Site-Specific Targeting of Nanoparticle Prednisolone Reduces In-Stent Restenosis in a Rabbit Model of Established Atheroma

Michael Joner, Katsumi Morimoto, Hiroaki Kasukawa, Kristin Steigerwald, Sabine Merl, Gaku Nakazawa, Michael C. John, Aloke V. Finn, Eduardo Acampado, Frank D. Kolodgie, Herman K. Gold, Renu Virmani

**Objective**—TRM-484 is a novel drug consisting of nanoparticles of prednisolone with high affinity to chondroitin sulfate proteoglycans (CSPGs). This may allow for neointimal suppression via directed targeting to areas of injury at systemic concentrations low enough to avoid adverse side effects known to occur with oral delivery of steroids.

**Methods and Results**—Atherosclerotic New Zealand white Rabbits were implanted with bare metal stents and randomized to receive intravenous TRM-484 at doses of 1 mg/kg or 0.32 mg/kg starting at the day of stenting and continuing 3 times a week for the duration of the study. Control animals received empty liposomes (placebo) or saline infusion. Stented arterial segments were harvested at 42 days and processed for histomorphometry and immunohistochemistry. Tissue and plasma levels were determined along with confocal microscopic analysis to determine distribution of rhodamine-labeled TRM-484 at various time points. TRM-484 was exclusively observed at sites of stent-induced injury, with absence of drug in contralateral nonstented arteries. Tissue concentration of stented arteries exceeded that of contralateral nonstented arteries by 100-fold 24 hours after administration of 1 mg/kg TRM-484 and resulted in significant reduction of percent stenosis compared to saline and placebo treated rabbits (22.5±4.4 versus 31.0±8.4 and 29.5±8.1%, P<0.03).

**Conclusion**—TRM-484 at doses of 1 mg/kg resulted in significant suppression of in-stent neointimal growth in atherosclerotic rabbits. Site-specific targeting by this nanoparticle steroid in injured atherosclerotic areas might be a valuable and cost-effective approach for the prevention of in-stent restenosis. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

**Key Words:** stent ■ inflammation ■ steroids ■ atherosclerosis ■ restenosis

Systemic pharmacological treatment to prevent restenosis has been limited by the inability to yield high enough drug concentrations at the site of injury without increasing blood levels and therefore the risks of systemic toxicity. While local delivery of drugs via stent platform avoids this shortcoming, this approach may also be hampered by local toxicity from overdosage of antiproliferative agents and polymer-induced inflammatory responses, resulting in delayed vascular healing including reendothelialization and increased rates of late thrombotic events.1

Glucocorticoids might have advantageous effects on neointimal growth after vascular injury because they have anti-inflammatory effects on vascular cells but do not seem to affect reendothelialization of injured surfaces.2 Unfortunately, the oral doses needed to produce this effect also result in significant side effects.3–5 Increasing the affinity of steroids for injured surfaces may allow for systemic administration at doses low enough to avoid side effects but high local concentrations to prevent restenosis.

TRM-484 is prednisolone phosphate encapsulated in pegylated 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) liposomes which specifically binds to chondroitin sulfate proteoglycans (CSPGs) expressed within the subendothelial matrix but not vascular endothelial cells.6 The cationic lipid component of TRX-20 is subsequently endocytosed by cellular mechanisms resulting in directed drug release inside the cell. It has previously been shown that systemically delivered TRM-484 has high enough affinity for injured sites, to be delivered at doses far lower than that of free prednisolone phosphate, thereby minimizing the risk of systemic side-effects.7

The aim of this study was to demonstrate the efficacy of TRM-484 in reducing in-stent restenosis in a rabbit model of established atheroma and to demonstrate that site-specific steroid targeting to stented arteries was feasible.
Methods

This protocol was approved by the Institutional Animal Care and Use Committee of the Medstar Research Institute and conformed to the position of the American Heart Association on use of animals in research and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Synthesis of TRM-484

The preparation of the liposomes was described previously; however, we modified this method to produce the liposomes used in the present study. In short, TRM-484 encompasses a lipid mixture of HSPC, cholesterol, and TRX-20 at a molar ratio of 50:42:8 and is hydrated with prednisolone disodium phosphate (PSLP) to obtain an expected diameter of around 100 nm. For a detailed description of the synthesis, please see supplemental materials (available online at http://atvb.ahajournals.org).

