Synthesis, Characterization and Evaluation of Anticancer Activity of (5-(Benzo[d][1,3]Dioxol-5-yl)-3-(2-Methoxyphenyl)-4,5-Dihydro-1H-Pyrazol-1-yl)(Furan-2-yl)Methanone

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Abstract

Compound (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(furan-2-yl)methanone was prepared using chalcones. The compound produced was subjected to different spectroscopical analysis like IR, ^{1}H-NMR, ^{13}C-NMR and mass spectra for characterization. The compound was subjected to cytotoxicity activity testing by MTT assay using MCF-7 cell line. The compound is also subjected to cell cycle analysis by Flow cytometry. The compound has shown cytotoxicity effect and also has the ability to cause arrest of cells in cell cycle at the G2/M phase of the MCF-7.

Keywords: Chalcones, pyrazoline, cytotoxicity, anticancer, MTT assay, flow cytometry

1. Introduction

Pyrazoline, a heterocyclic compound with adjacent nitrogen atoms 2 numbers at 1st and 2nd position of its structure. It also has three carbon atoms in its structure. Based on the double bond positions in the pyrazole, three reduced forms are identified. They are 1- Pyrazoline, 2-Pyrazoline and 3-Pyrazolines, which shows many biological properties based on the groups substituted in them. The derivatives of pyrazoline had been reported with many biological activities making them a pharmocophore for further studies in them [1]. Pyrazolines and their derivatives are interesting compounds since they posses many biological activities like antibacterial, antifungal, antitumor, anticonvulsant, and anti-inflammatory properties [2]. The new drugs are designed, containing different pharmacophores in a single entity and this will lead to the development of hybrid compounds. Many researchers have developed new lead molecules by combining chalcones with other active molecules. High mortality in people occurs due to cancer [3]. Even though different types of treatment is available for the cancer patients treatment, the efficacy is still not achieved due to the adverse effects produced by the chemotherapeutic agents and drug resistance seen. So, there is always a need to find a safe and more effective alternate treatment options. The occurrence of antibiotic resistance and adverse effects of the drugs have always led to the use of herbal products [4]. Many compounds are tested and available as effective chemotherapeutic agents, has they are able to effectively control the signaling pathways due their structure [5]. A compound is said to be cytotoxic when it stops the growth and division of the cells or death of the cell by either

apoptosis or necrosis [6]. We have tried a new hybrid compound by combining chalcone with furan-2 carbohydrazide. As a first step, the compound is tested for its cytotoxicity effect by MTT assay. The cell cycle analysis is performed using Flow Cytometry.

2. Experimental

2.1 General

General laboratory principles and techniques were followed. Aseptic techniques were carried out in a vertical biohazard compatible, laminar air flow chamber (Clean Air, India). Analytical reagent (AR) grade chemicals were used to prepare solutions required for routine molecular biology experiments.

2.2 Synthesis of chalcones

Chalcones were synthesized in the presence of 40% Sodium hydroxide (NaOH) by mixing piperanol **1** (1 mmol) and 2-methoxy acetophenone **2** (1 mmol) present in solvent ethanol (20 mL). The product resulted, was subjected to stirring at room temperature for 4 hours. Then the product was neutralized with 1N Hydrochloric acid (HCl). This results in formation of a precipitate like product. The resultant precipitate was filtered, followed by washing with hot ethanol and was dried under room temperature. Progression of the reaction can be done checked by Thin layer chromatography (TLC) [7].

2.3 Synthesis of (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(furan-2-yl)methanone

A mixture of chalcone **3** (0.250 g, 1 mmol) and furan-2-carbohydrazide **4** (0.111 g, 1 mmol) was homogenized by thorough grinding. The resulting solid was then transferred to a round-bottom flask. It was then heated to 160°C and after the heating process; it is allowed to melt for 30 minutes. The product was washed with a mixture of Ethyl acetate/hexane (1:3) after cooling to obtain product **5** in a high yield of 96%.

2.4Characterization of (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)(furan-2-yl)methanone

The compound was subjected to several spectroscopic analyses, including FTIR, 1H-NMR, ^{13}C-NMR, and Mass spectrometry. The compound was analyzed using FTIR spectroscopy (Perkin Elmer fourier transform infrared spectroscopy). To prepare the sample, the powdered compound was mixed with KBr and pressed into pellets. The FTIR spectrum was collected in the range of 4000 – 400 cm-1. The ^{1}H-NMR and ^{13}C-NMR spectra were collected using $CDCl_3$ as the solvent on a Bruker Advance II 400 spectrometer. Additionally, mass spectrometry was performed using a high resolution Schimadzu mass spectrometer.

