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Nature and Position of Functional Group on Thiopurine Substrates Influence Activity of Xanthine Oxidase – Enzymatic Reaction Pathways of 6-Mercaptopurine and 2-Mercaptopurine Are Different

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Abstract—Xanthine oxidase-catalyzed hydroxylation reactions of the anticancer drug 6-mercaptopurine (6-MP) and its analog 2-mercaptopurine (2-MP) as well as 6-thioxanthine (6-TX) and 2-thioxanthine (2-TX) have been studied using UV-spectroscopy, high pressure liquid chromatography, photodiode array, and liquid chromatography-based mass spectral analysis. It is shown that 6-MP and 2-MP are oxidatively hydroxylated through different pathways. Enzymatic hydroxylation of 6-MP forms 6-thiouric acid in two steps involving 6-TX as the intermediate, whereas 2-MP is converted to 8-hydroxy-2-mercaptopurine as the expected end product in one step. Surprisingly, in contrast to the other thiopurines, enzymatic hydroxylation of 2-MP showed a unique hyperchromic effect at 264 nm as the reaction proceeded. However, when 2-TX is used as the substrate, it is hydroxylated to 2-thiouric acid. The enzymatic hydroxylation of 2-MP is considerably faster than that of 6-MP, while 6-TX and 2-TX show similar rates under identical reaction conditions. The reason why 2-MP is a better substrate than 6-MP and how the chemical nature and position of the functional groups present on the thiopurine substrates influence xanthine oxidase activity are discussed.

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Mammalian xanthine oxidase (XOD; EC 1.1.3.22) catalyzes the oxidative hydroxylation of hypoxanthine and xanthine to uric acid [1, 2]. Oxidation of the substrate takes place at the molybdopterin (MoPt) center of the enzyme and electrons from the substrate are introduced into the enzyme and rapidly distributed through the internal electron transfer chain to different motifs, i.e. MoPt, iron-sulfur (Fe-S), and flavin (FAD) [3-6]. The electrons are taken up by oxygen at the FAD site of the enzyme to generate superoxide ($1e^-$ product of oxygen) and hydrogen peroxide ($2e^-$ product of oxygen) as common reaction products from all different purine substrates [7, 8]. In contrast to other oxidoreductases, XOD uses a water molecule as the source of oxygen for the hydroxylation reaction [9, 10].

Rastelli et al. [11] reported in their molecular modeling work that positioning of C-6, C-2, C-8, N-7, N-3, and N-9 of xanthine or hypoxanthine in the vicinity of MoPt and the active side residues like lysine, glutamate, and phenylalanine determine the productive interaction of the substrate with XOD. Thus, the presence of a thiol group in the mercaptopurine substrates in place of a carbonyl group of the natural purine substrates is expected to alter the binding affinity and productive interaction of these thiopurine substrates with XOD. Rastelli et al. [11] also mentioned that the functional group present in the

Abbreviations: e^-) electron; 2-MP) 2-mercaptopurine; 6-MP) 6-mercaptopurine; 8-OH-2-MP) 8-hydroxy-2-mercaptopurine; P_{max}) maximum level of product formed; PDA) photodiode array; R_t) retention time; 2-TX) 2-thioxanthine; 6-TX) 6-thioxanthine; 2-TUA) 2-thiouric acid; 6-TUA) 6-thiouric acid; XOD) xanthine oxidase.

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C-6 position of purine substrates plays important role for anchoring with the XOD active site and enzyme activity. From this perspective, 2-mercaptopurine (2-MP) is supposed to be a very interesting substrate because out of all the thiopurines used in this study, only 2-MP did not bear any functional group at the C-6 position.

The analog compound of 2-MP, i.e. 6-MP, is an anticancer drug, which bears the functional group at C-6 position. The target enzyme for 6-MP action is hypoxanthine-guanine phosphoribosyltransferase (HGPRT) forming thioinosinic monophosphate, which is cytotoxic. It has been reported that 6-mercaptopurine (6-MP) is metabolically oxidized to 6-thiouric acid (6-TUA) by XOD and excreted in the urine. When administered orally, 6-MP is eliminated very rapidly by liver and intestinal XOD, and the presence of XOD in the cancer cell is expected to depotentiate its therapeutic activity [12-15].

Surprisingly, even though work on XOD has been continued for several decades, thiopurines as substrates of this enzyme have received almost no attention. Therefore, in the present study, we have investigated XOD-catalyzed hydroxylation kinetics and reaction pathways using the anti-cancer drug substrate 6-MP and its analog substrate 2-MP. 6-Thioxanthine (6-TX) and 2-thioxanthine (2-TX) were used as control substrates for the study of the enzymatic reaction pathways of 6-MP and 2-MP. Furthermore, kinetic studies with all these substrates were performed with a view to obtain ideas about how the chemical nature and the position of the functional groups present on these thiopurine substrates influences the activity of XOD.

