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A randomized, open-label, multicentre study to evaluate plasma atherosclerotic biomarkers in patients with type 2 diabetes mellitus and arteriosclerosis obliterans when treated with Probucol and Cilostazol

Xiao-Wei Ma[1], Xiao-Hui Guo[1], Xin-Hua Xiao[2], Li-Xin Guo[3], Xiao-Feng Lv[4], Quan-Min Li[5], Yan Gao[1]

[1]*Department of Endocrinology, Peking University First Hospital, No.7 of Xishiku Street, Xicheng District, Beijing 100034, China*
[2]*Department of Endocrinology, Peking Union Medical College Hospital, No.1 Shuaifuyuan, Dongcheng District, Beijing 100005, China*
[3]*Department of Endocrinology, Peking Hospital, No.1 Dahua Road, Dongdan, Dongcheng District, Beijing 100730, China*
[4]*Department of Endocrinology, Military General Hospital of Peking PLA, No.5 Nanmencang, Dongsishitiao,Dongcheng District, Beijing 100070, China*
[5]*Department of Endocrinology, Second Artillery Forces General Hospital of Peking PLA, No.16 Xinjiekouwai Street, Xicheng District, Beijing 100088, China*

Abstract

Objectives To evaluate the plasma atherosclerotic biomarkers in patients with type 2 diabetes mellitus (T2DM) and arteriosclerosis obliteran (ASO) when treated with Probucol plus Cilostazol in combination and individually. **Methods** In this open-label study, patients aged 40–75 years were randomized to receive conventional therapy alone, or with Cilostazol 100 mg bid, or with Probucol 250 mg bid, or with both in combination. Endpoints included changes in plasma biomarker and safety at 12 weeks. **Results** Of the 200 randomized patients, 165 for per-protocol and 160 for the safety (QTc intervals) were set, respectively. Probucol significantly reduced total cholesterol ($P < 0.001$), low-density lipoprotein cholesterol (LDL-C), ($P = 0.01$), and high-density lipoprotein cholesterol (HDL-C) ($P < 0.001$) compared with conventional therapy. Cilostazol was effective in increasing HDL-C ($P = 0.002$) and reducing triglycerides levels ($P < 0.01$) compared with conventional therapy. A trend towards significance was observed for the difference between conventional therapy alone and Probucol plus Cilostazol group for the change in oxidized low-density lipoprotein (Ox-LDL, $P = 0.065$). No significant effects on the majority of the remaining biomarkers were found across the treatment groups. **Conclusions** We have confirmed that Ox-LDL could be a possible plasma atherosclerotic biomarker among the evaluated biomarkers, which reflected the synergetic effect of Cilostazol plus Probucol in patients with T2DM and ASO shown previously in preclinical studies.

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Keywords: Cilostazol; Probucol; Combination treatment; Atherosclerosis; Biomarkers; Synergetic effect

1 Introduction

Atherosclerosis is a major cause of stroke and coronary artery disease (CAD) and is associated with inflammation and oxidative stress.[1] Development of atherosclerotic lesions may result in myocardial infarction (MI), angina pectoris, ischemic stroke, and arteriosclerosis obliterans (ASO). Because these diseases are mostly related to the severity of atherosclerosis, preventing progression of atherosclerotic lesions and inhibiting thrombus formation are key therapeutic goals.[2] Therefore, the earlier detection of vascular damage, and possibly clinical events, predicted by plasma atherosclerotic biomarkers may be anticipated.

Correspondence to: Xiao-Wei Ma, MD, Department of Endocrinology, Peking University First Hospital, No.7 of Xishiku Street, Xicheng District, Beijing 100034, China. E-mail: maxiaowei_bjufh@yahoo.com
Telephone: +86-10-83575103 **Fax:** +86-10-66552395
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We have focused on the effect of the Cilostazol plus Probucol combination with conventional therapy due to their anti-atherosclerotic properties. Probucol is a unique cholesterol lowering drug with antioxidant, anti-inflammatory and anti-atherogenic properties.[3] Probucol, which acts by increasing the rate of low-density lipoprotein (LDL) catabolism, reduces not only LDL-cholesterol (LDL-C), but also high-density lipoprotein cholesterol (HDL-C) and enhances reverse cholesterol transport through the activation of reverse cholesteryl ester transport protein (CETP) and class B type1 scavenger receptors (SR-B1).[4–6] Recently, it was reported HDL-C lowering by Probucol may be due to changes in the composition of HDL subtypes, including an increase in pre-beta 1 (β1)-HDL, which participates in cholesterol efflux.[7] Rather than having a detrimental effect, this enhanced HDL remodeling and associated pre-β1-HDL dissociation from large HDL particles may partially explain the HDL-lowering ability and anti-atherogenic action of the agent.[8] Matsuzawa *et al.*[9]

reported close correlation between the extent of regression in Achilles tendon xanthoma and Probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia. Probucol has also been proven to reduce the intima-media thickness of the carotid artery and the incidence of cardiovascular events.[10]

