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Synthesis, Spectral Characterization and *In vitro* Anti Cancer Activity of Pyimidine – Imidazole coupled Heterocyclic compounds against Human Lung Cancer Cell Line

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Abstract

To Explore two heterocyclic based scaffolds; Pyrimidine and Imidazole for Anticancer activity targeting Human Lung cancer cell lines. The designed compounds were synthesized and evaluated for their *invitro* Anticancer activity against Human Lung Cancer Cell line A549. *In vitro* anticancer activity revealed that 14h showed more potent anticancer activity as compared to the standard drug Sunitinib. This compound exhibited apoptosis thus, arresting the cell cycle at G0/G1 phase itself. To conclude, the above findings clearly demonstrated that the compound 14h may serve as good anticancer agent for further development.

Keywords: Pyrimidine, Imidazole, Human Lung Cancer Cell line.

Introduction

Today, cancer is becoming a common word, with each one of us closely related with at least one nearby or a relative or a friend or a colleague, diagnosed with cancer (1). It can occur at any age, but it is more common among the people over 65 years old (2). It has been reported that cancer has become the major cause of death worldwide. As per WHO, Cancer is leading cause of death worldwide, accounting nearly 10 million deaths in 2020. The most common in 2020 (in terms of new cases of cancer) were Breast (2.26 million cases), lungs (2.21 million cases), Colon and rectum (1.93 million cases), Prostate (1.41 million cases), Skin (1.20 million cases) and stomach (1.09 million cases). The most common causes of cancer death in 2020 were lungs, colon and rectum, liver, stomach and breast (3). This increasing burden opens the window for the research in newer anticancer molecules. Tremendous efforts are taken to combat cancer over the past few years, but there is still a demand for new and better drugs (4). Heterocycles are a good choice when designing molecules that will interact with targets and influences the biological pathways involved in cancer progression, as many of the protein targets are habited to interact with heterocyclic moieties (5). With a vision of novel drug design, the concept of molecular coupling is a significant approach which involves combination of two structurally diverse moiety in a single framework. It is well evident from the literature survey that Pyrimidine scaffold possesses significant anticancer activity.

Pyrimidine substituted with another heterocycle is widely used in the design and discovery of anticancer drugs. Pyrimidine is a versatile heterocycle and possesses many sites which enable it to combine with other potent moieties to construct a coupled framework. Such framework has multifaced mode of action or may bind to various targets to get desired activity. Imidazole ring have been lately gaining much attention due to their roles as attractive scaffolds for biologically active heterocyclic drugs. In general terms, physicochemical properties like π-π stacking interactions, co-ordination bonds with metals as ligands, hydrogen bond donor-acceptor

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capability, van der Waals, polarization and hydrophobic forces have caused the increasing interest in these fragments. These properties liable for their reactivity enable derivatives to willingly bind with a series of biomolecules, including several enzymes and nucleic acids (6-9).

In the present work, we designed and synthesized Pyrimidine coupled with imidazole to explore the anticancer potential of the hybrid moieties in a single framework. The rationale behind the design of these coupled framework is to get maximum activity against Human Lung cancer.

Materials and Methods

Chemicals, Reagents and Cell Lines:

All the chemicals used were of analytical grade and procured from Sigma-Aldrich, Loba chemie Pvt Ltd, India and Sisco Research Laboratories Pvt. Ltd., India. o-Toluidine, Sulphuric acid, Nitric acid, n-Butanol, 50% aqueous solution of cyanamide, 50% Ethanol, Stannous chloride dihydrate, Methanol, Diethyl ether, N, N - Dimethylformamide dimethyl acetal, Cyclohexane, Hydrochloric acid, Ethyl acetate, Sodium sulfate, Dichloromethane, Thionyl chloride, Sodium hydroxide, Tetra Hydro Furan (THF), Tri Ethyl Amine (TEA), Propyl amine, Ammonia, and 2-Acetyl Imidazole. Cell culture was obtained from Future Bioscience, Korea. Fetal Bovie Serum, Antibiotics, Dulbecco's Modified Eagle's Medium (DMEM), Phosphate Buffered Saline, Trypsin-EDTA was purchased from HiMedia. RNase A, Propidium Iodide was purchased from HiMedia. Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) was obtained from Sigma. Annexin V-FITC/PI apoptosis detection kit was procured from BD Biosciences (Cat.No. 556547). Human lung cancer cell line A549 was procured from National Centre for Cell Science (NCCS). A549 cells were preserved in cryoperservation medium, which contains 10% DMSO, 30% Fetal Bovine Serum and 60% DMEM.

