SUPPLEMENT MATERIAL

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Detailed Methods

Animals

Male and female mice homozygous null for the apolipoprotein E (apoE) gene, were derived from a closed outbred colony housed within the Animal Unit of the University of Bristol (originally provided by Dr J Breslow, Rockefeller University, New York, New York). The strain background of the animals was 71% C57BL/6, 29% 129, as determined by fingerprinting of tail-tip DNA. The housing and care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office. The investigation conforms with 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).

MMP-12 Activity and Dose Ranging

Heparinised blood samples were added to a reaction mixture containing mouse recombinant MMP-12 (0.2 nmol/L final concentration) and a fluorogenic substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, 15 μmol/L). MMP-12 activity in absence or presence of various concentrations of RXP470.1 was measured in the reaction mixture by fluorimetry. Using this methodology, an inhibition calibration curve was obtained. According to Devel et al [1], the required plasma concentration of RXP470.1 (referred to previously as compound 1) to completely block MMP-12 but spare other MMPs and cathepsins, is 100 nmol/L. We therefore carried out a pilot study to determine the concentration of RXP470.1 that would need to be loaded into osmotic minipumps in order to achieve this plasma concentration. ApoE knockout mice (n=6) were implanted with Alzet model 2004 osmotic minipumps (pumping rate 0.25 μL/h for 4 weeks; Charles River Ltd, Margate, UK) containing a range of RXP470.1 concentrations from 10 to 50 μg/μL. Plasma RXP470.1 concentration was measured after 2 days dosing, a sufficient period to reach a constant infusion of inhibitor in animals. Using the assay described above, MMP inhibitory activity measured in RXP470.1 treated animal plasmas allowed to determine the inhibitor concentration, using the calibration inhibition curve. Furthermore, the presence of intact RXP470 was confirmed in plasma extraction mixtures with μHPLC coupled to mass spectrometry.

Drug Administration

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A total of 64 apoE knockout mice (47 male and 17 female) , aged 8 weeks at the start of the study, were fed a high-fat rodent diet containing 21% (w/w) fat from lard and supplemented with 0.15% (w/w) cholesterol (Special Diets Services, Witham, UK) for a period of 8 weeks to develop mature atherosclerotic plaques in the brachiocephalic artery as previously described [2]. At this stage, 14 mice (10 male and 4 female) were terminated as a satellite group to evaluate atherosclerosis size and composition at the commencement of RXP-470.1 infusion ('Control - 8 weeks'). The mice were then anaesthetised by inhalation with isofluorane and Alzet osmotic minipumps (model 2004, Charles River Ltd, Margate, UK) with an infusion rate of 0.25 μL/h for 4 weeks were implanted subcutaneously in the interscapular region. The reservoir of each pump was preloaded with 200 μL of either sterile phosphate-buffered saline ('Control - 12 weeks'; n=27, 20 male and 7 female) or the MMP-12 specific inhibitor RXP470.1 solution at a concentration of 4.6mg/kg of bodyweight/day in sterile PBS ('RXP470.1'; n=23, 17 male and 6 female), and returned to a high-fat diet for a further 4 weeks. The above protocol is outlined in Additional Figure I. Analysis of blood samples from RXP470 treated animals after 4 weeks of infusion confirm that the RXP470.1 plasma concentration was 100± 10 nmol/L.

Apolipoprotein E/MMP-12 Double Knockout Mice

We generated both apolipoprotein E (apoE)/MMP-12 double knockout mice (apoE/MMP-12^{DKO}) and apoE KO/MMP-12 wild-type (WT) controls (apoE^{KO}/MMP-12^{WT}) as described previously [3]. Mice were placed on a high-fat diet for 8 weeks (n=27 per group).

Termination

Animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone, before exsanguination by perfusion via the abdominal aorta with PBS at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. This was followed by constant pressure perfusion with 10% formalin.

