our studies one of the molecules, **4a** was identified as the most promising lead compound.

2. Chemistry

We have synthesized series of 14 derivatives of imidazo[2,1-b][1,3,4]thiadiazoles containing aralkyl group at 2nd position by reacting 2-amino-5-aralkyl-1,3,4-thiadiazoles **X** with 4-fluoro phenacyl bromide as depicted in Scheme 1. Further compounds **SCR2**, **SCR3** and **SCR4** were obtained by bromination, formylation and thiocyanation respectively (Table 1). Structures of the synthesized compounds were established on the basis of IR, ^{1}H NMR and mass spectra analysis (Supplementary Figs. 1–3). All synthesized compounds showed absorption bands ranging from 3288 to 3025 cm^{-1} for C–H aromatic stretching, 2980 to 2840 cm^{-1} for C–H aliphatic stretching. Compounds **3a** and **3b** showed vibration bands at 1653–1676 cm^{-1} for C=O stretching. Compounds **4a**–**4c** showed vibration bands at 2163–2159 cm^{-1} for SCN in their respective IR spectra. Rf values of all the compounds were determined (Supplementary Table 1)

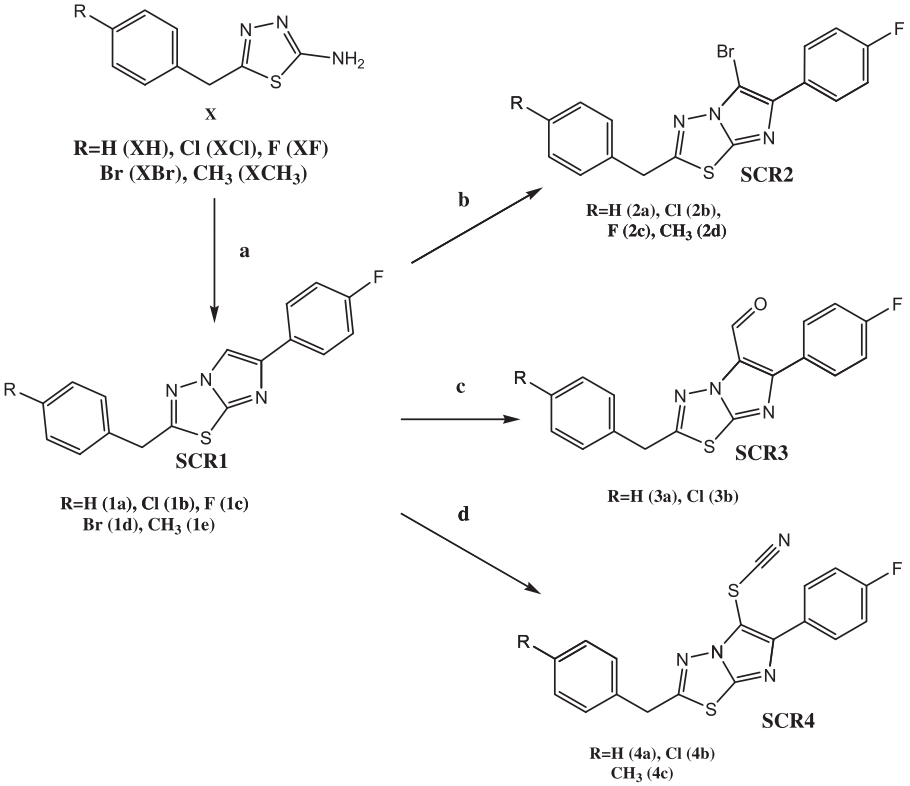
In ^{1}H NMR, the presence of singlet between δ 7.83 and 8.65 ppm for imidazole proton (C_5–H) confirmed the cyclization of **X** with 4-fluoro phenacyl bromide. All the electrophilic substitution reactions carried out on imidazo[2,1-b][1,3,4]thiadiazole derivatives (**SCR1**) and afforded the expected 5-substituted derivatives (**SCR2**, **SCR3**, and **SCR4**). All these 5-substituted derivatives showed the absence of C_5–H in their respective spectra, confirming the substitution at 5th position. Compounds **3a** and **3b** showed a singlet between δ 9.98 to 9.96 ppm for CHO proton respectively. All the compounds showed prominent signals for aromatic protons around δ 7.00–8.05 ppm. Methylene proton at C_2 appeared between δ 4.21–4.58 ppm for all synthesized derivatives. Compounds **1e**, **2d** and **4c** showed a singlet at δ 2.29 ppm for presence of methyl proton on phenyl ring. The structures of all the compounds were finally ascertained by mass spectra analysis (Table 1).

3. Pharmacology

The human T-cell leukemia cells, CEM were used for the study of preliminary anti-cancer screening of newly synthesized compounds. To assess the cytotoxicity, we used trypan blue dye exclusion and MTT assays. To test this, cells growing in log phase were treated with different concentrations of **SCR1** (**1a**–**1e**), **SCR2** (**2a**–**2d**), **SCR3** (**3a** and **3b**) and **SCR4** (**4a**–**4c**)). Further studies on lead cytotoxic compound, **4a**, in CEM cells were assessed by tritiated thymidine incorporation, cell cycle analysis, measurement of mitochondrial transmembrane potential ($\Delta\psi m$) and DNA fragmentation assays.

