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Beneficial effects of quinoline-3-carboxamide (ABR-215757) on atherosclerotic plaque morphology in S100A12 transgenic ApoE null mice

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Abstract

Objective—There is an emerging widespread interest in the role of damage-associated molecular pattern molecules (DAMP) S100A8, S100A9 and S100A12 in cardiovascular and other diseases. In this study we tested the efficacy of ABR-215757, a S100 protein binding immuno-modulatory compound to stabilize atherosclerosis in transgenic ApoE null mice that express the human pro-inflammatory S100A12 protein within the smooth muscle cell (SM22 α -S100A12).

Methods—Twelve-week old S100A12 transgenic/ApoE^{-/-} and WT/ApoE^{-/-} mice were treated with ABR-215757 for 5 weeks and were analyzed 4 month later.

Results—Surface plasmon resonance analysis demonstrated that S100A12 interacts with ABR-215757 in a zinc dependent manner *in vitro*. *In vivo*, ABR-215757 administration reduced features of advanced plaque morphology resulting in smaller necrotic cores, diminished intimal and medial vascular calcification, and reduced amount of infiltrating inflammatory cells. ABR-215757 normalized aortic expression of RAGE protein and normalized experimentally-induced delayed hypersensitivity. The effect of ABR-215757 was more prominent in ApoE^{-/-} mice expressing S100A12 than in ApoE^{-/-} animals lacking expression of human S100A12 protein.

Conclusion—Our data suggest that S100A12 is important for progression of atherosclerosis and can be targeted by the small molecule ABR-215757. The specific binding of quinoline-3-carboxamides to S100A12 attenuates S100A12-mediated features of accelerated murine atherosclerosis.

Keywords

atherosclerosis; S100 proteins; RAGE; Quinoline-3-carboxamides

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Introduction

Atherosclerosis is a chronic inflammatory process regulated by a complex network of cytokine and chemokine involving key cells such as endothelial cells, monocytes, dendritic cells, lymphocytes and smooth muscle cells. Vascular injury from a variety of insults leads to expression and release of danger signals including S100 proteins, HMGB1, heat shock proteins, and micro particles containing phosphatidylserine¹. These damage associated danger molecules (DAMPs) are up regulated in atherosclerosis and activate inflammatory pathways. S100 proteins belong to the family of the calcium binding EF-hand proteins, and participate in a large number of cellular functions such as calcium homeostasis, cell growth and differentiation, dynamic regulation of the cytoskeleton, as well as control of inflammation and immune responses². Besides their intracellular functions, the S100/calgranulins are also secreted under certain conditions and exert cytokine-like effects in an autocrine and/or paracrine manner through the activation of cell surface receptors. For example, we showed that S100A12 binds and activates the receptor for advanced glycation endproducts (RAGE), and Vogl et al. showed activation of toll-like receptor (TLR) 4 by S100A8/9^{3, 4}. S100A12 (also known as EN-RAGE or Calgranulin C), S100A8 (MRP 8, Calgranulin A) and S100A9 (MRP14, Calgranulin B) share structural and functional similarities and are often called S100/calgranulins.

Epidemiological studies have demonstrated a strong association of S100/calgranulins and chronic inflammatory disorders, including coronary artery disease. Indeed, peripheral blood mononuclear cell-derived expression of S100A12 was found to be one of the most predictive factors for obstructive coronary disease upon angiography^{5, 6}. Moreover, S100A8/9 is strongly linked to myocardial infarction^{7, 8}, and S100A12 is expressed in the vulnerable coronary plaques associated with sudden cardiac death⁹, in carotid artery plaques of patients with recent trans-ischemic attacks¹⁰, and in dissecting ascending thoracic aortic aneurysms¹¹; this suggests a potential role of S100/calgranulins in atherothrombosis. A disease-causing effect of S100A12 was recently shown by our laboratory where transgenic expression of human S100A12 in vascular smooth muscle accelerated atherosclerosis and calcification in atherosclerosis prone Apolipoprotein E deficient mice¹². Taken together, his data support the hypothesis that S100A12 could serve as a therapeutic target to alleviate vascular disease and remodeling.

The quinoline-3-carboxamide ABR-215757 small molecule was recently demonstrated as an inhibitor of the Zn-dependent interaction of S100A9 with RAGE and TLR4 resulting in reduced cytokine release and inflammation in a mouse model of sepsis¹³. Its *in vivo* activity has been studied in a number of experimental models of autoimmunity and efficacy has been demonstrated in collagen-induced arthritis, acute and chronic experimental autoimmune encephalomyelitis and type 1 diabetes, as well as in experimental lupus in MRL-lpr/lpr mice^{14, 15}. ABR-215757 and other quinoline-3-carboxamide derivatives are currently examined as novel treatments for autoimmune / inflammatory diseases in humans, such as multiple sclerosis¹⁶ and systemic lupus erythematosus (SLE)¹⁴. Because S100/calgranulins are endogenously expressed at high levels in myeloid cells, and are up-regulated in serum and affected tissue of patients with chronic inflammatory diseases such as rheumatoid arthritis, Kawasaki arteritis, inflammatory bowel disease, and SLE¹⁷, they might mediate autoimmune disease.

