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Pharmacomodulation of Broad Spectrum Matrix Metalloproteinase Inhibitors Towards Regulation of Gelatinases

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1. Introduction

Matrix metalloproteinases (MMP) constitute a family of 23 zinc- and calcium-dependent endopeptidases that play pivotal functions in several physiological processes such as embryogenesis, wound healing, vasculogenesis or stem cell mobilization (Nagase et al., 2006). These enzymes were originally defined as matrix-degrading proteases, but a myriad of other substrates have been discovered including cytokines, chemokines, growth factors and their receptors, cell adhesion molecules and angiogenic factors. MMP were first described to exert their degradative function extracellularly against matrix macromolecules or at the pericellular microenvironment. Recently, MMP proved to cleave intracellular substrates belonging to any subcellular compartments (Cauwe & Opdenakker, 2010). Among them were notably apoptotic regulators, signal transducers, molecular chaperones or transcriptional and translational regulators. Therefore, MMP can be considered as proteases mainly controlling signaling events through processing cytokines, chemokines and degrading matrix, liberating matrikines in the extracellular space, or in turn cleaving enzymes involved in signal transduction inside the cells. MMP are regulated at distinct levels including gene expression, compartmentalization, proenzyme activation, enzyme inhibition, endocytosis, and finally substrate availability and affinity. MMP up-regulation participates in tumor progression and metastasis, inflammatory disorders, cardiovascular and autoimmune diseases (Hu et al., 2007; López-Otín & Matrisian, 2007; Mandal et al., 2003; Murphy & Nagase, 2008).

All MMP are produced as proenzymes *i.e.* zymogen; enzyme latency is due to the formation of a coordinated bond between the zinc atom in the active site and an amino

acid residue cysteine present in a consensus PRCGXPD sequence in MMP prodomain. Proteolysis of the prodomain, action of reactive oxygen species (O_2^-, NO) on the amino acid residue cysteine and allosteric perturbation (Sela-Passwell et al., 2010) of the prodomain can disrupt this Cys-Zn bond, a process named "cysteine switch" (Van Wart & Birkedal-Hansen, 1990). In the active enzyme, the zinc atom is linked to three histidine residues and a water molecule. A conserved glutamic acid residue (Glu) in the catalytic domain HEBXHXBGBXHS polarizes the water molecule (Gomis-Rüth, 2009; Lovejoy et al., 1994). This ligated water molecule attacks the carbonyl carbon of the scissile bond and transfers a proton to Glu and then to the scissile nitrogen atom. Then Glu releases the second proton from the water molecule to the scissile nitrogen atom and the peptide bond is cleaved (Figure 1).

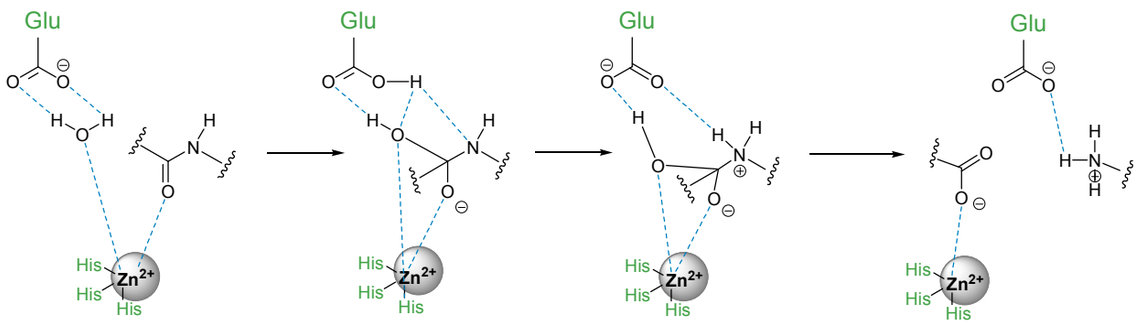


Fig. 1. Mechanism of action of MMP (adapted from Lovejoy et al., 1994).

Historically, MMP were named according to their preferential action on matrix components: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), proteoglycanases or stromelysins (MMP-3, MMP-10), macrophage elastase (MMP-12).

To date, a classification based on their domain organization is favoured: five of them are secreted and the others are transmembrane proteins (MT-MMP) based on their structure similarities (Table 1) (Egeblad & Werb, 2002).

MMP family is constituted by: a pre-domain involved in enzyme secretion, a pro-domain including a cysteine residue interacting with the zinc atom in the catalytic domain that maintains the inactive enzyme form. The catalytic domain is responsible of the MMP activity. All MMP, except MMP-7, MMP-26 (28 kDa) and MMP-23 (56 kDa) possess a hemopexin-like domain involved in the substrate interactions. The gelatinases (MMP-2 (72 kDa) and MMP-9 (92 kDa)) contain a gelatin-binding type II domain with three fibronectin (Fn(II))-like repeats. MMP-11 (51 kDa) and MMP-28 (59 kDa) contain a furin motif for recognition by furin-like serine proteinases. This motif is also present in MMP containing a vitronectin-like domain (MMP-21 (70 kDa)) and membrane-type MMP (MT-MMP). In addition, MT-MMP have a transmembrane domain and a short cytoplasmic domain or a glycosylphosphatidylinositol anchored (MMP-17 (57 kDa) and MMP-25 (63 kDa)). Finally, MMP-23 is a type II transmembrane MMP with a cysteine array and immunoglobulin-like domain.

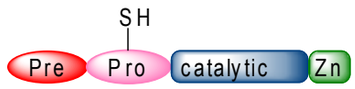
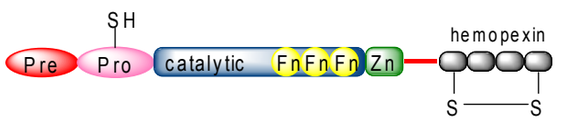
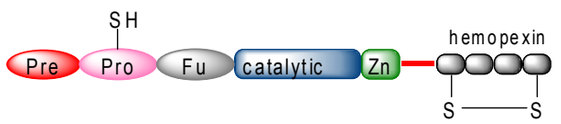
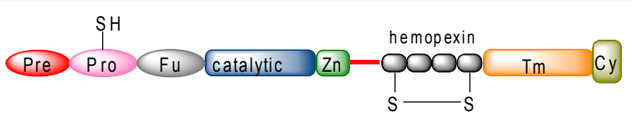
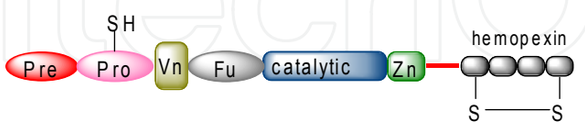
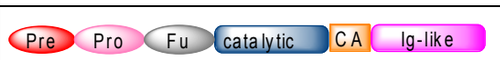
Designation	Main structure	Name
MMP-7 MMP-26		Matrilysin Matrilysin-2
MMP-1 MMP-3 MMP-8 MMP-10 MMP-12 MMP-13 MMP-18 MMP-19 MMP-20		Collagenase-1 Stromelysin-1 Collagenase-2 Stromelysin-2 Metalloelastase Collagenase-3 Collagenase-4 Enamelysin
MMP-2 MMP-9		Gelatinase A Gelatinase B
MMP-11 MMP-28		Stromelysin-3 Epilysin
MMP-14 MMP-15 MMP-16 MMP-24		MT1-MMP MT2-MMP MT3-MMP MT5-MMP
MMP-17 MMP-25		MT4-MMP MT6-MMP
MMP-21		XMMP (*Xenopus*)
MMP-23		Femalysin

Table 1. MMP family. Pre: signal peptide, Pro: propeptide, Fn: fibronectin type II domain, Fu: furin recognition site, Vn: vitronectin-like domain, TM: transmembrane domain, Cy: cytoplasmic domain, GPI: glycosylphosphatidylinositol, CA: cysteine array, Ig-like: immunoglobulin-like domain.

