

5-(4-Hydroxy-2,3,5-trimethylbenzylidene) thiazolidine-2,4-dione attenuates atherosclerosis possibly by reducing monocyte recruitment to the lesion

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<http://dx.doi.org/10.3858/emmm.2011.43.8.053>

Accepted 20 June 2011

Available Online 21 June 2011

Abbreviations: 5-LOX, 5-lipoxygenase; BHB-TZD, 5-(3,5-di-tert-butyl-4-hydroxybenzylidene) thiazolidin-2,4-dione; COX, cyclooxygenase; HMB-TZD, 5-(4-hydroxy-2,3,5-trimethylbenzylidene) thiazolidin-2,4-dione; ICAM-1, intercellular adhesion molecule-1; Ldlr, low density lipoprotein receptor; TNF- α , tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1

Abstract

A variety of benzylidenethiazole analogs have been demonstrated to inhibit 5-lipoxygenase (5-LOX). Here we report the anti-atherogenic potential of 5-(4-hydroxy-2,3,5-trimethylbenzylidene) thiazolidin-2,4-dione (HMB-TZD), a benzylidenethiazole analog, and its potential mechanism of action in LDL receptor-deficient (*Ldlr*^{-/-}) mice. HMB-TZD Treatment reduced leukotriene B₄ (LTB₄) production significantly in RAW264.7 macrophages and SVEC4-10 endothelial cells. Macrophages or endothelial cells pre-incubated with HMB-TZD for 2 h and then stimulated with lipopolysaccharide or tumor necrosis factor-alpha (TNF- α) displayed reduced cytokine production. Also, HMB-TZD reduced cell migration and adhesion in accordance with decreased proinflammatory molecule production *in vitro* and *ex vivo*. HMB-TZD treatment of 8-week-old male *Ldlr*^{-/-} mice resulted in significantly reduced atherosclerotic lesions without a change to plasma lipid profiles. Moreover, aortic expression of pro-atherogenic molecules involved in the recruitment of monocytes to the aortic wall, including TNF- α , MCP-1, and VCAM-1, was downregulated. HMB-TZD also reduced macrophage infiltration into atherosclerotic lesions. In conclusion, HMB-TZD ameliorates atherosclerotic lesion formation possibly by reducing the expression of proinflammatory molecules and monocyte/macrophage recruitment to the lesion. These results suggest that HMB-TZD, and benzylidenethiazole analogs in general, may have therapeutic potential as treatments for atherosclerosis.

Keywords: antioxidants; arachidonate 5-lipoxygenase; atherosclerosis; endothelial cells; macrophages

Introduction

Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) produce prostaglandins, thromboxanes, and leukotrienes (LTs) from arachidonic acid. These metabolites have been shown to play important roles in inflammation, thrombosis, allergy, and cancer (Vila, 2004). 5-LOX is a key enzyme involved in the production of proinflammatory LTs and has been linked to the pathogenesis of various inflammatory disorders (Vila, 2004). LTs are produced by cells critical to the inflammatory response, namely macrophages, neutrophils, eosinophils, mast cells, and endothelial cells (Lewis *et al.*, 1990; Zou *et al.*, 2009; Kim *et al.*, 2010). In response to a diversity of immune and inflammatory stimuli, LTs are secreted into the extracellular space where they bind receptors, including BLT₁, BLT₂, CysLT₁, and CysLT₂, and induce inflammation (Yokomizo *et al.*, 1997, 2000; Vila, 2004; Lötzer *et al.*, 2007; Sánchez-Galán *et al.*, 2009).

Atherosclerosis is a chronic inflammatory vascular disease involving inflammatory mediators, monocytes, endothelial cells, and smooth muscle cells (Ross, 1999). In atherosclerotic lesions, the number of 5-LOX-expressing cells, which are primarily macrophages, is markedly increased (Spanbroek *et al.*, 2003). In fact, a polymorphism in the 5-LOX promoter has been linked to atherosclerosis (Dwyer *et al.*, 2004). Therefore, inhibition or genetic ablation of the 5-LOX pathway has been reported to decrease the size of atherosclerotic lesions by reducing the production of inflammatory arachidonic acid metabolites such as LTB₄ (Mehrabian *et al.*, 2002; Heller *et al.*, 2005).