S100A12/ApoE-null mice develop accelerated and calcified atherosclerosis with histological features similar to human plaque instability. Here we investigated the effect of quinoline-3-carboxamide ABR-215757 administration on S100A12-mediated atherosclerosis in ApoE-null mice. We found that treatment with quinoline-3-carboxamide ABR-215757 reduced atherosclerotic lesion size and improved features of plaque remodeling with reduced

inflammation, smaller necrotic cores, less calcification and a lesser degree of elastic fiber degradation in the S100A12/ApoE^{-/-} mice. ABR-215757 had some anti-inflammatory effects in WT/ApoE^{-/-} mice, and we found reduced progression to complex atherosclerotic lesions, and a reduction of atherosclerotic lesion size of the innominate artery. Taken together, the effect of ABR-215757 was most evident in mice expressing S100A12, and therefore may at least in part be attributed to an interaction of S100A12 with ABR-215757 that affects activation of RAGE and TLR's in a manner different from that of S100A9.

Materials and Methods

Small molecule

ABR-215757 (N-ethyl-N-phenyl-1,2-dihydro-5-ethyl-4-hydroxyl-1-mehtyl-2-oxo-quinoline-3-carboxamide) was provided by Active Biotech (Lund, Sweden). ABR-215757 was dissolved in sterile PBS to a final concentration of 0.1µg/µl and preloaded syringes (0.1ml) were stored at -20°C until used.

Surface plasmon resonance (SPR)

Binding of S100A12 to RAGE and quinoline-3-carboxamide ABR-215757 was studied using the Biacore 3000 system (GE Healthcare), in a similar manner as previously published for S100A9¹³. In a first experimental setup, recombinant human or murine RAGE, both fused with a human IgG₁ Fc tag (hRAGE and mRAGE; R&D Systems), and ABR-215757, with a primary amino group linked to the amide nitrogen position, were immobilized to a CM5 chip using amine coupling. Recombinant *E. coli* derived S100A12 (R&D Systems) was injected over RAGE and quinoline-3-carboxamide compound in a buffer containing physiological concentration of Ca²⁺ and Zn²⁺ to study molecular interactions. Like S100A9, S100A12 coordinates Zn²⁺ with high affinity but differs from S100A9 in lacking a heparin-binding motif important for the interaction of S100A19 with RAGE. Therefore, the influence of Zn²⁺ and heparan sulfate (Sigma H7640; from bovine kidney) on S100A12 binding to RAGE and ABR-215757 was studied for comparison with S100A9. In a next series of experiments, mRAGE was injected over S100A12 or human S100A9 (produced in *E. coli* at Active Biotech) randomly coupled via primary amines at similar densities. HEPES buffered saline (10 mM Hepes, 0.15 M NaCl, 0.005% v/v Surfactant P20, pH 7.4; HBS-P) containing 1 mM Ca²⁺ and varying concentrations of Zn²⁺ was used as sample and running buffer. Binding data was evaluated using GraphPad Prism and BIAevaluation software.

Mice

Transgenic mice containing the SM22α promoter driving expression of human S100A12 protein were generated in C57BL6 background as previously reported¹⁸, and backcrossed for >5 generations into the atherosclerosis prone ApoE^{-/-} mice. S100A12 and ApoE genotypes were confirmed as described¹². Heterozygote S100A12/ApoE^{-/-} and wild type littermate mice lacking the S100A12 gene¹⁹ (WT/ApoE^{-/-}) were studied. Starting at age of 3 month, mice were treated with ABR-215757 (10 µg/g body weight, i.p.) or vehicle (100µl PBS), i.p., 5 days a week for 5 weeks. After completion of treatment, mice were kept for 4 month, and sacrificed at age 8 months. Mice were housed in specific pathogen-free barrier facility and fed normal rodent chow with free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of The University of Chicago following the Guide of the Care and Use of Laboratory Animals published by the United States National Institute of Health.

In vivo ultrasound

Mice were anesthetized with inhaled 1% -2% isoflurane titrated to a heart rate of 470-500 beats per minute and shaved. The ascending aorta was visualized in one plane from the aortic valve to the transverse aorta in a parasternal long axis view using a 40MHz high frequency Visual Sonics Vevo 660 ultrasound machine. The diameter of the ascending aorta 2 cm above the sinus of Valsalva and the diameter of the proximal innominate artery (brachiocephalic artery) was measured by the leading edge method. Two-dimensional guided M-mode imaging was used to measure the left ventricular end systolic (LVESD) and end-diastolic (LVEDD) diameter and posterior wall thickness during diastole. Systolic left ventricular function is expressed as fractional shortening using the equation: $FS (\%) = [(LVEDD-LVESD)/LVEDD] \times 100$.