2. Structures and properties of gelatinases

2.1 Structure of gelatinases active sites

Gelatinases A (MMP-2) and B (MMP-9), as classified as both collagenases and elastases, are involved to a great extent in pathologies affecting major elastic tissues (lung, arteries). Among the MMP family members, gelatinases subclan, MMP-2 and MMP-9, do exhibit several originalities that could be taken into account for the design of inhibitors.

MMP family proved to have a great homology of sequence and the zinc-containing catalytic site is surrounded by subsite pockets named S1, S2, S3 for non-primed and S'1, S'2, S'3 for the primed side (Terp et al., 2002).

The conserved amino acid residues in gelatinases active-site region (Cuniasse et al., 2005; Kontogiorgis et al., 2005; Nicolotti et al., 2007; Rao, 2005) are given in Table 2.

The structural amino acid sequence of MMP is mainly similar except for the loop region (S'1 pocket), which displays different length and is composed of distinct amino acid composition. The similarities are ordered as S'1 > S2 > S'3 > S1, S3 > S'2.

Selective and/or combined occupancy of these pockets were believed to direct selectivity of inhibitor. More generally, it has been determined that such subsites display distinct potency in driving selectivity in order S'1 > S2, S'3, S3 > S1 > S'2.

S'1 pocket located immediately to "the right" of the catalytic site differs notably in size and shape among MMP and has been named specific pocket.

The S'1 pocket is deep, presenting an elongated and hydrophobic shape with an amino acid residue Leu at position 197 for all MMP except MMP-1 and MMP-7. The variation of amino acid residues among MMP, within this pocket, might be important. It adopts an extended shape in both gelatinases, but S'1 pocket in MMP-2 forms a large channel nearly bottomless, while it is slightly flexible in MMP-9 presenting a real pocket-like subsite.

The S'2 pocket is shallow, partly solvent-exposed and delimited on the top face by the amino acid residue 158 and on the bottom face by the amino acid residue 218. Its size is affected by the amino acid residues 162 (Asn), which is a Leu for both gelatinases, 163 (Val) which is an Ala for both gelatinases and 164 (Leu).

The S'3 pocket is neutral and partly solvent-exposed and delimited by the amino acid residues 222 (Leu) and 223 (Tyr). The size of this pocket is dependent on the amino acid residue 193, which is a Tyr for both gelatinases (Table 2).

As a rule, the substrates bind weakly with the unprimed subsites; however, some differences could be assigned between gelatinases.

The S1 pocket is shallow and hydrophobic. The same triad is pinpointed for MMP-2 and MMP-9 (His166-Phe168-Tyr155 and His183-Phe185-Tyr172, respectively). The amino acid residue 163 and to a lower extent the amino acid residue 155 influence the S1 subsite interactions with an inhibitor. The amino acid residue 163 is a Leu for both gelatinases.

The S2 pocket is solvent-exposed and the amino acid residues 86, 169 and 210 are poorly conserved in MMP family and affect the shape and the properties of this pocket. Its shape is

N°		S3		S2				S'2					S1									S'1 bottom			S'3
		155	168	Catalytic domain (S2 + S'2)									155	163	166	168	172	183	185	197	220	222	193
				86	165	169	210	158	162	163	218												
MMP-2		Y	F	F	E	A	A	G	L	A	I		Y	L	H	F				L	T	R	Y
		FAPGTGVGGDS		HEFGHAMGLEH									AHA							PIY-TYT-KNFRLSQ			DGLL
		Small		Small, + charge /H bond acceptor				Shallow Solvent-exposed					Hydrophobic							Channel with no bottom			Neutral Partly solvent-exposed
MMP-9		Y	F	-	D	P	P	G	L	A	M		-	L	H	Y	Y	H	F	L	T	T	Y
		FPPGPGIGGDA		HEFGHALGLAH									AHA							PMY-RFT-EGFPLHK			DGLL
		Small		Big, hydrophobic				Shallow Solvent-exposed					Hydrophobic							Pocket-like subsite			Neutral Partly solvent-exposed

Table 2. Overview of favorable ligand properties and conserved domains of gelatinases (adapted from Cuniasse et al., 2005; Nicolotti et al., 2007; Terp et al., 2002).

dependent on the amino acid residue Pro at the position 87, then the MMP-2 has its Phe87 leading to a small and hydrophobic pocket and MMP-2 interacts with positive charge probes. When the Pro87 is lacking, another conformation was observed. The amino acid residue at the position 169 is a Pro for MMP-9 and defines a large hydrophobic pocket.

Finally, the amino acid residue 210, Asn in MMP-9 and Glu in MMP-2, leads to a less exposed pocket and notably plays a crucial role in enzyme selectivity. The S2 pocket is important and differentiates both gelatinases.

The S3 pocket is composed of a hydrophobic cleft delimited by the amino acid residues 155 and 168. The pocket shape and size are influenced by the amino acid residue 155 which is a Tyr and 168 which is a Phe for both gelatinases.

Although these subsites might direct enzyme specificities, interaction of gelatinases with macromolecular substrates also relies on the presence of remote binding sites named exosites that also notably act in driving enzyme action (Figure 2).

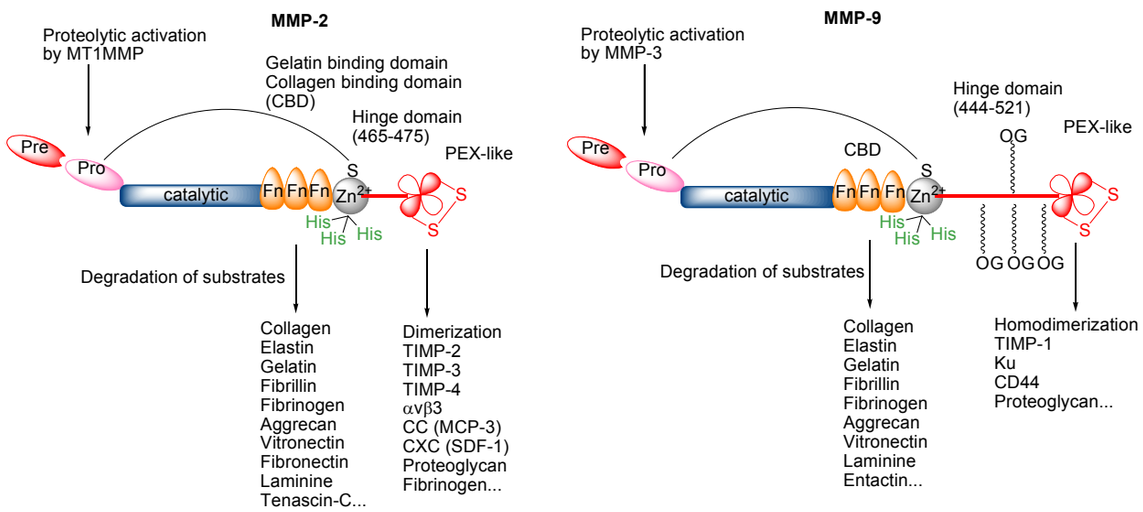


Fig. 2. Functions of gelatinases domains.

2.2 Biological properties of gelatinases

Gelatinases are most often associated to the cell plasma membrane of normal or transformed cells, thus targeting the proteolytic activity of invasive cells. Enzyme tethering to cell periphery requires the carboxy terminal hemopexin-like domain, designated as PEX of 200 amino acid residues on average forming a four bladed β-propeller structure.

In pro-MMP-2, the PEX domain interacts with the C-terminal domain of TIMP-2 (Tissue Inhibitors of Matrix MetalloProteinase-2 (Brew & Nagase, 2010)) which allowed the complex to tether to plasma membrane through interaction of the N-terminal part of the inhibitor to a MT1-MMP homodimer: one molecule of MT1-MMP acting as a docking molecule, the other catalyzing pro-MMP-2 activation (Itoh et al., 2006, 2011; Sato & Takino, 2010).

Of note, this PEX domain also reacts with TIMP-3 and TIMP-4 but no MMP-2 activation was noted in such case.

