Effect of Doxycycline on Atherosclerosis: From Bench to Bedside

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Abstract: Matrix metalloproteinases (MMPs) have a pivotal role in the natural history of atherosclerosis and its cardiovascular consequences. Non-selective MMP inhibition with doxycycline appears as a potential strategy to reduce the residual risk observed in patients already at intensive lipid lowering strategies. However, specific MMPs have different and even contradicting roles in the natural history of atherosclerosis, rendering broad spectrum MMP inhibition an important yet somewhat simplistic approach towards residual risk reduction in coronary atherosclerosis. Overall, the balance of non-selective MMP inhibition might shift to the favorable side in particular settings such as in acute coronary syndromes, where in addition to its potential plaque stabilization properties, doxycycline shows promise in preventing ischemia-reperfusion injury and left ventricular remodeling. Nevertheless, to date, most animal models used do not represent advanced coronary atherosclerosis seen in humans, and large and well-designed clinical studies are lacking. We discuss the available evidence and recent patents supporting the role of doxycycline in atherosclerosis.

Keywords: Inflammation, coronary, metalloproteinase, remodeling, atherothrombosis, MMP inhibitor, apo E deficient mice.

INTRODUCTION

Cardiovascular disease is the main cause of morbidity and mortality in the Western Hemisphere, accounts for > 500,000 deaths each year in the US alone, and doubles the mortality attributed to cancer [1]. Histopathological studies have established that atherosclerotic plaque composition as well as coronary artery remodeling patterns have a pivotal role in the etiology of acute coronary thrombosis, independently of the underlying stenosis [2].

Several systemic strategies have demonstrated their effectiveness in primary and secondary prevention of coronary artery disease (CAD). Among them, statins have shown a consistent decline in low density lipoprotein cholesterol (LDL-C) levels between 25% and 35%, with a significant reduction in the relative risk of myocardial infarction (MI) and death ranging between 29% and 35% [3-5]. Nevertheless, in spite of significant improvement in prevention, diagnosis and treatment of cardiovascular disease, sudden cardiac death or unheralded acute coronary syndromes (ACS) remain common initial manifestations of coronary atherosclerosis [6, 7]. These events are mainly attributed to a significant residual risk observed in approximately 70% of patients under optimal anti-atherosclerotic therapies with statins, angiotensin-converting enzyme inhibitors and aspirin, among others. Although, implementation of aggressive lipid lowering therapies with target LDL-C levels < 70mg/dl in high risk patients have demonstrated an additional risk reduction of 16%, even in these cases a 22% residual risk is observed [8]. Furthermore, a recent pooled analysis of 7 serial intravascular ultrasound trials including 3437 patients with CAD demonstrated that despite achieving a LDL-C < 70mg/dl level, more than 20% of patients continued to show plaque progression, high-lighting the multifactorial nature of atherosclerosis and the need for improvement in primary and secondary prevention strategies, with the incorporation of alternative drugs that contribute to reduce the significant residual risk [9].

Matrix metalloproteinases (MMPs) are a family of endopeptidases that act as regulators of the extracellular matrix (ECM), playing an essential role in the evolution of inflammatory processes and hence, in the natural history of atherosclerosis [10]. We therefore review the available evidence about the effect of a broad-spectrum MMP inhibitor (MMPI), doxycycline, on atherosclerosis.

MMPs AS VALID BUT COMPLEX CLINICAL TARGETS

Human MMPs are a family of at least 23 endopeptidases (although previously considered to be more, 23 human MMPs are currently identified by the Universal Protein Resource, www.uniprot.org) involved in the remodeling of several components of the ECM [11-14]. They participate in almost every biological process involving ECM remodeling, such as angiogenesis, embryogenesis, tissue remodeling and wound healing [15]. Although it was originally believed that the role of MMPs was essentially to degrade the ECM, it is

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now well-known that the function of MMPs is far more complex [13]. In fact, it has been shown that MMPs can act on as many non-ECM substrates as ECM substrates [16]. MMP substrates include ECM molecules and regulators, chemokines, cytokines, growth factors, angiogenic factors, receptors, proteases, metabolic enzymes, and proteins involved in cell adhesion and motility [13]. Therefore, MMPs should be considered as cell-signaling regulators rather than as solely destructive proteases [17].

Under normal physiological conditions, MMP activity is tightly regulated at the transcriptional and post-translational levels, by zymogen activation and by endogenous inhibitors [15]. Tissue inhibitors of MMPs (TIMPs) are specific, potent and natural MMPIs that bind to these enzymes and block their activity. Disruption of this balance, which results in an overexpression of MMPs, has been associated with severe human pathologies including cardiovascular diseases, cancer, rheumatoid arthritis, neurological disorders and periodontitis. Thus, considerable efforts have been made to develop potent and selective MMPIs to treat these diseases [18-21]. However, after 30 years of extensive research, only doxycycline (Periostat®, CollaGenex Pharmaceuticals) has been clinically approved as a broad-spectrum MMPI for the treatment of periodontal disease [22, 23]. Previous clinical trials with synthetic MMPIs were disappointing because of severe side effects and poor survival rates [12, 24-27]. This failure has been mainly due to the complex biology of the MMPs, the use of broad-spectrum MMPIs, and limitations in the design of the clinical trials [26, 27].

