Probucol in Albuminuric Type 2 Diabetes Mellitus Patients on Renin–Angiotensin System Blockade

A 16-Week, Randomized, Double-Blind, Placebo-Controlled Trial

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Objective—To determine the effect of probucol on urine albumin excretion in type 2 diabetes mellitus patients with albuminuria using angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

Approach and Results—This was a 16-week, phase II, randomized, placebo-controlled, parallel-group study in type 2 diabetes mellitus patients with a urinary albumin/creatinine ratio of ≥300 mg/g using angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, conducted in 17 tertiary referral hospitals. Eligible patients were randomized to probucol 250 mg/d (n=44), probucol 500 mg/d (n=41), and placebo (n=41) groups in a ratio of 1:1:1 after block randomization procedures, keeping the treatment assignment blinded to the investigators, patients, and study assistants. The primary end point was change in the geometric mean of urinary albumin/creatinine ratio from baseline to week 16 (ClinicalTrials.gov identifier NCT01726816). The study was started on November 8, 2012, and completed on March 24, 2014. The least squares mean change±SE from baseline in urinary albumin/creatinine ratio at week 16 was −7.2±639.5 mg/g in the probucol 250 mg/d group (n=43; P=0.2077 versus placebo group), 9.3±587.4 mg/g in the probucol 500 mg/d group (n=40; P=0.1975 versus placebo group), and 259.0±969.1 mg/g in the placebo group (n=41). Although the majority of subjects were on statins, probucol treatment significantly lowered total cholesterol and low-density lipoprotein cholesterol levels. QT prolongation occurred in one and two subjects in control and probucol 250 mg/d groups, respectively.

Conclusions—Four months of probucol up to 500 mg/d failed to reduce urinary albumin excretion. (Arterioscler Thromb Vase Biol. 2016;36:2108-2114. DOI: 10.1161/ATVBAHA.116.308034.)

Key Words: albuminuria • antioxidants • diabetic nephropathies • probucol • type 2 diabetes mellitus

Despite the proven efficacy of renin–angiotensin–aldosterone system blockade, there is still a strong clinical need for additional therapy to reduce the residual risk for progression of diabetic kidney disease. Although multiple novel agents exerting vascular protective factors and antifibrotic, antioxidant, anti-inflammatory, and intracellular metabolic modulatory properties have been developed, none of these novel agents have shown clear efficacy for preventing or delaying diabetic kidney disease. Among the multiple mechanisms involving the chronic complication of hyperglycemia, oxidative stress has been suggested as one of the key players because it promotes protein kinase C and mitogen-activated protein kinase activation and the formation of advanced glycation end products. In streptozotocin-induced diabetic rats, lipid peroxidation products, 8-hydroxydeoxyguanosine, and compensatory enhancement of antioxidative enzymes is found in the kidney. Induction of the antioxidant enzyme heme oxygenase-1, which is completely normalized.

Received on: March 25, 2016; final version accepted on: July 25, 2016.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.308034/-/DC1.

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Arterioscler Thromb Vase Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.116.308034

2108
by antioxidants such as vitamin E and probucol, has also been observed in the glomeruli of rats with diabetes mellitus. In normoalbuminuric and microalbuminuric patients with type 2 diabetes mellitus, probucol significantly decreases the urinary type IV collagen excretion rate.

Such evidence has prompted clinical trials to prove the clinical benefit of probucol in diabetic kidney disease. In a single-center, open-label study, probucol decelerated the increase in the extent of proteinuria and delayed the decline in glomerular filtration rate in patients with baseline serum creatinine ≥2 mg/dL (n=40). A larger scale (n=162), single-center, open-label study showed that probucol treatment delayed the initiation of chronic dialysis and renal dysfunction-related death in diabetes mellitus patients with urinary albumin excretion of >300 mg/g creatinine, with no significant difference in the extent of proteinuria between groups. However, only a minority (<30%) of the study subjects in these clinical trials used 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins), and use of angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II type I receptor antagonist (ARBs) was not included in the eligibility criteria. Therefore, the benefit of probucol in patients with diabetic kidney disease has not been examined by a multicenter, double-blind, placebo-controlled trial in a population in which most subjects use both renin–angiotensin–aldosterone system blockade and statins.

This study aimed to determine the effect of probucol on urine albumin excretion in type 2 diabetes mellitus patients with albuminuria using ACEIs or ARBs.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

Among the eligible patients (n=126), 112 completed the study up to week 16 (placebo group, n=40; probucol 250 mg/d group, n=38; probucol 500 mg/d group, n=34; Figure 1). Demographics and baseline characteristics were well balanced between the groups (Table). The proportion of subjects with glycated hemoglobin <7.0% (53 mmol/mol) was numerically

