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Design, Synthesis, Antitumor activity, cell cycle analysis and ELISA assay for Cyclin Dependant Kinase-2 of a new (4-aryl-6-flouro-4*H*-benzo [4, 5]thieno[3, 2-*b*]pyran) derivatives

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Abstract: A series of benzo[b]thiophene and their benzo[4,5]thieno[3,2-b]pyran derivatives **(3a-f)**, **(4a-f)**, **(5a-f)** and 6 were synthesized and characterized by spectroscopic and elemental analysis. All compounds were subjected to one dose anticancer screening in NCI- America, but only the compounds gave high percent growth inhibition were further subjected to five dose screening. A good result of compound **4f** with GI_{50} = 0.15 µmol, TGI= 1.14 µmol and **4c** with GI_{50} = 1.09 µmol, TGI = 10.19 µmol, LC50 = 100 µmol on HT-29 cell line. To explore mechanism of cytotoxicity, compound **4f** and **4c** were allowed to affect cell cycle progression using HT-29 cell line (human colon cancer) in two-time interval (24 and 48 hr). The cytotoxicity of **4f** and **4c** was correlated with induction of apoptosis causing pre-G_1apoptosis and cell growth arrest at G_2/M in a time dependant manner through inhibition of CDK-2. For exploring the SAR for all synthesized compounds, IC_{50} of **5d** was determined which was equal to 0.32 ±0.05 µmol, IC_{50} of **6** was equal to be 0.15 ±0.01 µmol while IC_{50} of **erlotinib** reference was equal to 0.3±0.02 µmol. Finally we were able to synthesize a series of benzo[b] thiophene, benzo[4,5]thieno[3,2-b]pyran having a good cytotoxic activity suggesting promising anticancer derivatives.

Keywords: benzo[4,5]thieno[3,2-b]pyran, anticancer, cell cycle, apoptosis, CDK-2.

Introduction

The process of programmed cell death [1], or apoptosis, is generally characterized by distinct morphological characteristics. Apoptosis is considered a vital component of various processes including normal cell turnover and proper development. Inappropriate apoptosis (either too little or too much) is a factor in types of cancer. Light microscopy has identified the various morphological changes that occur during apoptosis [2]. During the early process of apoptosis, cell shrinkage and psychosis (is result of chromatin condensation and this is the most characteristic feature of apoptosis). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. The apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments, all these changes found to be irreversible. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [3]. Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional. In fact, suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers [4]. Searching previous literatures, we found that aurones [5, 6] (Z)-2-benzylidenebenzofuran-3-(2H)-ones and flavopridol[7] constitute two subtypes of flavonoid, both of them have remarkable antiproliferative activity. Flavopridol is active against leukemia L1210 (IC_{50} = 0.15 µmol) causing cell cycle arrest at G_2 phase and breast cancer MCF-7 (IC50 = 0.03 µmol). Aurones inhibit both cyclindepen dentkinases (CDK's) [8] and tyrosine kinases [9] Figure 1. Although biological activity of thioaurones [10] was not explored like aurones, they proved to have a similar anticancer activity. 2-(2-Hydroxy-5-nitro-benzylidene)benzo[b]thiophen-3-one [11] was found to inhibit DHHC-mediated palmitoylation *invitro* which is necessary for the proper activity of oncoproteins such as H-Ras (involved in regulating cell division in response to growth factors stimulation [12]). Also we found that 2-amino-4-aryl-9-flouro-4,5-dihydrobenzo[b]pyrano-[4,3-b]pyran-3-carbonitriles were special active agents against lung, breast and CNS cancer (NCI-H460, MCF-7 and SF-268 cell lines). Bcl-2 protein [regulate apoptosis by inducing pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis] binding compounds also provides a satisfactory lead compound for the development of potential anticancer agent. Ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-

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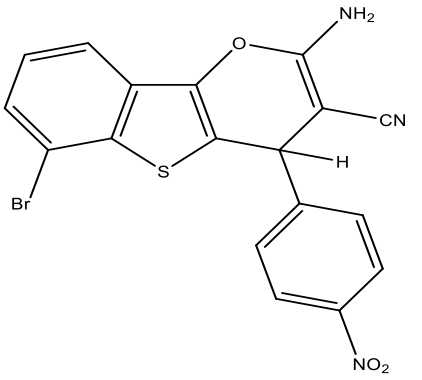
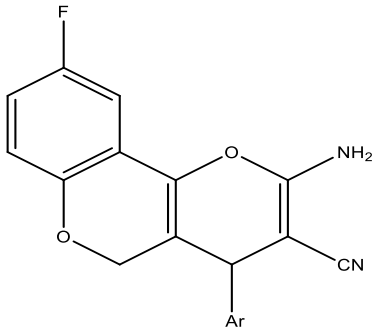
carboxylate (HA14-1) an antagonist for ant apoptotic Bcl-2 proteins was used to overcome drug resistance in cancer. Recently, screening anticancer activity of 2-amino-6-bromo-4-(4-nitrophenyl)-4H-benzo[4,5]-thieno[3,2-b]pyran-3-carbonitrile using HCT-116 cell line produced a good anticancer activity and also proved to induce apoptosis at G_0/G_1 phase [13].The present work is aimed towards construction of novel heterocyclic compounds of anticipated anticancer activity and to compare anticancer screening result of 2-amino-6-bromo-4-(4-nitrophenyl)-4H-[1]benzo-thieno[3,2-b]pyran-3-carbonitrile with our target compounds by replacing bromine atom at position 6 with fluorine atom which possess higher electronegativity, higher thermal stability and higher lipophilicity than bromine atom [14].



2-amino-6-bromo-4-(4-nitrophenyl)-4H-[1]benzothieno [3,2-b]pyran-3-carbonitrile



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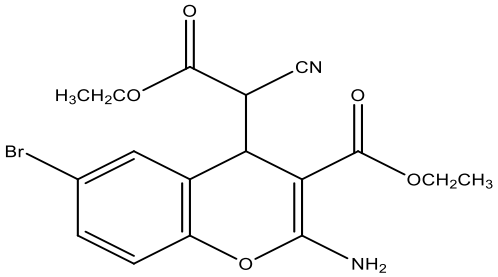
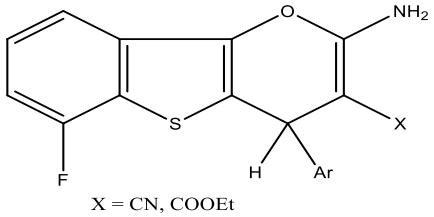


Ethyl 2-Amino-6-bromo-4-(1-cyano-2- ethoxy-2-oxoethyl) -4H-chromene-3-carboxylate



X = CN, COOEt

2-amino-6-flouro-4-aryl-4H-[1]benzothieno [3,2-b]pyran-3-carbonitrile

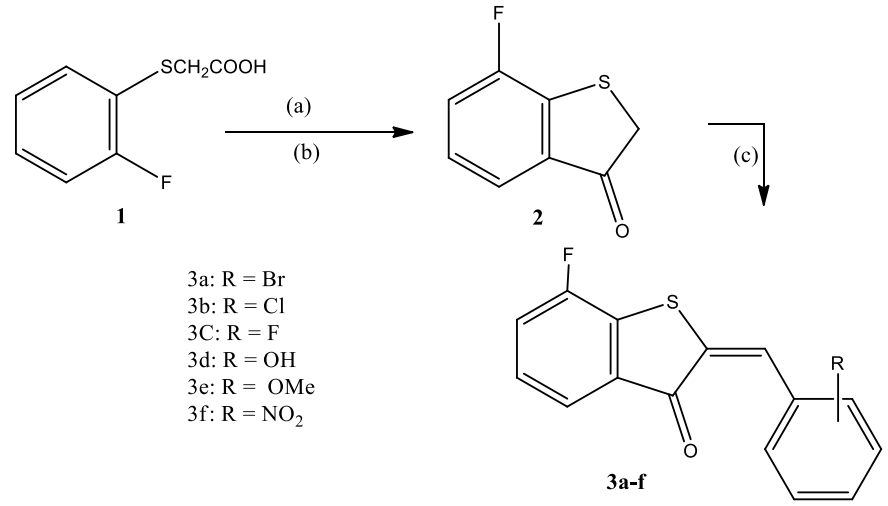
Figure 1. Design of target compounds (3a-f), (4a-f) and (5a-f).

