Atherosclerosis 281 (2019) 189–197

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Review article

# Potential epigenetic therapeutics for atherosclerosis treatment

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## HIGHLIGHTS

- Epigenetic modifications are increasingly being associated with atherosclerosis.
- Reversing these modifications by using epigenetic inhibitor drugs hold the promise for treating atherosclerosis.

## ARTICLE INFO

*Keywords:*
Atherosclerosis
Inflammation
Immune cells
Epigenetic
Histone modification
DNA methylation
Epigenetic inhibitors

## ABSTRACT

Notwithstanding the intense efforts into the understanding and prevention of cardiovascular disease (CVD), its complex pathology remains the leading cause of mortality worldwide. The pivotal role of epigenetic changes in the control of gene expression has been profiled in several diseases, such as cancer and inflammatory disorders. In the last decade, increasing evidence has also linked aberrant epigenetic modulation as a contributor to CVD development. Differential profiles of DNA methylation, histone methylation and acetylation have consistently been observed in tissues and cells (comprising the aortic lesions, vascular endothelium and monocytes) from patients with CVD. This highlights the therapeutic potential of epigenetic drugs for cardiovascular treatment.

## 1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall. Atherosclerotic plaques are characterized by the accumulation of lipids together with infiltration of various immune cells. Macrophages are the most abundant inflammatory cells present in atherosclerotic plaques [1].

Dyslipidemia and disbalance in cellular cholesterol influx and efflux lead to accumulation and oxidation of low-density lipoprotein (LDL) and LDL-like particles, which subsequently activates the endothelial cells (ECs) at atherosclerotic lesion-prone sites. Activated ECs then up-regulate cell adhesion molecules and chemokines and mediate the recruitment of circulating monocytes [2]. Being in the arterial lumen, monocytes and resident macrophages take up the oxidized particles, then transform these cells into foam cells and form early plaques. Foam cells further secrete pro-inflammatory cytokines (e.g. interleukins and tumor necrosis factor-α (TNF-α)) and chemokines (e.g. monocyte chemoattractant protein-1(MCP-1)). This amplifies the local inflammatory response [3]. Despite efforts to lower the levels of proatherogenic stimuli, the immune cells such as monocytes and macrophages remain in the pro-inflammatory state within the plaque environment [4].

Recent studies have suggested that pro-atherogenic stimuli, such as LDL cholesterol and oxidized low-density lipoprotein (Ox-LDL) can induce a long-term epigenetic reprogramming of cells of the innate immune system. This triggers their continuous activation even after the removal of the pro-atherogenic stimuli [5,6]. This concept which has been termed “trained immunity” was first introduced by Netea et al. and suggests that innate immune cells such as monocytes and natural killer (NK) cells can gain a nonspecific memory via epigenetic modulation and alter their response toward the subsequent stimuli [6].

In this review, we focus on potential epigenetic compounds that could be utilized to prevent or treat atherosclerosis based on the epigenetic concept.

## 2. Role of epigenetic modulation in cardiovascular disease

Traditionally, epigenetics is described as any inheritable chromatin modification, controlling gene expression by changing DNA accessibility or chromatin structure [7]. These modifications include DNA methylation and posttranslational modifications of histone proteins in

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https://doi.org/10.1016/j.atherosclerosis.2018.10.006
Received 27 June 2018; Received in revised form 18 September 2018; Accepted 4 October 2018
Available online 08 October 2018
0021-9150/ 

the nucleus. Despite the heritable nature of the epigenetic modifications, these alterations can be reversed through the use of pharmaceutical agents. In the following section, we briefly explain the contribution of these modifications to the initiation and progression of atherosclerosis. The recent advances in the development of epigenetic inhibitors and their potential application in the cardiovascular area will also be discussed. An overview of the subsequently described inhibitors and their mechanism of action can be found in Table 1 and Fig. 1.

### 2.1. DNA methylation

DNA methylation was the first described epigenetic modification. This process occurs by the addition of a methyl group to the C5 position of cytosine at CpG dinucleotides and is regulated by DNA methyltransferases (DNMTs).

These modifications at regulatory regions of genes result in heterochromatin formation which subsequently suppresses gene expression [8]. Dysfunctional epigenetic enzymes and differential DNA methylation patterns are hallmarks of many abnormalities, including CVD [9]. In atherosclerotic conditions, both states of DNA hypomethylation and hypermethylation have been described. In the study by Stenvinkel et al., patients at various stages of chronic kidney disease (CKD), a population with a high risk for CVD, were screened for DNA methylation patterns. Peripheral blood leukocytes from these patients showed global DNA hypermethylation, which was associated with increased inflammation [9]. Whole-genome shotgun bisulfite sequencing of atherosclerotic areas of the aorta also revealed hypermethylation across a wide array of genomic loci, particularly at genes involved in endothelial and smooth muscle cell functions [10].

