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RESEARCH PAPER

Perfecting a high hypoxanthine phosphoribosyltransferase activity–uricase KO mice to test the effects of purine- and non-purine-type xanthine dehydrogenase (XDH) inhibitors

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Background and Purpose: Purine metabolism in mice and human differ in terms of uricase (*Uox*) activity as well as hypoxanthine phosphoribosyltransferase (HPRT) activity. The aim of this study was the establishment of high HPRT activity–*Uox* knockout (KO) mice as a novel hyperuricaemic model. Then to investigate the effects of purine-type xanthine dehydrogenase (XDH) inhibitor, allopurinol, and non-purine-type XDH inhibitor, topiroxostat, on purine metabolism.

Experimental Approach: A novel hyperuricaemic mouse model was established by mating B6-ChrXC^{MSM} mice with uricase KO mice. The pharmacological effects of allopurinol and topiroxostat were explored by evaluating urate, hypoxanthine, xanthine and creatinine in the plasma and urine of these model mice. Furthermore, we analysed the effect of both drugs on erythrocyte hypoxanthine phosphoribosyltransferase activity.

Key Results: Plasma urate level and urinary urate/creatinine ratio significantly decreased after administration of allopurinol 30 mg·kg^{-1} or topiroxostat 1 mg·kg^{-1} for 7 days. The urate-lowering effect was equivalent for allopurinol and topiroxostat. However, the urinary hypoxanthine/creatinine ratio and xanthine/creatinine ratio after treatment with topiroxostat were significantly lower than for allopurinol. In addition, the urinary oxypurine/creatinine ratio was significantly lowered after treatment with topiroxostat, but allopurinol elicited no such effect. Furthermore, allopurinol inhibited mouse erythrocyte hypoxanthine phosphoribosyltransferase, while topiroxostat did not.

Conclusions and Implications: High hypoxanthine phosphoribosyltransferase activity– uricase KO mice were established as a novel hyperuricaemic animal model. In addition, topiroxostat, a non-purine-type xanthine dehydrogenase inhibitor, elicited a potent plasma urate-lowering effect. However, unlike allopurinol, topiroxostat did not perturb the salvage pathway, resulting in lowered total oxypurine excretion.

Abbreviations: BUN, blood urea nitrogen; CRP, C-reactive protein; FE, fractional excretion; HPRT, hypoxanthine phosphoribosyltransferase; IMP, inosine 5′-monophosphate; KO, knockout; PRPP, 5-phosphoribosyl-1-pyrophosphate; ULE, urate-lowering effect; *Uox*, urate oxidase (uricase); WT, wild-type; XDH, xanthine dehydrogenase.

Takuji Hosoya and Makoto Hosoyamada contributed equally to this work.

1 | INTRODUCTION

Hyperuricaemia, a purine metabolic disorder, contributes to the development of gout, urolithiasis, and kidney disease and is associated with multiple complications including hypertension, hyperlipidaemia and diabetes (Bonakdaran & Kharaqani, 2014; Li, Hsieh, & Chang, 2013; Lu et al., 2018; Merriman & Dalbeth, 2011). Humans lack uricase (*Uox*) activity, which is responsible for the degradation of **urate** to allantoin. Consequently, urate is the end product of purine metabolism in humans (Alvarez-Lario & Macarron-Vicente, 2010). By contrast, rodents, such as rat and mouse, excrete allantoin because they possess *Uox* activity. Consequently, the serum urate level of rodents is markedly lower than that of humans, resulting in a hypouricaemic state. In addition, rodents are also known to have a low activity of hypoxanthine phosphoribosyltransferase (HPRT) contributing to the purine salvage pathway compared with humans (Tax, Veerkamp & Trijbels, 1976). Rodents serve an important role as model organisms in preclinical drug development and for routine testing of candidate compounds before clinical trials. The differences in purine metabolism between humans and rodents are major concerns for research of hyperuricaemia and metabolic syndrome and for the development of drugs.

In the purine salvage pathway, HPRT recycles **hypoxanthine** using 5-phosphoribosyl-1-pyrophosphate (PRPP) for re-synthesising **inosine 5′-monophosphate** (IMP). Hypoxanthine phosphoribosyltransferase in mice comprises HPRT A protein, with an "alanine" as the second amino acid residue, and HPRT B protein, with a "proline" at the same position (Johnson & Chapman, 1987; Johnson, Kronert, Bernstein, Chapman, & Smith, 1988). Interestingly, there are subspecies differences in mice. It has been reported that wild-type (WT)-derived strains of mice (e.g. *Mus musculus castaneus*, *Mus spretus* or *Mus musculus molossinus*) have HPRT A protein, which is found to be more highly active than C57BL/6J mice with hypoxanthine phosphoribosyltransferase B protein (Johnson & Chapman, 1987). There are also species differences between human and mouse (e.g. C57BL/6J mice). For example, the erythrocyte HPRT activity of human erythrocytes is approximately 10-fold higher than that of mice (Tax et al., 1976). HPRT in human is known to have "alanine" as the second amino acid residue, like HPRT A protein, which is highly active in mice (Wilson, Tarr, Mahoney, & Kelley, 1982). B6-ChrXC^{MSM} is a consomic mouse strain with the chromosome portion of *Mus m. molossinus* (Oka et al., 2007). We recently reported that B6-ChrXC^{MSM} mice possess the highly active HPRT A protein similar to that found in humans (Watanabe et al., 2016). The *Uox* knockout (KO) mouse has a pure C57BL/6J genetic background with lower HPRT activity (Chen et al., 2013; Lu et al., 2018; Lu et al., 2019; Lu et al., 2019; Wu et al., 1994). Thus, to date, there is no useful animal model that has a purine metabolic system akin to that of human. We reasoned that if we generated *Uox* KO mice using the B6-ChrXC^{MSM} strain, the resultant mouse line may be a useful animal model that is close to the human purine metabolic system.

Thus, in the present study, we established a high HPRT activity–*Uox* KO mouse line as a novel hyperuricaemic animal model. The pharmacological effects of **allopurinol**, a purine-type **xanthine dehydrogenase** (XDH) inhibitor and topiroxostat, a non-purine-type XDH inhibitor, as urate-lowering drugs on purine metabolism were then investigated.

What is already known

- Purine metabolism in mice and human differ in terms of *Uox* activity and HPRT activity.
- In conventional hyperuricaemic model animals, urinary oxypurine excretion was not reduced by XDH inhibitors.

What this study adds

- High HPRT activity–*Uox* KO mouse was established as a novel hyperuricaemic animal model.
- Unlike allopurinol, topiroxostat was efficiently lowered urinary oxypurine excretion without HPRT inhibition.

What is the clinical significance

- Topiroxostat, a non-purine-type XDH inhibitor, may be more beneficial than allopurinol, a purine-type XDH inhibitor.
- The novel hyperuricaemic mouse model generated may contribute to the development of novel treatments.

