Supplemental Data

Pharmacokinetics of HMB-TZD in Ldlr$^{-/-}$ mice

To investigate the pharmacokinetics of HMB-TZD, *Ldlr*$^{-/-}$ mice were administered 3 mg of HMB-TZD, equivalent to the amount of HMB-TZD in the diet per day, once orally. Blood samples were collected at 12, 24, and 48 hours. High performance liquid chromatography (HP1090 HPLC/DAD) was used to analyze the plasma HMB-TZD level. 5-(3,5-di-tert-butyl-4-hydroxybenzylidene) thiazolidin-2,4-dione was used as an internal control. The standard and plasma samples were separated using the X-Terra RP18 (2.1 ×-150 mm, 5-μ50 mm, 5P18 (2.1 e separated using A diode array detector (HP 1090 Series) was used to quantitate the peak area of HMB-TZD at 350 nm. The standard and plasma samples were extracted with ethyl acetate with shaking for 20 minutes, and then centrifuged at 2000 rpm for 10 minutes. The organic layer was dried using an evaporator. After adding methanol and filtration, 10 μl of the solution was applied to the HPLC.

Measurement of plasma lipid peroxide

The lipid peroxidation product malondialdehyde (MDA) was measured as thiobarbituric acid-reactive substances as previously described [1]. Thiobarbituric acid-reactive substances were extracted in a mixture of butanol and pyridine, which was separated by centrifugation. The fluorescence intensity of the butanol/pyridine solution was measured at 553 nm with excitation at 513 nm. The plasma MDA contents of the control and HMB-TZD groups were expressed as μmol/ml.

Measurement of superoxide in lesion

Three tissue sections from each animal were incubated with 5 μM dihydroethidium (Molecular Probes, Eugene, OR) in a 37°C humidified chamber protected from light for 30 minutes. After the incubation, images were obtained using a laser scanning confocal microscope (LSM 510; Carl Zeiss GmbH, Jena, Germany). For ethidium bromide detection, a 543-nm He-Ne laser combined with a 585-nm long-pass filter was used. The average fluorescence intensity at the maximal response time period (approximately 30 seconds) was presented as fluorescence unit $\times 100/mm^2$. Quantitative analysis of the average fluorescence intensity was determined using Zeiss LSM 510 software.

Histopathology

Fatty streak lesions were quantified by evaluating the lesion size in the aortic sinuses as previously described [2]. Briefly, we obtained consecutive 5-μm-thick frozen sections through the aortic sinus, recognized by the cusps of the three aortic valves at the junction of the aorta and the left ventricle. Sections were stained by the Oil Red O method and counterstained with Harris hematoxylin. Fatty steak lesions were measured by computer-assisted morphometry (Image-Pro Plus), and the average lesion size of six sections at intervals of 30 μm was calculated for each animal. To stain macrophages, the sections were incubated with anti-monocyte/macrophage antibody (MOMA-2, AbD Serotec, Oxford, UK) and then subsequently with biotinylated anti-rabbit IgG antibody. Following incubation with peroxidase-conjugated Avidin D, antigens were visualized using a diaminobenzidine (DAB) substrate kit. Positively-stained areas were analyzed by computer-assisted morphometry (Image Pro Plus).

Quantitative real-time RT-PCR

Total aortic RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the Superscript III First-strand synthesis system (Invitrogen). Quantitative real-time RT-PCR was performed using a 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with primers and probes against mouse MCP-1, VCAM-1, and TNF-α (Applied Biosystems, Assay-on-Demand™). GAPDH primers (forward, 5on-Demandis system (Invitrogen). Quantitative real-time RT-PCR was performed using a 7700 body (MOMA-2, tained with Harris hematoxylin. Fatty steak lesions wBiosystems). All data were analyzed using SDS software (Applied Biosystems) with TAMRA as the reference dye.

