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RESEARCH NOTE

Febuxostat inhibited axillary osmidrosis risk factor ATP-binding cassette transporter C11 *in vitro*

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Dear Editor,

Human ATP-binding cassette transporter C11 (ABCC11) is a risk factor of axillary osmidrosis (AO) that is characterized by unpleasant body odor from the armpits. The inhibition of ABCC11 is expected to be useful to overcome AO; however, no ABCC11 inhibitor is currently used clinically.

Humans produce body odor like other animals. Nonetheless, in human culture, strong or specific body odors are sometimes perceived as undesirable. Among them, AO is a chronic skin condition characterized by unpleasant body odors and excessive sweating from the armpits.[1] As is often the case in Asian countries such as Japan and China, in which people with strong body odor comprise a minor section of the population, AO tends to be more strongly disliked and is recognized as a disease.[2] However, except for surgical treatments, there has been no causal therapy for AO. Thus, there is an unmet medical need for the development of a therapy for this unpleasant condition.

Axillary osmidrosis risk is determined by a non-synonymous single nucleotide polymorphism, c.538G>A (p.Gly180Arg), in the *ABCC11* gene.[1,3,4] Biochemical analyses have revealed that this amino acid substitution (variant type, Arg180) enhances proteasomal degradation of *de novo* synthesized ABCC11 protein, which results in the loss of the cellular function of ABCC11.[3] As genetically ABCC11-deficient subjects have little risk of AO,[2] and functional ABCC11 (wild-type, Gly180) is present in the axillary apocrine glands that secrete a variety of odor precursors,[5] it seems that the inhibition of ABCC11 function may be able to contribute to the prevention and treatment of AO. However, this notion has not yet been demonstrated because clinical investigations have been difficult due to the absence of ABCC11 inhibitors that have been approved for clinical use. Additionally, contrary to humans, rodents such as mice and rats have no *Abcc11* gene;[2] thus, it is almost impractical to examine the physiological impact of ABCC11 inhibition through *in vivo* experiments. Therefore, the investigation of ABCC11 inhibitors with biological safety is an important issue.

In this study, we conducted an *in vitro* transport assay using ABCC11-expressing plasma membrane vesicles to explore ABCC11 inhibitors. We identified febuxostat, a non-purine selective inhibitor of xanthine oxidase developed for hyperuricemia management, as a novel ABCC11 inhibitor (Fig. 1). Detailed information on our methods, including critical materials and resources we used (Table S1), is available in Appendix S1. Briefly, plasma membrane vesicles were prepared from adenovirus-mediated transiently ABCC11-expressing 293A cells. Immunoblotting and an *in vitro* transport assay for [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone sulfate (DHEA-S) an ABCC11 substrate,[6] were conducted as described previously.[5] To confirm the experimental system, we first examined ABCC11 expression on the plasma membrane vesicles (Fig. 1a), ATP-dependent DHEA-S transport into the ABCC11-expressing plasma membrane vesicles (Fig. 1b) and time profile for ABCC11-mediated DHEA-S transport (Fig. 1c). As febuxostat inhibited ABCC11 in our preliminary screening, we further examined the concentration-dependent inhibitory effects of febuxostat on ABCC11 (Fig. 1d). The half maximal inhibitory concentration value of febuxostat against the ABCC11-mediated DHEA-S transport activity was 3.26 μmol/L. Additionally, at this concentration (3.26 μmol/L), febuxostat had little effect on the transport activity of ABCC2 (Fig. S1), which belongs to the same protein family as ABCC11.

We herein demonstrated that febuxostat, a globally used drug for hyperuricemia treatment, is a novel ABCC11 inhibitor (Fig. 1). To the best of our knowledge, this is the first report of ABCC11 inhibition by a drug approved for clinical use on the market. As human axillary apocrine glands open into the hair follicles, the dermal administration of febuxostat onto the armpits, such as through a medical cream, may inhibit ABCC11 in humans. For this to be effective, such treatment must ensure appropriate levels of febuxostat in the ABCC11-expressing apocrine glands. On the other hand, a previous study has shown that the maximum plasma unbound concentration of febuxostat is 0.126 μmol/L when administrated to healthy subjects at a dose of 40 mg once daily for up to 12 consecutive days,[7] given that the unbound fraction of febuxostat is 0.022. This suggests that p.o. administrated febuxostat has little inhibitory effect on ABCC11 in current clinical situations under the assumption of a uniform of febuxostat level throughout the body. Based on this, our data should be interpreted carefully. However, despite the limitation on its practical use, our discovery will pave the way for the first proof of concept study in humans to investigate the effect of ABCC11 inhibition on the AO phenotype. Such study will be worth consideration in the