Due to their strong anti-inflammatory properties, modifiers of the 5-LOX pathway have been considered potential therapeutics for treating cardiovascular disease (Funk, 2005). Many multi-substituted benzylidenethiazole analogs derived from 2,6-di-tert-butylphenol have been found to reduce inflammatory processes *in vivo* and *in vitro* by inhibiting both COX and 5-LOX (Unangst *et al.*, 1994). Previously, we reported that one such analog, 5-(3,5-di-tert-butyl-4-hydroxybenzylidene) thiazolidin-2,4-dione (BHB-TZD), also possesses strong anti-atherogenic activity (Choi *et al.*, 2010). In this study, we examined the anti-atherogenic activity of another benzylidenethiazole analog, 5-(4-hydroxy-2,3,5-trimethylbenzylidene) thiazolidin-2,4-dione (HMB-TZD). Interestingly, although the structure of HMB-TZD is highly similar to that of BHB-TZD, HMB-TZD inhibits 5-LOX strongly but blocks COX weakly. Moreover, HMB-TZD exhibits anti-oxidative activity, preventing oxidative modification of low-density lipoprotein (LDL) (Jeong *et*

al., 2004). These characteristics of HMB-TZD prompted us to investigate the effect of this analog on atherogenesis and its mechanism of action in *Ldlr*^{-/-} mice.

Results

A potent binding mode of HMB-TZD in the catalytic channel of 5-LOX

To elucidate a potential mode of binding of HMB-TZD to the active site of mouse 5-LOX, an *in silico* docking experiment was performed. Since a crystal structure of mouse 5-LOX is unavailable, we modeled this enzyme on the crystal structure of 8R-LOX from *P. homomalla* (Neau *et al.*, 2009) using MODELLER. The root-mean-square deviation of 577 C α -atom pairs between the final model of mouse 5-LOX and 8R-LOX is 0.340 Å, suggesting that these structures are similar. Our model of the structure of mouse 5-LOX consists of an N-terminal C2-like domain and a larger C-terminal catalytic domain composed mostly of α -helices. Within the center of the catalytic domain is a putative arachidonate binding channel connected to the catalytic iron as found in other lipoxygenases (Neau *et al.*, 2009). Our docking experiment reveals that HMB-TZD binds to the catalytic channel of 5-LOX (Figures 1A and 1B). The hydrophobic HMB moiety of HMB-TZD is positioned in the hydrophobic portion of the channel lined by Phe170, Phe178, Tyr182, Ile407, Ala411, Leu415, and Leu608. In addition, one oxygen atom of the HMB moiety forms a hydrogen bond with the nitrogen atom of the Ala411 backbone. The polar TZD moiety is located adjacent to the catalytic iron and is stabilized by hydrogen bonding between one of its oxygen atoms and His373 and Asn408. The catalytic iron is coordinated by the side chains of four amino acids (i.e., His368, His373, His551, and Asn555) and the main carboxylate of I674 located in the C-terminus. Interestingly, these results demonstrate that one oxygen atom of the TZD moiety occupies the sixth position of the catalytic iron with an approximate distance of 3.17 Å. In the purple oxygenase structure, the peroxide crucial for the catalytic activity of the enzyme occupies the sixth ligand position in the iron coordination sphere (Skrzypczak-Jankun *et al.*, 2001). Structural comparison of several soybean 3-LOX complex structures with inhibitors such as 4-nitrocatechol (1NO3), 4-hydroperoxy-2-methoxyphenol (1HU9), protocatechuic acid (1N8Q), and epigallocatechin (1JNQ) showed a similar mode of binding between their analogous oxygen atom to the catalytic iron. Therefore, the mechanism under-

