Regular Article

Synthesis and Anti-cancer Activity of Novel Thiazolidinone Analogs of 6-Aminoflavone

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Novel heterocyclic analogs were synthesized by combining a flavone nucleus and thiazolidinone ring in an effort to potentiate the existing anti-cancer activity of flavone. The syntheses of 6-aminoflavone, 6-amino-3-methoxyflavone, 6-amino-3-methoxy-3′,4′-dimethxyflavone and their corresponding thiazolidinone analogs were performed. Fifteen novel analogs were synthesized and evaluated for their anti-cancer activity using cell-based assay techniques and *in vivo* testing. As expected, the analogs improved cytotoxicity and were shown to increase the life span of cancer-bearing mice. Cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays in HeLa, MDA-MB-435, and Vero cell lines. *In vivo* evaluation of anti-cancer activity performed in albino mice bearing Dalton's ascites carcinoma showed that the new analogs enhanced life span and prevented increases in body weight owing to tumor volumes. Moreover, cell-cycle analysis and Hoechst staining analysis proved the apoptotic potential of these analogs. Preliminary pharmacokinetic evaluation was carried out on the synthesized compounds to determine the lipophilicity and pK_a. Lipophilicity was determined using high-performance liquid chromatography and the results showed a direct correlation between the observed anti-cancer activity and log *P* value, while pK_a values indicated the ionizing range which is a prediction tool for solubility and permeability.

Key words aminoflavone; thiazolidinone; anticancer; cytotoxicity; lipophilicity; *in vivo* activity

Polyphenols such as flavonoids that contain chromone pharmacophore are known for their wide range of pharmacological activities. It is important to note that these compounds are non-toxic at concentrations that are normally consumed in a normal diet. A flavonoid-rich diet is also considered as a prophylactic treatment for cancer. Various clinical studies performed on flavonoids indicate that these molecules are potent chemotherapeutic agents against cancer. The anti-cancer activity of flavonoids is mainly by the inactivation of carcinogens, cell-cycle arrest, and by the induction of apoptosis.[1] The approach to anticancer drug discovery is to develop cytotoxic agents that can differentiate cancer cells and normal cells. One way of synthesizing molecules with lesser toxicity would be to adopt the molecularly targeted therapeutics, which will provide sufficient selectivity to a receptor.[2] The role of flavonoids in chemoprevention of cancer is well established.[3] Major highlight of flavonoids is that they act through multiple mechanisms such as angiogenesis inhibition, inactivation of carcinogen,[4–7] arresting the cell cycle and apoptosis induction.[8–10] Thiazolidinones are another class of heterocycle that are reported to possess anti-inflammatory,[11] anti-microbial,[12] anti-proliferative,[13,14] anti-viral,[15] anti-convulsant,[16,17] anti-fungal,[18] and anti-bacterial properties.[19] Recent reports indicate that these compounds possess anticancer properties.[20]

Aminoflavones are a class of compounds that do not have any natural occurrence are reported to be highly selective towards breast cancer, and are considered as a new class of anti-cancer agents.[21] These compounds are reported to undergo metabolic activation inside cancer cells to form a DNA-damaging metabolite.[22] This selectivity is due to the activation of the aryl hydrocarbon receptor, which in turn induces CYP1A1.[23] Other reported mechanisms of anti-cancer activity of aminoflavones suggest cross-linking between protein and DNA, and phosphorylation of histone H2AX.[24] A recent report indicates that aminoflavones are not cytotoxic to MCF10A non-malignant cells.[25] Flavopiridol is a potential drug candidate from the flavonoid family having inhibitory activity against kinases such as CDK-1, 2, and 4.[26] Hydroxyflavone and methoxyflavone are also reported to possess this inhibitory activity.[27] One of our earlier works to potentiate the anti-cancer activity of aminoflavone by incorporating imidazolidinone heterocycle has yielded promising activity.[28] It was also noticed that incorporation of methoxy groups has better activity than other analogs in the same series. The present study discusses the synthesis of novel thiazolidinone analogs of aminoflavone and evaluates their anti-cancer potential. Three series of thiazolidinone analogs of 6-aminoflavone were synthesized, as shown in Charts 1, 2, and 3. A structure–activity relationship (SAR) study was conducted by incorporating methoxy, hydroxyl, and chloro groups on the phenyl ring, and the anti-cancer effect of the addition of a methoxy group in the flavone nucleus was also evaluated. Preliminary pharmacokinetic evaluation of the newly synthesized molecules was performed to predict their absorption behavior.

Results and Discussion

Aminoflavones such as 6-aminoflavone, 6-amino-3-methoxyflavone, and 6-amino-3-methoxy-3′,4′-dimethoxyflavones were synthesized from paracetamol using modifications of several reported procedures.[29,30] The 6-aminoflavone (**5**) was synthesized as shown in Chart 1. Paracetamol was first acetylated and then condensed with benzaldehyde to obtain the chalcone.

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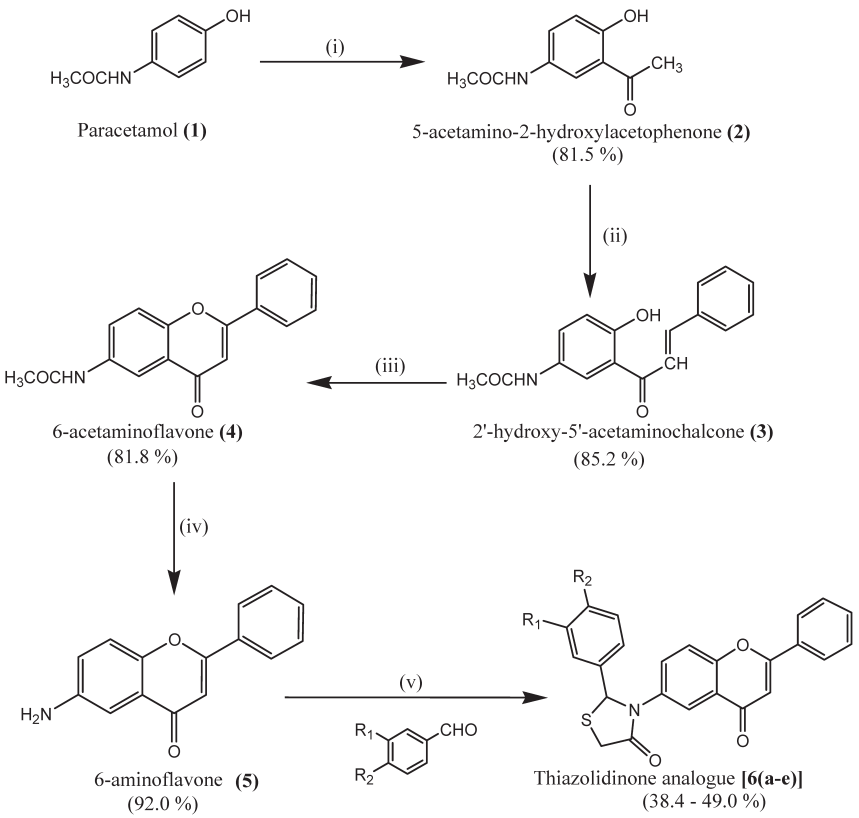


	6a	6b	6c	6d	6e
R_1	H	H	Cl	OCH_3	H
R_2	H	OCH_3	Cl	OCH_3	OH

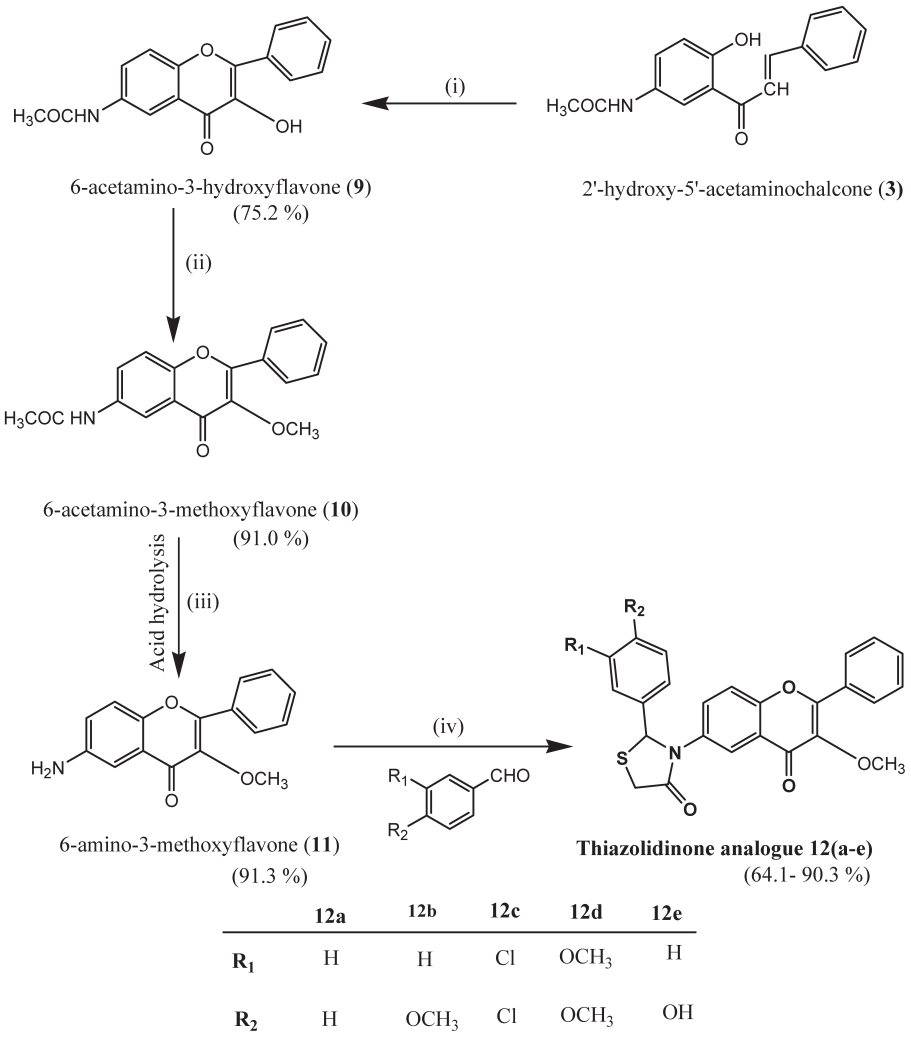
Reagents and conditions: (i) $AlCl_3$, CH_3COCl, nitrobenzene, 130°C, 2.5 h; (ii) 10% KOH (aq), benzaldehyde in ethanol, 30°C, 12 h; (iii) DMSO, I_2, 175°C, 30 min; (iv) conc. HCl–water (1 : 1), boil, 30 min; (v) substituted Ar-CHO, thioglycollic acid, 0°C, 5–15 min; DCC, 30 min to 1 h at r.t.