Rabbit Model of Experimental Atherosclerosis

New Zealand white rabbits (3.0 to 4.0 kg), 3 to 4 months of age, were fed an atherogenic diet (1% cholesterol and 6% peanut oil, F4366-CHL, Bio-Serv Inc) for 1 week to induce hyperlipidemia. Balloon injury of iliac arteries was performed at 1 week, and animals were continued on the high cholesterol diet (1% cholesterol) for another 4 weeks as described previously and then switched to reduced cholesterol (containing 0.025% cholesterol) until euthanasia (total 10 weeks, 6 weeks after stent implantation). Blood was collected for measurement of serum cholesterol and CRP at 0, 7, 35, 63, 84, and 105 days. Animal weights were recorded on the day of stent deployment (day 63) and at day 70, 77, 91, 98, and 105 (euthanasia; Figure 1).

**Stent Placement, Drug Treatment, and Tissue Harvest**

Premounted Express 2 stents (3.0×16 mm, Boston Scientific Corporation) were deployed at their nominal pressure (9 to 11 ATM, 30-second balloon inflation) in each iliac artery under fluoroscopic guidance 63 days after study initiation (Figure 1). After stent implantation, angiography was performed to document vessel patency. All animals received aspirin 40 mg/d orally until euthanasia. In addition, heparin (150 IU/kg) was administered intraarterially before catheterization procedures. Animals were randomized to 4 different groups: group 1, TRM-484 at doses of 1 mg/kg (n = 7) and group 2, TRM-484 at doses of 0.32 mg/kg (n = 5). All drugs were administered intravenously through subcutaneously implanted port systems connected to the jugular vein for 42 days after stenting. Drug administration was started 2 hours after stent implantation and given 3 times per week until euthanasia. This particular dosing regimen was chosen after completion of a pilot study to evaluate the plasma half-life time of TRM-484 in serum of healthy rabbits. Forty-two days poststenting, animals were euthanized. The stented segments were perfusion-fixed, removed, and submitted for histopathologic and immunohistochemical evaluation as previously described. All sections were stained with hematoxylin-eosin, Movat pentachrome, and Carstairs stain. Sections were also stained with antibodies to RAM11 (Dako Corp) to identify macrophages and for smooth muscle actin.
muscle actin (HHF35, Enzo) to identify SMCs. To assess cellular proliferation, animals received bromodeoxyuridine (BrdU) before euthanasia, as previously described.10

Another 3 animals received a single Express 2 stent in one iliac artery, whereas the contralateral nonstented artery served as control. After stent deployment, these animals were administered rhodamine-labeled TRM-484 at 1 mg/kg for the assessment of arterial drug distribution. Arterial segments were pressure-perfused with Ringer Lactate, and specimens were harvested at 24 hours, 3 days, and 7 days after stenting. Vessels were cut longitudinally to expose the luminal surface, stent wires were removed, and arteries were examined by confocal microscopy.

Proof-of Concept Study
To prove the concept of site-directed delivery of nanoparticles, arterial denudation was performed unilaterally using a 3 F Fogarty catheter, while the contralateral iliac artery served as noninjured control (n=2 for each group). Animals were euthanized at 24 hours, and specimens were harvested after rinsing in Ringer Lactate and dissected longitudinally to expose the luminal surface for ex vivo incubation with 100 μg Rhodamine-TRM-484/mL or 10 μg/mL Dil-Ac-LDL for 24 hours. Alternatively, arteries were first digested with Chondroitinase ABC (20 μU/mL) for 60 minutes at 37°C and subsequently incubated with 100 μg Rhodamine-TRM-484/mL or 10 μg/mL Dil-Ac-LDL for 24 hours.