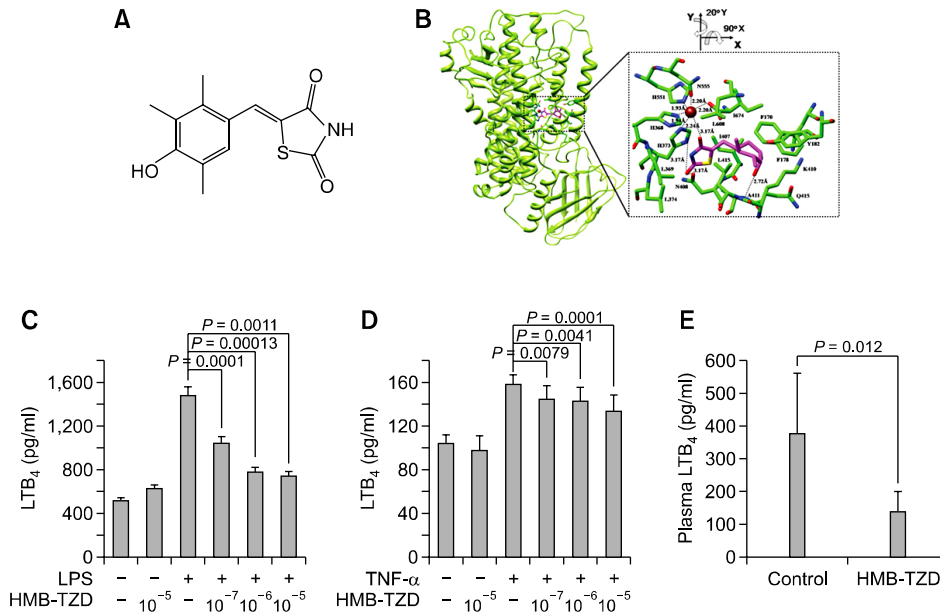


Figure 1. HMB-TZD reduces LTB₄ production *in vitro* and *in vivo*. (A) Structure of HMB-TZD. (B) HMB-TZD embedded in the catalytic channel of 5-LOX. The best position for HMB-TZD within the 5-LOX catalytic channel as modeled from the docking experiment was drawn using Chimera. The zoomed image shows surrounding residues interacting with HMB-TZD. 5-LOX is colored in green and HMB-TZD in pink. The residues are represented by ball-and-stick models with yellow representing sulfur, blue for nitrogen, and red for oxygen. (C and D) The concentrations of LTB₄ in RAW264.7 macrophage (C) and SVEC4-10 endothelial cell (D) culture supernatant were measured by ELISA. RAW264.7 macrophages and SVEC4-10 endothelial cells were pre-incubated with HMB-TZD (10⁻⁵, 10⁻⁶, or 10⁻⁷ M) for 2 h, and then stimulated with LPS (1 μg/ml) or TNF-α (20 ng/ml) for 24 h, respectively. (E) Effect of HMB-TZD treatment on the plasma LTB₄ level in *Ldlr*^{-/-} mice.

lying HMB-TZD inhibition of 5-LOX may be similar to these soybean 3-LOX inhibitors.

HMB-TZD reduced LTB₄ production *in vitro* and *in vivo*

To determine the effect of HMB-TZD on arachidonic acid metabolite production, we first assessed the effect of the analog on the production of LTB₄, a representative proatherogenic arachidonic acid metabolite produced by the 5-LOX pathway. In RAW264.7 macrophages and SVEC4-10 endothelial cells, lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) stimulated LTB₄ production was reduced significantly by HMB-TZD treatment in a dose-dependent manner, respectively (Figures 1C and 1D). To examine the effect of HMB-TZD on plasma LTB₄ levels, *Ldlr* knockout (*Ldlr*^{-/-}) mice were fed a western diet supplemented with 1% (w/w) HMB-TZD. After 8 weeks, the LTB₄ level was 63% lower in HMB-TZD-treated animals than in control mice (Figure 1E). Since many derivatives of 2,6-di-tert-butylphenol have been shown to have an inhibitory effect on COX (Unangst *et al.*, 1994), we measured plasma prostaglandin E₂ (PGE₂) levels to assess whether HMB-TZD also inhibits the COX pathway *in vivo*. We found no significant difference between the control and HMB-TZD-

treated groups (311 ± 36 vs. 325 ± 21 pg/ml, respectively). Taken together, our data demonstrate that HMB-TZD reduces LTB₄ production effectively in *Ldlr*^{-/-} mice.

