Novel Keap1-Nrf2 Protein-protein interaction inhibitor UBE-1099 Ameliorates Progressive Phenotype in Alport Syndrome mouse model

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*Keap1-Nrf2 protein-protein interaction inhibitor could be a therapeutic drug for glomerulosclerosis and chronic kidney disease

**Abstract:**

Background: Bardoxolone methyl activates nuclear factor erythroid 2 related factor 2 (Nrf2) via covalent binding and irreversible inhibition of Kelch-like ECH-associated protein-1 (Keap1), the negative regulator of Nrf2. Ongoing clinical trials of Bardoxolone methyl show promising effects for patients with chronic kidney disease (CKD). But the direct inhibition of Keap1-Nrf2 protein-protein interaction (PPI) as an approach to activate Nrf2 is less explored. Methods: We developed a non-covalent Nrf2 activator UBE-1099, which highly selectively inhibits Keap1-Nrf2 PPI, and evaluated its efficacy on progressive phenotype in Alport syndrome mouse model (Col4a5-G5X). Results: Similar to Bardoxolone methyl, UBE-1099 transiently increased proteinuria and reduced plasma creatinine in Alport mice. Importantly, UBE-1099 improved the glomerulosclerosis, renal inflammation and fibrosis, and prolonged the lifespan of Alport mice. UBE-1099 ameliorated the dysfunction of Nrf2 signaling in renal tissue of Alport mice. Moreover, transcriptome analysis in glomerulus showed that UBE-1099 induced the expression of genes associated with cell cycle and cytoskeleton, which may explain its unique mechanism of improvement such as glomerular morphological change. Conclusions: UBE-1099 significantly ameliorated the progressive phenotype in Alport mice. Our results firstly revealed the efficacy of Keap1-Nrf2 PPI inhibitor for glomerulosclerosis and presents a potential therapeutic drug for CKD.

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Novel Keap1-Nrf2 Protein-protein interaction inhibitor UBE-1099 Ameliorates Progressive Phenotype in Alport Syndrome mouse model

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Key Points

- UBE-1099 inhibits Keap1-Nrf2 protein-protein interaction and induces Nrf2 activation
- UBE-1099 ameliorates progressive phenotype of Alport syndrome mouse model
- Keap1-Nrf2 protein-protein interaction inhibitor could be a therapeutic drug for glomerulosclerosis and chronic kidney disease

Abstract

Background  Bardoxolone methyl activates nuclear factor erythroid 2 related factor 2 (Nrf2) via covalent binding and irreversible inhibition of Kelch-like ECH-associated protein-1 (Keap1), the negative regulator of Nrf2. Ongoing clinical trials of Bardoxolone methyl show promising effects for patients with chronic kidney disease (CKD). But the direct inhibition of Keap1-Nrf2 protein-protein interaction (PPI) as an approach to activate Nrf2 is less explored.

Methods  We developed a non-covalent Nrf2 activator UBE-1099, which highly selectively inhibits Keap1-Nrf2 PPI, and evaluated its efficacy on progressive phenotype in Alport syndrome mouse model (Col4a5-G5X).

Results  Similar to Bardoxolone methyl, UBE-1099 transiently increased proteinuria and reduced plasma creatinine in Alport mice. Importantly, UBE-1099 improved the glomerulosclerosis, renal inflammation and fibrosis, and prolonged the lifespan of Alport mice. UBE-1099 ameliorated the dysfunction of Nrf2 signaling in renal tissue of Alport mice. Moreover, transcriptome analysis in glomerulus showed that UBE-1099 induced the expression of genes associated with cell cycle and cytoskeleton, which may explain its unique mechanism of improvement such as glomerular morphological change.
Conclusions  UBE-1099 significantly ameliorates the progressive phenotype in Alport mice. Our results firstly revealed the efficacy of Keap1-Nrf2 PPI inhibitor for glomerulosclerosis and presents a potential therapeutic drug for CKD.
Introduction

Nuclear factor erythroid 2 related factor 2 (Nrf2) is an essential transcription factor that regulates various genes involved in biological defense (1,2). Under basal condition, Nrf2 is anchored in the cytoplasm by Kelch-like ECH-associated protein-1 (Keap1), an adaptor protein for Cullin-3 ubiquitin ligase, and undergoes proteasomal degradation (3,4). But in the presence of oxidative stress, electrophiles or itaconate, the reactive cysteine residues of Keap1 are modified. This results in a structural change at the Nrf2 binding site, which allows Nrf2 to translocate to the nucleus and induces target gene transcription (5,6). Enhancement of biological defense via activation of Nrf2 is expected as a possible novel therapeutic strategy for various intractable diseases (7).