Organ Sections
To examine whether there was significant accumulation of TRM-484 in lymphatic organs, portions of liver and spleen were taken from 8 rabbits (n=2 for 1 mg/kg TRM-484, n=2 for 0.32 mg/kg TRM-484, n=2 for placebo, and n=2 for control) and submitted for histopathologic evaluation 42 days after onset of treatment. Arterial segments were examined with the observer blinded to the treatment group. Stents were evaluated for thrombus formation, inflammation, and macrophage accumulation. Computerized planimetry was performed (IP Laboratory, Scanalytics, BD Biosciences) on all stented sections similar to previously described methods10 (also see supplemental materials). Fibrin was identified on H&E and Carstairs stained sections and semiquantified as described previously. The percentage of the neointima occupied by RAM11-positive macrophages or smooth muscle actin-positive cells were determined by digital color (intensity) threshold imaging.

Immunofluorescence Microscopy
Serial cryostat sections were prepared at 6 μm and mounted on charged slides. Single antibody markers for SMCs (HHF35, Enzo) and macrophages (RAM11, Dako) were incubated for 1 hour. Secondary labeling was performed with a donkey antimouse conjugated to Alexa Fluor 488. For dual labeling studies, a polyclonal SMC antibody was substituted (Abcam). TOTO-3 (Invitrogen) was used as a counterstain. The sections were viewed on a LSM Pascal confocal microscope (Zeiss).

Pharmacokinetics of TRM-484
Twelve additional rabbits (1 mg/kg TRM-484) were implanted single stents in one iliac artery, while the contralateral nonstented artery served as control. TRM-484 was administered as single intravenous injection at 1 mg/kg 2 hours after stent implantation, and arteries were harvested at 1, 24, 72, and 120 hours (n=3 at each time point) as described previously. Blood samples were drawn from the jugular vein at the corresponding time points. The concentration of prednisolone and prednisolone-phosphate in plasma as well as stented and nonstented arteries was determined by liquid chromatography and mass spectrometry and expressed per weight of tissue.

Chemokine Production Assay
To determine the antiinflammatory activity of TRM-484 toward SMCs, the in vitro release of proinflammatory cytokines was measured in the presence or absence of different concentrations of TRM-484 (also see materials).

Cell Proliferation Assay
To directly assess the antiproliferative effects of TRM-484, human aortic SMCs were cultured with platelet-derived growth factor–BB (PDGF-BB) in the presence or absence of different concentrations of TRM-484 (also see materials).

Data Analysis
All arterial segments were examined with the observer blinded to the treatment group. Stents were evaluated for thrombus formation, inflammation, and macrophage accumulation. Computerized planimetry was performed (IP Laboratory, Scanalytics, BD Biosciences) on all stented sections similar to previously described methods (also see supplemental materials). Fibrin was identified on H&E and Carstairs stained sections and semiquantified as described previously. The percentage of the neointima occupied by RAM11-positive macrophages or smooth muscle actin-positive cells were determined by digital color (intensity) threshold imaging.

Statistical Analysis
Numeric data are presented as mean±SD. Continuous variables were first evaluated for normal distribution by Shapiro-Wilk-Goedes- of-fit test and analysis of variance (ANOVA) or Wilcoxon rank-sum test performed where appropriate. Dunnett’s post hoc adjustment was used to determine significant differences, and a probability value <0.05 was considered statistically significant.

Results
Twenty-eight rabbits were included into the study; however, three rabbits were euthanized before schedule because of cholesterol diet-induced liver failure and excluded from analysis. The 25 rabbits received bilateral implants of Express bare metal stents (50 stents). Two rabbits died the day after stent deployment because of arterial dissection. All other animals remained healthy throughout the study, and there were no signs of drug toxicity (weight loss, lethargy, icterus; Figure 1). Animal weights were similar among groups at sacrifice (3.6±0.3 kg, 1 mg/kg TRM-484; 3.5±0.4 kg, 0.32 mg/kg TRM-484; 3.5±0.4 kg, placebo; 3.7±0.6 kg, control group, P=ns). At the time point of stent implantation, serum cholesterol levels were similar among groups (1 mg/kg TRM-484; 578±273 mg/dL, 0.32 mg/kg TRM-484 group; 573±222 mg/dL, placebo; 831±433 mg/dL, control; 637±287 mg/dL, P=ns). Serum CRP levels were <0.2 mg/dL throughout the entire study. Pre euthanasia angiography showed widely patent stents in all treatment groups without stent migration or aneurysm formation.

