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NecroX-7 reduces necrotic core formation in atherosclerotic plaques of Apoe knockout mice

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Abstract: Objective: A large necrotic core is a key feature of atherosclerotic plaque instability. Necrotic cellular debris accumulates in the lipid-rich core and promotes inflammation, destabilization and ultimately rupture of the plaque. Although the role of necrosis in atherosclerosis is rather clear-cut, not many strategies have been performed up till now to specifically target plaque necrosis. In the present study, we tested the plaque stabilizing potential of NecroX-7, a novel necrosis inhibitor that targets mitochondrial reactive oxygen species (ROS).

Methods: Male apolipoprotein E (ApoE) knockout mice were treated with NecroX-7 (30mg/kg) or vehicle 3 times per week via intraperitoneal injections for 16 weeks. Meanwhile, mice were fed a western-type diet to induce plaque formation.

Results: NecroX-7 reduced total plaque burden in the thoracic aorta as compared to vehicle-treated mice without affecting total plasma cholesterol. Plaques in the aortic root of NecroX-7-treated mice showed a significant decrease in necrosis, oxidative stress, inducible NO synthase (iNOS) and matrix metalloproteinase 13 (MMP13) expression while collagen content and fibrous cap thickness were increased. Moreover, plaques of NecroX-7-treated mice were characterized by reduced mRNA expression of multiple inflammation markers such as TNF α , IL1 β , iNOS, high mobility group box 1 (HMGB1) and receptor for advanced glycation products (RAGE). In vitro, NecroX-7 prevented tert-butyl hydroperoxide (tBHP)-induced ROS formation, necrosis, iNOS expression and HMGB1 release in primary macrophages.

Conclusion: NecroX-7 exerts not only antioxidative and anti-necrotic potential, but also anti-inflammatory effects. Therefore, NecroX-7 could be a promising pleiotropic drug for the treatment of atherosclerosis.

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Highlights

- NecroX-7 inhibits oxidative stress-induced necrosis in macrophages
- NecroX-7 inhibits atherosclerotic plaque necrosis in ApoE knockout mice
- NecroX-7 lowers plaque oxidative stress and inflammation
- NecroX-7 increases plaque collagen content and fibrous cap thickness

The necrosis inhibitor NecroX-7 increases features of plaque stability in ApoE knockout mice

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Abstract

Objective: A large necrotic core is a key feature of atherosclerotic plaque instability. Necrotic cellular debris accumulates in the lipid-rich core and promotes inflammation, destabilization and ultimately rupture of the plaque. Although the role of necrosis in atherosclerosis is rather clear-cut, not many strategies have been performed up till now to specifically target plaque necrosis. In the present study, we tested the plaque stabilizing potential of NecroX-7, a novel necrosis inhibitor that targets mitochondrial reactive oxygen species (ROS).

Methods: Male apolipoprotein E (ApoE) knockout mice were treated with NecroX-7 (30mg/kg) or vehicle 3 times per week via intraperitoneal injections for 16 weeks. Meanwhile, mice were fed a western-type diet to induce plaque formation.

Results: NecroX-7 reduced total plaque burden in the thoracic aorta as compared to vehicle-treated mice without affecting total plasma cholesterol. Plaques in the aortic root of NecroX-7-treated mice showed a significant decrease in necrosis, oxidative stress, inducible NO synthase (iNOS) and matrix metalloproteinase 13 (MMP13) expression while collagen content and fibrous cap thickness were increased. Moreover, plaques of NecroX-7-treated mice were characterized by reduced mRNA expression of multiple inflammation markers such as TNF α , IL1 β , iNOS, high mobility group box 1 (HMGB1) and receptor for advanced glycation products (RAGE). *In vitro*, NecroX-7 prevented *tert*-butyl hydroperoxide (tBHP)-induced ROS formation, necrosis, iNOS expression and HMGB1 release in primary macrophages.

Conclusion: NecroX-7 exerts not only antioxidative and anti-necrotic potential, but also anti-inflammatory effects. Therefore, NecroX-7 could be a promising pleiotropic drug for the treatment of atherosclerosis.