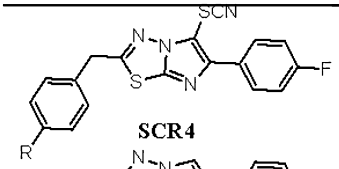
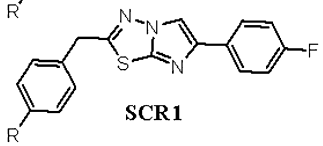
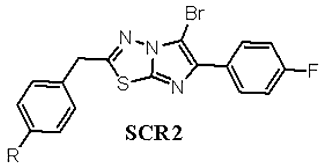
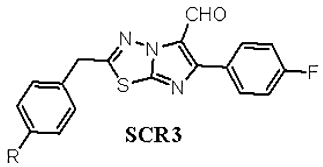
4. Results and discussion

In the present study we have investigated the cytotoxic effect of **1a**, **1b**,**1c**, **1d**, **1e** (**1a**–**1e**), **2a**, **2b**, **2c**, **2d** (**2a**–**2d**), **3a**, **3b** (**3a** and **3b**), **4a**, **4b**, **4c** (**4a**–**4c**) on T-cell leukemic cell line, CEM (Table 1). In order to estimate the effect of compounds, CEM cells were treated with increasing concentrations of compounds (10, 50, 100 and 250 μM)



Scheme 1. Reagents and conditions: (a) (i) F–C6H4–COCH2Br, EtOH, (ii) Na_2CO_3; (b) Br2, gl. acetic acid; (c) (i) POCl3, DMF, 80–90 °C, (ii) Na2CO3; (d) KSCN, gl. acetic acid, Br2 in gl. acetic acid, 0–5 °C.

Table 1
Structure and molecular weight of novel imidazo[2,1-b]thiazole derivatives of Levamisole, 2-aralkyl-6-(4′-flurophenyl)-imidazo[2,1-b][1,3,4]thiadiazoles (**SCR1**), bromo (**SCR2**), formyl (**SCR3**) and thiocynate (**SCR4**) derivatives.

Structure	R	Code no.	Molecular formula	Molecular weight	Melting point (°C)
 **SCR4**	H	**1a**	$C_{17}H_{12}FN_3S$	309.40	152–155
	Cl	**1b**	$C_{17}H_{11}ClFN_3S$	343.81	167–170
	F	**1c**	$C_{17}H_{11}F_2N_3S$	327.35	149–152
	Br	**1d**	$C_{17}H_{11}BrFN_3S$	388.26	118–120
	CH_3	**1e**	$C_{18}H_{14}FN_3S$	323.39	149–150
 **SCR1**	H	**2a**	$C_{18}H_{11}BrFN_3S$	338.30	122–125
	Cl	**2b**	$C_{17}H_{10}BrClFN_3S$	422.70	155–157
	F	**2c**	$C_{17}H_{10}BrFN_3S$	406.25	89–90
	CH_3	**2d**	$C_{18}H_{13}BrFN_3S$	402.28	92–95
 **SCR2**	H	**3a**	$C_{18}H_{12}FN_3OS$	337.40	112–114
	Cl	**3b**	$C_{18}H_{11}ClFN_3OS$	371.82	138–140
 **SCR3**	H	**4a**	$C_{18}H_{11}FN_4S_2$	366.40	133–135
	Cl	**4b**	$C_{18}H_{10}ClFN_4S_2$	371.82	148–150
	CH_3	**4c**	$C_{19}H_{13}FN_4S_2$	380.46	100–102

and cell viability was determined by trypan blue assay (Fig. 1, Supplementary Fig. 8). The 5-fluorouracil treated cells were used as positive control. Since the compounds were dissolved in DMSO, cells treated with DMSO alone served as vehicle control. The cells were counted at intervals of every 24 h, till they attained stationary phase and the data was plotted. Results showed that addition of compound to the CEM cells affected viability of cells in a dose-dependent manner, in the case of almost every compound (Fig. 1, Supplementary Fig. 8). However, the levels of sensitivity differed drastically among compounds. **3a**, **4a** and **4c** induced maximum toxicity on leukemic cells (Fig. 1), while the effect was moderate in the case of **1a**, **1c**, **2c**, **3b** and **4b** (Table 2, Supplementary Fig. 8). However, the compounds **1b**, **1d**, **1e**, **2a** and **2b** were least sensitive (Table 2, Supplementary Fig. 8). IC_{50} value of each compound was calculated for 48 and 72 h based on trypan blue assay (Table 2).

The effect of **1a**–**1e**, **2a**–**2d**, **3a** and **3b**, and **4a**–**4c** on cell proliferation was further tested using MTT assay. CEM cells treated with 10, 50, 100 and 250 μM of compounds were harvested after 24, 48 and 72 h and subjected to MTT assay. Results suggested that **4a** treatment lead to the reduction in cell proliferation in a dose-dependent manner (Fig. 2, Supplementary Fig. 9). Data from MTT assay in conjunction with trypan blue assay suggest that **4a** showed maximum cytotoxicity with an IC_{50} value of ~8 μM and hence it was selected as the lead compound (Table 2).