Bardoxolone methyl (CDDO-Me) is an electrophilic agent that induces Nrf2 activation by irreversibly and covalently binding to the cysteine residue of Keap1. (8). Multiple clinical studies showed that CDDO-Me significantly increases the estimated glomerular filtration rate (eGFR) in patients with stage 3-4 chronic kidney disease (CKD) (9–11). Although heart failure (HF) events most likely caused by fluid overload occurred in patients with BNP levels > 200 pg/mL and in patients with history of hospitalization for HF (11,12), CDDO-Me was well tolerated and no serious side effects were observed in patients without these risk factors (10). In this context, CDDO-Me is currently in clinical trials for different forms of CKD with careful monitoring and patient selection. Importantly, based on the efficacy and safety data from the CARDINAL phase 3 clinical trial, the Food and Drug Administration has now accepted for filing the New Drug application of CDDO-Me for the treatment of patients with Alport syndrome. Thus, there is a global trend to apply Nrf2 activators to CKD treatment. However, since covalent Keap1 inhibitors such as CDDO-Me has many off-targets involved in the efficacy, it is unclear to what extent its efficacy depends on Nrf2 activation. This is one of the compelling reasons to explore other strategies to activate Nrf2.
The development of Keap1-Nrf2 protein-protein interaction (PPI) inhibitor has been regarded as an innovative strategy to activate Nrf2 (13,14). Unlike the traditional Nrf2 activators such as electrophilic agents, this type of compound induces Nrf2 activation by competitively and directly inhibiting Keap1-Nrf2 PPI in a non-covalent manner (15). This mechanism of action can enhance the selectivity of the agents and reduce the risk of side effects (8).

In this study, we developed UBE-1099, a novel reversible and orally available Keap1-Nrf2 PPI inhibitor and evaluated its efficacy in a CKD mouse model. Most previous reports conducted short-term studies with an acute model to evaluate the effect of rodent tolerable CDDO-Me analogue (CDDO-Im or RTA 405) (16,17) or Keap1-Nrf2 PPI inhibitor (CPUY192018) (18). Here, to assess the long-term effect of Keap1-Nrf2 PPI inhibitor, we utilized the Alport syndrome mouse model, which has a nonsense mutation at glycine 5 in type IV collagen alpha 5 (Col4a5-G5X) and exhibits clinical phenotypes of CKD (19). Alport mice spontaneously start to show proteinuria and glomerulosclerosis at 8-12 weeks old and renal inflammation and fibrosis at 16 weeks old. These mice die at 35 weeks old. Here, we show that, delivered orally, UBE-1099 strongly induced Nrf2 activation in mice kidney. Notably, treatment with UBE-1099 ameliorated renal pathologies such as glomerulosclerosis, inflammation and fibrosis, and prolonged the lifespan of Alport mice.
Materials and methods

Fluorescence polarization assay

Seventy microliters of varying concentrations of UBE-1099, CDDO-Me or CDDO-Im was added to 350 μL of buffer solution (20 mM Tris-HCl; 150 mM NaCl; 0.05 % Tween 20; 5 mM DTT) containing 6 nM FITC-Labeled Nrf2 peptide (Invitrogen) and 0.2 mg/mL BSA. Then, 120 μL of solution was added into 96-well plate and mixed with 80 μL of buffer solution containing 2.5 nM human KEAP1-GST fusion protein (Ag0779, Proteintech). After incubation at room temperature for 30 min, fluorescence polarization was measured at λ_ex = 482 nm and λ_em = 530 nm. Inhibition rate was calculated using the following equation.

\[
\text{Inhibition rate (\%) = 100 - \left[ \frac{(A_{\text{sample}} - A_{\text{negative control}})}{(A_{\text{positive control}} - A_{\text{negative control}})} \right] \times 100}
\]

Wells without compound were used as positive control, and wells without Keap1 were used as negative control.

Animals and in vivo treatment

X-linked Alport syndrome mouse model (*Col4a5tm1Yseg* G5X mutant) was described previously (19). These mice were obtained from the Jackson Laboratory (Bar Harbor, USA). Age-matched wild-type (WT) C57BL/6 mice (Charles River Laboratories) were used for experiments as control to compare with Alport mice. Mice were housed in clean vivarium and fed with food and water ad libitum. For experiments, male mice were used to eliminate sex difference due to sex-linked inheritance of *Col4a5tm1Yseg* G5X mutation as we did previously (20–24). Six-week-old WT and Alport mice were treated with methylcellulose (Vehicle) or UBE-1099 (30 mg/kg/day, p.o.) for 16 weeks. UBE-1099 was synthesized by Pharmaceuticals Research Laboratory in UBE Industries LTD. (Yamaguchi, Japan).
Plasma pharmacokinetics

Plasma samples were collected 1, 4, 6, 14, 24 hours after compound administration in BALB/c mice. After adding 1 volume acetonitrile and 4 volumes acetonitrile containing an internal standard, mixture was shaken at 750 rpm for 1 min and centrifuged at 3,700 rpm for 2 min to remove proteins. The compound concentration in plasma was measured under the following conditions using LC-MS/MS (Shimadzu Corporation). Column = Phenomenex Kinetex C18 (50×2, 1 mm, 2.6 μm), Column temperature = 40 °C, Flow velocity = 0.3 mL/min, Mobile phase A = 0.1% formic acid/MiliQ aqueous solution, Mobile phase B = 0.1% formic acid, 50% acetonitrile/methanol solution, Gradient = 0-2 min; A / B = 90/10 to 10/90, 2-3 min; A / B = 10/90, 3-3.01 min; A / B = 10/90 to 90/10, MS = 3200QTrap (Sciex), Ionization = ESI, Mode = Positive.

