J Mol Med (2014) 92:509–521
DOI 10.1007/s00109-013-1118-x

JMolMed

ORIGINAL ARTICLE

A novel gene regulator, pyrrole–imidazole polyamide targeting ABCA1 gene increases cholesterol efflux from macrophages and plasma HDL concentration

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Received: 28 June 2013 / Revised: 29 November 2013 / Accepted: 16 December 2013 / Published online: 25 January 2014

Abstract
Pyrrole–imidazole (PI) polyamides are nuclease-resistant novel compounds that inhibit transcription factors by binding to the minor groove of DNA. A PI polyamide that targets mouse ABCA1 and increases ABCA1 gene expression was designed and evaluated as an agent to increase plasma HDL concentration. A PI polyamide was designed to bind the activator protein-2 binding site of the mouse ABCA1 promoter. The effect of this PI polyamide on ABCA1 expression was evaluated by real-time RT-PCR and Western blotting using RAW264 cells. In vivo effects of this polyamide on ABCA1 gene expression and plasma HDL level were examined in C57B6 mice. One milligram per kilogram of body weight of PI polyamide was injected via the tail veins every 2 days for 1 week, and plasma lipid profiles were evaluated. PI polyamide showed a specific binding to the target DNA in gel mobility shift assay. Treatment of RAW264 cells with 1.0 μM PI polyamide significantly increased ABCA1 mRNA expression. PI polyamide also significantly increased apolipoprotein AI-mediated HDL biogenesis in RAW264 cells. Cellular cholesterol efflux mediated by apolipoprotein AI was significantly increased by the PI polyamide treatment. PI polyamide significantly increased expression of ABCA1 mRNA in the liver of C57B6 mice. Plasma HDL concentration was increased by PI polyamide administration. All of the HDL sub-fractions showed a tendency to increase after PI polyamide administration. The designed PI polyamide that targeted ABCA1 successfully increased ABCA1 expression and HDL biogenesis. This novel gene-regulating agent is promising as a useful compound to increase plasma HDL concentration.

Key messages

- A novel pyrrole–imidazole (PI) polyamide binds to ABCA1.
- PI polyamide interfered with binding of AP-2 protein to the ABCA1 gene promoter.
- PI polyamide inhibited the AP-2 -mediated reduction of ABCA1 gene and protein expression.
- PI polyamide increased ABCA1 protein and apolipoprotein AI mediated HDL biogenesis.
- PI polyamide is a new gene regulator for the prevention of atherosclerotic diseases.

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Keywords ABCA1 · PI polyamide · HDL · AP-2

Introduction

Epidemiological evidence has confirmed the inverse relationship between high-density lipoprotein (HDL) cholesterol and coronary heart disease [1, 2]. Major clinical guidelines for the prevention and treatment of cardiovascular disease recognize HDL cholesterol as an independent risk factor [3–5]. The overloading of cholesterol in macrophages, regarded as foam cell formation, is one of the crucial steps in the early stage of atherosclerosis. Cholesterol molecules are not catabolized in mammalian somatic cells and must be transported to the liver for its degradation to bile acids. HDL is thought to play a central role in this pathway. The removal of excess cholesterol from macrophage foam cells by HDL and its principal apolipoprotein, apolipoprotein AI, is thought to be one of the key mechanisms underlying the atheroprotective properties of HDL [6, 7]. ATP binding cassette transporter A1 (ABCA1) plays a pivotal role in cholesterol efflux from macrophage foam cells [8]. Overexpression of ABCA1 in mice resulted in an increase of HDL and reduction of atherosclerosis [9, 10]. Accordingly, therapies that increase ABCA1 expression are a promising strategy for prevention and treatment of atherosclerosis. Expression of ABCA1 is highly regulated. Loading cholesterol into macrophages results in enhanced transcription of ABCA1 gene by a reaction mediated by oxysterol-activated liver X receptor (LXR) [11–13]. ABCA1 expression can also be increased by peroxisome proliferator-activated receptor (PPAR)α or PPARγ activators [14, 15]. Recently, Iwamoto et al. reported that activator protein (AP)2α negatively regulates the ABCA1 gene transcription to decrease HDL biogenesis [16, 17].

Pyrrole–imidazole polyamide (PI polyamide) compounds are a new class of synthetic DNA-binding ligands principally composed of N-methylpyrrole and N-methylimidazole amino acids. A binary code has been developed to correlate DNA-binding sequence specificity with antiparallel side-by-side ring pairings in the minor groove of DNA [18, 19]. A pairing of imidazole opposite pyrrole targets the G–C base pair, and pyrrole opposite imidazole targets the C–G base pair. Pyrrole–pyrrole degenerately targets T–A and A–T base pairs [20]. Initiation of transcription requires binding of transcription factors to the cognate DNA response elements in the gene promoter. PI polyamides do not require vectors or any other delivery devices to distribute into tissues or cells. They have superior cell and nuclear membrane permeability and bind the minor groove and block binding of transcription factors, inhibiting gene expression. PI polyamides may therefore be a new transcriptional gene regulating agents for the treatment of diseases [21–23].

In this study, we designed a PI polyamide targeting the ABCA1 promoter adjacent to the AP2 binding site to increase the ABCA1 gene expression; we then examined the effect of this polyamide on ABCA1 gene expression and plasma HDL levels.

Methods

Synthesis of PI polyamides

PI polyamide targeting ABCA1 was designed to span the boundary of the AP2 binding site of the ABCA1 promoter. PI polyamides were synthesized according to previously described methods [24, 25]. A mismatch polyamide was also designed and synthesized that did not bind to the transcription binding sites of ABCA1 promoter. The structural formula of PI polyamides used in this study are shown in Fig. 1.

DNA binding assay

Fluorescein isothiocyanate (FITC)-labeled oligonucleotides including AP-2 binding sequence were synthesized for a gel mobility shift assay. Four picomoles of the FITC-labeled oligonucleotides was incubated with 4.0 pmol of PI polyamide for 1 h at 37 °C. The resulting complexes were separated by electrophoresis and visualized with the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP)

For in vitro experiments, RAW264 cells (Health Science Research Resource Bank, Han-nan, Japan) were cultured in RPMI1640 medium supplemented with 10 % fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA) and 10 mg/dl streptomycin (Invitrogen).

Preparation and immunoprecipitation of chromatin was performed using a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology) according to manufacturer's instructions and monoclonal anti-AP-2α antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA). DNA samples were analyzed by 2 % agarose gel electrophoresis and real-time PCR using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Cells were treated with 0, 0.1, or 1.0 μM PI polyamide targeting ABCA1 for 24 h before immunoprecipitation. Primers used in this assay were 5′-AACGAGCTTTTCCCCTTTCCT-3′ and 5′-TGCTAGCCTTCGGGAAACG-3′.

Determination of mRNA expression

To determine the effect of PI polyamide on RAW264 ABCA1 mRNA expression, RAW264 were incubated with 1.0, 0.1, or 0.01 μM PI polyamide targeting ABCA1 or 1.0 μM mismatch

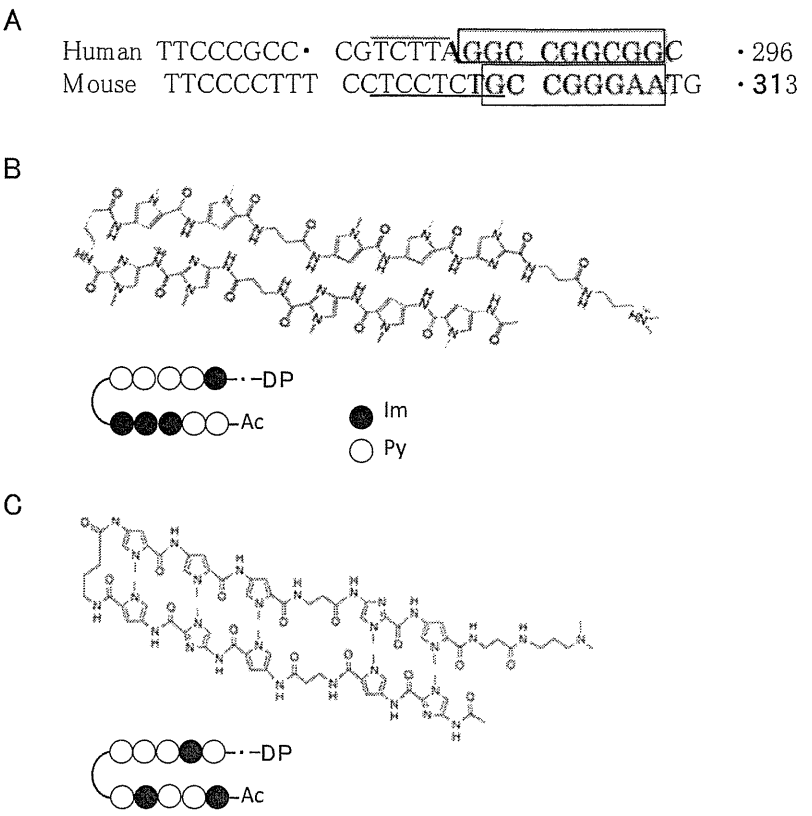


Fig. 1 Target sequence and the structure of PI polyamide targeting ABCA1 gene promoter. **a** Target sequence of the PI polyamide targeting human ABCA1 gene. The target sequence of PI polyamide is underlined. AP-2 binding site is indicated by black rectangle. **b** Structure of the PI polyamide targeting human ABCA1 gene. **c** Structure of the mismatch PI polyamide

PI polyamide in the presence of 1.0 μM phorbol-12-myristate-13-acetate in Dulbecco's modified Eagle's medium (DMEM) with 0.5 % FCS for 8 h. To determine the effect of PI polyamide on NCTC Clone 1469 cells ABCA1, ABCG1, and scavenger receptor B1(SR-B1) mRNA expression, NCTC Clone 1469 cells were incubated with 1.0 μM PI polyamide targeting ABCA1, 1.0 μM mismatch PI polyamide, or 10 μM doxazosine in DMEM with 0.5 % FCS for 8 h. Total RNA was isolated and reverse-transcribed to cDNA as described previously [26]. Real-time quantitative PCR was performed with the cDNA, diluted four times, using TaqMan Universal Master Mix (Applied Biosystems) and an ABI 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Assay-on-Demand primers and probes were purchased from Applied Biosystems (ABCA1 Mm00442646_m1, ABCG1 Mm00437390_m1, and SRB1 Mm00450234_m1). 18S rRNA expressions (Applied Biosystems 4319413E) were quantified for sample normalization. Real-time PCR data were analyzed using a standard curve. In all cases, the correlation coefficients for the standard curves were >0.90.

