Translating lipid-driven inflammation in atherosclerosis

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Publication date
2016

Document Version
Final published version

Citation for published version (APA):

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CHAPTER 11

MULTIPLE PATHWAY SCREENING TO PREDICT ANTI-ATHEROGENIC EFFICACY OF DRUGS TARGETING MACROPHAGES IN ATHEROSCLEROTIC PLAQUES


*Authors contributed equally

Vascul Pharmacol. 2016;82:51-9
ABSTRACT

Rationale: Macrophages play a central role in atherosclerosis development and progression, hence targeting macrophage activity is considered an attractive therapeutic. Recently, we documented nanomedicinal delivery of the anti-inflammatory compound prednisolone to atherosclerotic plaque macrophages in patients, which did however not translate into therapeutic efficacy. This unanticipated finding calls for in-depth screening of drugs intended for targeting plaque macrophages.

Methods and Results: We evaluated the effect of several candidate drugs on macrophage activity, rating overall performance with respect to changes in cytokine release, oxidative stress, lipid handling, endoplasmic reticulum (ER) stress, and proliferation of macrophages. Using this in vitro approach, we observed that the anti-inflammatory effect of prednisolone was counterbalanced by multiple adverse effects on other key pathways. Conversely, pterostilbene, T0901317 and simvastatin had an overall anti-atherogenic effect on multiple pathways, suggesting their potential for liposomal delivery.

Conclusion: This dedicated assay setup provides a framework for high-throughput screening. Further in vivo studies are warranted to determine the predictive value of this macrophage-based screening approach and its potential value in nanomedicinal drug development for cardiovascular patients.
INTRODUCTION

Atherosclerosis is a multifaceted disease of the arterial wall, underlying the vast majority of cardiovascular diseases[1]. Triggered by endothelial cell dysfunction, circulating lipids accumulate in the arterial wall and become modified through oxidation. Recruited macrophages become foam cells when taking up these oxidized lipids, which is a hallmark of initial atherosclerotic lesions. Over time, a complex interplay of maladaptive responses contributes to atherosclerosis progression, including, amongst others, chronic local inflammation, oxidative stress, impaired cholesterol efflux and excessive cell proliferation[2].

Past decades, the widespread use of statin-based lipid lowering strategies has revolutionized cardiovascular disease management, reducing the risk of an acute event by 25-35%[3]. Nonetheless, a considerable residual risk remains[4], driving the pursuit for novel anti-atherosclerotic strategies. Since plaque macrophages are crucial in atherogenesis, main mechanisms related to macrophage activity, including inflammation, oxidative stress, lipid metabolism and proliferation, are considered potential therapeutic targets[5]. Nanomedicine offers an attractive strategy to locally target macrophage activity within an atherosclerotic plaque[6]. In addition to promising results in experimental models[7,8], we recently reported successful targeting of plaque macrophages in patients with atherosclerosis using a liposomal delivery platform for prednisolone[9]. However, the unexpected lack of anti-inflammatory efficacy strongly argued for a more in-depth characterization of drug effects on plaque macrophages[9].

Therefore, we set up a dedicated series of in vitro assays to rapidly screen drug compounds for their effects on multiple key pathways of macrophage activity. Seven compounds recognized for their beneficial modulating effect on one of these pathways were selected to evaluate their effect on all other aforementioned macrophage pathways (Table 1). To facilitate potential nanomedicinal development, we aimed to screen drugs and compounds that have a good safety profile in humans and are suitable for liposomal encapsulation. We demonstrate here that we can rapidly assess overall performance of drug candidates to identify those likely to exert anti-atherogenic effects on lesional macrophages.
TABLE 1. Selected drug compounds for multi-pathway screening