GFR and blood pressure measurements

Glomerular filtration rate in 22-week-old mice was assessed via measurement of the clearance of FITC-sinistrin using a transdermal probe as previously described (16). In brief, mice anesthetized with isoflurane were injected with 7.5 mg/100 g body weight FITC-sinistrin (Medibeacon GmbH, Germany) through the subclavian vein. A transdermal GFR monitor was affixed directly to shaved skin on the dorsum of the animal (Medibeacon, Germany) and levels of FITC-sinistrin were measured. Calculation of GFR was performed with Medibeacon software according to previously published methods (25). Heart rate (HR), Systolic blood pressure (SBR), Diastolic blood pressure (DBP) and Mean blood pressure (MBP) were measured using BP-98A-1 (Softron) according to the manufacturer’s protocol.

Measurement of proteinuria and albuminuria score
Mouse urine samples were collected for 24 hr once every two weeks using metabolic cage (AS ONE Corporation, Japan). Urinary protein, albumin and creatinine were measured by Bradford method (Bio-Rad), ELISA method (Fujifilm) and Jaffe’s method (Wako Pure Chemicals), respectively, as described previously (22). Urinary protein and albumin concentration were normalized with urinary creatinine concentration, and presented as proteinuria and albuminuria.

**Measurement of plasma creatinine and BUN**

Mouse blood samples obtained from abdominal aorta were centrifuged at 3,000 rpm, 4 °C for 15 min, and blood plasma was collected. Plasma creatine and BUN were measured by DRI-CHEM (Fujifilm, Japan) and 7180 biochemistry automatic analyzer (Hitachi), respectively.

**Histological analysis**

Kidney tissues were fixed in 10 % formalin and embedded in paraffin. Tissue blocks were sliced into 4-µm thickness using microtome and stained with Periodic acid-Schiff (PAS) and Masson’s trichrome (MT) as previously described (20,22,23). We evaluated the glomerulosclerosis score as we did previously. More than 100 random glomeruli per mouse were scored based on the following criteria, 0: no lesion, 1: expansion of mesangial area, 2: expansion of Bowman’s epithelial cells, adhesion of glomeruli and Bowman’s capsule and partial sclerosis, 3: sclerotic area in 50–75% of glomeruli, and 4: sclerotic area in 75–100% of glomeruli. For renal fibrosis, MT-positive area was quantified using BZ-X700 microscope and image analysis software (KEYENCE, Osaka, Japan).

**Immunostaining**
For immunohistochemistry, 4-µm paraffin sections were stained with anti-F4/80 antibody (clone Cl:A3-1, BIO-RAD) and anti-WT1 antibody (clone C-19, Santa Cruz Biotechnology, CA, USA) as previously described (22). F4/80-positive area in cortex of kidney tissue was quantified using BZ-X700 microscope and image analysis software (KEYENCE, Osaka, Japan). WT1-positive cells in more than 100 random glomeruli per mouse were counted and expressed as average per glomerulus.

**Immunoblotting**

To isolate the whole cell protein, kidney samples were lysed in T-PER Tissue Protein Extraction Reagent (Thermo Scientific Inc., USA) according to the manufacturer’s protocol. To isolate the nuclear protein, kidney samples were homogenized with Dounce homogenizer (D8938, Sigma) in 2 ml ice-cold Nuclei EZ Lysis buffer (NUC101, Sigma) and incubated on ice for 5 min with an additional 2 ml of lysis buffer. The digested kidneys were passed through a 40-µm cell strainer and centrifuged at 500 G, 4 °C for 5 min. The pellet was resuspended and washed with 4 ml of the lysis buffer and incubated on ice for 5 min. After passing through a 20-µm cell strainer and centrifugation at 500 G, 4 °C for 5 min, the pellet was lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% w/v Nonidet P-40, 0.5 % w/v Sodium deoxycholate, 0.5 % w/v SDS). Protein lysates were subjected to SDS-PAGE and Western blot analysis as described previously (20,22,23). Anti-NRF2 (ab31163) and anti-NQO1 antibodies (ab34173) were from Abcam. Anti-vinculin (ab129002; abcam) and anti-HDAC2 antibodies (sc-7899; SantaCruz) were used as loading control. The above primary antibodies were detected using their respective HRP-conjugated secondary antibodies. Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific Inc., USA) were used for visualizing the blots.
Real-time quantitative RT-PCR

Total RNA was isolated from mice kidneys using NucleoSpin RNA (Takara, Japan) with homogenization. Reverse transcription and PCR amplification were performed using PrimeScript RT Reagent Kit with gDNA Eraser and SYBR Premix ExTaq II (Takara, Japan), respectively, according to the manufacturer’s recommended protocol. The sequences of primers used for Q-PCR are listed in Table 1.

