Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

Kokje, V.B.C.

Chapter 5

INHIBITION OF CYSTEINE PROTEASE ACTIVITY REDUCES ANEURYSM EXPANSION THROUGH DECREASED MATRIX DEGRADATION

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ABSTRACT

Aim

Abdominal aortic aneurysm (AAA) is a common vascular dilatation disorder in elderly people that may become lethal after aortic rupture. Current treatment exclusively relies on preventive surgical repair of larger AAA. Pharmaceutical intervention decreasing aneurysm progression to reduce the need for surgical repair is currently missing. The pathology of AAA is best described as a chronic inflammatory condition of the vessel wall accompanied by enhanced protease activity. Cysteine proteases are associated with matrix remodeling and are abundantly expressed in aneurysms. To test whether cysteine proteases constitute a target for pharmaceutical AAA stabilization, we evaluated the efficacy of the broad-spectrum cysteine protease inhibitor E64.

Methods and Results

Human AAA tissue shows enhanced cysteine protease expression and a strong increase (10-fold) in protease activity as illustrated by the increased presence of cysteine protease-mediated collagen (CTX)-fragments compared to control age-matched aortic tissue. In the angiotensin-II (AngII) and elastase AAA mouse model, we showed that E64 treatment reduced aneurysm severity of the ascending and descending aorta or aneurysm formation, respectively. We revealed inhibited breakdown of the elastic laminae and prevented loss of collagen in the vessel wall, promoting integrity of the aorta. In the elastase model, in which the aorta is locally treated with elastase, E64 was superior to doxycycline ($p=0.04$) in inhibition of aortic dilatation (ultrasound, $p<0.001$).

Conclusion

This study showed that the broad-spectrum cysteine protease inhibitior, E64, decreased aneurysm formation in two established mouse models of the disease, indicating that cysteine proteases are suitable potential targets for pharmaceutical AAA stabilization in humans.

INTRODUCTION

An abdominal aortic aneurysm (AAA) is a dilatation of the terminal aortic segment. Small AAAs are generally clinically silent, yet larger aneurysms can rupture, causing an often fatal bleeding. The current approach towards AAAs is surveillance of smaller aneurysms, and preventive surgical elimination of larger aneurysms once the diameter has become larger than 55 mm. Accordingly, pharmaceutical intervention inhibiting AAA progression may have major advances, both from patients' and from a socio-economical perspective[1].

Although elastin degradation is considered the hallmark of AAA-disease, collagen degradation is held responsible for the actual aneurysmal dilatation and ultimate rupture of the aneurysm[2]. The vascular type collagens type-I and -III are highly resistant towards proteolytic degradation, and specific collagenolytic enzymes are required for initiation of collagen degradation. Others and we previously identified the cysteine proteases, cathepsin K, -L and -S as prominent collagenolytic enzymes in human AAA samples[3-8]. Moreover, deficiency of cystatin C, the primary endogenous inhibitor of cathepsins, has been associated with human aneurysm formation[8, 9]. Genetic mouse models with cathepsin deficiency underline their importance in this pathology. Mice do not develop aneurysms spontaneously and the two most established murine AAA models are the angiotensin II- (AngII) induced and the elastase-induced aneurysm model[10-12]. Mice lacking the protease cathepsin K, -L, or -S are resistant to AAA formation[13-15]. Significantly, cystatin C deficiency in mice recapitulates many features of human aortic aneurysms[9]. Together these observations pose the cysteine collagenase network as an important pharmaceutical target to stabilize AAAs. To test the validity of this approach, we here employ the broad-spectrum cysteine protease inhibitor E64[16], in two established murine AAA models.

METHODS

Patients

The investigation conforms the principles outlined in the Declaration of Helsinki (59th, October 2008). Human AAA tissue was obtained during surgery for asymptomatic AAA. Peri-renal aortic patches of age-matched organ donors were used as controls. Sample collection was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center.

