Chapter 1

Evaluation of novel synthetic derivatives of coumarins for their anticancer activity in the A549 and NIH3T3 cell lines

Cancer is a chronic disease with high heterogenicity, which makes it uncurable with available medication. Lung cancer is the most frequently occurring cancer type with a high mortality rate, especially in men and characterized by two main types *viz.*, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The NSCLC type has the highest incident rate (80-85%) of all the lung cancers, and the sub-types adenocarcinoma of NSCLC, further accounts for approximately 40% of all the listed NSCLC which positioned it high in the priority list of drug discovery. Treatment of NSCLC type cancer often fails due to drug insusceptibility to advanced lung cancer stages (Musa et al., 2012). However, some synthetic, as well as natural coumarins, are believed to have significant anticancer properties against it.

Coumarin represents a large class of naturally occurring secondary metabolites with a broad spectrum of the pharmacological and biochemical properties, including anticancer. It not only acts as an anticancer agent to eradicate it but also combats its side-effect even at advanced stages of malignancies (Myers et al., 1994). The substitution pattern on the basic coumarin ring also plays a vital role in its targeted therapeutic interventions. Due to it's promising biological profile and diverse function, it attracted the medicinal chemists to synthesize and design a novel coumarin based anticancer agents with high potency. It is well proven that coumarin with different substitutions can act on various tumor types, including that of the breast, ovarian, lung, renal, prostate, hepatic, colon, and also leukemia. However, some of them were having excellent selectivity toward it (Klenkar and Molnar, 2015). According to a report, when coumarin was given with troxerutin, in combinational therapy, it protected the salivary gland and mucosa from radiogenic sialadenitis and mucositis in the patient's undergone with head and neck radiotherapy (Mahler et al., 1992).

Molecular hybridization techniques are frequently used to synthesize novel compounds by a conglomeration of two or more pharmacophores in one molecular scaffold to form a multi-targeted hybrid molecule. On the primary coumarin nucleus, six substitutions can be made,

which are at the position C-3, C-4, C-5, C-6, C-7, and C-8. However, only C-3, C-4, and C-7 positions reported being involved in anticancer activity when exploited for functional group substitution (Kaur et al., 2015). There is a report that coumarin prevents the rapid proliferation of cancer cells and intercalate between the bases of DNA via C-3 and C-4 position (Li et al., 2015). Therefore, coumarin with different pharmacophore at these positions on the core coumarin ring (2H-1-benzopyran-2-one) was synthesized and evaluated for its anticancer property with enhanced potency and minimized side-effects.

C-7 substituted coumarin derivatives (Figure 1.1), e.g.,7-hydroxycoumarin, the first metabolite of coumarin, Osthole (7-methoxy-8-(3- methyl-2-butenyl)coumarin), Umbelliprenin(7-{[(2E,6Z)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]oxy}-2H-coumarin) and daphnetin (7,8 – dihydroxy coumarin) (Wang et al., 2013) a naturally distributed coumarin and 7-[(6-chloropyridin-2-yl)sulfanyl]-4-methyl-2H-coumarin (Chen et al., 2012), a synthetic coumarin both caused cell cycle arrest, induced apoptosis and influenced the vital pathway (Khaghanzadeh et al., 2012, Xu et al., 2011) in the A549 cell line.

Similarly, C-3 position substituted coumarin also induced apoptosis via Bax/Bcl2 in the A549 cell line (Musa et al., 2012); one such example includes 8–(acetyloxy)-3-(4-methanesulfonyl phenyl)-2-oxo-2H chromen-7-yl acetate (Figure 1.1) (Musa et al., 2015).

