Prevention of Abdominal Aortic Aneurysm Progression by Targeted Inhibition of Matrix Metalloproteinase Activity With Batimastat-Loaded Nanoparticles

Nasim Nosoudi, Pranjal Nahar-Gohad, Aditi Sinha, Aniqa Chowdhury, Patrick Gerard, Christopher G. Carsten, Bruce H. Gray, Naren R. Vyavahare

Rationale: Matrix metalloproteinases (MMPs)–mediated extracellular matrix destruction is the major cause of development and progression of abdominal aortic aneurysms. Systemic treatments of MMP inhibitors have shown effectiveness in animal models, but it did not translate to clinical success either because of low doses used or systemic side effects of MMP inhibitors. We propose a targeted nanoparticle (NP)–based delivery of MMP inhibitor at low doses to the abdominal aortic aneurysms site. Such therapy will be an attractive option for preventing expansion of aneurysms in patients without systemic side effects.

Objective: Our previous study showed that poly(d,l-lactide) NPs conjugated with an antielastin antibody could be targeted to the site of an aneurysm in a rat model of abdominal aortic aneurysms. In the study reported here, we tested whether such targeted NPs could deliver the MMP inhibitor batimastat (BB-94) to the site of an aneurysm and prevent aneurysmal growth.

Methods and Results: Poly(d,l-lactide) NPs were loaded with BB-94 and conjugated with an elastin antibody. Intravenous injections of elastin antibody–conjugated BB-94-loaded NPs targeted the site of aneurysms and delivered BB-94 in a calcium chloride injury-induced abdominal aortic aneurysms in rats. Such targeted delivery inhibited MMP activity, elastin degradation, calcification, and aneurysmal development in the aorta (269% expansion in control versus 40% elastin antibody–conjugated BB-94-loaded NPs) at a low dose of BB-94. The systemic administration of BB-94 alone at the same dose was ineffective in producing MMP inhibition.

Conclusions: Targeted delivery of MMP inhibitors using NPs may be an attractive strategy to inhibit aneurysmal progression. (Circ Res. 2015;117:e80-e89. DOI: 10.1161/CIRCRESAHA.115.307207.)

Key Words: abdominal aortic aneurysm • batimastat • cardiovascular diseases • drug delivery systems • matrix metalloproteinase inhibitors • nanoparticles • vascular remodeling

Abdominal aortic aneurysm (AAA) characterized by dilation of the abdominal aorta is one of the top 10 causes of death among older men.1 Although the cause of AAA remains unknown in the majority of cases, several key regulators of AAA pathogenesis are known. Matrix metalloproteinases (MMPs) have been shown to play a major role in progressive extracellular matrix (ECM) degradation in AAA.2,3 Under inflammatory conditions, infiltrating macrophages, vascular smooth muscle cells, endothelial cells, and adventitial fibroblasts secrete pro-MMPs; cleavage of the pro-MMP subunit activates the MMPs, causing ECM degradation. MMP activity may be naturally suppressed by tissue inhibitors of MMPs (TIMPs), which comprise a family of 4 protease inhibitors: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.4 An improper balance between MMPs and TIMPs shifts equilibrium toward matrix degradation in several vascular conditions, such as AAA, atherosclerosis, hypertension, and calcification.5 Several types of MMPs are expressed in AAA tissue, including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12.6 MMP-9 and MMP-2 knockout mice do not develop aneurysms, suggesting that these MMPs play a major role in development of AAA.7 Several synthetic MMP inhibitors (eg, doxycycline and hydroxamate based) are known to decrease MMP activity4 and prevent medial destruction.8 Synthetic MMP inhibitors with a hydroxamate (−CONHOH) group bind zinc atoms and suppress MMP enzymes.10 Among the many hydroxamate-based MMP inhibitors, marimastat, solimastat, prinomastat, cipemastat, and batimastat (BB-94).11,12 It has been shown that BB-94 was one of the first synthetic MMP inhibitors to be tested clinically to reduce MMPs in cancers with advanced malignancies.13

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However, its effectiveness is limited by its poor water solubility when administered orally, requiring parenteral administration. In several studies, systemic administration of MMP inhibitors effectively reduced aneurysmal onset in animal models, but systemic delivery can cause off-target inhibition of the MMP activities essential for normal homeostasis. Although there are no in vivo studies of targeted delivery of MMP inhibitors to the aneurysms site, a recent in vitro study showed the potential of delivering doxycycline-loaded nanoparticles (NPs) for localized elastic matrix stabilization and regenerative repair in AAA. Doxycycline was also shown to reduce mRNA stability for MMP-2 and inhibit MMP activity that way.

In this study, we tested the hypothesis that systemic delivery of elastin antibody–conjugated NPs loaded with BB-94 would be targeted specifically to the aneurysm site, would slowly release the drug there, and would inhibit local MMP activity and subsequent aneurysm expansion. Such targeted MMP inhibition would require smaller and less frequent drug doses than systemic administration, and thus systemic side effects would be minimized.

Methods

Synthesis of Batimastat-Loaded NPs

Poly(ol,lactide) NPs were prepared using the solvent diffusion–based nanoprecipitation method (Online Data Supplement).

NP Yield

The total final dry weight of the NPs was recorded, and NP yield was calculated using the formula shown below:

\[
\text{NP Yield}\% = \frac{\text{Final dry weight}}{\text{Initial dry weight}} \times 100
\]

NP Characterization

Particle size, ζ-potential and transmission electron microscopy were performed to characterize the NPs (details are given in Online Data Supplement).

NP Degradation Studies

Blank NPs were suspended in PBS and stirred at 37°C to test polymer degradation. NP degradation was monitored by measuring the weight of the remaining NPs at different time points (1, 7, 14, 21, and 28 days) after lyophilization. The percent weight loss was calculated using the difference in the remaining dry weight and the initial weight. We also tested blank NPs degradation by gel permeation chromatography (details are given in Online Data Supplement).

