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Synthesis, structure activity relationship and mode of action of 3-substitutedphenyl-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-propenones as novel anticancer agents in human leukaemia HL-60 cells

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ABSTRACT

A novel class of 3-substitutedphenyl-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-propenones were designed, synthesized and evaluated for their antiproliferative activity against the human cancer cell lines of diverse origin. Structure activity relationship was elucidated with various substitutions on the benzene ring and these variations significantly affected the potency. Most of the twelve tested compounds inhibited the growth of aggressive cancer cell lines. Moreover, three compounds **4j**, **4k** and **4l** displayed excellent cytotoxic profile by inhibiting >90% cell proliferation in HL-60 and Caco-2 cells at 50 μM concentration. Further studies to elucidate the mode of action revealed that these three compounds induced G0/G1 cell cycle arrest and apoptosis, which was accompanied by loss of mitochondrial membrane potential, DNA fragmentation and nuclear morphology in HL-60 cells.

1. Introduction

Cancer has become the major cause of death in both developed and developing countries with the changes in the living habitat of people and environment [1]. Accordingly, much attention has been triggered throughout the world research laboratories to develop new classes of chemotherapeutic agents [2].

Chalcones or 1,3-diaryl-2-propen-1-ones are cancer-preventive food components found in human diet rich in fruits and vegetables [3]. In general, natural or synthetic chalcones, many reports have documented their biologically active properties such as anticancer [4], antibacterial [5], antimalarial [6], antileishmanial [7], antitumour [8], anti-inflammatory [9], immunomodulatory [10] and antimitotic [11]. A number of reports focused on their synthesis, bio-evaluation [12] and mechanism of action [13]. Their simple structure and the ease of preparation make chalcones as an attractive drug scaffold with important therapeutic potential [14].

On the other hand, chroman, or 3,4-dihydro-2*H*-1-benzopyran, is itself not found in nature, but the chroman unit is an important core structure in many biologically active natural products [15]. Chroman derivatives are interesting due to their significant pharmacological activities such as anticancer [16], antioxidant [17], antiestrogen [18], anticonvulsion [19], antirhinovirus [20], neuroprotection [21], etc. Due to the various biological functions associated with this skeleton, it has been frequently employed as a key scaffold in the drug research [22] and assorted pharmaceuticals [23].

The ultimate biological and pharmaceutical importance of both chromans and chalcones, incited us to envisage a possibility of pharmacomodulation, by incorporating the above two active pharmacophores in a single molecular frame work to yield a set of new chemical entities i.e., 3-substitutedphenyl-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-propenones or chromanochalcones (**4a**–**l**) and to examine their efficacy on a panel of human cancer cell lines.

To establish more advanced structure activity relationship around chromanochalcones, chroman ring A was fixed, and more diverse substituents (both electron-releasing and electron-withdrawing groups) such as methoxy, methyl, halogen, nitro and cyano groups were introduced on ring B of chalcone at different

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positions (Fig. 1) with a goal to explore whether the electron density on chalcone may influence the cytotoxicity. To assess this, the compounds were evaluated for their antiproliferative potential in various human cancer cell lines *viz*., prostate cancer PC-3, breast cancer MCF-7, human leukaemia HL-60, pancreatic cancers MIA PaCa-2 and AsPC, melonoma MDA-MB-431 and colon Caco-2 *in vitro* by means of MTT assay.

Most of the tested compounds being active cytotoxic agents, three of them that exhibit efficient antiproliferative activity in the preliminary screening were further evaluated against four reprehensive human cancer cell lines of HL-60, PC-3, MIA PaCa-2 and Caco-2. Interestingly, all the three compounds displayed substantial cytotoxicity towards the leukaemia cell line HL-60 with IC_{50} values in the range of 6–15 μM and the modality of cell death induced by these compounds was investigated in detail with HL-60 cells in terms of cell cycle analysis and mitochondrial potential assay by flow cytometry, DNA fragmentation assay on agarose gel electrophoresis and nuclear morphology under fluorescent microscope.

2. Results and discussion

2.1. Chemistry

With the objective of developing effective new anticancer agents, a series of twelve chromanochalcones (**4a**–**l**) were synthesised in quantitative yields from the commercially available 2,4-dihydroxy acetophenone **1** in two steps. In the first step, 1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-ethanone **2** was obtained by the reaction of **1** with 2-methyl-3-buten-2-ol in the presence of polyphosphoric acid (PPA) in excellent yield. Coupling of **2** with different substituted benzaldehydes **3a**–**l** was accomplished with borontrifluoride etherate in dioxane afforded the target compounds **4a**–**l** *via* Claisen Schmidt condensation in subsequent step. The synthetic steps are depicted in Scheme 1. The structures of compounds **4a**–**l** were confirmed by ^{1}H NMR, ^{13}C NMR, EI-MS, IR and elemental analyses. The configuration of compounds **4a**–**l** is of the *trans* form, as the coupling constants of two protons on vinyl bond is above 15.0 Hz evidenced in ^{1}H NMR.

2.2. Cancer biology

2.2.1. In vitro antiproliferative activity and structure activity relationship (SAR) of chromanochalcones (*4a*–*l*)

The *in vitro* cytotoxicity of all the synthesised compounds **4a**–**l** was evaluated by performing a comprehensive screen at a single dose of 50 μM in various human cancer cell lines encompassing prostate cancer cell line PC-3, breast cancer MCF-7, promyelocytic human leukaemia HL-60, pancreatic cancer cell lines MIA PaCa-2 and AsPC, melanoma MDA-MB-435 and colon cancer cell line Caco-2 by employing MTT assay with mitomycin (Miot-C)/adriamycin (Adria.)/5-fluorouracil (5-FU) as positive controls. The results are summarised in Table 1 and expressed as percentage (%) of growth inhibition.

The data shown in Table 1 demonstrate the effects of different substituents on the phenyl ring B for activity and selectivity against the different cancer cell lines. With compounds **4a**–**l**, the substitution pattern significantly affected potency against different cell lines, as evident from the percentage (%) of growth inhibition from Table 1. Precursor compound **2** was also included in the screening to allow for the direct comparison of cytotoxic effect of **4a**–**l**.

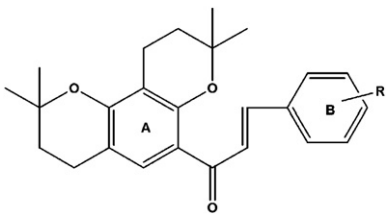


Fig. 1. Basic structure of chromanochalcones.

