An efficient platform to study metalloproteases

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Introduction

Metalloproteases are a class of proteins involved in diverse and important biological phenomena such as cell proliferation, multiplication and migration, hormonal signalisation, angiogenesis, pathogen growth. Numerous inhibitors of this enzyme class are described and some are important therapeutic classes: such as ACE or NEP inhibitors. This context justifies the systematic search of metalloproteases implication in orphan pathologies, as well as their expression, purification and crystallisation, or the design and synthesis of targeted libraries of inhibitors. Having a strong background in the inhibition of parasitic metalloproteases (PfA-M1: Flipo et al. *J Med Chem* 2007) and their human orthologues (APN: Flipo et al. *Bioorg. Med. Chem.* 2007) our group has designed and synthesized Zinc ligands aimed at being tested on a panel of metalloproteases.
The goals of the project are **1/** finding inhibitors of the chosen targets, **2/** validating these targets *in vitro* and *in vivo* in cellular or animal models, **3/** developing new drug lead series.
The designed panel of metalloproteases from different sub-classes allows us to elucidate the essential features of binding of a chemical series to this family of targets. Furthermore, the matrix of results helps finding the determinants of selectivity within a chemical class. At last all these results help to understand the function of the protein and hopefully to identify leads.

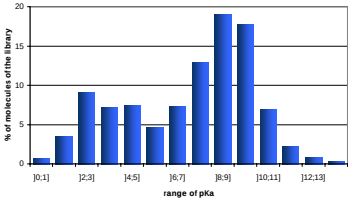

Figure1: pKa repartition for molecules in the Current Medicinal Chemistry database (CMC). **[1]**

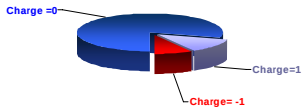

Figure 2: Acidic compounds in commercial libraries as the number of compounds with a given charge at physiological pH. **[2]**

Library of potential metalloprotease inhibitors

When targeting metalloproteases, a Lewis base (Zinc Binding Group ZBG) is critical for binding to Zinc ion.
One of the most classical ZBG is the carboxylate ion. The carboxylic acid function and its bioisosters like tetrazole is a key pharmacophore of numerous drug families including NSAIDs, Sartans (AT1antagonists) and Glitazones (PPARgamma agonists) that target different protein families. Hadjuk & coll. have identified indeed carboxylic acid function to be a privileged structure.
Nevertheless, despite the functional importance of this pharmacophore, analysis of a bioactive compounds database (MDL® CMC)**1** reveals that acidic molecules are underrepresented (Figure 1). This is also the case in the commercial libraries (Figure 2)**2**. This may in part be due to the relative difficulty of synthesis of such compounds.

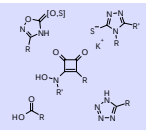

Figure 3 : Our chemical sub-library of Zinc-Binding Groups.

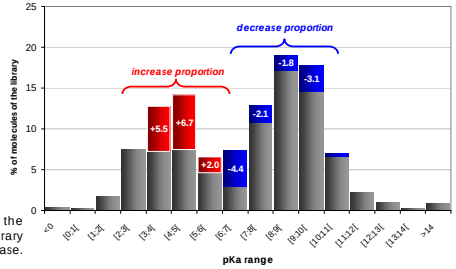

Figure 4 : pKa ranges for the molecules of our whole library compared to CMC database.

In this context, we designed and synthesized a library of 2,000 metalloproteases inhibitors, containing carboxylic acids and compounds bearing non conventional Zinc-binding groups (Figure 3).
This allowed enhancing the proportion of acidic compounds in our global library compared to commercial databases (Figure 4). Only the sublibray of 2,000 acidic compounds was tested in the following metalloprotease panel. Enhancement of the proportion of acidic compounds is delimited by white dots in the corresponding pKa range series. As can be seen increase range from 2 to 6%.

	ACE	ACE2	ADAMTS-5	NEP	IDE
ECNumber	3.4.15.1	3.4.24.B1	3.4.24.B12	3.4.24.11	3.4.24.56
Enzymatic class (MEROPS)	M2	M2	M12	M13	M16
Type	dicarboxypeptidase	carboxypeptidase	endopeptidase	endopeptidase	endopeptidase
Some synonyms	angiotensin-converting enzyme; CD143	angiotensin-converting enzyme-2	ADAMTS-11 ; aggrecanase-2	CD10 ; atriopeptidase; enkephalinase; kidney-brush-border neutral peptidase	insulin protease; insulin-degrading enzyme; insulinase; insulysine
Therapeutic interest	Renin-Angiotensin system	Renin-Angiotensin system & SARS virus entry receptor	Osteoarthritis	CV diseases and many others like FSAD	to be explored
Inhibitors	Marketed : Enalapril, Captopril,...	MLN4760	Numerous series under developement	Marketed : thiorphan	None disclosed
RX	✓	✓	✓	✓	✓
Collaboration			Pr. Nagase (Imperial College, London)		Pr. Tang (U. Chicago)

Table 1 : 5 first targets of our metalloprotease panel.