Results and discussion

Chemistry

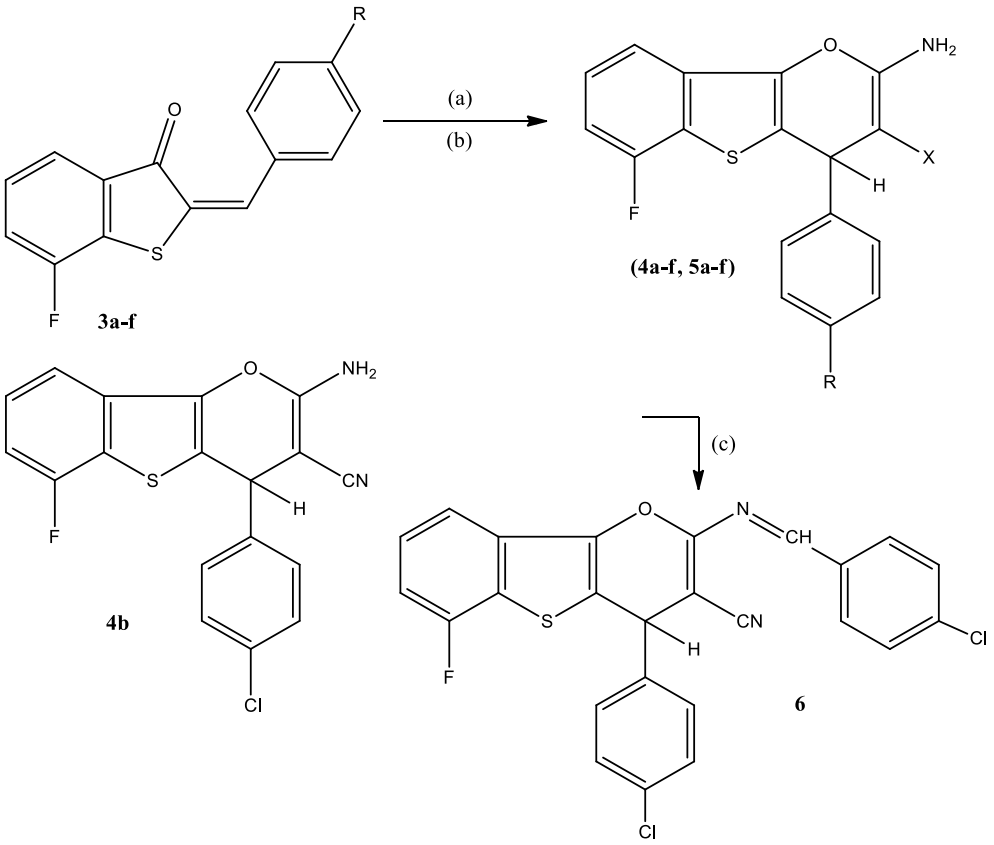
As shown in scheme **(1)**, the first target compounds 2-arylidene-7-flourobenzo[b]thiophen-3(2*H*)-ones **(3a-f)** were synthesized by condensation of 7-flourobenzo[b]hiophen-3-one (2) and different aromatic aldehydes which itself were synthesized according to reported methods [15,16]. The synthetic pathway of the second target compounds **(4a-f)** were outlined in scheme **(2)** where [17] the activated methylene compounds, containing electron-withdrawing groups, such as malononitrile and ethyl cyanoacetate reacted with α, β-unsaturated ketones by 1,4-Michael addition reaction followed by intramolecular cyclization to produce novel compounds **(4a-f)** and **(5a-f)**. Compound **(6)** was synthesized by refluxing **(4b)** with p-chlorobenzaldhyde in dry toluene. The structures of newely synthesized compounds **(3a-f), (4a-f), (5a-f)** and **6** (Figure 2) were confirmed by spectral and elemental analysis. IR spectra of **(3a-f)** revealed the appearance of the conjugated C=O band at arrange of 1673-1680 cm^{-1}. ^{1}HNMR revealed the appearance of methine proton (=CH) at δ 8-8.09 ppm together with the introduced aromatic protons, ^{13}CNMR of this series showed C=O signal at δ 185 ppm. Further, IR spectra of **(4a-f)** revealed forked peak of NH_2 at 3345-3460 cm^{-1} and CN group at 2190 cm^{-1}. ^{1}HNMR spectra of **(4a-f)** revealed the appearance of the CH aliphatic characteristic signal of the pyran ring at δ 5.03-5.10 ppm as well as an exchangeable singlet signal at δ 7.10-7.35 ppm corresponding to the NH_2 protons. ^{13}CNMR spectra indicated the disappearance of C=O carbon signal around δ 188 ppm and the appearance of CH aliphatic of the pyran ring at δ 58-60 ppm and CN at δ 120 ppm. In addition, IR spectral analysis of Structures **(5a-f)** showed CH aliphatic at 2930-2890 cm^{-1} and carbonyl group of the ester at 1850 cm^{-1} beside forked peak of NH_2 at 3300- 3400 cm^{-1}. ^{1}HNMR of **(5a-f)** showed triplet signal for CH_3 at range (1.10-1.20) ppm and quartet signal for CH_2 group at range (4.01 - 4.20) ppm beside pyran H-4 at 5.56 ppm.^{13}CNMR spectra showed CH_3, CH_2 in aliphatic area at 14.51 and 25.92 ppm and carbonyl of ester at (168) ppm. IR of **compound 6** showed disappearance of forked NH_2 and instead, appearance of N=CH at 1630 cm^{-1} beside CH aliphatic at 1920

cm^{-1} and =CH at 3100 cm^{-1}. ^{1}HNMR showed N=CH singal at 9.30 ppm beside pyran proton at 5.58 ppm and aromatic protons at 7.10-7.95 ppm. Mass spectra of all synthesized compounds showed molecular ion peaks (M^+) in addition compounds **3a, 3b, 4a, 4b, 5a, 5b**and **6** showed M+2 peaks confirming presence of bromine chlorine atom respectively.



Reagents and conditions: a) PCl_3/Chlorobenzene, stirring at 80 °C, 2 hr
b) $AlCl_3$ (F.C), stirring at 60 °C, 1 hr
c) Knoevenagel condensation, ArCHO, 50 ml gl.acetic acid, reflux 5 hr

Scheme 1. Synthetic pathway for the preparation of compounds **(3a-f).**



Reagents and conditions: a) $(CH_2)(CN)_2$, 50ml abs EtOH, 4 dps piperdine, reflux 5 hr
b) $(CH_2)(CN)(COOEt)$, 50 ml abs. EtOH, 7dps piperdine, reflux 5 hr
c) *p*ClC_6H_4CHO, 10 ml dry toluene, reflux 24 hr

Scheme 2. Synthetic pathway for the preparation of compounds (4a-f), (5a-f) and compound 6.

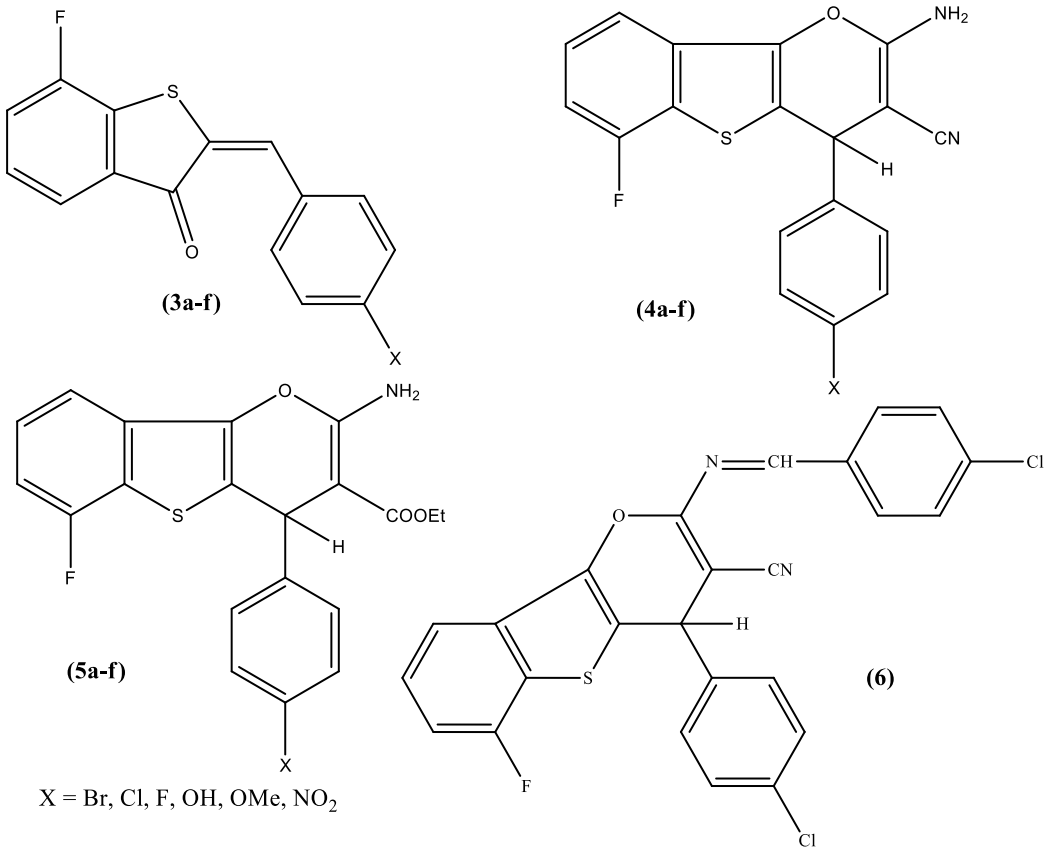


Figure 2. Schematic representation of the synthesized compounds.