2 | METHODS

2.1 | Animals

Male C57BL/6J mice as WT were purchased from Sankyo Lab. Service Corporation (Tokyo, Japan). B6-ChrXC^{MSM} mice (RRID: IMSR_NIG:264; with the central part of the X chromosome derived from *Mus m. molossinus*) were purchased with a C57BL/6J genetic background from the National Institute of Genetics (Shizuoka, Japan; Oka et al., 2007). *Uox* KO mice (C57BL/6J genetic background, RRID: IMSR_JAX:000664) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). High hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice were established by mating B6-ChrXC^{MSM} mice (C57BL/6J genetic background) with *Uox* KO mice (C57BL/6J genetic background). Profiles of WT mice (10 weeks) weighing 22–23 g and low and high HPRT activity–*Uox* KO mice (12–-15 weeks) weighing 22–31 g were examined by genotyping of the *Hprt* and *Uox* genes and measuring plasma and urine purine metabolites and erythrocyte HPRT activity.

Mice were housed in sterile and ventilated cages and kept in an air-conditioned room with a standard 12-hr light/dark cycle. The mice were fed a standard diet and water ad libitum throughout the acclimatisation and experimental periods. All animal experiments were approved by the animal ethics committee of Teikyo University. Animal

studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Group size

Equal group sizes were employed for all *in vivo* and *in vitro* experiments in this study. For studies using the *in vivo* model, the group size for each experimental condition was five mice. For *in vitro* studies, five independent tests were used for each experimental condition.

2.3 | Randomisation and blinding

Low and high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice were divided into groups according to the random comparison group methods in pharmacology. Vehicles and drugs were randomly assigned to each group prior to the start of the experiment. Data were analysed by observers who were blinded to the animal group assignment. Data files were labelled with the date and sample identifier. The data were analysed in this file format and subsequently assigned to the respective experimental condition using lab records.

2.4 | Genotyping of the *Hprt* and *Uox* genes

Allelic discrimination of the *Hprt* gene was conducted using TaqMan® GTXpressTM Master Mix (Applied Biosystems, Foster City, CA, USA; Watanabe et al., 2016) with the following oligonucleotide primers and minor groove binder (MGB) probes: F-CCT CCT CAG ACC GCT TTT TG; R-TCT GCT GGA GTC CCC TTG; VIC-CCCgTCA TgCCgAC-MGB; fluorescein amidite (FAM)-CCCgTC ATggCgAC-MGB. PCR amplifications were carried out in a final volume of 10 μl consisting of a master mix, 0.9 $\mu mol \cdot L^{-1}$ of each primer, and 0.2 $\mu mol \cdot L^{-1}$ of 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC)-labelled and FAM-labelled probes, respectively. The holding stage before PCR was performed at 25°C for 1 min. PCR cycling was conducted first at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s each, and finally at 60°C for 30 s. The holding stage after PCR was performed at 25°C for 1 min.

Genotyping of the *Uox* gene was performed by PCR of genomic DNA templates extracted from an ear punch according to the dilution protocol of Phire Animal Tissue Direct PCR Kit (Thermo Scientific, Waltham, MA, USA; Hosoyamada et al., 2016). To avoid carry-over contamination, Takara PCR Carryover Prevention Kit (Takara, Otsu, Japan) was used. The PCR mixture contained 0.8 μl of dU plus dNTP mixture, 0.3 μl of 25 $mmol \cdot L^{-1}$ of $MgCl_2$, 0.1 μl of UNG (reagents from Takara PCR Carryover Prevention Kit), 1 μl of 10× Standard Taq Reaction buffer, 0.2 μl of Hot Start Taq DNA polymerase (NEB), 0.5 μl of template, 0.3 μl of three mixed primers, and 6.8 μl of distilled water in a total volume of 10 μl. Using sense primers (5′-tcagaaacatcgagacctttgc-3′ for the WT allele and 5′-atcgccttctatcgccttctt-3′ for the targeted allele) and a common antisense primer (5′-taccgtttctcatctgctccac-3′), we amplified 291 and 158 bp of amplicons from WT and targeted *Uox* alleles, respectively.

2.5 | *In vitro* xanthine dehydrogenase (XDH) enzyme assay

Xanthine dehydrogenase (XDH) activity was assessed using the modified method described by Matsumoto, Okamoto, Ashizawa, & Nishino, 2011. The conversion of xanthine to urate was monitored at 295 nm using a spectrophotometer (U-3200; Hitachi, Tokyo, Japan). The assay mixture comprised various concentrations of **xanthine** and inhibitor (allopurinol 0.3–30 $\mu mol \cdot L^{-1}$ or topiroxostat 0.001–0.3 $\mu mol \cdot L^{-1}$) in 0.1 $mol \cdot L^{-1}$ of pyrophosphate buffer (pH 8.5) containing 0.2 $mmol \cdot L^{-1}$ of EDTA. The mix was pre-incubated for several minutes before initiating the reaction by addition of bovine milk XDH purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) under aerobic conditions at 25°C. Inhibitory activity was calculated as inhibition potency against the maximum activity in the absence of inhibitor. The IC_{50} values were obtained using probit analysis.

2.6 | *In vivo* experiments protocol

For the in vivo studies, we used male high and low hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice (12–15 weeks) weighing 25–35 g. The doses of allopurinol and topiroxostat were set to 30 and 1 $mg \cdot kg^{-1}$, respectively, as the doses at which the serum urate-lowering effect (ULE) of both compounds were comparable based on the report of Matsumoto et al. (2011). The model mice were treated with allopurinol and topiroxostat by dietary administration. Allopurinol and topiroxostat were adjusted to allopurinol 30 mg per 100-g feed and topiroxostat 1 mg per 100-g feed. Study 1: Low HPRT activity–*Uox* KO mice were randomly divided into three groups and fed standard powder chow, Charles River Formula-1 (control, $n = 5$), allopurinol (30 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 5$), and topiroxostat (1 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 5$) for 7 days. Study 2: High HPRT activity–*Uox* KO mice were randomly divided into three groups and fed standard powder chow, Charles River Formula-1 (control, $n = 5$), allopurinol (30 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 5$), and topiroxostat (1 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 5$) for 7 days. Feed intake was adjusted to each daily dose. Blood and urine samples were collected on the seventh day after administration of each drug.

2.7 | Purine metabolite (oxypurine as urate, hypoxanthine, and xanthine, and allantoin) and creatinine analysis by HPLC

Blood was collected using a heparinised 32 mm hematocrit capillary (Drummond, Broomall, PA, USA) from the end of the tail punctured with a 27-gauge needle. A 2-μl aliquot of the separated plasma was deproteinised with 80% acetonitrile by centrifugation at 12,000× *g*, 4°C for 4 min using an Ultrafree-MC 0.22 μm PVDF membrane filter unit (Millipore, Billerica, MA, USA). The deproteinised sample was

resuspended with 10 μl of HPLC mobile phase (10 mmol·L^{-1} of ammonium formate) after evaporation. Urine samples were collected as spot urine and diluted 50 times in HPLC mobile phase (10 mmol·L^{-1} of ammonium formate). Separation of the resuspended and the 50-fold diluted samples was achieved at a flow rate of 0.200 ml·min^{-1} on a 250 × 2 mm, 5 μm particle size octadecyl silica (ODS) column, Unison US-C18 (Imtakt, Kyoto, Japan) at 25°C using a Hitachi pump (Hitachi Chromaster; Hitachi High-Tech Science Corp, Japan). Peaks corresponding to allantoin, oxypurine (urate, hypoxanthine, and xanthine), and creatinine were detected at 234 nm. The creatinine ratio and fraction excretion (FE) were calculated using a previously described method (Hosoyamada et al., 2016; Liu, Sun, Zhang, Li, & Zhang, 2015) as follows:

Urinary urate/creatinine ratio = urine urate (μmol·L^{-1})/urine creatinine (μmol·L^{-1}).

