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Synthesis, *In- vivo* and *In-silico* anti-inflammatory studies of substituted fluoro pyrazole

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ABSTRACT

N-phenyl-5-substituted –aryl-3-p-(fluorophenyl) pyrazoles have been synthesized from cyclization of 4-fluoroacetophenone (1) with various benzaldehydes (2) to give 4-fluorophenylstyrylketone (3) followed by treatment with phenyl hydrazine. The title compounds and their derivatives have been characterized by their elemental and spectral analysis. The newly synthesized compounds are screened for anti-inflammatory activity. All substituted 4-fluorophenylstyrylketones (250mg/kg orally p.o.) possessed anti-inflammatory activity against carrageenan-induced paw oedema in rat. Indomethacin (10mg/kg) was used as standard drug. And all compounds (0.20mM) showed ability to denature bovine serum albumin as observed in vitro inhibition studies. No correlation was found between the anti inflammatory activity and inhibition of bovine serum albumin denaturation. The docking studies were carried out for these compounds against the protein NFκB which is involved in inflammation signal cascade. Some of them showed good activity and molecular binding. Compounds such as 3b, 5b and 5e have exhibited comparative results in both in vivo and in silico studies.

Keywords: anti inflammatory, fluoro pyrazole, molecular docking, protein denaturation

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INTRODUCTION

Non steroid anti-inflammatory drugs (NSAIDS) under current clinical usage are highly acidic in nature and suffer from common adverse side effects like allergic reaction, gastro intestinal disturbance, irritation, nausea, dizziness etc. In order to overcome these problems, extensive search for an ideal non acidic non steroidal anti-inflammatory agent still continues. Pyrazoles and its derivatives represent one of the most active classes of compounds, which possess wide range of biological activities like antibacterial [1], antifungal [2], analgesic [3], antipyretics [4], anti-inflammatory [5], antidiabetic [6], sedative [7], antirheumatic [8], anticholinesterase [9], antitubarcular [10] activities. Recently research and development of new pyrazoles for cancer therapy have been one of a major focus in anticancer drug design. In view of these and our continuing interest in the synthesis of biologically active compounds, we undertook the synthesis of the title compounds and studied their anti inflammatory activity. Since, molecular approaches are used in modern drug design we made an attempt to know their in silico binding through docking studies.

Protein denaturation is one of well documented causes of inflammation [11, 12, 13]. Production of auto antigens in certain rheumatic diseases may be due to in vivo protein denaturation [14] and also some anti inflammatory drugs inhibit protein denaturation [15]. Mizushima and others have used protein denaturation as an in vitro screening model for anti inflammatory compounds [16, 17]. Compound containing phenylstyrylketone inhibit bovine serum albumin (BSA) denaturation [18-20] and possess anti inflammatory activity [21].

The NFκB family of transcription factors is involved in a myriad of cellular activation events that are critical for development, proper control of cell growth and proliferation, the immune response, control of apoptosis and survival, and stress responses to a variety of noxious stimuli [22, 23]. Proper function of the NFκB network is therefore critical for human health. In fact, aberrant NFκB activity plays a role in chronic inflammatory diseases [24, 25]. A deep understanding of the interaction with this protein will perhaps lead to modulators of chronic inflammation. Nowadays, molecular docking approaches are routinely used in modern drug design to understand drug–receptor interaction. The most recent phase in the new drug discovery process is utilizing the knowledge of the three dimensional structures of target macromolecules or of related proteins. The same strategy is used in present study to know the binding of the newly synthesized pyrazoles to selected protein sites. It was theoretically predicted that how inhibitors bind to the molecular targets, specific interactions that are important in the molecular recognition [26, 27].

MATERIALS AND METHODS

Melting points were determined in open capillaries and are uncorrected. IR spectra were recorded Shimadzu FTIR5400 infrared spectrophotometer using KBr.[1] HNMR spectra were

recorded on Perkin-Elmer, 90MHz Spectrometer; using TMS as standard (chemical shift in δppm).

General procedure for the preparation of 4-fluorophenyl styryl ketones (3a-3e):

A mixture of 4-fluoroacetophenone (0.01mol) and substituted benzaldehydes (0.02mol) in 10% NaOH and ethanol was refluxed for 2 hrs, after 2hrs mixture was allowed to stand 1 hr. The precipitate obtained was filtered and recrystallised from ethanol. Reaction was monitored by TLC and characterized by elemental and spectral analysis. The spectral data of 3a and 3c are given below.

