**Regular Article**

**In Vitro Study of 6-mercaptopurine Oxidation Catalysed by Aldehyde Oxidase and Xanthine Oxidase**

Mohammad-Reza RASHIDI¹,*, Christine BEEDHAM², John S. SMITH³ and Soodabeh DAVARAN⁴

¹Drug Applied Research Center, Research and Development Complex, Tabriz University of Medical Sciences, Tabriz, Iran
²Department of Clinical Sciences, ³Department of Pharmaceutical Chemistry, School of Life Sciences, University of Bradford, Richmond Road, Bradford, West Yorkshire, UK
⁴Pharmaceutical Nonotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

**Summary:** In spite of over 40 years of clinical use of 6-mercaptopurine, many aspects of complex pharmacology and metabolism of this drug remain unclear. It is thought that 6-mercaptopurine is oxidized to 6-thiouric acid through 6-thioxanthine or 8-oxo-6-mercaptopurine by one of two molybdenum hydroxylases, xanthine oxidase (XO), however, the role of other molybdenum hydroxylase, aldehyde oxidase (AO), in the oxidation of 6-mercaptopurine and possible interactions of AO substrates and inhibitors has not been investigated in more details. In the present study, the role of AO and XO in the oxidation of 6-mercaptopurine has been investigated. 6-Mercaptopurine was incubated with bovine milk xanthine oxidase or partially purified guinea pig liver molybdenum hydroxylase fractions in the absence and presence of AO substrates, and the reactions were monitored by spectrophotometric and HPLC methods. According to the results obtained from the inhibition studies, it is more likely that 6-mercaptopurine is oxidized to 6-thiouric acid via 6-thioxanthine rather than 8-oxo-6-mercaptopurine. The first step which is the rate limiting step is catalyzed solely by XO, whereas both XO and AO are involved in the oxidation of 6-thioxanthine to 6-thiouric acid.

**Key words:** 6-mercaptopurine; metabolism; aldehyde oxidase; xanthine oxidase

**Introduction**

6-Mercaptopurine (6-MP) is a thiopurine drug in which carbon 6 of purine has been substituted with a sulphur-containing group (Fig. 1). This drug with methotrexate are the drugs of choice in the treatment of childhood acute lymphoblastic leukemia, especially in those cases where prolonged duration of remission is required.¹⁻³ 6-MP together with its prodrug azathio-prine are used as immunomodulatory agents in the treatment of inflammatory bowel disease.⁴ Although 6-MP has been used for over 40 years, many aspects of its complex pharmacology and metabolism remain unclear.¹,⁵ However, it has been suggested that in the cell, 6-MP is first converted to thioinosinic acid, by hypoxanthine-guanine phosphoribosyl-transferase and then metabolised to thiguanine ribonucleotide and deoxyribonucleotide; incorporation of these compounds into RNA and DNA results in the antitumour effect of the drug.⁶⁻⁸ 6-MP after administration, may enter into either anabolic or catabolic metabolic pathways.¹⁷ The anabolic pathway is responsible for conversion of 6-MP to its active form, whereas the drug is degraded to inactive forms in the catabolic pathway via two enzymatic sequences. The first route is thought to involve the initial oxidation of 6-MP to 8-oxo-6-mercaptopurine followed by conversion to 6-thiouric acid.¹⁵ However, Zimm et al. have identified 6-thioxanthine in the urine samples of some patients who received 6-MP by intravenous infusion which may indicate that 6-MP is converted to 6-thiouric acid via 6-thioxanthine.⁹ 6-Methylmercapto-8-hydroxypurine has been identified by others¹⁰ as a major metabolite of 6-MP in plasma during intravenous administration. The oxidative inactivation of 6-MP has been attributed to xanthine oxidase (XO) activity in intestinal mucosa and liver.¹,⁶ The presence of large amounts of this enzyme in the liver has been considered as a major reason for the low
bioavailability of 6-MP.\textsuperscript{11} Accordingly, administration of methotrexate as an inhibitor of XO alongside with 6-MP has been used to increase the bioavailability of the later drug.\textsuperscript{11,12} The \textit{Km} value for the oxidation of 6-MP by calf liver and bovine milk xanthine oxidase (BMXO) is 20–30 \textit{\mu}M.\textsuperscript{13,14} 6-MP is also a substrate for rabbit liver aldehyde oxidase (AO) with a reported \textit{Km} of 1.6 mM.\textsuperscript{15} The second catabolic pathway for 6-MP is methylation of the sulphur atom catalysed by thiopurine methyltransferase. 6-Methylmercaptopurine then undergoes oxidation at position 8. Although the oxidation at 8 position could be catalysed by XO and/or AO,\textsuperscript{11} there is some indirect evidence indicative of the significant role of AO in 8-hydroxylation.\textsuperscript{16} The results obtained from \textit{in vitro} studies are also controversy. Thiouric acid, 8-hydroxy-6-mercaptopurine and 6-methylmercaptopurine have been identified after incubation of 500 \textit{\mu}M 6-MP with human liver cytosol.\textsuperscript{17} To our knowledge, there is not a direct and comparative study of the involvement of molybdenum hydroxylases in this oxidative reactions. Furthermore, 8-hydroxy-6-mercaptopurine\textsuperscript{1,8,10,17} and 6-thioxanthine\textsuperscript{6,10} have been suggested to be the intermediate in the conversion of 6-MP to 6-thiouric acid. Therefore, in the present study, the oxidation of 6-MP by BMXO and partially purified guinea pig liver molybdenum hydroxylases have been investigated. Guinea pig liver enzyme fraction was chosen because of its resemblance to human liver AO.\textsuperscript{18,19}

