Zoledronate attenuates angiotensin II-induced abdominal aortic aneurysm through inactivation of Rho/ROCK-dependent JNK and NF-κB pathway

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Aims
Abdominal aortic aneurysm (AAA) is a life-threatening disease affecting almost 10% of the population over the age of 65. Nitrogen-containing bisphosphonates (N-BPs) have been shown to exert anti-atherogenic and anti-angiogenic effects, but the potential effects of N-BPs on AAA remain unclear. Here, we tested whether a potent N-BP, zoledronate, can attenuate the formation of Angiotensin II (Ang II)-induced AAA in hyperlipidemic mice.

Methods and results
Low-density lipoprotein receptor−/− (LDLR−/−) mice infused for 28 days with Ang II were treated with placebo and 100 μg/kg/day zoledronate. Continuous Ang II infusion in LDLR−/− mice exhibited a 59% incidence of AAA formation, and treatment with zoledronate decreased AAA formation (21%). Compared with the saline group, administration of zoledronate in Ang II-infused LDLR−/− mice attenuated the expansion of the suprarenal aorta (maximal aortic diameter), reduced elastin degradation in the media layer of the aorta, and significantly diminished vascular inflammation by reduction in vascular cell adhesion molecule expression and macrophage accumulation. Treatment with zoledronate decreased matrix metalloproteinase-2 (MMP-2) in aortic tissues. Zoledronate-treated mice had significant down-regulation of JNK, NF-κB, and reduced Ang II-induced Rho/ROCK activation. Zoledronate reduced macrophages adherence to human aortic endothelial cells in vitro.

Conclusion
Zoledronate-attenuated Ang II induced AAA formation by suppression of MMP-2 activity and suppressed vascular inflammation and Ang II-induced Rho/ROCK activation.

Keywords
Abdominal aortic aneurysm • Angiotensin II • Zoledronate • Bisphosphonate • Low-density lipoprotein receptor−/− mice • Rho • MAPK • NF-κB

1. Introduction
Rupture of abdominal aortic aneurysms (AAAs) can be life threatening and is one of the most common causes for sudden death among the elderly people.¹,² Epidemiological studies indicated that up to 13% of men over the age of 75 years have AAA, and the risk of rupture is determined by the size of the AAA. Clinical reports have shown that patients with small AAA (3–5 cm in diameter) who were followed for up to 6 years, had AAA diameter increase in 55% of patients.³ Moreover, the rate of expansion in diameter was >1 cm per year in 23% of patients, and AAA diameter had expanded to 6 cm in 9% of patients, at which point, the rupture risk of AAAs significantly increases.³–⁵ Most of the AAAs are detected incidentally, and 90% of these aneurysms are below the threshold for intervention at the time of detection. Although AAAs can be detected in humans by non-invasive imaging techniques, there are no pharmacological treatments currently available to prevent the progression at any stage of the disease.