Chart 1. Synthesis of 6-Aminoflavone and Its Thiazolidinone Analogs

Iodine-catalyzed cyclization of this chalcone in dimethyl sulfoxide (DMSO) yielded 6-acetamidoflavone, which was then hydrolyzed to obtain the 6-aminoflavone (**5**). Syntheses of 6-amino-3-methoxyflavone (**11**) and 6-amino-3-methoxy-3′,4′-dimethoxyflavone (**17**) were performed as discussed in Charts 2 and 3, respectively. 2′-Hydroxychalcone obtained in Chart 1 was converted to 6-acetamido-3-hydroxyflavone through treatment with sodium hydroxide and hydrogen peroxide by employing Algar–Flynn–Oyamada oxidation.[31,32] Methylation using dimethyl sulfate in dry acetone resulted in the formation of 6-acetamino-3-methoxyflavone; acid hydrolysis of the latter generated a good yield of 6-amino-3-methoxyflavone (**11**). The 6-amino-3-methoxy-3′,4′-dimethoxyflavone (**17**) was obtained similarly in a step wise synthesis by employing 3,4-dimethoxybenzaldehyde for the synthesis of chalcone followed by cyclization, methylation and hydrolysis steps. The synthesis of aminoflavone was confirmed by the use of various spectral techniques; the peak at δ 5.5 in the ^{1}H-NMR spectrum corresponds to the two amino protons, while the presence of methoxy group at the third position of compound **11** was confirmed by the NMR signal at δ 3.77 for three protons, and that in compound **17** was confirmed by the presence of signals at δ 3.77 and 3.85 corresponding to nine protons. The structures were further confirmed by the presence of molecular ion peaks in the mass spectra of these compounds.

The synthesis of thiazolidinone analogs from a primary aromatic amine and aromatic aldehyde was reported to be through the formation of a Schiff's base, followed by cyclization into the thiazolidinone using thioglycolic acid.[33] However, the Schiff's base could not be isolated in its pure form because of a lack of stability. Hence, an alternate single-step procedure of treating the starting materials with thioglycolic acid in the presence of *N*,*N*′-dicyclohexyl carbodiimide (DCC) was adopted,[34] and was successful in providing a fairly good yield. The formation of thiazolidinones was confirmed by the presence of the respective molecular ion peaks in the mass spectra and by the absence of the primary amino-protons of the parental aminoflavone at δ 5.5 in the proton NMR spectra. The ^{1}H-NMR spectra of the thiazolidinones also showed the characteristic double doublet at δ 4 accounting for the two protons at C-5 of the thiazolidinone nucleus.

In vitro cytotoxicity of these novel analogs and the corresponding aminoflavones were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in HeLa and MDA-MB-435 cells. In these assays, succinate dehydrogenase present in the mitochondria of living cells converts the methyl tetrazolium to a purple-colored complex. The color is proportional to the number of living cells and the assay provides information on the number of living and viable cells. This method was therefore used to measure cytotoxicity,



	12a	12b	12c	12d	12e
R_1	H	H	Cl	OCH_3	H
R_2	H	OCH_3	Cl	OCH_3	OH

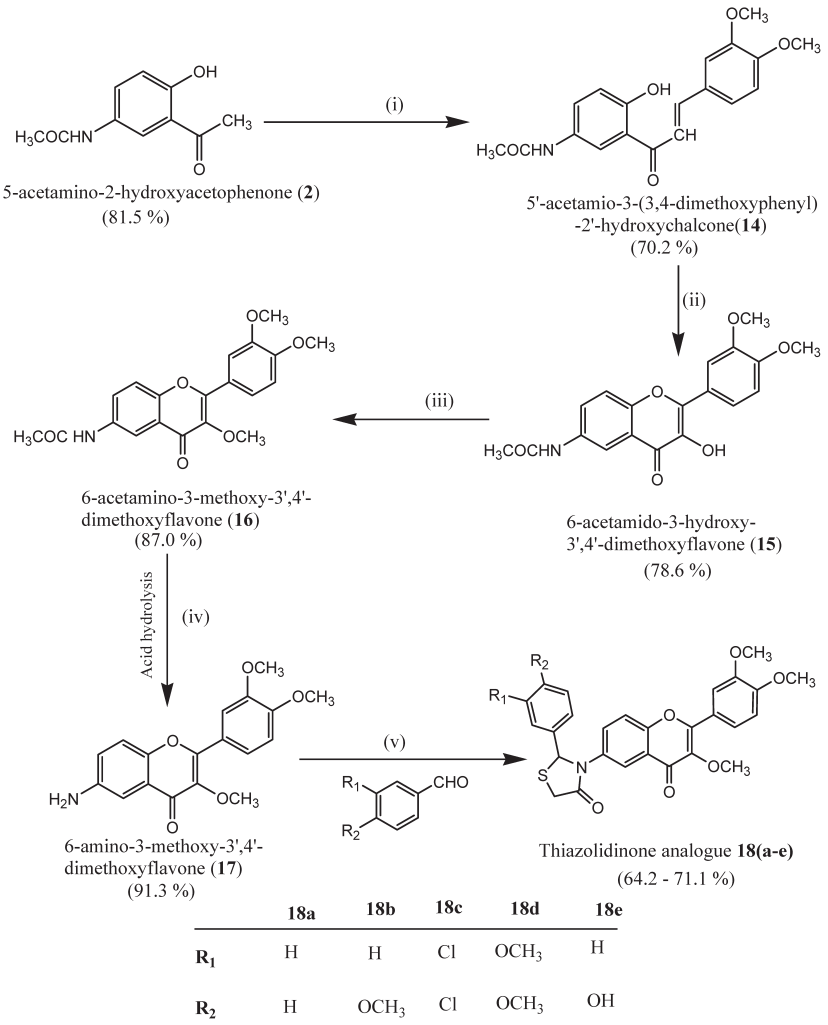
Reagents and conditions: (i) NaOH (20%), H_2O_2 (20%), 30 °C, 3 h; (ii) dry acetone, K_2CO_3 dimethyl sulfate, reflux, 5 h; (iii) conc. HCl–water (1 : 1), boil, 30 min; (iv) substituted ArCHO, thioglycolic acid, 0°C, 20–50 min; DCC, 30 min to 1 h at r.t. Yield is shown as percentage in brackets.

Chart 2. Synthesis of 6-Amino-3-methoxyflavone and Its Thiazolidinone Analogs

proliferation, or activation.[35] The results of the MTT assay are detailed in Table 1. All the aminoflavones showed cytotoxic activity towards both cell lines. Among the aminoflavones, it was observed that the presence of a methoxy group at the third position (compound **11**) and at the 3′,4′-positions (compound **17**) resulted in the enhancement of cytotoxic activity. Conversion to thiazolidinone analogs resulted in a synergistic effect as expected, which was evidenced by the increase in the cytotoxic potential. It was also found that compound **18d**, which is a thiazolidinone analog with five methoxy groups, was the most active in the series. Interestingly, it was also noticed that the compounds showed selective cytotoxicity, as evidenced by the high IC_{50} values against Vero cells. A diagrammatic representation of the results of MTT assay results of selected compounds in HeLa and MDA-MB-435 cells are provided in Fig. 1.

To evaluate apoptotic potential, Hoechst 33342 staining was performed in HeLa cells treated with synthesized compounds, doxorubicin, and untreated cells. Nuclear condensation and chromatic fragmentation was observed under fluorescent microscopy. Condensation and fragmentation of nuclei characteristic of apoptotic cells were evident in HeLa cells treated with newly synthesized compounds, which was comparable to the standard drug doxorubicin. The percentage of apoptotic cells increased significantly in cells exposed to the newly synthesized compounds, and there was membrane blebbing, fragmentation of DNA, and condensed nuclei in the treated cells (Fig. 2). In comparison with normal cells, Hoechst 33342 uniformly stained DNA so that the condensed chromatin was clearly visible as crescents around the nuclear surface or as spherical beads. It was also observed that the apoptotic cells had stronger blue fluorescence compared with non-apoptotic cells. The nuclear condensation was very prominent in compound **18d** [2-(3,4-dimethoxyphenyl)-3-(2-(3,4-dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)-thiazolidin-4-one], the thiazolidinone analog of 6-amino-3-methoxy-3′,4′-dimethoxyflavone.

