International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.4, pp 1397-1406, Aug-Sept 2014

Synthesis and Cell line study of Pyrazole Substituted Coumarin Derivatives

Twara Thakor, Jignasa Savjani*

Department of Pharmaceutical Chemistry, Institute of Pharmacy, Nirma University, Ahmedabad-382481, Gujarat, India.

***Corres. Author: jignasasavjani@gmail.com**
Tel.: +91-2717-241900-04, Mobile: +91-9824550737

Abstract: Pyrazole substituted coumarin derivatives were synthesised via multi-component reaction namely 3-(4-substituted phenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde containing 4-hydroxy-2H-chromen-2-one and 4-methyl-2-oxo-2H-chromen-7-yl acetate by conventional synthetic route. The structure of the synthesised compounds was characterized by FTIR, Mass, ^{1}H NMR spectral analysis. The synthesized compounds were screened for anticancer activity against the Lung cancer cell line (NCIH-522 cell line) using anticancer drug doxorubicin as a standard. Cell line studies revealed that compound 2-amino-4-(3-(4-chloro phenyl)-1-phenyl-1H-pyrazol-4-yl)- 5-oxo-4, 5-dihydropyrano[3,2-c] chromene-3-carbonitrile found to be active against the lung cancer cell line as compared to doxorubicin.
Key words: Cell line study, Coumarin, Doxorubicin, Lung cancer, Pyrazole.

1. Introduction

Cancer is the second leading cause of death worldwide next to cardiovascular diseases. It is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases. In the last few decades, human immortal cancer cell lines have aggregated an accessible, easily usable set of biological models in which residents of cells from a multicellular organism which would normally not proliferate indefinitely but, due to mutation have evaded normal cellular sequence and instead can keep undergoing division. Drug resistance is also one of the hefty hindrances to chemotherapy of cancer. Studies with cell lines can serve as an initial screen for agents that might regulate drug resistance. To establish more appropriate models of drug resistance and explore whether the differences exist in the different drug resistant cell lines selected by different treatments.

The utility of cell lines acquired from tumours allows the investigation of tumor cells in a simplified and controlled environment. There are specific advantages and disadvantages to exploit cancer cell lines over animal models. Firstly, the cost involved with sustaining them is significantly less than maintaining animal subjects. They are promptly available and research studies can be implemented relatively quickly. Large quantities and volumes of cells may be propagated to create high-throughput studies. Cell lines are exceptionally versatile in the types of studies in which they may be used. They cannot only build *in vitro* but can also be injected into mice to form xenograft models of prostate cancer progression.

Disadvantages related to cell lines are that they do not represent the heterogeneity of the tumour microenvironment as well as the necessarily heterogeneous nature of tumours with a patient and between patients. As a result multiple cell lines may be required to address the full heterogeneity seen in a tumour

phenotype. Cell lines are also subject to genetic alterations in culture that may alter their phenotype over the course of a long experiment. The path to the progression of the tumour is lost and does not provide insight in the pathogenic process significantly [1].

Cell line study can be used as a primary screening method in distinct forms of cancers influencing vital organs like lung, prostate, breast, ovarian, colorectal, salivary, epithelial, larynx, liver etc. For cell line study MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) assay used to determine *in vitro* inhibitory effects of test compounds on cell growth which was developed by Mosmann [2]. This colorimetric assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate, MTT into an insoluble, purple colored formazan product which is measured spectrophotometrically. Growth of cancer cells was quantitated by the ability of living cells to reduce the yellow MTT to purple formazan products. The amount of formazan product formed is directly proportional to the number of living cells [3].

Various drugs are available in the market; a therapeutic search for novel, potent anti-cancer agents is under process to combat the dreadful disease. Several coumarin derivatives have also been shown to be potent inhibitors of tumour induction by carcinogenic polycyclic aromatic hydrocarbons. Coumarin is a chemical compound which is found naturally in some plants, although it can be synthetically produced as well. Coumarin is a natural substance that has shown anti-tumour activity *in vivo*, with the effect believed to be due to its metabolites i.e.7-hydroxycoumarin. Therefore, the focus was on these relevant compounds and their therapeutic importance [4]. Coumarin is classified as a member of the Benzopyrone family of compounds, all of which consist of a benzene ring joined to a pyrone ring [5]. The investigations on coumarin derivatives have revealed their potentials as versatile biodynamic agents [6]. Along with coumarin ring, the pyrazole ring is also a prominent structural motif found in numerous pharmaceutically active compounds. Pyrazole framework also plays an essential role in biologically active compounds along with coumarin analogues and therefore represents an interesting scaffold for combinatorial as well as medicinal chemistry. Pyrazole and fused heterocyclic pyrazole derivatives constitute an interesting class of heterocycles due to their synthetic versatility and effective biological activities. Pyrazole derivatives are also known for their antibacterial, anti HIV, anticancer, anti-inflammatory, analgesic and hypoglycaemic activities. In recent years, a significant portion of research in heterocyclic chemistry has been devoted to pyrazole containing different aryl groups [7]. These finding encouraged us to synthesize the pyrazole and Coumarin containing compounds for biological interest.