Pathological features of aneurysm include smooth muscle cell (SMC) apoptosis, local vascular inflammation, increased oxidative stress, and significant matrix degradation.⁶ Matrix metalloproteinases (MMPs) are...
part of an endogenous family of enzymes responsible for extracellular collagen degradation and remodelling. Dysregulation of MMPs in aortic tissues was shown to play a critical role in the initiating and progression of AAA. Furthermore, extensive proliferation of the vasa vasorum and enhanced new capillary formation in rupture sites suggest that neovascularization might play a role in the pathogenesis of AAA. Bisphosphonates are widely used in the treatment of resorptive and metastatic bone diseases. Nitrogen-containing bisphosphonates (N-BPs) have been shown to exert anti-atherogenic, MMP inhibitory effects, and anti-angiogenic effects. N-BPs can inhibit farnesyl pyrophosphate synthase (FPPS), a crucial enzyme in the mevalonate pathway and further interfere with post-transcriptional modification of small GTPase proteins. Our recent study demonstrated that zoledronate exerts anti-angiogenic effects through the modulation of pro-angiogenic cells after tissue ischaemia by down-regulation of MMP-9 activity. Additionally, zoledronate had been shown to inhibit the development of experimental atherosclerosis and attenuate neointimal hyperplasia. Angiotensin II (Ang II)-induced AAA is a validated model of aneurysm formation in mice. Like human AAAs, Ang II-induced AAA tissues exhibit progressive changes and considerable heterogeneity. Osteoporosis and atherosclerosis have epidemiological similarities, and bisphosphonates seem to have an inhibitory effect on the atherosclerotic process. Previous studies regarding the use of bisphosphonates in patients with diabetes, postmenopausal osteoporosis, multiple myeloma, and Paget’s diseases had found that bisphosphonates seemed to modify lipid profiles towards decreased apoB/apoA1 or low-density lipoprotein (LDL)/high-density lipoprotein (HDL)-cholesterol ratio and exert insignificant anti-atherogenic effects (decreased carotid intimal thickness, decreased prevalence of cardiovascular calcification in older women). Taken together, the anti-angiogenic, anti-atherosclerotic, and MMP inhibitory effects of N-BPs have made itself a potential candidate in the medical treatment of AAA. However, the therapeutic effect of a potent N-BP, zoledronate on Ang II-induced AAA in hyperlipidaemic mice remains unclear. Therefore, we investigated whether a potent N-BP, zoledronate, can attenuate the progression of Ang II-induced AAA through inhibition of MMP activity and reduction of vascular inflammation.

2. Methods

2.1 Ang II-induced AAA mouse model

This is a prospective interventional animal study. Eight-week-old male mice were purchased from the Jackson Laboratory (low-density lipoprotein receptor-deficient (LDLR−/−) mice: C57Bl/6 background, Bar Harbor, ME, USA). Mice were fed with normal chow and ad libitum. Alzet osmotic minipumps (model 2004: ALZET Scientific Products, Mountain View, CA, USA) were implanted into mice at 10–12 weeks of age. Pumps were filled with solutions of Ang II (Sigma Chemical Co., St Louis, MO, USA) that delivered 1000 ng/kg/min of Ang II for 28 days, as previously described. The pumps were placed into the subcutaneous space of ketamine/xylazine anaesthetized mice through a small incision in the back and then were closed with surgical stitches. Zoledronate was given 100 μg/kg i.p. daily, as previously described to inhibit MMP activities and neointima formation. The animals were monitored during the treatment for their body weight to assess side-effects. At the end of the study, mice were euthanized by exsanguination under anaesthesia (ketamine-HCl 100 mg/kg and xylazine 20 mg/kg given i.p., animals were considered as adequately anaesthetized when no attempt to withdraw the limb after pressure could be observed). Blood was withdrawn from the right heart ventricle for analysis. All experimental protocols and procedures were approved by the institutional animal care committee of the National Defense Medical Center (Taipei, Taiwan) and complied with the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Determination of blood pressure and serum levels of total calcium, creatinine, and lipid profiles

Systolic blood pressure and lipid profiles were determined 1 week prior to the implantation of the osmotic minipump and weekly during the study period. Systolic blood pressure was measured in conscious mice using a tail-cuff apparatus (Softron BP-98A tail blood pressure system, Tokyo, Japan). Mice were initially acclimated to the device for two consecutive days before the formal measurement. Previous 10 blood pressure measurements were discarded. Values of the following five consecutive records were obtained and then averaged. Serum was obtained by centrifugation at 1400 g for 10 min at room temperature. Serum total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, total calcium, and creatinine were determined using atomic absorption spectrophotometry (SPOTCHEM II, Arkray Kyoto, Japan).

2.3 Characteristics and quantification of AAA

After perfusion fixation with cold 4% paraformaldehyde, the aorta was exposed under a dissecting microscope, and the periadventitial tissue was carefully removed from the aortic wall. The gross appearances of the aorta were photographed digitally and the maximal external diameter of the suprarenal aorta was measured using the imaging processing software (Multi Gauge, Fujifilm). A definition of increased in aortic outer diameter of >50% indicated the development of aortic aneurysm, as previously described.