Loading Efficiency and Release Profile of BB-94

Loading efficiency was calculated by dissolving NPs in dimethyl sulfoxide, determining BB-94 concentration at λ (max)=285 nm using UV spectrophotometry and using the equation shown below (details are given in Online Data Supplement):

\[
\%\text{Loading} = \frac{\text{BB-94 weight}}{\text{total weight of NPs}} \times 100
\]

Percent release was calculated as

\[
\%\text{BB-94 Released} = \frac{(\text{Initially loaded BB-94}) - (\text{Residual BB-94})}{\text{Initially loaded BB-94}} \times 100
\]

Conjugation of Elastin Antibody to NPs

Trast reagent (34 μg; G-Biosciences, Saint Louis, MO) was used for thiolation of 10 μg of rabbit anti-rat elastin antibody (United States Biological, Swampscott, MA), and the mixture was incubated in HEPES buffer (20 mmol/L, pH=9.0) for an hour at room temperature. Thiolated antibodies were rinsed with HEPES buffer and added to the NPs (4 μg antibody per 1-mg NPs) and then incubated overnight for conjugation. After incubation, antibody-conjugated NPs were washed twice with PBS, centrifuged (10000 rpm for 10 minutes, g force 9177), and suspended in 0.3% rat serum albumin for an in vivo animal study.

BB-94 Activity

To test if the BB-94 loaded in the NPs was still in active form, SDS-PAGE zymography was performed (details are given in Online Data Supplement).

Reverse Zymography for TIMP Activity

Equal amounts of protein from rat aortic smooth muscle cell (RASMC)–conditioned cell culture media (BB-94 treated or control) were loaded in 15% reverse zymogram gel containing 1.5% gelatin and collagenase (20 U) under non-denaturing and nonreducing conditions (details are given in Online Data Supplement).

NP Toxicity for RASMCs and Rat Aortic Endothelial Cells

A standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli-um bromide reduction) assay was performed to measure the cytotoxicity of NPs to RASMCs and rat aortic endothelial cells (details are given in Online Data Supplement).

NP Uptake by Macrophages

Elastin antibody–conjugated NPs were loaded with fluorescent dye, 1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide (DIR), as described previously. Macrophages (RAW 264.7, ATCC TIB-71) were grown in 24-well tissue culture plates followed by incubation with EL-DIR-NPs (1 mg/mL) at 37°C in 5% CO2 for 4 hours and 24 hours. Cells were washed 3× with sterile PBS and imaged before and after washing using EVOS XL Cell Imaging System to determine NP uptake. As controls for charge and size, we prepared 2 different batches of NPs, 1 with positive surface charge and 1 with smaller size NPs. To create a positive surface charge, NPs were coated with chitosan (molecular weight, 100000 to 300000 Da; ACROS, Morris, NJ). To study cytotoxicity, rat bone marrow macrophage cells (Cell Biologics, Inc, RA-6030F) were grown in 24-well tissue culture plates followed by incubation with blank and BB94-NPs (0.5, 1, and 2 mg/mL). A standard live/dead assay was performed after 24 hours.

Feasibility of NP Targeting In Vivo

Local elastin damage in the rat abdominal aortic region was induced by perivascular application of calcium chloride. Briefly, 19 male Sprague-Dawley rats (5–6 weeks old) were placed under general anesthesia (2%–3% isoflurane). The infrarenal abdominal aorta was exposed and treated periadventitially by placing 0.50 mol/L CaCl2-soaked sterile cotton gauze on the aorta for 15 minutes. The treated

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AAA</td>
<td>abdominal aortic aneurysm</td>
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<tr>
<td>BB-94</td>
<td>batimastat</td>
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<tr>
<td>DIR</td>
<td>1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EL-NP-BB94</td>
<td>elastin antibody-conjugated BB-94-loaded nanoparticles</td>
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<tr>
<td>EL-NP-Blank</td>
<td>elastin antibody-conjugated blank nanoparticles</td>
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<tr>
<td>EL-NP-DIR</td>
<td>elastin antibody-conjugated DIR-loaded nanoparticles</td>
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<td>IP</td>
<td>intraperitoneal</td>
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<td>MMPs</td>
<td>matrix metalloproteinases</td>
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<td>NPs</td>
<td>nanoparticles</td>
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<td>RASMC</td>
<td>rat aortic smooth muscle cell</td>
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<td>TIMPs</td>
<td>tissue inhibitors of MMPs</td>
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area was flushed with warm saline, and the abdominal incision was closed with sutures. Animals were allowed to recover and given a normal diet for 10 days. Advenital inflammation and elastic lamina degradation have previously been shown to occur within 10 days in this model. After 10 days, rats were divided into 2 groups and put through treatment with BB-94-loaded NPs or blank NPs. Elastin antibody–conjugated BB-94-loaded NPs (named EL-NP-BB94 hereafter) at ≈10 mg/kg body weight were suspended in 200 μL of 0.3% rat serum albumin (Sigma Aldrich, St. Louis, MO) and were injected through the tail vein (n=5). Control animals received elastin antibody–conjugated blank NPs (named EL-NP-Blank hereafter; n=5). Three additional rats were injected with elastin antibody–conjugated and DIR dye–loaded NPs (named EL-NP-DIR hereafter) to monitor delivery of the NPs by in vivo imaging. As a negative control, IgG antibody–conjugated NPs loaded with DIR dye were injected through the tail vein (n=3). To study if the systemic delivery of low-dose BB-94 affects MNP activity in the aorta, 3 rats received the same amount of BB-94 (600 μg of BB-94 per kg body weight) dissolved in 200-μL PBS solution with 0.01% Tween 20 (Merck, Germany) by intraperitoneal (IP) injection.