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**Figure 1.** Patient disposition. *Excluded by investigator decision because of hyperkalemia (n=3), abnormal level of creatine phosphokinase (n=1), abnormal platelet count (n=1), and lost to follow-up before randomization (n=1). UACR indicates urinary albumin/creatinine ratio.
The mean changes from baseline in the geometric mean of urinary albumin/creatinine ratio (UACR) from baseline to week 16 were 259.0±969.1 (least squares mean±SE, 259.8±116.4), −7.2±639.5 (least squares mean±SE, 4.8±113.9), and 9.3±587.4 (least squares mean±SE, −4.5±118.1) mg/g for placebo, probucol 250 mg/d, and probucol 500 mg/d groups, respectively (Figure 2). The difference (least squares mean±SE) from the placebo group was −255.0±162.8 mg/g for the probucol 250 mg/d group and −264.3±165.8 mg/g for the probucol 500 mg/d group (P=0.2077 for probucol 250 mg/d, and P=0.1975 for probucol 500 mg/d groups). There was no difference in changes from baseline in urinary protein-to-creatinine ratio, urinary transferrin (not shown), urinary fibronectin (not shown), serum creatinine, estimated glomerular filtration rate, or cystatin-C levels among the groups.

The total cholesterol, low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol levels of the probucol 250 mg and 500 mg/d groups during the study period were lower than those of the placebo group (all were P<0.001 for both probucol 250 and 500 mg/d groups; Figure 3). Triglyceride (P=0.8704 for probucol 250 mg/d group and P=0.8819 for probucol 500 mg/d group) and oxidized LDL-C (P=0.5411 for probucol 250 mg/d group and P=0.2700 for probucol 500 mg/d group) levels were not different between groups (Figure 3).

No differences in primary and secondary outcomes between groups were observed in subjects who did not use statins (Figure II in the online-only Data Supplement). No differences in primary and secondary outcomes between groups were observed in the prespecified subgroup analyses according to statin use, baseline UACR, estimated glomerular filtration rate, serum creatinine, sex, and glycated hemoglobin (not shown). No differences in primary or secondary outcomes between the groups were observed when the diabetes mellitus duration was included as a covariate in the ANCOVA models as an exploratory analysis (not shown).

All 126 patients who participated in the study were included in the safety assessment, and 58 (46%) reported ≥1 adverse event (AE). The between-group differences were statistically insignificant for AEs and adverse drug reactions. AEs related to probucol occurred in 8 subjects in total, comprising 8 events. By treatment group, these events occurred in 4 subjects (9.09%; 4 events) in the probucol 250 mg/d group and 4 subjects (9.76%; 4 events) in the probucol 500 mg/d group. QT prolongation in ECG, which occurred in 2 subjects in probucol 250 mg/d group (4.55%; 2 events) and none in probucol 500 mg/d group was the most frequent adverse drug reaction in the probucol groups. Other adverse drug reactions developed in the probucol groups were “enteritis,” “rash,” “diarrhea,” “nausea,” and “dizziness.” AEs causing death did not occur in this study and additional serious AEs occurred in 8 subjects (6.35%), comprising 16 events, and all were judged unrelated to the IPs (Table I in the online-only Data Supplement).
The plasma concentration of probucol was obtained in 34 subjects in the probucol 250 mg/d group and 29 subjects in the probucol 500 mg/d group. After repeated oral administration of probucol for 16 weeks, the mean plasma probucol concentration at week 16 was 18,979±8291 ng/mL in the probucol 250 mg/d group and 34,232±17,150 ng/mL in the probucol 500 mg/d group (coefficient of variation, 0.44 for 250 mg/d group and 0.58 for 500 mg/d group; median [range], 16,130 [8,662–41,480] for 250 mg/d group and 33,520 [8,851–75,370] for 500 mg/d group). There was no significant correlation between the plasma concentration of probucol at week 16 and the primary end point ($r = 0.162$, $P = 0.361$ in probucol 250 mg/d group; $r = 0.071$, $P = 0.714$ in probucol 500 mg/d group).

Discussion
To the best of our knowledge, this was the first multicenter, double-blind, randomized, placebo-controlled trial to examine the efficacy and safety of probucol in patients with diabetic kidney disease in which the majority of the subjects used statins and all subjects used ACEIs/ARBs. Although lower levels of total and LDL-C were achieved in subjects allocated to the probucol 250 and 500 mg/d groups, there
no difference in the rate of progression of albuminuria between groups.

The results of this multicenter, double-blind, placebo-controlled study are in contrast with previous open-label studies in which probucol suppressed the progression of proteinuria and decline in renal function. In addition to the open-label study design of the previous studies, the different proportions of subjects who used ACEIs/ARBs could explain the discrepancy. In a previous study, 17.6% of subjects in probucol group and 21.6% in the control group used ACEIs/ARBs, and a significant reduction in urinary protein excretion in the probucol group was observed. No significant change in UACR was observed in another study, in which 86% of the subjects in the probucol group and 83% in the control group used ACEIs/ARBs. Because all subjects in the current study used ACEIs/ARBs with no change in dose for at least 3 months before screening, the use of ACEIs/ARBs could have masked the benefit of probucol in terms of UACR. The study period of 4 months in the current study could have been too short to evaluate the long-term benefits of probucol, such as the delay in chronic hemodialysis therapy initiation seen in previous studies.