Biological screening result

As shown in Table 1, most of the synthesized compounds were exposed to one dose screening using sulforhoodamine B (SRB) assay at concentration (10^{-5} mol) in NCI- America against different 60 cell lines (Breast, colon, prostate, ovarian, renal, CNS, Non-small cell cancer, leukemia and melanoma). **2-arylidene-7-flourobenzo[b]thiophen-3(2*H*)-ones (3a-f)**, showed no activity with a mean growth percentage (80 to 95%) while tri cyclic series **2-amino-4-aryl-6-flouro-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4a-f)**, enhanced the activity with a mean growth (-29 to 86.5%) and series **ethyl 2-amino-4-aryl-6-flouro-4*H*-benzo[4,5]hieno[3,2-*b*]pyran-3-carboxlates (5a-f),** showed low activity except **5d** which showed high activity (38.3 %). Depending on the previous results compound **4f** and **4c** were selected for five dose screening at concentration (0.01, 0.1, 1, 10 and 100 µmol). A good result of the two compounds expressed in three parameters, median growth inhibition (**GI_{50}**, concentration of compound that cause 50% inhibition in growth of cells), total growth inhibition (**TGI**, concentration of compound that cause 100% inhibition in growth of cells) and median lethal concentration (**LC_{50}**, concentration of compound that cause 50% loss of intact cells at the end of growth period), suggesting a promising anticancer motifs as shown in Table (2) and also summarized in Figure (3, 4). From these result compound 4f (GI_{50} = 0.079 µmol, TGI= 1.21 µmol, LC_{50} = 3.48 µmol) was found to be more active than the structurally related compound 2-amino-6-bromo-4-(4-nitrophenyl)-4H[1]benzo-thieno[3,2-b]pyran-3-carbonitrile (3e) (GI_{50} = 0.11 µmol, TGI= 7.94 µmol, LC_{50} = 42.66 µmol) using HCT-116 cell line [13]. IC_{50} of compound **5d** was found 0.3±0.05µg/ml and IC_{50} of compound **6** was found 0.15±0.01µg/ml while IC_{50} of Erlotinib reference was found 0.3±0.02 µg/ml using HT-29 cell line, when measured in VACERA Egypt expecting that compound **6** has a comparable anticancer activity as 4f compound Figure (5).

Table 1. Single dose results of all compounds.

Compound No.	Ar	%mean of growth	GI_{50}	TGI	LC_{50}
3b	4-ClC_6H_4	95.95	n.t	n.t	n.t
3c	4-FC_6H_4	94.86	n.t	n.t	n.t
3d	4-OHC_6H_4	94.95	n.t	n.t	n.t
3e	4-$CH_3OC_6H_4$	80.73	n.t	n.t	n.t
4b	4-ClC_6H_4	28.33	n.t	n.t	n.t
4c	4-FC_6H_4	26.18	1.09	10.19	100

4d	4-OHC_6H_4	86.50	n.t	n.t	n.t
4e	4-$CH_3OC_6H_4$	27.15	n.t	n.t	n.t
4f	4-$NO_2C_6H_4$	-29.85	0.15	1.14	0
5b	4-ClC_6H_4	96.70	n.t	n.t	n.t
5d	4-OHC_6H_4	38.36	0.3	n.t	n.t
5e	4-$CH_3OC_6H_4$	92.20	n.t	n.t	n.t
5f	4-$NO_2C_6H_4$	95.25	n.t	n.t	n.t
6	4-ClC_6H_4	n.t	0.15	n.t	n.t

GI_{50}, TGI and LC_{50} are calculated in µmol unit, n.t (not tested).

Table 2. Five dose results of selected NCI compounds.

Cell line	**4f**			**4c**		
	GI_{50}	**TGI**	**LC_{50}**	**GI_{50}**	**TGI**	**LC_{50}**
Leukemia						
CCRF-CEM	0.119	1.02	100	0.418	12.4	100
HL-60(TB)	0.167	0.735	52.8	0.247	0.669	100
K-562	0.00494	1.79	100	0.353	10.43	100
MOLT-4	0.0926	1.96	100	0.579	10.26	100
RPMI-8226	0.206	1.63	100	1.36	10.32	100
SR	0.00541	10.08	100	0.516	20.39	100
Non- Small Cell Lung Cancer						
A549/ATCC	0.112	1.78	8.51	0.888	10.71	67.8
EKVX	0.386	2.26	7.12	4.26	20.10	40.93
HOP-62	0.155	1.58	5.83	0.638	10.72	48.8
HOP-92	0.0717	0.7	4.65	2.02	6.96	30.46
NCI-H226	0.195	1.51	4.2	2.41	10.77	60.21
NCI-H23	0.276	1.50	6.55	1.50	15.30	46.9
NCI-H322M	0.576	2.10	5.79	2.44	1.74	40.65
NCI-H460	0.0788	5.09	8.02	0.409	50.35	33.90
NCI-H522	0.017	0.541	4.93	0.374	8.65	80.40
Colon Cancer						
COLO 205	0.217	0.721	4.04	2.33	10.6	48.10
HCC-2998	0.335	1.59	4.72	2.05	10.14	35.5
HCT-116	0.0792	1.21	3.48	0.417	12	46.1
HCT-15	0.0682	1.41	5.23	0.409	11.20	37.60
HT-29	0.155	1.14	3.8	1.09	11.90	100
KM12	0.094	1.48	4.2	0.444	12.60	46.80
SW-620	0.047	1.68	6.55	0.553	16.2	48.30
CNS Cancer						
SF-268	0.292	1.75	8.29	0.921	13.5	43.6
SF-295	0.173	1.12	6.43	0.369	11.3	38.3
SF-539	0.172	0.54	2.22	0.439	2.46	15.1
SNB-19	0.191	1.30	5.21	0.582	14.1	40.3
SNB-75	0.052	0.718	2.34	0.338	14.9	48.6
U251	0.15	1.21	3.90	0.55	13.9	39.8
Melanoma						
LOX IMVI	0.132	1.14	5.83	0.853	12.1	38.8
MALME-3M	0.36	2.17	5.62	0.60	22.2	52
M14	0.11	0.64	3.48	0.38	3.37	28.2
MDA-MB435	0.025	0.076	2.77	0.22	0.74	5.19
SK-MEL-2	0.097	1.01	5.55	0.55	17.7	54.8
SK-MEL-28	0.249	2.02	5.84	1.81	19.8	51.3
SK-MEL-5	0.085	1.16	3.41	0.648	14.4	39.5
UACC-257	0.87	2.84	8.34	17.3	39.1	88.4
UACC-62	0.058	1.39	4.06	5.79	14.6	42
Ovarian cancer						
IGROV1	0.172	1.56	9.43	0.68	15.4	47.7
OVCAR-3	0.142	1.28	4.85	0.38	1.94	20.3

OVCAR-4	0.237	1.90	100	1.0	14.2	45.3
OVCAR-5	0.56	2.42	7.44	2.87	15.5	41.7
OVCAR-8	0.19	1.24	5.52	1.52	14.20	68.7
NCI/ADRRES	0.04	0.58	100	0.35	7.50	66.50
SK-OV-3	0.29	3.04	27.4	1.37	17.5	49.1
Renal Cancer						
786-0	0.26	1.11	4.13	0.53	10.2	33.4
ACHN	0.37	2.42	17.4	0.87	16.2	40.3
CAKI-1	0.25	1.78	6.07	0.50	16.3	43.3
RXF 393	0.15	0.62	3.16	1.10	5.82	27.1
SN 12C	0.27	1.31	4.83	0.88	15.7	41.2
TK-10	0.50	2.71	30.10	6.54	24.40	69.10
UO-31	0.23	1.44	5.34	1.49	15.60	40.50
Prostate Cancer						
PC-3	0.16	1.29	7.49	0.85	14.80	46.20
DU-145	0.35	1.45	4.84	1.74	12.50	36.50
Breast Cancer						
MCF7	0.031	1.47	45.10	0.37	11.00	53.80
MDA-MB 231/ATCC	0.16	1.12	4.97	1.54	12.80	41.20
HS 578T	0.18	0.93	100	0.48	12.00	100
BT-549	0.18	1.05	3.55	0.44	10.70	34.60
T-47D	0.18	2.52	64.00	0.51	22.60	100
MDA-MB468	0.04	0.08	4.24	0.51	17.70	52.60

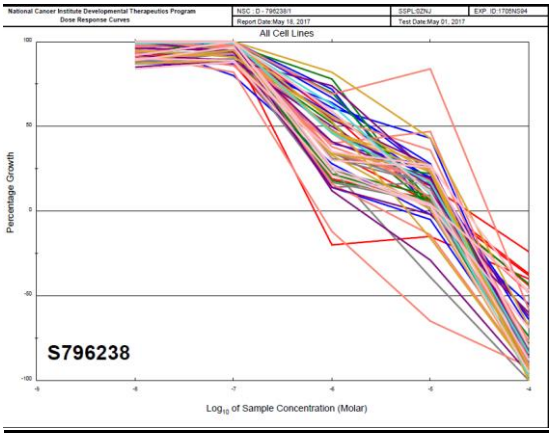


Figure 4. Dose response curve of 4c against 60 cell line

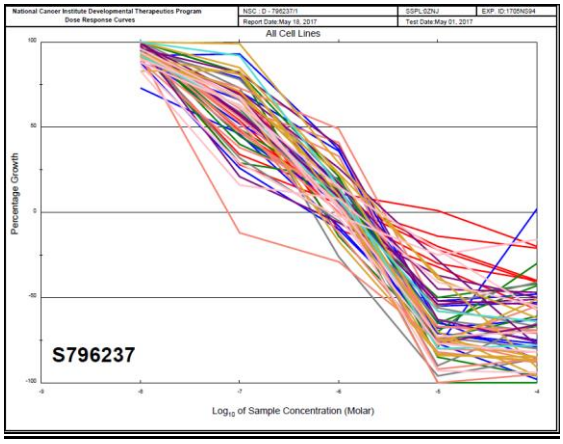


Figure 3. Dose response curve of 4f against 60 cell line.

