Sac-1004, a Pseudo-Sugar Derivative of Cholesterol, Restores Erectile Function through Reconstruction of Nonleaky and Functional Cavernous Angiogenesis in the Streptozotocin Induced Diabetic Mouse

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Purpose: We examined whether and how Sac-1004, a vascular leakage blocker, would restore erectile function in an animal model of diabetic erectile dysfunction.

Materials and Methods: Eight-week-old C57BL/6J mice were used. Diabetes was induced by intraperitoneal injection of streptozotocin. Eight weeks after diabetes induction the animals were divided into 6 groups, including controls, diabetic mice that received repeat intracavernous injections of phosphate buffered saline (20 μl) on days −3 and 0, and diabetic mice that received repeat intracavernous injections of Sac-1004 on days −3 and 0 (1, 2, 5 and 10 μg, respectively, in 20 μl phosphate buffered saline). One week after injection erectile function was measured by cavernous nerve stimulation. The penis was then harvested for histological examinations and Western blot analysis.

Results: Local delivery of Sac-1004 in the corpus cavernosum restored erectile function in diabetic mice. The highest erectile response was noted at a dose of 5 μg with a response comparable to that in the control group. Sac-1004 significantly increased cavernous endothelial and smooth muscle contents, and induced endothelial nitric oxide synthase phosphorylation (Ser1177). Sac-1004 decreased extravasation of oxidized low density lipoprotein by restoring endothelial cell-cell junction proteins and pericyte content. Sac-1004 also promoted tube formation in primary cultured mouse cavernous endothelial cells in vitro. Sac-1004 mediated cavernous angiogenesis and erectile function recovery was abolished by inhibiting angiopoietin-1-Tie2 signaling with soluble Tie2 antibody.

Abbreviations and Acronyms
Ang1 = angiopoietin-1
EC = endothelial cell-cell
ED = erectile dysfunction
eNOS = endothelial NO synthase
ICP = intracavernous pressure
LDL = low density lipoprotein
MCEC = mouse cavernous endothelial cell
MSBP = mean systolic blood pressure
NO = nitric oxide
PBS = phosphate buffered saline
PDE5 = phosphodiesterase-5
PECAM-1 = platelet/endothelial cell adhesion molecule-1
phospho- = phosphorylated
STZ = streptozotocin
Tie2 = tyrosine kinase with Ig and epidermal growth factor homology domain-2
VE = vascular endothelial
ZO-1 = zonular occludens-1

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Conclusions: With the effects of angiogenesis and antipermeability Sac-1004 reestablishes structural and functional cavernous sinusoids. This is highly promising for future treatment of erectile dysfunction from vascular causes.

Key Words: penis, erectile dysfunction, diabetes mellitus, Sac-1004, angiogenesis inducing agents

A variety of pathological conditions, including vascular risk factors or diseases, neurological abnormalities and hormonal disturbances, are known to be involved in the pathogenesis of ED. Diabetes mellitus is strongly associated with an increased risk of ED and about 50% to 75% of diabetic men experience ED. Although oral PDE5 inhibitors are generally effective and considered a first line treatment modality for ED, men with diabetic ED respond more poorly to these agents than nondiabetic ED patients. Diabetes induced severe endothelial dysfunction is responsible for poor responsiveness to PDE5 inhibitors. Because the action of PDE5 inhibitors relies on endogenous NO production, these agents may fail to increase the cGMP concentration if bioavailable NO does not reach the necessary threshold in cases of severe endothelial dysfunction from diabetes. Moreover PDE5 inhibitors are not cures for ED and they are used on demand prior to sexual intercourse, which greatly limits the spontaneity of the sexual act. Therefore new therapeutic approaches are necessary for diabetes associated ED.