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mode of action</th>
<th>Clinical use</th>
<th>Status for atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>Glucocorticoid receptor agonist</td>
<td>Inflammatory, oncological and hematological disorders</td>
<td>Phase I/II: Liposomal formulation of prednisolone phosphate showed no efficacy9</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>Folic acid antagonist</td>
<td>Neoplastic diseases, rheumatoid arthritis, psoriasis</td>
<td>Phase III: Systemic low dose MTX trial in progress (CIRT)40</td>
</tr>
<tr>
<td>T0901317 (T09)</td>
<td>Liver X receptor (LXR) agonist</td>
<td>Only preclinical use</td>
<td>Preclinical: Systemic dosing reduces atherosclerosis in animal models, but promotes hepatic lipogenesis24-29</td>
</tr>
<tr>
<td>Pterostilbene</td>
<td>Free radical scavenging</td>
<td>No clinical indications; available as dietary supplement</td>
<td>Preclinical: Long term oral dosing of resveratrol (analogue) reduces atherosclerosis in mice and rabbits31-35</td>
</tr>
<tr>
<td>Mercaptopurine (6-MP)</td>
<td>Purine antagonist</td>
<td>Organ transplantation, leukemia, auto-immune disorders</td>
<td>Preclinical: Drug-eluting cuff reduces atherosclerosis in mice26</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>HMG-CoA reductase inhibitor</td>
<td>Primary and secondary prevention of atherosclerosis</td>
<td>Preclinical: rHDL-vehicle delivery reduced atherosclerosis in mice7</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>mTOR inhibitor</td>
<td>Organ transplantation, drug-eluting stents</td>
<td>Preclinical: Oral dosing reduces atherosclerosis in mice43-50, local delivery strategies are being developed40-42</td>
</tr>
</tbody>
</table>

*CIRT indicates cardiovascular inflammation reduction trial; MTX, methotrexate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; rHDL, recombinant high-density lipoprotein; mTOR, mammalian target of rapamycin.

METHODS

Materials

All chemicals were purchased from Sigma unless mentioned otherwise. T0901317 (T09) was purchased from Cayman Chemical. The compounds were dissolved in dimethyl sulfoxide (DMSO), yet ensuring that in all experiments the final DMSO fraction in culture wells was below 0.05% (v/v). Lipoprotein depleted serum (LPDS) was prepared from fetal calf serum by ultracentrifugation in KBr at a density of 1.21 g/ml. After centrifugation at 50000 RPM and 4°C for 50000 RPM, the lipoprotein layer was removed by aspiration. The bottom fraction was dialysed against phosphate buffered saline (PBS) and sterile filtered. The purity is determined via HPLC.
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Cell culture
Human monocytic THP-1 cells, and RAW264.7 murine macrophages and murine bone marrow derived macrophages (BMDM) are widespread models to study macrophage function in atherosclerosis. THP-1 cells and RAW264.7 macrophages were obtained from the American Type Culture Collection. RAW264.7 cells stably transfected with the 3x-NF-κB-luc plasmid were kindly provided by Prof. M.P.J. de Winther.

THP-1 cells and RAW264.7 were cultured in RPMI-1640 and DMEM-high glucose, respectively. Both media were supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS; GIBCO Invitrogen). THP-1 cells were differentiated into macrophages with 50ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 hours, after which cells were washed and left in PMA-free medium for another 24 hours before adding the compounds. Bone marrow cells were isolated from both femurs and tibiae of wild-type mice (C57BL/6). Cells were cultured in RPMI-1640 with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS and 15% L929 conditioned medium for 8 days to generate BMDM according to a method previously described. For the oxidative burst assay, polymorph-nuclear neutrophils (PMNs) were isolated by Ficoll centrifugation of buffy coats purchased from Sanquin (Amsterdam) blood supply.

Cell viability
THP-1, and RAW264.7 and BMDM cells were seeded in 96-well plates (5 x 10⁴ cells/well). The next day, cells were treated with the compounds in concentrations ranging from 0.3 to 30 μM for 24 hours. The toxicity of compounds was determined by colorimetric MTT cell viability assay as described previously.

NF-κB transcriptional activity
RAW264.7 NF-κB-luc macrophages were seeded in 96-well plates (7 x 10⁴ cells/well). After 24 hours, cells were washed and treated with the compound for 2 hours after which cells were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for another 18 hours. NF-κB luciferase activity was determined by the ONE-Glo™ Luciferase Assay System (Promega).

