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Research Article

IN VIVO BIO-ACTIVITY OF PITC-II DERIVATIVE (PART II)

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ABSTRACT

PITC-2, a new thiophene contain compound obtained from P.indica tissue cultured plant. Its mono amine derivative has been synthesaised; In vitro studies showed that the synthesized compounds proved there anticancer property and cytotoxic effect against human leukemic cell lines U937, K562 and HL60. Semi synthetic derivatives of PITC-2 significantly inhibited the cell proliferation (in the case of U937 - 93.01%, K562 - 78% and HL60- 89.525%) and showed a higher cytotoxic effect than free PITC-2 and tissue-cultured *P. indica* root extract (TCPIRE) in vitro.

Keywords: PITC-II, Cell lines U937, K562 and HL60, Semi synthetic derivatives.

INTRODUCTION

Chemotherapeutic treatment of neoplastic diseases is often restricted by their severe adverse systemic toxicity which limits the dose of drugs. Administration of these drugs has some complications like drug resistance, inability to access the target site and drug metabolism[1]. PITC-2 is a new thiophene derivative isolated from root extract of tissue-cultured *Pluchea indica* (L.) Less. (Patent application no: 1990/KOL/2008). Because of its poor hydrophobic property, PITC-2 is being encapsulated in liposome to enhance its therapeutic effects and reduce the toxicities. In our earlier study, it was found that tissue-cultured *P. indica root* extract inhibited Ehrlich Ascites Carcinoma (EAC) in mice [2]. It is well known that tumor promoters recruit inflammatory cells to the relevant site and aggravation of inflammation in the tissue causes cancer enlargement and vice versa (Ghosh et al. 2006). So it may be concluded that anti-inflammatory agents may possess anticancer activity. We have already reported in our earlier studies that *Pluchea indica* root extracts and PITC-II it self has potent anti-inflammatory and anti-oxidant properties[3,4]. In the present study we have synthesized and investigated a mono amine derivative of PITC-2 for anticancer property and cytotoxic effect on different cell line (U937, a human leukemic cell line, K562, an erythroleukemic cell line and HL60, a human leukemic cell line).

MATERIALS AND METHODS

General experimental procedures and Chemicals

Melting point was determined using a Sturat SMP heating stage microscope and was uncorrected. Nuclear magnetic resonance (NMR) 1H spectra were recorded on a Bruker AV300 Supercon NMR System with chemical shifts being represented in parts per million (ppm) and with tetramethylsilane (TMS) as an internal standard. Reactions were monitored by thin layer chromatography (TLC) and the spots were visualized by spraying the TLC plates with 2% ninhydrin/acetic acid w/v solution. The TLC employed pre-coated silica gel plates (aluminum sheets 20×20 cm, silica gel 60 F254 of Merck K GaA). All solvents and reagents used were of analytical grade and obtained from Merck, India. All solvents used were of spectral grade or distilled prior to use. PITC-2 was isolated from root extract of tissue cultured medicinal plant P. indica and all other reagents like chloroform, methanol, EDTA, Triton X-100, etc. of high purity grade were purchased from a local manufacturer. Phosphatidyl choline (PC), cholesterol, glutaraldehyde, RPMI 1640 medium, fetal bovine serum, streptomycin and penicillin, Lglutamine, HEPES, Ara-c, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO).

6-(5-(prop-1-yn-1-yl)tetrahydrothiophen-2-yl)-1-(tosyl-peroxy)hexa-3,5-diyn-2-yl acetate (i)

98mg of PITC-II was taken in a RB containing 5mL of dry Aceto nitryl (MeCN) and Tryethyl amine 3.1 equivalent, to it TsCl 1.2 equivalent was added drop wise. The reaction mixture was allowed to stand for 6.0 hr with continuous steering. After those 1.3 equivalent Acetyl chlorides was added drop wise in room temperature and allow standing for additional 12hr. The reaction mixture was quenched using 0.01N HCl solution and extracted with Di-ethyl ether (3×20mL). The organic layer was evaporated to obtain a yellowish white solid (42mg). This was used in next step with out purification. The analytical sample was used by purified the aforesaid solid using column chromatography (Silicagel; 2-5% EtOAc: Hexane). **^{1}H NMR** (300MHz, $CDCl_3$) **δ** 7.91 – 7.73 (m, 2H), 7.52 – 7.37 (m, 2H), 5.43 (s, 1H), 3.87 (dd, *J* = 78.9, 43.8 Hz, 4H), 2.44 – 1.63 (m, 13H).