2. Results and Discussion

2.1 Chemistry

Different series of compounds were synthesised by using different target moiety like 4-hydroxy coumarin and 7-hydroxy 4-methyl coumarin. For synthesis of first series, 4-hydroxy coumarin was reacted with substituted pyrazole aldehyde by using different reaction condition. For synthesis of second series, 7-acetyloxy 4-methyl coumarin was reacted with substituted pyrazole aldehyde.

Two series of pyrazole substituted coumarin derivatives were synthesized namely 2-amino-4- (3-(4-substituted phenyl)-1-phenyl-1H-pyrazol-4-yl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile and 2-amino-4-(3-(4-substituted phenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy) nicotino nitrile.

Precursors of pyrazole aldehyde were synthesised by reacting substituted acetophenone **(2)** and phenyl hydrazine **(1)**. In literature survey we found significant application in heterocyclic chemistry is the synthesis of 4-formylpyrazoles from the double formylation of hydrazones with Vilsmeier-Haack (VH) reagent ($DMF/POCl_3$). These observations, coupled with the recent developments encouraged us to the synthesis of pyrazole derivatives without much effort [8].

In first series, 4-hydroxy coumarin **(7)** and substituted pyrazole aldehyde **(4a-e)** were refluxed in presence of ethanol for 4 hours with the use of piperidine as base catalyst. Reaction of Phenol with malonic acid **(6)** in presence of zinc chloride and phosphorous oxychloride yielded 4-hydroxy coumarin **(7)** [9]. During the synthesis Knoevenagel condensation occurs between pyrazole-4-carbaldehyde and malanonitrile **(8)** by loss of water molecules and finally, Michael addition occurs to afford the desired products **9a-e**.

Second series were synthesised with the use of 7-acetyloxy 4-methyl coumarin **(13).** 7-acetyloxy 4-methyl coumarin was synthesized by previously reported method with the use of resorcinol **(10)** and ethyl acetoacetate **(11)** [10]. The synthesized 7-acetyloxy 4-methyl coumarin **(13)** was coupled with pyrazole aldehyde **(4a-e)** to afford the desired compounds **14a-e**.

2.2 *In vitro* cell line study

Characterization of cell lines was performed for detection of microbial and cross contaminations. Cell lines used in the experiments were free from any kind of microbial or fungal contaminations (Table 1), which is essential in order to continue the screening experiments.

Culture media were also tested for microbial contaminations. To prevent microbial contamination, 2.5 % Amphotericin B (µg/ml) was supplemented to media which act as working concentration. Bacterial contamination was prevented by addition of 1 % of antibiotic, 100 X (10000 U/mL Penicillin G, 10000 µ g/mL Streptomycin) into culture media. All subculturing activities were done under class – II Biosafety cabinet. (Esco, Singapore).

Cross contamination of cell line was tested by direct observation under inverted microscope and photodynamic therapy (PDT) for specific cell line was determined. From viability studies and PDT, it has been concluded that the cell line derived from ATCC was initially free from cross contamination.

To prevent the cross contamination of cell line during experimental work, separate pipettes and plastic tips were used. Along with that, particular cell line was used at the time under Class – II Bio safety cabinet. These were proving to be valid steps to prevent cross contamination of cell line throughout the experiment.

In the present investigation, all the synthesized compounds as well as standard anticancer drug doxorubicin were evaluated against NCIH-522 cell line. Dose Response Curve (DRC) against the cell line was plotted with 10 analysis point i.e. with 10 different drug concentrations. The concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using *Graph Pad Prism* software (Ver. 5.04) (Graph Pad Software, Inc., USA) and Micorsoft Excel 2007 (Microsoft Corporation, USA) application.

Table 1. Results of Characterization of cell lines

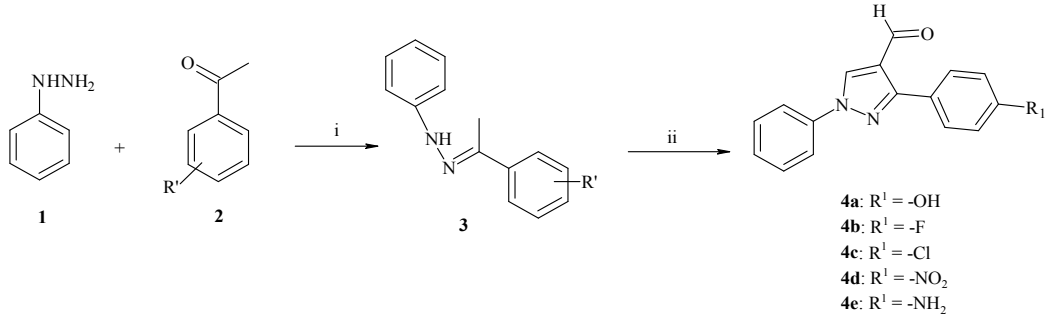
cell line	%viability		PDT(h)	Microbial Contamination	Cross Contamination	pH
	stock	After		No contamination	No	7
NCIH-522	64.54	82.8	32.9	No contamination	No	7.5