Histology

The brachiocephalic artery, proximal aorta, descending aorta and the heart of each mouse was embedded in paraffin. Sections were cut at 3 μm from the atherosclerosis-prone areas of these vascular beds as previously described (as reviewed by van der Laan and colleagues [4]). Sections were cut at 3 μm and stained using

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haematoxylin and eosin or Miller's elastin/van Gieson. Additionally, immunohistochemistry for MMP-12 (Abcam), cleaved caspase-3 (R&D), cleaved poly ADP ribose polymerase (PARP)-1 (Abcam), proliferating cell nuclear antigen (PCNA) (Abcam), von Willebrand factor (Dako), isolectin B4 (Vector Labs) and histochemistry for fibrillar collagens (Sirius red) and calcification (von Kossa) was performed. The detection of apoptotic macrophages was performed by dual immunohistochemistry using in situ end-labelling (ISEL).

Smooth Muscle Cell and Macrophage Density

Immunohistochemistry for smooth muscle cells (α-smooth muscle actin) and macrophages (F4/80) were each performed on four 3 μm paraffin sections respectively from proximal brachiocephalic plaques of both RXP470.1-treated (n=23) and control mice (n=27). Cells stained positive with the cell-specific markers were counted and density expressed as the percentage of total nucleated cells stained positive.

Plaque Morphometry

Up to five vessel cross-sections were quantified per mouse. Analysis was performed using a computerised image analysis program (Image Pro Plus, DataCell, Maidenhead, UK). The lengths of the internal and external elastic lamellae were recorded by image analysis. These were used to derive the total vessel area and the (lumen + plaque) area, by assuming them to be the circumferences of perfect circles. Plaque area was measured directly, and was subtracted from the area enclosed by the internal elastica to derive the lumen area.

In-Situ End Labelling

Apoptotic cells were identified by *in situ* end labelling (ISEL), performed as previously described[5]

In situ zymography

Elastinolytic activity was localised in brachiocephalic arteries removed from apoE knockout mice that had received high-fat diet for 12 weeks, using a modification of the in situ zymography method[6] as previously described[7]. In brief, frozen 8 μm cryostat sections were incubated overnight at room temperature in a humidified dark chamber with 20 μg/mL fluoroscein (FAM)-conjugated elastin (AnaSpec, Cambridge Bioscience Ltd, Cambridge, UK) dissolved in developing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM

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$CaCl_2$, 0.2 mM sodium azide). Cleavage of the substrate by proteinases results in unblocking of quenched fluorescence and in an increase in fluorescence intensity. Sections were incubated for 24 hours in developing buffer alone, or in the presence of either the MMP-12 inhibitor RXP470.1 (100 nmol/L), the non-selective MMP inhibitor BB-94 (British Biotechnology Ltd, Oxford, UK) (1 μmol/L); or EDTA (20 mmol/L).Sections were washed in PBS, fixed with 4% paraformaldehyde, and mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen, Paisley, UK). Using fluorescence microscopy, elastinolytic activity was identified as green fluorescence.

Purification and culture of mouse blood monocytes

Monocytes were purified from mouse peripheral blood by a Ficoll-Hypaque gradient (Ficoll-Paque Plus: Amersham Biosciences), followed by differential adherence and cultured in 20ng/mL M-CSF for 7-10 days to induce differentiation into macrophages.

Purification of rabbit foam cell-macrophages

New Zealand White rabbits (Harlan, UK) fed a 1% cholesterol-supplemented diet had sterile sponges placed under the dorsal skin to generate foam cell macrophages, as described previously[8].

In vivo monocyte/macrophage invasion assay

Ten week old, chow fed, male C57Bl/6;Sv129 mice (n=14) were anaesthetised by inhalation with isofluorane and Matrigel™ (BD Biosciences, Oxford, UK) infused sponges placed under the dorsal skin for 4 weeks to accumulate monocyte/macrophages as previously described[9]. Additionally, Alzet osmotic minipumps (model 2004, Charles River Ltd, Margate, UK) with an infusion rate of 0.25 μL/h for 4 weeks were implanted subcutaneously in the interscapular region. The reservoir of each pump was preloaded with 200 μL of either sterile phosphate-buffered saline (‘Control’; n=7) or the MMP-12 specific inhibitor RXP470.1 solution at a concentration of 23 mg/mL in sterile PBS (‘RXP470.1’; n=7), and returned on a chow diet for a further 4 weeks. Mice were terminated and the sponges retrieved and either monocyte/macrophages isolated as described previously[8], or the sponges were fixed in 10% formalin. Fixed sponges were then processed and wax-embedded before eight 3 μm sections were taken and subjected to immunohistochemistry for PCNA to detect proliferative rates and cleaved PARP-1 to determine apoptotic frequencies.