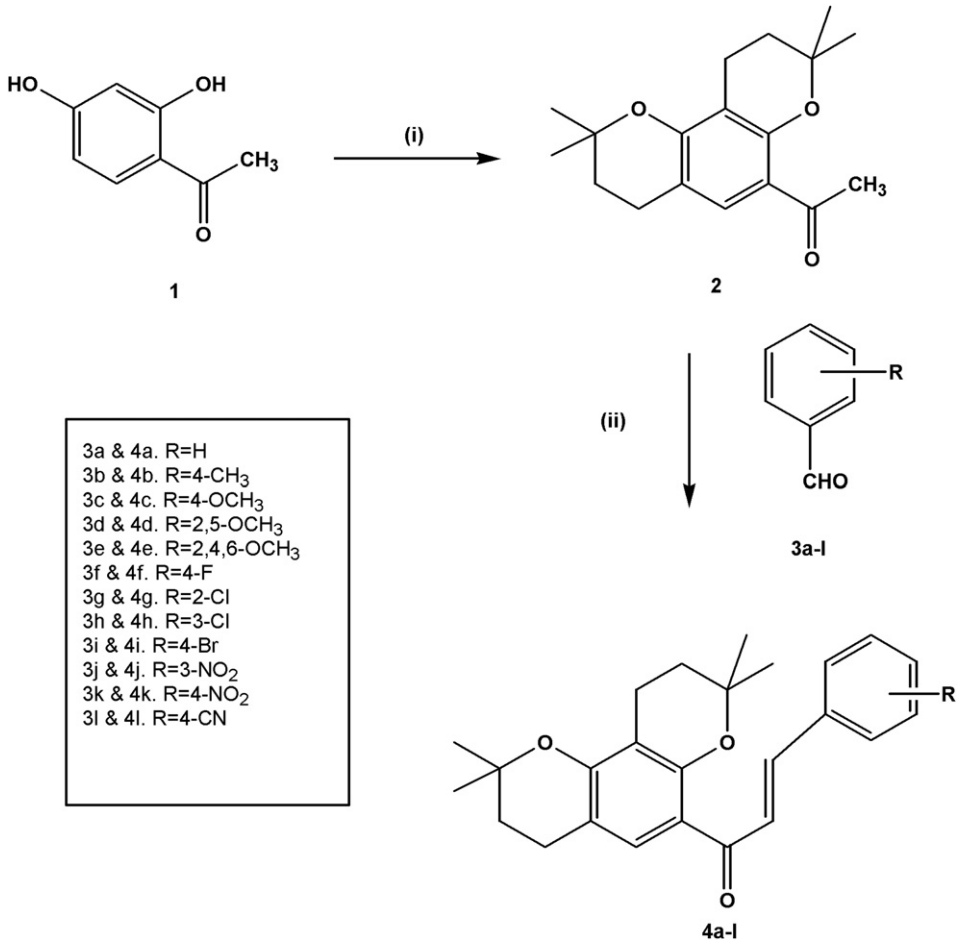
Among the precursor compound **2** and unsubstituted parent compound **4a**, it was noticeable that the introduction of the 'enone' moiety in **4a** improved the activity against all the cell lines except MCF-7 and MIA PaCa-2. Comparing the methyl derivative **4b** with the unsubstituted parent compound **4a**, the latter was more active in five (PC-3, HL-60, AsPC, MDA-MB-435 and Caco-2) of the seven cell lines examined, with the greatest difference observed in the Caco-2 cells. Turning specifically to **4c**–**e**, having electron releasing groups, the cytotoxicity was increased with the number of methoxy groups against MCF-7, HL-60 cancer cell lines and was dramatically diminished against MDA-MB-435. In some extent, the *in vitro* antiproliferative activity did not differ greatly with the number of methoxy groups and not significant. Indeed, compounds with electron withdrawing groups, **4f**–**l** showed highly variable potencies. Among the halogen derivatives (**4f**–**i**), the *meta*-chloro derivative **4h** was much more active than its *ortho*-congener **4g** except in MIA PaCa-2. *meta*-Chloro derivative **4h** has comparable antiproliferative activity with the *para*-bromo derivative **4i** against four (MCF-7, AsPC, MDA-MB-435 and Caco-2) of the seven cell lines tested. From the results, the small substituent like fluorine, seemed to have the moderate activity among the halogen derivatives. In case of the nitro substituted compounds, *para*-position is less favoured than *meta*-position. This is supported by comparing the activity of *meta* substituted compound **4j** and *para* substituted compound **4k** against all the cell lines screened. The antiproliferative activity of the *meta*-nitro derivative **4j** was not greatly different from that of the *para*-cyano derivative **4l** towards all the cell lines tested. However, **4l** is more effective among all the compounds tested (**4a**–**l**), as the cyano group is more electron withdrawing and also much more polar than the other groups. A closure look into the structure activity relationship (SAR) studies, indicated that the hydrophobic substituents such as halogens, nitro and cyano groups are better substituents to inhibit the cell proliferation and electron donating substituents are unfavourable.

Over all, it can be seen that three chalcones in particular **4j** (R = 3-NO_2), **4k** (R = 4-NO_2) and **4l** (R = 4-CN) have shown excellent cytotoxic profile (>90%) against HL-60, Caco-2 cell lines and appreciable level of cell growth inhibition (>50%) in rest of cells except pancreatic cell line AsPC (Table 1) at 50 μM concentration level. Further, the compounds that exhibited >50% inhibition were repeated screening at lower concentration (30 μM), which resulted in the identification of compounds **4j**, **4k** and **4l** are the most potent among all the compounds (**4a**–**l**). These encouraging results prompted us to determine their concentration needed for 50% of inhibition (IC_{50} value) *in vitro* against HL-60, PC-3, MIA PaCa-2 and Caco-2 cells at different concentrations between 1 and 100 μM (Fig. 2). Interestingly, compounds **4j**, **4k** and **4l** were more pronounced against HL-60 cells and resistant to pancreatic cancer cells MIA PaCa-2 in terms of their cytotoxic profile (Fig. 2).

A clear cytotoxic effect of **4j**, **4k** and **4l** was observed on cell proliferation of HL-60 cell line upon 24 h and 48 h exposure at different concentrations (Fig. 3). The IC_{50} values of these compounds for 24 h are in range of 16–25 μM which further decrease to 6–15 μM after 48 h exposure of compounds. Further experiments were aimed to examine the mechanistic details of compounds **4j**, **4k** and **4l** principally in HL-60 cells.

2.2.2. Cell cycle analysis of chromanochalcones

With a view to investigate the mode of action underlying the anti-proliferative nature of the chromanochalcones, the cell cycle



Scheme 1. Steps involved in the synthesis of **4a**–**l**. Reagents and conditions: (i) 2 methyl-3-buten-2-ol, methylene chloride, stirring, rt, 4 h. (ii) boron trifluoride etherate, dioxane, stirring, rt.

distribution of HL-60 cells was analyzed by flow cytometry. The HL-60 cells were exposed to **4j**, **4k** and **4l** compounds at 5, 10, 15 and 20 μM concentrations for 24 h, stained with propidium iodide and analyzed by Modfit software to determine the distribution of the total population in different phases (G0/G1, S, and G2/M) of cell cycle. Fig. 4 represents the percentage of HL-60 cells in each stage of the cell cycle following incubation with **4j**, **4k** and **4l** for a period of 24 h. Untreated cells used as negative control and camptothecin treated cells used as positive control (Fig. 4, Panel A). Treated HL-60 cells resulted in the accumulation of cells in the G0/G1 phase of cell cycle with a concomitant decrease in the number of cells in both the S and G2/M phases in a dose dependent manner. The

Table 1
In vitro cytotoxic profile of **4a**–**l** at a single concentration (50 μM) against different panel of human cancer cell lines.

Compound	Conc. (μM)	% Growth inhibition ± SD[a]						
		PC-3 prostate	MCF-7 breast	HL-60 leukaemia	AsPC pancreatic	MIA PaCa pancreatic	MDA-MB-43 melanoma	Caco-2 colon
2	50	19 ± 0.9	18 ± 2	25 ± 1	5 ± 1.1	25 ± 2.4	2 ± 0.6	20 ± 2
4a	50	51 ± 0.8	14 ± 3	32 ± 1.5	17 ± 0.6	24 ± 0.5	47 ± 1.1	34 ± 1
4b	50	48 ± 1.8	15 ± 1.4	18 ± 0.5	2 ± 0.7	41 ± 2	38 ± 0.7	11 ± 1.5
4c	50	63 ± 1.1	7 ± 0.7	5 ± 1	12 ± 0.9	30 ± 3	53 ± 0.6	16 ± 2
4d	50	36 ± 1.2	15 ± 1.5	27 ± 1.2	2 ± 1.7	17 ± 1.1	52 ± 0.9	23 ± 2
4e	50	62 ± 1	26 ± 1.2	77 ± 1.3	5 ± 1.2	27 ± 1.3	32 ± 1.2	17 ± 1.3
4f	50	59 ± 0.9	17 ± 1.1	38 ± 2.6	28 ± 1.2	38 ± 1	52 ± 1.1	12 ± 1.8
4g	50	52 ± 2	9 ± 0.5	41 ± 1.5	15 ± 1.3	31 ± 1.8	33 ± 2.6	11 ± 1.2
4h	50	59 ± 3	16 ± 1	60 ± 1.2	24 ± 1	24 ± 1.2	54 ± 3.4	31 ± 1.5
4i	50	33 ± 0.7	16 ± 1.3	25 ± 1	27 ± 1.3	45 ± 0.73	52 ± 1.9	28 ± 3
4j	50	72 ± 1.5	89 ± 1.3	92 ± 1.9	48 ± 1.5	80 ± 0.9	88 ± 1.2	93 ± 1.4
4k	50	61 ± 2	52 ± 2.8	91 ± 1.9	27 ± 1.2	74 ± 1.3	78 ± 1.2	73 ± 1.6
4l	50	76 ± 1.3	78 ± 1	93 ± 2	48 ± 0.8	76 ± 1.5	88 ± 0.9	93 ± 2
5-FU	10	72 ± 0.33	N.D[b]	84 ± 0.7	N.D	N.D	N.D	N.D
Mito-C	10	68 ± 0.88	N.D	N.D	N.D	N.D	N.D	N.D
Adria.	02	N.D	90 ± 3	N.D	N.D	N.D	N.D	N.D

[a] Data are presented as the mean of three independent experiments ± standard deviation.
[b] N.D not determined.