It needs to be emphasized that interaction of PEX domain in MMP-2 with MT3-MMP also leads to enzyme activation that can be enhanced by the prior binding of pro-MMP-2 to chondroitin-4 sulfate chains-containing proteoglycan. PEX domain of MMP-2 was also reported to bind to $\alpha_v\beta_3$, CC chemokine as monocyte chemoattractant protein-3 (MCP-3), CXC chemokine as stromal cell derived factor-1 (SDF-1) and fibrinogen.

Such PEX domain is also important in driving the pro-MMP-9 activation; in human neutrophils formation of pro-MMP-9-lipocalin complex favours enzyme activation by kallikrein. It is also important for localizing the enzyme at the cell periphery through interaction with low density lipoprotein-receptor related protein (LRP), CD-91 or different isomer forms of CD-44, protein Ku, and is involved in the formation of covalent complexes with proteoglycans (Malla et al., 2008; Monferran et al., 2004).

An important property of this carboxy terminal domain, in association with an unstructured, hydrophilic and flexible long-O-glycosylated domain (OG) in pro-MMP-9 which contains 11 repeats of the sequence T/SXXP (Figure 2), relies on its ability to catalyze the intracellular formation of enzyme dimers (Van den Steen et al., 2001). Importantly, the dimer form of pro-MMP-9 is more resistant to MMP-3 activation.

Gelatinases also appear unique in MMP family in exhibiting fibronectin type II domains which are also designated as collagen binding domains (CBD) (Shipley et al., 1996). Indeed, deletion of these domains in both enzymes led to protease devoided of collagen(s)-gelatin(s)- or elastin-degrading capacity (Allan et al., 1995). Recent data also indicated that OG could also mediate MMP-9 gelatin interaction by allowing the independent movement of enzyme terminal domain (Vandooren et al., 2011).

3. Design of MMP inhibitors (MMPI)

Up to now, a myriad of MMPI has already been synthesized (Table 3).

The most important studies focus on the combinations of diverse structural modifications; three classes of compounds have been developed: combined inhibitors, right hand side and left hand side inhibitors based on the scissile bond in the catalytic site (Skiles et al., 2004; Whittaker et al., 1999). Some of them have been used as potential therapeutic agents to limit tumor progression. Instead of using MMP inhibitors as therapeutic treatment, they might be also useful as preventive drugs or as biomarkers in early stage of cancer. Up to now, most of the clinical trials in cancer were rather disappointing (Abbenante & Fairlie, 2005; Dormán et al., 2010; Fingleton, 2007; Gialeli et al. 2011).

The first generation of MMPI was based on peptidomimetic skeleton containing a succinic acid motif and a hydroxamic acid as zinc binding group (ZBG) (batimastat®, marimastat®, solimastat®, galardin®, trocade®). Hydroxamic acid is a bidentate chelator, but it has also a good binding affinity to other ions (Cu^{2+}, Fe^{2+} and Ni^{2+}). They are broad spectrum inhibitors and led to musculoskeletal syndrome side effect mainly due to the presence of hydroxamic acid.

Prinomastat® contains a ring embedded sulfo-succinic acid motif, which increases oral availability and water solubility. Nevertheless, clinical trials have been discontinued after phase III for musculoskeletal toxicity and poor survival rate.

Tanomastat® has a thioether function increasing the oral activity and selectivity for MMP-2, MMP-3 and MMP-9. Unfortunately, clinical trials are discontinued for haematological toxicity and poor survival rate.

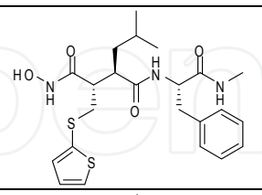
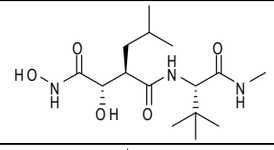
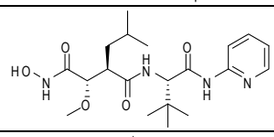
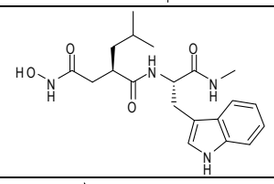
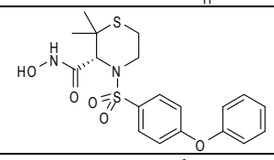
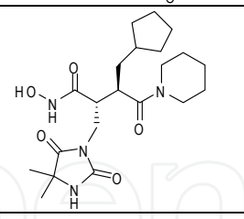
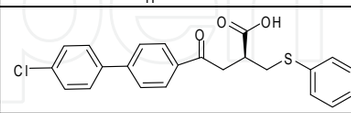
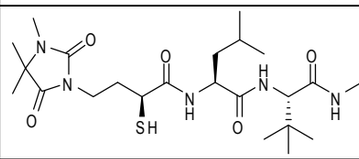
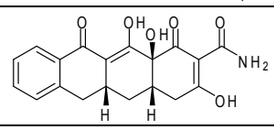
MMP Inhibitors	Indication	Clinical trials development	Structure
Batimastat® (BB-94) British Biotech	Cancer Broad spectrum (MMP-1, -2, -3, -7, -9)	Phase I (Discontinued)	
Marimastat® (BB-2516) British Biotech	Cancer Broad spectrum (MMP-1, -2, -7, -9)	Phase III (Discontinued)	
Solimastat® (BB-3644) British Biotech	Cancer Broad spectrum (MMP-1, -7)	Phase I (Discontinued)	
Galardin® (GM-6001) Glycomed	Eye disease, COPD Broad spectrum (MMP-1, -2, -9, -12)	Phase I (Discontinued)	
Prinomastat® (AG-3340) Agouron	Cancer Macular degeneration Broad spectrum (MMP-2, -3, -7, -9, -13)	Phase III (Discontinued)	
Trocade® (Ro32-3555) Roche	Rheumatoid arthritis (MMP-1, -8, -13)	Phase III (Discontinued)	
Tanomastat® (BAY 12-9566) Bayer	Cancer Arthritis (MMP-2, -3, -8, -9)	Phase II Phase III (Discontinued)	
Rebimastat® (BMS-275291) Bristol-Myers Squibb	Cancer Broad spectrum (MMP-1, -2, -7, -9, -14)	Phase III (Discontinued)	
Metastat® (CMT-3) Collagenex	Cancer (MMP-2, -9)	Phase II	

Table 3. Main MMPI in clinical development.

The thiol zinc binding group of rebimastat® is a weak monodentate ligand and the musculoskeletal toxicity and its poor response led clinicians to stop treatment.

Finally, metastat® is a second generation of tetracyclines still in phase II clinical trials (Acne, AIDS-related Kaposi's sarcoma and mainly used in cancer). The only detected side effect is its photosensibility. This inhibitor is selective of MMP-2 and MMP-9 and crosses the blood-brain barrier. Actually, it is the most promising MMPI.

These failures are mainly due to their broad spectrum MMP inhibitory activity, the similarity of their active sites with those of other metalloproteinases (ADAM, ADAMT…), the poor selectivity of the chelating group, the administration of MMPI in late disease stage, their poor pharmacokinetics, unavoidable side effects (musculoskeletal pain), toxicity and limited oral bioavailability.

However, these efforts led to pinpoint the importance of MMPI selectivity and allowed the identification of MMP as target and anti-target in various diseases progression (Overall & Kleifeld, 2006). Certain MMP are both targets and anti-targets depending on the stage of the disease.

4. Galardin® pharmacomodulation as a tool for designing specific gelatinases inhibitors

For a decade, we have been involved in the pharmacomodulation of galardin®, a powerful broad spectrum MMPI (Figure 3).

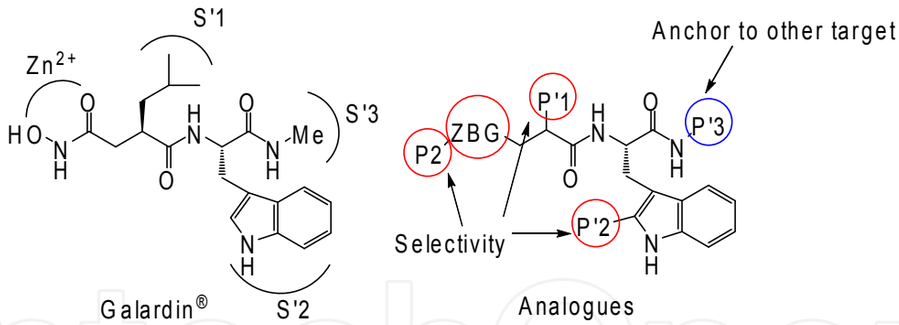


Fig. 3. Galardin® and analogues.

