
SCREENING OF SYNTHETIC NEW HETEROCYCLIC DERIVATIVES OF 3-FORMYL-4-HYDROXYCOUMARIN FOR ANTI-INFLAMMATORY ACTIVITY IN ALBINO RATS

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ABSTRACT

Coumarins have multiple biological activities; various coumarin-related derivatives are recognized as inhibitors of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism. Several natural or synthetic coumarins with various hydroxyl and other substitutes were found to inhibit lipid peroxidation and to scavenge hydroxyl radical and superoxide anion and to influence processes involving free radical mediated injury. The heterocyclic derivatives of 3-formyl-4-hydroxycoumarin were found to present significant anti-inflammatory effect, the compounds inhibited formalin induced hind paw edema and they also significantly suppressed the formation of granuloma tissue in cotton pellet induced chronic model of inflammation. The result showed that the anti-inflammatory (both acute & chronic) effect of the test compound **Ib** is comparable to that of standard Antiinflammatory drug Diclofenac Sodium.

Keywords: 3-Formyl-4-hydroxycoumarin derivatives; Coumarin; Anti-inflammatory.

1. INTRODUCTION

Coumarins show activities such as antifungal (Sangwan et al., 1990), anticoagulant (Stahman et al., 1941), antibacterial (Honmantgad et al., 1985), analgesic, antipyretic, anti-inflammatory and anti-arthritic (Santagati et al., 1993; Kontogiorgis and Hadjipavlou-Litina, 2005). Coumarins are reported to be used in the treatment of vitiligo, psoriasis and other dermal diseases. The physiological properties of natural and synthetic Coumarins have been reviewed by various workers (Soine, 1964). In recent times [1]benzopyran-2[H]-ones have been extensively used as intermediates for dyes, pesticides and pharmaceuticals (Hagen and Kohler, 1981) as well as in perfume formulations (Pozdnev, 1987; Pozdnev, 1990) and in enzymology as biological probes (Tamura et al., 1982). The objective of the present study was to screen three, 3-(3-methyl pyrazol-5-yl)-pyrano[3,2-c][1] benzopyran 2,5-dione, 3-(3-methyl-1-phenyl pyrazol-5-yl)-pyrano [3,2-c] benzopyran-2, 5-dione and -(3-methyl-1-benzothiazolopyrazol-5-yl)-pyrano[3,2-c][1] benzopyran-2,5-dione (synthesized by us) for anti-inflammatory activity in albino rats.

2. MATERIALS AND METHODS

2.1 Chemicals and test compounds

Following heterocyclic compounds were synthesized in the research laboratory of Department of Chemistry and studied for their physiochemical and spectral properties (Siddiqui and Asad, 2006). They were tested for acute and chronic anti-inflammatory activity in animal models.

1. 3-Acetoacetyl pyrano [3,2-c] [1] benzopyran 2,5-dione (fig.1)

Was prepared from intramolecular translactonization of 4-hydroxycoumarins and triacetic acid lactone. The resulting compound which possessed a 1,3-diketone unit in its structure were

converted to pyrazoles by treatment with hydrazine, phenylhydrazine and hydrazinobenzothiazole to afford.

