



Theobromine Inhibits Uric Acid Crystallization. A Potential Application in the Treatment of Uric Acid Nephrolithiasis

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Abstract

Purpose: To assess the capacity of methylxanthines (caffeine, theophylline, theobromine and paraxanthine) to inhibit uric acid crystallization, and to evaluate their potential application in the treatment of uric acid nephrolithiasis.

Materials and Methods: The ability of methylxanthines to inhibit uric acid nucleation was assayed turbidimetrically. Crystal morphology and its modification due to the effect of theobromine were evaluated by scanning electron microscopy (SEM). The ability of theobromine to inhibit uric acid crystal growth on calculi fragments resulting from extracorporeal shock wave lithotripsy (ESWL) was evaluated using a flow system.

Results: The turbidimetric assay showed that among the studied methylxanthines, theobromine could markedly inhibit uric acid nucleation. SEM images showed that the presence of theobromine resulted in thinner uric acid crystals. Furthermore, in a flow system theobromine blocked the regrowth of post-ESWL uric acid calculi fragments.

Conclusions: Theobromine, a natural dimethylxanthine present in high amounts in cocoa, acts as an inhibitor of nucleation and crystal growth of uric acid. Therefore, theobromine may be clinically useful in the treatment of uric acid nephrolithiasis.

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Competing Interests: The authors have read the journal's policy and have the following competing interests: The authors declare that they are the inventors of a pending patent based on some aspects of this work. It is a national (Spanish) patent, which has not been yet licensed. (Patent name: La teobromina y sus derivados para el tratamiento o prevención de la litiasis renal. Patent number: P2014300819.) The authors confirm that this patent does not alter their adherence to all PLOS ONE policies on sharing data and materials.

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Introduction

Renal lithiasis is a highly prevalent condition, currently affecting about 10% of the worldwide population [1] and estimated to affect 30% by 2050 [2]. Since most renal calculi consist of calcium oxalate, some calcium oxalate crystallization inhibitors with medical application are well known, such as magnesium, citrate and phytate [3–7]. Other renal calculi consist of uric acid, but, except for one in vitro study of some glycosaminoglycans and saponins [8], no uric acid crystallization inhibitors have been described to date.

Uric acid is the final product of purine catabolism in humans. In most other mammals, such as rats and dogs, uric acid is further degraded to allantoin by the enzyme uricase [9]. In humans, a high level of urate in blood is a pathophysiological condition, which, in patients with gout, can result in the formation of monosodium urate monohydrate crystals in the synovial fluid [10].

Uric acid nephrolithiasis accounts for 7–10% of kidney stones [11–15]. This frequency varies with age and gender, affecting men

more frequently than women, and older individuals more frequently than younger persons [16,17]. The frequency also varies with geographic localization, with uric acid nephrolithiasis affecting 1% of patients with kidney stones in India, 4% in Sweden and Turkey and 17% in Germany [18–21]. The metabolic abnormality most frequently associated with uric acid nephrolithiasis is low urinary pH, followed by hyperuricosuria and low diuresis [22–25]. Furthermore, uric acid can induce calcium oxalate monohydrate nephrolithiasis through a heterogeneous nucleation mechanism [26,27]. Due to the lack of uric acid crystallization inhibitors, the treatment of patients prone to the formation of uric acid stones is based on urine alkalization, and the administration of allopurinol to patients with hyperuricemia.

Theobromine is a dimethylxanthine present in high amounts in chocolate and cocoa [28]. Theobromine has been less well studied than other natural methylxanthines (figure 1) because it stimulates the central nervous system in a lesser degree [29]. Nevertheless, theobromine consumption has health benefits, including protec-