By the beginning of 1994, galardin® was in phase I clinical trials for age related macular degeneration (ARMD) and as chronic obstructive pulmonary disease (COPD) by Glycomed.

In order to increase selectivity, the synthesis of analogues of galardin®, has been achieved. The modifications have been focused on the P'1, P'2, P'3 groups and ZBG.

4.1 Influence of the S'1 subsite: Modulation of galardin® in gelatinases inhibition

Our first experiments started with the insertion of one unsaturation in P'1 position to increase the hydrophobicity of the new compounds and to study the effect of substitution on S'1 pocket specificity (Figure 4). For this purpose, replacement of isobutyl group by an isobutylidene group of *E* geometry enhanced by 100-fold MMP-2 selectivity *versus* MMP-3

(IC_{50} = 1.3 and 179 nM, respectively) (Marcq et al., 2003). The double bond geometry was found important for potency and selectivity as shown with the equimolar *E/Z* mixture which displayed lower activity.

Pursuing these pharmacomodulations aiming at better MMP-2 selectivities, we planned to increase hydrophobicity and rigidity with the dehydro and didehydro analogues which were synthesized (analogue **2a-d** and **3a-h**).

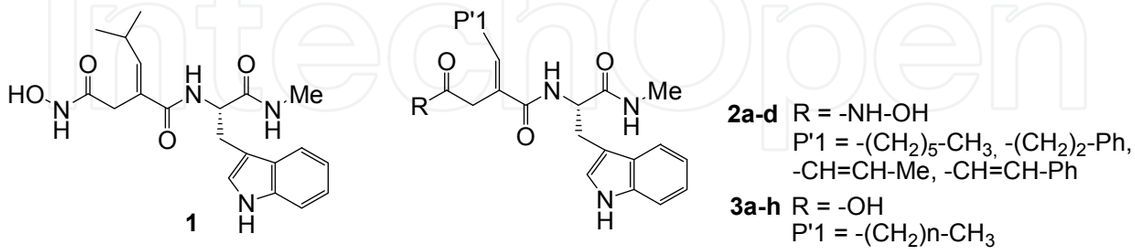


Fig. 4. Pharmacomodulation of the P'1 group.

Introduction of either one or two unsaturations decreased their potent MMP inhibitory activity as compared to parent molecule over all MMP (Moroy et al., 2007). However, the presence of a phenyl group at the end of alkyl chain (**2b** and **2d**) led to inhibitors with a good activity and selectivity for MMP-9 (IC_{50} = 38 and 45 nM, respectively).

In parallel, C7 long alkyl chain containing galardin® **2a** displayed a MMP-2 selectivity comparing to MMP-9 (IC_{50} = 123 nM) (Table 4).

Compounds	ZBG	P'1	MMP-1	MMP-2	MMP-9	MMP-14
Galardin®	-NH-OH		1.5	1.1	0.5	13.4
1	-NH-OH	-*i*Pr	18	9.2	17	10
2a	-NH-OH	$-(CH_2)_5$-Me	32000	123	$> 10^4$	2660
2b	-NH-OH	$-(CH_2)_2$-Ph	9130	280	45	53100
2c	-NH-OH	-CH=CH-Me	984	78500	974	913
2d	-NH-OH	-CH=CH-Ph	1240	120	38	2490
3a	-OH	$-(CH_2)_6$-Me	$> 10^5$	7570	838	$> 10^5$
3b	-OH	$-(CH_2)_7$-Me	$> 10^5$	458	241	$> 10^5$
3c	-OH	$-(CH_2)_8$-Me	$> 10^5$	247	173	$> 10^5$
3d	-OH	$-(CH_2)_9$-Me	$> 10^5$	249	450	$> 10^5$
3e	-OH	$-(CH_2)_{10}$-Me	$> 10^5$	351	211	$> 10^5$
3f	-OH	$-(CH_2)_{11}$-Me	$> 10^5$	655	673	$> 10^5$
3g	-OH	$-(CH_2)_{14}$-Me	$> 10^5$	762	582	$> 10^5$
3h	-OH	$-(CH_2)_{18}$-Me	$> 10^5$	$> 10^5$	$> 10^5$	$> 10^5$

Table 4. Influence of the P'1 chain length on the selectivity and potency of MMP inhibitors. IC_{50} values are expressed in nM.

AutoDock 4.0 program (Huey et al., 2007; Morris et al., 1998) was used to perform the computational molecular docking. AutoDockTools package was employed to prepare the

input files necessary to the docking procedures and to analyze the docking results. The figures have been done using Pymol program (DeLano, W. L. 2002. PyMol Molecular Graphics System, Palo Alto, CA. http://www.pymol.org).

Molecular modelling experiments confirmed the importance of the insertion of the alkyl chains in the S'1 pocket, supporting the observed biological data for **2a** (Figure 5).

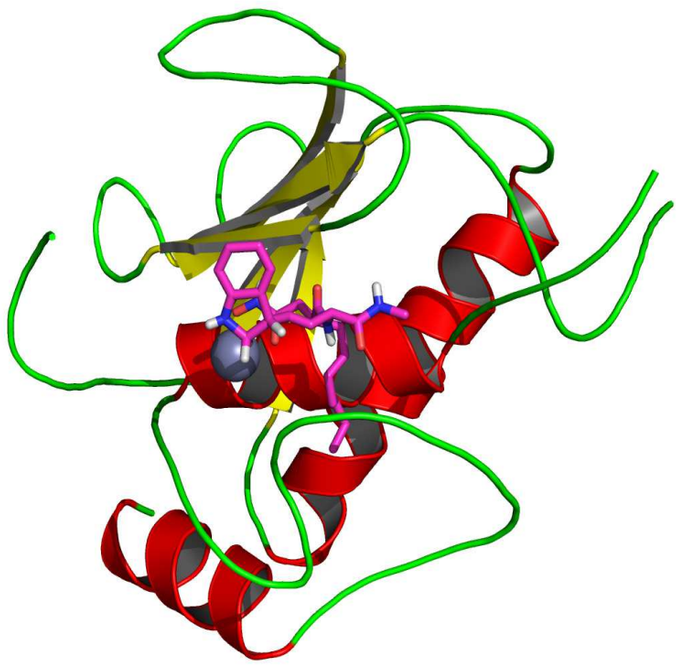


Fig. 5. Complex between MMP-2 and analogue **2a**. The MMP-2 secondary structure was represented in cartoon. The analogue **2a** is shown with sticks in which the C atoms are colored in magenta. Zn atom is displayed as a grey sphere.

The S'1 pocket of MMP-2 is sufficiently deep to accommodate the long alkyl chain. On the contrary, the S'1 pocket of MMP-9 at the end of the tunnel is restrained, like a funnel by the amino acid residues Glu233, Arg241, Thr246 and Pro247. The S'1 pocket of MMP-9 is large enough to accept a phenyl group at the entrance of the pocket such as compounds **2b** and **2d**.

In order to increase hydrophobicity, aiming at a better MMP-2 selectivity, the alkyl chain was elongated with n = 8 to 20 carbon atoms as described in the literature (Levy et al., 1998; Miller et al., 1997; Whittaker et al., 1999). Using galardin® as template, the activity and selectivity were not really increased with the length of the linear chain. Batimastat® inhibitors increase activity for MMP-2 with C8 long alkyl chain (IC_{50} = 0.6 nM) and a C12 analogue displays a MMP-2 selectivity comparing to MMP-1 (IC_{50} = 1 and 50000 nM, respectively). Finally, a good activity for MMP-2 is obtained for C9 long chain marimastat® analogues (IC_{50} < 0.15 nM) and a maximal selectivity occurs with a C16 for MMP-2 *versus* MMP-1 (IC_{50} = 0.6 and 5000 nM, respectively).