Two days (48 hours) after treatment, the rats were euthanized and thoracic and abdominal aortic tissue samples were explanted and snap frozen in liquid nitrogen. Total protein from the aortic samples was extracted by pulverizing liquid nitrogen-frozen tissue samples and homogenizing them in radio-immuno-precipitation extraction buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS with protease inhibitor cocktail; Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer’s protocol. Sections of thoracic and abdominal aortic tissue were embedded in optimal cutting temperature compound for cryostat sectioning. For DIR-loaded NP groups, whole fresh aortas and all organs were harvested and imaged using Caliper In Vivo Imaging System Lumina XR (Hopkinton, MA) with Ex/Em of 745/795 nm for studying targeting and biodistribution of NPs.

MMP Activity in Rat Aorta

The total protein from the harvested aortic tissue after 48 hours of treatment was quantified using the BCA Protein Assay Kit (Pierce, IL). An internally quenched peptide substrate that is specific to MMP-2 and 9 (excitation 280 nm, emission 360 nm, MMP Substrate III; Anaspec, San Jose, CA) was used to measure MMP activity. One milligram of substrate dissolved in 50-μL dimethyl sulfoxide was diluted in 10 mL of development buffer (50 mmol/L Tris Base, 5 mmol/L CaCl2, 2H2O, 200 mmol/L NaCl, and 0.02% brij 35). Development buffer (96 μL) was mixed with 2 μL of the extracted protein along with 2 μL of substrate stock solution and incubated for 1 hour at 37°C. End point fluorescence intensity was read using a fluorescence plate reader.

NP Targeting to Inhibit Aneurysm Progression

To study long-term aneurysmal inhibition, another set of 13 rats was used. AAAs were induced by perivascular application of calcium chloride22 as shown above. Ten days after injury, NPs (≈10 mg/kg body weight) were injected via tail vein once a week for 4 weeks (n=5 EL-NP-BB94, n=5 EL-NP-Blank, and n=3 EL-NP-DIR). Rats were euthanized on day 38, and organs and aortae were harvested. In vivo Lumina XR imaging system was used to confirm targeting and bio distribution in the EL-NP-DIR group. Aortic tissues from the EL-NP-Blank and EL-NP-BB94 groups were harvested as described above.

In Situ Zymography

To examine activity of MMPs in the aortic tissue samples in situ, zymography was performed on histological sections of aortae isolated at 48 hours and 38 days after NP injection. Gelatinolytic activity was demonstrated in fixed cryostat sections (8-μm thick) using dye-quenched (DQ) gelatin as a substrate (Life Technologies, Northbrook, IL). Cryostat sections of abdominal aorta were air dried for 10 minutes. One part of DQ gelatin (1 mg/mL of distilled water) was mixed with 9 parts of 1% agarose (Promega, WI) in PBS containing 4,6-diamidino-2-phenylindole (1 μg/mL; Thermo Fisher Scientific, Rockford, IL). A drop of the mixture was added to each section; these were then incubated in development buffer for 1 hour at 37°C. As a positive control, in 1 sample MMP activity was blocked by MMP inhibitor 1, 10-phenanthroline monohydrate (0.2 mmol/L; Life Technologies, OR). Images were captured using EVOS XL Cell Imaging System.

Histological Analysis

Formalin-fixed samples were embedded in paraffin, and 5-μm sections were mounted on glass slides and heated overnight to adhere the tissues to the slides and melt the paraffin. Subsequently, the slides were deparaffinized with xylene and graded ethanol and stained with hematoxylin and eosin for tissue morphology, Verhoeff-van Gieson for elastic fibers, and Alizarin Red S with a Light Green SF counterstain for calcification.

Immunostaining for Macrophages (CD80)

Aortic sections from the 38-day aneurysm study were used. Tissues preserved with formalin were embedded in paraffin and sectioned as previously described. Subsequently, the slides were deparaffinized with xylene and graded ethanol, and antigen retrieval was done using the HCl method. The slides were incubated overnight at room temperature with the primary antibody, Mouse Anti Rat CD80 (Bio-Rad, Hercules, CA). Subsequently, the slides were incubated for 1 hour with the secondary antibody, Cy7 goat antimouse IgG (H+L; Bioss Inc, Woburn, MA), and 4,6-diamidino-2-phenylindole nucleic acid stain and then imaged with a fluorescent microscope, EVOS XL Cell Imaging System.

Aneurysmal Development

Initial external diameter was measured at the time of the calcium chloride (CaCl2) application. Final aortic diameter was recorded before euthanasia on day 38. Aneurysmal development was calculated as shown below:

\[
\text{Aneurysmal development \%} = \frac{\text{Final external diameter} - \text{Initial external diameter}}{\text{Initial external diameter}} \times 100
\]

Statistical Analysis

In vitro experiments were performed in triplicate and repeated twice; for in vivo studies, 5 animals were used per group per time point. Student t test was performed using Microsoft Excel, and exact permutation test was performed using the NPAR1WAY procedure in SAS. The data are expressed as the mean±SD; results were considered to be significant when \( P \leq 0.05 \).