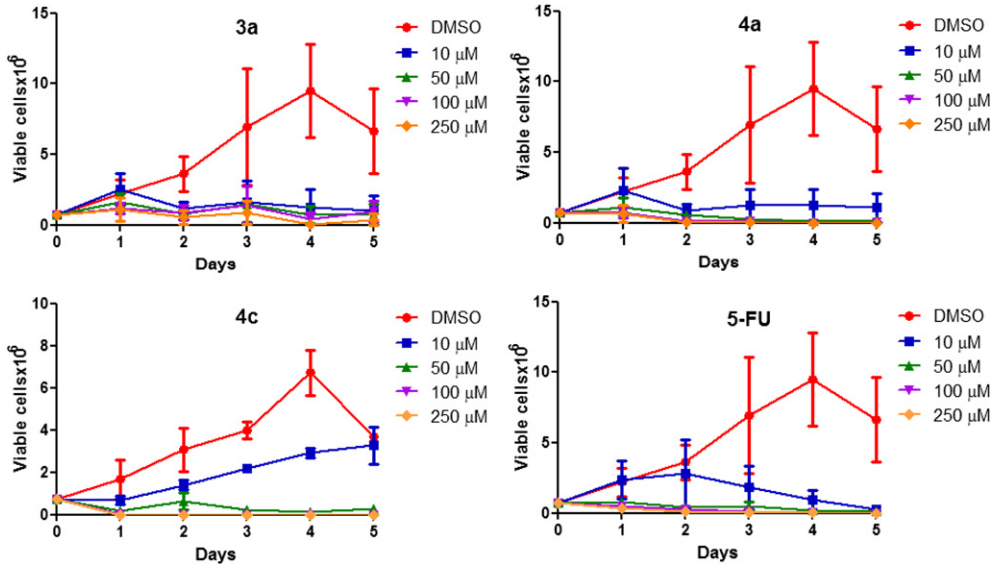


Fig. 1. Dose- and time-dependent effects of **3a**, **4a** and **4c** on cell viability in T-cell leukemic cells. CEM cells were treated with the compounds (10, 50, 100 and 250 μM) and cell viability was determined by Trypan blue assay. 5-flurouracil was used as the positive control. DMSO treated cells were used as vehicle control. The data shown is derived from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
IC50 value of **1a**–**4a** and 5-FU was calculated based on Trypan blue and MTT assay at 48 h and 72 h in CEM cell lines.

Compounds	IC_{50} value (μM)			
	Trypan blue assay		MTT assay	
	48 h	72 h	48 h	72 h
1a	75	60	60	50
1b	220	100	>250	>250
1c	50	100	110	120
1d	100	100	220	>250
1e	160	100	>250	>250
2a	200	100	>250	>250
2b	240	120	>250	>250
2c	58	40	80	60
2d	40	52	90	80
3a	20	16	35	26
3b	90	80	105	110
4a	10	10	8	8
4b	75	40	50	40
4c	18	14	10	10
5-FU	15	8	40	30

Based on above results, we were interested in determining whether the observed cytotoxicity by **4a** was due to effect of **4a** on cell division. In order to test this, CEM cells were cultured in presence of **4a** (10 μM) and [^{3}H] thymidine. Incorporation of tritiated thymidine to the DNA was determined following 12, 24 and 48 h. Data showed reduction in the incorporation in a dose-dependent manner (Fig. 3). These results suggest that the **4a** reduced incorporation of [^{3}H] thymidine into DNA of dividing cells at both 24 and 48 h treatment, which indicates that **4a** inhibits DNA replication and can also induce apoptosis.

Based on the above studies, we wondered whether the effect of **4a** on cell division could lead to cell cycle arrest. In order to test this, CEM cells cultured with **4a** (1, 5, 10 and 20 μM) for 24 and 48 h were stained with propidium iodide and subjected to flow cytometry. Results showed no arrest in cell cycle progression during the time period analyzed (Fig. 4). However, we could observe the accumulation of cells at sub G1 phase in a concentration- and time-dependent manner (Fig. 4). Thus, the observed accumulation of cells in sub G1 peak indicated cell death mediated by apoptosis. Therefore, our results suggest that **4a** induces apoptosis, but not cell cycle arrest.

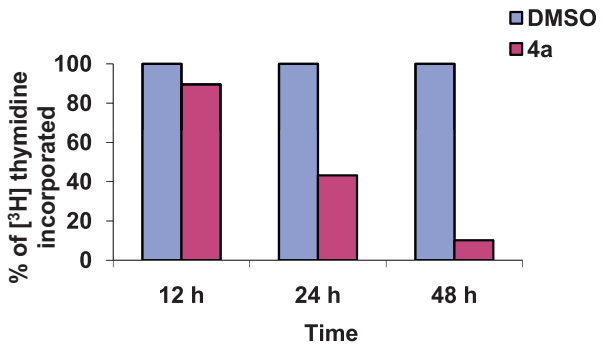


Fig. 3. Tritiated thymidine assay to determine the effect of **4a** on cell proliferation. **4a** treated (10 μM, for 12, 24 and 48 h) CEM cells were subjected to tritiated thymidine assay. DMSO treated cells were used as control. Radioactive counts in each sample was determined and presented as bar diagram.

The mitochondrial transmembrane potential is an important step in the induction of cellular apoptosis. During apoptosis, the electrochemical gradient ($\Delta\Psi$) across the mitochondrial membrane collapses. To test whether **4a** affected the mitochondrial membrane potential, CEM cells treated with the compound (1, 5, 10 and 20 μM) for 48 h, were subjected to JC-1 dye staining and FACS analysis. In healthy non-apoptotic cells, the dye stains the mitochondria and gives bright red fluorescence. However, in apoptotic cells, the mitochondrial membrane potential collapses and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm resulting in a green fluorescent monomeric form. The aggregate red form has absorption/emission maxima of 585/590 nm and green monomeric form has absorption/emission maxima of 510/527 nm. Results showed that mostly DMSO treated cells showed red fluorescence while **4a** treated cells showed green fluorescence in a concentration dependent manner (Fig. 5) similar to positive control, 2,4-DNP. Thus, our results indicate that **4a** altered mitochondrial membrane potential in dose-dependent manner resulting in cytosolic accumulation of monomeric JC-1, which further suggests the activation of apoptosis.

Since **4a** could induce apoptosis, DNA fragmentation assay was performed to detect the DNA double-strand breaks. Multiple DNA breaks on genomic DNA can lead to smearing on an agarose gel.