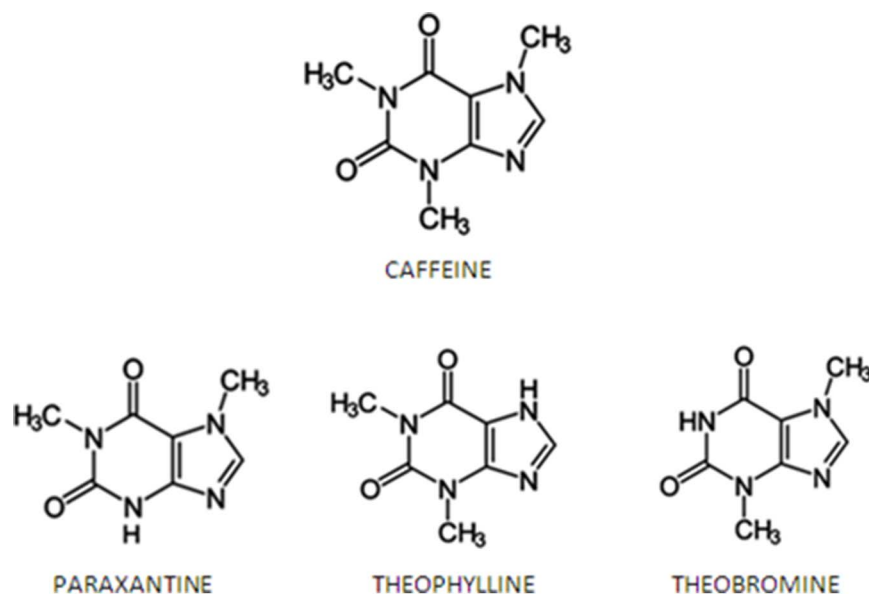


Figure 1. Chemical structure of methylxanthines: caffeine, theobromine, theophylline and paraxanthine. Caffeine is the 1, 3, 7-trimethylxanthine. The other three compounds are dimethylxanthines, which differ in the position of the two methyl groups.
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tion of the enamel surface [30] and cough suppression [31]. Furthermore, theobromine has been shown to increase plasma HDL cholesterol and decrease plasma LDL cholesterol concentrations, conferring cardiovascular protection and reducing the risk of coronary heart disease [32,33].

Studies in healthy volunteers showed that 50% of administered theobromine is recovered in urine after 8–12 h, and 100% is recovered after three days, suggesting that this compound is completely or almost completely absorbed [34]. The primary metabolites of theobromine were 3-methylxanthine, 7-methylxanthine, 7-methyluric acid and 3,7-dimethyluric acid, with 18–21% remaining unchanged [35,36].

The aim of the present work is to study the inhibitory effect of theobromine on uric acid crystallization in synthetic urine, using different *in vitro* models. Theobromine concentrations used in the present study were selected according to its normal levels in urine after consumption of theobromine.

Materials and Methods

Reagents and solutions

Uric acid, theobromine, theophylline, caffeine and paraxanthine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Synthetic urine components were obtained from Panreac (Montcada i Reixac, Barcelona, Spain). Chemicals of analytical

reagent-grade purity were dissolved in ultra-pure deionized water from a Milli-Q system and filtered through 0.45 μm pore filters before use. Uric acid stock solution was prepared daily by dissolving 1 g uric acid in 0.5 L of water with 1 M NaOH addition. Synthetic urine was prepared by mixing equal volumes of A and B solutions (Table 1), neither of which contained calcium or oxalate, thus preventing the crystallization of calcium oxalate. The pH of both solutions was adjusted depending on the experiment.

Turbidimetric assay

Uric acid crystal formation in synthetic urine and the effects of potential crystallization inhibitors were assessed using a kinetic turbidimetric system, consisting of a photometer (Metrohm 662) equipped with a fiber-optic light-guide measuring cell with an attached light path 2 \times 10 mm reflector and monochromatic light (550 nm). Crystallization was assessed at constant temperature (37°C) with magnetic stirring.

Synthetic urine solutions were adjusted to pH 5.40, and 2 g/L uric acid solution was adjusted to pH 10.70. Synthetic urine (160 mL) was added to a crystallization flask, followed by 40 mL uric acid solution. When the resulting solution reached a temperature of 37°C, 1.4–1.6 mL HCl was added to achieve the desired supersaturation of uric acid (pH 4.40–4.70), and the timer was switched on. These pH values were selected in order to have a

Table 1. Composition of synthetic urine.*

Solution A (mM)		Solution B (mM)	
Na ₂ SO ₄ · 10H ₂ O	19.34	NaH ₂ PO ₄ · 2H ₂ O	15.45
MgSO ₄ · 7H ₂ O	5.93	Na ₂ HPO ₄ · 12H ₂ O	15.64
NH ₄ Cl	86.73	NaCl	223.08
KCl	162.60		