Collagen Degradation Assay

Homogenates of aortic wall tissues (ICTP assay; N=3 control and N=8 AAA, CTX assay; N=12 both groups) were normalized for protein content. Collagen degradation was measured by CTX-assay (Serum Cross laps; Nordic Biosciences, Milsbeek, The Netherlands) and ICTP assay (Uniq ICTP RIA, Orion Diagnostica Oy, Espoo, Finland). The CTX epitope reflects cathepsin K-mediated collagen turn-over and the ICTP assay the matrix metalloproteinase-mediated collagen degradation[17].

Murine Aneurysm Models

All murine investigations were performed conform the Directive 2010/63/EU of the European Parliament or conform the *Guide for the Care and Use of Laboratory Animals* published by the US

National Institutes of Health (NIH Publication No. 85-23, revised 1996). Local approval was obtained from the institutional animal welfare committees.

Angiotensin II – induced model of AAA

This AAA-model relies on angiotensin II (AngII) infusion (1.44 mg/kg/day) via an osmotic minipump for 4 weeks[10]. ApolipoproteinE deficient (ApoE-/-) male mice of approximately 8 months old were used for the aneurysm formation experiment. The mice treated with the cysteine protease inhibitor E64 ($C_{15}H_{27}N_5O_5$; N-(trans-epoxysuccinyl)-L-Leucine-4-guanidinobutylamide: Sigma Aldrich Zwijndrecht, The Netherlands)[16], received 0.24 mg/kg/day E64 for 4 weeks via the minipump that also contained AngII. Mice were primed through a bolus injection of E64 (0.48 mg/mouse i.p.) on day one. As an extra control group for aortic dilatation, five ApoE-/- mice were given a minipump containing saline, which did not lead to aneurysm formation. After 4 weeks the mice were harvested and their aortas analyzed.

Aneurysms were classified according to Daugherty *et al*[10], measuring the maximal abdominal aortic diameter macroscopically and taking into account thrombus formation in an aneurysm or if multiple aneurysms/dissections were present per aorta. In addition, the circumference (to calculate diameter) of the ascending aorta was measured where the aorta leaves the heart.

Cytokine levels in serum

Murine monocyte chemoattractant protein 1 (JE), murine interleukin-8 (KC), interleukin-6 (IL-6), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) were determined in serum of the control and E64-treated mice by specific ELISAs.

Elastase-induced model of AAA

In male (8-12 weeks old) wild-type (WT; C57BL/6) mice aneurysms were generated via porcine pancreatic elastase (PPE) infusion as previously described[12]. In short, an isolated aorta segment was perfused with elastase solution for 5 minutes, after which the flow was restored. The experimental E64-treated mice (n=13: 0.48mg E64/kg/day) received E64 via an osmotic minipump placed one day prior to elastase infusion. A control group received a saline containing minipump (n=17), and served as positive control, where maximal aortic expansion could be determined. A second group was treated with doxycycline (in drinking water; n=12), an established inhibitor of aneurysm growth in mice and decreased vascular inflammation in human aneurysm vessel wall[18-21]. Ultrasound measurements were made at baseline and at day 7 and 14 after the infusion by means of the Vevo 770 Imaging system using an RMV 704 microvisualization scan head (Visualsonics, Ontario, CA).

(Immuno)Histochemistry

Upon harvest, human or murine aortic segments were fixed, embedded in paraffin and sectioned for histological analysis. To detect the different cysteine proteases in human AAA tissue, sections were incubated with primary antibodies against cathepsin K, -L, or -S (generous gift from Dr. Weber, Institute of Biochemistry, Martin-Luther University of Halle-Wittenberg, Halle, Germany (cathepsin K and S), or R&D systems Abbingdon, UK (Cathepsin L)).

To visualize elastic laminae and quantify breaks, Lawson-staining was performed. To detect collagen, sections were stained with Picro Sirius Red solution. Three sections per mouse were blindly evaluated and mean values for elastic laminae breaks or collagen area was calculated for each animal. Collagen area was measured using Leica QWin software.

Statistical analysis

The data are expressed as mean ± the standard error of the mean (SEM). The non-parametric Mann-Whitney U-test was used to compare the human or murine aneurysm data.

A 2-tailed Student t test was used to compare elastic lamina breaks, collagen content, and cytokine data from the two groups of mice. The level of significance was set at $p \leq 0.05$.

