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Renierol from marine sponge Haliclona.SP.: A natural inhibitor of xanthine oxidase with hypouricemic effects

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Abstract

The purpose of this study was to evaluate the inhibitory effect of renierol, extracted from marine sponge Halicdona.SP., on xanthine oxidase (XO) and its hypouricemic effect *in vivo*. Renierol and a positive control, allopurinol, were tested for their effects on XO activity by measuring the formation of uric acid and superoxide radical from xanthine. Renierol inhibited XO in a concentration-dependent and competitive manner. IC_{50} value was 1.85 $\mu g \cdot ml^{-1}$ through the measuring of uric acid and was 1.36 $\mu g.ml^{-1}$ through the measuring of superoxide radical. Renierol was found to have an *in vivo* hypouricemic activity against potassium oxonate-induced hyperuricaemia in mice. After oral administration of renierol at doses of 10, 20 and 30 $mg.kg^{-1}$, there was a significant decrease in the serum urate level (4.08 ± 0.09 $mg.dl^{-1}$, $P < 0.01$), (3.47 ± 0.11 $mg.dl^{-1}$, $P < 0.01$) and (3.12 ± 0.08 $mg.dl^{-1}$, $P < 0.01$), when compared to the hyperuricaemic control (6.74 ± 0.23 $mg.dl^{-1}$). Renierol was a potent XO inhibitor with hypouricemic activity in mice.

Keywords: *Renierol, Allopurinol, xanthine oxidase, hypouricemic activity*

Introduction

Xanthine oxidase (XO) is a highly versatile enzyme, which is widely distributed among species and within various mammals [1]. It catalyzes the oxidation of hypoxanthine and xanthine to uric acid, yielding superoxide radical (O_2^-). The active form of XO is that of a homodimer of molecular weight 290 Kd with each monomer independently catalyzing the reaction. Each subunit contains one molybdopterin cofactor, two distinct [2Fe-2S] centers, and one FAD cofactor. The co-crystal structure of salicylate-xanthine oxidase complex was first reported by Enroth et al.[2].

XO is considered to be an important biological source of reactive oxygen species (ROS), which induce oxidative stress and are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging [3]. It is also known that an extensive metabolism of xanthine by XO will increase body uric acid levels. The abnormal amount of uric acid in the body results in the deposition of urate crystals in the joints and kidneys, causing inflammation as well as gouty arthritis and uric acid nephrolithiasis, [4–6] which suggests that a compound inhibiting XO could have a benificical effect not only on the treatment of hyperurieaemia and gout, but also on the alleviation of inflammation.

The aim of this study was to evaluate the inhibitory activity of renierol (Figure 1) on XO *in vivo* through measuring the formation of uric acid and superoxide radical and hypouricemic effects *in vivo*.

Materials and methods

Reagents

Xanthine, allopurinol, Nitro Blue Btetrazolium, potassium oxonate, β-nicotinamide-adenine dinucleotide (β-NADH), phenazine methosulfate (PMS) and superoxide dismutase were purchased from

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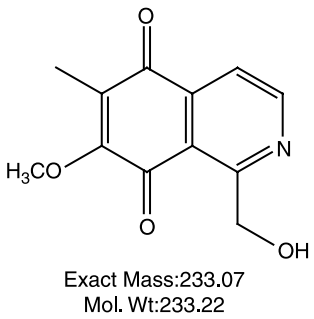

Figure 1. Structure of renierol.

Sigma. XO was purchased from Roche and other analytical reagents were made in China.

Animals

Male ICR mice (26–30 g) were purchased from the Laboratory Animal Center (Shanghai, China) and maintained on a 12-h light/dark cycle in a temperature-and humidity-controlled room for 1 week prior to the experiment.

XO inhibitory activity through measuring the formation of uric acid

The XO inhibitory activity was assayed spectrophotometrically at 295 nm under aerobic condition as previously described [7–9]. The reaction mixture contained 80 mM sodium pyrophosphate buffer (PH7.5), 120 M xanthine, 0.1 U XO, with or without renierol (100 μL, dissolved in DMSO in various concentrations). Allopurinol was used as a positive control.