Previous groups have reported functional and structural derangements in cavernous endothelium in animal models of diabetic ED. The generation of reactive oxygen species is regarded as an important cause of endothelial cell damage and reduced NO bioavailability in the diabetic condition. The EC junction, which serves as a barrier by regulating paracellular permeability, has a key role in vascular formation, networking and remodeling. Recently we reported an increase in cavernous endothelial permeability with a concurrent decrease in cavernous EC junction proteins in animal models of diabetes. These findings suggest that derangements in both cavernous endothelial cells and EC junctions are major pathophysiological mechanism involved in diabetic ED. Therefore restoration of the functional and structural integrity of the cavernous endothelium may be a promising therapeutic strategy for curing ED.

Sac-1004, a pseudo-sugar derivative of cholesterol known to block vascular leakage by enhancing endothelial integrity, has a protective effect on endothelial cells. Moreover Sac-1004 was reported to induce normalization of tumor blood vessels and increase perfusion.

In the current study we determined the effectiveness of Sac-1004 in promoting nonleaky and functional cavernous angiogenesis, and restoring erectile function in STZ induced diabetic mice in vivo. We also determined the angiogenic potential of Sac-1004 in primary cultured MCECs in vitro.

METHODS

Sac-1004 Compound Formulation
Sac-1004 was synthesized as previously described. Briefly pregnenolone was reacted with dihydropyran in the presence of p-toluenesulfonic acid to prepare the tetrahydropyran analogue. After Wittig olefination with 4-(carboxybutyl) triphenyl phosphonium bromide the acid moiety was methylated by trimethylsilyl-diazomethane. Sac-1004 was synthesized via tetrahydropyran deprotection and subsequent glycosidation with 4,6-di-O-acetyl-2,3-dideoxyhex-2-enopyran in the presence of acid (fig. 1). Stock solution of Sac-1004 (50 mg/ml) was prepared in dimethyl sulfoxide and dilutions were made in PBS.

Animals and Treatment
Eight-week-old C57BL/6 mice were used in this study. The experiments were approved by the Inha University institutional animal care and use subcommittee. Diabetes was induced by intraperitoneal injections of STZ (50 mg/kg) for 5 days consecutively as previously described. Eight weeks after the induction of diabetes the animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) intramuscularly, and the penis was exposed by sterile technique. A 30-gauge insulin syringe was used to administer repeat injections of PBS (20 µl on days −3 and 0) or Sac-1004 compound (1, 2, 5 and 10 µg, respectively, in 20 µl PBS on days −3 and 0) in the mid portion of the corpus cavernosum.

Figure 1. Sac-1004 structure
We compressed the penis at the base with a vascular clamp immediately before injection. The clamp remained in place for 30 minutes to restrict blood flow out of the penis.

One week after treatment we evaluated erectile function during electrical stimulation of the cavernous nerve in 6 mice per group. The highest erectile response was noted in diabetic mice that received Sac-1004 at the concentration of 0.25 mg/ml (5 µg in 20 µl PBS). Based on these functional results the mice were divided into the 3 groups for histological examination and Western blot analysis, including age matched control, diabetic mice that received repeat intracavernous injections of PBS (20 µl on days −3 and 0) and diabetic mice that received repeat intracavernous injections of Sac-1004 compound (5 µg in 20 µl PBS on days −3 and 0). Fasting and postprandial blood glucose levels were determined with an Accu-Chek® blood glucose meter before the mice were sacrificed.

**Physiological Erection and Inhibition Studies**

ICP and systemic blood pressure were measured during electrical stimulation of the cavernous nerve as previously described. For the inhibition study a separate group of diabetic mice was subcutaneously given soluble antibody to Tie2 (4 µg/20 µl, R&D Systems®), a receptor tyrosine kinase for Ang1. This was done to examine the role of Ang1 in Sac-1004 mediated enhancement in angiogenesis and improvement in erectile function. Soluble Tie2 was administered immediately before intracavernous injection of Sac-1004.

