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ANTITHROMBOTIC EFFECT OF THIOPURINOL*

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ABSTRACT

Antithrombotic activity of ten pyrazolo pyrimidine derivatives was tested in mouse pulmonary thromboembolism model. Out of these compounds, Thiopurinol ($C_5H_4N_4S$) showed dose-dependent protection in mice from death/paralysis induced by collagen + adrenaline. It also caused dose-dependent inhibition of thrombus formation in the cat. Thiopurinol inhibited aggregation of platelets induced by ADP and arachidonic acid but did not inhibit superoxide generation. It had no antiinflammatory activity nor any effect on cardiovascular system. The results indicate that the antithrombotic activity of the compound is mediated via inhibition of platelet aggregation.

INTRODUCTION

Pyrazolo [3,4-d] pyrimidine ring system has received renewed interest in recent years, owing to the discovery of allopurinol (4-hydroxypyrazolo[3,4-d]-pyrimidine) possessing potent antigout (1) as well as antiparasitic activities (2). The mechanism of action of allopurinol is mediated via its free radical scavenging and xanthine oxidase inhibitory activities. It has earlier been reported from our laboratory that allopurinol also possesses antithrombotic activity in animal models (3).

Free radicals are involved in the genesis of thrombosis (unpublished observations) and their involvement in arterial thrombosis has been suggested (4). It was, therefore, decided to study the antithrombotic activity of some purine analogs with structural similarity to allopurinol. One of the compounds 4(5H)-thiopyrazolo [3,4-d]pyrimidine, the thioanalog of allopurinol, showed potent antithrombotic activity, the results of which are discussed in this communication.

MATERIALS AND METHODS

Materials: Male albino mice of Swiss strain and male cats were obtained from CDRI animal colony. Collagen, adrenaline, adenosine diphosphate sodium salt,

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arachidonic acid sodium salt, phenazinemethosulfate, nitroblue tetrazolium were purchased from Sigma Chemical Co. (USA).

Methods

1. **Synthesis of 4(5H)-thiopyrazolo [3,4-d]pyrimidine:** Thiopurinol was synthesized according to the technique of Robins (5). Briefly, phosphorus pentasulfide (10 g, 52 mMol) was mixed portion wise into dry pyridine (30 ml) at 65°-70°C and allopurinol (5 g, 36 mMol) was added to the above suspension. The reaction mixture was gently refluxed for 4 hours. The excess of solvent was removed *in vaccuo*. The hot syrup was poured into boiling water (100 ml). The resulting solution was then allowed to stand overnight at 4°C. The crystallized product was filtered. Yield (4 g, 64%), m.p. 250°, M.S. (m/z) 152 (M^+), PMR (CDCl3): 8.25 (S,IH,H-6) 8.05 (S,IH,H-3).

	Found	Requires
Anal :	C,39.0	39.4
	H, 2.9	2.6

Analogues IV, VII, X and XI were prepared by reported techniques (6,7,8,9) while other analogues were synthesized at our laboratory.

2. **Antithrombotic activity:**

a) **Mouse pulmonary thromboembolism:** Male albino mice (20-25 g) of Swiss strain were used in groups of at least ten animals each. A mixture of 15 ug collagen and 30 uM adrenaline in a volume of 100 ul was injected into the tail vein of each mouse. This caused either death or paralysis of the hind limbs of almost 100% of the animals (10).

The compound was administered intraperitoneally (i.p.) one hour prior to the thrombotic challenge. The antithrombotic effect of the compound was assessed by calculating the percentage of animals protected from death or paralysis which occured due to thrombotic challenge.

b) **Thrombosis in extracorporal circulation:**

Cats weighing 3.5-4 kg were anaesthetized with pentobarbitone sodium (40 mg/kg i.p.). Extracorporeal circulation was created by connecting left jugular vein and the right carotid artery, as described earlier (3). The compound was administered intravenously through a cannulated femoral vein. The percent inhibition of the wet weight of the thrombus was calculated.

3. **Antiinflammatory activity:** Antiinflammatory activity of the compound was assessed in carrageenin edema model of Winter *et al* (11) in rat and by the method of Srimal and Dhawan (12) in mouse.

