Research article

**Preferential inhibition of xanthine oxidase by 2-amino-6-hydroxy-8-mercaptopurine and 2-amino-6-purine thiol**

Sukirti Kalra*¹, Gopabandhu Jena², Kulbhushan Tikoo² and Anup Kumar Mukhopadhyay³

Address: ¹Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, Phase X, S.A.S Nagar, Mohali, 160062 India, ²Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, Phase X, S.A.S Nagar, Mohali, 160062 India and ³Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, Phase X, S.A.S Nagar, Mohali, 160062 India

Email: Sukirti Kalra* - skalra11@yahoo.com; Gopabandhu Jena - gbjena@niper.ac.in; Kulbhushan Tikoo - tikoo@niper.ac.in

* Corresponding author

**Abstract**

**Background:** The anticancer drug, 6-mercaptopurine (6MP) is subjected to metabolic clearance through xanthine oxidase (XOD) mediated hydroxylation, producing 6-thiouric acid (6TUA), which is excreted in urine. This reduces the effective amount of drug available for therapeutic efficacy. Co-administration of allopurinol, a suicide inhibitor of XOD, which blocks the hydroxylation of 6MP inadvertently enhances the 6MP blood level, counteracting this reduction. However, allopurinol also blocks the hydroxylation of hypoxanthine, xanthine (released from dead cancer cells) leading to their accumulation in the body causing biochemical complications such as xanthine nephropathy. This necessitates the use of a preferential XOD inhibitor that selectively inhibits 6MP transformation, but leaves xanthine metabolism unaffected.

**Results:** Here, we have characterized two such unique inhibitors namely, 2-amino-6-hydroxy-8-mercaptopurine (AHMP) and 2-amino-6-purine thiol (APT) on the basis of IC⁵₀ values, residual activity in bi-substrate simulative reaction and the kinetic parameters like $K_m$, $K_i$, $k_{cat}$. The IC⁵₀ values of AHMP for xanthine and 6MP as substrate are $17.71 ± 0.29 \text{ µM}$ and $0.54 ± 0.01 \text{ µM}$, respectively and the IC⁵₀ values of APT for xanthine and 6MP as substrate are $16.38 ± 0.21 \text{ µM}$ and $2.57 ± 0.08 \text{ µM}$, respectively. The $K_i$ values of XOD using AHMP as inhibitor with xanthine and 6MP as substrate are $5.78 ± 0.48 \text{ µM}$ and $0.96 ± 0.01 \text{ µM}$, respectively. The $K_i$ values of XOD using APT as inhibitor with xanthine and 6MP as substrate are $6.61 ± 0.28 \text{ µM}$ and $1.30 ± 0.09 \text{ µM}$. The corresponding $K_m$ values of XOD using xanthine and 6MP as substrate are $2.65 ± 0.02 \text{ µM}$ and $6.01 ± 0.03 \text{ µM}$, respectively. The results suggest that the efficiency of substrate binding to XOD and its subsequent catalytic hydroxylation is much superior for xanthine in comparison to 6MP. In addition, the efficiency of the inhibitor binding to XOD is much more superior when 6MP is the substrate instead of xanthine. We further undertook the toxicological evaluation of these inhibitors in a single dose acute toxicity study in mice and our preliminary experimental results suggested that the inhibitors were equally non-toxic in the tested doses.

**Conclusion:** We conclude that administration of either APT or AHMP along with the major anti-leukemic drug 6MP might serve as a good combination cancer chemotherapy regimen.
Background

6MP, an analog of hypoxanthine was first among the thio-purine series found to be useful as an anticancer drug to treat ALL, the most common malignancy affecting children and other leukemias [1,2]. 6MP is taken up by the cell and is transformed into an active metabolite 6-thiopurine monophosphate, an inhibitor of DNA synthesis, by the target enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) using phosphoribosyl-pyrophosphate (PRPP) as a co-substrate [2,3]. 6MP is also inadvertently being utilized by another enzyme XOD leading to the generation of an inactive metabolite, 6TUA which is excreted in urine [4,5]. Levels of XOD expression varies from tissue to tissue and is also known to be over-expressed in tumors [6-10]. So, in such cases, when the anticancer drug 6MP is administered, it would possibly lead to more metabolic transformation of 6MP. XOD catalyzes a two-step hydroxylation reaction of 6MP, leading to formation of 8-OH-6MP first and subsequently to 6TUA (Figure 1).

The wasteful degradation of 6MP by XOD suggested that it is highly essential to minimize this catabolic pathway by the use of a XOD inhibitor. Previous reports indicated that semicarbazide could inhibit XOD and XDH activities in vitro as well as in vivo but the inhibition in vivo was less than 50% at doses that produced significant toxic effects [6].

Then, allopurinol, a pyrazolopyrimidine derivative and an analog of hypoxanthine, was employed as a part of combination cancer therapy along with 6MP and was found to result in a noticeable drop in the pace of catabolism of 6-substituted purines including 6MP as well as potentiate the antitumor and immunosuppressive properties of 6MP up to three to four-folds [6,11,12]. Allopurinol is a non-specific suicide inhibitor of XOD available in market for the treatment of gout, caused by the accumulation of uric acid crystals in the joints and tissues [13-15]. XOD is an important purine metabolism pathway enzyme which catalyzes the oxidative hydroxylation of the natural purine, hypoxanthine to xanthine to finally uric acid which is excreted in the urine [16-18] (Figure 2). Allopurinol usage in the combination chemotherapy with 6MP increases plasma concentration of the anticancer drug allowing the reduction in the large 6MP dose to almost 25% [19-21]. On the other hand, allopurinol displays certain biochemical complications; the most important amongst these is leading to the accumulation of natural purines hypoxanthine and xanthine, as along with 6MP, allopurinol also inhibits the natural hydroxylation pathway of hypoxanthine and xanthine to uric acid. This accumulation of natural purines leads to xanthine nephropathy [22]. Furthermore, a huge amount of reactive oxygen species (ROS) is generated with the administration of allopurinol [23].