Key words: Atherosclerosis, inflammation, mitochondrial ROS, necrosis, NecroX-7

1. Introduction

Formation and enlargement of a necrotic core plays a key role in the pathology of unstable atherosclerotic plaques [1]. In a detailed morphometric analysis of advanced human lesions, 80% of necrotic cores appeared to be larger than 1 mm² and comprised >10% of the plaque area [2]. However, in two thirds of the examined ruptured plaques, the necrotic core occupied >25% of the plaque [2]. Cellular debris of necrotic cells as well as the accumulation of lipids, most likely released by necrotic foam cells, contributes to necrotic core formation [3]. Necrotic cell death is morphologically distinguishable from other forms of cell death (e.g. apoptosis) by a gain in cell volume (oncosis) and swelling of organelles, followed by rupture of the plasma membrane and release of intracellular content [4]. The necrotic debris is a major source of damage-associated molecular patterns (e.g. high mobility group box 1 [HMGB1]), pro-inflammatory cytokines (e.g. IL6), proteases (e.g. matrix metalloproteinases [MMPs]) and pro-thrombotic factors (e.g. tissue factor) [5] and thus can jeopardize plaque stability by promoting inflammation, plaque rupture (e.g. by thinning of the fibrous cap) and subsequent thrombosis [2]. Necrosis in atherosclerotic plaques is triggered by various stimuli including high levels of reactive oxygen species (ROS), ATP depletion and elevated intracellular calcium levels [6]. Moreover, impaired phagocytic clearance of apoptotic cells promotes secondary necrosis and expansion of the necrotic core [5, 7]. Only since the late 1990's, the first conclusive evidence for necrotic cell death in human plaques was published [8, 9]. Histological analysis of advanced lesions showed that the necrotic core is surrounded by dying macrophages and contains predominantly macrophage debris [3, 8].

Despite the importance and potential therapeutic relevance of necrosis in human atherosclerosis, plaque necrosis has not been a subject of interest for many years. Possible reasons are the lack of reliable markers to detect necrosis and the poor availability of therapeutic agents to specifically target necrosis. Essentially, necrosis has long been considered as an accidental and uncontrolled form of cell death. However, this concept

1 changed when researchers identified receptor-interacting protein (RIP) kinases, in particular
2 RIPK1 and RIPK3, as key regulators in mediating programmed necrosis (also called
3 necroptosis) [10-12]. RIPK3-deficient low density lipoprotein receptor (LDLR) knockout mice
4 show a significant reduction in the size of advanced plaques due to inhibition of macrophage
5 necrosis [13], while early lesions are not affected. This finding suggests that RIPK3-mediated
6 necrosis promotes plaque instability in advanced plaques, but does not play a role in early
7 plaque development.
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16 Given that necrosis is a poorly investigated yet crucial phenomenon in
17 atherosclerosis, we aimed to inhibit necrosis by targeting one of its main inducers, namely
18 mitochondrial ROS. Numerous studies have demonstrated the importance of mitochondrial
19 ROS in the progression of atherosclerosis both in patients and animal models [14-18]. High
20 levels of ROS may lead to necrosis by causing irreversible damage of vital cellular
21 components such as DNA, proteins and lipids. Recently, several groups reported promising
22 results with a small molecule necrosis inhibitor, termed NecroX-7, that exhibits both
23 antioxidative and cytoprotective properties [19-21]. NecroX-7 protects cells against oxidative
24 stress-induced necrotic cell death through ROS and RNS scavenging, NADPH oxidase
25 inhibition and inhibition of mitochondrial ROS generation [19-21], and inhibits HMGB1-
26 mediated inflammatory responses [22]. Furthermore, NecroX-7 is currently being tested in
27 ST-segment elevated myocardial infarction (STEMI) patients undergoing percutaneous
28 coronary intervention. Based on these findings, we were encouraged to evaluate the
29 potential plaque stabilizing effects of NecroX-7 in atherosclerosis. Our data indicate that
30 NecroX-7 increases features of plaque stability in western-type diet fed apolipoprotein E
31 knockout (ApoE^{-/-}) mice by inhibiting necrosis, suppressing oxidative stress, lowering plaque
32 inflammation and increasing collagen deposition and fibrous cap thickness.
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2. Materials and Methods

2.1 Mice

Male ApoE^{-/-} mice (C57BL/6, Jackson Laboratory, stock number 002052) were fed a western-type diet (4021.90, AB Diets) for 16 weeks. Simultaneously, mice were treated with NecroX-7 (LG Life Sciences, 30mg/kg body weight) (n=25) or vehicle (water for injection) (n=26) via intraperitoneal injections 3 times per week. Mice were housed in a temperature-controlled room with a 12h light/dark cycle and food and water ad libitum. At the end of the experiment, mice were fasted overnight and blood was collected by cardiac puncture. Total cholesterol, LDL cholesterol and triglyceride levels were measured on a Dimension Vista® System (Siemens Healthcare Diagnostics) with reagents from the same manufacturer. Tissues were imbedded in Neg-50 Frozen Section Medium and stored at -80°C or fixed in formalin 4% for 24h before paraffin imbedding. For *in vitro* experiments, wild-type mice (C57BL/6, Jackson Laboratory, stock number, 000664) and RIPK3^{-/-} mice were used. RIPK3^{-/-} mice, produced by targeted gene deletion [23], were a generous gift from K. Newton and V. Dixit (Genentech, San Francisco). All experiments were approved by the Ethical Committee of the University of Antwerp.

