injection of methyl cellosolve also sensitized the mice to endotoxin. If the methyl cellosolve and endotoxin were preincubated (15 min, 37 °C) prior to injection, the sensitization still occurred. However, methyl cellosolve administered 4 h prior to lipopolysaccharide failed to sensitize the mice. Methyl cellosolve treatment 4 h after the endotoxin increased sensitivity to endotoxin to approximately the same degree as the simultaneous injection.

Effect of methyl cellosolve on endotoxin lethality in mice

Treatment	No. of mice	Endotoxin[a] LD_{50}	95% (confidence limits)
Endotoxin	122	17	(15 -20)
Propylene glycol + endotoxin	125	16	(14 -19)
Methyl cellosolve + endotoxin	105	0.25	(0.19-0.33)[c]
Methyl cellosolve (i.p.)[b] + endotoxin	82	1.5	(1.0 -2.2)[c]
Methyl cellosolve preincubated with endotoxin	65	1.6	(1.2 -2.2)[c]
Methyl cellosolve 4 h before endotoxin	82	15	(12 -20)
Methyl cellosolve 4 h after endotoxin	44	0.87	(0.49-1.5)[c]

[a] Values are expressed as mg endotoxin per kg. [b] Methyl cellosolve was injected i.p. in this group. All other groups received i.v. injection of drug and endotoxin. [c] Significantly different from propylene glycol control at $p < 0.01$.

Methyl cellosolve is a volatile, organic liquid which has a LD_{50} in mice of 2150 mg/kg[4]. There have been a number of reports of methyl cellosolve intoxication in humans [5-7] and the symptoms include metabolic acidosis, which may play a role in sensitization to endotoxin. Another possibility is that methyl cellosolve may inhibit RNA or protein synthesis and thereby increase the lethality of endotoxin. Further studies to clarify the mechanism by which this agent sensitizes mice to endotoxin may prove to be useful in characterizing the toxic mechanism of endotoxin action.

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Potentiation of the inhibition of xanthine oxidase by a combination of 6-mercaptopurine and 6-thioguanine

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Summary. Carcinostatic agents, 6-mercaptopurine and 6-thioguanine inhibited the in vitro and in vivo activity of the enzyme xanthine oxidase (xanthine-oxygen oxidoreductase, E.C. 1.2.3.2.). Simultaneous addition of a mixture of the 2 antimetabolites produced a synergistic effect on the inhibition of the enzyme activity.

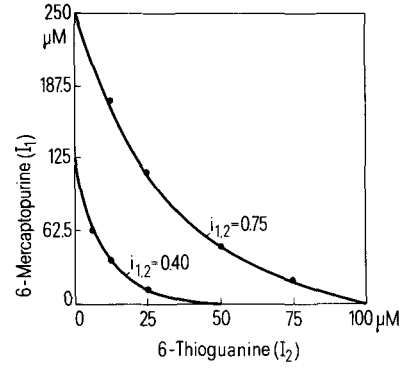
Various purine analogs, including 6-mercaptopurine and 6-thioguanine which are active as carcinostatic agents, have been shown to act as potent inhibitors of xanthine oxidase (xanthine-oxygen oxidoreductase, EC 1.2.3.2.)[3-6]. 6-Mercaptopurine is also a substrate for this enzyme[7] and consequently its pharmacological efficacy and its toxicity are related to the activity of xanthine oxidase. However, this enzyme is not involved in the metabolic degradation of 6-thioguanine[8].

The current emphasis in cancer chemotherapy is on the use of combinations of drugs, taking into account the mode of action of the agents, their synergistic properties and the part of the cell cycle on which they act. The fact that 6-thioguanine and 6-mercaptopurine are both used for the treatment of leukemias[9] prompted us to study the interaction of the combination of the 2 antimetabolites on the activity of xanthine oxidase.

The in vitro studies on milk xanthine oxidase revealed a definite synergistic effect of the combination of the 2 drugs on the inhibition of the enzyme activity. In an attempt to understand further the effect of simultaneous administration of the 2 drugs on tissue xanthine oxidase activity, the response of this enzyme to the in vivo administration of the combination of 6-thioguanine and 6-mercaptopurine was studied in the liver of rats.

Xanthine, 6-mercaptopurine, 6-thioguanine and calcium phosphate gel were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,3,5-Triphenyltetrozolium chloride was obtained from British Drug House (London). All other reagents were of A.R. Grade or the purest grade commercially obtainable.

Milk xanthine oxidase was prepared and purified by the method of Massey et al.[10]. The specific activity was deter-



Isobologram for simultaneously acting inhibitors, 6-mercaptopurine (I_1) and 6-thioguanine (I_2) on xanthine oxidase.