The IR Spectra of 3a showed characteristic absorption peaks at 1510cm^{-1}(C=C) 1460-1490 (C=N), 1220-1250cm^{-1}(C-F), 660-690cm^{-1}(arom).^{1}HNMR ($CDCL_3$): (δppm) =6.95. (s, C-4'H,Ch),7.3-7.6(m,14H,arom).

The IR Spectra of 3c showed characteristic absorption peaks at 1650cm^{-1} (C=O), 990-850 (CH=CH), 1220-1250cm^{-1}(C-F), 660-690 cm^{-1}(arom). ^{1}HNMR ($CDCL_3$): (δppm)=6.6-6.8(2H)and, 7.2-8.2(m,9H,arom).

General procedure for the preparation of 4-(fluorophenyl) Di bromo styryl ketones (4a-4e):

These compounds were prepared by vigorous stirring for 1 hr of a mixture of 4 fluorophenylstyryl ketones (0.01mol) in chloroform (10ml) while bromine (0.01mol) was added in a drop wise manner and allowing the reaction mixture to stand for 1 hr. the precipitate obtained was filtered, washed with ether to remove excess bromine and recrystalized from ethanol. The substituted 4-fluorophenyl dibromo styryl ketones were characterized by M.P. and IR spectra showed characteristic bands due to C=O attached to phenyl, C-F,C-Br.and aromatic groups at 1680,1210,560-580, and 690cm^{-1} respectively.

General procedure for Preparation of N-phenyl-5-substituted-aryl-3p (fluorophenyl) pyrazoles (5a-5e):

The title compounds were synthesized by cyclization of 4-fluorophenyl styryl ketones. A mixture of the 4-fluorophenyl styryl ketones (0.01mol) was refluxed on an oil bath in the presence of drug pyridine and (0.02mol) and phenyl hydrazine for about 8 hrs. The cooled product was triturated with glacial acetic acid, precipitate obtained was separated, recrystallised from chloroform and characterized by spectral analysis. Spectral data of 5a are given below.

1510cm^{-1}(C=C) 1460-1490 (C=N), 1220-1250cm^{-1}(C-F), 660-690cm^{-1}(arom).^{1}HNMR ($CDCL_3$): (δppm) =6.95. (s, C-4'H,Ch),7.3-7.6(m,14H,arom).

Inhibition of BSA Denaturation:

Inhibition of protein denaturation was studied according to the method of Elias and Rao [18].The test compounds were dissolved in a minimum amount of DMF and diluted with phosphate buffer (0.2M pH 7.4). The test sample solution (1ml) containing different concentration of drug was mixed with 1ml of a 1mM BSA solution in phosphate buffer and incubated $27^{o}C \pm 1^{o}C$ for 15 min. Denaturation was induced by keeping the reaction mixture at $60^{o}c$ for 10 min in a water bath. After the mixture was cooled turbidity was measured at 660nm.The percent inhibition of BSA denaturation was calculated with reference to control samples to which drugs was added. Each experiment was done in duplicates and average value was reported. The percent inhibition was determined by using the formula:

$$\% \text{ inhibition} = (1-Vt/Vc) \times 100,$$

where Vt is the absorbance of test sample and Vc is the absorbance of control sample.

Carrageenan induced edema Test:

Adult albino rats of either sex, weighing 150-200g were used. The rats were divided into 5 groups of 6 animals each and initial paw volume was measured at 0 hr. Animals were allowed free access to standard pellet diet (Sai Durga Feeds, Bangalore) and water ad libitum. Food was withdrawn 2 hrs before and during experimental duration. All experimental protocols were prepared and performed based on ethical guidelines of Institutional Animal Ethics Committee (No. 1051/ac/07/CPCSEA/24Apr2007). Freshly prepared 1% suspension of carrageenan in 0.9% saline (0.1ml) was injected into the planter aponeurosis of the left hind paw [14]. 1 hr after the oral (p.o) administration of test compounds which were dissolved in 1ml of 2% gum acacia, the rats were treated p.o with test samples 1 hr before the injection of carrageenan. The control group received an equivalent amount of the gum acacia suspension used to dissolve the test compounds. The rats in the standard reference group received indomethacin (10mg/kg p.o) dissolved in 2% gum acacia. The increase in paw volume was measured by a plethysmograph (mercury displacement is the index of edema and was measured before and 4 hr after the administration of carrageenan). The anti inflammatory activities of test compounds and standard reference drug were determined by using the following formula. % inhibition = (1-Vt/Vc) x 100, where Vt is the edema volume in the test drug treated animals, Vc is the edema volume in the control group animals.