For in vivo experiments, peripheral blood was collected from C57BL/6 mice in heparinized tubes, layered on a Ficoll-Paque density gradient (Sigma-Aldrich, St Louis, MO, USA), and centrifuged at 400×g for 30 min at room temperature to isolate peripheral blood mononuclear cells. Peripheral blood mononuclear cells were then removed and total RNA isolated and reverse-transcribed. Real-time quantitative PCR was performed with the cDNA. Also, total RNA was isolated from 20 mg of liver tissue of a C57BL/6 mouse and reverse-transcribed.

Western blotting analysis

Immunoblotting of ABCA1, ABCG1, SR-B1, and apolipoprotein AI were performed as previously described by Arakawa et al. [27]. Total membrane fraction was isolated by RIPA buffer [Nacalai, Kyoto, Japan; 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 % Nonident P140, 0.5 % sodium deoxycholate, and protease inhibitor cocktail] containing 1 % Triton X-100 (Roche Applied Science). After transfer, the membrane was blocked in 5 % skim milk and incubated with polyclonal goat anti-ABCA1 antibodies (Santa Cruz Biotechnology), anti-ABCG1 antibody (Santa Cruz), anti-SR-B1 (Santa Cruz) antibody, anti-apolipoprotein AI antibody (Santa Cruz), or mouse monoclonal anti a-tubulin antibody (Sigma-Aldrich) for 1 h. After washing three times with 0.02 m Tris-buffered saline containing 0.1 % Tween 20 (Sigma-Aldrich), the membrane was incubated with horseradish peroxidase-conjugated anti-goat IgG antibody (Bio-Rad Laboratories, Hercules, CA, USA) or anti-mouse IgG

antibody (Zymed, San-Francisco, CA, USA) for 1 h. Bands were visualized by a chemiluminescence method (ECL plus Western blotting detection system; GE healthcare, Buckinghamshire, UK) using the luminescent image analyzer LAS-3000.

Cellular lipid release

Apolipoprotein AI was isolated from the human HDL fraction as described previously [28]. RAW264 cells were preloaded with 1.0 μM mismatch polyamide, 10 μM doxazasin, 0.1 μM ABCA1 polyamide, or 1.0 μM ABCA1 polyamide and incubated with 10 μg/ml of apolipoprotein AI in RPMI 1640 medium containing 0.1 % BSA for 16 h. Free cholesterol and phospholipid in the medium were measured by an enzymatic method [27, 29].

Agarose gel electrophoresis

Plasma lipoprotein profile was evaluated by thin-layer agarose gel electrophoresis. The elution pattern was scanned and densitometric patterns were analyzed by the image-J software (INH, Bethesda, MD, USA, http://imagNihonej.nih.gov/ij/, 1997–2011).

Gel permeation high-performance liquid chromatography (HPLC)

Plasma lipoprotein profiles were analyzed by HPLC using gel-permeation column(s) (Lipopropak XL, 7.8 mm× 300 mm; Tosoh, Tokyo, Japan) eluted with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05 % sodium azide, and 0.005 % Brij-35 at a flow rate of 0.7 ml/min and an online enzymatic lipid-detection system [30]. Gel permeation HPLC analysis was performed in three rats in PI polyamide and control group.

In vivo experiments

This study conformed to the guidelines published in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by Nihon University Institutional Animal Care and Use Committee. Male C57BL/6 mice (Charles River Breeding Laboratories, Stone Ridge, NY, USA) were used in all of the experiments.

Mice were divided into two groups and were fed normal chow diet (Oriental Yeast, Tokyo, Japan) ad libitum for 2 weeks. PI polyamide (1 mg/kg body weight) targeting ABCA1 or mismatch polyamide was dissolved in 100 μl of 0.1 % acetic acid and injected via the tail vein every 2 days for 1 week (total 7 mg of polyamide per mouse, n=6 for each group).

The mice were euthanized by a lethal injection of sodium pentobarbital (IP, 100 mg/kg body weight) 14 days after PI polyamide administration.

Statistical analysis

Results are given as the mean±standard error of mean. The significance of differences between mean values was evaluated by Student's t test for unpaired data.

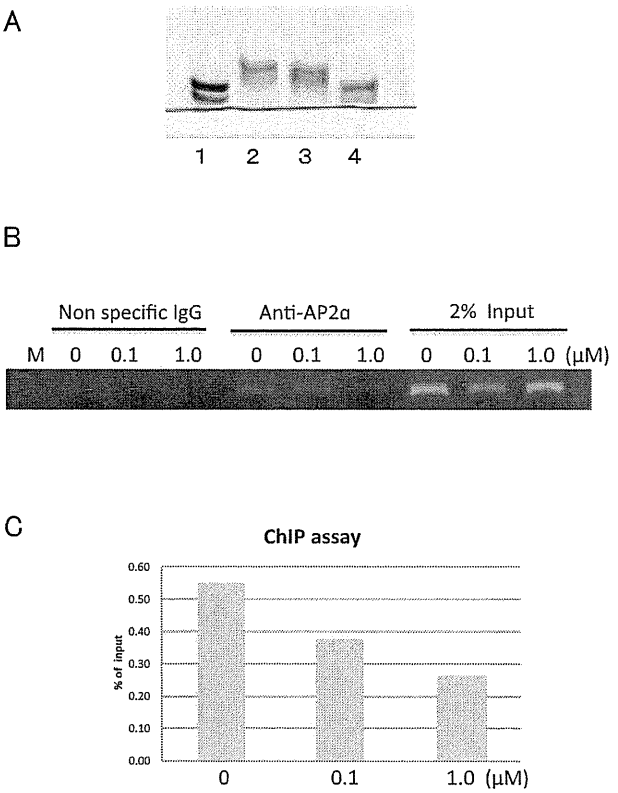


Fig. 2 Binding of PI polyamide to the target sequence. Binding of PI polyamide to the target sequence assessed by the gel shift assay. **a** Lane 1 FITC-conjugated synthetic double-strand DNA corresponding to the target sequence, 2 FITC-conjugated target double-strand DNA+PI polyamide (1:1), 3 FITC-conjugated target double-strand DNA+PI polyamide + unlabeled double-strand DNA (1:1:10), 4 FITC-conjugated target double-strand DNA + mismatch polyamide (1:1). **b**, **c** Inhibition of AP-2 binding to the target sequence by PI polyamide assessed by ChIP assay. Anti-AP-2 antibody was used to immunoprecipitate the protein–DNA complex from cellular lysates of RAW264 cells, and results were evaluated by agarose gel electrophoresis (**b**) and bar graph of signals relative to input (**c**) to detect the protein DNA binding. Cells were treated with 0, 0.1, or 1.0 μM PI polyamide targeting ABCA1 prior to the assay. Non-specific non-specific rabbit IgG, AP2: anti AP-2 antibody, 2 % Input 2 % of input sample (samples before immunoprecipitation)