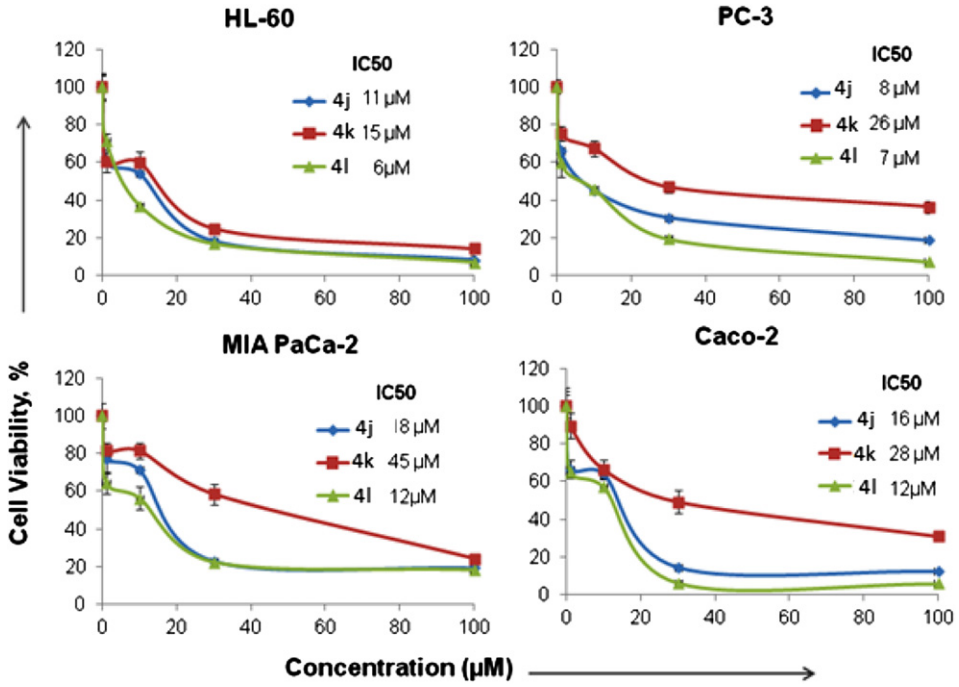
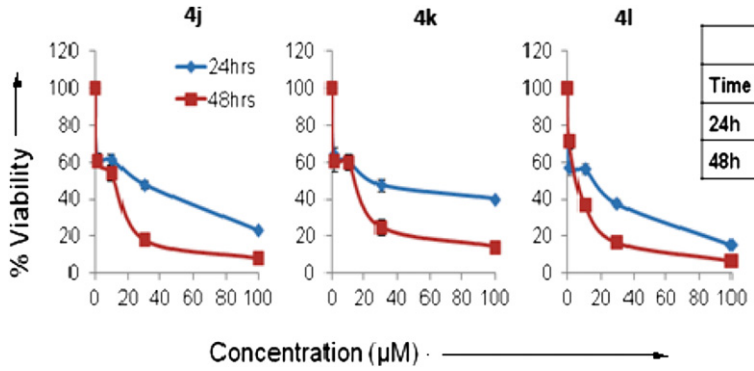


Fig. 2. Inhibition of cell proliferation by compounds **4j**, **4k** and **4l** on HL-60, PC-3, MIA PaCa-2 and Caco-2 cancer cell lines. Mitochondrial competence of MTT reduction as an index of cell viability determined. Optical density (OD) of untreated control was taken as 100% viability. IC_{50} values of the compounds are depicted in the figure. Data are presented as the mean ± SD (n = 8 wells) of three independent experiments.

population changes were observed even at the lowest concentration (5 μM) used, and doubling the concentration of chromanochalcones also doubled the sub-G0/G1 population. In particular, compound **4j** increases G0/G1 population of HL-60 cells to 86% at 20 μM concentration which is nearly equal to positive control camptothecin (88%). Sub-G0/G1 population (<2n DNA), an indicative of apoptosis also increased in **4j** in dose dependent manner up to 58% as compared to control (<1%). (Fig. 4, Panel B). While **4k** showed a less pronounced G0/G1 arrest (59%) in comparison with **4j** (86%) and **4l** (88%) in the same cell line accompanied with a significant reduction of cells in both S and G2/M phases at 20 μM concentration. The sub-G0/G1 population is 32% in **4k** at 20 μM concentration as compared to control (<1%). (Fig. 4, Panel C). Among the three tested compounds, **4l** is most potent in inducing apoptosis indicated by sub-G0/G1 fraction with 71% cells, which is more than positive control camptothecin (69%) at 20 μM concentration as compared to control (<1%) and the G0/G1 population is 88% at 20 μM as compared to control that is 48% (Fig. 4, Panel D). Thus the exposure of HL-60 cells to compounds **4j**, **4k** and **4l** leads to an accumulation of cells in G0/G1 phase with increase in hypo diploid population (sub-G0/G1) in a concentration dependent manner. It indicates that during the rise in concentration from 5 to 20 μM, an increase in the population of G0/G1 phase was associated with the population increase in the sub-G0/G1 phase, indicating that the arrested cells entered into apoptosis. However, further studies were conducted to confirm the induction of apoptosis by these novel compounds, as the presence of hypo diploid cells is not conclusive proof of apoptotic death [24].

2.2.3. DNA fragmentation

The apoptotic potential of **4j**, **4k**, and **4l** was further confirmed through induction of DNA fragmentation in HL-60 cells (Fig. 5), which is known as the biochemical hallmark of apoptosis [25]. HL-60 cells (2×10^6/3 ml/well) were treated with above compounds for



	IC50 (μM)		
Time	4j	4k	4l
24h	25	23	16
48h	11	15	6

Fig. 3. Inhibition of cell proliferation by compounds **4j**, **4k** and **4l** on human leukaemic HL-60 cell line. MTT assay was performed at various concentrations of compounds. OD of untreated control was taken as 100% viability. Data are presented as the mean ± SD (n = 8 wells) of three independent experiments.