Histopathology

The left ventricle was cannulated and the aorta was perfused in situ with PBS (2 min) followed by 4% paraformaldehyde (5 min) under physiologic pressure (~45 cm water). After dissection, the aorta was fixed in 10% formalin for 12 hours prior to tissue processing. Seven μ m serial sections were obtained from the proximal aorta (sinus of Valsalva), the aortic arch and from the origin of the innominate artery. Sections were stained with H&E, Masson Trichrome, Verhoeff-Van Gieson stain (VVG), Alizarin Red S, and immunostained for SM α actin (Sigma), CD-3 (abcam, 27-3B19) and F4/80 (Serotec, MCA497). Spleen from C57BL6/J strain was used as positive control tissue for CD-3 and F4/80 immunohistochemistry, and non-immune rat IgG was used as a negative control IgG. The mean value of plaque cross sectional areas was used to estimate the extent of atherosclerosis for each animal and histological grading of medial elastin degradation was performed on VVG stained slides as previously reported¹². Briefly, the lesion area containing calcium in the tissue sections as indicated by the red color upon Alizarin Red S staining was quantified as “calcific area” which may also include necrotic tissue. In contrast, lesions without calcification and with the typical presence of cholesterol clefts and cell debris were quantified as “necrotic area”. Vessel morphometry and areas of positive staining were quantified using Image-Pro Plus software (Media Cybernetics). For detection of positive staining, the portion of the color spectrum representing the positive signal for each stain was defined and then applied to the digitized images to determine the total positive area within the 40x power field region of interest containing atherosclerotic plaque. Briefly, four 7 μ m serial sections placed on 4 slides (total of 16 sections) were examined and mean positive lesion area was calculated by using the most advanced lesion of 1 section/slide for a total of 4 slides. For the CD-3 stained slides, only cells with the characteristic appearance of lymphocytes, i.e. small cells with scant cytoplasm within the vessel/plaque were counted (and not the total brown-stained area), since we noticed staining of foam cells and endothelium upon incubation with anti CD-3 IgG, which however was not observed upon incubation with non-immune IgG.

Protein analysis

Unfixed thoracic aorta was dissected for whole tissue lysates using T-PER-tissue lysis buffer (Pierce). The following primary antibodies were used for western blotting: S100A12 (Abcam 37657), mS100A8 (R&D Systems), mRAGE IgG (R&D Systems), and β -actin (Sigma). Blood was collected at the end of the study by direct cardiac puncture. Serum was stored at -80°C until used for the measurement of interleukin 6 (R&D Systems) and serum amyloid A (SAA ELISA, Tridelta Development).

RNA isolation and quantitative RT-PCR

Total RNA was isolated from the thoracic aorta with the Qiagen-connective tissue RNA mini kit including a DNA-removal step. cDNA was synthesized from 1 µg RNA using Superscript first strand synthesis system (Invitrogen) and used as template for PCR amplification using SYBR GreenER (Invitrogen) and an IQ5 cycler (BioRAD). Primers were designed on the primer bank MGH library. The ΔC_T value was used to describe the difference between the ABR-215757 treatment group and the placebo group, normalized to the house keeping gene β -actin. Relative mRNA expression was estimated as $2^{\Delta C_T}$ (target gene- ΔC_T housekeeping gene).

T-cell mediated response (delayed hypersensitivity, DTH)

One week after completed treatment with ABR-215757 or PBS as described above, mice were sensitized by subcutaneous injection over the left inguinal lymph nodes with 10 µg ovalbumin (ova) (grade V, Sigma) emulsified in 2 mg alum (AlumImuject, Pierce). Seven days after sensitization, one ear of each mouse was challenged on both sides by topical application of 20 µl of 2% ova dissolved in olive oil. The other ear was used as control (olive oil only) and ear thickness was measured 24 hours after challenge using calipers. The DTH reaction was defined as the ear thickness of the challenged ear minus the control ear thickness (modified from ¹⁵).

Statistics

At least 10 mice were analyzed per group for the ultrasound and histology studies. Slides were graded and measured in a blinded manner. For immunoblotting and qRT-PCR at least 5 mice per group were analyzed and experiments were performed three independent times. Student's t-test and one-way analysis of variance were used for mean comparison between two or multiple groups. The Bonferroni correction was used to adjust for multiple comparisons.

Results

Human S100A12 is a target of quinoline-3- carboxamides

First, we wanted to test whether S100A12 interacts with ABR-215757 and human RAGE in the presence of Ca^{2+} and Zn^{2+} . In Figure 1A, S100A12 was injected over amine coupled ABR-215757 and demonstrates binding of S100A12 to the surface in a similar manner as the positive control S100A9, although with somewhat different kinetics. Interestingly, ABR-215757 turned out to be a much weaker competitor of S100A12 interaction with solid phase ABR-215757, than was observed with S100A9 (Figure 1B). As both, S100A9 and S100A12 are known ligands to RAGE we then compared their interaction with immobilized human RAGE. Both proteins demonstrated binding to hRAGE with different kinetics (Figure 1C) and with the S100A12-RAGE complex displaced ten times less effectively by ABR215757 than S100A9 (Figure 1D). This suggests different binding domains to RAGE and ABR-215757. Next, we tested heparan sulfate (HS) as an inhibitor of S100A12 and S100A9 interaction with human RAGE and ABR-215757. HS is a potent inhibitor of S100A9 binding to both human RAGE (Figure 1E) and ABR-215757 (Figure 1F) but was unable to displace S100A12 binding to these surfaces.