Pro-inflammatory cytokine production
Quantitation of secreted cytokine concentrations of tumor necrosis factor alpha (TNF-α) and interleukin(IL)-6 was performed by using the Cytometric Bead Array Human Inflammation Kit (BD Biosciences). THP-1 and BMDM cells were seeded in 96-well plates (5 x 10⁴ cells/well). After differentiation with PMA, cells were treated with each compound for 2 hours. Thereafter, LPS was added at final concentration of 100 ng/ml for another 22 hours. Before analysis, cellular debris was removed from the supernatants of treated cells by centrifugation (400g 5’5 minutes at 500G).
RNA isolation, cDNA synthesis and qRT-PCR

THP-1 cells were seeded into 12-well plates (5 x 10^5 cells/well). After differentiation with PMA, cells were first incubated with oxidized low-density lipoprotein (oxLDL; 50 μg/ml; Alfa Aesar) for 24 hours, then treated with the compounds for an additional 18 hours. Total RNA was extracted using Trizol and cDNA was synthesized from 1μg RNA with the iScript cDNA Synthesis kit (BioRad). Semi-qQuantitative real-time PCR (qRT-PCR) was performed using SensiFAST™ SYBR® (BC Biotech) and measured with the CFX384 system (BioRad). Specific primers for human CD36, ATP-binding cassette transporter A1 (ABCA1), fatty acid binding protein 4 (FABP4), C/EBP homologous protein (CHOP), inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1) and ribosomal protein 36B4 were designed (Table S1).

Nitric oxide (NO) production

In vitro evaluation of NO production of THP-1 macrophages using the Griess assay is not achievable. RAW264.7 macrophages and BMDM were seeded in 96-well plates (1 x 10^5 cells/well) and next day washed and pre-treated with each compound for 2 hours followed by 100 ng/ml LPS for another 22 hours. Nitrite (NO₂⁻) concentrations in the supernatants were measured by adding 100 μl freshly made Griess reagent ((0.1% N-(1-Naphtyl)ethylenediamine dihydrochloride (Merck), 2,5% pPhosphoric acid (Merck), 1% sulfanilamide (Sigma))) to 100 μl culture supernatant. Serial dilutions of nitrite standard solution were used to generate a standard curve ranging from 0 to 100 μM of nitrite. The absorbance was measured at 550 nm with a microplate reader (SPECTROstar Nano).

Production Reactive Oxygen Species (ROS)

PMNs were suspended in Hank’s’ Buffered Saline Solution (HBSS) substituted with 1% gelatine solution in deionized H₂O (HBSS-gel.). Cells were counted and diluted in the HBSS-gel to a concentration of 1x10^6 cells/ml. Zymosan A from Saccharomyces cerevisiae was used as ROS inducer. Luminol, dissolved in DMSO and diluted in HBSS resulting in a final DMSO concentration lower than 0.1% (v/v), was used as luminescence enhancer and was added to the PMNs in a 1:1 volume ratio in white 96-well plates. Compounds were added to the previously indicated final concentrations and zZymosan was added to final concentration of 0.2 mg/ml. Luminescence was measured using Titertek Luminoskan (TechGen International). The assay was repeated using buffy coats from different donors.

OxLDL uptake

Human oxLDL (Alfa Aesar) was labelled with DyLight 488 NHS Ester (#46402 Thermo Scientific). The manufacturer has oxidized the LDL via copper sulphate oxidation and the degree of oxidation was 99% (determined via the TBARS assay). Briefly, 0.43 μL Dylight 488 was added
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per 250 μL of 2 mg/ml oxLDL. The solution was protected from light and incubated on a
shaker at RT for 1 hour. Subsequently, unbound dye was washed away by dialysis at 4°C
overnight. THP-1 complete medium was replaced by medium containing 10% lipoprotein
depleted serum (LPDS) and pre-treated overnight with each compound. Subsequently, 25
μg/ml oxLDL-Dylight 488 was added to treated or non-treated cells for 6 or 24 hours. Finally,
cells were washed once with ice cold phosphate buffered saline (PBS) containing 5% FBS,
twice with ice cold PBS and lysed using radio-immunoprecipitation assay buffer (TEKnova).
Fluorescence was measured with a Typhoon scanner (GE Healthcare).

Cholesterol efflux

THP-1 cells were seeded in 24-wells plates at a density of 5 x 10⁵ cells/ml. Macrophages were
treated with compounds for 8 hours and loaded with cholesterol overnight using 30 μg/
ml cholesterol mixed with 0.5 μCi/ml tTritium-labeled cholesterol (Amersham) in RPMI with
100 U/ml penicillin-/streptomycin (100 U/ml-100 μg/ml) and 0.2% free fatty acid (FFA)-free
bovine serum albumin (BSA). After washing with PBS containing 0.2% FFA-free BSA, cells
were incubated for 4 hours with 20 μg/ml apolipoprotein-A1 (apoA-1; Calbiochem) in RPMI
(without FBS) containing 100U/ml penicillin-/streptomycin (100 U/ml - 100 μg/ml) and 0.2%
FFA-free BSA (Sigma). Cells were lysed using isopropanol 100%. Tritium was measured for
both cell culture medium and cell lysates using a scintillation counter.