Isolation of Glomeruli

Glomeruli were isolated using magnetic beads after administration of the compound to Alport mice for 4 weeks from 6 to 10 weeks of age. Briefly, mice were perfused with prewarmed 38 mL Hank’s balanced salt solution (HBSS) and 2 mL HBSS with enzymatic digestion solution [300U/mL Collagenase type II (Sigma), 1 mg/ml Proteinase E (Sigma) and 50 U/mL Dnase I (Invitrogen)]. Then, kidneys were removed and minced into 1 mm³ pieces, and digested in 2 mL enzymatic digestion buffer at 37 °C for 20 min on a rotator. The digested kidneys were passed through a 200-µm cell strainer, and glomeruli were washed four times and collected using Dynabeads (Invitrogen) according to the manufacturer’s recommended protocol.

RNA sequencing

Total RNA from glomeruli was isolated and purified using RNeasy plus Mini Kit (QIAGEN) according to the manufacturer’s instructions. The purity and integrity of the isolated RNA was checked by Epoch Microplate Spectrophotometer (BioTek) and Agilent 2100 BioAnalyzer. Poly(A)-selected cDNA libraries were generated using the TruSeq Stranded mRNA Library Prep kit (Illumina). Sequencing was performed using NextSeq500 system (Illumina) in 76-bp single-end read. After adaptor trimming and quality
check by Trim Galore (v0.5.0), sequencing reads were aligned to the mouse reference genome (mm10) using STAR (v2.6.0a). Gene expression profiles for each sample were measured as transcripts per million (TPM) using RSEM (v1.3.1). Differentially expressed genes, fold change of >1.2 or < -1.2, p<0.05 (WT vs Alport vehicle, Alport vehicle vs Alport UBE-1099), were measured using DESeq2 and subjected to heatmap analysis. TPM data were subjected to Gene Set Enrichment Analysis (v4.1.0). The RNA-seq data have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number DRA012265.

**Statistical analysis**

All data are presented as mean ± SE. The significance of the difference between two groups was assessed using Student’s unpaired two-tailed t-test. For three- or four-group comparisons we used analysis of variance (ANOVA) with the Dunnett test. P-values <0.05 were considered to be statistically significant.

**Study approval**

Animal experiments for Fig. 1 and 7 were reviewed and approved by the Animal Care and Use Committee of the Pharmaceutical Research Laboratory of UBE Industries Ltd, Yamaguchi, Japan (P-20032, P20170). Animal experiments for Fig. 2-6 and Fig. S1-11 were approved by the Animal Care and Use Committee of Kumamoto University, Kumamoto, Japan (A2020-020).
Results

UBE-1099 induced Nrf2 activation through the inhibition of Keap1-Nrf2 protein-protein interaction

For the purpose of developing a non-covalent Nrf2 activator, we performed fluorescence polarization-based screening using the previously developed Keap1-Nrf2 PPI inhibitor (26) as the lead compound, and optimized the structure based on structure activity relationship (SAR). We successfully identified UBE-1099 that markedly inhibited Keap1-Nrf2 interaction (Fig. 1A, B). CDDO-Me and CDDO-Im did not show inhibitory effect (Fig. 1B) because this evaluation system used the human Keap1-Kelch domain, which does not contain BTB and IVR domain with the cysteine residue that is the target of covalent Nrf2 activator. The maximum blood concentration of UBE-1099 is much higher than that of CDDO-Im when orally administrated in mice, and UBE-1099 strongly induced the Nrf2-target molecule, NAD(P)H: quinone oxidoreductase 1 (Nqo1) expression in mouse renal tissue (Fig. 1C, D).

The effects of UBE-1099 on the renal dysfunction in Alport mouse model

The Nrf2-activating effect of UBE-1099 lasted up to 24 hr after administration at a concentration of 30 mg/kg as detected from the elevated expression of Nqo1 mRNA compared to pre-treatment (Fig. 1D). Therefore, we set the dosing condition at 30 mg/kg/day, p.o. to maintain a constant effect, and assessed the effect of UBE-1099 on the renal function in Alport mice at 6 to 22 weeks old. Slight weight loss and increased urine volume were observed in the UBE-1099-treated Alport mice, but no noticeable toxicity was suspected (Fig. S1). UBE-1099 also did not induce changes in heart rate and blood pressure in Alport mice over time (Fig. S2). Similar to the results in clinical trials of CDDO-Me (27), UBE-1099 transiently increased the proteinuria, but not albuminuria in Alport mice at 2 to 4 weeks after
the start of administration (Fig. 2A, B; 8 and 10 weeks old). However, at the late stage of the disease, UBE-1099 slightly, though not statistically, reduced the proteinuria and albuminuria in Alport mice (Fig. 2A, B; 22-week-old). Although clear improvement of GFR was not confirmed, UBE-1099 significantly decreased plasma creatinine and blood urea nitrogen (BUN) in Alport mice (Fig. 2C-E). Moreover, the mRNA expression level of the renal injury marker Lipocalin-2 (Lcn2; also known as NGAL) was statistically decreased by treatment with UBE-1099 (Fig. 2F).