2.2 Cell culture

Bone marrow-derived macrophages (BMDM) were harvested by flushing bone marrow from the hind limbs of mice. Cells were cultured for 7d in RPMI medium (Gibco Life Technologies) supplemented with 15% L-cell conditioned medium (LCCM) containing monocyte colony stimulating factor (M-CSF). To polarize BMDM into M1 and M2 macrophages, cells were incubated for 24h with 100ng/ml LPS (Sigma-Aldrich) or 10ng/ml IL4 (Peprotech) and 10ng/ml IL13 (Peprotech), respectively. To induce oxidative stress-mediated necrosis, BMDM were treated with 0.5mol/l and 1mmol/l *tert*-butyl hydroperoxide (tBHP, Sigma Aldrich) for 2h and 4h. Cellular ROS was measured using the fluorogenic marker 2',7'-dichlorofluorescein diacetate (DCFDA, Molecular Probes). After incubation with 25μmol/l

1 carboxy-H₂DCFDA in HBSS (Gibco Life Technologies) for 1h, cells were collected and
2 centrifuged. Cell pellet is then resuspended in FACS-buffer (PBS containing 0.1% BSA and
3 0.05% sodium azide) and analyzed with BD Accuri flow cytometer. Necrosis was monitored
4 by propidium iodide (PI, Molecular Probes) labeling and fluorescence microscopy. To induce
5 apoptosis and necroptosis, BMDM were treated for 24h with 30µg/ml cycloheximide (Sigma-
6 Aldrich) or 10ng/ml LPS and 20µmol/l zVAD-fmk (Enzo Life Sciences), respectively.
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8 Necrostatin-1 (Enzo Life Sciences) was used as RIP1K inhibitor.
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10 11 12 13 14 15 16 *2.3 Western blotting*

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19 Cells were lysed in Laemmli sample buffer containing β-mercaptoethanol (Sigma-Aldrich)
20 and boiled for 4 min. Samples were loaded on Bolt 4-12% Bis-Tris gels (Life Technologies)
21 and after electrophoresis transferred to Immobilon-P membranes (Millipore). Membranes
22 were probed with rabbit anti-cleaved caspase-3 (9661, Cell signaling Technologies) or
23 mouse anti-β-actin (A5441, clone AC-15, Sigma-Aldrich) primary antibodies. Subsequently,
24 membranes were incubated with HRP-conjugated secondary antibodies (Dako) to allow
25 chemiluminescent detection. In some experiments, supernatant was collected and the
26 extracellular proteins were precipitated with 10% trichloroacetic acid (Sigma-Aldrich). After
27 incubation on ice for 15min, supernatant was centrifuged for 10min at 2500g. The protein
28 pellet was resuspended in Laemmli sample buffer containing β-mercaptoethanol. The
29 release of HMGB1 in supernatant was detected via western blotting using rabbit anti-HMGB1
30 (ab18256, Abcam) antibody.
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46 47 *2.4 Real time RT-PCR*

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50 Total RNA was prepared using the Nucleospin RNA Kit (Filter Service). Cell culture samples
51 were analysed for iNOS using a TaqMan gene expression assay (Mm00440502_m1, Applied
52 Biosystems). β-actin (Mm00607939_s1) was used as reference gene. Tissue samples were
53 analyzed for TNFα, IL6, IL1β, iNOS, HMGB1, RAGE and TLR4 expression using SYBR
54 Green (GC Biotech) and the following gene-specific primers (Sigma-Aldrich): 5'-
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GGCAGGTCTACTTTGGAGTCATTG-3' (FW, TNF α) and 5'-
ACATTCGAGGCTCCAGTGAATTCG-3' (RV, TNF α); 5'-
CTGGTGACAACCACGGCCTTCCCTA-3' (FW, IL6) and 5'-
ATGCTTAGGCATAACGCACTAGGTT-3' (RV, IL6); 5'-TGAAATGCCACCTTTTGACAG-3'
(FW, IL1 β) and 5'-CCACAGCCACAATGAGTGATAC-3' (RV, IL1 β); 5'-
CCCTTCCGAAGTTTCTGGCAGC-3' (FW, iNOS) and 5'-
GGCTGTCAGAGCCTCGTGGCTTTGG-3' (RV, iNOS); 5'-TGGCAAAGGCTGACAAGGCTC-
3' (FW, HMGB1) and 5'-GGATGCTCGCCTTTGATTTTGG-3' (RV, HMGB1); 5'-
CACTTGTGCTAAGCTGTAAGGG-3' (FW, RAGE) and 5'-CATCGACAATTCCAGTGGCTG-
3' (RV, RAGE); 5'-GCCTTTCAGGGAATTAAGCTCC-3' (FW, TLR4) and 5'-
GATCAACCGATGGACGTGTAAA-3' (RV, TLR4). GAPDH (5'-
CCAGTATGACTCCACTCACG-3' (FW), 5'-GACTCCACGACATACTCAGC-3' (RV)) and
PPI α (5'-GAAGCCATGGAGCGTTTTGG-3' (FW), 5'-CAGATGGGGTAGGGACGCTC-3'
(RV)) were used as reference genes. Real time RT-PCR was performed on a ABI Prism
7300 sequence detector system (Applied Biosystems). The parameters for PCR amplification
were 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min. In case of
SYBR Green analysis, an additional step of 72°C for 30sec and a dissociation stage were
added. Relative expression of mRNA was calculated using qBase+ software (Biogazelle).