So, alternatively a substrate specific inhibitor along with administration of this multipotential drug, 6MP must be used. Diphenyleneiodonium chloride has been reported as the preferential inhibitor of NAD(P)H oxidase as it inhibits the NADH oxidation more effectively than of NADPH [24]. Human PC4 is also been reported as a substrate specific inhibitor of the RNA Polymerase II phosphorylation [25]. Inspite of the humungous progress towards the development of XOD inhibitors to date [6,11,26-30], there has been no report of any such substrate specific inhibitor that selectively prevents 6MP from being wastefully transformed into 6TUA against natural purines. In the present work, we have discussed the action of two such preferential XOD inhibitors.

Results

A preferential XOD inhibitor is the one which will inhibit 6MP from being metabolized by the action of XOD while enzymatic hydroxylation of xanthine to uric acid is continued. The hydroxylation reaction of hypoxanthine forming uric acid and 6MP forming 6TUA has no common intermediates (Figures 1 and 2).

IC50 determination

A few purine and pyrazolo pyrimidine-based compounds were screened for inhibitory property against XOD-catalyzed hydroxylation using xanthine and 6MP as substrates (data not shown). 10 μM of either of the substrates and 2.8 U/ml of XOD were taken for the IC50 determination

---

**Figure 1**
Scheme showing oxidative hydroxylation of 6-mercaptopurine to 6-thioxanthine to 6-thiouric acid.

**Figure 2**
Scheme showing oxidative hydroxylation of hypoxanthine to xanthine to uric acid.
(Table 1). IC$_{50}$ values of allopurinol for the enzymatic conversion of xanthine to uric acid and 6MP to 6TUA are found to be almost same (2.36 ± 0.03 and 1.92 ± 0.03 μM) and therefore, suggest that allopurinol is equally efficient in inhibiting enzymatic conversion of both xanthine to uric acid and 6MP to 6TUA formation. Since allopurinol is a suicide inhibitor of XOD and the inhibition develops time-dependently [31], so the IC$_{50}$ value of allopurinol was calculated for the late phase as well by carrying out the reaction for 60 min and the IC$_{50}$ values of allopurinol for XOD corresponding to the substrates hypoxanthine and xanthine were 0.55 ± 0.45 and 1.65 ± 0.25 μM, respectively. While for 6MP, the IC$_{50}$ values were already calculated from 60 min reaction. Among many compounds tested, two purine-based compounds namely, AHMP and APT demonstrated a lower IC$_{50}$ value for the 6MP to 6TUA reaction (0.54 ± 0.01 μM and 2.57 ± 0.08 μM, respectively), although showed a reasonably higher IC$_{50}$ value for the xanthine to uric acid formation reaction (17.71 ± 0.29 μM and 16.38 ± 0.21 μM, respectively). The lower IC$_{50}$ values for 6MP hydroxylation than xanthine indicate that AHMP and APT inhibit hydroxylation of 6MP and xanthine with variable efficiencies. These two purine-based compounds are found to be showing no such difference in the IC$_{50}$ value in the first and the late phases and none were found to be XOD substrates. However, like allopurinol, the recently discovered XOD inhibitors, 6-(N-benzoylamino)purine [29] and also 4-aminopyrazolo 3, 4-d pyrimidine (APP) [30] did not exhibit any preferential inhibition of the 6MP hydroxylation over xanthine. The results imply that the structure of the inhibitor plays a major role in regulating this preferential inhibition mechanism.

We also monitored the substrate-induced enzymatic electron transfer to the acceptors like 2,6-dichlorophenol indophenol (DCPIP) and cytochrome c and found that these two preferential inhibitors did not release either any electrons or generate any ROS on interaction with XOD while allopurinol led to substrate-induced enzymatic electron transfer via DCPIP (data not shown) [29,30]. Some researchers report the generation of superoxides from allopurinol in large amounts [23].

**In vitro bi-substrate-inhibitor-enzyme simulation**

In order to determine the extent of enzymatic hydroxylation of xanthine and 6MP into their corresponding products when the two substrates were present together in the same reaction mixture, we carried out a bi-substrate simulative experiment.

Percent residual activity for generation of uric acid and 6TUA was determined in the presence of various concentrations of AHMP, APT and allopurinol (Table 2). XOD-mediated hydroxylation reaction of xanthine and 6MP (bi-substrate) led to generation of uric acid and 6TUA with progress of time, as shown in Figure 3. Absorption spectra of the time dependent XOD-mediated bi-substrate reaction in the presence of APT and AHMP are depicted in Figure 4 and Figure 5, respectively. Almost complete inhibition of 6MP hydroxylation while little inhibition of the xanthine hydroxylation reaction was achieved in the presence of 5 μM APT and 2 μM AHMP (Here, variable inhibitor concentrations were chosen depending on different IC$_{50}$ values for the two substrates). Accounting for the percentage inhibition using these inhibitors, we found that using 2.5 μM allopurinol, the uric acid and 6TUA forming residual enzyme activity was 30% and 16%, respectively. Using 2.5 μM APT, the residual enzyme activity for xanthine and 6MP hydroxylation was reduced to 80% and 25%, respectively and while using 2.5 μM AHMP it resulted in residual 64% uric acid formation activity and 18% 6TUA formation.

Therefore, the results suggest that when the two substrates, 6MP and xanthine are present together along with either of the preferential inhibitors, the conversion of xanthine to uric acid is more favored than 6MP to 6TUA conversion. The results also suggest that a critical concentration of the inhibitor could be used to effectively inhibit the enzymatic conversion of 6MP to 6TUA in the branched pathway. A similar invitro tri-substrate simulative studies was carried out with three substrates of XOD namely, hypoxanthine, xanthine and 6MP in the absence and presence of preferential inhibitors. Identical results corresponding to the preferential inhibitors were obtained indicating the discriminatory inhibition of the 6MP to 6TUA conversion and not hypoxanthine to uric acid conversion by the action of AHMP and APT (data not shown).