The structural redundancy but functional diversification among the different subclasses of MMPs is the main challenge when developing selective MMPIs. Although significant overlap in the substrates that MMPs can cleave *in vitro* [11], the efficiency, as also the expression patterns and turnover in a given tissue, can vary [28, 29]. Therefore, for a given pathology, some MMPs might act as drug targets and others as anti-targets [12], underscoring the importance of MMPI specificity.

THE STRUCTURAL AND FUNCTIONAL COMPLEXITY OF MMPs

MMPs are zinc (Zn)-dependant enzymes comprised of shared structural modules [30]. The interaction between these domains is crucial for specific substrate binding and processing [30]. Based on domain organization and substrate specificity, MMPs can be clustered into several groups Fig. (**1**). [31]. The archetypical domain arrangement consists of a N-terminal propeptide, a catalytic MMP domain, a linker region (hinge) and a hemopexin-like C-terminal domain. The catalytic domain contains the characteristic Zn-binding sequence with three conserved histidine (His) residues, which serve as the Zn-ligands, and one glutamic acid (Glu), which facilitates catalysis, and is stabilized by a structural Zn and up to three calcium (Ca) ions. The catalytic domains of all MMPs are essentially superimposable containing a shallow active-site cleft that binds a peptide-substrate [32]. Substrate binding is dictated by the structure of this active site, including a pocket called the S_1' pocket, a main determining factor for substrate specificity [31].

All MMPs are either secreted or anchored to the plasma membrane. A hallmark of the MMP family is its regulation by zymogen (pro-MMP) activation. Most MMPs exist in an inactive or latent form inside the cells, in which the prodomain interacts with the catalytic domain, blocking the active site. The enzymes are activated when this interaction is relieved, upon removal of the propeptide or a conformational change [33], once they are secreted. Exceptions are MMP-11 (stromelysin-3), -21, -23, -28 (epilysin) and the six membrane-type (MT)-MMPs, which are activated by furin in the endosomal pathway [15]. The extracellular activation of most MMPs can be initiated by other already active MMPs or by several serine proteinases [11].

MMPs also share a common reaction mechanism in which the peptidic substrate is cleaved. During the proposed proteolytic mechanism Fig. (**2**). the carbonyl group of the scissile peptide bond of the substrate is directed towards the catalytic Zn and becomes polarized [18]. The Zn-bound water molecule is activated by the catalytic Glu properly oriented to attack the electrophilic carbonyl carbon. The resulting tetrahedral intermediate is presumably stabilized by the Zn ion. Additionally, one water proton is shuttled via the Glu carboxylate to the amino group of the scissile bond. After the simultaneous break of the peptide bond and the transfer of another proton to the amino group, the two product fragments leave the active site.

NATURAL HISTORY OF ATHEROSCLEROSIS

Since atherosclerosis is primarily an inflammatory disease, MMPs play a pivotal role in the development and natural history of atherosclerotic plaques [34].

In human atherosclerotic plaques, the intima comprises a hyaluronan-rich matrix with sparse vascular smooth muscle cells (VSMC) [35]. Basement membranes contain type IV collagen, laminin, and heparan sulfate proteoglycans such as perlecan [36] and syndecans [37]. The media comprises contractile VSMC surrounded by a basement membrane [38], few macrophages and fibroblasts [39]. The medial interstitial matrix contains types I and III collagen, elastin, and a number of glycoproteins, namely fibronectin, vitronectin, tenascin, and thrombospondin, along with chondroitin/dermatan sulfate proteoglycans, such as versican [40]. Finally, the adventitia comprises fibroblasts and *vasa vasorum* within a loose interstitial matrix [41].

In response to a vascular insult or biochemical stimuli, intimal thickening occurs mediated by a variety of cells along with accumulation of new ECM [42]. Provided that the stimuli remains, uptake of LDL which will subsequently transform into oxidized LDL is followed by infiltration by circulating monocytes, which convert to macrophages and incorporate oxidized LDL to become foam cells [43]. Later, apoptosis of macrophages and dumping of their lipid contents results in the formation of a fibrous cap overlying a large lipid core [44]. The fibrous cap is originated by migration of contractile VSMC from the media, which explains the medial thinning commonly observed in atherosclerotic plaques [45].