Instrumentation:

Melting points was measured in open capillaries and were uncorrected. The purity of all the newly synthesized compounds were checked by TLC with silica gel glass plates and the spots were detected by exposure to iodine and viewed under UV light at λ 254 nm. The infrared (FT-IR) spectra were recorded using Perkin Elmer Spectrum one FT-IR instrument at a resolution of 1.0cm^{-1} and in the range of 4000-450cm^{-1}. Proton Nuclear Magnetic Resonance (H^1 NMR) spectra were recorded in $CDCl_3$ as solvent on a Bruker Avance™ III 500MHz NMR Spectrometer using Tetra Methyl Silane (TMS) as an internal standard. Chemical shifts (δ) are expressed in parts per million (ppm). Mass spectra for the newly synthesized compounds were obtained from the Q-TOF Mass Spectrometer (Micromass). ELISA well plate reader (Robonik India pvt Ltd.,), Flow cytometer BD FACS VERSE (BD BioSciences) was used for in vitro anticancer studies.

Synthesis:

2-Methyl-5-nitroaniline (2)

51.1mL of o-toluidine (1) was added dropwise to 122.3mL of sulphuric acid cooled to ice cold condition with vigorous stirring. Mixture of 8.9mL of 65% nitric acid and 27.2mL of sulphuric acid was cooled to ice cold condition and added16, 17 to the above mixture for a period of 2h. Finally, it was poured into crushed ice. It was made alkaline, with aqueous sodium hydroxide. The product formed was filtered and dried in air. Recrystallization was done using 50% ethanol.

N-(2-Methyl-5-nitrophenyl)guanidinium nitrate (3)

2-methyl-5-nitroaniline (2, 25g) in n-butanol (120mL) was taken. 65% aqueous nitric acid (10.5mL) was added dropwise. 50% aqueous solution of cyanamide (22.7mL) was added to the above mixture. The mixture was refluxed for 12h and subsequently it was cooled to 0ºC. The precipitate formed was collected by filtration and washed with an ice

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cooled solution of 50% ethanol and diethyl ether taken in equal quantity. The product was dried in air for further use.

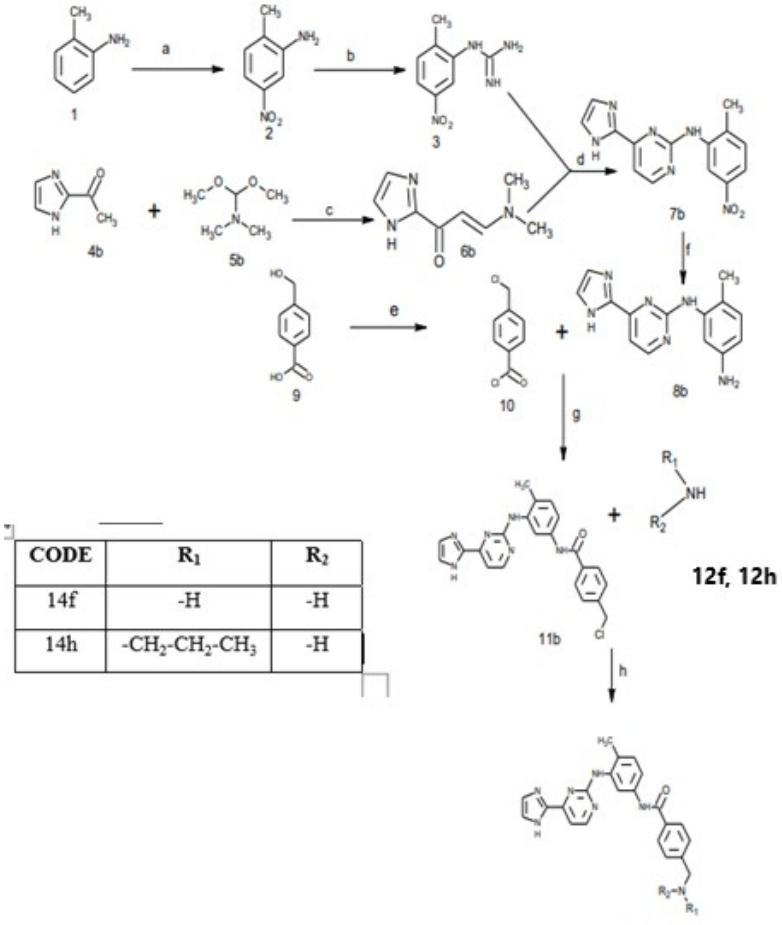
4-(Chloromethyl) benzoyl Chloride (10)

