



Synthesis and Anticancer Evaluation of *Bis* Furfurylidene-4-piperidone Analog of 5-Fluorouracil

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5-Fluorouracil (5-FU) displayed several side effects due to its nonspecific cytotoxicity for tumour cells. In order to overcome these disadvantages, numerous modifications of the 5-fluorouracil structure have been performed. In the present study, *bis* furfurylidene-4-piperidone substituted 5-fluorouracil derivative is prepared and evaluated against various cancer cells. The resultant derivative is characterized by spectral data. This compound having 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore which interact with cellular thiols and produces cytotoxic activity whereas 5-fluorouracil part showed anticancer activity by interacting with nucleic acid. The resulting synthesized compound has been screened for *in vitro* cytotoxic property by SRB assay method against breast and colon cancer cell lines. The results indicate that, this compound showed equipotent cytotoxicity against human breast cell lines as compared to standard, 5-fluorouracil and doxorubicin. Acute toxicity was determined by OECD-423 guidelines. *In vivo* anticancer activity was evaluated in Swiss albino mice bearing Ehrlich Ascites Carcinoma (EAC). This compound showed significant anticancer activity against Ehrlich Ascites Carcinoma in Swiss albino mice. This study revealed the potentiality of this molecule for further development as anticancer agents.

Keywords: 4-Piperidones, 5-Fluorouracil derivatives, Cytotoxic agents, α,β -unsaturated ketones, Alkylating agents and Anticancer agents.

INTRODUCTION

5-Fluorouracil (5-FU) is one of the most potent antimetabolites, which have been widely used in the treatment of advanced solid tumours. As an anticancer agent, because of its low efficacy and high toxicity, numerous modifications of 5-fluorouracil structure have been performed [1]. The most of currently available anticancer alkylating agent suffer from a number of significant disadvantages because of their interactions with nucleic acids [2]. In contrast, various α,β -unsaturated ketones react preferentially or exclusively with thiols but not with amino or hydroxy groups present in nucleic acid [3,4]. Since thiols are not part of the nucleic acid structures, hence such conjugated enones may be having less mutagenic and carcinogenic than conventional alkylating agents [5,6].

The theory of sequential cytotoxicity state that successive chemical attack may lead to greater damage in cancer cell compared to normal cell [7]. Initially only one conjugated arylidene keto group was utilized in the molecules. However, recently the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has been incorporated into various cyclic systems which permit two successive alkylations of thiols [8-10]. The pH of a number of tumours is lower than the corresponding normal cells [11]

and hence an increase in the electrophilicity of the olefinic carbon atoms in enones under acidic conditions should enable greater thiolation in cancerous cells to occur. A number of studies have demonstrated that depletion of thiol concentrations prior to treatment with various anticancer drugs has increased cell killing compared to the use of the drug alone [12,13]. In addition, various conjugated enones inhibit the p isozyme of glutathione-S-transferase [14] and such inhibition has been shown to enhance the cytotoxicity of the cancer chemotherapeutic alkylating agent thiotepa [15].

Thus, it was predicted that different pathway of the cancer could be effectively targeted by using only one molecule, which might be increasingly its therapeutic effectiveness. In view of this, *bis* furfurylidene 4-piperidone analog of 5-fluorouracil was prepared and evaluated for cytotoxic properties.

EXPERIMENTAL

All the chemicals were procured from local distributors and were of LOBA Chemie Pvt Ltd, Mumbai, India. Melting points were uncorrected. The ¹H NMR spectra were carried out using VARIAN MERCURI YH-300 NMR spectroscopy machine. TMS was used as internal standard. CHNS analysis was carried by Thermo finnigan, FLASH EA 1112 series. IR spectra were recorded on a BRUKAR FTIR alpha ATR

spectrometer. The microwave was used for synthesis of final compound. The TLC was employed to monitor the progress of the reaction. The observation was made under UV light and iodine chamber. Protocol used in this study for the use of mice as an animal model for cancer was approved by the Institutional Animal Ethical Committee.