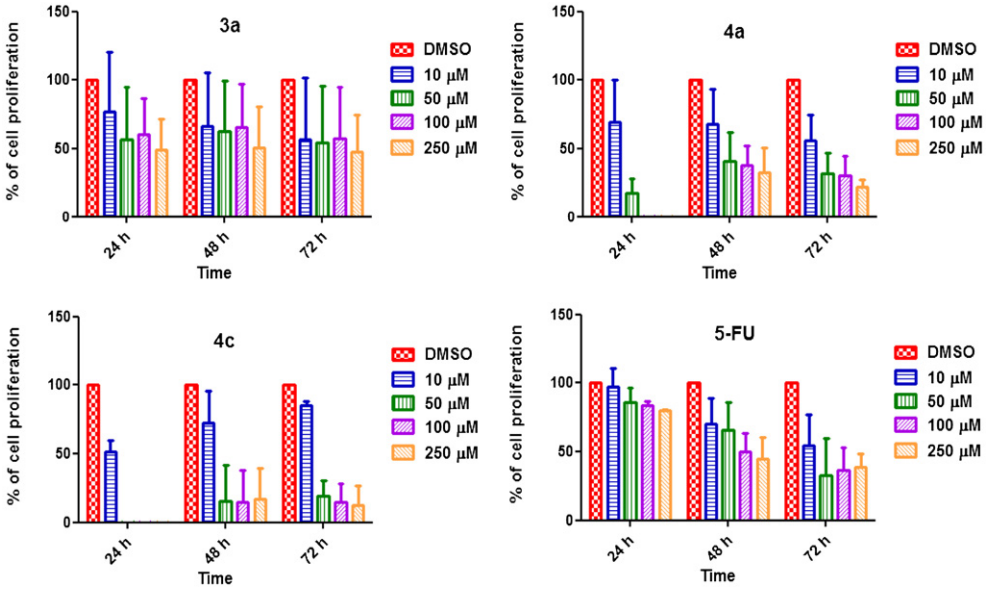


Fig. 2. Determination of effect of **3a**, **4a** and **4c** on proliferation of CEM cells. Cells treated with the compounds (10, 50, 100 and 250 μM for 24, 48 and 72 h) were subjected to MTT assay and results were expressed as percentage of cell proliferation.

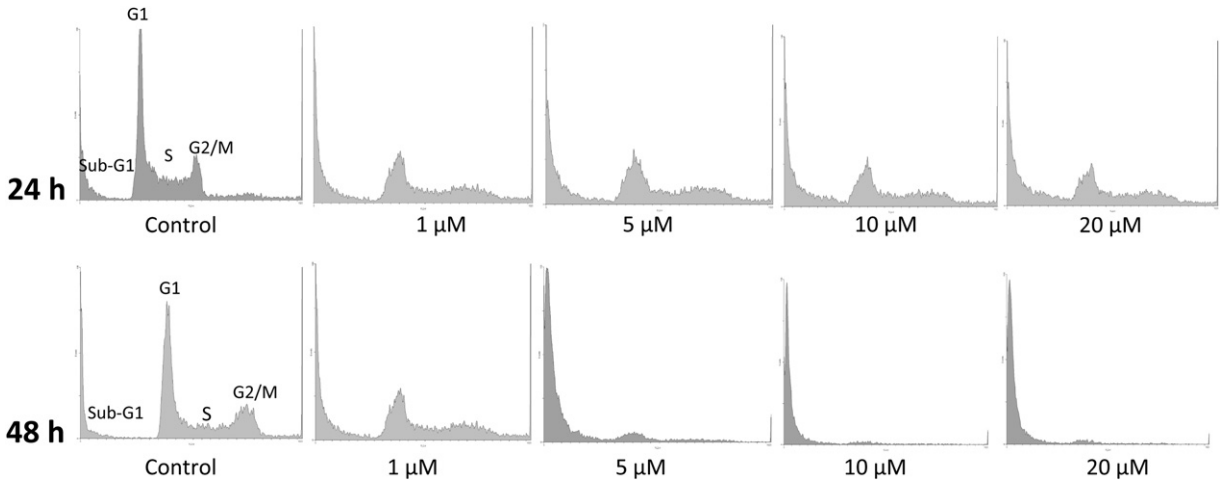


Fig. 4. Analysis of cell cycle progression followed by **4a** treatment. CEM cells treated with **4a** (1, 5, 10 and 20 μM) for 24 and 48 h, were harvested, stained with propidium iodide and DNA content of 10, 000 cells were analyzed by flow cytometry. Histogram representing cell population of different phases of cell cycle (sub G1, G1, S and G_0/M) is represented.

CEM cells treated with different concentrations of **4a** (1, 5, 10 and 20 μM) were harvested using nondenaturing method for 48 h and subjected to gel electrophoresis. Results showed an increase in DNA fragmentation with increase in concentration of the compound (Fig. 6). Therefore, different lines of evidences suggest that **4a** induces cytotoxicity by activating apoptosis.

Based on the IC_{50} values of the molecules studied (Table 2), it appears that when the substituents at the 5th position of imidazo [2,1-b][1,3,4]thiadiazole group are thiocyanate (**4a** and **4c**) and formyl (**3a**) groups, the compounds exhibit maximum cytotoxicity. However, the introduction of bromine or hydrogen substituents results in lesser cytotoxicity.

