The Anti-atherogenic Activity of Beauveriolide Derivative BVD327, a Sterol O-Acyltransferase 2-Selective Inhibitor, in Apolipoprotein E Knockout Mice

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INTRODUCTION

Beauveriolide III (BeauIII) (Fig. 1), a fungal 13-membered cyclodepsipeptide, was originally isolated along with a known, structurally related molecule, beauveriolide I (BeauI), as an inhibitor of lipid droplet formation in mouse macrophages. These compounds exhibited anti-atherogenic activity in mouse models by inhibiting sterol O-acyltransferase (SOAT). Based on these results, a number of derivatives were synthesized combinatorially on the basis of BeauI and BeauIII as a lead scaffold.

Our current understanding of SOAT is that two isozymes, SOAT1 and SOAT2, exist in mammals. SOAT1 is expressed ubiquitously, particularly at a higher level in sebaceous glands, steroidogenic tissues and macrophages, whereas SOAT2 is expressed predominantly in the small intestine and the liver (hepatocytes). In addition, studies using knockout mice have indicated that SOAT1 inhibition has detrimental effects, whereas SOAT2 inhibition has atheroprotective effects. In fact, clinical studies established method. Importantly, we observed that these inhibitors are orally active in atherogenic mouse models with no side effects. Furthermore, PPPA has become not only a candidate for the treatment of atherosclerosis, but also a useful tool for elucidating the mechanisms underlying important biological activity. In fact, we established an assay system using PPPA to evaluate the ratio of SOAT1 and SOAT2 activity in total SOAT activity.

Recently, we demonstrated that BeauI and BeauIII selectively inhibit SOAT1 in intact mammalian cells. Because their efficacy was shown in vivo in atherogenic mouse models, a myriad of beauveriolide derivatives (BVDs) have been synthesized. We initially searched for BVDs with SOAT2-selective inhibitory activity because of the reasons given above.

In this study, we first describe the in silico absorption, distribution, metabolism and excretion (ADME) analysis and SOAT1/SOAT2 inhibitory activity of 86 BVDs. Next, we describe the in vivo efficacy of BVD327 in an atherogenic mouse model because this compound showed an excellent ADME score and is selective for SOAT2.

MATERIALS AND METHODS

Materials BVD327 was synthesized according to an established method.

In Silico ADME Analysis In silico ADME of beauveriolides I and III, as well as 149 beauveriolide derivatives, was analyzed by PharmaDesign Inc. (Tokyo, Japan) using...
an Admensa Interactive system (Inpharmatica Ltd., London, U.K.). In brief, ADME scores were calculated from *in silico* data of log S, log P (log [octanol]/[water]), log D, human intestinal absorption (HIA), blood–brain barrier (BBB) log([brain]/[blood]), human ether-a-go-go related gene (hERG) pIC50, CYP2C9 pKi, CYP2D6 affinity, P-glycoprotein substrate, and plasma protein binding (PPB) as oral non-central nervous system (CNS) scoring profiles.

**Mice and Diet** Male apolipoprotein E knockout (Apoe<sup>−/−</sup>) mice were purchased from Jackson Laboratory. Two diets were used, namely regular chow (CE-2, Crea Japan, Tokyo, Japan) and a Western-type diet containing 21% fat and 0.2% cholesterol by weight (D12079B, Research Diet, New Brunswick, NJ, U.S.A.). All mice had unrestricted access to their respective diet and water. Mice were ensured to be in the fed state at the time of study. All *in vivo* mouse studies were approved by the Animal Care Committees of Jichi Medical University and Kitasato University.

**Anti-atherosclerotic Activity in Apoe<sup>−/−</sup> Mice** This *in vivo* test was carried out using previously established methods. Male Apoe<sup>−/−</sup> mice at 10 weeks of age were switched from regular chow to a Western-type diet and orally given a drug (BVD327, 50 mg/kg/d, n = 8) suspended in 0.5% carboxy methyl cellulose sodium (CMC-Na) or 0.5% CMC-Na (control, 0 mg/kg/d, n = 8) for 12 weeks. Blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) from the retro-orbital venous plexus every two weeks. After centrifugation at 2700 rpm at 4°C, plasma was stored at −80°C until use. At the end of the 12-week treatment period, blood was collected from heart puncture and tissues and whole aortae were removed and stained with Sudan IV (Wako, Tokyo, Japan), and proximal aorta cross-sections were prepared and stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, U.S.A.) as previously described. The luminal side of the stained aortae was photographed. Image capture and analysis were performed using Adobe Photoshop CS5. The hearts were perfused with phosphate buffered saline (PBS) containing 4% (w/v) formalin, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Tissue-Tek, Tokyo, Japan), and 6 μm-thick serial sections were cut using a Cryostat (LEICA, Wetzlar, Germany). Three sections, each separated by a distance of 60 μm, were used to evaluate the lesions: three at the end of the aortic sinus, at the junctional site of the aortic sinus, and the ascending aorta. The sections were counterstained with Oil Red O and hematoxylin. Images of the sections were captured using a digital camera (DP70, Olympus, Tokyo, Japan) mounted on a light microscope (PROVIS AX80, Olympus) and analyzed with Adobe Photosho CS5.