MATERIALS AND METHODS

Materials. Bovine milk xanthine oxidase, xanthine, hypoxanthine, 2-MP, 6-MP, 6-TX, uric acid, cytochrome *c*, peroxidase, and *o*-dianisidine were purchased from Sigma (USA). Hydrogen peroxide (30% w/v) was obtained from Merck (Germany). 2-Thioxanthine was purchased from Lancaster (UK).

Enzyme activity unit definition. One unit of XOD is defined as the amount of enzyme that produces 1 μmol of uric acid per minute (which corresponds to ΔOD 0.011 at 293 nm in 1 ml reaction mixture) at 25°C using 10 μM xanthine as the substrate. Concentration of XOD was calculated using the molar extinction coefficient of 36 $mM^{-1}\cdot cm^{-1}$ at 450 nm [16].

Spectral analysis and molar extinction coefficient of 8-hydroxy-2-mercaptopurine (8-OH-2-MP). Due to the unavailability of authentic 8-OH-2-MP (enzymatic product of 2-MP) from any commercial source, the following experiment was performed to calculate the molar extinction coefficient of 8-OH-2-MP. 2-MP (100 μM) was subjected to exhaustive enzymatic hydroxylation by using excess XOD (0.061 μM) in phosphate buffer (0.05 M, pH 7.4) for 45 min at 30°C. Heating the mixture at 60°C for 10 min terminated the reaction. The solution was immediately kept in ice for 10 min, centrifuged at 8000*g* for 10 min at 4°C, and filtered through 0.22 μm Amicon (USA) membrane filter for subsequent HPLC analysis. The confirmation of completion of the enzymatic conversion of 2-MP was judged by HPLC. The absorption spectrum of the reaction mixture was taken at different concentrations. The molar extinction coefficients of 2-MP and its hydroxylation product, 8-OH-2-MP were calculated to be 16,000 and 30,000 $M^{-1}\cdot cm^{-1}$, respectively.

Kinetic experiments. All the kinetic experiments were performed using a Perkin Elmer (USA) Lambda 25 UV-Visible double beam spectrophotometer at 25°C. The reaction was carried out in a total volume of 1 ml containing 980 μl of 100 mM sodium phosphate buffer (pH 7.5) and 10 μM of substrate [16]. With the addition of XOD to the reaction mixture, time dependent absorbance change was monitored in the wavelength range of 200 to 400 nm.

Superoxide assay. Superoxide formation was measured by monitoring the reduction of cytochrome *c* [17]. The extinction coefficient of reduced cytochrome *c* at 550 nm was taken as 21,000 $M^{-1}\cdot cm^{-1}$.

Hydrogen peroxide assay. Hydrogen peroxide formation was measured using a coupled assay system containing *o*-dianisidine and peroxidase [18]. The reaction was terminated using 50% H_2SO_4 (v/v). The pink color so formed was measured at 510 nm.

Sample preparation for HPLC experiments. The standard reaction mixture in the volume of 1 ml contains 980 μl of 100 mM sodium phosphate buffer (pH 7.5) and 10 μl of 1 mM substrate. The reaction was initiated with the addition of 1.6 U (0.005 μmol) of XOD and terminated after 10 min, by heating the mixture at 60°C for another 10 min. The mixture was then centrifuged at 8000*g* for 10 min at 4°C, filtered through 0.22 μm Amicon membrane filter, and 20 μl of the supernatant was used for HPLC (Shimadzu 10A VP) analysis using a Merck C8 OS column (4.6 × 250 mm). The mobile phase was 100 mM sodium phosphate buffer, pH 4.0, and UV-detection of appropriate wavelength was used.

Photodiode array (PDA) analysis of the compounds. Substrates and products were separated from the HPLC column based on their polarity and thus retention time (R_t) values and spectrum of each of the compound was taken using the UV6000LP (Spectra Systems, USA) PDA spectrometer for estimating the λ_{max} of the respective compound.

Sample preparation for LC/MS experiments. The standard reaction mixture in final volume of 1 ml contains 500 μl of 100 mM sodium phosphate buffer (pH 7.5) and 500 μl of 1 mM substrate. The reaction was initiated with the addition of 2.25 U of XOD and terminated after 10 min, by heating the mixture at 60°C for 10 min. The mixture was then centrifuged at 8000*g* for 10 min at 4°C,

filtered through membrane filter, and 20 μl of the supernatant was used for LC/MS analysis (LCQ Finnigan (UK) Matt Model) with Merck C8 OS column (4.6 × 250 mm), and triple distilled water was used as the mobile phase. The instrument was operated in the positive ion mode (APCI) with voltage of 4.2 kV, scan range 50-200 amu, and temperature 200°C using the capillary method. MS/MS data were simultaneously acquired for the selected parent ions. Helium was used to fragment both the selected ions of *m/z* 169 with collision energy of 35 eV.