Figure 3. Effect of probucol treatment on lipid and oxidation parameters. A, Total cholesterol (mg/dL). B, LDL-cholesterol (mg/dL). C, HDL-cholesterol (mg/dL). D, Triglyceride (mg/dL). E, Oxidized LDL (U/L). Error bars represent standard deviation. *P < 0.05; ***P < 0.001. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.
superior efficacy of probucol plus telmisartan to that of telmisartan alone in terms of proteinuria extent. In the study by Zhu et al.,12 a fixed dosage of telmisartan (80 mg once daily) was given with or without probucol after a 4-week run-in period during which a fixed dosage of telmisartan (80 mg once daily) was given and other ACEIs/ARBs were stopped if they were used before the study period. Therefore, the study was not designed to examine the additional benefit of probucol when added to the optimized dosage of ACEIs/ARBs, and the proportion of subjects who used ACEIs/ARBs before the run-in period was not provided.12

In the current study, probucol did not decrease the oxidized LDL level (Figure 3). However, this finding does not preclude an antioxidative effect of probucol because it has been suggested that the protective effect of probucol depends not on its ability to inhibit lipid oxidation, but on its ability to induce the stress-induced anti-inflammatory enzyme heme oxygenase-1.13,14 Alternatively, suboptimal concentrations in this study might have affected the observed lack of difference in oxidized LDL-C level. In a recent study that showed the superior efficacy of probucol plus telmisartan to that of telmisartan alone in terms of proteinuria extent, the dosage of probucol was 1000 mg/d (500 mg twice daily) for the first 24 weeks and then 500 mg/d (250 mg twice daily) for an additional 24 weeks.15 The protective effect of probucol in contrast-induced acute kidney injury has also been reported15–17 presumably because of the antioxidative effect of probucol. The dosage of probucol in these studies, 1000 mg orally before the procedure and then 500 mg twice daily for 3 days, was much higher than that of the current study.

In the current study, 86 of 126 (68%) subjects used statins, whereas a minority (14%–26%) of study subjects in the previous studies used statins.10,11 However, a significant additional LDL-C–lowering effect of probucol was observed in this study. As probucol upregulates the fractional catabolic rate of LDL-C via an LDL receptor–independent pathway and increasing bile acid synthesis,18,19 its effect on LDL-C lowering might be complementary to that of statins. Indeed, a recent animal study showed that a combination of probucol and atorvastatin produced a significantly greater reduction in area of the atherosclerotic lesion in high fat–fed rabbits than that of atorvastatin monotherapy.20 To date, however, it is unclear whether the combination of probucol and statins has additional lipid-lowering and antiatherosclerotic effects compared with statin monotherapy in diabetic kidney disease because of the limited clinical use of probucol after the introduction of statins. Nevertheless, the additional LDL-C–lowering effect of probucol observed in the current study is noteworthy given the inadequate power of this study.

Administration of probucol up to 500 mg/d was well tolerated. All adverse drug reactions resolved after appropriate treatment and were predictable. Producibol may be safely administered in patients with diabetic nephropathy, with a monitoring of QT prolongation by conducting a periodic ECG.

Several limitations of this study should be discussed. First, the sample size was calculated based on a previous randomized, open-label study, which is supposed to have overestimated the cause and effect relationship because of potential biases underlying the open-label design.10 Therefore, the current study might be underpowered to demonstrate the benefit of probucol in albuminuric type 2 diabetes mellitus patients. Second, the proportion of patients on statins was also greater than those in previous studies. In fact, the benefit of statins and their antioxidative/anti-inflammatory effects in diabetic nephropathy has been well-demonstrated.15,22,23 Therefore, the results of this study do not preclude the benefit of probucol in statin-intolerant or statin-naïve patients with diabetic kidney disease, and the sample size calculation based on the previous studies including limited proportions of statin-using patients also support the inadequate power of this study.

In conclusion, treatment with probucol up to 500 mg/d for 4 months failed to reduce urinary albumin in type 2 diabetes mellitus patients on ACEI/ARBs with a baseline UACR of >300 mg/g. This regimen neither changed the rate of decline in renal function nor suppressed levels of oxidized LDL although it did significantly lower total and LDL-cholesterol levels. In type 2 diabetes mellitus patients with diabetic nephropathy using ACEI/ARBs and statins, demonstration of a protective effect on the nephropathy may require adequately powered further study with longer-term treatment using a higher dosage sufficient to exert antioxidative effects.

Sources of Funding
This study was supported by Korea Otsuka Pharmaceuticals, Seoul, Republic of Korea.

Disclosures
None.

References


**Highlights**

- This was the first multicenter, double-blind, randomized, placebo-controlled trial to examine the efficacy and safety of probucol in patients with diabetic kidney disease in which the majority of the subjects used statins and all subjects used angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

- Although lower levels of total and low-density lipoprotein-cholesterol were achieved in subjects allocated to the probucol 250 and 500 mg/d groups, there was no difference in the rate of progression of albuminuria between groups.