### Table 1. Characterization of NPs and BB-94 Loading

<table>
<thead>
<tr>
<th>Polymer:BB-94 Ratio</th>
<th>% Yield, n=4</th>
<th>% Loading, n=3</th>
<th>NP size (nm), n=3</th>
<th>( \zeta )-Potential (mV), n=3</th>
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<tbody>
<tr>
<td>15:1</td>
<td>62.9±33.9</td>
<td>5.7±2.5</td>
<td>123.8±24.9†</td>
<td>−83.1±2.8*,†</td>
</tr>
<tr>
<td>10:1</td>
<td>44.2±3.3</td>
<td>8.4±2.7</td>
<td>153.5±26.8*</td>
<td>−47.2±5.5*,†</td>
</tr>
<tr>
<td>5:1</td>
<td>38.4±18</td>
<td>6.7±0.4</td>
<td>196.3±3.3</td>
<td>−29.1±5.1</td>
</tr>
</tbody>
</table>

†Statistical significance (Student unpaired t test, \( P < 0.05 \)).

*Statistical significance (Student unpaired t test, \( P < 0.05 \)).

BB-94 indicates batimastat; and NP, nanoparticles.
Results

NP Characterization
NPs were prepared with 3 different initial BB-94 concentrations (5:1, 10:1, and 15:1 polymer:drug ratio). No significant differences were found for the percent yield of NPs among the 3 batches. Increasing the initial BB-94 concentration during NP preparation did not increase the final BB-94 loading in the NPs. Particle surface charge was dependent on initial polymer to BB-94 ratios. All BB-94-loaded NPs were negatively charged; however, a higher polymer to BB-94 ratio led to a more negative surface charge (Table).

NP Degradation and BB-94 Release Study
A higher polymer:BB-94 ratio led to a smaller NP size, which was confirmed visually by transmission electron microscopy (Figure 1A). When suspended in distilled water, blank NPs lost substantial weight (65±4.5%) in 4 weeks; the majority of weight loss occurred in the first week (45.6±6.8; Figure 1B). These data were corroborated by NP degradation seen in the gel permeation chromatography study (Online Data Supplement).

In vitro BB-94 release from the NPs was gradual and continued ≤8 days. Although final BB-94 loading was similar for all polymer:BB-94 ratios, the BB-94 release profile varied with the starting BB-94 concentration (Figure 1C). Slower release was observed in NPs with a 5:1 polymer:BB-94 ratio, whereas significant burst release was observed when the initial BB-94 concentration was lower (10:1 or 15:1 polymer:BB-94 ratio). BB-94 release was more controlled for the batch with a higher starting BB-94 concentration (5:1 polymer:BB-94 ratio), so these NPs were chosen for further study.

Activity of BB-94
The activity of the BB-94 loaded in NPs was examined using gel zymography. When extracted BB-94 was added to the development buffer, pro-MMP-2 (72 kDa), active MMP-2 (62 kDa), and MMP-9 (92 kDa) activities were inhibited completely (Figure 2A). When BB-94 extracted from the NPs was added to the RASMC cultures, 90% of MMP-9 (92 kDa) and 10% of MMP-2 (62 kDa) activities were inhibited.

Figure 1. Transmission electron microscopy (TEM), degradation profile of nanoparticles (NPs) and release of batimastat (BB-94) from NPs. A, TEM images of 15:1, 10:1, and 5:1 polymer to BB-94 ratio NPs. B, Weight loss of blank NPs during 4 weeks. Weight loss showed the gradual polymeric degradation from week 1 to week 4. (n=3). C, Release kinetics of BB-94 for 8 days. This 5:1 polymer:BB-94 ratio showed a more controlled and longer release profile (n=3).

Figure 2. Activity of batimastat (BB-94) in nanoparticles (NPs). A, Gel zymography study, BB-94 extracted from NPs was added to the development buffer (+) or no BB-94 (-). Relative activity of pro and active forms of matrix metalloproteinase (MMP)-2 and MMP-9 was totally inhibited by BB-94. B, Cell-culture study, rat aortic smooth muscle cells (RASMCs) treated with medium containing 500 nmol/L extracted BB-94 in dimethyl sulfoxide (+), RASMCs treated with dimethyl sulfoxide alone (-), MMP-9 and active MMP-2 were inhibited 90% and 10%, respectively. C, Reverse gel zymography showing no significant changes in tissue inhibitors of MMP (TIMP)-2 activity present in the cell culture medium of RASMCs either in the presence (+) or in the absence (-) of BB-94. *Student unpaired t test, P<0.05 and #exact permutation test, P<0.05; n=6 for zymography and n=4 for reverse zymography. Dashed line represents the mean value.
(Figure 2B). Notably, more MMP-2 remained in the proform (inactivated, 72 kDa) when BB-94 was added to cell cultures (Figure 2B). There was no significant difference in the expression of TIMP-2 (21 kDa) between control and BB-94-treated cells (Figure 2C). These results suggest that BB-94-loaded NPs inhibited MMPs without affecting TIMP-2 levels.

**NP Cytotoxicity and Cellular Uptake**

Rat aortic endothelial cells and RASMC viability showed no significant change in 24 hours in the presence of NPs when compared with the control (Figure 3A and 3B). We have already shown that such NPs are not taken up by vascular smooth muscle cells.19 Inflammatory cells such as macrophages are commonly present at the site of AAA. Thus, we determined whether our NPs were resistant to macrophage uptake. NPs labeled with a DIR dye were incubated with macrophage cell cultures for 24 hours (ζ-potential: −29.1±5.1, size: 196.28±3.2 nm). Because of the negatively charged surface of the chosen NPs, no macrophage uptake was observed (Figure 3C–a1, a2, and a3). When the surface charge was changed to positive with the addition of chitosan (ζ-potential: +20.28±4.93), NPs could be seen in the cell cytoplasm; this suggests that

