Antihyperuricemic effects of thiazolopyrimidin-5-one analogues in oxonate treated rats

Introduction

Hyperuricemia is one of the most important metabolic diseases. Hyperuricemia (>7 mg/dl of uric acid in serum) results from the overproduction or under excretion of uric acid (UA) and is an established risk factor for ‘gout’ (Choi et al., 2005a; Duskin-Bitan et al., 2014). The UA is synthesized in liver and elevated UA levels are associated with a wide variety of disorders that include renal dysfunction, cardiovascular diseases, hypertension, diabetes (Dalbeth et al., 2010; Choi et al., 2007; Johnson et al., 2005; Short et al., 2005), cancer, obesity, and hyperlipidemia (Lin et al., 2000). High intake of nucleic acids in the diet also results in hyperuricemia (Choi et al., 2004). The increase in uric acid concentration in the body ultimately leads to the deposition of monosodium urate crystals (MSU) in the joints and kidneys leading to inflammation as well as gouty arthritis and UA nephrolithiosis (Kramer et al., 2002; Tomita et al., 2000; Huang et al., 2011). Hyperuricemia does not always results in gout, but gout is always preceded by hyperuricemia. It is the most common form of inflammatory arthritis in men, especially affecting 1-2% of adult men in Western countries (Terkeltaub., 2003). An increasing number of postmenopausal women are also affected by gout (Wallace et al., 2004). Compounds that have the ability to enhance the urinary excretion of UA or those that can inhibit the UA biosynthesis have been employed for the treatment of gout (Ishibuchi et al., 2001). Gout is clinically manifested in four stages namely asymptomatic hyperuricemia, acute gouty arthritis, intercritical gout and chronic tophaceous gout (Wortman., 2002). These stages of gout are extensively described previously in chapter 1. There are several causes for hyperuricemia, they are diet, environment and genetics. Excess alcohol consumption (particularly beer), purine-rich diet (particularly red meat),
metabolic syndrome, use of diuretics and chronic renal failure are some of the causes of hyperuricemia, (Roddy et al., 2007). High intake of nucleic acids in the diet particularly through red meat, fish and shellfish has long been known to increase hyperuricemia and risk of gout (Choi et al., 2004; Choi et al., 2005b). Hereditary renal disorders, such as autosomal dominant familial juvenile hyperuricemic nephropathy or medullary cystic kidney disease (type 1 and 2), predispose individuals to hyperuricemia and gout (Choi et al., 2010a). Interestingly chronic diuretic therapy is associated with reduced excretion of UA. This is because the UA reabsorption in the proximal tubule secondary to volume depletion is increased and there is a competition between the diuretic action and UA reabsorption for the organic acid secretary mechanism in the proximal tubule (Spieker et al., 2002). However, low-dose diuretic therapy in hypertensive patients does not seem to modify serum urate levels significantly (Spieker et al., 2002; Bagatin et al., 1998). Low-dose diuretic therapy may be effective in hypertension but ineffective in patients with chronic heart failure who often additionally suffer from chronic renal failure.
Figure 1. Dietary impact on the risk of gout and their implications within a healthy eating guideline pyramid (Source: Choi, 2010b).

The presence of hyperuricemia in humans has raised questions about its origin and evolutionary advantages. Its association with the diseases requires an understanding of how it can become harmful at high concentrations. Initially, UA was believed to be considered an inert waste product that crystallizes at high concentrations to form renal stones and initiate gouty arthritis. Subsequently, UA was found to be a powerful antioxidant that scavenges singlet oxygen, oxygen radicals, and peroxynitrite and also chelates transition metals, to reduce, metal catalysed oxidation. Urate thus accounts for approximately half of the antioxidant capacity of human plasma, and its antioxidant properties are as strong as those of ascorbic acid (Ames et al., 1981; Davies et al., 1986).
Figure 2. Pathways of urate homeostasis. Pathways of production and degradation of uric acid. Hyperuricemia can lead to gout and possibly to cardiovascular effects, whereas hyperuricosuria may lead to uric acid crystal-induced kidney pathologies. (Source: Alexander et al., 2010).

The prevalence and incidence of gout and hyperuricemia is on the rise worldwide. Although gout affects mainly males, it affects post-menopausal women also. There is a clear increase in prevalence in both sexes with aging. The tissue deposition of urate crystals elicits an intense self-limiting inflammatory reaction.

The epidemiological link between hyperuricemia and gout was established more than 150 years ago (Campion et al., 1987). These aspects have been described previously in chapter 1.