**UBE-1099 suppressed the glomerulosclerosis in Alport mouse model**

We investigated the effect of UBE-1099 on glomerulosclerosis, which is one of the hallmarks of Alport syndrome. While vehicle-treated Alport mice had typical glomerulosclerosis with formation of glomerular tuft adhesion in Bowman’s capsule, UBE-1099 inhibited the collapse of glomeruli and ameliorated the characteristic glomerulosclerosis (Fig. 3A; PAS). While more than 50% of glomeruli showed the highest glomerulosclerosis score (injury score of 4) in vehicle-treated Alport mice, only less than 30% of glomeruli had severe glomerulosclerosis in UBE-1099-treated Alport mice and 40% of these glomeruli presented less severe injury score of 2 (Fig. 3B). Because podocyte is the most important cell in glomerular filtration and its injury is associated with glomerulosclerosis (28–30), we evaluated the number of podocytes by staining its nuclear marker Wilms’ tumor 1 (WT1). We found that UBE-1099 suppressed the reduction of WT1-positive cells in glomeruli (Fig. 3A, C). These results collectively revealed that UBE-1099 ameliorated the glomerulosclerosis that is characteristically seen in Alport mice.

**UBE-1099 suppressed the renal tissue inflammation and fibrosis in Alport mouse model**
Along with glomerulosclerosis, Alport mice overtly exhibit renal inflammation and fibrosis (20–24). Immunostaining of F4/80 showed that macrophage infiltrated the cortical area of kidney tissue in Alport mice, but UBE-1099 suppressed it to the level of the wild-type mice (Fig. 4A, B). Masson-trichrome (MT) staining revealed that the fibrotic region in kidney tissue of Alport mice was reduced by treatment with UBE-1099 (Fig. 4A, C). Consistent with the staining results, the expression levels of inflammatory cytokines (Mcp1, Il-1β, Il-6 and Tnf-α) and fibrosis-related genes (Tgf-β and Mmp9) were down-regulated by treatment with UBE-1099 (Fig. 4D-I). Moreover, kidney injury marker-1 (Kim1) was down-regulated by UBE-1099 (Fig. 4J). These results suggest that UBE-1099 suppressed renal tissue injury, inflammation and fibrosis in Alport mice.

**UBE-1099 induced Nrf2 activation and ameliorated the dysfunction of Nrf2 signaling in renal tissue of Alport mouse model**

Oxidative stress is one of the leading causes of progression of CKD (31,32), and Nrf2 signaling pathway is an evolutionally conserved intracellular defense mechanism against oxidative stress (1,2), so we isolated whole cell protein or nuclear fraction protein from mice kidney tissues and assessed the expression level of Nrf2 and its downstream targets. Compared with wild-type, Alport mice kidney exhibited dysregulation of the protein expression of Nrf2 and its downstream molecules, NQO1 (Fig. 5A, C, D). The protein expression level of Nrf2 was slightly increased, and NQO1 was highly increased in the kidney of UBE-1099-treated Alport mice compared with vehicle-treated Alport mice. Because active Nrf2 localizes to the nucleus, we examined Nrf2 expression in kidney nuclear extracts. Nrf2 protein expression was increased in the nuclear extracts of UBE-1099-treated Alport mice to the level similar to that of wild-type mice while nuclear Nrf2 protein level remained low in untreated Alport mice (Fig. 5B, E). The mRNA expression level of Nrf2 was
reduced and normalized to wild-type level in Alport mice treated with UBE-1099 compared with vehicle (Fig. 5F). Notably, the expression of the Nrf2-induced anti-oxidant molecules were also increased at the transcription level (Fig. 5G, H). Collectively, these data suggest that UBE-1099 activates Nrf2 signaling and ameliorated the dysregulation of Nrf2 signaling pathway in Alport mice.

**Transcriptome analysis reveals the comprehensive effects of UBE-1099 in the glomeruli of Alport mouse model**

To clarify the molecular effects of UBE-1099 on the glomerulosclerosis in Alport mice, we performed RNA sequencing on the glomeruli of wild-type and Alport mice treated with vehicle or UBE-1099 (30 mg/kg/day, p.o.) for 4 weeks. Transcriptome analysis showed 3511 genes with differential expression between wild-type (WT) vs Alport/vehicle, 633 genes between Alport/vehicle vs Alport/UBE-1099 (fold change <-1.2 or >1.2, \( P \) value < 0.05, Fig. 6A). Importantly, more than half of the fluctuated genes between Alport/vehicle vs Alport/UBE-1099 are not fluctuated genes between WT vs Alport/Vehicle (Fig. 6A, and Fig. S3). Therefore, we performed Gene Set Enrichment Analysis (GSEA) to characterize the altered genes. We detected Gene Ontology (GO) terms associated with ER stress, cell adhesion and transporter as the main abnormalities in the Alport/vehicle group (Fig. S4-S6). On the other hand, we detected GO terms associated with the cell cycle, Nrf2 signaling, cytoskeleton and mitochondria as the target of UBE-1099 (Fig. 6B, and Fig. S7-S9). It is considered that UBE-1099 activated Nrf2 and acted on the downstream pathway (33–38). It had been suggested previously that the effect of CDDO-Me in increasing the GFR involves changes in glomerular structure (17). Based on this notion and the differential expression of cell cycle and cytoskeletal pathways without changes in cell composition in the glomerulus (Fig. S10), it is possible that UBE-1099 may also induce glomerular structure change.
UBE-1099 prolonged the lifespan of Alport mouse model