HMB-TZD reduced atherosclerotic lesion formation in *Ldlr*^{-/-} mice

Initially, to investigate the pharmacokinetics of HMB-TZD, *Ldlr*^{-/-} mice were administered 3 mg of HMB-TZD once orally. This dose was equivalent to the daily amount ingested from the diet used in this study (i.e., 1% w/w HMB-TZD as mentioned above). The plasma HMB-TZD level in the treated animals increased up to 1.9 × 10⁻⁶ M at 2 h and then decreased dramatically, indicating that HMB-TZD is metabolized and excreted normally.

To determine the effect of HMB-TZD on the development of atherosclerosis, we analyzed atherosclerotic lesions in aortic vessels from animals that were fed the western diet with and without HMB-TZD for 8 weeks. HMB-TZD supplementation did not affect plasma concentrations of total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol (Supplemental Data Figure S1). Necropsy and histopathological examinations did not reveal notable lesions in the parenchymal organs of both groups of mice (data not shown). In

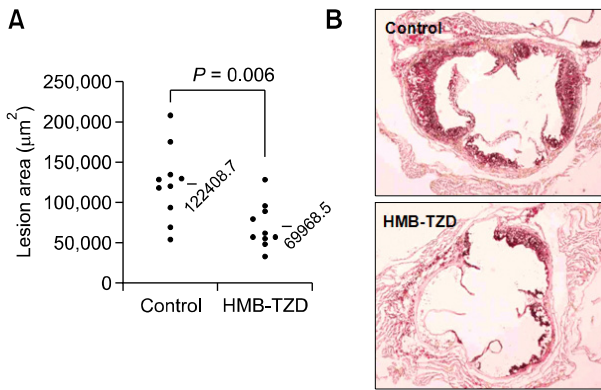


Figure 2. HMB-TZD treatment reduced fatty streak lesions in *Ldlr*^{-/-} mice. (A) The mean size of fatty streak lesions in HMB-TZD-treated (*n* = 10) and control (*n* = 10) mice. (B) Representative oil red O staining of atherosclerotic lesions in control and HMB-TZD-treated mice.

addition, daily food intake and body weight gain was similar between the groups (data not shown). Measurement of the lesion size by computer-associated morphometry revealed that the mean lesion area of the aortic sinus in HMB-TZD-treated mice was smaller than that of control animals by 43% (Figures 2A and 2B).

Reactive oxygen species play an important role in atherogenesis through multiple mechanisms, including LDL oxidation. The lipid peroxidation product, malondialdehyde (MDA), may also be critical to atherogenesis. Previously, HMB-TZD had been reported to reduce LDL oxidation (Jeong *et al.*, 2004). To determine the antioxidative activity of HMB-TZD, the levels of plasma lipid peroxide and lesional superoxide were measured. Although not statistically significant, the average lipid peroxide and lesional superoxide levels were lower in the HMB-TZD-treated group (Supplemental Data Figure S2).

HMB-TZD reduced the expression of vascular proatherogenic genes in *Ldlr*^{-/-} mice

To understand the mechanism underlying the anti-atherogenic activity of HMB-TZD, we measured the expression of atherosclerosis-related genes, including *MCP-1*, *VCAM-1*, and *TNF-α*, in the aortas of HMB-TZD-treated and control mice. Using real-time reverse transcription-polymerase chain reaction (RT-PCR), we found that *MCP-1* and *VCAM-1* mRNAs were decreased by 75% and 41%, respectively, in the HMB-TZD-treated group compared to control animals (Figure 3A). In addition, *TNF-α* mRNA and plasma *TNF-α* levels were also reduced significantly by HMB-TZD administration (Figures 3A and 3B).