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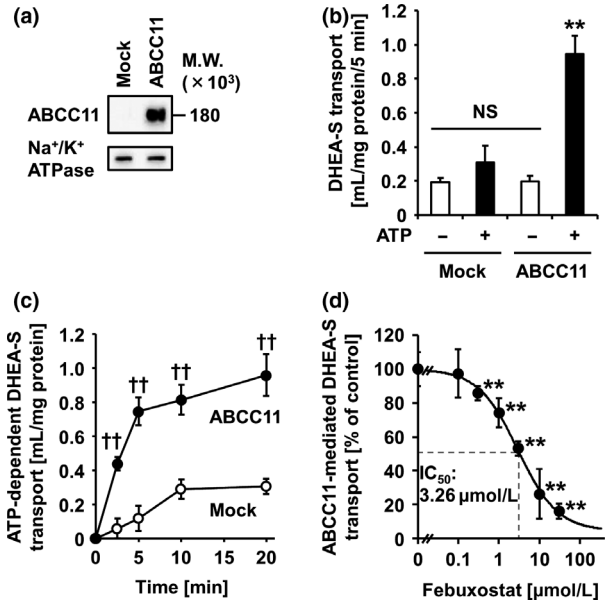

Figure 1. Febuxostat inhibited ATP-binding cassette transporter C11 (ABCC11) *in vitro*. (a) Expression of ABCC11 on plasma membrane vesicles. Plasma membrane vesicles were subjected to immunoblot analysis using an ABCC11 antibody or anti-Na^+/K^+-ATPase antibody. Na^+/K^+-ATPase, loading control. (b) [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone sulfate (DHEA-S) transport activities. Plasma membrane vesicles were incubated with or without ATP for 5 min. (c) Time-dependent increase in DHEA-S transport by ABCC11. DHEA-S transport into membrane vesicles was measured at the indicated periods with or without ATP; the ATP-dependent DHEA-S transport was calculated by subtracting the DHEA-S transport activity in the absence of ATP from that in the presence of ATP. (d) Concentration-dependent inhibition of ABCC11-mediated DHEA-S transport by febuxostat. The DHEA-S transport activities were measured in the presence of febuxostat at the indicated concentrations for 5 min. Data are expressed as the mean ± standard deviation. (b,c) $n = 3$ and (d) 4. Statistical analyses for significant differences were performed using Bartlett's test, followed by a parametric Tukey–Kramer multiple comparison test (**$P < 0.01$ vs the other groups; NS, not significantly different among groups in [b]) or Dunnett's test (**$P < 0.01$ vs 0 μmol/L control in [d]); statistical analyses for significant differences between groups in each time point were performed using a two-sided *t*-test ($^{\dagger\dagger}P < 0.01$ in [c]). M.W., molecular weight.

near future. We believe that our results shed light on a new possibility of overcoming AO by drug repurposing.

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CONFLICT OF INTEREST: The authors have a patent pending related to the work reported in this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary Methods.
Figure S1. Febuxostat hardly affected ATP-binding cassette transporter C2 (ABCC2) function.
Table S1. Key resources.

Supporting Information

Febuxostat inhibited axillary osmidrosis risk factor ATP-binding cassette transporter C11 *in vitro*

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Appendix S1: Supplementary Methods

Materials

Critical materials and resources used in this study are summarized in **Supplementary Table S1**. Stock solution of febuxostat (Tokyo Chemical Industry, Tokyo, Japan) was prepared with dimethyl sulfoxide (DMSO). Recombinant adenoviruses for the expression of human ABCC11 wild-type (NCBI accession; NM_033151) or EGFP as a control were from our previous study.[1] After purification by a CsCl gradient ultracentrifugation method, the adenovirus titer was determined using an Adeno-X™ Rapid Titer Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. The adenoviruses were stored at −80°C until use. All other chemicals used were commercially available and of analytical grade.

Cell culture

Human embryonic kidney 293 (HEK293)-derived 293A cells were maintained in Dulbecco's Modified Eagle's Medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 1% penicillin-streptomycin (Nacalai Tesque), 2 mM L-Glutamine (Nacalai Tesque), and 1 × Non-Essential Amino Acid (Life Technologies, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. Adenovirus infection was performed as described previously.[1]

Preparation of ABCC11-expressing plasma membrane vesicles

Plasma membrane vesicles were prepared from 293A cells infected with the ABCC11-expressing or EGFP-expressing (control) adenovirus as described previously.[1] Obtained plasma membrane vesicles were rapidly frozen in liquid N_2 and stored at −80°C until use. Protein concentration of plasma membrane vesicles was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard according to the manufacturer's protocol.