5. Conclusion

In the present study we have synthesized 14 novel 2-aralkyl-5-substituted-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivatives, which showed strong and moderate cytotoxicity on leukemia cell line, CEM. The derivates **3a**, and **4a** with formyl (−CHO) and thiocyanate (−SCN) substitutions at 5th position showed highest cytotoxicity. Another derivative, **4c** with thiocyanate (−SCN) and methyl ($-CH_3$) substitution on imidazo[2,1-b] [1,3,4]thiadiazole at 5th and 4th positions of benzyl group, respectively also showed higher effect on cell viability. Based on IC_{50} values, **4a** (8 μM) was selected as the lead compound and its

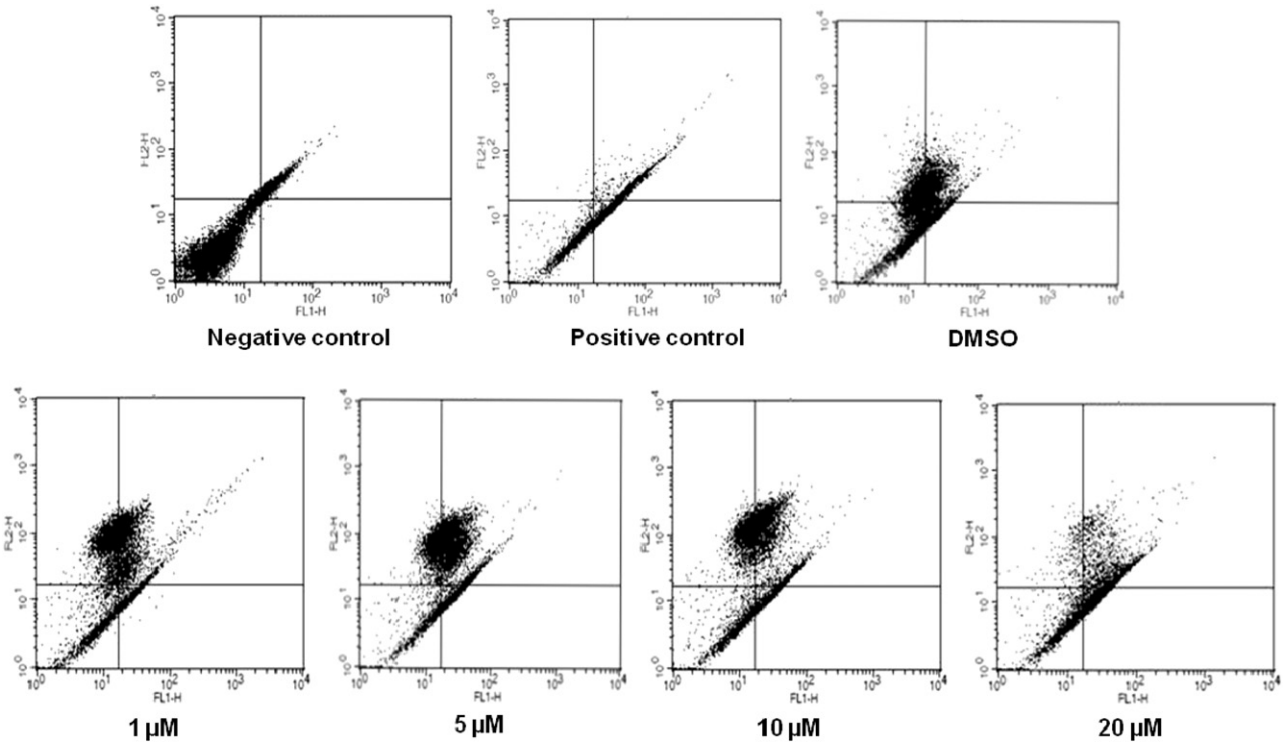


Fig. 5. Detection of loss of mitochondrial membrane potential followed by **4a** treatment. CEM cells were treated with **4a** (1, 5, 10 and 20 μM) for 48 h and analyzed by flow cytometry following staining with JC-1 dye. 2,4-DNP treated CEM cells were used as positive control.

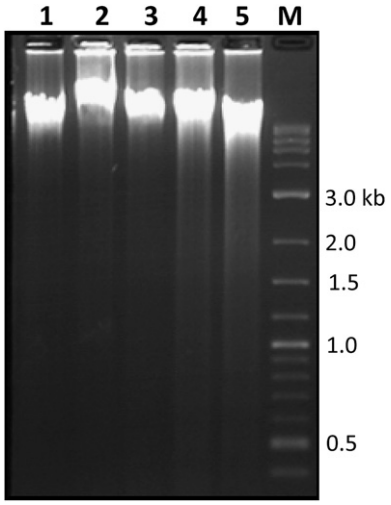


Fig. 6. Detection of DNA breaks induced by **4a** in CEM cells. The genomic DNA was extracted from CEM cells following treatment with **4a** (1, 5, 10 and 20 μM) for 48 h and resolved on an agarose gel. DNA from DMSO treated cells served as vehicle control.

biological activity was tested. Initial studies indicate that **4a** induced cytotoxicity is mediated through apoptosis; however, it does not affect cell cycle progression.

6. Experimental protocols

6.1. Chemicals and reagents

All the chemicals used in the present study were analytical grade and purchased from Sigma–Aldrich, USA. Tritiated thymidine ([^{3}H]) was purchased from BARC, India and antibodies were purchased from Santa Cruz Biotechnology, USA. The IR spectra were recorded in KBr on a Jasco 430+; the ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker (400 Mz), and *J* values are reported in hertz (Hz), 2-amino-5-benzyl-1,3,4-thiadiazole (**XH**) was prepared according to literature [18]. The melting points are uncorrected. Silica gel plates were used for the TLC by using $CHCl_3$: MeOH.