RESULTS

Cysteine protease activity in human AAA

Although the main focus in AAA research has been on the role of matrix metalloproteinases (MMPs), cysteine proteases are now recognized as key factors in vessel wall destabilization[20]. We previously determined that expression of members of both classes are increased in aneurysm tissue, compared to aortic (atherosclerosis) tissue from age-matched control individuals[21]. To establish to what extent MMPs and cysteine proteases are involved in AAA, we now determined their activity, by measuring their specific collagen degradation products. The ICTP assay (MMP-activity)[17], showed minimal levels of MMP-specific fragments in control and AAA tissue (Fig.1A). However, we observe abundant CTX fragments (cathepsin K activity) in AAA tissue compared with control aortic wall (10-fold increase; $p<0.001$)(Fig.1A). Cathepsin K, L and S are each abundantly expressed in human AAA tissue, predominantly by macrophages (Fig.1B), and are probably all involved in generation of CTX fragments. In conclusion, macrophage-derived cysteine proteases contribute significantly to matrix degradation in human AAA.

E64 in the AngII-model decreased AAA severity

The importance of cysteine proteases in aneurysm formation was first investigated in the AngII-induced AAA model by blocking their activity via cysteine protease inhibitor E64[22, 23]. Mice were treated with AngII without or with E64, and after four weeks the aorta was analyzed for aortic dilatation. Dilatation of the ascending aorta is a typical feature in the AngII model[24]. To assess the normal aortic diameter of ApoE-/- mice, we incorporated the analysis of mice that received saline (N=5) instead of AngII for 4 weeks. The normal wideness of the ascending aorta is 1.1 ± 0.1 mm (Fig. 2A). However, in response to AngII infusion (N=11) the ascending aorta diameter expands significantly to a diameter of 1.6 ± 0.4 mm ($p<0.03$). E64 treatment (N=11) reduced the aortic diameter to 1.3 ± 0.3 mm (saline vs E64 is not different; $p=0.08$).

In addition, aneurysms in the descending aorta were typed according to the scoring system as described before[10]. We observed 4 severely affected aortas with multiple aneurysms per aorta (scored as type IV; Fig. 2B) in the control group, compared with 2 milder phenotype type II (dilated aorta) and type III (dilated with thrombus) aneurysms in the E64 treated group (Fig. 2B). Cross sections of these aneurysms were stained to visualize collagen in red or elastic laminae in purple,

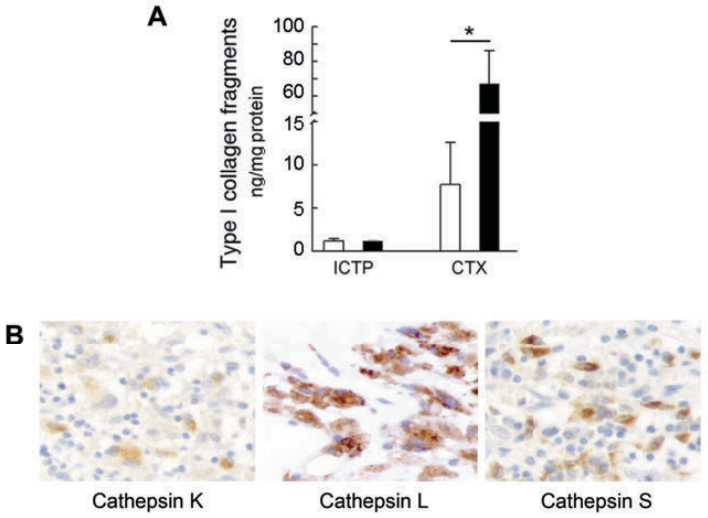

Figure 1. Collagen degradation and cathepsin expression in human aneurysm. Collagen degradation was measured in human aortic tissue from control (white) and AAA specimens (black) (A). The levels of collagen-I fragments derived by MMP-mediated (ICTP) or cysteine proteases (CTX) degradation were determined (*p<0.001) (B).