Immunoblotting

Expression of ABCC11 protein in plasma membrane vesicles was examined by immunoblotting as described previously,[1, 2] with minor modifications. Briefly, the prepared samples were electrophoretically separated on poly-acrylamide gels and transferred to a Hybond® ECL™ nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) by electroblotting at 15 V for 70 min. For blocking, the membrane was incubated in Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk (TBST-skim milk), at 4°C overnight. Blots were probed with a rat monoclonal anti-ABCC11 antibody (M8I-74; Abcam, Cambridge, MA, USA; diluted 200 fold) and a rabbit polyclonal anti-Na^+/K^+-ATPase α antibody (sc-28800; Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1,000 fold) followed by incubation with a goat anti-rat immunoglobulin G (IgG)-horseradish peroxidase (HRP)-conjugated antibody (NA935V; GE Healthcare; diluted 2,000 fold) and a donkey anti-rabbit IgG-HRP-conjugated antibody (NA934V; GE Healthcare; diluted 3,000 fold), respectively. All antibodies were used in TBST-skim milk. HRP-dependent luminescence was developed using the ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and detected using a multi-imaging Analyzer Fusion Solo 4™ system (Vilber Lourmat, Eberhardzell, Germany).

Vesicle transport assay

Experiments to study the *in vitro* transport of [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone sulfate (DHEA-S) (PerkinElmer, Waltham, MA, USA), an ABCC11 substrate,[3] into ABCC11-expressing and control plasma membrane vesicles were performed by using a rapid filtration technique,[1, 4] with a minor modification. Briefly, the plasma membrane vesicles were incubated with 100 nM of [1,2,6,7-^{3}H(N)]-DHEA-S in the reaction mixture (10 mM Tris/HCl, 250 mM sucrose, 10 mM $MgCl_2$, 10 mM creatine phosphate, 1 mg/mL creatine phosphokinase, and 50 mM ATP or AMP as a substitute of ATP at pH

7.4) for the indicated periods at 37°C with or without febuxostat at the indicated concentrations. Then, the radioactivity derived from the incorporated DHEA-S was measured. To reduce background signals derived from nonspecifically adsorbed radiolabeled DHEA-S on the filter membrane (MF-Millipore Membrane, HAWP02500; 0.45 μm pore size and 25 mm diameter; Millipore, Tokyo, Japan) for the trapping of plasma membrane vesicles, the filter membranes were incubated with 2 μM of cholesterol (Wako Pure Chemical Industries, Tokyo, Japan) in ice-cold Stop buffer (250 mM Sucrose, 0.1 M NaCl, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.4) that contained 0.2% (v/v) DMSO before use. In this transport experiment, the transport activity in each group was calculated as incorporated clearance [mL/mg protein/min = incorporated level of DHEA-S (disintegrations per minute (DPM)/mg protein/min) / DHEA-S level in the incubation mixture (DPM/mL)]. ATP-dependent DHEA-S transport was calculated by subtracting the DHEA-S transport activity in the absence of ATP from that in the presence of ATP; ABCC11-mediated DHEA-S transport activity was calculated by subtracting the ATP-dependent DHEA-S transport activity for control plasma membrane vesicles from that for ABCC11-expressing plasma membrane vesicles.

To examine the effect of febuxostat on the function of ABCC2 (also known as MRP2), a similar vesicle transport assay was conducted. For this purpose, we used ABC Transporter Vesicles for human ABCC2 and control (GenoMembrane, Yokohama, Japan) of which function was confirmed in our previous study,[5] and estradiol 17β-D-glucuronide, [estradiol-6,7-^{3}H(N)] (PerkinElmer) (100 nM in the reaction mixture that was incubated for 5 min at 37°C) was employed as an ABCC2 substrate.

Calculation of the half-maximal inhibitory concentration (IC_{50}) values

To calculate the IC_{50} values of febuxostat against DHEA-S transport by ABCC11, the DHEA-S transport activities were measured in the presence of febuxostat at several concentrations. Then, the ABCC11-mediated transport activities were expressed as a percentage of the control (100%). Based on the calculated values, fitting curves were obtained according to the following formula using the least-squares methods with Excel 2019 (Microsoft, Redmond, WA, USA) as described previously:[6]

$$\text{Predicted value [\%]} = 100 - \left({E_{max} \times C^{n}} \Big/ {EC_{50}^{n} + C^{n}} \right)$$

where, E_{max} is the maximum effect, EC_{50} is the half maximal effective concentration, C is the concentration of test compound, and n is the sigmoid-fit factor. Finally, based on the results, IC_{50} was calculated.