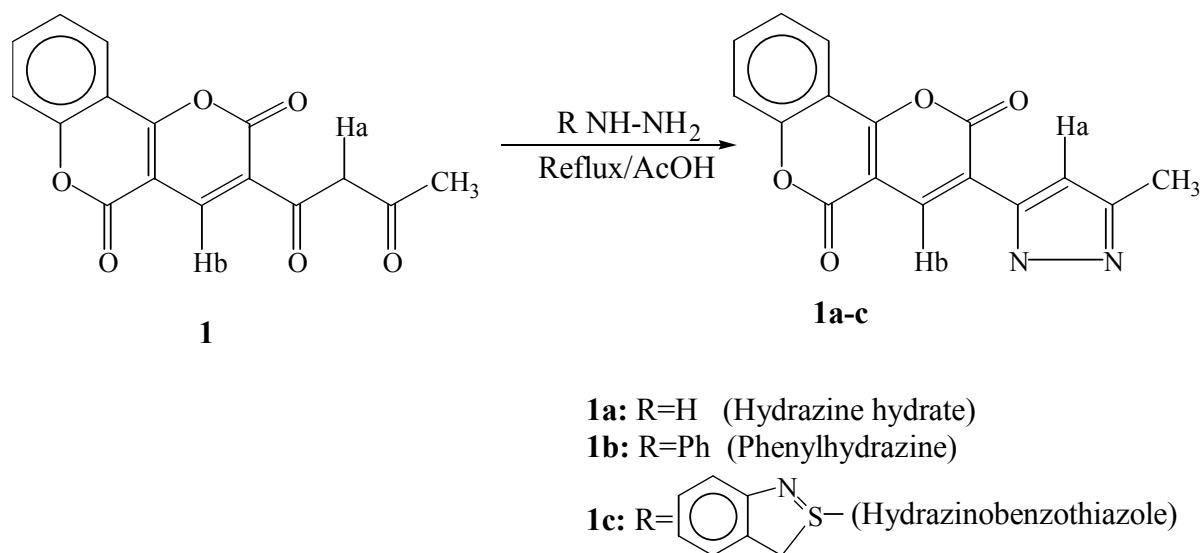


Fig 1: Structure of some novel new heterocyclic derivatives of 3-formyl-4-hydroxycoumarines synthesized and screened.

1a. 3-(3-methyl pyrazol-5-yl)-pyrano[3,2-c][1] benzopyran 2,5-dione.

1b. 3-(3- methyl-1-phenyl pyrazol-5-yl)- pyrano [3,2-c] benzopyran-2, 5-dione and

1c. 3-(3-methyl-1-benzothiazolopyrazol-5-yl)-pyrano[3,2-c][1] benzopyran-2,5-dione

The test compounds were dissolved in 2.5% DMSO (Dimethyl sulphoxide) prior to administration in different concentration so that animal received equal volume each time (5 ml/kg). Dose selection of the test compound was based on preliminary trial carried out in our laboratory over a dose range 5 mg/kg to 40 mg/kg in geometric increasing order and maximal effect was found at the dose of 20 mg/kg.

Drugs used:

Formalin (Merck, India)

Diclofenac (Novartis)

2.2. Experimental Animals

Male Wistar Albino rats (weight 100–150 g) obtained from Laboratory Animal Breeding and Research Center Jamia Hamdard University New Delhi were used for the present study. The animals were given a week time to get acclimatized with laboratory conditions. The animals were housed in polypropylene cage (4 per cage) with sterilized paper cuttings as bedding material under laboratory conditions with control environment of temperature 22 ± 3 °C, humidity (60 % \pm 10 %) an 12 h light/dark cycle. They were given free access to food with standard rodent pellet diet (from Lipton India) and drinking water.

The study protocol was approved by the institutional ethical committee.

2.3. Experimental Protocol

The following experimental models were used for test compounds.

a. Acute anti-inflammatory model (Paw edema induced by Formalin)

Method describe by Northover and Subramanian (1961) in which 0.05 ml of 3.5% Formalin in Normal Saline was injected in the subcutaneous tissue of the planter surface of right hind paw produced sub maximal degree of swelling. The paw volume was measured at 0, 0.5, 1, 2, 3, 4 and 5 hour after injection of Formalin. The volume (in milliliters) of the inflamed paw was measured by standard volumetric technique, using a calibrated plethysmometer. The paw was immersed upto the tibiotarsic articulation (marked with ink) in a cylinder filled with mercury. The increased level, consequent on the increase of the mercury meniscus, was measured from the increase of dyed ethanol in a glass tube connected to the surface of the mercury so that variation of the mercury level corresponded to increases in the dyed ethanol in a

calibrated glass tube. The increase in volume of the paw was calculated by subtracting the initial volume from the volume obtained after formalin administration and expressed as paw volume increase over time (ml ± SD) and effect (percent of negative control) for each rat and each group was obtained as follows.

$$\text{Percentage of inhibition} = \frac{(\text{Vt} - \text{Vo}) \text{ control} - (\text{Vt} - \text{Vo}) \text{ treated}}{(\text{Vt} - \text{Vo}) \text{ control}} \times 100$$

(Vo = is average volume of right paw before injection of formalin i.e. at 0 h and Vt = is average volume of right paw after injection of formalin)

Experimental design and drug treatment: Rats were divided into three groups of six rats each.