Table 2. Quantity of selected synthesized compounds taken for cell line study

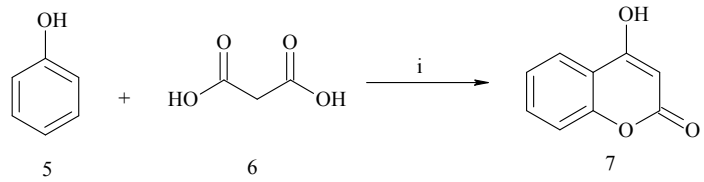
Compounds	Mol.wt	1M=xmg/mL	1 mM soln. (mg/5mL)
9c	527	527	2.635 mg
9d	529	529	2.645 mg
14a	493	493	2.465 mg
14b	503	503	2.515 mg

3. Conclusion

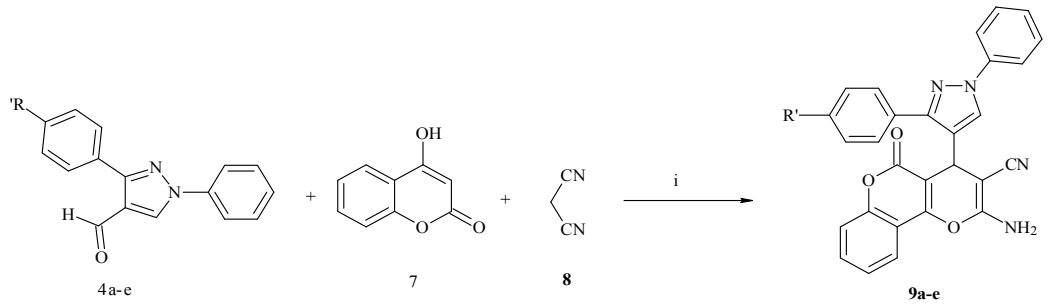
Pyrazole substituted coumarin derivatives were synthesized and *in vitro* cell line study was performed on NCIH-522 cell line used for lung cancer. Compounds **9c** and **9d** had shown good anticancer activity against the selected cell line. For this NCIH-522 cell line selected which was used to check cytotoxicity of compounds in Lung cancer and doxorubicin was selected as standard. From the investigation it was found that **9c** showed very good cytotoxicity against NCIH-522 lung cancer cell line, while compound **14a** showed lowest activity in comparison with standard.



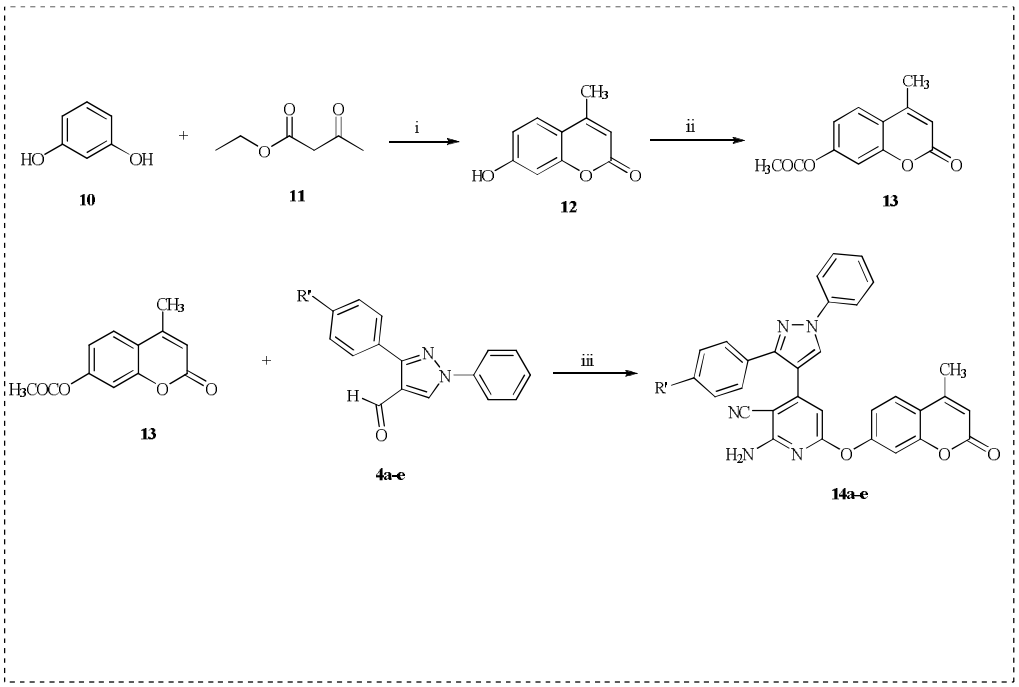
Scheme 1. Reagents and conditions: (i) Ethanol, CH_3COOH, stirring at rt, 1 h; (ii) DMF, $POCl_3$, reflux, 6 h