BrdU incorporation

Proliferation was primarily evaluated in RAW264.7 cells, since differentiation of THP-1 cells with
PMA is known to halts proliferation¹⁵. RAW264.7 and THP-1 cells were seeded in 96-well plates
(3 x 10³ cells/well), washed the next day with serum free medium and subsequently serum
starved for 24 hours. Thereafter, serum free medium was replaced with complete medium
reconstituted with each compound except for the control, in which only complete medium
was added. After 24 hours, proliferation was measured using the 5-bromo-2'-deoxyuridine
(BrdU) ELISA kit (Roche) according to the manufacturer’s recommendations.

Overall performance score

The experiments provided outcomes known to have either an atheroprotective or atherogenic
effect, which was expressed in fold change compared to the untreated control. A scoring
system was applied where a statistical significant change compared to control was scored
as +1 or -1 point depending on whether the effect was atheroprotective or atherogenic,
respectively. A two-fold change or more was scored by adding or subtracting 2 points. The
total score was tallied and compounds were ranked accordingly. Subsequently, a summarizing
heatmap was generated in MultipleExperiment Viewer (MeV 4.9.0., Microarray Software Suite,
Statistical analysis

All experiments assays were at least performed in triplicate three independent experiments. Statistical analysis was performed using GraphPad Prism 5 software. Statistical significance was calculated using the unpaired Student’s T-test (Welch corrected when necessary). Values are represented as mean ± SEM. The significance level was set at p<0.05.

RESULTS

Cell viability

Prior to evaluating the chosen macrophage pathways relevant to atherosclerosis, cell viability was assessed after treatment with drug concentrations ranging from 0.3 μM to 30 μM to assess the non-toxic dose to be used for the other assays. MTT cell viability assays showed that none of the compounds affected THP-1 cell viability, apart from rapamycin, which was toxic at 30 μM. In addition to human cells, viability assays of murine RAW264.7 cells and BMDM showed comparable results (data not shown Figure S1). Thus, for each compound a concentration of 30 μM was applied, whereas for rapamycin a concentration of 3 μM was used in the screening assays.

Anti-inflammatory potency

Anti-inflammatory effects of the selected compounds were first evaluated with LPS-induced NF-κB activation in RAW264.7 NF-κB-luc macrophages (Figure 1A). A strong (>50%) inhibition of LPS-induced NF-κB activity was observed after prednisolone, T09 and 6-MP treatment compared to LPS stimulation only (all p>0.001). MTX, pterostilbene and simvastatin showed a modest (25%) reduction on NF-κB activity (all p<0.01), whereas rapamycin increased NF-κB activity (p=0.006). The subsequent release of pro-inflammatory cytokines following LPS was determined in THP-1 macrophages. Prednisolone, T09 and pterostilbene strongly (>50%) reduced the production of TNFα and IL-6. Simvastatin did repress TNFα, but had no statistically significant effect on IL-6 (Figure 1B&C-E). MTX, 6-MP and rapamycin did not significantly influence cytokine release, apart from a strong reduction (>50%) in IL-6 by rapamycin (p=0.046). Generally similar effects for cytokine release were observed for BMDM (Figure S2A-B).
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FIGURE 1. Inflammation

RAW264.7 NF-κB-luc cells (A) or THP-1 macrophages (B,C) were treated with the compounds and stimulated with LPS (100 ng/ml) for 24 hrs. (A) NF-κB activity was determined by luciferase assay and fold change was calculated relative to LPS stimulation only from three independent experiments (n=6). (B,C) TNF-α and IL-6 production was measured using a cytometric bead array. Data are presented as mean±SEM; *p<0.05, **p<0.01, ***p<0.001 compared to LPS stimulation only. 6-MP indicates 6-mercaptopurine; c, control; MTX, methotrexate; pred, prednisolone; ptero, pterostilbene; rapa, rapamycin; simva, simvastatin; T09, T0901317.