Group I: received 2.5% DMSO 1 h prior to formalin and served as control.

Group II: received test compound (20mg/kg, orally) 1 h before injection of formalin.

Group III: received Diclofenac (5 mg/kg, orally) 1 h before injection of formalin.

b. Chronic anti-inflammatory activity (Cotton pellets-induced granuloma test)

The method described by D'Arcy et al., 1960 (Turner, 1965) was used. Under light ether anesthesia, sterile cotton pellets (10 ±1 mg) were implanted subcutaneously in the groin regions of the rats. The test compounds (20mg/kg), Diclofenac (5 mg/kg) and control vehicle 2.5% DMSO (5ml/kg) were administered once daily orally for seven consecutive days from the day of cotton pellet implantation. The animals were anesthetized on the eighth day and cotton pellets were removed surgically and made free from fat and extraneous tissues. The wet weights of granuloma were estimated and than pellet were dried overnight at 60 °C in hot-air oven to constant weight. The weight of the cotton pellet before implantation was subtracted from the

weight of the dried, dissected pellet. The mean weight was calculated for the pellets from a group of rats, and compared with the mean for a group of controls. Increment in the dry weight of the pellets was taken as measure of granuloma formation. The difference in wet weight and dry weights of granuloma from control group to that of treated group indicated the anti-inflammatory activity.

Experimental design and drug treatment: Rats were divided into three groups of six rats each.

Group I: received 2.5% DMSO (5ml/kg, orally) daily for seven days and served as control.

Group II: received test compound (20mg/kg, orally) daily for seven days.

Group III: received Diclofenac (5 mg/kg, orally) daily for seven days

c. Acute Toxicity study

The acute oral toxicity was carried out as per the guideline set by the organization for the economic co-operation and development (OECD) received from the committee for the purpose of control and supervision of experimental animals (CPCSEA).

Experimental design and drug treatment: (Bruce. R-D et al),

Two rats (one from either sex) were dosed at predetermined [250, 500 and 1000 mg /kg dissolved in fixed amount (1.5 ml) of DMSO] and administered by stomach feeding cannula. They were observed continuously for the first 2 h for toxic symptoms and up to 24 h for mortality (Litchfield et al., 1949). If there was no mortality or if no more than one rat of either sex died at the highest level tested (1000 mg/kg) with the total of 10 rats (5/sex) dosed at 1000 mg/kg and monitored for 7 days period, LD50 was considered to more than 1000 mg/kg.

f. Statistical analysis

All values are presented as mean \pm S.E.M. of six rats and difference between means were assessed by one-way analysis of variance (ANOVA), followed by student's *t* test. Difference between means were considered to be significant at $P < 0.05$ as compare to control.

3. RESULTS

a. *Acute inflammation model (formalin induced paw oedema)*

The results of the anti-inflammatory effect of the test compounds on Formalin induced oedema in rat's right hind paws are presented in Table 1&2. There was a gradual increase in oedema paw volume of rats in the control (Formalin treated). However, in the test groups, the compounds showed a significant reduction in the oedema paw volume. As indicated in Table 2, the significant antiinflammatory effect induced by test compounds Ia, Ib & Ic appeared at 1-2 h and progressively increased and reached a maximum 46.6%, 88.5% & 65.5% respectively at five hours, while the maximum Antiinflammatory effect of test compound I appeared at I h (60%).

The Antiinflammatory effect induced by Diclofenac sodium progressively increased and reached a maximum (70.8%) at two hours. It was maintained up to five hours.

b. *Cotton pellet induced granuloma (Chronic model)*

In the chronic model (cotton pellet induced granuloma) the test compounds and Diclofenac sodium significantly ($P < 0.05$) reduced both wet as well as dry weights in cotton pellet granuloma (Table 3). The effect of test compound Ib in both reducing wet weight and dry weight of cotton pellet induced granuloma was similar to that of Diclofenac sodium.

Table 1: Effects of test compounds and Diclofenac on acute inflammation of paw oedema induced by formalin.