Molecular Docking Studies:

The newly synthesized compounds were subjected for molecular docking by calculating the minimum energy by inhibiting the target protein involved in the inflammation pathway. The ligands were drawn in ChemDraw Ultra 6.0 assigned with proper 2D orientation (Chem Office

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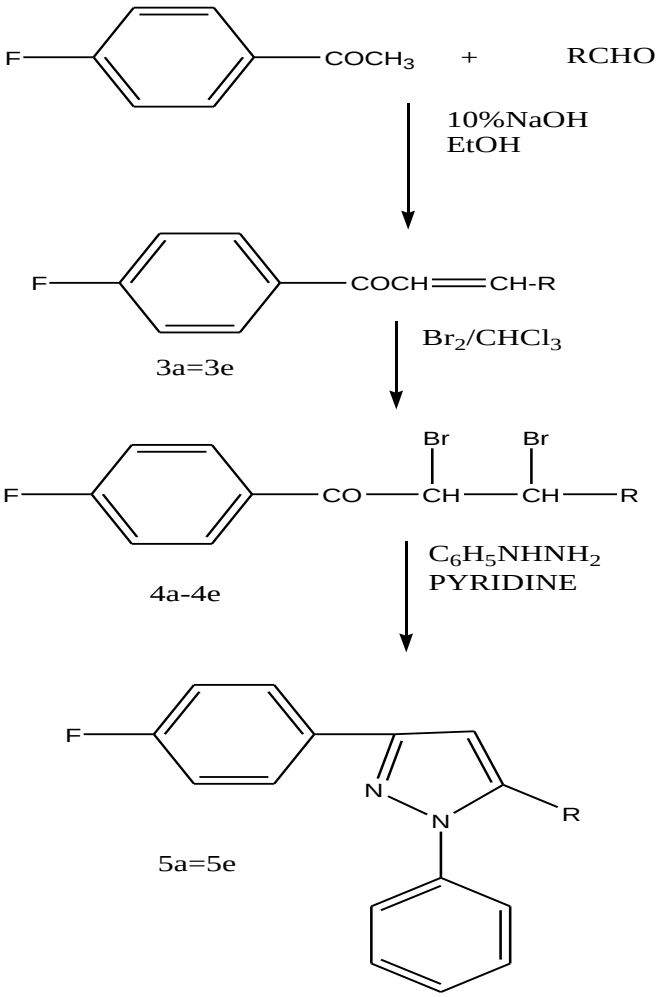
package) and the structure of each ligand was analyzed by using Chem-3D Ultra 6.0 (ChemOffice package) and was checked for the connection error in bond order. ADMET property was achieved through Pre ADMET server- a web-based application for predicting ADMET data and building drug-like library using in silico method. Energy of the molecules was minimized using Dundee PRODRG2 Server. Then the file was opened in SPDB viewer and C-terminal Oxygen was added using fit module property. CASTp (Computed Atlas of Surface Topography of proteins) server was used to identify the active pockets on target protein molecules. Autodock V3.0 was used to perform Molecular Docking.



R=3a=4a=5a=C_6H_5
R=3b=4b=5b=4-OH,3-$OCH_3C_6H_5$
R=3c=4c=5c=4-ClC_6H_5
R=3d=4d=5d=4-$N(CH_3)_2C_6H_5$
R=3e=4e=5e=2-$NO_2C_6H_5$

RESULTS AND DISCUSSION

The 4-fluorophenyl styryl ketones (3a-3e) possess anti inflammatory activity. The degree of protection afforded by 3a-3e (250mg/kg.p.o) against carrageenan induced edema in rat paw ranged from 57-70%. The abilities of indomethacin (10mg/kg p.o) used as standard reference drugs. The maximum protection was observed in 3b (70%), whereas the minimum protection

(57%) observed with 3d. Copmpounds 3a-3e (0.20mM) inhibited BSA denaturation by 7-59%. Indomethacin used as standard reference inhibited in vitro BSA denaturation by 79%.