The reaction was started by addition of XO. The formation of uric acid was detected by the absorption increments at 295 nm.

XO inhibitory activity through measuring the formation of superoxide radical

The reaction mixtures contained the same proportion of components as those in the measurement of the uric acid. Superoxide was detected by the reduction of nitroblue tetrazolium (NBT) at 100 μM, followed spectrophotometrically at 560 nm[10].

Additionally, this procedure was repeated with different concentrations of xanthine (60, 120, 180, 240 and 300 μM). The Lineweaver-Burk plot was used to evaluate the type of inhibition. Renierol was assayed for XO inhibitory activity at the concentrations of 1.2, 2.4 and 3.6 $\mu g.mL^{-1}$. IC_{50} values were calculated by linear regression analysis.

Direct superoxide radicals scavenging effect of renierol

The antiradical activity of renierol was determined according to previous reports [11–13], in which superoxide radicals were generated in the system β-NADH/PMS (NBT 25 μM, MS 10 μM, β-NADH 156 μM). The reaction was conducted at room temperature for 2 min and initiated by the addition of PMS (SOD as positive control).

Hypouricemic effect on potassium oxonate-treated mice

The mice were divided into six groups (n = 10): Group I, control group; Group II, hyperuricemic control; Group III-IV respectively received renierol of 10 $mg.kg^{-1}$, 20 $mg.kg^{-1}$ and 30 $mg.kg^{-1}$ and reference drug allopurinol (10 $mg.kg^{-1}$) orally. The uricase inhibitor potassium oxonate was used to induce hypouricemia in mice as described previously[14,15]. Briefly, the mice were injected intraperitoneally with potassium oxonate (250 $mg.kg^{-1}$) 1 h before the administration of the tested drug to increase serum urate. The blood was obtained from the mice via tail tip cuts 1 h after the drug administration. Uric acid levels were determined by the phosphotungstic acid method. All procedures were carried out in accordance with the Chinese legislation on the use and care of laboratory animals and were approved by the respective University Committees for Animal Experi ments.

Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M) for each group. They were analyzed for the significance of intergroup differences utilizing two-tailed, paired Student's-tests and $P < 0.05$ was considered as significant.

Results

XO inhibitory activity through measuring the formation of uric acid

Renierol potently inhibited the formation of uric acid under the assay condition with an IC_{50} at 1.85 $\mu g.mL^{-1}$ (Figure 2). To further characterize the binding region on XO, Lineweaver-Burk double reciprocal plots were done as shown in Figure 3. Renierol was exhibited to be a competitive inhibitor to XO.

XO inhibitory activity through measuring the formation of superoxide radical

Renierol inhibited the formation of superoxide radical under the assay conditions with an IC_{50} at 1.36 $\mu g.mL^{-1}$ (Figure 4). A Lineweaver-Burk double reciprocal showed that renierol was a competitive

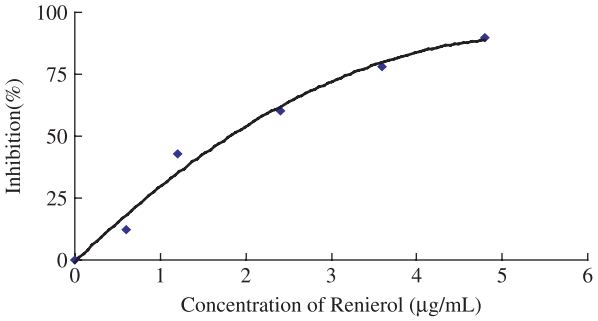

Figure 2. Effect of renierol on XO through measuring the formation of uric acid. Values showed mean ± SE from four experiments performed in triplicate.

inhibitor to XO (Figure 5), which was consistent with that through detecting the formation of uric acid.