Nevertheless, in our case no better IC_{50} were found for MMP-2 with increasing chain length when an unsaturation was incorporated. However, a good selectivity for MMP-2 *versus* MMP-1 and MMP-14 is observed. Also, carboxylic derivatives **3c** and **3e** displayed a good selectivity for MMP-9 *versus* MMP-1 and MMP-14.

As stated previously, S'1 pocket is generally quite large in all MMP. However, the amino acid residue Arg214 redefines the bottom of the pocket in MMP-1 leading to a small and restricted S'1 pocket. The amino acid residue Arg214 can be flexible but in most cases, a large P'1 group inhibitor is expected to bind weakly to MMP-1. Thus, it is not surprising, to find that most of the MMP-1 inhibitors have relatively small P'1 groups.

MMP-14 appears as one key-proteolytic enzyme to promote cancer invasion and metastasis (Hernandez-Barrantes et al., 2002). Up to now, only one pentacyclic sterol sulphate MMP-14 inhibitor was described to display MMP-14 selectivity while it exhibits only low potency (Fujita et al., 2001).

S'1 pocket of MMP-14 was found to be two amino acid residues longer than those of gelatinases. The amino acid residue Met237 allows favourable interactions with the hydrophobic substituents at the bottom of the pocket, but unfavourable interaction with the positively charged substituents.

Therefore, lack of inhibition of this enzyme by long alkyl chain (Table 4) is rather surprising. The unsaturation might disturb the entrance of the S'1 pocket, but that is purely speculative. Finally, it seems to be more difficult to fit MMP-14 pockets, perhaps in keeping with its transmembrane localization and domain structure.

4.2 Influence of the S'2 subsite on gelatinases inhibition

Introduction of an alkyl chain in the P'2 position of the indole ring leads to lower gelatinase inhibitory activity. Nevertheless, the selectivity for MMP-2 was pinpointed (Marcq et al., 2003).

On the contrary, the introduction of a phenyl group at the P'2 position (analogue **4**) enhanced the selectivity towards MMP-2 maintaining a high potency (IC_{50} = 0.092 nM). To the best of our knowledge, the large and solvent-exposed S'2 pocket could accommodate large and hydrophobic groups (LeDour et al., 2008). A good activity was found for MMP-1 and MMP-14 (IC_{50} = 0.244 and 0.601 nM, respectively).

4.3 Influence of the unprimed subsites on gelatinases inhibition

It is well documented that the hydroxamic acid is one of the most powerful ZBG, but its toxicity and low bioavailability triggered tremendous efforts to design other ZBG (Jacobsen et al., 2007, 2010). Of note, the zing binding group affinity is as follows: hydroxamate > retrohydroxamate > sulfhydryl > phosphinate > carboxylate > heterocyclic core. Nevertheless, a zinc binding group with lower affinity may be advantageous.

In this line, we have proposed various hydrazide and sulfonylhydrazide-type functions as potential ZBG. The sulfonylhydrazide derivative is responsible for the increased acidity of the NH close to SO_2 function allowing the H-bond to be formed with the catalytic glutamate residue (Augé et al., 2003, 2004).

The hydroxamate acts as a bidentate ligand with the zinc ion to form the distorded trigonal-bipyramidal coordination geometry. With respect to the design of new ZBG, a DFT (Density Functional Theory) study revealed different modes of chelation of the sulfonylhydrazide group (Rouffet et al., 2009).

The zinc ion was found to be ligated to three 4-Me-imidazoles used as mimetics of histidine imidazole moieties located in the MMP catalytic site in physiological conditions. The sulfonylhydrazide group could chelate the zinc ion in two different manners either in a bi- or tri-dentate mode.

In all cases, interaction between

i. the Zn^{2+} ion and the sulfonamide nitrogen was observed as well as,
ii. the Zn^{2+} ion with the oxygen atom of the sulfonylhydrazide carbonyl.

In the case of the tridentate mode, the third interaction involves one of the sulfonyl oxygen atom and the Zn^{2+} ion. Consequently, the bidentate conformation was more favourable (4 to 5 kcal/mol) and the sulfonylhydrazide function seemed to possess ideal zinc binding properties and also biodisponibility and stability.

Following these investigations, the sulfonylhydrazide group was incorporated into the galardin® backbone as zinc binding group. Among the synthesized subtituents, the *p*-bromobiphenyl group displayed a good potency for MMP-2 (LeDour et al., 2008). Then, based on our preliminary results further modifications of the P'1 (long alkyl chain) and the P'2 (phenyl group) substituents were introduced to increase the selectivity for MMP-2 (Figure 6).

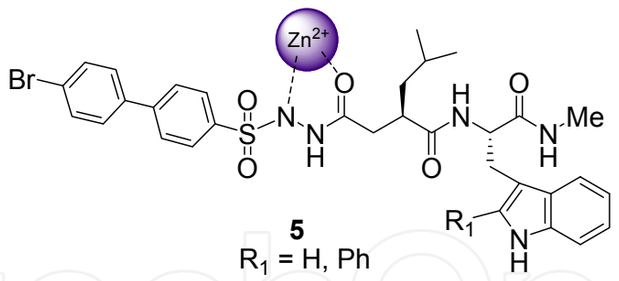


Fig. 6. Modifications of the P'2 group and sulfonylhydrazide function as ZBG.

Finally, a high potency for MMP-9 was obtained with the compound **5a** (or **5b**) with a small group such as an isobutyl (Table 5). Introduction of a phenyl group at the position 2 of the indole ring did not modify the activity. This could easily be explained taking into account the S'2 solvent-exposed pocket.

Compounds	R_1	MMP-1	MMP-2	MMP-3	MMP-9	MMP-14
5a	H	30	98	5800	3	20000
5b	Ph	350	247	53	18	1237

Table 5. Influence of the P'2 group and ZBG on the selectivity and potency of MMP inhibitors. IC_{50} values are expressed in nM.

Docking studies of **5a** confirmed the occupancy of the MMP-9 S'1 pocket by the isobutyl group, the S2 subsite by the *p*-bromobiphenyl group and the chelation of the sulfonylhydrazide to the catalytic site (Figure 7). It is known that the amino acid residues Glu412 and Asp410, located in the S2 subsite control the selectivity of MMP-2 and MMP-9, respectively.

In MMP-2, the amino acid residue Glu412 is able to form an H-bond with the substrate which could not be formed in MMP-9 presenting the amino acid residue Asp410 (Chen et al., 2003).

In our case, no H-bond can be formed with the hydrophobic *p*-bromobiphenyl group and the compound **5a** displayed selectivity for MMP-9.

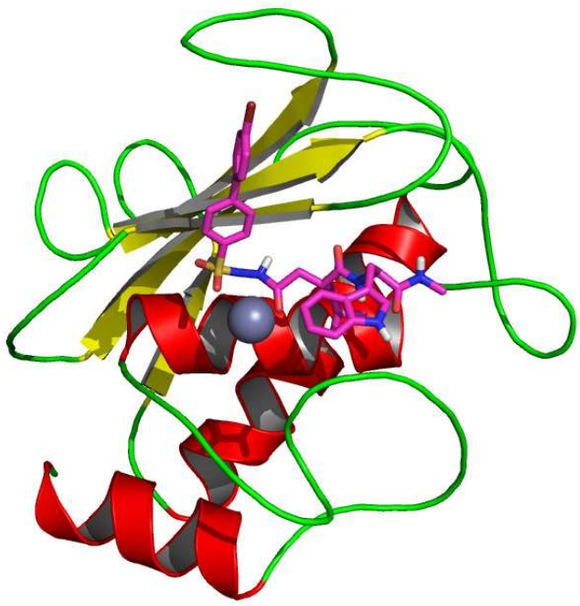


Fig. 7. Complex between MMP-9 and analogue **5a.** The MMP-9 secondary structure was represented in cartoon. The analogue **5a** is shown with sticks in which the C atoms are colored in magenta. Zn atom is displayed as a grey sphere.