It has been established that coronary plaque rupture, resulting in ACS, is the cause of death in a large proportion

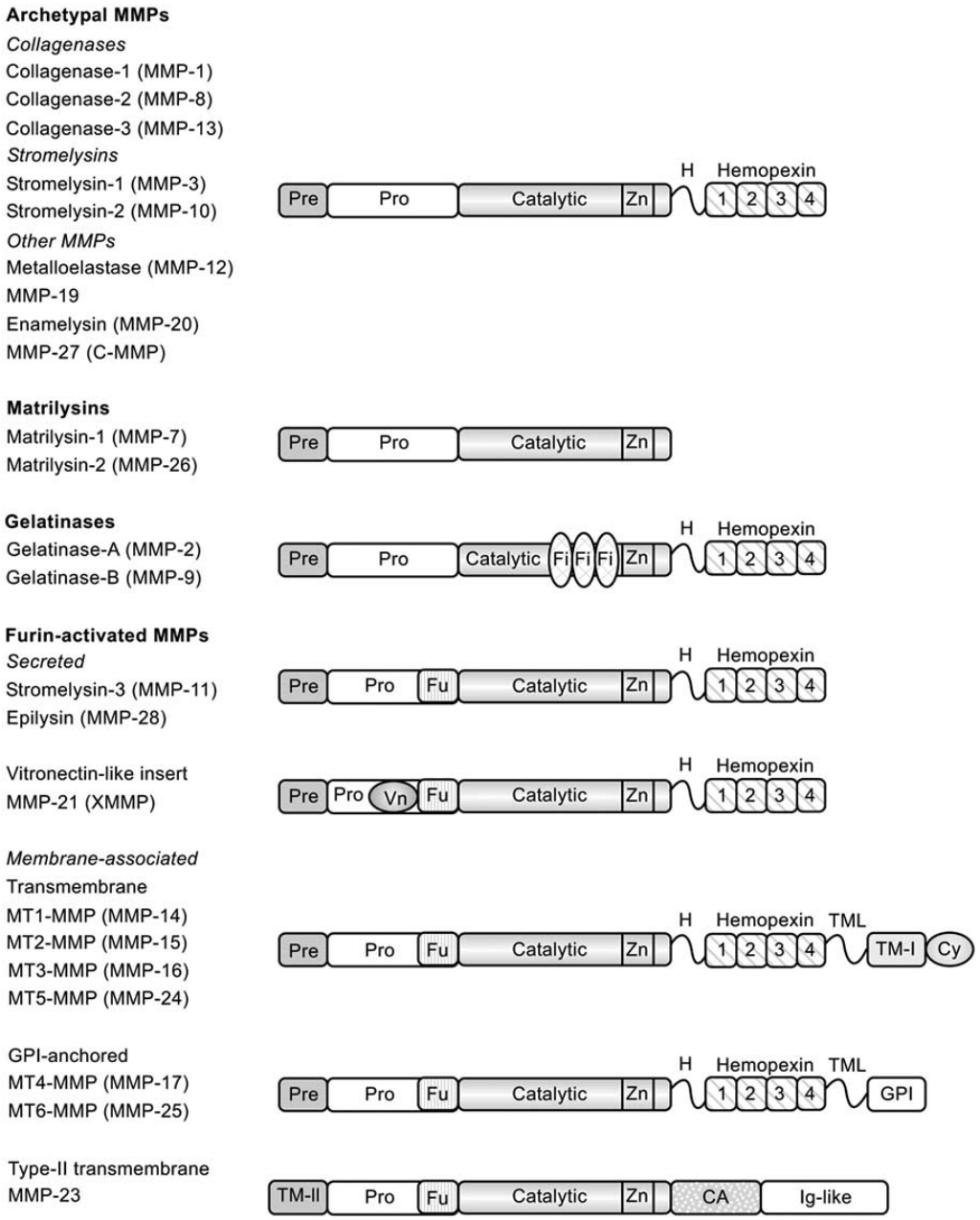


Fig. (1). Schematic representation of the structure of the 23 human MMPs. They are classified into four different groups on the basis of domain organization. Archetypal secreted MMPs contain a signal peptide (Pre), a propeptide (Pro), a catalytic domain that binds Zn, a linker (H), and a hemopexin C-terminal domain. Matrilysins contain the minimal domain organization that is required for function. Gelatinases incorporate three fibronectin (Fi) type II modules that improve collagen and gelatin degradation efficiency. Convertase-activatable MMPs contain a basic insert in the propeptide domain that is cleaved by furin-like proteases (Fu). This group includes the three secreted MMPs (MMP-11, MMP-21, MMP-28), the six membrane-type (MT)-MMPs and an unusual type-II transmembrane (TM) MMP (MMP-23). MMP21 contains a vitronectin-like (Vn) insert in the propeptide. MT-MMPs are inserted in the membrane by a type-I TM or GPI (glycosylphosphatidylinositol) anchor. Another linker (TML) connects these segments with the soluble archetypal core. MT-MMPs can also have a cytoplasmatic (Cy) tail. MMP-23 contains a unique cysteine array (CA) and immunoglobulin (Ig)-like domains in its C-terminal region.

of sudden death patients [46]. Despite its pre-conceived dire prognosis, retrospective studies have determined that plaque rupture is a common finding in both coronary and non-coronary sudden death patients [46, 47]. In addition, clinically silent plaque rupture has been identified as a cause of plaque progression [48, 49]. The fate of a given atherosclerotic plaque is linked not only to its severity but also to its histological composition, and the presence of a lipid-rich necrotic core has been consistently related to plaque fissuring [50, 51]. Plaque rupture typically occurs in regions of high mechanical stress and where collagen is depleted by matrix destruction, weakening the fibrous cap to the point where it can no longer resist the cyclical strain caused by the cardiac cycle [52, 53].