* Synthetic urine was obtained by mixing equal volumes of solutions A and B.
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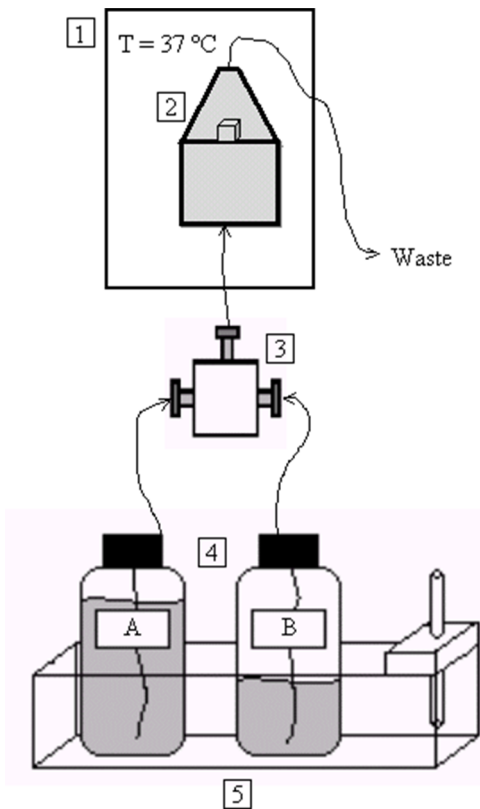


Figure 2. Diagram of the experimental flow device used to assess uric acid renal calculi regrowth. The device shown here is used to study the regrowth of post ESWL fragments of uric acid calculi. 1. Temperature-controlled chamber; 2. Chamber containing post-ESWL uric acid calculi fragments solutions; 3. Three-way T mixing chamber for solutions A and B; 4. Uric acid (B) and synthetic urine (A) solutions; 5. Temperature controller.

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short crystallization time (6–10 minutes without inhibitor). The pH of the final solution was measured at the beginning of each experiment. The absorbance of the solution (550 nm) was measured every two minutes, until the end of the kinetic assay.

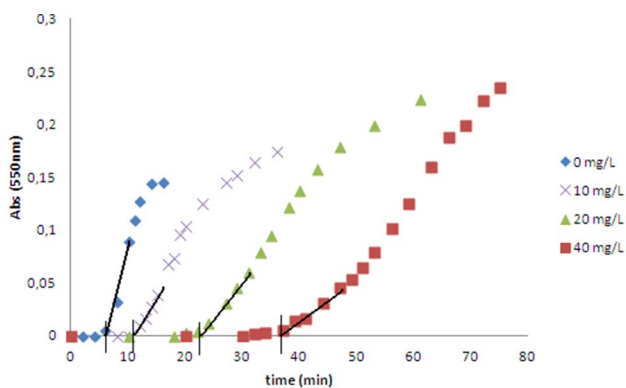


Figure 3. Kinetic curves for uric acid crystallization. The curves were recorded for uric acid crystallization using a turbidimeter in synthetic urine, without and with 10, 20 and 40 mg/L theobromine at pH 4.65. The calculation of induction time is represented by a solid black line.

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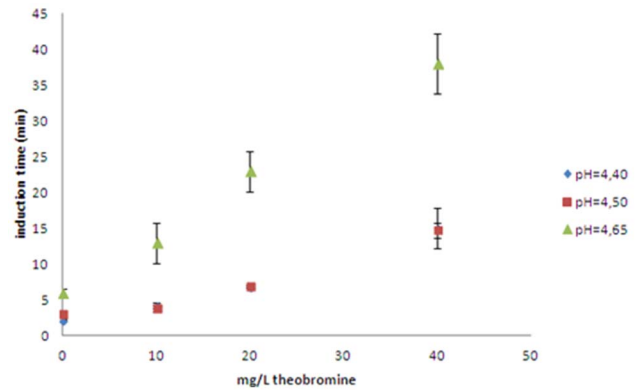


Figure 4. Uric acid crystallization induction times (mean \pm SD) in synthetic urine. The induction times were calculated from the crystallization curves, recorded turbidimetrically, at four theobromine concentrations (0, 10, 20 and 40 mg/L), and three pH values (4.40, 4.50 and 4.65). The results are expressed as mean of duplicates and standard deviation.