The ability of compounds (5a-55) at 250mg/kg p.o. to protect against carrageenan induced edema in rat paw ranged from 30-47%. This anti inflammatory activity is much less than those of indomethacin (10mg/kg p.o) which gave (97%).

Among substituted phenyl styryl ketones, the presence of fluorine in phenyl nucleus increased the anti inflammatory activity. Cyclization of the substituted phenyl styryl ketone into their corresponding substituted aryl-3-p-(fluoprophenyl) pyrazoles decrease their anti-inflammatory activity. The inhibition of BSA denaturation by substituted 4-fluorophenyl styryl ketone (3a-3e) and their corresponding cyclized pyrazoles (5a-5e) did not correlate with anti inflammatory effectiveness. An increase in BSA denaturation activity was observed with only 3a and 3b. The anti inflammatory and BSA denaturation activities of 3a-3e and 5a-5e showed no structure-activity relationship and failed to establish BSA denaturation activity as the cellular basis for the anti-inflammatory properties (table 1).

Table 1: Anti inflammatory and BSA denaturation activities of synthesized pyrazoles.

Compound No.	Mean increase in paw Volume ±SE	Anti inflammatory activity % inhibition	Inhibition of BSA denaturation % protection
3a	0.17 ± 0.02	70.6	59.5 ± 0.0092
3b	0.24 ± 0.01	58.2	22.0 ± 0.0039
3c	----	----	----
3d	0.28 ± 0.01	57.7	7.6 ± 0.00032
3e	----	----	----
5a	0.38 ± 0.03	32.1	12.6 ± 0.00014
5b	0.34 ± 0.02	39.2	22.7 ± 0.0016
5c	----	----	----
5d	0.39 ± 0.03	30.3	14.8 ± 0.00018
5e	0.34 ± 0.01	39.2	----
Indomethacin	0.56 ± 0.02	---	----

n=6 in each group

The docking results for ligand pyrazole derivatives against NFκB are shown in table 2. Some of the compounds showed minimum docking energy, binding energy, inhibition constant intermolecular energy and RMS with 0.0. Among them 3b showed minimum docking energy and maximum hydrogen bonds which is followed by 5b and 5e. The docking postures are shown in fig 1. Results obtained from the compounds are comparable with the standard indomethacin (fig 2). The theoretical outcome highlight that the minimum binding energy of the molecules with the targeted protein may make these newly synthesized pyrazoles as good inhibitors for inflammation. However, these promising results are reliable and further can be subjected for preclinical studies to arrive at the conclusion on these molecules for their clinical use.

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Table 2: Molecular docking details of all the compounds along with the standard indomethacin.

Molecule	Binding energy	Docking energy	Inhibitory constant	Intermol energy	H-bonds	Bonding
3a	-5.23	9.42	0.000146	-5.86	-	----------
3b	-0.57	0.33	0.38	-1.5	3	NFkB:A:GLU222:OE2::3c:DRG1:HAA NFkB:A:LYS221:HN::3c:DRG1:OAS NFkB:A: LYS221:HZ1::3c:DRG1:OAR
3c	-5.43	-6.25	0.000105	-6.05	-	----------
3d	-5.19	-6.29	0.000158	-6.12	-	----------
3e	-5.57	8.08	8.26e-005	-6.5	-	----------
5a	-5.91	-7.17	4.68e-005	-6.84	1	NFkB:A:GLN247:HE22::4a:DRG1:NAI
5b	-4.42	-5.8	0.000571	-5.67	2	NFkB:B:LYS218:HZ2::4c:DRG1: NAJ,NAI NFkB:B:ARG246:HH21::4c:DRG1:OAZ
5c	-5.81	-6.95	5.53e-005	-6.74	1	NFkB:A:GLN247:HE22::4d:DRG1:NAI
5d	-5.08	-6.45	0.000188	-6.33	1	NFkB:A:GLN247:HE22::4e:DRG1:NAI
5e	-6.25	-7.6	2.63e-005	-7.49	2	NFkB:A:ARG246:HH12::4f:DRG1:OAZ NFkB:B:LYS218:HZ3::4f:DRG1:OBA
IDM	-6.29	-8.2	2.46e-005	-7.84	3	NFkB:A:LYS218:HZ2::IDM:DRG1:OAC NFkB:A:ARG246:HH12::IDM:DRG1:OAD NFkB:B: ARG246:HH21::IDM:DRG1:OAC