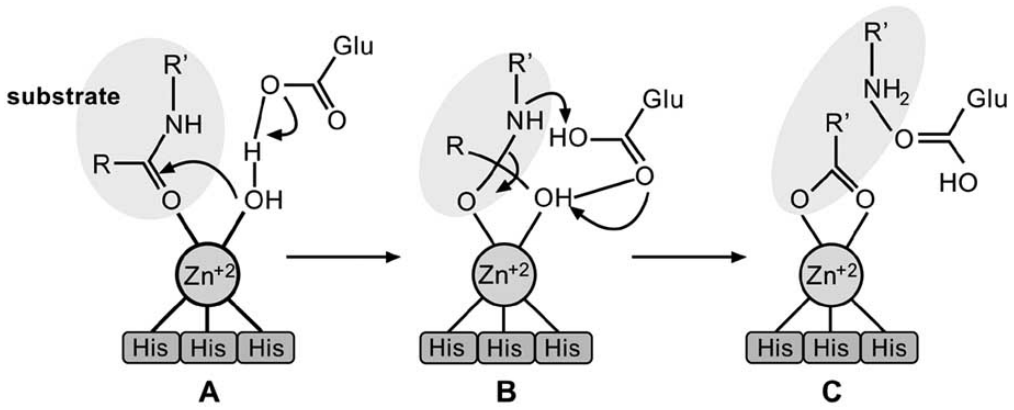


Fig. (2). Proposed reaction mechanism of peptide hydrolysis for MMPs. Within the MMP's catalytic domain, the catalytic Zn that is coordinated by three histidine (His) residues binds the carbonyl group of the scissile peptide bond of the substrate (**A**). The Zn-bound water molecule is activated by the catalytic glutamic acid (Glu), which is properly oriented to attack the electrophilic carbonyl carbon of the substrate, leading to a tetrahedral intermediate (**B**). Next, one water proton is shuttled via the Glu carboxylate to the amino group of the scissile bond. After the simultaneous break of the peptide bond and the transfer of another proton to the amino group (**C**), the two product fragments leave the active site.

ROLE OF MMPs IN INFLAMMATION, CORONARY REMODELING, AND PLAQUE INSTABILITY

MMPs activity is important for all the phases of an inflammatory response, including initiation, execution and resolution [25]. ECM remodeling by MMPs aids in the initial stages of the inflammatory response by allowing migration of leukocytes into the injured site [54-57]. At the same time, MMPs are also cell-signaling regulators [17] that modify cytokines, chemokines and receptors [58-64] and are able to release antiapoptotic or antiangiogenic factors from the ECM, which may help in the resolution of an inflammatory response [13].

The ECM provides the structural and functional platform of the arterial wall, so it is not surprising that alterations in its turnover, mediated by the activity of MMPs, play a key role throughout the natural history of coronary atherosclerosis, from plaque development and progression to fibrous cap disruption [65, 66]. In particular, MMP-1 (collagenase-1) [65-72], MMP-2 (gelatinase-A) [73, 74], MMP-3 (stromelysin-1) [67, 71, 75, 76], MMP-7 (matrilysin-1) [29], MMP-8 (collagenase-2) [77, 78], MMP-9 (gelatinase-B) [67, 71, 78, 79], MMP-10 (stromelysin-2) [80], MMP-11 [81], MMP-12 (metalloelastase) [29, 72, 82], MMP-13 (collagenase-3) [70, 72], MMP-14 (MT1-MMP) [83, 84], and MMP-16 (MT3-MMP) [85] levels are increased in human atherosclerotic plaques, especially at the macrophage-rich shoulder regions. Interestingly, plaque shoulders and regions of foam cell accumulation contain the highest levels of MMP-9 [67, 86].

As aforementioned, intimal thickening implies the generation of new tissue at least in part by means of hyperplasia and migration of VSMC derived from the media. Removal of the basement membrane and subsequent exposure of the interstitial matrix to the VSMC is promoted by MMPs. This seems to enable a shift from quiescent, contractile VSMC to cells able to migrate and proliferate and eventually mediate repair. The generation of new ECM that favors VSMC migration and proliferation is also promoted by specific MMPs.