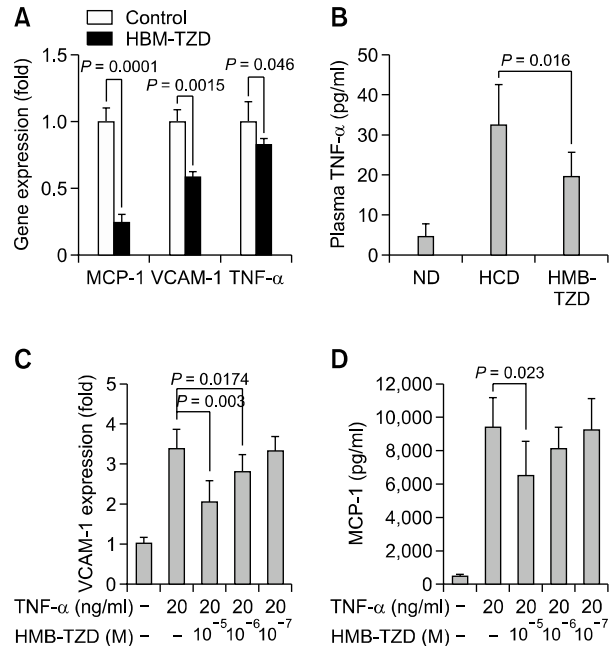


Figure 3. Effect of HMB-TZD on inflammatory gene expression in the aorta and endothelial cells. Total RNA was isolated from five pooled thoracic aortas from control and HMB-TZD-treated mice. (A) The expression of *MCP-1*, *VCAM-1*, and *TNF-α* mRNA was quantitated by real-time RT-PCR and normalized to the expression of *GAPDH*. Each PCR reaction was performed in triplicate, and gene expression levels and standard errors were determined. (B) Reduction in plasma *TNF-α* level in HMB-TZD-treated animals. (C, D) The effect of HMB-TZD on *VCAM-1* expression and *MCP-1* production in HUVECs stimulated with *TNF-α*. HUVECs were pre-treated with HMB-TZD at the indicated concentration for 2 h, and then stimulated with 20 ng/ml of *TNF-α* for 24 h.

Since HMB-TZD treatment reduces *VCAM-1* expression in the aortic wall, we investigated whether this compound affects cultured endothelial cells. Our data demonstrate that HMB-TZD reduced *VCAM-1* expression significantly in *TNF-α*-stimulated endothelial cells in a dose-dependent manner. Moreover, *MCP-1* production was decreased (Figures 3C and 3D), indicating that HMB-TZD reduced pro-inflammatory gene expression directly in endothelial cells.

HMB-TZD inhibited monocyte adhesion to endothelium and transmigration

Since HMB-TZD reduced the production of inflammatory molecules in cytokine-stimulated endothelial cells directly, we performed *in vitro* cell migration and adhesion assays, as well as *ex vivo* adhesion assays using aortic organ culture. These studies revealed that monocyte adhesion and migration diminished significantly with HMB-TZD treatment *in vitro* (Figures 4A and 4B). Furthermore, monocyte attachment to the aortic lumen after