- The results of this study indicate that demonstration of protective effect of probucol as an add-on therapy to statins on the diabetic nephropathy may require adequately powered further study with longer-term treatment of a higher dosage sufficient for antioxidative effect.
Probucol in Albuminuric Type 2 Diabetes Mellitus Patients on Renin–Angiotensin System Blockade: A 16-Week, Randomized, Double-Blind, Placebo-Controlled Trial
Sang-Man Jin, Kyung Ah Han, Jae Myung Yu, Tae Seo Sohn, Sung Hee Choi, Choon Hee Chung, Ie Byung Park, Eun Jung Rhee, Sei Hyun Baik, Tae Sun Park, In-Kyu Lee, Seung-Hyun Ko, You-Cheol Hwang, Bong Soo Cha, Hyoung Woo Lee, Moon-Suk Nam and Moon-Kyu Lee

Arterioscler Thromb Vasc Biol. 2016;36:2108-2114; originally published online August 4, 2016;
doi: 10.1161/ATVBAHA.116.308034
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/10/2108

Data Supplement (unedited) at:
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Albuminuric type 2 diabetes patients on renin-angiotensin system blockade (n=126)
✓ 86 (69%) used statins

1:1:1 Block randomization (blinded to the investigators, patients, and study assistants)

- Placebo for 16 weeks (n = 41)
- Probucol 250mg/day for 16 weeks (n = 43)
- Probucol 500mg/day for 16 weeks (n = 43)

✓ No difference in primary outcome (change in the geometric mean of UACR from baseline to Week 16)
✓ Significantly lower total and low-density lipoprotein cholesterol levels in probucol 250mg/d and 500mg/d groups
Supplementary Appendix

Supplementary Table I. Summary of adverse events (safety population)

<table>
<thead>
<tr>
<th></th>
<th>Placebo (N=41)</th>
<th>Probucol 250 mg/day (N=44)</th>
<th>Probucol 500 mg/day (N=41)</th>
<th>Total (N=126)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AEs</td>
<td>16/41 (39.02%)</td>
<td>23/44 (52.27%)</td>
<td>19/41 (46.34%)</td>
<td>58/126 (46.03%)</td>
</tr>
<tr>
<td>Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>16 (39.02%)</td>
<td>20 (45.45%)</td>
<td>17 (41.46%)</td>
<td>53 (42.06%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>2 (4.88%)</td>
<td>4 (9.09%)</td>
<td>4 (9.76%)</td>
<td>10 (7.94%)</td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
<td>2 (4.55%)</td>
<td>1 (2.44%)</td>
<td>3 (2.38%)</td>
</tr>
<tr>
<td>Severe AEs</td>
<td>1 (2.44%)</td>
<td>3 (6.82%)</td>
<td>4 (9.76%)</td>
<td>8 (6.35%)</td>
</tr>
<tr>
<td>Adverse events leading to discontinuation</td>
<td>1 (2.44%)</td>
<td>2 (4.55%)</td>
<td>6 (14.63%)</td>
<td>9 (7.14%)</td>
</tr>
<tr>
<td>Adverse drug reactions</td>
<td>1 (2.44%)</td>
<td>4 (9.09%)</td>
<td>4 (9.76%)</td>
<td>9 (7.14%)</td>
</tr>
</tbody>
</table>

AE, adverse events
### Supplementary Table II. Changes in eligibility criteria by protocol amendments

<table>
<thead>
<tr>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inclusion Criteria:</strong></td>
<td><strong>Inclusion Criteria:</strong></td>
</tr>
<tr>
<td>The subject is male or female diagnosed with type 2 diabetes mellitus (duration of prevalence more than 5 years) and must be aged 20 to 75 years at the time of screening visit</td>
<td>The subject is male or female diagnosed with type 2 diabetes mellitus and must be aged ≥20 and &lt;80 years at the time of screening visit</td>
</tr>
<tr>
<td>15 mL/min/1.73m² ≤ eGFR ≤ 50 mL/min/1.73m²</td>
<td>15 mL/min/1.73m² ≤ eGFR &lt; 90 mL/min/1.73m²</td>
</tr>
<tr>
<td></td>
<td>• Reasons: The criteria for chronic kidney disease (CKD) of Japanese Diabetes Association is different from that of the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) guideline. To secure future application of the results to the Japanese population, the inclusion criteria was changed to stage 3A/3B CKD in Japanese criteria.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac damage (abnormal levels of Troponin I or Troponin T)</td>
<td>Cardiac damage (abnormal levels of Troponin I)</td>
</tr>
<tr>
<td></td>
<td>• Reasons: Consideration of the high false positive rate (27 to 78%) of Troponin T in patients with decreased renal function</td>
</tr>
<tr>
<td>HbA1c &lt;6.5% or HbA1c &gt; 9%</td>
<td>HbA1c &gt; 9%</td>
</tr>
<tr>
<td></td>
<td>• Reasons: Although exclusion of severe hyperglycemia is reasonable, the exclusion of cases with HbA1c &lt;6.5% is not reasonable because it would restrict the extrapolation of the results to considerable proportion of the adequately-controlled type 2 diabetes.</td>
</tr>
</tbody>
</table>
Supplementary Table III. Analytical conditions of high-performance liquid chromatography