Synthesis of furfurylidene derivative [16]: A mixture of piperidin-4-one (2.51 g, 0.01 mol) and furfuraldehyde (1.92 mL, 0.02 mol) in alcoholic NaOH (50 mL, 10 %) was stirred at room temperature. The reaction was monitored on TLC. After completion of reaction, the separated solids were filtered off and recrystallized from the ethanol solvent.

(3E,5E)-3,5-bis(furan-2-ylmethylene)piperidin-4-one (2): Yield: 65 %; m.p.: 215 °C; ¹H NMR: δ: 7.26 (s, 2H, furfurylidene H), δ: 6.5-7.2 (m, 6 H, furfuryl H), δ: 4.29 (s, 4H, piperidyl H), δ: 2.1 (br, 1H, NH). IR: 1469 (aliphatic CH), 1651 (CO), 837 (alkene), 1247 (R-O-R) & 1595 (NH). Anal. calcd. (%) for C₁₅H₁₃NO₃: C-70.40, H-5.13, N-5.49, Found (%): C-70.26, H-5.10, N-5.70 %; TLC: 0.81 (Chloroform).

Synthesis of N-substituted derivative: Bis furfurylidene 4-piperidone (2.5 g, 0.01 mol) and 5-fluorouracil (1.3 g, 0.01 mol) were dissolved in pyridine (20 mL). 2 mL thionyl chloride was added drop wise. The flask was irradiated in microwave for 30 s. After completion of reaction, flask was removed. Pyridine was removed by vacuum. The product was recrystallized from rectified spirit (Scheme-I).

((3E,5E)-3,5-bis(furan-2-ylmethylene)-4-oxopiperidin-1-ylsulfonyl)-5-fluoropyrimidine-2,4(1H,3H)-dione: Yield: 65 %; m.p.: 260 °C; ¹H NMR: δ: 8.6 (t, 2H, furfuryl H), δ: 7.6 (d, 4H, furfuryl H), δ: 8.07 (s, 2H, furfurylidene H), δ: 8.74 (s, 1H NH in 5-fluorouracil), δ: 8.03 (s, 1H, CH in 5-fluorouracil), δ: 4.70 (s, 4H, piperidyl H). IR: 2997 (aliphatic CH), 3066 (aromatic CH), 1651 (CO), 808 (alkene), 1240 (R-O-R), 1311 (t-amine), 1072 (sulfonylamine). Anal. calcd for C₁₉H₁₄N₃O₆SF: C-52.90, H-3.27, F-4.44, N-9.74, S-7.43, Found-C-54.00, H-3.00, N-10.24, S-6.32 %, TLC: 0.55 [water: methanol (40:60)].

Bioevaluation

Cytotoxicity evaluation [17,18]: SRB assay was used for determination of cytotoxicity and performed according to the procedure given in the literature. 5-Fluorouracil and doxorubicin were used as standards.

Acute toxicity study [19]: OECD guideline-423 was used for performing oral acute toxicity. The toxicological effects

were observed in terms of mortality and expressed as LD₅₀. The number of animals dying during the period was noted.