Outward (positive) remodeling of coronary vessels has been initially regarded as beneficial by preventing lumen encroachment owing to plaque growth, and hence improving coronary flow [87]. Notwithstanding, several studies have subsequently shown increased levels of inflammatory markers, larger lipid cores and pronounced medial thinning in positive remodeled vessels; being all factors related to the tendency of plaques to undergo rupture [88-91]. Vascular remodeling implies the degradation and reorganization of ECM lead by MMPs [92]. Such phenomenon is already evident at very early stages of atherosclerosis [93] and has a key role in the pathogenesis of plaque disruption [7].

Several clinical studies have established that genetic polymorphism in a variety of MMPs might be useful in determining individual susceptibility to ACS and the extent of coronary atherosclerosis, and are associated with MMPs plasma levels [94-96]. Indeed, Liu *et al.* demonstrated that MMP-3 5A/6A polymorphism was independently associated with the risk of ACS, MMP-3 activity and angiographical severity of coronary atherosclerosis [97, 98].

In parallel, MMP-8 gene variation has also been associated with the extent of coronary atherosclerosis and VCAM-1 levels [99]. Furthermore, Cheng *et al.* recently established that intra-plaque hemorrhage and collagen breakdown in vulnerable atherosclerotic lesions is mediated by activation of MMP-8 and MM-P13 [100].

MMP INHIBITION AS A POTENTIALLY EFFECTIVE ANTIATHEROSCLEROTIC STRATEGY

Specific MMPs have different roles in the development and phenotype of atherosclerosis, and sometimes also contradicting roles, being both beneficial and detrimental [25]. For example, MMP-9 appears as harmful and protective depending on the site and the experimental setup [101]. This paradox complicates the use of broad-spectrum MMPIs [102, 103]. MMPs may contribute to the formation and growth of atherosclerotic lesions by facilitating migration of VSMC through the internal elastic lamina into the intima space [104], by favoring monocyte infiltration of the

vascular wall [66], and by triggering fibrous cap rupture. Conversely, MMPs may also aid in the resolution of a plaque by degrading ECM in the intima [104-107].

The expression of MMPs can be induced by a number of inflammatory cytokines, hormones, growth factors and thrombin [76, 85, 108-114]. In particular, C-reactive protein (CRP), an inflammatory marker of atherosclerotic risk, induces the expression of MMP-1 and MMP-10 in macrophages [115] and endothelial cells, contributing to plaque vulnerability [80]. MMP-10 also appears as a useful marker of subclinical atherosclerosis in asymptomatic patients, because its levels are associated with inflammatory markers, increased thickness of the intima media of the carotid artery and presence of atherosclerotic plaques [116]. Furthermore, overexpression of MMP-1 and MMP-9 by macrophages and VSMC has been associated with the pathology and progression of vulnerable lesions [67, 70].

There is abundant evidence that suggests MMP-12 is a harmful protease that favors atherosclerotic plaque development and destabilization. Overexpression of MMP-12 in transgenic rabbits promotes macrophage infiltration and disruption of the internal elastic lamina, accelerating the atherosclerotic process [117]. Animal models have also shown a role of MMP-12 in macrophage recruitment to sites of preinduced inflammation, suggesting MMP-12 as a key player of inflammation [118-121]. Moreover, studies on apolipoprotein E-deficient (apoE(-/-)) and MMP-12 double-knockout mice suggest that MMP-12 may act as a destructive protease that promotes plaque instability by increasing the atherosclerotic lesion size and macrophage content, and decreasing the number of VSMC [122].

On the other hand, there is overwhelming evidence of gelatinase induction and activation in animal models of neointima formation after vascular injury that correlates with the activation and migration of VSMC [10]. These studies have shown upregulation of both MMP-2 and MMP-9 after balloon injury in rat [123, 124], pig [125], baboon [126], rabbit [127], and mouse [128, 129] arteries. Migration and proliferation of VSMC can favor fibrous cap formation and plaque stability [101, 130]. By using apoE/MMP-2 knockout mice it has been demonstrated that MMP-2 contributes to the formation and growth of the fibrous cap in the aortic root [131]. In agreement, increased MMP-2 activity levels were associated with VSMC content and a fibrous phenotype in carotid arteries, suggesting that MMP-2 expression is associated with a stable lesion phenotype [78].

Less clear is the role of MMP-9 in plaque stability based on apoE/MMP double-knockout mice models, suggesting a dual effect [101]. MMP-9 increases lesion size, macrophage content and medial destruction at the base of plaques in the descending aorta, suggesting that MMP-9 promotes instability at this site [132]. However, MMP-9 appears as a protective protease in mouse brachiocephalic arteries, because its presence results in smaller lesions with more smooth muscle content and less macrophage infiltration, promoting plaque stability [122].

Immunopositive MMP-2 and MMP-9 are increased in positive remodeled sections compared to negative remodeled sections [133]. Similarly, increased MMP-2 and MMP-9 levels were found in abdominal aortic aneurysms, an extreme kind of positive remodeling [134, 135]. In parallel, matrix degradation of the fibrous cap shoulder was concomitantly associated with overexpression of MMPs, thereby promoting the vulnerability of atherosclerotic plaques [67].