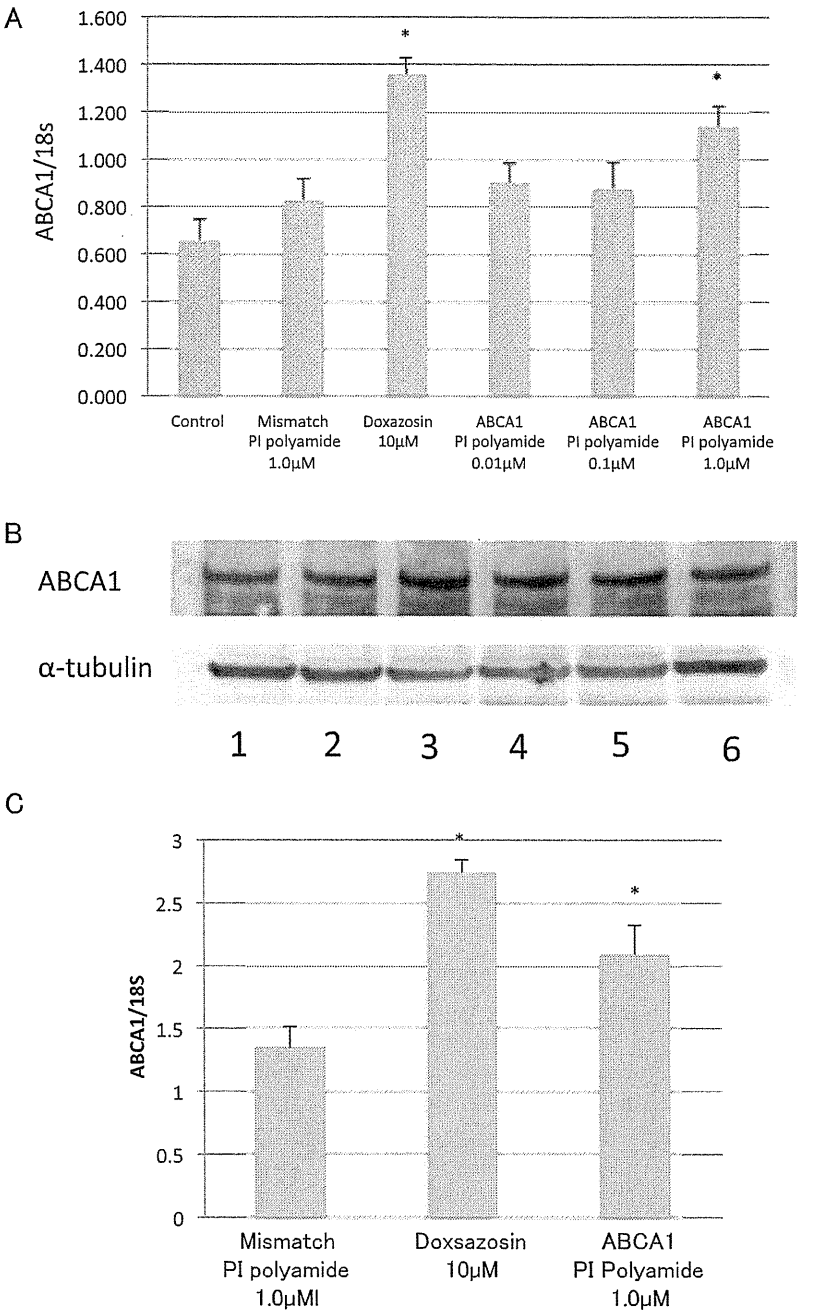


Fig. 3 Effect of PI polyamide on ABCA1 mRNA and protein expression in RAW264 cells. **a** Effect of PI polyamide on mRNA levels in RAW264 cells. Bars represent mean value and standard error of ABCA1 mRNA/18S rRNA ratio for each condition from six independent experiments. *$p < 0.05$ vs. control. **b** Effect of PI polyamide on protein levels in RAW264 cells. Lanes 1–2 control, 3–4 10 μM doxazosin, 5·6 1 μM PI polyamide. **c** Bars represent mean value and standard error of ABCA1/ tubulin ratio for each condition from six independent experiments. *$p < 0.05$ vs. control

Results

Design and synthesis of PI polyamide

Since the AP-2-induced enhancement of ABCA1 gene expression was reported, a PI polyamide was designed so as to partially overlap the homologous region of the human AP2 consensus sequence located at ·321 base pair of the mouse ABCA1 promoter sequence (Fig. 1a). This PI polyamide had a U shape with a molecular weight of 19,911.01 Da and increased the expression of ABCA1 gene. The structural formula of ABCA1 PI polyamide and the mismatch PI polyamide are presented in Fig. 1b and c.

Binding of PI polyamide to the target sequence

A synthetic oligonucleotide with a sequence corresponding to positions ·310 base pair to ·340 base pair of the ABCA1 gene promoter was labeled with FITC. Double-

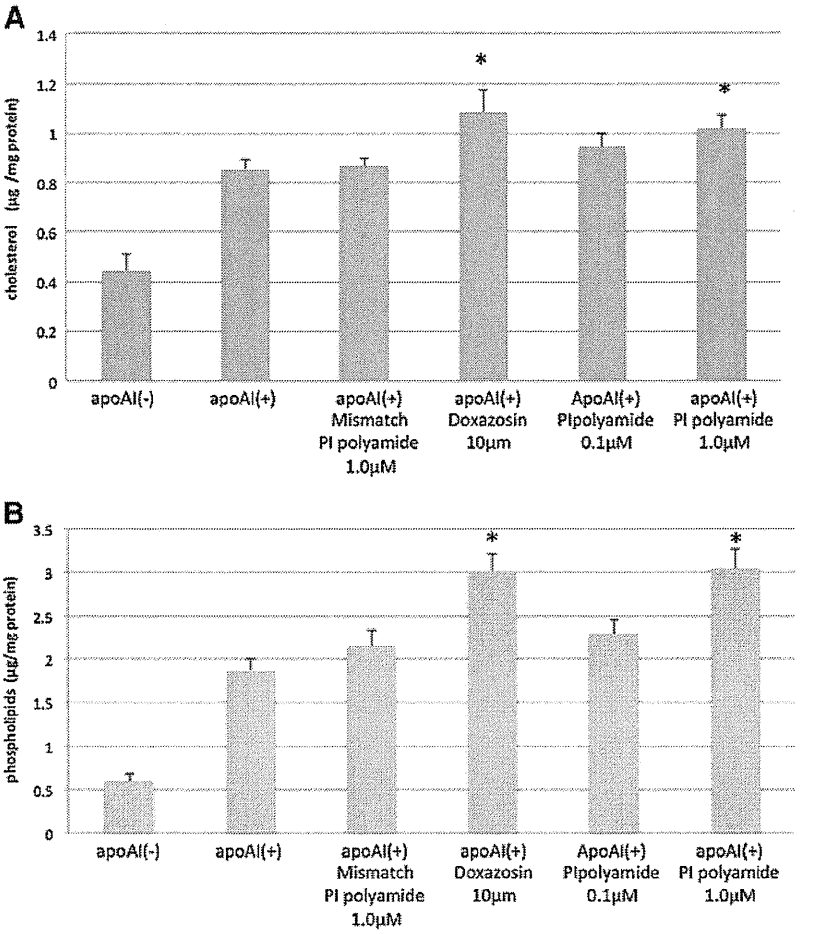


Fig. 4 Effect of PI polyamide on the apolipoprotein AI-mediated HDL biogenesis in RAW264 cells. RAW264 cells were preloaded with 10 μM doxazasin, 1.0 μM mismatch polyamide, 0.1 μM ABCA1 polyamide, or 1.0 μM ABCA1 polyamide and incubated in media containing 0.1 % BSA for 16 h. Free cholesterol (**a**) and phospholipid (**b**) in the medium were measured by an enzymatic method. Bars represent mean value and standard error of phospholipids or free cholesterol release from six independent experiments. *p < 0.05 vs. control. apoAI apolipoprotein AI

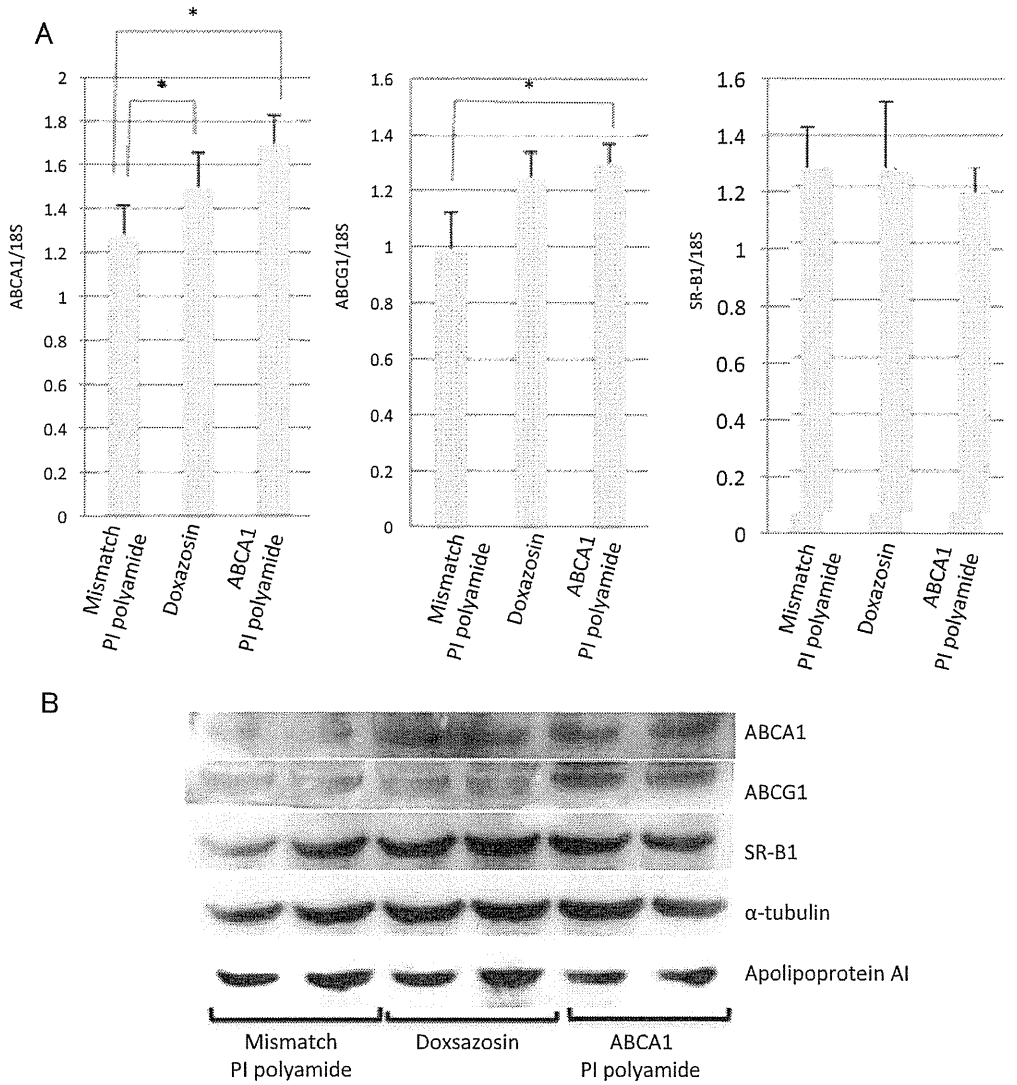
stranded DNA was prepared using the sense and antisense strands of the oligonucleotide, and binding of the synthetic PI polyamide to this DNA was investigated using a gel shift method (Fig. 2a). Lane 1 contained double-stranded DNA alone. Lane 2 contained double-stranded DNA incubated with an equivalent amount of the PI polyamide. Compared with the band in lane 1 containing the double-stranded DNA alone, the band in lane 2 had a slower mobility, showing that PI polyamide bound the synthetic oligonucleotide. The band shift upwards was inhibited by adding an excess amount of unlabeled double-stranded DNA (lane 3). When the DNA was incubated with the mismatch PI polyamide, no band sift was noted (lane 4), showing that the binding was specific for the target sequence.