#### 2.1.1. DNA methyltransferase inhibitors in clinic

DNA methyltransferase inhibitors (DNMTi) are promising therapeutics for cancer therapy. They are divided into two categories: nucleoside analogs inhibitors and non-nucleoside analogs inhibitors. Nucleoside analog inhibitors are cytosine analogs. They incorporate into newly synthesized DNA during the S phase of the cell cycle, and sequester DNMTs by mediating their proteasomal degradation [11]. 5-azacytosine (azacytidine) and 5-Aza-2′-deoxycytidine (decitabine) are two prototypes of DNMTis with FDA approval for treatment of hematologic malignancies [12], which are now gaining more attention for treatment of solid tumors (NCT00748553, NCT00005639, NCT00529022, NCT00075634). Interestingly, they were also found to have beneficial effect in experimental CVD and inflammatory disorders [13–15].

In the study by Coa et al., it has been shown that treatment of *Ldlr*$^{-/-}$ mice with decitabine significantly decreased atherosclerosis by suppression of macrophage migration and adhesion to ECs. Reduced macrophage infiltration into atherosclerotic plaques also was observed [16]. These macrophages showed reduced expression of inflammatory genes including *Tnf-α*, Interleukin 6 *(Il-6),* Interleukin 1 beta *(Il*-1b) and also chemokines such as Mcp-1, Chemokine (C−C motif) ligand 5 (Ccl5), Ccl9, and C−C chemokine receptor type 2 (Ccr2). Furthermore, it also suppressed macrophage endoplasmic reticulum (ER) stress, which contributes to macrophage activation and induction of apoptosis [16].

In another study by Zhuang et al., decitabine treatment significantly attenuated atherosclerotic lesions (20.1 ± 2.2% *versus* 30.8 ± 7.5%; $p = 0.016$) in *Apoe*$^{-/-}$ mice and promoted a global reduction in 5-methylcytosine content at the site of lesions [17]. Genome-wide epigenetic and mRNA expression screens led to the identification of 344 regions in the genome with changed DNA methylation state upon replicative aging of smooth muscle cells (SMCs). *COL15A1* is a gene with atheroprotective property located in these regions. *In vivo* data from *Apoe*$^{-/-}$ mice fed a Western diet showed increased *Col15A1* expression in atherosclerotic lesions. Genotyping of 88 human aorta showed that a polymorphism in this gene, rs4142986, is associated with atherosclerosis. This SNP falls within a CpG site and therefore has the potential to be methylated. This methylation led to decreased expression of *COL15A1,* which enhanced the chance of disease. Treatment of the cells with decitabine reversed the methylation effect and increased transcript as well as protein level of COL15A1 in SMC [18]. A similar effect was also observed on the expression of monocarboxylate transporter3 (*MCT3*) gene. MCTs are involved in the transport of lactate, pyruvate, and ketone bodies across the plasma membrane of SMC [19]. Methylation of a CpG island located in exon 2 of *MCT3* led to its downregulation which was associated with impaired lactate transport in SMCs. More importantly, suppression of *MCT3* using siRNA resulted in increased proliferation of SMCs, which provides a link between MCT3 expression and atherosclerosis. In agreement with these findings, mRNA an protein analysis of 23 aorta with varying degree of atherosclerosis showed downregulation of MCT3. Interestingly, decitabine treatment restored MCT3 expression at both mRNA and protein level and normalized lactate transport [20]. However, it is still unclear whether restored function of MCT3 using decitabine can prevent atherosclerosis development *in vivo*.

Despite these observations, both azacytidine and decitabine have low specificity and stability. This led to the development of their prodrugs with an increased bioavailability, solubility and stability over the parental compound [21]. Among them, dinucleotide decitabine-p-deoxyguanosine (Guadecitabine) and 4′-Thio-2′-deoxycytidine (TdCyd) are currently being evaluated in clinical trials for the treatment of hematological malignancies and advanced solid tumors (NCT01261312, NCT02901899, NCT01752933, NCT02423057) [22,23]. To our knowledge, there is no data available on their use in atherosclerosis or cardiovascular disease.

Non-nucleoside analogs were developed to overcome the non-specificity and cytotoxicity of the nucleoside inhibitors. They show high affinity for CpG-rich regions of DNA, where they can inhibit the effect of DNMTs and reactivate the hypermethylated, silenced genes. The potential of some of these compounds, such as RG108 and hydralazine has been tested in reversing hypermethylation.

RG108 is a non-nucleoside inhibitor which directly binds to the active site of DNMT1 and suppresses its activity. Considering the study by Yu et al. [24] on the role of DNMT1 in atherosclerosis progression, inhibition of this enzyme by RG108 can be considered as a potential strategy to prevent or treat cardiovascular disease [24]. RG108 has been shown to effectively block DNMTs without covalent enzyme trapping in the human cell lines. Additionally, in contrast to decitabine, the activity of RG108 is associated with a lower level of toxicity [25,26]. Beside inhibiting DNMT1, RG108 also showed an inhibitory effect toward DNMT3A, another DNA methyltransferase which has been shown to be associated with coronary heart disease [27]. Using porcine aorta as well as cultured human aortic endothelial cells, Jiang et al. [29] demonstrated that reduced shear stress increased the expression of DNMT3A. This leads to DNA methylation of CpG islands within the kruppel-like factor 4(KLF4) promoter and subsequent suppression of *KLF4* transcription that can directly impact atherosclerosis susceptibility. This effect can be reversed by the DNMT inhibitors, azacytidine and RG108 [28,29].