4. **Antiaggregatory activity:** Venous blood was collected from healthy donors in 3.8% sodium citrate and *in vitro* platelet aggregation studies were carried out as described earlier (13). Aggregation was induced by ADP (5 uM) and arachidonic acid (50 uM), in platelet rich plasma.

5. **Superoxide radical generation:** Effect of thiopurinol was studied on the non-enzymatic superoxide radical generation (14). Briefly, 10 uM phenazine methosulfate (PMS), 78 uM NADH and 25 uM nitrobluetetrazolium in 0.1 M phosphate buffer, pH 7.4, were incubated with and without the compound at different concentrations for 2 minutes at room temperature. Optical density was recorded at 560 nm on Pyeunicam spectrophotomer (PU8600) immediately afterwards. Blank tubes contained no PMS.

6. **Cardiovascular activity:** Cats were anaesthetised with sodium pentobarbitone (40 mg/kg i.v.). Blood pressure, respiration and contraction of the nictitating

membrane to electrical stimulation of preganglionic sympathetic nerve were recorded on a kymograph. Blood pressure was recorded from carotid artery through a mercury manometer, while respiration was recorded by cannulating the trachea. The pressor and depressor effects of adrenaline, isoprenaline, acetylcholine and histamine were observed prior to and after the administration of the compound (i.v.).

RESULTS

I. Antithrombotic activity:

a) Mouse pulmonary thromboembolism: Of the twelve purine analogs with structural similarities tested, thiopurinol possessed 47.5% antithrombotic activity at 10 mg/kg i.p. dose. The activity was dose dependent and was more potent than allopurinol (ED_{50} 17.50 mg/kg) (3) but less than indomethacin (ED_{50} 6.7 mg/kg) (3). Of the other analogs tested, the antithrombotic effect was either not present or was much less compared to thiopurinol (Table 1).

TABLE 1

Effect of Purine Analogs on Mouse Pulmonary Thromboembolism

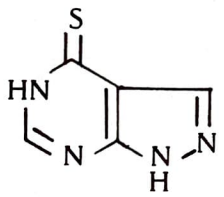
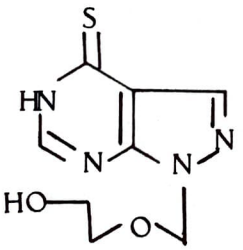
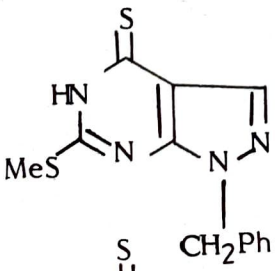
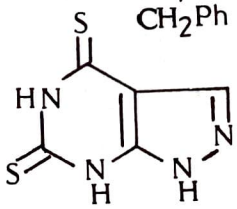
No.	Structure	Dose (mg/kg ip)	Activity (% protection)
I		5 10 20	20.0 47.5 90.0
II		10	37.5
III		10	0
IV		10	0

Table 1 contd.

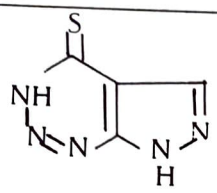
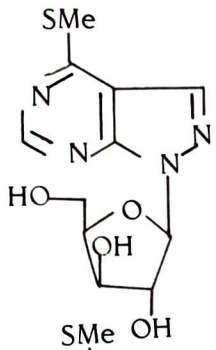
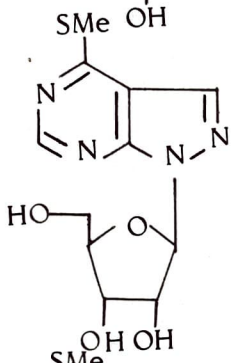
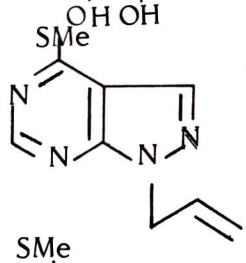
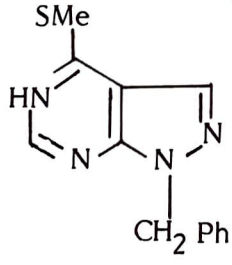
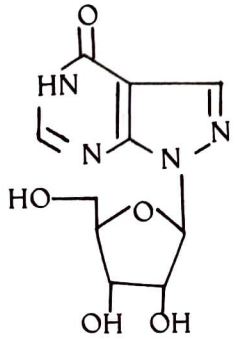
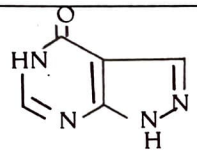
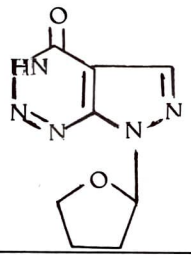
No.	Structure	Dose (mg/kg ip)	Activity (% protection)
V		10	0
VI		10	10
VII		10	0
VIII		10	10
IX		10	12.5
X		10	0

Table 1 contd.