Cilostazol, another drug in the combination treatment in this study, is a type 3 phosphodiesterase inhibitor that displays antiplatelet and vasodilating activity by suppressing the degradation of cyclic adenosine monophosphate. [11] Cilostazol also improves lipid parameters and vascular endothelial function.[12] Decreased triglyceride (TG) levels and increased HDL-C levels have been reported in clinical studies,[13] possibly due to an increase in the activity of lipoprotein lipase and the promotion of reverse cholesterol transport through increased ABCA1/G1 expression in macrophages.[14,15] In a recent study, Katakami *et al*.[16] reported Cilostazol reduced the intimamedia thickness of the carotid artery in patients with diabetes mellitus (DM). Cilostazol also has pleiotropic effects against oxidative stress on vascular function and atherosclerosis.[17]

Plasma biomarkers recommended for use in global risk assessment of CAD by the National Cholesterol Education Program (NCEP)-Adult Treatment Panel (ATP III) Guidelines are LDL-C, HDL-C and TG levels.[18] However, there are a number of new markers associated with inflammation and oxidative stress that show clinical promise and are currently being investigated, including oxidized LDL (Ox-LDL), and the leukocyte adhesion molecules, soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), monocyte chemotactic protein-1 (MCP-1), soluble platelet (sP-selectin) and soluble CD40 ligand (CD40L).[19] Specifically, Ox-LDL is thought to play a crucial role in the inflammatory response in the arterial vessel wall.[20] It is of particular interest as a potential biomarker because lipid oxidation is an important aspect in the metabolism of reactive oxygen species (ROS).[21] Research to date suggests that oxidative stress, through ROS, or via modification of LDL, plays an integral role in the initiation of atherosclerosis, its progression, and the resulting atherosclerotic events. [22]

Accordingly, we have tried to find appropriate plasma atherosclerotic biomarkers in T2DM patients with ASO to evaluate the effectiveness of Cilostazol and Probucol, along with their combination, because these drugs have anti-oxidative activities.[3,17] The previous *in vitro* study with cultured human coronary artery endothelial cells,[23] *in vivo* studies in rats and in LDL receptor-deficient mice revealed the synergetic effect of Cilostazol plus Probucol against oxidative stress and atherosclerotic lesions.[24,25] Therefore, these results suggested possible synergetic effects of these drugs when the two are in combination in patients with T2DM and ASO. Therefore, the aim of this study was to find the plasma atherosclerotic biomarkers which could reflect the synergetic effect of Cilostazol and Probucol in a 12-week treatment in T2DM patients with ASO. The safety of Cilostazol plus Probucol in combination was also assessed.

2 Methods

2.1 Patients

Patients aged 40–75 years with a diagnosis of T2DM and ASO were eligible for enrolment, and both inpatients and outpatients were eligible. A diagnosis of ASO included at least one of the following criteria: ankle brachial pressure index (ABI) < 1.0 (the normal range in China definite as 1.0 ≤ ABI ≤ 1.4, 0.9 ≤ ABI < 1.0 was recognized as a critical value) in the past year, a significantly weakened popliteal or dorsalis pedis artery pulse or a difference in left- and right-sided pulse, intermittent claudication, diagnosed as ASO, or the presence of atherosclerotic plaque in a lower limb within the previous year. A diagnosis of diabetes was followed by the Chinese T2DM guidelines (2007) at the study. In the Chinese Guideline, the DM diagnosis criteria of WHO 1999 were adopted, which is defined as fasting blood glucose (FBG) ≥ 7.0 mmol/L, or two hour postprandial blood glucose ≥ 11.1 mmol/L, and hemoglobin A1c (HbA1c) > 6.5 % by HPLC (Bio-Rad D10/Bio-Rad Variant 2, Bio-rad Laboratories, Inc. Hercules, CA).

Exclusion criteria included a known allergy to either of the study drugs, the use of anti-platelet, or anti-coagulant agents (except aspirin), or any lipid-lowering agent (except statins), DM other than type 2, hemorrhagic disease, pre-existing cardiovascular conditions, pregnancy, abnormal hepatic or renal function, severe pre-existing conditions, and any other condition(s) which, in a physician's opinion, would deem the subject unable to participate in the study. This study was conducted according to the 'Declaration of Helsinki', and approved by the Institutional Review Board of every participating institution with signed informed consent by the patient before enrolment. This trial is registered with ClinicalTrials.gov, NO.: NCT00823849.