Ex vivo and *in vivo* studies have shown that both rupture-prone plaques and plaque rupture are highly prevalent even in stable patients [46, 47, 136, 137]. During the past 10 years, the role of MMPs in plaque rupture has become focus of attention since the proteolytic disruption of the fibrous cap overlying a lipid-rich plaque has been established as the most common physiopathological substrate of sudden cardiac death [7, 70, 138].

It has been suggested that one of the main circulating markers of ECM breakdown is MMP-9 [66]. MMP-9 plasma levels are significantly higher in patients with ACS, and correlate with a narrowing of the arterial lumen and restenosis after stent deployment [139, 140]. The serum levels of MMP-9 are significantly higher in patients with CAD with respect to control patients, and correlate directly with those of CRP, interleukin-6 and fibrinogen [141]. To note, MMP-9 expression is increased in plaques of patients with unstable angina with respect to those with stable angina [142, 143]. Furthermore, MMP-9 has been demonstrated to be a predictor of cardiovascular death in patients with coronary heart disease [144], and of ischemic heart disease and high pressure in patients with no history of cardiovascular disease [145]. Altogether, these results place MMP-9 as a possible marker of inflammation in patients with known CAD [141], and suggest a positive relationship between MMP-9 expression and plaque instability and rupture [10]. However, a clinical trial with 389 patients showed that TIMP-1 and not MMP-9 was able to independently predict death and MI [146]. Interestingly, others have suggested that the increase in plasma MMP-9 concentration after an acute coronary event might represent a healing response that involves the recruitment of VSMC rather than the initial of plaque rupture [122].

INHIBITION OF MMPs WITH SMALL MOLECULES

Most synthetic MMPIs present some cross-reactivity because they competitively target the structurally-conserved substrate binding pocket [18]. They generally contain a Zn chelating group (hydroxamate, carboxylate, thiolate, phosphinate) and a peptidomimetic moiety that mimics the peptide backbone of the substrate that interacts with the active site [18, 147]. Third generation MMPIs have been designed with new Zn-binding groups (pyrone, thiirane) and even without any Zn-coordinating element [148]. The later were designed to target allosteric sites of the MMPs structures by exploiting the recognized flexibility of the MMPs active site [149]. However, they still bind to the S_1' pocket and compete for substrate binding regions [148].

An important and more recent group of MMPIs corresponds to tetracyclines and chemically modified tetracyclines (CMTs), which can exhibit antimicrobial or non-antimicrobial activities [25]. Tetracyclines and CMTs can inhibit MMP activity and connective tissue breakdown both *in vitro* and *in vivo* [150]. They have been found to inhibit gelatinases, stromelysins, collagenases and MT-MMPs [150]

from numerous tissue and cellular sources [151]. Because of their chemical nature, these compounds may be able to cross anatomical barriers such as the blood brain barrier and blood retina barrier [25]. CMTs have been extensively studied in a number of animal models of periodontitis [152], metastasis [153], multiple sclerosis [154], and adjuvant arthritis [155].

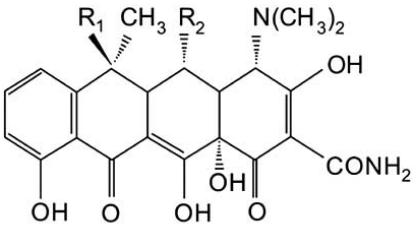
Nearly 60 MMPIs have been developed and tested as clinical candidates over the past 30 years, but, except doxycycline, all of them have failed due to poor safety and a lack of efficacy [147, 156-158]. For example, batimastat (British Biotech), ilomastat (GlycoMed), solimastat (British Biotech) and marimastat (British Biotech) are all broad-spectrum hydroxamate-based peptidomimetic MMPIs that were discontinued in Phase I, II or III studies due to severe side effects, including musculoskeletal syndrome [158]. On the other hand, Other MMPIs are currently being tested in clinical trials, such as incyclinide (Metastat®), S-3304 (Shionogi) and CPA-926 (Kureha Chemical Industry) [147]. Incyclinide is a CMT MMPI that inhibits MMP-2 and MMP-9, and is currently in Phase II trials for the treatment of acne, brain tumors, solid tumors, and HIV-related Kaposi's sarcoma, and presents mild to moderate side effects [147, 159]. S-3304 is a novel D-tryptophan derivative MMPI that inhibits most potently the activities of MMP-2 and MMP-9 without inhibiting MMP-1, -3 and -7, and has shown promising results in Phase I and II trials for the treatment of lung cancer and solid tumors [147, 160]. Finally, CPA-926 (a pro-drug of Esculetin) is a non-peptidomimetic MMPI that is currently in Phase II trials for the treatment of osteo-arthritis [161, 162].

