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# 3,4-Dihydroxy-5-nitrobenzaldehyde (DHNB) is a potent inhibitor of xanthine oxidase: A potential therapeutic agent for treatment of hyperuricemia and gout

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ABSTRACT

Hyperuricemia, excess of uric acid in the blood, is a clinical problem that causes gout and is also considered a risk factor for cardiovascular disease. The enzyme xanthine oxidase (XO) produces uric acid during the purine metabolism; therefore, discovering novel XO inhibitors is an important strategy to develop an effective therapy for hyperuricemia and gout. We found that 3,4-dihydroxy-5-nitrobenzaldehyde (DHNB), a derivative of the natural substance protocatechuic aldehyde, potently inhibited XO activity with an $IC_{50}$ value of 3 μM. DHNB inhibited XO activity in a time-dependent manner, which was similar to that of allopurinol, a clinical XO inhibitory drug. DHNB displayed potent mixed-type inhibition of the activity of XO, and showed an additive effect with allopurinol at the low concentration. Structure–activity relationship studies of DHNB indicated that the aldehyde moiety, the catechol moiety, and nitration at C-5 were required for XO inhibition. DHNB interacted with the molybdenum center of XO and was slowly converted to its carboxylic acid at a rate of $10^{-10}$ mol/L/s. In addition, DHNB directly scavenged free radical DPPH and ROS, including $ONOO^-$ and HOCl. DHNB effectively reduced serum uric acid levels in allantoxanamide-induced hyperuricemic mice. Furthermore, mice orally given a large dose (500 mg/kg) of DHNB did not show any side effects, while 42% of allopurinol (500 mg/kg)-treated mice died and their offspring lost their fur. Thus, DHNB could be an outstanding candidate for a novel XO inhibitory drug that has potent activity and low toxicity, as well as antioxidant activity and a distinct chemical structure from allopurinol.



## 1. Introduction

Uric acid is a product of purine catabolism and is excreted in the urine. Overproduction or under excretion of uric acid in humans could lead to hyperuricemia and gout, which is caused by crystallization and deposition of uric acid in joints and surrounding tissues [1]. Unlike most other mammals, humans lack the enzyme uricase [2], which converts uric acid to the more soluble allantoin [1]. Gout affects 1–2% of adults in developed countries and represents the most common case of inflammatory arthritis in men [1,3]. Furthermore, hyperuricemia and gout are also associated with chronic diseases such as hypertension, diabetes mellitus, metabolic syndrome, and renal and cardiovascular disease [1].

Xanthine oxidase (XO) is a form of the molybdoflavin protein xanthine oxidoreductase (XOR) [4] that plays an important role in the catabolism of purines in humans. XO first catalyzes the oxidation of hypoxanthine to xanthine, and then catalyzes the oxidation of xanthine to uric acid [4,5]. Because overproduction of uric acid is the primary cause of hyperuricemia [1], XO is considered the most promising target for therapeutic treatment of this condition. Currently, the drugs allopurinol and febuxostat are available to reduce serum uric acid levels by inhibiting XO. Allopurinol is the most commonly used therapy for chronic gout and has been used clinically for more than 40 years. However, allopurinol cannot be used when the patient is hypersensitive or intolerant to the drug, or when the treatment fails. While rare, allopurinol has life-threatening side effects such as hypersensitivity syndrome consisting of fever, skin rash, eosinophilia, hepatitis, and renal toxicity, for which the mortality rate approaches 20% [1]. More recently, febuxostat, a new non-purine XO inhibitor, has been approved for the management of gout in the European Union and USA [6]. Many side effects of febuxostat have been reported [7]. Both allopurinol and febuxostat are not recommended for the treatment of asymptomatic hyperuricemia because of concern for their potential side effects [8–10]. Beyond gout, recent studies have indicated that asymptomatic hyperuricemia is associated with or may have a causal relationship with cardiovascular disease [11]. New XO inhibitors with more specific effects and fewer side effects than allopurinol and

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febuxostat are needed for preventing and treating gout and cardiovascular disease associated with hyperuricemia.