2.4 Histology and immunohistochemistry

The perfusion-fixed aorta were embedded, cut in cross section (5 μm), and stained with haematoxylin and eosin and Verhoeff-Van Gieson for elastin and CD-68 (Thermo Scientific, MS-1808) for macrophages. The severity of elastin degradation was semi-quantified, as previously described (grade 1, no degradation; grade 2, mild degradation; grade 3, severe degradation; and grade 4, presence of aortic rupture). Macrophage infiltration in the intima was quantified by counting the involved circumference of the abdominal aorta. Macrophage-positive areas were determined and related to the total intimal surface area in three serial sections. Vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz, sc-1504) expression was quantified by sum of the areas. These vessels were imaged and measured using Aperio ScanScope (Aperio Technologies, Vista, CA, USA).

2.5 Immunoblotting

After carefully removing the peri-aortic soft tissue, the whole aorta was perfused with saline and excised. The aorta was homogenized and protein lysates were subjected to SDS–polyacrylamide (PAGE) followed by electroblotting onto a polyvinyl difluoride membrane. Membranes were probed with monoclonal antibodies against p-p38 (Cell Signaling, #9211), p38 (Cell Signaling, #9212), p-JNK (Cell Signaling, #9251), JNK (Cell Signaling, #9252), p-ERK 1/2 (Cell Signaling, #9106), p-p65 (Cell Signaling, #3033), p65 (Cell Signaling, #3034), vascular endothelial growth factor (Santa Cruz, SC7269), Rho (Cell Signaling, #2117), Ras (Cell Signaling, #3965), Rac (Cell Signaling, #2465), p-myosin phosphatase target subunit-1 (p-MYPT-1, Cell Signaling, #5163), MYPT-1 (Cell Signaling, #2634), intercellular adhesion molecule (ICAM, R&D Systems, BBA-3), and VCAM (R&D Systems, BBA-5).

2.6 Measurement of MMP-2 and MMP-9 activities

Gelatin zymography was used to determine the gelatinolytic activities of MMP-2 and MMP-9 in homogenates of aortas and in conditioned medium,
as previously described. Briefly, equivalent amounts of sample were electro-
phoresed under non-reducing conditions on 7.5% SDS–PAGE gels contain-
ning 0.1 mg/mL gelatin as a substrate. The gels were washed in a buffer
containing 2.5% Triton X-100 for 1 h to remove SDS and then incubated
with a substrate buffer at 37 °C for 18 h. MMP activities were quantified by
densitometry scanning. Data obtained from densitometric analysis were
expressed as fold-change in activity relative to controls.

2.7 The in vitro effects of zoledronate on
vascular cells
Endothelial cells (ECs), rather than SMC-specific depletion of angiotensin re-
ceptor type 1a (AT1a), attenuated Ang II-induced aortic aneurysm.26 To de-
termine the effects of zoledronate on vascular cell biology, human aortic
endothelial cells (HAECs) and human aortic smooth muscle cell
(HASMCs) were purchased from Life technologies. Cultured HAECs and
HASMCs were treated with various concentration of zoledronate and
then challenged with Ang II 1 μM.

2.8 Monocytes adherence assay and
macrophage chemotaxis assay
A human monocyte cell line, THP-1 cells, was obtained from the American
Type Culture Collect. Confluent HAECs were plated on 6-well dishes and
were incubated with zoledronate for 48 h. Thereafter, THP-1 cells [labelled
with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl
ester, BCECF-AM, Molecular Probes] were added to each monolayer at a
density of 5 × 10^5 cells/mL. The THP-1 cells were dissolved in RPMI1640
containing 1% foetal bovine serum and incubated for 60 min at 37 °C.
Non-adherent cells were removed by carefully washing with cold phos-
phate-buffered saline twice. The number of adherent cells was counted by
microscope (× 100). The average number obtained from four fields was
used as an average value. The fluorescent intensities were determined by
excitation and emission at 490 and 535 nm, respectively.