Urinary hypoxanthine/creatinine ratio = urine hypoxanthine (μmol·L^{-1})/urine creatinine (μmol·L^{-1}).

Urinary xanthine/creatinine ratio = urine xanthine (μmol·L^{-1})/urine creatinine (μmol·L^{-1}).

Urinary oxypurine/creatinine ratio = urine oxypurine (μmol·L^{-1})/urine creatinine (μmol·L^{-1}).

FE_{UA} (%) = [urine urate × plasma creatinine/(plasma urate × urine creatinine)] × 100.

FE_{HX} (%) = [urine hypoxanthine × plasma creatinine/(plasma hypoxanthine × urine creatinine)] × 100.

FE_{XA} (%) = [urine xanthine × plasma creatinine/(plasma xanthine × urine creatinine)] × 100.

FE_{OP} (%) = [urine oxypurine × plasma creatinine/(plasma oxypurine × urine creatinine)] × 100.

2.8 | Measurement of renal damage and inflammatory biomarkers

The plasma cystatin C, blood urea nitrogen (BUN), and C-reactive protein (CRP) were measured using Mouse Cystatin C ELISA Kit (BioVendor—Laboratory Medicine, Inc., Brno, Czech Republic), BUN Colorimetric Detection Kit (Arbor Assays, Inc., Ann Arbor, MI, USA), and Mouse C-Reactive Protein/CRP Immunoassay Kit (BioVendor—Laboratory Medicine, Inc.).

2.9 | Mouse erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) activity assay

Assay mixtures comprised 2 mmol·L^{-1} of PRPP, 6 mmol·L^{-1} of $MgCl_2$, and 100 μmol·L^{-1} of hypoxanthine in 50 mmol·L^{-1} of Tris–HCl buffer (pH 7.4). The reaction was initiated by addition of 20 mg·ml^{-1} of erythrocyte extract containing hypoxanthine phosphoribosyltransferase (HPRT) to each mixture at 37°C for 5 min. The reaction was then quenched, and the mixtures were deproteinised by addition of 80% acetonitrile. Samples were subjected to centrifugation at 20,000× *g*, 4°C for 15 min, and the supernatant was collected. The supernatant was further clarified by centrifugation at 12,000× *g*, 4°C for 4 min using an Ultrafree-MC 0.22 μm PVDF membrane filter unit (Millipore) to remove proteins. The deproteinised sample was resuspended with 10 μl of HPLC mobile phase (50 mmol·L^{-1} of ammonium formate, pH 4.1) after evaporation.

Assay samples were analysed by HPLC at a flow rate of 0.250 ml·min^{-1} on a 250 × 2 mm, 5 μm particle size ODS column, using a Unison US-C18 system (Imtakt) at 40°C (Hitachi). The peak corresponding to IMP was detected at 260 nm. Each HPRT activity was expressed as product nmol·min^{-1}·mg^{-1} erythrocyte.

2.10 | Metabolic assay using mice erythrocyte HPRT

An assay mixture comprising 2 mmol·L^{-1} of PRPP and 6 mmol·L^{-1} of $MgCl_2$ and each drug (100 μmol·L^{-1} of allopurinol and 100 μmol·L^{-1} of topiroxostat) in 50 mmol·L^{-1} of Tris–HCl buffer (pH 7.4) was prepared, and reactions were started by addition of 20 mg·ml^{-1} of erythrocyte extract containing hypoxanthine phosphoribosyltransferase (HPRT). Reactions were performed at 37°C for 0 to 120 min before quenching and deproteinising by addition of 80% acetonitrile. Samples were centrifuged at 20,000× *g*, 4°C for 15 min to obtain the supernatant. The supernatant was further clarified by centrifugation at 12,000× *g*, 4°C for 4 min using an Ultrafree-MC 0.22 μm PVDF membrane filter unit (Millipore) to remove proteins. The deproteinised sample was resuspended in 10 μl of HPLC mobile phase (50 mmol·L^{-1} of ammonium formate, pH 4.1) after evaporation. Allopurinol assay samples were analysed by HPLC at a flow rate of 0.250 ml·min^{-1} on a 250 × 2 mm, 5 μm particle size ODS column using a Unison US-C18 system (Imtakt) at 40°C (Hitachi). Peaks corresponding to allopurinol and its corresponding ribonucleotide were detected at 260 nm. Topiroxostat assay samples were analysed by HPLC at a flow rate of 1.0 ml·min^{-1} on a Mightysil RP-18 GP column (Kanto Chemical, Tokyo, Japan) at 40°C (Waters Alliance HPLC System; Waters, MA, USA). The mobile phase for the analysis of topiroxostat consisted of 50 mmol·L^{-1} of ammonium formate/acetonitrile (75:25). Peaks corresponding to topiroxostat were detected at 273 nm.

2.11 | *Ex vivo* inhibitory assay on mice erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) activity

For *in vivo* studies, we used male high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice weighing 26–34 g, which were randomly divided into four groups for assessment of allopurinol and topiroxostat: control (n = 5), allopurinol (30 and 100 mg·kg^{-1}, n = 5, respectively), and topiroxostat (1 mg·kg^{-1}, n = 5). Allopurinol and topiroxostat were orally administered. The drug volume administered corresponded to 10 ml·kg^{-1} body weight in each case. Control mice received the same volume of drug vehicle (0.5% methylcellulose) via the same administration route. Blood was collected from the postcaval vein with a

heparinised 24-gauge needle and syringe under isoflurane inhalation anaesthesia after 30 min of drug administration. The assay mixture comprised 2 mmol·L^{-1} of PRPP, 6 mmol·L^{-1} of $MgCl_2$, and 10 μmol·L^{-1} of hypoxanthine in 50 mmol·L^{-1} of Tris–HCl buffer (pH 7.4). Reactions were started by addition of 20 mg·ml^{-1} of erythrocyte extract containing HPRT to each mixture at 37°C for 1 min. The reactions were then stopped and deproteinised by addition of 80% acetonitrile. Samples were centrifuged at 20,000× *g*, 4°C for 15 min to obtain the supernatant. The obtained supernatant was centrifuged at 12,000× *g*, 4°C for 4 min on an Ultrafree-MC 0.22 μm PVDF membrane filter unit (Millipore) to remove proteins. The deproteinised sample was resuspended with 10 μl of HPLC mobile phase (50 mmol·L^{-1} of ammonium formate, pH 4.1) after evaporation. Samples were assayed by HPLC at a flow rate of 0.25 ml·min^{-1} on a 250 × 2 mm, 5 μm particle size ODS column, using a Unison US-C18 system (Imtakt) at 40°C (Hitachi). The peak corresponding to IMP was detected at 260 nm. Each HPRT activity was expressed as product nmol·min^{-1}·mg^{-1} erythrocyte.