Unfortunately, none of the P'1 (with an unsaturation and long alkyl chain or bulky substituent) and P'2 (with a phenyl group) modified galardin® derivatives exhibited increased inhibitory capacity and selectivity. Our docking data indicated that these compounds adopted a conformation in which sp2-hybridized carbon atom of the alkylidene side-chain led to steric hindrance impeding the entrance in the S'1 subsite. Consequently, when the S2 pocket is occupied, the primed subsites could not tolerate any large substituent and no synergistic effect could be obtained.

5. Control of elastolytic cascade by oleoyl-galardin®

Elastin degradation is at the genesis of cardiovascular disease as athero-arteriosclerosis and aneurysm formation, and pulmonary diseases as chronic obstructive pulmonary disease or lung cancer (Moroy et al., 2012; Muroski et al., 2008; Thompson & Parks, 1996).

Elastolysis requires the participation of serine- and metallo-elastases (Figure 8) which act through proteolytic cascades.

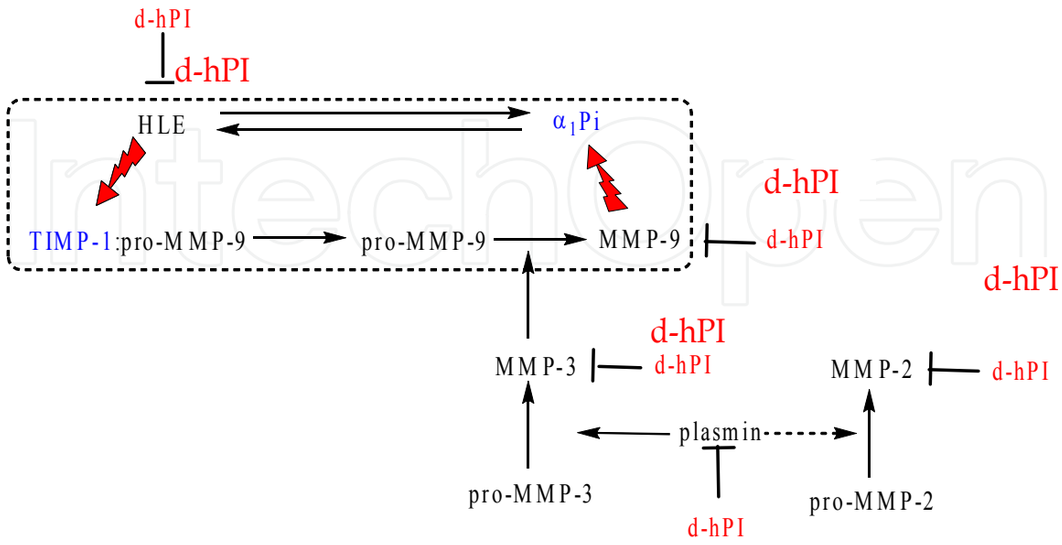


Fig. 8. Control of the serine (HLE)- and metallo-elastases (MMP-2, MMP-9) crosstalk in elastolysis by double-headed protease-MMP inhibitor (d-hPI).

Besides, a serine elastase as human leucocyte elastase (HLE) can degrade TIMP, and reversely MMP can hydrolyse serine protease inhibitor as α1 proteinase inhibitor (Nunes et al., 2011).

To control elastolysis, we thus attempted to design substances that could interfere with all actors of the depicted cascade. For that purpose, long chain-unsaturated fatty acids, as oleic acid, have been described to inhibit HLE (Hornebeck et al., 1985; Shock et al., 1990; Tyagi & Simon, 1990) and to impede plasmin-mediated prostromelysin-1 activation (Huet et al., 2004) as well as gelatinases activities (Berton et al., 2001). To that respect, we envisaged the synthesis of a double-headed protease-MMP inhibitor (d-hPI) able to block elastase and MMP activities. To that end, an oleoyl group was incorporated to galardin® at the P'3 position (Figure 9) (Moroy et al., 2011).

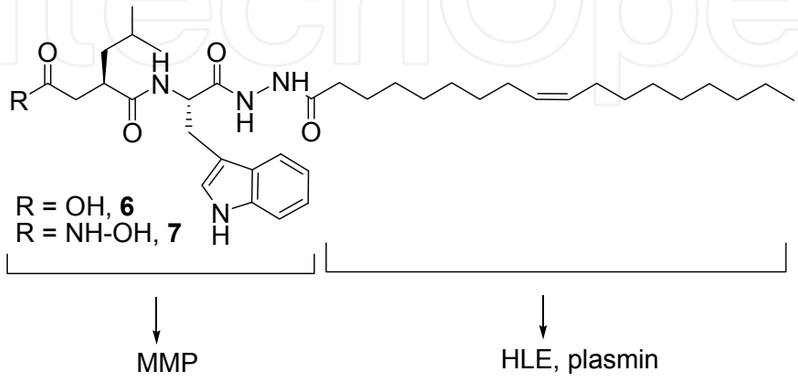


Fig. 9. Double-headed protease-MMP inhibitor (d-hPI).

Oleoyl analogues (carboxylic **6** or hydroxamic **7** acids) are more potent than oleic acid to inhibit MMP (Table 6). The hydroxamic acid **7** was found to improve the inhibitory capacity toward MMP-2 comparing to oleic acid.

Compounds	MMP-2	MMP-3	MMP-7	MMP-9	HLE	Plasmin
Oleic acid	4.3	-	6.5	6.4	3.0	3.5
6	0.5	0.5	0.07	0.7	0.6	0.7
7	0.1	0.05	0.6	0.04	8.7	8.3

Table 6. Inhibition of MMP and serine elastases by oleic acid and oleoyl-galardin® derivatives. MMP (*Kis* values are expressed in μM), HLE and plasmin (IC_{50} values are expressed in μM).

The molecular docking computations indicated that compound **7** is able to bind MMP-2 active site and unable to chelate Zn^{2+} ion in the active site (Figure 10).

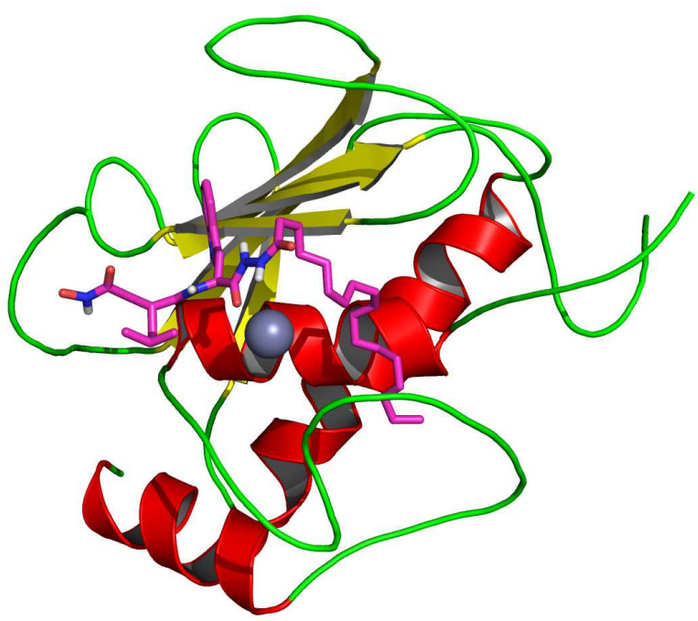


Fig. 10. Complex between MMP-2 and analogue **7**. The MMP-2 secondary structure was represented in cartoon. The analogue **7** is shown with sticks in which the C atoms are colored in magenta. Zn atom is displayed as a grey sphere.

Instead, the hydroxamic acid function forms a salt bridge with the N-terminal end of the amino acid residue Tyr110, while the long alkyl chain was inserted into the S'1 pocket as

was already demonstrated. The heterocycle is inserted into the S1 subsite where it interacts *via* an H-bond with the carbonyl group of the amino acid residue Ala194 peptide bond.