4-(hydroxyl methyl)-benzoic acid (9, 15.2g), dichloromethane (50mL), and thionyl chloride (50 mL) was added into a round bottomed flask and it was refluxed for 5h. The excess thionyl chloride and dichloromethane were removed. The residue was cooled to ice cold condition for 12h. White crystals. mp 30-32°C.

Scheme:

3-Dimethylamino-1-(1H-imidazol-2-yl)prop-2-en-1-one (6b)

A mixture of 2-acetyl Imidazole (4b, 24.21g) and N, N-dimethylformamide dimethyl acetal (5, 34.4mL) were refluxed for 16h and then concentrated to get residue. Cyclohexane (100 mL) was added to the residue and it was cooled



CODE	R_1	R_2
14f	-H	-H
14h	$-CH_2-CH_2-CH_3$	-H

Reagents and conditions: (a) H_2SO_4, HNO_3, Ice cold condition (b) 50% aqueous H_2NCN, HNO_3, n-butanol, 100 °C (c) 100 °C (d) NaOH, n-butanol, 100 °C (e) $SOCl_2$, CH_2Cl_2 (f) $SnCl_2{\cdot}2H_2O$, HCl, Ice cold condition (g) THF, TEA, Ice cold condition, 3h (h) Reflux 3h

Scheme

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in ice. The precipitate formed was filtered and dried in air. Yellow solid. *mp* 84-86ºC.

N-(2-methyl-5-nitrophenyl)-4-(1H-imidazol-2-yl)pyrimidin-2-amine (7b)

To a mixture of 3-Dimethylamino-1-(1H-imidazol-2-yl)prop-2-en-1-one (6b, 26.96g) and N-(2-methyl-5-nitrophenyl) guanidinium nitrate (3, 51.40g) in n-butanol (200mL), sodium hydroxide (8.63g) was added in solid form and it was refluxed for 16h. After the completion of 16h it was cooled in ice. The precipitation formed was filtered. It was washed with methanol and diethyl ether. The product was dried in air for further use. Yellow solid. *mp* 199-201ºC.

4-methyl-N-[4-(1H-imidazol-2-yl)pyrimidin-2-yl]benzene-1,3-diamine (8b)

Stannous chloride di hydrate (11.29 g in 30mL of hydrochloric acid) was cooled to 0 ºC. N-(2-methyl-5-nitrophenyl)-4-(1H-imidazol-2-yl)pyrimidin-2-amine (7b, 3.69g) in portions was added to the above mixture and stirred vigorously for 6h. The mixture was poured into crushed ice and it was made alkaline using solid sodium hydroxide. Extracted three times with ethyl acetate (100mL). The combined organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. Yellow solid. mp 145-147°C

4-(chloromethyl)-N-(3-{[4-(1H-imidazol-2-yl)pyrimidin-2-yl]amino}-4-ethylphenyl) benzamide(11b)

4-methyl-N-[4-(1H-pyrrol-2-yl)pyrimidin-2-yl]benzene-1,3-diamine (0.277g), Tetra hydro furan (5mL), and Triethylamine (0.29mL) were refluxed. The reaction mixture was cooled to 0°C and maintained for 10 min. 4-(chloromethyl)benzoyl chloride (0.217g) in Tetra Hydro Furan (2mL) was added dropwise to the above mixture within 10min. TLC of reaction mass indicated the absence of starting compound. After stirring the mixture at 0°C for 3h, 15mL of water was added dropwise. The resultant precipitate was collected by filtration and washed with 100mL of water. The product was dried at 75-80°C. Light yellow crystals. mp 271-273°C