**Mechanism of inhibition of Xanthine oxidase by AHMP and APT**

To study mechanism of inhibition and distinguish the interaction of XOD with the substrates, xanthine and 6MP and inhibitors, AHMP and APT; we studied the steady-state kinetics of XOD with respect to the substrates and inhibitors. The double reciprocal Lineweaver Burk (LB) plots were drawn to find the mechanism of inhibition of XOD by APT and AHMP (Figure 6, 7, 8, 9). The $K_m$, $K_i^1$, $K_i^2$, $K_i^3$ and $k_{cat}$ values, and inhibitory mechanism exhibited by APT and AHMP are all depicted in Table 3.

Figure 6 depicts the LB plot of the steady-state inhibition with increasing APT concentrations, which show straight lines meeting at a point on Y-axis. Mechanistically, this type of inhibition is interpreted as competitive. Structurally speaking, we observe that the 8th position in both xanthine and APT is free for hydroxylation facing the Mo site of XOD. Also, both APT and xanthine has substitution
at the same positions, i.e. 2nd and 6th. Therefore, it seems that xanthine and APT compete for XOD active site. Consequently, it becomes evident that APT will bind to XOD at its substrate binding site. The Ki1 value estimated from the LB plot using the equation (1) [mentioned in Methods section] were equivalent to 6.6 ± 0.28 \( \mu \text{M} \) with respect to xanthine (Table 3).

Figure 7 projects a LB plot of steady-state inhibition of XOD using 6MP as substrate with increasing APT concentrations, suggesting a conventional competitive inhibition mechanism. Structural explanations illustrate that 8th position is open for action in both 6MP and APT in front of the Mo site of XOD. The \( K_{i1} \) value determined from the LB plot using the equation (1) [mentioned in Methods section] was found to be 1.30 ± 0.09 \( \mu \text{M} \) (Table 3).

AHMP also showed signs of efficiently inhibiting the XOD-catalyzed 6MP catabolic reaction than the xanthine hydroxylation reaction. Figure 8 demonstrates the LB plot of the steady-state inhibition of XOD using xanthine as substrate with increasing AHMP concentrations, illustrating the mixed type of inhibition like known XOD inhibitors, BOF-4272, Pd\textsuperscript{2+}, Y-700, Morin and Galagin, [32-35]. The \( K_{i2} \) and \( K_{i2} \) values of 5.78 ± 0.48 \( \mu \text{M} \) (competitive) and 6.24 ± 0.04 \( \mu \text{M} \) (non-competitive) were calculated from the LB plot using the equation (4) and (5) [mentioned in Methods section]. Its non-competitive inhibition property can be explained on the basis that 8th position is occupied by SH group in AHMP, which is found free in xanthine. Besides, the presence of NH\textsubscript{2} group at 2nd position in AHMP in place of an OH group in xanthine makes it faultless contender to bind at any site other than active site on XOD. And its competitive inhibition property may be due to the presence of OH group present at the 6th position in both AHMP and xanthine and thus, they compete for the enzyme active site.

For XOD-catalyzed 6MP catabolic reaction, AHMP shows evidence of a mixed inhibition mechanism (Figure 9).
indicating that native XOD is capable of forming two inhibitory complexes with AHMP. Occurrence of SH group at positions 6th in 6MP and at 8th in AHMP makes the latter an ideal applicant to compete with 6MP for active site and consequent inhibition of XOD. However, the existence of extra groups (OH and NH2) in AHMP at two positions makes it attach at a site other than the active site of the enzyme. \( K_i^1 \) and \( K_i^2 \) values for inhibition were calculated out to be 0.96 ± 0.01 μM (competitive) and 0.98 ± 0.06 μM (non-competitive) using the equations (4) and (5) [mentioned in Methods section]. The values of the two inhibition constants of AHMP do not differ much, indicating that the nature of the coordination of the AHMP for the competitive and non-competitive binding sites is similar.

The reaction mixture comprised of 1 ml of 0.2 M sodium phosphate buffer pH 7.4 containing 10 μM of both xanthine and 6MP. The concentration range of allopurinol was 0–2.5 μM. The concentration range of AHMP was 0–3 μM. The concentration range of APT was 0–10 μM. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD.

Comparison and evaluation of acute toxicity

Single dose acute toxicity was evaluated for AHMP and APT and compared with allopurinol, the standard drug used in the present investigation. No differences in general behavioral toxicity and no mortality were observed after the administration of the compounds. No significant changes in the initial and final body weight were observed for any of the compounds. The weekly food intake decreased for the groups treated with AHMP, APT and allopurinol as compared to vehicle treated groups. However, AHMP and APT treated groups food intake was marginally less as compared to allopurinol treated groups. Gross necropsy revealed no changes in vital organs. No difference in organ weight was observed as compared with the control groups.

Table 2: Bi-substrate simulative experiment.

| Inhibitor (μM) | % Activity with xanthine as substrate | % Activity with 6MP as substrate |
|---------------|--------------------------------------|---------------------------------|
| ALLOPURINOL   |                                      |                                 |
| 0.5           | 95 ± 1.09                            | 83 ± 1.05                       |
| 1.0           | 68 ± 3.51                            | 48 ± 1.07                       |
| 2.5           | 30 ± 1.04                            | 16 ± 1.05                       |
| APT           |                                      |                                 |
| 0.5           | 96 ± 1.00                            | 73 ± 2.50                       |
| 1.0           | 90 ± 0.51                            | 66 ± 3.01                       |
| 2.5           | 80 ± 2.50                            | 25 ± 2.06                       |
| AHMP          |                                      |                                 |
| 0.5           | 90 ± 1.09                            | 59 ± 4.01                       |
| 1.0           | 85 ± 2.06                            | 47 ± 4.01                       |
| 2.5           | 64 ± 1.02                            | 18 ± 2.08                       |

The reaction mixture comprised of 1 ml of 0.2 M sodium phosphate buffer pH 7.4 containing 10 μM of both xanthine and 6MP. The concentration range of allopurinol was 0–2.5 μM. The concentration range of AHMP was 0–3 μM. The concentration range of APT was 0–10 μM. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD.