Test Compound	Dose/kg	Before Formalin	Mean paw volume \pm SEM (ml)					
			0.5 h	1 h	2 h	3h	4 h	5 h
DMSO	5 ml	0.71 \pm 0.02	0.76 \pm 0.02	0.86 \pm 0.03	0.95 \pm 0.02	1.02 \pm 0.02	1.07 \pm 0.04	1.23 \pm 0.01
1	20 mg	0.70 \pm 0.01	0.73 \pm 0.01	0.76 \pm 0.01*	0.83 \pm 0.02*	0.89 \pm 0.02*	0.98 \pm 0.02	1.09 \pm 0.02*
1a	20 mg	0.71 \pm 0.02	0.76 \pm 0.03	0.80 \pm 0.03	0.85 \pm 0.03*	0.90 \pm 0.02*	0.93 \pm 0.02	0.99 \pm 0.04*
1b	20 mg	0.67 \pm 0.02	0.70 \pm 0.02	0.74 \pm 0.01*	0.76 \pm 0.02*	0.76 \pm 0.03*	0.75 \pm 0.02*	0.73 \pm 0.03*
1c	20 mg	0.67 \pm 0.02	0.70 \pm 0.02	0.74 \pm 0.01*	0.76 \pm 0.02*	0.76 \pm 0.03*	0.75 \pm 0.02*	0.73 \pm 0.03*
Diclofenac Sodium	5mg	0.70 \pm 0.02	0.73 \pm 0.02	0.75 \pm 0.02*	0.77 \pm 0.02*	0.80 \pm 0.02*	0.82 \pm 0.03*	0.88 \pm 0.04*

The results given are mean \pm S.E.M; number of animals used (n=6) *P value of < 0.05 was considered as significant in comparison to control

c. Acute Toxicity study evaluation

In acute toxicity study the test compounds did not show any toxicity and mortality up to maximum dose of 1000 mg/kg body weight in rats. No gross change in behavior was observed at this dose. Weight of rats had a normal variation after 7 days of observations.

Table 2: Percentage Inhibition in formalin induced paw edema by test compounds and Diclofenac.

Test Compounds	Dose/kg	% Inhibition					
		0.5 h	1h	2h	3h	4h	5h
DMSO	5ml	-	-	-	-	-	-
1	20 mg	40	60*	45.8*	38.7	22.2	25
1a	20 mg	00	40	41.6*	38.7	38.8	46.6*
1b	20 mg	40	53.3*	62.5*	71*	77.7*	88.5*
1c	20 mg	00	46.6*	58.3*	45.2*	50*	65.4*
Diclofenac Sodium	5 mg	40	66.6*	70.8*	67.7*	66.6*	65.4*

Table-3: Effects of test compounds and Diclofenac on cotton pellet induced granuloma.

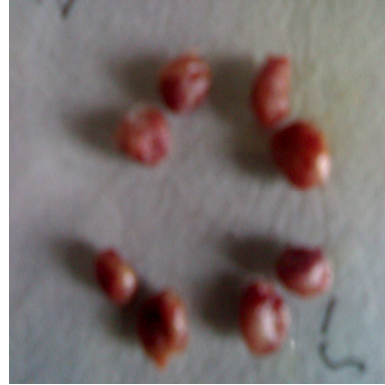
Compounds	Dose/kg	Weight of cotton pellets			
		Wet weight	% inhibition	Dry weight	% inhibition
Control (DMSO)	5ml	183.7 ± 11.5	-	79.3 ± 3.5	-
1	20 mg	100 ± 9.5*	45.56	39.6 ± 2.6*	50.06
1a	20 mg	99.5 ± 8.2*	45.83	42.0 ± 2.3*	47.03
1b	20 mg	79.0 ± 4.5*	56.99	30.35 ± 1.6*	61.72
1c	20 mg	115.0 ± 10.2*	37.39	44.0 ± 2.9*	44.51
Diclofenac Sodium	5 mg	84.5 ± 6.3	54.00	30.0 ± 1.8*	62.17

The results given are mean ± S.E.M; number of animals used (n=6)

*P value of < 0.05 was considered as significant in comparison to contro



Control (DMSO)



Test compound (I)



Test compound (1_a)



Test compound (1_b)



Test compound (1_c)



Diclofenac sodium

Fig.2: Cotton pellet surrounded by granuloma tissue.