UA formed by the oxidation of hypoxanthine to xanthine and then to UA by the enzyme XO (Okamoto et al., 2008). This reaction also produces ROS that initiate tissue damage.
in a range of pathophysiological states (Bonomini et al., 2008). Therefore, XO inhibitors such as allopurinol are in use in treating both hyperuricemia and other related disorders where ROS are implicated (Riegersperger et al., 2011). Allopurinol has been considered as the drug of first choice for hyperuricemia because it needs to be administered only once daily and it also prevents urolithiasis. The average dose is 300 mg/day, although initial, prescribed at low doses with a slow intake increase of 100 mg every 2-4 weeks until reaching the minimum dose necessary to maintain the target level of UA in serum. The maximum recommended dose (800 or 900 mg/day) is administered while constantly monitoring serum urate and creatinine clearance (Harris et al., 1999; Khanna et al., 2012). However, use of allopurinol is associated with undesired effects such as gastrointestinal upset, skin rashes, hypersensitivity reactions, liver dysfunction, exfoliative dermatitis, vasculitis, eosinophilia, acute interstitial nephritis (Emmerson., 1996; Wang et al., 2004), Stevens-Johnson syndrome, renal toxicity and fatal liver necrosis (Wallach et al., 1998). Recently discovered drug febuxostat, an alternative to allopurinol also showed higher incidence of hepatotoxicity in clinical trials (Hair et al., 2008).

I have previously described the synthesis of twenty pyrimidine analogues in chapter 2, among which four of them (6a, 6b, 6d & 6f) showed inhibition against XO using three XO sources (Sathisha et al., 2011). Docking studies showed important interactions of these molecules in the active site of XO (Sathisha et al., 2011). In the current investigation, antihyperuricemic effects of synthetic analogs in potassium oxonate treated rats is described.
Materials and methods

Chemicals

Xanthine, allopurinol and potassium oxonate (oxonic acid potassium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals and reagents, which were used in this study, were of analytical grade.

Preparation of test Samples

The detailed synthesis of the test compounds (7-methyl-2-(phenoxyethyl)-5H[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one derivatives of 6a, 6b, 6d and 6f compounds) is reported in our previous communication (Sathisha et al., 2011). These compounds were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 1 mg/ml.

Animals

Male wistar rats (8-10 weeks old) were obtained from the central animal facility, Department of Studies in Zoology, University of Mysore, after obtaining the ethical clearance from the Institutional Animal Ethical Committee (IAEC) of University of Mysore (UOM/IAEC/17/2011). The rats were housed on a constant 12 hour light/12 hour dark cycle in a temperature and humidity controlled room and were allowed free access to food and water.

Induction of hyperuricemia in rats

Rats were randomly divided into twelve groups: Group 1 received DMSO solution (vehicle control). Group 2 was administered with potassium oxonate (250 mg/kg) – an uricase inhibitor, injected intraperitoneally to overnight fasted rats to increase serum urate levels and served as hyperuricemic rats. Group 3 was administered with standard drug allopurinol (10 mg/kg) alone. Hyperuricemic rats receiving allopurinol were put under group 4. The groups 5-12 were all hyperuricemic rats receiving four test compounds at two doses (6a, 6b, 6d and 6f at 50 and 100 mg/kg respectively). The test compounds were
dissolved in DMSO and were administered intraperitoneally to the rats, 1 hour after the potassium oxonate injection (B-Rao et al., 2012; Nepali et al., 2011).

Blood was collected from the rats by cardiac puncture 2 hours after the administration of the test compounds and were sacrificed after the blood collection. Kidney and liver tissues were collected and were used for histological/enzyme analyses. The blood was allowed to clot for approximately 1 hour at room temperature and then centrifuged at 1000xg for 20 min to obtain the serum. The serum was stored at -20°C until assayed.

**Measurement of serum uric acid and creatinine levels**

Serum uric acid and creatinine levels were determined by using assay kits from Randox (Bangalore, India) in an autoanalyzer (Beckman-Coulter, AU480 ISE) as per manufacturer’s instructions. The results were expressed in mg/dl.