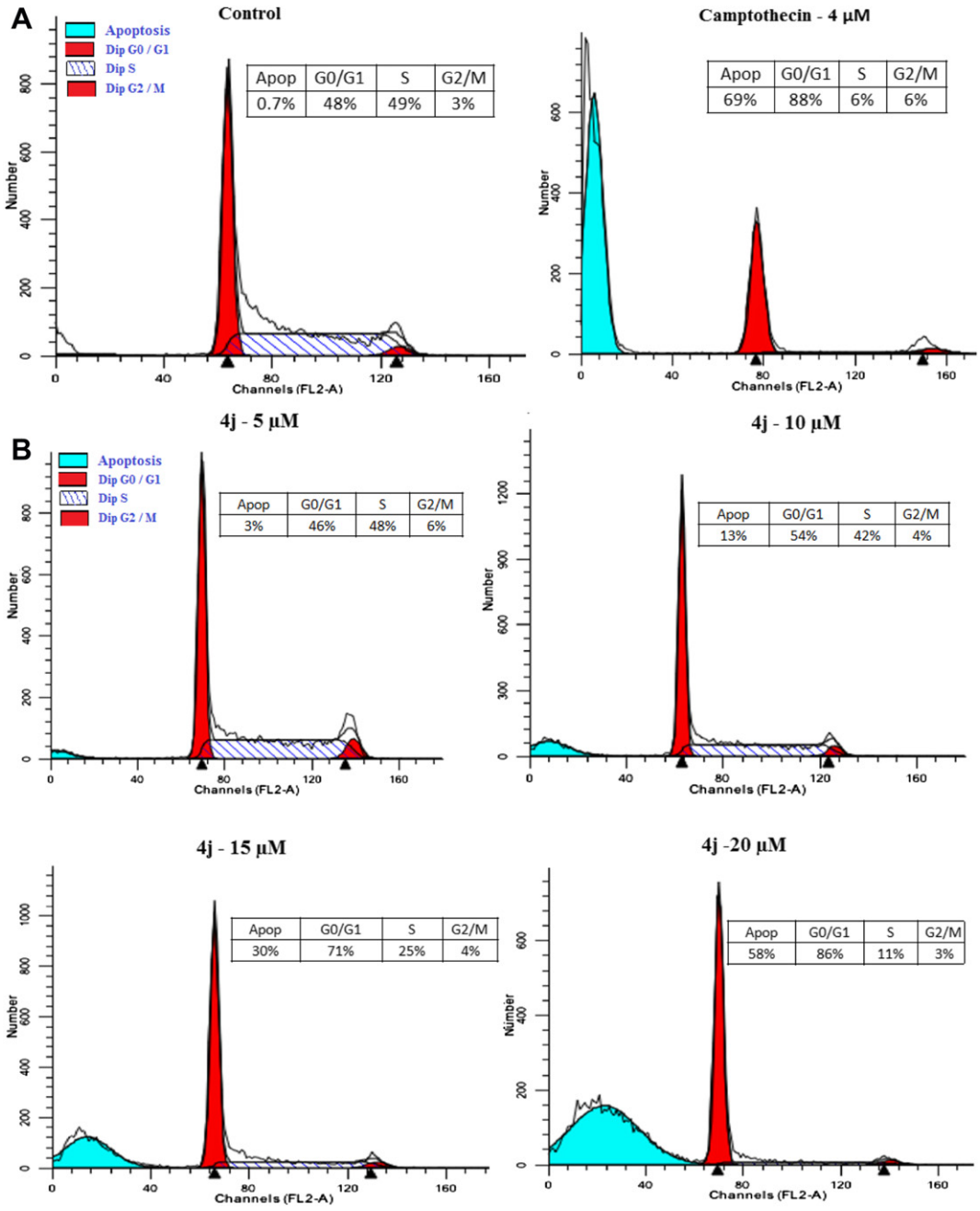


Fig. 4. Representative graphs of HL-60 cell cycle distribution after 24 h, Panel A: control and positive control camptothecin (4 µM, for 6 h). Panel B: **4j** at 5 µM, **4j** at 10 µM, **4j** at 15 µM, **4j** at 20 µM. Panel C: **4k** at 5 µM, **4k** at 10 µM, **4k** at 15 µM, **4k** at 20 µM. Panel D: **4l** at 5 µM, **4l** at 10 µM, **4l** at 15 µM, **4l** at 20 µM. The ModFit software calculates the number of diploid cells in G0/G1, S and G2/M phases of the cell cycle, hypo diploid cells appear as a sub-G0/G1 peak and indicated as apoptosis.

24 h, then the genomic DNA was isolated and electrophoresis is performed. Results obtained clearly showed that DNA from compounds treated cells exhibited such fragments typical of apoptosis. The DNA fragmentation analysis revealed that compounds **4j**, **4k** and **4l** induced a discrete ladder pattern in HL-60 cell line at 20 µM after 24 h of incubation thereby showing significant fragmentation, while no fragmentation was observed in untreated cells (Fig. 5).

2.2.4. *Mitochondrial membrane potential ($\Delta\Psi m$) loss*

Apoptotic stimuli results the convergence of many signals at mitochondria and most of these stimuli trigger a change of the mitochondrial membrane permeability [26] resulting in the release of several mitochondrial proteins into the cytoplasm, which constitutes the key event of mitochondrial mediated apoptosis. Loss of mitochondrial membrane potential leads to depolarization of mitochondrial membrane which results less uptake of rhodamine-123, a fluorescent dye by mitochondria. Compounds **4j**, **4k** and **4l** caused concentration dependent mitochondrial damage and hence decrease in mitochondrial membrane potential (Fig. 6). They showed 48%, 56% and 61% dissipation of mitochondrial membrane potential when used at concentration of 20 µM in HL-60 cells for 24 h (Fig. 6). Hence, treated HL-60 cells in the presence of **4j**, **4k** and **4l** exhibited a marked shift in fluorescence compared with untreated control cells (7%) and the positive control camptothecin (51%).

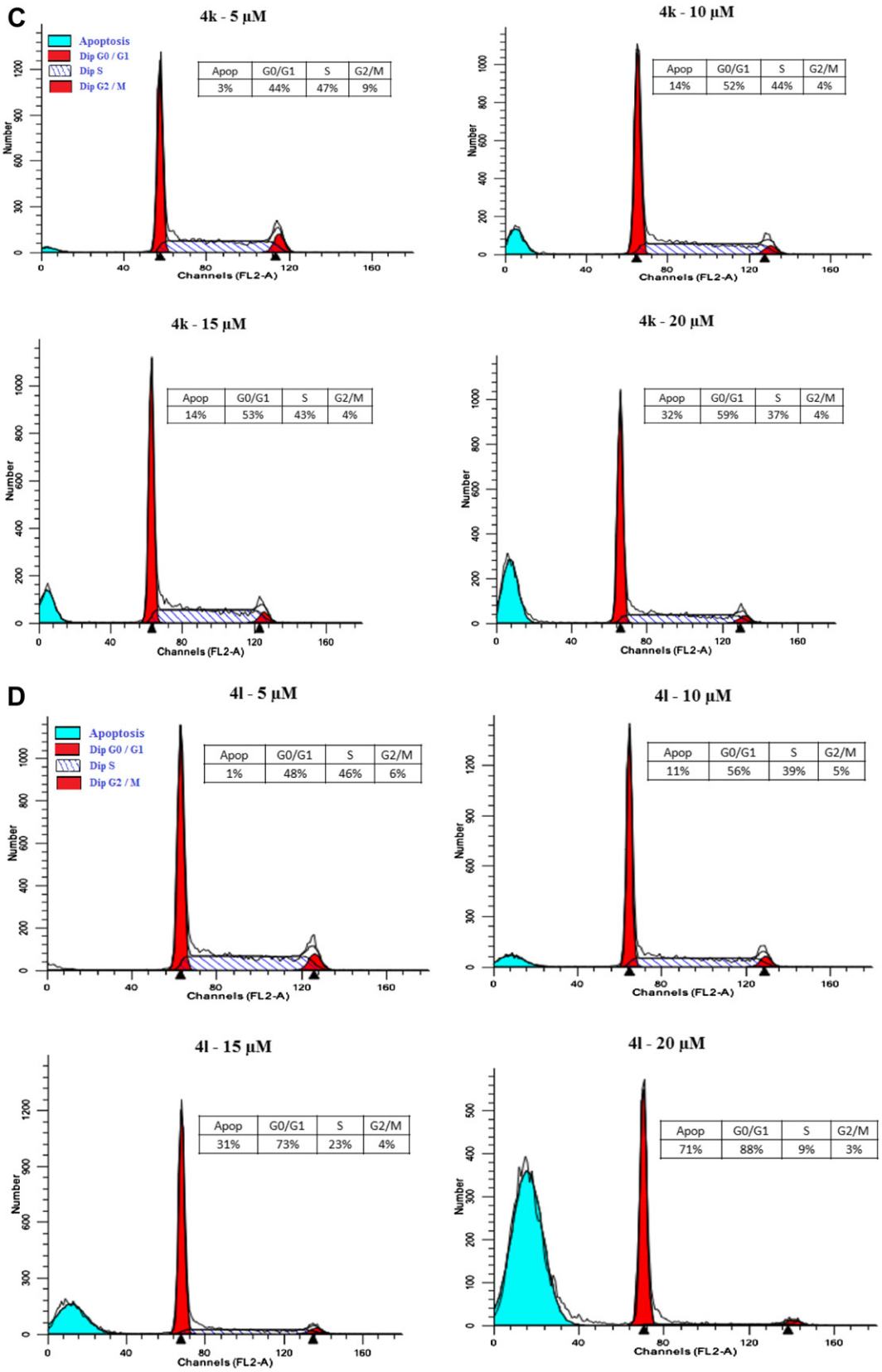


Fig. 4. (*continued*).