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Caffeine, paraxanthine and theophylline were assayed at 40 mg/L, and theobromine at concentrations of 10, 20 and 40 mg/L, each at three different pHs (4.40, 4.50 and 4.65). Each experiment was repeated twice in order to assess its reproducibility. In some cases, the crystals formed during the turbidimetric assay were filtered through a 0.45 μ m pore filter, dried in a desiccator, and examined by scanning electron microscopy.

Post-extracorporeal shock wave lithotripsy regrowth fragments

Fifteen post extracorporeal shock wave lithotripsy (ESWL) fragments of uric acid calculi, of similar weight and morphology, were obtained from a single patient. This study was a retrospective evaluation of a clinical patient sample. The volunteer had provided written informed consent for its clinical samples to be used in scientific studies. This study was approved by the “Comitè d’Ètica de la Recerca (CER)” of the University of the Balearic Islands.

The fragments were not pre-treated and were placed in a hermetic flow chamber. This chamber was placed in a larger, temperature-controlled (37°C) chamber, and incubated for 48 h. The experimental flow device used (Figure 2) was similar to that used to study the regrowth of calcium oxalate dihydrate (COD) crystals on COD calculi [37,38] and calcium oxalate monohydrate crystals on uric acid calculi [27]. Using a multichannel peristaltic pump, freshly prepared synthetic urine (pH 3.00), with or without theobromine (10 or 20 mg/L), was introduced into the flow chamber at a rate of 600 mL/day. Using a second pump, 2 g/L uric acid (pH 10.70) was introduced into the flow chamber at a rate of 150 mL/day. Both solutions were at 37°C and were mixed in a three-way T mixing chamber. Thus, 750 mL of synthetic urine every 24 hours passed through the chamber, which is approximately the volume of urine that passed through one kidney per day. We measured the pH of the waste solution, after passing through the stone fragments, to check that it was the same pH value in all the experiments.

The calculi were dried in a desiccator before and after each experiment until they reached constant weight, as determined using a precision balance, and fragment growth was evaluated by measuring the difference in weight. Mean growth rates were determined, and fragment growth was standardized by calculating