**Plasma Lipids, Blood Urea Nitrogen (BUN), and Alanine Transaminase (ALT)** Colorimetric assays were used to measure total plasma cholesterol (TPC) (Determiner TC555, Kyowa Medex, Japan) and triglyceride (TG) (Triglyceride G-test, Wako) levels. BUN and ALT levels in plasma were deter-
mained by using commercially available kits, Urea N B (Wako) and Transaminase CII (Wako), respectively.

**Lipoprotein Profile** Lipoproteins were fractionated by HPLC as previously described (Skylight Biotech, Akita, Japan), and the cholesterol content of each lipoprotein fraction was measured.

**Hepatic Lipid Contents** Hepatic lipid contents in drug-treated A\textsubscript{po}e\textsuperscript{−/−} mice were estimated after performing chloroform-methanol extraction on 100 mg of liver tissue according to the method of Bligh and Dyer.\textsuperscript{42} An aliquot of liver lipid extract was solubilized in 1% Triton X-100 solution and lipid concentrations (total cholesterol [TC], free cholesterol [FC], TG, and phospholipid [PL]) were determined by using commercial kits. [\textsuperscript{14}C]cholesteryl ester (CE) contents were obtained by subtracting FC from TC, and the difference was multiplied by 1.67 to convert it to CE mass.

**Analysis of SOAT Activity in Small Intestines and Livers** Preparations of whole tissue homogenates and microsome fractions from the tissue of drug-treated A\textsubscript{po}e\textsuperscript{−/−} mice, as well as SOAT activity, were carried out by a previously described method.\textsuperscript{34,35,41} After the 12-week treatment, small intestines and livers were removed from drug-treated A\textsubscript{po}e\textsuperscript{−/−} mice for analysis. The intestines were divided into four equal segments that were classified from the proximal to the distal end as I-1, I-2, I-3, and I-4. Segments I-1 and I-2 were pooled and used for this study. After washing with saline, enterocytes were scraped from segments I-1 and I-2 (hereafter referred to as SI) and livers were stored at −80°C until use. All small intestines and livers were homogenized in 1.0 mL buffer A (100 mM sucrose, 50 mM KCl, 40 mM KH\textsubscript{2}PO\textsubscript{4}, 30 mM EDTA, and protease inhibitors (SIGMA), pH 7.2) in a Teflon homogenizer. After homogenization, all samples were centrifuged at 10000 \times g for 10 min at 4°C to remove cell debris. Five hundred microliters samples of whole homogenate were stored at −80°C until use. The resulting supernatant was centrifuged at 100000 \times g for 1 h at 4°C. The microsomal fraction of liver and SI from this spin was resuspended in the same buffer A and stored at −80°C until use. SOAT activity of the whole homogenate and the microsomal fraction of liver and SI was determined by [\textsuperscript{1-14}C]oleoyl-CoA and excess free cholesterol as substrates. The reaction mixture containing 2.5 mg/mL bovine serum albumin (BSA) in buffer A, [\textsuperscript{1-14}C]oleoyl-CoA (18.5 kBq, PerkinElmer, Inc., U.S.A.) and cholesterol, and the intestinal or hepatic sample in a total volume of 200 µL, were incubated at 37°C for 30 min. The reaction was started by adding [\textsuperscript{1-14}C]oleoyl-CoA and stopped by adding 1.2 mL chloroform:methanol (2:1). The product [\textsuperscript{14}C]cholesteryl ester was extracted by the method of Bligh and Dyer.\textsuperscript{42} After the organic solvent was removed by evaporation, lipids were separated on a TLC plate by using hexane:ethyl ether:acetic acid (70:30:1) as a developing solvent. The band corresponding to [\textsuperscript{14}C]CE on the TLC plate was scraped and resuspended in scintillation fluid for radioactivity measurements using a liquid scintillation counter (LS6500, Beckman Coulter, U.S.A.).