Because UBE-1099 improved the glomerulosclerosis, renal inflammation and fibrosis in Alport mice, we investigated whether it prolongs the lifespan of these mice. We treated Alport mice with UBE-1099 (30 mg/kg/day, p.o.) from 6 weeks old and monitored their survival. While half of vehicle-treated Alport mice had died by 210 days, half of UBE-1099-treated mice survived beyond 250 days (Fig. 7). This result suggests that UBE-1099 delayed the onset of end-stage renal disease.
Discussion

This study firstly revealed the efficacy of Keap1-Nrf2 PPI inhibitor on glomerulosclerosis in CKD. Interestingly, while UBE-1099 ameliorated the renal pathology and prolonged the lifespan of Alport syndrome model mice, transient increase in proteinuria was confirmed after the start of administration. This is similar to clinically observed phenomenon in patients receiving CDDO-Me (27). It has been suggested that changes in glomerular structure are involved in the improvement of GFR and increase in proteinuria by CDDO-Me (17), but the detailed mechanism is unknown. Our transcriptome analysis of glomeruli revealed that UBE-1099 increased the expression of genes related to cell cycle and cytoskeleton in the glomerulus of Alport mice. These pathways may contribute to the unique mechanism of CKD improvement by UBE-1099. UBE-1099 increased the antioxidant activity and detoxification process (Fig. 6B), which was expected considering that these are target processes of Nrf2, and indicated that UBE-1099 activated the Nrf2 pathway. However, it is surprising that UBE-1099 downregulated the processes associated with ATP production and mitochondrial function (Fig. 6B). We previously observed that treatment with MitoQ, which is a mitochondria-targeted anti-oxidant supplement (39,40), did not improve the glomerulosclerosis of Alport mice (data not shown). Together, these data suggest that the ameliorative effect of UBE-1099 is not merely via the suppression of oxidative stress but also involves the regulation of glomeruli cell cycle and cell structure. Moreover, considering that the renal tubules reabsorb most of the primitive urine containing small proteins, UBE-1099 may also act on renal tubules and increase the urine volume and urinary protein by inhibiting reabsorption.

While there are studies showing the efficacy of activating Nrf2 (41–43), other reports show that Nrf2 activation exacerbates glomerular diseases (16,44). Especially, Rush et al indicated that genetic or pharmacological Nrf2 activation worsened proteinuria and glomerulosclerosis
in experimental model induced by adriamycin and angiotensin II while Nrf2 knockout mice were protected against proteinuria (16). These results suggest that under certain conditions such as adriamycin-induced injury and high level of angiotensin II, Nrf2 activation exacerbates renal pathology. However, at least to date, there have been no reports of deterioration of renal function in clinical trials in patients with diabetic kidney disease (AYAME), autosomal dominant polycystic kidney disease (FALCON), IgA nephropathy, type I diabetic nephropathy, focal segmental glomerulosclerosis (PHOENIX) and Alport syndrome (CARDINAL). This suggests that exacerbation events that are provoked in experimental mouse model cannot be extrapolated to humans. Because Alport mouse model exhibits phenotype close to clinical phenotype in that renal function gradually declines over a period of more than half a year, we may have confirmed the ameliorative effect of Nrf2 activator against glomerulosclerosis. We also confirmed that UBE-1099 did not worsen the early renal pathology and did not affect the food intake and muscle weight in Alport mice (Fig. S11).

Glomerular diseases including Alport syndrome are primarily treated with renin-angiotensin system (RAS) inhibitors (45). Moreover, recent clinical studies show efficacy of sodium/glucose cotransporter 2 (SGLT2) inhibitors for non-diabetic glomerular disease (46). However, since these medicines are symptomatic treatments, their therapeutic effects are limited and only suppress the decline in renal function. Therefore, the development of drugs that improve renal function, such as CDDO-Me, is essential to prevent the progression of CKD to end-stage renal disease. Longer period of treatment with CCDO-Me will clarify its effect on GFR in patients with kidney disease. Considering that the direct effect of Nrf2 and GFR increase was observed using Nrf2 genetically modified mice in diabetes model (47), development of Keap1-Nrf2 PPI with higher Nrf2 activity may be expected to further improve GFR.
Importantly, based on the efficacy and safety data from the CARDINAL phase 3 clinical trial, the Food and Drug Administration has now accepted for filing the New Drug application of CDDO-Me for the treatment of patients with Alport syndrome. Thus, there is a global trend to apply Nrf2 activators to CKD treatment. Exploring other strategies of activating Nrf2, such as by using highly specific Keap1-Nrf2 PPI inhibitors that are less likely to cause off target side effects could provide safer and more options clinically. The endothelin signaling is suggested to be involved in the side effect of CDDO compounds because heart failure and fluid retention observed in BEACON trial were also observed in the clinical trial of endothelin receptor antagonist Avosentan in patients with stage 3-4 CKD (12,48). Here, we confirmed that CDDO-Im significantly and dose-dependently decreased the endothelin-1 receptor expression. However, UBE-1099 did not affect the endothelin-1 expression in proximal tubular epithelial cells (Fig. S12). This result suggests that UBE-1099 does not produce an off-target effect similar to CCDO-Im.