Recent studies have searched for XO inhibitors in natural compounds ranging from flavonoids to a host of natural plant products [12–19]. For example, nitro-oleic acid inhibits purine oxidation more potently than allopurinol does [14]. Caffeic acid shows variable XO inhibitory effects [20–24]. In this study, we intended to develop a novel XO inhibitor that is derived from natural substances, has potent activity and low toxicity, and whose chemical structure is distinct from allopurinol and febuxostat. Our goal was to have a novel XO inhibitor that could be used to treat gout patients that failed to allopurinol and febuxostat therapies, or had hypersensitivity or intolerance to these drugs. In addition, we wanted a drug that could be used in combination with allopurinol or febuxostat in order to increase therapeutic efficacy and reduce the potential toxicity of these drugs. Furthermore, we wanted a drug that could be used to treat patients with asymptomatic hyperuricemia to prevent cardiovascular disease and other hyperuricemia-associated diseases.

Protocatechuic aldehyde can be obtained from plants such as *Salvia miltiorrhiza Bunge*, and leaves of *Stenoloma chusanum* (L.) *Ching* and *Ilex chinensis Sims*. Protocatechuic aldehyde has an anti-inflammatory effect and increases coronary artery blood flow [5]. It is the most effective component of the leaves of *Ilex chinensis Sims* for treating angina, and of *Herb of Common Edelweiss* for treating nephritis. Protocatechuic aldehyde is an important intermediate in the synthesis of various antibiotics and anti-inflammatory drugs. In the present study, we explored the inhibitory effects of 14 catechol compounds on XO activity, and found that protocatechuic aldehyde has limited inhibitory activity. However, its 5-nitro derivative, 3,4-dihydroxy-5-nitrobenzaldehyde (DHNB), is a potent XO inhibitor in a cell-free system. In this study, we determined the potency and potential mechanism of XO inhibition by DHNB in a cell-free system and in a mouse model of hyperuricemia, as well as its toxicity *in vivo*. DHNB could be a potential novel therapeutic agent for the treatment of gout and hyperuricemia.

## 2. Materials and methods

### 2.1. Chemicals and reagents

XO from bovine milk, xanthine, allopurinol, 3,4-dihydroxybenzaldehyde (DHB-CHO), gallic acid, phosphate buffered saline (PBS) solution, potassium nitrite ($KNO_2$), polyethylene glycol 400, sodium carboxymethyl cellulose (CMC-Na), sodium phosphotungstate hydrate, dioxide manganese ($MnO_2$), diethylene-triamine-pentaacetic acid (DTPA), EDTA, ferrous ammonium sulfate, hydrogen peroxide ($H_2O_2$), sodium hypochlorite, DPPH, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sodium borohydride, potassium persulfate, ascorbic acid and (±)-α-tocopherol were obtained from Sigma Chemical Co (Saint Louis, MO). 3,4-Dihydroxy-5-nitrobenzaldehyde (DHNB), 3,4-dimethoxybenzyl alcohol (DMB-$CH_2OH$), 3,4-dihydroxyphenyl ethanol (hydroxytyrosol), caffeic acid, 3,4-dihydroxybenzyl alcohol (DHBA), 3,4,5-trihydroxybenzaldehyde hydrate (THB-CHO), 4-hydroxy-3-methoxybenzyl alcohol, vanillin, 3,4-dihydroxybenzoic acid (DHB-COOH) and dihydrorhodamine were obtained from VWR Inc (Radnor, PA). 3,4-Dihydroxy-6-nitrobenzaldehyde (DH6NB) was obtained from Oakwood Products Inc (West Columbia, SC). Entacapone was obtained from Toronto Research Chemicals (Ontario, Canada). 3,4,5-Trihydroxybenzyl alcohol (THB-$CH_2OH$) and 3,4-dihydroxy-5-nitrobenzyl alcohol (DHNB-$CH_2OH$) were prepared by reducing 3,4,5-trihydroxybenzaldehyde and 3,4-dihydroxy-5-nitrobenzaldehyde with sodium borohydride, respectively, according to the literature [25]. Allantoxanamide (purity >98% by HPLC) was purchased from Nanjing Chemlin Chemical Industry Co., Ltd., Nanjing, China.