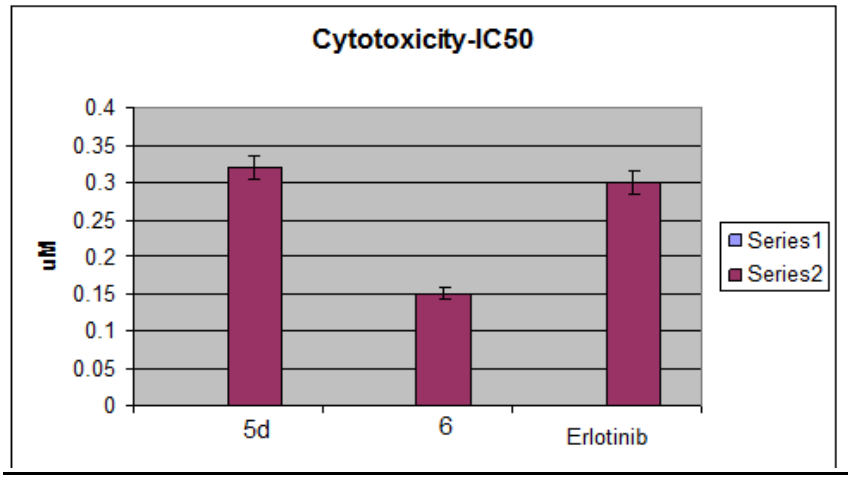


Figure 5. IC_{50} of compounds 5d and 6 compared with erlotinib reference.

***SAR* result according to NCI**

1- Compounds containing aminocyanogroup **(4a-f)** are more active than compounds containing aminoester group **(5a-f)** using the same cell line.

2- Presence of fluorine atom instead of bromine or chlorine at positions 6 **(4a-f)** results in higher anticancer activity [13].

3- Presence of electron withdrawing group as nitro group at position 4 in phenyl ring give higher activity compared with electron donating group as methoxy group.

4- 4-Subsituted Phenyl ring more active than heterocyclic ring as naphthyl ring [14].

5- Amino group in compound **4b** and imine group in compound **6** at C- 2 give a comparable activity.

Cell cycle analysis

The cell cycle is comprised of four ordered, strictly regulated phases referred to as G_1, S (DNA synthesis), G_2, and M (mitosis). Normal cells grown in culture will stop proliferating and enter aquiescent state called G_0 once they become confluentor are deprived of serum or growth factors [18]. The first gap phase (G_1) prior to the initiation of DNA synthesis represents the period of commitment that separates M and S phases as cells prepare for DNA duplication. Cells in G_0 and G1 are receptive to growth signals, but once they have passed a restriction point, they are committed to enter DNA synthesis. Cells demonstrate arrest at different points in G1 in response to different inhibitory growth signals [19]. Therefore, the effect of 4f and 4c on cell cycle was studied in order to elucidate their mechanism of action. Figure 6 and Table 3 displayed that exposure of HT-29 cells to compound 4f and 4c for 24 and 48 hr, induced a significant disruption in the cell cycle profile at pre- G_1 phase. This might indicate an ability of compounds 4f and 4c to reduce the cellular proliferation and to induce cell cycle arrest at G_2/M phase and induce DNA fragmentation, the hallmark of cell death, preventing the cells from further replication and proliferation.

As indicated in Table 4, compound 4f showed decreased apoptotic cells by 50 % for 24 hr exposure compared to reference drug. The early apoptotic cells declined by 26.5 % for 48 hr exposure period compared to erlotinib. When the cells treated with 4c compound for 24 hr, apoptotic cells decreased by 39 % whereas the cells declined by 17.6 % for 48 hr exposure level compared to reference drug.

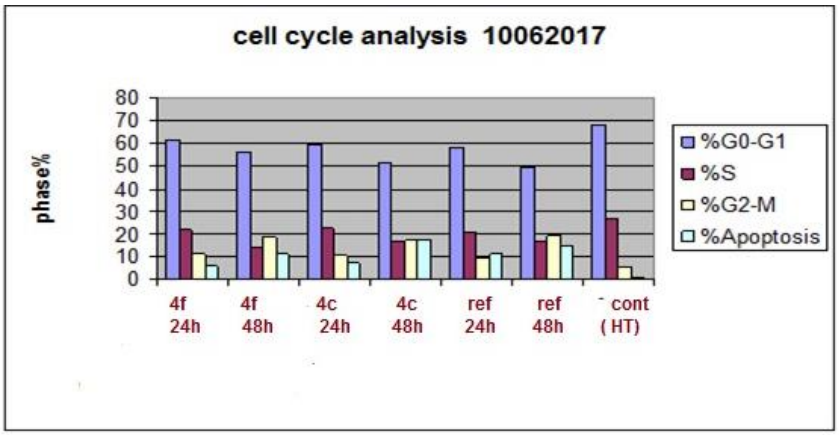


Figure 6. Cell cycle analysis for HT-29 cells treated with 4f, 4c and erlotinib.

Table 3. Effect of 4f and 4c compounds on cell cycle profile of HT-29 cells.