RESULTS

Time-dependent spectral scans and mechanistic implications on the reaction pathways. XOD-catalyzed hydroxylation of 6-MP, 2-MP, 6-TX, and 2-TX was observed by monitoring time-dependent absorption spectra between 200 and 400 nm. Enzymatic hydroxylation reaction of 6-MP displayed a time dependent decrease in the absorbance at 320 nm (λ_{max} of 6-MP) and a corresponding increase in the absorbance at 350 nm suggesting the formation of 6-TUA as the expected end product. Initial (6-MP) and final (6-TUA) scans of reaction are depicted in Fig. 1a as curves *1* and *3*. The observed isobestic point at 330 nm in these spectra suggested that the intermediate product 6-TX is not accumulated and is immediately converted to 6-TUA. Therefore, we concluded that the enzyme catalyzed hydroxylation of 6-MP follows a two-step reaction process with the formation of 6-TUA as the end product (Scheme).

The enzymatic hydroxylation of the 6-TX exhibited a decrease in the absorbance at 330 nm (λ_{max} of 6-TX) and a concomitant increase in the absorbance at 350 nm (λ_{max} of 6-TUA) (Fig. 1a and Scheme). Initial (6-TX) and final (6-TUA) scans of reaction are depicted in Fig. 1a as curves *2* and *3*.

On the contrary, enzymatic hydroxylation of 2-MP (Fig. 1b) demonstrated no observable rise in the absorbance at 330 or 350 nm (λ_{max} of 2-TX and 2-TUA, respectively). Instead, the absorbance at 264 nm (λ_{max} of 2-MP) increased rather than decrease with time. We interpret from the results that the enzymatic product of 2-MP has the same λ_{max} (264 nm) as that of 2-MP, but the product has higher molar extinction coefficient value than the substrate. Therefore, from mechanistic argument, as elaborated below, we propose that enzymatic hydroxylation of 2-MP leads to the probable formation of 8-OH-2-MP and not 2-TX or 2-TUA as end product (Scheme). Initial (2-MP) and final (8-OH-2-MP) scans of reaction are depicted in Fig. 1b as curves *1* and *2*. Similarly, enzymatic hydroxylation of 2-TX (λ_{max} = 330 nm) yielded a product with an absorbance peak at 350 nm (λ_{max} of 2-TUA) (Fig. 1b and Scheme). Initial (2-TX) and final (2-TUA) scans of reaction are depicted in Fig. 1b as curves *3* and *4*. Accordingly, it is proposed that XOD-catalyzed hydroxylation reaction of 2-MP is one-step and markedly different from that of 6-MP.

The nature of the enzymatic hydroxylation kinetics of 2-MP and 6-MP is also different. The initial reaction rate of 6-MP measured using substrate concentrations of 10 and 20 μM was found to be 15 times slower than that of 2-MP. Since 10-μM substrate concentration is at least 5 times higher than the K_m values of all these substrates (table), identical initial rates of the product formation were obtained from 10 μM and further even at 20 μM substrate concentration, rates were found to be identical (data not shown). Hence, 10 μM of substrate concentration was used for the subsequent experiments. The turnover number (k_{cat}) of 2-MP is lower as compared to 2-