Apoptosis is accompanied by DNA fragmentation, which can be identified by analyzing DNA content in cell-cycle analysis.[36–38] Figure 3 shows the DNA content histogram obtained for compound **18d** and for the standard drug doxorubicin along with untreated cells. All the compounds showed a DNA histogram characteristic of apoptosis. The thiazolidinone analogs showed an increased percentage of DNA in the G_2/M phase, characteristic of G_2/M phase arrest, as seen with doxorubicin treatment. The presence of the sub-G_0 phase DNA



	18a	18b	18c	18d	18e
R_1	H	H	Cl	OCH_3	H
R_2	H	OCH_3	Cl	OCH_3	OH

Reagents and conditions: (i) 3,4-dimethoxybenzaldehyde, ethanol, 10% KOH, 30°C , 14 h; (ii) ethanol, 20% NaOH, 20% H_2O_2, 30°C , 3 h; (iii) dry acetone, dimethyl sulfate, reflux, 7 h; (iv) conc. HCl–water (1 : 1), boil, 30 min; (v) substituted Ar-CHO, thioglycolic acid, 0°C , 20–50 min; DCC, 30 min to 1 h at r.t. Yield is shown as percentage in brackets.

Chart 3. Synthesis of 6-Amino-3-methoxy-3′,4′-dimethoxyflavone and Its Thiazolidinone Analogs

content is a parameter indicative of fragmented DNA and thus apoptosis. In growing and dividing cells, the sub-G_0 phase will be zero, as seen in the untreated HeLa control. It was also observed that compound **18d** showed an increase in DNA content from 18.65% (untreated control) to 28.73% in the G_2/M phase, which indicated that compound **18d** caused the G_2/M phase arrest. An increase in the content of DNA in the sub-G_0 phase and G_2/M phase is a characteristic of increased apoptotic cell death.

Based on the results of the cytotoxicity assays and apoptotic evaluation, an *in vivo* evaluation of the anti-cancer potential of the synthesized compounds was planned. In the *in vivo* study, parameters such as mean survival time, percentage increase in life span (% ILS), and increase in body weight were measured and compared with that of the standard drug and control. A safe dose was selected from the data obtained from acute toxicity studies.[39] Accordingly, a dose of 100 mg/kg body weight per day was selected as the safe dose. The mean survival time and % ILS were studied in Ehrlich's ascites tumor induced mice in two doses of 100 and 50 mg/kg/d.[40] The treated mice showed an increase in survival time (mean) from 17 d (for the untreated control) to 27.5 d (Table 2). The standard drug cisplatin showed a mean survival time of 34 d. Among the tested compounds, compound **18d** was found to be most active in terms of the survival time of 27.5 d at a dose of 100 mg/kg/d. The dosage of 50 mg/kg/d showed a similar result with a maximum survival time of 26.5 d for compound **18d**. The % ILS also was calculated and ranged from 32.72% to a maximum of 58.68% at a dose of 100 mg/kg/d, and from 28.87 to 50.99% at a dose of 50 mg/kg/d (Table 2). Compound **18d** demonstrated the maximum % ILS of 58.68% at a dose of 100 mg/kg/d and 50.99% at a dose of 50 mg/kg/d. The statistical significance of the results were analyzed using the Dunnett's test for one-way ANOVA and was found to be significant in comparison with the control ($p<0.01$). Another parameter measured was the increase in tumor volume. There was a rapid increase in the tumor volume in the untreated mice. It was also observed that treatment with the novel analogs resulted in a reduction of tumor volume in comparison with the control. The reduction in the tumor volume in turn resulted in the reduction of body weight. The percentage increase in body weight at a dose of 100 mg/kg body weight was determined as per the standard procedure.[41] There was an average increase of 58.8% in body weight for the untreated mice, whereas the positive control al-

Table 1. Results of MTT Assay in HeLa, MDA MB 435 and Vero Cells

Compounds screened	R_1	R_2	IC_{50} Value* (μg/mL)		
			HeLa	MDA-MB-435	Vero
5	6-Aminoflavone		55.75±5.30	59.48±0.56	>300
6a	H	H	17.39±0.67	20.06±1.00	>300
6b	H	OCH_3	13.89±1.30	16.27±0.90	>200
6c	Cl	Cl	24.91±1.00	25.83±0.66	>200
6d	OCH_3	OCH_3	11.89±0.66	15.43±0.68	>200
6e	H	OH	15.52±0.87	17.89±0.68	>200
11	6-Amino-3-methoxyflavone		42.34±0.48	46.41±0.52	>300
12a	H	H	12.75±0.80	14.36±1.10	>200
12b	H	OCH_3	8.99±0.69	9.97±0.96	>200
12c	Cl	Cl	18.37±0.97	21.14±0.40	>200
12d	OCH_3	OCH_3	4.20±0.84	9.66±0.96	>200
12e	H	OH	11.80±0.42	13.69±0.70	>200
17	6-Amino-3-methoxy-3′,4′-dimethoxyflavone		28.87±0.89	33.18±0.36	>300
18a	H	H	2.37±0.49	4.71±0.40	>150
18b	H	OCH_3	1.91±0.30	3.05±1.10	>100
18c	Cl	Cl	13.47±0.47	15.84±0.88	>150
18d	OCH_3	OCH_3	1.19±0.96	2.36±0.70	>100
18e	H	OH	2.08±1.00	3.93±0.15	>150
Doxorubicin			0.69±0.12	0.77±0.19	>300

*Values are the average of three determinations±SDEV (Dox: doxorubicin standard).

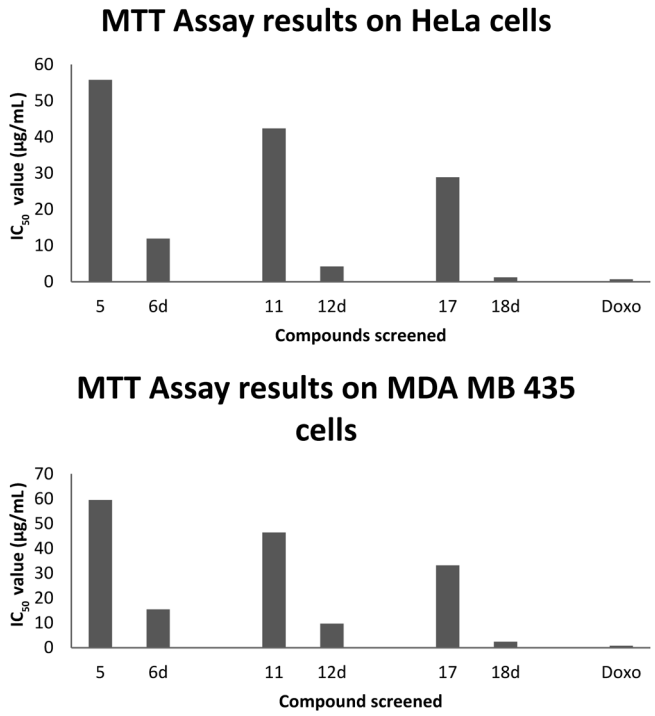
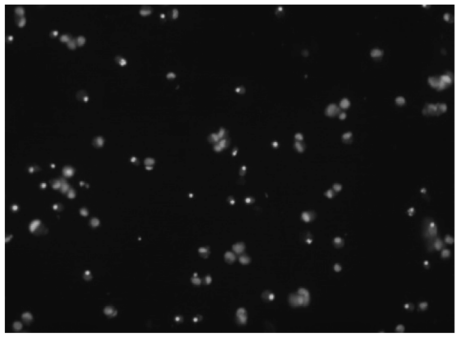


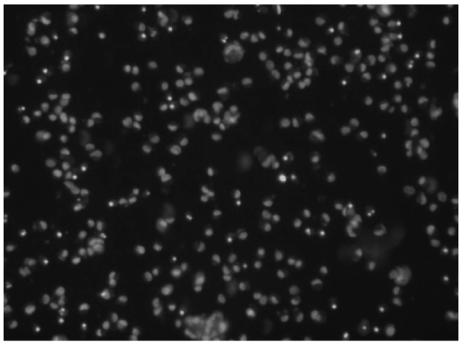
Fig. 1. Cytotoxic Potential of Selected Compounds against HeLa and MDA-MB-435 Cells in Comparison with Doxorubicin

lowed only an increase of 9.81%. The percentage increase in body weight for the mice treated with synthesized compounds ranged from 19.73 to 10.98%, proving the anti-cancer potential of these novel analogs (Table 2). The highly active compound among the tested compounds was found to be compound **18d**, which allowed only an increase of 10.99% on day 15 of treatment. The activity of this compound was comparable to that of cisplatin at a dose of 3.5 mg/kg/d (9.81±0.48). It is clear from the results that this treatment did not allow a drastic increase in body weight in contrast to the untreated negative control. The results were analyzed for the level of significance as per Tukey's multiple comparisons test for one-way ANOVA, and a satisfactory result was obtained.