Importantly, we next studied the interaction between S100A12 and murine RAGE. mRAGE was injected over S100A12 and S100A9 coupled to the sensor chip via their lysine side-chains at similar densities. As is shown in Figure 2A-C, we found that mRAGE demonstrated significantly higher binding to solid-phase presented S100A12 than S100A9 (B_{max} 260 ± 27 RU vs. 162 ± 5 RU, $p < 0.01$) although they interact with similar affinities (K_D 60 ± 10 nM and 47 ± 3). Finally, we examined the influence of Zn^{2+} on binding of

S100A12 to immobilized mRAGE and ABR-215757. As shown in Figure 2D, addition of Zn^{2+} results in strongly enhanced binding of S100A12 but unlike S100A9, where previous studies demonstrated optimal binding already at 10-20 μM Zn^{2+} ¹³, the SPR signal for S100A12 increased in the entire concentration range used (<200 μM Zn^{2+}). This suggests that although Zn^{2+} is required for both proteins to interact with ABR-21557 and RAGE it does so in a qualitative different way, probably affecting the oligomer status and the confirmation of S100A9 and S100A12 differently. Taken together, our data indicate that S100A12 binds to ABR-215757 with different kinetics than S100A9; ABR-215757 is a poor competitor of S100A12 binding to ABR-215757 and RAGE; S100A12 binding is strongly enhanced by increasing Zn^{2+} concentrations; and heparan sulfate does not interfere with the binding of S100A12 to RAGE or ABR-215757.

Quinoline-3-carboxamides alleviate S100A12-mediated accelerated atherosclerosis

The ability of S100A12 to strongly bind ABR-215757 *in vitro* prompted us to assess whether ABR-215757 would ameliorate S100A12-mediated atherosclerosis *in vivo*. We treated 12 week-old S100A12/ApoE^{-/-} transgenic mice and control wild type WT/ApoE^{-/-} littermates with established early atherosclerosis, i.e. fatty streak lesions (data not shown) with ABR-215757 or vehicle, and analyzed disease state 4-month after completion of treatment (Figure 3). The treatment was well tolerated, and survival, body weight, and appearance were normal in all four groups until sacrifice, similarly to that reported in a murine model of lupus¹⁵. Figures 4 A-D and 4I-L show representative lesions in the innominate artery of a vehicle-treated transgenic S100A12/ApoE^{-/-} mouse and WT/ApoE^{-/-} littermate. Consistent with our previous findings¹², vehicle treated S100A12/ApoE^{-/-} mice have 20-40% increase in aorta atherosclerotic plaque size at the sinus of Valsalva ($215 \pm 17 \mu\text{m}^2$ vs. $181 \pm 21 \mu\text{m}^2$, $p=0.03$), the aortic arch ($437 \pm 31 \mu\text{m}^2$ vs. $352 \pm 28 \mu\text{m}^2$, $p=0.03$) and at the innominate artery ($165 \pm 14 \mu\text{m}^2$ vs. $117 \pm 17 \mu\text{m}^2$, $p=0.01$) compared to vehicle treated WT/ApoE^{-/-} mice (Table 1). Importantly, plaques in the innominate artery of vehicle S100A12/ApoE^{-/-} mice demonstrated accelerated plaque remodeling with large necrotic cores occupying 19 % of the lesion (Table 1, and a representative histology shown in Figure 4D and quantified in Figure 4Q), enhanced intima and media calcification (Table 1 and Figure 4B, and quantified in Figure 4R), significantly enhanced degradation of elastic fibers with grade 3-4 elastolysis (Table 1 and Figure 4C and quantified in Figure 4S), and outward remodeling of the vasculature with a significant increase in the circumference of the external elastic membrane, compared to control treated WT/ApoE^{-/-} lesions ($2210 \pm 360 \mu\text{m}$ vs. $1256 \pm 254 \mu\text{m}$, $p=0.05$, Table 1). Consistent with these findings of advanced plaque remodeling in vehicle-treated mice, the amount of smooth muscle is strikingly reduced in the atherosclerotic plaques of S100A12/ApoE^{-/-} compared to WT/ApoE^{-/-} (Figure 4T). Using this model of S100A12-mediated accelerated atherosclerosis we next analyzed pathologic features in mice treated with ABR-215757, a compound recently shown to inhibit *acute* inflammation in LPS-treated mice by binding to S100A9¹³. Compared to vehicle, ABR-215757 treatment reduced atherosclerotic lesion size in the innominate artery and in the aortic root of S100A12/ApoE^{-/-} mice by 20% (Table 1). Despite this rather small difference in lesion size, morphometric analyses shown in Table 1 and Figures 4 Q-T revealed markedly diminished necrotic core size (5 % vs. 19%), decreased intima and media calcification (11 vs. 36%), minimal elastic fiber disruption (grade 1.4 vs. 3.4), and more plaque area covered with smooth muscle cells (2.8% vs. 0.5%). A representative lesion of an ABR-215757-treated transgenic S100A12/ApoE^{-/-} animal is shown in Figure 4E-H. Interestingly, ABR-215757 had lesser effect in WT/ApoE^{-/-} mice and the lesion size was reduced by 10% in the innominate artery and unchanged in the proximal aortic root (Table 1). As in the case with the S100A12/ApoE^{-/-} mice, ABR-215757 did also affect plaque remodeling in WT/ApoE^{-/-} mice, which exhibit features of early fatty streak lesions with foam cell accumulation and lacking the cellular complexity of infiltrating smooth muscle