Macrophage adhesion assay

Adhesion of THP-1 cells to a HUVEC monolayer was assayed as described previously [3]. Briefly, HUVECs were plated on 6-well plates at a density of 1×10^5 cells per well and cultured to 90% confluence in EGM-2 Bullet kit media. After 2 hours of HMB-TZD treatment, the cells were incubated with 20 ng/ml TNF-α for another 12 hours. THP-1 cells in the exponential growth phase were washed with serum-free DMEM and then resuspended in the same medium to 2×10^4cells/ml. The THP-1 cell suspension (1 ml) was added into the wells containing HUVEC monolayers and incubated for 20 minutes at 37°C. Unbound cells were washed three times with serum-free DMEM. The total number of adherent cells was counted in five randomly selected optical fields per well.

Mouse aorta isolation and ex vivo adhesion assay

Aortas were harvested from C57BL/6 mice, opened up longitudinally, and pinned onto sterile agar. Aortas were pre-treated for 2 hours with 0.1% DMSO or HMB-TZD (10^{-5}, 10^{-6}, or 10^{-7}M) prior to incubation with 20 ng/mL TNFα for 12 hours. After washing, aortas were incubated immediately for 30 min with 1 mm10^{6} $CD11b^{+}GFP^{+}$ cells isolated from the bone marrow of green fluorescence protein (GFP) transgenic mice. Bone marrow cells are maintained in w of gsupplemented with 10% heat-inactivated fetal bovine serum prior to use. After incubation, unbound macrophages were removed by washing, and the number of macrophages firmly bound to six pieces of aorta was counted in four consistent fields using fluorescence microscopy.

Cell migration

Migration of THP-1 monocytes was performed using Corning 3421 (Corning, NY) in a 24-well plate fitted with a collagen-precoated polycarbonate filter with 5.0-μm pores according to the manufacturerilter with 5.0 w The lower chambers were filled with 600 μl of medium obtained from cultured HUVECs treated with HMB-TZD for 2 hours and subsequently stimulated with 20 ng/ml TNF-α. The filter membranes were loaded with 1.25×10^{5} cells in 100 μl. After incubation for 6 hours at 37°C, the number of cells that had migrated into the lower chamber was estimated using a cell counter.

References

[1] Ohkawa, H., Ohishi, N. and Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

[2] Choi, J. H., Jeong, T. S., Kim, D. Y., Kim, Y. M., Na, H. J., Nam, K. H., Lee, S. B., Kim, H. C., Oh, S. R., Choi, Y. K., Bok, S. H. and Oh, G. T. Hematein inhibits atherosclerosis by inhibition of reactive oxygen generation and NF-kappaB-dependent inflammatory mediators in hyperlipidemic mice. J Cardiovasc Pharmacol 2003; 42: 287-295.

[3] Kim, J., Nam, K. H., Kim, S. O., Choi, J. H., Kim, H. C., Yang, S. D., Kang, J. H., Ryu, Y. H., Oh, G. T. and Yoo, S. E. KR-31378 ameliorates atherosclerosis by blocking monocyte recruitment in hypercholestrolemic mice. Faseb J 2004; 18: 714-716.