2.5 Biological activities

2.5.1 Cell line and culture:

MTT assay was performed according the standard procedure. MCF-7- is a Human breast cancer cell line was ordered and received from National Centre for Cell Sciences, Pune (NCCS). MCF-7 cell line has to be maintained using a medium and this assay Dulbecco's Modified Eagle Medium (DMEM) was used to maintain the cell line. Fetal Bovine Serum (FBS) 10% was used to supplement the cells. At humidified atmosphere of 50 µg/ml CO_2, 37 °C, streptomycin at a concentration of 100µg/ml and penicillin at a concentration of 100 U/ml were added to the cells.

***In Vitro* assay for Anticancer activity: (MTT assay)**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was done in order to assess the cytotoxicity or antiproliferative or anticancer activity of the compound using 24-well plates. In the well plates, cells at the concentration of 1×10^5/well were plated and incubated in 37^0C with 5% CO_2 condition. The compound at different concentrations was added to the plates and then the same was incubated for 24hrs, once the cell reaches the confluence. Once incubation was over, the compound was taken away from the well. Then without serum, the cells were washed with phosphate-buffered saline (pH 7.4) or DMEM. To the compound, concentration of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) 100µl/well (5mg/ml) was added and then incubated for 4 hours. After incubation was over, the well was taken out and Dimethyl sulfoxide (DMSO) liquid 1ml was added in the entire well. DMSO was used a blank and the absorbance was measured at 570nm in the UV-Spectrometer [8]. Measurements were performed in triplicates and the average value is calculated. The concentration that is needed for inhibition of 50% (IC50) of the cells was determined graphically. The formula that was used to calculate the viability of cells in percentage is as follows:

% Cell viability = A570 of treated cells / A570 of control cells × 100

In the graph, X-axis was plotted with concentrations of the compound and Y –axis was plotted with % of Cell Viability. The full cell viability assessments were compared by including cell control and sample control.

2.5.2 Cell Cycle Analysis by Flow Cytometry

Six well plates were used for seeding the cells. Cells were seeded (2 x 10 5 cells/ml) and incubated for 24 hours of time at 37 °C in a humidified atmosphere of 5% CO_2. After attaining the confluence, one ml of sample was added (IC50 conc.) and again incubated for 24hrs of time. On completion of incubation the compound was washed with PBS. Then, the attached cells were subjected to trypsinization and centrifugation. Then the attached cells are collected and made into pellet. The obtained pellet was suspended in PBS. The cells were incubated for 30 minutes after adding, 0.2 ml of Propidium Iodide at a concentration of 10 µg/ml. Then the cells were observed at 488 nm of excitation wavelength by flow cytometry [9].

3. Results andDiscussion

The yellow color Pyrazoline compound was purified by recrystallization using a 1:1 mixture of ethyl alcohol and ethyl acetate. This resulted in the isolation of a pure compound with a yield of 96%. The compound synthesized was (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(furan-2-yl)methanone, with melting point of 116-118°C and Rf of 0.70 which was characterized using analytical techniques such as FTIR, 1H NMR, 13C NMR, and mass spectral studies to determine its structure.

3.1 Synthesis of (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)(furan-2-yl)methanone

Pyrazole derivatives can be synthesized by many methods like microwave irradiation, ultrasonic irradiation, ionic liquid and grinding technique. The selection of the method should be in such a way that, there should be maximum product yield of the compound (Meilinda Setya Praceka et al). Synthesis of pyrazoline derivatives takes place in two stages – First step involves the synthesis of chalcones by combining piperanol **1** (1 mmol) and 2-methoxy

acetophenone **2** and second step involves the reaction of chalcones **3** with furan -2-carbohydrazide **4** to produce the pyrazoline compound.

Figure 1 Schematic representation of (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)(furan-2-yl)methanone synthesis

3.2 Characterization

The pyrazoline compound was characterized using various analytical techniques such as FTIR, NMR, and mass spectrometry. The characteristic peaks for FTIR spectrum were seen at 1632 cm^{-1} for C=O, 1591 cm^{-1} for C=N, and 1324 cm^{-1} for C-N, which are indicates the presence of pyrazoline rings.

The 1H NMR spectrum provided further confirmation of the formation of the pyrazoline ring. The appearance of doublets of doublets at 3.25 and 3.75 ppm (with 2JAM = 18.5 and 3JAX = 4.3 Hz, respectively) indicated the presence of the pyrazoline and methoxy protons. The presence of H-5 proton as a double doublet at 5.58 ppm (with 3JMX= 11.6 Hz and 3JAX= 4.3 Hz), affirms the existence of an AMX type of coupling system in the pyrazoline ring. The methylene dioxy protons were observed at 5.82 ppm singlet, and the aromatic protons were observed in the 7.83-6.46 ppm region.

In the 13C NMR spectrum, the absence of the carbonyl carbon signal and the appearance of signals at 43.49 and 59.74 ppm for methylene and methine, respectively, confirmed the formation of the pyrazoline ring. The methoxy and methylenedioxy carbons were observed at 55.40 and 101.10 ppm, respectively, while the carbonyl carbon attached to the furan ring was observed at 161.23 ppm.