\textbf{Materials and Methods}

\textbf{Chemicals:} Phthalazine and phenanthridine were purchased from Aldrich Chemical Company (UK). 6-MP, 6-thioxanthine, allopurinol, menadione, isovanillin, xanthine, hypoxanthine, uric acid and BMXO (Grade I) were obtained from Sigma Chemical Company (UK).

\textbf{Animals:} Mature male or female Dunkin-Hartley guinea pigs (400–600 g, University of Bradford, Bradford, UK) were used in this study. Animals were housed
in pairs in plastic cages, maintained in controlled temperature (18–19°C), humidity (50%), and lighting cycle of 07:00–19:00 hours light, 19:00–07:00 hours dark. They were fed with a standard laboratory diet and allowed food and water ad libitum.

Preparation of molybdenum hydroxylase fractions: The guinea pigs were killed between 9.00 am and 10.00 am by cervical dislocation, their livers were immediately excised, placed in ice-cold isotonic potassium chloride solution (1.15% KCl w/v) containing 0.1 mM EDTA and the gall bladder and excess fat were removed. Partially purified molybdenum hydroxylase fractions were prepared from liver homogenates by heat treatment and ammonium sulphate precipitation according to the method of Johnson et al. The final EDTA suspension was stored in liquid nitrogen until required.

Spectrophotometric measurement of enzyme activity: All spectrophotometric determinations were carried out at 37°C using a Shimadzu 2101 UV/VIS spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia USA) which was computer controlled by the Shimadzu UV-210 personal spectroscopy software package with additional kinetics software. The instrument was connected to a Pye-Unicam cell temperature control unit. The cuvettes used had a path length of 1 cm and total cuvette volume was constant at 3.0 mL. 6-MP, 6-thioxanthine and hypoxanthine (100 µM) was separately incubated with BMXO or partially purified guinea pig liver fractions in Sorenson’s phosphate buffer pH 7.0 containing 0.1 mM EDTA at 37°C and the enzymatic oxidation of each compound was monitored spectrophotometrically by repetitive scanning of the UV spectra between 190–400 nm up to 60 min. The final spectrum was compared with that of 6-thioxanthine and those reported13) for 8-oxo-6-MP and 6-thiouric acid as authentic samples were not commercially available. This method was also used to determine if substrate oxidation or product formation could be measured at a single wavelength where there was maximal difference between the UV spectra of substrate and product.

The initial oxidation rates of 100 µM 6-MP catalysed by BMXO or partially purified guinea pig liver fractions were measured up to 5 minutes at 350 nm by following the metabolite production using molecular oxygen as electron acceptor. The reactions were also monitored in the presence of AO inhibitors, 100 µM menadione, isovanillin and chlorpromazine,18,21–23) and XO inhibitor, allopurinol.24) All reactions were carried out in 67 mM Sorenson’s phosphate buffer pH 7.0 containing 0.1 mM EDTA at 37°C.