Hydralazine, another non-nucleoside DNMT inhibitor, has been approved by the FDA as an anti-hypertensive drug. Interestingly, angiotensin II (Ang II) - induced hypertension in *Apoe*$^{-/-}$ mice and was found to accelerate the initiation and progression of atherosclerosis [30,31]. Using the C57/BL6 mouse model of angiotensin II infusion, Qi et al. [32] reported the anti-inflammatory property of hydralazine. Administration of hydralazine in drinking water of these mice (250 mg/l per day) suppressed angiotensin II (Ang II)-induced fibrosis, reduced Mac-2(+) inflammatory cell infiltrationand proinflammatory cytokine expression, such as Il-1b and Il-6 [32]. Besides its antihypertensive effect, hydralazine also received renewed recognition for its capacity to inhibit DNA methylation and reactivate several tumor suppressor genes [33,34]. Considering both anti-hypertensive and demethylating activity

**Table 1**
Overview of the epigenetic inhibitors and their clinical applications.

| Class | | Compound | Condition and disease | Clinical trial phase |
|---|---|---|---|---|
| DNA methyltransferase (DNMT) inhibitors | Nucleoside analogs | Azacytidine | AML[f,] MDS[f],CMML[f], solid tumors[I] | FDA approved, Phase I |
| | | Decitabine | AML[f], MDS[f],CMML[f], solid tumors[I] | FDA approved, Phase I |
| | | Guadecitabine (SGI-110) | MDS[f], AML[f] | FDA approved |
| | | Thio-2′-deoxycytidine (TdCyd) | Solid tumors[I] | Phase I |
| | Non-nucleoside analogs | Hydralazine | Hypertension[f], lung tumor[I,] polycystic kidney diseases[I], chronic hemodialysis [IV] | FDA approved<br>Phase I/IV |
| | | MG98 | Solid tumor[I] | Phase I |
| | Small molecule inhibitors | C646 | Prostate cancer cell line, human lung (carcinoma) cell line, murine macrophages RAW264.7 cell line | Preclinical (*in vitro*) |
| Histone deacetylase inhibitors (HDACi) | | Vorinostat (SAHA) | CTCL[f,]<br>Neimann-Pick disease[I/II]<br>Soft tissue sarcoma [II] | FDA approved<br>Pahse I/II |
| | | Valproate | Toxoplasmosis [IV],schizophrenia [IV], glycogen storage disease type V[II], CLL[I/II], neuroectodermal tumor[I], brain metastases[I] | Phase I/II/IV |
| | | Romidepsin | CTCL[f], solid tumors[I], lymphoma[I] | FDA approved, Phase I |
| Histone methylation inhibitors | | EPZ-5676 | Leukemia[I]<br>Acute myeloid leukemia (AML)[I]<br>Acute lymphocytic leukemia(ALL)[I] | Phase I |
| | | GSK126 | Diffuse large B-cell lymphoma (DLBCL), mouse primary macrophages and microglia | Preclinical |
| Bromodomain and extra-terminal motif (BET) inhibitors | | JQ1 | Hematologic malignancies, lung cancer, breast cancer, prostate cancer, colon cancer, hepatocellular cancer | Preclinical |
| | | RVX208 | Diabetes [II], atherosclerosis [II], coronary artery disease [II], dyslipidemia[I/II], cardiovascular disease[I/II] | Phase I/II |
| | | MS436 | Not applicable | Compound discovery |
| | | I-BET151(GSK1210151A) | MLL, myeloma, AML, melanoma | Preclinical |
| | | I-BET762(GSK525762) | Solid tumor[I], hematologic malignancies[I] | Phase I |

F, FDA approved; I, PhaseI; II, Phase II; III, Phase III; IV, Phase IV, AMl, acute myeloid leukemia; MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; MCI, mild cognitive impairment; CTCL, cutaneous T cell lymphoma; CLL, chronic lymphocytic leukemia; MLL, mixed lineage leukemia.