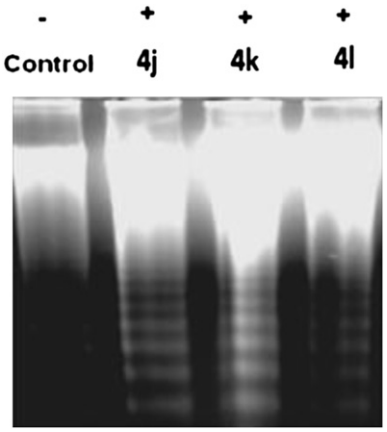


Fig. 5. DNA fragmentation of compounds **4j**, **4k** and **4l** in HL-60 cancer cells. Lane-1: control. Lane-2: **4j** at 20 μM. Lane-3: **4k** at 20 μM. Lane-4: **4l** at 20 μM.

2.2.5. Morphological appearance of HL-60 cancer cells treated with *4j*, *4k* and *4l*

To confirm the apoptosis-induction of **4j**, **4k** and **4l** compounds, morphological analysis was done by Hoechst 33258 staining that selectively binds DNA and allows monitoring of nuclear morphological changes under fluorescence microscopy. In Fig. 7, control cells showed an even distribution of staining of homogeneous nuclei features. Compounds **4j**, **4k** and **4l** caused nuclear condensation and blebbing in HL-60 cells after 24 h of treatment in concentration dependent manner. At 20 μM concentration all the three compounds showed apoptotic bodies in most of cells indicated by arrows in Fig. 7, confirming these compounds trigger cell demise by apoptosis.

3. Conclusion

In conclusion, a series of twelve novel 3-substitutedphenyl-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-propenones (**4a**–**l**) have been designed, synthesised and evaluated for their cytotoxic potential against seven human cancer cell lines (PC-3, MCF-7, HL-60, MIA PaCa-2, AsPC, MDA-MB-435 and Caco-2). A comprehensive structure activity relationship (SAR) study established the substitutional requirements that have a positive impact and those that have a negative impact on the biological activity of these novel compounds. Most of the twelve tested compounds exhibited significant *in vitro* antiproliferative activity at micromolar (μM) concentration. Three compounds, **4j**, **4k** and **4l** demonstrated the most marked effect in the human leukaemia HL-60 cancer cell line with IC_{50} values of 6–15 μM range. With regard to the proposed mode of action, cell cycle analysis in the HL-60 cell line has shown a significant increase in the G0/G1 and sub-G0/G1 population, which is suggestive of induction of cell cycle arrest and apoptosis. Moreover, DNA fragmentation, mitochondria depolarization and nuclear morphology studies evidenced that these compounds inhibit the cancer cell growth through apoptosis. Therefore, it was confirmed that **4j**, **4k** and **4l** are an excellent scaffolds for further study in the field of cancer chemotherapy.

4. Experimental section

4.1. Chemistry

All the chemicals and solvents used in this work were of analytical reagent grade (anhydrous) and purchased from Sigma Aldrich. Organic solutions were dried over Na_2SO_4. Melting points were determined on an electrothermal apparatus in an open capillary tube and are uncorrected. The ^1H NMR and ^{13}C NMR were recorded on Bruker Avance-II 300 MHz in $CDCl_3$ using tetramethylsilane (TMS) as internal reference. Chemical shifts (δ) are given in ppm (parts per million). Infrared spectra were recorded from KBr discs on Thermo Nicolet Avatar 330 spectrometer. The main bands are given in cm^{-1}. Electron induced (EI) mass spectra were recorded on an Ionspec QFT FT-ICR mass spectrometer. Elemental analyses were performed on an Elementar Vario EL elemental analyzer. All tested compounds yielded data consistent with a purity of at least 98% as compared with the theoretical values. Reaction mixtures were monitored by TLC on silica gel (precoated F_{254} Merck plates), and compounds were visualized with aqueous $KMnO_4$. Flash chromatography was performed using 230–400 mesh Merck silica gel and the indicated corresponding solvent system.

4.1.1. General procedure for synthesis of compound *2*

To a solution of 2,4-dihydroxy acetophenone **1** (1.52 g, 10 mmol) in methylenedichloride (20 ml) was added polyphosphoric acid (0.2 equiv) followed by 2-methyl-3-buten-2-ol (1.72 g, 20 mmol) and stirred for 4 h at room temperature. The solvent was evaporated under reduced pressure and the compound was purified by flash chromatography using benzene/ether (5:9.5) (v/v) as an eluent.

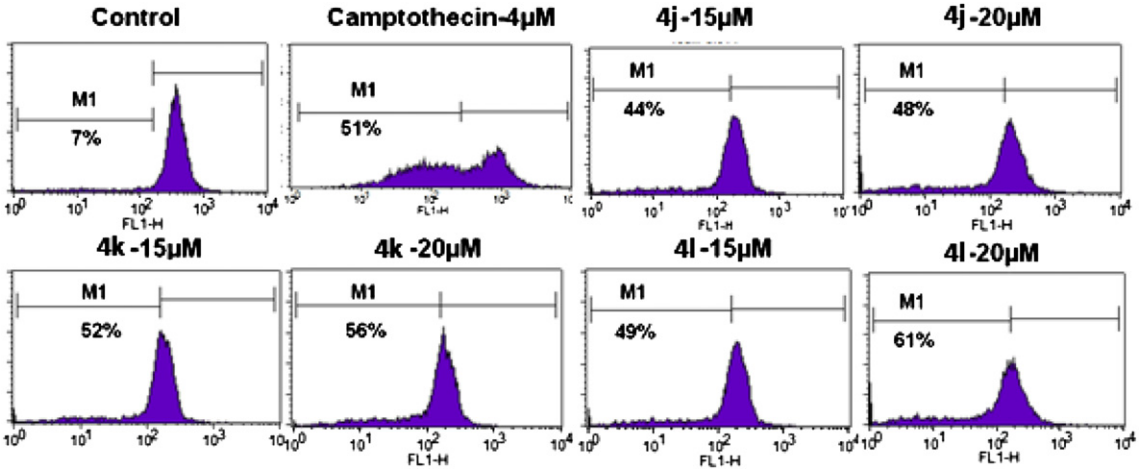


Fig. 6. Compounds **4j**, **4k** and **4l** induced loss of mitochondrial membrane potential in HL-60 cells at 15 μM and 20 μM concentrations. Figures are representative of one of three similar experiments.

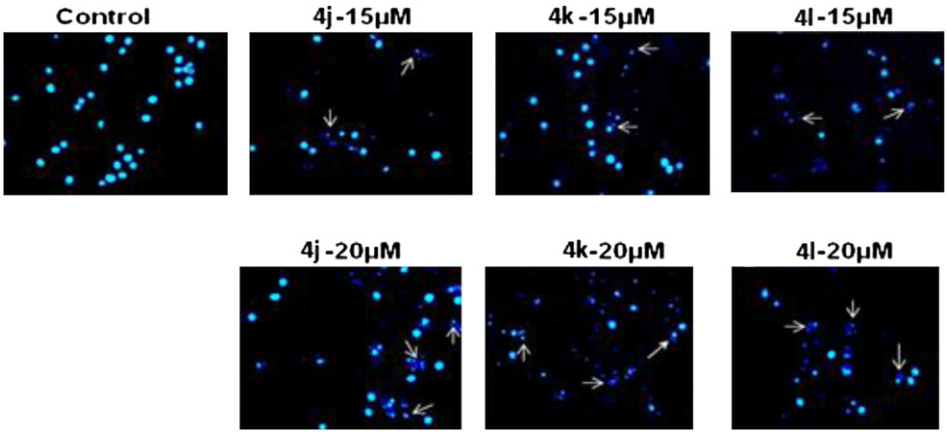


Fig. 7. Nuclear morphological changes of compounds **4j**, **4k** and **4l** at 15 μM and 20 μM concentrations towards HL-60 cells.