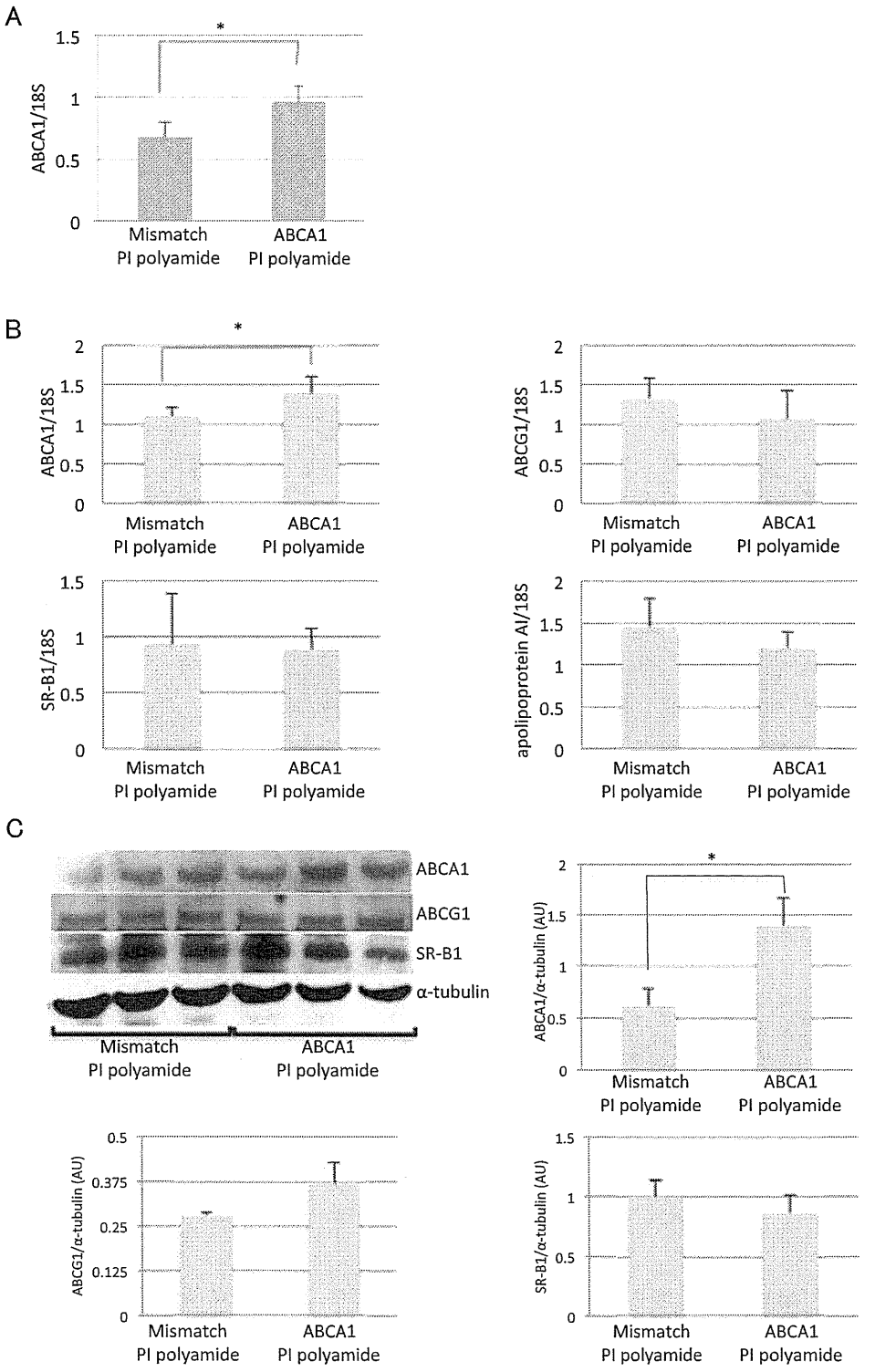
We then performed a ChIP assay to assess whether PI polyamide inhibit AP-2α binding to the ABCA1 promoter in RAW264 cells. We used AP-2α antibody to immunoprecipitate the protein–DNA complex from cellular lysates of RAW264 cells, and performed agarose gel electrophoresis (Fig. 2b) and RT-PCR (Fig. 2c) to detect the protein DNA binding. PI polyamide decreased anti-AP-2 antibody-mediated precipitation of DNA–chromatin complex, suggesting that there is direct binding of PI polyamide with the ABCA1 promoter.

Increase of ABCA1 mRNA expression by PI polyamide in RAW264 cells

Treatment of RAW264 cells with 10 μM doxazosin increased the ABCA1 mRNA expression level by about 2.2-fold. When 1.0 μM PI polyamide was added, the ABCA1 mRNA expression level was significantly increased by about 1.9-fold compared with the control cells. Cells treated with 0.01 and 0.1 μM PI polyamide or mismatch PI polyamide did not show a significant difference in the

Fig. 5 Effect of PI polyamide on ABCA1, ABCG1, and SRB1 in NCTC Clone 1469 cells. **a** Effect of PI polyamide on mRNA levels in NCTC Clone 1469 cells. Bars represent mean value and standard error of ABCA1, ABCG1, SR-B1, and apolipoprotein AI mRNA/18S rRNA ratio for each condition from six independent experiments. *p < 0.05 vs. control. **b** Effect of PI polyamide on protein levels in NCTC Clone 1469 cells. Lanes 1–2 mismatch PI polyamide, 3–4 10 μM doxazosin, 5–6 1 μM PI polyamide


A
ABCA1/18S
ABCG1/18S
SR-B1/18S
Mismatch PI polyamide
Doxazosin
ABCA1 PI polyamide
B
ABCA1
ABCG1
SR-B1
α-tubulin
Apolipoprotein AI
Mismatch PI polyamide
Doxsazosin
ABCA1 PI polyamide
ABCA1/ α-tubulin (AU)
ABCG1/α-tubulin (AU)
SR-B1/α-tubulin (AU)
ApoA1 (AU)



ABCA1 mRNA expression level compared with the control cells (Fig. 3a). Doxazosin at 10 μM and PI polyamide at 1.0 μM increased the production of ABCA1 protein, but this was not seen in control cells (Fig. 3b, c).

Fig. 6 Effect of PI polyamide on mRNA and protein expression in peripheral blood mononuclear cells and liver of C57BL/6 mice. **a** Effect of PI polyamide on ABCA1 mRNA expression in peripheral blood mononuclear cells of C57BL/6 mice. Bars represent mean value and standard error of ABCA1 mRNA/18S rRNA ratio from six independent experiments. MisPI mismatch PI polyamide. ABCA1PI PI polyamide targeting ABCA1. *p < 0.05 ABCA1 PI polyamide vs. mismatch PI polyamide. **b** Effect of PI polyamide on ABCA1, ABCG1, SR-B1, and apolipoprotein AI mRNA expression in liver of C57BL/6 mice. Bars represent mean value and standard error of ABCA1 mRNA/18S rRNA ratio from six independent experiments. MisPI mismatch PI polyamide. ABCA1PI PI polyamide targeting ABCA1. *p < 0.05 ABCA1 PI polyamide vs. mismatch PI polyamide. **c** Effect of PI polyamide on ABCA1, ABCG1, and SR-B1 protein expression in liver of C57BL/6 mice

Enhancement of cholesterol and phospholipid efflux from RAW264 cells by PI polyamide

Treatment of RAW264 cells with 10 μM doxazosin increased the apolipoprotein AI-mediated release of free cholesterol and phospholipids from RAW264 cells. In cells treated with 1.0 μM PI polyamide, cholesterol and phospholipid efflux from RAW264 cells increased relative to control cells. Treatment with mismatch polyamide did not have any effect on cholesterol and phospholipid efflux from RAW264 cells (Fig. 4).