6.2. Chemistry

2-amino-5-aralkyl-1,3,4-thiadiazole **X** was prepared from aryl acetic acid and thiosemicarbazide in presence of H_2SO_4 at 70 °C for 7 h. The 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazoles **SCR1** reported in Scheme 1 was prepared by reaction of 2-amino-5-aralkyl-1,3,4-thiadiazole **X** with the 4-fluoro phenacyl bromide, and neutralization with cold aqueous sodium carbonate to get the free base in 50–60% yield. The 4-fluoro phenacyl bromide was prepared by bromination of the 4-fluoro acetophenone using the Friedel–Crafts reaction [19]. The 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivative, **SCR1**, thus obtained was subjected to electrophilic substitution reaction [20] at the 5th position with bromine in the presence of CH_3COONa in acetic acid to obtain the 5-bromo derivative, **SCR2** in good yield. Appropriate aldehyde, **SCR3** were obtained in turn by means of the Vilsmeier reaction on the corresponding 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole, **SCR1**. Introduction of thiocyanate functional group at the 5th position was carried out by reaction between 2-phenyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole, **SCR1** and potassium thiocyanate in glacial acetic acid by drop wise addition of bromine in glacial acetic acid to get **SCR4** in good yield.

6.2.1. General procedure for preparation of X

The mixture of 4-substituted/phenyl acetic acid (0.1 M) and thiosemicarbazide (0.15 M) was added slowly to the round bottom flask containing concentrated H_2SO_4 (30 ml) with constant stirring in ice bath. After complete addition, ice bath was replaced by water bath and slowly heated to 70–80 °C and maintained at that temperature for 7 h. After cooling to room temperature, the contents of the reaction were poured into ice water and made basic with ammonia and the precipitate was filtered, washed with water and recrystallized from ethanol.

6.2.2. 2-Amino-5-(4-chlorobenzyl)-1,3,4-thiadiazole (*XCl*)

Yield 50%. M P. 181–182 °C FT IR (λ cm^{-1}) 3262, 3100, 2971, 2915, 1698, 1519, 1491, 1332, 1091, 1016. ^{1}H NMR δ: 4.30 (s, 2H. $-CH_2-$), 7.06 (s, 2H, $-NH_2$), 7.55 (d, 2H, $J = 8$ Hz), 8.20 (d, 2H, $J = 8$ Hz).

6.2.3. 2-Amino-5-(4-fluorobenzyl)-1,3,4-thiadiazole (*XF*)

Yield 47%. M P. 205–207 °C. FT IR (λ cm^{-1}) 3265, 3107, 2950, 1685, 1515, 1334, 1232, 1148, 1092, 1052. ^{1}H NMR δ: 4.14 (s, 2H. $-CH_2-$), 7.04 (s, 2H, $-NH_2$), 7.13–7.18 (m, 2H, ar), 7.30–7.34 (m, 2H, ar).

6.2.4. 2-Amino-5-(4-bromobenzyl)-1,3,4-thiadiazole (*XBr*)

Yield 55%. M P. 200–202 °C. FT IR (λ cm^{-1}) 3395, 3275, 2986, 2948, 1598, 1511, 1349, 1305, 1047, 1012. ^{1}H NMR δ: 4.14 (s, 2H. $-CH_2-$), 7.05 (s, 2H, $-NH_2$), 7.25 (d, 2H, $J = 8$ Hz), 7.53 (d, 2H, $J = 8$ Hz).

6.2.5. 2-Amino-5-(4-methylbenzyl)-1,3,4-thiadiazole (*XCH$_3$*)

Yield 52%. M P. 208–212 °C. FT IR (λ cm^{-1}) 3396, 3270, 3126, 2922, 1605, 1516, 1430, 1342, 1147, 1048, 906. ^{1}H NMR δ: 2.27 (s, 3H, CH_3), 4.08 (s, 2H. $-CH_2-$), 7.03 (s, 2H, $-NH_2$), 7.14 (s, 4H, ar).

6.2.6. Synthesis of the 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivatives (*SCR1*)

The appropriate 1,3,4-thiadiazole **X** (30 m mol) was treated with the 4-fluoro phenacyl bromide (32 m mol) in ethanol (150 ml). The mixture was refluxed for 10–12 h. Excess of solvent was removed under reduced pressure and the solid hydrobromide separated was filtered, washed with cold ethanol and dried. Neutralization of hydrobromide salts with cold aqueous solution of sodium carbonate yielded the corresponding free base, which was filtered, with a yield of 50–60%.

*6.2.6.1. 2-Benzyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**1a**).* Yield 52%. FTIR (λ cm^{-1}): 3136, 3065, 2923, 1598, 1470, 1422, 1222. ^{1}H NMR δ: 4.21 (2H, s, CH_2), 7.00 (2H, t, $J = 16$), 7.23–7.32 (5H, m, ar), 7.68 (2H, q, $J = 12$), 7.83 (IH, s, im). +ESI: 310 (309.36).

*6.2.6.2. 2-(4-Chlorobenzyl)-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**1b**).* Yield 55%. FTIR (λ cm^{-1}): 3115, 3042, 2917, 2882, 1537, 1483, 1413, 1227, 1092. ^{1}H NMR δ: 4.27 (2H, s, CH_2), 7.07–7.12 (2H, m, ar), 7.25–7.27 (2H, m, ar), 7.34–7.37 (2H, m, ar), 7.73–7.78 (2H, m, ar) 7.91 (1H, s, im). +ESI: 344 (343.81).

*6.2.6.3. 2-(4-Fluorobenzyl)-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**1c**).* Yield 50%. FTIR (λ cm^{-1}): 3126, 3047, 2916, 1601, 1515, 1478, 1229, 1150. ^{1}H NMR δ: 4.45 (2H, s, CH_2), 7.20–7.26 (4H, m, ar), 7.45–7.48 (2H, m, ar), 7.86–7.89 (2H, m, ar), 8.65 (1H, s, im). +ESI: 328 (327.35).