Synthetic coumarin with substitution at the C-4 position (Figure 1.1), e.g., 6-chloro-4-[(4-iodophenoxy)methyl]-2H-chromen-2-one, where iodinated-4-aryloxy methyl was added at the C-4 position and chlorine at sixth position (Basanagouda et al., 2014) and 4-(1,2,3-triazole-1-yl) coumarin derivative; 4-(4-((4-fluorophenoxy)methyl)-1,2,3-triazol-1-yl)-7 methoxy coumarin (Zhang et al., 2014a) were also reported to have an excellent anti-proliferative effect on A549 cell line. One of the compound 4-[(E)-2-(2,4-dimethoxy phenyl)ethenyl]-7-methoxy-2H-chromen-2-one, when administered orally in patients with lung carcinoma (H460), inhibited proliferation of cancer cells, without any toxicity (Belluti et al., 2010). Therefore, coumarins, either synthetic or natural analogs, can act as a promising lead compound for future NSCLC treatment.

In this chapter, we auenre presenting the initial screening results of 4-aminomethyl substituted and 3,7-disubstituted novel series of coumarin derivatives in the A549 cell line for their anticancer activity, which has a substitution at C-4 and C-3, C-7 positions (di-substitution). The half-maximal inhibitory concentration (IC_{50}) was calculated for each derivative of both the

series and the derivative, which showed the lowest IC_{50} concentration, was further analyzed at different time-points to predict the time at which the derivative is maximum effective. Subsequently, for the most active derivative, as a part of safety evaluation, the cytotoxicity was also checked in the NIH3T3 cell line, a non-cancerous mouse fibroblast cell line.

MATERIAL AND METHOD

Chemical and Reagent

The reagents were purchased from Sisco Research Laboratories (India), Gibco (USA), or Sigma-Aldrich (USA). Chemicals and solvents were used after purification wherever necessary. A stock solution of the coumarin derivatives was prepared in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO). Various dilutions of the derivative were prepared in phosphate buffer saline (PBS), wherein the final concentration of DMF/DMSO was not more than 0.5% in any of the chosen aliquots.

Cell line procurement and maintenance

Human lung adenocarcinoma cell line A549 and mouse fibroblast cell line NIH3T3 were purchased from National Centre for Cell Science (Pune, India). Cell lines were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 2mM of l-glutamine and 10% fetal bovine serum (FBS) with 1% antibiotic solution (Penicillin and streptomycin). The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 incubator (Thermo Fisher Scientific, USA).

MTT assay

The MTT assay was used to screen the derivatives listed in Table 1 for their cytotoxicity toward A549 and NIH3T3 cell lines. It is a quantitative assay widely used in *in vitro* evaluation of cytotoxic potency of any drug, by quantifying the cytotoxicity index in a cell population using a 96-well plate format. For the assay, A549 cells were seeded in a 96-well plate ($1x10^3$ cells/well) in 100µl DMEM media supplemented with 10% FBS and kept overnight. Each derivative was added in 0.5, 1, 10, 25, 50, 75, 100µM concentrations and incubated for 48h (for preliminary screening). 20µl of MTT solution (5mg/ml prepared in PBS) was added and incubated for 4h. 5-Fluorouracil was taken as a positive control (reference/standerd control). Following incubation, the supernatant was removed, and the formazan crystals were dissolved in 100µl of Isopropanol (acidified). The absorbance (abs.) was measured using a microplate reader at 570nm (Metertech Σ960), and cell viability was calculated using the following formula.

$$\% \text{ Cell Viability} = \frac{\text{Average abs. of treated group}}{\text{Average abs. of control group}} \text{ X } 100$$

IC_{50} concentration was calculated by GraphPad Prism 5 software (GraphPad Software Inc., USA). Derivative showed the lowest IC_{50} concentration was further analyzed at various time-points namely 6h, 12h 24h and 72h with narrow concentration (0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5nM) range. Since derivative 2 (4-FPAC) showed the lowest IC_{50} concentration at 48h among all listed derivatives, hence it was further analyzed for its cytotoxicity in NIH3T3 cell line at 48h.

STATISTICAL ANALYSIS

All values are reported as Mean ± Standerd Error of Mean (SEM). Experiments were performed in triplicates. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., USA). The difference between groups was analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. The level of significance was kept at 95%.