To determine the direct effects of zoledronate effects on macrophage, the
macrophage chemotaxis assay was performed using the transwell method as
previously described.27 Briefly, phorbol 12-myristate 13-acetate (PMA,
Sigma, P1585) was used to differentiate THP-1 into macrophage; 2 × 10^5
THP-1 macrophages were placed in the upper chamber of 24-well transwell
plates with 8 μm pore filter. Macrophages were treated with zoledronate at
a concentration of 5, 10, 20 μM for 24 h. Ang II (10^{-6} M) was then added to
stimulate macrophage migration. After incubation for 4 h at 37 °C, migrated
macrophages were stained with haematoxylin and counted. The average
number obtained from four fields was used as an average value.

2.9 TUNEL assay
Detection of cell apoptosis was performed with a terminal deoxynucleotidyl
transferase-mediated dUTP nick end-labelling (TUNEL) assay (In Situ Cell
Death Detection Kit, Fluorescein, Roche Applied Science) according to
the manufacturer’s protocol. Apoptotic cells in the media layer were visua-
lized under a fluorescence microscope and cells were then counterstained
with 4′,6-diamidino-2-phenylindole. Apoptotic cells in the media layer
were determined as the number of positive cells per high-power field.

2.10 Statistical analysis
All continuous variables were presented as the means ± standard deviation.
Student’s t-test was used to compare continuous variables. Categorical
variables were compared by using the $\chi^2$ or Fisher’s exact test. Statistical significance was defined as a $P$-value of $<0.05$. Analyses were performed using a statistical software package (SPSS version 16.0 for Windows; SPS, Inc., Chicago, IL, USA).

3. Results

3.1 Zoledronate reduced Ang II-induced aneurysm formation and attenuated the expansion of the aorta

To evaluate the possible effect of zoledronate on aneurysm formation, we induced AAA by continuous Ang II infusion in LDLR$^{-/-}$ mice. These mice were treated with normal saline and zoledronate (100 μg/kg/i.p) per day. In addition, administration of zoledronate did not alter systolic blood pressure, serum cholesterol, creatinine, and total calcium levels between groups. As shown in Figure 1, consistent with previous studies, continuous infusion of Ang II in LDLR$^{-/-}$ mice induced AAAs development in the suprarenal aorta, but treatment with zoledronate significantly reduced the incidence of AAA formation (Ang II vs. Ang II+Zol: 59 vs. 21%, $P < 0.01, n = 29$ each group; Figure 1B). The external diameter of the aorta was also significantly attenuated by administration of zoledronate from 1.33 ± 0.28 to 0.98 ± 0.18 mm ($P < 0.01, Figure 1C$). However, as demonstrated in Figure 1D, there was no significant difference on the mortality rate between groups. There was no significant difference in body weight change (defined as 10% of body weight loss) among the two groups during the study period. These findings suggest that zoledronate attenuated Ang II-induced aneurysm formation and decreased the expansion of the aorta, which were independent of changes in blood pressure and plasma lipids (see Supplementary material online, Table S1).

3.2 Zoledronate attenuated elastin degradation, suppressed MMP-2 activity, and decreased macrophage infiltration in the aortic tissues

As depicted in Figure 2, continuous Ang II infusion caused degradation of elastin in the media layer of AAAs. Administration of zoledronate significantly reduced the grading of elastin degradation (Ang II vs. Ang II+Zol: 3 ± 2.34 vs. 1.6 ± 1.25, $P < 0.01$). Additionally, continuous Ang II infusion in LDLR$^{-/-}$ mice stimulated MMP-2 and MMP-9 up-regulation in homogenates of aneurysms for 2.16 ± 0.25-fold ($P < 0.05$) and 2.23 ± 0.31-fold ($P < 0.01$) compared with those in saline mice, respectively. As shown in Figure 2B, zoledronate significantly down-regulated MMP-2 activity (Ang II vs. Ang II+Zol: 2.16 ± 0.25 vs. 1.34 ± 0.16, $P < 0.05$) in the AAA tissues, but did not alter MMP-9 activity ($n = 6–8$ per group). However, we did not observe statistically significant difference in apoptotic cells in the media layer among the Ang II- and Ang II+Zol-treated groups (see Supplementary material online, Figure S1).