cells and chondrocyte-like cells (Figure 4 M-P, and quantified in Figure 4T). We did observe no overt calcification or elastolysis in the abdominal aorta of either S100A12 or WT ApoE^{-/-} mice, and we observed no differences in those features of advanced atherosclerosis between the treatment groups (data not shown). The improved plaque morphology in the innominate artery did not arise from altered lipid levels because there was no significant difference in plasma cholesterol and triglyceride content among the four groups. Total cholesterol was 432± 53 mg/dl in S100A12/ApoE^{-/-} mice and 447± 39 mg/dl in WT/ApoE^{-/-} mice receiving vehicle (p=0.39), and this was not different from ABR-215757-treated mice (411± 47 mg/dl and 427± 47 mg/dl). We conclude that ABR-215757 reduces atherosclerosis and promotes plaque remodeling by reducing their necrotic core size and calcification in S100A12/ApoE^{-/-} mice, and prevents progression to complex and smooth muscle cell-enriched atherosclerotic lesions in WT/ApoE^{-/-} mice. This suggests that ABR-215757 treatment may be sufficient to halt S100/calgranulin-mediated acceleration of atherosclerosis.

We next corroborated these histopathological findings using high frequency ultrasound. Vascular lumen diameter was increased by 17% at the level of the ascending proximal aorta, and to 25% at the origin of the innominate artery in placebo treated S100A12/ApoE^{-/-} mice compared to WT/ApoE^{-/-} (p=0.03, Figure 5 A-C). However, the outward remodeling and dilatation of ABR-215757-treated S100A12/ApoE^{-/-} mice was abolished to the level observed in WT/ApoE^{-/-} mice. No further effect on aortic diameter was seen in ABR-215757-treated WT/ApoE^{-/-} mice. Moreover, ABR-215757 had no effect on myocardial systolic performance as calculated as left ventricular fractional shortening (FS) and wall thickness as shown in Figure 5D-F.

Quinoline-3-carboxamides attenuates vascular S100/RAGE cascade and systemic inflammation

Based on the significant effects of ABR-215757 on plaque progression, we analyzed expression of genes previously linked to S100A12/RAGE signaling immediately after completion of the 5 weeks treatment with ABR-215757 or vehicle. Since S100A12 is a chemo-attractant for inflammatory cells^{3, 20}, we measured gene expression for leukocyte markers in the aortic tissue. Figure 6A shows a 3.5 fold increase in CD 68 (monocytes/macrophage marker), 2.6 fold increase in CD4 (T helper, monocytes, macrophages, and dendritic cell marker) and 3.2 fold increase in CD11c (neutrophils, monocytes, macrophage and dendritic cell marker) mRNA abundance in the aorta of S100A12/ApoE^{-/-} mice compared to WT/ApoE^{-/-} mice. Importantly, expression of leukocytes markers was reduced by 55-60% in the S100A12/ApoE^{-/-} aorta after treatment with ABR-215757 (p<0.01). In aorta from WT/ApoE^{-/-} mice, ABR-215757 administration reduced CD11c by 25% (p=0.05) but had no effect on CD4 and CD68 expression. This data suggest that ABR-215757 dampens S100A12-mediated infiltration of inflammatory cells to the vasculature, and reduces CD-11c positive cells in WT/ApoE^{-/-} mice. Consistently, gene expression of MCP-1, VCAM-1, ICAM-1 and RAGE were reduced in aorta from S100A12/ApoE^{-/-} mice treated with ABR-215757 by 56%, 39%, 42%, 63%, respectively compared to vehicle-treated S100A12/ApoE^{-/-} mice (Figure 6B and p<0.01 for each gene tested). In WT/ApoE^{-/-} mice, ABR-215757 reduced VCAM-1 and RAGE mRNA by 20-30% (p=0.05), and had no significant effect on MCP-1 and ICAM-1 expression. Next, we examined protein expression of human S100A12, murine S100A8 and their receptor RAGE in aorta of WT/ApoE^{-/-} and S100A12/ApoE^{-/-} mice immediately after 5-weeks of treatment with ABR-215757 or vehicle. S100A12 was not expressed in WT/ApoE^{-/-} aorta as expected, and it was reduced in S100A12/ApoE^{-/-} treated with ABR-215757 compared to vehicle-treated mice (Figure 6C). Moreover, compared to vehicle, ABR-215757 attenuated the expression of endogenous S100A8 and RAGE in mice of either genotype.

Consistent with our findings of reduced expression of genes linked to leucocyte recruitment as shown in Figure 6, there were significantly reduced number of CD3- positive lymphocytes in the vessel wall of ApoE^{-/-} mice treated with ABR-215757 (Figure 7E-H, quantified in 7L), suggesting that ABR-215757 prevents T-cell accumulation in atherosclerotic lesions. This effect was more pronounced in S100A12 transgenic mice. In contrast, macrophage accumulation, as detected by staining with F4/80 marker, was not significantly different among the four groups. (Figure 7 A-D, quantified in 7K). These findings suggested that the beneficial effect of ABR-215757 on atherosclerotic lesion morphology and cellular composition is at least in part, by reducing the influx of T cells into the vessel wall. Furthermore, we found significantly reduced serum interleukin-6 and serum amyloid A in S100A12/ApoE^{-/-} mice treated with ABR-215757 compared to control treated mice (for IL6: 174±12 vs. 45±11 pg/ml; p<0.01 and for SAA: 35±3 vs. 19±4; p=0.02). There was no significant difference in WT/ApoE^{-/-} receiving ABR-215757, although the absolute amount of cytokines were near the lower detection limit of the assay in WT/ApoE^{-/-} serum (Figure 7E-F).