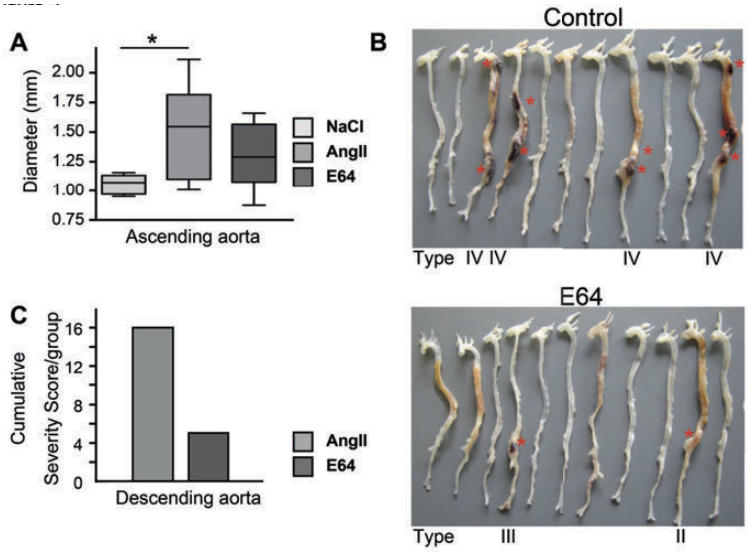

Figure 2. E64 reduces aneurysm formation in the AngII-model. Increased aortic dilatation is observed in the ascending aorta upon AngII-treatment when comparing with saline-treated mice (p<0.03) (A). In addition, 4 (out of 11) mice showed severe aorta pathology (type IV), which is reduced to 2 (out of 11) mice with minor aneurysm formation in the E64-treated group (type II and III) (B). The cumulative aneurysm severity score for the descending aorta is shown (C).

and revealed extensive medial degradation (Supplemental Fig. I). The cumulative aneurysm score of 16 in control mice and 5 in the E64-treated group revealed protection by E64 (Fig. 2C). Taken together, the E64-treated mice showed decreased aneurysm pathology.

E64 reduced several specific circulating pro-inflammatory factors

AngII infusion has a pro-inflammatory effect, which is causative in induction of aneurysms[10]. Therefore, we determined the effect of E64 on systemic inflammatory responses by measuring a panel of hallmark cytokines/chemokines in the circulation of the mice. E64 treatment reduced circulating KC ($p<0.03$) and IFNγ ($p<0.04$) levels, whereas no effect was found on JE, IL-6 and TNFα serum levels (Fig. 3).

E64 protects against extracellular matrix degradation

In the AngII-model the vessel wall was severely damaged at the site of the macroscopically visible aneurysms (Supplemental Fig. I). Consequently, we decided to measure elastic lamina breaks only in macroscopically *un*-affected aortas at the location where AAA normally form (N=7 control and N=9 E64), to analyze aortic damage in an early stage of the disease. Elastic lamina degradation was observed in the aortas of all AngII-treated mice, yet the number of breaks per aorta was significantly decreased in the E64-treated group (Fig. 4A; $p=0.05$). The analysis also revealed that the elastic lamina breaks often occur at sites where atherosclerotic lesions have developed (Fig. 4B).

Quantification of the abundance of the collagen matrix in the media of the vessel wall showed that aortas from E64-treated mice contained 50% more collagen compared to untreated mice (Fig. 4C; $p<0.02$), indicating that collagen had disappeared from the media to a higher extent in