**Assay for XO activity in rat liver**

XO activity was assayed by monitoring uric acid formation using a spectrophotometric method described previously (Sathisha et al., 2011; Kadam et al., 2007). Briefly, 2 g of the liver from respective groups of rats was homogenized (Remi, India) with 5 volumes of 10 mM Tris-HCL buffer, pH 8.0 containing 1 mM EDTA. The homogenate was then heated to 55°C for 5 min and then cooled rapidly on an ice bath with constant stirring. This was then subjected to centrifugation at 16,000xg for 15 min. Ammonium sulphate was added to the resulting supernatant to a final concentration of 30% saturation and the mixture was centrifuged at 16,000xg for 15 min. XO in the supernatant was precipitated by further addition of ammonium sulphate to a final concentration of 60% saturation. The resulting precipitate was collected by centrifugation at 16,000xg for 15 min and the pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.5 containing 300 mM EDTA and stored at -80°C until use.
The enzyme assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.5) containing 300 mM EDTA and the enzyme source from the respective treatment groups in a total volume of 2 ml. The reaction was initiated by the addition of xanthine (50 µM) as the substrate to the above assay mixture. The change in absorbance at 292 nm was recorded in a spectrophotometer (Biomate 3S, Thermo Scientific, USA). The absorption rate at a wavelength of 292 nm indicates the formation of uric acid. The assays were carried out in duplicates and were repeated three to four times. The protein concentration of the tissue homogenates were determined by the Lowry’s method, using bovine serum albumin as standard (Lowry et al., 1951). XO activities were expressed as nmoles of uric acid formed/ min/ mg protein. The percentage inhibition of XO activity was calculated.

**Histological analyses of kidney and liver**

Tissues collected from the above experimental rats were fixed in Bouin’s solution (picric acid: formaldehyde: glacial acetic acid 30:10:2) for 24 hours and then subjected to dehydration with increasing concentrations of ethanol and embedded in paraffin wax. Sections of 5 µm were taken using microtome (R. Jung AG, Germany) and stained with classical hematoxylin and eosin staining protocol.

**Statistical Analysis**

The data were expressed as mean ± SEM. Statistical comparisons were performed by one way ANOVA.

**Results**

XO is a one of the key enzyme in purine catabolism. The elevated activity of this enzyme leads to hyperuricemia. Therefore, traditionally inhibiting this enzyme by drugs such as allopurinol is a way to control hyperuricemia. However, undesired effects associated with the use of allopurinol have forced scientific community to formulate new chemical entities. Previously, we had reported the synthesis of twenty pyrimidine analogues (7-
methyl-2-(phenoxymethyl)-5H [1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one derivatives) of which 4 showed significant XO inhibitory activity (6a, 6b, 6d and 6f) (Sathisha et al., 2011). The structures of these analogues are shown in fig-3.

**Figure 3.** Structures of the four thiazolopyrimidin-5-one derivatives employed in this study.

**Effect of test compounds on serum uric acid and creatinine levels in oxonate treated rats**

Administration of the uricase inhibitor - potassium oxonate resulted in significantly increased serum uric acid levels when compared to the control group. However, marked increase in serum creatinine level was noticed (Table.1). Basal serum uric acid level in rats was 2.52±0.00 mg/dl. Intraperitonial injection of potassium oxonate (250 mg/kg) increased the serum uric acid level to 4.85±0.03 mg/dl at the end of the experiment.
Treatment with the test compounds reduced the serum urate and creatinine levels when compared with the hyperuricemic group. The reduction of urate and creatinine levels in hyperuricemic groups exerted by 100 mg/kg dosage of the test compound was more potent than that of 50 mg/kg dosage (Table 1). Among the test compounds employed, 6a was more potent while, 6f was with the least effect at the doses tested (Table 1).

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Dose (mg/kg)</th>
<th>Serum levels</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uric acid (mg/dl)</td>
<td>Creatinine (mg/dl)</td>
<td></td>
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<tr>
<td>Control</td>
<td>-</td>
<td>2.52 ± 0.00</td>
<td>0.81 ± 0.01</td>
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<tr>
<td>Potassium oxonate</td>
<td>250</td>
<td>4.85 ± 0.03</td>
<td>0.92 ± 0.02</td>
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<tr>
<td>Allopurinol</td>
<td>10</td>
<td>1.53 ± 0.01</td>
<td>0.57 ± 0.00</td>
<td></td>
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<tr>
<td>Allo + Pot. Oxonate</td>
<td>50</td>
<td>1.63 ± 0.01</td>
<td>0.56 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.21 ± 0.01</td>
<td>0.40 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>6a + Pot. Oxonate</td>
<td>50</td>
<td>2.92 ± 0.02</td>
<td>0.59 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.56 ± 0.01</td>
<td>0.65 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>6b + Pot. Oxonate</td>
<td>50</td>
<td>2.10 ± 0.00</td>
<td>0.57 ± 0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.64 ± 0.01</td>
<td>0.48 ± 0.02</td>
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</tr>
<tr>
<td>6d + Pot. Oxonate</td>
<td>50</td>
<td>3.65 ± 0.02</td>
<td>0.71 ± 0.06</td>
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<tr>
<td></td>
<td>100</td>
<td>3.12 ± 0.02</td>
<td>0.63 ± 0.02</td>
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Table 1. Effects of thiadiazolopyrimidin-5-one derivatives (6a, 6b, 6d and 6f) and allopurinol on serum uric acid and creatinine levels in hyperuricemic and normal rats. Serum uric acid and creatinine were measured in an autoanalyzer using kits from Randox (Bangalore, India). The data were expressed as mean ± SEM.