*4.1.1.1. 1-(2,2,8,8-Tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-ethanone (**2**).* White sticky solid; yield 68%; mp 76 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.32 (s, 6H, 2 × CH_3), 1.35 (s, 6H, 2 × CH_3), 1.77 (t, 4H, J = 6.9 Hz, 2 × CH_2), 2.57 (s, 3H, CH_3), 2.70 (t, 4H, J = 6.6 Hz, 2 × CH_2), 7.48 (s, 1H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.0, 21.6, 26.7, 26.8, 27.0, 31.6, 32.6, 74.5, 75.1, 104.5, 109.2, 111.7, 119.8, 129.0, 153.6, 156.0, 198.5; IR (KBr) ν_{max}: 881, 921, 1121, 1151, 1118, 1299, 1358, 1456, 1579, 1660, 2931, 2973 cm^{-1}; MS: *m/z* 288 (M^+), 273, 245, 219, 203, 177, 165, 149, 123, 109. Anal. Calcd for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39. Found: C, 74.95; H, 8.41.

*4.1.2. General procedure for the synthesis of compounds (**4a**–**l**)*

To a stirred solution of 1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2*H*,8*H*-pyrano[2,3-*f*]chromen-6-yl)-ethanone **2** (2.88 g, 10 mmol) and corresponding substituted benzaldehyde **3a**–**l** (10 mmol) was added gradually $BF_3 \cdot Et_2O$ (0.71g, 5 mmol) at room temperature in dioxane. The reaction mixture was stirred at ambient temperature till the disappearance of the starting material **2**. After completion of reaction, the reaction mixture was diluted with moist ether (50 ml), washed with water to discharge the colour and the $BF_3 \cdot Et_2O$ complex. The extracted ethereal solution was dried over anhydrous Na_2SO_4, concentrated and purified by column chromatography over SiO_2 using hexane/ethylacetate (1:9) as an eluent to yield the final products **4a**–**l**.

*4.1.2.1. 3-Phenyl-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4a**).* Yellow crystals; yield 72%; mp 127 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 6H, 2 × CH_3), 1.37 (s, 6H, 2 × CH_3), 1.71–1.81 (m, 4H, 2 × CH_2), 2.34 (s, 3H, CH_3), 2.67 (t, 2H, J = 6.9 Hz, CH_2), 2.73 (t, 2H, J = 6.9 Hz, CH_2), 7.18 (d, 2H, J = 7.8 Hz, Ar), 7.45 (s, 1H, Ar), 7.48 (m, 3H, Ar), 7.61 (d, 1H, J = 15.9 Hz, H_α), 7.69 (d, 1H, J = 15.6 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 20.3, 21.6, 26.9, 27.5, 31.3, 32.6, 74.4, 75.2, 108.5, 112.9, 120.7, 127.39, 127.9, 129.2, 133.3, 139.9, 141.2, 152.0, 154.7, 190.5; IR (KBr) ν_{max}: 819, 887, 987, 1125, 1135, 1293, 1337, 1493, 1529, 1608, 1654, 2935, 2965 cm^{-1}; MS: *m/z* 376.2 (M^+), 320.1, 265.0, 217, 178, 103.0. Anal. Calcd for $C_{25}H_{28}O_3$: C, 79.75; H, 7.50. Found: C, 79.70; H, 7.48.

*4.1.2.2. 3-(4-Methylphenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4b**).* Pale yellow crystals; yield 73%; mp 122 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.37 (s, 6H, 2 × CH_3), 1.71–1.82 (m, 4H, 2 × CH_2), 2.36 (s, 3H, CH_3), 2.64 (t, 2H, J = 6.9 Hz, CH_2), 2.73 (t, 2H, J = 6.9 Hz, CH_2), 7.18 (d, 2H, J = 7.8 Hz, Ar), 7.46 (s, 1H, Ar), 7.48 (d, 2H, J = 7.8 Hz, Ar), 7.61 (d, 1H, J = 15.9 Hz, H_α), 7.69 (d, 1H, J = 15.6 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.3, 21.6, 26.8, 27.0, 31.7, 32.6, 74.5, 75.1, 109.3, 112.0, 120.7, 127.0, 127.9, 129.6, 133.1, 139.7, 140.2, 153.0, 155.8, 190.8; IR (KBr) ν_{max}: 817, 892, 999, 1117, 1139, 1287, 1328, 1450, 1577, 1610, 1650, 2929, 2973 cm^{-1}; MS: *m/z* 390.2 (M^+), 334.1, 279.1, 217.0, 178.2, 117.0. Anal. Calcd for $C_{26}H_{30}O_3$: C, 79.97; H, 7.74. Found: C, 79.95; H, 7.78.

*4.1.2.3. 3-(4-Methoxyphenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4c**).* Yellow oil; yield 78%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 6H, 2 × CH_3), 1.35 (s, 6H, 2 × CH_3), 1.70–1.81 (m, 4H, 2 × CH_2), 2.62 (t, 2H, J = 6.9 Hz, CH_2), 2.72 (t, 2H, J = 6.9 Hz, CH_2), 3.83 (s, 3H, OCH_3), 6.89 (d, 2H, J = 8.1 Hz, Ar), 7.03 (s, 1H, Ar), 7.27 (d, 2H, J = 8.1 Hz, Ar), 7.40 (d, 1H, J = 15.9 Hz, H_α), 7.57 (d, 1H, J = 15.6 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.3, 20.3, 21.2, 25.6, 24.3, 30.7, 32.5, 74.3, 74.8, 109.2, 119.9, 120.7, 127.3, 127.9, 129.8, 132.1, 139.7, 140.0, 152.7, 155.3, 188.8; IR (KBr) ν_{max}: 818, 892, 929, 1110, 1139, 1229, 1319, 1436, 1529, 1604, 1636, 2935, 2936 cm^{-1}; MS: *m/z* 406.2 (M^+). Anal. Calcd for $C_{26}H_{30}O_4$: C, 76.82; H, 7.44. Found: C, 75.95; H, 7.40.

*4.1.2.4. 3-(2,5-Dimethoxyphenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4d**).* Light brown oil; yield 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.70–1.81 (m, 4H, 2 × CH_2), 2.62 (t, 2H, J = 6.9 Hz, CH_2), 2.75 (t, 2H, J = 6.9 Hz, CH_2), 3.84 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.83 (dd, 2H, J = 8.2 and 2.3 Hz, Ar), 6.99 (s, 1H, Ar), 7.37 (m, 2H, Ar and H_β), 7.18 (d, 1H, J = 15.9 Hz, H_α); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.8, 21.2, 26.9, 26.2, 29.7, 30.3, 32.2, 55.3, 54.3, 74.3, 75.2, 96.3, 108.2 110.1, 112.9, 116.5, 120.1, 124.8, 128.1, 135.5, 143.3, 150.2, 154.3, 155.3, 189.9; IR (KBr) ν_{max}: 831, 929, 1026, 1113, 1227, 1223, 1348, 1436, 1539, 1527, 1606, 1633, 1732, 2822, 2934, 2948 cm^{-1}; MS: *m/z* 436.2 (M^+), 380.1, 325.1, 273.1, 217.0, 178.9, 163.0. Anal. Calcd for $C_{27}H_{32}O_5$: C, 74.29; H, 7.39. Found: C, 74.25; H, 7.34.

*4.1.2.5. 3-(2,4,6-Trimethoxyphenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano [2,3-f]chromen-6-yl)-propenone (**4e**).* Deep brown oil; yield 72%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33 (s, 6H, 2 × CH_3), 1.35 (s, 6H, 2 × CH_3), 1.78–1.79 (m, 4H, 2 × CH_2), 2.63 (t, 2H, J = 7 Hz, CH_2), 2.73 (t, 2H, J = 7Hz, CH_2), 3.86 (s, 6H, 2 × OCH_3), 3.92 (s, 3H, OCH_3), 6.50 (s, 1H, Ar), 7.14 (s, 1H, Ar), 7.45 (s, 1H, Ar), 7.60 (d, 1H, J = 15.9 Hz, H_α), 8.01 (d, 1H, J = 15.9 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.6, 26.8, 27.0, 31.8, 32.7, 55.9, 56.1, 56.4, 74.3, 75.0, 109.2, 110.1, 112.0, 116.5, 121.1, 125.8, 129.6, 135.2, 143.1, 151.5, 152.7, 153.9, 155.5, 190.9; IR (KBr) ν_{max}: 849, 926, 1033, 1117, 1209, 1296, 1316, 1454, 1513, 1564, 1603, 1640, 1735, 1773, 2851, 2926, 2972 cm^{-1}; MS: *m/z* 467.3 (M^+), 411.2, 355.1, 273.1, 217.0, 178.9, 193.0, 160.0; Anal. Calcd. for $C_{28}H_{34}O_6$: C, 72.08; H, 7.35. Found: C, 71.96; H, 7.37.