Compound 14f

4-[aminomethyl]-N-(3-{[4-(1H-imidazol-2-yl)pyrimidin-2-yl]amino}-4-methylphenyl)benzamide

4-(chloromethyl)-N-(3-{[4-(1H-imidazol-2-yl)pyrimidin-2-yl]amino}-4methylphenyl) benzamide (0.429g) and ammonia(12f, 11.1mL) were added in a round bottomed flask and refluxed for 3h. The reaction mass was checked for the absence of compound (11b). The resultant mixture was cooled to room temperature and 10mL of water was added to it. The precipitate was collected by filtration and washed with 100mL of water. The product was dried at 75-80°C. Recrystallized using methanol. White solid. Yield 94.21%, Melting range 220-222°C; **FTIR (cm^{-1})** 3456.09 (Ar N-H str), 3066.09 (Ar C-H str), 2948.10 (-C-H-str), 2790.01(N-H str), 1648.42 (amide -C=O str), 1578.98 (-C=C-, -C=N-ring str), 1181.66 (-C-C-str), 1081.77 (C-N str), 805.79 (N-H wag), 705.60 (C-H out of plane bending), 665.20 (N-H out of plane bend). **^{1}H NMR ($CDCl_3$)** δ 8.64 (m, 1H), 8.09 (s, 1H), 7.92 – 7.65 (m, 3H), 7.46 – 7.07 (m, 7H), 4.76 (s, 1H), 4.10 (m, 2H), 2.29 (s, 3H), 1.35 (s, 3H). **m/z** [M^+ +1] 400.5 Peak and 382.4 Base Peak observed.

Compound 14h

4-[(propylamino)methyl]-N-(3-{[4-(1H-imidazol-2-yl)pyrimidin-2-yl]amino}-4-methylphenyl) benzamide

4-(chloromethyl)-N-(3-{[4-(1H-imidazol-2-yl)pyrimidin-2-yl]amino}-4-methylphenyl)benzamide (0.429g) and propylamine (12h, 11.1 mL) were added in a round bottomed flask and refluxed for 3h. The reaction mass was checked for the absence of compound (11b). The resultant mixture was cooled to room temperature and 10mL of water was added to it. The precipitate was collected by filtration and washed with 100mL of water. The product was dried at 75-80°C. Recrystallized using methanol [10,11]. White

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powder. Yield 92.70%, Melting point 226-228°C. **FTIR (cm^{-1})** 3430.48 (Ar N-H str), 3055.01 (Ar C-H str), 2878.57 (-C-H-str), 2797.23 (N-H str), 1648.42 (amide -C=O str), 1576.66 (-C=C-, -C=N- ring str), 747.66 (C-H out of plane bending), 1050.62 (C-N str), 809.16 (N-H wag), 611.96 (N-H out of plane bend). **^{1}H NMR ($CDCl_3$)** δ 8.64(m, 1H), 8.16 – 7.73 (m, 5H), 7.47 – 7.03 (m, 8H), 4.78 (m, 2H), 2.83 – 2.49 (m, 4H), 2.29 (s, 3H), 1.94 (m, 2H), 1.20 (s, 2H). **m/z** 442.5 (M^+ +1) Peak, 440.2 (M^+-1) Peak, 398.4 (Base Peak).

In vitro Anticancer Studies:

Cytotoxic Activity

The cytotoxic potential of the synthesized compounds 14f and 14h was screened *invitro* against Human Lung cancer cell line A549 according to procedures described in the literature. Sunitinib was used as standard drug. The results are summarized in **Table 1** Compound 14f and 14h showed a cytotoxic effect of 10.14% and 51.04% compared to the standard exhibiting 35.20% at the concentration of 25μM for 24h. At 48h compounds 14f and 14h showed 19.24% and 53.28% of inhibitory effect at 25 μM concentration. The IC_{50} value was calculated using the obtained results.