1-azido-6-(5-(prop-1-yn-1-yl) tetrahydrothiophen-2-yl) hexa-3, 5-diyn-2-yl acetate (ii)

Using literature methodology [5, 6] the corresponding azideo-acetate compound has been prepared[7]. A simple borosil glass conical flask was used as a reaction vessel and no stirrer used. To a 1:1Water/DMF solution 30mg compound **i** and 1.6 equivalents Sodium Nitrite was added. It was irradiate under microwave system with occasional steering and cooling at room temperature for 1hr. The reaction was monitored by TLC in every 3 min interval. Then it was extracted with di-ethyl ether (3×20mL). The combined organic layer was collected and purified by column chromatography (10-20% EtOAc/Haxene) to prepare analytical sample yield 12.1 mg of compound **ii**. In practice the crud reaction mixture was obtained after evaporation of organic layer was used in next step with out purification. ^{1}H NMR (300 MHz, $CDCl_3$) **δ** 5.43 (s, 2H), 3.87 (dd, *J* = 77.8, 44.2 Hz, 4H), 2.44 – 1.63 (m, 10H)

1-amino-6-(5-(prop-1-yn-1-yl) tetrahydrothiophen-2-yl) hexa-3, 5-diyn-2-ol (iii)

18 mg of dry reaction mixture was taken in 20mL THF and heated up to 80°C for 6 hours. To the resulting mixture, were added PPh3 (3.2 equiv.) and H_2O 10mL at room temperature. The resulting mixture was stirred at room temperature for additional 12 hours. Then 10mL 0.1N HCl was added and refluxed for 4hr. to it 15mL 0.1N Sodium bicarbonate solution was added and extracted with Di-chloromethen (DCM, 3 × 20mL). It was dried using sodium sulfate. The organic layer was evaporated under reduced pressure and purified by column chromatography (0.5-2% Methanol/Chloroform) to yield 4.6 mg of compound **iii** as a brownish white solid. M.P: 178°C, **1H** NMR (300 MHz, $CDCl_3$) δ 5.43 (s, 2H), 3.87 (s, 1H), 3.31-2.86 (dd, *J* = 72.5, 40.6 Hz, 4H), 2.44 – 1.63 (m, 7H).

Leukemic cell lines

Human leukemic cell lines U937, K562 and HL60 were purchased from the National Facility for Animal Tissue and Cell Culture, Pune, India. Cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 in air. In all the experiments untreated leukemic cells were termed as control group.

Determination of cell proliferation in vitro

Log phase cells (U937, K562 and HL60) were taken at a concentration of 106/ml in RPMI 1640 (with 10% FCS) in multiple well sterile plastic plate. TCPIRE, PITC-2 and Liposome encapsulated PITC-2 (10, 20, 30 μg/ml) were added to the neoplastic cells and viable cells were counted by Trypan blue exclusion principle (Sur et al. 2000) after 24 hours and values were presented graphically[8].

Cytotoxic effect of Amine derivatives of PITC-2 on U937 K562 and HL60

Cytotoxicity was evaluated after 24 hrs by means of MTT assay[9]. This method estimates the capacity of living cells to reduce the tetrazolium salt – MTT by means of mitochondrial dehydrogenase enzyme. The amount of the blue dye production is proportional to the number of living cells in the culture. Cells in logarithmic phase of proliferation were seeded in 96-well micro titer plates. Control conditions comprised of (1) cells with medium alone and (2) cells with medium and vehicle. Cells were cultured both under normoxia and hypoxia conditions in a final volume of 100 μL in replicates of 4 wells per condition. Control of the O_2 tension in an atmosphere of 5% CO_2 in air was achieved using N_2. 20% O_2 (termed as normoxia) and 3% O_2 (termed as hypoxia) were the test atmospheres. Plates were assayed at 72 hrs after initiation of compound exposure. Next, 10μL of stock MTT solution were added to each well (0.5 mg/ml) for an additional 4 hrs incubation (37°C, 5% CO2). After 4 hrs incubation, 100 μL of DMSO is added to each

well and the optical density was measured at 570 nm. The IC_{50} values were calculated using the Prism Pad computer program (GraphPad Software) [2].