HPLC analysis of 6-MP oxidation: HPLC analysis was carried out using a system supplied by Waters Associates, Northwich, Cheshire which consisted of a 510 pump, 710 B WISP automatic injector, Lambda-Max 481 LC Spectrophotometer and 740 data module. Chromatographic separation was achieved using a Spherisorb ODS2 5 µm (25 cm x 4.6 mm, i.d.) column with a µBondapak C18 Guard-Pak insert and 0.5 mM ammonium acetate, pH 4.65/1.5% acetonitrile as the mobile phase at a flow rate of 1.0 mL/min. The UV detection was at 335 nm.

6-MP (50 µM) was incubated with BMXO or partially purified guinea pig liver fractions at 37°C in a total volume of 3 mL 67 mM phosphate buffer pH 7.0 containing 0.1 mM EDTA up to 90 minutes. Incubations were performed in 10 mL closed vials which were placed in a shaking water bath and pre-warmed to 37°C. Aliquots (200 µL) were removed at 1, 5, 10, 15, 30, 45, 60 and 90 minutes and added to 100 µL 20% trichloroacetic acid to terminate the reaction. Samples were centrifuged in a Beckman bench-top microcentrifuge for 3–5 minutes at 3000 rpm and the supernatants were subsequently analysed by HPLC.

Incubations were also carried out in the presence of 100 µM of chlorpromazine, isovanillin and allopurinol.

Protein determination: Protein concentrations of partially purified enzyme fractions were determined spectrophotometrically using a Pierce BCA Protein assay kit with bovine serum albumin as a protein standard.25)

Results

Spectrophotometric measurement of 6-MP oxidation

UV spectral analysis: The incubation of 100 µM 6-MP with 0.08 mg/mL bovine milk XO resulted in disappearance of absorption maximum of 6-MP spectrum at 321 nm and appearance of two new peaks at 349 nm and 241 nm within 30 minutes (Fig. 2). The final spectrum was compared with that of 6-thioxanthine and those reported13) for 8-oxo-6-MP and 6-thiouric acid as authentic samples were not commercially available. This method was also used to determine if substrate oxidation or product formation could be measured at a single wavelength where there was maximal difference between the UV spectra of substrate and product.

The spectrum of 8-oxo-6-MP contains two peaks at 349 and 308 nm and 241 nm within 30 minutes (Fig. 2). The final spectrum was compared with that of 6-thioxanthine and those reported13) for 8-oxo-6-MP and 6-thiouric acid. The spectrum of 8-oxo-6-MP contains two peaks at 312–313 nm and 330 nm13) which indicates that this compound was not formed during 6-MP oxidation. However, the resulting spectrum was similar to those of 6-thioxanthine and 6-thiouric acid which have similar UV spectra13) indicating that either of these two compounds could be the oxidation product. It was thus difficult to determine whether oxidation proceeded through an intermediate metabolite. When the experiment was repeated with lower XO concentration (0.016 mg protein/mL) to reduce the reaction rate, a similar profile was obtained. However, when the spectral changes of the oxidation of hypoxanthine by the same concentrations of BMXO were analysed, sequential formation of xanthine and uric acid could be clearly observed. This indicates that either a single oxidation product is formed from 6-MP or that oxidation of an intermediate, possibly 6-thioxanthine, is faster than its formation.

When 100 µM 6-thioxanthine was incubated with
BMXO (0.08 mg protein/mL), the absorption maxima at 341 nm and 250 nm in the spectrum of 6-thioxanthine disappeared over <7 minutes and two new peaks appeared at 349 nm and 241 nm (Fig. 3).

Therefore, it seems more likely that 6-MP is converted to 6-thiouric acid via 6-thioxanthine in which the oxidation of 6-thioxanthine is faster than its formation. This will be further discussed later. As 6-MP is reported to be converted to 6-thiouric acid by BMXO,\textsuperscript{1,6,13} it is likely that the final oxidation product is 6-thiouric acid although the possibility of the metabolite being 6-thioxanthine cannot be discounted.

6-MP was slowly metabolised by high concentrations (1.7 mg protein/mL) of guinea pig enzyme. A slow reduction in the peak at 321 nm was observed with a shoulder appearing at 350 nm which could indicate the gradual formation of the same product as that formed in BMXO incubations. Therefore, it is possible to monitor the oxidation of 6-MP by following metabolite production at 350 nm where there is no interference from 6-MP.