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**Figure 3.** Macrophage uptake of elastin antibody–conjugated nanoparticles (NPs). A, No change in rat aortic endothelial cells (RAOECs) viability after incubation with blank NPs, 70% ethanol as positive control and no NPs as negative control (n=6). B, No change in rat aortic smooth muscle cell (RASMC) viability after incubation with blank NPs, 70% ethanol as positive control and no NPs as negative control (n=6). C, 1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide (DIR) dye-loaded NPs (purple, size: 196 nm and ζ-potential: −29.1±5.1) after 24-hour incubation with macrophages showing no uptake (a1 shows DIR-loaded NPs, a2 shows white light image, and a3 shows merged image). NPs were taken up by cells either when the surface charge was positive (b1, b2, and b3) or the size was small (c1, c2, and c3). Bar, 25 μmol/L. D, Live/dead assay for macrophages incubated with batimastat (BB-94) NPs for 24 hours: 0.5, 1, 2, and 2 mg/mL Blank NPs. None of the concentrations above showed any toxicity to the macrophages. *Student unpaired t test, P<0.05 and #exact permutation test, P<0.05; n=6. Dashed line represents the mean value.
they were taken up by macrophages (Figure 3C–b1, b2, and b3). When NP size was changed to ≈125 nm and the surface charge remained negative (ζ-potential: −83 ± 2.8), there was again significant uptake by macrophages (Figure 3C–c1, c2, and c3). Overall, ≈200-nm NPs with negative surface charge that were chosen for further studies were resistant to macrophage uptake. Bone marrow macrophage viability showed no significant change in 24 hours for blank and BB-94-loaded NPs at the 13-fold concentration used in vivo (Figure 3D).

**In Vivo NP Targeting and MMP Inhibition**

We have shown that NPs with a surface-conjugated antielastin antibody can target the site of aortic injury in this animal model.19 We confirmed in vivo targeting by observing localized fluorescence at the injury site of the abdominal aorta for the EL-NP-DIR group, this suggests excellent targeting to the injured aorta (Figure 4A), whereas no fluorescence was observed in the injured aorta for control IgG antibody–conjugated NPs loaded with DIR dye group (Figure 4A).

We next examined whether NPs loaded with BB-94 would target the injury site and inhibit local MMP activity. When normalized to total protein content fluorescence intensity data showed that the abdominal aorta had =50% higher MMP activity than the thoracic aorta in the EL-NP-Blank group (Figure 4B). This result suggests that MMPs were activated by CaCl2-mediated injury and that blank NPs targeted to the abdominal aorta did not suppress MMP activity. The EL-NP-BB94 group showed 56% lower MMP activity in the abdominal aorta in comparison with the thoracic aorta, suggesting that MMP activity was completely suppressed at the injury site (Figure 4B). When the same concentration of BB-94 was delivered systemically by IP injection, it was ineffective in suppressing local MMP activity in the abdominal aorta (Figure 4B). These results were further confirmed by in situ zymography studies on histological sections of abdominal aorta in different groups. The green fluorescence in this assay is caused by the enzymatic degradation of dye-quenched gelatin, which directly corresponds to the MMP activity in the sections. Intense green fluorescence corresponding to higher MMP activity was found in the sections of the abdominal aorta in the control animals receiving EL-NP-Blank or IP-injected BB-94 (IP-BB94); a significant suppression of MMP activity was observed in the BB-94 NP group (EL-NP-BB94; Figure 4C). This was similar to the positive controls, where MMP activity was inhibited by the addition of 1, 10-phenanthroline during in situ zymography.

**Long-Term Targeting and Biodistribution of NPs**

With encouraging results in the 48-hour targeting experiment, we next determined if such targeting could inhibit MMP activity and aneurysmal development for prolonged periods. Because our BB-94 release study showed slow release for ≤7 days, we decided to inject elastin antibody–conjugated NPs once weekly for 4 weeks. After injections (4 injections during a total of 38 days after the first CaCl2 injury), a 3-fold increase (from 26.3% to 78.7%) was seen in the fluorescence signal in the abdominal aorta for the EL-NP-DIR group when compared with a single injection (Figure 5A compared with Figure 4A), indicating more NPs accumulated at the injury site. Bio distribution of NPs (Figure 5B) showed NPs in lung, liver, kidneys, and spleen in addition to the aorta at 48 hours after injection. After 38 days and 4 weekly injections, the signal decreased from 0.78% to 0.003% for the kidneys, from 15% to 2.7% for the liver, and from 48% to 18% for the spleen in comparison with the 2-day study (Figure 5B). These data show that additional NPs accumulated at the injury site, whereas other organs were clearing nontargeted NPs. More importantly, the NPs were seen infiltrating from the adventitial side of the aneurysmal aorta through the vasa vasorum rather

![Figure 4. In vivo targeting of batimastat (BB-94)-loaded nanoparticles (NPs). A. Ten days after CaCl2 injury, NPs were injected systemically (tail vein) and allowed to target for 48 hours. Fluorescent image of whole aorta taken by Lumina XR in vivo imaging system. Left, elastin antibody–conjugated DIR-loaded NPs (EL-NP-DIR) group showing NPs targeting aneurysmal aorta while sparing healthy thoracic aorta. Right, IgG antibody–conjugated NPs loaded with DIR dye (IgG-NP-DIR) group as control showing no targeting of NPs. B, Matrix metalloproteinase (MMP) activity as measured by fluorogenic substrate assay showing >150% increase in systemic BB-94 (at the same dose as BB-94 NPs and IP-BB94) and blank NP (EL-NP-Blank) groups in the abdominal aorta region (CaCl2-mediated injury) compared with the thoracic aorta (noninjured, healthy) showing MPPs were not inhibited in the abdominal aorta by these treatments. Only the elastin antibody–conjugated BB-94-loaded NPs (EL-NP-BB94) group showed a significant decrease in MMP activity in the abdominal aorta. *Student unpaired t test, P < 0.05 and #exact permutation test, P < 0.05; n = 5. Dashed line represents the mean value. C, MMP activity in abdominal aortic sections by in situ zymography. Green signal corresponds to active MMPs and blue signal corresponds to 4′,6-diamidino-2-phenylindole staining for cell nuclei. Only EL-NP-BB94 (ii) group shows suppression of MMPs, whereas high MMP activity is seen in EL-NP-Blank (i) and IP-BB94 (iii). Positive control: abdominal aorta section incubated with MMP inhibitor shows minimal fluorescence (iv). *Lumen. Bar, 100 μm. DIR indicates 1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide; and IP, intraperitoneal.
than from the luminal side and then attaching to the degraded elastic lamina deep within the medial layer (Figure 5C).