2.12 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. All data were expressed as the mean ± SEM. Statistical analysis was performed using SPSS Statistics Version 24 (SPSS, RRID:SCR_002865; IBM, Armonk, NY, USA). Differences between groups were performed using one-way ANOVA followed by Tukey's post hoc test. *P* values of <.05 were considered significant.

2.13 | Drugs and reagents

Allopurinol was obtained from Wako Pure Chemical Inc., Ltd. (Tokyo, Japan). Topiroxostat was manufactured and supplied by Fuji Yakuhin Co., Ltd. (Saitama, Japan). The chemical structures of allopurinol and topiroxostat are shown in Figure S1. For in vitro studies, allopurinol and topiroxostat were dissolved in 0.1 N of NaOH and 0.1 N of HCl, respectively. For *in vivo* studies, allopurinol and topiroxostat were adjusted to allopurinol 30 mg per 100-g feed and topiroxostat 1 mg per 100-g feed. For the ex vivo study, allopurinol and topiroxostat were suspended in 0.5% methylcellulose solution at a constant volume of 10 ml·kg^{-1} body weight. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), Wako Pure Chemical Inc., Ltd., or Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.14 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Profiles of low and high HPRT activity–*Uox* KO mice

Firstly, we investigated the construction of high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice as a novel mouse model of human hyperuricaemia. The model mice were established by mating B6-ChrXC^{MSM} mice having high HPRT activity with *Uox* KO mice. The gender rate of mice born in the low HPRT activity–*Uox* KO group was 55.6% male (79 of 142) and 44.4% female (63 of 142). By contrast, the gender rate of mice born in the high HPRT activity–*Uox* KO group was 44.4% male (55 of 124) and 55.6% female (69 of 124). Growth curves for the *Uox* KO mice from 6 to 15 weeks were similar between the low and the high HPRT group (Figure S2).

Secondly, we examined the profile of purine metabolism in high HPRT activity–*Uox* KO mice. Specifically, we determined the levels of urate, creatinine, allantoin, hypoxanthine, xanthine and erythrocyte HPRT activity in the plasma and urine of WT mice as well as low and high hypoxanthine phosphoribosyltransferase activity–*Uox* KO mice. The plasma urate level in WT mice and low and high HPRT activity–*Uox* KO mice was 0.37 ± 0.10, 5.12 ± 0.39 and 4.51 ± 0.66 mg·dl^{-1}, respectively (Figure 1a). Compared with WT mice, the plasma urate level was significantly increased by approximately 13-fold to 15-fold in low and high HPRT activity–*Uox* KO mice. Moreover, compared with WT mice, the urinary urate/creatinine ratio was also significantly increased by approximately 40-fold to 43-fold in low and high HPRT activity–*Uox* KO mice, respectively (Figure 1b). Urinary allantoin/creatinine ratio in WT mice was 6.40 ± 0.91, but urinary allantoin was not detected in these model mice (Figure 1c), confirming that *Uox* was knocked out. Urinary hypoxanthine and xanthine were also undetectable in the WT and *Uox* KO mice (data not shown). There was no significant difference in the plasma creatinine in these *Uox* KO mice (Figure 1d), while blood urea nitrogen and plasma cystatin C were significantly elevated in these *Uox* KO mice (Figure 1e,f). In addition, there was no significant difference in the plasma C-reactive protein in both *Uox* KO and WT mice (Figure 1g). HPRT activity in the erythrocytes of high HPRT activity–*Uox* KO mice was markedly elevated by approximately 33-fold compared with WT mice and low HPRT activity–*Uox* KO mice (Figure 1h). Thus, we established high HPRT activity–*Uox* KO mice as a novel hyperuricaemic animal model.

3.2 | In vitro inhibitory effects of allopurinol and topiroxostat on xanthine dehydrogenase (XDH)

We demonstrated the in vitro inhibitory effect of purine-type xanthine dehydrogenase (XDH) inhibitor, allopurinol, and non-purine-type xanthine dehydrogenase inhibitor, topiroxostat, on bovine milk XDH.

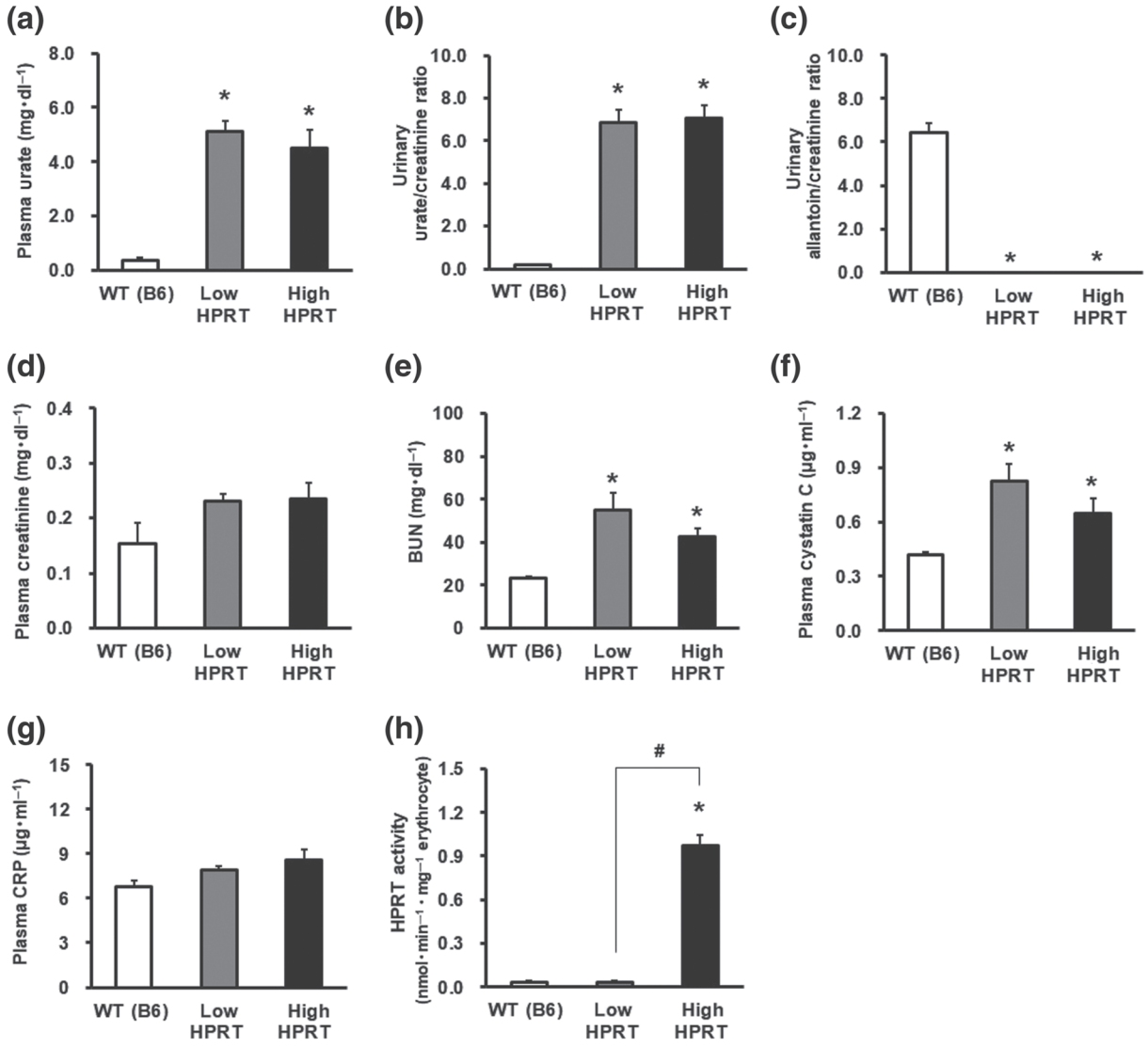
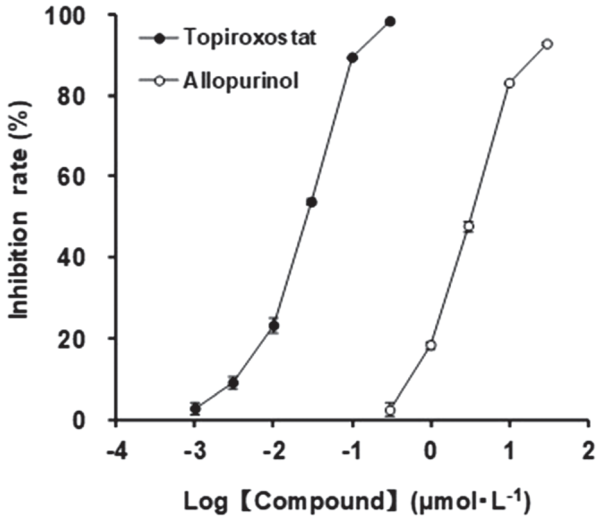