**Immunoblotting Analysis of SOAT2 in Livers** An immunoblotting analysis of SOAT2 in livers was performed by our established method.\textsuperscript{36,44} Briefly, whole homogenate (75 µL) prepared from livers were added to 4x Laemmli sample buffer and 100 mM dithiothreitol (DTT, SIGMA), and then incubated at room temperature for 30 min. Furthermore, 400 mM iodoacetamide (IAA, Wako) was added. After a 30-min incubation at room temperature, 10 µL of 1 M Tris–HCl (pH 9.2) was added to the mixture, a part of which was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel, e-PAGEL, ATTO, Japan) at a constant current of 20 mA for 1 h. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, U.S.A.) for 1 h at 100 V with a Western blot apparatus (Mini Trans-Blot Cell, Bio-Rad, U.S.A.). After transfer, the PVDF membrane was blocked with 5% skim milk in TBST buffer (20 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 0.10% (v/v) Tween 20) at 4°C overnight. The PVDF membrane was washed with TBST buffer and then soaked in the primary antibody (anti-SOAT2 rabbit polyclonal antibody,\textsuperscript{41} 1:2000 dilution) in TBST buffer at room temperature for 2 h. The primary antibody was then removed, and the PVDF membrane was washed and soaked in the secondary antibody (anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP), 1:2000 dilution) for 1 h. After removal of the secondary antibody, the blot was visualized using enhanced chemiluminescence (ECL) Western blotting Detection Reagents (Clarity, Bio-Rad) and detected using ChemiDoc Imaging system with Image Lab (Bio-Rad).

**Statistical Analysis** Experimental data are expressed as the mean ± standard deviation (S.D.). Statistically significant differences between the non-treatment group and treatment group were analyzed with a t-test (p < 0.05) using GraphPad Prism Software (GraphPad Software, INC., U.S.A.).

**RESULTS**

**Evaluation of Beauveriolide Derivatives by in Silico ADME Scores and SOAT2 Selectivity** We previously synthesized 149 BVDs using combinatorial chemistry\textsuperscript{6,7} and reported the structure–activity relationship (SAR) of SOAT isozyme inhibition.\textsuperscript{37} Among them, 86 BVDs showed inhibitory activity against SOAT1, SOAT2, or both isozymes (IC\textsubscript{50} = <20 µM).

First, we calculated the selectivity index (SI = log(IC\textsubscript{50} for SOAT1/IC\textsubscript{50} for SOAT2)) of the 86 BVDs.\textsuperscript{37} We defined compounds with an SI value of more than +1.00 as...
SOAT2-selective inhibitors. Eleven BVDs had SI values of more than +1.00, and their chemical structures are shown in Fig. 1. Second, the in silico ADME analysis for all BVDs was carried out using an Admensa Interactive system.\[^{38-40}\]

In silico ADME analysis included log S, log P, log D, HIA, BBB log(BB), hERG pIC\(_{50}\), CYP2C9 pKi, CYP2D6 affinity, P-glycoprotein substrate, and PPB, and finally, ADME scores were calculated (Supplementary Table 1). An ADME score (range, 0 to 1.0) with a score near 1.0 means that a compound is more druggable.

The SI values (X axis) and ADME scores (Y axis) for the 86 BVDs are plotted in Fig. 2. We found that only 11 BVDs (Fig. 1) are SOAT2-selective inhibitors; of these 11, BVD327 had the highest ADME score (0.323).

Thus, BVD327, which has an SI value of +1.22 SI and an ADME score of 0.323, was selected for our in vivo atherogenic mouse study using apolipoprotein E knockout mice.

**Anti-atherosclerotic Activity of BVD327 in Apoe\(^{-/-}\) Mice**

We used Apoe\(^{-/-}\) mice as an atherogenic mouse model. Control mice were fed a cholesterol-enriched diet containing 0.2% cholesterol and 21% fat for 12 weeks. Under these conditions, BVD327 was administered orally at 50 mg/kg/d for 12 weeks.

Toxicity of BVD327 in Apoe\(^{-/-}\) Mice

During our in vivo tests, body weight, ALT and BUN of BVD327-treated Apoe\(^{-/-}\) mice were measured as toxicity markers. No significant changes in these tests were observed (Figs. 3A, B, C), which indicates that BVD327 had no toxic effects on the liver, kidney and intestine.

**Plasma Lipids Levels and Lipoprotein Profile in Apoe\(^{-/-}\) Mice**

As shown in Fig. 3D, the total plasma cholesterol (TPC) levels of the BVD-treated group showed no significant dif-
ferences compared with the control group for 12 weeks. Although the levels of chylomicron, very low density lipoprotein (VLDL), and high density lipoprotein (HDL) showed no significant differences between the two groups, low density lipoprotein (LDL) was decreased slightly (Fig. 3E).

Lipid Accumulation in the Liver of Apoe−/− Mice

As shown in Fig. 4A, hepatic cholesteryl ester (CE) levels were significantly reduced in BVD327-treated mice. In contrast, free cholesterol (FC), TG and phospholipid levels did not change between the two groups (Fig. 4).