Doxycyline: Pharmacokinetics, Pharmacodynamics and Adverse Effects

Doxycycline (Periostat®) (Fig. (**3**)), an antimicrobial CMT, is the only FDA-approved MMPI used for the treatment of periodontitis [23]. Doxycycline is a reversible, noncompetitive and broad-spectrum MMPI that binds to allosteric sites proximal to the structural Zn and/or Ca atoms [163, 164]. The proposed mechanism of action results from its ability to chelate these structural ions, which are required to maintain proper enzyme conformation and activity [150, 164]. In the case of MMP-7, it has been reported that doxycycline binds to the enzyme *in vitro* with a stoichiometry of 2.3 ± 0.2 and a dissociation constant of 73 ± 8μM [164] . Its binding in both pro and active MMPs results in the disruption of the normal conformation of the protein structure [164], leaving the enzymes inactive [150].

Doxycycline inhibitory effect has been tested in both humans and animals in a number of conditions associated with elevated MMP activity, including arthritis [165-168], periodontitis [169, 170] and chronic wounds [171].

Doxycycline is available in oral and intravenous formulations [172], and it is believed to be almost completely absorbed in the duodenum [173], with a bioavailability between 73 and 95% [173, 174], significantly more than other tetracyclines. The half-life absorption ranges from 1 to 2 hours when administrated while fasting [175], and the peak concentration varies with dose, being 15.3mg/L (~30 μM) after an oral dose of 500mg [176].



	R_1	R_2
Tetracycline	OH	H
Doxycycline	H	OH

Fig. (3). Chemical structure of tetracycline and doxycycline.

Regarding tissue penetration, doxycycline levels are poor in saliva, below those of serum in bone, skin, fat, tendons and muscle [172, 177], and highest in the liver, kidney and digestive tract [173]. Doxycycline is eliminated unchanged by both the renal and biliary routes [172]; bile concentrations may be 10-25 times greater than serum [178]. About 35-60 % is excreted in urine and the remainder in faeces [179-182]. Doxycycline is slowly absorbed orally. It reaches peak concentration in 2-3 hours, and the elimination half-life ranges between 12 to 25 hours [172]. The area under the serum time curve for a 200mg/day oral dose varies from 41-123mg.h/L and 61-112mg.h/L for intravenous doses [172].

Different studies have been performed to assess the pharmacodynamics of doxycycline as an antimicrobial drug using the minimum inhibitory concentration as a measure of drug potency [172]. With respect to its inhibitory effect on MMPs, it has been shown that doxycycline inhibits collagenase activity more effectively than tetracycline or other CMTs [151]. For example, the concentration of doxycycline required to inhibit 50 % of collagenase activity (IC50) *in vitro* was ~13 and ~23 times smaller than minocycline and tetracycline, respectively [183]. *In vitro*, the doxycycline IC50 against a colorimetric peptide substrate was reported to be 28 ± 5 μM for MMP-7 [164] and 90 ± 13μM for MMP-2 [184].

The inhibitory effect of doxycycline varies between different MMPs, as tested *in vitro* by Smith *et al*. In this study, 30μM doxycycline (value comparable to the concentrations achieved in serum after oral administration) was able to inhibit MMP-1, MMP-8 and MMP-13 activity against type II collagen by 5, 50 and 60% [163].

Mild but relatively common adverse effects of oral doxycycline include hives, shortness of breath, swelling of the face, lip, tongue or throat, headache, dizziness, fever, chills, rash, nausea, vomiting, diarrhea, thrush, vaginitis and photosensitivity [185].

EFFECT OF DOXYCYCLINE ON ATHEROSCLEROSIS AND DIVERSE INFLAMMATORY PROCESSES

A link between chronic infection, the associated inflammatory processes in the periodontal tissue and cardiovascular disease has been undoubtedly established. The presence of carotid artery plaque is associated with

periodontitis and tooth loss [186, 187]. Indeed, using serial carotid ultrasound, Schillinger *et al*. demonstrated that a variety of markers of periodontal disease predict carotid plaque progression, independently of traditional cardiovascular risk factors and the baseline degree of stenosis [188]. Furthermore, a Danish investigation has shown a six-fold increase in the risk of coronary artery disease in individuals with more than 4mm of alveolar bone loss [189]. The beneficial effects of doxycycline in periodontal disease, along with the reported association between periodontitis and atherosclerosis, warrant further research evaluating the effect of doxycycline on atherosclerosis.

There is robust evidence about the detrimental role of positive remodeling in the natural history of coronary atherosclerosis [190]. Several studies have demonstrated an association between coronary remodeling and plaque composition [88, 89, 91]. Indeed, positive remodeling has been established as a major criteria of plaque vulnerability. Since MMPs play a major role in vascular remodeling, MMP inhibition with doxycycline shows promise towards plaque stabilization.

Abdominal aortic aneurysm is an extreme form of vascular remodeling and, as such, nonspecific MMP inhibition has been shown to retard expansive aortic remodeling [102].