2.5 Histological analysis

The thoracic aorta was stained *en face* with ORO (Sigma-Aldrich) to determine plaque
burden. Atherosclerotic plaques located in the aortic root were analyzed by
immunohistochemistry at 3 different sections sliced at equally spaced intervals (every 50 μ m).
Necrosis was measured on a hematoxylin-eosin (H&E) staining according to a standard
method [24]. A 3000 μ m² minimum threshold was implemented in order to avoid counting of
regions that likely do not represent substantial areas of necrosis. A Sirius red (Sigma-Aldrich)
staining was used for detection of total collagen and collagen type I under polarized light.
Apoptosis was determined by anti-cleaved caspase-3 (9661, Cell signaling Technology) and

1 TUNEL (S7101, Millipore) staining. Plaques were further analyzed by immunohistochemistry
2 with the following primary antibodies: mouse anti- α -SMC-actin (A2547, Sigma-Aldrich) and
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4 rabbit anti-MMP13 (ab39012, Abcam). Frozen sections of the plaques were analyzed with
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6 rabbit anti-Moma2 (MCA519, Serotec), rabbit anti-8-oxodG (orb10011, Biorbyt) and rabbit
7
8 anti-iNOS (BML-SA200, Enzo Life Sciences). Thereafter, tissue sections were incubated with
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10 species-appropriate HRP-conjugated secondary antibodies followed by 60min of reactive
11
12 ABC. 3,3'-diaminobenzidine or 3-amino-9-ethyl-carbazole were used as a chromogen. All
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14 images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope
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16 and quantified with Image J software.
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21 *2.6 Statistical analysis*

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24 All data were analyzed with SPSS 22.0 software (SPSS Inc.) and presented as mean $\pm$ SEM.
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26 Statistical tests are mentioned in the figure legends. Differences were considered significant
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28 at $P < 0.05$.
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3. Results

3.1 NecroX-7 reduces total aortic plaque burden in ApoE^{-/-} mice without affecting total plasma cholesterol levels

ApoE^{-/-} mice were treated with NecroX-7 (30mg/kg body weight) or vehicle 3 times per week and fed a western-type diet for 16 weeks to induce atherosclerotic plaque formation. Oil Red O staining of the thoracic aorta showed a significant decrease in atherosclerotic plaque formation, particularly in the aortic arch of NecroX-7-treated mice as compared to vehicle-treated control mice (Fig. S1). Body weight, total cholesterol, LDL cholesterol and triglyceride plasma levels were not significantly altered in NecroX-7-treated mice as compared to controls (Table 1).

3.2 NecroX-7 increases features of atherosclerotic plaque stability in ApoE^{-/-} mice

To evaluate the effect of NecroX-7 on plaque size and composition, atherosclerotic plaques located in the aortic root were analyzed. NecroX-7 treatment significantly reduced plaque necrosis as illustrated by smaller and less abundant necrotic cores (Fig. 1A), but did not affect plaque apoptosis (Fig. 1B and Table 1). However, neither plaque size nor macrophage content were altered between both groups (Table 1). Because reduced plaque necrosis is a key event in plaque stabilization, other features of plaque stability were examined. Total collagen, in particular collagen type I, was significantly increased in plaques of NecroX-7-treated versus vehicle-treated mice (Fig. 2A and 2B). Moreover, smooth muscle cell content and fibrous cap thickness were significantly elevated in plaques of NecroX-7-treated mice (Table 1 and Fig. 2C). Interestingly, MMP13 expression, also known as collagenase 3, was significantly decreased in plaques of NecroX-7-treated mice (Fig. 2D). Further analysis showed that there was a significant correlation between the amount of collagen and the number of smooth muscle cells ($P=0.025$) (data not shown) as well as an inverse association between collagen amount and MMP13 expression in the plaque ($P=0.015$) (Fig. 2D). These data suggest that the increase in collagen content in plaques of NecroX-treated ApoE^{-/-} mice

is due to a combination of increased smooth muscle cell content and decreased MMP13-mediated degradation.