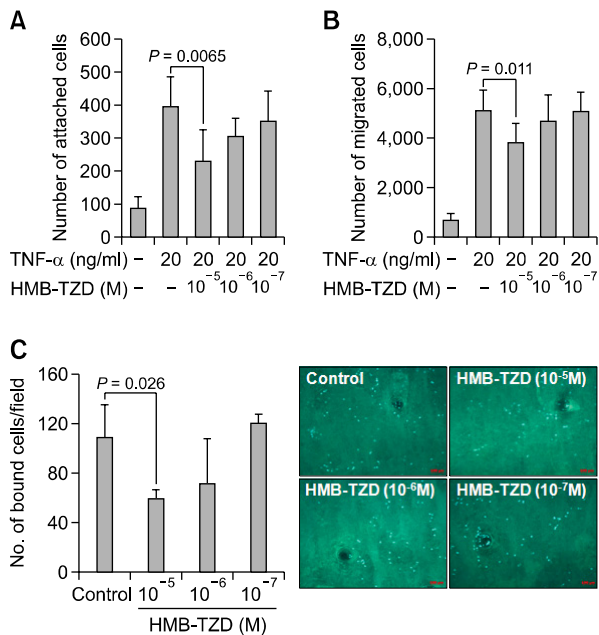


Figure 4. HMB-TZD reduced macrophage accumulation in atherosclerotic lesions. (A) Monocyte adhesion assay. HUVECs were pre-incubated with HMB-TZD (10^{-5} , 10^{-6} , or 10^{-7} M) for 2 h, and then stimulated with TNF- α for 12 h. Next THP-1 monocytes were added to the culture. After 20 min, unbound cells were washed and the remaining attached cells were counted in five randomly selected optical fields per well. (B) Monocyte migration assay. THP-1 monocytes were loaded into the upper chamber of Transwell plates and the lower chambers were filled with conditioned medium from HUVECs treated with HMB-TZD (10^{-5} , 10^{-6} , or 10^{-7} M) or left untreated for 2 h and subsequently activated with 20 ng/ml TNF- α for 12 h. (C) Monocyte adhesion assay in aortic organ culture. Aortas were pre-treated for 2 h with 0.1% dimethyl sulfoxide or HMB-TZD (10^{-5} , 10^{-6} , or 10^{-7} M) prior to incubation with 20 ng/ml TNF- α for 12 h. After washing, aortas were incubated for 30 min with 1×10^6 CD11b⁺ GFP⁺ cells, and fluorescence microscopy was used to count the bound cells.

TNF- α treatment was also reduced by HMB-TZD (Figure 4C).

HMB-TZD reduced macrophage accumulation in atherosclerotic lesions

Finally, we measured the area occupied by macrophages in the aortic sinus to determine whether HMB-TZD treatment reduces monocyte recruitment to atherosclerotic lesions. HMB-TZD administration reduced the macrophage area significantly compared to untreated animals (Figure 5), suggesting that this compound affects macrophage infiltration in atherosclerotic lesions.

Discussion

In this study, we show that HMB-TZD ameliorates

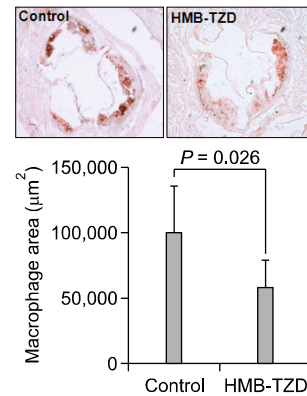


Figure 5. HMB-TZD treatment reduced macrophage infiltration into the aortic sinus of *Ldlr*^{-/-} mice. Representative immunohistochemical staining of macrophages in control and HMB-TZD-treated mice are shown (upper). The graph shows the mean size of macrophage positive areas in each group (lower).

the formation of typical atheromatous lesions in *Ldlr*^{-/-} mice without changing the plasma concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides. HMB-TZD also reduced the plasma level of LTB₄, suggesting that this analog inhibits the 5-LOX pathway *in vivo*. Our data also demonstrate that this compound reduces the macrophage accumulation in atherosclerotic lesions. LTB₄ is well-established as an inducer of several inflammatory processes, including chemotaxis, leukocyte adhesion to the vascular endothelium, and gene expression (Yokomizo *et al.*, 1997, 2000; Vila, 2004; Sánchez-Galán *et al.*, 2009). Reduced LTB₄ production due to inhibition of 5-LOX activity can reduce macrophage adhesion and transmigration into the subintimal space, leading to a reduction in atherosclerotic lesion size (Aiello *et al.*, 2002; Friedrich *et al.*, 2003; Huang *et al.*, 2004). Results from this study demonstrate that HMB-TZD may reduce macrophage accumulation in vessel walls, in part, by inhibiting the 5-LOX pathway, which results in downregulation of LTB₄.