Figure 3

Bi-substrate reaction of XOD-mediated hydroxylation of xanthine and 6MP. The reaction mixture comprised of 1 ml of 0.2 M sodium phosphate buffer pH 7.4 containing 10 μM of both xanthine and 6MP. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD and observed for 20 min.

Figure 4

Bi-substrate reaction of XOD-mediated hydroxylation of xanthine and 6MP in presence of APT. The reaction mixture comprised of 1 ml of 0.2 M sodium phosphate buffer pH 7.4 containing 10 μM of both xanthine and 6MP. 5 μM APT was taken. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD and observed for 20 min.
Discussion

Inhibition mechanism of Xanthine oxidase by AHMP and APT

Mixed-type inhibition includes competitive and non-competitive inhibition. Competitive inhibition is exhibited when the substrate and the inhibitor compete for the substrate binding active site but non-competitive inhibition is exhibited when the inhibitor binds at a site other than the active site. Following the crystallographic studies performed on XOD, no peripheral binding site has been reported in XOD catalytic subunits [36,37]. For the past 50 years, it was assumed that the two XOD subunits carry out catalysis independently but a recent report [38] on the cooperative binding of the two subunits of XOD may support the non-competitive inhibition mechanism exhibited by AHMP. AHMP possibly interacts with the XOD domains distal to the substrate binding site also other than the substrate binding site, thereby possibly resulting in allosteric effects that attenuate the enzymatic activity.

Since XOD acts via a ping-pong mechanism, alternating between the oxidized and reduced forms [39], so another plausible explanation for the mixed inhibition exhibited by AHMP is that AHMP binds to both the oxidized and reduced form of XOD. Allopurinol (a known weak competitive inhibitor) and nitric oxide are known to strongly bind to the reduced state of XOD [40,41]. APT being a competitive inhibitor thus, might not be binding to the oxidized state but to the reduced state of XOD. The difference in the mechanism of inhibition exhibited by AHMP and APT must be possible due to the structural dissimilarities between the two inhibitors.

Biochemical explanations of the efficiency of binding of substrate and inhibitor to Xanthine oxidase

The $K_m$, $k_{cat}$ and $K_i$ values of XOD corresponding to the substrates, 6MP and xanthine and the inhibitors, APT and AHMP behave as the factors indicating the type of their binding and catalytic interactions with the enzyme. In the biochemical reaction,

$$E+S \overset{k_1}{\longrightarrow} ES \overset{k_2}{\longrightarrow} E+P$$

where, $E$ is enzyme, $S$ is substrate, $P$ is product, $k_1$ and $k_3$ are the rate constants for the forward reactions and $k_2$ is the rate constant for the backward reaction.

By definition, $K_m$ of any biochemical reaction can be mathematically expressed in terms of rate constant [42] as

$$K_m = \frac{(k_2 + k_3)}{k_1}$$

Lower $K_m$ implies a lower dissociation of ES into E+S or E+P while higher $K_m$ implies higher dissociation of ES into E+S or E+P. While looking at the turnover number ($k_{cat}$) of xanthine and 6MP, we found that it is the dissociation of the corresponding ES complexes into E+P and not E+S.

The inhibition constant $K_i$ on the other hand can be defined [42] as:

| Inhibitor | Substrate | $K_m$ (μM) | Mechanism of inhibition | $K_i$ (μM) | $k_{cat}(min)^{-1}$ |
|-----------|-----------|------------|-------------------------|------------|---------------------|
| APT       | Xanthine  | 2.65 ± 0.02| Competitive             | 6.61 ± 0.28| 114.54 ± 0.24       |
|           | 6MP      | 6.01 ± 0.03| Competitive             | 1.30 ± 0.09| 2.85 ± 0.02         |
| AHMP      | Xanthine  | 2.71 ± 0.05| Mixed                  | 5.7 ± 0.48 (K_i^2) (Competitive) | 128.57 ± 0.45 |
|           | 6MP      | 4.89 ± 0.04| Mixed                  | 6.24 ± 0.04 (K_i^2) (Non-competitive) | 3.64 ± 0.04     |

The reaction mixture comprised of 1 ml of 0.2 M, sodium phosphate buffer pH 7.4 containing 10 μM of either xanthine or 6MP. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD.

$K_i^2$ = Inhibition constant for competitive inhibition mechanism exhibited by AHMP

$K_i^2$ = Inhibition constant for non-competitive inhibition mechanism exhibited by AHMP

Table 3: Table showing kinetic parameters of APT and AHMP.

Figure 5
Bi-substrate reaction of XOD-mediated hydroxylation of xanthine and 6MP in presence of AHMP. The reaction mixture comprised of 1 ml of 0.2 M, sodium phosphate buffer pH 7.4 containing 10 μM of both xanthine and 6MP. 2 μM AHMP was taken. The reaction was initiated by the addition of 2.8 U/ml of bovine XOD and observed for 20 min.
where, I is inhibitor, \( k_1 \) is the rate constant for the forward reaction and \( k_2 \) is the rate constant for the backward reaction.

\[
K_i = \frac{k_2}{k_1}
\]

Lower \( K_i \) implies lower dissociation of EI complex into E + I and higher association of E and I, while higher \( K_i \) means higher dissociation of EI complex and lower association of E and I.