2.2 Study design

This randomized, controlled, open-label, 12-week clinical study was conducted at five sites in China, from October 2008 until August 2009. Two weeks after an initial screening visit, eligible patients were randomized to one of four treatment

groups (n = 50 patients per group): Group A received 'Conventional' therapy; Group B: the 'Cilostazol group', received conventional therapy and Cilostazol; Group C: the 'Probucol group', received conventional therapy and Probucol; and Group D: the 'combination therapy group', received conventional therapy and Cilostazol and Probucol. 'Conventional therapy' was defined as basic therapeutic regimens prescribed by the doctor, according to the patient's condition. Patients were treated for 12 weeks.

Cilostazol was initially dosed at 50 mg (one tablet, orally) twice daily (after breakfast and dinner). After one week treatment, if patients had no significant study drug-related discomfort, the dose could be titrated to 100 mg (two tablets) twice daily. Probucol was administered orally at 250 mg (one tablet) twice daily (after breakfast and dinner).

2.3 Efficacy endpoints and safety assessments

The primary endpoint was shown as the mean change from baseline to week 12 in atherosclerosis-related biomarker indices. Candidate biomarkers were chosen for investigation based on evidence of their involvement in various areas of the pathobiology of atherothrombosis, including inflammation, hemostasis, thrombosis and oxidative stress.[3,11] The biomarker indices used were: (1) Lipid Index: total cholesterol (TC), TG, LDL-C, HDL-C, and apolipoprotein B (apoB) (Roche Diagnostics, Penzberg, Germany); (2) Oxidation Index: Ox-LDL (Mercodia, Uppsala, Sweden); (3) sICAM-1, sVCAM-1 (R&D Systems, Minneapolis, MN), MCP-1 (LINCO Research, Inc., St. Charles, MO) and Von Willebrand Factor (vWF) (Instrumentation Laboratory, Lexington, MA); (4) sP-selectin, CD40 ligand and thrombomodulin (TM) (R&D Systems, Minneapolis, MN); and (5) Inflammation index: high-sensitivity C-Reactive Protein (hs-CRP) and interleukin 6 (IL-6) (Siemens Healthcare Diagnostics, Deerfield, IL).

Blood samples were taken from a peripheral vein under fasting condition at morning as baseline before administrating the study drug and after 12 weeks of treatment. The plasma or serum samples were separated at the sites. All the samples were shipped to central lab of Quintiles (Quintiles Medical Research and Development Co., Ltd., Beijing, China) under dry ice conditions on the same day of sample collection.

Safety related endpoints included the incidence of adverse events, clinically relevant abnormal laboratory results (before and after treatment), vital signs, physical evaluation of QTc intervals examination, and 12-lead electrocardiograph (ECG) investigations. Adverse events were grouped according to their relationship to the study drug(s), and stratified by disease severity (mild, moderate, or severe). QTc interval data was evaluated by the same cardiology ECG specialist.

2.4 Statistical analysis

According to the intent-to-treat principle, all subjects who were randomized and received at least one dose of the study drug(s) and who had at least one post-treatment evaluation were included in the full analysis set (FAS). The per-protocol (PP) data set included all patients in the FAS without a major protocol violation. This study is an exploratory study to explore atherosclerosis-related biomarkers in patients with atherosclerotic cerebral infarction. To evaluate the relationship between the treatment of investigational drugs and the change of biomarkers, efficacy analysis performed on the PP data set are reported in this paper.

Differences between the means and proportions of the baseline clinical characteristics and biomarkers were compared using analysis of variance, Cochran-Mantel-Haenszel test, respectively.

The difference of biomarkers between baseline and after 12 weeks treatment in each group was analysed using Student's t-test.

The difference between each group was analysed using Student's t-test or Wilcoxon test, depending on their distribution. To evaluate any exploratory relationship, no multiplicity adjustment was done.

Safety analyses were performed on the safety data set. This included all patients who had received at least one dose of the randomized study drug (s) and later discontinued.

For Bazett QTc interval (QTcB) and Fridericia QTc interval (QTcF), differences between groups were analyzed using Student's t-test.

3 Results

3.1 Patient disposition and baseline characteristics

A total of 200 patients were randomly assigned in the study: 50 subjects in each group. Overall, 178 of 200 patients (89%) completed the study through week 12. 47, 37, 43 and 38 in Group A, B, C and D, respectively. The major reason for discontinuation among the study group was treatment-emergent adverse events. Of the 22 patients who discontinued from the study, one withdrew informed consent, 15 experienced adverse events leading to study withdrawal, three missed a visit, and three withdrew for other reasons.

A summary of patient baseline demographics and clinical characteristics is presented in Table 1. There were no significant differences among the treatment groups in baseline efficacy parameters or demographics, except for age, which was lower in the combination therapy group. As the basal marker of T2D in this study, HbA1c levels were not different among the four groups. Patient adherence to the study protocol was similar across treatment groups.