FIGURE 1 Profiles of purine metabolism in wild-type (WT) mice and low and high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* knockout (KO) mice. (a) Plasma urate (mg·dl^{-1}), (b) urinary urate/creatinine ratio, (c) urinary allantoin/creatinine ratio, (d) plasma creatinine (mg·dl^{-1}), (e) plasma blood urea nitrogen (BUN; mg·dl^{-1}), (f) plasma cystatin C (μg·ml^{-1}), (g) plasma C-reactive protein (CRP; μg·ml^{-1}), and (h) erythrocyte HPRT activity (nmol·min^{-1}·mg^{-1} erythrocyte) in WT mice (C57BL/6J) and low and high HPRT activity–*Uox* KO mice. Data represent means ± SEM of five mice per group. Significantly different from WT mice ($^{*}P < .05$) or low HPRT activity–*Uox* KO mice ($^{\#}P < .05$)

Allopurinol and topiroxostat dose-dependently inhibited XDH activity, with IC_{50} values of 3.40 and 0.022 μmol·L^{-1}, respectively (Figure 2). These results suggest that the *in vitro* inhibitory effect of topiroxostat is approximately 150-fold more potent than that of allopurinol.

3.3 | Effects of allopurinol and topiroxostat on the purine metabolites of low and high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice

We measured purine metabolites in the plasma and urine of low and high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice treated with allopurinol (30 mg·kg^{-1}) or topiroxostat (1 mg·kg^{-1}) for 7 days (Figure 3a–j). In the low HPRT activity–*Uox* KO mice, allopurinol 30 mg·kg^{-1} significantly decreased the plasma urate level and urinary urate/creatinine ratio (Figure 3a,c) and increased the hypoxanthine/creatinine ratio and xanthine/creatinine ratio compared with the control group (Figure 3d,e). By contrast, allopurinol had no significant effect on the oxypurine/creatinine ratio (Figure 3f). The topiroxostat 1 mg·kg^{-1} group showed a significant reduction in plasma urate level compared with the control group (Figure 3a), although the urinary urate/creatinine ratio did not significantly decrease (Figure 3c). By contrast, there was an increase in both the hypoxanthine/creatinine ratio and the xanthine/creatinine ratio (Figure 3d,e). No difference in the oxypurine/creatinine ratio was detected after treatment with topiroxostat for 7 days compared with the control group (Figure 3f). The plasma creatinine level for the allopurinol and topiroxostat groups



Compound	IC_{50} (μmol·L^{-1})
○ Allopurinol	3.40
● Topiroxostat	0.022

FIGURE 2 *In vitro* inhibitory effects of allopurinol and topiroxostat on bovine milk xanthine dehydrogenase (XDH). Inhibition curves of allopurinol (open circles) and topiroxostat (closed circles) on XDH activities with values of % inhibition. Allopurinol and topiroxostat inhibited XDH activity in a concentration-dependent manner. Data represent means ± SEM from five experiments

was similar to that of the control group (Figure 3b). Moreover, FE_{HX} and FE_{XA} increased in both the allopurinol and topiroxostat groups compared with the control group (Figure 3h,i), while FE_{UA} and FE_{OP} showed no significant difference (Figure 3g,j).

In the high HPRT activity–*Uox* KO mice, the allopurinol 30 mg·kg^{-1} group gave a significantly decreased plasma urate level and urinary urate/creatinine ratio (Figure 3a,c) and increased hypoxanthine/creatinine ratio and xanthine/creatinine ratio compared with the control group (Figure 3d,e). By contrast, the oxypurine/creatinine ratio did not significantly change (Figure 3f). The topiroxostat 1 mg·kg^{-1} group showed a significantly decreased plasma urate level, urinary urate/creatinine ratio, and oxypurine/creatinine ratio (Figure 3a,c,f), while the urinary hypoxanthine/creatinine ratio and xanthine/creatinine ratio increased compared with the control (Figure 3d,e). The plasma and urinary urate-lowering effect observed for the topiroxostat 1 mg·kg^{-1} group were equivalent to that seen in the allopurinol 30 mg·kg^{-1} group (Figure 3a,c). The urinary hypoxanthine/creatinine ratio and xanthine/creatinine ratio were significantly lower in the topiroxostat 1 mg·kg^{-1} group than in the allopurinol 30 mg·kg^{-1} group (Figure 3d,e). No significant differences were detected in the plasma creatinine level between the allopurinol, topiroxostat, and control groups (Figure 3b). FE_{HX} and FE_{XA} in the allopurinol and topiroxostat groups increased compared with those of the control group (Figure 3h,i). Interestingly, FE_{HX} for the topiroxostat group was significantly lower than for the allopurinol group (Figure 3h). No significant difference in FE_{UA} was detected in the allopurinol, topiroxostat, and control groups (Figure 3g). However, FE_{OP} of the topiroxostat group was significantly lower than for the allopurinol group (Figure 3j).

3.4 | *In vitro* metabolic assay using erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) of high HPRT activity mice

We investigated whether allopurinol and topiroxostat were metabolised by erythrocyte hypoxanthine phosphoribosyltransferase (HPRT)of high HPRT activity–*Uox* KO mice. Allopurinol (100 μmol·L^{-1}) and topiroxostat (100 μmol·L^{-1}) were mixed aerobically with 20 mg·ml^{-1} of erythrocyte extract containing HPRT and the mixture was incubated at 37°C. The reaction mixture was analysed for metabolites of allopurinol by HPLC immediately after mixing (0 min of incubation) and 120 min after incubation. The peak for allopurinol (peak A) after 120 min of incubation (Figure 4b) was reduced compared with that immediately after mixing (Figure 4a). Moreover, there was a corresponding increase in the level of allopurinol ribonucleotide (peak B) upon incubation. By contrast, topiroxostat was not converted by HPRT after 120 min of incubation (Figure 4c,d).