RESULTS

The MTT-based assay relies upon the cellular reduction of tetrazolium salts to their colored formazans and is an index on cell respiration. The amount of formazan formed is proportional to the number of living cells present and an increase or decrease in cell number, indicating the degree of cytotoxicity caused by the drug. The result of the test can be extrapolated to derive the IC_{50} concentration of the tested drug and can be used as a predictive index of the cytotoxic effect. The lower the value, the more cytotoxic, is the substance.

In the current study, we tested nine 7-substituted-3-acetyl coumarin (derivatives 1 to 9), and ten 4-amino benzocoumarin derivatives (derivatives 10 to 19) for their potential anticancer property. 5-Fluorouracil, a reported lung cancer chemotherapeutic, was used as a reference to compare the anticancer activity of the derivatives, as mentioned earlier. The subsequent probit analysis revealed that derivative 2 (Figure 1.2A), namely 4-Fluorophenylacetamide-acetyl coumarin (4-FPAC) of 7-hydroxy 3-acetyl coumarin series has the lowest IC_{50} concentration of 0.16±0.02nM for A549 cells incubated for 48h (Figure 1.3A). The 5-fluorouracil, which was taken as a standerd drug for comparing the anticancer activity of derivatives, showed an IC_{50} concentration of 11.13±2.39µM in the A549 cell line (Figure 1.2B).

As 4-FPAC showed lowest IC_{50} concentration, narrow concentration range study was performed to reaffirm the above findings in A549 cell line. The concentration range used was0,

0.05, 0.1, 0.5, 1, 1.5, 2 and 2.5nM for narrow range as well as for time-point study, and found that IC_{50} concentration at 6h (Figure 1.3B), 12h (Figure 1.3C), 24h (Figure 1.3D), 48h (Figure 1.3E) and at 72h (Figure 1.3F) are 1.19±0.02nM, 0.75±0.08nM, 0.67±0.013nM, 0.161±0.003nM and 0.1927±0.019nM respectively.

Parallelly, the MTT assay for cytotoxicity toward the non-cancerous normal cell line was performed using the NIH3T3 cell line, and after 48h of incubation with varying concentrations of 4-FPAC, theIC_{50} concentration was computed to be 79.59±10.24μM (Figure 1.2B).

DISCUSSION

The anticancer property of coumarin derivatives is affected by the pharmacophore substitutions on the C-3, C-4 and C-7 positions, of the primary coumarin ring, since, these positions directly interact with DNA and hamper the replication process (Li et al., 2015). Hence, coumarin derivatives with different pharmacophore substitutions on C-3 C-4 and C-7 position were synthesized and evaluated for their anticancer activity. There were two series of derivatives one has substitution on C-4, and the other has di- substitution on C-3, C-7 position. Amongst the first series (from derivative 1 to 9), derivative 2 *viz.*, 4-fluorophenylacetamide-acetyl coumarin showed the lowest IC_{50} concentration of 0.16nM, whereas derivative 16 *viz.*, 4-morpholinomethyl benzocoumarin of 4- amino benzocoumarin series showed the lowest IC_{50} concentration of 0.32μM. Most of the derivatives were found to have lower IC_{50} concentrations than 5-Fluorouracil *viz.*,11.13μM, a known combinational drug used in NSCLC treatment. As derivative 2 has shown the lowest IC_{50} value among both the series, it was further analyzed on different time-points and at a narrow concentration range.

At 48h, it showed an IC_{50} concentration of 0.17nM, nearly equivalent to the IC_{50} concentration obtained with a broad concentration range. Therefore, a concentration of 0.16nM was taken for further analysis. At 6h, 12h, and 24h, the derivative (4-FPAC) showed an IC_{50} concentration of 1.19nM, 0.75nM, and 0.67nM, respectively, which is much higher than the IC_{50} concentration of 48h. However, at 72h, the IC_{50} concentration was 0.197nM, which showed an increase in comparison to the IC_{50} concentration of 0.16nM at 48h. Therefore, it confirms that the 4-FPAC is maximum effective at 48h and displaying time-dependent as well as dose-dependent cytotoxicity.

