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Multiple pathway assessment to predict anti-atherogenic efficacy of drugs targeting macrophages in atherosclerotic plaques

Amr Alaarg [a,b,1], Kang He Zheng [c,1], Fleur M. van der Valk [c], Acarilia Eduardo da Silva [b], Miranda Versloot [d], Linda C. Quarles van Ufford [e], Dominik M. Schulte [c,f], Gert Storm [a,b,1], Josbert M. Metselaar [b,g], Erik S.G. Stroes [c,*], Anouk A.J. Hamers [d]

[a] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands
[b] Department of Biomaterials Science and Technology, Targeted Therapeutics section, MIRA Institute, University of Twente, Enschede, The Netherlands
[c] Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[d] Department of Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[e] Medicinal Chemistry & Chemical Biology – Biomolecular Analysis, Department of Pharmaceutical Sciences, Utrecht University, The Netherlands
[f] Department of Internal Medicine I, UKSH, 24105 Kiel, Germany
[g] Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH-Aachen University, Aachen, Germany

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ABSTRACT

Background: 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 assessment. 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.

1. 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

Abbreviations: Pred, prednisolone; MTX, methotrexate; T09, T091317; Ptero, pterostilbene; 6-MP, mercaptopurine; Simva, simvastatin; Rapa, rapamycin; LXR, liver receptor X; CIRT, cardiovascular inflammation reduction trial; rHDL, recombinant high-density lipoprotein; ER, endoplasmic reticulum; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PMA, phorbol-12-myristate-13-acetate; PMN, polymorph-nuclear neutrophils; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; IL, interleukin; oxLDL, oxidized low-density lipoprotein; qPCR, quantitative real-time polymerase chain reaction; ABCA1, ATP-binding cassette transporter *A1*; FABP4, fatty acid binding protein 4; CHOP, C/EBP homologous protein; IRE1, inositol-requiring transmembrane kinase/endonuclease 1; NO, nitric oxide; ROS, reactive oxygen species; HBSS, Hanks' Buffered Saline Solution; LPDS, lipoprotein depleted serum; BMDM, bone marrow derived macrophage; FFA, free fatty acid; BSA, bovine serum albumin; apoA-1, apolipoprotein-*A1*; BrdU, 5-bromo-2′-deoyxuridine.

* Corresponding author at: Department of Vascular Medicine, F4.211, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands.
E-mail addresses: a.m.s.a.alaarg@uu.nl (A. Alaarg), k.h.zheng@amc.nl (Z. Kang He), f.m.valkvander@amc.nl (F.M. van der Valk), acariliasilva@gmail.com (A.E. da Silva), m.versloot@amc.nl (M. Versloot), h.c.quarlesvanufford@uu.nl (L.C.Q. van Ufford), dominik.schulte@uksh.de (D.M. Schulte), bart@enceladus.nl (J.M. Metselaar), e.s.stroes@amc.nl (E.S.G. Stroes), a.a.hamers@amc.nl (A.A.J. Hamers).
[1] Authors contributed equally.

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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.

2. Materials and methods

2.1. 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 calve serum by ultracentrifugation in KBr at a density of 1.21 g/ml. After centrifugation at 50,000 RPM and 4 °C for 50,000 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.

2.2. Cell culture

Human monocytic THP-1 cells [10], RAW264.7 murine macrophages [11] 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 3×-NF-κB-*luc* plasmid were kindly provided by Prof. M.P.J. de Winther [11].

THP-1 cells and RAW264.7 were cultured in RPMI-1640 and DMEM-high glucose, respectively. Both media were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS; GIBCO Invitrogen). THP-1 cells were differentiated into macrophages with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 h, after which cells were washed and left in PMA-free medium for another 24 h 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 [12].

For the oxidative burst assay, polymorph-nuclear neutrophils (PMNs) were isolated by Ficoll centrifugation of buffy coats purchased from Sanquin (Amsterdam) blood supply.

2.3. Cell viability

THP-1, RAW264.7 and BMDM cells were seeded in 96-well plates (5×10^4 cells/well). The next day, cells were treated with the compounds in concentrations ranging from 0.3 to 30 μM for 24 h. The toxicity of compounds was determined by colorimetric MTT cell viability assay as described previously [13].