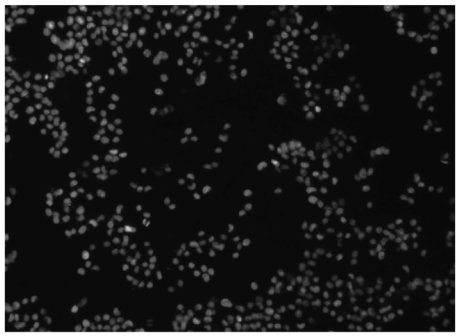
The determination of lipophilicity and pK_a gave an insight into the permeability and ionization behavior of the synthesized compounds (Table 3). The aminoflavones (compounds **5**, **11** and **17**) showed a low log$P$ value. This can be attributed to the presence of polar amino group in these molecules, which makes them impermeable through the cell membrane. The thiazolidinone analogs that were devoid of these polar substituents demonstrated higher log$P$ values, but the values were within the limits of the Lipinski scale. The anti-cancer activity evidenced by the MTT assays was in support of this observation. The aminoflavones with lower log$P$-values showed a higher IC_{50} value and the analogs with higher log$P$-values showed lower IC_{50} values and higher activity. A plot showing the relationship between log$P$-value and anti-cancer activity is shown in Fig. 4. A log$P$ value of 4.43 for compound **18d** suggests that it is highly lipophilic which supports the observed potency for this compound. This also suggests the possibility of high hepatic clearance through CYP450 metabolism, lower solubility, and elevated plasma binding. Determination of pK_a values indicated the ionization range of the synthesized compounds (Table 3). pK_a affects the solubility and permeability of acidic and basic compounds. This in turn depends on the ratio of the ionized and un-ionised species. The ratio of the amount of the drug in ionized and unionized form at different pH conditions of stomach and lumen can be arrived at based on the Henderson–Hasselbalch equation. The pK_a value of 5.96 to 7.97 suggests the title compounds are highly ionized in the acidic conditions of the stomach and will be highly permeable across the lipid membrane of the stomach. The preliminary solubility study data suggests that the newer analogs have poor aqueous solubility. The information obtained on pK_a value can be used in the salt selection process for improving



Doxorubicin treated cells



Compound **18d** treated cells



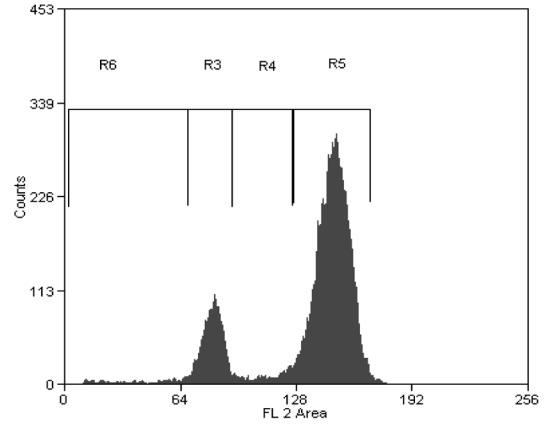
Untreated cells

Fig. 2. Hoechst Staining Picture of HeLa Cells Treated with Doxorubicin, Compound **18d** and Untreated HeLa Cells

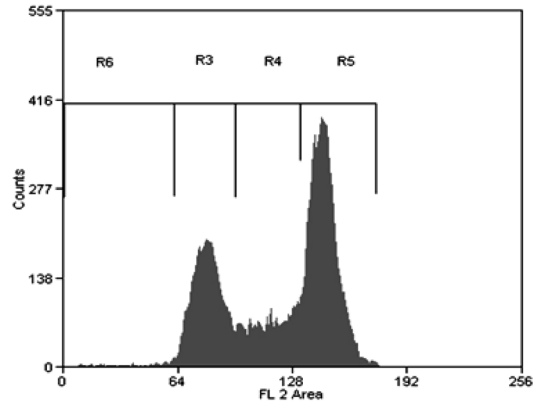
the solubility of these compounds.

Experimental

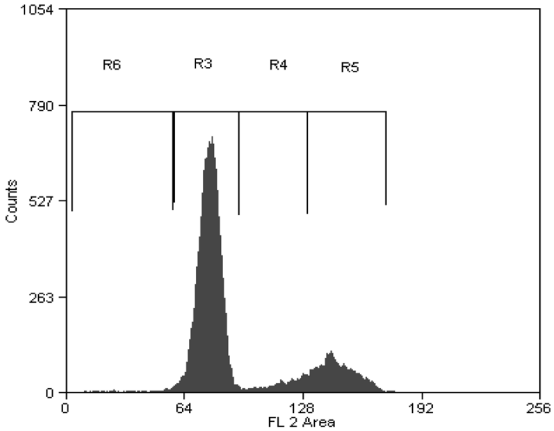
Chemicals such as aldehydes, paracetamol, thioglycollic acid, *N*,*N*′-dicyclohexyl carbodiimide, MTT Hoechst 33342 stain and doxorubicin were obtained from Sigma-Aldrich, U.S.A. The anticancer drug cisplatin was obtained as a gift sample from Dabur India Limited. The melting points of the synthesized compounds were determined using a digital melting point apparatus. *Rf* values of the synthesized compounds were determined using pre-coated silica gel plates (Merck) using a mobile phase composition consisting of hexane and acetone in the ratio 6 : 4. Shimadzu FTIR 8310 instrument was used to record the IR spectra of the synthesized compounds. NMR analyses were performed using AMX Bruker 400 MHz instrument. GCMS QP 5050 instrument was used to obtain the electron ionization-mass spectra (EI-MS) data. Electro-



Doxorubicin treated



Compound **18d** treated



Untreated cells

Fig. 3. DNA Histogram of HeLa Cells Treated with Doxorubicin, Compound **18d** and Untreated Cells

spray ionization (ESI)-MS spectra were obtained using the instrument Equire 3000 plus, Bruker Daltonics, Germany. UV spectra were recorded using a UV-Visible spectrophotometer, Shimadzu UV-2400PC. The cell lines used in this study such as MDA MB 435 and HeLa were maintained by the Department of Molecular Reproduction and Developmental Genetics (MRDG) of Indian Institute of Science in Bangalore. Olympus fluorescent microscope was used for the Hoechst staining analysis and the MoFlo FACS with 488 nm solid state lasers

Table 2. Mean Survival Time and Percentage Increase in Body Weight

Compound code	Dose (mg/kg/d)	Mean survival time (d)†	Mean % ILS	Average % increase in body weight in grams±S.E.M.‡
- ve control	—	17.33±0.21	—	58.67±1.46[a)]
Cisplatin	3.50	34.00±0.63 [a)]	96.19	9.81±0.48[a)]
6d	100	23.00±0.36 [a)]	32.72	19.73±1.69[a)]
	50	22.33±0.33 [a)]	28.87	—
12d	100	24.00±0.36 [a)]	38.49	16.79±1.43[a)]
	50	23.00±0.36 [a)]	32.72	—
18b	100	25.83±0.30 [a)]	49.07	13.62±0.52[a)]
	50	24.50±0.42 [a)]	41.37	—
18d	100	27.50±0.42 [a)]	58.68	10.99±0.40[a)]
	50	26.16±0.30 [a)]	50.99	—

†n=6 animals in each group, *a*) $p<0.01$ *vs.* control as per Dunnet's multiple comparisons test for one-way ANOVA. Days of treatment=9, values are expressed as mean±S.E.M. ‡n=6 animals in each group, *a*) $p<0.001$ *vs.* control as per Tukey's test for one-way ANOVA. Days of treatment=9, values are expressed as mean±S.E.M.

Table 3. Log *P*-Value, pK_a and Preliminary Solubility Data of Synthesized Aminoflavones and Selected Compounds

Compound	Log *P* value	pK_a*	Solubility
5	0.84	7.97	Partially soluble in water, freely soluble in methanol, DMSO
11	1.23	6.34	Partially soluble in water, freely soluble in methanol, DMSO
17	1.88	7.12	Partially soluble in water, freely soluble in methanol, DMSO
6d	3.28	6.09	Insoluble in water, partially soluble in methanol, freely soluble in chloroform, DMSO
12d	3.84	5.96	Insoluble in water, partially soluble in methanol, freely soluble in chloroform, DMSO
18b	4.09	5.34	Insoluble in water, partially soluble in methanol, freely soluble in chloroform, DMSO
18d	4.43	6.72	Insoluble in water, partially soluble in methanol, freely soluble in chloroform, DMSO

*pK_a value calculated using Henderson–Hasselbalch equation.

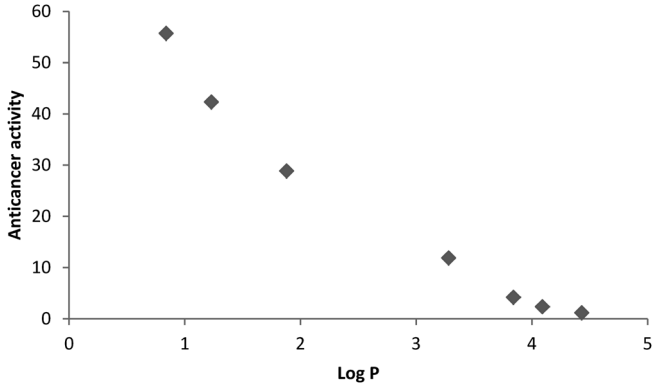


Fig. 4. Anticancer Activity *vs.* Log *P*-Value of Selected Compounds

was used for cell cycle analysis.