Long-Term Inhibition of MMPs and Aneurysmal Development

When NPs were injected weekly for 4 weeks after CaCl$_2$ injury, MMP activity was still suppressed in animals receiving EL-NP-BB94 NPs (similar to the 48-hour study), whereas MMPs remained elevated in the EL-NP-Blank group (Figure 5D).

Hematoxylin and eosin staining showed significant inflammation in the adventitia in the EL-NP-Blank group, whereas the EL-NP-BB94 group maintained greater structural integrity and had little inflammation (Figure 6A and 6E, respectively). Verhoeff-van Gieson staining revealed that the elastic lamina was broken and damaged in the EL-NP-Blank group, but elastin preservation was observed in the EL-NP-BB94 group (Figure 6B and 6F). Similarly, Alizarin Red S staining showed heavy medial calcification in the (EL-NP-Blank) group and a substantial reduction in calcification in the BB94-treated group (EL-NP-BB94; Figure 6C and 6G). Macrophage immunohistochemistry revealed that the blank NP group had a higher density of M1 macrophages in the adventitia and ruptured media in comparison with the BB-94 group (Figure 6D and 6H).

We next determined aneurysmal expansion by measuring the external-aortic diameter (Figure 7A and 7B). When control blank NPs were injected (EL-NP-Blank), a large increase in diameter compared with the size before injury was observed (269.5±56%), suggesting that targeted blank NPs did not inhibit aneurysmal development. However, when BB-94-loaded NPs were injected, significant suppression of aneurysms was observed (40.25±26% increase in diameter; Figure 7).

Discussion

This study demonstrates that the use of targeted NPs carrying a potent MMP inhibitor can successfully inhibit local MMP activity in inflammatory vascular conditions, such as AAA. We optimized NP size, surface charge, and BB-94 loading. Our data agree with the literature: during NP preparation, the particle size increased with increasing drug concentration and the negative surface charge decreased.23 Particle size and drug loading are important parameters that dictate the release of a drug from NPs. The negative surface charge facilitates electric repulsion among NPs, thus increasing NP stability. 24 Surprisingly, increasing initial BB-94 concentration did not lead to a higher amount of BB-94 loading; the maximal loading was only 6% to 8% of BB-94 in poly($\alpha$-$\delta$-lactide). This may have occurred because of the physical properties of the original drug.25 Although drug loading was similar, drug dispersion within NPs, and thus ultimate release, varied with initial drug concentration. Our study shows that higher polymer content (15:1 and 10:1 polymer:BB-94 ratio) resulted in burst release on day 1. This may have occurred because of the smaller particle size with higher surface area for drug diffusion. NPs in the 5:1 polymer:BB-94 ratio bath had the biggest particle size and showed a more controlled release because of either lower surface area or better drug encapsulation. Our NP degradation data with blank NPs showed significant weight loss after 7 days and an increase in polymer degradation products as assessed by gel permeation chromatography (Online Data Supplement), suggesting that drug diffusion and polymer degradation occurred simultaneously.

Maintaining drug activity during NP synthesis is necessary for success in targeting. We showed that BB-94 extracted from NPs suppressed MMP activity both in gel zymography and in...
cell cultures. We found that adding BB-94 to the development buffer during gel zymography was more effective in inhibiting MMPs (both the active and pro forms) than adding to cell culture media. It is possible that BB-94 dissociated during gel electrophoresis when the cell culture media was loaded in gels and, thus caused lower MMP inhibition. The findings from reverse zymography showed no significant difference in the expression of TIMP-2 protein within the groups; this is consistent with the literature showing that BB-94 has no effect on TIMPs.26

Particle size and charge are important criteria in targeting NPs to the vasculature. It has been shown that NP targeting of vascular ECM in an aneurysmal site occurred for particle sizes <200 nm.19 That study also showed that a negatively charged NP surface prevented NP uptake by vascular smooth muscle cells. Because macrophages are phagocytic and present at the aneurysmal site, we determined whether a negative surface charge would also prevent the uptake of NPs by macrophages. We showed that macrophages do not take up ≈200 nm BB-94-loaded NPs (5:1 polymer:BB-94 ratio, negative surface charge). On the basis of these results and previous vascular smooth muscle cell uptake results, these NPs would remain in the ECM.

Elastin antibody conjugation on the NP surface also allowed us to target these NPs to the site of elastin damage found in the aneurysm site because this antibody recognizes only core amorphous elastin that is exposed during elastic lamina degradation.19 Our data with DIR dye–loaded NPs confirmed that they targeted only the injury site while sparing the healthy aorta. More importantly, we found that NPs entered preferentially from the adventitial side through the vasa vasorum and lodged deep in the media when delivered systemically. This is clinically advantageous because the intraluminal thrombus generally present in an aneurysm can obstruct NP targeting from the luminal side. Notably, more degradation and inflammation are seen clinically in the adventitia.27

MMPs, in particular MMP-9 (gelatinase B/92 kDa) and MMP-2 (gelatinase A/72 kDa), play a significant role in AAA development and progression.6 The goal of targeted treatment is to suppress MMP activity at the site of AAA, so that further ECM degradation can be prevented. Our MMP fluorogenic assay showed that the MMP-2 and MMP-9 activities (represented as a ratio of abdominal aorta over thoracic aorta in the same animal) were 50% higher in the EL-NP-Blank and BB-94 IP