Table 1. Baseline characteristics of study patients according to treatment group in the per-protocol (PP) set (*n* = 165).

Baseline characteristics	Conventional Group A (*n* = 47)	Cilostazol Group B (*n* = 37)	Probucol Group C (*n* = 43)	Combination Group D (*n* = 38)
Male, *n* (%)	29 (61.7)	22 (59.5)	31 (72.1)	22 (57.9)
Age, yrs	64 ± 8	63 ± 6	63 ± 8	59 ± 8
Body weight, kg	68 ± 12	70 ± 11	73 ± 10	71 ± 11
Height, cm	166 ± 8	164 ± 8	166 ± 7	166 ± 9
Body mass index, kg/m^2	25 ± 3	26 ± 3	26 ± 3	26 ± 4
Race, *n* (%)				
Han Chinese	46 (97.9)	32 (76.2)	41 (83.7)	35 (83.3)
Other Chinese	1 (2.1)	5 (11.9)	2 (4.1)	3 (7.1)
Heart rate, beats/minute	73 ± 8	73 ± 9	71 ± 7	73 ± 8
Seated systolic blood pressure, mmHg	128 ± 13	131 ± 13	127 ± 13	128 ± 16
Seated diastolic blood pressure, mmHg	75 ± 8	76 ± 8	77 ± 8	77 ± 8
HbA1c (%)	7.3 ± 1.4	7.4 ± 1.4	7.4 ± 1.5	7.3 ± 1.2
Medical history, *n* (%)				
Coronary Heart Disease	8 (17.0)	7 (18.9)	8 (18.6)	4 (10.5)
Stroke	3 (6.4)	5 (13.5)	9 (20.9)	4 (10.5)
Hyperlipidemia	29 (61.7)	27 (73.0)	28 (65.1)	24 (63.2)
Hypertension	26 (55.3)	28 (75.7)	31 (72.1)	24 (63.2)
Atherosclerosis-related biomarkers				
TC, mmol/L	4.69 ± 1.07	4.84 ± 0.89	4.68 ± 0.96	4.50 ± 0.87
TG, mmol/L	1.49 ± 0.95	1.45 ± 0.93	1.51 ± 0.84	1.61 ± 1.13
LDL-C, mmol/L	2.93 ± 0.89	2.93 ± 0.81	2.86 ± 0.83	2.62 ± 0.79
Ox-LDL, U/L	47.7 ± 15.3	47.1 ± 16.5	48.7 ± 16.6	46.6 ± 14.3
HDL-C, mmol/L	1.23 ± 0.32	1.32 ± 0.37	1.19 ± 0.30	1.22 ± 0.30
apoB, g/L	0.87 ± 0.24	0.86 ± 0.20	0.86 ± 0.23	0.81 ± 0.21
sICAM-1, μg/mL	190.4 ± 64.7	206.8 ± 55.0	187.3 ± 59.5	196.1 ± 74.0
sVCAM-1, ng/mL	831 ± 218	792 ± 250	848 ± 232	855 ± 337
MCP-1, ng/mL	122 ± 37	124 ± 29	139 ± 46	133 ± 72
sP-selectin, ng/mL	75.8 ± 21.1	71.4 ± 19.5	72.7 ± 23.4	71.3 ± 18.2
sCD40L, pg/L	5997 ± 2940	5720 ± 3193	5742 ± 3354	5297 ± 3382
TM, ng/mL	3.68 ± 1.28	3.98 ± 1.37	4.01 ± 1.63	3.55 ± 1.19
IL-6, ng/L	3.74 ± 2.93	3.57 ± 1.92	4.50 ± 6.43	2.94 ± 1.83

HbA1c: hemoglobin A1c; IL-6: interleukin 6; MCP-1: monocyte chemotactic protein-1; Ox-LDL: oxidized LDL; sCD40L: soluble CD40 ligand; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1; TC: total cholesterol. TG: triglyceride; TM: thrombomodulin.

3.2 Primary endpoint data

The results of primary endpoint biomarkers for the four groups are shown in Table 2 and in Figure 1 & 2.

3.3 Lipid index

Overall, there were significant differences among treatment groups in the change in TC ($P < 0.001$), TG ($P < 0.012$), LDL-C ($P = 0.002$), and HDL-C ($P < 0.001$) levels from baseline to week 12 in the PP data set. Differences among groups trended towards significant for the change in apoB levels from baseline to week 12 ($P = 0.052$).

Regression analysis showed the difference in the least squares (LS) mean reduction in TC was significant between the conventional therapy and Probucol group ($P < 0.001$), the conventional therapy and combination therapy group ($P < 0.001$) and between the Cilostazol and the combination therapy group ($P < 0.001$).

Figure 1 shows the mean differences between the

Table 2. Biomarker levels at baseline and 12 weeks in the per protocol set according to treatment group.