*6.2.6.4. 2-(4-Bromobenzyl)-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**1d**).* Yield 57%. FTIR (λ cm^{-1}): 3141, 3066, 2943, 2879, 1535, 1479, 1227, 1011. ^{1}H NMR δ: 4.43 (2H, s, CH_2), 7.20–7.25 (2H, m, ar), 7.36 (2H, d, $J = 8$), 7.56 (2H, d, $J = 8$), 7.84–7.88 (2H, m, ar), 8.61 (1H, s, im). +ESI: 389.9 (388.26).

*6.2.6.5. 2-(4-Methylbenzyl)-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**1e**).* Yield 60%. FTIR (λ cm^{-1}): 3110, 3025, 2909, 1522, 1478, 1228, 1055. ^{1}H NMR δ: 2.29 (3H, s, CH_3), 4.37 (2H, s, CH_2), 7.17–7.24 (4H, m, ar), 7.26 (2H, d, $J = 8$), 7.84–7.88 (2H, m, ar), 8.60 (1H, s, im). +ESI: 324 (323.39).

*6.2.7. Synthesis of the 2-aralkyl-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivatives (**SCR2**)*

2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole **SCR1** (5 mmol), sodium acetate (10 mmol) and 10 ml of glacial acetic acid (stirred together at room temperature) was added drop wise 0.98 g of bromine (6 mmol). After the addition, stirring was continued for 30 min. The mixture was poured into 100 ml water from which a solid separated. The solid was collected by filtration and washed with water, dried and recrystallized from EtOH–$CHCl_3$ with a yield of 65–75%.

*6.2.7.1. 2-Benzyl-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**2a**).* Yield 68%. FTIR (λ cm^{-1}): 3140, 3055, 2933, 1600, 1468, 1412 and 1224. ^{1}H NMR δ: 4.35 (2H, s, CH_2), 7.12 (2H, t, $J = 16$), 7.32–7.41 (5H, m, ar), 7.96 (2H, m, ar).+ESI: 389 (388.26).

*6.2.7.2. 2-(4-Chlorobenzyl)-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**2b**).* Yield 75%. FTIR (λ cm^{-1}): 3063, 2923, 2853, 1601, 1528, 1481, 1226, 1092. ^{1}H NMR δ: 4.50 (2H, s, CH_2), 7.28–7.33 (2H, m, ar), 7.45 (4H, s, ar), 7.95–7.98 (2H, m, ar). +ESI: 423.9 (422.7).

*6.2.7.3. 2-(4-Fluorobenzyl)-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**2c**).* Yield 65%. FTIR (λ cm^{-1}): 3066, 2938, 1602, 1527, 1488, 1224, 1100, 1018. ^{1}H NMR δ: 4.49 (2H, s, CH_2), 7.19–7.23 (2H, m, ar), 7.28–7.33 (2H, m, ar), 7.45–7.48 (2H, m, ar), 7.95–7.98 (2H, m, ar). +ESI: 407.9 (406.25).

*6.2.7.4. 2-(4-Methylbenzyl)-5-bromo-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**2d**).* Yield 67%. FTIR (λ cm^{-1}): 3288, 3034, 2922, 1525, 1477, 1227. ^{1}H NMR δ: 2.29 (3H, s, CH_3), 4.43 (2H, s, CH_2), 7.18 (2H, d, $J = 8$), 7.28–7.33 (4H, m, ar), 7.94–7.98 (2H, m, ar). +ESI: 403.2 (402.28).

*6.2.8. Synthesis of the 2-aralkyl-5-formyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole derivatives (**SCR3**)*

The Vilsmeier reagent was prepared at 0–5 °C by dropping $POCl_3$ (2.3 g, 15 mmol) into a stirred solution of DMF (10 ml). The 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole **SCR1** (4 mmol) was added slowly to the Vilsmeier reagent while maintaining stirring and cooling for 2 h. Further stirring was continued for 6 h at 80–90 °C. The resulting reaction mixture was poured into 100 ml of water; the precipitate was filtered, pressed, suspended in water and neutralized to pH 7 with cold aqueous solution of sodium carbonate. The solid that separated was filtered, washed with water, dried and recrystallized from EtOH–DMF with a yield 55–60%.

*6.2.8.1. 2-Benzyl-5-formyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**3a**).* Yield 55%. FTIR (λ cm^{-1}): 3081, 3028, 2951, 2864, 2777, 1676, 1595, 1522, 1482, 1315. ^{1}H NMR δ: 4.56 (2H, s, CH_2), 7.32–7.45 (7H, m, ar), 8.01–8.05 (2H, m, ar), 9.98 (1H, s, CHO). +ESI: 338 (337.37).

*6.2.8.2. 2-(4-Chlorobenzyl)-5-formyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**3b**).* Yield 60%. FTIR (λ cm^{-1}): 3053, 2980, 2916, 2840, 1653, 1604, 1492, 1388, 1230, 1090. ^{1}H NMR δ: 4.56 (2H, s, CH_2), 7.32–7.36 (2H, m, ar), 7.45 (4H, s, ar), 7.99–8.03 (2H, m, ar), 9.96 (1H, s, CHO). -ESI: 370 (371.82).

*6.2.9. Synthesis of the 2-aralkyl-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4] thiadiazole derivatives (**SCR4**)*

To a mixture of 2-aralkyl-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazoles **SCR1** (10 mmol) and potassium thiocyanate (1.56 g, 16 mmol) in glacial acetic acid (50 ml) was added bromine (1.6 g, 10 mmol) in glacial acetic acid (20 ml) at 0–5 °C drop wise with stirring. Stirring was continued for 30 min at 15–20 °C and then at room temperature for 1 h. The reaction mixture was poured into ice water, filtered, washed with water and recrystallized from EtOH–$CHCl_3$ with a yield of 55–65%.