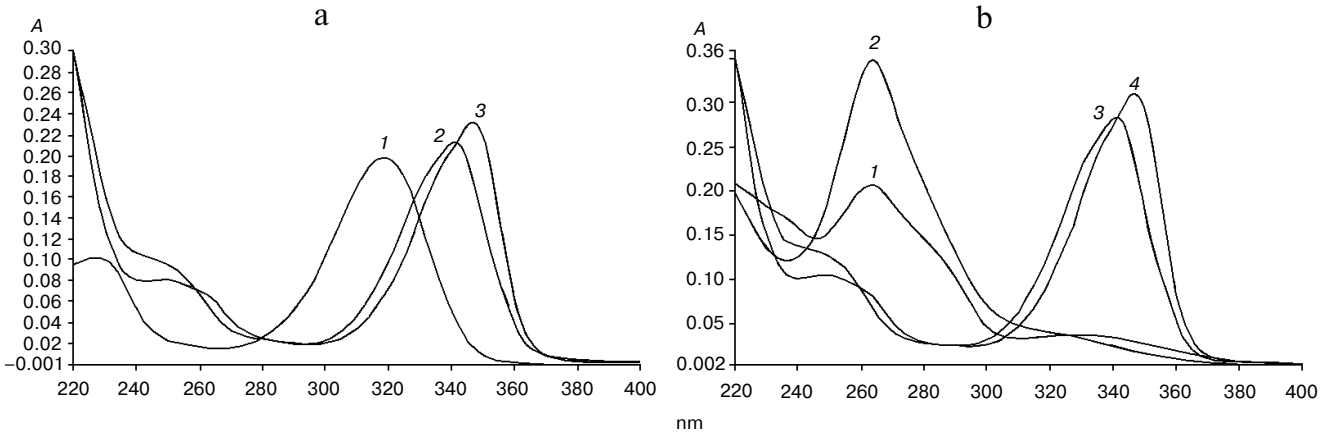
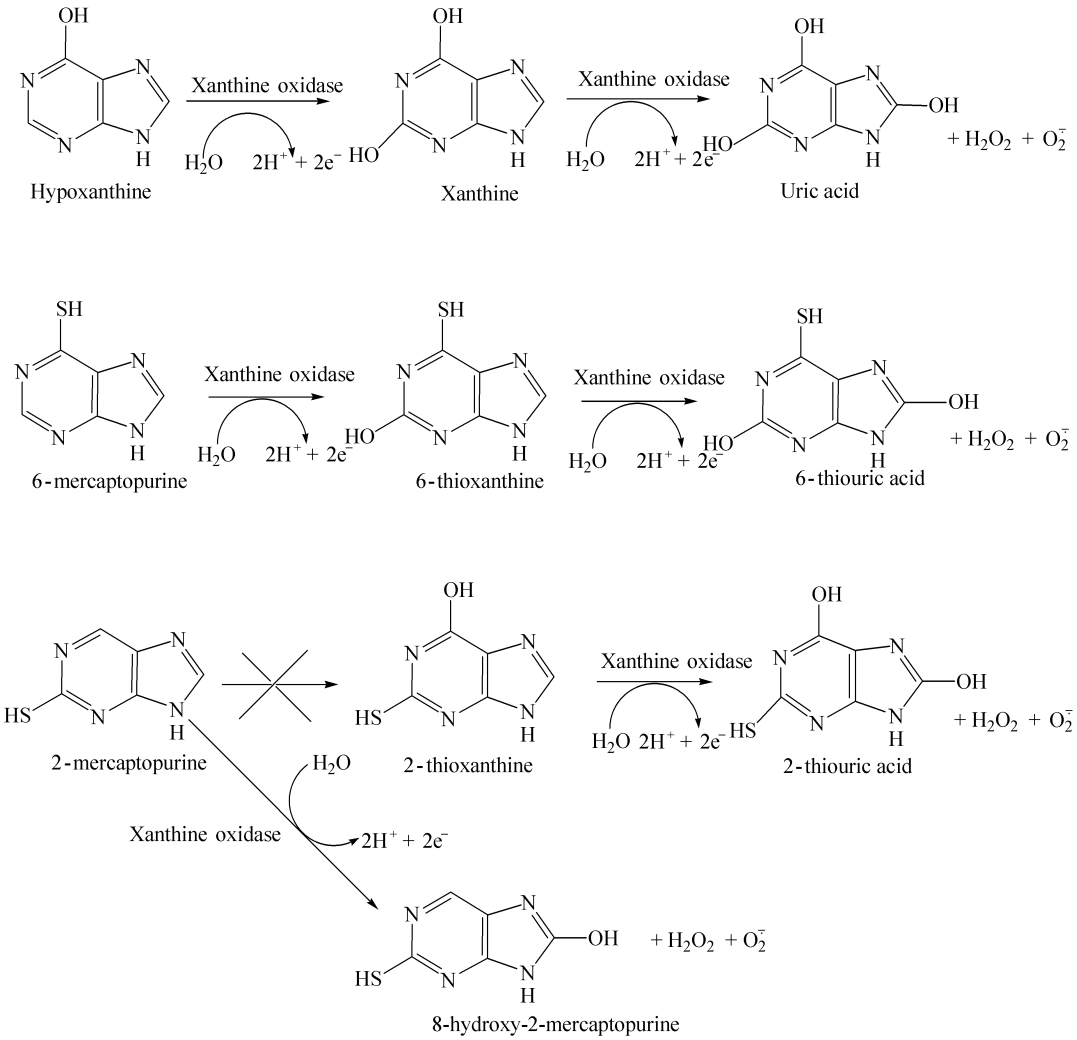


Fig. 1. a) Initial and final absorption spectra (200 to 400 nm) during enzymatic hydroxylation of 6-MP (*1*) forming 6-TUA (*3*) and of 6-TX (*2*) forming 6-TUA (*3*). The reaction mixture contained 10 μM of substrate, 1.6 U (equivalent to 0.005 μmol) of XOD, and 0.2 M sodium phosphate at pH 7.4. b) Initial and final absorption spectra during enzymatic hydroxylation of 2-MP (*1*) forming 8-OH-2-MP (*2*) and 2-TX (*3*) forming 2-TUA (*4*). Reaction conditions are same as for panel (a).