Apoptosis Detection Assay

Human lung cancer cell A549 was treated with 25μM concentrations of synthesized compounds14f, 14h and standard, diluted in DMSO for 18h. After treatment, cells were washed with cold Phosphate Buffer Solution and then resuspended cells in 1X Binding Buffer (dilute 1 part of the 10X annexin V Binding Buffer to 9 parts of distilled water) at a concentration of 1×10^6 cells/ml. One hundred micro liter (100μl) of the solution was transferred to a 5ml culture tube. Five micro liters (5μl) of FITC Annexin V and 5μl of Propidium Iodide were added. The cells were vortexed gently and incubated for 15min at room temperature (25ºC) in the dark. After incubation, 400 μl of 1X Binding Buffer was added to each tube and analysis was carried out within 1h using flow cytometer.

Apoptosis Detection assay was used to elucidate the mode of cell death caused by the newly synthesized compounds 14f, 14h and standard drug sunitinib in Human lung cancer cell line (A549) after treatment with IC_{50} concentration of 25μM for a period of 18h. The disruption of cell membrane phospholipid asymmetry, evidenced by Phosphatidyl Serine (PS) externalization, was examined by utilizing annexin V-FITC and propidium iodide assay and monitored via the flow cytometer protocol. The cell distributions in flow cytometric histograms are as follow: Cells in the lower left quadrant (Q3) represented live cells. Cells that are viable shows Annexin V-FITC and PI negative. The lower right quadrant (Q4) represented early apoptotic cells. Early apoptotic cells show Annexin V-FITC positive and PI negative. The upper right quadrant (Q2)

Table 1. Percentage of Scavenging Activity at 24h

Comp Code	5μM	25μM	50μM	75μM
14f	10.08	10.14	19.80	26.91
14h	42.08	51.04	54.08	59.20
Standard	26.08	35.20	44.48	59.20
Percentage of scavenging activity at 48h				
14f	28.28	19.24	26.77	30.67
14h	33.16	53.28	56.12	67.38
Standard	27.84	29.79	53.90	67.02

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represented late apoptotic cells. Cells that are in late apoptosis shows Annexin V-FITC and PI positive and the necrotic cells show PI positive only. The upper left quadrant (Q1) represents necrotic cells. The necrotic cells show PI positive only (Fig.1).

The addition of both early and late apoptotic cells (annexin V-FITC positives) was defined as the total percentage value of apoptotic cells. When Human A549 lung cancer cells were treated with the newly synthesized compounds 14f, 14h and Standard drug sunitinib (25µM) for 18h, the total percentage of apoptotic cells increased up to 28.96%, 38.01% and 21.03% at 25µM respectively (**Table.2**). This indicates that the newly synthesized compounds 14f, 14h and Standard drug sunitinib was able to induce apoptosis of Human A549 lung cancer cells.

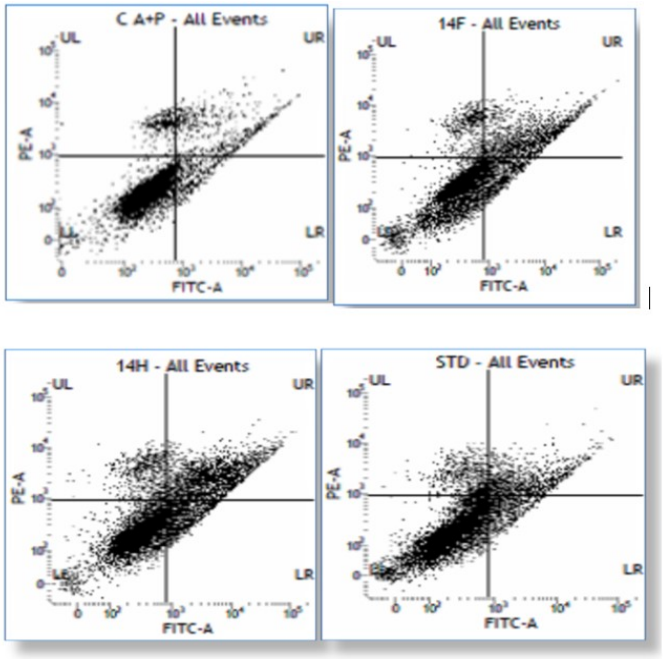

Fig 1. Apoptosis Detection Assay

Apoptosis Detection Assay - Flow cytometric analysis of the newly synthesized compounds 14f, 14h and Standard drug sunitinib in Human A549 lung cancer cells using the Annexin V-Fluorescein isothiocyanate / Propidium Iodide method. (i) Control group, (ii-iv) treatment with the newly synthesized compounds 14f, 14h and Standard drug sunitinib