	Conventional (47)		Cilostazol (*n* = 37)		Probucol (43)		Combination (*n* = 38)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
TC, mmol/L	4.69 ± 1.07	4.95 ± 1.07	4.84 ± 0.89	4.97 ± 1.12	4.68 ± 0.96	4.01 ± 1.02*	4.50 ± 0.87	3.96 ± 0.75*
TG, mmol/L	1.49 ± 0.95	1.65 ± 1.18*	1.45 ± 0.93	1.20 ± 0.69	1.51 ± 0.84	1.45 ± 0.91	1.61 ± 1.13	1.27 ± 0.95*
LDL-C, mmol/L	2.93 ± 0.89	2.97 ± 0.93	2.93 ± 0.81	2.97 ± 0.92	2.86 ± 0.83	2.49 ± 0.82*	2.62 ± 0.79	2.41 ± 0.68
Ox-LDL, U/L	47.7 ± 15.3	50.2 ± 14.0	47.1 ± 16.5	49.1 ± 18.5	48.7 ± 16.6	49.1 ± 16.3	46.6 ± 14.3	42.2 ± 15.2
HDL-C, mmol/L	1.23 ± 0.32	1.25 ± 0.35	1.32 ± 0.37	1.47 ± 0.44*	1.19 ± 0.30	0.91 ± 0.30*	1.22 ± 0.30	0.96 ± 0.25*
apoB, g/L	0.87 ± 0.24	0.86 ± 0.24	0.86 ± 0.20	0.81 ± 0.25	0.86 ± 0.23	0.77 ± 0.21*	0.81 ± 0.21	0.73 ± 0.19*
sICAM-1, ug/L	190 ± 65	203 ± 67*	207 ± 55	217 ± 58	187 ± 60	198 ± 64*	196 ± 74	212 ± 73*
sVCAM-1, ng/mL	831 ± 218	877 ± 188	792 ± 250	872 ± 225*	848 ± 232	981 ± 272*	855 ± 337	959 ± 382*
MCP-1, pg/mL	122 ± 37	116 ± 38	124 ± 29	116 ± 39	139 ± 46	122 ± 40*	133 ± 72	118 ± 46
sP-selectin, ng/mL	75.8 ± 21.1	70.6 ± 16.9*	71.4 ± 19.5	67.3 ± 25.0	72.7 ± 23	63.1 ± 19.0*	71.3 ± 18.2	60.6 ± 20.6*
sCD40L, ng/L	5997 ± 2940	6249 ± 3351	5721 ± 3193	7183 ± 3761*	5743 ± 3354	6074 ± 3935	5297 ± 3382	6135 ± 2538
Thrombomodulin ng/mL	3.68 ± 1.28	4.62 ± 2.60*	3.98 ± 1.37	4.06 ± 1.57	4.01 ± 1.63	4.94 ± 1.74*	3.55 ± 1.19	4.68 ± 2.45*
IL-6, ng/L	3.74 ± 2.93	3.74 ± 5.14	3.57 ± 1.92	3.86 ± 2.73	4.50 ± 6.43	3.66 ± 3.96	2.94 ± 1.83	2.73 ± 1.11

*P < 0.05 Intragroup comparison (compare pretreatment with after 12 weeks treatment). Pre means base line and Post means at 12 week. apoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin 6; LDL-C: low-density lipoprotein cholesterol; MCP-1: monocyte chemotactic protein-1; Ox-LDL: oxidized LDL; sCD40L: soluble CD40 ligand; sICAM-1: soluble intercellular adhesion molecule-1; sP-selectin: soluble platelet selectins; VCAM-1: soluble vascular cell adhesion molecule-1; TC: total cholesterol; TG: triglyceride.