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**Figure 6.** Histological analysis of abdominal aorta after 38 days. Hematoxylin and eosin staining (A and E) and Verhoeff-van Gieson staining for elastin (B and F) of aortic sections showing medial elastic lamina damage in the elastin antibody–conjugated blank nanoparticles (EL-NP-Blank) group with the presence of a significant adventitial inflammatory capsule, whereas the media is intact in the elastin antibody–conjugated BB-94-loaded NPs (EL-NP-BB94) group. Alizarin Red S staining for calcification (red stain for calcium) with a Light Green SF counterstain (C and G) showing heavy calcification in the EL-NP-blank group while there is no calcification in EL-NP-BB94-treated group. Immunohistochemistry for M1 macrophages (purple color) showing high activity in the EL-NP-Blank group while there is little macrophage activity in EL-NP-BB94 group (D and H). A, B, C, and D are EL-NP-Blank group and E, F, G, and H are EL-NP-BB94 group; *Lumen.

**Figure 7.** Inhibition of aneurysm development by targeted therapy. Targeted nanoparticles (NPs) with no drug (elastin antibody–conjugated blank NPs [EL-NP-Blank]) or with drug (elastin antibody–conjugated BB-94-loaded NPs [EL-NP-BB94]) were intravenously administered once a week for 4 weeks. Aneurysmal development in the blank NP group was significantly higher in comparison with the group receiving batimastat (BB-94) NPs. *Student unpaired $t$ test, $P<0.05$ and #exact permutation test, $P<0.05$. A and B, Representative images of aorta at euthanization. C, External diameter in 2 groups (n=5).
groups, suggesting that sustained MMP activation was induced by CaCl₂ injury and was not inhibited in the abdominal aorta by these treatments. Only when BB-94 was delivered by targeted NPs (EL-NP-BB94) was a significant decrease in MMP activity seen in the abdominal aorta for prolonged periods, suggesting that targeted NPs delivered BB-94 at the site of injury and inhibited local MMP expression. This can either occur by the suppression of macrophage recruitment as we have observed which is consistent with previous study⁵⁸ or because of inhibition of MMPs secreted by vascular smooth muscle cells. These data are corroborated by the qualitative in situ zymography findings on histological sections of the abdominal aorta, which show suppression of gelatinolytic activity in tissues in the EL-NP-BB94 group alone.⁵⁹ Others have shown that a daily IP injection of 15 mg of BB-94 inhibited the expansion of AAA in elastase-induced aneurysms in rats.¹⁵ However, the study lasted for only 7 days, and AAA was treated only by systemic side effects and reduced patient compliance,³³,³⁴ thus β-temic delivery of, due to a 580-fold lower BB-94 concentration than others have used (equivalent to 25 μM), has not been achieved systemically.³⁹ Several clinical trials for systemic MMP inhibition as a cancer treatment failed because of poor bioavailability, dose limiting toxicity, and systemic side effects, such as musculoskeletal problems, that limited the dose that could be tolerated.³⁰ The side effects caused by MMP inhibitors in cancer patients led to the use of substantially lower doses of MMP inhibitors in clinical trials for aneurysm prevention.¹⁸ The propanolol treatment was successful in aneurysm-prone transgenic mice and caused an increase in the tensile strength of tissue rings from the abdominal aorta.³² However, clinical trials of the systemic delivery of, β-blockers—like propranolol, did not show significant changes in the growth rate of aneurysms, but caused systemic side effects and reduced patient compliance,³³,³⁴ thus dampening enthusiasm for pharmacological therapy for AAA. Because our targeted treatment’s site-specificity permits low doses of the drug, such therapy would be an attractive option for preventing the expansion of aneurysms in patients without causing systemic side effects.

In conclusion, we demonstrate that targeted delivery of small doses of MMP inhibitor BB-94 by NPs is an effective way to suppress MMP activity and aneurysmal development in an experimental rat model of AAA.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

• No pharmacological treatments are currently available to halt abdominal aortic aneurysm (AAA) growth.
• Systemic drug therapies often have side effects.

What New Information Does This Article Contribute?

• Smart nanoparticles (NPs) were designed to carry drugs to the AAA site.
• Systemic injection of NPs led to delivery of the drug to the site of the aneurysm at low drug doses, and the treatment inhibited aneurysmal growth.
• AAA is an abnormal focal bulging of a vessel because of structural weakness. Currently, there are few options to treat diagnosed AAA other than regular monitoring of its growth. Surgical placement of a vascular graft is not recommended until the diameter of the AAA reaches ≥5 cm as the risk associated with the surgery outweighs the benefit; however, 10% of deaths occur with AAA below that diameter. Systemic pharmacological treatments administered clinically to halt aneurysmal growth have not been successful. Matrix metalloproteinase-mediated elastic lamina degradation is the main cause of aneurysm formation, but global inhibition of matrix metalloproteinases is detrimental to healthy tissue.