The mass spectrum of the pyrazoline compound showed a molecular ion peak at 390.1232, which is in agreement with the molecular weight of the compound calculated. The fragmentation pattern in the mass spectrum had provided additional information on the molecular structure and characteristic functional groups that were present in the compound.

3.3 Biological activity

3.3.1 Cytotoxicity of the compound by MTT assay

MTT assay is a type of colorimetric assay which detects the growth of the cells and survival of the cells *in vitro.* Live cells reduce MTT to an insoluble formazan product. The number of live cells in the sample can be easily detected and it helps in determining the cytotoxicty of a compound. The cyotoxic effect of any compound can be predicted by evaluating the IC_{50}. The concentration of any compound which has the ability to cause 50% death of the cells is called the IC^{50} of that compound. Increased concentration of the compound is expected to produce more cytotoxic effect. The concentration of the compound (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol- 1-yl)(furan-2-yl) methanone was increased and that has caused increased cytotoxicity (Table 1). The extent of cytotoxicity can

be assessed by the optical density reduction which is directly proportional to the number of viable cells in relation to the cell control (100%). IC_{50} value, which exhibits 50% cell viability for MCF-7 cell line is calculated graphically. It indicates the cytotoxic effect of a compound [10]. The IC_{50} value for the compound is 250µg/ml which is determined graphically (Figure 2).

3.3.2 Cell Cycle Analysis

Scattering of light and emission of fluorescence by the cells which are fluorescent probe-labeled, when they pass through a laser beam is the basic principle of flow cytometry technique. Many parameters of a single cell can be analyzed. The cell cycle of a compound can be analyzed and can be done by using a fluorescent dye which can stain DNA. The intensity can be measured easily has the fluorescent dye stains the cells in different phases of cell cycle differently. In order to assess the DNA content and distribution of the cell cycle, the dye propidium iodide is commonly used. In P_1slot 0.32% of cells is gated. P_2 slot-Sub G0 phase 3.60% are dead cells that undergone apoptosis. 71.78% of cells were seen in P_3 slot – G0-G. In the P_4 slot of the analysis – 18.29% of cells had entered S phase. 1.32% of cells seen in the P_5 slot had entered the G2 – M phase of the cell cycle. The compound (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(furan-2-yl)methanone is able cause cell cycle arrest at this phase. This has shown that the tested compound has anticancer activity.

Table 1 Anticancer effect of the Compound *on MCF 7* cell line

S.No	Concentration (µg/ml)	Dilutions	Absorbance(O.D)	Cell Viability (%)
1	**1000**	**Neat**	**0.302**	**32.57**
2	**500**	**1:1**	**0.375**	**40.45**
3	**250**	**1:2**	**0.448**	**48.32**
4	**125**	**1:4**	**0.522**	**56.31**
5	**62.5**	**1:8**	**0.595**	**64.18**
6	**31.2**	**1:16**	**0.669**	**72.16**
7	**15.6**	**1:32**	**0.742**	**80.04**
8	**7.8**	**1:64**	**0.815**	**87.91**
9	**Cell control**	**-**	**0.927**	**100**

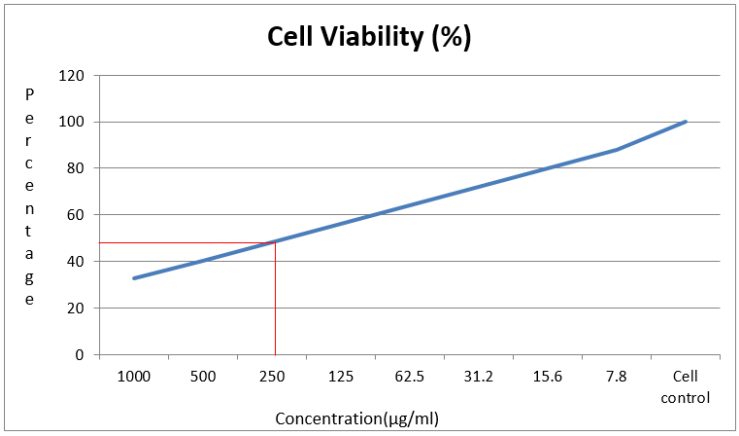
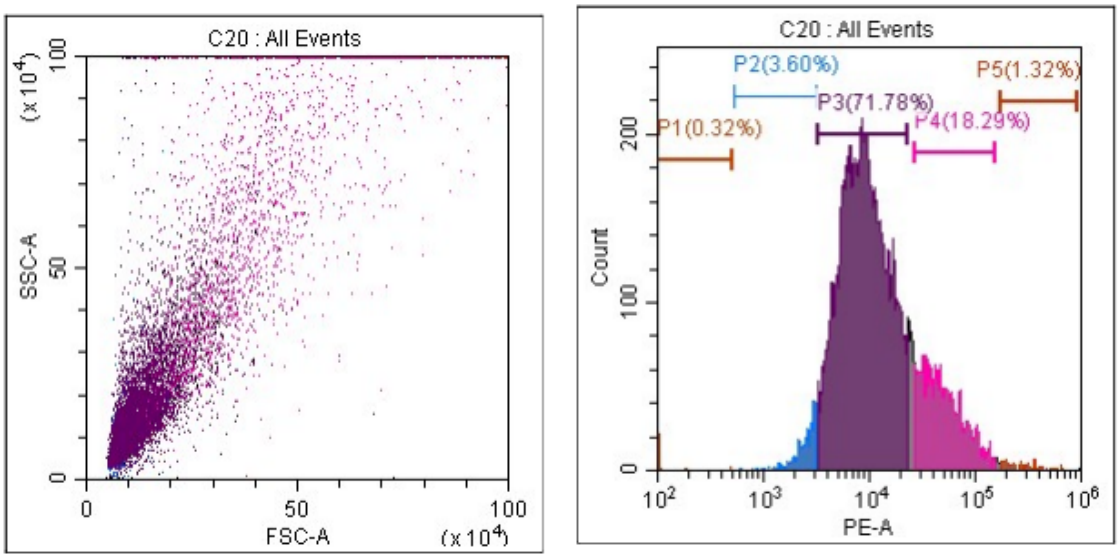


