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# Inhibiting epigenetic enzymes to improve atherogenic macrophage functions

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ABSTRACT

Macrophages determine the outcome of atherosclerosis by propagating inflammatory responses, foam cell formation and eventually necrotic core development. Yet, the pathways that regulate their atherogenic functions remain ill-defined. It is now apparent that chromatin remodeling chromatin modifying enzymes (CME) governs immune responses but it remains unclear to what extent they control atherogenic macrophage functions.

We hypothesized that epigenetic mechanisms regulate atherogenic macrophage functions, thereby determining the outcome of atherosclerosis. Therefore, we designed a quantitative semi-high-throughput screening platform and studied whether the inhibition of CME can be applied to improve atherogenic macrophage activities.

We found that broad spectrum inhibition of histone deacetylases (HDACs) and histone methyltransferases (HMT) has both pro- and anti-inflammatory effects. The inhibition of HDACs increased histone acetylation and gene expression of the cholesterol efflux regulators *ATP-binding cassette transporters* ABCA1 and ABCG1, but left foam cell formation unaffected. HDAC inhibition altered macrophage metabolism towards enhanced glycolysis and oxidative phosphorylation and resulted in protection against apoptosis. Finally, we applied inhibitors against specific HDACs and found that HDAC3 inhibition phenocopies the atheroprotective effects of pan-HDAC inhibitors.

Based on our data, we propose the inhibition of HDACs, and in particular HDAC3, in macrophages as a novel potential target to treat atherosclerosis.



## 1. Introduction

Current atherosclerosis medication efficiently lowers plasma cholesterol levels, but reduces the risk for cardiovascular disease only partially. Therefore, alternative treatment strategies are needed [1]. During atherosclerosis initiation, accumulation of *low-density lipoproteins* (LDL) and their modifications (e.g. oxidation to oxLDL) in the arterial wall activate endothelial cells. Attracted monocytes adhere to and migrate into the vessel wall, where they differentiate into macrophages and can become lipid-loaded foam cells. Upon atherosclerosis progression, additional immune cells propagate chronic inflammation and proliferating smooth muscle cells enclose the lesion with a fibrous cap. Apoptotic foam cells can be eliminated by neighboring macrophages through efferocytosis or can form a necrotic core. Additionally, macrophage-derived metalloproteases can induce thinning of the fibrous cap and when so-called 'vulnerable' plaques rupture this can cause myocardial infarction and stroke [2].

While macrophages clearly play a central role in different stages of atherogenesis, surprisingly little is known about the molecular mechanisms that regulate their phenotype within the plaque [3]. Macrophages display high heterogeneity and in response to the microenvironment adopt different polarization states [4]. Classically activated (M1) macrophages, activated by Toll-like-receptor (TLR) triggers and Th1 cytokines, are pro-inflammatory and are therefore regarded as pro-atherogenic. Interleukin-4 (IL-4)/IL-13-induced alternatively activated (M2) macrophages are considered

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anti-atherogenic by counteracting inflammation and by efferocytosis. However, M2 macrophages can also aggravate atherogenesis through oxLDL-uptake [1–3]. Oxidized phospholipids, produced in atherosclerotic plaques, induce Mox macrophages that are characterized by an anti-oxidant response [5]. A strategy that promotes anti-inflammatory M2 features and/or inhibits pro-inflammatory, pro-atherogenic macrophage actions could be envisaged as an atheroprotective treatment [3].

Histone modifications by CME govern multiple aspects of inflammation and immunity [6]. Histone acetylation on lysine (K) residues by histone acetyltransferases (HATs) is associated with transcriptional activation and is counteracted by HDACs. Histone methylation is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). Di- or tri-methylation of histone H3K4, H3K36 and H3K79 is associated with activation of transcription, whereas H3K9me2/3 and H3K27me3 constitute repressive marks. In macrophages, the histone demethylase Jmjd3 regulates responses to various stimuli, and HDAC3 promotes inflammatory M1 activation while inhibiting M2 [7]. Pharmaceutical companies are currently pursuing CME-inhibitors as a therapy against inflammatory diseases and we consider such approaches to be valuable for atherosclerosis treatment as well.

