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

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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.

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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.