Our present study has some limitations. Firstly, we were not able to fully clarify whether our results depend solely on Nrf2. UBE-1099 highly selectively inhibits Keap1-Nrf2 PPI, but we cannot rule out the possibility that it interacts with other targets. To resolve this issue, the use of genetically modified mice, such Nrf2 knockout mice, is necessary. Additionally, in order to avoid affecting the renal pathology, we measured blood pressure (BP) with noninvasive tail cuff, which may fail to identify small to moderate changes in BP. Therefore, in the future, it may be necessary to measure BP using other methods.

In conclusion, we show that Nrf2 activation by Keap1-Nrf2 PPI inhibitor improved renal pathology and prolonged the survival of Alport mice. These results not only present the efficacy of Keap1-Nrf2 PPI inhibitor for therapeutic application for Alport syndrome and CKD, but also support the current trend toward considering the use of Nrf2 activators for patients with kidney disease.
Disclosures

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Author contributions

S.K. and K.O. designed the research, conducted experiments, and wrote the manuscript. S.O. designed the research and conducted experiments. M.A.S. designed the research and wrote the manuscript. F.N., T.S. and H.K. designed the research. Y.S., J.K., R.S., H.S., H.F., M.K., S.N., J.H. and Y.K. conducted experiments. H.N. contributed to the synthesis of UBE-1099. All authors discussed the results and provided input on the manuscript.
Data Sharing Statement

All data is included in the manuscript and/or supporting information

Supplementary Materials

Supplementary Method

Endothelin-1 concentration measurement

Supplementary Figures

Figure S1. UBE-1099 slightly reduced the body weight and increased the urine volume in Alport mice

Figure S2. UBE-1099 did not affect the heart rate and blood pressure in Alport mice

Figure S3. Transcriptome analysis reveals the comprehensive effects of UBE-1099 in the glomeruli of Alport mice

Figure S4. Dysregulated GO terms in the glomeruli of Alport mice

Figure S5. Up-regulated GO terms for Alport vehicle vs WT

Figure S6. Down-regulated GO terms for Alport Vehicle vs WT

Figure S7. Up-regulated GO terms for Alport UBE-1099 vs Alport Vehicle

Figure S8. Down-regulated GO terms for Alport UBE-1099 vs Alport Vehicle

Figure S9. UBE1099 altered genes in each condition

Figure S10. Expression level of cell specific markers in the glomerular cell

Figure S11. UBE-1099 did not affect the food intake and muscle weight in Alport mice and did not worsen the early renal pathology

Figure S12. UBE-1099 did not affect the endothelin expression

Figure S13. Full length blots for Figure 5A, B
References


Table 1. Primers used in quantitative RT-PCR

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*Used in Fig. 1; #Used in Figs. 2, 4, 5
Figure Legends

Figure 1. UBE-1099 induced Nrf2 activation through the inhibition of Keap1-Nrf2 protein-protein interaction

(A) Chemical structure of UBE-1099. (B) Keap1-Nrf2 protein-protein interaction was measured by fluorescence polarization assay. (C) Plasma samples were collected at the indicated time points, and concentration of CDDO-Im and UBE-1099 were measured by LC-MS/MS. (D) Total RNA was isolated from renal tissues at the indicated time points. The level of the Nqo1 mRNA was measured and normalized to the level of Gapdh mRNA (internal control). Data are presented as mean ± SE (n = 3 per group). P values were assessed by Dunnett’s test. (*p<0.05, **p<0.01, ***p<0.001 vs Pre).

Figure 2. The effects of UBE-1099 on the renal dysfunction in Alport mouse model

(A, B) Urine samples were collected at the indicated time points. Urinary protein and albumin were measured by Bradford method and ELISA method, respectively. Urinary creatinine was measured by Jaffe’s method. Urinary protein and albumin concentration were normalized with urinary creatinine concentration, and presented as proteinuria and albuminuria, respectively. (C) GFR was measured by Transdermal GFR Monitor (MediBeacon) in 22-week-old wild-type and Alport mice. (D, E) Plasma creatinine and BUN were measured by DRI-CHEM (Fujifilm) and 7180 biochemistry automatic analyzer (Hitachi), respectively, in 22-week-old wild-type and Alport mice. (F) Total RNA was isolated from renal tissues of 22-week-old mice. The level of the Lcn2 mRNA was measured and normalized to the level of Gapdh mRNA (internal control). Data are presented as mean ± SE (n = 7-8 per group). P values were assessed by Dunnett’s test. (*p<0.05, **p<0.01, ***p<0.001).
Figure 3. UBE-1099 suppressed the glomerulosclerosis in Alport mouse model

(A) Renal sections of 22-week-old wild-type and Alport mice were analyzed by PAS staining and immunohistochemistry of WT1. Representative images are shown. Scale bars = 50 μm.