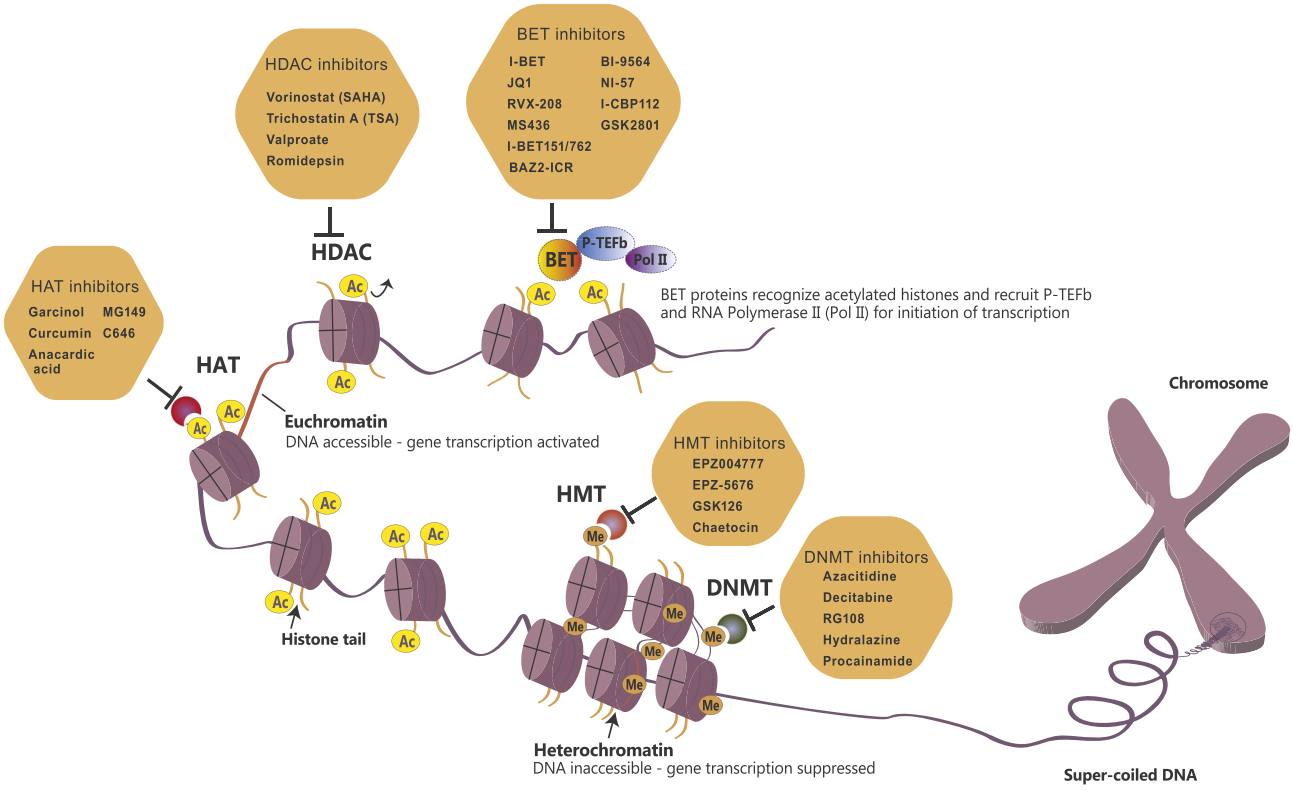


**Fig. 1.** Modification of histone proteins and DNA nucleotides by epigenetic modification enzymes and an overview of available epigenetic inhibitors.
The basic unit of chromatin is nucleosome, which contains eight histone proteins and about 146 base pairs of DNA. Post-translational modifications can regulate gene expression by changing chromatin structure. These modifications include DNA methylation (Me) and the methylation and acetylation (Ac) of histone tails. Addition of methyl groups at regulatory regions, such as promoters, by DNMT leads to heterochromatin formation, which represses transcription by preventing the binding of transcription complexes to the gene promoter. Post translational modification of histone proteins by HMTs, HDACs and HATs can also influence gene expression, depending on the site and number of modifications. Usually, addition of acetyl groups to histone tails associated with relaxed, open chromatin (euchromatin) promotes gene transcription while methylation can repress or promote gene expression. Acetyl groups can be recognized by BET reader proteins, which recruit additional transcription factors (P-TEFb) and RNA polymerase (RNA Pol II) to initiate the transcription process. Imbalance in the interplay between these factors can lead to aberrant epigenetic profiles, ultimately resulting in disease development, such as CVD and atherosclerosis. Interestingly, these modifications can be reversed by targeting epigenetic modifying enzymes using their specific inhibitors. This way, the expression of specific genes can be controlled. This figure illustrates several epigenetic compounds classified accordingly to their respective target.

of hydralazine, it might be a potential drug for treatment of atherosclerosis. More research on using this compound in the context of atherosclerosis may therefore be of interest.

Antisense oligonucleotides represent another example of non-nucleoside DNMT inhibitors. MG98 is a promising antisense inhibitor for human DNMT1 and several phase I/II clinical studies have investigated its effect on a variety of solid and hematopoietic cancers [16,35–37]. It reduces the cellular levels of DNMT1 by binding to *DNMT1* mRNA and preventing its further processing. Decreased DNMT1 enzyme reactivates several silenced tumor suppressor genes, including *p16*, in a variety of cancer cells lines. As mentioned earlier, DNMT1 also plays an important role in progression of atherosclerosis and targeting of DNMT1 could be utilized for treatment of atherosclerosis.

Beside synthetic DNMT inhibitors, natural DNA methylation inhibitors are also available in food. These inhibitors have been extensively reviewed elsewhere [38].

### 2.2. Histone modifications

Histone modifications are the key epigenetic marks that play a crucial role in the regulation of gene expression in many human diseases [39]. Beside involvement in disease progression, these modifications are also important in the early development. Histone modifications include methylation, acetylation, phosphorylation, ubiquitination, sumoylation and glycosylation which are collectively referred to as histone post-translational modifications (PTMs) [40]. Among these modifications, Histone acetylation and methylation are the most studied modifications in the context of inflammation and cardiovascular disease [41].