Scheme 2. Reagents and conditions: (i) $POCl_3$, anhydrous $ZnCl_2$, 70°C, 36 hr



Scheme 3. Reagents and conditions: (i) piperidine, reflux, 4 h, ethanol



Scheme 4. *Reagents and conditions*: (i) H_2SO_4, stirring, 2.5 h; (ii) acetic anhydride, reflux, 1.5 h; (iii) malanonitrile, ammonium acetate, CH_3COOH, reflux, 3 h

4. Experimental Section

4.1 General

Melting Point of the synthesised compounds was recorded on VEEGO Corporation Melting point apparatus and reported as uncorrected. All the chemicals were obtained from Merck, Spectrochem, CDH, RANKEM & Ureca and were used without purification. Precoated Silica Gel Plates (MERCK) was used for TLC to monitor progress of the reaction and visulization was achieved by UV light or iodine detector. IR spectra were recorded on JASCO FTIR by KBr dispersion method. Mass spectra were recorded, using ESI as ion source at Oxygen healthcare and at Synzeal Research lab Ahmedabad. The proton NMR spectra were recorded on Bruker 400 MHz instrument at Saurastra University, Rajkot.

4.2 General Procedures

4.2.1 Synthesis of 2-amino-4- (3-(4-hydroxyphenyl)-1-phenyl-1H- pyrazol-4-yl) -5-oxo-4,5-dihydro pyrano [3,2-c]chromene-3-carbonitrile (9a)

To a mixture of 4-hydroxy coumarin**(7)** (0.49g, 30 mmol), 3-(4-hydroxy phenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4a)** (0.79g, 30 mmol) and malanonitrile (0.19g, 30 mmol) in 25 mL ethanol was added three to four drops of piperidine and refluxed with stirring for 4 h. Completion of reaction was checked by TLC using ethyl acetate: n-hexane (50:50). The reaction mixture was cooled at room temperature and solid was collected by filtration.

Yield: 86.16%; mp:169-173°C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 8.5 (s, 2H, NH_2), 7.9 (d, *J* = 8.1 Hz, 2H), 7.82 (s, 1H), 7.76 (d, J = 7.2 Hz, 2H), 7.65 to 7.44 (m, 4H), 7.31 (dd *J* = 8.8, 2.2 Hz, 4H), 7.2 (t, *J* = 7.7 Hz, 1H), 4.7 (s, 1H, OH), 4.00 (s, 1H); MS (ESI) *m/z* 475.6 (M^++1)

4.2.2 Synthesis of 2-amino-4- (3-(4-flourophenyl) -1-phenyl-1H- pyrazol-4-yl) -5-oxo-4,5- dihydropyrano [3,2-c]chromene-3-carbonitrile (9b)

The compound **9b** was prepared according to the method described for the compound **9a** by using the compounds 4-hydroxy coumarin**(7)** (0.49g, 30 mmol) and 3-(4-flourophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4b)** (0.79g, 30 mmol).

Yield: 89.63%; mp:185-187°C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.0 (s, 2H, NH_2), 8.1 (d, *J* = 8.1 Hz, 2H), 7.9 (s, 1H), 7.86 (d, J = 7.2 Hz, 2H), 7.73 to 7.61 (m, 4H), 7.53 (dd *J* = 8.8, 2.2 Hz, 4H), 7.32 (t, *J* = 7.7 Hz, 1H), 3.34 (s, 1H); MS (ESI) *m/z* 477.3 (M^++1)

4.2.3 Synthesis of 2-amino-4- (3-(4-chlorophenyl) -1-phenyl-1H- pyrazol-4-yl) -5-oxo-4,5- dihydropyrano [3,2-c]chromene-3-carbonitrile (9c)

The compound **9c** was prepared according to the method described for the compound **9a** by using the compounds 4-hydroxy coumarin**(7)** (0.49g, 30 mmol) and 3-(4-chlorophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4c)** (0.84g, 30 mmol).

Yield: 91.02%; mp: 176-180°C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 8.8 (s, 2H, NH_2), 8.24 (d, *J* = 8.1 Hz, 2H), 8.15 (s, 1H), 7.94 (d, J = 7.2 Hz, 2H), 7.86 to 7.72 (m, 4H), 7.50 (dd *J* = 8.8, 2.2 Hz, 4H), 7.47 (t, *J* = 7.7 Hz, 1H), 4.02 (s, 1H); MS (ESI) *m/z* 494.7 (M^++2)

4.2.4 Synthesis of 2-amino-4- (3-(4-nitrophenyl) -1-phenyl-1H- pyrazol-4-yl) -5-oxo-4,5- dihydropyrano [3,2-c]chromene-3-carbonitrile (9d)

The compound **9d** was prepared according to the method described for the compound **9a** by using the compounds 4-hydroxy coumarin**(7)** (0.49g, 30 mmol) and 3-(4-nitrophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4d)** (0.87g, 30 mmol).