2.4. NF-κB transcriptional activity

RAW264.7 NF-κB-*luc* macrophages were seeded in 96-well plates (7×10^4 cells/well). After 24 h, cells were washed and treated with the compound for 2 h after which cells were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for another 18 h. NF-κB luciferase activity was determined by the ONE-Glo™ Luciferase Assay System (Promega).

2.5. 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 (5×10^4 cells/well). After differentiation with PMA, cells were treated with each compound for 2 h. Thereafter, LPS was added at final concentration of 100 ng/ml

Table 1
Selected drug compounds for multi-pathway screening.

Drug	Mode of action	Clinical use	Status for atherosclerosis
Prednisolone *Anti-inflammatory*	Glucocorticoid receptor agonist	Inflammatory, oncological and hematological disorders	Phase I/II: Liposomal formulation of prednisolone phosphate showed no efficacy [9]
Methotrexate (MTX) *Anti-inflammatory*	Folic acid antagonist	Neoplastic diseases, rheumatoid arthritis, psoriasis	Phase III: Systemic low dose MTX trial in progress (CIRT) [47]
T0901317 (T09) *Cellular cholesterol efflux stimulator*	Liver X receptor (LXR) agonist	Only preclinical use	Preclinical: Systemic dosing reduces atherosclerosis in animal models, but promotes hepatic lipogenesis [25–30]
Pterostilbene *Anti-oxidant*	Free radical scavenging	No clinical indications; available as dietary supplement	Preclinical: Long term oral dosing of resveratrol (analogue) reduces atherosclerosis in mice and rabbits [32–36]
Mercaptopurine (6-MP) *Anti-proliferative*	Purine antagonist	Organ transplantation, leukemia, auto-immune disorders	Preclinical: Drug-eluting cuff reduces atherosclerosis in mice [43]
Simvastatin *Lipid lowering* *Anti-inflammatory*	HMG-CoA reductase inhibitor	Primary and secondary prevention of atherosclerosis	Preclinical: rHDL-vehicle delivery reduced atherosclerosis in mice [7]
Rapamycin *Anti-inflammatory*	mTOR inhibitor	Organ transplantation, drug-eluting stents	Preclinical: Oral dosing reduces atherosclerosis in mice [50–57]; local delivery strategies are being developed [48,49]

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

for another 22 h. Before analysis, cellular debris was removed from the supernatants of treated cells by centrifugation (5 min at 500 *g*).

2.6. RNA isolation, cDNA synthesis and qPCR

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

2.7. Nitric oxide (NO) production

In vitro evaluation of NO production of THP-1 macrophages using the Griess assay is not achievable [14]. RAW264.7 macrophages and BMDM were seeded in 96-well plates (1×10^5 cells/well) and next day washed and pre-treated with each compound for 2 h followed by 100 ng/ml LPS for another 22 h. Nitrite (NO_2^-) concentrations in the supernatants were measured by adding 100 μl freshly made Griess reagent ((0.1% *N*-(1-Naphtyl)ethylenediamine dihydrochloride (Merck), 2.5% phosphoric 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).

2.8. Production reactive oxygen species (ROS)

PMNs were suspended in Hanks' Buffered Saline Solution (HBSS) substituted with 1% gelatine solution in deionized H_2O (HBSS-gel.). Cells were counted and diluted in the HBSS-gel to a concentration of 1×10^6 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 zymosan 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.

2.9. 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 per 250 μL of 2 mg/ml oxLDL. The solution was protected from light and incubated on a shaker at RT for 1 h. Subsequently, unbound dye was washed away by dialysis at 4 °C overnight. THP-1 complete medium was replaced by medium containing 10% 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 h. 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).

2.10. Cholesterol efflux

THP-1 cells were seeded in 24-wells plates at a density of 5×10^5 cells/ml. Macrophages were treated with compounds for 8 h and loaded with cholesterol overnight using 30 μg/ml cholesterol mixed with 0.5 μCi/ml tritium-labelled cholesterol (Amersham) in RPMI with 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 h with 20 μg/ml apolipoprotein-*A*1 (apoA-1; Calbiochem) in RPMI (without FBS) containing 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.