#### 2.2.1. Histone acetylation and deacetylation

Histone acetylation occurs by the addition of positively charged acetyl groups to amino acid residues on the histones, which neutralizes the negative charges of DNA. This modification reduces the affinity of the histone for DNA and consequently results in the formation of a more relaxed chromatin structure, increasing chromatin accessibility [42].

Comparable to DNA methylation, various acetylation patterns have been described at the promoter region of the genes involved in inflammation and atherogenesis [43]. Histone acetyltransferases (HATs) and Histone deacetylases (HDACs) are the two main enzymes that play a crucial role in determining histone acetylation state. *Ldlr*$^{-/-}$ mice lacking *Hdac9* (*Ldlr*$^{-/-}$*Hdac9*$^{-/-}$) showed reduced atherosclerotic lesions in both genders. This effect was accompanied by a down-regulation of inflammatory genes such as *Il*-1b and *Mcp-1*. More interestingly, these mice had increased *Abca1* and *Abcg1* mRNA expression, which resulted in increased cholesterol efflux [44]. Using conditional knockout mice, Hoeksema et al. [45] observed a more stable plaque phenotype in atherosclerotic lesions upon *Hdac3* deficiency. This beneficial effect was linked to epigenetic modulations at the transforming growth factor beta (*Tgf-b1*) locus, which drives vascular smooth muscle cells (VSMCs) to produce more collagen. This leads to a more stable plaque phenotype characterized by decreased lipid accumulation and a

switch in macrophages toward an anti-inflammatory phenotype [45]. A differential acetylation pattern was also observed in VSMCs in coronary atherosclerotic lesions, which was further accentuated by thrombin, an activator and proliferation inducer of VSMCs [46].

#### 2.2.2. Histone acetyl transferase inhibitor

Histone acetyltransferase inhibitors (HATi) are a class of compounds that interfere with the function of HATs. Several HAT inhibitors have been discovered with specificity toward HATs, known to be crucial in induction of inflammation and progression of atherosclerosis.

Garcinol, a polyisoprenylated benozophenone derived from the Garcinia indica fruit is a natural HAT inhibitor with specificity toward p300 HAT. It was used to investigate the role of histone acetylation in regulation of early growth response protein 1 (EGR1) gene. EGR1 has been implicated in several processes that are important for atherosclerosis progression including thrombus formation and endothelial cell migration [47,48]. Stimulation of SMCs with IL1-b resulted in *EGR-1* transcription through acetylation of Histone3 at the promoter of *EGR-1* which could be prevented by garcinol [49].

Anacardic acid (6-nonadecyl salicylic acid) is another HAT inhibitor with potency toward p300 and cellular p300/CBP associated factor (PCAF) [51]. Recently MG149, a novel analog of anacardic acid, has been developed as a potent and selective inhibitor of the MYST family (Tip60 or KAT5 and MOZ) of HATs [50]. Expression of several HATs including MYST1 was shown to correlate with the severity of atherosclerosis [51]. In addition DNA microarrays demonstrated that MG149 has the potency to inhibit the NF-κB pathway [50]. Acetylations of the NF-κB transcription factor as well as the histones play a crucial role in activation of this pathway [52]. Since the NFκB pathway is involved in the expression of various pro-inflammatory cytokines and plays a pivotal role in inflammatory diseases including atherosclerosis, MG149 could be an interesting candidate for further investigation.

High throughput screening enables the identification of some small molecule HAT inhibitors such as C646 which is the first potent, selective and cell-permeable inhibitor of p300[53]. One study on prostate cancer cell lines has shown that C646 treatment suppressed androgen receptor function and decreased the NF-κB subunit, p65. This in turn suppressed the expression of several NF-κB target genes. More interestingly, matrix metallopeptidase 2 (MMP2) and MMP9, two proteases suggested to be involved in destabilization of atherosclerotic lesions, showed significant reduction following p300 inhibition [54,55]. In a study by van den Bosch et al. [56], C646 treatment suppressed *Tnf-α* and Interleukin 12b (*Il*-12b) gene expression in RAW264.7 murine macrophages upon lipopolysaccharide (LPS) and interferon gamma (IFNγ) treatment [56]. Using an NF-κB reporter gene assay, they also showed the inhibitory effect of C646 on NF-κB activity. These data suggest that C646 can be a potential anti-inflammatory candidate for regulation of gene expression via the NF-κB pathway, which makes it a potential novel therapeutic compound [53].

#### 2.2.3. Histone deacetylase inhibitors

Histone deacetylase inhibitors (HDACi) can be structurally grouped into at least four groups: hydroxamates, cyclic peptides, aliphatic acids and benzamides [57].

To date, several HDAC inhibitors have been approved for cancer therapy by the FDA. Vorinostat, also known as SAHA, a compound derived from the hydroxamic acid class, was approved in October 2006 for treatment of relapsed and refractory cutaneous T cell lymphoma (CTCL). This compound inhibits HDACs and cell proliferation in nanomolar concentrations [58–60]. Besides its anti-tumor activity, it also possesses immunoregulatory properties at low non-cytotoxic concentrations and was shown to reduce graft versus host disease in mice [61,62]. In the study by Leoni et al. [63] it has been shown that a single oral administration of Vorinostat in mice reduced the circulation of TNF-α, IL-1b, IL-6, and IFN-γ which was induced by LPS in a dose dependent manner [63].