3.3 NecroX-7 reduces atherosclerotic plaque oxidative stress and inflammation

According to an immunohistochemical staining of 8-oxodG, a well-known marker of oxidative stress-induced DNA damage, 4 out of 11 control plaques showed signs of oxidative stress while none of the plaques of NecroX-7-treated mice stained positive for 8-oxodG (Fig. 3A). In control mice, 8-oxodG-positivity was mostly observed in the proximity of the necrotic core and revealed both nuclear and non-nuclear oxidative DNA damage. Also iNOS expression was significantly decreased in plaques of NecroX-7-treated mice, suggestive of reduced oxidative stress and inflammation (Table 1). Real time RT-PCR analysis of plaques from NecroX-7-treated mice revealed a significant decrease of multiple inflammation markers including TNF α , IL1 β , iNOS, high mobility group box 1 (HMGB1) and receptor for advanced glycation products (RAGE) as compared to vehicle-treated mice (Fig. 3B). There was also a trend ($P=0.06$) for reduced IL6 mRNA expression in NecroX-7-treated plaques.

3.4 NecroX-7 inhibits oxidative-stress induced necrosis in macrophages

To validate the necrosis inhibitory effect of NecroX-7, bone marrow-derived macrophages (BMDM) were treated with *tert*-butyl hydroperoxide (tBHP). High doses of tBHP (0.5 and 1mmol/l) induced macrophage necrosis after 2h and 4h treatment in a dose-dependent manner as shown by PI labeling (Fig. 4A), but failed to induce apoptosis and RIPK1/RIPK3-dependent necroptosis (Fig. S2A and S2B). Treatment with NecroX-7 reduced PI labeling significantly (Fig. 4A) and diminished HMGB1 levels in the supernatant of tBHP-treated BMDM (Fig. 4B). Morphological analysis of tBHP-treated BMDM confirmed a decrease in the number of oncotic cells and an overall improvement of cell viability after NecroX-7 treatment (Fig. 4C). Interestingly, M2-polarized BMDM were much more sensitive to oxidative-stress-induced necrosis as compared to M1-polarized BMDM (Fig. 4D). NecroX-7 strongly inhibited

1 tBHP-induced necrosis in M2-polarized BMDM (Fig. 4D), suggesting that NecroX-7 favors
2 the survival of anti-inflammatory BMDM.
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5 Next, we investigated whether the anti-necrotic effects of NecroX-7 were associated with its
6 antioxidative properties. High doses of tBHP (0.5 and 1mmol/l) generated high levels of ROS
7 after 2h and 4h treatment in a dose-dependent manner as shown by DCFDA labeling (Fig.
8 4E). NecroX-7 strongly abolished ROS generation in all conditions (Fig. 4E). Moreover,
9 NecroX-7 inhibited iNOS expression in M1-polarized BMDM exposed to tBHP (Fig. 4F).
10 These experiments indicate that NecroX-7 lowers oxidative stress in macrophages in the
11 presence of a powerful oxidant and prevents subsequent necrosis.
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21 *3.5 NecroX-7 inhibits neither RIPK1/3 dependent nor caspase-3 dependent cell death*

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24 To investigate whether NecroX-7 inhibits necroptosis, BMDM were treated with LPS
25 (10ng/ml) and zVAD-fmk (20μmol/l) for 24h. Necroptosis was induced in wild-type BMDM but
26 not in Necrostatin-1 (Nec-1, RIPK1 inhibitor) treated or RIPK3^{-/-} BMDM. However, NecroX-7
27 treatment did not affect LPS+zVAD-fmk induced necroptosis (Fig. S3A).
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33 To study whether NecroX-7 targets apoptosis, BMDM were treated with 30μg/ml
34 cycloheximide for 24h. According to immunocytochemistry and western blot analysis of
35 cleaved caspase-3, NecroX-7 did not inhibit cycloheximide-induced macrophage apoptosis
36 (Fig. S3B).
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4. Discussion

Recently, NecroX-7 has been successfully used to inhibit necrosis in several pathologies including oxidative stress-induced cardiomyopathy, cardiac and hepatic (ischemia-reperfusion) injury, nonalcoholic steatohepatitis, bone-destructive diseases, allergic lung inflammation and acute graft-versus-host-disease [20-22, 25-33]. As a result, this compound is currently being tested in ST-segment elevated myocardial infarction (STEMI) patients before percutaneous coronary intervention to reduce myocardial infarct size (<https://clinicaltrials.gov>, identifier NCT02070471). In the present study, we tested the potential plaque stabilizing effects of NecroX-7 in ApoE^{-/-} mice, a model of atherosclerosis.