Direct superoxide radicals scavenging effect

Superoxide radicals were generated in the β-NADH/PMS system but not in the xanthine/XO system, showing that renierol had no direct effect on the scavenging of superoxide radicals. However, the positive control SOD scavenged the superoxide radicals effectively under this condition (no date shown).

In vivo hypouricemic activity

The effects of potassium oxonate on the serum uric acid levels in mice are shown in Table I. The uric acid levels in non-hyperuricemic, vehicle-treated mice were $1.28 \pm 0.06\,mg.dL^{-1}$. Intraperitoneal injection of potassium oxonate markedly increased the serum uric acid levels ($6.74 \pm 0.23\,mg.mL^{-1}$). The animals treated with renierol ($10\,mg.kg^{-1}$, $20\,mg.kg^{-1}$ and $30\,mg.kg^{-1}$) showed a significant decrease in the serum urate levels down to $4.08 \pm 0.09\,mg.dL^{-1}$ ($P < 0.01$), $3.47 \pm 0.11\,mg.dL^{-1}$ ($P < 0.01$) and $3.12 \pm 0.08\,mg.dL^{-1}$ ($P < 0.01$), when compared to the hyperuricemic control. The reference drug allopurinol also significantly reduced the serum uric acid levels ($3.03 \pm 0.12\,mg.dL^{-1}$) ($P < 0.01$).

Discussion

XO oxidizes oxypurines (hypoxanthine and xanthine) to uric acid in the purine catabolic pathway (Figure 6).

The XO inhibitory activity could be detected through measuring the formation of uric acid or superoxide radicals. Whereas uric acid can be measured by spectrometer, superoxide radicals cannot be detected directly but indirectly through reaction with NBT. The inhibitory effect on XO activity was detected through measuring the formation of uric acid, because the inhibitor usually cannot react to uric acid. The formation of uric acid was significantly reduced through inhibiting XO activity. The superoxide radical is another product of XO and measuring the formation of superoxide radicals can also reflect the XO activity. In the present study, the inhibition of renierol on XO was detected through measuring the formation of uric acid and superoxide radicals. The IC_{50} value by measuring the formation of uric acid ($1.85\,\mu g{\cdot}mL^{-1}$) coincided with that by measuring the formation of superoxide radicals ($1.36\,\mu g{\cdot}mL^{-1}$), implying that renierol can only inhibit XO, and cannot scavenge the superoxide radicals directly; otherwise, the value of IC_{50} detected through measuring the formation of superoxide radicals should

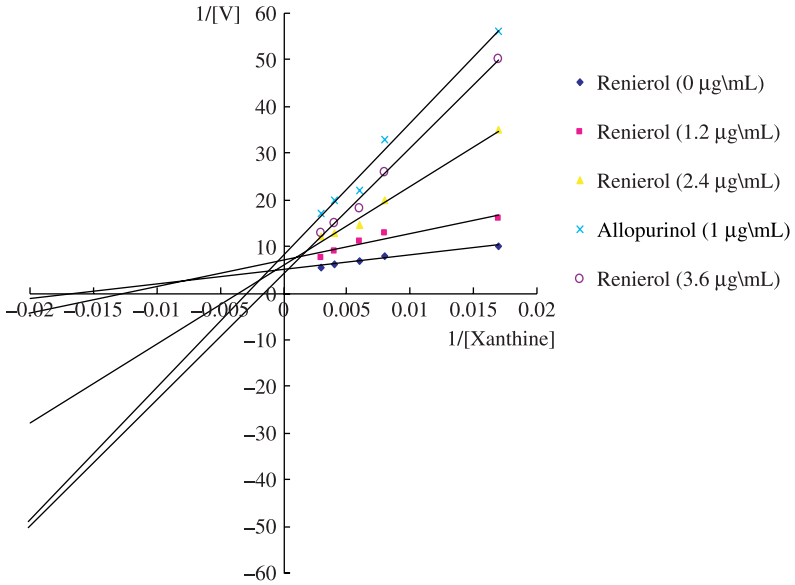

Figure 3. Lineweaver-Burk plots of the inhibition of XO. Enzyme activity was measured using 60,120,180, 240 and 300 μM of xanthine as a substrate. The date represented the mean ± SE from three experiment performed in triplicate.