Effect of test compounds on XO activities in rat liver

Animals treated with potassium oxonate produced a significant increase in XO enzyme activities in rat liver compared to control group. Pre-treatment of rats with the test compounds (6a, 6b, 6d and 6f) at a dose of 50 mg/kg produced significant inhibition while, at 100 mg/kg the effect was even more (Table 2). Among the test compounds
employed 6a exhibited more inhibition while, 6f was with the least effect at the doses tested (Table.2).

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Dose (mg/kg)</th>
<th>XO activity (nmol/min/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.094 ± 0.00</td>
<td>-</td>
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<tr>
<td>Potassium oxonate</td>
<td>250</td>
<td>0.118 ± 0.01</td>
<td>-</td>
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<tr>
<td>Allopurinol</td>
<td>10</td>
<td>0.052 ± 0.00</td>
<td>55.93</td>
</tr>
<tr>
<td>Allo + Pot. Oxonate</td>
<td>-</td>
<td>0.071 ± 0.01</td>
<td>39.83</td>
</tr>
<tr>
<td>6a + Pot. Oxonate</td>
<td>50</td>
<td>0.058 ± 0.00</td>
<td>50.84</td>
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<tr>
<td></td>
<td>100</td>
<td>0.038 ± 0.00</td>
<td>67.79</td>
</tr>
<tr>
<td>6b + Pot. Oxonate</td>
<td>50</td>
<td>0.072 ± 0.01</td>
<td>38.98</td>
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<tr>
<td></td>
<td>100</td>
<td>0.065 ± 0.00</td>
<td>44.91</td>
</tr>
<tr>
<td>6d + Pot. Oxonate</td>
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<td>0.068 ± 0.01</td>
<td>42.37</td>
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<td></td>
<td>100</td>
<td>0.060 ± 0.00</td>
<td>49.15</td>
</tr>
<tr>
<td>6f + Pot. Oxonate</td>
<td>50</td>
<td>0.081 ± 0.00</td>
<td>31.35</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.074 ± 0.01</td>
<td>37.28</td>
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</table>

**Table 2:** Effects of thiaadiazolopyrimidin-5-one derivatives (6a, 6b, 6d and 6f) and allopurinol on rat liver xanthine oxidase of hyperuricemic and normal rats. XO assays in liver homogenate were carried out as described in materials and method section. The data were expressed as mean ± SEM.

**Histological analyses of liver and kidney**

Histological examination of liver and kidney tissues of rats treated with oxonate developed significant hepatic damage characterized by swelling in hepatic cells with increasing granularity of the cytoplasm as compared to controls (Fig-4a). The administration of allopurinol alone showed the near normal architecture of the lobule and hepatocytes similar to the vehicle control treated rats. An ameliorative effect was obtained in hyperuricemic rats by intraperitoneal treatment with allopurinol (Fig-4a) and test compounds 6a, 6b, 6d and 6f (Fig-4b). Renal sections from the vehicle control treated rats (Fig.5a) showed normal renal architecture. While oxonate treated rats displayed
disappearance of brush border epithelial cells with shrunken renal tubules (Fig. 5a). These effects were diminished significantly in allopurinol treated and test compound treated groups (Fig. 5a-b).

**Figure 4a:** Histological observation of rat liver stained with H&E; Representative tissue sections from (A) Control, (B) Potassium oxonate (PO) (250 mg/kg), (C) Allopurinol (10 mg/kg), (D) PO + Allopurinol treated rats. The photographs were taken at 100X magnification.
Figure 4b: Histological observation of rat liver stained with H&E; Representative tissue sections from (E) PO + 6a (100 mg/kg), (F) PO + 6b (100 mg/kg), (G) PO + 6d (100 mg/kg), and (H) PO + 6f (100 mg/kg) treated rats. The photographs were taken at 100X magnification.
Figure 5a: Histological observation of rat kidney stained with H&E; Representative tissue sections from (A) Control, (B) Potassium oxonate (PO) (250 mg/kg), (C) Allopurinol (10 mg/kg), (D) PO + Allopurinol, (E) PO + 6a (100 mg/kg), (F) PO + 6b (100 mg/kg), treated rats. The photographs were taken at 200X magnification.
Figure 5b: Histological observation of rat kidney stained with H&E; Representative tissue sections from (G) PO + 6d (100 mg/kg), and (H) PO + 6f (100 mg/kg) treated rats. The photographs were taken at 200X magnification.