Figure 2 The IC_{50} value of the compound determined using MTT assay



Tube Name: C20
Sample ID:

Population	Events	% Total	% Parent
All Events	10000	100.00%	100.00%
P1	32	0.32%	0.32%
P2	360	3.60%	3.60%
P3	7178	71.78%	71.78%
P4	1829	18.29%	18.29%
P5	132	1.32%	1.32%

Figure 3 Cell cycle arrest effects of IC_{50} concentration of the compound in MCF-7 cell line

4. Conclusion

Cell cycle regulation is disrupted and it is responsible for the progression of many cancers. In order to inhibit the proliferation of cells, this process has to be disrupted. This can be achieved by apoptosis induction through cell cycle arrest at specific points. In our study we had successfully synthesized a compound and tested the anticancer activity of the same. The The compound (5-(benzo[d][1,3]dioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(furan-2-yl)methanone has shown cytotoxic or antiproliferative effect in the assay done. The produced compound had also shown its ability to arrest the multiplication of cells at G_2/M phase.

References

[1] Bhutani R, Pathak DP, Husain A, Kapoor G, Kant R. A Review on Recent Development of Pyrazoline as a Pharmocologically Active Molecule. International Journal of Pharmaceutical Sciences and Research. 2015 Oct 1;6(10):4113.

[2] Kumar S, Bawa S, Drabu S, Kumar R, Gupta H. Biological activities of pyrazoline derivatives-A recent development. Recent patents on anti-infective drug discovery. 2009 Nov 1;4(3):154-63.

[3] Venkatesan V, Rozario Cyril, Deepa. Antitubercular and anticancer activities of compounds obtained from methanol extract of *Toona ciliata* leaves. 2022 June(11) 906-920

[4] Anitha KV. Management of Denture Infection with Phytomedicine. Infectious Disorders Drug Targets. 2022 Aug 29.

[5] Hong SH, Cha HJ, Hwang-Bo H, Kim MY, Kim SY, Ji SY, Cheong J, Park C, Lee H, Kim GY, Moon SK. Anti-proliferative and pro-apoptotic effects of Licochalcone A through ROS-mediated cell cycle arrest and apoptosis in human bladder cancer cells. International journal of molecular sciences. 2019 Aug 5;20(15):3820.

[6] Kumari P, Mishra VS, Narayana C, Khanna A, Chakrabarty A, Sagar R. Design and efficient synthesis of pyrazoline and isoxazole bridged indole C-glycoside hybrids as potential anticancer agents. Scientific Reports. 2020 Apr 20;10(1):6660.

[7] Ganesan S, Sarangapani M, Doble M. An expedient, one-pot, stepwise sequential approach for the regioselective synthesis of pyrazolines. Journal of Chemical Research. 2021 Mar;45(3-4):326-33.

[8] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983 Dec 16;65(1-2):55-63.

[9] Darzynkiewicz Z, Juan G. DNA content measurement for DNA ploidy and cell cycle analysis. Current protocols in cytometry. 1997 Apr(1):7-5.

[10] Florento L, Matias R, Tuaño E, Santiago K, dela Cruz F, Tuazon A. Comparison of cytotoxic activity of anticancer drugs against various human tumor cell lines using in vitro cell-based approach. International journal of biomedical science: IJBS. 2012 Mar;8(1):76.