Yield: 77.23%; mp: 199-202°C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.3 (s, 2H, NH_2), 7.21 (d, *J* = 8.1 Hz, 2H), 7.10 (s, 1H), 6.97 (d, J = 7.2 Hz, 2H), 6.89 to 6.80 (m, 4H), 6.75 (dd *J* = 8.8, 2.2 Hz, 4H), 6.7 (t, *J* = 7.7 Hz, 1H), 3.46 (s, 1H); MS (ESI) *m/z* 504.7 (M^++1)

4.2.5 Synthesis of 2-amino-4- (3-(4-aminophenyl) -1-phenyl-1H- pyrazol-4-yl) -5-oxo-4,5- dihydropyrano [3,2-c]chromene-3-carbonitrile (9e)

The compound **9e** was prepared according to the method described for the compound **9a** by using the compounds 4-hydroxy coumarin**(7)** (0.49g, 30 mmol) and 3-(4-aminophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4e)** (0.78g, 30 mmol).

Yield: 81.25%; mp: 210-213℃; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.1 (s, 2H, NH_2), 8.8 (s, 2H, NH_2), 8.4 (d, *J* = 8.1 Hz, 2H), 8.24 (s, 1H), 8.1 (d, J = 7.2 Hz, 2H), 7.93 to 7.89 (m, 4H), 7.74 (dd *J* = 8.8, 2.2 Hz, 4H), 7.1 (t, *J* = 7.7 Hz, 1H), 4.12 (s, 1H); MS (ESI) *m/z* 474.5 (M^++1)

4.2.6 Synthesis of 2-amino-4-(3-(4-hydroxyphenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy)nicotinonitrile (14a)

A mixture of 7-acetyloxy 4- methyl coumarin **(7)** (0.21g, 1 mmol), 3-(4-substituted phenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4a)** (0.26g, 1 mmol), ammonium acetate (0.06g, 2 mmol) and malanonitrile **(8)** (0.07g, 1 mmol) were taken into round bottom flask. 5 mL glacial acetic acid was added to the reaction mixture and refluxed for 3.5 h. Completion of reaction was monitored by TLC using ethyl acetate:n hexane (30:70) mixture. The reaction mixture was allowed to cool at room temperature to solidify. The obtained solid was collected by filtration and washed with ethanol.

Yield: 59.37%; mp:179-182° C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.18 (s, 2H, NH_2), 8.53 (s, 1H, OH), 8.48(d, *J* = 8.7 Hz, 1H), 8.37 (s, 1H), 8.12 (s, 1H), 7.92 to 7.76 (m, 4H), 7.69 (dd *J* = 8.8, 2.2 Hz, 2H), 7.48 to 7.34 (m, 4H), 7.23 (t, *J* = 8.7 Hz, 1H), 3.30 (s, 1H), 2.21 (d, 3H); MS (ESI) *m/z* 528.4 (M^++1)

4.2.7 Synthesis of 2-amino-4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy)nicotinonitrile (14b)

The compound **14b** was prepared according to the method described for the compound **14a** by using the compounds 4-hydroxy coumarin **(7)** (0.21g, 1 mmol), 3-(4-fluorophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4b)** (0.26g, 1 mmol) and malanonitrile **(8)** (0.07g, 1 mmol).

Yield: 65.71%; mp: 160-164° C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.7 (s, 2H, NH_2), 8.27(d, *J* = 8.7 Hz, 1H), 8.10 (s, 1H), 7.91 (s, 1H), 7.82 to 7.76 (m, 4H), 7.51 (dd *J* = 8.8, 2.2 Hz, 2H), 7.43 to 7.32 (m, 4H), 7.20 (t, *J* = 8.7 Hz, 1H), 3.53 (s, 1H), 2.01 (d, 3H); MS (ESI) *m/z* 530.4 (M^++1)

4.2.8 Synthesis of 2-amino-4-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy)nicotinonitrile (14c)

The compound **14c** was prepared according to the method described for the compound **14a** by using the compounds 4-hydroxy coumarin **(7)** (0.21g, 1 mmol), 3-(4-chlorophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4c)** (0.28g, 1 mmol) and malanonitrile **(8)** (0.07g, 1 mmol).

Yield: 57.27%; mp: 171-175° C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.23 (s, 2H, NH_2), 8.3(d, *J* = 8.7 Hz, 1H), 8.22 (s, 1H), 7.94 (s, 1H), 7.25 to 7.78 (m, 4H), 7.64 (dd *J* = 8.8, 2.2 Hz, 2H), 7.57 to 7.42 (m, 4H), 7.22 (t, *J* = 8.7 Hz, 1H), 3.52 (s, 1H), 2.32 (d, *J* = 7.7 Hz, 3H); MS (ESI) *m/z* 546.2 (M^++2)

4.2.9 Synthesis of 2-amino-4-(3-(4-nitrophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy)nicotinonitrile (14d)

The compound **14d** was prepared according to the method described for the compound **14a** by using the compounds 4-hydroxy coumarin **(7)** (0.21g, 1 mmol), 3-(4-nitrophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4d)** (0.29g, 1 mmol) and malanonitrile **(8)** (0.07g, 1 mmol).