<table>
<thead>
<tr>
<th>Column</th>
<th>CAPCELL PAK C18 MGII 3 μm, 2.0 mm × 50 mm (SHISEIDO, Tokyo, Japan)</th>
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<tbody>
<tr>
<td>Guard column</td>
<td>GUARD CARTRIDGE CAPCELL C18 MGII 5 μm, 2.0 mm × 10 mm (SHISEIDO)</td>
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<td>Column temperature</td>
<td>40°C</td>
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<tr>
<td>Mobile phase A</td>
<td>10 mmol/L ammonium hydrogencarbonate</td>
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<tr>
<td>Mobile phase B</td>
<td>Acetonitrile</td>
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<tr>
<td>Mobile phase C</td>
<td>Ethanol / water (90:10, v/v)</td>
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<tr>
<td>Flow rate (mobile phase C)</td>
<td>0.5 mL/min</td>
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<tr>
<td>Gradient condition</td>
<td>Time (min)</td>
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<td>0.10</td>
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<td>17.60</td>
<td>90.0</td>
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<td>19.50</td>
<td>90.0</td>
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<td>20.00</td>
<td>90.0</td>
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<td>Linear gradient</td>
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<tr>
<td>Autosampler temperature</td>
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<tr>
<td>Needle wash solvent 1</td>
<td>Ethanol / water (90:10, v/v)</td>
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<tr>
<td>Needle wash solvent 2</td>
<td>Acetonitrile / water (80:20, v/v)</td>
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<tr>
<td>Using syringe type</td>
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<tr>
<td>Injection volume</td>
<td>5 μL (1st Injection and 2nd Injection)</td>
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<tr>
<td>Run time</td>
<td>20.5 min</td>
</tr>
<tr>
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<td>Divert Time (min)</td>
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<tr>
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<td>0.0</td>
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### Supplementary Table IV. Analytical conditions of mass spectrometry

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<th>Ionization mode</th>
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<td>Multiple reaction monitoring</td>
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<tr>
<td>Polarity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Period 1 : 0.00 – 10.00 min</td>
</tr>
<tr>
<td></td>
<td>Period 2 : 10.00 – 20.50 min</td>
</tr>
<tr>
<td>Collision gas</td>
<td>N₂, 6 (Number of valves opened)</td>
</tr>
<tr>
<td>Curtain gas</td>
<td>N₂, 10 psi</td>
</tr>
<tr>
<td>Ion source gas 1</td>
<td>Air, 45 psi</td>
</tr>
<tr>
<td>Nebulizer current</td>
<td>-5μA</td>
</tr>
<tr>
<td>Temperature</td>
<td>350°C</td>
</tr>
<tr>
<td>Channel Electron Multiplier</td>
<td>1500 to 3000 V</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monitoring ions</th>
<th>Period</th>
<th>Analyte</th>
<th>Monitoring ion</th>
<th>Dwell time (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period 1</td>
<td>OPC-13213* and OPC-13217*</td>
<td>m/z 384 → m/z 162</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>OPC-13015*</td>
<td>Cilostazol*</td>
<td>m/z 368 → m/z 161</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>OPC-13112 (internal standard)</td>
<td>OPC-13015*</td>
<td>m/z 366 → m/z 159</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>OPC-13112 (internal standard)</td>
<td>OPC-13217*</td>
<td>m/z 382 → m/z 161</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Period 2</td>
<td>Probufol OPC-13112 (internal standard)</td>
<td>m/z 515 → m/z 236</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>OPC-13112 (internal standard)</td>
<td>OPC-13217*</td>
<td>m/z 382 → m/z 161</td>
<td>100</td>
</tr>
</tbody>
</table>

*OPC-13015, OPC-13213 and OPC-13217 are cilostazol’s metabolites (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Cilostazol and its metabolites was monitored but was not analyzed.
### Supplementary Table V. Determination of probucol concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calibration curve range</strong></td>
<td>10.00 to 5000 ng/mL in human plasma (LLOQ, 10.00 ng/ml)</td>
</tr>
<tr>
<td><strong>Method of quantity determination</strong></td>
<td>Internal standard method</td>
</tr>
<tr>
<td><strong>Calibration curve regression</strong></td>
<td>Using least square linear regression analysis, $Y = aX + b$ (weighting = $1/X^2$)</td>
</tr>
<tr>
<td></td>
<td>$Y$: Analyte peak area ratio against the internal standard</td>
</tr>
<tr>
<td></td>
<td>$X$: Nominal concentration</td>
</tr>
<tr>
<td></td>
<td>Excluding BS and S0 from calibration curve regression</td>
</tr>
<tr>
<td><strong>r, slope, and intercept</strong></td>
<td>• $r$: expression to four significant figures</td>
</tr>
<tr>
<td></td>
<td>• slope and intercept: expression to three significant figures</td>
</tr>
<tr>
<td><strong>Calculation software for calibration curve regression</strong></td>
<td>“Analyst Version 1.5” which is adjunct to the mass spectrometer (AB SCIEX, Foster City, CA, USA)</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>Percentage of Accuracy (RE) is expressed to one decimal place after rounding off.</td>
</tr>
<tr>
<td></td>
<td>$RE = \frac{(determined \ value^* - nominal \ concentration)}{nominal \ concentration} \times 100$</td>
</tr>
<tr>
<td></td>
<td>$^*$: or back-calculation value of standard sample for calibration curve</td>
</tr>
<tr>
<td><strong>Calibration curve criteria</strong></td>
<td>• $r \geq 0.9800$</td>
</tr>
<tr>
<td></td>
<td>• RE: At least 75.0% of the standard samples for the calibration curve, including LLOQ and ULOQ, except for the blank and 0 ng/mL samples should meet the criteria that the RE should be within $\pm 15.0%$ ($\pm 20.0%$ at LLOQ) after points of which REs are not within $\pm 15.0%$ are excluded from the calibration curve regression; in this study, at least 6 out of the 8 points of the calibration curve should meet the criteria.</td>
</tr>
<tr>
<td><strong>Quality control (QC) samples criteria</strong></td>
<td>The RE of at least four out of the six QC samples should be within $\pm 15.0%$ of their nominal concentrations. Two out of the six QC samples may be outside $\pm 15.0%$, but not both at the same concentration.</td>
</tr>
</tbody>
</table>