Since there is a negligible yet significant increase in half-maximal inhibitory concentration at 72h after the most active stage, that is 48h, and there could be a probability that the derivative is showing phase-specific cytotoxicity. Phase-specific cytotoxicity can be seen since the

derivative is targeting a vulnerable phase of the cell cycle, and a fraction of tumor cells killed depend on the time of exposure (Alagkiozidis et al., 2011).

Most of the anticancer drugs adversely affect the neighboring non-cancer cell. However, it will be more effective and accepted as a chemotherapeutic agent if it poses negligible or no side-effects to the neighborhood cells. Therefore, the derivative was evaluated for its cytotoxicity in NIH3T3, a non-cancerous mouse fibroblast cell line. The IC_{50} concentration was found to be 79.58µM, which is very much higher than the IC_{50} concentration of 4-FPAC, which revealed that the 4-FPAC would show negligible side-effects to the nearby non-cancer cell if used at the selected concentration of 0.16nM. It was therefore taken further to comprehend the mechanism of its cytotoxicity in the A549 cell line.

SUMMARY

Novel coumarin derivatives were synthesized by adding different pharmacophore at the C-4 and C-3, C-7 position of the basic ring. Out of 18, the derivative 2, a C-4 substituted, 4-fluorophenylacetamide-acetyl coumarin derivative showed the lowest IC_{50} concentration of 0.16nM in A549 cell line, when screened at 48h post-incubation. The time-point study at 6h, 12h, 24h, 48h, and 72h at narrow concentration range confirmed that the derivative is showing maximum cytotoxicity at 48h, which is time as well as dose-dependent. When the derivative was analyzed for its cytotoxicity toward normal, non-cancerous mouse fibroblast cell line, NIH3T3, at 48h, it showed an IC_{50} concentration of 79.98µM, which is very much higher than the IC_{50} concentration in A549 cell line, which confirmed that derivative would have less side-effect to non-cancerous neighborhood cells. Since derivative 2, i.e., 4-FPAC showed the lowest IC_{50} concentration among all the derivatives screened, with minimal cytotoxicity toward non-cancerous cell line NIH3T3, it was taken further for the study to check its competency as an anticancer compound.