**Histological Examinations**

For fluorescence microscopy in 6 mice per group penile tissue was fixed in 4% paraformaldehyde for 24 hours at 4C. Frozen section tissues (8 µm) were incubated with antibodies to the endothelial cell marker PECAM-1 (Chemicon®, 1:50), FITC (fluorescein isothiocyanate) conjugated antibody to smooth muscle α-actin, a smooth muscle cell marker (Sigma®, 1:200), occludin (Invitrogen™, 1:50), oxidized LDL (Abcam®, 1:50), phospho-eNOS (Ser1177, Cell Signaling Technology, 1:200), Ang1 (NOVUS Biologicals, 1:200), ZO-1 (Zymed Laboratories, 1:200), occludin (Invitrogen, 1:1,000) or β-actin (Abcam, 1:6,000). Results were quantified by densitometry in 4 preparations per group.

**Tube Formation Assay**

MCECs were prepared and maintained as previously described. Cells at passages between 2 and 3 were used for experiments. Tube formation assay was performed using 48-well plates coated with 100 µl Matrigel® per well. MCECs were seeded on coated plates at 2 × 10^4 cells per well in complete Medium 199 (GIBCO®). Samples were divided into 3 groups, including MCECs exposed to a normal glucose condition (5 mmol), MCECs exposed to a high glucose condition (30 mmol) and treated with PBS, and MCECs exposed to a high glucose condition (30 mmol) and treated with Sac-1004 (0.1 µg/1 ml). The assay was performed in a CO₂ incubator and the plates were incubated at 37°C for 24 hours. Images were obtained with a phase contrast microscope. The number of tubes in each well of the plate was counted at a screen magnification of 40×. Only integrated tubes were counted.

**Statistical Analysis**

Results are expressed as the mean ± SE. Group comparisons of parametric data were made by 1-way ANOVA followed by the Newman-Keuls post hoc test. We used the Kruskal-Wallis test for nonparametric data. Statistical analysis was performed with SigmaStat® 3.5 software with p <5% considered significant.

**RESULTS**

**Metabolic Variables**

Body weight was significantly lower in diabetic mice than in control mice. Fasting and postprandial blood glucose concentrations were significantly higher in diabetic mice than in controls. No significant differences in body weight or blood glucose levels were found between the diabetic groups regardless of the treatment given (see table).

**Sac-1004 in Diabetic Mice**

**Restored Erectile Function.** Figure 2, A shows a representative intracavernous tracing after stimulation of the cavernous nerve (5 V and 12 Hz for 1 millisecond) for 1 minute in age matched control and diabetic mice 1 week after treatment. Ratios of maximal ICP and total ICP to MSBP were significantly lower in PBS treated diabetic mice than in age matched controls. Repeat intracavernous injections of Sac-1004 significantly improved erection parameters at the concentrations of 0.1 mg/ml (2 µg in 20 µl), 0.25 mg/ml (5 µg in 20 µl) and 0.5 mg/ml (10 µg in 20 µl). The highest erectile...
function recovery was observed at the concentration of 0.25 mg/ml (5 μg in 20 μl), which reached up to 96% of control values (fig. 2, B and C). No detectable differences were found in MSBP among the 6 experimental groups (see table). However erectile function recovery was not obvious 2 weeks after Sac-1004 administration (fig. 2, D to F).

**Restored Cavernous Endothelial and Smooth Muscle Contents.** Immunohistochemical staining of cavernous tissue with antibody to PECAM-1 or smooth muscle-α actin was performed in age matched control and diabetic mice 1 week after treatment. We found significantly lower cavernous endothelial and smooth muscle contents in PBS

<table>
<thead>
<tr>
<th>Physiological and metabolic parameters in 6 STZ induced diabetic mice per group</th>
<th>Mean ± SE Sac-1004 (mg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.05</td>
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<tr>
<td><strong>Body wt (gm)</strong></td>
<td>31.8 ± 0.43</td>
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<tr>
<td><strong>Glucose (mg/dl):</strong></td>
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<tr>
<td>Fasting</td>
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<tr>
<td>Postprandial</td>
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<tr>
<td><strong>Blood pressure (mm Hg):</strong></td>
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<tr>
<td>Mean</td>
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<tr>
<td>Diastolic</td>
<td>61.9 ± 1.4</td>
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*p <0.01 vs control.