4. DISCUSSION

The results of this study indicate the synthetic new heterocyclic derivatives of 3-formyl-4-hydroxycoumarin with addition of different groups possess both acute and chronic antiinflammatory activity.

It is well known that inhibition of edema induced by formalin in rats is one of the most suitable test procedures to screen anti-inflammatory and antiarthritic agents, as it closely resembles human arthritis. (Greenwald RA et al; 1991) Arthritis induced by formalin is a model used for the evaluation of an agent with probable antiproliferative activity (Banerjee S. et al; 2000).

The formalin-induced inflammation in the rats foot may be conveniently divided into two parts, the first involving 5-hydroxytryptamine as mediator and the second some mediator which is unrelated to 5-hydroxytryptamine. The portion of the total response, which is due to the release of 5-hydroxytryptamine, can be prevented by either depleting the skin of 5-hydroxytryptamine or by giving the rats an antagonist of 5-hydroxytryptamine. It also seems probable that the portion of the total response which is due to the " second mediator" can be prevented by treatment with certain analgesic-antipyretic drugs (acetylsalicylic acid) and other substances like the hydroxybenzoates, the pyrazolones, the flavone and flavanone glycosides are inactive against 5-hydroxytryptamine-induced inflammation but they produce their action against an formalin-induced inflammation by inactivating the second factor (NORTHOVER B.J et; 1962). Another possibility is that an antiinflammatory agent might operate by releasing or activating some endogenous factor, which is antiinflammatory. It is well known that the Salicylates, for example, release both adrenal cortical (Smith, 1953) and adrenal medullary hormones (Smith, 1955), and although the adrenal cortical hormones are inactive against

formalin-induced inflammation in the rats foot (Northover & Subramanian, 1961a) the adrenal medullary are active (NORTHOVER B. J. et al; 1962)

The test compound 3-acetoacetyl pyrano [3,2-c] [1] benzopyran 2,5-dione (test compound **1**) exhibited significant antiinflammatory activity with maximum effect at 3 h. Addition of phenylhydrazine (Test compound 3-acetoacetyl pyrano [3,2-c] [1] benzopyran 2,5-dione with phenylhydrazine, **1b**) markedly improved the antiinflammatory activity, which was as good as Diclofenac sodium, the addition of hydrazine hydrate (test compound 3-acetoacetyl pyrano [3,2-c] [1] benzopyran 2,5-dione with hydrazine hydrate, **1a**) and hydrazinobenzothiazole (test compound 3-acetoacetyl pyrano [3,2-c] [1] benzopyran 2,5-dione with hydrazinobenzothiazole **1c**) also exhibited significant antiinflammatory activity but to a lesser extent.

As the test compounds significantly inhibited this model of inflammation, it can be thought to possess antiproliferative and antiarthritic activities similar to Diclofenac and Salicylates the cyclooxygenase inhibitors.

The cotton pellet method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. Inflammation and granuloma develops during the period of several days. The Inflammation involves proliferation of macrophages, Neutrophils and fibroblasts, which are basic sources of granuloma formation. The wet weight of the cotton pellets correlates with the transuda; the dry weight of the pellets correlates with the amount of the granulomatous tissue [Olajide OA et al; 1999 & Olajide OA et al 2000]. Hence, the decrease in the weight of granuloma indicates the ability of the test compounds in reducing the synthesis of proteins, collagen and infiltration of macrophages. Administration of test compounds (20mg/kg) and Diclofenac sodium (5 mg/kg) appear to be effective in inhibiting both the wet weight and dry

weight of cotton pellet (table-3). The test compound **Ib** (20 mg/kg) appears to be equally effective to that of Diclofenac sodium (5 mg/kg) in inhibiting both the wet weight and the dry weight of cotton pellets

5. CONCLUSION:

Based on the results it can be concluded that the new heterocyclic derivatives of 3-formyl-4-hydroxycoumarin possess significant role in inhibition of both acute and chronic phases of inflammation. Additions of different functional groups have varying effects. Significant increase in anti-inflammatory effect of compound I was observed after addition of phenylhydrazine. Further studies to determine the relevance of this finding in humans should be undertaken that might result in the development of a potent antiinflammatory and antiarthritic agent with low toxicity and better therapeutic index

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