Chemistry

Synthesis of 6-Aminoflavone (**5**)

To a suspension of paracetamol (**1**) (66.00 mmol) and anhydrous aluminium chloride (16.00 mmol) in nitrobenzene (50 mL) was added acetyl chloride (0.013 mol) over a period of 0.5 h. The temperature was gradually raised to 130°C over a period of 0.5 h and then maintained for 2.5 h. The mixture was then cooled to 40°C within 0.5 h and poured into a mixture of 350 g crushed ice and 30 mL conc. hydrochloric acid with vigorous stirring and filtered. The crude product thus obtained was washed with water till free from acid followed by toluene and crystallized from isopropanol to yield light brown needle shaped crystals of 5-acetamino-2-hydroxyacetophenone (**2**). Yield 81.50%. mp 160–164°C. This product (**2**) was then mixed with benzaldehyde (55.00 mmol) in ethanol (150 mL) and a 10% aqueous KOH solution was added with stirring. The reaction mixture was stirred at 30°C for 12 h and poured on to crushed ice containing 5 N hydrochloric acid. The precipitated product was washed with cold water, dried and crystallized from methanol to yield dark yellow crystals of 5′-acetamino-2′-hydroxychalcone (**3**). Yield 85.16%. mp 197°C. The compound (**3**) obtained above was added to a solution of iodine in 10 mL of DMSO and this mixture was refluxed for 30 min at 175°C. The reaction mixture was then cooled, poured on ice cold water (20–30 mL) and added 10% sodium thiosulphate solution to remove the excess iodine and then purified by recrystallization from methanol to obtain 6-acetaminoflavone (**4**). Yield 81.81%. mp 272°C. This compound (**4**) was then hydrolyzed using a mixture of conc. HCl–water (1 : 1) and boiled for 30 min until the compound got completely dissolved. At the end of 30 min ice cold water was added to separate the crude aminoflavone. The crystals obtained were dissolved in water and basified by adding 10% $NaHCO_3$ solution to obtain a yellow colored precipitate of aminoflavone (**5**). The crude product obtained was washed with water, dried, and recrystallized from ethanol. Yield 92.00%. mp 196–199°C. *Rf* value 0.347. UV λ_{max} (MeOH) nm: 282, 374. IR (KBr) cm^{-1}: 1614, 3342, 3404, 3036. MS (EI) *m*/*z*: 237 (M^+), 135, 209, 102, 79. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 5.5 (s, 2H, NH_2 at C-6), 6.86 (s, 1H, H-3), 7.48 (d, 1H, H-5, J=8.8 Hz), 8.03 (m, 2H, H-7, 8), 7.10 (m, 2H, H-2′, 6′), 7.57 (m, 3H, H-3′, 4′, 5′).

Synthesis of 6-Amino-3-methoxy Flavone (**11**)

To a suspension of 2′-hydroxy-5′-acetaminochalcone (**3**) (10.00 mmol) in ethanol was added 20% NaOH with stirring followed by the addition of 20% H_2O_2 over a period of 30 min keeping the temperature of the reaction below 30°C (Algar–Flyn–Oyamada oxidation). After the complete addition of H_2O_2, the temperature was maintained at 30°C for 3 h and

Table 4. Reaction Conditions for Synthesis of Thiazolidinone Analogs and Their Yield

Compound	Amine used (2.50 mmol)	Aldehyde used (5.00 mmol)	Optimized reaction conditions		Yield mg (%)
			Stage 1	Stage 2	
6a	6-Aminoflavone	Benzaldehyde	0°C for 5 min	50 min at r.t.	750 (44.1)
6b		4-Methoxybenzaldehyde	0°C for 10 min	30 min at r.t.	650 (40.3)
6c		3,4-Dichlorobenzaldehyde	0°C for 15 min	1 h at r.t.	850 (49.0)
6d		3,4-Dimethoxybenzaldehyde	0°C for 15 min	1 h at r.t.	580 (38.4)
6e		4-Hydroxybenzaldehyde	0°C for 15 min	1 h at r.t.	600 (47.1)
12a	6-Amino-3-methoxyflavone	Benzaldehyde	0°C for 20 min	30 min at r.t.	870 (64.1)
12b		4-Methoxybenzaldehyde	0°C for 30 min	30 min at r.t.	920 (80.0)
12c		3,4-Dichlorobenzaldehyde	0°C for 30 min	30 min at r.t.	1120 (90.3)
12d		3,4-Dimethoxybenzaldehyde	0°C for 30 min	1 h at r.t.	840 (68.4)
12e		4-Hydroxybenzaldehyde	0°C for 50 min	1 h at r.t.	760 (68.0)
18a	6-Amino-3-methoxy-3′,4′-dimethoxyflavone	Benzaldehyde	0°C for 20 min	30 min at r.t.	900 (64.3)
18b		4-Methoxybenzaldehyde	0°C for 30 min	50 min at r.t.	950 (65.4)
18c		3,4-Dichlorobenzaldehyde	0°C for 30 min	30 min at r.t.	1210 (71.1)
18d		3,4-Dimethoxybenzaldehyde	0°C for 30 min	1 h at r.t.	890 (59.1)
18e		4-Hydroxybenzaldehyde	0°C for 50 min	1 h at r.t.	810 (66.0)

the reaction mixture was poured into crushed ice (500–800 g) containing 5 N HCl (30–35 mL). The precipitated product was filtered, washed with cold water to remove the excess of acid and allowed to dry. The crude product obtained was recrystallized from chloroform–acetone (8 : 2) to get colorless crystals of 6-acetamino-3-hydroxyflavone (**9**). Yield 75.24%. mp 279–282°C. The flavone (**9**) obtained above was then suspended in dry acetone containing powdered anhydrous (K_2CO_3) and dimethyl sulfate and refluxed for 5 h. The solvent was evaporated under reduced pressure and the residue diluted with water, filtered, washed well with water and dried. This was later recrystallized from MeOH–chloroform (8 : 2) to obtain pure 6-acetamino-3-methoxyflavone (**10**). Yield 91.00%. mp 205°C. This methoxy flavone (**10**) was subjected to acid hydrolysis with a mixture of conc. HCl–water (1 : 1) by boiling it for 30 min. At the end of 30 min ice cold water was added and cooled to room temperature; a yellow crystalline solid separated from the clear solution. The crystals were dissolved in water and basified by adding 10% $NaHCO_3$ solution to obtain 6-amino-3-methoxyflavone (**11**), which was then washed with water, dried, and recrystallized from ethanol (95%). Yield 91.30%. mp 174–176°C. *Rf* value 0.593. UV λ_{max} (MeOH) nm: 250, 280. IR (KBr) cm^{-1}: 1629, 1494, 1602, 3450. MS (EI) *m/z*: 266 (M^+), 220, 196, 180, 135, 105, 77. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 3.77 (s, 3H, H-OCH_3), 5.51 (s, 2H, H-NH_2), 7.05 (dd, 1H, H-7, J=2.8 Hz), 7.12 (d, 1H, H-8, J=2.8 Hz), 7.44 (d, 1H, H-5, J=8.8 Hz), 7.58 (m, 3H, H-3′, 4′, 5′), 7.99 (dd, 2H, H-2′, 6′, J=2.63 Hz).

Synthesis of 6-Amino-3-methoxy-3′,4′-dimethoxyflavone (**17**)

A 10% aqueous KOH solution was added dropwise with stirring to a mixture of 5-acetamino-2-hydroxylacetophenone (**2**) (83.00 mmol) and 3,4-dimethoxy benzaldehyde (91.00 mmol) in ethanol. The reaction mixture was stirred at 30°C for 14 h and poured into crushed ice containing 5 N hydrochloric acid. The precipitated product was washed with cold water, dried and recrystallized from methanol to obtain 5′-acetamino-3-(3,4-dimethoxyphenyl)-2′-hydroxychalcone (**14**). Yield 70.20%. mp 184°C. This product (**14**) was taken in a dry round bottom flask and added ethanol (200 mL) to make a suspension. To this was added 20% NaOH with stirring followed by the addition of 20% H_2O_2 over a period of 30 min. Temperature of the reaction was kept below 30°C and then maintained at 30°C for 3 h. The reaction mixture was poured on to crushed ice (500–800 g) containing 5 N HCl (30–35 mL). The precipitated product was filtered, washed with cold water to remove the acid and dried. Recrystallization using chloroform–methanol (8 : 2) yielded 6-acetamino-3-hydroxy-3′,4′-dimethoxyflavone (**15**). Yield 78.60%. mp 250°C. The compound (**15**) was then suspended in dry acetone containing powdered anhydrous K_2CO_3 and dimethyl sulfate. The suspension was refluxed with stirring for 7 h. As the reaction proceeded, the suspension dissolved. The solvent was evaporated under reduced pressure and the residue diluted with water. The precipitate obtained was filtered, washed well with water and dried. Recrystallization of this precipitate from methanol–chloroform (8 : 2) yielded colorless crystals of 6-acetamino-3-methoxy-3′,4′-dimethoxyflavone (**16**). Yield 87.00%. mp 228–232°C. Compound (**16**) was subjected to acid hydrolysis by boiling for 30 min with a mixture of conc. HCl–water (1 : 1). Ice cold water was added to this mixture to separate the crystals of free amine from the clear solution. The crystals obtained were again dissolved in water and basified by adding 10% $NaHCO_3$ solution to obtain yellow colored precipitate of 6-amino-3-methoxy-3′,4′-dimethoxyflavone (**17**). The crude product obtained was washed with water, dried, and recrystallized from ethanol. Yield 91.30%. mp 186°C. *Rf* value 0.38. UV λ_{max} (MeOH) nm: 248, 286, 342. IR (KBr) cm^{-1}: 1624, 1494, 3441. MS (EI) *m/z*: 327(M^+), 312, 296, 280, 284, 165, 135, 107, 79. ^{1}H-NMR (DMSO, 400 MHz) δ: 3.77 (s, 3H, H-OCH_3 at $C_{3'}$), 3.84 (s, 6H, H-OCH_3 at C-3, C-4′), 5.48 (s, 2H, H-NH_2), 7.45 (d, 1H, H-5, J=8.8 Hz), 7.03 (dd, 1H, H-7, J=2.8 Hz, J=2.8 Hz), 7.14 (s, 1H, H-2′), 7.12 (d, 1H, H-8, J=8.7 Hz), 7.61 (d, 1H, H-5′), 7.65 (d, 1H, H-6′, J=8.5 Hz).