Yield: 62.6%; mp: 189-193° C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.17 (s, 2H, NH_2), 8.01(d, *J* = 8.7 Hz, 1H), 7.92 (s, 1H), 7.64 (s, 1H), 7.25 to 7.18 (m, 4H), 7.0 (dd *J* = 8.8, 2.2 Hz, 2H), 6.87 to 6.72 (m, 4H), 6.64 (t, *J* = 8.7 Hz, 1H), 3.22 (s, 1H), 2.05 (d, *J* = 7.7 Hz, 3H); MS (ESI) *m/z* 557.3 (M^++1)

4.2.10 Synthesis of 2-amino-4-(3-(4-aminophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(4-methyl-2-oxo-2H-chromen-7-yloxy)nicotinonitrile (14e)

The compound **14e** was prepared according to the method described for the compound **14a** by using the

compounds 4-hydroxy coumarin **(7)** (0.21g, 1 mmol), 3-(4-aminophenyl)-1-phenyl -1H-pyrazole-4-carbaldehyde **(4e)** (0.26g, 1 mmol) and malanonitrile **(8)** (0.07g, 1 mmol).

Yield: 71.87%; mp: 195-198° C; ^{1}H NMR: (DMSO, 400MHz) : δ ppm 9.73 (s, 2H, NH_2), 8.71 (s, 2H, NH_2), 8.49 (d, *J* = 8.7 Hz, 1H), 8.32 (s, 1H), 8.14 (s, 1H), 7.95 to 7.98 (m, 4H), 7.69 (dd *J* = 8.8, 2.2 Hz, 2H), 7.51 to 7.72 (m, 4H), 7.24 (t, *J* = 8.7 Hz, 1H), 3.31 (s, 1H), 2.21 (d, *J* = 7.7 Hz, 3H); MS (ESI) *m/z* 515.6 (M^++1)

4.3 Anticancer activity

Anticancer activity of synthesised compound was evaluated by performing *in-vitro* cell line study using Lung cancer (NCIH-522) by employing MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) assay [2, 3]. Three fold serial dilution of cytotoxic drugs in growth medium was prepared to give eight concentrations. Doxorubicin, a cytotoxic anticancer substance used in antineoplastic therapy was used as reference standard. The anticancer activity of synthesised compound was evaluated by *in-vitro* cell line study which was performed at S.K Patel Institute of pharmaceutical research, Kherva, Gujarat.

4.3.1 Selection of Assay Parameters and Methodology

Under in-*vitro* assay conditions, exposure to an antitumor agent may decrease the number of viable tumor cells by direct cell killing or by simply decreasing the rate of cellular proliferation. Many *in-vitro* assays of drug sensitivity typically employ relatively low initial cell inoculation densities (e.g., a few hundred cells/well) followed by relatively long continuous drug exposure times (e.g.6-7 d or considerably longer than the doubling times of many tumor cell lines). Selection of such assay parameters, although favouring the detection of anticancer effects (i.e., growth inhibition), might, however, obscure otherwise potentially interesting patterns of differential cytotoxicity (e.g., net cell killing). Moreover, with an anticancer or growth inhibition end point, cell lines with very short doubling times (e.g., leukaemia's) might appear hypersensitive in comparison to more slowly growing tumor cell lines (e.g., from solid tumours). Additionally, potential problems of nutrient deprivation, as well as practical limitations on the use of pulse drug exposures in the course of an assay may necessitate removal and replacement of medium. On the other hand, longer assay duration could facilitate the detection of activity of relatively insoluble compounds or active trace constituents in mixtures or extracts. Furthermore, the longer assay format is essential for detection of agents that required several cell cycles for expression of lethal drug effects. An alternative selection of assay parameters was considered in order to enhance the screen's ability to discern interesting differences in net cell killing (i.e., actual reduction of biomass) among the sensitive panel lines. This required the use of a relatively large initial cell inoculums (e.g., 20,000 cells/well), and a relatively short drug exposure per incubation time (e.g., 1-2 d). Optimal exploitation of this format required a high level of sensitivity and reproducibility of the assay methodology, along with the capability to measure reliably the initial viable cell densities ("t_0" values) just prior to drug introduction. This selection was based principally on the desire to minimize the effects of variable doubling times of the diverse cell lines in the panel, to optimize the chances of detection of cell-line-specific or subpanel-specific cytotoxins, and to minimize the chances of obscuring such activities by nonspecific anticancer effects. This choice of assay parameters was also emphatically endorsed by the Harrap committee, after much discussion, debate, and extensive review of the relevant available experimental data. Given the above decisions concerning assay parameters, the optimal choice of a tetrazolium assay (e.g., MTT or XTT (Tetrazolium salt assay)) vs. the sulphorhodamine B (SRB) assay had to be determined for the desired application to a large-scale screening operation employing simultaneously many diverse tumor lines. Pilot-screening studies were performed on a common set of compounds using both MTT and SRB, along with the selected assay parameters. Under the experimental conditions employed and within the limits of the data analyses applied, the assays gave quite comparable results. However, the SRB assay had important practical advantages for large-scale screening, as the study required small scale *in-vitro* analysis and hence the MTT assay method was used for initial screening.