We have developed a novel targeted, NP-based drug therapy for AAA. Using NPs surface conjugated with an elastin antibody, which recognizes only degraded elastin, and loaded with a matrix metalloproteinase inhibitor batimastat, we show that batimastat could be delivered to the site of the aneurysm in a calcium chloride injury model of AAA in rats. We found that NP injection inhibited AAA growth at a low dose. Such targeted therapy could potentially be a useful pharmacological therapy for treating patients with early stage AAA.
Prevention of Abdominal Aortic Aneurysm Progression by Targeted Inhibition of Matrix Metalloproteinase Activity With Batimastat-Loaded Nanoparticles
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*Synthesis of batimastat-loaded nanoparticles (EL-NP-BB 94)*

PLA (10 mg, average MW 75k-120k) (Sigma Aldrich, St. Louis, MO) was dissolved in 1 ml acetone (VWR International, Radnor, PA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol) - 2000] (2 mg, DSPE-PEG (2000) Maleimide, Avanti Polar Lipids, Inc., Alabaster, AL) and BB-94 (Sigma Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO), and this solution was then added to the PLA solution. The polymer solution was added drop-wise (16 µl/sec) to water and kept under sonication (Omni Ruptor 4000) for 20 minutes at 4°C. Following sonication, the particles were washed twice with distilled water by centrifugation at 14000x g for 30 minutes at 4°C and then re-suspended in distilled water. The non-solvent (water) to solvent (acetone) ratio was 1:15 for all experiments. Three different batches containing ratios of 5:1, 10:1, and 15:1 polymer to BB-94 were prepared in which the ratio between the two polymers (PLA: DSPE-PEG(2000) Maleimide) was 4:1.

*Nanoparticles characterization*

The NP suspension (1 mg) was diluted in HPLC-grade water, and the ζ-potential and particle size were measured with a 90Plus particle size analyzer (Brookhaven Instruments Co, Holtsville, NY). Transmission Electron Microscopy (TEM) was used to study NPs morphology. A drop of 0.1 mg/ml NPs-water suspension was placed on a formvar-coated copper grid and dried overnight in vacuum desiccator. Grid-mounted samples were imaged using a Hitachi H7600 TEM.

*Nanoparticle degradation study as assessed by gel permeation chromatography (GPC)*
Nanoparticles (5 mg) were suspended in 5 mL of DI water at stirred at 37°C. At week 1 and week 4, NPs were isolated by filtration and lyophilized. NPs were dissolved in chloroform at a concentration of 1mg/ml. The solution was filtered directly into chromatography vials using 0.2µm PTFE filters. Polymer molecular weight was assessed by Size Exclusion Chromatography/ Gel Permeation Chromatography (SEC/GPC) using a Shodex KF-804L column on a Waters HPLC/GPC system equipped with an Autosampler, column heater, and refractive index (RI) Detector. Chloroform was used as the mobile phase at a flow rate 0.65 ml/min, and the column was kept at 30°C during all separation runs. The injection volume for GPC analysis was 50 µL. Polystyrene standards of 9, 35, 50, 100 and 200 kDa were used as markers, and a control sample of PLA (Average Mₘ 75,000-120,000 kDa) was used to validate the calibration curve reliability.

Polymer nanoparticles as prepared showed one broad peak at retention time at ~9 min. With time, low molecular weight products (with retention time ~12-13 min) appeared suggesting polymer degradation (Figure I).
Figure I: GPC curves show peaks for degradation products at retention time (~13 min) at week 1 and 4.

**Release profile of BB-94**

To study release kinetics, a known amount of NPs was suspended in phosphate buffered saline (PBS) and incubated at 37°C on a shaker. Suspensions were centrifuged for 30 minutes at 10000×g at room temperature (RT). The supernatant was removed, and the sediment was freeze-dried and characterized for BB-94 concentration by dissolving it in 100 µl DMSO and measuring absorbance by UV spectrophotometry.

**BB-94 activity**

Briefly, NPs were dissolved in dichloromethane (Aldrich, MO) and DI water at a ratio of 3:1. This mixture was mixed vigorously and centrifuged for 10 minutes at 10000×g to extract BB-94 from the NPs. The DI water containing BB-94 was subsequently collected and lyophilized. The activity of the extracted BB-94 from the NPs was tested by two different methods. In the first approach, culture medium collected from rat aortic smooth muscle cell (RASMC) cultures was loaded into a SDS-PAGE zymography gel, and extracted BB-94 was added to the development buffer(500ng/ml) (50 mM Tris Base, 5 mM CaCl2•2H2O, 200 mM NaCl, 0.02% brij 35). Gels were stained with 0.5% coommasie blue for an hour at room temperature and were destained with 5% water, 40% methanol, 10% acetic acid. After this, the gels were photographed using epi-illuminated white light in a Bio-Rad Gel Doc instrument. Zymography bands were quantified using Image J software. In the second approach, BB-94 activity was tested in RASMCs cultures. Briefly, RASMCs were grown to 80% confluency at 37°C and 5% CO2 in DMEM containing 10% FBS and 1% penicillin-streptomycin (ScienCell Research Laboratories, Carlsbad, CA). After 24 hours, cells were treated with extracted BB-94 (500 nM) in serum-free medium for 24 hours. After treatment, conditioned medium was collected. The total protein of the culture medium was
quantified using Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL) with 10 µg total protein loaded per well and prestained molecular weight standards (Precision Plus Protein Standard, Bio-Rad, Hercules, CA). Gel zymography was performed as described in the previous section.

Reverse zymography for TIMP activity

After 24 hours incubation in the development buffer, gels were stained with 0.5% coommasie blue for an hour and were destained. The gel pictures were captured using epi-illuminated white light and a Bio-Rad Gel Doc instrument; bands were analyzed and quantified using Image J, and data was reported as RDU.

NP toxicity for rat aortic smooth muscle cells (RASMCs) and rat aortic endothelial cells (RAOEC)

RASMCs and RAOECs were cultured with NPs for 24 hours. MTT colorimetric assays (Sigma Aldrich, St. Louis, MO) were performed according to the manufacturer’s protocol to confirm cell viability. Control and NP-treated cells were washed with PBS and incubated for 3 h with 55 µl of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml). Formazan crystals formation was detected at 570 nm and viability was shown as % MTT reduction compared to control. MTT activity was normalized to control.