General Procedure for Synthesis of Thiazolidinone Analogues [6a–e, 12a–e, and 18a–e] This is a two-stage reaction process carried out as a one-pot synthetic procedure. In the first stage of the reaction, thioglycollic acid was added to an ice cold mixture of aminoflavone (**5**/**11**/**17**) and appropriate aldehyde (**a–e**) in dry tetrahydrofuran with stirring. To this reaction mixture was added DCC and was stirred further for

another 50 min at room temperature to complete the reaction, which forms the second stage. The reaction conditions for various derivatives are depicted in Table 4. The precipitated dicyclohexyl urea was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue obtained was taken up in chloroform and the organic layer was washed successively with 5% $NaHCO_3$ and then finally with brine. The organic layer was dried over sodium sulphate and the solvent was removed under reduced pressure to get crude product, which was then purified by column chromatography on silica gel using $CHCl_3$–MeOH (8 : 2) as the mobile phase to obtain the title compounds [**6a–e, 12a–e, and 18a–e**]. The novel analogues were characterized by a combination of spectral techniques.

3-(4-Oxo-2-phenyl-4*H*-chromen-6-yl)-2-phenylthiazolidin-4-one (**6a**)

Yield 44.1% as micro-crystalline powder. mp 225°C. *Rf* value 0.439. UV λ_{max} (MeOH) nm: 298. IR (KBr) cm^{-1}: 1670, 1654, 1132, 692. MS (ESI) *m/z*: 400 (M+1). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.00 (s, 1H, H-Ar-CH- of thiazolidinone ring), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=14.3 Hz, J=14.5 Hz), 6.70 (s, 1H, H-3), 7.94 (s, 1H, H-5), 7.80 (dd, 2H, H-7, 8, J=9.3 Hz, J=8.7 Hz), 7.40 (d, 2H, H-2′, 6′, J=6.6 Hz), 8.10 (d, 2H, H-2″, 6″, J=6.3 Hz), 7.30 (m, 3H, H-3′, 4′, 5′), 7.50 (m, 3H, H-3″, 4″, 5″). ^{13}C-NMR (300 MHz, $CDCl_3$) δ: 162.65 (C-2), 106.68 (C-3), 176.47 (C-4), 119.27 (C-5), 134.9 (C-6), 128.64 (C-7), 119.27 (C-8), 153.35 (C-9), 123.4 (C-10), 130.96 (C-1′), 126.37 (C-2′, C-6′), 128.7 (C-3′, C-5′), 131.44 (C-4′), 63.43 (C-2*), 170.80 (C-4*), 32.96 (C-5*), 139.5 (C-1″), 129.07 (C-2″, C-6″), 128.77 (C-5″, C-3″), 127.2 (C-4″).

2-(4-Methoxyphenyl)3-(4-oxo-2-phenyl-4*H*-chromen-6-yl)-thiazolidin-4-one (**6b**)

Yield 40.3%. Colorless microcrystalline powder. mp 225°C. *Rf* value 0.469. UV λ_{max} (MeOH) nm: 260, 298. IR (KBr) cm^{-1}: 1670, 1642, 1143, 777, 1452, 1442. MS (ESI) *m/z*: 430 (M+1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 3.65 (s, 3H, H-OCH_3 at C-4″), 6.99 (s, 1H, H-Ar-CH- of thiazolidinone ring), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=13.34 Hz, J=13.51 Hz), 6.60 (s, 1H, H-3), 7.91 (s, 1H, H-5), 7.73–7.86 (d, 2H, H-7, 8, J=26.7 Hz), 8.03 (d, 2H, H-2″, 6″, J=5.4 Hz), 7.32 (d, 2H, H-2′, 6′, J=7.2 Hz), 6.8 (d, 2H, H-3′, 5″, J=7.2 Hz), 7.56 (m, 3H, H-3′, 4′, 5′).

2-(3,4-Dichlorophenyl)3-(4-oxo-2-phenyl-4*H*-chromen-6-yl)-thiazolidin-4-one (**6c**)

Yield 49.0%. Colorless microcrystalline powder. mp 200°C. *Rf* value 0.551. UV λ_{max} (MeOH) nm: 260, 288. IR (KBr) cm^{-1}: 1672, 1641, 1134, 677, 813. MS (ESI) *m/z*: 469 (M+1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.99 (s, 1H, H-Ar-CH- of thiazolidinone ring), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.9 Hz, J=15.6 Hz), 6.98 (s, 1H, H-3), 7.97 (s, 1H, H-5), 8.05 (d, 2H, H-2′, 6′, J=6.3 Hz), 7.46 (d, 1H, H-4′, J=8.4 Hz), 7.80 (m, 3H, H-7, 8, 2″), 7.5 (m, 4H, H-3′, 5′, 5″, 6″).

2-(3,4-Dimethoxyphenyl)3-(4-oxo-2-phenyl-4*H*-chromen-6-yl)thiazolidin-4-one (**6d**)

Yield 38.4%. Off white powder. mp 186°C. *Rf* value 0.429. UV λ_{max} (MeOH) nm: 262, 296. IR (KBr) cm^{-1}: 1693, 1649, 1141, 700, 1454. MS (ESI) *m/z*: 460 (M+1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 3.66 (d, 6H, H-OCH_3, J=14.4 Hz), 7.00 (d, 2H, H-Ar-CH- of thiazolidinone ring, H-2″, J=9.3 Hz), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=12.9 Hz, J=12.6 Hz), 6.57 (s, 1H, H-3), 7.94 (s, 1H, H-5), 7.7–7.8 (m, 2H, H-7, 8), 8.00 (d, 2H, H-2′, 6′, J=6.9 Hz), 6.90 (d, 1H, H-5″, J=9 Hz), 6.77 (d, 1H, H-6,″ J=8.4 Hz), 7.50 (m, 3H, H-3′, 4′, 5′).

2-(3,4-Dimethoxyphenyl)3-(4-oxo-2-phenyl-4*H*-chromen-6-yl)thiazolidin-4-one (**6e**)

Yield 47.1%. Colorless microcrystalline powder. mp 162°C. *Rf* value 0.347. UV λ_{max} (MeOH) nm: 260, 298. IR (KBr) cm^{-1}: 3404, 3313, 1674, 1629, 1139, 709, MS (ESI) *m/z*: 416.1 (M+1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.98 (s, 1H, H-Ar-CH- of thiazolidinone ring), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.9 Hz), 6.52 (s, 1H, H-3), 7.887 (s, 1H, H-5), 7.77 (s, 2H, H-7, 8) 8.04 (d, 2H, H-2′, 6′, J=6.3 Hz), 7.2 (d, 2H, H-2″, 6″, J=8.4 Hz), 6.60 (d, 2H, H-3″, 5″, J=8.4 Hz), 7.53 (m, 3H, H-3′, 4′, 5′).

3-(3-Methoxy-4-oxo-2-phenyl-4*H*-chromen-6-yl)-2-phenylthiazolidin-4-one (**12a**)

Yield 64.1%. White powder. mp 185°C. *Rf* value 0.596. UV λ_{max} (MeOH) nm: 306, 258. IR (KBr) cm^{-1}: 1679, 1631, 1452, 1159, 690. MS (EI) *m/z*: 428 (M^+), 410, 354, 292, 178, 251, 222, 135. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.80 (s, 3H, H-OCH_3), 4.00 (dd, 2H, H- CH_2 of thiazolidinone ring, J=18 Hz, J=15.6 Hz), 6.7 (s, 1H, H-CH of thiazolidinone ring), 8.00 (m, 3H, H-5, 2″, 6″), 7.68 (d, 1H, H-8, J=8.7 Hz), 7.70 (d, 1H, H-7, J=9.3 Hz), 7.50 (d, 3H, H-3″, 4″, 5″), 7.41 (d, 2H, H-2′, 6′), 7.29 (m, 3H, H-3′, 4′, 5′). ^{13}C-NMR (DMSO, 300 MHz) δ: 173.33 (C-4), 170.85 (C-4*), 155.2 (C-2), 152.5 (C-9), 140.6 (C-3), 139.6 (C-1″), 134.5 (C-6), 131.3 (C-1′), 130.9 (C-4′), 130.3 (C-4″), 128.8 (C-7), 128.65 (C-3″, C-5″), 128.3 (C-3′, C-5′), 128.1 (C-2″, C-6″), 127.2 (C-2′, C-6′), 123.63 (C-10), 121.1 9C-5), 119.25 (C-8), 63.42 (C-2*), 59.82 (C-OCH_3), 33.0 (C-5*).