Morphometry and Inflammatory Response
Fourteen stents from the 1 mg/kg TRM-484 group were compared to 10 stents from the 0.32 mg/kg TRM-484 group, to 12 stents from the placebo group, and to 10 stents from the control group. There were no significant differences among treatment groups with respect to stent area and plaque area (Table 1). Treatment with 1 mg/kg TRM-484 resulted in a significant 24% reduction of percent area stenosis compared to control animals and a significant 27% reduction of percent area stenosis compared to placebo animals (Table 1; supplemental Figure I). Semiquantitative assessment of fibrin deposition and endothelialization were similar among groups (Table 1). The overall inflammation score and giant cell infiltration were significantly lower in 1 mg/kg and 0.32 mg/kg TRM-484–treated compared to control or placebo animals (Table 1).

Immunohistochemistry Analysis
RAM11 immunostaining demonstrated a significant 42% decrease in the percentage of the neointima occupied by macrophages in the 1 mg/kg TRM-484 group compared to...
Importantly, macrophage infiltration was diminished in the areas surrounding stent struts in 1 mg/kg TRM-484–treated animals, whereas there was more pronounced macrophage accumulation observed in the adventitial layer in placebo treated animals. There was no significant difference in smooth muscle actin staining and neointimal proliferation index among groups (see supplemental Table I).

**Organ Sections**
Sections of liver and spleen showed minor foam cell reaction within a normal structure. Inflammatory cells were seen within the expected range.

**Confocal Microscopy**
Cryosections showed TRM-484 within the superficial neointimal tissue layer at 24 hours and close to the internal elastic lamina at 3 and 7 days after stent implantation (Figures 3 and 4). Immunofluorescent staining revealed absence of SMCs within the neointima at 24 hours in stented and contralateral nonstented arteries (Figure 3). In contrast, there was an abundance of neointimal macrophages in stented and nonstented arteries. At 7 days, neointimal SMCs and macrophages were present, and TRM-484 showed a layered distribution within the neointimal tissue of stented arteries (Figure 4). No drug was observed in contralateral nonstented arteries at any time point.

**Proof-of Concept Study**
Twenty-four hours after ex vivo incubation of denuded arteries, TRM-484 was predominantly located within the superficial subendothelial matrix (supplemental Figure IIA through IIC), whereas there was absence of TRM-484 in control (nominjured) arteries. Interestingly, when arteries were digested with Chondroitinase ABC before incubation with TRM-484, no relevant uptake of the drug could be observed.

**Table 1. Cross-Sectional Vessel Areas, Percent Stenosis, and Inflammatory Comparison Between Treatment Groups 42 Days After Stent Placement**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Stent Area mm²</th>
<th>Plaque Area mm²</th>
<th>% Stenosis</th>
<th>% Fibrin deposition</th>
<th>Inflammation Score</th>
<th>% Giant Cells</th>
<th>% Endothelialization</th>
<th>% Neointimal RAM11</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRM-484 1 mg/kg</td>
<td>5.6±1.8</td>
<td>1.5±0.5</td>
<td>22.5±4.4</td>
<td>21.7±5.4</td>
<td>0.3±0.2</td>
<td>10.2±7.3</td>
<td>88.5±5.3</td>
<td>2.9±1.2</td>
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<tr>
<td>n=14</td>
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</tr>
<tr>
<td>TRM-484 0.32 mg/kg</td>
<td>5.2±1.1</td>
<td>1.3±0.3</td>
<td>28.0±8.2</td>
<td>22.8±10.5</td>
<td>0.3±0.3</td>
<td>24.5±8.3</td>
<td>87.5±7.3</td>
<td>4.1±2.5</td>
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<td>n=10</td>
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<tr>
<td>Placebo n=12</td>
<td>5.0±0.9</td>
<td>1.3±0.4</td>
<td>31.0±8.4</td>
<td>21.4±13.4</td>
<td>1.0±0.6</td>
<td>32.8±18.6</td>
<td>85.0±21.9</td>
<td>5.1±1.0</td>
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<td>Control n=10</td>
<td>6.4±0.9</td>
<td>1.6±0.4</td>
<td>29.5±8.1</td>
<td>28.5±10.5</td>
<td>0.7±0.4</td>
<td>15.2±7.4</td>
<td>81.0±6.9</td>
<td>3.3±1.0</td>
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<td>P Value</td>
<td>ns</td>
<td>ns</td>
<td>0.03</td>
<td>ns</td>
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<td>0.0001</td>
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<td>Post hoc correction</td>
<td>* †</td>
<td>* †§</td>
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</tbody>
</table>