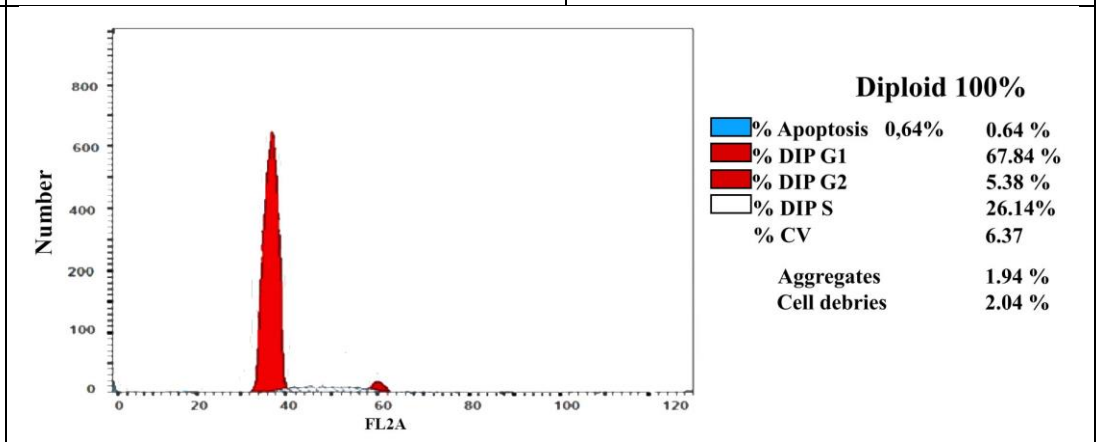
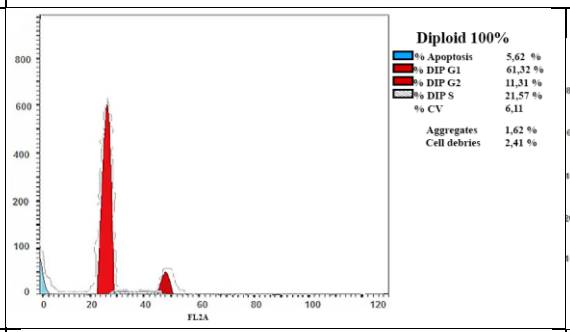
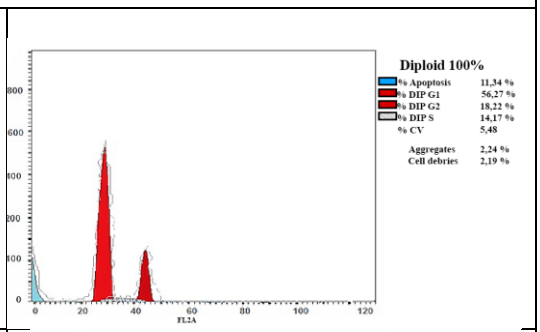
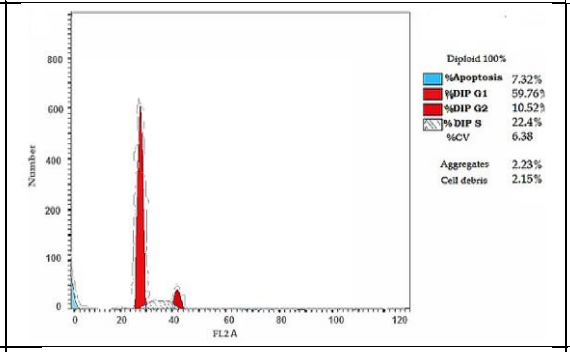
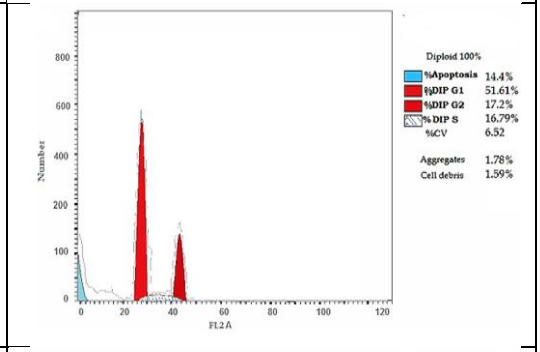
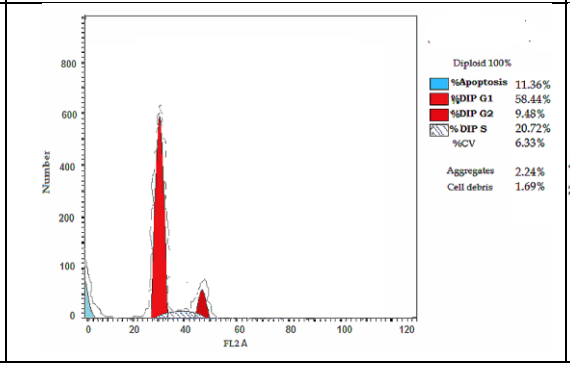
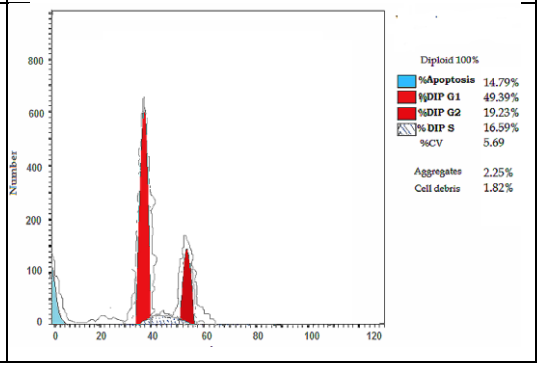
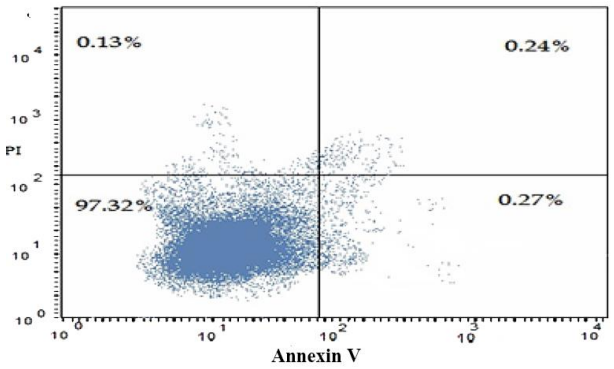
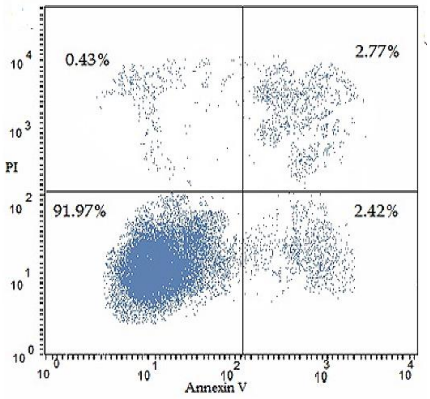
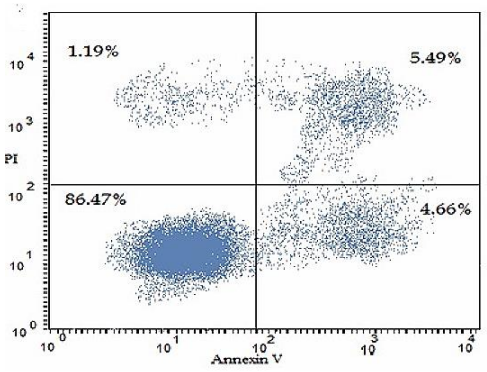
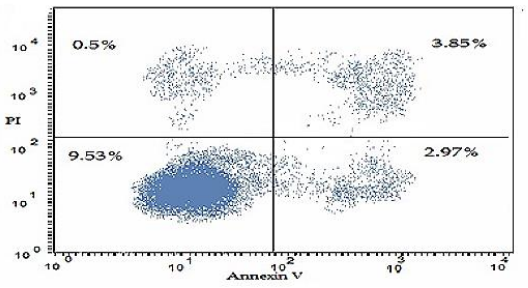
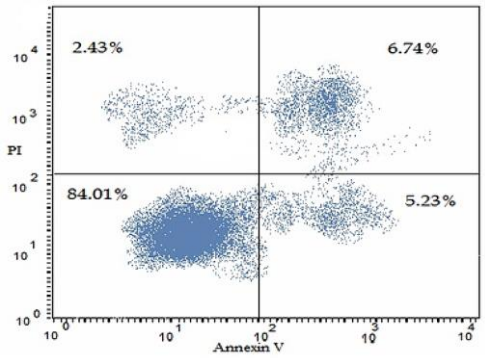
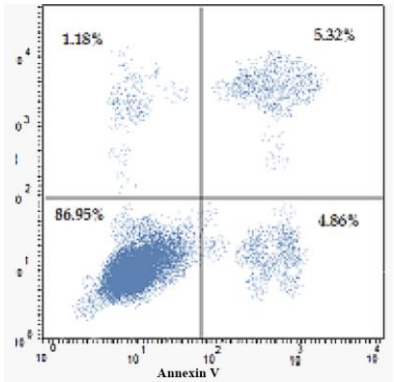
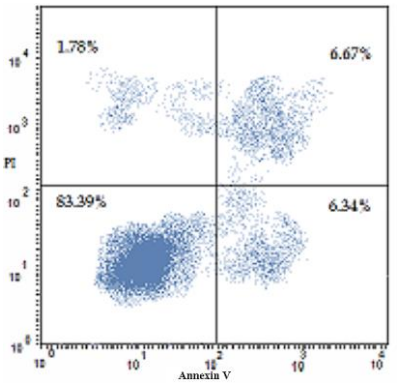
	24 hr	48 hr
Control		
Compound 4f		
Compound 4c		
Reference Erlotinib Tarceva®		

Table 4. Effect of 4f and 4c compounds on apoptosis of HT-29 cells.

	24 hr	**48 hr**
Control		
Compound 4f		
Compound 4c		
Reference Erlotinib Tarceva®		

Effect on CDK2 level:

Compound 4f increased CDK level by 24% for 24hr exposure compared to reference drug (erlotinib). When the cells treated with 4f for 48hr, the enzyme concentration decreased by 14.5 % compared to the reference. Regarding to 4c compound, enzyme concentration declined by 2% for 24 hr exposure whereas the enzyme level increased by 6.7 % for 48 hr exposure compared to reference drug **Figure (7)**.

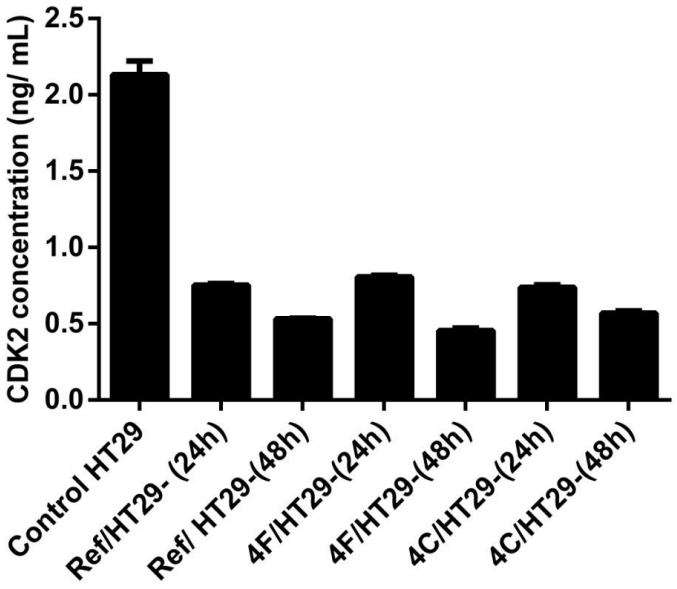


Figure 7. Effect of 4f, 4c and erlotinib on CDK2 of HT-29 cells.

Conclusion

This study represents the synthesis of novel benzo[b]thiophene, benzo[4,5]thieno[3,2-b]pyran derivatives. Most of the studied compounds exhibited a good growth inhibitory effect, especially compounds **4f**, **4c**, **5d**, and **6**, which were active in the low micro molar range, and therefore, they were submitted to further evaluations. Spectroscopic analysis of these compounds confirmed their structures. Cell cycle study showed that exposure of HT-29 cells to compound 4f and 4c for 24 and 48 hr, induced a significant disruption in the cell cycle profile at pre-G1 phase and arrest at G2/M phase, which could be a consequence of several stresses, such as CDK-2 inhibition. In conclusion, the here-presented novel compounds possess high potential as novel leads with anticancer potentials; however, the most intriguing result is the observation that exchanging amino group in compound **4f** with imines group in **6** led to a comparable cytotoxic activity which need further investigation in future research.