No.	Structure	Dose (mg/kg ip)	Activity (% protection)
XI		10	0
XII		10	0

b) Thrombosis in extracorporal circulation: Thiopurinol caused dose dependent inhibition of thrombus formation in extracorporeal circulation in cat. However, it was less potent than indomethacin (ED_{50}, 0.44 mg/kg) as well as allopurinol (ED_{50} 0.39 mg/kg) (3) in this test. The results are summarised in Table 2.

TABLE 2

Effect of thiopurinol, allopurinol and indomethacin on thrombosis in extracorporeal circulation in cat

Compound	Dose (mg/kg iv)	Percent inhibition of thrombus formation
Thiopurinol (I)	2	6.25
	5	34.38
	10	65.63
Allopurinol	1	59.16
	3	64.05
Indomethacin	1	66.80
	3	77.87

Values are mean of two or more experiments.

2. Antiinflammatory activity: Thiopurinol was devoid of any significant anti-inflammatory activity when tested on carrageenin induced edema in mouse as well as rat. At 60 mg/kg p.o. dose in mice it produced 15% antiiflammatory effect and at 30 mg/kg p.o. in rats it caused no significant antiinflammatory effect.

3. **Antiaggregatory activity:** Thiopurinol caused concentration dependent inhibition of ADP induced human platelet aggregation in vitro (Fig. Ia). However, significant antiaggregatory effect was observed only at the highest concentration used (1 mM). It also caused concentration dependent inhibition of arachidonic acid induced platelet aggregation (Fig. Ib). Effect on arachidonic acid induced aggregation was more pronounced than on ADP induced aggregation.

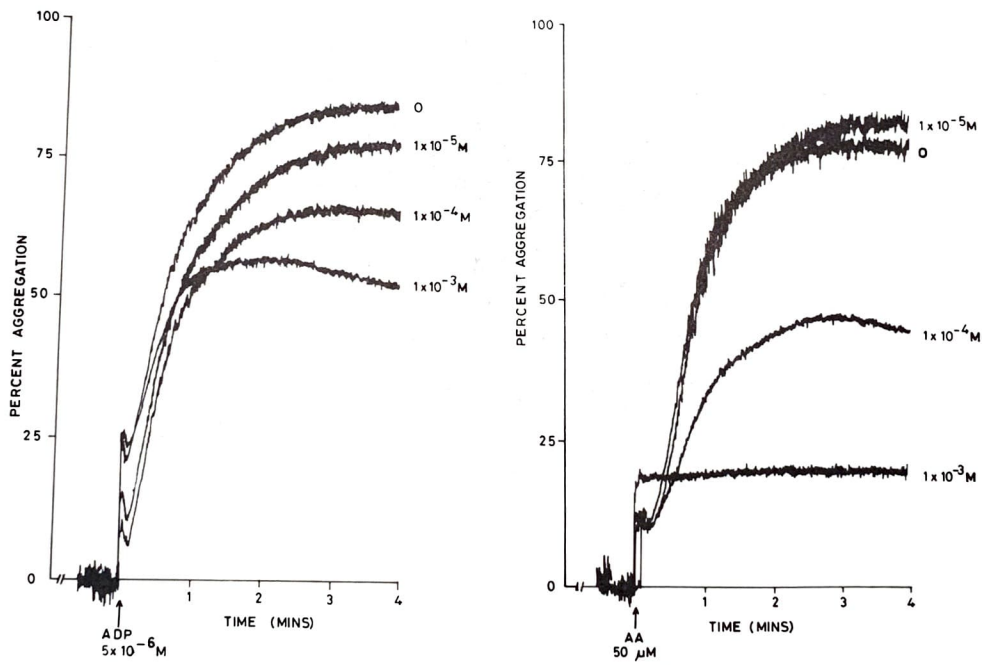


FIG. 1

Antiaggregatory effect of thiopurinol on human platelet rich plasma. ADP (a) and arachidonic acid (b) were used to stimulate platelets at 5 uM and 50 uM concentrations, respectively.