2.11. BrdU incorporation

Proliferation was primarily evaluated in RAW264.7 cells, since differentiation of THP-1 cells with PMA is known to halt proliferation [15]. RAW264.7 and THP-1 cells were seeded in 96-well plates (3×10^3 cells/well), washed the next day with serum free medium and subsequently serum starved for 24 h. 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 h, proliferation was measured using the 5-bromo-2′-deoxyuridine (BrdU) ELISA kit (Roche) according to the manufacturer's recommendations.

2.12. 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, www.tm4.org).

2.13. Statistical analysis

All assays were at least performed in 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 $p < 0.05$.

3. Results

3.1. 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 (Supplemental Fig. 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.

3.2. 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 (Fig. 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 (Fig. 1B + C). MTX, 6-MP and rapamycin did not significantly influence cytokine release, apart from a reduction (>50%) in IL-6 by rapamycin ($p = 0.046$). Generally similar effects for cytokine release were observed for BMDM (Supplemental Fig. S2).

3.3. 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 >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 (Fig. 2A). In BMDM, NO production was reduced the most (>50%) by prednisolone, pterostilbene and T09 (Supplemental Fig. S3). 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 (Fig. 2B).

3.4. 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 reduced oxLDL uptake by 45% (both $p < 0.001$) compared to oxLDL stimulation only (Fig. 3A), whereas MTX, simvastatin and rapamycine decreased oxLDL uptake to lesser extent (all $p < 0.001$). Similar results were observed when oxLDL treatment was extended to 24 h (Supplementary Fig. S4). In line with the diminished oxLDL uptake, both T09 and pterostilbene markedly downregulated the expression of the lipid uptake receptor CD36 (both $p < 0.001$; Fig. 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 augmented cholesterol efflux, whereas the other compounds did not affect this process (Fig. 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) (Fig. 3D).

3.5. 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

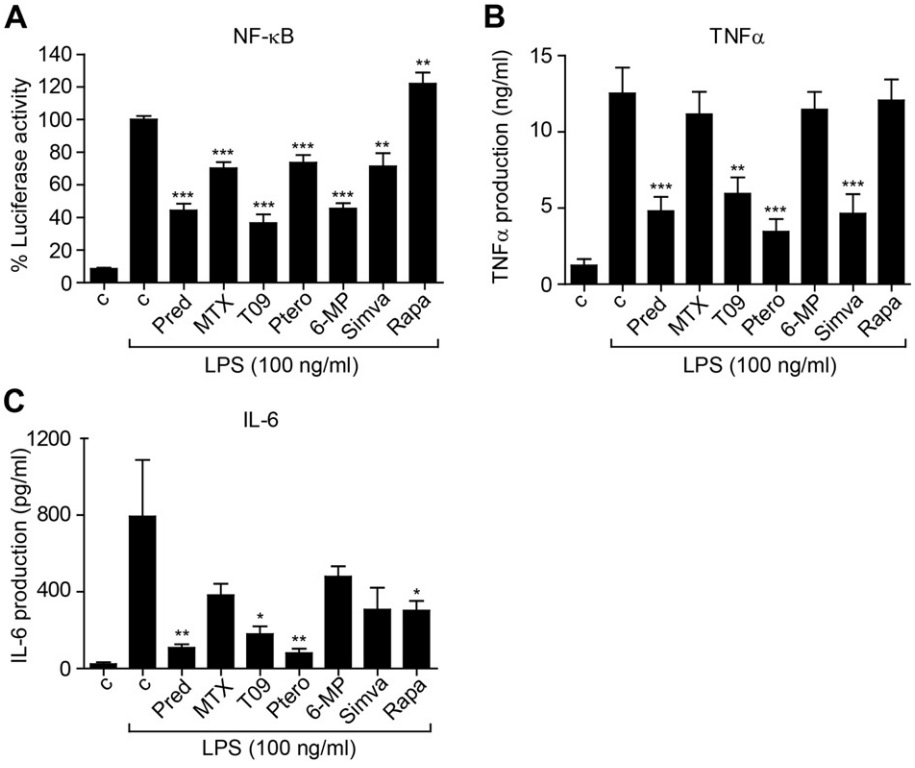

Fig. 1. Inflammation. RAW264.7 NF-κB-*luc* cells (A) or THP-1 macrophages (B + C) were treated with the compounds and stimulated with LPS (100 ng/ml) for 24 h. (A) NF-κB activity was determined by luciferase assay and fold change was calculated relative to LPS stimulation only by combining data from three independent experiments (n = 6/experiment). (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. c = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin.