*4.1.2.6. 3-(4-Fluorophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4f**).* Light brown crystals; yield 65%; mp 122 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.36 (s, 6H, 2 × CH_3), 1.76–1.82 (m, 4H, 2 × CH_2), 2.63 (t, 3H, J = 6.6 Hz, CH_2), 2.73 (t, 3H, J = 6.9 Hz, CH_2), 7.34 (d, 2H, J = 8.4 Hz, Ar), 7.50 (d, 2H, J = 8.4 Hz, Ar), 7.48 (s, 1H, Ar), 7.57 (d, 1H, J = 15.6 Hz, Ar), 7.70 (d, 1H, J = 15.9 Hz, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.6, 26.8, 27.0, 31.7, 32.6, 74.6, 75.2, 109.4, 112.2, 120.4, 128.6, 128.9, 129.0, 129.7, 134.4, 135.1, 138.4, 153.2, 156.1, 190.2; IR (KBr) ν_{max}: 834, 998, 1117, 1142, 1284, 1327, 1450, 1577, 1607, 1650, 2972, 2932 cm^{-1}; MS: m/z 394.1 (M^+), 338.1, 283.0, 217.0, 178.9, 121.0. Anal. Calcd. for $C_{25}H_{27}FO_3$: C, 76.12; H, 6.90. Found: C, 76.10; H, 6.91.

*4.1.2.7. 3-(2-Chlorophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4g**).* Pale yellow crystals; yield 68%; mp 132 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.36 (s, 6H, 2 × CH_3), 1.76–1.82 (m, 4H, 2 × CH_2), 2.63 (t, 2H, J = 6.6 Hz, CH_2), 2.74 (t, 2H, J = 6.9 Hz, CH_2), 7.24–7.28 (m, 2H, Ar and H_α), 7.40 (dd, 1H, J = 6.9 and 2.4 Hz, Ar), 7.48 (s, 1H, Ar), 7.68–7.73 (m, 2H, Ar), 8.03 (s, 1H, J = 15.9 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.6, 26.8, 27.0, 31.7, 32.6, 74.6, 75.2, 109.3, 112.2, 120.4, 126.7, 127.2, 129.8, 130.0, 130.3, 134.1, 135.1, 135.7, 153.1, 156.1, 190.3; IR (KBr) ν_{max}: 866, 978, 1138, 1118, 1277, 1329, 1448, 1586, 1608, 1660, 2931, 2974 cm^{-1}; MS: m/z 411.2 (M^+), 413.3 (M+2), 353.0, 299.0, 271.1, 233.0, 217.0, 178.9, 137.0. Anal. Calcd. for $C_{25}H_{27}ClO_3$: C, 73.07; H, 6.62. Found: C, 73.01; H, 6.66.

*4.1.2.8. 3-(3-Chlorophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4h**).* Yellow crystals; yield 68%; mp 125 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35 (s, 6H, 2 × CH_3), 1.37 (s, 6H, 2 × CH_3), 1.75–1.85 (m, 4H, 2 × CH_2), 2.63 (t, 2H, J = 6.9 Hz, CH_2), 2.75 (t, 2H, J = 6.6 Hz, CH_2), 7.32 (s, 1H, Ar), 7.39 (d, 1H, J = 8.1 Hz, Ar), 7.51 (d, 1H, J = 15.6 Hz, H_α), 7.62 (d, 1H, J = 7.5 Hz, Ar), 7.90 (d, 1H, J = 15.9 Hz, H_β), 8.17–8.20 (m, 1H, Ar), 8.49 (s, 1H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.6, 26.8, 27.1, 31.7, 32.6, 74.8, 75.2, 109.3, 112.4, 119.9, 121.5, 123.4, 129.6, 129.8, 130.5, 134.2, 135.36, 137.6, 147.3, 153.6, 156.3, 189.5; IR (KBr) ν_{max}: 839, 984, 1126, 1268, 1343, 1450, 1575, 1611, 1650, 2986, 2977 cm^{-1}; MS: m/z 411.2 (M^+), 413.3 (M+2), 353.0, 299.0, 271.1, 233.0, 217.0, 178.9, 137.0. Anal. Calcd. for $C_{25}H_{27}ClO_3$: C, 73.07; H, 6.62. Found: C, 73.01; H, 6.64.

*4.1.2.9. 3-(4-Bromophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4i**).* Dark yellow crystals; yield 66%; mp 138 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.36 (s, 6H, 2 × CH_3), 1.76–1.82 (m, 4H, 2 × CH_2), 2.63 (t, 2H, J = 6.9 Hz, CH_2), 2.73 (t, 2H, J = 6.6 Hz, CH_2), 7.43 (d, 2H, J = 8.7 Hz, Ar), 7.50 (d, 2H, J = 8.7 Hz, Ar), 7.48 (s, 1H, Ar), 7.55 (d, 1H, J = 15.9 Hz, H_α), 7.71 (d, 1H, J = 15.9 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.1, 21.6, 26.8, 27.0, 31.7, 32.6, 74.6, 75.2, 109.4, 112.2, 120.4, 123.4, 128.6, 129.2, 129.7, 131.9, 134.8, 138.5, 153.2, 156.2, 190.3; IR (KBr) ν_{max}: 817, 981, 1117, 1138, 1290, 1324, 1448, 1574, 1611, 1649, 2922, 2970 cm^{-1}; MS: m/z 454.1 (M^+), 456.8 (M+2), 398.0, 342.9, 217.0, 180.9, 178.9. Anal. Calcd for $C_{25}H_{27}BrO_3$: C, 65.94, H, 5.98. Found: C, 65.95; H, 5.95.

*4.1.2.10. 3-(3-Nitrophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4j**).* Deep yellow crystals; yield 63%; mp 161 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.41 (s, 6H, 2 × CH_3), 1.77–1.85 (m, 4H, 2 × CH_2), 2.64 (t, 2H, J = 6.9 Hz, CH_2), 2.74 (t, 2H, J = 6.6 Hz, CH_2), 7.53 (s, 1H, Ar), 7.57 (d, 1H, J = 8.1 Hz, Ar), 7.64 (d, 1H, J = 15.6 Hz, H_α), 7.82 (d, 1H, J = 7.5 Hz, Ar), 7.90 (d, 1H, J = 15.9 Hz, H_β), 8.17–8.20 (m, 1H, Ar), 8.49 (s, 1H, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.0, 21.6, 26.8, 27.0, 31.7, 32.6, 74.9, 75.4, 109.5, 112.4, 119.9, 121.5, 123.5, 129.7, 129.9, 130.9, 134.0, 136.6, 137.8, 148.6, 153.5, 156.6, 189.5; IR (KBr) ν_{max}: 836, 980, 1116, 1139, 1277, 1330, 1452, 1527, 1574, 1609, 1650, 2936, 2977 cm^{-1}; MS: m/z 421.1 (M^+), 365.1, 310.0, 217.0, 178.9, 148.0. Anal. Calcd for $C_{25}H_{27}NO_5$: C, 71.24; H, 6.46; N, 3.32. Found: C, 71.22; H, 6.49; N, 3.30.