Taken together, this data demonstrate attenuation of S100/RAGE axis and reduced vascular and systemic inflammation in ABR-215757 treated animals, and may provide a possibly mechanistic link to the observed anti-inflammatory effects of ABR-215757.

Quinoline-3-carboxamides modulates the adaptive immunity

We and others have showed an important role of RAGE and S100 proteins in mediating delayed hypersensitive inflammation and adaptive immune responses^{3, 21}. To assess whether the observed reduced vascular inflammation in ABR-215757-treated mice could stem from systemic immune-modulatory effects, we assessed experimentally-induced delayed-type hypersensitivity. Ear thickness was measured 24 hours after epicutaneous challenge with ovalbumin (ova) in mice sensitized to ova. As expected, S100A12/ApoE^{-/-} mice showed increased ear thickness compared to WT/ApoE^{-/-} mice (2.4 ± 0.3 mm vs. 1.4 ± 0.3 mm, p=0.01). In contrast, S100A12/ApoE^{-/-} treated with ABR-215757 had significantly reduced ear thickness compared to placebo treated S100A12/ApoE^{-/-} mice (1.3 ± 0.2 mm and 2.4 mm± 0.4 mm, respectively, p=0.01).

Discussion

The focus of this study was to identify a pharmacological treatment to reduce S100A12-mediated accelerated atherosclerosis. We selected ABR-215757, a known anti-inflammatory small molecule candidate for the treatment of autoimmune diseases, because of its known ability to bind S100A9 and consequent impairment of S100A9-mediated activation of RAGE and Toll like receptors¹³. RAGE is involved in many inflammatory processes including atherosclerosis, and studies utilizing mice lacking RAGE provided strong evidence that RAGE mediates vascular inflammation and atherosclerosis²². Our data here show that S100A12 binds to ABR-215757 and recombinant RAGE in a Zn²⁺-dependent manner. However, unlike S100A9, these interactions were not affected by pre-incubation of S100A12 with heparan sulfate ABR-215757. The biological importance of these different binding characteristics are not well understood. It is possible that S100A12 plays a more important role under conditions of increased Zn²⁺ concentrations. In this context, a recent paper reported on an association of elevated serum Zn²⁺ (77-200 μM) with up to 1000-fold increase in S100A8/A9 in five patients with systemic inflammation. S100A12 was not reported in this case series²³. *In vivo*, we demonstrate that ABR-215757 treatment reduced vascular inflammation, atherosclerotic lesion size, and improved atherosclerotic plaque morphology in a murine model of S100A12-mediated accelerated vascular disease. In regular chow fed transgenic S100A12/ApoE^{-/-} mice, ABR-215757 administration reduced necrotic core size and atherosclerotic calcification, two histological features of advanced

atherosclerosis not present in chow fed WT/ApoE^{-/-} mice. Therefore, future studies utilizing a western high fat diet (20% fat, 0.1-0.2% cholesterol) or adding other metabolic de-arrangements that accelerate atherosclerosis in WT/ApoE^{-/-} such as hyperglycemia, would allow evaluation of ABR-215757 on advanced atherosclerosis independently of transgenic expression of human S100A12. In support of ABR-215757 having a beneficial effect on remodeling of advanced atherosclerotic lesions, our data suggest that ABR-215757 attenuates early atherosclerosis even in the absence of S100A12, as shown by a 10% reduction in lesion size in the brachiocephalic artery, and reduced cellular complexity within the atherosclerotic lesions. We hypothesize that the effect of ABR-215757 in WT mice is mediated by binding to murine S100A9, and thereby impairing its pro-inflammatory function. This view is in agreement with other animal models, and Croce and colleagues demonstrated attenuated atherosclerosis in ApoE^{-/-} mice lacking S100A9²⁴. Moreover, S100A9 neutralizing antibodies similarly reduced atherosclerosis in ApoE^{-/-} mice²⁵.

Although attractive, anti-inflammatory or immunomodulatory therapy for atherosclerosis may result in unexpected outcomes. For example, ApoE^{-/-} mice lacking the receptor for interleukin-1 were recently shown to develop enhanced plaque vulnerability²⁶. Here we show that ABR-215757 has a beneficial effect on atherosclerotic plaque morphology in ApoE^{-/-} mice. Mechanistically, ABR-215757 treatment normalized experimentally induced delayed hypersensitivity in S100A12/ApoE^{-/-} mice and attenuated the enhanced chemo-attractant properties of the S100A12-expressing vasculature. Interestingly, this effect of ABR-215757 was long lasting and even 4 month after the last injection, the atherosclerotic lesions were smaller and exhibited less CD 3 positive cells compared to vehicle-treated animals. We speculate that this could be caused by the early reduction in infiltrating inflammatory cells preventing a vicious cycle of vascular inflammation. Another, although not exclusive, explanation of the beneficial effects of ABR-215757 is an acceleration in the reduction of S100A12-transgene levels in the vasculature of aged ApoE^{-/-} mice¹². Future studies are needed to define dosing and treatment duration of ABR-215757. Moreover, ABR-215757 may have effects independent of S100A9 or S100A12, since recent work identified quinoline-3-carboxamides as potent inhibitors of phosphodiesterase 4, a cAMP metabolizing enzyme highly expressed in inflammatory and immune cells²⁷, in addition to antioxidant activity and effect calcium channel activity²⁸.