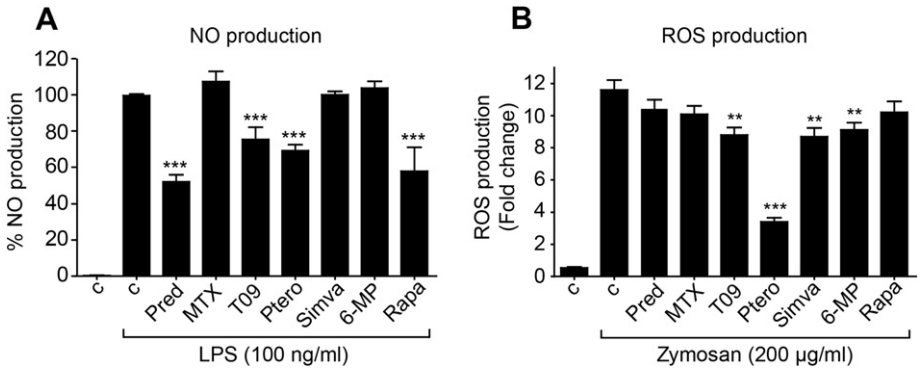

Fig. 2. Anti-oxidant properties. RAW264.7 cells were treated with the compounds and stimulated with LPS (100 ng/ml) for 24 h after which nitric oxide (NO) production was determined with Griess reagent (A). PMNs were treated with the compounds and stimulated with zymosan to measure the inhibition of reactive oxygen species (B). Fold change was calculated relative to zymosan stimulation only (c) by combining data from three independent experiments (n = 6/experiment). Data are presented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to LPS or zymosan stimulation only. c = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin.

macrophages pre-exposed to oxLDL were assessed. As regulator of intracellular lipid trafficking [16–18] and lipid-induced ER stress [16,19], 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 ($p < 0.001$) treatment compared to oxLDL stimulation only, whereas T09 and pterostilbene decreased FABP4 expression by 30–70% (both $p < 0.001$) (Fig. 4A).

Prolonged or unresolved ER stress can induce apoptosis mediated through CHOP and IRE1 [20]. 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 (Fig. 4B). IRE1 mRNA expression was upregulated (50%) in response to