*Significance between TRM-484 1 mg/kg and control after Dunnett’s post hoc correction. †Significance between TRM-484 0.32 mg/kg and control after Dunnett’s post hoc correction. ‡Significance between TRM-484 1 mg/kg and placebo after Dunnett’s post hoc correction. §Significance between TRM-484 0.32 mg/kg and placebo after Dunnett’s post hoc correction.
In contrast, Dil-Ac-LDL–incubated arteries showed presence of the drug within the endothelial monolayer of control (noninjured) arteries, whereas no uptake was observed in denuded arteries (supplemental Figure IID through IIF). Digestion with Chondroitinase ABC did not have any effect on the endothelial uptake of Dil-Ac-LDL.

**In Vivo Pharmacokinetics**

At 24 hours, the concentration of prednisolone-phosphate was approximately 100-fold higher in stented compared to nonstented arteries after single injection of TRM-484 at 1 mg/kg and remained significantly higher in stented arteries throughout various time points (Table 2; also see supplemental materials).

**In Vitro Chemokine Release and Cell Proliferation**

TRM-484 dose-dependently diminished the production of MCP-1 and interleukin (IL)-8 in PDGF-stimulated SMCs. On the other hand, TRM-484 and PSLP did not suppress cell proliferation in PDGF-stimulated SMCs (supplemental Figures III and IV). Accordingly, no significant effect on cellular proliferation could be observed 42 days after stent implantation (supplemental Table I).

**Discussion**

In the current study, TRM-484 when given at 1 mg/kg 3 times a week resulted in a significant reduction of percent stenosis at 42 days after implantation of bare metal stents without any relevant impairment in arterial healing. Drug uptake was observed in significantly higher concentrations in regions of strut-induced arterial injury, resulting in markedly increased tissue concentrations of TRM-484 in stented as compared to nonstented arteries. This is the first study to demonstrate that site-directed targeting of nanoparticle prednisolone diminishes neointimal growth in an animal model of established atheroma. Neointimal inflammation was notably reduced in animals treated with 1 mg/kg TRM-484 and signs of systemic toxicity such as weight loss, lethargy, icterus, or accumulation of liposomes in lymphatic organs were absent.

**Antirestenotic Effects**

In-stent restenosis follows a “response-to-injury” cascade of events that results in proliferation and migration of vascular SMCs and synthesis of extracellular matrix. Indeed, greater inflammatory response after stent implantation in animal models and humans have been strongly correlated with the degree of neointimal formation, which suggests that antiinflammatory drugs such as prednisolone should exert profound antirestenotic effects by limiting injury-related inflammation. In our study, we observed a mitigation of neointimal inflammation in rabbits treated with 1 mg/kg TRM-484, which was associated with significant suppression of neointimal growth. Conversely, neointimal macrophages and giant cells were greatest in placebo animals,

**Figure 3.** Cryostat sections 24 hours after stenting with staining for SMCs (A, B), macrophages (C, D), or Movat-Pentachrome (E, F). TRM-484 (red, arrowheads) was exclusively observed within the neointimal tissue of stented-arteries (A), which is distinguishable from media with abundant SMCs (green). Macrophages (green) were abundant in the neointima with proximity to TRM-484 (red) of stented arteries (C). No specific staining within nonstented section (D).