**Figure 2.** Sac-1004 restored erectile function. A, representative ICP responses 1 week after treatment. Solid bar indicates 1-minute stimulus interval. B and C, ratio of mean maximal ICP and total ICP (AUC) to MSBP in 6 mice per group. DM, diabetes mellitus. Asterisk indicates p <0.01 vs control. Single pound sign indicates p <0.05 vs control and diabetic plus PBS. Double pound signs indicate p <0.05 vs control and diabetic plus PBS. D, representative ICP responses of 2 weeks after treatment. E and F, ratios of mean maximal ICP and total ICP to MSBP in 6 mice per group. Single asterisk indicates p <0.01 vs control.
treated diabetic mice than in control mice. Repeat intracavernous injections of Sac-1004 restored cavernous endothelial and smooth muscle contents in diabetic mice. However cavernous endothelial and smooth muscle contents returned to baseline 2 weeks after Sac-1004 administration (fig. 3, A to C).

Figure 3. Sac-1004 restored cavernous endothelial and smooth muscle contents. A, immunohistochemical staining of cavernous tissue with antibodies to PECAM-1 (red areas) and smooth muscle α-actin (green areas) 1 or 2 weeks after treatment. Reduced from ×200. B and C, quantitative analysis of endothelial and smooth muscle contents in 6 mice per group. DM, diabetes mellitus. B, asterisk indicates p < 0.05 vs control. Pound sign indicates p < 0.01 vs diabetic plus PBS. C, asterisk indicates p < 0.01 vs control. Pound sign indicates p < 0.05 vs diabetic plus PBS. D, tube formation assay in MCECs exposed to normal glucose (NG, 5 mmol) or high glucose (HG, 30 mmol) condition and treated with 1 ml PBS or 0.1 μg/ml Sac-1004. E, number of tubes per field at 40× screen magnification. Asterisk indicate p < 0.01 vs NG group. Pound sign indicates p < 0.01 vs high glucose plus PBS.
We further examined the angiogenic effect of Sac1004 in primary cultured MCECs. In vitro Matrigel assay revealed impairments in tube formation in MCECs exposed to high glucose conditions, which were completely restored by treatment with Sac1004 (0.1 μg/ml) (fig. 3, D and E).

**Figure 4.** Sac-1004 induced cavernous eNOS phosphorylation. A, immunohistochemical staining of cavernous tissue performed with antibodies to phospho-eNOS (P-eNOS) and eNOS 1 week after treatment. Reduced from ×200. B and C, quantitative analysis of phospho-eNOS or eNOS positive area in 6 mice per group. DM, diabetes mellitus. Pound sign indicates p < 0.01 vs diabetic plus PBS. B, asterisk indicates p < 0.05 vs control. C, Asterisk indicates p < 0.01 vs control. D, Western blot results were similar in 4 independent samples.
**Induced Cavernous eNOS Phosphorylation.** Cavernous phospho-eNOS or total eNOS expression as assessed by immunohistochemical staining and Western blot analysis was significantly lower in PBS treated diabetic mice than in age matched controls. Repeat intracavernous injections of Sac-1004 significantly increased cavernous phospho-eNOS and total eNOS expression in diabetic mice (fig. 4).

**Restored Cavernous EC Junction Proteins.** We performed immunohistochemical staining and Western blot to evaluate cavernous expression of adherens junction protein (VE-cadherin) and tight junction proteins (ZO-1 and occludin) in age matched control and diabetic mice 1 week after treatment. Cavernous expression of EC junction proteins was significantly lower in PBS treated diabetic mice than in age matched controls. Repeat intracavernous injections of Sac-1004 significantly restored EC junction proteins in the corpus cavernosum tissue of diabetic mice (fig. 5).