Acknowledgements

The authors would like to thank NCI- America for carrying anti-cancer screening of the synthesized compounds.

Experimental Section

General

Melting points were determined using a Griffin apparatus and were uncorrected. All chemicals and reagents were obtained from Aldrich (Sigma-Aldrich), and were used without further purification. Reactions were monitored by TLC, performed on silica -gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. IR spectra were determined on Shimadzu IR 435 spectrophotometer (KBr, cm^{-1}). ^{1}HNMR and ^{13}CNMR spectra were carried out using Bruker 400 MHZ and 100MHZ spectrophotometer using TMS as internal standard faculty of pharmacy Cairo University, Egypt. Chemical shifts (δ) were recorded in ppm on δ scale. Mass spectra and Elemental analysis were carried out at the regional center for mycology and biotechnology, Al-Azhar University, Cairo, Egypt.

General procedure for the synthesis of 2-arylidene-7- flourobenzo[b]thiophen-3(2*H*)-ones (3a-f).

7-flourobenzo[b]thiophen-3(*2H*)-one (**2**) (1.68gm, 10mmol) was added to an appropriate amount of aromatic aldhyde (10mmol) in 50ml glacial acetic acid and the mixture was refluxed for 5hr. The product was obtained after evaporation of half amount of glacial acetic acid, dried and crystallized from isopropanol.

2-(4-bromobenzylidene)-7-Flourobenzo[b]thiophen-3(2*H*)-one (3a)
Yield: 78%; m.p 180 °C; IR (KBr) cm^{-1}: 1685 (C=O), 1589 (C=C);
^{1}H NMR (400 MHz, $CDCl_3$) ppm δ: 7.65-7.87 (m, 7H, CH aromatic), 7.93(s, 1H =CH methine);
^{13}CNMR(100 MHz, $CDCl_3$) ppm: 121.2, 122.7, 125, 127.2, 130.1, 132, 132.2, 132.2, 132.4, 132.8, 133.1, 133.1, 133.4 (CH aromatic,benzylic), 158.9 (C-7), 187.5 (C=O);
MS: m/z (% rel. Abundance): 334 (M^+) (100%), 336 (M+2) (78.48%);
Anal. Calcd for $C_{15}H_8BrFOS$: C, 53.75; H, 2.41; S, 9.57; Found C, 53.94; H, 2.39; S, 9.58.

2-(4-chlorobenzylidene)-7-Flourobenzo[b]thiophen-3(2*H*)-one (3b)
Yield: 72 %; m.p 180 °C; IR (KBr) cm^{-1}: 1687 (C=O), 1583 (C=C);
^{1}H NMR (400 MHz, DMSO-d_6) ppm δ: 7.40- 7.80 (m, 7H, CH aromatic), 8.03 (s, 1H,=CH methine);
^{13}CNMR(100 MHz, DMSO) ppm: 122.4, 123.3,124, 125, 125.5, 126, 126.8, 127.6, 130, 132.7, 132.9, 133, 133, 133.7, 136 (aromatic C, benzylic C) , 187 (C=O);
MS: m/z (% rel. Abundance): 290 (M^+) (60.41%), 292 (M+2) (23.58%);
Anal. Calcd for $C_{15}H_8ClFOS$: C, 61.97; H, 2.72; S, 11.03; Found C, 62.23; H, 2.82S, 11.01.

7-Flouro-2-(4-flourobenzylidene)-benzo[b]thiophen-3(2*H*)-one (3c)
Yield: 60%; m.p 180 °C; IR (KBr) cm^{-1}: 1693 (C=O) ,1581 (C=C);
^{1}H NMR (400 MHz, DMSO- d_6) ppm δ: 7.40-7.90 (m, 7H, CH aromatic), 8.02 (s, 1H,=CH methine);
^{13}CNMR(100 MHz, DMSO) ppm: 117,117.2, 122.3, 122.5, 123.2,124, 128.8, 130.4, 131.20, 132.4, 133.3, 133.6, 134, 134.1(CH aromatic,benzylic), 186.8 (C=O);
MS: m/z (% rel. Abundance): 274 (M^+) (100%);
Anal. Calcd for $C_{15}H_8F_2OS$: C, 65.68; H, 2.94; S, 11.69 Found: C, 65.89; H, 2.98; S, 11.69.

7-Flouro-2-(4-hydroxybenzylidene)benzo[b]thiophen-3(2*H*)-one (3d)
Yield: 70%; m.p 249 °C; IR (KBr) cm^{-1}: 3336.85 (OH), 1655 (C=O), 1604 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) ppm δ: 6.91-7.70 (m, 7H, CH aromatic), 7.90 (s, 1H, =CH methine), 10.49(s,1H, OH, D_2O exchangeable);
MS: m/z (% rel. Abundance): 272 (M^+) (100%);
Anal.Calcd for $C_{15}H_9FO_2S$: C, 66.16; H, 3.33; S, 11.78; Found C, 66.42; H 3.41; S, 11.75.

7-Flouro-2-(4-Methoxybenzylidene)benzo[b]-thiophen-3(2*H*)-one (3e)
Yield: 78 %; m.p 165 °C; IR (KBr) cm^{-1}: 1672 (C=O), 1583 (C=C), 2920-2846 (CH aliphatic);
^{1}HNMR (400 MHz, DMSO-d_6) ppm δ: 3.80(s, 3H, OCH_3), 7.10-7.80 (m, 7H, CH aromatic), 7.90 (s, 1H,=CH methine);
MS: m/z (% rel. Abundance): 286 (M^+)(100%);
Anal. Calcd for $C_{16}H_{11}FO_2S$: C, 67.16; H, 3.87; S, 11.20; Found C, 67.41; H, 3.93; S, 11.22.

7-Flouro-2-(4-Nitrobenzylidene)benzo[b]-thiophen-3(2*H*)-one (3f)
Yield: 82 %; m.p 230 C°; IR (KBr) cm^{-1}: 1686 (C=O), 1523 (C=C);
^{1}H NMR (400 MHz, DMSO-d_6) ppm: 7.50-8.30 (m, 7H, CH aromatic), 8.40(s,1H,=CH methine);
MS: m/z (% rel. Abundance):301(M^+) (46.24%);
Anal. Calcd for $C_{15}H_8FNO_3S$: C, 59.8; H, 2.68; N, 4.65; S, 10.64; Found C, 59.97; H, 2.65; N, 4.77; S, 10.65.

General procedure for the synthesis of 2-amino-4-aryl-6-flouro-4*H*- benzo[4,5]thieno[3, 2-*b*] pyran-3-carbonitrile (4a-f)

A mixture of 2-arylidene-**7**-flourobenzo[b]thiophen-3-ones **3a-f** (1 mmol), malononitrile (0.07 g, 1 mmol) and piperidine (4 dps) in absolute ethanol (50 ml) was heated under reflux for 5 hr. The reaction was cooled, and the separated solid was filtered, dried and crystallized from isopropanol.

2-Amino-4-(4-bromophenyl)-6-flouro-4*H*-benzo[4,5] thieno[3, 2-*b*] pyran-3-carbonitrile (4a)
Yield: 50%; mp: 201 °C; IR (KBr) cm^{-1}: 3468, 3321 forked peak (NH_2), 2886 (CH-aliphatic), 2198 (CN);
^{1}HNMR (400 MHz, DMSO-d_6) ppm: 5.12 (s,1H, H-4), 7.31-7.32 (s, 2H, NH_2, D_2O exchangeable), 7.33–7.35 (d, 2H, *J = 8 HZ)*, 7.42-7.44 (d, 2H, *J = 8HZ)*, 7.51-7.57 (m, 3H, CH aromatic);
^{13}CNMR(100 MHz, DMSO) ppm: 55.94 (aliphatic C), 111.5, 111.7, 116.4, 116.4, 119, 121.2, 122.3, 122.5, 127.8, 127.9, 130.2, 132.2, 132.3, 132.4, 139.4, 143.5, (aromatic C), 120.2 (CN), 160.7 (C-2);
MS: m/z (% rel. Abundance): 400 (M^+) (51.34%) , 402 (M+2) (7.65%);
Anal. Calcd for $C_{18}H_{10}BrFN_2OS$: C, 53.88; H, 2.51; N, 6.98; S, 7.99; Found C, 54.12; H, 2.50; N, 7.21; S, 8.00.