Xanthine oxidase-catalyzed reaction products of different substrates as proposed from our experimental results

Scheme

TX and the specificity constant (k_{cat}/K_m) of 2-MP is almost three times lower than that for 2-TX indicating that 2-TX is a better substrate than 2-MP. The k_{cat} of 6-MP is 22 times lower than that for 6-TX and the k_{cat}/K_m of 6-MP is almost a hundred times lower than that for 6-TX, indicating that 6-TX is a better substrate than 6-MP. Furthermore, estimations of enzyme turnover number, specificity constant, and maximum level (P_{max}) of product formation under normalized substrate concentration (table) of all these substrates also indicated that 2-MP is a

Kinetic parameters of the xanthine oxidase-catalyzed hydroxylation reaction of thiopurines

Substrate	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	P_{max}, μM
6-TX	0.46 ± 0.01	166 ± 4.17	360.86 ± 5.23	6.90 ± 0.10
2-TX	0.47 ± 0.01	199 ± 5.51	423.40 ± 6.78	6.60 ± 0.08
2-MP	0.88 ± 0.01	119 ± 4.89	135.22 ± 3.20	4.38 ± 0.12
6-MP	2.0 ± 0.004	7.6 ± 0.51	3.80 ± 0.14	0.99 ± 0.04

more efficient substrate than 6-MP, while 2-TX and 6-TX are almost equally efficient substrates of XOD.

HPLC analysis for characterization of reaction pathways. In order to further confirm that the enzymatic end products of 6-MP and 6-TX are same but that of 2-MP and 2-TX are completely different (Scheme), we separated and characterized the enzymatic products of 6-TX, 2-TX, 6-MP, and 2-MP using HPLC. 6-TX and 6-MP had R_t values of 17.23 and 12.13 min while their products had the same R_t value of 6.18 min suggesting that these two substrates generated the common end product, 6-TUA, on enzymatic hydroxylation. On the other hand, 2-TX and its product 2-TUA had R_t values of 17.35 and 6.23 min, respectively (Figs. 2a and 2b). It was found that pure 2-MP (R_t value of 6.01 min (Fig. 2c)) and the enzymatic product of 2-MP, i.e. 8-OH-2-MP (R_t value of 5.49 min), were well separated (Fig. 2d). The results show that the R_t value of 8-OH-2-MP is distinctly different from that of 2-TUA and 2-TX. Furthermore, 2-TUA and 2-TX were monitored at 350 nm and could not be detected at 264 nm (λ_{max} of 2-MP or 8-OH-2-MP) due to absence of any spectral overlapping. On the other hand, 2-MP and 8-OH-2-MP were monitored at 264 nm, and since these compounds did not have any absorbance at 350 nm, they could not be detected at 350 nm. Therefore, HPLC data together with spectral and kinetic data provide strong evidence that the enzymatic reaction product of 2-MP is neither 2-TX nor 2-TUA, but 8-OH-2-MP as proposed in the Scheme.

Photodiode array (PDA) and simulative spectroscopy experiments for confirming reaction pathways. In order to further confirm that the end product formations of 2-MP and 2-TX were different, PDA experiments were performed. In this experiment, residual 2-MP and its enzymatic product were separated in a HPLC column and the spectrum of each of these separated products was taken individually using the attached PDA spectrometer with the HPLC instrument. It was observed that the residual 2-MP and its enzymatic product have common λ_{max} and almost identical spectrum, differing in the blue region confirming the two different conformers. The increase in λ_{max} to 284 nm instead of 264 nm is because of the change in buffer condition and pH in the mobile phase of the HPLC method. To rule out the possibility that 2-TX and 2-TUA were not formed in the enzymatic reaction of 2-MP, the residual 2-TX and its enzymatic product 2-TUA in another reaction mixture were studied. The PDA spectrometer-derived results revealed that the spectrum of 2-TX and 2-TUA were quite different from that of 2-MP or its product 8-OH-2-MP. A closer inspection of these spectra confirmed that 2-MP and its product 8-OH-2-MP also did not have any significant absorbance contribution at 350 nm. Similarly, 2-TX and 2-TUA did not show any significant absorbance contribution at 264 nm, and hence any spectral overlapping was absent. It was therefore concluded that both 2-MP and its enzymatic product had identical λ_{max} (264 nm), which is different from the λ_{max} of 2-TX (330 nm) or 2-TUA (350 nm).