In this study, we show that HMB-TZD reduces LTB₄ production effectively in RAW264.7 macrophages and SVEC4-10 endothelial cells. Moreover, HMB-TZD reduced plasma LTB₄ levels significantly. A previous study demonstrated that benzylidene-thiazole analogs derived from 2,6-di-tert-butylphenol could also inhibit COX activity (Unangst *et al.*, 1994). Therefore, we tested the effect of HMB-TZD on PGE₂ production in RAW264.7 macrophages. Our data show that HMB-TZD inhibition of PGE₂ production was weak (data not shown). Furthermore, we also found that the plasma PGE₂ level did not change with HMB-TZD supplementation. This result indicates that the attenuated atherosclerosis

observed in the HMB-TZD-treated group was not mediated by COX inhibition.

In addition to anti-diabetic effects such as reducing the plasma glucose level and acting as PPAR γ agonists, thiazolidinediones (TZDs) exhibit anti-atherogenic activity (Tontonoz and Spiegelman, 2008). HMB-TZD has a structure similar to TZDs and was demonstrated previously to react with PPAR γ (Momose *et al.*, 2002). Therefore, HMB-TZD is considered a PPAR γ agonist. However, we found that HMB-TZD did not increase luciferase reporter activity when controlled by a PPAR γ -responsive element, whereas troglitazone, which was used as a positive control, increased this activity dramatically (data not shown). Furthermore, HMB-TZD supplementation did not affect plasma glucose levels (data not shown). These results indicate that HMB-TZD is not a PPAR γ agonist, and that PPAR γ is not involved in the attenuation of atherosclerosis by HMB-TZD.

Inhibitors of 5-LOX have been demonstrated to attenuate airway inflammation by reducing LT production (Berger *et al.*, 2007). 5-LOX inhibitors have been used as therapeutics for asthma patients. In addition to their potent proinflammatory properties, leukotrienes have been demonstrated recently to affect atherosclerosis. Inhibitors of the 5-LOX pathway have been shown to possess anti-atherogenic activity (Aiello *et al.*, 2002; Jawien *et al.*, 2006). Our data support the hypothesis that inhibiting the 5-LOX pathway is a potential therapeutic strategy for treating atherosclerosis.

In conclusion, our study demonstrates that HMB-TZD reduces monocyte adhesion and infiltration. We also show that this compound attenuates atherosclerosis in LDL receptor-deficient mice. Furthermore, using *in silico* docking, we demonstrated that HMB-TZD can bind to the catalytic channel of 5-LOX. By reducing the LTB $_4$ level, HMB-TZD decreased monocyte adhesion and transmigration *in vitro*, thereby inhibiting monocyte recruitment into the plaque. Our findings clearly support that this group of benzylidene derivatives are potential therapeutic candidates for treating atherosclerosis.

Methods

Chemicals and cell culture

HMB-TZD (Figure 1A) was obtained from the Korea Research Institute of Bioscience and Biotechnology. RAW264.7, SVEC4-10 and THP-1 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. RAW264.7 macrophages were pre-incubated for 2 h with HMB-TZD at concentrations that did not cause

cytotoxicity as determined by the MTT assay (10^{-5} , 10^{-6} , or 10^{-7} M; data not shown). Then RAW264.7 macrophages and SVEC4-10 endothelial cells were activated with 1 μ g/ml LPS or 20 ng/ml TNF- α for 24 h. The level of LTB $_4$ in tissue culture supernatants from these cultures was subsequently measured by enzyme-linked immunosorbent assay (ELISA). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (BioWhittaker, Walkersville, MD) and maintained in EGM-2 Bullet kit media (BioWhittaker) at 37°C and 5% CO $_2$. All HUVECs used in this study were passaged 3 to 7 times.