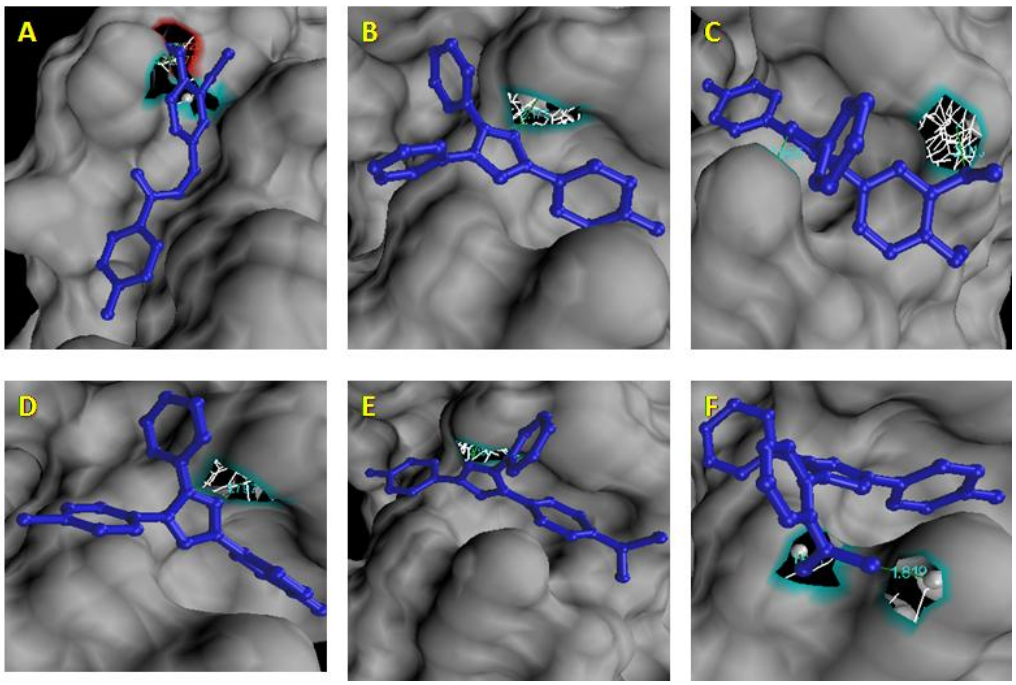

Fig 1: Docking postures of compounds, 3b with NFkB (A); 5a with NFkb (B); 5b with NFkB (C); 5c with NFkB (D); 5d with NFkB (E); 5e with NFkB (F).

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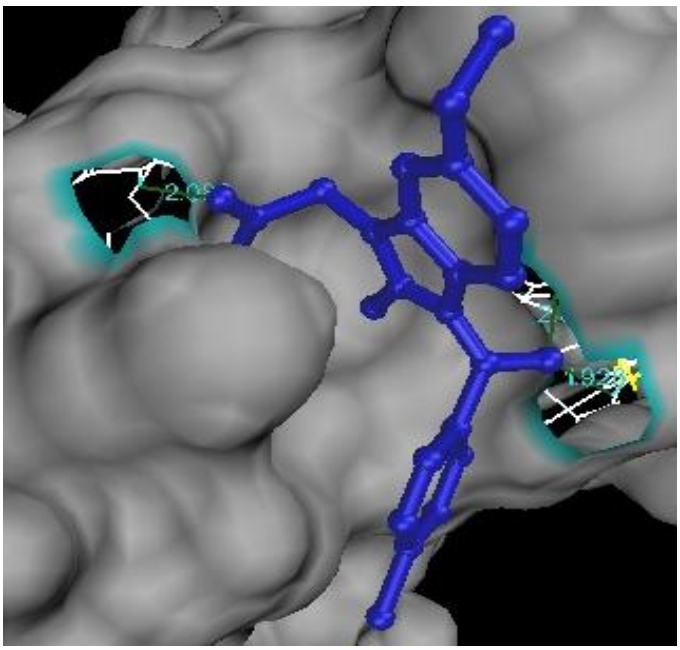

Fig 2: Docking posture of the standard drug indomethacin with protein NFkB.

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