We analyzed the inhibitory capacity of oleic acid, analogues **6** and **7** towards HLE and plasmin activities. The compound **6** displayed high potency (IC_{50} = 0.6 μM) against HLE, but lower inhibition was observed with oleic acid and the analogue **7** (IC_{50} = 3.0 and 8.7 μM, respectively). Molecular docking computations indicated that the carboxylic function of compound **6** and oleic acid can form a salt bridge with the amino acid residue Arg217, but not compound **7** (not shown).

Almost the same values are found in the same order for the plasmin–mediated pro-MMP-3 activation. The lowest energy model of oleic acid with the kringle 5 domain is characterized by the presence of a salt bridge between the carboxylic function and the amino acid residue Arg512 of plasmin (not shown).

6. Control of gelatinases through impeding enzyme-substrate interaction

Another approach to control the activity of those enzymes consists in the regulation of exosite protein-ligand interaction. To that respect both Fn(II) [or CBD] and PEX domains are involved and, in keeping with data presented on Figure 2, blocking either Fn(II) or PEX function will prevent the catalytic function of gelatinases on protein substrates selectively. For instance, the proteolysis of collagen and elastin might be inhibited while maintaining intact the potential of gelatinases to cleave proteoglycans or several growth factors.

6.1 Fn(II) domains

Using recombinant Fn(II) domain as bait, a one bead one-peptide combinatorial peptide library was screened (Xu et al., 2007). A peptide displaying high sequence identity with the segment 715-721 in human α1(I) collagen chain was identified and proved to inhibit by > 90% gelatinolysis catalyzed by MMP-2 (Xu et al., 2009).

The unsaturated fatty acid such as oleic acid inhibited MMP-2 with K_i = 4.3 μM. Molecular modelling studies focus on the interactions localized at two sites on MMP-2: the fatty chain filled the S'1 pocket while the carboxylic acid group was exposed to the solvent. This result is in agreement with our previous works showing that the S'1 pocket could accommodate long alkyl side chains (LeDour et al., 2008; Moroy et al., 2007). The molecular docking computations identified the second site of the oleic acid interactions as the 3rd Fn(II) domain. The carboxylic acid function interacts *via* an H-bond with the phenolic group from the amino acid residue Tyr381, *via* a salt bridge with the guanidinium group of the amino acid residue Arg385 and *via* van der Waals interactions with the amino acid residue Leu356 while the unsaturated bond forms van der Waals interaction with the amino acid residues Phe355, Trp374, Tyr381 and Trp387 (Figure 11).

Another approach could be the use of a more specific inhibitor directed against the 3rd Fn(II) domain.

For this latter, an inhibitor with two carboxyl groups at each end of the alkyl chain should be efficient for targeting respectively the amino acid residue Arg385, as it was observed for

oleic acid, and the amino acid residue Arg368 that is also present on the rim of the hydrophobic pocket.

Interestingly, in the full MMP-2, the amino acid residue Arg368 forms a salt bridge with the amino acid residue Asp40 belonging to the propeptide domain. According to the binding mode of oleic acid, the size of the alkyl chain should be composed by 15 or 16 atoms of carbon such as (7*Z*)-hexadec-7-enedioic acid and (6*Z*)-pentadec-6-enedioic acid.

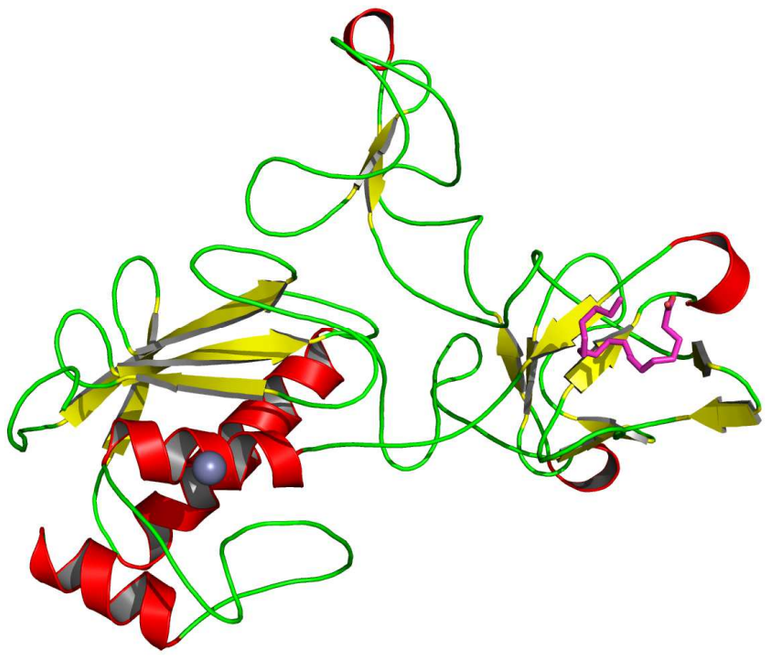


Fig. 11. Complex between MMP-2 and oleic acid. Oleic acid is anchored in the binding pocket at the surface of the 3rd Fn(II) domain.

6.2 Dual occupancy of the enzyme active site and Fn(II) domains

Another more complex strategy relies on the design of a dual occupancy of the enzyme active site and the exosite as Fn(II). That has been originally attempted with a coupled hydroxamate-based inhibitor to gelatin-like structures (Jani et al., 2005). No increase in selectivity or potency of those compounds towards gelatinases could be attained; it was attributed to the possibility that the Fn(II) and catalytic domains of enzyme are tumbling independently.

Thus, we have built a hypothetical inhibitor from the inhibitors previously studied. We have added alkyl groups until the hydrophobic pocket of the 3rd Fn(II) was reached: 19 are needed to interact with its rims, *i.e.* the amino acid residues Arg368 and Trp387. If we want to reproduce the binding mode of the oleic acid, 32 alkyl groups should be added (Figure 12). However, the size and the high flexibility of this kind of inhibitor could be problematic.

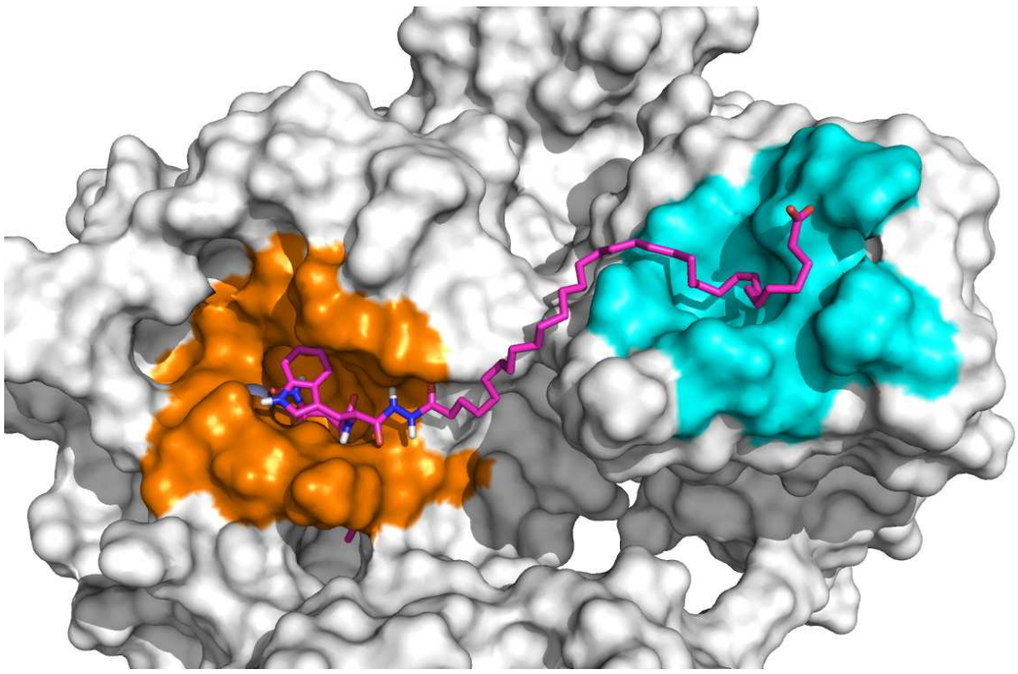


Fig. 12. Hypothetical model of an inhibitor able to bind both the catalytic site and the Fn(II) domain of the human MMP-2. The MMP-2 is displayed with its accessible surface area. The active site and the binding pocket on the 3rd Fn(II) domain are coloured in orange and in cyan, respectively.