Determination of initial oxidation rates: Table 1 summarises the inhibitory effects of 100 \(\mu\text{M}\) allopurinol, menadione and isovanillin on the initial oxidation rate
of 100 μM 6-MP catalysed by BMXO or guinea pig liver fractions. As expected, the oxidation of 6-MP by BMXO was completely inhibited by 100 μM allopurinol. The reaction rate was increased in the presence of 100 μM menadione as this compound has an enhancement effect on XO activity. However, with guinea pig enzyme fractions, 6-MP oxidation was only partially inhibited with 100 μM allopurinol with both menadione and isovanillin inhibiting the reaction by 40–50%. This indicates that both AO and XO are important in 6-MP oxidation in guinea pig liver. Menadione had a dual effect on the reaction; it initially reduced the oxidation rate but quietly caused an enhancement of the rate. The reaction was completely inhibited in the presence of both menadione (100 μM) and allopurinol (100 μM).

**Oxidation of 6-MP by BMXO:** When 50 μM 6-MP (Rt = 9.8 minutes) was incubated with BMXO (0.028 mg/mL), it almost completely disappeared within 90 minutes producing only a single peak (metabolite 1: M-1) with a retention time of 4.4 minutes. The retention time of 6-thioxanthine under these conditions was 11.5 minutes. Therefore, although the UV spectrum of the oxidation product of 6-MP was similar to that of 6-thioxanthine, the latter compound does not appear to be present in these incubations. Furthermore, when 50 μM 6-thioxanthine was incubated with BMXO (0.017 mg/mL), a product with the same retention time (Rt = 4.4 minutes) as M-1 was obtained. There was no 6-thioxanthine remaining after 15 minutes which indicates that 6-thioxanthine is oxidised by BMXO at a faster rate than 6-MP. Therefore, it appears that metabolite M-1 is not 6-thioxanthine. Zimm *et al.* have separated 6-thiouric acid, 6-MP and 6-thioxanthine using an isocratic HPLC system which is very similar to the HPLC system employed in the present study. The chromatographic conditions developed by Zimm *et al.* consisted of a C-18 column (4.6 mm × 25 cm), water (98.8%), acetonitrile (1%), and acetic acid (0.2%) as a mobile phase, flow rate of 1.0 mL/min. Using this HPLC system, 6-thiouric acid, 6-MP and 6-thioxanthine chromatographed at 9.9, 12.5 and 16.8 minutes respectively. It, thus, appears that 6-MP is oxidised to 6-thiouric acid by BMXO. This is in agreement with those results indicating that 6-thiouric acid is the major metabolite following 6-MP administration.

However, as stated above, only one peak (Rt = 4.4 minutes) was obtained from HPLC analysis of the oxidation of 6-MP by BMXO, whereas the conversion of 6-MP to 6-thiouric acid must proceed via an intermediate either 6-thioxanthine or 8-oxo-6-mercaptopurine. The relative oxidation rate of 6-thioxanthine to 6-MP oxidation rate with BMXO is ~8, whereas the oxidation rate of 8-oxo-6-mercaptopurine is only 1.2-fold higher than that of 6-MP reported by Krenitsky *et al.* Taking into account the results obtained from UV spectral analysis of the reaction, it may be suggested that 6-MP is oxidised in a sequential pathway to 6-thiouric acid via 6-thioxanthine in which the first reaction is the rate limiting step.

### Table 2

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Allopurinol (100 μM)</th>
<th>Menadione (100 μM)</th>
<th>Allopurinol (100 μM)</th>
<th>Menadione (100 μM)</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig liver fraction</td>
<td>66 ± 10</td>
<td>39 ± 9</td>
<td>99 ± 3</td>
<td>ND</td>
<td>52 ± 11</td>
</tr>
<tr>
<td>Bovine milk xanthine oxidase</td>
<td>98 ± 2</td>
<td>22 ± 5 (t)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*aThe results are expressed as percentage inhibition. (mean ± (SD, n = 3)

*bThe extent of the inhibition declined to zero after 2 minutes.

(1) The oxidation rate increased, ND: not determined.
Table 2. Effects of 100 \( \mu \text{M} \) xanthine oxidase and aldehyde oxidase inhibitors on the formation of M-1 from 50 \( \mu \text{M} \) 6-mercaptopurine catalysed by partially purified guinea pig liver fractions and bovine milk xanthine oxidase monitored by HPLC at 335 nm

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Guinea pig liver fraction</th>
<th>Bovine milk xanthine oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>90±2</td>
<td>91±6</td>
</tr>
<tr>
<td>Isovanillin</td>
<td>43±6</td>
<td>64±3</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>45±3</td>
<td>54±3</td>
</tr>
</tbody>
</table>

*The results are expressed as percentage inhibition. (mean ± SD, n = 3)
(1): The metabolite production increased.