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In vitro studies on macrophages and macrophage-derived foam cells

The effect of 24 hours incubation of 100 nmol/L RXP470.1 in serum-free RPMI on proliferation and apoptosis were determined in M-CSF differentiated mouse monocyte-derived macrophages and rabbit experimental foam cells as described previously[5].

In vitro studies on smooth muscle cells

Mouse aortic smooth muscle cells were cultured in serum-free DMEM media supplemented with 100 nmol/L RXP470.1 and 300 ng/ml recombinant Fas Ligand (FasL). Apoptosis was assessed by measurement of cleaved caspase-3 levels using the Caspase-Glo luminescent assay (Promega). Samples were incubated with an equal volume of the mixed Caspase-Glo reagent and luminescence measured using a Veritas microplate luminometer from 0-90 minutes until peak luminescence was achieved. Immunocytochemistry for cleaved caspase-3 was performed as described previously[7].

Quantification of Apoptosis

To assess the effect of deletion of MMP-12 on in vitro macrophage apoptosis, terminal whole blood samples (1ml) were taken from both apoE/MMP-12^{DKO} mice (n=7) and apoE^{KO}/MMP-12^{WT} control mice (n=7). Monocytes were isolated using a Ficoll-paque density gradient and adherence. To differentiate the monocytes into macrophages, cells were cultured in 6-well plates (1 x 10^5 cells/well) in RPMI 1640 media supplemented with 10% foetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L L-glutamine and treated with 40ng/ml macrophage-colony stimulating factor (M-CSF) for 96 hours, then replaced with fresh media and M-CSF and cultured for a further 72 hours. To induce apoptosis, media was removed and replaced with serum-free media and cells cultured for 60 hours. Apoptosis was measured by immunocytochemistry for cleaved caspase-3 (R&D Systems, Abingdon, UK) as previously described [7], although is must be noted that necrosis was not evaluated by this method and may contribute to overall numbers of cell death.

Western blotting

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Total cell lysates were subjected to Western blotting using 0.1 μg/ml mouse anti-N-cadherin antibody (clone 32, Transduction Labs) or 1 μg/ml anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB374, Chemicon International). Detected bands were quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad, Hemel Hempstead, UK) and were normalised by GAPDH values.

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). Treatment group values were compared with their controls using the computer programs InStat and Prism (both GraphPad Software, San Diego, California, USA). For the comparison of group means, a check was first made for similar variances: if this was passed then an unpaired two sample two-tailed Student's t-test was carried out. If the variances were significantly different, then an unpaired two sample two-tailed t-test with Welch's correction was used. For the comparison of the longer term groups (12 weeks) and the 8 week group, an ANOVA test was used. Discontinuous data (incidence of buried fibrous layers or presence of calcification) were analysed using Fisher's exact test. In all cases, statistical significance was concluded where the two-tailed probability was less than 0.05.

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Table I: Effect of MMP-12 inhibition on plaque characteristics

	Control		RXP470.1	
	Male	Female	Male	Female
Lesion area ($x10^3$ μm^2)	234±34	223±41	186±37	120±43
Plaque macrophage density (%)	42.1±2.1	44.1±2.1	36.3±1.9	35.9±1.7
Plaque SMC density (%)	29.9±6.7	20.1±4.4	40.6±3.5	41.7±6.5
Plaque apoptosis (%)	38.2±5.3	47.2±5.3	12.8±2.5	25.2±13.4
Plaque collagen (%)	5.2±0.8	6.0±1.4	6.8±0.7	10.8±6.0
Plaque elastin (%)	10.7±2.1	14.4±1.7	12.2±3.0	13.9±13.0
Plaque calcification (%)	6.0±2.8	5.6±2.5	0.3±0.1	1.8±1.8
Plaque lipid (%)	59.8±3.0	59.4±7.3	62.1±3.8	69.3±10.2