As shown in Figure 3A, Ang II infusion promoted macrophage infiltration in aneurysm tissues, and treatment with zoledronate decreased macrophage infiltration in the media layer of AAAs from 50.5 ± 11.2 to 11.3 ± 8.4% ($n = 6$ per group; $P < 0.05$). Zoledronate inhibited Ang II infusion-induced VCAM expression (sum of areas) from 35753.3 ± 5109.3 to 17821 ± 2909.0 μm$^2$ in the endothelium (Figure 3B, $n = 5$ per group).
Moreover, Ang II activated phosphorylation of p65, a marker of vascular inflammation, and zoledronate markedly downregulated phosphorylation of p65 in AAA tissues (Ang II vs. Ang II + Zol: 3.1 ± 0.5 vs. 1.47 ± 0.39, *P* < 0.01; Figure 3C, *n* = 6 per group). These findings indicate that zoledronate attenuated aortic elastin degradation, down-regulated MMP-2 activity, and decreasing vascular inflammation by suppression of macrophage infiltration.

### 3.3 Zoledronate attenuates phosphorylation of JNK and ERK1/2 MAPK

The MAPK signalling pathway plays a pivotal role in mediating vascular inflammation. As shown in Figure 4A–D, Ang II-induced activation of JNK and ERK1/2, but not p38. Administration of zoledronate significantly decreased Ang II-induced phosphorylation of JNK (Ang II vs. Ang II + Zol: 5.09 ± 0.73 vs. 1.14 ± 0.60, *P* < 0.01) and ERK1/2 (Ang II vs. Ang II + Zol: 2.05 ± 0.1 vs. 1.29 ± 0.01, *P* < 0.01, *n* = 6 per group) in the homogenates of aortas. However, there were no significant differences in the phosphorylation of p38 with zoledronate treatment. These results suggest that treatment with zoledronate suppressed activation of JNK and ERK1/2 in aortic tissues.

### 3.4 Zoledronate reduces Ang II-induced Rho/ROCK activation

Small GTPase proteins, including RhoA, Ras, and Rac, are required for AT1 receptor activation. Rho-associated kinase (ROCK) mediates Rho signalling and reorganizes the actin cytoskeleton through phosphorylation of several substrates. Specific phosphorylation of MYPT1 at threonine residue 696 is a surrogate marker of Rho/ROCK activation. As shown in Figure 4E and F, we assessed the RhoA and pMYPT1 protein levels in aortic tissues and observed that zoledronate attenuated Ang II-induced phosphorylation of MYPT-1 (Ang II vs. Ang II + Zol: 2.34 ± 0.59 vs. 1.25 ± 0.27, *P* < 0.05) but not RhoA protein levels (Ang II vs. Ang II + Zol: 1.20 ± 0.07 vs. 1.23 ± 0.03, *P* = 0.50, *n* = 6 per group). These results indicate that zoledronate alleviated Ang II-induced Rho/ROCK activation.

### 3.5 Zoledronate inhibits Ang II-induced adhesion molecules expression and suppresses monocytes adherence to human endothelial cells in vitro