Generation of reactive oxygen species

Potential anti-oxidative effects were studied using LPS-induced NO production and zymosan-induced PMN ROS generation. After treatment with prednisolone and rapamycin, a substantial reduction in NO production of more than 40% compared to LPS stimulation only (both p<0.001) was observed, while pterostilbene and T09 reduced NO production in the order of 25 to 30% (both p<0.001). MTX, simvastatin, and 6-MP did not affect NO production at all (Figure 2A). In BMDM, NO production was reduced the most (>50%) by prednisolone, pterostilbene and T09 (Figure S2C). With respect to ROS, pterostilbene was the strongest inhibitor of ROS production (8 fold change). T09, 6-MP and simvastatin diminished ROS production to a less extent, while prednisolone, MTX and rapamycin did not affect ROS production (Figure 2B).
Lipid handling by macrophages

Important changes in macrophage lipid handling with regard to atherosclerosis comprise the uptake, efflux and intracellular trafficking of lipids. First, the uptake of fluorescently labelled oxLDL was assessed in THP-1 macrophages. Correcting for background fluorescence measured in control cells without oxLDL, T09 and pterostilbene modestly reduced oxLDL uptake by 25-45% (both p<0.001) compared to oxLDL stimulation only (Figure 3A), whereas MTX, simvastatin and rapamycin decreased oxLDL uptake to a somewhat lesser extent (all p<0.001). Similar results were observed when oxLDL treatment was extended to 24 hours (Figure S2D). In line with the diminished oxLDL uptake, both T09 and pterostilbene markedly down-regulated the expression of the lipid uptake receptor CD36 (both p<0.001; Figure 3B).

Next, treatment effects on cholesterol efflux from THP-1 cells to apoA-1 were assessed. As expected, the LXR-agonist T09 greatly increased cholesterol efflux by 2.5-fold (p<0.001), in respect to untreated control cells. Simvastatin (p=0.005) and rapamycin (p=0.017) also strongly augmented cholesterol efflux, whereas the other compounds did not affect this process (Figure 3C). mRNA expression of the cholesterol efflux transporter ABCA1 was significantly (>2.5-fold) elevated by T09 and pterostilbene, while it was reduced in response to prednisolone (45%) and simvastatin (20%) (all p<0.001 compared to oxLDL stimulation only) (Figure 3D).
FIGURE 3. Macrophage lipid handling
THP-1 macrophages were treated with compounds and oxLDL-Alexa488 for 6 hrs (A) or oxLDL for 24 hrs (B, D). (A) OxLDL uptake was determined in protein lysates and by (B) mRNA expression of CD36 using qPCR. (C) Cholesterol efflux was measured by Tritium-labelled cholesterol loading and using ApoA1 as acceptor. (D) mRNA expression of ABCA1 was determined using qRT-PCR. Total mRNA input was corrected for the housekeeping gene 36B4. Fold change was calculated relative to oxLDL stimulation only from three independent experiments (n=3). Data are presented as mean±SEM; *p<0.05, **p<0.01, ***p<0.001 compared to oxLDL stimulation (A, B, D) or untreated cells (C). 6-MP indicates 6-mercaptopurine; c, control; MTX, methotrexate; pred, prednisolone; ptero, pterostilbene; rapa, rapamycin; simva, simvastatin; T09, T0901317.

Endoplasmic reticulum (ER) stress
To examine whether the compounds affected macrophage ER stress, changes in mRNA expression of ER stress related proteins in THP-1 macrophages pre-exposed to oxLDL were assessed. As regulator of intracellular lipid trafficking and lipid-induced ER stress, mRNA expression of lipid chaperone FABP4 was found to be significantly increased by 6-MP (p=0.002) and up to almost 2-fold by prednisolone (p<0.001) and 6-MP (p=0.002) treatment compared to oxLDL stimulation only, whereas T09 and pterostilbene markedly decreased FABP4 expression by 30-70% (both p<0.001) (Figure 4A).
Prolonged or unresolved ER stress can induce apoptosis mediated through CHOP and IRE1. Compared to oxLDL treated control, mRNA expression of CHOP was most notably increased by treatment with T09 (>1000%), pterostilbene (>200%), 6-MP (75%) (all p<0.001); whereas prednisolone modestly decreased CHOP by 20% (p=0.002). MTX and simvastatin had no significant effects on CHOP expression (Figure 4B). IRE1 mRNA expression was strongly upregulated (50%) in response to prednisolone, T09, pterostilbene and rapamycin compared to oxLDL stimulation only, while modestly upregulated by MTX and 6-MP. Simvastatin treatment did not change IRE1 levels. (Figure 4C)