Fig. 4. Proposed metabolic pathway for the oxidation of 6-mercaptopurine by partially purified guinea pig liver fractions.

Discussion

Although XO is considered as the major enzyme in the oxidation of 6-MP, there is some evidence which indicates that oxidation can also be catalysed by another source. In vivo studies have shown that there is a marked increase (>4-fold) in the amount of free 6-MP in the urine and 8-fold decrease in the amount of 6-thiouric acid excreted during the first 12 hours following oral co-administration of 150 mg 6-MP and 75/300 mg allopurinol in man. In spite of this reduction in 6-thiouric acid excretion, the amounts of the metabolite detected in urine were still 39 and 14% of the control values with 75 and 300 mg allopurinol administration respectively. The catabolic oxidative routes of 6-MP other than those catalyzed by XO have been attributed to the action of AO.16) According to in vitro studies,14) 6-MP is a substrate for AO, however, there is no direct in vivo evidence or detailed comparative studies indicating the involvement of molybdenum hydroxylases in the metabolism of this purine-based drug. Similar results with other compounds have been also published. Auscher et al.29) have shown that, in vivo, the rate of the oxidation of allopurinol and its 6-mercapto analogues, thiopurinol, in one xanthinuric subject was as rapid as in controls. Interestingly, in vitro, the oxidation of thiopurinol observed by Auscher et al.29) is due to AO activity. Kitchen et al.31) have shown the oxidation of thioguanine which is not a
substrate for XO\textsuperscript{14} following intravenous infusion of the drug. Based on some in vitro data, they attributed this catabolic pathway to aldehyde oxidase activity. Nevertheless, the in vivo contribution of AO to the oxidation of thiopurines is worthy of further investigation.

The major metabolite of 6-MP oxidation is 6-thiouric acid, however, this oxidative route passes via 6-thioguanine or 8-hydroxy-6-mercaptopurine is not clear. According to some reports, 6-MP is converted to 6-thiouric acid through 6-thioxanthine,\textsuperscript{6,32} whereas, other authors have suggested that the oxidation intermediate is 8-hydroxy-6-mercaptopurine.\textsuperscript{1,9,10,17} The reason for this controversy could be due in part to the difference in metabolite profile between oral and intravenous administration of the drug.\textsuperscript{16,33} It is more likely that following oral administration, 6-MP is predominantly oxidized to 6-thiouric acid via 6-thioxanthine. The first step is catalyzed by XO, in particular in the gut. However, during intravenous infusion, which bypasses intestinal XO, either 6-MP or 6-thioguanine are mostly oxidized at 8 position.\textsuperscript{10} Although these reactions could be oxidized by either XO or AO, the difference in metabolite profile between oral and intravenous administration indicates that AO may be the major enzyme in 8-hydroxylation.\textsuperscript{16} In addition, the results obtained in the present study and a higher capability of AO to produce 8-hydroxy metabolites from guanine and purine analogs compared with XO\textsuperscript{16,23} provide more support for the involvement of AO in the metabolism of thioguanine to thiouric acid as the major enzyme. This may be account for the results obtained by Keuzenkamp-Jansen et al.\textsuperscript{32} These authors indicated that co-administration of allopurinol and high dose 6-MP infusion to patients with non-Hodgkin lymphoma resulted in a relatively higher plasma levels of 6-MP, 6-thioxanthine and methylpurine and a lower thiouric acid plasma level compared to those who did not receive allopurinol.

Conclusions

Although the conversion of 6-MP to thiouric acid could be either via 8-hydroxy-6-mercaptopurine or 6-thioxanthine, it appears that with both BMXO and partially purified guinea pig liver molybdenum hydroxylases, 6-MP is oxidised to 6-thiouric acid via 6-thioxanthine in which the first reaction is the rate limiting step. However, in order to clarify this proposed metabolic pathway, it seems necessary that the oxidation of both 8-oxo-6-mercaptopurine and 6-thioxanthine by guinea pig liver fractions are investigated in the absence and presence of specific inhibitors of AO and XO.

With guinea pig liver fractions, XO is the major enzyme contributing to the conversion of 6-MP to 6-thiouric acid, whereas both molybdenum hydroxylases are involved in 6-thiouric acid formation from 6-thioxanthine.

References

13) Chalmers, A. H., Knight, P. R. and Atkinson, M. R.: 6-Thiopurines as substrates and inhibitors of purine