4. O_2^- **radical generation:** Thiopurinol did not cause any inhibition of O_2^- radicals generation in vitro, while allopurinol caused 30.26 % inhibition at 1 mM concentration (Table 3).

TABLE 3

Effect of Thiopurinol and Allopurinol on Non-enzymatic O_2^- Radical Generation In vitro

Compound	Final concentration (molar)	Percent inhibition (-) or potentiation (+) of O_2^- generation ± S.E.
Thiopurinol	10^{-4}	+ 32.08±8.12
	10^{-5}	+ 18.11±4.33
	10^{-6}	+ 6.45±4.66
Allopurinol	10^{-3}	- 30.26±2.06
	10^{-4}	- 13.26±3.92
	10^{-5}	- 8.03±3.36
	10^{-6}	0±0

5. **Effect on cardiovascular activity:** Thiopurinol caused no significant alteration in blood pressure in cat at 1 and 5 mg/kg i.v. There was a transient (8 min) decrease in heart rate and respiration (7 min) at 5 mg/kg dose. The compound did not modify responses to adrenaline, histamine, acetylcholine and isoprenaline. No change in nictitating membrane contraction was observed.

DISCUSSION

Free radicals have been implicated in the genesis of thrombosis and their removal could be beneficial in preventing it (3,4). Studies in this laboratory have indicated that the enzymes responsible for the generation and scavenging of free radicals, play an important role in pulmonary thromboembolism in mice (unpublished observations). Since allopurinol, a xanthine oxidase inhibitor and free radical scavenger, proved highly potent in preventing pulmonary thromboembolism in mice as well as in inhibiting thrombus formation in extracorporeal circulation in cat (3), we studied the effect of some analogs of allopurinol for their antithrombotic activity in two animal models.

Of the 12 analogs, the thio-analog of allopurinol (I) was observed to be most potent, exhibiting 90% antithrombotic effect at 20 mg/kg i.p. The thio group at position 5 appears to be responsible for its superior antithrombotic activity when compared to allopurinol. Also the glycoside of this analog, (II) was found to be effective at 10 mg/kg i.p. However, thio substitution at 5 position in III, IV and V were ineffective. Thiomethyl substitution is also not effective (VI, VII, VIII and IX).

Thio analog of allopurinol, 4 (5H)-thiopyrazolo (3,4-d) pyrimidine (I) not only caused dose-dependent inhibition of thromboembolism in mice, but also reduced thrombus formation in extracorporeal circulation. However, it was less potent than either allopurinol or indomethacin in the latter, but showed higher activity than them in the former.

Since non-steroidal antiinflammatory drugs have proved very effective in mouse pulmonary thromboembolism, the antiinflammatory effect of (I) in carrageenin

edema in mouse and rats was examined. The compound was, however, devoid of any antiinflammatory effect. Allopurinol, also has no effect on carrageenin foot paw edema in rats (15).

Both allopurinol and its thioanalogue (I) are potent inhibitors of xanthine oxidase. The IC_{50} values of this purinol and allopurinol for xanthine oxidase inhibition as reported by Robins et al (16) are 8.3 and 5.9. (I) failed to inhibit non-enzymatic superoxide radical generation. Allopurinol has earlier been reported to inhibit xanthine oxidase-dependent superoxide radical generation (17) but not NADPH-oxidase-dependent superoxide radical generation, as in neutrophils (15).

The study of the anti-aggregatory activty of (I) revealed that it caused inhibition of ADP induced aggregation of human platelets at 1 mM concentration in vitro. The antiaggregatory effect on arachidonic acid induced aggregation was more pronounced and concentration dependent, indicating that its antiaggregatory effect is mediated via inhibition of arachidonic acid metabolism. The antiaggregatory activity of thiopurinol could be responsible for its potent antithrombotic effect.

The results suggest that thioanalogue of allopurinol can be developed as a potential therapeutic agent for treating thrombotic disorders and its antithrombotic activity is mediated via inhibition of aggregation of platelets, besides inhibition of xanthine oxidase enzyme.

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