**FIGURE 4.** Macrophage oxLDL-induced ER-stress and macrophage proliferation

(A-C) To measure ER stress, THP-1 macrophages were treated with compounds and oxLDL for 24 hrs. mRNA was isolated, cDNA was made and qRT-PCR was performed for FABP4 (A), CHOP (B) and IRE1 (C). Total mRNA input was corrected for the housekeeping gene 36B4. (D) Macrophage proliferation was determined in RAW264.7 cells by BrdU-incorporation after an initial serum starvation. Proliferating cells were expressed as a percentage relative to untreated controls. Three independent experiments (each n=6) were performed. Data are presented as mean±SEM (A-D); *p<0.05, **p<0.01, ***p<0.001 compared to oxLDL stimulation (A-C) or untreated control (D). 6-MP indicates 6-mercaptopurine; c, control; MTX, methotrexate; pred, prednisolone; ptero, pterostilbene; rapa, rapamycin; simva, simvastatin; T09, T0901317.
Macrophage proliferation

The proliferative capacity of macrophages after treatment was assessed using BrdU incorporation in RAW264.7 cells (Figure 4D). Simvastatin and pterostilbene demonstrated the strongest anti-proliferative effect by reducing the BrdU incorporation with 75% and 55% (both p < 0.001), respectively. T09, 6-MP and rapamycin also significantly inhibited cell proliferation, though to a less extent. MTX did not affect macrophage proliferation. In THP-1 cells fluorescence intensity was generally lower, as may be expected with this cell line. Still, a reduction pattern (≥50%) similar to RAW264.7 was observed for T09, pterostilbene and 6-MP (Figure S2E).

Overall drug performance

Finally, the outcomes of the assays were expressed in an atheroprotective or atherogenic score. The overall performance of each compound was assessed, with heterogeneous results between the different compounds and assays, largely in concordance with their mode of action. The constructed heatmap illustrates that T09, pterostilbene, and simvastatin were identified as highest scoring compounds for anti-atherosclerotic impact on plaque macrophages (Figure 5).

**FIGURE 5.** Summary

Results from each macrophage assay were expressed in fold change compared to stimulated controls to reflect an atheroprotective or atherogenic effect. A heatmap was generated in MultiArray viewer (MeV4.9.0). 6-MP indicates 6-mercaptopurine; MTX, methotrexate; pred, prednisolone; ptero, pterostilbene; rapa, rapamycin; simva, simvastatin; T09, T0901317.
DISCUSSION

In this study, we assessed multiple macrophage-related pathways to evaluate the overall anti-atherogenic impact of several drug candidates. We focused on key mechanisms of macrophage activity in atherosclerotic plaques: inflammation, oxidative stress, lipid handling, ER stress and proliferation. Using a dedicated *in vitro* approach, we rated the overall performance of the 7 candidate drugs known to interfere in one or more of these pathways. The overall performance of the well-known anti-inflammatory corticosteroid prednisolone was found to be counterbalanced by pro-atherogenic effects, leading to a low anti-atherogenic score. Conversely, pterostilbene, T09 and simvastatin exhibited a strong overall anti-atherogenic performance in macrophages, exerting beneficial effects in multiple pathways at the same time, suggesting their potential for plaque macrophage-targeted liposomal delivery. *In vivo* validation studies are warranted to corroborate the predictive value of this macrophage-based screening approach and its potential value in compound selection for nanomedicinal delivery in cardiovascular patients.