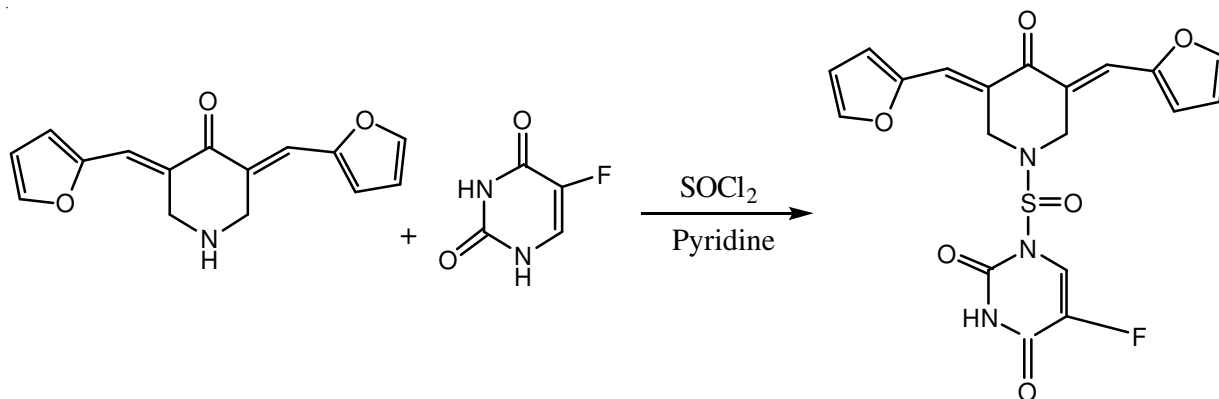
In vivo anticancer procedure [20,21]: In vivo anticancer activity was performed according to the procedure given in the literature. Three groups of eight animals (n = 8) each of Swiss albino mice were used for the activity. 5-Fluorouracil (5-FU) and test compound at a dose level of 20 mg/kg body weight were used. The antitumour activity of the test compound and standard were measured in Ehrlich Ascites Carcinoma animals with respect to the following parameters: tumour volume, viable/non-viable tumour cell count, tumour cell count, percentage increase life span (% ILS), body weight and glutathione concentration.

RESULTS AND DISCUSSION

Bis furfurylidene derivative of 4-piperidone was prepared by Claisen-Schmidt condensation between piperidin-4-one and furfuraldehyde in the basic medium. N-5-Fluorouracil derivative was prepared by reacting furfurylidene piperidin-4-one with 5-fluorouracil in the presence of thionyl chloride.

The evidence from TLC and ¹H NMR spectroscopy revealed the compound to be isometrically pure. The ¹H NMR spectra of synthesized products showed that all furfurylidene protons were found in between the 7.2-7.8 ppm and piperidyl in 4.2-4.7 ppm. In case of final compound, NH and CH of 5 fluorouracil were highly deshielded and observed above the δ 8.0 ppm. In a present study, fingerprint region was used for characterization of molecule. The compounds having aromatic CH gave strong band at 700-680 cm⁻¹. Bending of aliphatic CH was observed in between 1465-1440 cm⁻¹. Carbonyl group of most molecules was observed around 1651, because conjugation of carbonyl group with carbon-carbon double bond lowers the stretching frequency to near about 1680 cm⁻¹. The alkene groups were observed in between 808 cm⁻¹. The *tert*-amine group (=N-), which absorbed IR frequency around 1311 cm⁻¹. The signal of sulfonamide group was observed around 1072 cm⁻¹. C-O-C linkage showed bands in between 1300-1200 cm⁻¹.

Final compound was evaluated for cytotoxicity towards breast cancer cell line MCF-7 and colon cancer cell lines COLO-205 by SRB assay method. Doxorubicin and 5-fluorouracil were used as standards. The concentration of synthesized product and 5-fluorouracil causing 50 % MCF-7 breast cancer cell kill are 80 µg/mL and more than 100 µg/mL for COLO-205 colon cancer cell line. The concentration of test compound causing



Scheme-I

total inhibition of both cell groups are superior than 5-fluorouracil (Table-1). The GI_{50} values less than 10 $\mu\text{g/mL}$ indicate equipotent cytotoxicity of test compound as compared to standard against both cell lines (Table-1).

Acute oral toxicity of final compound was determined according to the OECD guide line 423. The dose level to be used as the starting dose was selected from fixed level 300 mg/kg body weight of flow chart of annex. 2C. The final compound showed Globally Harmonized Classification System (GHS) category 3 *i.e.* LD_{50} value in between 50 to 300 mg/kg.

The parameters such as tumour volume, packed cell volume, cell count, mean survival time and increase of life span were assessed in the *in vivo* antitumour activity of final compound against Ehrlich Ascites Carcinoma tumour bearing mice. The synthesized molecule treated animals at the doses 20 mg/kg have significantly inhibited the tumour volume, packed cell volume, tumour (viable) cell count and brought back the hematological parameters to more or less normal levels as compared to the standard.