LLOQ, lower limit of quantification, ULOQ, upper limit of quantification
Supplementary Figure I. Summary of the study procedure.
Supplementary Figure II. Effect of probucol treatment on proteinuria and renal function in subjects who did not use statins. (A) Urinary albumin-creatinine ratio (UACR, mg/g). (B) Change from baseline in UACR levels (Full analysis set population). (C) Urinary protein-creatinine ratio (mg/g). (D) Serum creatinine (mg/dL). (E) Estimated glomerular filtration rate (eGFR, ml/min/1.73 m²). (F) Cystatin C (mg/L). Error bars represent standard deviation.
Materials and Methods

Study Design
This was a phase II, multi-center, double-blind, double-dummy, randomized, placebo-controlled, parallel group study conducted in 17 tertiary referral hospitals in the Republic of Korea. Every patient who participated in this study provided written informed consent. The study was approved by the independent institutional review boards of each study center and was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Participants
Patients were screened for eligibility on Visit 1 (Week -4). The included subjects met all of the following criteria: 1) type 2 diabetes outpatient ≥ 20 years and < 80 years of age at screening; 2) urinary albumin/creatinine ratio (UACR) ≥ 300 mg/g Cr at the time of screening; 3) administration of angiotensin converting enzyme inhibitor (ACEI) and/or angiotensin receptor blocker (ARB) with no change in dose for at least three months prior to screening; 4) administration of statins with no change in dose for at least three months prior to screening, or not taking statins at the time of screening with no plan to begin statins during the study period; 5) estimated glomerular filtration rate (eGFR) calculated by the Chronic Kidney Disease Epidemiology Collaboration formula of ≥ 15 mL/min/1.73 m² and < 90 mL/min/1.73 m² at screening; and 6) written consent to participate in the study before screening. Patients with either type 1 diabetes or gestational diabetes were excluded from the study. Patients who had a history of dialysis or renal transplantation were also not eligible. Other exclusion criteria included glycated hemoglobin (HbA1c) > 9% (75 mmol/mol) and/or systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 100 mmHg at screening, congestive heart failure, myocardial infarction or cerebral infarction within 6 months prior to screening, myocardial damage (an abnormal troponin I level), ventricular arrhythmia (multiple multifocal premature ventricular contraction), unconscious patient of unknown or cardiovascular cause, abnormally prolonged QT interval at screening (QT interval > 450 msec for male, and QT interval > 470 msec for female), hyperkalemia, urinary tract disease, renal artery stenosis, renal disease other than diabetic nephropathy, active hepatitis or hepatocirrhosis, a positive
pregnancy test at screening, and administration of probucol within 3 months prior to screening. Doppler ultrasound was performed for exclusion of renal artery stenosis at the screening visit. Several protocol amendments which include changes in eligibility criteria had been made during the study period. The details of these changes are summarized in the Supplementary Table II.

Randomization and Masking
Eligible subjects were randomized into the probucol 250 mg per day, probucol 500 mg per day, or placebo groups in a ratio of 1:1:1 following block randomization procedures via an Interactive Web Response System, which was stratified by institution. Block randomization was by a computer generated random number list prepared by an independent statistician with no clinical involvement in the trial. The randomization code was supplied to the Interactive Web Response System provider by an independent statistician following procedures prepared by the project statistician or designee. The monitors, central laboratory staff, the investigators, study pharmacists and study subjects were unaware of the treatment assignment.

The probucol and placebo were in white or near white, round, film coated tablet and identical in appearance. They were prepacked in bottles and consecutively numbered for each study participant according to the randomization schedule. Each study participant was assigned an order number and received the tablets in the corresponding prepacked bottle.

Treatment codes were not to be unblinded except for medical emergencies requiring knowledge of treatment assignment. Only the principal investigator could request unblinding with an explanation to the sponsor. If decision was made to unblind, the investigator informed the sponsor’s Pharmacovigilance Department using the immediate safety report template within three working days. The unblinding was documented in the subject’s medical chart along with the date and time of the unblinding and name of the investigator who carried out the unblinding.