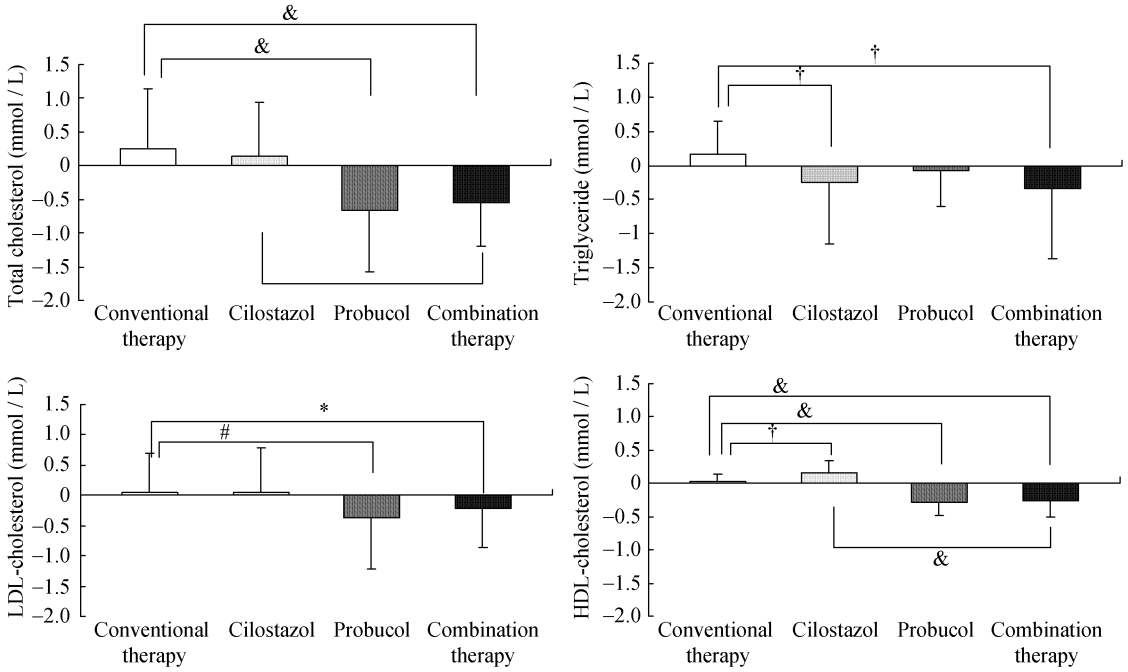

Figure 1. Changes of lipid profiles from baseline to 12 weeks in the per-protocol (PP) set in the four study groups. Data are expressed as the mean ± SD. *P < 0.1, #P < 0.05, †P < 0.01, &P < 0.001. HDL: high-density lipoprotein; LDL: low-density lipoprotein.

conventional therapy and the study groups. A significant difference in the LS mean reduction in LDL-C was observed between the conventional therapy and Probucol group (P = 0.01), and was very close to significant for the conventional therapy and combination therapy comparison (P = 0.052). The mean change in LDL-C from baseline to week 12 was 0.05 ± 0.64, 0.04 ± 0.74, −0.37 ± 0.84 and −0.21 ± 0.65 mmol/L for groups A, B, C and D, respectively. Importantly, Cilostazol therapy showed a significant increase in HDL-C versus conventional therapy (P = 0.002). Furthermore, the decrease in HDL-C observed with Probucol (–0.29 ± 0.19 mmol/L) was slightly offset by the addition of Cilostazol (–0.26 ± 0.24

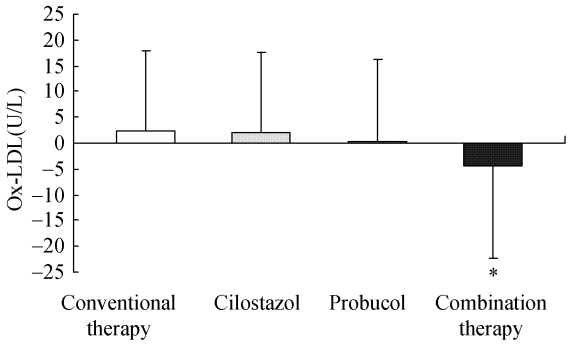

Figure 2. Changes of Ox-LDL from baseline to 12 weeks in the per-protocol set in the four study groups. Data are expressed as mean ± SD. *: The difference in the LS mean change from baseline to week 12 was close to significant for combination therapy *vs.* conventional therapy, $P = 0.065$. Ox-LDL: oxidized LDL.

mmol/L, no significance difference) as shown in the preclinical study.[25] Multiple comparisons showed the LS mean change from baseline in HDL-C was significant between both Probucol and combination therapy compared with conventional therapy ($P < 0.001$ for both), and for Cilostazol versus conventional therapy ($P < 0.001$).

The LS mean reduction in TG levels was significantly different between conventional and combination therapy groups ($P = 0.018$), and conventional therapy and Cilostazol groups ($P = 0.037$). No difference in TG level changing was observed with combination therapy versus either Cilostazol or Probucol, or conventional therapy versus Probucol. The LS mean difference in apoB levels from baseline to week 12 was close to significant for the comparison between the conventional therapy and combination therapy groups (LS mean difference 0.096, 95% confidence interval (95% CI): –0.001–0.193, $P = 0.054$). There were significant differences in the change in apoB levels between the convention therapy and Probucol alone and the combination therapy groups as shown in LDL-C.

3.4 Oxidation index

Overall, after 12 weeks of treatment, the difference in the mean change from baseline in Ox-LDL trended towards significance in the PP data set ($P = 0.075$). Furthermore, the difference in the LS mean change from baseline in Ox-LDL was close to significant for the combination therapy (Group D) after 12 weeks of treatment versus the conventional therapy (Group A; treatment difference, LS mean is 7.489 U/L; $P = 0.065$) after 12 weeks of treatment. The LS mean change in Ox-LDL from baseline to week 12 in Groups A, B, C and D was 2.47 ± 15.54, 2.05 ± 15.61, 0.37 ± 15.83 and 4.47 ± 17.84 U/L, respectively (Figure 2).