To further elucidate the mechanism of zoledronate of Ang II-meditated vascular inflammation, we tested the effect of zoledronate on Ang II challenged HAECs and HASMCs. In in vitro studies, zoledronate decreased Ang II-induced phosphorylation of MYPT, JNK, and ERK1/2 in a dose-dependent fashion in HAECs (Figure 5). Moreover, incubation of zoledronate in cultured HAECs significantly reduced Ang II-induced phosphorylation of p65, and expression of ICAM and VCAM. Using a monocyte adhesion assay, Ang II-promoted monocyte adhesion, and administration of zoledronate significantly reduced Ang II-induced monocyte adhesion (Figure 6). However, zoledronate could not inhibit
Ang II-induced macrophage migration \((n = 5, \text{ Supplementary material online, Figure S2})\). In addition, we found that zoledronate could not reverse Ang II-induced Rho/ROCK activation and MAPK, JNK, ERK1/2 phosphorylation in comparable doses in HASMCs \((n = 5, \text{ Supplementary material online, Figure S3})\). These findings suggest that zoledronate decreased Ang II-induced vascular inflammation and attenuated monocyte adhesion by down-regulation of adhesion molecule expression in cultured HAECs, rather than direct effects on macrophage or VSMCs.

4. Discussion

To the best of our knowledge, this is the first study to report a positive effect of zoledronate on suppression of Ang II-induced AAA formation in LDLR \(^{-/-}\) mice. We showed that administration of zoledronate decreased aortic elastin destruction, reduced MMP-2 activity, and attenuated vascular inflammation by suppression of macrophage infiltration in aortic aneurysmal tissues. Additionally, continuous infusion of Ang II-enhanced activation of JNK and ERK1/2 in aneurysmal tissues, and treatment with zoledronate attenuated vascular inflammation and reduced Ang II-stimulated Rho/ROCK activation (as indicated by lower the p-MYPT/RhoA ratio). In \textit{in vitro} studies, we further determined the effects of zoledronate on Ang II-treated HAECs, and showed that zoledronate decreased Ang II-promoted vascular inflammation and diminished monocyte adhesion through down-regulation of adhesion molecule expression. These findings suggest that zoledronate has direct beneficial effects on Ang II-induced AAA formation; this may provide a therapeutic rationale for clinical protection in patients with AAA.

AAA is a potentially fatal disorder that epidemiological studies have detected in 2–9% of the general population. Because of their silent nature, the likely sequelae of undiagnosed AAAs include rupture and sudden death. Knowledge about the mechanism of AAA formations is incomplete, mainly because of the lack of an adequate animal model that closely mimics human pathology. Three key processes contribute to the AAA phenotype. These processes include vascular inflammation, enhanced proteolysis, and SMC apoptosis, although the well-defined initiating mechanisms are not fully understood. On the basis of the loss of extracellular matrix (ECM), especially elastin and accumulation of proteolytic enzymes in the aneurysmal tissues, proteolysis has been regarded as the critical pathogenesis of AAAs. Accumulating evidence has indicated that dysregulation of MMPs and other proteinases in aortic tissues play an essential role in the initiation and progression of AAA. The ECM degradation by both pre-dominant proteolytic enzymes MMP-2 and MMP-9, which were synthesized and released by the vascular ECs, SMCs, and infiltrating inflammatory cells, such as macrophages, contribute to the apoptosis of vascular-SMCs. Degradation of elastin and apoptotic cell death of the medial vascular SMCs destroy the aortic wall integrity, weaken the wall tensile strength, and consequently facilitate

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**Figure 4** Zoledronate alleviates Ang II-induced phosphorylation of JNK, ERK 1/2, and p38 on the aorta through inactivation of RhoA/ROCK. (A) Phosphorylation of JNK, ERK 1/2, and p38 was determined by western blotting on tissues from the aneurysm wall. (B–D) Zoledronate significantly decreased Ang II-induced phosphorylation of JNK \((P < 0.01)\) and ERK1/2 \((P < 0.01)\) in the homogenates of aortas but not p38. (E and F) ROCK inactivates myosin phosphatase through the specific phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at threonine residue 696. RhoA protein levels and phosphorylation of MYPT1 were determined by western blotting \((n = 6 \text{ per group})\).
the development of AAAs. Because of the high mortality rate associated with AAA, it is important to seek pharmacological approaches to stop the expansion of AAAs.