Increase of ABCA1 mRNA expression by PI polyamide in NCTC Clone 1469 cells

Treatment of NCTC Clone 1469 cells with 10 μM doxazosin increased the ABCA1 mRNA expression level by about 1.2-fold. When 1.0 μM PI polyamide was added, the ABCA1 mRNA expression level was significantly increased by about 1.4-fold, compared with the control cells. Cells treated with mismatch PI polyamide did not show a significant difference in the ABCA1 mRNA expression level compared with the control cells. PI polyamide also significantly increased ABCG1level after 8 h of treatment. PI polyamide targeting ABCA1 did not

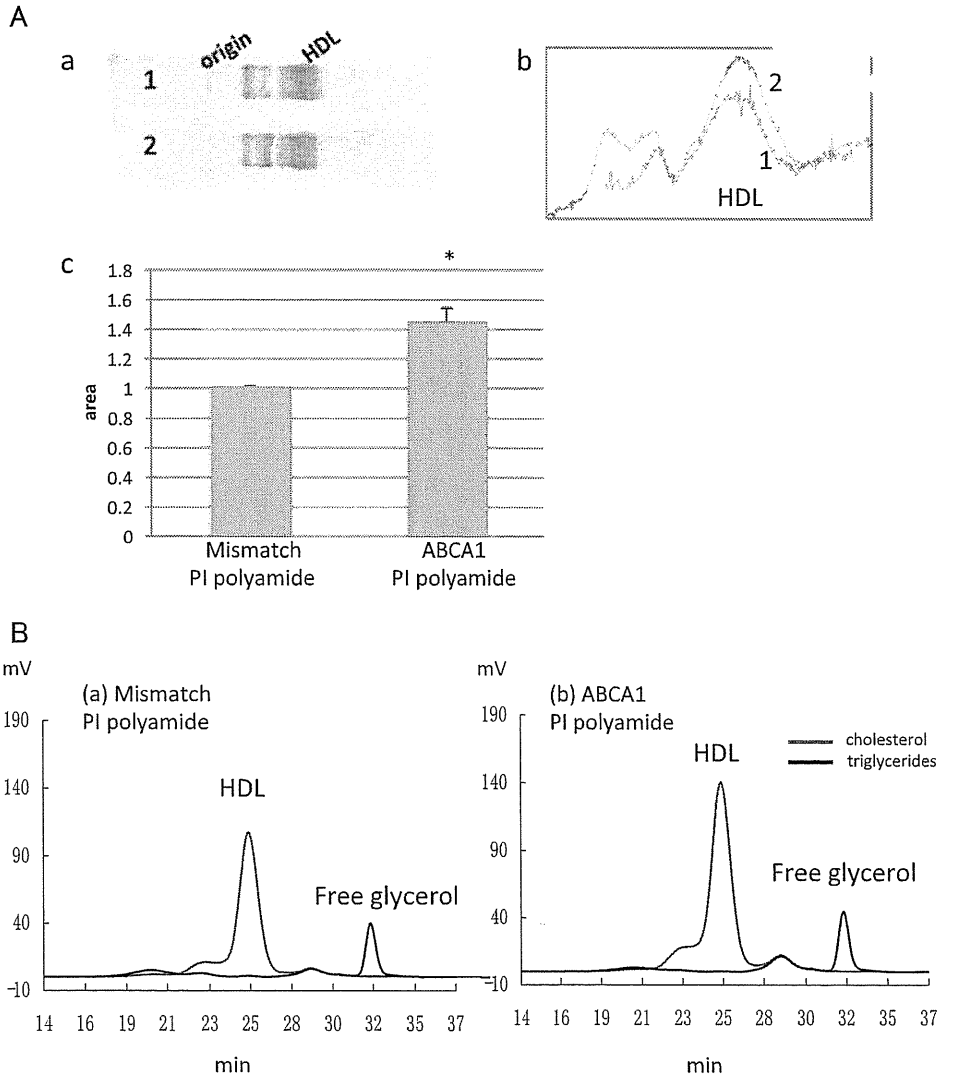


Fig. 7 Effect of PI polyamide on plasma HDL in C57B6 mice. **a** Effect of PI polyamide on plasma HDL evaluated by agarose gel electrophoresis. A typical gel picture of plasma lipoprotein is shown (a), densitometric pattern (b), and average area under the curve of HDL densitometric patterns (c). *p < 0.05 vs. control. **b** Effect of PI polyamide on plasma HDL evaluated by gel permeation HPLC. Typical elution pattern of HDL fraction evaluated by gel permeation HPLC. A typical elution pattern in mismatch PI polyamide-treated mouse (a) and ABCA1 PI polyamide-treated mouse (b). Pink line indicates elution pattern of gel permeation HPLC by cholesterol monitoring. Blue line indicates elution pattern of gel permeation HPLC by triglyceride monitoring

Table 1 Effect of PI polyamide on plasma lipoprotein fractions evaluated by gel permeation HPLC

	Total	CM (>80 nm)	VLDL (30–80 nm)	LDL (16–30 nm)	HDL (8–16 nm)
Mismatch PI polyamide	87.63±7.80	0.10±0.13	2.92±0.43	13.30±1.94	71.31±5.79*
ABCA1 PI polyamide	108.48±4.22*	0.08±0.03	2.14±0.21	20.59±4.83	85.67±2.04

Cholesterol (mg/dl), mean±SD of three rats

*p < 0.05 ABCA1 PI polyamide vs. mismatch PI polyamide

show any significant effect o the expression levels of SR-B1 mRNA in NCTC Clone 1469 cells (Fig. 5a). PI polyamide at 1.0 μM increased the production of ABCA1 protein but this was not seen in control cells whereas the treatment with PI polyamide did not alter ABCG1, SR-B1, or apolipoprotein AI (Fig. 5b).

Increase of HDL cholesterol by PI polyamide in vivo

Effects of PI polyamide on ABCA1 and plasma HDL were evaluated in mice. The ABCA1 mRNA in peripheral blood mononuclear cells was increased, but the difference was not statistically significant relative to controls (Fig. 6a). In the liver, the expression of ABCA1 mRNA was significantly increased by PI polyamide treatment (Fig. 6a). Liver ABCG1, SR-B1, and apolipoprotein AI mRNA expression levels did not show any significant alterations after PI polyamide treatment (Fig. 6b). Liver ABCA1 protein expression increased after PI polyamide treatment whereas ABCG1 and SR-B1 protein expression did not increase after the treatment (Fig. 6c).

Increased HDL plasma concentration by treatment with PI polyamide was confirmed by agarose gel electrophoresis (Fig. 7a) and by the elution profiles of plasma lipoprotein (Fig. 7b). Plasma HDL increased by 20 % after PI polyamide administration. Plasma very-low-density lipoprotein (VLDL) concentration was also decreased, and plasma low-density lipoprotein was increased in PI-polyamide-treated animals (Table 1). Effect of PI polyamide on plasma HDL sub-fraction was determined by quantification of the lipids by gel permeation HPLC of HDL sub-fractions. In all HDL sub-fractions, HDL concentration was increased by treatment with PI polyamide. Very large, large, and very small HDL sub-fractions showed statistically significant increases after PI polyamide treatment (Table 2).

Discussion

PI polyamide is an organic compound consisting of repeating pyrrole and imidazole groups. PI polyamide recognizes nucleic acid base sequences by a unique recognition rule and specifically binds to the side groove of DNA double helix. PI polyamide is designed to bind to a site close to the binding sequence for a transcription factor in the promoter region of a target gene, and it binds to DNA with a higher affinity than the transcription factor thus interfering with binding of the transcription factor and changing the expression of the target gene. We have previously reported several distinct PI polyamides targeting expression of transforming growth factor β1 gene [31, 32] or lection-like oxidative LDL receptor 1 gene [33]. The strategy in all of our previously reported examples of this gene-regulating method was "gene silencing". We have successfully inhibited target gene expression in each study by inhibiting transcription factor which enhance expression of the target gene. In a converse situation, we have designed PI polyamide targeting the repressor-binding site of the ABCA1 promoter to enhance the gene expression. Iwamoto et al. reported that phosphorylated AP-2α negatively regulates expression of ABCA1 gene, and the binding sequence spans from ·305 base pair to ·310 base pair of human ABCA1 promoter. The homologous region of the human AP-2 binding site in mice is located from ·321 base pair to ·327 base pair of the mouse ABCA1 promoter sequence [34]. Knowing this, we designed PI polyamide to bind to a proximal site (i.e., upstream) of the AP-2 binding region in the mouse ABCA1 gene promoter. This molecule interfered with binding of AP-2 protein to the ABCA1 gene promoter, and inhibited the AP-2 -mediated reduction of ABCA1 gene expression. This is the first in vivo example of evaluation of PI polyamide which

Table 2 Effect of PI polyamide on plasma HDL sub-fractions evaluated by gel permeation HPLC

Fraction (average particle diameter)	Very large HDL (15 nm)	Very large HDL (13.5 nm)	Large HDL (12.1 nm)	Medium HDL (10.9 nm)	Small HDL (9.8 nm)	Very small HDL (9.8 nm)	Very small HDL (7.6 nm)
Mismatch PI polyamide	2.63±0.31	5.59±0.66	29.65±1.49	1.84±0.06	1.73±0.13	1.84±0.06	1.73±0.13
ABCA1 PI polyamide	4.27±0.43	8.29±0.40*	36.08±0.51*	2.15±0.07*	1.85±0.11	2.15±0.07*	1.85±0.11

Cholesterol (mg/dl), mean ± SD of three rats

*p < 0.05 ABCA1 PI polyamide vs. mismatch PI polyamide

enhanced the expression of a target gene. In order to increase the specificity for ABCA1 gene, the PI polyamide was designed to bind to the region including the ABCA1 gene promoter-specific sequence, not the AP-2 consensus sequence. The PI polyamide sequence was designed such that it partially overlapped the AP-2 consensus sequence.