**Restored Cavernous Pericyte Content.** Pericytes are known to have a crucial role in vascular development and the regulation of vascular permeability. We performed immunohistochemical staining of cavernous tissue with antibody to NG2 in age matched control and diabetic mice 1 week after treatment. We observed a significantly lower NG2 positive cavernous pericyte content in PBS treated diabetic mice than in controls. Repeat intracavernous injections of Sac-1004 completely restored the cavernous pericyte content in diabetic mice (fig. 6).

**Decreased Cavernous Oxidized LDL Extravasation.** Immunohistochemical double staining of cavernous tissue with antibodies to PECAM-1 and oxidized LDL was performed in age matched control and diabetic mice 1 week after treatment. Extravasation of oxidized LDL was significantly higher in the corpus cavernosum tissue of PBS treated diabetic mice than in control mice. Repeat intracavernous injection of Sac-1004 significantly reduced cavernous oxidized LDL leakage in diabetic mice (fig. 7).

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**Figure 5.** Sac-1004 restored cavernous EC junction proteins. A, immunohistochemical staining of cavernous tissue with antibodies to occludin and VE-cadherin 1 week after treatment. Reduced from ×200. B and C, quantitative analysis of occludin and VE-cadherin positive area in 6 mice per group. DM, diabetes mellitus. Pound sign indicates p < 0.01 vs diabetic plus PBS. Asterisk indicates p < 0.05 vs control. C, asterisk indicates p < 0.01 vs control. D, Western blot. E to G, relative density of each protein compared to β-actin in 4 mice per group. E, asterisk indicates p < 0.05 vs control. Pound sign indicates p < 0.05 vs diabetic plus PBS. F and G, asterisk indicates p < 0.01 vs control. Pound sign indicates p < 0.01 vs diabetic plus PBS.
Sac-1004 Induced Cavernous Angiogenesis and Recovery of Erectile Function was Ang1-Tie2 Pathway Dependent

The Ang1-Tie2 signaling pathway has an important role in generating a nonleaky, stable and functional vasculature.22 Cavernous Ang1 protein expression was significantly lower in PBS treated diabetic mice than in control mice. The expression of Ang1 protein returned to control values after treatment with Sac-1004 (fig. 8, A and B).

Cavernous nerve mediated erection studies indicated that recovery of erectile function was attenuated after treatment with Sac-1004 in diabetic mice treated with soluble Tie2 (fig. 8, C to E). Moreover Sac-1004 induced restoration of cavernous endothelial cell and pericyte content. The subsequent decrease in extravasation of oxidized LDL was diminished by treatment with soluble Tie2 in diabetic mice (fig. 8, F and G).

DISCUSSION

The major findings of this study are that 2 successive injections of Sac-1004 in the corpus cavernosum of diabetic mice significantly increased cavernous endothelial and smooth muscle contents, and induced eNOS phosphorylation (Ser1177) and decreased extravasation of oxidized LDL by restoring EC junction proteins and pericyte content. These changes restored erectile function in diabetic mice. Sac-1004 mediated cavernous angiogenesis and erectile function recovery depended on the Ang1-Tie2 pathway. Sac-1004 also promoted tube formation in primary cultured MCECs in vitro. Figure 9 summarizes a proposed mechanism of action by which Sac-1004 restores erectile function.

In agreement with the findings of our previous studies in STZ induced mice15,23,24 we found a significant decrease in cavernous endothelial area in PBS treated diabetic mice compared with that in age matched controls. Intracavernous injection of Sac-1004 completely recovered cavernous endothelial content in diabetic mice. The presence of normal corporeal smooth muscle content is a prerequisite for a normal erectile response and a reduction in smooth muscle content leads to ED in the diabetic condition.8,9 In the current study intracavernous injection of Sac-1004 also restored cavernous smooth muscle content in diabetic mice.
although it did not reach the level in age matched controls.