LC/MS analysis of 2-mercaptopurine, 2-thioxanthine, and their reaction products. That the enzymatic product of 2-MP (153.2 amu) is 8-OH-2-MP (169 amu)

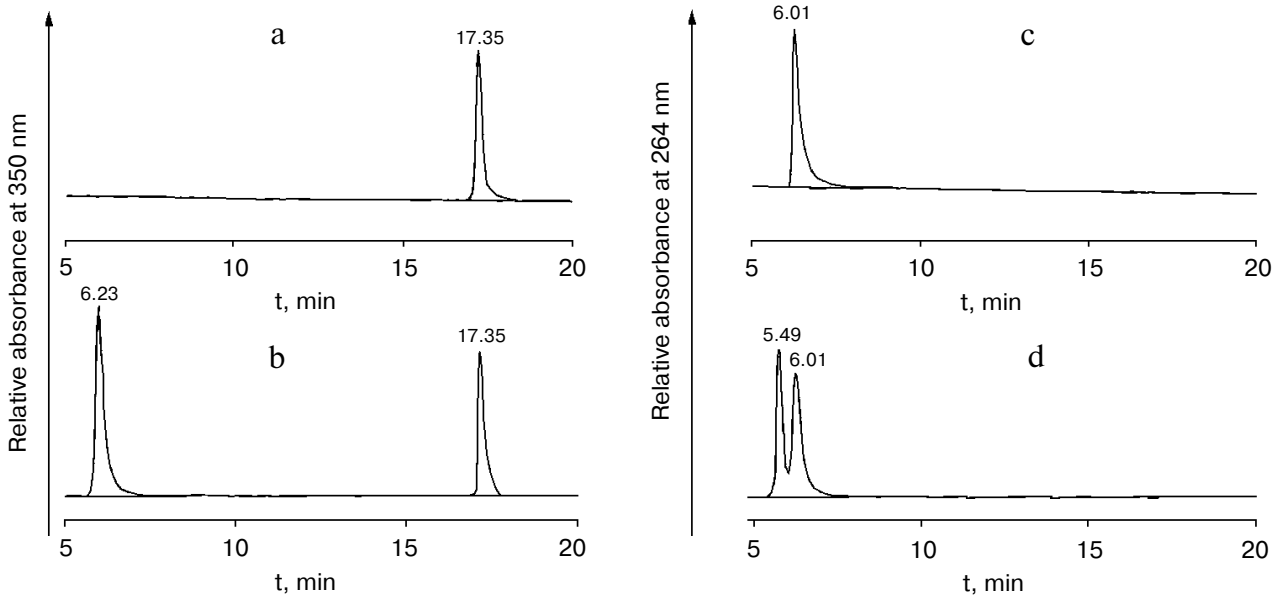


Fig. 2. HPLC chromatograms of thiol substrates and products. Substrate and products were separated on C8 OS column at a flow rate of 1 ml/min. a) 2-TX (control); b) 2-TX (residual) and 2-TUA (product) at 350 nm; c) 2-MP; d) 2-MP (residual) and 8-hydroxy-2-mercaptopurine (product) and 264 nm.

and not 2-TX (169 amu) was further supported from LC/MS experiments. Figures 3a and 3b show the mass spectra of the enzymatic product of 2-MP. Two major molecular ion peaks of 153.2 and 169 in the MS analysis were detected, which were equivalent to the mass of residual 2-MP and its mono-hydroxylated product, respectively. Figure 3a shows that 2-MP (m/z = 153.2) has been converted to its mono hydroxylation product (m/z = 169) and not the dihydroxylated product. Thus, 2-TUA (m/z = 185) is not generated as the enzymatic product of 2-MP. To rule out the possibility that 2-TX (169 amu) is generated as the enzymatic product of 2-MP, the molecular ion of 169 amu generated as a mono-hydroxylated product of 2-MP (153 amu) was selected and further subjected to MS/MS analysis. This fragmentation profile (Fig. 3b) identified several species that are different from that of the fragmentation profiles of pure 2-TX which exhibited a molecular mass of 169 (Fig. 3c). This molecular ion from 2-TX was then selected and subjected to MS/MS and MS/MS/MS analysis. The MS/MS and MS/MS/MS analysis yielded two fragmented species of the mass 152 and 141.9 (data not shown). Since the fragmentation profile of pure 2-TX (m/z = 169) is markedly different from that of enzymatic reaction product of 2-MP (identical mass of m/z = 169), it is concluded that the enzymatic reaction product of 2-MP is different from that of 2-TX. Therefore, the putative enzymatic product of 2-MP is now confirmed as 8-OH-2-MP and not 2-TX.