ABR-215757 has been tested in other mouse models. For example, in a study of MRL/l mice, which serves as a model for autoimmune diseases similar to SLE, continuous administration of ABR-215757 in the drinking water for 20 weeks at high (75 µg/mouse) or low (7.5 µg/mouse) dose markedly improved survival and reduced SLE associated kidney failure¹⁵. Although this study did not measure S100 proteins or RAGE, other studies in humans demonstrated elevated S100A8/9 serum levels^{29, 30} or increased S100A8/9 expression in dendritic cells³¹ from SLE patients with augmented disease activity. Importantly, an elegant study by Chen et al. utilizing myeloid cells with forced over expression of S100A9 demonstrated a defect in dendritic cell differentiation with increased production of myeloid suppressor cells³².

In summary, our data demonstrates beneficial effects of ABR-215757 on the course of atherosclerosis with improved features of plaque morphology in a mouse model of accelerated disease caused by transgenic expression of human S100A12. Our data suggests that progression/regression of atherosclerosis could be studied in the ongoing clinical trials in the patients with rheumatoid arthritis treated with ABR-215757.

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Highlights

S100A12 protein is highly up-regulated in the vasculature of human atherosclerotic plaques and was previously shown to accelerate atherosclerosis in apolipoprotein E deficient mice.

Here we show that Quinoline-3-carboxamide (ABR-215757) binds S100A12 in vitro and treatment with ABR-215757 attenuates accelerated atherosclerosis in apolipoprotein E deficient mice with transgenic expression of human S100A12 in smooth muscle.

Quinoline-3-carboxamide (ABR-215757) is currently in clinical testing for the treatment of systemic lupus erythematosus (SLE), and may have beneficial effects on human atherosclerosis.

Figure 1. S100A12 and S100A9 interact differently with immobilized ABR-215757 and hRAGE
A. Binding to immobilized ABR-215757. Sensorgrams obtained after injection (1) of 100 nM S100A12 or S100A9 (positive control) for 3 min at 30 $\mu\text{L}/\text{min}$ over amine-coupled immobilized ABR-215757 in the presence of 1 mM Ca^{2+} and 20 μM Zn^{2+} . Sample buffer was pumped over the surface (2) and final regeneration was performed with a 60 s pulse of 3 mM EDTA (3). **B.** Binding of S100A12 and S100A9 after pre-incubation with ABR-215757. 100 nM S100A12 or S100A9 injected with 7.81-1000 μM ABR-215757 shown as the response during the late dissociation phase in % of response without competitor. Data were fit to a sigmoidal dose-response curve using GraphPad Prism. Fifty percent inhibition (IC₅₀) was obtained at 80 μM and > 1 mM ABR-215757 for S100A9 and S100A12, respectively. **C.** Binding to immobilized hRAGE. RAGE was immobilized at a density of 3 kRU. Other conditions are as in A. **D.** Inhibition of RAGE binding with ABR-215757. IC₅₀ values of 85 μM and > 1000 μM were calculated for S100A9 and S100A12 binding to RAGE which demonstrate that S100A12-hRAGE association is weakly displaced by ABR-215757 in solution. **E/F.** Effects of heparan sulfate (HS) on S100A12 and S100A9 binding to RAGE and ABR-215757. 100 nM S100A12 (■), S100A9 (◆) or S100A8/9 (▲) + HS was injected (3 min at 30 $\mu\text{L}/\text{min}$) over immobilized human RAGE (left panel) or ABR-215757 (right panel) in HBS-P containing 1 mM Ca^{2+} and 20 μM Zn^{2+} . Displacement curves were fit to a sigmoidal dose-response model in GraphPad Prism for calculation of IC₅₀. HS inhibited S100A9 binding to RAGE and ABR-215757 to 50% at 20

and 24 ng/mL, respectively, whereas inhibition of S100A8/9 required 96 and 44 times higher concentrations. S100A12 was not displaced even at the highest HS concentration, 5 μ g/mL.

Figure 2. S100A12 interacts with mRAGE in a Zn²⁺-dependent manner similar to S100A9

A. Sensorgrams obtained after injection of 12.5-50 nM mRAGE over immobilized S100A12 (A) or S100A9 (B) in HBS-P containing 1 mM Ca²⁺ and 50 μM Zn²⁺. In C, signals at late association phase were plotted versus concentration of mRAGE. B_{max} was calculated to 260 ± 27 and 162 ± 5 RU and K_D to 60 ± 10 and 47 ± 3 nM for S100A12 and S100A9, respectively, after fit of curves to a 1:1 model in Graph Pad Prism. Recombinant human IgG1 Fc (from R&D Systems) demonstrated no binding to immobilized S100A12 or S100A9 (data not shown). **D.** S100A12 interacts with ABR-215757 and mRAGE in a Zn-dependent manner. 100 nM S100A12 was injected (2 min at 30 μL/min) over amine coupled mRAGE or ABR-215757 in a buffer containing 1 mM Ca²⁺ in the absence or presence of 3.125-200 μM Zn²⁺. Responses at late association phase were plotted versus Zn²⁺ concentration and show enhanced binding of S100A12 to both mRAGE and ABR-215757 in the entire concentration range. Even in the absence of Zn²⁺, S100A12 was able to associate with both mRAGE (51 RU) and ABR-215757 (195 RU).