Table 2. Total Percentage of Apoptotic Cells Exhibited by the Newly Synthesized Compounds at IC_{50} Concentration

S. No.	Compound Code	Total Apoptotic Cells in % (at 25µM)
1	14f	28.96
2	14h	38.01
3	Standard	21.03

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Table 3. Percentage of Cells in each phase of Cell Cycle after Treatment with 25μM of the Newly Synthesized Compounds and Standard

Compound	P7 (G0/G1)	P8 (S)	P9 (G2)
Control	100	100	100
14f	25.14	257.13	176.57
14h	73.89	10.03	9.29
Standard	60.47	13.79	5.78

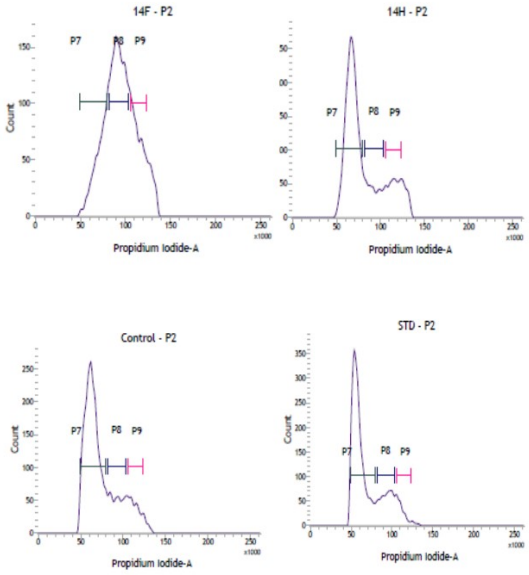

Fig 2.. Graph Showing Result Generated for Cell Cycle Analysis using Flow Cytometer

Cell Cycle analysis

Changes in cell cycle due to the synthesized compounds 14f, 14h and standard were estimated using flow cytometry. Briefly, after treatment of cells with 25μM concentration of the synthesized compounds14f, 14h and standard for 18h, cells were harvested and washed with 1X Phosphate Buffer Solution. The cells were treated with 75μg of RNase for 30 minutes at room temperature. After treatment, cells were washed with 1X Phosphate Buffer Solution. The cells were then incubated with propidium iodide (1mg/mL) for 15 minutes. After 15 minutes, the cells were washed and resuspended in 1X Phosphate Buffer Solution. Cell cycle analysis was estimated in flow cytometry. From the flow cytometry data, the control cells in each phase of the cell cycle were considered as 100% and changes in treated cells were calculated with respect to control cells[12-18].

Further, cell cycle analysis was carried on BD FACS VERSE (BD BioSciences) flow cytometer using propidium iodide. Human A549 lung cancer cell lines was treated with 14f, 14h and standard drug sunitinib at a concentration of 25μM for a period of 15min (Fig. 2).

Treatment of newly synthesized compounds with 14h and standard drug sunitinib at 25μM showed increase in fraction of cells 73.89% and 60.47% respectively arrested in G1 phase from control (50.23%). The DNA contents of the live population (control) were 50.23%, 11.99% and 7.81% for untreated cells at 25μM, respectively for G0/G1, S and G2/M phase. Human A549 cell line treated with compound 14h showed 73.89%, 10.03% and 9.29%, standard drug sunitinib showed 60.47%, 13.79% and 5.78% respectively for G0/G1, S and G2/M phase. compound 14f showed 25.14%, 257.13% and 176.57% respectively for G0/G1, S and G2/M phase (**Table. 3**). Taken together, the results of apoptosis and cell cycle analysis suggest that the compound 14h delay the cell cycle progression by arresting the cell cycle at G1phase and Compound 14f at S phase.

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Conclusion

The designed compounds were synthesized using chemicals of synthetic grade and obtained a good yield. The spectral data of the synthesized compounds were consistent with the assigned structure. *In vitro* anticancer activity revealed that 14h showed more potent anticancer activity as compared to the standard drug Sunitinib. This compound exhibited apoptosis thus, arresting the cell cycle at G0/G1 phase itself. To conclude, the above findings clearly demonstrated that the compound 14h may serve as good anticancer agent for further development.

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