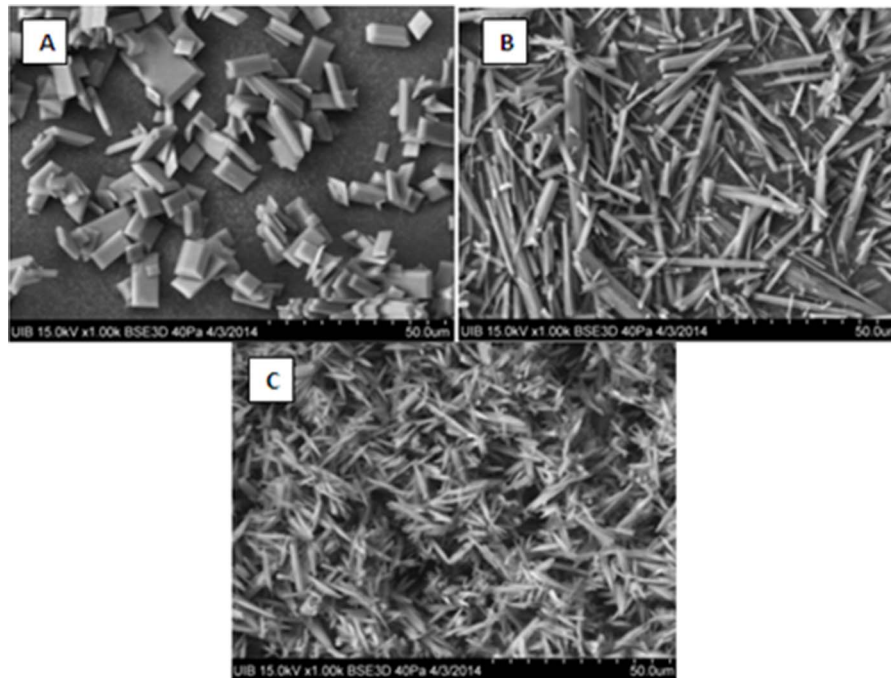


Figure 5. Scanning electron microscopy of uric acid crystals. After finishing the turbidimetric assay, some of the solutions were filtered through a 45 μm filter, and uric acid crystals were observed by scanning electron microscopy. These images correspond to uric acid crystals formed at pH = 4.65, in the absence (A) and presence of 20 mg/L (B) and 40 mg/L (C) theobromine. doi:10.1371/journal.pone.0111184.g005

the relative mass increase, thus avoiding the effects of surface area on growth rate.

The morphology of the growing calculi was assessed by scanning electron microscopy.

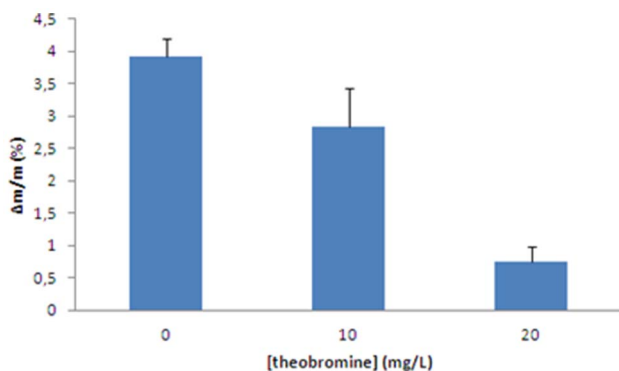


Figure 6. Increased relative weight of post-ESWL uric acid calculi fragments. The fragments were placed into a temperature-controlled flow chamber and incubated for 48 h. A constant flow of 400 mg/L uric acid in synthetic urine, with 0, 10 and 20 mg/L theobromine, passed through the flow chamber containing the calculus fragment. A total volume of 750 mL synthetic urine passed every 24 hours, which is approximately the volume that passes through one kidney per day. The calculi fragments were weight before and after the experiment, and the relative weight increase was calculated for each fragment. In this figure, the relative weight variation are represented as a mean \pm SEM for 5 calculi fragments. doi:10.1371/journal.pone.0111184.g006

Results

Figure 3 shows the kinetic curves for the formation of uric acid crystals at three theobromine concentrations, with the induction time for each concentration calculated as shown. Caffeine, theophylline and paraxanthine at concentrations of 40 mg/L did not alter the induction time. Thus, the effects of theobromine on uric acid crystallization were assessed at different pHs and different theobromine concentrations. Induction time increased as pH and theobromine concentration increased (Figure 4). Scanning electron microscopy of crystals formed at pH 4.65 showed that theobromine inhibited uric acid crystallization in a concentration-dependent manner (Figure 5). Moreover, the uric acid crystals were thinner and longer in the presence than in the absence of theobromine. We selected the crystals formed at pH 4.65 because, as the induction time was longer, the crystals were formed slower, leading to bigger crystals, allowing us to see better in which faces the inhibitor acts.

Figure 6 shows the effects of theobromine on the regrowth of post-ESWL uric acid calculi when incubated in a flow chamber for 48 h with 400 mg/L of uric acid in synthetic urine. Theobromine showed a concentration dependent inhibition of calculi regrowth. The pH of the waste solution was around 5.50, which is a common urinary pH value in uric acid stone former patients. Moreover, scanning electron microscopy of these calculi fragments showed that the new uric acid crystals were smaller in the presence than in the absence of theobromine (Figure 7).