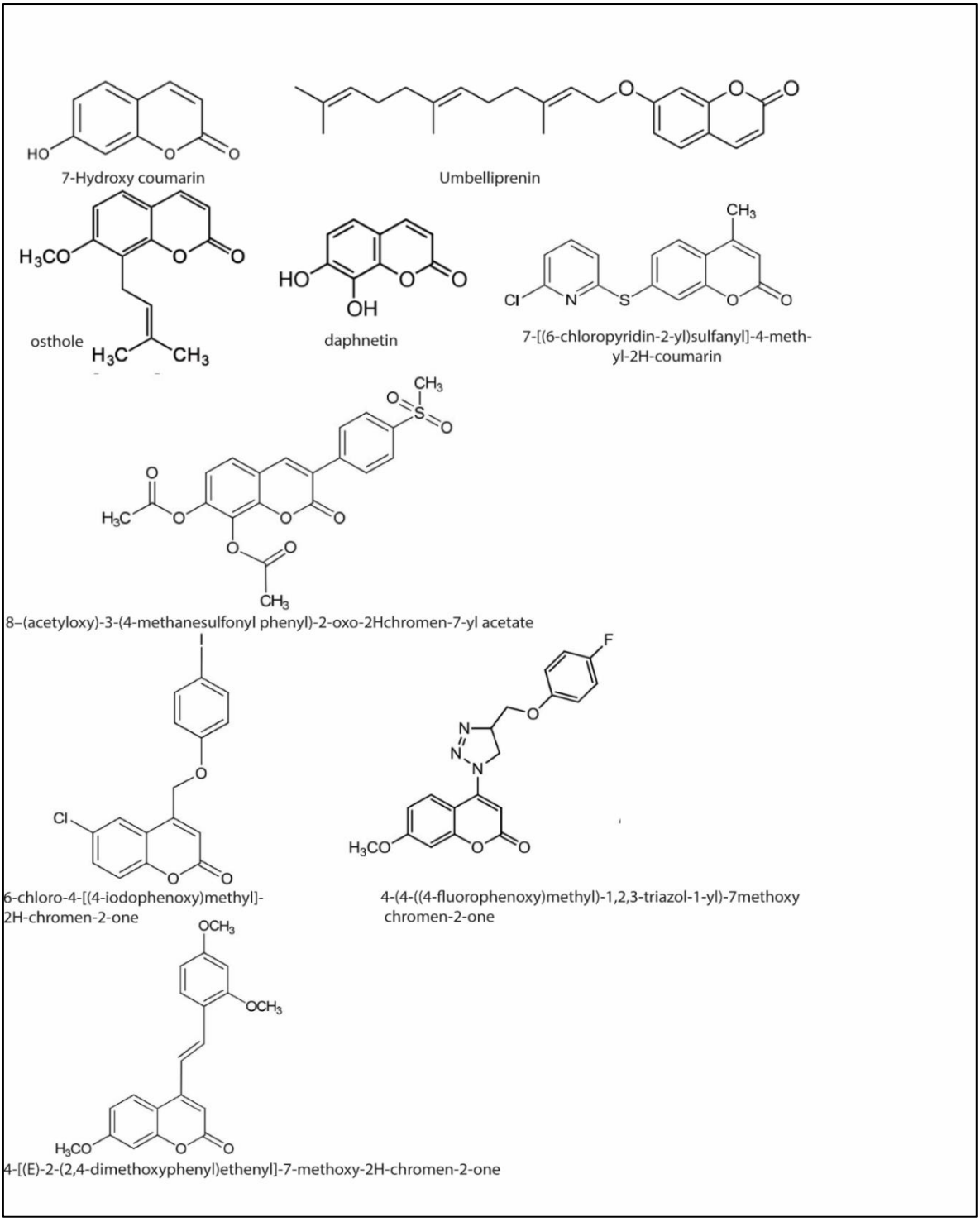


Figure 1.1. Structure of Coumarin derivatives: **C-7 substituted**; 7 hydroxy coumarin, umbelliprenin, osthole, daphnetin 7-[(6-chloropyridin-2-yl)sulfanyl]-4-methyl-2H-coumarin, **C-3 substituted**; 8–(acetyloxy)-3-(4-methanesulfonyl phenyl)-2-oxo-2Hchromen-7-yl acetate, **C-4 substituted**; 6-chloro-4-[(4-iodophenoxy)methyl]-2H-chromen-2-one, 4-(4-((4-fluorophenoxy)methyl)-1,2,3-triazol-1-yl)-7methoxycoumarin, 4-[(E)-2-(2,4-dimethoxyphenyl)ethenyl]-7-methoxy-2H-chromen-2-one.