Figure 3. Experimental design

Transgenic S100A12/ApoE^{-/-} mice and WT/ApoE^{-/-} littermates with established fatty streak atherosclerotic lesions were treated for 5 weeks with i.p injections of ABR-215757 or PBS, and analyzed 4 month later.

Figure 4. Treatment with ABR-21757 improves features of atherosclerotic plaque morphology Representative images of innominate (brachiocephalic) artery lesions from S100A12/ApoE^{-/-} (A-H) and WT/ApoE^{-/-} mice (I-P) stained with Masson trichrome (A,E,I,M), Alizarin Red S (calcium phosphate in red, B,F,J,N), Verhoeff -Van Gieson (elastic fibers in black, C,G,K,O), and Hematoxylin & Eosin (D, H,L,P). Original magnification, 10x, scale bar, 10 μ m. Quantification of necrotic area within plaques (Q). Quantification of Alizarin Red stained area within plaques (R). Quantification of elastic fiber degradation (S); grade 1=intact fibers, grade 2=disruption of one fiber with intact neighboring fibers, grade 3=disruption of two or more fibers in direct contact, grade 4=disruption of all fibers from internal elastica lamina to external elastic lamina. Total plaque smooth muscle (SMC) content based on SMC α -actin staining (T). * $p < 0.05$, 10 animals per group.

Figure 5. Treatment with ABR-215757 reduces aortic dilatation

A. *In vivo* ultrasound for measurement of the diameter of the ascending aorta 2 cm above the aortic valve (AV, arrow) and at the origin of the brachiocephalic (BC) artery were quantified in **B** and **C** (n=5 male mice). **D.** M-Mode derived measurement of LV dimension at end-systole and end-diastole, and thickness of the LV posterior wall, and quantified in **E** and **F**.

Figure 6. ABR-215757 reduces vascular inflammation

Expression of markers of inflammatory cells (**A**) and inflammatory mediators (**B**) mRNAs as well as protein expression of S100 and RAGE (**C**) was analyzed in whole lysates prepared from the thoracic aorta of S100A12/ApoE^{-/-} and WT/ApoE^{-/-} mice immediately after 5 weeks of ABR-215757 or vehicle treatment. * $p < 0.05$ vs. vehicle group, 10 animals per group.

Figure 7. ABR-215757 reduces local and systemic inflammation

Representative sections of the innominate artery stained with anti F4/80 (A-D) and anti CD3 (E-H) in S100A12/ ApoE^{-/-} (left column) and in WT/ApoE^{-/-} (right column) with control treatment (A, B, E, G), or treated with ABR-215757 (C, D, F, H). Scale bar =10μm. Inserts of serial sections stained with control non-immune IgG are shown in the right lower corner in A-D. Mouse spleen is shown as positive tissue control for F4/80 (I) and for CD3 (J) immunohistochemistry.

Plaque macrophage content based on F4/80 staining (K) and number of infiltrating T cells based on CD-3 staining (L) were quantified in serial sections of atherosclerotic plaques in the innominate artery. Serum IL-6 (M) and serum amyloid A (N) was measured at study end. (n=10 animals for each group).

Table 1

Analysis of atherosclerotic plaques in transgenic S100A12/ApoE^{-/-} or WT/ApoE^{-/-} mice treated with vehicle or ABR-215757.

	Ascending aorta (Sinus of Valsalva)			
	S100A12/ApoE ^{-/-}		WT/ApoE ^{-/-}	
	vehicle	ABR-215757	vehicle	ABR-215757
Plaque area (×10 ³ , μm ² /section)	215±17	172±31 *	181±21	182±16
EEL circumference (μm)	5084±411	4057±469 *	4162±454	3967±346
Grade of elastin degradation	2.7	1.6 *	1.3	1.2
Calcified area, %	12±4	5±3 *	7±6	6±5
Necrotic area, %	15±3	7±5	5±4	3±4
	Innominate artery (brachiocephalic artery)			
Plaque area (x10 ³ , μm ² /section)	165±14	131±21 *	117±17	105±23 *
EEL circumference (μm)	2210±360	1536±257 *	1256±254	1350±236
Grade of elastin degradation	3.4	1.4 **	1.4	1.2
Calcified area, %	36±8	11±6 **	5±3	6±5
Necrotic area, %	19±8	5±5 **	5±6	6±4

Data are expressed as mean ± SEM; External elastic laminae (EEL); calcified area and necrotic area are shown as % of total plaque area; and

* p<0.05 and

** p<0.01 compared to respective vehicle group. (n=10 animals for each group)