In the Framingham study, Gordon et al. showed that low HDL cholesterol is a risk factor for coronary heart disease [2]. This important finding has given rise to a large number of diverse HDL studies over the last few decades. Because of a general consensus that HDL protects against atherosclerosis, strategies have been developed to increase plasma HDL levels. There are many potential aspects of HDL that appear to be important in reducing atherosclerosis. Approaches targeting the macrophage include LXR agonists and up-regulation of ABCA1 and other receptors that are involved in improving efflux of cholesterol from the macrophage. Other therapeutic strategies to enhance reverse cholesterol transport include infusing reconstituted HDL, delipidated HDL, apolipoprotein AI peptides [35], and cholesteryl ester transfer protein inhibitors [36]. ABCA1 mediates and is rate limiting in the biogenesis of HDL from helical apolipoprotein acceptors, such as apolipoprotein AI, and cellular cholesterol and phospholipids. Mutations of ABCA1 gene have been found in patients with Tangier disease, in which the lack of ABCA1 leads to impairment of cellular cholesterol removal and absence of HDL in plasma [37]. Accordingly, therapies that increase ABCA1 expression are promising strategies for increasing plasma HDL and preventing atherosclerosis. Several pharmacological agents that modify of ABCA1 gene expression have been reported. LXR agonists and retinoid X receptor (RXR) agonists improve the efflux potential of plasma from treated animals by altering HDL composition. However, stimulation of LXR and RXR in vivo can lead to a hypertriglyceridemic state and hepatic steatosis [38, 39]. Thus, these agents are not suitable for clinical application because of their adverse effects on plasma triglycerides. Doxazosin [16] and PPAR agonists [14, 15] were also reported to stimulate the ABCA1 gene expression. However, these agents have other distinct effects such as anti-hypertensive, anti-diabetic, or anti-hyperlipidemic effects. Therefore, developing novel agents to increase ABCA1 expression by gene targeting medicine is an attractive and important challenge. PI polyamide targeting ABCA1 increased plasma HDL by 20 % without any adverse effect in mice. However, we administrated PI polyamide only for 2 weeks. Further evaluation of the long-term effects and side effects of this compound is necessary for its developments as a potential novel anti-atherosclerotic agent.

Nucleic acid-based medicines such as antisense DNA and ribozymes are easily degraded by nucleases in vivo. However, PI polyamides are non-nucleic acid organic chemical compounds that are resistant to nucleases and stable in vivo [19, 40]. Therefore, it is possible that PI polyamides could be used as novel, orally administrated gene-regulating agents.

Two steps have been reported to occur in ABCA1-mediated cholesterol efflux from macrophage and binding to apolipoprotein AI. The first step is that apolipoprotein AI forms complexes with phospholipids and cholesterol at the cell surface in a process promoted by ABCA1 activity [41, 42]. The next step is that apolipoprotein AI binds ABCG1 at the cell surface and is subsequently internalized and targeted to late endosomes, where apolipoprotein AI picks up lipids and the apolipoprotein–lipid complexes are then re-secreted from cells [43–45]. Therefore, ABCA1 mediates the early step of HDL biogenesis and increases small HDL particles. In this study, very small HDL fraction was significantly increased after PI polyamide treatment, suggesting a direct effect on ABCA1 up-regulation. However, in our results, very large and large HDL also increased after PI polyamide treatment. Because ABCA1-mediated HDL formation in the early step of HDL biogenesis is a rate-limiting step, following HDL maturation step seems to occur rapidly after the initial cholesterol efflux mediated by ABCA1.

According to the traditional model of reverse cholesterol transport, HDL cholesterol originates from peripheral tissues and subsequently transferred to the liver. However, recent studies challenged this model based on the finding of overexpression of ABCA1 by the liver, which raised plasma HDL concentrations and suggested that significant HDL particle assembly occurred at the hepatocyte surface [46–48]. Using liver-specific ABCA1 knockout mouse model, hepatic ABCA1 is essential for approximately 80 % of the steady-state pool of plasma HDL. They also indicated that lipidation by hepatocytes is necessary for maintaining plasma HDL concentration by prolonging the circulation time of HDL apo AI in vivo [49]. In this study, PI polyamide targeting ABCA1 increased Abca1 mRNA expression in both murine macrophage and hepatocyte cell line, and increased mRNA expression in mouse liver and peripheral blood macrophage. From these data, suggested mechanisms of HDL increase after PI polyamide treatment are increased phospholipid assembly at liver surface induced by the increased hepatic ABCA1 expression, apolipoprotein AI stabilization induced by the phospholipid assembly in the former step, and increased cholesterol assembly at macrophage surface induced by the increased macrophage ABCA1 expression.

In conclusion, the synthetic PI polyamide designed to bind the ABCA1 promoter increased ABCA1 gene and protein expression, increased HDL biogenesis, and increased plasma HDL levels. To our knowledge, this is the first report of a drug that increases plasma HDL level developed by a gene-targeting approach. PI polyamide targeting ABCA1 gene may be a novel gene regulator for the prevention of atherosclerotic diseases.

Acknowledgment This work was partially supported by a grant from the "Strategic Research Base Development" Program for Private Universities subsidized by MEXT (2011).

Conflict of interest There is no conflict of interest to disclose for any of the authors.

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Effects on Serum Uric Acid by Difference of the Renal Protective Effects with Atorvastatin and Rosuvastatin in Chronic Kidney Disease Patients

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Received May 29, 2013; accepted October 29, 2013

Hyperuricemia and hyperlipidemia have attracted attention as progression factors for chronic kidney disease (CKD). In the drug treatment of hyperuricemia and hyperlipidemia complications, Atorvastatin (ATV), which inhibits urinary protein, increases glomerular filtration rate (GFR) and has renal protective effects, and Rosuvastatin (ROS) were found be suitable because they promote serum uric acid (SUA) excretion. However, these drugs were administered at very high doses in previous studies. In this study, we have investigated the effects of ATV or ROS on renal protective effects and their SUA levels before and three months after each drug administration in CKD patients. We retrospectively investigated outpatients presenting with CKD (stages 3) on the basis of their electronic medical records as subjects. Estimated GFR (eGFR) was significantly increased after ATV administration, whereas no change in eGFR was observed following ROS administration. Furthermore, SUA levels significantly decreased after ATV administration, whereas no changes were observed following ROS administration. Therefore, it may be not necessary to administer drugs that lower the SUA levels to patients presenting with hyperuricemia and hyperlipidemia complications associated with moderate renal failure, such as patients with at least stage 3 CKD. We consider that, by selecting ATV, the renal protective effects and SUA-lowering effect would be sufficient.

Key words chronic kidney disease (CKD) stages 3; hyperuricemia; hyperlipidemia; Atorvastatin; Rosuvastatin

Hyperuricemia and hyperlipidemia have attracted attention as progression factors for chronic kidney disease (CKD).[1] In healthy subjects, approximately 10% of uric acid is excreted in the urine. Thus, serum uric acid (SUA) levels increase with decreasing renal function, and a high SUA level is a risk factor for developing cardiovascular disease (CVD). That is, increased SUA levels directly lead to vascular disorder by promoting the progression of atherosclerosis.[2] Therefore, CKD patients presenting with hyperuricemia complications have a high risk of developing CVD, and it is necessary to intervene as soon as possible by administering drugs that lower the SUA levels. However, serious side effects such as toxic epidermal necrolysis have been found to be associated with allopurinol administration at the time of renal failure, and there have been reports of serious liver failure associated with benzbromarone administration; thus, these drugs should be administered with caution. On the other hand, the effect of hyperlipidemia on CKD has also been shown in various studies. Low-density lipoprotein cholesterol (LDL-C) invades tissues owing to the increase in LDL-C levels in the blood. LDL-C is oxidized and oxidized LDL-C is incorporated into mesangial cells and causes glomerular sclerosis lesions. It has been found to cause renal artery atherosclerosis and a decrease in renal blood flow. In the Atherosclerosis Risk in Communities (ARIC) Study, increased triacylglycerol (TG) and total cholesterol (TC) levels in CKD patients have been reported to be risk factors for developing CVD.[3] In the drug treatment of hyperuricemia and hyperlipidemia complications, Atorvastatin (ATV),[4] which inhibits urinary protein, increases the glomerular filtration rate (GFR), and has renal protective effects, and Rosuvastatin (ROS) were found to be suitable because they promote uric acid excretion.[5,6] However, these drugs were administered at very high doses in previous studies. For example, the dose of the ATV group exceeded the maximum dose of 20 mg/d in Japan. In this study, we have investigated the effects of ATV or ROS on their renal protective effects and SUA levels in CKD patients. In addition, the solubility of the urate crystal in body fluid is lower than 7.0 mg/dL. But it is said to be important to maintain SUA levels at less than 6.0 mg/dL. Pascual and Sivera maintained SUA levels at less than 6.0 mg/dL and could not detect the urate crystal from the intra-articular area for 3–33 months.[7] Sarawate *et al.* reported that a gout attack was unlikely to occur in patients whose SUA levels were maintained at less than 6.0 mg/dL.[8] Therefore, in addition, we investigated ATV and ROS in terms of their ability to achieve SUA levels of less than 6.0 mg/dL.