A regular rapid increase in ascetic tumour volume was observed in the Ehrlich Ascites Carcinoma tumour bearing mice (Table-2). Ascetic fluid is the nutritional source for tumour cells and regular increase in ascetic fluid volume with tumour growth would be a means to meet the nutritional requirement of tumour cells [22]. The criteria for judging the significance value of any anticancer drugs is the prolongation of the life span of animals [23]. This *in vivo* study reflects decreasing the nutritional fluid volume and arresting the tumour growth, increases the life span of Ehrlich Ascites Carcinoma-bearing mice.

Hematological parameters of tumour bearing mice and normal mice on day 14 were determined. As compared to the normal animals, the 5-fluorouracil, test compound treated animals and Ehrlich Ascites Carcinoma bearing mice, the

erythrocyte and hemoglobin count was found to be decreased. The RBC parameters like mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration and leucocytes were found to be normal as compared to the 5-fluorouracil treated and/or normal animal except Packed Cell Volume. In differential count of WBC, the percentage of eosinophils and neutrophils increased, while lymphocyte count decreased of Ehrlich Ascites Carcinoma bearing mice. All hematological parameters of test compound treated mice were found to be similar as compared to the 5-fluorouracil treated animals (Table-2).

Myelo-suppression and anemia are usually the major problems in cancer chemotherapy [24,25]. The anemia encountered in tumour bearing mice is mainly due to reduction in RBC or hemoglobin (Hb) percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions [26]. Present study indicates that synthesized molecule treatment brought back the hemoglobin content, RBC and WBC count more or less to normal levels. This clearly indicated that synthesized molecules were also possessing protective action on the hemopoietic system.

Conclusion

The evidence from TLC and spectroscopic analysis revealed that the compound synthesized and it is isometrically pure. SRB cytotoxic investigation has indicated that final compound showed equipotent cytotoxicity against breast and colon cell lines as compared to the standards 5-fluorouracil and doxorubicin. The GI_{50} value less than 10 $\mu\text{g/mL}$ indicate retention of cytotoxicity of test compound after modification. The LD_{50} value of final compound was observed at 100 mg/kg dose level. *In vivo* anticancer results reflect that, synthesized product showed equipotent anticancer activity as compared to the standard 5-fluorouracil. But, further optimization is required for development of potent anticancer agent.

TABLE-1
CYTOTOXICITY DATA

Cell lines	Parameters	5-Fluorouracil ($\mu\text{g/mL}$)	Test compound ($\mu\text{g/mL}$)	Doxorubicin ($\mu\text{g/mL}$)
Human breast cancer cell line MCF-7	LC_{50}	80	80	10
	TIG	80	69	10
	GI_{50}	10	10	10
Colon cancer cell lines COLO-205	LC_{50}	NE	80	NE
	TIG	NE	76.6	10
	GI_{50}	10	10	10

Note: LC_{50} value is the concentration of drug causing 50 % cell kill. TIG value is the concentration of drug causing total inhibition of cell growth. GI_{50} value is concentration of drug causing 50 % inhibition of cell growth, GI_{50} value below $< 0.1 \mu\text{M}$ or $< 10 \mu\text{g/mL}$ is considered as active, NE indicate non evaluate in this concentration level.

TABLE-2
in vivo ANTICANCER DATA

Treatment	Body wt. variation data	Tumour data		Tumour cell count		Hematological data		Life span	
		Tumour volume	Packed cell volume	Viable (%)	Non-viable (%)	Hb	Erythrocytes	Median survival time	Increased life span (%)
Normal	2	—	—	—	—	13.2	8.80	41.5 ± 0.08	—
EAC	13	3.33 ± 0.73	1.6 ± 0.11	99.26	0.73	10.2	6.68	24.5 ± 0.12	—
EAC + Test	2.9	—	—	—	—	11.1	7.0	36 ± 0.22	48.12
EAC + 5-Fluorouracil	2.3	—	—	—	—	11.6	7.09	40.0	52.26

Note: Values are mean SEM, n = 6.

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