Discussion

Hyperuricemia is the most important biochemical basis of gout. Overproduction or under excretion of uric acid leads to hyperuricemia, which is present in 5-30% of the general population and is an increasing global concern (Shimoto et al., 2005). It is estimated that 10% of hyperuricemic patients suffer from gout (Chih-Yi Kuo et al., 2012). Clinical and experimental evidences suggest that hyperuricemia is also a predictive factor for the initiation of reno-cardiovascular diseases resulting in hypertension and renal damage (Sundstrom et al., 2005; Kanellis et al., 2003). Fig-6 summarizes the sources and excretion route for UA.
Figure 6: Crystallization of MSU occurs when uric acid levels exceed the saturation point, through inefficient elimination or excessive production of uric acid. The multiple risk factors for the formation of urate crystals are shown in yellow boxes at their sites of action. Abbreviation: MSU, monosodium urate (Rees et al., 2014)

Uric acid is generated when hypoxanthine is converted to xanthine and then to uric acid by XO (Okamoto et al., 2008). Therefore XO inhibitors are widely used against hyperuricemia. Among the XO inhibitors, allopurinol has been the most commonly used antigout agent (Riegersperger et al., 2011), although its use is associated with a number of side effects (Emmerson., 1996; Wallach et al., 1998; Wang et al., 2004). Febuxostat is also used as a XO inhibitor, but it also shows higher incidence of hepatotoxicity in clinical trials (Hair et al., 2008). Therefore, there is an urgent need to develop new XO inhibitors with least toxicity.

Previous studies have also reported XO inhibition from natural derivatives like flavonoids (Zhu et al., 2004; Lin et al., 2002), folates (Lewis et al., 1984) and lithospermic acid (Liu et al., 2008). However, none of these are potent enough to show clinical efficacy (Kumar et al., 2011). In an attempt to inhibit XO, small molecular weight inhibitors such as pyrazoles (Ishibuchi et al., 2001; Fukunari et al., 2004), isoxazoles (Wang et al., 2010), chalcones (Niu et al., 2011), triazoles (Sato et al., 2009a; 2009b), thiazolopyrimidin-5-ones (Sathisha et al., 2011), pteridines (Oettl et al., 1999), purine derivatives (Hsieh et al., 2007), isocytosine derivatives (B-Rao et al.,
2012), salicylic acid derivatives (Masuoka et al., 2004), curcumin analogues (Shen et al., 2009), oxopropylamides (Nepali et al., 2011), dihydropyrazoles (Nepali et al., 2011), triazolopyrimidinones (Tomohisa et al., 2000) and pyrazolopyrimidine (Gupta et al., 2008) also showed reasonable XO inhibition.

Mention must be made about the use of pyrimidines and ring fused derivatives that showed broad spectrum of biological activities which include antiviral, antibacterial, anticancer (Kappe et al., 2000; Krakoff et al., 1966; Rashad et al., 2011), antioxidant, immunotrophic, anti-inflammatory and membrane stabilizing properties (Krivonogov et al., 1983, 1987; Myshkin et al., 1994) and also ability to inhibit XO.

Of the twenty thiadiazolopyrimidin-5-one derivatives were synthesized in our laboratory, four of them (6a, 6b, 6d and 6f) (Fig.3) showed potent XO inhibitory activity, better than that of standard drug allopurinol (Sathisha et al., 2011). Docking studies showed important interactions of these molecules in the active site of XO (Sathisha et al., 2011).

In the present study, these compounds are used to test antihyperuricemic effects in oxonate treated rats. Potassium oxonate treated rats can serve as a useful animal model of hyperuricemia to evaluate drugs that affect serum uric acid levels (B-Rao et al., 2012; Nepali et al., 2011). Four compounds (6a, 6b, 6d and 6f) when administrated at the dose of 50 and 100 mg/kg exhibited their antihyperuricemic effect in potassium oxonate treated hyperuricemic rat model. The histopathological observations of liver (Fig.4a and 4b) and kidney (Fig.5a and 5b) sections were consistent with the biochemical changes seen in this current study. Finally, the present results clearly demonstrate the beneficial and potent antihyperuricemic effect of thiadiazolopyrimidin-5-one analogues. These effects may be mediated, in part, by inhibiting XO activity in the liver. These compounds can be further explored for the effective therapeutic potential in the treatment of hyperuricemia.
References