MATERIALS AND METHODS

Subjects Among the 268 CKD patients who were outpatients in Yokosuka Kyousai Hospital from 2006.11 to 2011.10, we included 29 CKD patients (stage 3) as subjects. These patients had hyperuricemia and hyperlipidemia complications and were administered ATV or ROS (Fig. 1). We considered losartan, irbesartan, loop diuretics, thiazide diuretics, drugs that lowering the uric acid level, and fenofibrate as drugs that affect the uric acid levels[9–15]; thus, we excluded the patients who were administered a combination of these drugs. This study complied with the Declaration of Helsinki and we paid sufficient attention to the "Ethical Guidelines for Clinical Research." 13 patients (male: 8, female: 5), aged 57–87 years old (mean: 67.5±8.4 years old), were included in the ATV group. On the other hand, 16 patients (male: 9, female: 7), aged 41–77 years old (mean: 64.8±11.5 years old), were included in the

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ROS group. Average doses of ATV and ROS were 8.8±2.2 and 2.7±0.7 mg/d, respectively.

Materials and Methods The research items were age, sex, systolic and diastolic blood pressure (SBP, DBP) clinical laboratory values (hemoglobin (Hb), TC, TG, LDL-C, SUA, and serum creatinine (SCr) levels and estimated GFR (eGFR)), combination drugs (immunosuppressants or anti-platelet agents), and the presence or absence of diabetes. We collected information from medical records retrospectively. For each patient, we compared the measured values of TG, LDL-C, TC, SBP, DBP, SUA levels, and eGFR before and three months after ATV or ROS administration. In addition, we analyzed the patients in whom the SUA levels decreased to less than 6.0 mg/dL after the administration of ATV or ROS, and we compared and investigated the achievement rate of less than 6.0 mg/dL SUA levels.

Statistical Analysis The results are given as mean± standard deviation (S.D.). We carried out the normality test to compare the data volume between the two groups. We used the unpaired *t*-test after we confirmed that it showed a normal distribution. We used the χ^2 test or Fisher's exact test to compare the categorical data and we used paired *t*-test to compare the results obtained before and after drug administration for each group. The significance level was 5% ($p<0.05$). In addition, the statistical analysis was performed using JMP® (Version 10, SAS Institute Inc.).

Ethics Regulation This study was conducted with the approval of the Yokosuka Kyousai Hospital Ethics Committee (Approval number: 12–10).

RESULTS

Subjects Figure 1 shows the flow chart of the selection of subjects in this study. Among them, 27 patients took a combination of losartan and irbesartan, 102 patients took a combination of loop and thiazide diuretics, 114 patients took drugs that lower the SUA levels, and 3 patients took fenofibrate. When we categorized the patients by CKD stage, 29 patients (10.8%) belonged to stage 3 and 48 patients (17.9%) were non-CKD stage 3 patients. There are reports that lipid-rich plaque formation progresses easily in patients presenting with CKD equivalent to stage 3[16,17]; thus, we surveyed the stage 3 CKD patients.

Comparison of Patient Characteristics before ATV or

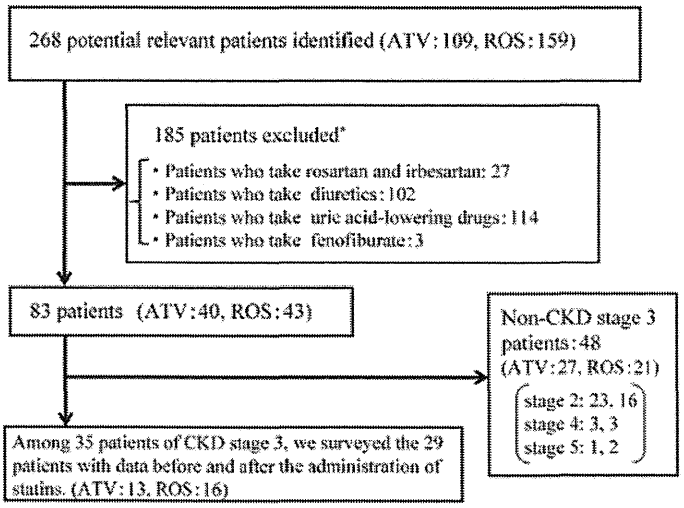

Fig. 1. Flow Chart of the Trial Selection Process

*: A total of 246 patients were included in the combinations.

Table 1. Demographic and Clinical Characteristics of Patients before ATV and ROS Administration

	ATV (n=13)	ROS (n=16)	p Value
Age	67.5±8.4	64.8±11.5	0.46
Sex (male/female)	8/5	9/7	0.78
SBP (mmHg)	136.7±21.6	132.9±15.7	0.58
DBP (mmHg)	78.2±10.8	80.0±15.4	0.74
Diabetes number (%)	6 (46.2%)	7 (43.8%)	0.89
Immunosuppressants use	2 (15.4%)	2 (12.5%)	0.62
Anti-platelet agents use (%)	6 (46.2%)	5 (31.3%)	0.33
Hb (mg/dL)	14.0±1.8	13.6±1.8	0.51
TC (mg/dL)	233.1±19.8	330.5±186.6	0.95
TG (mg/dL)	217.9±171.5	219.2±90.2	0.31
LDL-C (mg/dL)	141.3±29.7	200.2±122.6	0.95
SUA (mg/dL)	6.4±1.1	6.7±1.2	0.40
SCr (mg/dL)	1.0±0.2	1.1±0.2	0.22
eGFR (mL/min/1.73 m^2)	51.4±7.8	46.5±7.7	0.08

Mean±S.D. or number (percentage).

ROS Administration Patient characteristics before ATV or ROS administration are shown in Table 1. We investigated 13 patients in the ATV group and 16 patients in the ROS group. When we compared the two groups, there were no significant differences in TG, LDL-C, SUA levels, eGFR, and so on.

Effects of ATV and ROS on Serum Lipids and Blood Pressure Changes in clinical laboratory values, TG, LDL-C, TC, SBP and DBP before and after ATV or ROS administration are shown in Table 2. For ATV group, TG, LDL-C, and TC levels significantly decreased from 217.9±171.5, 141.3± 29.7, and 233.1±19.8 mg/dL to 114.5±50.4, 91.7±32.1, and 174.2±30.8 mg/dL, respectively. For ROS group, TG, LDL-C, and TC levels significantly decreased from 219.2±90.2, 200.2±122.6, and 330.5±186.6 mg/dL to 153.8±73.6, 93.7± 21.9, and 175.6±29.6 mg/dL, respectively. We did not observe significant differences in TG, LDL-C, and TC levels after administration between ATV and ROS administration. SBP and DBP were also not observed to have significant differences before (136.7±21.6 and 78.2±10.8 mmHg) and after (135.8± 26.4 and 77.2±13.9 mmHg, respectively) ATV administration as well as before (132.9±15.7 and 80.0±15.4 mmHg) and after (127.1±16.1 and 77.2±13.9 mmHg, respectively) ROS administration.

Effects of ATV and ROS on Renal Function In this study, we have investigated eGFR as a marker of renal function. For ATV group, eGFR was significantly increased from 51.1±7.82 mL/min/1.73 m^2 to 61.8±13.3 mL/min/1.73 m^2, whereas no significant change was observed before (47.7± 7.04 mL/min/1.73 m^2) and after (52.5±18.4 mL/min/1.73 m^2) ROS administration (Fig. 2).

Effects of ATV and ROS on SUA Levels For ATV group, SUA levels significantly decreased from 6.38 ±1.11 mg/dL to 5.48±1.44 mg/dL (Fig. 3), whereas no change was observed before (6.69±1.25 mg/dL) and after (6.71±1.36 mg/dL)

Table 2. Effects of ATV and ROS on Serum Lipids and Blood Pressure

	ATV (n=13)			ROS (n=16)		
	Pre-drug	Post-drug	p Value	Pre-drug	Post-drug	p Value
TG (mg/dL)	217.9±171.5	114.5±50.4	0.018	219.2±90.2	153.8±73.6	0.045
LDL-C (mg/dL)	141.3±29.7	91.7±32.1	0.001	200.2±122.6	93.7±21.9	0.001
TC (mg/dL)	233.1±19.8	174.2±30.8	<0.001	330.5±186.6	175.6±29.6	0.011
SBP (mmHg)	136.7±21.6	135.8±26.4	0.500	132.9±15.7	127.1±16.1	0.128
DBP (mmHg)	78.2±10.8	77.2±13.9	0.373	80.0±15.4	77.2±13.9	0.201

Pre-drug and post-drug show laboratory data before and three months after administration of ATV and ROS.

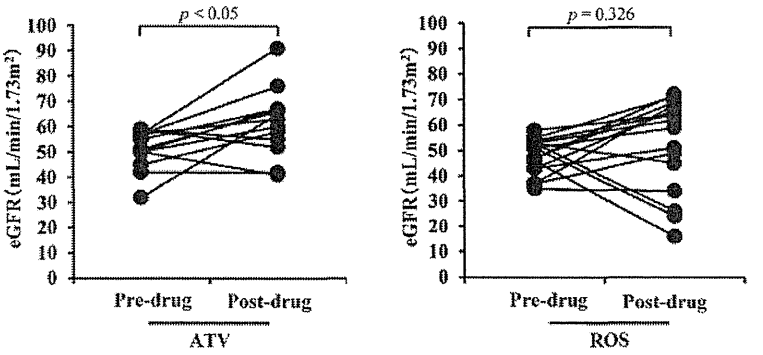

Fig. 2. Change in eGFR by ATV and ROS

We compared the measured values of eGFR before and three months after ATV and ROS administration.