3.5 Endothelium, fibrinolysis and inflamation index

There were no significant differences among the treatment groups in the change of endothelium index from baseline to week 12 in sICAM-1, sVCAM-1, MCP-1 in the PP data set, and no significant differences were observed between the treatment groups after multiple logistic regression analysis (Table 2). Also the change from baseline to week 12 in vWF levels was not consistent and could not compare across all four treatment groups in the PP set.

There were no significant differences among the treatment groups in the change of fibrinolysis index from baseline in sP-selectin, CD40L and TM from baseline to week 12 in the PP data set (Table 2). Similarly, no significant differences were observed in the between group multiple logistic regression analyses.

There were no significant differences among the treatment groups in the change in IL-6 of the inflammation index from baseline to week 12 in the PP data set (Table 2). Similarly, no significant differences in hsCRP were observed in the between group multiple logistic regression analyses.

3.6 Safety data

The overall treatment-emergent adverse event (TEAE) rate was 59.6%. A total of 26%, 77%, 60% and 76% of patients experienced a TEAE in Group A, B, C and D, respectively. All adverse events were mild-to-moderate in nature, with the exception of one severe adverse event (both blood glucose fluctuations) in the conventional therapy and Probucol group, respectively. Adverse drug reactions were reported in 56.3%, 26.0% and 60.0% of patients in the Cilostazol, Probucol and combination therapy groups, respectively, with an overall adverse drug reaction rate of 35.4%. The type and incidence of adverse events were generally the same across all four treatment groups. There were six serious adverse events in five patients during the study: one with conventional therapy (acute myocardial infarction), two with Cilostazol (fever and abdominal pain), one with Probucol (blood glucose fluctuation), and two in the combination therapy group (pancreatic cancer and lumbar spinal canal stenosis). None were deemed related to the study drugs. A number of clinically significant abnormalities in laboratory test parameters were reported; these occurred rarely and were mostly mild in nature. Twenty-four adverse events in 15 patients (7.6 %; 11 adverse events each in the Group B and D and one each in the Group A and C) led to withdrawal from the study. There were no deaths during the study.

The mean QTc interval at baseline and week 12 for the 160 evaluable readings is shown in Table 3. In the Probucol and combination therapy groups, a slight prolongation of the

Table 3. Mean QTc interval at baseline and week 12 in each of the four treatment groups.

QTc, ms	Conventional Group A	Cilostazol Group B	Probucol Group C	Combination Group D
n	43	36	41	40
QTc at baseline	417 ± 21	407 ± 17	419 ± 21	422 ± 23
QTc at week 12	414 ± 20	404 ± 24	426 ± 24	430 ± 22
Mean change from baseline	–3	–3	7	8

Data from 160 patients was evaluable for QTc analysis, and are presented as mean ± SD.

QTc interval by 7 ms and 8 ms, respectively, was observed at week 12. A change in the QTc interval of ≥ 30 ms was observed in 1, 0, 2 and 1 patient, in Group A, B, C, and D, respectively.[26]

4 Discussion

The changes of plasma atherosclerotic biomarkers at combination treatment with Cilostazol plus Probucol in patients with T2D and ASO were consistent with similar results of preclinical data previously reported.[23–25] Preclinical studies showed the low concentrations of Cilostazol and Probucol act synergistically to significantly reduce oxidative stress marker such as NAD(P)H-dependent $O_2^{\cdot-}$ production in human coronary artery endothelial cells.[23] Also in a rat model of cerebral ischemic infarct and in LDL receptor-deficient mice,[24,25] low doses of concurrent Cilostazol and Probucol showed beneficial synergetic effects when compared with those of either agent alone.

The potential of Ox-LDL as a biomarker for oxidation index and lipid parameters in atherosclerotic disease patients have been shown in this study. After 12-weeks treatment, a trend towards significance was observed for the difference between the conventional and the combination treatment group for the change in Ox-LDL ($P = 0.065$). Namely, combination treatment appeared to have a synergetic effect on Ox-LDL reduction, with a decrease from baseline of –4.47 ± 17.84 U/L for the combination treatment versus 0.37 ± 15.83 and 2.05 ± 15.61 with Probucol and Cilostazol, respectively. However Probucol alone, as noted, was less.

