Pharmacological Stabilization of Intracranial Aneurysms in Mice
A Feasibility Study

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Background and Purpose—An increasing number of unruptured intracranial aneurysms are being detected, partly due to the increased use of brain imaging techniques. Pharmacological stabilization of aneurysms for the prevention of aneurysmal rupture could potentially be an attractive alternative approach to clipping or coiling in patients with unruptured intracranial aneurysms. We have developed a mouse model of intracranial aneurysm that recapitulates key features of intracranial aneurysms. In this model, subarachnoid hemorrhage from aneurysmal rupture causes neurological symptoms that can be easily detected by a simple neurological examination. Using this model, we tested whether anti-inflammatory agents such as tetracycline derivatives, or a selective inhibitor of matrix metalloproteinases-2 and -9 (SB-3CT), can prevent the rupture of intracranial aneurysms.

Methods—Aneurysms were induced by a combination of induced hypertension and a single injection of elastase into the cerebral spinal fluid in mice. Treatment with minocycline, doxycycline, or SB-3CT was started 6 days after aneurysm induction. Aneurysmal rupture was detected by neurological symptoms and confirmed by the presence of intracranial aneurysms with subarachnoid hemorrhage.

Results—Minocycline and doxycycline significantly reduced rupture rates (vehicle versus doxycycline=80% versus 35%, P<0.05; vehicle versus minocycline=73% versus 24%, P<0.05) without affecting the overall incidence of aneurysms. However, SB-3CT did not affect the rupture rate (62% versus 55%, P=0.53).

Conclusions—Our data established the feasibility of using a mouse model of intracranial aneurysm to test pharmacological stabilization of aneurysms. Tetracycline derivatives could be potentially effective in preventing aneurysmal rupture.

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Key Words: animal model ▪ inflammation ▪ intracranial aneurysm ▪ intracranial hemorrhage ▪ matrix metalloproteinase ▪ subarachnoid hemorrhage ▪ tetracycline

An increasing number of unruptured intracranial aneurysms are being detected, partly due to the increased use of brain imaging techniques. In patients with unruptured aneurysms, surgical clipping or endovascular coiling is performed to prevent future aneurysmal rupture. However, the morbidity and mortality associated with clipping and coiling of unruptured aneurysms are not negligible.1,2 In addition, there are limited treatment options for a subset of patients with giant aneurysms. Therefore, pharmacological stabilization of aneurysms as a means of rupture prevention may be an attractive alternative approach. However, currently there is no known pharmacological stabilization of aneurysms in the prevention of aneurysmal rupture. This is partly due to the lack of appropriate animal models for conducting preclinical studies in the pharmacological stabilization of aneurysms.

The potential role of inflammation in the pathophysiology of intracranial aneurysms has been suggested by both clinical and animal studies.3-8 Tetracycline derivatives such as doxycycline and minocycline are clinically available antibiotic agents that possess anti-inflammatory effects. In addition, these agents can exert weak inhibitory effects on matrix metalloproteinases (MMPs).9,10 Pharmacotherapy using tetra-
cyccline derivatives as anti-inflammatory agents or broad-spectrum MMP inhibitors have been proposed for various vascular diseases.\(^8\)\(^9\)\(^1\)

Recently, we have developed a mouse model of intracranial aneurysm that recapitulates key features of intracranial aneurysms, including spontaneous rupture.\(^7\)\(^\text{–}^\text{13}\) In this model, subarachnoid hemorrhage as a result of aneurysmal rupture causes neurological symptoms that can be easily detected by a simple neurological examination. As a first step in the process of testing the pharmacological stabilization of aneurysms in this model, we examined whether tetracycline derivatives can prevent aneurysmal rupture.

Methods

Animal Model

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Intracranial aneurysms were induced in 8- to 10-week-old male mice (C57BL/6J; Jackson Laboratory, Bar Harbor, ME) using a previously described method with modifications.\(^7\)\(^\text{–}^\text{13}\) We combined induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid at the right basal cistern. Detailed methods are presented in the online-only Data Supplement.