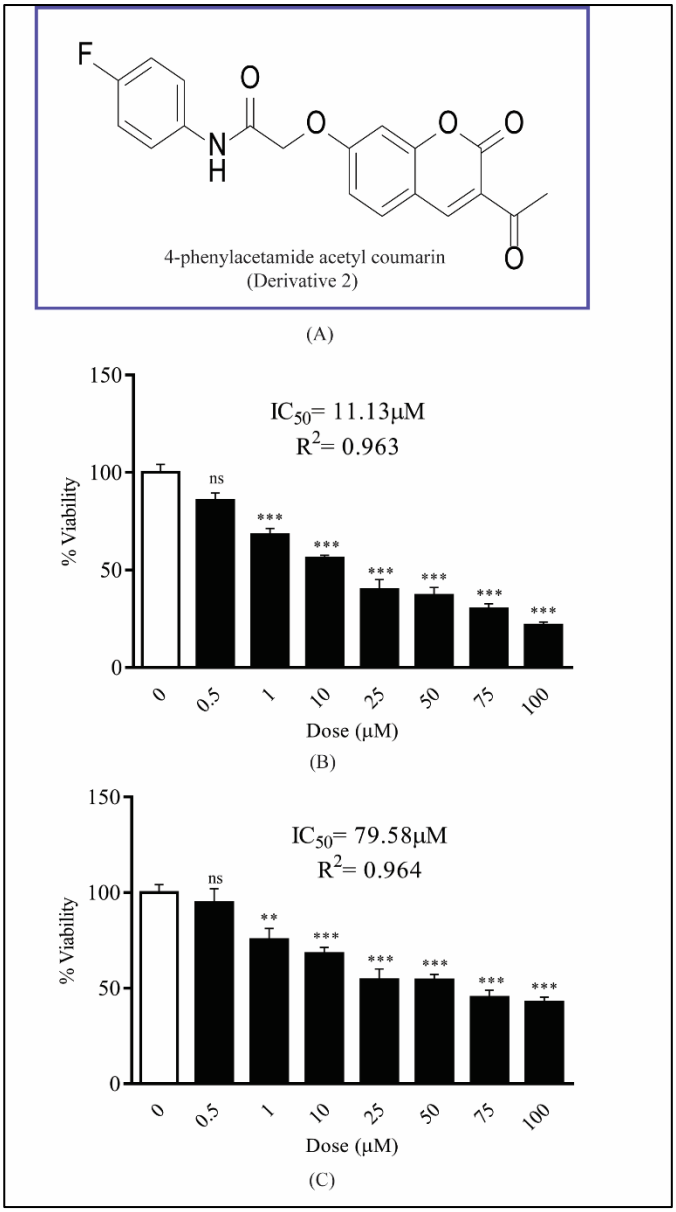


Figure 1.2. Structure of Derivative 2 (4-FPAC) (A), Graph representing the IC_{50} concentration of; 5-Fluorouracil in the A549 cell line (B), 4-FPAC in NIH3T3 cell line (C). Data are represented as Mean ± Standard Error of Mean (SEM), ***p≤0.001, **p≤0.01, ns=not significant.