To study which CME might be favorable to inhibit in macrophages during atherogenesis, we designed a screening that addresses all major atherogenic macrophage features. Based on our data, we propose macrophage-specific inhibition of HDAC3 as a potential therapeutic target in atherosclerosis.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Femurs and tibia from C57Bl/6 mice were flushed with PBS and bone marrow cells were cultured 8 days in RPMI-1640 2 mM L-glutamine, 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco) and 15% L929-conditioned medium. Unless otherwise indicated, the resulting bone marrow-derived macrophages (BMMs) were plated in 150 μl at $10^6$/ml in a confluent monolayer in 96-well microplates (Greiner). Cells were left untreated or were pretreated 30 min with the following commonly used [8–11] pan-inhibitors (Sigma): 50 nM trichostatin A (TSA; HDAC inhibitor [HDACi]), 50 μM epigallocatechin-3-gallate (EGCG; HATi), 10 μM pargyline (HDMi), 0.5 mM 5′-methylthioadenosine (MTA, HMTi) or 0.5% DMSO as a control for HMTi. Inhibitor concentrations were selected based on previous studies [8–11] and a MTT toxicity assay (data not shown). The pan-HDAC inhibitor ITF2357 (250 nM) and small molecule inhibitors with distinct specificities against particular HDACs were synthesized at Italfarmaco (Milan, Italy) [12]. The HDAC8i retains high selectivity for HDAC8 up to 10 μM and all information about the specificity of the other inhibitors is provided in Supplementary Table 1. BMMs were activated with 10 ng/ml LPS or 2 μg/ml LTA (lipoteichoic acid, Sigma) for 6 or 24 h, or were stimulated 24 h with 10 U/ml interferon-γ (IFNγ, Peprotech) + 10 ng/ml LPS, 100 U/ml IL-4 (Peprotech) or 50 μg/ml oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC, Invivogen) to elicit M1, M2 or Mox macrophages, respectively.

### 2.2. Gene expression analysis

RNA was isolated with High Pure RNA Isolation kits (Roche), cDNA was synthesized from with iScript (Bio-rad) and real-time PCR was performed using Sybr Green Fast mix (Applied Biosystems) on a ViiA7 apparatus (Applied Biosystems). Gene expression was normalized to the mean expression of housekeeping genes *Rplp0* (Arbp) and *Ppia* (CypA). Primer sequences are available on request.

### 2.3. Foam cell formation

After overnight inhibitor pretreatment and DiI-oxLDL (Biotrend) treatment (3 h, 10 μg/ml), oxLDL-uptake was measured by flow cytometry (BD Canto II). For lipid staining, BMMs were pretreated with the inhibitors for 30 min, stimulated with 50 μg/ml oxLDL (BTI) and stained with LipidTOX Red (Invitrogen) according to the manufacturer's protocol. The median fluorescence intensities (MFI) were calculated with FlowJo. Alternatively, cells were lysed in water with 1% Triton and free and total cholesterol was determined using an established enzymatic fluorometric assay [13]. Protein concentrations in the cell lysates were measured with a Pierce BCA assay and used to normalize cholesterol concentrations.

### 2.4. Apoptosis

After 30 min inhibitor pretreatment, BMMs were left untreated or were stimulated overnight with 20 μg/ml 7-ketocholesterol (7KC; Sigma), 50 μg/ml oxLDL or 10 μg/ml 25-hydroxycholesterol (25OHC; Sigma) and stained with propidium iodide (PI)/Annexin-V-Alexa-Fluor647 (Invitrogen) according the manufacturer's protocol. The percentage living (Annexin-$V^-$/$PI^-$), early apoptotic (Annexin-$V^+$/$PI^-$), late apoptotic (Annexin-$V^+$/$PI^+$) and necrotic (Annexin-$V^-$/$PI^+$) macrophages was measured in a FACS Canto II.

### 2.5. Metabolic assay

BMMs were plated in XF-96 cell culture plates and either left untreated or pretreated with the CME-inhibitors, followed by M1 or M2 polarization. Metabolic fluxes were analyzed in an XF-96 Flux Analyzer (Seahorse Bioscience) three times with 5 min intervals per the manufacturer's protocol.

### 2.6. Statistical analysis

Data represent the mean ± SEM of one representative experiment. Data were evaluated with GraphPad Prism4 using one-way ANOVA and values represent the mean ± SEM of 3 replicates; $^*P < 05$; $^{**}P < 01$; $^{***}P < 001$.

## 3. Results

### 3.1. Study design: a phenotypic in vitro screening platform to identify compounds that improve atherogenic macrophage functions

To identify CME-inhibitors that affect atherogenic macrophage activities, we designed a phenotypic screening that addresses major atherogenic macrophage features, including adhesion to endothelial cells, activation and inflammatory cytokine production, foam cell formation, apoptosis and efferocytosis. All assays were designed in a quantitative 96-well-plate-based semi-high-throughput format, providing a new tool to screen large numbers of modulators for their involvement in atherogenic macrophage functions in a fast manner and requiring minimal amounts of materials. First we performed a cell adhesion assay and found that macrophage/endothelial cell-adhesion was not affected by pre-treatment of BMMs with CME-inhibitors (Fig. S1).

### 3.2. HDAC and HMT inhibitors dampen inflammatory macrophage responses in M1 and regulate M2 and Mox macrophage polarization

To test the involvement of epigenetic enzymes in macrophage activation, we pretreated BMMs with the different CME-inhibitors and challenged them with LPS for 6 or 24 h. Blocking HDACs and