TC, TG, and LDL-C were all significantly improved from baseline after 12-weeks treatment with Probucol plus Cilostazol ($P < 0.001$, $P = 0.012$ and $P = 0.002$, respectively). Probucol alone was significantly more effective than conventional therapy in reducing TC ($P < 0.001$) and LDL-C ($P < 0.010$) after 12-weeks treatment; however, a significant reduction in HDL-C was also seen with Probucol *vs.* conventional therapy as previously reported,[3–6] and no significant difference was observed between the two groups in terms of reducing TG. Cilostazol was effective in increasing HDL-C ($P = 0.002$) and reducing TG ($P = 0.037$) compared with conventional therapy, and was the only treatment group to be associated with a significant increase in HDL-C, as previously reported.[13] Importantly, the addition of Cilostazol to Probucol slightly reversed the decrease in HDL-C produced by the latter agent in the preclinical study.[25]

The majority of other atherosclerosis biomarkers for inflammation and oxidative stress tested in this study (sICAM, sVCAM, MCP, sP-selectin, CD40L, TM, IL-6) showed no significant differences among the treatment groups in changes from baseline to week 12. Additionally, although vWF and hs-CRP were also assessed for validity as biomarkers of atherosclerosis in this study, the results are not reported in tables due to large inter- and intra-patient variation. Therefore those biomarkers are not appropriate biomarkers for the evaluation of the effect of Probucol plus Cilostazol combination therapy.

Ox-LDL has been found in the plasma of patients with CAD, and is thought that it may play a vital role in the generation of inflammatory processes involved in atherosclerosis.[20–22] A prospective, nested, clinical study in apparently healthy middle-aged men, demonstrated that elevated levels of Ox-LDL were predictive of future CAD events. Ox-LDL was investigated in 88 men with incident CAD and 258 age- and survey-matched controls. Baseline mean plasma Ox-LDL concentrations were significantly higher in subjects who subsequently experienced a CAD event compared with controls ($P < 0.001$). Furthermore, the hazard ratio for a future CHD event was 4.25 (95% CI: 2.09–8.63, $P < 0.001$). Elevated plasma Ox-LDL was the strongest predictor of CHD events compared with a conventional lipoprotein profile and other traditional risk factors for CAD.[27]

Several clinical studies have demonstrated that statin therapy, including atorvastatin, simvastatin, pravastatin, and fluvastatin, lower Ox-LDL levels.[28] Importantly, the increased incidence of coronary in-stent restenosis in T2DM was shown to be significantly related to elevated serum malondialdehyde-modified LDL (MDA-LDL) levels, a kind of Ox-LDL.[29]

These results reveal the possibility that increased Ox-LDL levels in plasma may reflect the presence of vulnerable atherosclerotic plaques on arterial vessel walls. Vulnerable plaques may shed Ox-LDL or MDA-LDL from the atherosclerotic lesion as biomarkers, which may be associated with the risk of plaque raptures. [30]

In this study of patients with T2DM and ASO, most adverse events were mild or moderate with the study drugs generally well tolerated and all adverse reactions were as expected; no new adverse events were reported. The type, severity and incidence of adverse events were similar across the combination treatment, Cilostazol, and Probucol group. These results are similar to those demonstrated in previous studies of Probucol and Cilostazol.[31–34]

Double-blind, randomized trial–the Synergistic Effect of Combination Therapy with Cilostazol and Probucol on Plaque Stabilization and Lesion REgression (SECURE) study–is investigating the mechanisms underlying the prevention of atherosclerosis progression by Cilostazol and Probucol.[35] A clinical study[36] has also demonstrated that combined Probucol/Cilostazol therapy is safe and effective in the prevention of acute post-stent complications, including chronic restenosis. Rates of restenosis were significantly lower with Cilostazol or Probucol monotherapy, or both agents combined ($P < 0.05$, all *vs*. control).

Potential limitations of this current study include the small sample size ($n = 200$) that was randomized across four study arms, and the short duration of observation. These trial limitations may account for the lack of statistically significant differences between the conventional therapy arm and the drug treatment groups in terms of the changes from baseline observed in some of the investigated biomarkers.

To further confirm the preliminary positive results observed in this study, larger trials of longer duration are required to fully investigate the effects of Cilostazol plus Probucol on potential biomarkers of atherosclerosis, particularly Ox-LDL and lipid parameters, and the impact of these changes on disease diagnosis and long-term clinical outcomes.

In conclusion, the plasma biomarkers after 12-week treatment with Cilostazol, Probucol, or a combination of the two agents in patients with T2DM and ASO were compared. The differences in the mean change from baseline to 12 weeks in the levels of atherosclerosis-related biomarkers were shown to be close to significant in Ox-LDL, LDL-C, TC, TG, and HDL-C. The effect of Cilostazol plus Probucol on Ox-LDL confirmed the preclinical data and suggests Ox-LDL to be a potential biomarker for the detection of synergetic effect of two agents in atherosclerotic disease. Therapies were generally well tolerated and no new serious adverse events occurred in patients receiving combination treatment compared with either agent with conventional therapy alone.

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