From a general point of view, unstable plaques are characterized by a highly inflammatory cell content, a large necrotic core and a thin fibrous cap consisting of a low amount of smooth muscle cells and extracellular matrix (e.g. collagen) [34]. NecroX-7 was able to improve plaque stability at different levels. Firstly, NecroX-7 reduced plaque necrosis, albeit this effect did not result in smaller plaques. Secondly, we observed an increase in collagen content. A correlation analysis showed that the increase in total collagen content is most likely due to an increase in collagen deposition (owing to increased smooth muscle cell amount) and a decrease in MMP13-mediated collagen degradation. Thirdly, even though the amount of macrophages was not different after NecroX-7 treatment, plaques developed a less inflammatory phenotype. Multiple inflammation markers such as TNF α , IL1 β , iNOS, HMGB1 and RAGE were decreased in plaques of NecroX-7-treated versus vehicle-treated ApoE^{-/-} mice. These data emphasize the important link between necrosis and inflammation in atherosclerosis. During necrotic cell death, the plasma membrane ruptures and releases intracellular components including several pro-inflammatory molecules that are considered detrimental for plaque stability [6]. Most well-known examples are pro-inflammatory mediators (e.g. TNF α , IL6, HMGB1), proteases (e.g. MMPs) and pro-thrombotic factors (e.g. tissue factor). Of all pro-inflammatory mediators, HMGB1 is one of the most studied

1 molecules associated with necrosis. HMGB1 is a nuclear protein that is passively released
2 from necrotic or damaged cells, but not during apoptosis and secondary necrosis [35]. Once
3 released, HMGB1 acts as a danger-associated molecular pattern (DAMP) signal and
4 stimulates macrophages to produce pro-inflammatory cytokines such as TNF α , IL6 and IL1 β
5 through binding with the receptor for advanced glycation end products (RAGE) or members
6 of the toll-like receptor family (e.g. TLR4) [36, 37]. Binding of HMGB1 to RAGE results in
7 activation of NF- κ B and MAPK signaling pathways [38]. Thus, during necrosis, pro-
8 inflammatory molecules are not only released in a passive manner, but can also be actively
9 released upon HMGB1-mediated NF- κ B-activation. On the other hand, pro-inflammatory
10 cytokines may stimulate HMGB1 mRNA expression in macrophages [39]. HMGB1 is
11 released from TNF α -stimulated macrophages in an active manner that requires translocation
12 of HMGB1 from the nucleus into secretory lysosomes [40]. These findings demonstrate that
13 HMGB1 may act both as an early initiator and late promoter of inflammation [37]. In the
14 present study, we provide evidence that NecroX-7 treatment reduces HMGB1 and RAGE
15 mRNA expression in plaques of ApoE^{-/-} mice and therefore attenuates both HMGB1-
16 mediated inflammatory responses and inflammatory cytokine-induced HMGB1 expression.
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36 Our *in vitro* experiments demonstrated that NecroX-7 inhibits oxidative stress-induced
37 necrosis in primary macrophages, but targets neither RIPK1/RIPK3-mediated necroptosis
38 nor caspase 3-mediated apoptosis. According to previous evidence, the anti-necrotic effect
39 of NecroX-7 relies on its ability to target ROS [19]. Indeed, NecroX-7 lowers oxidative stress
40 via 3 different mechanisms: ROS scavenging, inhibition of NADPH oxidase and inhibition of
41 mitochondrial ROS (mtROS) generation [19, 21]. Mitochondria and NADPH oxidase
42 enzymes are the most important sources of ROS in cardiovascular disease [41, 42]. Several
43 lines of evidence indicate that excessive mtROS promotes progression of atherosclerosis in
44 both patients and mouse models [14-18]. mtROS are by-products of the electron transport
45 chain generated during the oxidative phosphorylation process in the mitochondria to produce
46 ATP [43]. The imbalance between mtROS production and mtROS elimination may lead to
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excessive mtROS levels and subsequent cell death [44]. We could observe that NecroX-7 lowered oxidative stress in plaques in ApoE^{-/-} mice as detected by 8-oxodG. This structure is a typical oxidative modification of nuclear DNA and mtDNA [45], and recently accepted as marker of mtROS in murine atherosclerotic lesions [17].