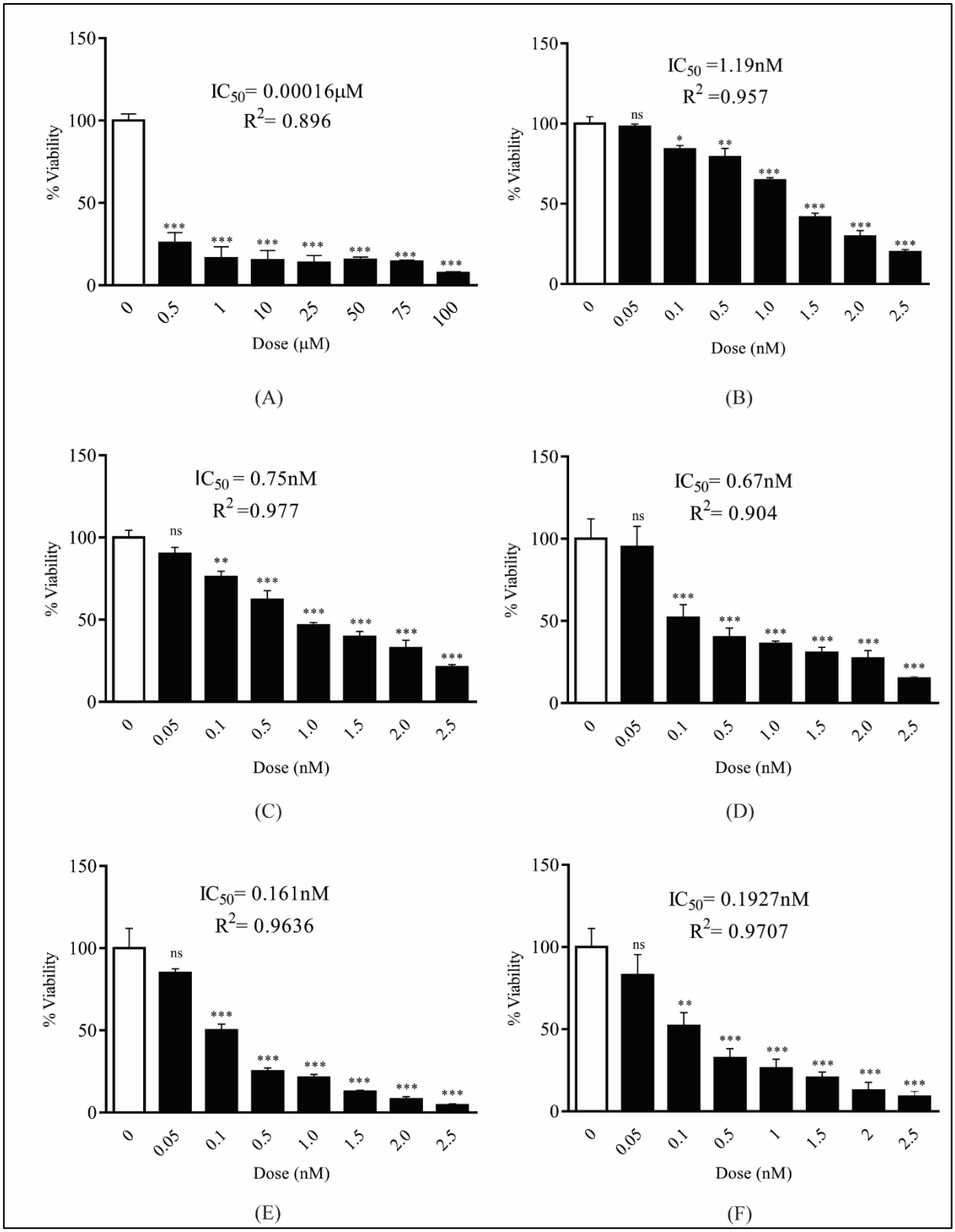
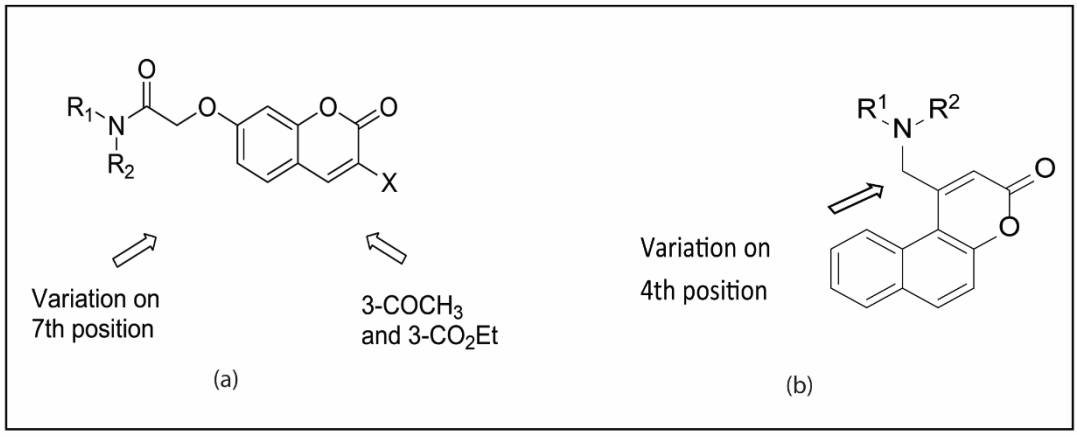
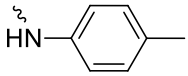
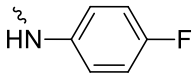
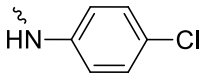
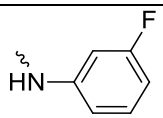
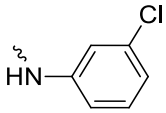
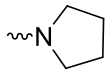
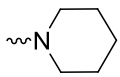
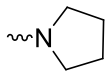
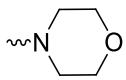


Figure 1.3. MTT assay graph of A549 and NIH3T3 cell line; graph representing the IC_{50} concentration of 4-FPAC, at broad concentration range at 48h (A), at narrow concentration range at 6h (B), 12h (C), 24h (D), 48h (E) and 72h (F) in A549 cell line. Data are represented as Mean ± Standard Error of Mean (SEM), ***p≤0.001, **p≤0.01, *p≤0.1, ns=not significant.