Bisphosphonates are a class of pharmacological compound which are commonly used to treat postmenopausal osteoporosis and osteolytic diseases such as multiple myeloma and complications associated with cancer metastasis to bone.29 Newly developed N-BPs, such as zoledronate, have been shown to have more potent therapeutic effects. Zoledronate is very effective at suppressing osteoclastic activity and resorptive bone loss and is, therefore, widely used to treat various bone diseases. N-BPs can inhibit FPPS, a crucial enzyme in the mevalonate pathway and further interfere with post-transcriptional modification of small GTPase proteins.9 Furthermore, N-BPs have been shown to exert anti-atherogenic, anti-MMPs, and anti-angiogenic effects.10,11,24

On the basis of these findings, we designed a study to investigate whether zoledronate may exert protective effects on AAA formation by continuous Ang II infusion in hyperlipidaemia mice.

Numerous human and experimental studies have confirmed the presence of MMP-2 and MMP-9 in the aneurismal wall.30 Their principal action seems related to the elastolytic activity and modulation of arterial wall remodelling.31 In the AAA model, MMP-2 and MMP-9 were elevated, but showed different patterns of behaviours during aneurysm development. The MMP-9 peak coincided with the inflammatory cell influx (especially neutrophils) and with the elastolytic activity burst, but interestingly seemed to remain active even after most of the elastic fibre degradation. In contrast, MMP-2 showed a progressive increase during the process of AAA formation. In our study, we found that both MMP-2 and MMP-9 were activated in aneurysm tissues, but treatment with zoledronate inhibited MMP-2, but not MMP-9 activity. These findings are in line with previous reports showing that MMP-2 deficiency protects mice from AAA formation.32 These results suggest that selective MMPs exert interdependent behaviour and may have different roles in aneurysmal wall remodelling.33 However, further studies are needed to clarify the functional regulation of MMPs in AAA formation.

It is also known that the AAA have been considered historically as a focal manifestation of advanced atherosclerosis.34 Transmural inflammatory cell infiltration is an important feature of AAAs. Infiltrating leukocytes are thought to be a major source of proteases directed against aortic ECM in human and experimental AAAs.35 N-BPs can inhibit FPPS in the mevalonate pathway, and this pathway is important for the production of small G-proteins, such as Ras, Rac, and Rho, that are important for intracellular structure, signalling, and transport. Small GTPase proteins are required for AT1 receptor activation. Rho-kinase activation regulated TNF-α-induced early and late JNK activation in pulmonary ECs.36 Atorvastatin has been shown to block the RhoA-JNK-MMP pathway in osteosarcoma.37 The RhoA/ROCK signalling pathway participates in the activation of NF-κB, the expression of adhesion molecules and leucocyte adhesion to the microcirculation.38–40 A Rho-kinase inhibitor, fasudil, has been shown to attenuate Ang II-induced AAA through the inhibition of both apoptosis and proteolysis pathways.41 Zoledronate elicits cell-cycle prolongation that seems to be associated with alterations in the levels of certain cyclins and cyclin-related regulatory proteins.42