\( K_m \) and \( k_{cat} \) values of XOD using 6MP as substrate implied that the conversion of XOD-6MP (ES) complex into its product 6TUA is slower as its turnover number is much lower (2.85 ± 0.02 \( \mu \)M/min) than the conversion of XOD-xanthine complex into its corresponding product (Table 3). Additionally, XOD-6MP (ES) complex is also less favored as the \( K_m \) in this case is high (6.01 ± 0.03 \( \mu \)M). Considering xanthine as substrate of XOD however, the situation is different. The high \( k_{cat} \) (114.54 ± 0.24 \( \mu \)M/min) indicates that the forward reaction of xanthine-XOD into uric acid (ES into E+P) is faster as compared to its dissociation into other counterpart (E+S) and lower \( K_m \) (2.65 ± 0.02 \( \mu \)M) indicates that xanthine-XOD (ES) complex formation is also more favored. Therefore, the higher turnover of xanthine to uric acid conversion in comparison to 6MP to 6TUA conversion is favored only if the ES complex formation is favored rather than its dissociation into E+S which does not occur in case of 6MP-XOD interaction.

Now, we can presume a correlationship of \( K_m \) and \( K_i \) of XOD with respect to xanthine and 6MP, respectively. When 6MP and APT (or AHMP) interact with XOD, lower \( K_i \) implies stronger XOD-AHMP (EI) complex formation and AHMP will more efficiently disassociate 6MP from XOD active site while higher \( K_m \) implies less favored XOD-6MP (ES) complex formation and subsequently, slower 6TUA formation, further supporting that XOD and 6MP (E+S) formation is much preferred in 6MP as compared to xanthine. However, when xanthine and APT (or AHMP) interact with XOD, higher \( K_i \) implies weaker and slower rate of formation of XOD-AHMP (EI) complex and the inhibitor AHMP is not efficient in displacing xanthine from XOD and thus, the enzymatic hydroxylation of xanthine continues while lower \( K_m \) implies more favored formation of XOD-xanthine (ES) complex and subsequently, higher formation of uric acid.

It will be right to say that these preferential inhibitors, AHMP and APT displace 6MP efficiently from weak XOD-6MP complex while AHMP and APT are incapable of displacing xanthine from strong xanthine-XOD complex.

**Figure 6**
Lineweaver-Burk plot of inhibition of XOD-mediated xanthine hydroxylation by APT. The reaction mixture comprised of 1 ml of 0.2 M, sodium phosphate buffer pH 7.4 containing varying concentrations of xanthine ranging from 2, 5, 8, 10 and 20 \( \mu \)M. APT concentrations were [I] = 0 \( \mu \)M (●), [I] = 5 \( \mu \)M (○), [I] = 10 \( \mu \)M (▼), [I] = 15 \( \mu \)M (▼). The reaction was initiated by the addition of 2.8 U/ml of bovine XOD.

**Figure 7**
Lineweaver-Burk plot of inhibition of XOD-mediated 6MP hydroxylation by APT. The reaction mixture comprised of 1 ml of 0.2 M, sodium phosphate buffer pH 7.4 containing varying concentrations of 6MP ranging from 2, 5, 8, 10, 15 and 20 \( \mu \)M. APT concentrations were [I] = 0 \( \mu \)M (●), [I] = 2 \( \mu \)M (○), [I] = 4 \( \mu \)M (▼). The reaction was initiated by the addition of 2.8 U/ml of bovine XOD.
In other words, substrate hit and inhibitor displacement takes place from XOD active or non-active site for xanthine-XOD reaction but inhibitor hit and substrate displacement for 6MP-XOD reaction. It can be stated that the 6MP to 6TUA hydroxylation is discriminately inhibited in contrast to xanthine to uric acid hydroxylation by these preferentially inhibitors.

**Conclusion**

In the present study, we have attempted to search a preferential XOD inhibitor which would discriminately inhibit the 6MP to 6TUA reaction rather than xanthine to uric acid reaction because allopurinol employed in combination with 6MP as cancer chemotherapy inhibits enzymatic hydroxylation of hypoxanthine, xanthine, and 6MP with equal efficiency, which leads to the development of some biochemical problems like xanthinuria [43] and xanthine nephropathy [44]. Furthermore, hypoxanthine when accumulated in the body competes with 6MP for the action by HGPRT [45]. Additionally, it has been observed that the administration of allopurinol, as a component of combination chemotherapy leads to the depletion in the intracellular pool of phosphoribosyl pyrophosphate (PRPP) as allopurinol is converted to allopurinol ribonucleotide by HGPRT using PRPP. The allopurinol ribonucleotide possesses further the potential for inhibition of PRPP amidotransferase and orotidyl acid decarboxylase [45-49]. Conversion to the allopurinol nucleoside by purine nucleoside phosphorylase has also been reported [50].

We found two such preferential inhibitors namely, APT and AHMP. The extent of inhibition is dependent on the binding interactions of the respective substrates and inhibitors with the enzyme.

It is important to mention here that APT is a known anti-cancer drug with HGPRT as its target enzyme. Therefore, use of APT as a preferential XOD inhibitor along with 6MP may increase the efficiency and reduce the dosage of 6MP. We conclude that administration of either APT or AHMP along with the major anti-leukemic drug 6MP might serve as a good combination cancer chemotherapy regimen. To the extent that adverse consequences of allopurinol therapy may relate to actions of this purine analog compound exclusive of XOD inhibition, the selectivity of AHMP and APT may lessen the risk for at least some of the untoward effects of current combined chemotherapy in patients with cancer. To the best of our knowledge, we have for the first time developed such preferential inhibitors of XOD based on the difference in the interactions with its substrate and inhibitor.

**Methods**

AHMP and APT both were purchased from Lancaster, UK. Bovine milk XOD was procured from Calbiochem (La
Jolla, CA, USA). Hypoxanthine, xanthine and 6MP were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate and Sodium dihydrogen phosphate were procured from Merck Inc. Ltd (Germany). The spectrophotometric experiments were performed on Perkin Elmer's Lambda 25 dual beam spectrophotometer.