DISCUSSION

Since 6-MP is an anticancer drug and its target enzyme is HGPRT rather than XOD, the action of XOD on 6-MP is expected to depotentiate the anticancer action of the drug. Since 2-MP is an analog substrate of 6-MP, it was also interesting to verify the effect of XOD on 2-MP in regard to the structure–function relationship. In the present study, it has been shown that the nature of enzymatic hydroxylation of the substrates 6-MP and 2-MP is different. Spectroscopy experiments indicated that the enzymatic hydroxylation of 2-MP does not take place at the C-6 position or simultaneously at the C-6 and C-8 position, but only at the C-8 position. If there had been any enzymatic hydroxylation at the C-6 position or C-6 and C-8 position simultaneously, then the corresponding products would be 2-TX and 2-TUA, respectively. But neither of these products was detected. Instead, an increase in the absorbance at 264 nm during the enzymatic hydroxylation of 2-MP was seen, which was the genesis of the notion that probably the enzymatic product of 2-MP is 8-OH-2-MP. 6-MP was found to form 6-TX, which finally gave rise to 6-TUA. HPLC results also supported that 2-TX or 2-TUA was not the enzymatic product of 2-MP. Once again, here it was

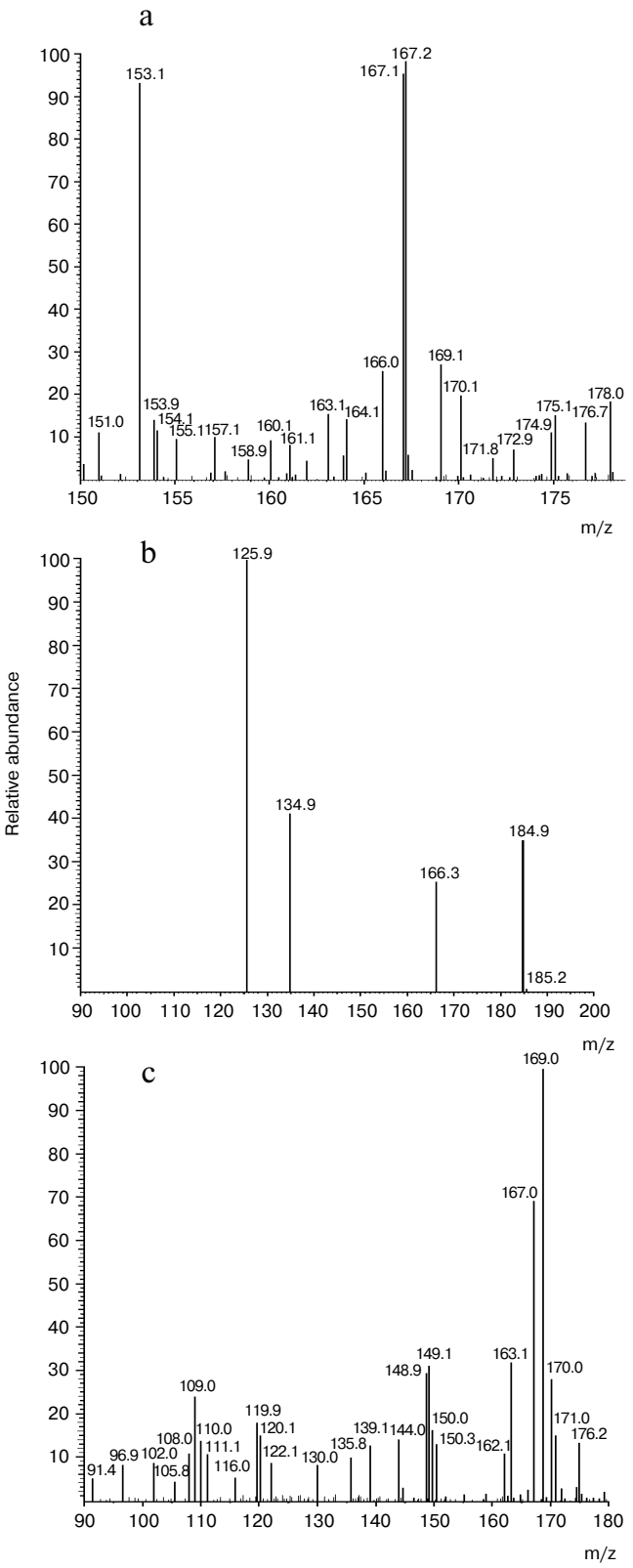


Fig. 3. LC/MS spectra of thiol substrates and products. Substrate and products were separated on C8 OS column at a flow rate of 1 ml/min. a) Mass spectrum (MS) of 2-MP (153 amu) reaction products; b) MS/MS analysis of m/z = 169.3 species of 2-MP reaction product; c) mass spectrum (MS) of pure 2-TX (169 amu).

proved that 6-MP forms 6-TUA via the intermediate 6-TX. PDA data analysis suggested that the enzymatic product of 2-MP was 8-OH-2-MP, whose molar extinction coefficient was higher than of 2-MP. LC/MS data also suggested that XOD converts 2-MP (153 amu) into a monohydroxylated product of the molecular mass of 169, which is not 2-TX because in that case the final enzymatic product will be 2-TUA. Therefore, it can be concluded that while 6-MP is a two-step substrate of XOD, 2-MP is a one-step substrate.