EC junction proteins have an important role in regulating vascular permeability and derangements since these proteins are involved in a variety of diabetes related microangiopathies, such as diabetic retinopathy\textsuperscript{25} and diabetic ED\textsuperscript{15,16}. Similar to these findings cavernous expression of EC junction proteins was significantly decreased in the diabetic condition. Intracavernous injection of Sac-1004 restored cavernous EC junction proteins in diabetic mice.

A previous study showed that Sac-1004 blocks vascular leakage by enhancing endothelial integrity via the cAMP/Rac/cortactin pathway\textsuperscript{17}. Therefore we speculate that the cAMP/RAC/cortactin pathway may also be involved in Sac-1004 mediated restoration of endothelial junctional integrity in the diabetic penis. Furthermore pericytes, which were discovered as a population of contractile cells surrounding endothelial cells, are also known to be involved in the regulation of vascular or cavernous endothelial permeability\textsuperscript{20,21}. Pericyte loss or dropout and subsequent capillary leakage are the main features of diabetic retinopathy\textsuperscript{26}. Intracavernous injection of Sac-1004 significantly restored the NG2 positive cavernous pericyte content in diabetic mice so that it was comparable to levels in age matched controls.

In the current study the extravasation of oxidized LDL in the corpus cavernosum was significantly increased in diabetic mice. The vascular endothelium serves as a barrier to LDL in the physiological condition\textsuperscript{27}. Diabetes mellitus is known to promote LDL oxidation, which induces inflammatory responses, oxidative stress and endothelial cell apoptosis\textsuperscript{28}. It was also reported in patients with diabetic retinopathy that the severity of retinopathy is proportional to the extent of oxidized LDL leakage\textsuperscript{29}. Sac-1004 significantly decreased oxidized LDL leakage in the corpus cavernosum tissue of diabetic mice. We believe that the restoration of cavernous EC junction proteins and pericytes is responsible for the Sac-1004 mediated decrease in oxidized LDL extravasation.

NO is a major mediator of penile erection\textsuperscript{30}. In the current study eNOS phosphorylation (Ser1177) was significantly decreased in PBS treated diabetic

![Figure 7. Sac-1004 decreased cavernous oxidized LDL extravasation. A, immunoflourescent double staining of cavernous tissue with antibodies to oxidized LDL (green areas) and PECAM-1 (red areas) 1 week after treatment. Reduced from ×200. B, quantitative analysis of oxidized LDL positive area in 6 mice per group. Asterisk indicates p < 0.01 vs control. Pound sign indicates p < 0.01 vs diabetic plus PBS and control. DM, diabetes mellitus.](image-url)
mice compared with the control group. Increased reactive oxygen species generation is known to inactivate eNOS in the penis of STZ induced diabetic mice. Sac-1004 restored endogenous eNOS phosphorylation in diabetic mice. Regeneration of cavernous endothelial cell content and protection of endothelial cell damage by reducing oxidized LDL expression may account for the restoration of eNOS activity.

A lack of long-term efficacy of Sac-1004 to improve erectile function is a limitation of this study. Further studies are necessary to determine whether periodic injections or chronic administration of Sac-1004 as an oral formulation would induce long-lasting recovery of erectile function.

CONCLUSIONS
Intracavernous administration of Sac-1004 successfully restored erectile function in diabetic mice via healthy cavernous angiogenesis as evidenced by the restoration of cavernous endothelial cells and EC junction proteins, smooth muscle cells and pericytes as well as decreased vascular permeability. Reestablishment of functional and structural cavernous vasculature using the anti-permeability factor Sac-1004 may be a highly promising treatment modality for ED from vascular causes.
REFERENCES