Values are mean±SEM

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Figure Legends and Figures

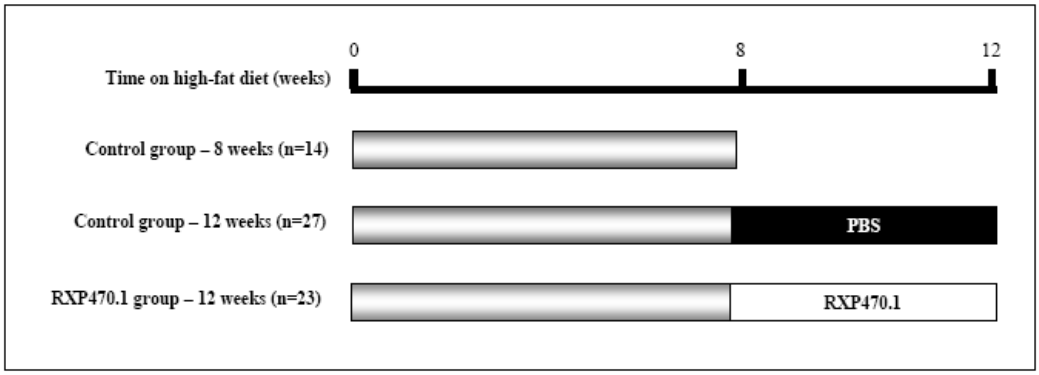

Figure I: Schematic presentation of the study design

Horizontal bar represents apoE-/- mice, placed on study at 8 weeks of age (n=64). After 8 weeks of high-fat feeding mice received subcutaneous implantation of osmotic mini-pumps filled with RXP470.1 (□), or PBS to act as a control (■), and maintained on high-fat diet for a further 4 weeks before termination.

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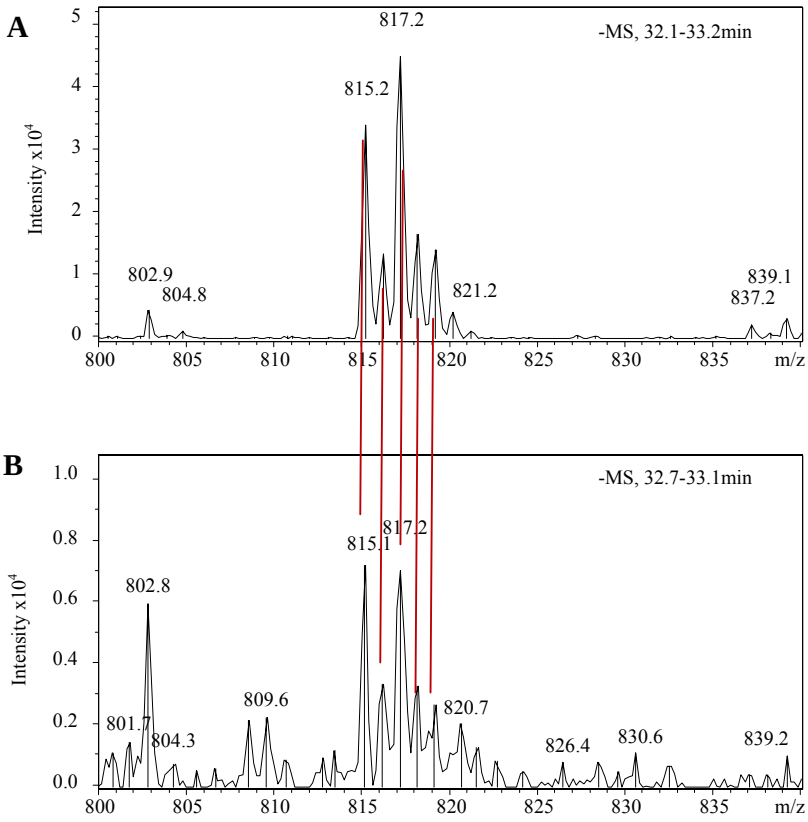

Figure II: Determination of the stability of RXP470.1 in plasma of mice

Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) finger-print of (A) pure 100nM RXP470.1 and (B) blood from infused mice with osmotic mini-pumps filled with 100nM RXP470.1 inhibitor.