*4.1.2.11. 3-(4-Nitrophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f]chromen-6-yl)-propenone (**4k**).* Yellow crystals; yield 69%; mp 179 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35 (s, 6H, 2 × CH_3), 1.37 (s, 6H, 2 × CH_3), 1.62–1.82 (m, 4H, 2 × CH_2), 2.64 (t, 2H, J = 6.9 Hz, CH_2), 2.74 (t, 2H, J = 6.6 Hz, CH_2), 7.51 (s, 1H, Ar), 7.62 (d, 1H, J = 15.9 Hz, H_α), 7.70 (d, 2H, J = 8.7 Hz, Ar), 7.84 (d, 1H, J = 15.9 Hz, H_β), 8.24 (d, 2H, J = 8.7 Hz, Ar); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.0, 21.6, 26.8, 27.0, 31.7, 32.6, 74.8, 75.4, 109.5, 112.5, 119.9, 122.9, 124.0, 128.2, 128.6, 129.9, 132.0, 136.5, 142.3, 146.1, 153.4, 156.6, 189.5; IR (KBr) ν_{max}: 846, 989, 1119, 1140, 1283, 1340, 1453, 1516, 1577, 1601, 1650, 2930, 2974 cm^{-1}; MS: m/z 421.1 (M^+), 365.1, 310.0, 217.0, 178.9, 148.0. Anal. Calcd for $C_{25}H_{27}NO_5$: C, 71.24, H, 6.46; N, 3.32. Found: C, 71.26; H, 6.44; N, 3.30.

*4.1.2.12. 3-(4-Cyanophenyl)-1-(2,2,8,8-tetramethyl-3,4,9,10-tetrahydro-2H,8H-pyrano[2,3-f] chromen-6-yl)-propenone (**4l**).* Bright yellow crystals; yield 62%; mp 209 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 6H, 2 × CH_3), 1.36 (s, 6H, 2 × CH_3), 1.77–1.83 (m, 4H, 2 × CH_2), 2.63 (t, 3H, J = 6.9 Hz, CH_2), 2.74 (t, 3H, J = 6.6 Hz, CH_2), 7.50 (s, 1H, Ar), 7.57 (d, 1H, J = 15.9 Hz, H_α), 7.63 (d, 2H, J = 8.4 Hz, Ar), 7.67 (d, 2H, J = 8.4 Hz, Ar), 7.80 (d, 1H, J = 15.9 Hz, H_β); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.0, 21.6, 26.8, 27.0, 31.7, 32.6, 74.8, 75.4, 109.4, 112.3, 112.4, 118.5, 120.0, 128.1, 129.9, 131.3, 132.4, 137.1, 140.4, 153.4, 156.5, 189.7; IR (KBr) ν_{max}: 830, 994, 1119, 1140, 1287, 1329, 1452, 1575, 1609, 1651, 2224, 2926, 2974 cm^{-1}; MS: m/z 401.2 (M^+), 345.1, 290.0, 217.0, 178.9, 163.0, 128.0. Anal. Calcd for $C_{26}H_{27}NO_3$: C, 77.78; H, 6.78; N, 3.49. Found: C, 77.75; H, 6.75; N, 3.44.

4.2. Cancer biology

4.2.1. Cancer cell lines

Human cancer cell lines for promyelocytic human leukaemia HL-60, prostate PC-3, pancreatic MIA PaCa-2 and AsPC, colon Caco-2 were procured from European Collection of Cell Cultures (ECACC). Breast cancer cell line MCF-7 and melanoma cancer cell line MDA-MB-435 were procured from National Cancer Institute, Frederick, U.S.A. Cells were grown in RPMI-1640/DMEM/MEM medium containing 10% FCS, 100 units penicillin and 100 μg of streptomycin per ml medium. Cells were grown in CO_2 incubator (Thermocon Electron Corporation, USA) at 37 °C with 95% humidity and 5% CO_2 gas environment. Cells were treated with tested compounds dissolved in DMSO, while the untreated control cultures received only the vehicle (DMSO, <0.2%).

4.2.2. Chemicals and reagents

RPMI-1640, minimal essential medium (MEM), Dulbecco's modified eagle medium (DMEM), rhodamine-123 (Rh-123), PBS, proteinase K, agarose, propidium iodide (PI), DNase-free RNase, Hoechst-33258, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil, mitomycin C, adriamycin and camptothecin were purchased from Sigma Chemical Company. Other reagents used were of analytical grade and available locally.

4.3. MTT cell proliferation assay

Cells from suspension and adherent cultures grown in 96-well plates were exposed to indicate concentrations of compounds for 48 h. MTT solution (2.5 mg/ml in PBS) was added to each well 3 h

before termination. The plates were centrifuged and the supernatant was discarded while the MTT-formazan crystals [27] were dissolved in 100 μl DMSO. The OD measured at 570 nm with reference wavelength of 620 nm.

4.4. Cell cycle analysis

Cells (0.5×10^6/ml) were treated with different concentrations of **4j**, **4k** and **4l** for 24 h and collected at 300 *g* for 5 min. Cell pellets were washed with PBS twice and fixed in 70% alcohol for overnight at 4 °C. Cells were again washed, suspended in 250 μl of PBS and incubated with RNase A (400 μg/ml) at 37 °C for about 1 h and stained with propidium iodide [28] (10 μg/ml) for 30 min. Cells were analyzed for PI-DNA fluorescence by flow cytometry using FACS CALIBUR (Becton Dickinson, USA). The fluorescence intensity of sub-G0/G1 cell fraction represents the apoptotic cell population.

4.5. DNA agarose gel electrophoresis

DNA fragmentation typical of apoptosis was analysed by electrophoresis [29] of extracted genomic DNA from HL-60 cells. After various treatments, HL-60 cells were centrifuged at 300 *g* for 5 min, washed twice with PBS containing 10 mM EDTA. The pellets were lysed in 250 μl of lysis buffer (100 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 8.0, and 5% Triton X-100, 0.25% SDS) containing 400 μg/ml DNase-free RNase and incubated at 37 °C for 90 min followed by 1 h incubation with proteinase-K (200 μg/ml) at 50 °C for 1 h. DNA extraction was done with phenol/chloroform/iso-amyl alcohol (1:24:1) and then centrifuged at 13,000 *g* for 2 min. DNA was precipitated from aqueous phase with 3 volumes of chilled alcohol and 0.3 M sodium acetate at 4 °C overnight. The precipitate was centrifuged at 13,000*g* for 10 min. The DNA pellet was washed in 80% alcohol, dried, dissolved in 50 μl TE buffer and electrophoresed in 1.8% agarose gel at 70 V for 1 h. Ethidium bromide used for staining of DNA and to observe bands under transilluminator.

4.6. Assessment of mitochondrial potential loss

Changes in mitochondrial transmembrane potential as a result of mitochondrial perturbation were measured after staining with rhodamine-123 [30]. HL-60 cells (1×10^6/2 ml/well) after indicated treatments in 12-well plate were incubated with medium containing rhodamine-123 (200 nM) for 40 min. Cells were washed in PBS and centrifuged at 300 *g* for 5 min and suspended in PBS. The intensity of fluorescence from 10,000 events was analyzed in FL-1 channel on flow cytometer. Camptothecin was (4 μM for 6 h) used as positive control.

4.7. Hoechst 33258 staining of cells for nuclear morphology

Human leukaemia HL-60 cells (1×10^6cells/2 ml/12 well plate) treated with **4j**, **4k** and **4l** compounds for 24 h at concentrations of 15 and 20 μM. Cells were centrifuged at 300 *g* for 5 min and washed twice with PBS. Cells were gently suspended in 100 μl PBS and fixed in 400 μl cold acetic acid/methanol (1 + 3, v/v) overnight at 4 °C. Cells were washed again in 1 ml of fixing solution, suspended in the residual volume of about 50 μl, spread on a clean slide and dried overnight at room temperature. One millilitre of staining solution (Hoechst 33258, 10 μg/ml of 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween-20) [31] was poured on each slide and stained for 30 min under subdued light at room temperature. Slides were washed under gentle low of tap water, rinsed in distilled water followed by in PBS. While wet, 50 μl of mounting fluid (PBS/glycerol, 1:1) was poured over the centre of and covered with glass cover slip. The slides were sealed with nail polish and observed for any nuclear morphological alterations and apoptotic bodies under inverted fluorescence microscope (Olympus 1X70, magnification 10×) using UV excitation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.01.027.

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