XOD activity was determined spectrophotometrically by measuring uric acid formation at 293 nm with xanthine as substrate [51] and 6TUA formation at 350 nm with 6MP as substrate. One enzyme unit is defined as amount of enzyme required to produce 1 μM uric acid per min per ml reaction mixture from 10 μM of xanthine at 30°C, pH 7.4. Equivalent concentration of XOD was found to be 35 nM based on the molar extinction coefficient (Δε) of 36 mM⁻¹cm⁻¹ at 450 nm [51]. In all the experiments, the enzyme was added lastly to initiate the reaction. Δε value of uric acid is 11 mM⁻¹cm⁻¹ at 293 nm [52] and Δε value of 6TUA is 22 mM⁻¹cm⁻¹ at 350 nm.

The IC₅₀ values were determined for all the purine and pyrazolopyrimidine-based compounds. Percent inhibition of XOD was studied in terms of decrease in uric acid and 6TUA formation as compared to the product formation in absence of inhibitor. GRAFIT software procured from Erithacus Software Limited, UK, written by Dr R.J. Leatherbarrow, was employed for calculating the IC₅₀ values.

To appreciate the actual biochemical interactions carried out by XOD in vivo with respect to its natural (xanthine) and synthetic substrates (6MP), we designed a combined competitive in vitro simulative experiment wherein we determined the percent residual activity for the formation of uric acid and 6TUA by the co-presence of equimolar concentrations (10 μM, which is higher than the KM of the two substrates) of both the substrates of XOD (xanthine and 6MP) and either of the two preferential inhibitors (AHMP and APT). Percent residual activity of formation of uric acid and 6TUA was determined by subtracting the percentage inhibition from 100%. KM of XOD was determined for 6MP and xanthine from the Michaelis Menten plot and the kcat was determined by using the formula

$$k_{cat} = V_{max}/E_t,$$

where Vmax is the maximum velocity attained by a reaction and Et is the total enzyme concentration used in the reaction. KM and kcat were determined in the absence of the inhibitors. To determine the mechanism of inhibition exhibited, LB plots were drawn using Sigma Plot 9.01 version with Enzyme Kinetics module and the Ki was calculated using the following formulae,

For competitive inhibition,

$$-1/K_{m_{app}} = -1/K_m (1 + [I]/K_i) \tag{1}$$

For non-competitive inhibition,
\[
\frac{1}{V_{\text{max app}}} = \frac{1 + \frac{[I]}{K_i}}{V_{\text{max}}} \tag{2}
\]

The modified LB equation for the mixed inhibition is

\[
\frac{1}{v} = \frac{1}{V_{\text{max app}}} + \frac{K_{\text{m app}}}{V_{\text{max app}}}[S_0] \tag{3}
\]

where, \(K_{\text{m app}}\) and \(V_{\text{max app}}\) are defined by equations (4) and (5).

\[
K_{\text{m app}} = K_m \left\{\frac{1 + ([I_0]/K_i)}{1 + ([I_0]/K_i)}\right\} \tag{4}
\]

\[
V_{\text{max app}} = \frac{V_{\text{max}}}{1 + ([I_0]/K_i)} \tag{5}
\]

\([I_0]\) is the total concentration of the inhibitor, \([S_0]\) is the total concentration of substrate, \(K_m\) is the Michaelis constant and \(V_{\text{max}}\) is the maximum velocity of the reaction of XOD with the substrates 6MP or xanthine. \(K_{\text{m app}}\) is the apparent Michaelis constant and \(V_{\text{max app}}\) is the apparent maximum velocity of the reaction of XOD with its substrates. \(K_i\) and \(K_i\) are the inhibition constants of the inhibitors [53,54].

**Evaluation of acute toxicity of two discriminatory inhibitors**

In order to evaluate the toxicity of the preferential inhibitors of XOD, the single dose acute toxicity study was performed for both the preferential inhibitors on swiss male mice following a fourteen days observation period. The body weight of the animal ranged from 25–30 gm. Three different doses (2.5, 5, 10 mg/kg) for the two compounds were selected, using allopurinol as the standard drug and administered intravenously [55]. Parameters, e.g., body weight, food intake, mortality, general behavioral toxicity were observed after the administration of the compounds for fourteen days. Gross necropsy of each animal of all groups was conducted and organ weight was recorded after the termination of the study.

**Abbreviations**

XOD, Xanthine oxidase; 6MP, 6-Mercaptopurine; 6TUA, 6-thiouric acid; AHMP, 2-amino-6-hydroxy-8-mercaptopurine; API, 2-amino-6-purinethiol, HGPR, Hypoxanthine-guanine phosphoribosyl transferase; \(K_i^1\), Inhibition constant of 2-amino-6-purinethiol; \(K_i^2\), Competitive inhibition constant of 2-amino-6-hydroxy-8-mercaptopurine; \(K_i^2\), Non-competitive constant of 2-amino-6-hydroxy-8-mercaptopurine; LB, Lineweaver Burk.

**Authors’ contributions**

SK carried out the biochemical characterization of the preferential inhibitors by screening the various purine and pyrimidine based compounds and studied the mechanism of the inhibitors. SK assisted in the toxicological studies and also drafted the manuscript. GBJ and KT carried out the toxicological studies of the two preferential inhibitors. AKM conceived the study and participated in its design; principal investigator. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Council of Scientific and Industrial Research (CSIR, New Delhi, Govt. of India) (Grant No. 01(1823)/02/EMR-II) for funding the project to Dr Anup Kumar Mukhopadhyay.