We recently demonstrated successful liposomal delivery of prednisolone to plaque macrophages in patients with advanced atherosclerotic disease. Prednisolone is a potent, anti-inflammatory compound, exerting pleiotropic effects on many signalling pathways via the glucocorticoid receptor, which is widely used to reduce inflammatory activity in both acute and chronic inflammatory diseases. In patients with atherosclerosis we were, however, unable to show a reduction in overall plaque inflammatory activity with even a small increase in FDG-PET/CT signal, despite successful plaque delivery of the liposomal payload. In our *in vitro* assays, we corroborate that prednisolone indeed potently inhibits inflammatory activity, including NF-κB activity and attenuation of inflammatory cytokines. Concomitantly, prednisolone has a strong adverse impact on lipid handling, as it reduced expression of ABCA1 and upregulated the lipotoxicity mediator FABP4. Moreover, prednisolone activated the ER stress pathways by increasing IRE1 levels. These findings imply unsuitability of prednisolone to exert an overall anti-atherogenic effects on macrophages in an atherosclerotic, lipid-rich environment.

From the selected variety of drug candidates, simvastatin, T09 and pterostilbene were identified to have a broad anti-atherogenic effect in our assays, attributed to their beneficial impact on inflammation, oxidative stress, lipid handling and proliferation.

Simvastatin has been widely acknowledged for its marked LDL-c lowering effect. Previous studies have emphasized non-lipid *pleiotropic* effects of statins. In the present study, we corroborate a spectrum of anti-atherogenic effects on macrophages. The potential relevance of these macrophage-related effects are supported by our recent findings using targeted delivery of statins to macrophages. Namely, statins packaged within reconstituted HDL were found to be delivered effectively to plaque macrophages, where statins were shown to exert potent anti-inflammatory as well as anti-proliferative effects.

The LXR-agonist T09 has been previously shown to have strong anti-atherogenic effects mediated by both enhanced cholesterol efflux and suppression of inflammation. Here, we
indeed establish these beneficial effects on both lipid handling and inflammation, as attested by the decrease in NF-κB activity, reduction of inflammatory cytokines, decrease in oxLDL uptake and increase in cholesterol efflux. The anti-atherogenic effect of T09 is further favoured by the observed reduction of oxidative stress and decrease in macrophage proliferation. However, clinical development has been hampered by adverse effects on hepatic lipogenesis, leading to hepatic steatosis and dyslipidemia. Local delivery to macrophages and/or development of novel LXR-agonists may overcome these drawbacks.

The evaluation of pterostilbene (and its analogue resveratrol) in atherogenesis has so far been limited. Pterostilbene has been attributed a wide range of beneficial effects in various medical settings, including cancer prevention and therapy, neurological decline, and metabolic syndrome. We here show the anti-atherogenic potential of pterostilbene on several macrophage activities. Pterostilbene suppresses inflammation by decreasing NF-κB activity strongly inhibiting the release of inflammatory cytokines, decreases oxidative stress by potently lowering NO and ROS production, beneficially affects lipid handling, and decreases macrophage proliferation. Together with some preliminary experimental studies showing that oral supplementation of resveratrol may reduce atherosclerosis, this favourable in vitro profile paves the way for further exploration of pterostilbene in a macrophage-targeted therapy approach.

Remarkably, we observed discrepancies between ABCA1 expression and cholesterol efflux after simvastatin and pterostilbene treatment. Although reduced ABCA1 expression may be expected based on statin-mediated inhibitory effects on LXR and SREBP-2 pathways, the increase in cholesterol efflux to apoA-1 was not expected. Previous studies do not corroborate such a divergent effect. These results underline the complexities of lipid metabolism in macrophages and that mRNA expression does not equal downstream functionality. In addition, pterostilbene treatment resulted in up regulation of ABCA1, although the cholesterol efflux assay unexpectedly did not show an increase. While data on pterostilbene in current literature is lacking, its analogue resveratrol has shown similar effects on ABCA1 expression, however this was indeed coupled with an increase in cholesterol efflux. Further investigations are warranted to assess whether pterostilbene has inherently different effects on cholesterol efflux than resveratrol, or that this is due to differences in experimental setup.

Despite demonstrating clear prednisolone-like anti-inflammatory effects, we find that 6-MP and MTX fail to convince in our in vitro screening as anti-atherogenic compounds, considering their lack of efficacy in non-inflammatory pathways. Preclinical work for 6-MP in atherosclerosis has so far been scarce and reports from clinical use in inflammatory disorders lean towards an increased cardiovascular risk. In contrast, MTX is seen as a viable compound for atherosclerosis. Currently, a low-dose of MTX is being evaluated as add-on therapy in cardiovascular patients in the Cardiovascular Inflammation Reduction Trial (CIRT). Even though MTX did not cumulate any points in our heatmap, overall it appears to be a relatively neutral compound without any excessive atherogenic pathway effects. With this
in mind, we await with interest the results of CIRT; although not specifically plaque targeted, these results will add to our understanding of the value and relative contribution of the separate pathways in our assays.