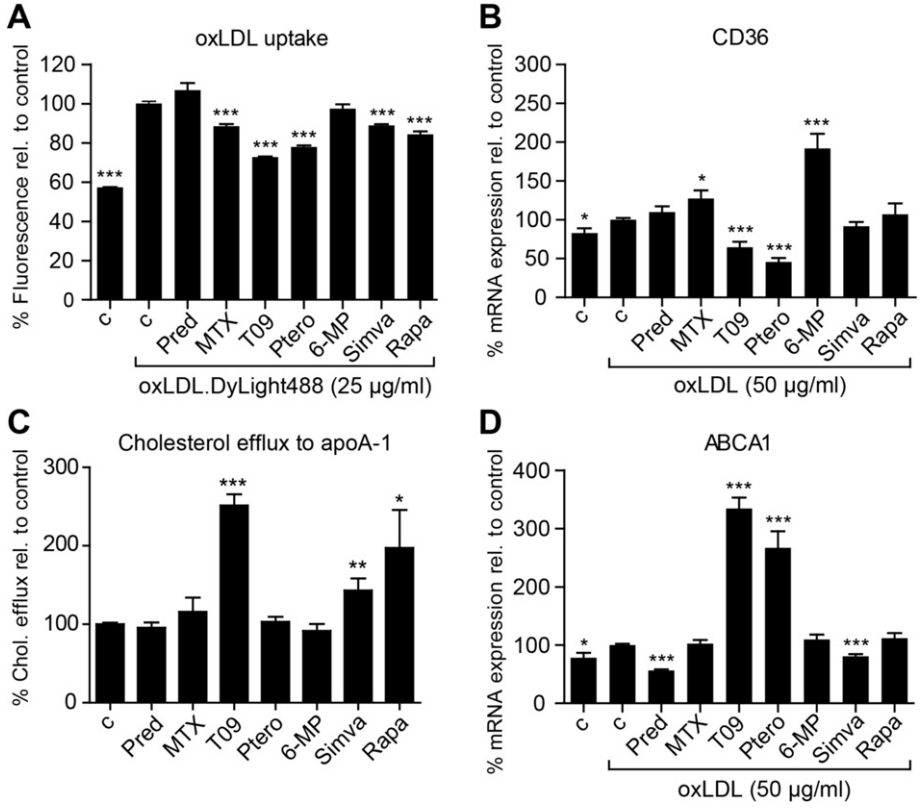

Fig. 3. Macrophage lipid handling. THP-1 macrophages were treated with compounds and oxLDL-DyLight488 for 6 h (A) or oxLDL for 24 h (B, D). (A) OxLDL uptake was determined in protein lysates and by (B) mRNA expression of CD36 using qPCR. (C) Cholesterol efflux was measured by Tritium-labelled cholesterol loading and using ApoA1 as acceptor. (D) mRNA expression of ABCA1 was determined using qPCR. Total mRNA input was corrected for the housekeeping gene 36B4. Fold change was calculated relative to oxLDL stimulation only by combining data from three independent experiments (n = 3/experiment). 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). c = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin; rel. = relative.

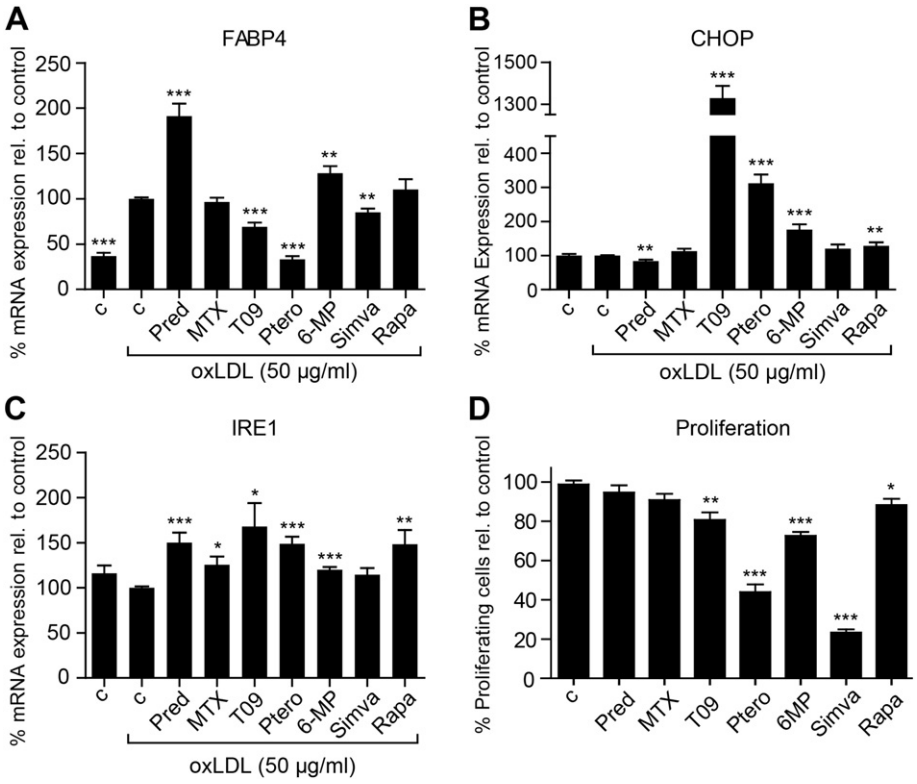

Fig. 4. Macrophage oxLDL-induced ER-stress and macrophage proliferation. (A–C) To measure ER stress, THP-1 macrophages were treated with compounds and oxLDL for 24 h. mRNA was isolated, cDNA was made and qPCR was performed for FABP4 (A), CHOP (B) and IRE1 (C). Total mRNA input was corrected for the housekeeping 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 (n = 6/experiment) were performed and data were combined. 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). c = control; Pred = prednisolone; MTX = methotrexate; T09 = T0901317; Ptero = pterostilbene; 6-MP = 6-mercaptopurine; Simva = simvastatin; Rapa = rapamycin; rel. = relative.