**References**

1. Pinkel D: Intravenous mercaptopurine: life begins at 40. J Clin Oncol 1993, 11:1826-1831.
2. Burchenal JH, Murphy ML, Ellison RR, Sykes MP, Tan TC, Leone LA, Karnafo dyski DA, Craver LF, Dargeon HW, Rhoads CP: Clinical evaluation of a new antimetabolite, 6-mercaptopurine in the treatment of leukemia and allied diseases. Blood 1953, 8:965-999.
3. Weigel G, Griesmacher A, De Abreu RA, Wolner E, Mueller MM: Azathioprine and 6-mercaptopurine alter the nucleotide balance in endothelial cells. Thromb Res 1999, 94:87-94.
4. Lennard L: The clinical pharmacology of 6-mercaptopurine. Eur J Clin Pharmacol 1992, 43:329-339.
5. Elion GB: Symposium on immunosuppressive drugs. Biochemistry and pharmacology of purine analogues. Fed Proc 1967, 26:898-904.
6. Elion GB, Callahan S, Nathan H, Bieber S, Rundles RW, Hitchings GH: Potentiation by inhibition of drug degradation: 6-substituted purines and xanthine oxidase. Biochem Pharmacol 1963, 12:85-93.
7. Reiger P, Edwards PN, Gbaj A, Cole C, Barry ST, Page KM, Ashton SE, Luke RW, Douglas KT, Stratford IJ, Jaffar M, Bryce RA, Freeman S: Aminimidazololylmethylyracil analogues as potent inhibitors of thymidine phosphorylase and their bioreductive nitroimidazoyl prodrugs. J Med Chem 2005, 48:392-402.
8. Anderson RF, Patel KB, Reghebi K, Hill SA: Conversion of xanthine dehydrogenase to xanthine oxidase as a possible marker for hypoxia in tumours and normal tissues. Br J Cancer 1989, 60:193-197.
9. Giler S, Sperling O, Brosh S, Urca I, De Vries A: Proceedings: Serum xanthine oxidase in hepatocellular damage. Br J Med Sci 1975, 11:1225.
10. Kokoglu E, Belce A, Ozyurt E, Tepeler Z: Serum xanthine oxidase levels in human brain tumors. Cancer Lett 1990, 50:179-181.
11. Vogler WR, Bain JA, Hugosly CM, Palmer HG, Lowrey ME: Metabolic and therapeutic effects of allopurinol in patients with leukemia and gout. Am J Med 1966, 40:548-559.
12. Elion GB, Callahan S, Rundles RW, Hitchings GH: Relationship between Metabolic Fates and Antitumor Activities of Thiopurines. Cancer Res 1963, 23:1207-1217.
13. Emmerson BT: The management of gout. N Engl J Med 1996, 334:445-451.
14. Rodnan GP: Treatment of the gout and other forms of crystal-induced arthritis. Bull Rheum Dis 1982, 32:43-53.
15. Massey V, Komai H, Palmer G, Elion GB: On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-d]pyrimidines. J Biol Chem 1970, 245:2837-2844.
16. Vorbach C, Scciven A, Capechci MR: The housekeeping gene xanthine oxidoreductase is necessary for milk fat droplet enveloping and secretion; gene sharing in the lactating mammary gland. Genes Dev 2002, 16:3223-3235.
17. Hille R, Nishino T: Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. Faseb J 1995, 9:1055-1003.
18. Eskoth C, Eger BT, Okamoto K, Nishino T, Nishino T, Pai EF: Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. Proc Natl Acad Sci USA 2000, 97:10723-10728.
19. Kaplan SR, Calabresi P: Drug therapy: immunosuppressive agents. N Engl J Med 1973, 289:1324-1326.
20. Venkat Raman G, Sharman VL, Lee HA: Azathioprine and allopurinol: a potentially dangerous combination. J Intern Med 1990, 226:69-71.
21. Zimm S, Collins JM, O’Neill D, Chabner BA, Poplack DG: Inhibition of first-pass metabolism in cancer chemotherapy: interaction of 6-mercaptopurine and allopurinol. Clin Pharmacol Ther 1983, 34:810-817.

22. Jezelewicz MM: Effect of allopurinol (4-hydroxyprazolo(3,4-d)pyrimidine) on xanthine accumulation by milk xanthine oxidase in vitro. Eur J Biochem 1974, 46:361-365.

23. Gilaberte C, Orts P, Fedida D, Spector T: Superoxide radical production by allopurinol and xanthine oxidase. Biochim Bio phys Acta 2006, 1717:1752.

24. Morre D: Preferential inhibition of the plasma membrane NADH oxidase (NOX) activity by diphenylethenoidium chloride with NADPH as donor. Antioxid Redox Sign 2002, 4:207-212.

25. Ishibuchi S, Morimoto H, Oe T, Ikebe T, Inoue H, Fukunari A, Kamezawa M, Yamada I, Naka Y: Synthesis and structure-activity relationships of 1-phenylpyrazoles as xanthine oxidase inhibitors. Bioorg Med Chem Lett 2001, 11:879-882.

26. Tamta H, Schang LM, Hwang GJ, Dynlacht BD, Speicher DW, Bantly A, Schaffer T, Pomales R, Bieber S, Friedman R, Hitchings GH: Differential nature of substrate affinity, kinetics and electron transfer reaction displayed by xanthine and 6-thioxanthine as substrates of milk xanthine oxidase. Biochem Pharmacology of drugs for hyperuricemia: Mechanisms, Kinetics and Interactions. Contrib Nephrol 2005, 147:35-46.

27. Tamta H, Kalra S, Anand GCS, Mukhopadhyay AK: The structure of the inhibitory complex of alloxanthine (1H-pyrazolo[3,4-d]pyrimidine) on 4,6-dilhydroxyprazolo[3,4-d]pyrimidine (Oxipurinol) in vivo in the absence of xanthine-oxen oxidoreductase. Biochim Biophys Acta 1963, 72:119-120.

28. Fox IH, Wyngaarden JB, Kelley WN: Depletion of erythrocyte phosphoribosylpyrophosphate in man. N Engl J Med 1970, 283:1177-1182.

29. Fathallah-Shaykh S: Xanthinuria. WebMD 2005 [http://www.emedicine.com/ped/bymame/xanthinuria.htm], last updated August 2006.

30. Ge H, Shiekhattar R, Siden H, Hille R, Massey V: The structure of the inhibitory complex of alloxanthine (1H-pyrazolo[3,4-d]pyrimidine) on xanthine oxidase: inhibition mechanism and molecular modeling studies. J Enzyme Inhib Med Chem 2005, 20:317-324.