Trichostatin A (TSA) is another HDAC inhibitor with an inhibitory effect toward HDAC I, IIA and IIB. However, TSA was shown to have adverse effects on atherosclerosis development. Treatment of $Ldlr^{-/-}$ mice with TSA, increased aortic root lesions and also increased macrophage scavenger receptor CD36 at both mRNA and protein level, which consequently enhanced the uptake of oxLDL [64]. It also resulted in 2.2 times more neointima/media ratio in a murine model of post-angioplastic restenosis. This was accompanied by enhanced platelet-derived growth factor (PDGF)-induced proliferation and migration of VSMCs [65]. Increasing the specificity of TSA may certainly help to improve its efficacy in arteriosclerosis.

Valproate is a class I selective HDAC inhibitor [66]. *In vivo* data showed that supplementation of a low level of valproate in hyperglycemic $Apoe^{-/-}$ mice diet, significantly attenuated atherogenesis in these mice. It also diminished pro-atherogenic ER stress signaling pathways *in vitro* [67].

Romidepsin is another structurally unique and selective inhibitor against HDAC1, HDAC2, HDAC3 and HDAC8 [68]. It received FDA approval in November 2009 for treatment of CTCL in patients who have received at least one prior systemic therapy [69]. It was also used in clinical trials for patients with solid tumors and lymphomas [70,71]. HDAC1/2 and 3 play important role in several cellular functions that are known to be crucial for athersoclrosis development. HDAC2, termed canonical HDAC, increases the histone deacetylation of *SM22α* (markers of differentiated SMCs). This results in SMC phenotype switch from the “contractile” phenotype to a “synthetic” phenotype and consequently progression of atherosclerosis [72]. It also regulates the deacetylation of several transcription factors including krüppel-like factor 5 (Klf5) and CREB binding protein (CBP) which play pivotal role in SMCs proliferation and acceleration of atherosclerosis development [73]. In addition, several roles for HDAC 1/2 have been described in inflammatory diseases [74,75]. Similar to HDAC1/2, a pro-inflammatory role was also described for HDAC3 [76]. Moreover, HDAC3 has been reported to be an important player in monocyte recruitment to sites of inflammation, and in macrophage cytokine production [77]. These data suggest that targeting these HDACs can help to improve athersosrotic condition. Furthermore, in a mouse model of autoantibody induced arthritis model, Romidepsin treatment was shown to reduce joint swelling and synovial inflammation. It also significantly reduced TNF-α and IL-1b level in synovial tissues via induction of p16 and p21 [78]. This also highlights the anti-inflammatory property of this drug.

#### 2.2.4. Histone methylation

Histone methylation is defined as the transfer of methyl group from S-adenosyl-L-methionine to lysine or arginine residues of histone proteins by histone methyltransferases (HMTs) [79]. While lysines may be mono-, di- or tri-methylated, arginines may be monomethylated or dimethylated (symmetrically or asymmetrically) [79]. Histone methylation is generally associated with the transcriptional repression. However, methylation of some lysine and arginine residues of histones results in transcriptional activation. Lysine methylation of H3 and H4 is implicated in both transcriptional activation and repression depending on the methylation site. However, arginine methylation promotes transcriptional activation [80]. Recent molecular biology studies have demonstrated that the function of histone methylation is more complex than previously thought.

Global evaluation of H3K27me3 levels in vessels showed an overall reduction of this modification in advanced atherosclerotic plaques. However, this effect was not accompanied by alterations in overall levels of the HMTs such as enhancer of zeste homolog 2 (EZH2) or the histone demethylase, JMJD3 [81]. Similarly, analysis of histone methylation/acetylation as well as DNA methylation, in arteries from atherosclerotic patients, revealed a global decrease in H3K9 and H3K27 dimethylation whereas H3K4 dimethylation was unchanged. Beside modifications at histone level, a reduction in DNA methylation was also

observed in both early and advanced atherosclerotic plaques. In accordance with these findings, DNMT1 level was decreased, while the expression of DNA-demethylase, ten-eleven translocation methylcytosine dioxygenase 1 (TET1) increased [82]. Histological analysis on carotid tissue samples from 80 patients with atherosclerosis also showed significant reduction in methylation of H3K9 and H3K27 in atherosclerotic plaques in SMCs and inflammatory cells. In contrast to H3K9 and H3K27, the methylation of H3K4 was significantly increased in advanced stages of atherosclerosis in SMCs. This was accompanied by an increased expression of methyltransferase MLL2/4. These differential modifications might be cell specific and each might have different effect on cellular activation. For example, while methylation of H3K9 and H3K27 may be important in macrophage activation; however, these modifications might have different effect in SMCs [51].

*In vitro* data also showed that atherogenic risk factors, such as oxLDL, can promote enrichment of H3K4me3 at the promoter of inflammatory genes such as *TNF- α, IL6, IL-18, MCP-1, MMP2* and *MMP9* in human monocytes. These modifications are characterized by increased proinflammatory cytokine production, which gives the monocytes a proatherogenic phenotype [5].