2-Amino-4-(4-chlorophenyl)-6-flouro-4*H*-benzo[4,5]thieno[3, 2-*b*] pyran-3-carbonitrile (4b)
Yield: 70%; mp: 203-205°C; IR (KBr) cm^{-1}: 3456,4329 forked peak (NH_2), 2924 (CH-aliphatic), 2194 (CN); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 5.10 (s, 1H, H-4), 7.26-7.27 (d, 2H, *J = 4 HZ)*, 7.29-7.30 (d, 2H, *J = 4 HZ)*, 7.32 (s, 2H, NH_2, D_2O exchangeable),7.53-7.58 (m, 3H, CH aromatic);
^{13}CNMR(100 MHz, DMSO) ppm: 56 (C-4), 111.5, 111.7, 116.4, 116.4, 119, 122.3, 127.9, 128.7, 129.3, 129.8, 132.3, 132.4, 132.7, 139.1, 139.1 (aromatic CH),120.2 (CN), 160.7 (C-2);
MS: m/z (% rel. Abundance): 356 (M^+) (3.45%), 358 (M+2) (1.20%);

Anal. Calcd for $C_{18}H_{10}ClFN_2OS$: C, 60.59; H, 2.82; N, 7.85; S, 8.99 Found: C, 60.81; H, 2.84; N, 8.01; S, 9.00.

2-Amino-6-flouro-4-(4-fluorophenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4c)
Yield: 80%; mp: 180 °C; IR (KBr) cm^{-1}: 3471, 3333 forked peak (NH_2), 2854 (CH-aliphatic), 2194 (CN); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 5.13(s, 1H, H-4), 7.32 (s, 2H, NH_2, D_2O exchangeable), 7.35-7.36 (d, 2H, *J = 2 HZ*), 7.37-7.38 (d, 2H, *J =2* HZ), 7.50 - 7.56 (m, 3H, CH aromatic);
MS: m/z (% rel. Abundance): 340 (M^+)(1.19%);
Anal. Calcd for $C_{18}H_{10}F_2N_2OS$: C, 63.52; H, 2.96; N, 8.23; S, 9.42; Found: C, 63.78; H, 2.99; N, 8.42; S, 9.44.

2-Amino-6-flouro-4-(4-hydroxyphenyl)-4*H*-benzo[4,5]thieno[3,2-*b*] pyran-3-carbonitrile (4d)
Yield: 80%; mp: 240 °C IR (KBr) cm^{-1}: 3471, 3329 forked peak (NH_2), 3210 (OH), 2854(CH-aliphatic), 2191 (CN); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 4.96(s, 1H, H-4), 6.70 (s, 2H, NH_2, D_2O exchangeable), 7.08-7.54 (m, 7H, CH aromatic), 9.43(s, 1H, OH, D_2O exchangeable);
MS: m/z (% rel. Abundance):338 (M^+) (43.45%);
Anal. Calcd for $C_{18}H_{11}FN_2O_2S$: C, 63.42; H, 3.28; N, 8.28; S, 9.48 Found: C, 63.42; H, 3.41; N, 8.17; S, 9.50.

2-Amino-6-flouro-4-(4-methoxyphenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4e)
Yield: 80%; mp: 210 C°; IR (KBr) cm^{-1}: 3460, 3345 forked peak (NH_2), 2960 (CH-aliphatic), 2189 (CN); ^{1} H NMR (400 MHz, DMSO-d_6) ppm: 3.71 (s, 3H, OCH_3) , 5.03(s,1H, H-4),), 7.15-7.17 (d, 2H, *J = 8* HZ), 7.23-7.21 (d, 2H, *J = 8* HZ), 7.32 (s, 2H, NH_2, D_2O exchangeable), 7.33-7.51 (m, 3H, CH aromatic) ;
^{13}CNMR(100 MHz, DMSO) ppm: 55.5 (OCH_3) , 56.7 (C-4), 111.4, 111.5, 114.6, 116.3, 120.4, 122.3, 122.5, 127.7, 127.8, 129, 132.4, 132.5, 136.2, 138.7, 138.8 (aromatic CH), 120.2 (CN), 160.5 (C-2),;
MS: m/z (% rel. Abundance): 352 (M^+) (48.78%);
Anal. Calcd for $C_{19}H_{13}FN_2O_2S$: C, 64.76; H, 3.72; N, 7.95; S, 9.10; Found: C, 64.95; H, 3.70; N, 8.12; S, 9.08.

2-Amino-6-flouro-4-(4-nitrophenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4f)
Yield: 95%; mp: 235 °C; IR (KBr) cm^{-1}: 3429,4329 forked peak (NH_2), 2924 (CH-aliphatic), 2198 (CN); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 5.34 (s, 1H, H-4), 7.30 (s, 2H, NH_2, D_2O exchangeable), 7.58-7.6- (d, 2H, *J = 8* HZ), 7.61-7.63 (d, 2H, *J = 8* HZ), 8.23-8.26 (m, 3H, CH aromatic);
MS: m/z (% rel. Abundance): 367 (M^+) (11.26%);
Anal. Calcd for $C_{18}H_{10}FN_3O_3S$: C, 58.85; H, 2.74; N, 11.44; S, 8.73; Found: C, 59.17; H, 2.86; N, 11.73; S, 8.74.

4. 4. General procedure for the synthesis of ethyl 2-amino-4-aryl-6-flouro-4*H*-benzo[4,5]thieno[3,2-*b*] pyran-3-carboxlates (5a-f).

A mixture of 2-arylidene-7-flouro-benzo[b]thiophen-3-ones **(3a-f)** (1mmol), ethylcyanoacetate (0.113 g, 1 mmol) and piperidine (7 dps) in absolute ethanol (50 ml) was heated under reflux for 5 hours. The reaction was cooled, and the separated solid was filtered, dried and crystallized from isopropanol.

Ethyl 2-Amino-4-(4-bromophenyl)-6-flouro-4*H*-benzo[4,5] thieno[3,2-*b*]pyran-3-carboxylate (5a)
Yield: 35%; mp: 115 °C; IR (KBr) cm^{-1}: 3468, 3317 forked peak (NH_2), 2974-2908 (CH-aliphatic), 1685 (C=O); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 0.97-1.01 (t, 3H, CH_3, *J = 7HZ*), 3.92-3.95 (q, 2H, OCH_2, *J = 7 HZ*), 5.1 (s, 1H, H-4), 7.45-7.47 (d, 2H, *J = 8* HZ), 7.51-7.52 (d, 2H, *J = 8* HZ), 7.53-7.81 (m, 3H, CH aromatic), 7.91 (s, 2H, NH_2, D_2O exchangeable);
MS: m/z (% rel. Abundance): 448 (M^+) (48.27 %), 450 (M+2) (3.79%);
Anal. Calcd for $C_{20}H_{15}BrFNO_3S$: C, 53.58; H, 3.37; N, 3.12; S, 7.15; Found: C, 53.72; H, 3.33; N, 3.19; S, 7.13.

Ethyl 2-Amino-4-(4-chlorophenyl)-6-flouro-4*H*-benzo[4,5]thieno[3, 2-*b*] pyran-3-carboxylate (5b)
Yield: 44%; mp: 125 °C; IR (KBr) cm^{-1}: 3363, 3271 forked peak (NH_2), 2958(CH-aliphatic), 1680 (C=O); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 0.99- 1.01 (t, 3H, CH_3, *J = 7 HZ*), 3.92- 3.95 (q, 2H, OCH_2, *J = 7 HZ*), 5 .17(s, 1H, H-4),7.28-7.26 (d, 2H, *J = 8 HZ*), 7.32-7.34 (*d, 2H, J = 8 HZ*), 7.52-7.90(m, 3H, CH aromatic),7.90 (s, 2H, NH_2, D_2O exchangeable);
MS: m/z (% rel. Abundance):403 (M^+), (7.72%), 405 (M+2) (2.93%);
Anal. Calcd for $C_{20}H_{15}ClFNO_3S$: C, 59.48; H, 3.74; N, 3.47; S, 7.94; Found: C, 59.76; H, 3.70; N, 3.56; S, 7.95.

Ethyl 2-Amino-6-flouro-4-(4-flourophenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carboxylate (5c)
Yield: 40%; mp: 150 °C; IR (KBr) cm^{-1}: 3368 , 3270 forked peak (NH_2), 2927 (CH-aliphatic), 1665 (C=O); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 0.97-1.006 (t, 3H, CH_3, *J = 7 HZ*), 3.89- 3.96 (q, 2H, OCH_2, *J = 7 HZ*), 5.17 (s, 1H, H-4), 7.07-7.06 (d, 2H, *J = 8* HZ), 7.57-7.55 (d, 2H, *J = 8* HZ), 7.26- 7.31 (m, 3H,CH aromatic), 7.89 (s, 2H, NH_2, D_2O exchangeable);
^{13}CNMR(100 MHz, DMSO) ppm: 14.5 (CH_3), 59.3(OCH_2), 75.4(CH-4), 111.1, 111.3, 115.5, 116.3, 122.4, 122.6, 122.6, 127. 6, 129.3, 129.3, 132.5, 132.5, 138.3, 138.4, 143.2, 143.2 (aromatic C), 168.7 (C=O); MS: m/z (% rel. Abundance): 387 (M^+) (17.5 %);
Anal. Calcd for $C_{20}H_{15}F_2NO_3S$: C, 62.01; H, 3.9; N, 3.62; S, 8.28; Found: C, 62.34; H, 3.85; N, 3.80; S, 8.30.