3.5 | *Ex vivo* inhibition assay for allopurinol and topiroxostat using mouse erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) in high HPRT activity–*Uox* KO mice

Here, we investigated whether allopurinol (30 and 100 mg·kg^{-1}) and topiroxostat (1 and 3 mg·kg^{-1}) inhibit erythrocyte HPRT of high HPRT activity–*Uox* KO mice (Figures 5 and S3). Allopurinol showed a dose-dependent and significant reduction of erythrocyte HPRT activity in high HPRT activity–*Uox* KO mice compared with that of the control group. By contrast, no such effect was observed with topiroxostat (Figures 5 and S3). In addition, HPRT activity in the topiroxostat 1 mg·kg^{-1} group was significantly higher than that of the allopurinol 100 mg·kg^{-1} group (Figure 5).

4 | DISCUSSION AND CONCLUSIONS

In this study, we established high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* KO mice as a novel hyperuricaemic animal model. This novel hyperuricaemic mouse model had an elevated urate state as confirmed from the plasma urate level and urinary urate/creatinine ratio by comparison with WT mice. Indeed, these values were lower than the reported plasma urate levels of *Uox* KO mice (Lu et al., 2018; Wu et al., 1994). We believe that these differences may be that in these KO mice there was not an induced false elevation of blood urate level due to high HPRT activity (Watanabe et al., 2016). By contrast, urinary allantoin was not detected in the

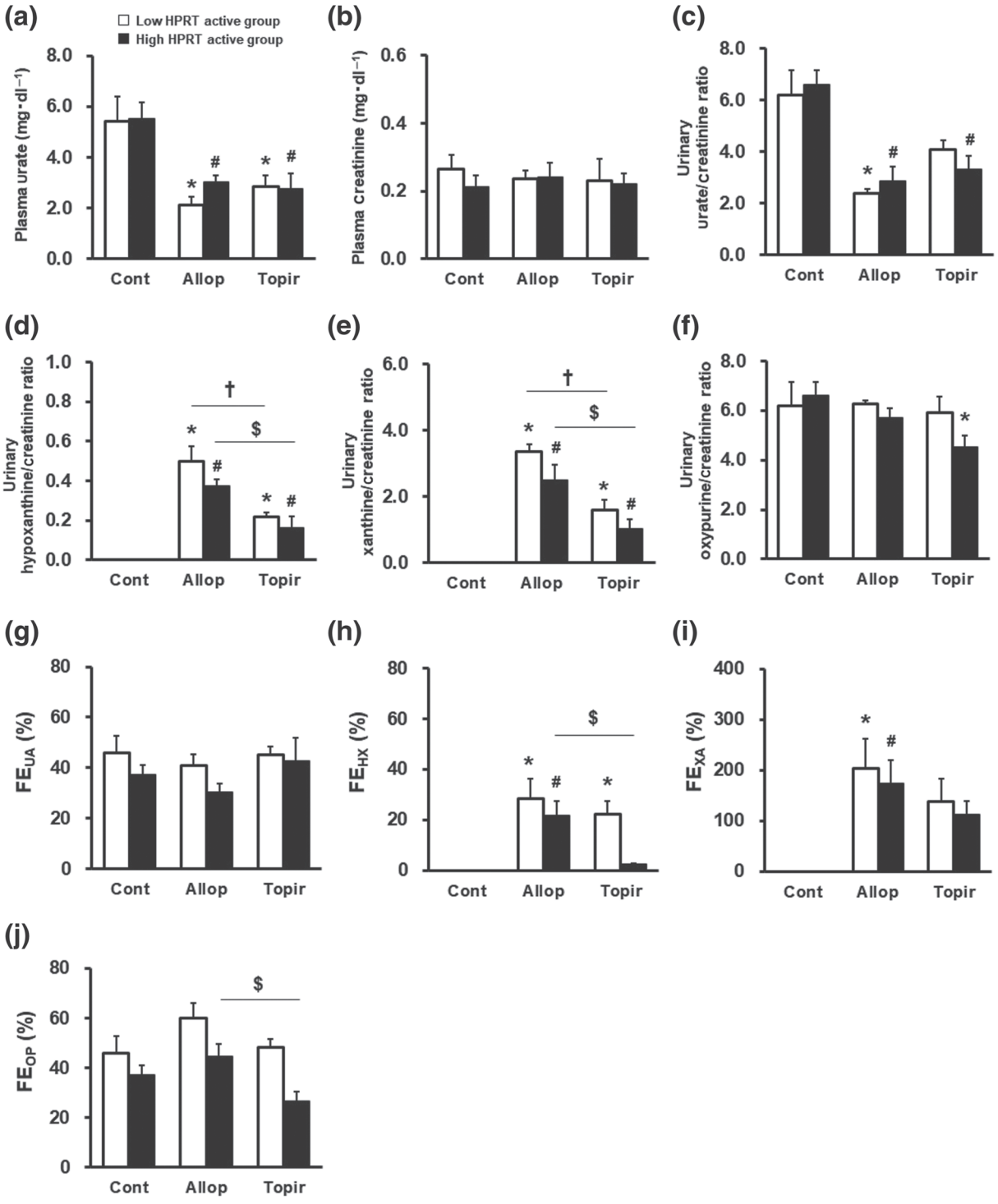


FIGURE 3 Effect of allopurinol (30 mg·kg^{-1}) and topiroxostat (1 mg·kg^{-1}) on the purine metabolites in the plasma and urine of low and high hypoxanthine phosphoribosyltransferase (HPRT) activity–*Uox* knockout mice. (a) Plasma urate (mg·dl^{-1}), (b) plasma creatinine (mg·dl^{-1}), (c) urinary urate/creatinine ratio, (d) urinary hypoxanthine/creatinine ratio, (e) urinary xanthine/creatinine ratio, and (f) urinary oxypurine/creatinine ratio, (g) fractional excretion of urate (FE_{UA}; %), (h) fractional excretion of hypoxanthine (FE_{HX}; %), (i) fractional excretion of xanthine (FE_{XA}; %), and (j) fractional excretion of oxypurine (FE_{OP}; %) in mice treated with allopurinol or topiroxostat by dietary administration. Data represent means ± SEM of five mice per group. Significantly different from the low HPRT active control ($^{*}P < .05$), high HPRT active control ($^{\#}P < .05$), allopurinol-treated low HPRT active group ($^{\dagger}P < .05$), or allopurinol-treated high HPRT active group ($^{\$}P < .05$)

model mice generated in this study. These results suggest that *Uox* is knocked out in this mouse model. It has been reported that *Uox* KO mice exhibit kidney damage from increased levels of serum creatinine and blood urea nitrogen (BUN) (Lu et al., 2018, Lu et al., 2019a; Guo et al., 2019). Our results showed no significant increase in plasma creatinine in these *Uox* KO mice but a significant increase in plasma