#### 2.2.5. Histone methyltransferase inhibitors

In contrast to other epigenetic inhibitors, histone methylation inhibitors (HTMi) have not been as extensively researched and remain an untapped source for pharmacological interventions. EPZ004777, introduced in 2011, was the first potent and selective methyltransferase inhibitor, which is able to selectively kill MLL-rearranged leukemia cells in culture, while having little effect on non-MLL translocated cells [83,84]. However, because of poor pharmacokinetic characteristics, this compound didn't enter clinical studies. EPZ-5676 is a novel derivative of EPZ004777 with improved pharmacokinetic properties. It is an inhibitor of disruptor of telomeric silencing 1-like (DOT1L), histone H3K79 methyltransferase, with sub-nanomolar affinity [85]. It showed complete tumor regressions in a nude rat subcutaneous xenograft model of MLL-rearranged leukemia [86]. However, not much known about the anti-inflammatory and anti-atherogenic property of this compound.

GSK126 is another potent methyltransferase inhibitor which is highly selective for the histone methyltransferase EZH2, with a half inhibitory concentration (IC50) of 9.9 nM. It showed more than 1000-fold selectivity for EZH2 over other human methyltransferases [87]. EZH2 has been shown to play an important role in atherosclerosis [88,89]. For example, it induced lipid accumulation in both THP-1 and RAW264.7-derived macrophages, upon treatment with ox-LDL, as well as macrophage activation and inflammation [90]. Moreover, overexpression of EZH2 increased atherosclerotic lesions in *Apoe*$^{-/-}$ mice. The authors concluded that EZH2 recruited DNMT1 to the promoter of *Abca1* gene, which in turn increased methylation of CpG dinucleotides at Abca1 promoter and consequently suppressed its transcription [89]. Additionally, EZH2 has been shown to play a crucial role in macrophages/microglial activation. It induced MyD88-dependent TLR ligands-induced proinflammatory gene expression such as *IL-6, IL*-12b, *TNF-α*, C−C motif chemokine ligand 2 *(CCL2)* and C-X-C motif chemokine 10 *(CXCL10)*. EZH2 ChIP-seq analysis also resulted in identification of Socs3, an anti-inflammatory gene, whose expression is fine-tuned by EZH2 mediated H3K27me3. EZH2 deficiency or inhibition of H3K27ac, using GSK126, severely attenuated the expression of proinflammatory genes at both mRNA and protein levels [91]. These data make GSK126 a potential drug candidate for atherosclerosis treatment.

Chaetocin is a promising epigenetic inhibitor for H3K9 methyltransferase, SUV39H, which is associated with pathogenesis of myocardial infarction. Yang et al. [92] showed that exposure of neonatal rat ventricular myocytes (NRVM) to ischemic and oxidative stress upregulated SUV39H, accompanied by downregulation of sirtuin 1(SIRT1), a lysine deacetylase with cardioprotective property. Furthermore, inhibition of SUV39H with intraperitoneal administration of chaetocin in C57/BL6 mice improved their survival and reduced infarct size. Chaetocin treatment also decreased expression of *MMP9*, which is known to play an important role in plaque destabilization [54,92]. Additional investigations are warranted for the use of this compound in CVD.

#### 2.2.6. Bromodomain and extra-terminal motif

The reader proteins are involved in the interplay between writing and erasing of histone marks. They bind to specific chromatin modifications and also associate with chromatin modifying proteins, such as HATs [93]. One group of reader proteins are called BET proteins, Bromodomain and ExtraTerminal domain-containing proteins (a family of BRD2, BRD3, BRD4 and BRDt) [94]. Bromodomains are structural motifs binding to acetylated lysines. BETs bind to specific acetylated lysines on histone tails, and thereby facilitate the assembly of the transcriptional machinery. BETs can interact both with promoter regions as well as enhancer regions [95]. Differential recruitment of BET proteins has been studied predominantly in cancer. Moreover, there is compelling evidence confirming the association of BET proteins and expression of inflammatory genes through translation of histone marks [17–19]. Since chronic inflammation is one of the pivotal factors aggravating atherosclerosis and CVD, BET proteins are potential targets for treatment and prevention of several diseases including atherosclerosis.

#### 2.2.7. Bromodomain and extra-terminal domain inhibitors

Over the last 6 years, several novel inhibitors of BETproteins have been developed as novel approaches for epigenetic anticancer therapy. These inhibitors may not only provide novel therapeutic approaches for cancer treatment but also for other types of diseases. I-BET, a benzodiazepine derivative, was the first type of BET inhibitor introduced in 2010 with anti-inflammatory potency [96]. After that JQ1 was introduced, which displaces the BRD4 domain of BET proteins from chromatin and consequently stops cell cycle progression. It induces apoptosis both in cancer cell line and patient-derived xenograft models [97]. In several studies, JQ1 showed potent anti-inflammatory properties. The study by Brown et al. [98] demonstrated that treatment of ECs with TNF-α resulted in co-localization of p65 and BRD4 to the enhancer and promoter regions marked by H3K27ac, and co-treatment of ECs with JQ1 reduced TNF-α-induced enrichment of BRD4 at NF-κB binding sites. It significantly attenuated endothelial activation during acute inflammation *in vitro* and *ex vivo* and also supresses TNF-α induced leukocyte rolling, adhesion and transmigration. JQ1 administration (50 mg/kg) reduced aortic plaque area by 40% in cholesterol fed *Ldlr*$^{-/-}$ mice [98]. It also suppressed p65 recruitment to the *IL-6* and *IL-8* promoters in human pulmonary microvascular endothelial cells (HPMEC) which inhibited their expression at both the mRNA and protein level [99].