Procedures
Once eligibility was confirmed, patients were randomized and treated with placebo (given twice daily in the morning and evening), probucol 250 mg per day (given as 125 mg twice daily in the morning and evening), or probucol 500 mg per day (given
as 250 mg twice daily in the morning and evening) from baseline to Week 16. Probucol (Lorelco™, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan; molecular formula, C$_{31}$H$_{48}$O$_2$S$_2$) and its placebo were provided by Korea Otsuka Pharmaceutical, Seoul, Republic of Korea. Efficacy and safety assessments were performed at baseline, Week 8 and Week 16, and at the last visit in cases of request for study discontinuation due to reasons such as safety issues. Plasma concentrations of probucol were determined at Week 16 (Figure S1). If the reason for withdrawal was an adverse event recognized as causally related to the investigational product (IP), the investigator was to ensure follow-up until the adverse event symptoms resolved or improved.

**Assessment**

The primary endpoint for the assessment of efficacy was change in the geometric mean of the two UACRs measured at Week 16 from the geometric mean of the two UACRs measured at baseline. Two first morning urines were used for urinalysis, and the mean of these two UACRs was used as a representative value. Secondary efficacy endpoints included change from baseline at Week 16 in the following variables: serum creatinine, eGFR, cystatin C, urinary albumin and protein-to-creatinine ratio, fasting lipid parameters (total cholesterol, triglyceride, low-density lipoprotein-cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C]), oxidized LDL, urinary fibronectin, urinary transferrin, fasting insulin and fasting C-peptide.

Planned subgroup analysis for primary and secondary endpoints was conducted for the pre-specified subgroups according to baseline UACR, eGFR, serum creatinine, sex, HbA1c, and statin use.

All adverse events (AEs) were recorded and their severity and causal relationship to the investigational products were monitored. Monitoring of vital signs as well as physical and medical examinations was performed at baseline, Week 8 and Week 16, and following request for study discontinuation due to reasons such as safety issues by the investigators. A telephone visit was made at 3 weeks after the last investigational product administration to obtain further safety information. A 12-lead electrocardiogram was measured at each visit after each subject rested in a lying
position for at least 10 minutes. Date and time of measurement, heart rate, RR interval, PR interval, QRS width, QT interval, QTc measurements, and normal or abnormal findings were recorded. The investigator or delegated person assessed the measured values and their changes, and recorded all abnormalities while paying special attention to the QTc interval.

**Laboratory Methods**

A central laboratory performed all the clinical biochemistry tests used to determine the primary and secondary efficacy endpoints at baseline, Week 8, and Week 16. The level of urinary albumin was determined using a turbidimetric immunoassay (Autokit Micro Albumin assay; Wako Pure Chemical Industry, Osaka, Japan) on the Hitachi 7600-010 analyzer (Hitachi, Tokyo, Japan). Intra- and inter-assay coefficients of variation were 0.77 and 1.50%, respectively. When a study participant was included in the study based on a local laboratory UACR ≥300 mg/g but did not meet the eligibility criteria of the central laboratory UACR at baseline, the patient was withdrawn from the study. The level of urinary protein was determined using Pyrogallol red method (Autokit Micro TP assay; Wako Pure Chemical Industry, Osaka, Japan; Intra- and inter-assay coefficients of variation, 0.8 to 1.8% and 1.2 to 1.6%) on the Hitachi 7600-010 analyzer (Hitachi). The level of urine and serum creatinine were determined using a kinetic colorimetric assay with the Jaffé method (Roche Diagnostics, Mannheim, Germany) on the Hitachi 7600-010 analyzer (Hitachi). Intra- and inter-assay coefficients of variation for serum creatinine were 0.6 to 0.7% and 1.5 to 2.3%, respectively. Intra- and inter-assay coefficients of variation for urine creatinine were 1.1 to 2.1% and 1.2 to 2.2%, respectively. The level of serum cystatin C was determined using the Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway; Intra- and inter-assay coefficients of variation, 1.29% and 2.34%) on the Hitachi 7600-010 analyzer (Hitachi). Fasting serum total cholesterol (Intra- and inter-assay coefficients of variation, 0.7 to 1.0% and 1.7 to 2.7%), triglycerides (Intra- and inter-assay coefficients of variation, 0.9 to 1.5% and 1.8 to 2.4%), LDL-C (Intra- and inter-assay coefficients of variation, 0.64 to 1.22% and 0.87 to 1.20%), and HDL-C (Intra- and inter-assay coefficients of variation, 0.60 to 0.95% and 1.1 to 1.8%) were measured using an enzymatic colorimetric method.
The plasma oxidized LDL (OxLDL) level was measured by the Mercodia Oxidized LDL ELISA kit (Mercodia AB, Uppsala, Sweden), according to the manufacturer’s protocol. This was a solid phase two-site enzyme immunoassay, based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. The coefficient of variation of the assay was 7.4 to 8.3%, and the assay was performed on a SpectraMax 190 analyzer (Molecule Devices, Sunnyvale, CA, USA). Urinary fibronectin was measured by an enzyme immunoassay (Takara Bio, Shiga, Japan; Intra- and inter-assay coefficients of variation, 3.9 to 7.1% and 3.1 to 6.0%), on a SpectraMax 190 analyzer (Molecule Devices, Sunnyvale, CA, USA). Urinary transferrin was measured by a turbidimetric immunoassay (Nittobo Medical, Tokyo, Japan; coefficients of variation, <5.0%), on a Hitachi 7600-010 analyzer (Hitachi). Fasting insulin was measured by an immunoassay (ADVIA Centaur Insulin IRI, Siemens Medical Solutions Diagnostics; Intra- and inter-assay coefficients of variation, 1.5 to 2.1% and 6.1 to 6.5%). C-peptide was measured by a chemiluminescence assay (Immulite 2000, Siemens Medical Solutions Diagnostics, LA, USA; Intra- and inter-assay coefficients of variation, 2.33 to 3.06% and 3.29 to 5.15%).