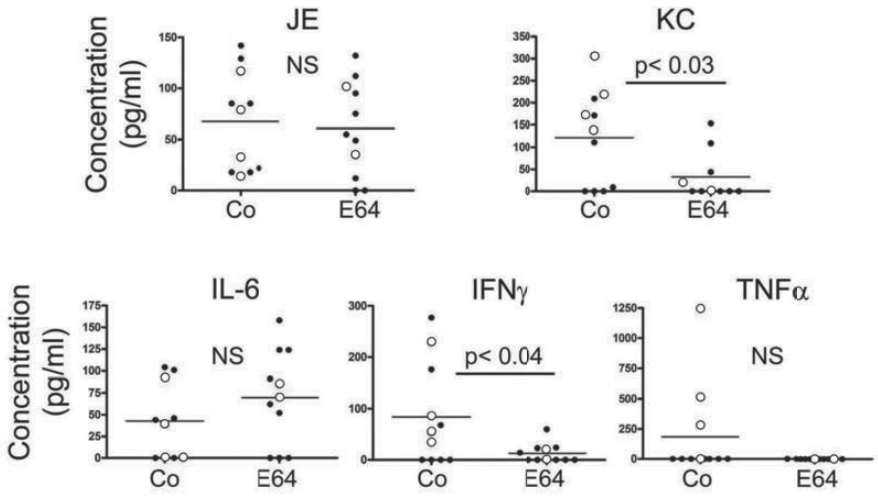

Figure 3. E64 reduces several specific cytokines. The expression level of the chemokines JE and KC and the cytokines IL-6, IFNγ and TNFα was determined in serum of control (Co) and E64-treated mice. White dots indicate mice with an aneurysm.

control mice (reduced red staining in media) (Fig. 4D). Thus, both the elastin and collagen matrix components are more intact in response to E64 treatment.

E64 in the Elastase model prevents AAA formation

To validate the seemingly protective effect of E64, in yet another AAA model, we performed aortic elastase infusion experiments. Mice that received additional saline developed an aneurysm (as defined by ≥50% increase in aortic diameter) within 14 days (85% ± 22% increased diameter) (Fig.5A). Mice that received E64-treatment, however, were protected from aortic dilatation ($p<0.001$). E64 was even more effective (32% ± 16% increased diameter) than the established inhibitor of aneurysm formation in mice, doxycycline (51% ± 21%), that we incorporated in our study as a control for treatment effectiveness ($p<0.05$). Representative ultrasound photographs depict the lumen of the abdominal aorta in the different groups, revealing the preserved vessel diameter in the E64- and doxycyclin-treated mice (Fig.5B).

DISCUSSION

This study shows that cysteine protease-mediated matrix degradation is prominent in human AAA, and that comprehensive cysteine protease inhibition prevents extensive degradation of the vessel wall and aortic dilatation in two established mouse models.

AAA pathology comprehends chronic inflammation accompanied by proteolytic imbalance, with ultimate aortic rupture due to loss of collagen fibers[25]. Cathepsins K, -L and -S exhibit collagenolytic enzyme activity, yet for a long time this was assigned to the MMP subfamily of collagenases[3]. To

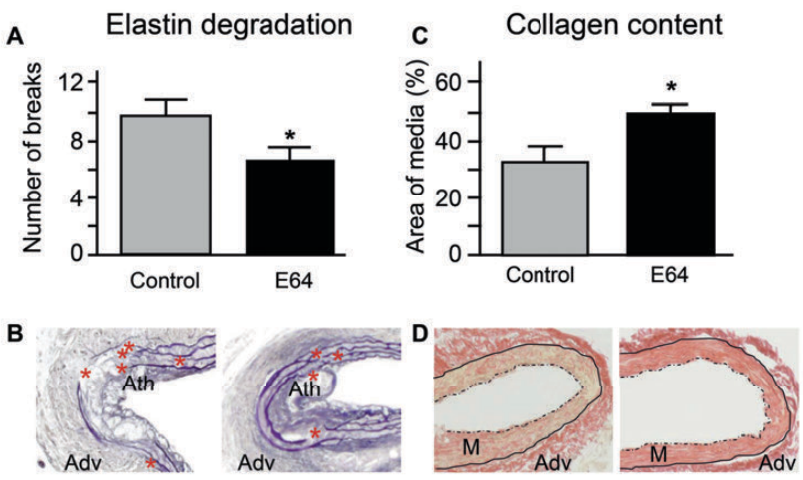

Figure 4. Reduced elastin breaks and enhanced collagen content after E64 treatment. Elastic lamina breaks were counted as a measure for medial integrity of the abdominal aorta (A;*$p=0.05$). Examples of elastic lamina disruptions (red star) in the media in the presence or absence of E64 are shown (B). Collagen content of the vessel wall was analyzed as a measure for vessel wall stability (C;*$p<0.02$). Collagen (red) degradation was most prominently observed in the media (D). Dotted line indicates internal elastic lamina and black line indicates outer elastic lamina. Adv, adventitia; Ath, atherosclerosis; M, media.