The fact that 2-MP is a one-step substrate is further supported by interaction energy calculations. The substrate–enzyme interaction energies were calculated using docking studies for various binding orientation (C-8 and C-6 for 2-MP). As compared to C-8 position (–47.48 kcal/mol), orientation of C-6 of 2-MP towards the metal center of the enzyme exhibited less favorable interaction (–24.50 kcal/mol) (data not shown). Therefore, the probability of the hydroxylation of 2-MP at the C-6 position is very remote.

We found from the measurements of initial rate, turnover number, and specificity constant values that 2-MP is a better substrate of XOD than 6-MP. The reason for 2-MP being a more efficient substrate of XOD than 6-MP is not very clear, but this can be explained from the molecular modeling work of Rastelli et al. [11] and also by applying mechanistic enzymology aspects of XOD.

Rastelli et al. [11] mentioned that the C-2 and C-8 positions of hypoxanthine and the C-8 position of xanthine are the susceptible sites for the enzymatic hydroxylation reaction, and the functional group present at the C-6 position of the purine is important for the anchoring with the enzyme. It has been shown that for the hydroxylation to take place, the six-membered ring of hypoxanthine with the C-2 site and five-membered ring of xanthine with the C-8 site should be in close proximity of the molybdenum center (active site of the enzyme). Therefore, it is likely that the C-2 site of the six membered ring of 6-MP and the C-8 site of five-membered ring of 2-MP are located towards the enzyme substrate-binding pocket. Thus, the -SH functional group present on the C-6 (6-MP) and C-2 (2-MP) sites in these substrates are expected to have different types of interaction with XOD.

Xia et al. [9] suggested that at the final stage of catalysis, hydride transfer takes place from C-8 of xanthine to the Mo=S center of the enzyme. Any mechanism that facilitates the hydride transfer from the C-8 site should enhance the hydroxylation of the substrate. Such type of preferable electronic transition at the C-8 site of 2-MP rather than at the C-2 site of 6-MP favors it to be a better substrate of XOD. As reported earlier, the presence of any substituent group in the C-6 position in a purine substrate is expected to provide an anchoring effect with the enzyme [11, 19, 20], but we found that 2-MP is a fairly good substrate of XOD in spite of the absence of any functional group at C-6 position. We believe that for 2-MP, -SH group present at the C-2 position is important both for anchoring and influencing higher enzymatic turnover into its corresponding product. Considering all these unexpected characteristics displayed by 2-MP, we conclude that 2-MP is a unique substrate of XOD.

In the two-step enzymatic hydroxylation reaction of 6-MP, the first step, i.e. 6-MP to 6-TX conversion, is much slower compared to the final step, i.e. 6-TX to 6-TUA. 2-TX and 6-TX exhibited almost similar spectra and also exhibited almost identical enzymatic turn over and kinetics. It is seen from the structure that interchanging of -OH and -SH groups between the C-6 and C-2 position in the six membered ring of these purine substrates did not change either their spectra or the substrate specificity. But, interchanging only -SH group between C-2 and C-6 in the case of 2-MP and 6-MP resulted in a dramatic difference in the enzymatic activity of these substrates. The differences in the reactivity of XOD for 2-MP and 6-MP can also be explained from H-bonding character of these substrates with the enzyme. Hernandez et al. [19] mentioned that H-bonding and keto–enol tautomerism also appear to be responsible for the substrate recognition by XOD. In case of xanthine, for example, N-3 and N-7 act as H-bond donors, while the N-9 and O-6 are H-bond acceptors.

The fact that XOD can discriminate different thiopurine substrates may be correlated with different types of H-bonding and anchoring effect. This also indicates that the position of the functional group present on the purine ring determines its substrate specificity and therefore serves as an important criterion in designing purine based anticancer drugs, as XOD can discriminate the hydroxylation site on the purine ring of different substrates depending on the positions of the functional groups.

We conclude that: i) XOD-catalyzed reaction pathways of 2-MP and 6-MP are different; ii) 2-MP is a better substrate of XOD than 6-MP, and iii) the structure of different purine substrates influences the activity of XOD.

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