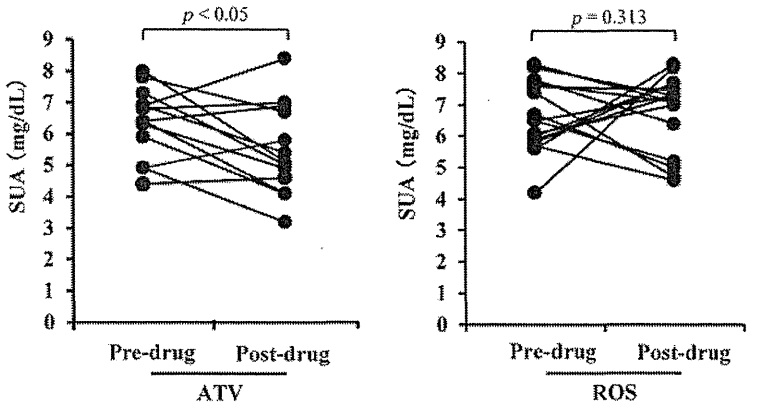

Fig. 3. Changes in SUA Levels by ATV and ROS

We compared the measured values of SUA before and three months after ATV and ROS administration.

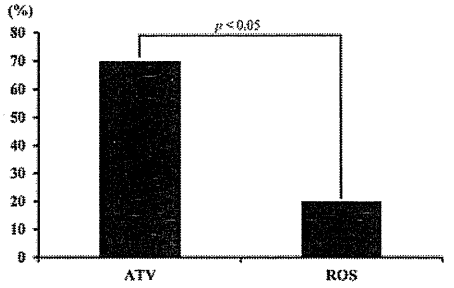

Fig. 4. Achievement Rate of Less than 6.0 mg/dL SUA Levels of ATV and ROS

Average doses of ATV and ROS were 8.8 and 2.5 mg/d, respectively. The achievement rate of ATV was significantly higher than that of ROS.

ROS administration (Fig. 3).

Next, we compared ATV and ROS in terms of their achievement rate of SUA levels of less than 6.0 mg/dL. The achievement rates of ATV (5–10 mg/d) and ROS (2.5 mg/d) were 70 and 20%, respectively (Fig. 4).

DISCUSSION

The most valuable result obtained from this study is that we could show renal protective effects in stage 3 CKD patients with hyperuricemia and hyperlipidemia complications after ATV administration. We could also show a significant decrease in SUA level after ATV administration. On the other hand, we could not show significant changes in SUA levels and eGFR after ROS administration.

In the meta-analysis by Sandhu *et al.*, wherein the renal protective effects of statin drugs were determined, eGFR improved to 3.1 mL/min/1.73 m^2 in one year for subjects with underlying diseases such as glomerulonephritis, diabetes, and hypertension.[18] In the subanalysis of the results of the Treating to New Target (TNT) trial that determined the second prevention of cardiovascular disease, eGFR increased approximately 2% after one year of ATV administration (80 mg/d) in CKD patients.[19] In this study, eGFR increased significantly in comparison with the result of the previous study. In addition, it is known that the improvement degrees of eGFR are different between statin drugs. In the PLANET study, in which the renal protective effects of ATV and ROS were observed in CKD patients, it was reported that ATV was superior to ROS in improving urinary protein levels and renal function.[20] We also determined the change in eGFR before and after ATV and ROS administration. Results showed that eGFR was increased significantly by ATV. On the other hand, eGFR before and after ROS administration was not significantly changed. In this study, we could obtain the result that was similar to that of the PLANET trial. We could demonstrate that the renal protective effects might be different between ATV and ROS. Concerning the mechanism of the improvement of renal function by ATV, we believe that it may be based on the effect of lowering the LDL-C level. In addition, it may also be necessary to consider the involvement of a multifaceted action, such as anti-platelet, anti-coagulation, and anti-inflammatory effects, and the favorable impact of statins on endothelial cells and vascular smooth muscle.

The effect of ATV on lowering the SUA level was shown. In the GREek Atorvastatin and Coronary heart disease Evaluation (GREACE) study, 1600 patients with coronary heart disease were administered ATV up to a maximum of 80 mg/d. As a result, SUA level decreased by 8.2% after 48 months (end of study).[21] In addition, Marais *et al.* administered ATV (80 mg/d) for 6 weeks to 22 patients with familial hypercholesterolemia, and reported that SUA levels decreased by approximately 10% from 4.87 to 4.36 mg/dL.[22] The SUA-lowering effect of ATV in these patients is the result of a dose that far exceeds the maximum daily dose of 20 mg allowed in Japan. In this study, we could demonstrate the SUA-lowering effect of ATV at the average dose of 8.8 mg/d, which is less than the dose reported previously. We suggested that SUA level decreased by renal protective effects of ATV. In this study, we have shown that ATV has the significant effect of improving hyperlipidemia and hyperuricemia complications. Moreover, it has a renal protective effect and it may be able to suppress the development of CVD. However, subjects who were taking calcium channel blockers (CCBs) (cilnidipine and efonidipine) and angiotensin-receptor blockers (ARBs) (telmisartan, valsartan, and olmesartan) were also included in this study. There are reports that these drugs increase eGFR. We consider the possibility that these drugs have an effect on the findings of this study. It is necessary to sufficiently lower the blood pressure to show the renal protective effects. However, these drugs were used in this study, and no significant difference in blood pressure before and after ATV or ROS administration was found. Therefore, we hypothesize that there is little effect of ARBs, except losartan and irbesartan, and CCBs. Furthermore, the primary disease of subjects is mostly chronic glomerulonephritis. Immunosuppressants and anti-platelet agents are used for the treatment of chronic glomerulonephritis. The excretion level of urinary protein decreases, and renal function is maintained by the action of these drugs. In other words, in some cases that eGFR increased significantly, we hypothesized that there was a possibility that these drugs may be related.

On the other hand, we could not show the SUA-lowering effect of ROS. Nezami *et al.* reported no change in SUA levels in 30 patients with type 2 diabetic nephropathy, who were administered 20 mg of ROS daily for 90 d.[23] The results of this study support these previous findings. We suggested that ROS has no SUA-lowering effect.

The reasons for the difference between the SUA-lowering effects of ATV and ROS have been reported. Athyros *et al.* reported that the SUA-lowering effect observed in the GREACE study has been attributed to an increase in renal blood flow owing to the improvement of endothelial function by ATV, which exerts a variety of pleiotropic effects in addition to lowering lipid levels.[21] In this study, we analyzed the correlation between the rate of increase in eGFR and the rate of decrease in SUA levels for patients with decreased LDL-C levels. However, the significant difference was not found in this study.

There are no reports on the target achievement rates of statins for the reduction of SUA levels. Sarawate *et al.* reported that a gout attack was unlikely to occur in patients whose SUA levels were maintained at less than 6.0 mg/dL.[8] We compared ATV and ROS in terms of their achievement rate of SUA levels of less than 6.0 mg/dL. As a result, that of ATV was 3.5 times higher than that of ROS. Our results are sufficient to indicate the effectiveness of ATV in lowering the

SUA levels in patients with up to moderate renal failure such as stage 3 CKD.

There has been much discussion on drug therapy to prevent renal failure, and the selection and dose adjustment of drugs that lower the SUA levels have become important issues in the medical field. In this study, the renal protective effects and SUA-lowering effect of ATV were observed, and we found that ATV was significantly better than ROS in terms of achieving SUA levels of less than 6.0 mg/dL. Therefore, it is not necessary to administer drugs that lower the SUA levels to patients presenting with hyperlipidemia and hyperuricemia complications associated with moderate renal failure, such as patients with at least stage 3 CKD. We consider that, by selecting ATV, the renal protective effects and SUA-lowering effect would be sufficient. Due to this, the number of drugs to be taken will be reduced, and this will lead to compliance for the patients. Moreover, the possible side effects caused by drugs that lower the SUA levels will be eliminated, and we consider that there are good benefits from the viewpoint of medical economics. However, it is necessary to be careful about the side effects of rhabdomyolysis when we administer ATV. Most of the reported cases of rhabdomyolysis are renal function disorder patients. In addition, it is recognized that rapid renal dysfunction is aggravated by rhabdomyolysis. Therefore, we consider that it is necessary to administer ATV carefully after having considered its usefulness and risk in CKD patients.

This study is a cross-sectional study. There are few subjects than *meta*-analysis by Sandhu *et al.* and GREACE study and also is short during the target period in this study.[18] In addition, there was a possibility that the drugs of subjects and the treatments for chronic glomerulonephritis may be related in eGFR. Furthermore, we did not consider the effects of diet and exercise. That is to say that we cannot deny the possibility those other factors may have affected the results of this study. We consider that it is necessary to confirm through observation period of one year or more by a large-scale prospective study to demonstrate the clinical utility of ATV. These are the limitations of this study.

In recent years, the number of patients with end-stage kidney disease (ESKD) has been increasing. Aggressive management and treatment of CKD in terms of reducing the risk of developing CVD and preventing the progression to ESKD should be carried out immediately.

In conclusion, we confirmed the clinical utility of ATV in CKD patients in a retrospective observational study. Through this study, we should consider the renal protective effects of ATV and the need for drugs that lower the SUA level to monitor the development of CVD.

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