**Figure 4.** Cryostat stented sections (7-day) were examined for TRM-484 after staining for SMCs (A) or macrophages (B). There is interstitial accumulation of TRM-484 (red) within a thin SMC-rich (A) neointimal layer underlying strut impressions (*). TRM-484 was also observed in macrophage-rich regions (B). Dual staining for SMCs (blue) and macrophages (green) showed no preference of TRM-484 (red) toward either cell type (C).
suggesting that empty liposomes (lacking prednisolone-phosphate) attract monocytes. It has previously been reported that nanoparticles tend to get tagged by opsonin proteins soon after systemic administration, facilitating recognition by macrophages.\(^6,18\) Although this issue has partly been resolved by modifications of nanoparticles with polyethylene glycol (PEG),\(^3\) an effect on macrophage recruitment might still be evident and deserves further investigation. It is also important to mention that, in the current trial, macrophage infiltration was predominantly observed in the adventitial layer in animals receiving placebo (empty liposomes), suggesting that opsonin tagged liposomes tend to accumulate through transport of vasa-vasorum, whereas intact steroid-containing liposomes deposit within the neointima. Further studies are needed to specifically address this issue.

### Targeted Delivery Allows for Reduced Dosing

The concept of systemic pharmacological treatment to reduce inflammation after stenting has been applied in many preclinical and clinical studies with varying success.\(^1\) Many of the studies have been performed using established tumors. Significant growth-reducing effects were achieved via a low-frequency dosing schedule. Using free glucocorticoids, the same effect required substantially higher dosing.\(^2,3\) These results support our findings that liposomal encapsulation of steroids may allow antirestenotic efficacy at levels far below those needed for oral administration of glucocorticoids.

### Antirestenotic Efficacy Without Delayed Healing

DES have dramatically reduced restenosis, but late stent thrombosis has emerged as a safety concern.\(^1\) Therefore, other approaches to prevent restenosis are warranted. Steroids seem to exert direct antiinflammatory and secondary antiproliferative effects on smooth muscle cells without affecting endothelial regrowth of injured vascular tissues. Site-specific targeting of steroids might be a valuable and cost-effective approach for the prevention of in-stent restenosis without the induction of delayed healing.

### Limitations

In the current study, atherosclerotic rabbits were used to confirm site-targeted binding of TRM-484. We have previously reported on the differences between atherosclerotic lesions seen in human disease and the predominantly foam cell–rich plaque in atherosclerotic rabbits.\(^24\) Prospective randomized trials in humans are needed to prove the significance of the findings reported in the current study.

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**Table 2. Tissue and Plasma Concentrations of Prednisolone and Prednisolone-Phosphate at Various Time Points at a Dosage of 1 mg/kg**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Artery (µg/g)</th>
<th>Plasma (µg/mL)</th>
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<tr>
<td></td>
<td>PSL (Nonstented)</td>
<td>PSL (Stented)</td>
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<tr>
<td>1</td>
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<td>0.0316</td>
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<tr>
<td>24</td>
<td>&lt;0.005</td>
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<tr>
<td>72</td>
<td>&lt;0.005</td>
<td>0.223</td>
</tr>
<tr>
<td>120</td>
<td>&lt;0.005</td>
<td>0.106</td>
</tr>
<tr>
<td>AUC (0–120 hr)</td>
<td>0.783</td>
<td>29.8</td>
</tr>
</tbody>
</table>

PSL indicates prednisolone; PSLP, prednisolone phosphate.

Area under the curve (AUC) unit: µg·hr/g or µg·hr/mL.
Conclusion

The present study supports proof of concept for site-specific steroid targeting to prevent neointimal growth in atherosclerotic rabbits at 42 days after stent implantation. The marked decrease of neointimal inflammation in animals treated with 1 mg/kg TRM-484 is likely an important mechanism for the suppression of neointimal growth.

Disclosures

None.

References

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Figure IIFigure II

TRM-484

non-digested
denuded
control
digested

Dil-Ac-LDL

non-digested
control
denuded
digested

Figure II
A. ANOVA with Dunnett’s post hoc comparison
* P < 0.05; ** P < 0.01 (v.s control)

MCP-1 (ng/mL)

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<td>2</td>
</tr>
<tr>
<td>TRM-484 (µM)</td>
<td>*</td>
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B.