6.3 PEX domains

Since gelatinases are critically involved in directing cellular invasion, interfering with PEX-integrins (receptors) interaction might be a nice alternative. As an example, the use of phage display identified a peptide that inhibits the association of MMP-9 PEX domain with the $\alpha_v\beta_5$ integrin, preventing proenzyme activation and cell migration (Björklund et al., 2004). More recently, a 20 mers peptide encompassing the PEX-binding tail region of C-TIMP-2 was found to inhibit the membrane-mediated activation in HT-1080 cells (Xu et al., 2011).

PEX domains play a crucial function in the non proteolytic function of gelatinases. As example, the PEX domain of MMP-9 is directly involved in the modulation of epithelial cell migration in a transwell chamber assay (Dufour et al., 2008). This domain also promotes B cell survival by interacting with $\alpha_4\beta_1$ and CD-44 receptors (Redondo-Muñoz et al., 2010).

7. Conclusion

General considerations need to be pinpointed at aims to give a novel expansion to the design of substances able to regulate MMP activity.

First, MMP as gelatinases are produced by nearly all cell types, but their cellular source may intervene in their function and activity. Proteolytic activity liberated by activated neutrophils is one pivotal element in the genesis and progression of aneurysms or chronic obstructive pulmonary diseases (Muroski et al., 2008; Thompson & Parks, 1996).

It has been demonstrated that pro-MMP-9 is produced by neutrophils as a free form *i.e.* not associated with TIMP-1 molecule, more readily activatable by enzyme as stromelysin-1 (Ardi et al., 2007). In addition, following activation, those cells release extracellular traps *i.e.* neutrophil extracellular traps (NET) formed by the association of chromatin and granule proteins; NET are enriched in neutral endopeptidases as neutrophil elastase and MMP-9 (Brinkmann et al., 2004). To that respect, the use of double-headed (HLE-MMP-9) inhibitors as oleoyl-galardin® might be of therapeutic value. Advantageously, as we recently documented, oleoyl moiety might be replaced by β-lactam (Moroy et al., 2012), a more potent and selective HLE inhibitor.

Although inflammation can orchestrate cancer (Kessenbrock et al., 2010), MMP-2 and MMP-9 intervene in several other stages of cancer progression. Both enzymes have been involved in promoting cell growth; MMP-2 is more linked to cancer cell invasiveness while MMP-9 may contribute to cell survival.

Up to now, one selective MMP-9 inhibitor is a monoclonal antibody binding to N-terminal part of catalytic domain (Martens et al., 2007). Thus, intuitively, in keeping with those distinct functions, the concept of selective inhibitor among gelatinases is emerging.

Their role in establishing a "metastatic niche" has also been delineated and their contribution in angiogenesis has been widely underlined.

However, paradoxically, MMP-9 can generate either pro- or anti-angiogenic signals (Figure 13).

On one side, it can

i. regulate VEGF bioavailability for VEGF-R2 receptor (Bergers et al., 2000),
ii. activate the basic fibroblast growth factor-2 (FGF-2) pathway (Ardi et al., 2009),
iii. generate elastin fragments *i.e.* elastokines with potent angiogenic activity (Robinet et al., 2005).

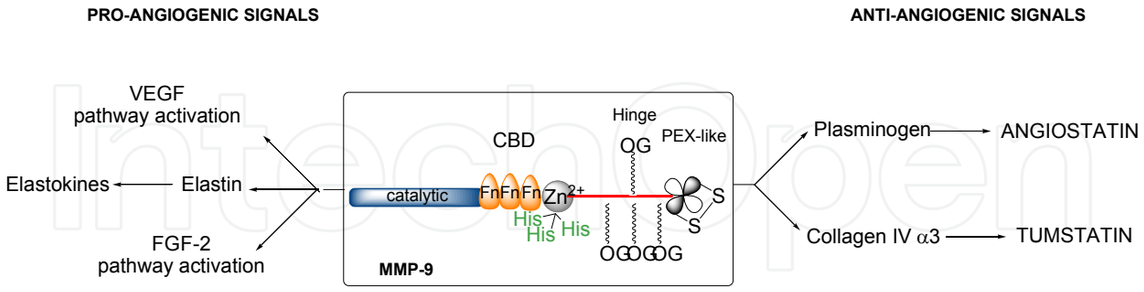


Fig. 13. The paradoxical function of MMP-9 in angiogenesis.

At the opposite, proteolysis of plasminogen and α_3 chain of collagen IV leads to the formation of angiostatin and tumstatin (Cornelius et al., 1998; Kessenbrock et al., 2010). Importantly, mice deficient in MMP-9 evidenced an increased-tumor growth which was attributed to lack of tumstatin formation (Hamano et al., 2003).

As mentioned, both gelatinases exerted their action at the pericellular environment, following binding of their PEX domain to receptors as $\alpha_v\beta_3$ for MMP-2 or CD-44 for MMP-9.

Peptide or chemical libraries can be developed at aims to impede MMP-2(PEX)-$\alpha_v\beta_3$ or MMP-9(PEX)-CD-44 interactions.

As one example, a bivalent derivatized dilysine tetraamide was isolated which proved to interfere with MMP-2/$\alpha_v\beta_3$ interaction and inhibit angiogenesis (Silletti et al., 2001). Possibly, this compound could be chemically modified to confer it additionally MMP-2 inhibitory activity (Bourguet et al., 2009).

One main problem related with the control of MMP in general and gelatinases in particular relies on the kinetics of production of those enzymes during the cancer course from initiation to metastasis formation. In other words, at what stage for one particular type of tumor do we need to incorporate MMPI in cancer treatment?

The development of imaging MMP activity using derivatized selective inhibitor will probably answer to this question. Several techniques have been already developed using Positron Emission Tomography (PET) with ^{18}F-labelled MMP-2 inhibitor (Furumoto et al., 2003), Single Photon Emission Computed Tomography (SPECT) with a ^{123}I gelatinases inhibitor (Schaffers et al., 2004), or the use of fluorogenic substrates bearing self quenched and near infrared FRET pairs (Scherer et al., 2008).

In our Federal Research Institute (IFR), we aim to develop hybrid nanoprobes build from MMPI and fluorescent nanocrystal quantum dots (QDs). Design and chemical synthesis of derivatives of galardin®, selective inhibitors of MMP-2, will be followed by their tagging with QDs. Photo- and chemical stability of QDs will enable long-term spatiotemporal tracking of the process of inhibition of MMP-2 with developed nanoprobe thus permitting understanding of physiological process of invasion of melanoma for example.

8. Acknowledgment

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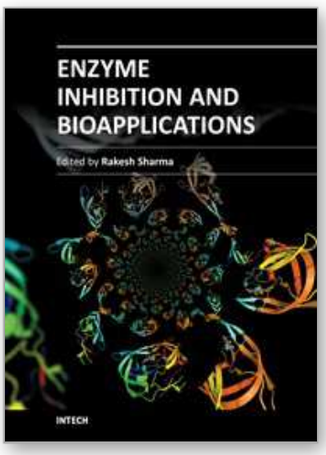

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Enzyme Inhibition and Bioapplications is a concise book on applied methods of enzymes used in drug testing. The present volume will serve the purpose of applied drug evaluation methods in research projects, as well as relatively experienced enzyme scientists who might wish to develop their experiments further. Chapters are arranged in the order of basic concepts of enzyme inhibition and physiological basis of cytochromes followed by new concepts of applied drug therapy; reliability analysis; and new enzyme applications from mechanistic point of view.

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