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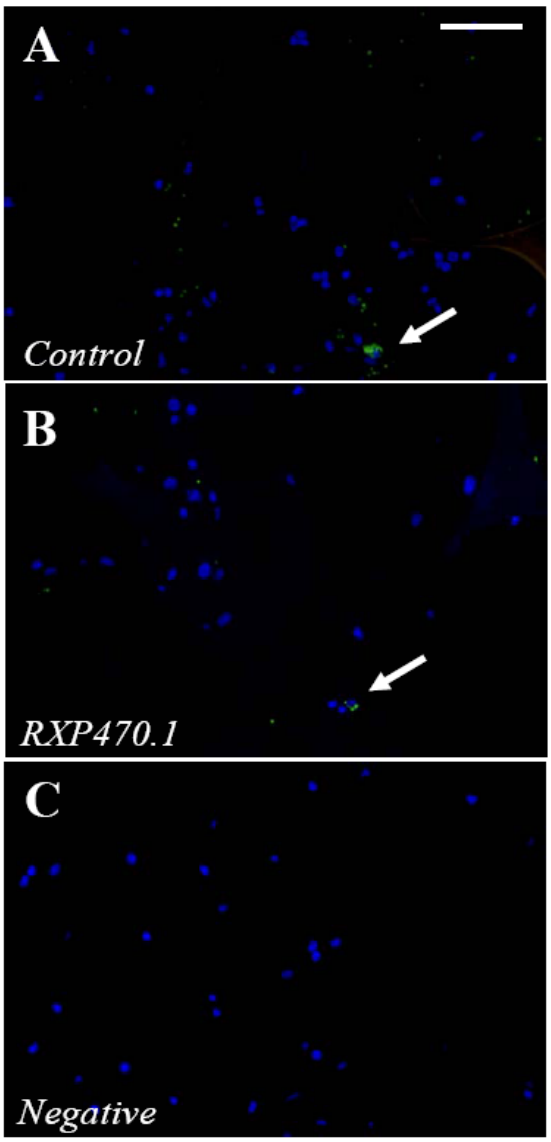

Figure III: Effect of RXP470.1 treatment on macrophage proliferation in subcutaneous sponges

Representative sections of PCNA immuno-labelled macrophages in subcutaneous sponges. Scale bar in panel (A) represents 50 μm and is applicable to all panels. White arrows indicate PCNA positive macrophages.

Panel A: Control

Panel B: RXP470.1 treated

Panel C: Rat IgG negative control

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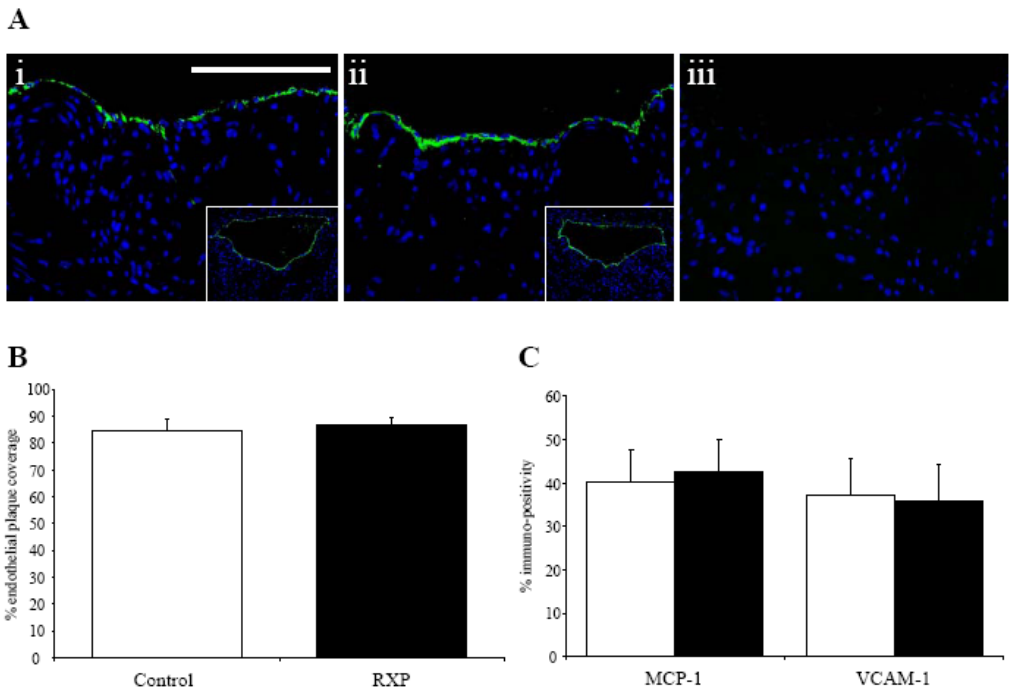