Doxycycline effect on vascular remodeling and atherosclerosis has been found to be independent of its antimicrobial properties, as suggested in clinical studies where sub-antimicrobial doses of doxycycline (SDD) decreased the growth rate of abdominal aortic aneurysms [191]. Indeed, Manning *et al.* have shown a significant reduction in abdominal aortic aneurysm formation and severity with doxycycline administration independently from lipid levels obtained or systolic blood pressure [192].

A recent prospective, randomized study including patients requiring carotid endarterectomy, has demonstrated that doxycycline penetrates atherosclerotic plaques at acceptable tissue levels and achieves a significant *in situ* MMP inhibition [193].

The effect of doxycycline on atherosclerosis has been explored by Bendeck *et al*. who demonstrated a significant effect on cell proliferation, migration and MMP activity [194], although it should be noted that a restenosis model (balloon injury in left common carotid of male Sprague-Dawley rats) was used in that study, while such models are not applicable to advanced natural history atherosclerosis, as commonly seen in human. More recently, Madan *et al*. demonstrated a significant decrease in pro-inflammatory cytokines resulting in reduction of atherosclerosis in apoE double knockout mice inoculated with *Porphyromonas gingivalis*, a pathogen related to periodontal disease and systemic inflammation [195].

The MIDAS trial was a prospective, randomized, double-blinded, placebo-controlled pilot study that evaluated the effect SDD in a very small population of ACS patients. At 6 months, although the study was unable to detect an effect on clinical outcome, high-sensitivity CRP was reduced by 46% and pro-MMP-9 activity was reduced by 50% with SDD, whereas no significant reductions were detected with placebo [196].

In the setting of acute MI and heart failure, upregulation of diverse MMPs has been associated with left ventricular remodeling [197, 198]. Furthermore, it has been ascribed to MMPs a rapid effect on cellular transduction processes before changes in ECM occur, particularly noted on MMP-mediated platelet aggregation [199, 200]. This has lead to the exploration and further confirmation that myocardium subjected to ischemia-reperfusion injury releases MMP-2 and that its liberation, of pathological significance for the development of mechanical dysfunction, might be mitigated by doxycycline [201].

CURRENT & FUTURE DEVELOPMENTS

Despite lasting debate about the role of infection in atherosclerosis [202], the largest antibiotic treatment trial to date has failed to show any benefit of azithromycin in post-MI patients with elevated *Chlamydia pneumoniae* titers [203]. In accordance, subsequent randomized trials failed to show benefit of antibiotics in both patients with stable CAD and ACS [204, 205]. These findings are highlighted by the fact that the cardiovascular effects observed with doxycycline were obtained using sub-antimicrobial doses.

Throughout this review, we have outlined the pivotal role of MMPs in the natural history of atherosclerosis and its cardiovascular consequences. Non-selective MMP inhibition with doxycycline appears as a potential strategy to reduce the residual risk observed in patients already at intensive lipid lowering strategies. However, as aforementioned, specific MMPs have different and even contradicting roles in the natural history of atherosclerosis, rendering broad spectrum MMP inhibition an important yet somewhat simplistic approach towards residual risk reduction in coronary atherosclerosis. The effect of a broad-spectrum MMP inhibition is probably expected to be dependent on its degree of inhibition of specific MMPs [103]. It should be stressed though that, overall, the balance of non-selective MMP inhibition might shift to the favorable side only in particular settings such as in ACS, where in addition to its potential plaque stabilization properties, doxycycline shows promise in preventing ischemia-reperfusion injury and left ventricular remodeling. In turn, it seems unlikely that long-term administration of doxycycline might be beneficial in stable angina patients.

Doxycycline appears to be tolerated well by both mice and human, with no significant changes in body weight, lipid metabolism, or blood pressure [192]. From January 1998 to August 2003, approximately 50 million new doxycycline prescriptions were dispensed in the United States, with an event rate of 13per million [206]. Indeed, long term administration of doxycycline appears to be safer than minocycline, which might be rarely associated with serious adverse events including hypersensitivity syndrome reaction and drug-induced lupus, whereas most common adverse events with doxycycline are mild and gastrointestinal [206, 207].

Nevertheless, to date, most animal models used do not represent advanced coronary atherosclerosis seen in humans,

and clinical studies exploring the role of doxycycline on atherosclerosis progression, as well as powered clinical outcome studies are lacking.

Finally, it is noteworthy that the complexity of MMPs as targets for inhibition, with some MMPs being attributed a potential beneficial effect particularly during the healing response, warrants further research towards the design of selective inhibitors of individual MMPs [208-222].

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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ABBREVIATIONS

MMPs	=	Matrix metalloproteinases
CAD	=	Coronary artery disease
MI	=	Myocardial infarction
ACS	=	Acute coronary syndromes
ECM	=	Extracellular matrix
TIMPs	=	Tissue inhibitors of MMPs
VSCM	=	Vascular smooth muscle cells
CRP	=	C-Reactive protein
SDD	=	Sub-antimicrobial doses of doxycycline
CMTs	=	Chemically modified tetracyclines

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