Although the detrimental role of oxidative stress in atherosclerosis is broadly accepted, the results of clinical trials investigating antioxidative compounds (e.g. vitamin E) are rather disappointing (reviewed in [46]). Possibly, these compounds convert into pro-oxidants when scavenging ROS and/or impair mitochondrial function [47]. Because most antioxidants do not preferentially accumulate in the mitochondria [44], the therapeutic efficacy of antioxidants to target mtROS is questionable. NecroX-7, however, inhibits the generation of mtROS and therefore prevents mitochondrial depolarization and swelling, ATP depletion and subsequent necrosis [21]. To our knowledge, these anti-necrotic effects have not been described for other currently available antioxidative compounds. Of note, given that mtROS can also drive pro-inflammatory cytokine production in a direct (but poorly defined) manner [17, 48, 49], we cannot exclude that the decrease in plaque inflammation in NecroX-7-treated mice is a direct result of reduced mtROS rather than a consequence of reduced necrosis. Furthermore, it is worthwhile to mention that comparable plaque stabilizing effects were observed in ApoE^{-/-} mice treated with MitoQ, one of the few antioxidants that selectively target the mitochondria. MitoQ does not affect total plaque size, smooth muscle content and apoptosis, but reduces the number of plaque macrophages [50]. The positive correlation between mtROS and the progression of human atherosclerosis, combined with our data and those from previous studies, suggests that specific targeting of mtROS may result in a better outcome of inflammatory diseases such as atherosclerosis.

In conclusion, NecroX-7 improves features of atherosclerotic plaque stability in 16 week western-type diet-fed ApoE^{-/-} mice by lowering plaque necrosis, oxidative stress and inflammation, and by increasing collagen deposition and fibrous cap thickness. Our study demonstrates that inhibition of important triggers of necrosis such as mitochondrial oxidative

stress attenuates the severity of atherosclerosis. Given the multifaceted pathophysiology of atherosclerosis and the pleiotropic efficacy of NecroX-7, this compound could be a new attractive therapeutic drug in the treatment of atherosclerosis.

Acknowledgements

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Conflict of interest

None.

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Table 1: Characteristics of vehicle-treated and NecroX-7-treated ApoE^{-/-} mice after 16 weeks on western-type diet

	Vehicle	NecroX-7
<i>General</i>		
Body weight (g) ^a	34±1	33±1
Total cholesterol ^{a,b}	482±25	465±28
LDL cholesterol ^{a,b}	349±19	315±17
Triglycerides ^{a,b}	154±13	122±21
<i>Aortic Root Plaque</i>		
Plaque area (x10 ³ μm ²) ^c	176±6	168±5
# TUNEL positive cells/mm ² plaque area ^c	46±10	52±7
Macrophages (%) ^c	1.2±0.1	1.4±0.1
Smooth muscle cells (%) ^c	2.5±0.2	3.2±0.2*
iNOS (%) ^c	1.6±0.1	1.0±0.1*

(^a, Student t test; ^b, mg/dl; ^c, Repeated Measure; *P<0.05 vs. Vehicle)

Figure legends

Figure 1: NecroX-7 reduces necrosis in atherosclerotic plaques of ApoE^{-/-} mice

(A) Sections of the aortic root of vehicle-treated (Veh) and NecroX-7-treated (NX-7) ApoE^{-/-} mice were stained with hematoxylin-eosin to quantify the percentage of necrosis (* $P < 0.05$ vs. vehicle; $n = 25$; Repeated Measure). Necrotic cores are indicated by an asterisk. Scale bar: 100 μ m. **(B)** Serial sections were immunostained for cleaved caspase-3 to measure the percentage of apoptosis (NS, not significant vs. vehicle; $n = 25$; Repeated Measure). Scale bar: 100 μ m.

Figure 2: NecroX-7 increases collagen deposition and formation of a thick fibrous cap in atherosclerotic plaques of ApoE^{-/-} mice

(A, B) Sections of the aortic root of vehicle-treated (Veh) and NecroX-7-treated (NX-7) ApoE^{-/-} mice were stained with Sirius red to quantify total collagen (A) and collagen type I (B) (red, indicated by white arrowheads) (*** $P < 0.001$, ** $P < 0.01$, vs. vehicle; $n = 25$; Repeated Measure). Scale bars: 100 μ m. **(C)** Serial sections were immunostained for α -SMC-actin to measure the fibrous cap thickness. (* $P < 0.05$ vs. vehicle; $n = 8$ measurements/mouse; Repeated Measure). **(D)** Serial sections were immunostained for MMP13 (* $P < 0.05$ vs. vehicle; $n = 10$; Repeated Measure). A correlation analysis was performed between total collagen content and MMP13 expression in plaques of vehicle-treated (blue dots) and NecroX-7-treated (green dots) ApoE^{-/-} mice (Spearman's rho, $P = 0.015$).