Statistical analysis

The data were subjected to one way ANOVA followed by Dunnett's test (GraphPad Prisn software) and values at $P \leq 0.001$ were considered to be statistically significant.

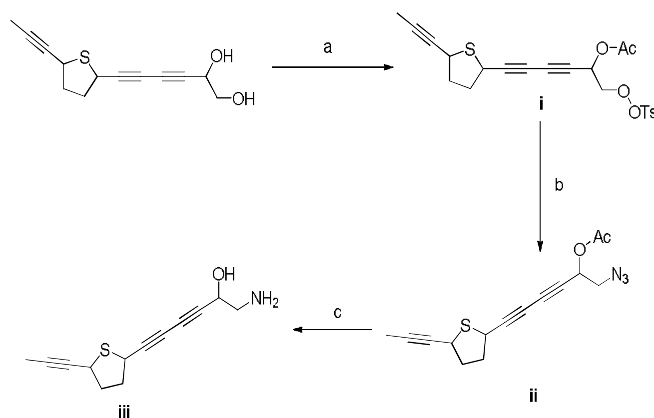
RESULT AND DISCUSSION

In the present study we synthesized and evaluated compound **iii** a mono amine derivatives of PITC II. For the synthesis of PITC II a cost effective and convenient methodology was adopted. We isolate and collected PITC II as per our previously reported methodology[10]. Till today there is no method available for the synthesis of PITC II. Selective Tosylation and Acetylation of the primary and secondary alcohol group helps for the preparation of azaide derivative, because OTs group is more labile in compare to OAc group. Using $NaNO_3$/ THF:Water system and micro wave irradiation the correspondind azaide compound was prepared. It is a less time consuming method. A Staudinger type of Reaction was used for the reduction of azaide to amino functionality. A low concentration of compound **iii** was sufficient to inhibit proliferation of U937, K562 and HL60 cells. The mechanism for this increased action is not yet known, however amino derivatisation may improve the effect of PITC-2, which is linked with the thiophin ring through an alkyne side chain, may encourage for developing more amino-derivatives of PITC II. Antiproliferative effects of mono amine derivative of PITC-2 (compound **iii)** were tested on three cancer cell lines. We got a very positive anti proliferative activity of compound **iii** (17 µg) on U937 and HL-60 cell lines. The proliferation of both cell lines was inhibited to a greater degree (in the case of U937 it was 95.72% and in the case of HL-60 it was 80.2%) when mono amine PITC-2 was applied (Table 1). We assessed the effect of mono amine PITC-2 (compound **iii**) on the proliferation of U937, K562 and HL60 cell lines using the MTT assay method. Significant dose-dependent inhibition of cell growth was observed in all three cell lines after treatment with compound **iii**, for 72 hrs. Compound **iii** at 17µg caused > 80% growth inhibition in three cell lines. However as in our previous study, PITC-2 and liposome-encapsulated PITC-2 infatuated much better the cytotoxic effect than TCPIRE on all three cell lines it is already proved. The cytotoxic effect was also confirmed by the result of the MTT assay which showed that TCPIRE, PITC-2 and mono amino PITC-2 (Compound iii). The present *in vitro* study confirmed that compound **iii** possess potent anti-leukemic activity.

CONCLUSION

A very efficient and cost effective method has been employed for the synthesis of PITC II amino derivatives. The bio activity study revealed that an amino derivative of PITC II shows impressive bio activity at µg level (IC_{50}: 17µg) on various cell lines like U937, K562 and HL6. In future we are planning to synthesized more amino derivatives to develop a potent anticancer agent.

Scheme 1



Scheme 1: (a) i) TsCl; 1.2eq, Et_3N;3.1eq,MeCN, ii) CH_3COCl; 1.3eq (b) NaN_3;1.6eq, H_2O, MW/1200w/50Hz (c) PPh_3; 3eq, THF, 0.1N Aquous HCl.

Table 1: Anti cancer effect activity

ID	Inhibition of proliferation		
	U937	K562	HL60
Compound **iii**	95.72%	77.21%	80.2%
PITC II[2]	90.39	55.71	69.52%
Liposome Encapsulated PITC II[2]	93.03%	97.48%	89.52%

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