Table 1. Effect of 6-mercaptopurine, 6-thioguanine and their combination on the activity of milk xanthine oxidase

Inhibitors (μM)	% Inhibition	Both inhibitors (μM)	% Inhibition	% Inhibition calculated on the basis of summation (Webb, 1963)
6-Mercaptopurine (6-MP)		6-MP+6-TG		
62.5	22.7	62.5+25	65.4	48.9
125	41.0	125 +25	80.0	54.4
250	76.7	250 +25	96.8	81.6
6-Thioguanine (6-TG)				
25	21.0			

All details are described in the materials and methods section.

mined as described by them. The method used for the assay of xanthine oxidase activity was modified from that described by Owen[11]. The final assay conditions were adjusted to maintain the enzyme activity linear for more than 5 min. The assay mixture, in a final volume of 5 ml contained, in the tube of the Thunberg apparatus: phosphate buffer (pH 7.4), 500 μmoles; 2,3,5 triphenyltetrazolium chloride, 5 μmoles; 0.05 ml of the diluted enzyme and different concentrations of the drugs as indicated. In the lid was placed xanthine, 1 μmole. The reaction was carried out under an anaerobic atmosphere for 5 min at 37 °C and then terminated by the addition of glacial acetic acid. The formazan produced was extracted with toluene and the absorbance measured at 495 nm. The change in absorbance of 0.1/min corresponded to 1 unit of enzyme activity.

The in vivo experiments were conducted on albino rats weighing 150–220 g. Different concentrations of the antimetabolites were dissolved in 0.05 N NaOH. For the determination of the rate of change of the enzyme activity, initially the animals were sacrificed at 30, 45, 60, 120, 240 min following the injection of the drugs. As the maximum change in the activity was found at 45 min, the animals were sacrificed at this period in all further experiments. The livers were rapidly removed and placed in ice-cold 0.1 M Tris-HCl buffer pH 8.1. The supernatants were prepared and the enzyme activity determined as described by Rowe and Wyngaarden[12]. The enzyme activity was measured by following the formation of uric acid, using the rate of change of absorbance at 292 nm in a 3 ml reaction mixture at 25 °C containing: 0.1 M Tris HCl buffer, pH 8.1, 60 μM xanthine and 0.2 ml of the supernatant. The enzyme activity is expressed as units 100 mg^{-1} of tissue. A unit of enzyme activity is defined as the formation of 1 nmole of uric acid min^{-1} (Stripe and Della Corte[13]).

The effect of 6-mercaptopurine and 6-thioguanine on milk xanthine oxidase activity, presented in table 1, shows a marked synergistic effect on the inhibition of the enzyme activity on simultaneous addition of the 2 drugs. Isobolograms[14,15] for the simultaneously acting drug pairs, 6-mercaptopurine and 6-thioguanine were prepared, to demonstrate further the nature of the interaction of the drug pair on enzyme inhibiton. The observations plotted in figure 1 also indicates a synergistic pattern for the inhibition.

The data presented in table 2, on the effect of in vivo administration of 6-mercaptopurine and 6-thioguanine, individually as well as in combination, show a significant inhibition of the enzyme activity of the liver after 45 min. The activity was found to decrease gradually with time. The results clearly demonstrate a greater inhibition of the enzyme activity when both the drugs were administered simultaneously as compared to either of the drugs given individually. The injection of a smaller dose of the drugs demonstrated that the degree of inhibition of enzyme activity was proportional to the magnitude of the dose since with the smaller dose, there was diminished inhibition of the enzyme activity.

Table 2. Effect of in vivo administration of 6-mercaptopurine, S-thioguanine and their combination on the xanthine oxidase activity of liver in rats

Treatment	Dose (mg/kg b.wt)	Xanthine oxidase activity (units/ 100 mg of tissue)
Control	–	17.0±2.3 (6)
6-Mercaptopurine	30	12.0±1.2 (6)
	60	10.6±1.6 (7)
6-Thioguanine	30	13.4±0.9 (6)
	60	11.5±0.8 (8)
Both drugs	30 each	9.2±1.4 (8)
	60 each	6.8±1.7 (7)

Conditions of assay and units of xanthine oxidase activity are as described in the materials and methods section. Mean±SD are given, with the number of independent determinations in parenthesis.

From the results of the present study it is apparent that the xanthine oxidase inhibition by 6-mercaptopurine and 6-thioguanine, both in vitro and in vivo is increased to a greater extent when they are both administered simultaneously in combination. The present study therefore suggests that a combination of 6-mercaptopurine and 6-thioguanine might be useful in potentiating the action of the former drug in therapeutics.

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