3-(3-Methoxy-4-oxo-2-phenyl-4*H*-chromen-6-yl)-2-(4-methoxyphenyl)thiazolidin-4-one (**12b**)

Yield 80.0%. Off white powder. mp 171°C. *Rf* value 0.553. UV λ_{max} (MeOH) nm; 306, 258. IR (KBr) cm^{-1}: 1687, 1626, 1491, 1161, 688. MS (ESI) *m/z*: 459 (M+1). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.65 (s, 3H, H-OCH_3 at C4″), 3.76 (s, 3H, H-OCH_3 at C3), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz), 6.60 (s, 1H, H-CH of thiazolidinone ring), 7.99 (s, 3H, H-5, 7, 8), 7.50 (s, 3H, H-3′, 4′, 5′), 7.36 (d, 2H, H-2′, 6′, J=8.7 Hz), 6.80 (d, 2H, H-2″, 6″, J=24.9 Hz), 7.70 (d, 2H, H-3″, 5″).

2-(3,4-Dichlorophenyl)-3-(3-methoxy-4-oxo-2-phenyl-4*H*-chromen-6-yl)thiazolidin-4-one (**12c**)

Yield 90.3%. White fine powder. mp 169°C. *Rf* value 0.617. UV λ_{max} (MeOH) nm: 306, 246. IR (KBr) cm^{-1}: 1672, 1640, 1481, 1155, 680, 819. MS (ESI) *m/z*: 498 (M+1). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.90 (s, 3H, H-OCH_3), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=18 Hz, J=15.7 Hz), 6.98 (s, 1H, H-CH of thiazolidinone ring), 7.86 (s, 1H, H-5), 8.01 (d, 2H, H-2′, 6′, J=6.2 Hz), 7.43 (d, 1H, H-4′, J=8.3 Hz), 7.70 (m, 3H, H-7, 8, 2″), 7.40 (m, 4H, H-3′, 5′, 5″, 6″).

2-(3,4-Dimethoxyphenyl)-3-(3-methoxy-4-oxo-2-phenyl-4*H*-chromen-6-yl)thiazolidin-4-one (**12d**)

Yield 68.4%. Off white fine powder. mp 203°C. *Rf* value 0.51. UV λ_{max} (MeOH) nm: 306, 246. IR (KBr) cm^{-1}: 1678, 1640, 1481, 1148, 698. MS (ESI) *m/z*: 490 (M+1). ^{1}H-NMR (DMSO, 300 MHz) δ: 3.64 (s, 3H, H- of OCH_3 at C4″), 3.69 (s, 6H, H-OCH_3 at C3 & C3″), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz), 6.70 (s, 1H, H-CH of thiazolidinone ring), 6.77 (d, 1H, H-6″, J=8.4 Hz), 6.90 (d, 1H, H-5″, J=7.8 Hz), 7.02 (s, 1H, H-2″), 7.68 (d, 1H, H-8, J=8.7 Hz), 7.77 (d, 1H, H-7, J=10.2 Hz), 8.00 (m 3H, H- 5′, 6′),

7.54 (m, 3H, H-3′, 4′, 5′).

2-(4-Hydroxyphenyl)-3-(3-methoxy-4-oxo-2-phenyl-4*H*-chromen-6-yl)thiazolidin-4-one (**12e**)

Yield 68.0%. White fine powder. mp 259°C. *Rf* value 0.426. UV λ_{max} (MeOH) nm: 306, 256. IR (KBr) cm^{-1}: 3292, 1670, 1639, 1481, 1148, 690. MS (ESI) *m/z*: 446 (M +1). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.76 (s, 3H, H-OCH_3), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz), 6.52 (s, 1H, H-CH of thiazolidinone ring), 6.60 (d, 2H, H-3″, 5″, J=20.4 Hz), 7.2 (d, 2H, H-2″, 6″, J=8.4 Hz), 7.53 (s, 3H, H-3′, 4′, 5′), 7.67 (m, 2H, H-2′, 6′), 7.94 (d, 3H, H-5, 7, 8), 9.50 (s, 1H, H-OH).

3-(2-(3,4-Dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)-2-phenylthiazolidin-4-one (**18a**)

Yield 64.3%. Off white powder. mp 174°C. *Rf* value 0.5. UV λ_{max} (MeOH) nm: 344, 252. IR (KBr) cm^{-1}: 1689, 1629, 1514, 1141, 696. MS (EI) *m/z*: 489 (M^+), 474, 414, 310, 208, 192, 178, 135. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.76 (s, 3H, H-OCH_3 at C3′), 3.82 (s, 6H, H-OCH_3 at C3 and C4′), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz), 6.65 (s, 1H, H-CH of thiazolidinone ring), 7.1 (d, 1H, H-5′, J=8.7 Hz), 7.41 (d, 2H, H-2′, 6′, J=7.2 Hz), 7.22 (m, 3H, H-7, 8, 4″), 7.70 (m, 4H, H-2″, 3″, 5″, 6″). ^{13}C-NMR (DMSO-d_6, 300 MHz) δ: 155.05 (C-2), 139.96 (C-3), 173.04 (C-4), 121.07 (C-5), 134.42 (C-6), 128.76 (C-7), 119.16 (C-8), 152.30 (C-9), 123.53 (C-10), 122.41 (C-1′), 111.36 (C-2′), 151.08 (C-3′), 148.37 (C-4′), 111.56 (C-5′), 122.01 (C-6′), 139.52 (C-1″), 128.65 (C-2″, C-6″), 127.18 (C-3″, C-5″), 128.76 (C-4″), 63.45 (C-2*), 170.79 (C-4*), 32.96 (C-5*), 59.58 (C-OCH_3), 55.75 (C-OCH_3 at 3′ and 4′).

3-(2-(3,4-Dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)-2-(4methoxyphenyl)thiazolidin-4-one (**18b**)

Yield 65.4%. White fine powder. mp 152°C. *Rf* value 0.456. UV λ_{max} (MeOH) nm: 342, 252. IR (KBr) cm^{-1}: 1701, 1648, 1508, 1143. MS (ESI) *m/z*: 519 (M+1). ^{1}H-NMR (DMSO, 300 MHz) δ: 3.60 (s, 3H, H-OCH_3 at C4′), 3.70 (s, 3H, H-OCH_3 at C3′), 3.80 (s, 6H, H-OCH_3 at C3, C4″), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=7.5 Hz, J=16.2 Hz), 6.60 (s, 1H, H-CH of thiazolidinone ring), 6.80 (d, 2H, H-2″, 6″, J=8.1 Hz), 7.10 (m, 2H, H-7, 8), 7.33 (d, 2H, H-3″, 5″, J=8.1 Hz), 7.70 (s, 2H, H-2′, 6′), 7.84 (d, 1H, H-5′), J=8.1 Hz, 7.94 (s, 1H, H-5).

2-(3,4-Dichlorophenyl)-3-(2-(3,4-dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)thiazolidin-4-one (**18c**)

Yield 71.1%. Off white powder. mp 193°C. *Rf* value 0.53. UV λ_{max} (MeOH) nm: 342, 252. IR (KBr) cm^{-1}: 1695, 1633, 1512, 1141, 705, 835. MS (ESI) *m/z*: 557.9 (M+1). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.77 (s, 3H, H-OCH_3 at C3′), 3.85 (s, 6H, H-OCH_3 at C3 and C4′), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz,) 6.67 (s, 1H, H-CH of thiazolidinone ring), 8.12 (s, 1H, H-5), 7.99 (d, 2H, H-2″, 6′), 7.14 (m, 2H, H-7, 5′), 7.77 (m, 4H, H-8, 2″, 5″, 6″).

2-(3,4-Dimethoxyphenyl)-3-(2-(3,4-dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)thiazolidin-4-one (**18d**)

Yield 59.1%. Off white powder. mp 217°C. *Rf* value 0.33. UV λ_{max} (MeOH) nm: 432. IR (KBr) cm^{-1}: 1683, 1627, 1516, 1141, 704. MS (ESI) *m/z*: 549.5 (M+1). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.64 (s, 3H, H- of OCH_3 at C3), 3.70 (s, 6H, H-OCH_3 at C3′ & C3″), 3.83 (s, 6H, H-OCH_3 at C4′ & C4″), 4.00 (dd, 2H, H-CH_2 of thiazolidinone ring, J=6.9 Hz, J=15.6 Hz), 6.56 (s, 1H, H-CH of thiazolidinone ring), 7.97 (s, 1H, H-5), 7.60 (m, 3H, H-5′, 6′, 2′), 7.13 (m, 1H, H-5″), 6.90 (d, 1H, H-7, J=7.8 Hz), 7.03 (d, 1H, H-8, J=8.4 Hz), 6.70 (m, 2H, H-2″, 6″). ^{13}C-NMR (DMSO-d_6, 300 MHz) δ: 155.01 (C-2), 139.51 (C-3), 179.03 (C-4), 121.02 (C-5), 135.35 (C-6), 128.16 (C-7), 115.37 (C-8), 151.96 (C-9), 124.18 (C-10), 122.42 (C-1′), 111.54 (C-2′), 149.78 (C-3′), 148.01 (C-4′), 111.49 (C-5′), 121.09 (C-6′), 137.53 (C-1″), 114.08 (C-2″), 139.65 (C-3″), 142.22 (C-4″), 115.38 (C-5″), 124.06 (C-6″), 69.98 (C-2*), 171.02 (C-4*), 33.18 (C-5*), 58.74 (C-OCH_3 at C-3), 56.08 (C-OCH_3 at 3′, 4′, 3″, 4″).