Figure 5 Zoledronate attenuates Ang II-induced activation of RhoA/ROCK and MAPK JNK, and ERK in cultured human aortic endothelial cells. (A) Phosphorylation of MYPT1, JNK, ERK 1/2, and RhoA protein levels was determined by western blotting in cultured human aortic endothelial cells (HAEC). (B–D) In vitro, zoledronate significantly reduced Ang II-induced phosphorylation of MYPT, JNK, and ERK 1/2 (n = 3–5 per group).
aneurismal tissues. Macrophages are sensitive to bisphosphonates and bisphosphonates can cause apoptosis of phagocytizing cells both in vitro and in vivo. Less immunoreactive macrophages were present in the atheromatous regions of the aorta of rabbits medicated with clodronate for 12 weeks. Once phagocytosed, bisphosphonates have been shown to affect the ability of macrophages to internalize atherogenic LDL-cholesterol and subsequently transformation into foam cells. In vitro studies, we found that zoledronate decreased Ang II-promoted vascular inflammation and diminished monocytes adhesion through down-regulation of adhesion molecules expression, suggesting that zoledronate may attenuate Ang II-induced AAA formation through anti-inflammatory effects. Since zoledronate did not exert significant effects on VSMC and macrophage on comparable dosages in vitro, we suggest that the AAA inhibitory effect is associated with the reduced endothelial activation, rather than a direct effect on macrophages. Although another N-BPs, alendronate, failed to improve aneurysm progression in Fbn1mgR/mgR mice, an animal model for severe Marfan syndrome. We speculate that the contradictory results raised from the basis of the involved mechanisms; mutations that affect the structure or expression of the ECM glycoprotein fibrillin-1 cause pleiotropic manifestations of Marfan syndrome by impairing connective tissue integrity and by promoting TGF-α activation in Fbn1mgR/mgR mice; whereas macrophage accumulation followed by vascular inflammation, elastin degradation, dissection, accelerated atherosclerosis, and neovascularization are classical pathological findings of Ang II-induced AAA.

Furthermore, angiogenesis is the new blood vessel formation from pre-existing blood vessels and is a prominent feature in both atherosclerosis and AAA. Thompson et al. reported that the density of the newly formed vessels was increased in all layers of the aneurysmal wall compared with control samples. The degree of neovascularization is correlated with the extent of the inflammatory infiltration. Moreover, angiogenesis is associated with the inflammatory responses in the aneurysmal wall and accelerates the aneurysm rupture. Misso et al. had demonstrated the potential anti-angiogenic and anticancer activity of zoledronate in vitro and in vivo studies. Our recent data also showed that zoledronate exerts anti-angiogenic effects through modulation of pro-angiogenic cells in ischaemic tissues. In the current study, treatment with zoledronate in hyperlipidaemia mice for 1 month did not alter lipid profiles, which implies zoledronate prevented AAA formation independent of lipid-lowering or modifying effects. These novel findings

Figure 6 Zoledronate suppresses Ang II-induced activation of NF-kB, the expression of adhesion molecules and monocytes adhesions to human aortic endothelial cells. (A) Phosphorylation of p65, ICAM-1, and VCAM-1 protein levels were determined by western blot in cultured human aortic endothelial cells (HAEC). (C–E) Zoledronate significantly reduced Ang II-induced phosphorylation of p65. (B) Confluent HAECs plated on 6-well dishes were incubated with zoledronate for 48 h, and the human monocyte cell line, THP-1 cells (labelled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, BCECF-AM, Molecular Probes) were added on each monolayer at a density of \(5 \times 10^5\) cells/mL dissolved in RPMI1640 containing 1% foetal bovine serum and incubated for 60 min at 37°C. Non-adherent cells were removed by carefully washing with cold phosphate-buffered saline twice. The number of adherent cells was counted by microscope (× 100). The average number obtained from four fields was used as an average value. The fluorescent intensities were determined by excitation and emission at 490 and 535 nm, respectively (n = 3–5 per group).
provide the rationale for using zoledronate, which stems from its ability to induce anti-angiogenic effects that delay AAA progression.

5.1 Limitations
Our study has several limitations. Although the therapeutic range of bisphosphonates is wide, the dose of zoledronate used in this concept-proving study was higher than conventional doses used in treating metastatic bone diseases and osteoporosis. Moreover, we did not feed the LDLR−/− mice a high-fat diet to avoid short-term mortality. We used one animal model to test the effects of zoledronate on AAA formation in the current study, and further studies are needed to assess the therapeutic effects in other animal AAA models.

6. Conclusions
This study showed that zoledronate attenuated abdominal Ang II-induced AAA formation by suppression of MMP-2 activity and inhibition of vascular inflammation by down-regulation of JNK, NF-kB, and reduction of Ang II-induced Rho/ROCK activation. These findings may provide a therapeutic rationale and a novel therapeutic approach for clinical protection in patients with AAA.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: None declared.

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