IL-8 (ng/mL)

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<td>TRM-484 (µM)</td>
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Figure III
Figure IV
Table I. Neointimal Proliferation Index Following BrdU Immunostaining

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<tr>
<th>Treatment Group</th>
<th>TRM-484 1mg/kg n=14</th>
<th>TRM-484 0.32mg/kg n=10</th>
<th>Placebo n=12</th>
<th>Control n=10</th>
<th>p-value</th>
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<td>Proliferation Index</td>
<td>0.1±0.05</td>
<td>0.15±0.01</td>
<td>0.14±0.09</td>
<td>0.13±0.03</td>
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<td>%Smooth muscle actin</td>
<td>7.3±2.9</td>
<td>17.1±16.3</td>
<td>14.1±10.5</td>
<td>13.8±6.2</td>
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</table>
Methods

Synthesis of TRM-484. The preparation of the liposomes was described previously \(^1\,^2\), however, we modified this method to produce the liposomes used in the present study. A lipid mixture of HSPC, cholesterol, and TRX-20 at a molar ratio of 50:42:8 was hydrated with prednisolone disodium phosphate (PSLP) in phosphate-buffered solution (pH 7.2) by sonication, and extruded through two stacked Nucleopore filters (pore sizes: 0.4, 0.2, and 0.1 µm), to obtain an expected diameter of around 100 nm. After PEG5000-PE solution was added at a ratio of 0.75 mol % to the total lipids in the liposomal solution, to modify the liposome surface, unencapsulated PSLP was removed by gel filtration using Sepharose 4FF (Amersham Biosciences Co., Piscataway, NJ). HSPC and PSLP concentrations were determined using a phospholipids determination kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and HPLC, respectively. The diameter of the liposomes was estimated using a dynamic light-scattering system (Zetamaster-S; Malvern Instrument, Malvern, UK). PSLP-encapsulating liposomes (TRM-484) with a diameter of about 100 nm contained approximately 0.1 mg PSLP per 1 mg HSPC. In addition, rhodamine-labeled TRM-484 was prepared using basically the same method as described above, except that rhodamine-DHPE was dissolved in the lipid mixture solution at a ratio of 0.2 mol % to total lipids.

Histomorphometric Processing and Analysis. The stented portion of the vessel was placed intact into processing vials and dehydrated with graded series of alcohols. Arteries containing stents were then infiltrated and embedded in methylmethacrylate plastic. Vials were sealed airtight and placed in a 38°C waterbath for polymerization. After polymerization, 1-mm segments from the proximal,
middle, and distal portion of the methacrylate blocks were cut using a diamond edge rotating precision saw. Plastic sections (4–5 µm thick) were then cut, adhered to glass slides, and allowed to dry. The plastic was then removed, and the sections were rehydrated and stained with hematoxylin–eosin and Movat pentachrome stains. A 3.0-mm segment just proximal and distal to the stents was processed for light microscopy to evaluate possible edge effects. All histologic sections were magnified and digitized with the observer blinded to the treatment group. The arterial injury score could not be determined due to the fact that the formation of an atherosclerotic plaque frequently disrupts the internal elastic lamina, and would, therefore, result in inappropriately high injury scores. The lumen area, adventitial area, and areas within the external elastic lamina (EEL), and stent were measured by computerized morphometry (IP Lab software for Macintosh, Scanalytics, BD Biosciences, Rockville, MD). The neointimal area was calculated (stent area minus lumen area) as was the plaque area (EEL area minus stent area). The percent luminal stenosis was calculated as:

\[1-\frac{\text{lumen area}}{\text{stent area}}\] \times 100. The neointimal thickness at and between each stent wire site was measured, and the mean neointimal thickness for each arterial segment was calculated. Intimal inflammatory cells were counted in 8 randomly selected high-power (400x) fields. Intimal areas were measured in arterial segments just proximal and distal to the stents. To assess intimal cellular proliferation (BrdU staining), midstent sections were incubated at room temperature for 24 hours in xylene, 20 minutes in methylacetate, and 4 minutes in acetone to remove the plastic. Antigen recovery was performed by heating the sections with steam for 20 minutes. The slides were cooled to room temperature and immersed in 2 N HCl for 15 minutes at 37°C. Sections were
rinsed and incubated with a mouse monoclonal anti-BrdU antibody (1:400 dilution, Dako) overnight at 4°C in a humidified chamber. After rinsing in phosphate-buffered saline (PBS), the primary antibody was labeled by a biotinylated link antibody directed against mouse using a peroxidase based labelled streptavidin-biotin (LSAB) kit. Positive staining (brownish-red reaction product) was visualized with a 3-amino-9-ethylcarbazole (AEC) substrate– chromogen system. After immunostaining, the sections were counterstained with Gill’s hematoxylin, washed, and mounted in glycerol gelatin. Intimal cell proliferation was determined by counting BrdU-positive intimal cells as a percent of total cells in 8 randomly selected 400x fields per section. A BrdU-labeling index was calculated using the following formula: total BrdU positive cells/total cells. Midstent sections were also stained with HHF35 (anti-muscle actin, Enzo, 1:20 dilution incubated overnight at 4°C) or RAM11 (Dako, 1:50 dilution incubated overnight at 4°C). Actin-positive or RAM11-positive intimal cells were determined by digital color (intensity) threshold imaging (IP Lab software for Macintosh, Scanalytics, BD Biosciences, Rockville, MD).

**Results**

**Pharmacokinetics of TRM-484.** To determine if systemic dose of TRM-484 can produce levels high enough to have local anti-inflammatory effects, pharmacokinetic studies were performed for the selected high dose of 1mg/kg TRM-484. Twelve additional rabbits were implanted single stents in one iliac artery, while the contralateral non-stented artery served as control. TRM-484 was administered as single intravenous injection at 1mg/kg 2 hours following stent implantation and arteries harvested at 1, 24, 72 and 120 hours (n=3 at each timepoint) as described previously. Blood samples (1.0 mL) were drawn from
the jugular vein at the corresponding time points. The concentration of prednisolone and prednisolone-phosphate in plasma as well as stented and non-stented arteries was determined by liquid chromatography and mass spectrometry and expressed per weight of tissue.

References


**Figure I.** Iliac artery morphology at 42 days following stent placement is shown at low power (A, C, E, G) and high power (B, D, F, H) magnification after staining with Movat Pentachrome. Note, percent stenosis is less in the 1mg/kg TRM-484 (A and B) as compared to the 0.32mg/kg TRM-484 (C and D), the placebo (E and F) and the control (G and H) group.

**Figure II.** Proof-of concept study. Twenty-four hours following ex vivo incubation of denuded arteries, TRM-484 was predominantly found to be located within the superficial subendothelial matrix (figure IIA, white arrowheads), while there was absence of TRM-484 in control (non-injured) arteries (figure IIB). In control arteries, endothelial cells were seen towards the luminal surface (figure IIB, white arrowhead). Interestingly, when denuded arteries were digested with Chondroitinase ABC prior to incubation with TRM-484, no relevant uptake of the drug could be observed (figure IIC). The IEL is outlined by white arrows.

In contrast, Dil-Ac-LDL-incubated arteries showed presence of the drug within the endothelial monolayer of control (non-injured) arteries (figure IID, white arrowheads), while no uptake was observed in denuded arteries (figure IIE). Digestion with Chondroitinase ABC did not have any effect on the endothelial uptake of Dil-Ac-LDL (figure IIF, white arrowheads). The IEL is outlined by white arrows.

**Figure III.** Effects of TRM-484 on chemokine production in PDGF-stimulated SMCs. Human aortc SMC’s were stimulated in the absence or presence of different concentrations of TRM-484. There was a dose-dependent reduction of MCP-1 (A) and
Interleukin-8 (B) following treatment with increasing TRM-484 doses. Statistical analysis was performed by ANOVA test with Dunnett’s post hoc comparison.

**Figure IV.** Effects of TRM-484 on cell proliferation in PDGF-stimulated SMC’s. Human aortic SMC’s were stimulated in the absence or presence of different concentrations of TRM-484 or prednisolone phosphate (PSLP). Cellular proliferation was determined utilizing a colorimetric absorbance assay 72 hours following initiation of the experiment. No significant difference could be observed between any of the treatment groups.

**Table I.** Neointimal cell proliferation index following immunostaining with BrdU and percent area occupied by smooth muscle actin positive cells.