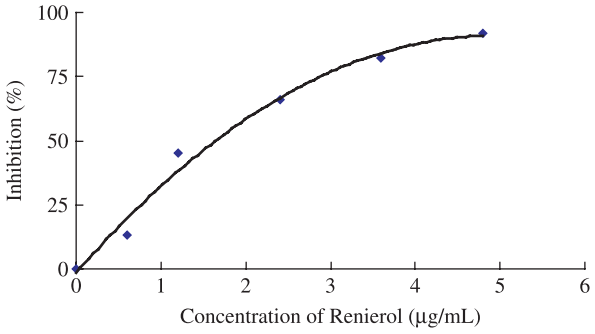

Figure 4. Effect of renierol on XO activity through measuring the formation of superoxide radical. Values showed mean ± SE from four experiments performed in triplicate.

have been much smaller than that detected through measuring the formation of uric acid. To confirm that renierol cannot scavenge superoxide radicals directly, we adopted the β-NADH/PMS system to produce superoxide radicals. Superoxide radicals were generated in the β-NADH/PMS system but not in the xanthine/XO system, showing that renierol had no direct scavenging effect on superoxide radicals.

There are much enzyme inhibitors found in marine sponge, such as acetylcholinesterase inhibitor, nitric oxide synthase selective inhibitor, sialidase inhibitor, etc, [16–18] but there have been few studies reporting the inhibitory effect on XO. To our knowledge, this is the first report about the inhibitor effect of renierol on XO. XO catalyzes the oxidation of hypoxanthine and xanthine to uric acid. The overproduction of this acid may lead to hyperuricemia such as that in gout [19]. One of the treatments for gout is the use of an XO inhibitor to block the production of uric acid. Allopurinol is the most common, and perhaps the only inhibitor of XO being used in clinical practice [20,21], but allopurinol has severe adverse effects in some patients, including hepatitis, nephropathy and allergic reactions[22]. Therefore, there is an urgent need to search for new XO inhibitors.

In this study, we found that renierol was a potent inhibitor of XO and the IC_{50} values were 1.85 $\mu g \cdot mL^{-1}$ and 1.36 $\mu g \cdot mL^{-1}$ through the two different methods. Compared to allopurinol (IC_{50} = 1.0 $\mu g \cdot mL^{-1}$), the inhibitory effect of renierol is commensurate with that of allopurinol. We also found

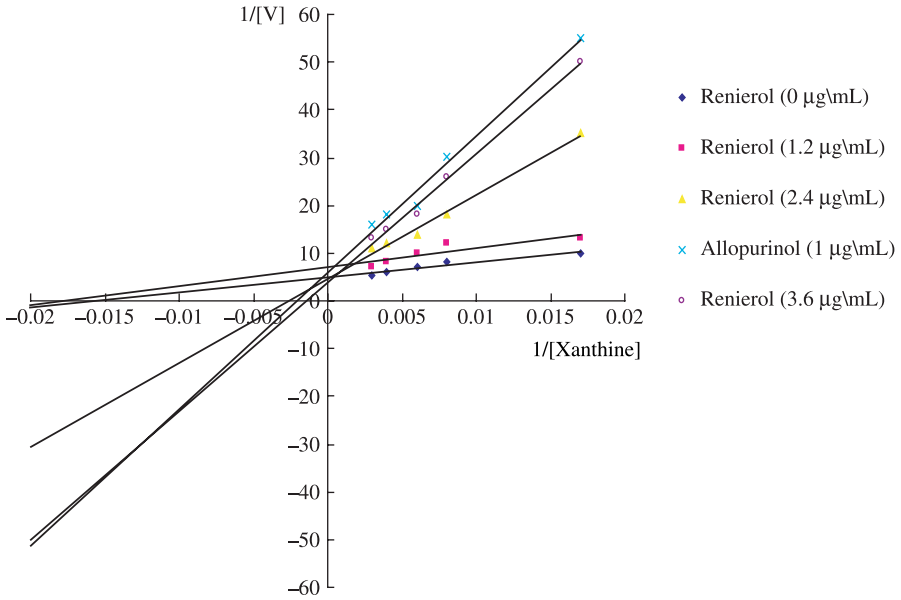

Figure 5. Lineweaver-Burk plots of the inhibition of XO. Enzyme activity was measured using 60, 120, 180, 240 and 300 μM of xanthine as a substrate. The data represented the mean ± SE from three experiments performed in triplicate.