Figure IV: Effect of RXP470.1 treatment on atherosclerotic lesion endothelial cell coverage and expression of MCP-1 and VCAM-1

(A) Immunohistochemical labelling of brachiocephalic artery lesions, for von Willebrand factor from control (i) and RXP470.1 treated mice (ii) and a relevant negative control where an isotype-matched rabbit IgG replaced the primary antibody is shown in panel (iii). Immunopositive endothelial cells appear as green fluorescence and nuclei blue.

Scale bar in (i) represents 100μm and is applicable to all panels. Insets represent lower power images of larger panel. Quantification is summarised in the adjoining graph (B) and depicts mean±SEM (n≥23 per group).

(C) Quantification of MCP-1 and VCAM-1 immunopositivity of endothelial cells overlying atherosclerotic lesions from control (white bars) and RXP470.1-treated mice (black bars).

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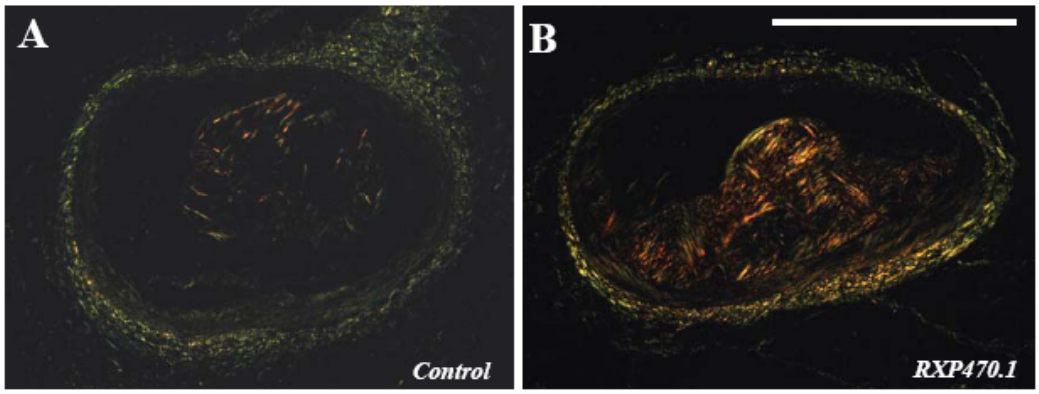

Figure V: Effect of RXP470.1 treatment on atherosclerotic lesion collagen content

Representative sections of Picrosirius red stained brachiocephalic atherosclerotic lesions viewed under polarised light. Scale bar in panel (A) represents 500 μm and is applicable to all panels.

Panel A: Control

Panel B: RXP470.1 treated

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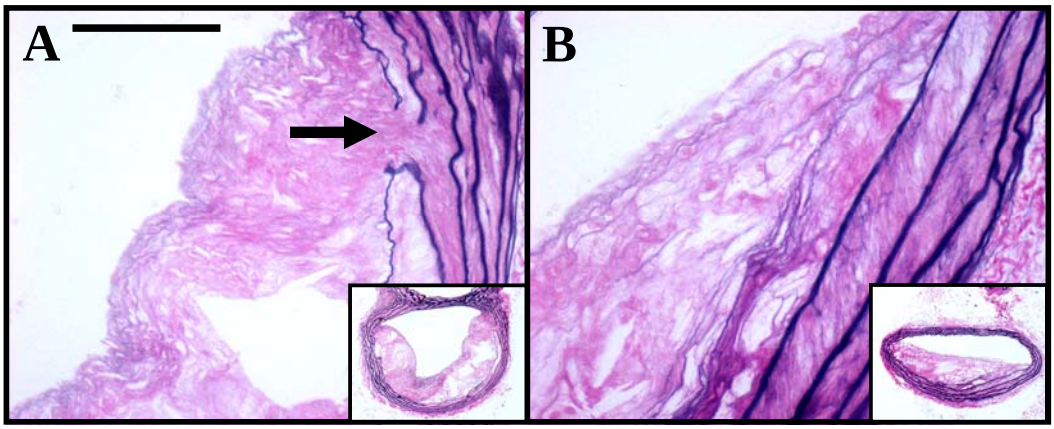

Figure VI: Effect of RXP470.1 treatment on atherosclerotic lesion elastin fragmentation

Representative sections of elastin van Gieson stained brachiocephalic atherosclerotic lesions. Arrow indicates area of elastin fragmentation. Scale bar in panel (A) represents 500 μm and is applicable to all panels. Insets represent lower power images of larger panel.