Statistical analyses

All statistical analyses were performed using Excel 2019 with Statcel4 add-in software (OMS publishing Inc., Saitama, Japan). Different statistical tests were used for different experiments as described in the figure legends. Briefly, when analyzing multiple groups, the similarity of variance between groups was compared using Bartlett's test. When passing the test for homogeneity of variance, a parametric Tukey–Kramer multiple-comparison test for all pairwise comparisons or a Dunnett's test for comparisons with a control group was used. In the case of a single pair of quantitative data, after comparing the variances of a set of data using an *F*-test, an unpaired Student's *t*-test was performed. Statistical significance was defined in terms of *P* values less than 0.05 or 0.01.

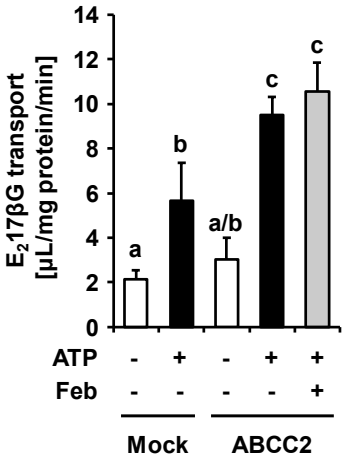
Data Availability Statement

Data supporting the results of this study are included in this published article and its Supplementary Information or are available from the corresponding author on reasonable request.

Supplementary Table S1. Key resources.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-MRP8 (ABCC11) antibody [M8I-74]	Abcam	Cat# ab91452; RRID: AB_2049125
Rabbit polyclonal anti-Na^+/K^+-ATPase α antibody	Santa Cruz Biotechnology	Cat# sc-28800; RRID: AB_2290063
Goat anti-rat IgG-horseradish peroxidase (HRP)-conjugate	GE Healthcare	Cat# NA935V; RRID: AB_772207
Donkey anti-rabbit IgG-horseradish peroxidase (HRP)-conjugate	GE Healthcare	Cat# NA934V; RRID: AB_772206
Chemicals, Peptides, and Recombinant Proteins		
ABC Transporter Vesicles (Human MRP2)	GenoMembrane	Cat# GM0001
ABC Transporter Vesicles (Control)	GenoMembrane	Cat# GM0003
Cholesterol	Wako Pure Chemical Industries	Cat# 034-03002; CAS: 57-88-5
Clear-sol II	Nacalai Tesque	Cat# 09136-83
Dehydroepiandrosterone sulfate, sodium salt, [1,2,6,7-^{3}H(N)] (60.0 Ci/mmol)	PerkinElmer	Cat# NET860
Dimethyl Sulfoxide	Nacalai Tesque	Cat# 13445-74; CAS: 67-68-5
Estradiol 17β-D-glucuronide, [estradiol-6,7-^{3}H(N)] (41.8 Ci/mmol)	PerkinElmer	Cat# NET1106
Febuxostat	Tokyo Chemical Industry	Cat# F0847; CAS: 144060-53-7
Critical Commercial Assays		
Pierce™ BCA Protein Assay Reagent A	Thermo Fisher Scientific	Cat# 23223
Pierce™ BCA Protein Assay Reagent B	Thermo Fisher Scientific	Cat# 23224
Virus strains		
ABCC11-expressing adenovirus	Toyoda et al, 2017	N/A
EGFP-expressing adenovirus	Toyoda et al, 2017	N/A
Recombinant DNA		
The complete human ABCC11 cDNA	Toyoda et al, 2009	NCBI Reference Sequence: NM_033151
Experimental Models: Cell Lines		
293A	Invitrogen	R70507
Software and Algorithms		
Excel 2019	Microsoft	https://www.microsoft.com/ja-jp/
Statcel4 add-in software	OMS publishing	http://www.oms-publ.co.jp/

Supplementary Figure S1.



Supplementary Figure S1. Febuxostat hardly affected ABCC2 function.
The estradiol 17β-D-glucuronide, [estradiol-6,7-^{3}H(N)] ($E_2$17βG) transport activities were measured in the presence or absence of febuxostat (Feb) at 3.26 μM for 5 min. Data are expressed as the mean ± SD. n = 3. Statistical analyses for significant differences were performed using Bartlett's test, followed by a parametric Tukey–Kramer multiple-comparison test. Different letters indicate significant differences between groups ($P < 0.05$).

Supplementary References

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