Model building and evaluation

The primary sequence of mouse arachidonate 5-LOX consists of 674 amino acids. The *Plexaura homomalla* 8R-lipoxygenase (8R-LOX) sequence exhibiting 60% homology with 5-LOX was obtained selected using BLAST (<http://blast.ncbi.nlm.nih.gov>) for use as a template. The 3D structure coordinates of 8R-LOX (PDB ID: 3DY5) were obtained from the Brookhaven Protein databank. MODELLER 9v8 (Eswar *et al.*, 2006) was used to build a computational homology model of 5-LOX that satisfies spatial restraints. The input files for MODELLER were the pdb file of the template, the alignment file of the template and target sequences, and Python command script files. Energy-minimized models were produced using CHARMM energy parameters. The most reasonable model was selected based on evaluation and quality assessment of the stereochemistry of the model by PROCHECK (Laskowski *et al.*, 1996). ProSa (Sippl, 1993) generating energy graphs based on interactions between C β -C β pairs and C β -surface was also used to check strained regions in the model.

In vivo docking

To probe the preferred binding mode of HMB-TZD to the active site of 5-LOX, *in silico* docking studies were performed using the modeled structure of 5-LOX. To accomplish this, the docking algorithm Surflex-Dock, which is part of the SYBYL software package (version SYBYL-X 1.1, Tripos, Inc.), was used. The active site of 5-LOX was defined using the SYBYL structure preparation tool based on the complex structures of 3-LOX from soybean with 1NO3, 1HU9, 1N8Q, 13(S)-hydroperoxy-9(Z), 11(E)-octadecadienoic acid, or 1JNQ. The HMB-TZD structure drawn in PubChem Sketcher v2.4 (<http://pubchem.ncbi.nlm.nih.gov>) was converted to a 3D structure and energy-minimized with the Gasteiger-Huckel force field and saved in the MOL format for docking.

Ldlr^{-/-} mice and HMB-TZD treatment

Eight-week-old male *Ldlr*^{-/-} mice ($n = 20$) were divided randomly into two equal-sized groups, control and HMB-TZD-treated. The control group was fed a western diet (CRF-1 supplemented with 0.15% cholesterol, 20% fat, and 0.05% Na-choleate, Oriental Yeast Co. Ltd., Tokyo, Japan), while the HMB-TZD-treated mice were fed the same diet supplemented with 1% (w/w) HMB-TZD. All mice were given water and food *ad libitum*. After 8 weeks, the mice were sacrificed, and plasma levels of various

molecules were determined using an automatic blood chemical analyzer (HITACHI, Japan). Other parenchymal organs were harvested for histopathological examination. This investigation conforms to the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. All animal study protocols were approved by the Institutional Animal Care and Usage Committee of Ewha Womans University (Seoul, Korea).

Measurement of LTB₄, PGE₂, MCP-1, and TNF- α levels

The levels of LTB₄, PGE₂, MCP-1, and TNF- α in plasma or cell culture supernatant were quantified by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Each measurement was performed in triplicate and three independent measurements were conducted.

Statistical analysis

Statistical significance between two groups was determined with the two-tailed Student's *t* test. Analysis of variance followed by Fisher's Protected Least Significant Difference was used for comparisons within a test group. All data in this study are expressed as the mean \pm standard deviation. Values of *P* < 0.05 were considered significant.

Supplemental data

Supplementary data were included with methods for establishing the pharmacokinetics of HMB-TZD in *Ldlr*^{-/-} mice, measurement of plasma lipid peroxide, measurement of superoxide in atherosclerotic lesions, histopathology, quantitative real-time RT-PCR, macrophage adhesion assay, mouse aorta isolation, and *ex vivo* adhesion assay and cell migration. Supplemental Data include two figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-8-05.pdf.

Acknowledgements

This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A090264).

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