Discussion

The results presented here indicate that, in vitro, theobromine can markedly inhibit uric acid crystallization. Turbidimetric experiments showed that theobromine can inhibit uric acid nucleation, since the delay in induction time was dependent on

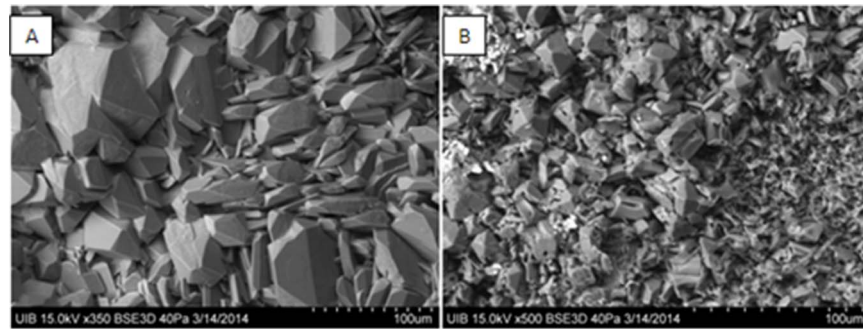


Figure 7. Scanning electron microscopy of uric acid crystal formation on a post-ESWL uric acid calculus fragment. The fragments were incubated in a temperature-controlled chamber during 48 h with a constant flow of 400 mg/L uric acid in synthetic urine, without (A) and with (B) 20 mg/L theobromine.

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theobromine concentration. In contrast, caffeine, theophylline and paraxanthine had no effects. This was surprising, inasmuch as their structures are very similar. However, crystallization inhibitors are extremely selective, such that slight modifications in chemical structure can render inhibitors ineffective.

Theobromine concentrations used in this study were similar to those observed in urine after consumption of theobromine [39–41]. Thus, following the administration of 300 mg theobromine, 60 mg will be excreted unchanged in the urine, resulting in urinary theobromine concentrations similar to those used in this study.

The theobromine-induced delay in induction time was higher at higher pH, due to the decreased supersaturation of uric acid at increased pH. Thus, the effect of theobromine at pHs between 5 and 5.5 (common urinary pHs values in uric acid stone former patients) will be even more important, since the supersaturation of uric acid decreases when urinary pH increases. The increased effect of theobromine at higher pH suggests that urinary alkalinization combined with theobromine may be effective clinically.

Theobromine also altered the morphology of uric acid crystals (figure 8), making them longer and thinner. Theobromine may inhibit growth at only one of the faces of the crystal, (210), but not at the others, (001) and (201). As uric acid and theobromine molecules have very similar structural patterns, it could be admitted that theobromine can substitute uric acid molecules in the corresponding uric acid crystals. The incorporation of this molecule to the uric acid crystal lattice will modify the structure of some layers that, as consequence, will increase their energy, decreasing thus their growth rate. So, it is very likely to think that the face (210) is affected by the incorporation of theobromine to the uric acid crystal lattice.

Using post-ESWL uric acid fragments, we found that theobromine also inhibits the regrowth of these calculi in a concentration dependent manner. Furthermore, the crystals formed on the surface of the uric acid were smaller in the presence than in the absence of theobromine. Thus, theobromine may be useful

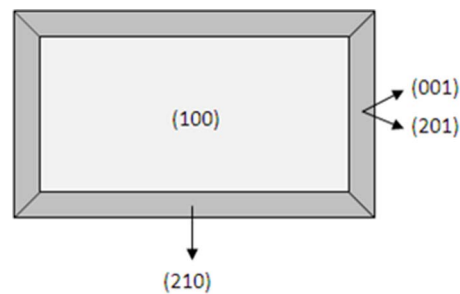


Figure 8. Morphology of an anhydrous uric acid crystal with its crystallographic planes. This morphology is the same that can be observed at the scanning electron microscopy when in vitro uric acid crystals are formed in the absence of admixtures (figure 5A). Due to the morphology of the uric acid crystals formed in the presence of theobromine in synthetic urine (figures 5B and 5C), we can conclude that the inhibited face is (210), which results in thinner and longer uric acid crystals.

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clinically in preventing the regrowth of uric acid calculi fragments after ESWL.

The experiments described in this paper were all performed in synthetic urine. The morphology of the crystals showed in this paper may change in real urine, due to the presence of other substances in urine, such as proteins.

In conclusion, theobromine acts as a uric acid crystallization inhibitor, with high clinical potential in the treatment and prevention of uric acid nephrolithiasis. Clinical trials are necessary to assess its inhibitory capacity in vivo.

Author Contributions

Conceived and designed the experiments: FG AR ACB. Performed the experiments: AR. Analyzed the data: AR. Wrote the paper: FG AR ACB.

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