Anti-atherosclerotic Activity of Aortae and Heart of Apoe−/− Mice

After 12 weeks of feeding with a Western-type diet, untreated Apoe−/− mice developed atherosclerotic lesions in their aortae (Fig. 5A, control) and in their heart valves (Fig. 5C, control), which were visualized by staining with Sudan IV and Oil Red O, respectively. The areas (% of the total surface) of the aortae in the BVD327-treated group were significantly reduced (12.2 ± 2.16% [control] vs. 9.12 ± 2.39% [50 mg/kg/d]), indicating 25.4 ± 6.9% inhibition by BVD327 (Fig. 5B). In particular, we observed a marked decrease in atherosclerotic lesion areas of the aortic arches in the treated group (Fig. 5A). Furthermore, from cross-sections of the aortic sinuses of the hearts, we observed a marked thickening of the intima due to atherosclerosis progression in the control mice (Fig. 5C, control). The thickening of the heart intima in the BVD327-treated group was, however, significantly reduced (5.97 ± 1.19 × 10^5 μm² [control] vs. 4.74 ± 0.493 × 10^5 μm² [50 mg/kg/d]), indicating 20.6 ± 2.9% inhibition by BVD327 (Fig. 5D). Thus, we have demonstrated that the SOAT2-selective inhibitor BVD327 shows in vivo atheroprotective activity.

SOAT Activity of the Liver and Small Intestine of Apoe−/− Mice

The livers and small intestines Apoe−/− mice treated with drug for 12 weeks were removed, and whole homogenates and microsomes were prepared to determine SOAT activity. SOAT activity in the liver microsome fraction (Fig. 6A) and in small intestine microsomes (Fig. 6B) showed no significant changes when compared with control. SOAT activities in whole homogenates prepared from the livers and small intestines decreased to 33 and 41% of control, respectively. Then, SOAT2
proteins in whole homogenates prepared from the livers were no significant changes (Fig. 6C).

**DISCUSSION**

Among the 149 BVDs synthesized combinatorially, we found 11 BVDs had SOAT2-selective inhibition (\(SI = > + 1.00\)) in our established cell-based assay using SOAT1- and SOAT2-CHO cells. A combination of \(l\)-alanine and \(l\)-diphenylalanine, and \(l\)-2-naphthylalanine or \(d\)-phenylalanine in these depsipeptides (BeauI/BeauIII) appears to be responsible for SOAT2 selectivity\(^{37}\) (Fig. 1). A subtle structural difference in BeauI/BeauIII-based stereoisomers may affect their biological activity.\(^{45}\)

In this study, BVD327 was selected for the mouse *in vivo* study because the derivative had the highest ADME score among the 11 BVDs (Fig. 2). As we have emphasized previously,\(^{24,25}\) SOAT2-selective inhibitors have a strong potential as a post-statin drug for treating and preventing atherosclerosis. Researchers in pharmaceutical companies have not pursued SOAT2-selective inhibitors so far. We found that fungal PPPA and certain semisynthetic derivatives are the first SOAT2-selective inhibitors with markedly high SI values (+3.00 to +4.00).\(^{29–33}\) BVD327 selectively inhibited SOAT2, but the SI value (+1.22) was not as high compared with those of PPPA and other derivatives as well as inhibitory parameters (for example, \(K_i\), etc.) for SOAT2 might be not effective. However, BVD327 has a 13-membered cyclodepsipeptide skeleton different from PPPA and PRDs. Therefore, it was worth determining the *in vivo* efficacy of BVD327.

We found that BVD327, when administered at 50 mg/kg/d, exhibited atheroprotective activity (20–25% reduction) (Fig. 5) without toxic effects (Figs. 3B, C) in the *Apoe\(^{-/-}\)* mouse model, but TPC levels were not reduced (Fig. 3D). The SOAT2 inhibitory activity of BVD327 may be too weak to reduce TPC levels. At the end of the *in vivo* study, LDL was significantly reduced (20% inhibition), though other lipoprotein levels showed no significant changes (Fig. 3E). The amount of CE in the livers was significantly reduced (24% inhibition, Fig. 4A). SOAT activity in whole homogenates of livers and intestines from BVD327-treated mice was lowered (33–40% inhibition, Figs. 6A, B), while the activity in microsomes prepared from livers and intestines was not inhibited. On the other hand, SOAT2 protein in whole homogenates of livers from BVD327-treated mice was not changed (Fig. 6C). Dr. Rudel’s group reported that SOAT activity in mouse livers was mainly regulated by SOAT2 from liver-specific SOAT2 antisense oligonucleotide (ASO) study and liver-specific SOAT2 knockout
mice study. These data might suggest that the binding of BVD327 to SOAT2 localized to the endoplasmic reticulum (ER) membrane is weak although BVD327 inhibit SOAT2 in the liver and intestine.

In conclusion, we found that BVD327, a SOAT2-selective inhibitor with a 13-membered depsipeptide skeleton different from PPPA, is orally active in an atherogenic mouse model.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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