Panel A: Control

Panel B: RXP470.1 treated

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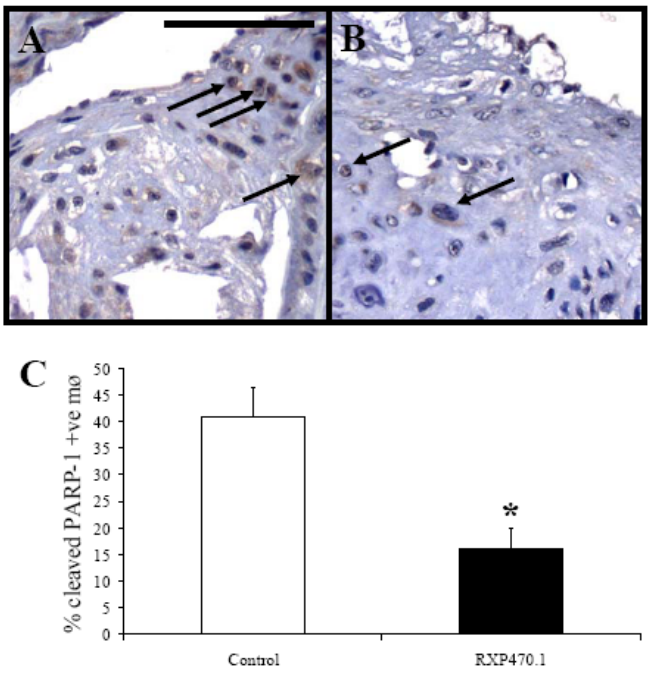

Figure VII: Effect of MMP-12 inhibition on apoptosis as assessed by cleaved PARP-1 IHC

Representative brachiocephalic artery lesions from control (A) and RXP470.1-treated (B) mice cleaved PARP-1 immunolabelled to detect apoptotic frequencies. Arrows indicate immunopositive cells (brown). Quantification is summarised in the adjoining graph (C), and ***** denotes $p<0.05$ ($n\geq23$ per group). Scale bar in A represents 100 µm and is applicable to A and B.

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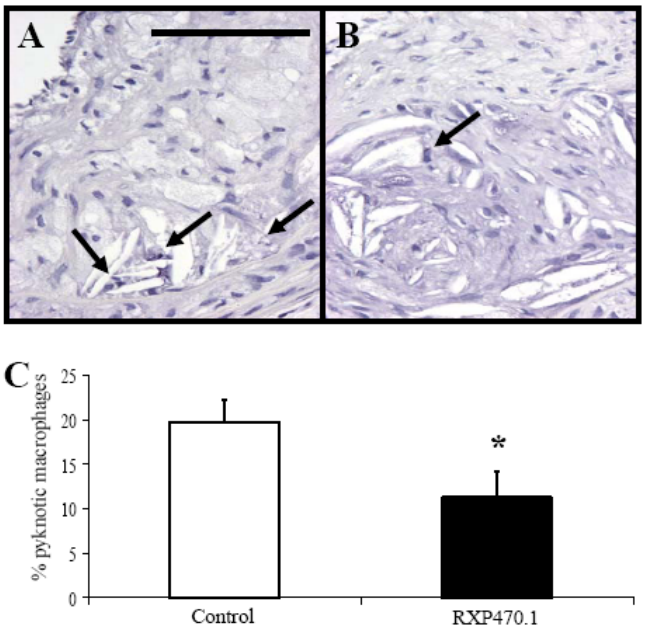

Figure VIII: Effect of MMP-12 inhibition on apoptosis as assessed under light microscopy

Representative haematoxylin stained brachiocephalic artery lesions from control (A) and RXP470.1-treated (B) mice. Arrows indicate cells with clear changes in nuclear shape and demonstrating pyknotic and/or fragmented nuclei. Quantification is summarised in the adjoining graph (C), and ***** denotes $p<0.05$ ($n\geq23$ per group). Scale bar in A represents 100 μm and is applicable to A and B.