4.3.2 Cell Cultures

The synthesized compounds were screened on cell line NCIH-522 [Human; Lung cancer cell line]. The cells were cultured in DMEM, supplemented with fetal calf serum (FCS; 5%), gentamycin sulphate (0.004%), glucose (0.57%), and $NaHCO_3$ (0.12%), in a humidified atmosphere with 5% CO_2 at 37°C until confluence reached. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 cm^2 tissue culture flasks, then in 75 cm^2, and finally in 150 cm^2 tissue culture flask and all the cytotoxicity experiments were carried out in 96-well microtiter plates. Doxorubicin was used as a reference standard for cytotoxic activity. The test compounds were prepared with different

concentrations using DMSO and IC_{50} values for the same were determined. IC_{50} is a drug concentration that causes a 50% inhibition of cell proliferation.

4.3.3 Cell Growth Inhibition Study and Cytotoxicity Study

NCIH-522 cell line [Human; Lung cancer cell line] was seeded in 96- well sterile plates and were treated with different concentrations of compound 9c, 9d, 14a, 14b for 24-72h and the cytotoxicity studies were performed by MTT assay.

Table 3. Percentage (%) cell inhibition

Conc. **µg/mL**	Log con.	% Cell Inhibition				
		9c	9d	14a	14b	Std. Doxorubicin
0.01	-2.29	-1.235	-11.560	15.410	13.530	-39.66495
0.02	-1.82	-2.354	-21.090	25.580	16.480	-35.52140
0.05	-1.34	1.365	-20.070	7.850	7.370	-22.98541
0.14	-0.86	2.563	-21.890	27.390	8.330	-19.35120
0.41	-0.39	4.850	-19.450	12.270	14.380	-13.89175
1.23	0.09	11.270	-17.610	9.440	34.830	-12.78351
3.70	0.57	31.550	5.650	37.010	15.000	-10.39691
11.11	1.05	43.200	20.520	24.440	35.940	48.80412
33.33	1.52	69.600	50.220	53.000	59.050	65.69072
100.00	2.00	78.650	71.630	79.140	73.240	78.20103
IC_{50} (µM/mL)		**8.126**	**16.97**	**71.69**	**24.97**	**9.034**
R^2		**0.9912**	**0.9849**	**0.8547**	**0.8981**	**0.9551**

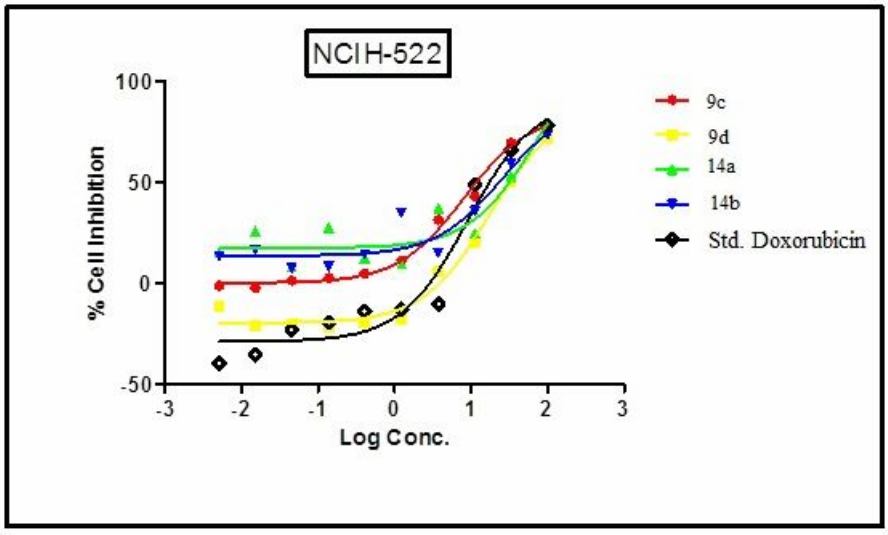


Fig. (1). Graph of % cell inhibition

4.3.4 Preparation of Dilution

A cells in growth medium which containing serum and trypsinize a sub confluent monolayer culture were collected. Suspension was centrifuged for 5 min at 200 g to pellet cells. The cells were counted after preparing re-suspension in growth medium. Cells were diluted to 2.5-50 × 10^3cells /mL, depending on the growth of cell line in which 20mL cell suspension per micro titration plate was allowed. Cell suspension was transferred to a 9 cm petridish and with the help of multichannel pipette 200 µL of suspension was added into each of central 10 columns of a flat bottomed 96 well pate (80 well per plate) starting with column 2 and ending with column 11 (0.5-10 × 10^3cells in each well). 200 µL of growth medium was added to the eight wells in column 1 and 12. Column 1 was used as blank plate reader and column 12 was used to maintain humidity for column 11 which minimize the edge effect and then all plates were incubated in the CO_2 incubator. For non-adherent cells, suspension was prepared in fresh growth medium. Cells were diluted to 5-100 × 10^3cells /mL

and only 100 µL of suspension was plate out into round-bottomed 96-well plates to which drug was added immediately.