Table I. Effect of renierol on serum urate level in oxonate hyperuricemia in mice.

Treatment	Dose(mg.kg^{-1})	Serum urate levels(mg.dL^{-1})
Control		1.28 ± 0.06
Hyperuricemic control (potassium oxonate)		6.74 ± 0.23[a]
Renierol	10	4.08 ± 0.09[b]
	20	3.47 ± 0.11[b]
	30	3.12 ± 0.08[b]
Allopurinol	10	3.03 ± 0.12[b]

Values were mean ± S.E.M. (n = 10). [a]$P < 0.01$ *vs* control, [b]$P < 0.01$ *vs* hyperuricemic control. All drugs were given orally except potassium oxonate injected i.p.

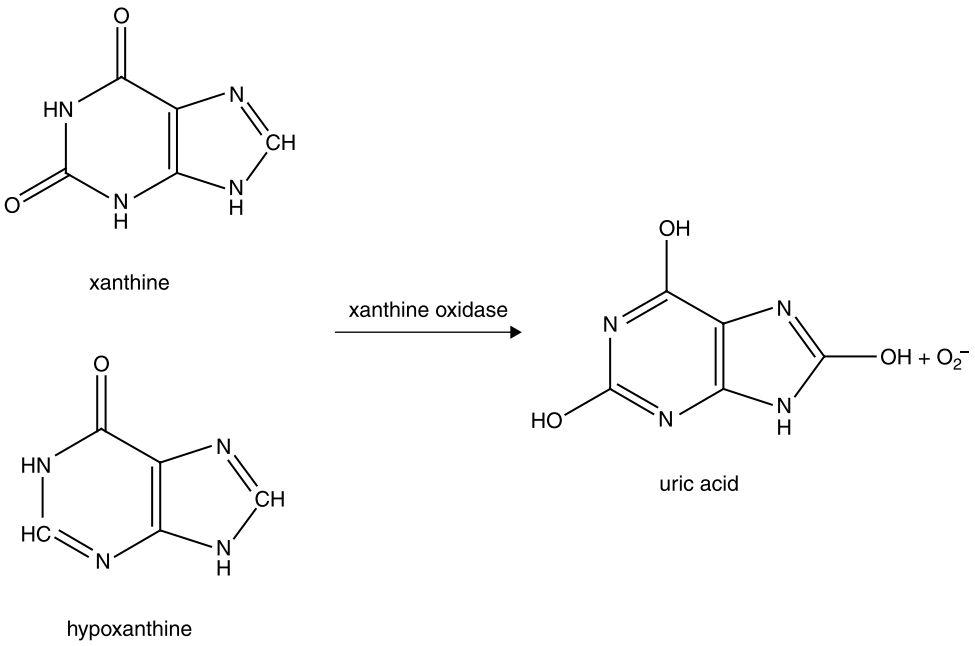

Figure 6. Catalytic reaction of XO.

that renierol significantly and in a dose-dependent manner, prevented the increase of serum uric acid levels induced by potassium oxonate. The hypouricamic activity *in vivo* may be related to the XO inhibition effect *in vitro*.

In conclusion, through measuring the formation of uric acid or superoxide radicals, renierol is found to be an effective inhibitor of XO *in vitro*, which is commensurate with allopurinol. Renierol cannot scavenge the superoxide radicals directly and reduce the serum uric acid levels *in vivo* in potassium oxonate-pretreated mice.

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