3-(2-(3,4-Dimethoxyphenyl)-3-methoxy-4-oxo-4*H*-chromen-6-yl)-2-(4-hydroxyphenyl)thiazolidin-4-one (**18e**)

Yield 66.0%. Off white powder. mp 191°C. *Rf* value 0.29. UV λ_{max} (MeOH) nm: 344, 240. IR (KBr) cm^{-1}: 3246, 1683, 1636, 1487, 1139, 700. MS (ESI) *m/z*: 505 (M+1). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.76 (s, 3H, H-OCH_3 at C3′), 3.83 (s, 6H, H-OCH_3 at C3 and C4′), 3.90 (dd, 2H, H-CH_2 of thiazolidinone ring, J=15.6 Hz, J=15.6 Hz), 6.51 (s, 1H, H-CH of thiazolidinone ring), 6.60 (d, 2H, H-3″, 5″, J=8.4 Hz), 7.20 (d, 2H, H-2″, 6″, J=8.4 Hz), 7.12 (d, 1H, H-5′), 7.90 (s, 1H, H-5), 7.60 (m, 4H, H-7, 8, 2′, 6′), 9.50 (s, 1H, H-OH).

Biological Activity Evaluation of cytotoxic potential by MTT assay: Cytotoxic potential of the synthesized compounds were evaluated using HeLa and MDA-MB-435 cell lines. The cell count was adjusted to 5×10^3 cells/mL in a 96 well micro-titer plate. Seeded plates were incubated at 37°C in 5% CO_2 atmosphere for 24 h. Cells were then exposed to the synthesized compounds at concentrations 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 μg/mL. Cytotoxicity to normal cells were evaluated using Vero cells, wherein cells were plated using a suspension of 1×10^5 cells/mL and exposed to concentrations 300, 200, 100, 50, and 25 μg/mL. Doxorubicin was used as positive control at a concentration of 1.6 μg/mL. Vehicle control containing 1% DMSO and blank wells were also kept to balance the effect of vehicle and media. After an incubation of 48 h (72 h for Vero cells), the solution in the wells were discarded and a 5 mg/mL MTT solution (50 μL) was added. These wells were then incubated at 37°C for 3 h in 5% CO_2 atmosphere. On completion of the reaction, the solutions in the wells were replaced by DMSO (200 μL) and the color intensity was measured at 540 nm using a micro-plate reader to calculate the IC_{50} values.

Cell cycle analysis (Flow cytometry): HeLa cells treated with the synthesized compounds at their IC_{50} value concentrations were incubated for 48 h at 37°C and then trypsinised and fixed overnight in fixation buffer. Doxorubicin was used as the standard drug at a concentration of 1.6 μg/mL. The cells were then spun at 1200 rpm for 5 min and washed with phosphate buffer saline (PBS) and re-suspended in 1 mL PBS and incubated at 37°C for 4 h after adding 50 μg/mL RNase A. At the end of incubation 25 μg/mL propidium iodide was added to stain the cells. Cells were then analyzed using a flow cytometer and the histogram was recorded.

Hoechst staining analysis using fluorescent microscopy: HeLa cells (Human cervical cancer) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in 37°C incubator and 5% CO_2. The newly synthesized compounds were added to the cells at their log phase cell growth at their IC_{50} concentration and incubated for 48 h. Cells were monitored in a phase contrast microscope at an interval of 12 h till the end of the treatment to evaluate compound mediated cell death. At the end of treatment, cell

culture media were removed and cells were washed with 1× concentration of phosphate buffer saline (pH 7.4) and incubated at 37°C under 5% CO_2 atmosphere for 3 min after adding 0.5 mL of trypisin–ethylenediaminetetraacetic acid (EDTA) solution. After trypsinisation cells were rounded off and detached from the cell culture plate. To this was added 5 mL of DMEM with 10% fetal bovine serum and the cell suspension was centrifuged at 1200 rpm for 4 min at room temperature. The pellet was then re-suspended in the media and cells were counted. Cell count was adjusted to 50000 cells/mL. Cells were washed twice with PBS and stained with Hoechst 33342 (2 μg/mL in PBS) for 10 min at 37°C. At the end of staining, cells were centrifuged at 1200 rpm for 4 min at 4°C. The pellet was washed twice with PBS and spotted on a cover slip and observed under fluorescent microscope to spot normal cells as well as cells showing apoptotic morphology. Doxorubicin was used as the positive control and 0.1% DMSO was used as the vehicle control.

In vivo anticancer study: Healthy adult Swiss albino mice weighing 20–25 g were procured from the central animal facility of the Manipal University. They were housed in polypropylene cages in a controlled environment (temperature 25±2°C, humidity (50±5% and 12 h dark and light cycle) with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance and following the ethical guidelines specified (Clearance certificate no: IAEC/KMC/02/2006-2007). Preliminary acute cytotoxicity evaluation was carried out to evaluate the maximum tolerated dose and to determine the safe dose by an up and down staircase method. Ehrlich's ascites carcinoma (EAC) cells were obtained through the courtesy of Amala Cancer Research Center, Thrissur. They were maintained by weekly intraperitoneal (i.p.) inoculation of 1×10^6 cells/ mouse. Albino mice were divided into six groups of 6 animals in each group. All the groups were inoculated with EAC cells (2×10^6 cells/mouse). Group I served as the tumor control and received an intraperitoneal injection consisting of 2% acacia suspension. Group II served as a positive control and was treated with the standard drug Cisplatin at 3.5 mg/kg/d i.p. on alternative days for three days. Groups III to VI were treated with the selected compounds (**6d**, **12d**, **18b**, **18d**) at a dose of 100 mg and 50 mg orally. All the treatments were given 24 h after tumor inoculation. The treatments were given once daily for 9 d. The mice were weighed every day till the 15th day. The antitumor efficacies of the compounds (mean survival time, % increase in life span, and % increase in body weight) were compared with that of the standard drug Cisplatin. The results obtained were analyzed statistically and the level of significance determined by one way ANOVA followed by Dunnet multiple comparisons test in case of mean survival time and by Tukey test in case of percentage increase in body weight.

Pharmacokinetic Evaluation

Lipophilicity

Reversed phase (RP)-HPLC was used to find out the log*P* value. Twenty millimeter morpholino propane sulphonic acid having a pH 7.3 and methanol containing 0.25% octanol was used as the mobile phase. The column used was Grace Vydac having C_{18} monomeric stationary phase (25×0.46, 5 μm). Column oven temperature was kept at 25°C and the UV detector was set at 254, 290 nm. The ratio of organic phase varied from 30 to 10% v/v, and the flow rate was 1 mL/min. The retention time data was used to calculate the retention factor (k'). The logarithmic retention factor was extrapolated to 0% methanol to obtain the log*P* value.

pK_a

RP-HPLC with a C_{18} column (25×0.46 cm column packed with 5 μm particle stationary phase) was used. UV detection was programmed at 254 and 290 nm. Column oven temperature was maintained at 25°C and the flow rate was maintained at 1.5 mL/min. The mobile phase consisted of solution A and solution B in the ratio 85 : 15. (Solution A is an aqueous buffer containing phosphoric acid, acetic acid and boric acid having pH 3, 7.4 and 9.5 and solution B was acetonitrile.) Retention time data of compounds were recorded after injecting 50 μL of 10 μg/mL solution of compounds in acetonitrile and calculated the retention factor. Retention factor was calculated for the compounds at pH 3 (k') at fully ionized condition (k'A) and at the unionized condition (k'HA). Using these data, pK_a was calculated using Henderson–Hasselbalch equation.

Conclusion

Fifteen novel thiazolidinone analogs of flavone were prepared in three different series, which differed in the number of methoxy groups in the flavone ring system. Each series contains five compounds that differed in the substituent groups attached to the thiazolidinone ring, such as methoxyl, hydroxyl, and chloro groups. Full characterization of these compounds was performed using NMR, MS, IR and UV spectral techniques. Cytotoxicity studies revealed that the novel analogs potentiated the existing cytotoxic potential of the flavonoids in MDA-MB-435 and HeLa cells. Interestingly, it was also noticed that the presence of methoxy groups on flavonoids improved cytotoxic potential without causing toxicity to normal Vero cells. The most active compound among the three series was found to be compound **18d**, which contained five methoxy groups. The appearance of condensed nuclei in the Hoechst staining analysis and the characteristic DNA content in cell-cycle analysis were features that suggested apoptosis. Compound treatment resulted in an increase in the lifespan and a reduction in the body weight of tumor-bearing mice compared with untreated mice. Determination of physicochemical properties such as pK_a and log*P*-value showed that the solubility and lipophilicity were adequate for the molecule to be drug like. Further studies are required to establish the site of action of these novel anti-cancer molecules.

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Conflict of Interest The author declares no conflict of interest.

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