31. Massey V, Komai H, Palmer G, Elion GB: On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-d]pyrimidines. J Biol Chem 2003, 278:1846-1855.

32. Tamta H, Thilagavathi R, Chakraborti AK, Mukhopadhyay AK: 6-(N-benzoylamine)purine as a novel and potent inhibitor of xanthine oxidase: inhibition mechanism and molecular modeling studies. J Enzyme Inhib Med Chem 2003, 20:317-324.

33. Tamta H, Kalra S, Mukhopadhyay AK: Biochemical characterization of some Pyrazolopyrimidine-based inhibitors of xanthine oxidase. Biochemistry (Mosc) 2005, 71:S49-554.

34. Hille R, Massey V: Tight binding inhibitors of xanthine oxidase. Pharmacol Ther 1981, 14:249-263.

35. McCallister RJ, Gilbert WR Jr, Ashton DM, Wyngaarden JB: Pseudofeedback Inhibition of Purine Synthesis by 6-Mercaptopurine Ribonucleotide and Other Purine Analogues. J Biol Chem 1964, 239:1560-1563.

36. McCallister RJ, Gilbert WR Jr, Ashton DM, Wyngaarden JB: Pseudofeedback Inhibition of Purine Synthesis by 6-Mercaptopurine Ribonucleotide and Other Purine Analogues. J Biol Chem 1964, 239:1560-1563.

37. Chen A, Okamoto K, Nishino T, Eger BT, Pai EF, Kamezawa M, Yamada I, Kato N: Y-700 [1-[3-Cyan-4-(2,2-dimethylpro- pheny1)]-1H-pyrazole-4-carboxylic Acid]: A potent Xanthine oxidoreductase inhibitor with hepatic excretion. J Pharmacol Exp Ther 2004, 311:519-528.

38. Sau AK, Mondal MS: Mixed inhibition of the oxidoreductase activity of xanthine oxidase by Pd2+ ion. J Chem Soc Chem Comm 1994, 13:1547-1548.

39. Hoorn DECV, Nijveldt RJ, Leeuwen PAMV, Hofman Z, Rabbet LM, Bont DBAde, Norren KV: Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids. Eur J Pharmacol 2002, 451:111-118.

40. Enroth C, Eger BT, Okamoto K, Nishino T, Nishino T, Pai EF: Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: Structure-based mechanism of conversion. Proc Nat Acad Sci USA 2000, 97:10723-10728.

41. Okamoto K, Masumoto K, Hille R, Eger BT, Pai EF, Nishino T: The crystal structure of xanthine oxidoreductase during catalysis: Implications for reaction mechanism and enzyme inhibition. Proc Nat Acad Sci USA 2004, 101:7931-7936.

42. Fersht A, ed: Enzyme Structure and Mechanism. 2nd edition. Cambridge: W.H. Freeman & Company; 1984.

43. Fathallah-Shaykh S: Xanthinuria. WebMD 2005 [http://www.emedicine.com/ped/bymame/xanthinuria.htm], last updated August 2006.

44. Pea F: Pharmacology of drugs for hyperuricemia: Mechanisms, Kinetics and Interactions. Contrib Nephrol 2005, 147:35-46.

45. Schang LM, Hwang GJ, Dynlacht BD, Speicher DW, Bantly A, Schaffer T, Pomales R, Bieber S, Friedman R, Hitchings GH: Augmentation of the incorporation of hypoxanthine into nucleic acids by the administration of an inhibitor of xanthine oxidase. Biochim Biophys Acta 1963, 72:119-120.

46. Fox IH, Wyngaarden JB, Kelley WN: Depletion of erythrocyte phosphoribosylpyrophosphate in man. N Engl J Med 1970, 283:1177-1182.

47. Krenitsky TA, Elion GB, Sretlitz RA, Hitchings GH: Ribonucleosides of allopurinol and oxoallopurinol. Isolation from human urine, enzymatic synthesis, and characterization. J Biol Chem 1976, 242:2675-2682.

48. Hille R, Massey V: Tight binding inhibitors of xanthine oxidase. Pharmacol Ther 1981, 14:249-263.

49. Krenitsky TA, Elion GB, Sretlitz RA, Hitchings GH: Ribonucleosides of allopurinol and oxoallopurinol. Isolation from human urine, enzymatic synthesis, and characterization. J Biol Chem 1976, 242:2675-2682.

50. Krenitsky TA, Elion GB, Sretlitz RA, Hitchings GH: Ribonucleosides of allopurinol and oxoallopurinol. Isolation from human urine, enzymatic synthesis, and characterization. J Biol Chem 1976, 242:2675-2682.

51. Shiekhattar R, Siden H, Hille R, Massey V: The structure of the inhibitory complex of alloxanthine (1H-pyrazolo[3,4-d]pyrimidine) on 4,6-dihydroxyprazolo[3,4-d]pyrimidine (Oxipurinol) in vivo in the absence of xanthine-oxen oxidoreductase. Biochim Biophys Acta 1963, 72:119-120.

52. Massey V, Komai H, Palmer G, Elion GB: On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-d]pyrimidines. J Biol Chem 2003, 278:1846-1855.

53. Tamta H, Thilagavathi R, Chakraborti AK, Mukhopadhyay AK: 6-(N-benzoylamine)purine as a novel and potent inhibitor of xanthine oxidase: inhibition mechanism and molecular modeling studies. J Enzyme Inhib Med Chem 2005, 20:317-324.

54. Tamta H, Kalra S, Mukhopadhyay AK: Differential nature of substrate affinity, kinetics and electron transfer reaction displayed by xanthine and 6-thioxanthine as substrates of milk xanthine oxidase. J Biol Phys Chem 2005, 5:89-99.

55. Krenitsky TA, Elion GB, Sretlitz RA, Hitchings GH: Ribonucleosides of allopurinol and oxoallopurinol. Isolation from human urine, enzymatic synthesis, and characterization. J Biol Chem 1976, 242:2675-2682.