To induce systemic hypertension, we used deoxycorticosterone acetate-salt hypertension.\(^14\) Mice underwent nephrectomy followed by an implantation of deoxycorticosterone acetate pellet 1 week later; 1% sodium chloride drinking water was started on the same day as the deoxycorticosterone acetate pellet implantation.\(^4\)\(^\text{–}^\text{15}\) Mice received a single injection of elastase (25–35 nM) into the cerebrospinal fluid at the right basal cistern on the same day as deoxycorticosterone acetate pellet implantation.\(^7\)\(^\text{–}^\text{13}\) Aneurysms were defined as a localized outward bulging of the vascular wall whose diameter was greater than the parent artery diameter.\(^7\)\(^\text{–}^\text{13}\)

Two blinded observers performed daily neurological examination using a previously described method with modifications.\(^6\)\(^\text{–}^\text{19}\) Neurological symptoms were scored as follows: 0, normal function; 1, reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (approximately 10% weight loss) over 24 hours; 2, flexion of the torso and forelimbs on lifting of the whole animal by the tail; 3, circling to one side with a normal posture at rest; 4, leaning to one side at rest; 5, no spontaneous activity. Mice were euthanized when they developed neurological symptoms (score 1–5). All asymptomatic mice were euthanized 28 days after aneurysm induction. The brain samples were perfused with phosphate-buffered saline followed by a gelatin containing blue dye to visualize cerebral arteries as well as to assess for aneurysm formation and subarachnoid hemorrhage.

In Situ Zymography

Gelatinase activity, primarily from MMP-2 and MMP-9, was assessed by gelatin in situ zymography.\(^11\)\(^\text{–}^\text{15}\) Pretreatment of the tissues with 1,10-phenanthroline monohydrate was used to confirm the MMP origin of the gelatinase activity.\(^11\)

Treatment With Doxycycline, Minocycline, or SB-3CT

To test whether doxycycline and minocycline, anti-inflammatory agents with broad-spectrum MMP inhibitory effects, could stabilize aneurysms, we started treatment with doxycycline, minocycline, or vehicle 6 days after aneurysm induction and continued the treatment for 3 weeks.

Twenty-five mice were treated with doxycycline dissolved in water (40 mg/kg/day through gavage) as described.\(^13\)\(^\text{–}^\text{20}\) Twenty-two mice in the doxycycline vehicle group received the vehicle (water). Twenty-six mice were treated with minocycline (45 mg/kg/day) through daily intraperitoneal injection; 21 mice in the minocy-
artery) showed limited gelatinase activity (Figure 2A). As shown in Figure 2B, gelatinase activity was restricted to the intracranial aneurysms and the adjacent parent artery (middle cerebral artery) had little gelatinase activity. There was a general trend of more prominent gelatinase activity in ruptured aneurysms than in unruptured aneurysms (Figure 2B–C). However, this might be due to inflammation caused by subarachnoid hemorrhage rather than the intrinsic property of rupture-prone aneurysms.

Effects of Doxycycline and Minocycline on Aneurysmal Rupture
To test whether tetracycline derivatives—doxycycline and minocycline—could prevent aneurysmal rupture, we initiated a pharmacotherapy with doxycycline, minocycline, or vehicle 6 days after aneurysm induction for a total treatment course of 3 weeks (Figure 1D).

Although there was no difference in the overall incidence of aneurysms (including both ruptured and unruptured) be-
between the vehicle and doxycycline group (68% versus 68%, P=0.62), the incidence of ruptured aneurysms was significantly lower in the doxycycline group than in the vehicle group (24% versus 55%, P<0.05). B, Rupture rate. The doxycycline treatment significantly reduced the rupture rate compared with the vehicle treatment (80% versus 35%, P<0.05). C, Symptom-free curve (Kaplan-Meier analysis curve). A log-rank test revealed a significant reduction of aneurysmal rupture by the doxycycline treatment (P<0.05). Mice that did not develop aneurysms were excluded from the survival analyses.

For the purpose of exploratory analysis, a symptom-free curve (Kaplan-Meier analysis curve) was created after excluding mice that did not have aneurysms (Figure 3C). A log-rank test revealed a significant reduction of aneurysmal rupture by the doxycycline treatment (P<0.05).

There was no difference in the overall incidence of aneurysms (both ruptured and unruptured) between the vehicle and minocycline group (71% versus 65%, P=0.45; Figure 4A). However, the incidence of ruptured aneurysms was significantly lower in the minocycline group than in the vehicle group (16% versus 52%, P<0.05; Figure 4A).

Furthermore, minocycline treatment significantly reduced the rupture rate when compared with the vehicle treatment (73% versus 24%, P<0.05; Figure 4B). A log-rank test for mice with aneurysms revealed a significant reduction of aneurysmal rupture with minocycline treatment (P<0.05; Figure 4C).

**Effects of SB-3CT on Aneurysmal Rupture**

As a next step, we tested whether the potent and selective inhibitor of MMP-2 and MMP-9, SB-3CT,22 can prevent aneurysmal rupture. There was no difference in the overall incidence of aneurysms (both ruptured and unruptured) between the vehicle and SB-3CT group (62% versus 55%, P=0.53; Figure 5A). In addition, there was no difference in the rupture rate between the vehicle group and SB-3CT group, with SB-3CT treatment significantly reducing the rupture rate compared to the vehicle group (73% versus 24%, P<0.05; Figure 5B). A log-rank test for mice with aneurysms revealed a significant reduction of aneurysmal rupture by the SB-3CT treatment (P<0.05; Figure 5C).
A log-rank test revealed no difference between the vehicle group and SB-3CT group ($P = 0.79$; Figure 5C). Treatment with tetracycline derivatives or SB-3CT did not affect blood pressure (Table).

### Discussion

In this study, we showed the feasibility of using a mouse model of intracranial aneurysm to test pharmacological stabilization of aneurysms as a prevention of subarachnoid hemorrhage. We demonstrated that doxycycline and minocycline, anti-inflammatory agents with weak broad-spectrum MMP inhibitory activity, reduced rupture of intracranial aneurysms in mice.

The mouse model of intracranial aneurysm used in this study had a predictable time course and a relatively high incidence of aneurysmal rupture. Aneurysmal formation occurred during the first week after aneurysm induction. Treatment with tetracycline derivatives 6 days after aneurysm induction reduced the rupture rates without affecting the overall incidence of aneurysms. This showed the feasibility of using this model for studying the mechanisms of aneurysmal rupture as well as testing pharmacotherapy as stabilization of aneurysms.

In our model, aneurysmal ruptures were detected by the presence of neurological symptoms, particularly motor deficit. The neurological signs that were observed were sensitive and specific for detecting aneurysmal subarachnoid hemorrhage. Because the neurological examination detects only symptomatic aneurysmal subarachnoid hemorrhage, this method may have missed asymptomatic or subtle subarachnoid hemorrhage. Nevertheless, when the brain samples from asymptomatic mice were examined, no apparent signs of large hemorrhage were observed. Some of the asymptomatic mice showed hemosiderin on the surface of the brain, possibly indicating minor hemorrhage that was not severe enough to cause a neurological deficit. Detecting only symptomatic subarachnoid hemorrhage may mimic an actual clinical setting. Alternatively, these hemosiderin deposits could possibly be due to tissue injury caused by the needle insertion at the time of elastase injection.

Treatment with doxycycline or minocycline was able to reduce the incidence of aneurysmal rupture and the rupture rate. However, SB-3CT, a potent and selective inhibitor of MMP-2 and MMP-9, did not affect aneurysmal rupture. Considering that both tetracycline derivatives and SB-3CT can inhibit MMPs, these results were somewhat surprising. There are several potential explanations.

Stabilization of aneurysms by doxycycline and minocycline could possibly be due to their general anti-inflammatory effects rather than their inhibitory properties on MMPs. Tetracycline derivatives can exert anti-inflammatory effects through their antiapoptosis effects and nonspecific protease inhibition, both of which could affect aneurysmal rupture. Tetracycline derivatives’ inhibitory effects on MMPs are rather weak with a high half-maximal inhibitory concentration ($>$100,000 nmol/L). The potential of anti-inflammatory agents to stabilize intracranial aneurysms has been suggested by a recent clinical report that showed the association between the use of an anti-inflammatory agent, aspirin, and a lower risk for aneurysmal rupture.

Although tetracycline derivatives represent an anti-inflammatory agent, SB-3CT is a selective inhibitor of...
MMP-2 and MMP-9. SB-3CT does not exert significant inhibitory effects against MMPs. Our results might argue for key roles of other MMPs or other effectors in aneurysmal rupture.

Finally, although animal studies suggest potential role of MMPs in the formation of intracranial aneurysms, MMPs may not play a significant role in the rupture of intracranial aneurysms. It has often been presumed that understanding the mechanisms of aneurysmal formation and growth provides insights into the mechanisms of aneurysmal rupture. This notion is based on an assumption that the processes of aneurysmal formation, growth, and rupture share the same or similar underlying mechanisms. However, there is no clear basis for such assumption. Mechanisms of aneurysmal rupture may be fundamentally different from those of formation and growth. Therefore, an animal model that allows us to directly study the mechanisms of aneurysmal rupture becomes important. Our results, along with previous studies on mechanisms of aneurysm formation,13 may indicate differential roles of MMPs between aneurysm formation and aneurysmal rupture.

A major limitation of this study is a lack of mechanistic investigation on how tetracycline derivatives prevented aneurysmal rupture. We did not compare inflammation or MMP activity in aneurysm tissues between the vehicle groups and the treatment groups. Comparison within ruptured aneurysms would be significantly confounded by the effects of hemorrhage that can cause significant activation of inflammation and MMPs. Unfortunately, comparison of unruptured aneurysms was not possible because of the extremely small number of specimens available at the end of observation period. One of the focuses of future studies should be on mechanisms by which aneurysmal rupture occurs. Preclinical studies using this model would be useful in refining the therapeutic targets and selecting optimal anti-inflammatory agents for the stabilization of aneurysms. This becomes especially important when chronic treatment with tetracycline derivatives may have adverse effects on the brain, possibly depending on the stages of injury and inflammation.24,25

Although this dose of SB-3CT has been used successfully to inhibit MMP activities in mice,21 further studies with a wide dose range of SB-3CT are needed to confirm the lack of protective effects of SB-3CT against aneurysmal rupture. The vehicle for SB-3CT—a combination of dimethyl sulfoxide and PEG-200—may have had potentially confounding effects.26 The mice that received the dimethyl sulfoxide-containing vehicle had a similar incidence of aneurysmal rupture to those receiving saline or water alone, indicating that the vehicle for SB-3CT itself did affect the processes leading to aneurysmal rupture.

Summary
In this study, we have shown the feasibility of using a mouse model of intracranial aneurysm to test pharmacotherapy in the stabilization of aneurysms. Anti-inflammatory therapy using tetracycline derivatives may be useful in preventing the rupture of intracranial aneurysms.

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Disclosures
None.

References
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Pharmacological stabilization of intracranial aneurysms in mice— a feasibility study

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Expanded method section

Pharmacological stabilization of intracranial aneurysms in mice— a feasibility study

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Methods

Animal model

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Intracranial aneurysms were induced in 8-10 week old male mice (C57BL/6J, Jackson Laboratory) using a previously described method with modifications.\textsuperscript{1,2} We combined systemic hypertension and a single injection of elastase into the cerebrospinal fluid at the right basal cistern as previously described.\textsuperscript{1,2}

To induce systemic hypertension, we used deoxycorticosterone acetate-salt hypertension (DOCA-salt hypertension).\textsuperscript{3} In order to induce DOCA-salt hypertension, we performed left nephrectomy followed by implantation of DOCA pellet one week later; 1% sodium chloride drinking water was started on the same day as the DOCA pellet implantation as previously described.\textsuperscript{3,4} Induction of aneurysm by disruption of elastic lamina was achieved by a single injection of elastase (25-35 milli-units) into the cerebrospinal fluid at the right basal cistern using a stereotaxic method on the same day as DOCA pellet implantation as previously described.\textsuperscript{1,2} Aneurysms were defined as a localized outward bulging of the vascular wall whose diameter is greater than the parent artery diameter.\textsuperscript{1,2} All surgical procedures were performed under general anesthesia with isoflurane (1.5-2%).
Two blinded observers performed daily neurological examination using a previously described method with minor modifications.\textsuperscript{5-8} Neurological signs were scored as followings; 0: normal function; 1: reduced eating or drinking activity demonstrated by the weight loss greater than 2 grams of body weight (approximately 10\% weight loss) over 24 hours; 2: flexion of torso and forelimb upon lifting of the whole animal by the tail; 3: circling to one side but normal posture at rest; 4: leaning to one side at rest; and 5, no spontaneous activity. Mice were sacrificed when the neurological score was 1-5. All asymptomatic mice were sacrificed 28 days after aneurysm induction. After euthanasia, mice were perfused with phosphate-buffered saline, then with gelatin containing blue dyes to visualize the cerebral arteries. Two blinded investigators assessed brains for aneurysm formation and subarachnoid hemorrhage. Systolic blood pressure was measured before the treatment (baseline) and at one, two, and four weeks after the elastase injection using the tail cuff method as previously described.\textsuperscript{1, 9}

\textbf{Statistical Analysis}

Primary outcomes of this study were the incidence of unruptured intracranial aneurysms and rupture rate (number of mice with ruptured aneurysm / number of mice with any aneurysms). For the analysis of primary outcomes, we used Fisher’s exact text. As an exploratory analysis, we plotted Kaplan-Meier survival curve, and the survival analysis was performed by Log-rank test. For the survival analyses, mice that did not develop aneurysms were excluded. For analyses of blood pressure and body weight, we used ANOVA. Statistical significance was taken at $P < 0.05$. 
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マウスの頭蓋内動脈瘤の薬理学的安定化 実現可能性の研究
Pharmacological Stabilization of Intracranial Aneurysms in Mice
A Feasibility Study

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Abstract

背景および目的：脳画像診断技術の使用が増加したこともあり、未破裂頭蓋内動脈瘤の発見数が増加している。動脈瘤破裂の予防を目的とした動脈瘤の薬理学的安定化は、未破裂頭蓋内動脈瘤を有する患者に対してクリーニングまたはコイリングに代わる魅力的な代替アプローチとなる可能性がある。我々は、頭蓋内動脈瘤の主要な特徴を再現した頭盖内動脈瘤のマウスモデルを開発した。このモデルでは、動脈瘤破裂によるくも膜下出血が神経学的症候を引き起こし、これが簡単な神経学的検査によって容易に検知できる。このモデルを用いて、トトラサイクリン誘導体などの抗炎症薬、またはマトリックスメタプロテーゼ-2および-9の選択的阻害薬（SB-3CT）が頭蓋内動脈瘤の破裂を予防できるかどうかを検討した。

方法：マウスに高血圧を誘導するとともに、脳脊髄液にエラスターゼを単回注射することにより、動脈瘤を誘発した。動脈瘤の誘発から6日後に、ミノサイクリン、ドキサイクリン、またはSB-3CTによる処置を開始した。動脈瘤破裂を神経学的症候によって検知し、くも膜下出血を伴う頭蓋内動脈瘤の存在によって確認した。

結論：ミノサイクリンおよびドキサイクリンは破壊率を有意に低下させたが（溶媒対ドキサイクリン＝80％対35％, p < 0.05；溶媒対ミノサイクリン＝73％対24％, p < 0.05）、動脈瘤の全体の発生率には影響を与えなかった。一方、SB-3CTは破壊率に影響を及ぼさなかった（62％対55％, p = 0.53）。

脳動脈瘤に対するミノサイクリンの効果：A、動脈瘤の発生率、動脈瘤の全体の発生率（破壊と未破裂の両方）には、溶媒群とミノサイクリン群の間に差はみられなかった（71％対65％, p = 0.45）しかし、破壊動脈瘤の発生率はミノサイクリン群が溶媒群より有意に低かった（16％対52％, p < 0.05）。B、破裂率。ミノサイクリン投与により破裂率は溶媒投与と比べて有意に低下した（24％対73％, p < 0.05）。C、無症状生存曲線（symptom-free curve）。動脈瘤が生じたマウスのロジスティック検定の結果、ミノサイクリン投与による動脈瘤破裂の有意な減少が明らかになった（p < 0.05）。