Nonetheless, the drug's half-life is only 1 h, thus limiting its clinical application. The use of this epigenetic inhibitor in cardiovascular diseases warrants further investigation.

RVX-208 is a more selective BET inhibitor and is currently under investigation in phase II clinical trials for cardiovascular diseases. The phase III clinical trial (BETonMACE) for patients with type 2 diabetes mellitus and low high-density lipoprotein cholesterol (HDL-C) has also been initiated. In the study by Gilham et al. [100] it has been shown that RVX-208 raises *APOA-I* gene transcription and protein level in human and primate primary hepatocytes. Furthermore, it repressed pro-inflammatory and pro-atherosclerotic pathways involved in CVD development [100,101]. *In vivo* data on *Apoe*$^{-/-}$ mice also showed that oral administration of RVX-208 (150 mg/kg) significantly reduced aortic lesion formation. It also reduced the pro-inflammatory cytokine production such as interferon gamma inducible protein 10 (Ip10) and macrophage inflammatory protein 1(Mip1). Moreover, it increased the levels of circulating HDL-C and decreased LDL-C by 50% [102]. These data agree with the outcomes from two clinical trials: SUSTAIN and ASSURE (NCT01423188, NCT01067820) [103]. Following that, a

pooled analysis was conducted with an aim to measure clinical outcomes on lipid parameters and coronary atherosclerosis after 3–6 month administration of RVX-208 in 798 CAD patients included in SUSTAIN and ASSURE studies. This analysis revealed that RVX-208 treatment increased APOA-I level (6.7%, $p < 0.001$), HDL-C (6.5%, $p < 0.001$) and HDL particle numbers (23%, $p < 0.001$). Moreover, treated patients experienced less major adverse cardiovascular events compared to placebo treated patients (5.9% *vs.* 10.4%, $p = 0.02$), after 26 week follow-up [104]. In addition, safety profile showed a relatively well-tolerated treatment.

MS436 is another BET inhibitor with more specificity toward BRD2 and BRD4. *In vitro* data, using murine macrophage RAW264.7 cells treated with MS436, showed suppression of NF-κB mediated nitric oxide production upon LPS stimulation in a dose-dependent manner. This suggests the anti-inflammatory property of this compound.

Recently, more derivatives of BET inhibitors such as I-BET151/762, BAZ2-ICR, BI-9564, NI-57, I-CBP112 and GSK2801 have been developed. They are still in the initial phases of pre-clinical testing and require further research for their application in the clinic [105].

## 3. Conclusions and future perspectives

Common epigenetic modifications such as DNA methylation and histone post-translational modifications are in the spotlight of epigenetic therapeutics and are show promise for the treatment of many diseases including cancer, diabetes, neurological disorders and autoimmune diseases [22,23]. Despite our significant progress in making systems that model atherosclerosis, much is left to do to accurately test all these potential epigenetic inhibitors for atherosclerosis treatment. Moreover, we also need a better understanding of epigenetic pathways and their regulation at different stages of plaques progression. Understanding this concept in combination with developing biomarker profiling of patients with high and low risk plaques can lead to considerable health benefits.

In addition, improvement in the pharmacology of the drugs themselves is also required. The specificity of epigenetic therapies is an important issue. Non-specific effects of compound may result in activation or suppression of a set of genes which adverse the treatment effect. Considering the fact that histone-modifying enzymes are highly homologous to their family members and share similar catalytic core pockets, it has been a difficult task to design selective inhibitors against them. Recently some success has been achieved in increasing the specificity of these compounds such as BET inhibitors. However, much is left to do in these areas. Among epigenetic compounds some have special properties that makes them more interesting for further improvement such as nucleoside DNA methyltransferases. They have been used as novel cancer therapeutic strategies. They are incorporated into DNA during replication. Since proliferation of VSMC are vital to the pathogenesis of atherosclerosis and plaque rupture, inhibiting the proliferation of these cells using nucleoside DNMTi can reduce atherosclerosis progression. On the other hand, delivery of these compounds to the site of leision remains an issue which can be solved by the use of nanoparticles. Building on the foundation of currently available knowledge will help us to take full advantage of the incredible therapeutic capacity of epigenetic drugs.

## Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

## Author contributions

All author participated actively in writing the manuscript, editing, and approving the final, submitted version.

## Acknowledgements

Funding was provided by European Union's Horizon 2020 research and innovation program under grant agreement No 667837 (REPROGRAM).

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