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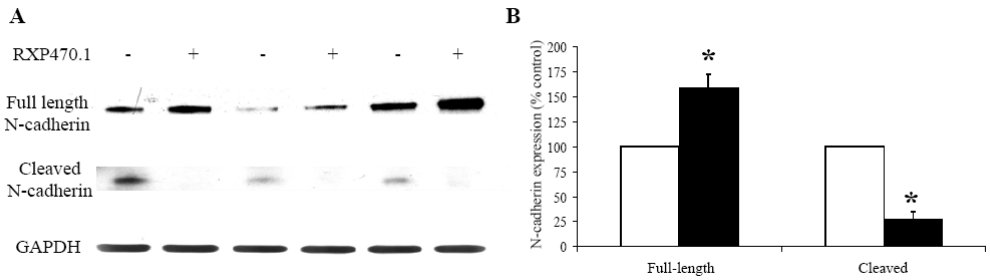

Figure IX: Effect of MMP-12 inhibition on in vitro macrophage and foam-cell macrophage N-cadherin cleavage

A and B; Representative western blots for full length (110kDa) and cleaved (35kDa) N-cadherin in RXP470.1-treated and control macrophages co-incubated with recombinant active MMP-12. GAPDH is shown as a loading control. Optical densities expressed as percentage of control are shown in adjoining graph (B) where white bars indicate control (-RXP470.1) and black bars denote treated macrophages (+RXP470.1). * denotes $p<0.05$ versus control.

All values are expressed as mean±SEM. All groups n=3.

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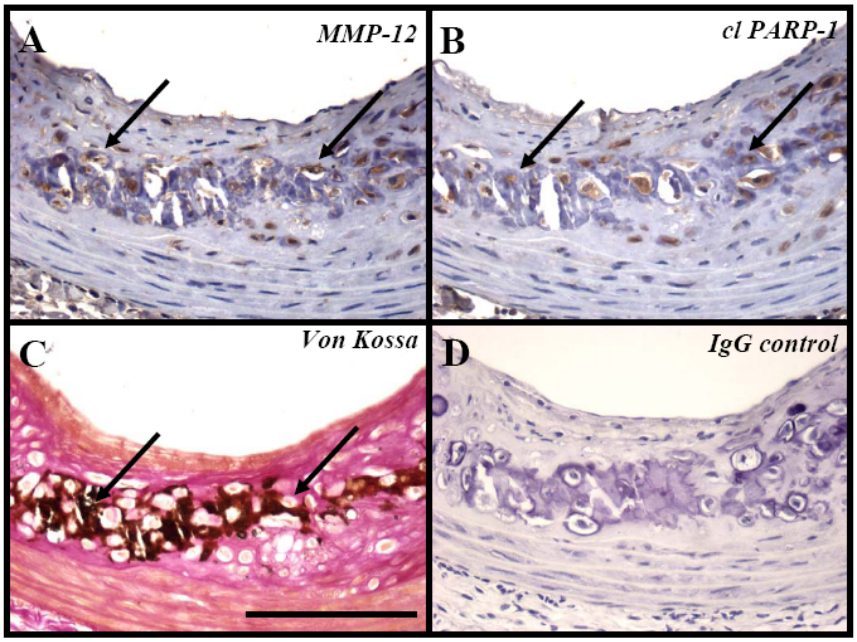

Figure X: Co-localisation of MMP-12, apoptosis and calcification in brachiocephalic atherosclerotic plaques

Immunohistochemical labelling of serial sections of brachiocephalic artery lesions for MMP-12 (A), cleaved PARP-1 (B) and von Kossa (C), counterstained with haematoxylin, from control mice. A relevant negative control where an isotype-matched rabbit IgG replaced the primary antibody is shown in panel (D). MMP-12 and PARP-1 positive labelling appear as brown. Areas of calcification appear as lakes of brown/black. Arrows indicate areas of co-localisation. Scale bar in A represents 100μm and is applicable to all panels.

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