### 2.2. XO inhibition assay

XO activity was determined using the method of continuous spectrophotometric rate measurements. The reaction mixture contained uric acid in 67 mM phosphate buffer (pH 7.4) and 20 nM XO with an activity of 5 mU/mL, with or without DHNB derivatives. After pre-incubating the mixture for 1–5 min at 25 °C, 50 μM xanthine was added to initiate the formation of uric acid, and the increase of absorption of uric acid at 295 nm was monitored. Allopurinol was used as a positive control. For the enzyme kinetic analysis, relatively low concentrations of xanthine were used. All test compounds, including allopurinol, were dissolved in $H_2O$ or in an aqueous solution, so $H_2O$ was used as the negative control.

### 2.3. Conversion of DHNB to products by XO

The kinetic reaction of DHNB with XO at different pH values was measured spectrophotometrically. The decay of DHNB was monitored at 327 nm in a system containing 30 nM XO with 30 μM DHNB in phosphate buffer, pH 6.5–8.5. The extinction coefficient of DHNB at 327 nm was measured as 15,600 $M^{-1}$ $cm^{-1}$. Samples for product analysis by mass spectroscopy and HPLC were prepared by mixing 0.3 U XO with 4 mg DHNB in 1 mL phosphate buffer (pH 7.4) for 3 days. The DHNB/XO samples were analyzed by HPLC (Bio-Rad BioLogic DuoFlow, Hercules, CA) equipped with a 250 × 4.6 mm, 5 μm Phenomenex C-18 (2) Luna column, with a mobile phase of 40% acetonitrile/water. DHNB and its product were monitored by the optical absorption at 327 nm and 279 nm, respectively.

### 2.4. Ultra performance liquid chromatography/mass spectroscopy (UPLC/MS)

Negative electrospray ionization-mass spectrometry (ESI-MS) and tandem (MS-MS) were used to detect and confirm the reaction products of DHNB with XO. All mass spectrometric experiments were performed on an API 3200-Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) equipped with a turbolonSpray™ source. The main working parameters for mass spectrometry were set as follows: ion-spray voltage, −4.5 kV; ion source temperature, 600 °C; gas 1, 40 psi; gas 2, 40 psi; curtain gas, 20 psi; and collision gas, high.

### 2.5. HOCl scavenging assay

HOCl was prepared immediately before use by adjusting the pH of a 1% (v/v) solution of NaOCl to pH 6.2 with 0.6 M sulfuric acid. The concentration was further determined spectrophotometrically at 235 nm using the molar extinction coefficient of 100 $M^{-1}$ $cm^{-1}$. 5-Thio-2-nitrobenzoic acid (TNB) was prepared by reducing 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) with sodium borohydride in phosphate buffer. The HOCl scavenging assay was based on the inhibition of TNB oxidation to DTNB induced by HOCl [26].

### 2.6. Peroxynitrite scavenging assay

Peroxynitrite ($ONOO^-$) was generated by mixing 5 mL acidic solution (0.6 M HCl) of $H_2O_2$ (0.7 M) and 5 mL of 0.6 M $KNO_2$ on ice bath for 1 s, and then quenching the reaction with 5 mL of ice-cold 1.2 M NaOH. Residual $H_2O_2$ was removed using granular $MnO_2$ pre-washed with 1.2 M NaOH, and the reaction mixture was then left overnight at −20 °C. Concentrations of $ONOO^-$ were determined before each experiment at 302 nm using a molar extinction coefficient of 1670 $M^{-1}$ $cm^{-1}$. The $ONOO^-$ scavenging assay was performed by monitoring the oxidation of dihydrorhodamine (DHR 123) by $ONOO^-$ spectrophotometrically at 500 nm [26]. The abilities