*6.2.9.1. 2-Benzyl-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole (**4a**).* Yield 62%. FTIR (λ cm^{-1}): 3089, 3068, 2917, 2159, 1604, 1475 and 1216. ^{1}H NMR δ: 4.41 (2H, s, CH_2), 7.19 (2H, t, $J = 16$), 7.35–7.41 (5H, m, ar), 7.95 (2H, dd, $J = 8, 8$). +ESI: 367 (366.44)

*6.2.9.2. 2-(4-Chlorobenzyl)-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole(**4b**).* Yiled 65%. FTIR (λ cm^{-1}): 3072, 2921, 2854, 2163, 1602, 1527, 1479, 1227, 1095. ^{1}H NMR δ: 4.58 (2H, s, CH_2), 7.36–7.40 (2H, m, ar), 7.47 (4H, m, ar), 7.98–8.08 (2H, m, ar). -ESI: 398.9 (400.88).

*6.2.9.3. 2-(4-Methylbenzyl)-5-thiocyanato-6-(4′-fluorophenyl)-imidazo[2,1-b][1,3,4]thiadiazole(**4c**).* Yield 55%. FTIR (λ cm^{-1}): 3059, 3032, 2920, 2869, 2163, 1603, 1520, 1468, 1228. ^{1}H NMR δ: 2.29 (3H, s, CH_3), 4.50 (2H, s, CH_2), 7.19 (2H, d, $J = 8$), 7.30 (2H, d, $J = 8$), 7.36–7.40 (2H, m, ar), 7.97–8.01 (2H, m, ar). +ESI: 381 (380.46).

6.3. Biology

6.3.1. Cell lines and culture conditions

Human T-cell leukemia cell line, CEM was used for the present study. CEM cell line was purchased from National Center for Cell Science, Pune, India. Cells were cultured in RPMI1640 (SERA LAB, UK) containing 10% FBS (GIBCO BRL, USA), 100 U of Penicillin G/ml and 100 μg of streptomycin/ml (Sigma–Aldrich, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

6.3.2. Trypan blue dye exclusion assay

The effect of **1a**–**1e**, **2a**–**2d**, **3a** and **3b** and **4a**–**4c** on cell viability of T-cell leukemic cells was determined by Trypan blue dye exclusion assay [4,21]. CEM cells were seeded at a density of 1×10^5 cells/ml, cultured for 24 h and compounds were added at a concentration of 10, 50, 100 and 250 μM. DMSO (Sigma–Aldrich, USA) treated cells were used as vehicle control. 5-fluorouracil treated cells were used as positive control. Cells were harvested at intervals of 24 h and resuspended in 0.4% Trypan blue (Sigma–Aldrich, USA) and the viable cells were counted using hemocytometer. IC_{50} values (50% inhibition of cell growth) were estimated after 48 and 72 h of treatment. Experiment was repeated atleast 3 times and the values obtained were plotted against respective time points.

6.3.3. MTT assay

The effect of **1a**–**1e**, **2a**–**2d**, **3a** and **3b** and **4a**–**4c** on cell proliferation of CEM cells was determined by MTT assay as described previously as in References [15,22]. In brief, cells were treated with increasing concentrations of compounds (10, 50, 100 and 250 μM) and incubated for 24, 48 and 72 h. MTT reagent (5 mg/ml, Sigma–Aldrich, USA) was added and the color change due to proliferating cells was estimated. 5-fluorouracil treated cells were used as positive control while DMSO treated cells were used as vehicle control. Each experiment was repeated a minimum of 3 independent times and the values obtained were plotted.

6.3.4. Tritiated thymidine incorporation assay

DNA synthesis was monitored by labeling cells using [^{3}H] thymidine as described [21]. The **4a** was added to CEM cells at a concentration of 10 μM after 24 h. The [^{3}H] thymidine (1 μCi) was added after 8 h of treatment and was harvested after 12, 24 and 48 h of incubation, processed and used for scintillation counting. The radioactivity count was expressed as disintegrations/min.

6.3.5. Cell cycle analysis

Cells were cultured and treated with **4a** at a concentration of 1, 5, 10 and 20 μM as described above and harvested after 24 h and 48 h of treatment. The cells were then processed as described earlier in Reference [5]. Briefly, propidium iodide stained cells were subjected to flow cytometric reading (FACScan, BD Biosciences, USA). A minimum of 10,000 cells were acquired per sample and histogram were analyzed by using WinMDI 2.8 software [15].

6.3.6. Measurement of mitochondrial membrane potential

Changes of mitochondrial transmembrane potential ($\delta\psi m$) were analyzed by flow cytometry using sensitive dye JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1, 3, 3′- tetraethylbenzimidazolcarbocyanamide iodided; Calbiochem, USA) [15,23]. Cells were cultured, treated with **4a** (1, 5, 10 and 20 μM) for 48 h, harvested, washed, resuspended in PBS and incubated with JC-1 (0.5 μM) at 37 °C for 15 min. The cells were then subjected to flow cytometric analysis, using CellQuest Pro software an excitation at 488 nm laser and emission at 530/30, 580/610 nm. JC-1 monomers emit at 530 nm (FL-1) and J-aggregates emit at 590 nm (FL-2). 10, 000 cells were acquired per sample and 2,4-dinitrophenol (2-4-DNP) was used as positive control for compensation [24].

6.3.7. DNA fragmentation assay

Fragmentation of chromosomal DNA was analyzed following treatment with **4a** (1, 5, 10 and 20 μM) for 48 h. The isolated genomic DNA was resolved on a 1% agarose gel [17] and visualized by ethidium bromide staining and analyzed.

6.3.8. Statistical analysis

Values were expressed as mean ± SEM for control and experimental samples and statistical analysis was performed by Student's *t*-test. The values were considered statistically significant, if the *p*-value was less than 0.05.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.02.064.

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