Ethyl 2-Amino-6-flouro-4-(4-hydroxyphenyl)-4*H*-benzo[4,5]thieno[3, 2-*b*]pyran-3-carboxylate (5d)
Yield: 30%; mp: 142 °C; IR (KBr) cm^{-1}: 3340, 3309 forked peak (NH_2), 3200 (OH), 2924-2854 (CH-aliphatic), 1712 (C=O);
^{1}HNMR (400 MHz, DMSO-d_6) ppm: 1.28-1.31 (t, 3H, CH_3, *J = 7 HZ*), 4.26-4.32 (q, 2H, OCH_2, *J = 7 HZ*), 5.02 (s, 1H, H-4), 6.94-6.96 (d, 2H, *J = 8 HZ*), 6.99-7.96 (m, 3H, CH aromatic), 7.99-8.01 (d, 2H, *J = 8 HZ), 8*.24 (s, 2H, NH_2, D_2O exchangeable),10.81(s, 1H, OH, D_2Oexchangeable);
MS: m/z (% rel. Abundance): 385 (M^+) (72.07 %);
Anal. Calcd for $C_{20}H_{16}FNO_4S$: C, 62.33; H, 4.18; N, 3.63; S, 8.32; Found: C, 62.59; H, 4.20; N, 3.81; S, 8.30.

Ethyl 2-Amino-6-flouro-4-(4-methoxyphenyl)-4*H*-benzo[4,5]thieno[3, 2-*b*] pyran-3-carboxylate (5e)
Yield: 40%; mp: 150 °C; IR (KBr) cm^{-1}: 3421, 3298 forked peak (NH_2), 2924 (CH-aliphatic), 1670 (C=O); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 1.05- 1-07 (t, 3H, CH_3, *J = 7 HZ*), 3.6(s, 3H, OCH_3) 3.95- 3.97 (q, 2H, OCH_2, *J = 7 HZ*), 5 .09 (s, 1H, H-4), 7.13-7.14 (d, 2H, *J = 8* HZ), 7.15-7.17 (d, 2H, *J = 8* HZ), 7.47-7.82 (m, 3H, CH aromatic),8.00(s, 2H, NH_2, D_2O exchangeable);
MS: m/z (% rel. Abundance): 399 (M^+) (13.38%);
Anal. Calcd for $C_{21}H_{18}FNO_4S$: C, 63.15; H, 4.54; N, 3.51; S, 8.03; Found: C, 63.49; H, 4.62; N, 3.80; S, 8.00.

Ethyl 2-Amino-6-flouro-4-(4-nitrophenyl)-4*H*-benzo[4,5]thieno [3, 2-*b*] pyran-3-carboxylate (5f)
Yield: 44%; mp: 125 °C; IR (KBr) cm^{-1}: 3460, 3309 forked peak (NH_2), 2981-2920 (CH-aliphatic), 1739 (C=O); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 0.96-0.997 (t, 3H, CH_3, *J = 7 HZ*), 3.9-3.96 (q, 2H, OCH_2, *J = 4HZ*), 5.36 (s, 1H, H-4), 7.55-7.57 (d, 2H, *J* =8 *HZ),* 8.15-8.17 (d, 2H, *J = 8* HZ), 7.29- 7.59 (m, 3H, CH aromatic),8.39 (s, 2H, NH_2, D_2O exchangeable);
MS: m/z (% rel. Abundance): 414 (M^+) (9.19 %);
Anal. Calcd for $C_{20}H_{15}FN_2O_5S$: C, 57.97; H, 3.65; N, 6.76; S, 7.74; Found: C, 58.21; H, 3.80; N, 6.89; S.7.70.

2-((4-chlorobenzylidene)amino)-4-(4-chlorophenyl)-6-flouro-4H-benzo[4,5]thieno[3,2-b]pyran-3-carbonitrile (6)

A mixture of 2-Amino-6-flouro-4-(4-chlorophenyl)-4*H*-benzo-[4,5]thieno[3,2-*b*] pyran-3-carbonitrile **(4b)** (0.2 gm, 1mmol) and 4-chlorobenzaldhyde (0.14 gm, 1mmol) in (10 ml) toluene was refluxed for 24 hr. The excess solvent was removed under vacuum, the solid remained was crystallized from benzene.
Yield: 80%; m.p: 220 °C; IR (KBr) cm^{-1}: 3066 (=CH), 2924 (CH), 2224 (CN), 1630 (N=CH), 1550 (C=C); ^{1}HNMR (400 MHz, DMSO-d_6) ppm: 5.58 (s, 1H, H-4), 7.34-7.36 (d, 2H, *J = 8* HZ, schiff's base H), 7.68-7.70 (d, 2H, *J = 8* HZ, schiff's base H), 7.98-8.00 (d, 2H, *J = 8* HZ), 8.09-8.11 (d, 2H, *J = 8* HZ), 7.38-7.70 (m, 3H, aromatic H), 9.3 (s, 1H, CH methine);
MS: m/z (% rel. Abundance): 478 (M^+) (20.78%), (480) (M+2) (22.37%);
Anal. Calcd for$C_{25}H_{13}Cl_2FN_2OS$: C, 62.64; H, 2.73; N, 5.84; S, 9.69; Found C, 62.89; H, 2.80; N, 6.02; S, 9.70.

Antiproliferative activity

Anticancer activity of the newly synthesized compounds was measured *invitro* utilizing 60 different human tumor cell lines representing lung, colon, CNS, ovarian, brain, prostate, kidney cancer, leukemia and melanoma at US National Cancer Institute according to the following standard procedures[20].

1- The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine.
2- For a typical screening experiment, cells are inoculated into 96 well micro titer plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines.
3- After cell inoculation, the micro titer plates are incubated at 37°C, 5% CO2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.
4- After 24 hr, two plates of each cell line are fixed *insitu* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addiction (Tz).
5- Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use.
6- At the time of drug growth, (C), and test growth in the presence of drug at the five additions, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 µg/ml gentamicin.
7- Additional four, 10-fold or ½ log serial dilutions are made to provide a total of five drug concentrations plus control.
8- Aliquots of 100 µl of these different drug dilutions are added to the appropriate micro titer wells already containing 100 µl of medium, resulting in the required final drug concentrations.
9- Following drug addition, the plates are incubated for an additional 48 h at 37°C, 5% CO2, 95% air, and 100% relative humidity.
10- For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed *in situ* by the gentle addition of 50 µl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C.
11- The supernatant is discarded, and the plates are washed five times with tap water and air dried.
12- Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature.
13- After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried.

14- Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm.
15- For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80 % TCA (final concentration, 16 % TCA).
16- Using the seven absorbance measurements [time zero, (Tz), control concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:
[(Ti-Tz)/(C-Tz)] × 100 for concentrations for which Ti ≥ Tz
[(Ti-Tz)/Tz] × 100 for concentrations for which Ti<Tz

Cell line:

HT-29 (ATCC® HTB-38™) is a human colon cancer cell line, and was purchased from American Type Culture Collection (ATCC, USA). The cells were grown as a monolayer sheet in 75 cm^2 culture flask in Dulbecco's Modified Eagle Medium (DMEM)supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 ug/ml of insulin; all were purchased from (ThermoFisher Scientific®, USA). The cells were incubated at 37°C and 5% CO_2 in air atmosphere until they are ready to be tested.

Cell Cycle Analysis:

To clarify the cytotoxic effect of the newly synthesized compounds, the effects of compounds 4f and 4c on the cell cycle progression[21] were examined against HT-29cells. At a density of 4 x 10^6 cell/T 25 flask, HT-29 cells were exposed to compound 4f and 4c at their GI_{50} concentration (0.05 µM) for 24 and 48 hr. The cells then were harvested by trypsinization, washed with phosphate buffered saline (PBS), and fixed in ice cold absolute alcohol. Thereafter, the cells were stained, using Cycle test TM plus DNA Reagent Kit (BD Biosciences, USA), according to the manufacturer's instructions. Cell cycle analysis was determined using a FACS Calibur flow cytometer (BD Biosciences, USA).

Detection of apoptosis:

Apoptosis of the cells was determined by fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit purchased from BioVision (USA) using flow cytometry (BD Biosciences, USA). FITC Annexin V is used to quantitatively determine the cells undergoing apoptosis[22]. It relies on the high affinity of Annexin V to bind to phospholipid phosphatidylserine (PS), which translocated from the inner leaflet of the plasma membrane to the outer surface in the cells undergoing apoptosis. Briefly, 4 x 10^6 cell/T 25 flasks were exposed to compound 4C and 4F at theirGI_{50} concentration (0.05 mM) for 24 and 48 hr. After incubation time, cells were harvested by trypsinization; centrifuged; and washed with phosphate buffered saline. Finally, cells were stained according to the manufacturer's protocol.

ELISA assay for Cyclin-Dependent Kinase 2 (CDK2):

CDK2 content [23] of HT-29 cells was assessed by ELISA kit purchased from Cloud-Clone Crop. (USA) according to the manufacturer's protocol, and the enzyme concentration was expressed as ng/mL.

Erlotinib

Erlotinib [24, 25] is a small-molecule inhibitor of the kinases enzymes that is approved by the US Food and Drug Administration for the treatment of NSCLC (Non-Small Cell Lung Cancer).

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