Similar to MTX, rapamycin also exhibited a generally neutral effect in our assays with a low total score. While already applied for over a decade in drug eluting coronary stents, targeted delivery strategies seem to be underway following anti-atherogenic efficacy in mice models of atherosclerosis.

Our study has several limitations. First, we assess the effects of the compounds only on macrophage activity, thereby disregarding potentially beneficial or harmful effects on other relevant plaque cell types, such as endothelial cells, smooth muscle cells and immune cells other than macrophages. However, since we intend to employ a targeted drug delivery system based on endothelial permeability and phagocytosis, it is reasonable to expect the largest and most relevant impact on plaque macrophages. In addition, we used compounds in their free (unencapsulated) form in our macrophage assays. As a next step, experiments using encapsulated formulations of promising compounds are to be performed to assess any additional effects of the delivery vehicle itself. Lastly, we use only *in vitro* tests, mimicking an atherogenic environment by adding atherogenic factors such as oxLDL to monocyte/macrophage cell lines. However, the extrapolation of *in vitro* tests towards clinical impact remains to be established. Hence, *in vivo* validation testing is warranted to determine the value of our screening assays as a translational tool in atherosclerotic disease. Thus far, earlier *in vivo* experiments with nanodelivery of simvastatin and prednisolone appear to be in agreement with our *in vitro* data, hinting at the feasibility of the currently employed *in vitro* testing strategy.

Nanomedicinal drug delivery has the potential to take cardiovascular disease management to the next level. Transitioning from previous work in which we focused on targeting inflammation as the main driver behind atherosclerosis, the current study clearly underlines the need to broaden our focus and take a wide array of macrophage-related processes into account for nanomedicinal drug development. Out of a variety of drug candidates, we identified T09, pterostilbene and simvastatin as potential anti-atherogenic compounds for plaque macrophage targeted therapy. Our current assay setup provides a promising framework for high-throughput screening, as it offers a rapid and comprehensive method to simultaneously screen multiple drug candidates for their anti-atherogenic potency. We envision our work will facilitate the process of bringing novel cardiovascular treatment strategies from bench to bedside.

**Acknowledgements:** We like to thank Alinda Schimmel for her assistance in the efflux experiments.
Disclosures and funding: This work was supported by a European Framework Program 7 grant (ESS, GS: FP7-Health 309820: Nano-Athero); the Dutch network for Nanotechnology NanoNext NL, in the subprogram “Drug Delivery”. All other authors declare that they have no conflict of interest and no relationships with industry relevant to this study.

REFERENCES


**TABLE S1.** Human primer sequences for semi-quantitative real-time PCR

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<th>Gene</th>
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<td>IRE1</td>
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**FIGURE S1.** Cell viability

Cell viability for (A) THP-1, (B) RAW cells and (C) BMDM was determined by measuring active mitochondria using MTT. Cell viability was calculated as a percentage from untreated cells. A 50% decrease in fluorescence was considered toxic. Data was combined from two independent experiments (n=5) and are presented as mean±SEM; *p<0.05, **p<0.01, ***p<0.001 compared to control. c = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin.
FIGURE S2. Pro-inflammatory cytokines

(A-B) Bone marrow derived macrophages (BMDM) were treated with the compounds and stimulated with LPS (100 ng/ml) for 24 hrs. TNF-α and IL-6 production was measured using a cytometric bead array, compared to LPS stimulation only. (C) BMDM were treated with the compounds and stimulated with LPS (100 ng/ml) for 24 hrs after which nitric oxide (NO) production was determined with Griess reagent, fold change was calculated relative to LPS stimulation. (D) THP-1 macrophages were treated with compounds and oxLDL-DyLight488 for 24 hrs and oxLDL uptake was determined in protein lysates, fold change was calculated relative to oxLDL stimulation only. (E) Macrophage proliferation was determined in THP-1 cells by BrdU-incorporation after an initial serum starvation. Proliferating cells were expressed as a percentage relative to untreated controls. Data are presented as mean±SEM. p<0.05, **p<0.01, ***p<0.001. C = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin.