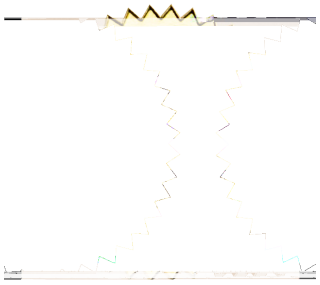

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If the application for this patent was led on or after December 12, 1980, maintenance fees are due three years and six months, seven years and six months, and eleven years and six

(54) **N ACYLA ED HYAL ONIC ACID FO HYPE CEMIA AND GO Y A H I**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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A61P 19/06 (2006.01)
A61P 37/06 (2006.01)

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CPC ***A61K 31/728*** (2013.01); ***A61P 19/06*** (2018.01); ***A61P 37/06*** (2018.01)

(58) **F e d of C ass cat on earc**
CPC A61K 31/728; A61P 19/06; A61P 37/06
See application file for complete search history.

(56) **eferences C ted**

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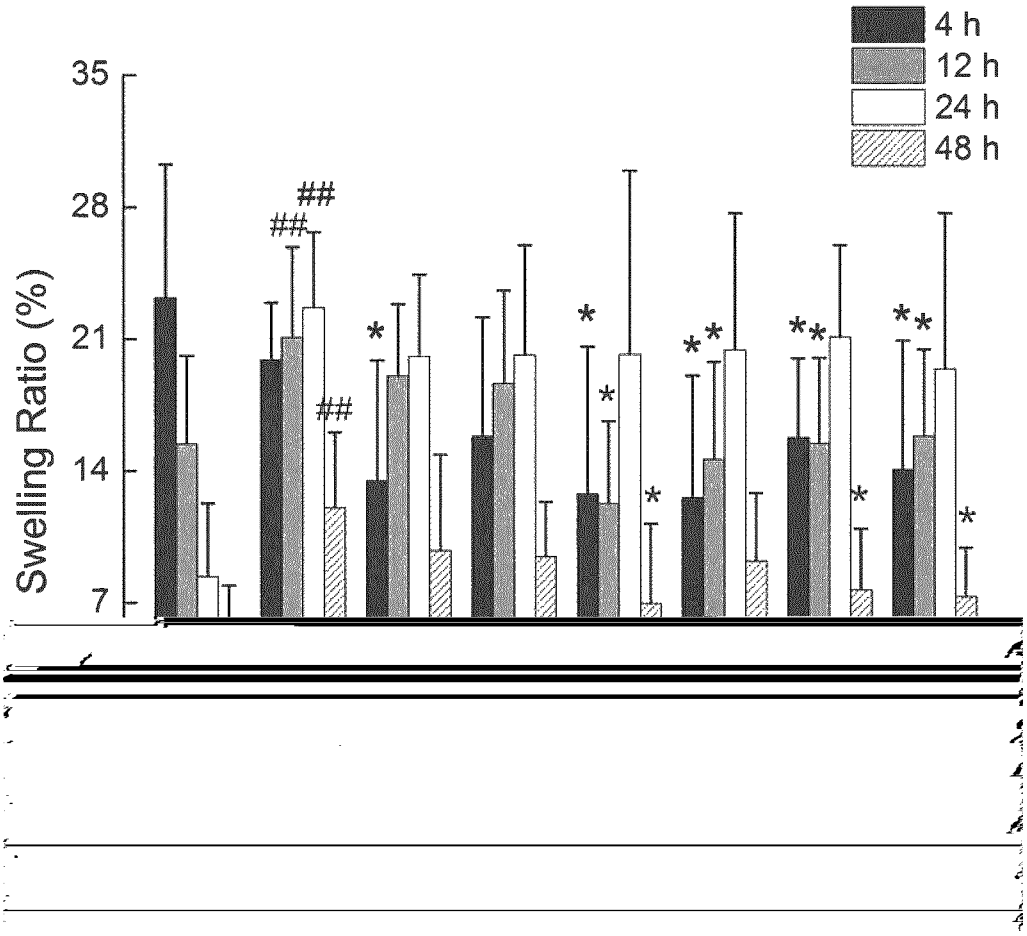
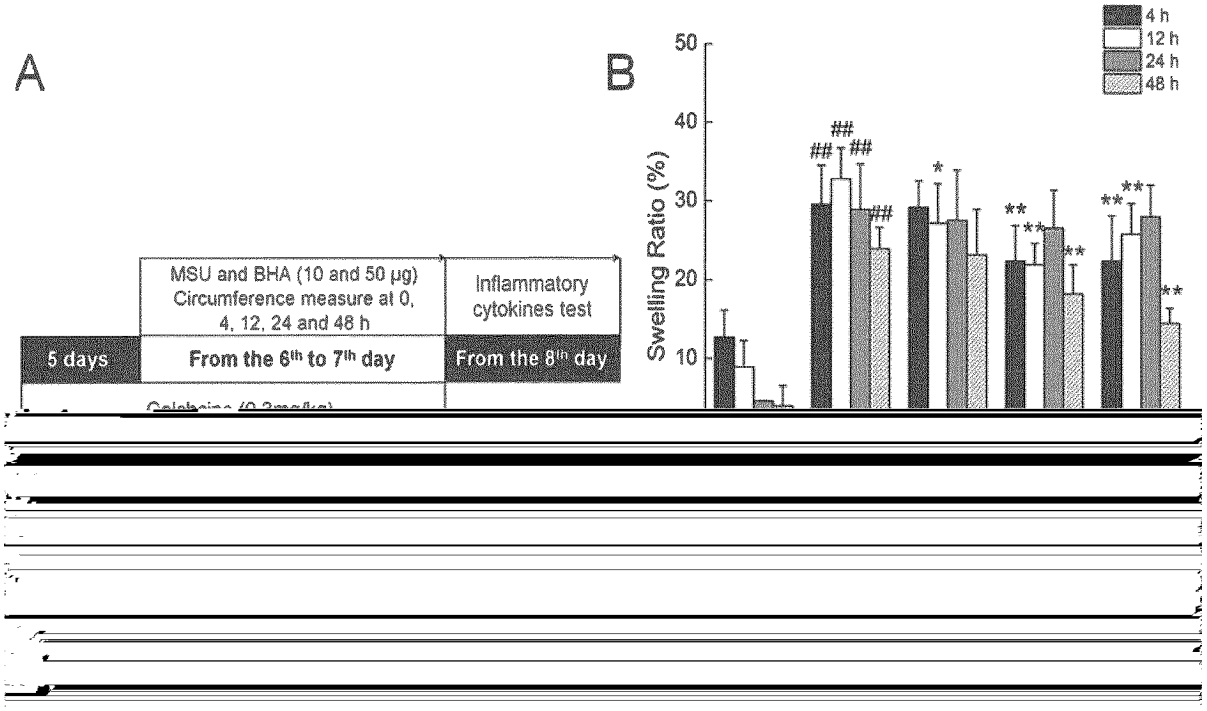
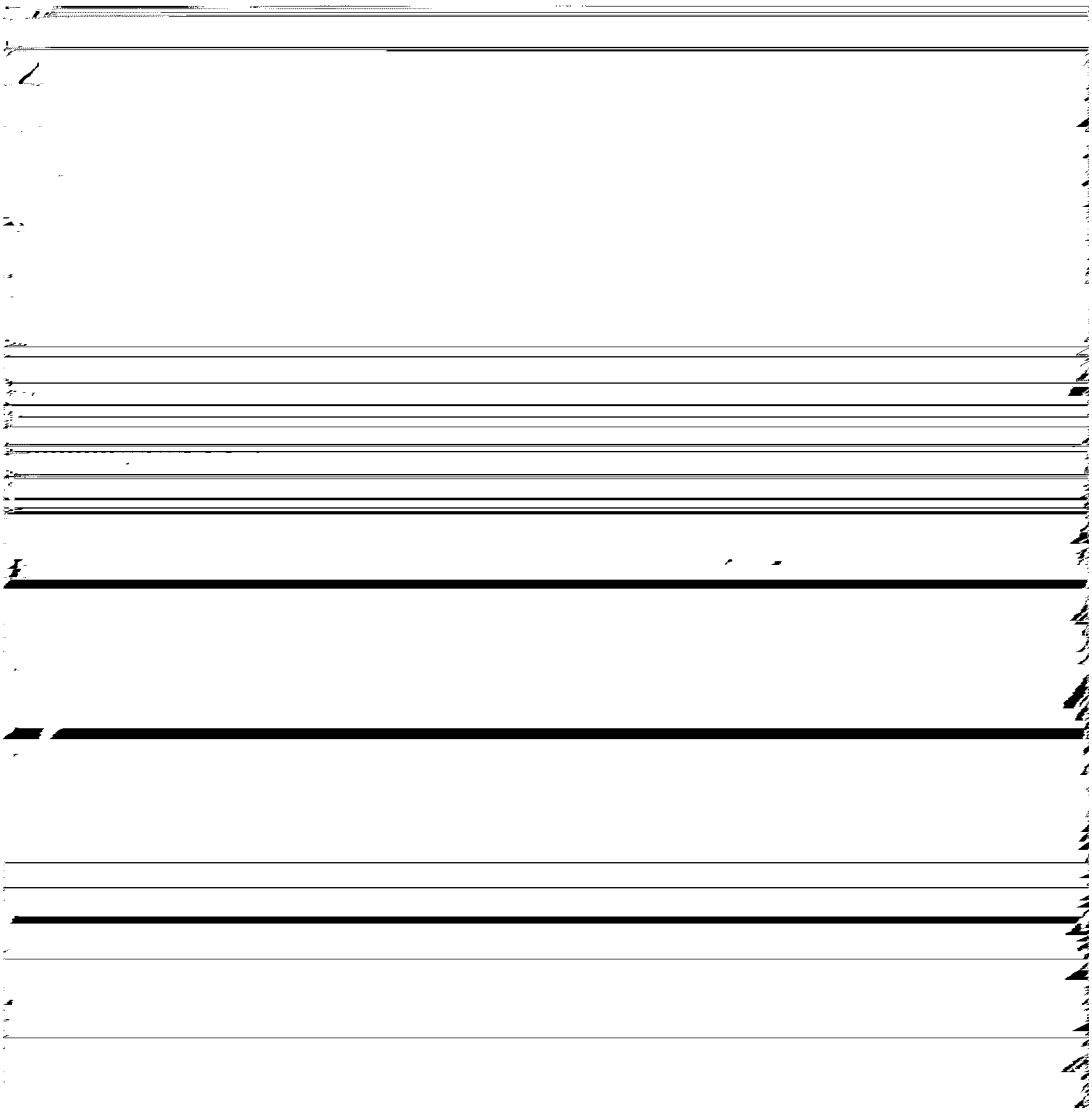
(57) **AB AC**

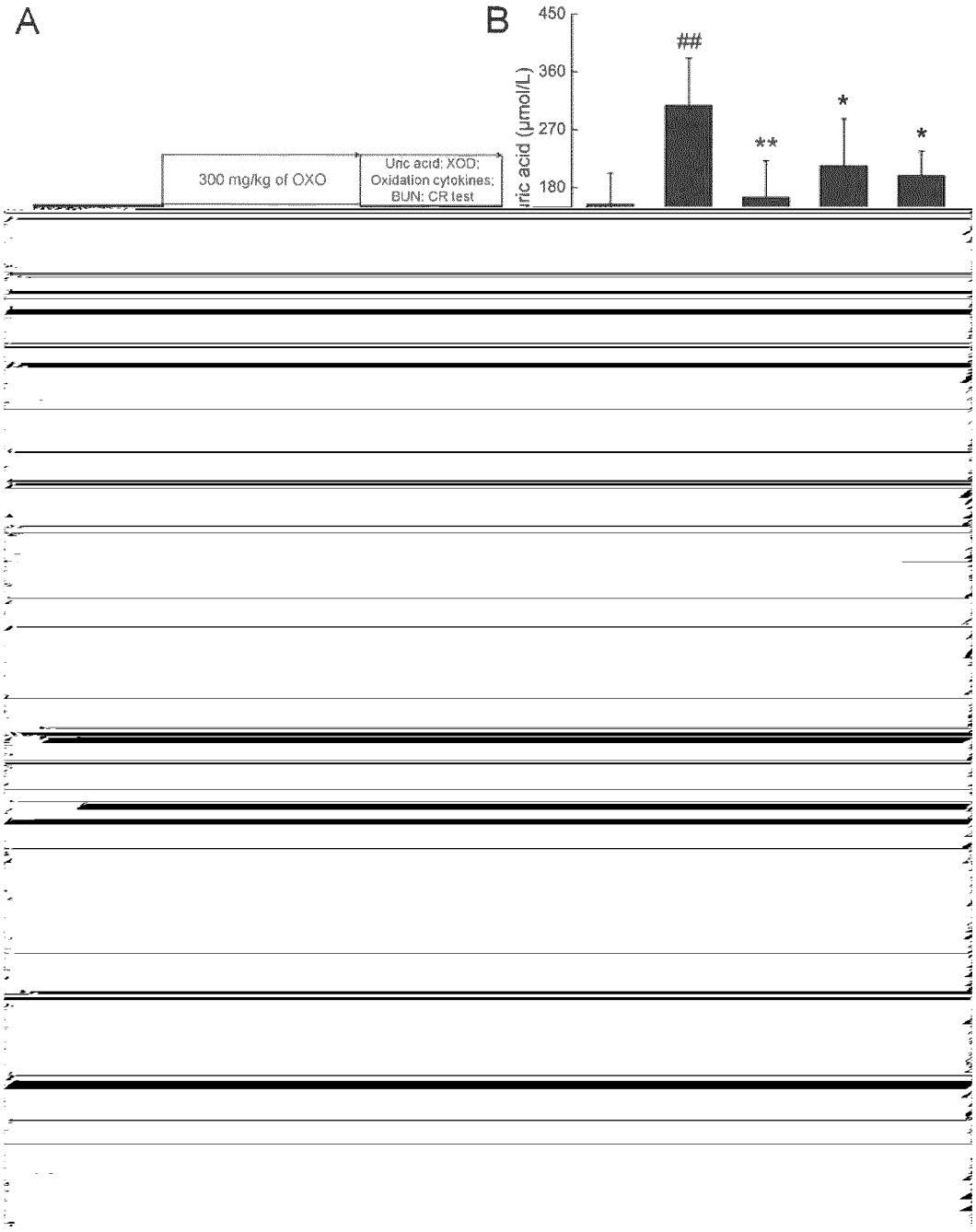
The invention provides a pharmaceut2.974(a)-482.95

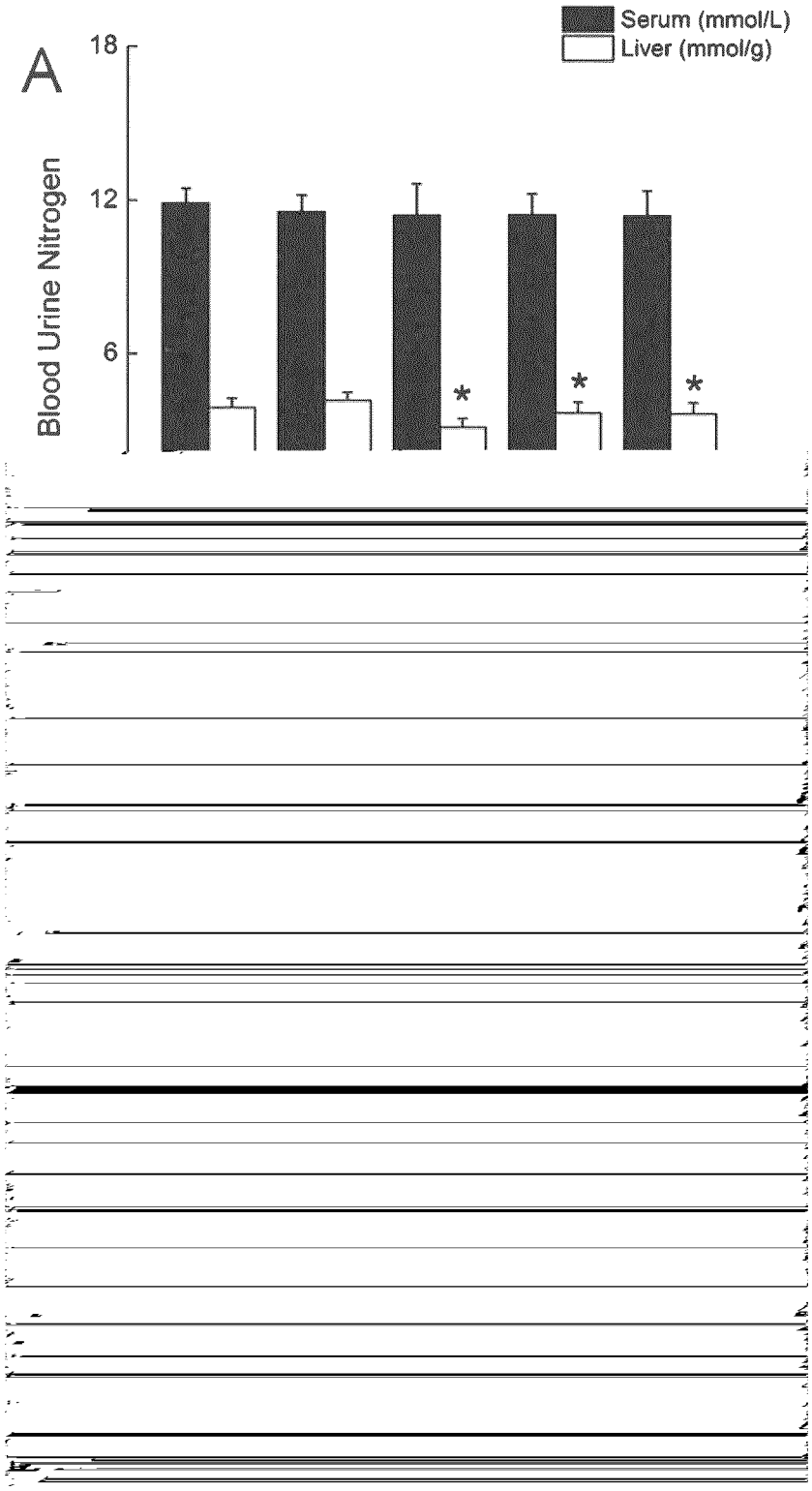

4 h
12 h
24 h
48 h
Swelling Ratio (%)
35
28
21
14
7
##
##
##
*
*
*
*
*
*
*
*
*
*
*
*


A
MSU and BHA (10 and 50 µg)
Circumference measure at 0, 4, 12, 24 and 48 h
Inflammatory cytokines test
5 days
From the 6th to 7th day
From the 8th day
B
Swelling Ratio (%)
4 h
12 h
24 h
48 h

C




A
300 mg/kg of OXO
Uric acid; XOD; Oxidation cytokines; BUN; CR test
B
450
360
270
180
uric acid (μmol/L)
##
**
*
*


A
Serum (mmol/L)
Liver (mmol/g)
Blood Urine Nitrogen
18
12
6
*
*
*

N ACYLA ED HYAL ONIC ACID FO HYPE CEMIA AND GO Y A H I

FIELD

This disclosure relates to hyaluronic acid derivatives, and in particular, derivatives in which the N-acetyl group of hyaluronic acid has been substituted, and methods and uses thereof.

BACKGROUND

Hyaluronan (hyaluronic acid) is a widely distributed glycosaminoglycan in animal tissues, composed of alternating monosaccharide units of N-acetyl glucosamine (N-acetyl-2-amido glucose) and glucuronic acid. Hyaluronan has multiple functions including hydration, provision of matrix for cell migration and lubrication of joints. Intact hyaluronan has a high molecular mass of greater than 1,000 kDa but can exist in lower molecular mass forms, for example, 100-250 kDa. Intact hyaluronanth2.194o-

Formula (II) wherein R is —C(O)—(C_2-C_4)-alkyl, or a pharmaceutically acceptable sodium- or potassium-salt, ester, or glucoside thereof,

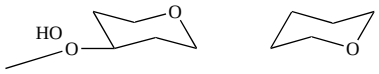


FIG. 1A shows a protocol summary and drug administration in hyperuricemic mice described herein.

FIG. 1B shows a bar graph displaying effects of AL and BHA on serum levels of UA in hyperuricemic mice.

FIG. 1C shows a bar graph displaying effects of AL and BHA on the XO levels in serum of hyperuricemic mice. Data are expressed as mean±S.D. (n=10) and were analyzed via a one-way ANOVA test followed by post-hoc Dunn's multiple comparison tests. #P<0.05 and ##P<0.01 versus normal control, * P<0.05 and ** P<0.01 versus model control.

FIG. 1D shows a bar graph displaying effects of AL and BHA on the XO levels in liver of hyperuricemic mice. Data are expressed as mean±S.D. (n=10) and were analyzed via a one-way ANOVA test followed by post-hoc Dunn's multiple comparison tests. #P<0.05 and ##P<0.01 versus normal control, * P<0.05 and ** P<0.01 versus model control.

FIG. 1E shows a summary of treatment.

FIG. A shows effects of AL and BHA on the serum and liver levels of urine nitrogen in hyperuricemic mice.

FIG. B shows effects of AL and BHA on the serum and liver levels of Cr in hyperuricemic mice.

DETAILED DESCRIPTION

Definitions

As used herein, the term "HA" refers to hyaluronic acid.

As used herein, the term "BHA" refers to N-butyrylated HA.

As used herein, the term "MSU" refers to monosodium urate.

As used herein, the term "ROS" refers to reactive oxygen species.

As used herein, the term "MDA" refers to malondialdehyde.

As used herein, the term "SOD" refers to superoxide dismutase.

As used herein, the term "NSAID" refers to non-steroidal inflammatory drugs.

As used herein, the term "acute gout" refers to pain and inflammation that may affect only one joint or more than one joint.

As used herein, the term "chronic gout" refers to repeated episodes of pain and inflammation at one joint or more than one joint.

Hyaluronic acid (HA), also called hyaluronan, is a linear polysaccharide belonging to the glycoamioglycan family, which is composed of simple repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid

As described herein, a MSU-induced gouty arthritis rat model and an oteracil potassium- and yeast extract-induced hyperuricemia mouse model were established. Partially butylated HA (see Formulas I and II above, wherein R is —C(O)—(C_3)-alkyl) was synthesized as a representative example of a compound of Formulas I and II above wherein R is —C(O)—(C_2-C_4)-alkyl. Therapeutic effects of partially butylated HA (BHA) were investigated with the animal models described herein. The expression level of cytokines and levels of oxidative stress markers were analyzed by ELISA.

Results described herein demonstrate therapeutic effects and suggest molecular mechanisms of BHA of approximately 30 kDa for treating hyperuricemia and gouty arthritis. Results demonstrate that intra-articular injection of BHA improved symptoms of ankle swelling in a rat model exhibiting MSU-induced gouty arthritis. Histological studies (H&E staining) indicated that intra-articular injection of BHA decreased the number of inflammatory cells and preserved joint space in comparison with untreated rats with MSU-induced gouty arthritis. Histopathologically, the injection of MSU crystals caused pronounced inflammatory cell infiltration in the synovium compared to the normal controls. Treatment with COL and the low dose of BHA attenuated the inflammation reaction in terms of that fewer inflammatory cells were observed in the groups of COL and 10BHA. The higher dose BHA (50BHA) was not as effective as the 10BHA dose. Furthermore, BHA reduced expression of pro-inflammatory cytokines including interleukin-1 beta (IL-1 beta), interleukin-8 (IL-8), and IFN-γ, down regulated the expression of monocyte chemotactic protein 1 (MCP-1) and increased the expression of anti-inflammatory cytokine interleukin-10 (IL-10). In addition, intraperitoneal injection of BHA significantly decreased serum level of uric acid and liver xanthine oxidase (XO) activity in mice with oteracil potassium- and yeast extract-induced hyperuricemia.

In one embodiment, a dose range of BHA if recommended of about 0.3 mg to about 40 mg per human for intra-articular injection, about 3 to about 400 mg/human for intraperitoneal injection, or about 25 mg/day to about 5 g/day for oral administration. In one embodiment oral administration is recommended as the route of administration. Notably, a

assessed for the degree of inflammatory cell infiltrate, by an experienced histopathologist. Microscopy at magnifications of 40×, 100×, 200× and 400× were investigated for typical areas for each of the five groups. Normal rats (NC), displayed normal synovium. Increased inflammatory cell infiltration was noted in the synovium of MSU crystal-injected rats (MC). Treatment with COL, and 10 μg of BHA partially prevented the pathological changes seen in the MSU crystal-injected rats.

The level of serum UA is considered to be a direct indicator of the clinical diagnosis of hyperuricemia, and XO is an enzyme that plays a key catalytic role in the process of UA production. Results suggest that the levels of serum UA and liver XO increased significantly in hyperuricemic mice. However, with the treatment of the XO inhibitor-AL, the serum UA levels were reduced to normal levels and serum XO activity was significantly inhibited in hyperuricemic mice. As was the case with AL, treatment with BHA significantly reduced the serum UA levels and administration with 10 μg BHA dramatically reduced liver XO activity while administration with 50 μg BHA did not significantly reduce XO activity beyond that obtained with 10 μg BHA. Notably, oral administration with AL resulted in reduction of the serum UA to normal levels and this was also the case with BHA, which significantly reduced the serum UA levels (FIG. 2B). The dose of 10 μg BHA intra-peritoneally dramatically reduced liver XO activity.

Therefore, BHA treatment showed potentials in treating gouty arthritis by acting as an anti-inflammatory agent. Anti-hyperuricemia activity of BHA was achieved at least partly by inhibiting the activity of XO and reducing the serum UA levels. Treatment with 50 μg BHA had no significant effect on XO activity in the livers of hyperuricemic mice, but significantly decreased the serum UA levels (see FIG. 2A-E). Therefore, it is clear that BHA treatment for hyperuricemia is not limited to inhibiting XO activity.