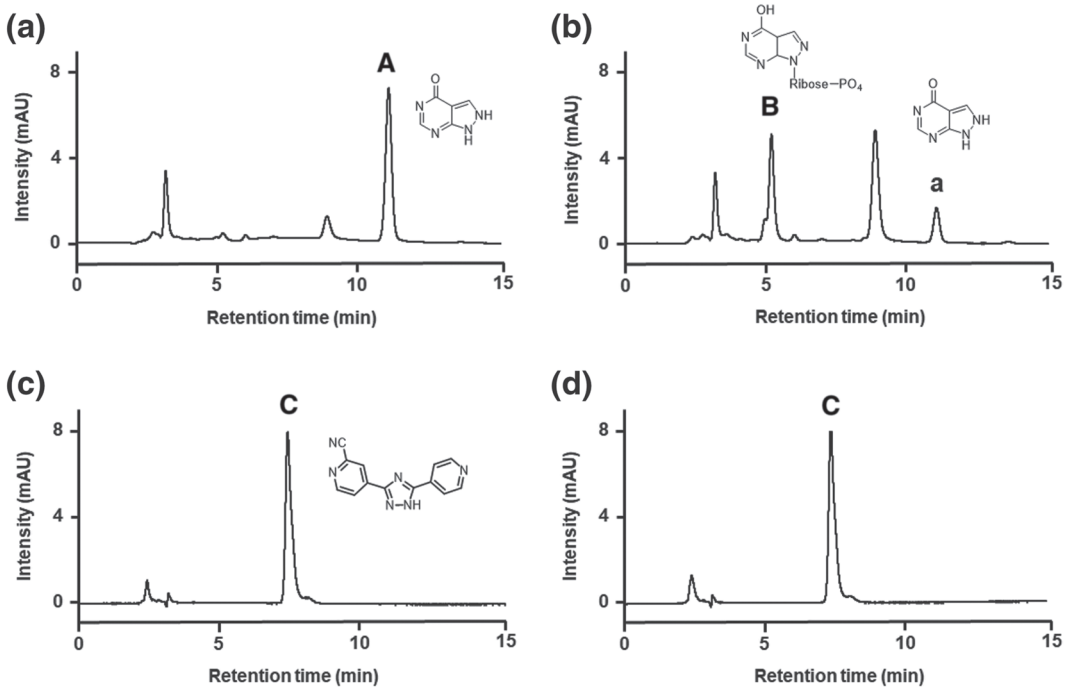


FIGURE 4 HPLC chromatogram showing conversion of allopurinol to its corresponding ribonucleotide by erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) of high HPRT activity–*Uox* knockout mice. Analysis of metabolites of allopurinol produced by mouse erythrocyte HPRT. Allopurinol (100 μmol·L^{-1}) and topiroxostat (100 μmol·L^{-1}) were each mixed aerobically with 20 mg·ml^{-1} of erythrocyte extract containing HPRT, and the mixture was incubated at 37°C. The reaction mixture was then analysed by HPLC: (a) immediately after mixing allopurinol (0 min of incubation); (b) 120 min after incubation allopurinol; (c) immediately after mixing topiroxostat (0 min of incubation); and (d) 120 min after incubation with topiroxostat. Peak A, allopurinol; peak B, allopurinol ribonucleotide (metabolite); peak C, topiroxostat

cystatin C, a renal functional marker, and BUN. Therefore, we consider that our novel model mice exhibit kidney damage as with conventional *Uox* KO mice. Moreover, we measured the plasma C-reactive protein (CRP) as inflammatory marker. However, there was no significant elevation in plasma CRP in our *Uox* KO mice. In clinical and non-clinical studies, increased serum CRP is observed in gouty arthritis (Inokuchi et al., 2006; Urano et al., 2002). Thus, our results suggest that our model mouse does not have gouty arthritis symptoms. In addition, we also showed that hypoxanthine phosphoribosyltransferase activity of high HPRT activity–*Uox* KO mice was significantly elevated by comparison with low HPRT activity–*Uox* KO mice. It has been reported that HPRT activity of mice are approximately 25-fold to 75-fold higher than that of C57BL/6J mice (Johnson & Chapman, 1987). The HPRT activity in *Mus m. sperutus* is 75-fold higher than low HPRT activity mice, which can be partly accounted for from the high reticulocyte count (Johnson & Chapman, 1987). A 25-fold increase in activity would be anticipated if the reticulocyte count was the same. Thus, our results are consistent with these previous reports. In conclusion, we have established a high HPRT activity–*Uox* KO mouse line as a novel hyperuricaemic animal model with high activity in the purine salvage enzyme HPRT that resembles purine metabolism in human, which contrasts with the conventional *Uox* KO mice.

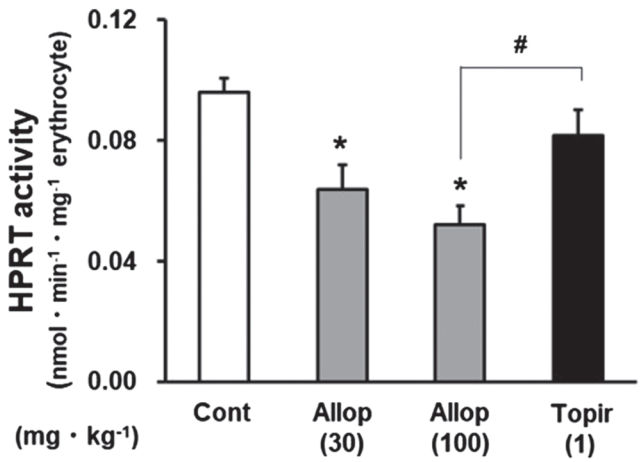


FIGURE 5 Effect of oral administration of allopurinol (30 and 100 mg·kg^{-1}) and topiroxostat (1 mg·kg^{-1}) on erythrocyte hypoxanthine phosphoribosyltransferase (HPRT) activity of high HPRT activity–*Uox* knockout mice. HPRT activity (nmol·min^{-1}·mg^{-1} erythrocyte) in mice after 30 min oral treatment with allopurinol or topiroxostat. Allopurinol dose-dependently inhibited mice erythrocyte HPRT activity, while topiroxostat did not. Data represent means ± SEM of five mice per group. Significantly different from control group ($^{*}P < .05$) or allopurinol 100 mg·kg^{-1} group ($^{\#}P < .05$)