4.3.5 Drug addition

Three fold serial dilutions of the synthesized compounds in growth medium were prepared to give eight concentrations. This set of concentrations should be chosen such that highest concentration of drug kills most of cells and lowest concentration of compound should kill none of cells. Once toxicity of drug was known smaller range of concentration could be used. Normally three plates were used for each drug to give triplicate determinations within one experiment. For adherent cells medium was removed from wells in columns 2 to 11.Cells were feed in eight wells in column 2 to 11 with 200 µL of fresh growth medium, these were taken as the controls. Compound was added to cells in columns 3 to 10. Only four wells were needed for each drug concentration, such that rows A-D can be used for one drug and rows E-H for second drug and again incubated in CO_2 incubator for 24 hours.

4.3.6 Reference substance

Doxorubicin, a cytotoxic anticancer substance used in antineoplastic therapy can be considered to classify the coumarin derivatives according to their relative toxicity. Molecular weight of doxorubicin is 545.3. Stock solution was prepared with 1 mL of DMSO (Dimethylsulphoxide), which produces stock solution of doxorubicin of 10 mM concentration and stock solution was further diluted to 10 times using DMSO to obtain 1 mM solution.

4.3.7 Growth period

At the end of drug exposure period, medium from all the wells was replaced with 200 µL of fresh medium. Non-adherent cell plates were centrifuged (5min at 200g) to pellet cells. Medium was removed, using a fine gauge needle to prevent disturbance of cell pellet. Plates were feed daily for 2-3 PDTs.

4.3.8 Estimation of surviving cell number

The plate was fed with 200 µL of fresh medium at end of growth period and 10 µL of MTT was added to all wells in columns 1 to 11. Plates were wrapped in aluminium foil, and incubated for 4 hrs in a CO_2 incubator at 37 °C and plates could be left for up to 8h. The medium and MTT were removed from the well and centrifuged for non-adherent cells. The remaining MTT formazan crystals were dissolved by adding 100 µL of MTT Solubilizer (90% acidified Isopropanol + 10% Triton – X) to all wells in columns 1 to 11. Absorbance was recorded at 540 nM immediately because product was unstable. Column 1 wells reading was recorded as blank plate reading and percentage of cell growth inhibition or percentage cytotoxicity was calculated using the following formula.

% viability = $(A_T - A_B) / (A_C - A_B)$ X 100

$A_{T=}$ Absorbance of treated cells (drug)

$A_{B=}$ Absorbance of blank (only media)

$A_{C=}$ Absorbance of control (untreated)

% cytotoxicity = 100 - % cell survival

References

1. Kashyap, C.P.; Tikka, B.; Sharma, S.; Arya V. Human Cancer Cell lines- A brief Communication. *J. Chem. Pharma. Res.* 2011, *3(6)*, 514-20.
2. Swarna, G.; Bhavani, S. *In vitro* Anticancer Activities of few Antihypertensive Agents Against Carcinoma of Scalp by MTT Assay. *J. Chem. Biolog. Phys. Sci.* 2011, *3*, 299-3.
3. Suvarna, G. K. Shivani, C. Muhammad, M. Synthesis, Docking Study and Anticancer Activity of Coumarin Substituted Derivatives of Benzothiazole. *J. Comput. Mol. Des.* 2012, *2(1)*, 51-60.
4. Moreno, N.P. The Science of Microbes. *BioEd.* , 2008, *2*, 4-8.
5. Rybicki, E.P. The Classification of Organisms at the Edge of Life or Problems with Virus Systematics. *S. Afr. J.Sci.* 1990, *86*,182–86.

6. Gen, J.; Lwoff, A. The Concept of Virus. *Microbiol Science.* 1957, *17(2)*, 239–53.
7. John, B.T.; David, J.T. Comprehensive Medicinal Chemistry II, Therapeutic Areas II: Cancer, Infectious Diseases, Inflammation and Immunology and Dermatology, Elsevier Science:Vol. 7, 2006.
8. Virsodia, V.; Manvar, A.; Upadhyay, K. Synthesis of substituted pyrazole aldehyde, *European J of Med. Chem.* 2008, 1-8.
9. Sehemi, Al.; Abdullah, G.; Sameh, R. Synthesis and Photo oxygenation of Furo[3,2-*c*]coumarin Derivatives as Antibacterial and DNA Intercalating Agent, *Chinese J. of Chem.* 2012, *30*, 316-20.
10. Kumar, T.; Dewangan, D.; Alexander, A.; Nagori, K. Synthesis and Characterization of 8-[(2-Amino-6aryl-Pyrimidin-4-Yl) Oxy]-4-Methyl- 2H-Chromen-2-Ol Derivatives. *Asian J. of Biochem. and Pharm. Res. 2011, 2(1),* 393-1.

*****