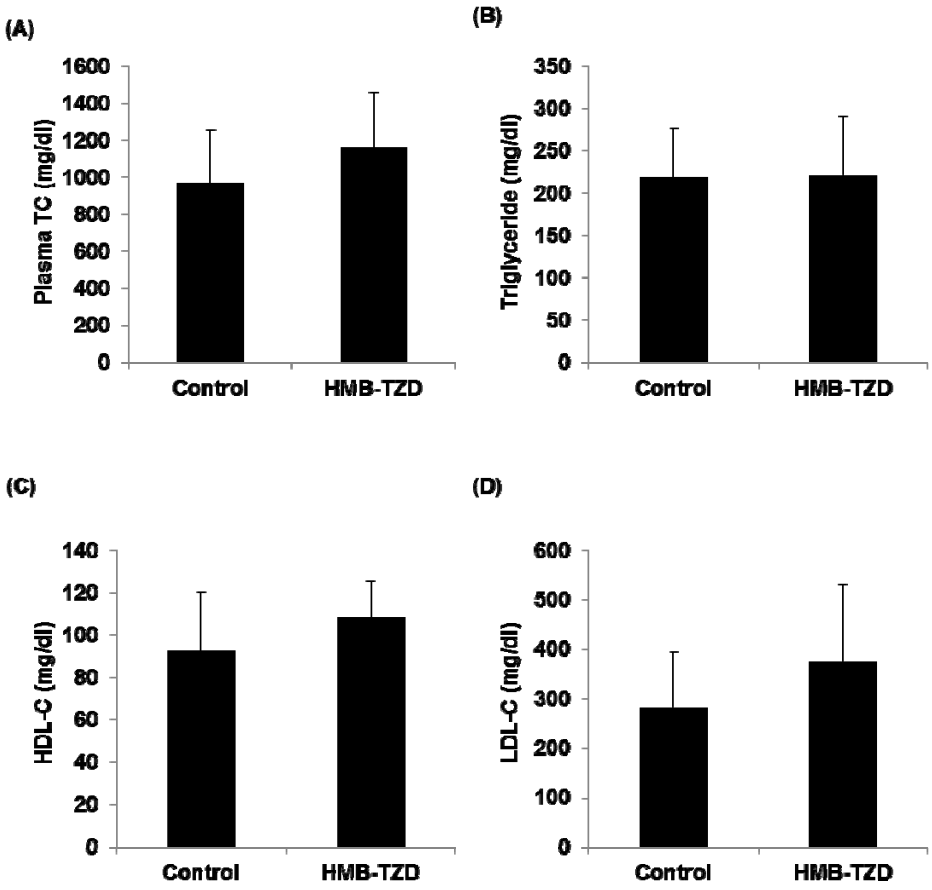

Figure S1. Plasma concentrations of (A) total cholesterol, (B) triglyceride, (C) HDL-cholesterol, and (D) LDL-cholesterol in control and HMB-TZD treated groups.

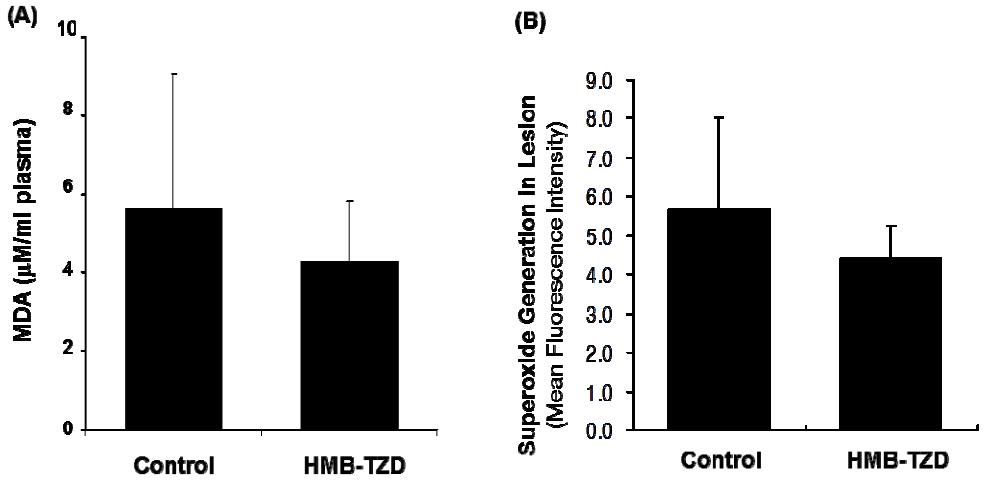

Figure S2. The effect of HMB-TZD treatment on plasma MDA level and lesional superoxide generation. (A) The effect of HMB-TZD on plasma MDA levels in control and HMB-TZD-treated animals. (B) The effect of HMB-TZD on superoxide generation in atherosclerotic lesions.