Plasma probucol concentration was determined by a liquid chromatograph-tandem mass spectrometer/mass spectrometer method at Week 16. Blood samples were collected in heparin–coated tubes. After centrifugation, the plasma was immediately stored in polypropylene tubes at –20°C until shipped for analysis. Bioanalytic assays were performed at Mitsubishi Chemical Medience Corporation, Tokyo, Japan. Plasma concentrations of probucol were quantitated using a validated HPLC method1 (Agilent 1200 HPLC system, Agilent Technologies, Santa Clara, CA, USA) with a L-6000 pump (Hitachi), coupled with tandem mass spectrometry (API 4000Q TRAP, AB SCIEX, Foster City, CA, USA). OPC-13112 (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan; Lot No. DS1828-139) was used as the internal standard for analytes. Plasma with internal standard was deproteinated in acetonitrile. A CAPCELL PAK C18 MG II column (2.0 × 50 mm, 3.0 μm; Shiseido Co., Ltd., Tokyo, Japan) was used with a gradient mobile phase (A: 10 mmol/L ammonium hydrogen carbonate; B: acetonitrile; C: ethanol/water [90:10 v/v]). The tandem mass spectrometry system was operated with atmospheric pressure chemical ionization.
Probucol concentration was determined by internal standard method. The lower limit of quantification was 10 ng/mL. The linear range for probucol was 10 to 5,000 ng/mL. The detailed chromatographic condition, mass spectrometer settings, and calculation method of probucol concentration are provided in the Supplementary Table III-V.

**Statistical Analysis**

Efficacy analysis was performed on the full analysis set (FAS), which included all subjects who received at least one dose of the study drug and had a post-baseline efficacy assessment available. The analysis of the primary efficacy endpoint was based on an analysis of covariance (ANCOVA) model in which the treatment group was designated as an effect and the baseline value as a covariate. The UACR values were log-transformed because they did not meet the assumption of normality of residuals. Secondary end points were analyzed in the FAS. The change in secondary efficacy endpoints was based on an ANCOVA model in which the treatment group was designated as an effect, and the baseline value as a covariate.

Pre-specified subgroup analysis of the primary efficacy endpoint was performed according to the stage of chronic kidney disease (CKD) based on eGFR calculated by the Chronic Kidney Disease Epidemiology Collaboration formula in the FAS population (CKD stage 2, 60 ≤ eGFR < 90 mL/min per 1.73 m² body surface area; CKD stage 3, 30 ≤ eGFR < 60 mL/min per 1.73 m² body surface area; CKD stage 4, 15 ≤ eGFR < 30 mL/min per 1.73 m² body surface area). Subgroup analysis was also performed according to baseline UACR, serum creatinine, sex, HbA1c, and use of statins. In subgroup analyses, treatment-by-factor interactions were evaluated and descriptive statistics were presented by treatment group for each factor.

Safety parameters were analyzed in the safety population set, which consisted of patients who were enrolled and received at least one dose of the IP. In case of an administration error, the analysis was performed based on the drug that was actually administered. The plasma concentration of probucol was determined at Week 16. Cases were removed from the analysis of the plasma concentration of probucol based on the following criteria: (1) Probucol concentration was “NS (no sample)” and “NA (not analyzed)”; (2) IP compliance was less than 80% (overall compliance). Correlation between plasma concentration of probucol and the primary endpoint was assessed by Spearman correlation analysis in probucol 250mg per day group and
Pearson correlation analysis in probucol 500mg per day group.

PASS software was used to calculate sample size. Based on a previous study\(^2\), the change in UACR was assumed to be +0.042 for the placebo group and -0.005 for both the probucol 250 mg per day and 500 mg per day groups, and the within-group standard deviation of the change in urinary protein was assumed to be 0.06 for all three treatment groups. The null hypothesis was that mean change in UACR was not different between the placebo, probucol 250 mg per day, and probucol 500 mg per day groups. The alternative hypothesis was that there was a difference in the mean change in UACR between any of the groups, with the placebo group as the smallest and the probucol 500mg per day group as the largest. Under the hypotheses above, with a 5% significance level (\(\alpha\)), 90% power (1-\(\beta\)), and 1:1:1 allocation to each group, at least 32 subjects were required for each group. Considering a 20% drop-out rate and protocol violations, 40 subjects per group for a total of 120 subjects were to be enrolled. Eligible subjects were randomized with stratification by center, using an interactive web response system. This trial is registered with ClinicalTrials.gov, number NCT 01726816.

**Role of Funding Source**
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.
References