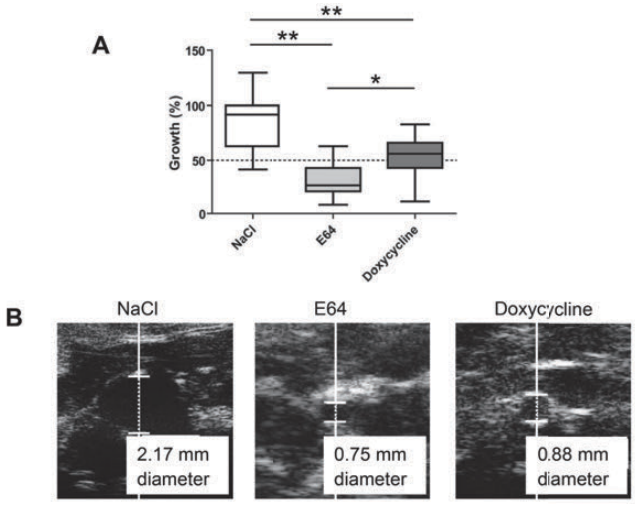

Figure 5. E64 prevents aortic dilatation in the elastase model. Upon elastase infusion more than 50% increase in aortic diameter is observed after 2 weeks, which is inhibited by E64 or doxycycline (**p<0.001)(A). The E64-treated mice revealed less diameter growth than the doxycycline group (*p<0.05). Representative ultrasound photographs are shown (B).

assess the relative contribution of cathepsins and MMPs in collagen degradation, we measured CTX and ICTP fragments and observed minimal levels of the MMP-mediated ICTP fragments, whereas CTX levels were 10-fold higher in aneurysm compared with normal aortic tissues. This may reflect a minor role for the MMPs in aneurysm formation, however, it should be noted that ICTP fragments are larger than CTX fragments and contain multiple cathepsin cleavage sites. As such, cysteine proteases degrade ICTP fragments, which may explain the relatively low levels of ICTP fragments[17].

Others and we previously demonstrated increased expression of cathepsin K, -L and -S in human aneurysm tissue, at both mRNA and protein level[3-8]. While the expression level of the proteases was increased, a reduction in the expression of its endogenous inhibitor cystatin C was correlated with aneurysm pathology in human and mouse[8,9]. We now investigated the effect of cysteine protease inhibitor E64 in two murine AAA models. E64 prevented AAA formation and severity in both models, yet more potently in the elastase AAA model. This may be due to differences between the AAA models, with the AngII model having a more systemic effect compared to the local focus in the elastase model[26], or more practical that the concentration used in the elastase model was more efficient.

AngII is a strong pro-inflammatory factor, mediating signaling via the AngII-receptor-1. We observed reduced levels of KC and IFNγ upon E64 treatment. We have previously shown in human AAA tissue that the monocyte/neutrophil chemo-attractant interleukin-8 (IL-8) is increased in the vessel wall[27]. In the current study, we demonstrated that KC, the murine equivalent of IL-8, is high in the serum of AngII-infused mice and decreased upon E64 treatment and correlates with a decrease in vessel wall damage. The role of IFNγ in development of AAA is controversial[28, 29]. In our study, increased IFNγ is associated with increased aorta pathology, which is in line with the findings of Xiong *et al*, who demonstrated that IFNγ-deficient mice do not develop AAA[29] and with Zhou

et al, who revealed IFNγ production promotes AAA formation[30]. The reduced cytokine levels may reflect decreased aorta wall damage upon E64, because elastic lamina breaks and collagen degradation products in itself are a pro-inflammatory stimulus and matrix degradation was reduced in E64-treated mice.

Our study, showing that E64 inhibits AAA development, is in line with observations that cathepsin K or -L deficiency results in decreased aneurysm formation in mice in the elastase model[13,14], suggesting that cathepsin K and L are prominent cathepsins in this model. In the AngII-mediated AAA model cathepsin S deficiency causes decreased AAA development[15] yet, Bai *et al* reported that cathepsin K gene disruption did not affect aneurysm formation[31], indicating that possibly redundancy by the other cathepsins compensates for the loss of cathepsin K in this model.