Derivatives	NR^1R^2	X	IC_{50} concentrations in A549
(1)		$-CH_3$	2.40 µM
(2)		$-CH_3$	0.16 nM
(3)		$-CH_3$	0.82 µM
(4)		$-CH_3$	9.16 µM
(5)		$-CH_3$	89.16 µM
(6)		$-CH_3$	23.9 µM
(7)		$-CH_3$	5.06 µM
(8)		$-OC_2H_5$	3.11 µM
(9)		$-OC_2H_5$	23.2 µM

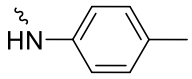
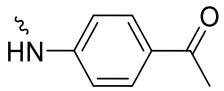
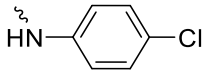
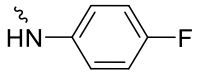
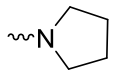
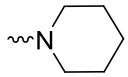
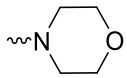
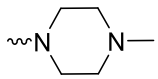
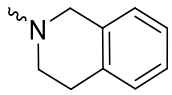
Derivatives	NR^1R^2	X	IC_{50} concentrations in A549
(10)			4.49 µM
(11)			2.32 µM
(12)			56.75 µM*
(13)			59.14 µM*
(14)			6.20 µM
(15)			1.12 µM
(16)			0.32 µM*
(17)			0.74 µM
(18)			19.98 µM*

Table 1.1. Derivative 1-9 has di-substitution at C-3 and C-7, position on basic coumarin ring (a) and derivative 10-18 has substitution on C-4 position on aminomethyl benzocoumarin (b). *IC_{50} values were determined using GraphPad Prism 5 software (GraphPad Software Inc., USA) by MTT assay using DMSO rest were prepared in DMF.

(1) *2-[(3-Acetyl-2-oxo-2H-chromen-7-yl)oxy]-N-(4-methylphenyl)-acetamide*

(2) *2-[(3-Acetyl-2-oxo-2H-chromen-7-yl)oxy]-N-(4-fluorophenyl)-acetamide*

(3) *2-[(3-Acetyl-2-oxo-2H-chromen-7-yl)oxy]-N-(4-chlorophenyl)-acetamide*

(4) *2-[(3-Acetyl-2-oxo-2H-chromen-7-yl)oxy]-N-(3-fluorophenyl)-acetamide*

(5) *2-[(3-Acetyl-2-oxo-2H-chromen-7-yl)oxy]-N-(3-chlorophenyl)-acetamide*

(6) *3-Acetyl-7-[2-oxo-2-(pyrrolidin-1-yl)ethoxy]-2H-chromen-2-one*

(7) *3-Acetyl-7-[2-oxo-2-(piperidin-1-yl)ethoxy]-2H-chromen-2-one*

(8) *Ethyl 2-oxo-7-[2-oxo-2-(pyrrolidin-1-yl)ethoxy]-2H-chromene-3-carboxylate*

(9) *Ethyl 7-[2-(morpholin-4-yl)-2-oxoethoxy]-2-oxo-2H-chromene-3-carboxylate*

(10) *1-((p-Tolylamino)methyl)-3H-benzo[f]chromen-3-one*

(11) *1-((4-Acetylphenyl)amino)methyl)-3H-benzo[f]chromen-3-one*

(12) *1-((4-Chlorophenylamino) methyl)-3H-benzo[f]chromen-3-one*

(13) *1-((4-Fluorophenylamino) methyl)-3H-benzo[f]chromen-3-one*

(14) *1-(Pyrrolidin-1-ylmethyl)-3H-benzo[f]chromen-3-one*

(15) *1-((Piperidine-1-ylmethyl)-3H-benzo[f]chromen-3-one*

(16) *1-(Morpholinomethyl)-3H-benzo[f]chromen-3-one*

(17) *1-((4-Methylpiperazin-1-yl)methyl)-3H-benzo[f]chromen-3-one*

(18) *1-((3,4-Dihydroisoquinolin-2(1H)-yl)methyl)-3H-benzo[f]chromen-3-one*