Next, we investigated whether allopurinol and topiroxostat elicit a different pharmacological effect by using low and high HPRT activity–*Uox* KO mice. The urate-lowering drugs were xanthine oxidoreductase inhibitors and uricosurics, which are used to treat hyperuricaemia. Xanthine dehydrogenase (XDH) inhibitors are classified as purine-type (allopurinol) and non-purine-type (febuxostat and topiroxostat) drugs. Non-purine-type XDH inhibitors are known to possess a stronger

serum urate-lowering effect than purine-type XDH inhibitors (Cutolo, Cimmino, & Perez-Ruiz, 2017; Hosoya, Ogawa, Hashimoto, Ohashi, & Sakamoto, 2016; Kamatani et al., 2011; Nakamura et al., 2016). Indeed, we showed that the *in vitro* inhibitory effect of topiroxostat was stronger than that of allopurinol on XDH inhibitor activity. Matsumoto et al. (2011) reported that the urate-lowering effect of topiroxostat is 30-fold stronger than that of allopurinol in a potassium oxonate-induced hyperuricaemic rat model. We also showed that the plasma urate-lowering effect in the presence of topiroxostat 1 $mg \cdot kg^{-1}$ was equivalent to that of allopurinol 30 $mg \cdot kg^{-1}$ in high HPRT activity–*Uox* KO mice. These results suggest that topiroxostat has about 30-fold stronger urate-lowering effect than that of allopurinol. The efficacy of XDH inhibitors in rodents has been evaluated using higher doses compared with clinical doses (Cutolo et al., 2017; Hosoya et al., 2016; Kamatani et al., 2011; Lu et al., 2018; Matsumoto et al., 2011; Nakamura et al., 2016). In fact, we used similar doses to those used in the *in vivo* studies. Xu, LaVallee, and Hoidal (2000) reported that the liver *Xdh* gene in mice is approximately 100-fold more transcribed than liver *XDH* gene in human. Therefore, we deduced that rodents have a higher XDH activity than humans, so that doses of XDH inhibitor exceeding the clinical dose are required for efficacy. In addition, we also speculate that the pharmacokinetic species differences of each drug may be a factor (absorption, distribution, metabolism and excretion).

In this study, there was no change in the urinary oxypurine/creatinine ratio within the allopurinol and topiroxostat groups compared with the control *Uox* KO mice with low HPRT. Interestingly, topiroxostat significantly lowered the urinary oxypurine/creatinine ratio in high HPRT activity–*Uox* KO mice, although allopurinol did not. These data suggest that purine- and non-purine-type XDH inhibitors elicit a distinct effect on HPRT and the purine salvage pathway. There was also no change in the FE_{HX} after treatment with topiroxostat compared with allopurinol in low HPRT activity–*Uox* KO mice. By contrast, although the urinary urate-lowering effect was equivalent for allopurinol and topiroxostat, FE_{HX} and FE_{OP} were also significantly lowered after treatment with topiroxostat compared with allopurinol in high HPRT activity–*Uox* KO mice. These data indicate that due to differences in HPRT activity, hypoxanthine is recycled by HPRT, thereby reducing the amount of hypoxanthine subject to glomerular filtration, resulting in decreased urinary excretion. HPRT converts hypoxanthine to inosine monophosphate (Gogia, Balaram, & Puranik, 2011). Interestingly, feedback inhibition of the purine de novo pathway occurs when the inosine 5'-monophosphate (IMP) and **GMP** level increases due to salvage mediated by HPRT (Hershfield & Seegmiller, 1976a; Hershfield & Seegmiller, 1976b). Therefore, we propose that in the high HPRT activity mouse model, hypoxanthine produced by treatment with topiroxostat is rapidly converted to IMP, resulting in decreased urinary oxypurine excretion via feedback inhibition of the *de novo* pathway. The repetitive administration of XDH inhibitors to rodents increases xanthine excretion and produces xanthine crystals, resulting in kidney damage (Shimo, Ashizawa, Moto, Iwanaga, & Nagata, 2011), but no xanthine crystals occur in humans. Allopurinol-treated $Hprt^{-/-}$ mice showed a marked increase in serum hypoxanthine and xanthine and urinary xanthine crystals compared with allopurinol-treated WT mice and $Hprt^{+/-}$ mice (Zennaro et al., 2017). These observations suggest that HPRT affects urinary xanthine excretion. We also showed that topiroxostat-treated high HPRT active mice displayed significantly decreased oxypurine excretion compared with topiroxostat-treated low HPRT active mice (Figure 3). Thus, we speculate that XDH inhibitor-induced xanthine crystals may be less likely to develop in high HPRT active mice than in low HPRT active mice.

Next, we investigated the effect of allopurinol and topiroxostat on HPRT. Allopurinol is a purine-like hypoxanthine and is metabolised by various pathways in humans and rodents (Kelly, 1975). It has been reported that allopurinol is converted to allopurinol ribonucleotide by HPRT in humans (Elion & Nelson, 1974; Krenitsky, Papaioannou, & Elion, 1969). Using erythrocytes, we also established that allopurinol is metabolised to its corresponding ribonucleotide in high HPRT activity mice but not after treatment with topiroxostat (Figure 4). Moreover, *in vivo* studies using this mouse model showed that allopurinol has an inhibitory effect on mice erythrocyte HPRT, while topiroxostat displays no such inhibition (Figures 5 and S3). Therefore, these results suggest that, unlike allopurinol, topiroxostat elicits an efficient urate-lowering effect without perturbing HPRT and the purine salvage (Figure S4).

Allopurinol is known to cause anaemia symptoms, such as macrocytic anaemia and aplastic anaemia, as side effects in humans and rodents (Chao, Yang, & Lee, 2005; Kim et al., 2009; Lin et al., 1999). In particular, aplastic anaemia which is a serious condition and is clinically problematic. Patients with Lesch–Nyhan syndrome who have HPRT deficiency show anaemic symptoms (Cakmakli et al., 2018; Hakoda et al., 1995; Hidalgo-Laos, Kedar, Williams, & Neiberger, 1997). Hakoda et al. (1995) reported that HPRT is important for early blood cell formation. Furthermore, purine nucleotide biosynthesis is also considered to play a role in cell viability of dividing erythroblasts (Fox, Wyngaarden, & Kelley, 1970). Thus, we speculate that allopurinol probably affects erythropoiesis by inhibiting HPRT, resulting in anaemic symptoms, while topiroxostat is unlikely to induce such a side effect because HPRT is not inhibited. The mechanism underlying these haematological disorders brought about by treatment with allopurinol warrants further investigation.

Interestingly, non-purine-type XDH inhibitor has been reported to improve the symptoms of amyotrophic lateral sclerosis model mice. The XDH inhibitor-induced increase in the level of hypoxanthine was found to be important in promoting ATP production via the purine salvage pathway (Kato, Kato, Kusano, & Nishino, 2016). Moreover, other groups have reported clinical studies where combined therapy with XDH inhibitor and inosine was found to be useful for disorders with ATP deficiency by efficiently increasing ATP production via the purine salvage pathway (Johnson, Jinnah, & Kamatani, 2019). From these reports and our own results, we speculate that this novel model mouse may contribute not only to hyperuricaemia but also to other diseases related to the salvage pathway. As such, the model mice will be useful in the development of novel therapeutic agents.

In conclusion, high HPRT activity–*Uox* KO mice were established as a novel hyperuricaemic animal model. Topiroxostat, unlike allopurinol,

was found to have a potent plasma urate-lowering effect without perturbing the salvage pathway to efficiently reduce total oxypurine excretion. These findings may be reproduced in hyperuricaemia patients. Consequently, the novel hyperuricaemic mouse model generated in this study may contribute to the development of novel treatments.

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AUTHOR CONTRIBUTIONS

T.H. and M.H. did the study conception and design. T.H. and M.H. performed experiments and data acquisition. T.H., N.H.T., and M.H. analysed and interpreted the data. T.H., N.H.T., and M.H. drafted the paper. M.H., N.H.T., S.S., and S.U. did the critical revision.

CONFLICT OF INTEREST

T.H. was an employee of Fuji Yakuhin Co., Ltd. at the time of this study.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers, and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

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