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. (Fig. 4C).

3.6. Macrophage proliferation

The proliferative capacity of macrophages after treatment was assessed using BrdU incorporation in RAW264.7 cells (Fig. 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 (Supplemental Fig. S5).

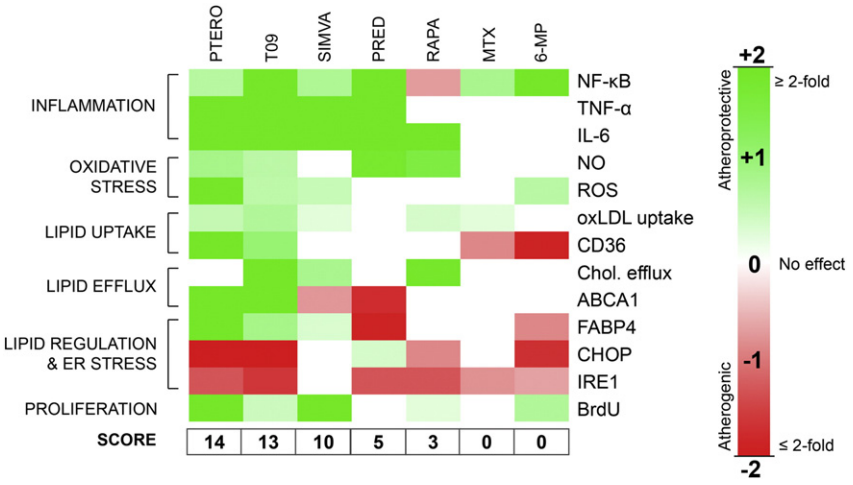

Fig. 5. Summary. Results from each macrophage assay were expressed in fold change compared to stimulated controls to reflect an atheroprotective or atherogenic effect. A heatmap was generated in MultiArray viewer (MeV4.9.0). Ptero = pterostilbene; T09 = T0901317; Simva = simvastatin; Pred = prednisolone; Rapa = rapamycin; MTX = methotrexate; 6-MP = 6-mercaptopurine.

3.7. 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 (Fig. 5).

4. 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 counter balanced 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.

4.1. Screening multiple pathways to estimate the anti-atherogenic impact

We recently demonstrated successful liposomal delivery of prednisolone to plaque macrophages in patients with advanced atherosclerotic disease [9]. 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 [21]. 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 [9]. 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 effect on macrophages in an atherosclerotic, lipid-rich environment.

4.2. Promising anti-atherogenic candidates

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 [22]. Previous studies have emphasized non-lipid *pleiotropic* effects of statins [23]. In the present study, we corroborate a spectrum of anti-atherogenic effects on macrophages. The potential relevance of these macrophage-related effects is 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 [7], where statins were shown to exert potent anti-inflammatory [7] as well as anti-proliferative effects [24].

The LXR-agonist T09 has been previously shown to have strong anti-atherogenic effects mediated by both enhanced cholesterol efflux and suppression of inflammation [25–30]. 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 [31]. 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 [32–36], 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 [37], the increase in cholesterol efflux to apoA-1 was not expected. Previous studies do not corroborate such a divergent effect [37–39]. 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 [40], however this was indeed coupled with an increase in cholesterol efflux [41,42]. 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.

4.3. Compounds potentially lacking anti-atherogenic impact

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 [43] and reports from clinical use in inflammatory disorders lean towards an increased cardiovascular risk [44–46]. 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) [47]. 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 [48,49] following anti-atherogenic efficacy in mice models of atherosclerosis [50–57].

4.4. Limitations

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 [2]. 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 [7] and prednisolone [9] appear to be in agreement with our *in vitro* data, hinting at the feasibility of the currently employed *in vitro* testing strategy.

4.5. Future perspectives

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 relatively high-throughput screening, as it offers a rapid and comprehensive method to simultaneously screen multiple drug candidates or genuine unknown putative candidates for their anti-atherogenic potency. We envision our work will facilitate the process of bringing novel cardiovascular treatment strategies from bench to bedside.

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Appendix A. Supplementary data

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