Our data suggests that the broad-spectrum cysteine protease inhibitor E64 is effective to reduce aneurysm formation through inhibition of matrix degradation. Inhibition of cysteine proteases has potential therapeutic value to treat AAA. At present, we do not propose the use of E64 in humans because of its limited cathepsin specificity, however, highly specific cathepsin K inhibitors have been developed to treat human osteoporosis and are currently tested in clinical trials[32]. Cathepsin S inhibitors have been generated, but need further validation in patients with autoimmune diseases, for whom they have been developed[33]. The application of such novel inhibitors in aneurysm patients holds promise for the future. In conclusion, cysteine protease inhibition results in decreased aneurysm formation, offering an attractive therapeutic approach to prevent AAA progression.

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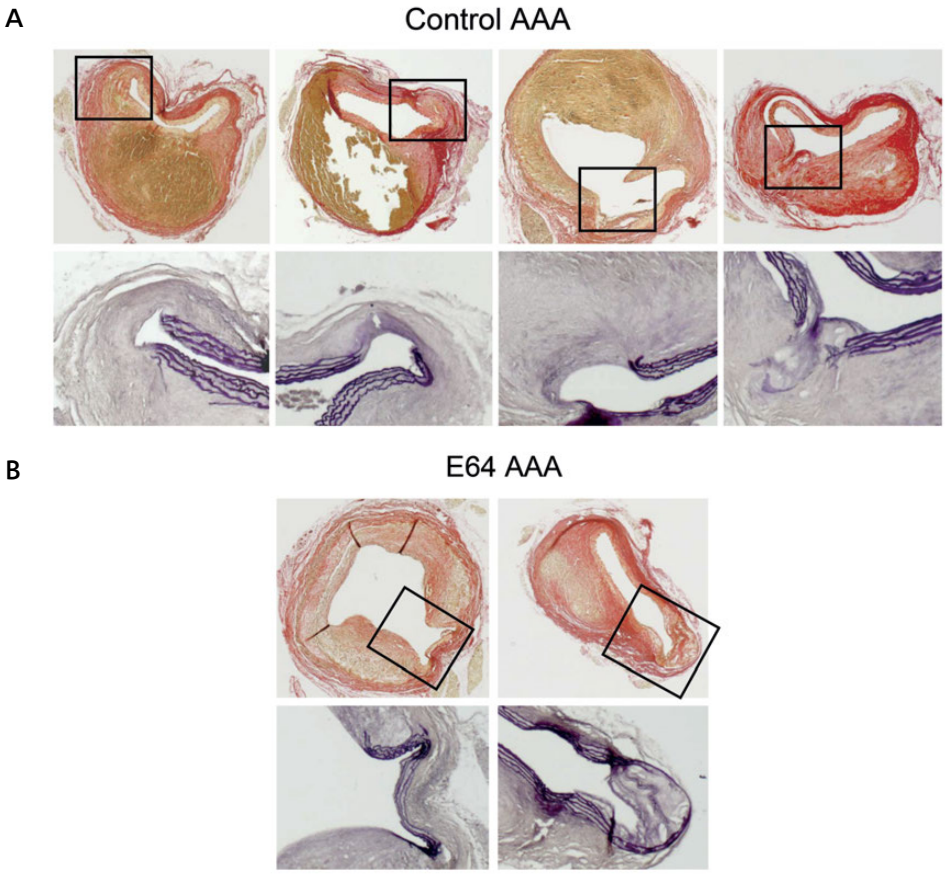
SUPPLEMENTAL DATA



Supplemental Figure 1. Aneurysms acquired in the AngII-AAA model. Collagen staining (red) reveals the collagen content and distribution throughout the aneurysm in control (A) and E64-treated (B) mice. The medial disruption is shown in more detail (box upper panel enlarged in lower panel) by staining of the elastic laminae (purple). Clearly, there is extensive aortic damage at the location of the aneurysm.