(B) Glomerulosclerosis scores were evaluated based on the PAS-stained sections. See Methods for assessment. (C) Quantification of WT1-positive cells in the glomerulus. Data are presented as mean ± SE (n = 8 per group). P values were assessed by Dunnett’s test. (*p<0.05, **p<0.01, ***p<0.001).

Figure 4. UBE-1099 suppressed the renal tissue inflammation and fibrosis in Alport mouse model

(A) Renal sections of 22-week-old wild-type and Alport mice were analyzed using F4/80 immunohistochemistry and Masson-trichrome (MT) staining. Representative images are shown. Scale bars = 200 μm. (B, C) F4/80-positive area and fibrotic region were evaluated based on the F4/80-stained section and MT-stained section, respectively, using Bio-Revo imaging and analysis software. (D-J) Total RNA was isolated from renal tissues of 22-week-old wild-type and Alport mice. The level of the indicated mRNA was measured and normalized to the level of Gapdh mRNA (internal control). Data are presented as mean ± SE (n = 7-8 per group). P values were assessed by Dunnett’s test. (*p<0.05, **p<0.01, ***p<0.001).

Figure 5. UBE-1099 induced Nrf2 activation and ameliorated the dysfunction of Nrf2 signaling in renal tissue of Alport mouse model

(A) Whole cell protein and (B) nuclear fraction protein were isolated from kidney tissue in the indicated group at 22-week-old and analyzed by immunoblotting. (C-E) The relative amount of proteins was quantified using Image Gauge software (Fujifilm) and normalized
with Vinculin and HDAC2 (internal control). Data are presented as mean ± SE (n = 3, 4 per group). \( P \) values were assessed by Dunnett’s test. (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \)). (F-H) Total mRNA was isolated from renal tissues of 22-week-old wild-type or Alport mice. The level of the indicated mRNA was normalized to the level of Gapdh mRNA (internal control). Data are presented as mean ± SE (n = 7-8 per group). \( P \) values were assessed by Dunnett’s test. (***\( p < 0.001 \)).

**Figure 6. Transcriptome analysis reveals the comprehensive effects of UBE-1099 in the glomeruli of Alport mouse model**

(A) Venn diagram shows the number of fluctuated genes in three comparisons (WT vs. Alport vehicle, Alport vehicle vs. Alport UBE-1099; fold change > 1.2 or < -1.2, \( p < 0.05 \)).

(B) Gene ontology (GO) analysis of the differentially expressed genes in UBE-1099-treated Alport mice vs Alport Vehicle analyzed using Gene Set Enrichment analysis software (v4.10). Colored graphs represent the terms as indicated. (p<0.01).

**Figure 7. UBE-1099 prolonged the lifespan of Alport mouse model**

(A) Kaplan-Meier survival curves. Results were derived from wild-type (n=12), vehicle-treated Alport (n=12) and UBE-1099-treated Alport mice (n=12). \( P \) values were assessed by log-rank test. (**\( p < 0.01 \)).
Figure 6

A

WT vs Alport Vehicle

Alport Vehicle vs Alport UBE1099

3244  295  338

(genres)

B

Up-regulated GO Terms
(Alport UBE1099 vs Alport Vehicle)

Normalized Enrichment Score

Mitotic sister chromatid segregation
Metaphase anaphase transition of cell cycle
Sister chromatid segregation
Cellular response to toxic substance
Regulation of chromosome separation
Regulation of chromosome segregation
Glutathione metabolic process
Nuclear chromosome segregation
Chromosome separation
Cell redox homeostasis
Condensed chromosome centromeric region
Chromosome centromeric region
Condensed chromosome
Spindle microtubule
Platelet alpha granule membrane
Kinetochore
Cyclin dependent protein kinase holoenzyme complex
Microtubule-associated complex
Spindle pole
Condensed nuclear chromosome
Antioxidant activity
Glutathione transferase activity
Microtubule motor activity
Histone kinase activity
Oxidoreductase activity acting on peroxide as acceptor

Normalized Enrichment Score

Mitochondrial ATP synthesis coupled proton transport
Aromatic amino acid family catabolic process
Cotranslational protein targeting to membrane
Mannosylation
Metanephric Epithelium Development
Metanephric tubule development
ATP synthesis coupled proton transport
Protein N-linked glycosylation
Regulation of ATP biosynthetic process
Proton transmembrane transport
NADH dehydrogenase complex
Proton transporting ATP synthase complex
Respirosome
Respiratory chain complex
Oxidoreductase complex
Inner mitochondrial membrane protein complex
Proton transporting two sector ATPase complex proton transporting domain
Endoplasmic reticulum protein containing complex
Proton transporting two sector ATPase complex proton transporting domain
Mitochondrial protein containing complex
Mannosyltransferase activity
Acid thiol ligase activity
Solute sodium symporter activity
Ligase activity forming carbon-sulfur bonds
Proton transporting ATP synthase activity rotational mechanism

Cell cycle  Keap1-Nrf2 pathway  Cytoskeleton  Mitochondria  Others
Figure 7: Survival rate (%) over age (Days) for different groups. The survival rate is compared between WT, Vehicle, and UBE-1099 groups. The graph shows a significant difference in survival rate between the groups, indicated by the ** symbol.