Figure 3: NecroX-7 reduces oxidative stress and inflammation in atherosclerotic plaques of ApoE^{-/-} mice

(A) Sections of the aortic root of vehicle-treated (Veh) and NecroX-7-treated (NX-7) ApoE^{-/-} mice were immunostained for 8-oxodG to detect oxidative stress ($P<0.05$; Chi square, 4/11 (Veh) vs. 0/9 (NX-7)). Scale bars: 150 μ m and 25 μ m (detail). **(B)** Real time RT-PCR analysis of plaques for TNF α , IL6, IL1 β , iNOS, HMGB1, RAGE and TLR4 mRNA expression. (* $P<0.05$, ** $P<0.01$; NS, not significant vs. vehicle; n=6 aortas/group; Student t test).

Figure 4: NecroX-7 inhibits oxidative-stress induced necrosis in macrophages

(A,B) Bone marrow-derived macrophages (BMDM) were exposed to 0.5mmol/l or 1mmol/l tBHP for 2h or 4h and treated with 10 μ mol/l NecroX-7 (NX-7) or left untreated (Ctrl). Necrosis was determined by (A) PI labeling (*** $P<0.001$ vs. Ctrl; n=2 experiments with 2 counting regions of 300 cells/region in duplicate; Factorial ANOVA) and (B) western blot analysis of HMGB1 release in supernatant. Corresponding cell lysates were analyzed for β -actin as internal control. Bands are shown in duplicate. **(C)** Representative images of BMDM exposed to 1mmol/l tBHP for 4h and treated with 10 μ mol/l NecroX-7 (NX-7) or left untreated (Ctrl). Necrotic BMDM (indicated by black arrows) were characterized by an increase in cell volume (oncosis) and a translucent cytoplasm. Scale bar: 25 μ m. **(D)** BMDM were polarized to M1 and M2 macrophages, then exposed to 1mmol/l tBHP for 4h and treated with 10 μ mol/l NecroX-7 (NX-7) or left untreated (Ctrl). Necrosis was determined by PI labeling (* $P<0.05$, *** $P<0.001$ vs. Ctrl; n=2 experiments with 3 counting regions of 200 cells/region in duplicate; Student t-test). **(E)** BMDM were exposed to 0.5mmol/l or 1mmol/l tBHP for 2h or 4h and treated with 10 μ mol/l NecroX-7 (NX-7) or left untreated (Ctrl). Cellular ROS generation was monitored by DCFDA labeling. (*** $P<0.001$ vs. Ctrl; n=2 experiments in duplicate; Factorial ANOVA). **(F)** BMDM were polarized to M1 macrophages, left untreated (Untr) or exposed to 1mmol/l tBHP for 4h in the presence (NX-7) or absence (Ctrl) of 10 μ mol/l NecroX-7. iNOS

expression was analyzed by real time RT-PCR ($***P<0.001$ vs. Ctrl; $###P<0.001$ vs. Untr; $n=2$
independent experiments in tetraplicate; One way ANOVA with Bonferroni post hoc test).

Universiteit Antwerpen

December 24, 2015

Prof. Steve Humphries
Editor-in-Chief *Atherosclerosis*

Dear Editor,

We have the pleasure to send you herewith our manuscript entitled "*The necrosis inhibitor NecroX-7 increases features of plaque stability in ApoE knockout mice*".

All authors have read and approved the submission of the manuscript. The manuscript has not been published and is not being considered for publication elsewhere.

Looking forward to hearing from you soon.

Yours sincerely,

Mandy O.J. Grootaert

Universiteit Antwerpen

December 24, 2015

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Editor-in-Chief *Atherosclerosis*

Dear Editor,

We have the pleasure to send you herewith our manuscript entitled "*The necrosis inhibitor NecroX-7 increases features of plaque stability in ApoE knockout mice*".

May we kindly ask you to consider this manuscript for publication in *Atherosclerosis* as a full-length basic research paper.

We suggest the following researchers as potential reviewers:

Ass. Prof. Danilo Giuseppe Norata (danilo.norata@unimi.it)

Prof. Patrizia Agostinis (patricia.agostinis@med.kuleuven.be)

Prof. Richard Kitsis (richard.kitsis@einstein.yu.edu)

Dr. Wim Declercq (Wim.Declercq@irc.vib-Ugent.be)

Looking forward to hearing from you soon.

Yours sincerely,

Mandy O.J. Grootaert

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript entitled "*The necrosis inhibitor NecroX-7 increases features of plaque stability in ApoE knockout mice*" has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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