XO could catalyze the oxidation of hypoxanthine and xanthine c to UA, which would be accompanied by a large amount of oxygen free radicals. In hyperuricemic mice the levels of ROS and MDA were significantly elevated in the liver, and the content of SOD in the serum was significantly diminished. MDA is formed by the degradation of polyunsaturated fat by ROS, and thus could be considered a biomarker for oxidative damage. BHA treatment significantly decreased the levels of ROS in the serum and liver and increased the level of SOD in the liver of hyperuricemia mice, which is consistent with the observation of lowered serum UA level. It has been reported that hyperuricemia is closely related to renal dysfunction and UA plays a major role in this pathology. Cr and urea nitrogen could be used as indicators of renal function evaluations. Both the Cr and urea nitrogen were measured in serum and in liver to access pathogenenic change in this hyperuricemia mouse model as well as to evaluate the safety of BHA treatment. Hyperuricemic mice exhibited a level of liver Cr that was significantly increased while there was no substantial increase of serum Cr. As was the case with treatment of the hyperuricemia mice with AL, treatment with BHA significantly

GlcNAc in the GlcNAc-GlcA unit was observed at 5.09-5.08 ppm as a doublet. The anomeric proton of GlcA in the GlcNAc-GlcA unit was also observed as a doublet at 4.94-4.93 ppm. The newly visible smaller peaks at 5.18-5.31 ppm corresponded to anomeric protons of GlcN-GlcA unit. The anomeric proton of GlcN in the GlcN-GlcA unit was observed at 5.09-5.08 ppm, doublet. The anomeric proton of GlcA in GlcN-GlcA unit was also observed as a doublet at 4.94-4.93 ppm. In the spectrum of DHA, the integration ratio of the three methyl protons to the anomeric protons was calculated to be 1.13. From this ratio, the percentage of deacetylation was calculated to be 24.8%. The spectrum of BHA shows additional —$CH_2CH_2CH_3$ proton signals indicating that a reacylation reaction was occurred and that the ratio of butylation to acetylation was calculated to be 25.4%.

Example 1D. Molecular Weight Estimation of HA, DHA and BHA

The molecular weights of DHA and BHA were estimated by electrophoresis. Briefly, samples were characterized using a 0.75 (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer, containing 400 mM Tris, 50 mM acetate acid and 9 mM EDTA, pH 8.0. A sample loading buffer was prepared

F=12.09 to 28.24) compared with control rats. Treatment with 0.3 mg/kg COL significantly suppressed swelling of the ankle ($P<0.05$, F=6.79) compared to MSU treated group at 4 h only. Treatment with BHA significantly suppressed swelling at 4 h ($P<0.05$, F=4.89 to 8.32), and 12 h ($P<0.05$, F=5.33 to 15.70). At doses of 10 μg, 50 μg and 100 μg, BHA showed significant suppressing of swelling effect at 48 h ($P<0.05$, F=4.97 to 7.29) compared to the MSU treated group.

Example 3A. Protocol for Inducing Acute Gout in Rats by MSU Crystals Injection and Treatment by BHA

Male Wistar rats (n=50, 8 weeks: 160-200 g) were purchased from Liaoning Changsheng Biotechnology Company Ltd, Jilin, China (SCXK (Liao)-2015-0001). These rats were housed in plastic cages and maintained on a 12-h light/12-h dark cycle (lights on 7:00-19:00 h) under standard laboratory conditions of 55% relative humidity and at 23° C.±1° C. They were given standard chow (Liaoning Changsheng Biotechnology Company Ltd, Jilin, China) and tap water ad libitum. All experimental procedures were approved by the Animal Ethics Committee of Jilin University (Reference NO. 201605).

An experimental model of MSU-induced gouty arthritis was used in order to evaluate the anti-inflammatory activities of BHA. Rats were randomly divided into five groups (n=10), include a control group (NC), a model group (MC), a 0.3 mg/kg colchicine group (COL), a 10 μg BHA group (10BHA) and a 50 μg BHA group (50BHA). MSU crystals were suspended in 0.9% sterile saline (30 mg/mL) prior to use. The colchicine group rats were orally administrated colchicine (0.3 mg/kg) for 8 days, and all rats except for the control group were injected with 3 mg of MSU (Sigma, USA) at the 6^{th}