Metalloproteases panel

An essential condition for the selection of an enzyme in our panel was its implication (known or putative) in the physiopathology of a disease for which study models exist (Table 1). Other parameters drove our choice such as

- 3D structure availability
- Knowledge of endogeneous or synthetic substrate
- Known inhibitors
- Commercial availability of the enzyme
- Various enzyme classes

We selected first our targets based on their involvement in the physiopathology of diseases for which a relevant biological model exists. In particular, ACE2 was selected since it is a new enzyme discovered in 2000 and its implication in the Renin Angiotensin system is not yet fully understood. As a consequence we also selected its homolog ACE. Moreover this enzyme serves for us as an index of promiscuity of our library.
ADAMTS-5 is a well known enzyme that is a validated target for osteoarthritis. NEP is a known enzyme for the treatment of cardiovascular diseases and more recently for ACNE and Femal Sexual Arousal Disorders. At last Insulysine, IDE, was selected as a potential target in Diabetes and Alzheimer's disease. Though it is known for long, no non peptidic inhibitors are so far described. It is an interesting protease since it has a promiscuous expression and cleaves several biologically important peptides.
IDE and NEP are of high interest because they constitute nice examples of the cryptidase structural family which stands for proteases with a large catalytic cavity (crypt) as defined by Malito et al.
Several other metalloproteases were included in the panel like APN (Neutral endo-peptidase) and carboxypeptidase A for validation of new Zinc-Binding Groups and selectivity. Other metalloproteases from other organisms can be incorporated as well in the panel, to target infectious diseases. That is the case with PfAM1, the plasmodial neutral aminopeptidase (Flipo et al. 2007).

Screening conditions

Screening conditions are summarized in Table 2. Various protocol types were chosen for the 5 enzymes. For example, ADAMTS-5 was screened in Elisa. **[3]**

Enzyme (Provider)	ACE hACE (R&D system ™)	ACE2 hACE 2 (R&D Systems™)	ADAMTS-5 hADAMTS-5 (Chemicon™)	NEP hNEP (R&D Systems™)	IDE hIDE (Pr Tang)
Enzyme Expression and Tissue	Human recombinant ACE, residues 30 to 1261) expressed in a murine myeloma cell line, NS0	Human amino acid residues 1 - 740 of the recombinant ACE (Tipnis; S.R. et al., 2000, J. Biol. Chem.) expressed as a secreted protein with a COOH-terminal His tag in a murine myeloma cell line, NS0.	Human ADAMTS-5 Δ625-930 (baculovirus expression system) contains the catalytic domain, the disintegrin domain and the thrombospondin type-1 motif of full-length ADAMTS-5 followed by a C-terminal His6-tag.	Human CD33 Signal Peptide (Met 1 - Ala 16); Mature Human Neprilysin (Tyr 45 - Trp 743)	Human recombinant wt insulysin
Enzyme concentration	1/2400 dilution from commercial stock	100 pM	10 ng/ml	200 ng / mL.	0.05 µg / 80 µL
Substrate	Abz-Gly-p-nitro-Phe-Pro-OH.TFA (Bachem™)	MCA-Ala-Pro-Lys(DNP)-OH (NeoMPS ™)	QTVTWPDMELPLPRNITEGEARGSVILTVKPIFEVSPSPL(biotinyl)K (Synthesized)	N-Dansyl-D-Ala-Gly-p-nitro-Phe-Gly (Sigma™)	ATTO 655- Cys-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Trp (Synthesized)
Km (µM)	180	50	NA	350	>100
Substrate Conc (µM)	100	50	NA	200	5µM due to low solubility
Reference Inhibitor	Captopril	EDTA / MLN4760	EDTA 50µM	DL thiorphan	EDTA 2mM
IC50 or Ximb mean	11 (± 7) µM	6.5 (±0.1) µM / 0.44 nM	80 (±1)%	1 (±0.3) nM	79 (±6)%
DMSO%	0.3	1.1	1	1	1
Test Conc (µM)	10	11	30	30	30
Z'Facteur	0.80	0.83	0.40	0.68	0.75
Assay type	homogeneous	homogeneous	Elisa	homogeneous	homogeneous
Detection	FRET	FRET	Absorbance	FRET	FRET
Wavelength ex	320	320	NA	340	635
Wavelength em	420	405	450	535	750
Pre-Incubation (min)	10	10	10	10	10
Rxn Time (min)	330	120	180	120	40
Temperature (°C)	37	rt	37	rt	37
Buffer	75 mM NaCl, 0.5 µM ZnCl2, pH 7.4	50 mM MES, 300 mM NaCl, 10µM ZnCl2, 0.01% Brij-35, pH 6.5	50 mM Tris, 5 mM CaCl2, 150 mM NaCl, 0.05% Brij35, pH 7.5	Hepes 50 mM, NaCl 100 mM, pH 7.4	Hepes 50 mM, NaCl 100 mM, pH 7.4

Table 2: Screening conditions

	ACE	ACE2	ADAMTS-5	NEP	IDE
N	1766	1766	2006	2051	2051
Nb of hits	23	8	59	11	24
Nb of clusters	3	1	17	1	3
ZBGs	3	4	5	6	3
Hit Confirmations	9	5	21	7	11
Validated hits	2	2	18	4	2

Table 3: Screening results

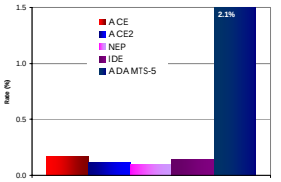

Figure 5: % of hits (above 80% inhibition) for each target

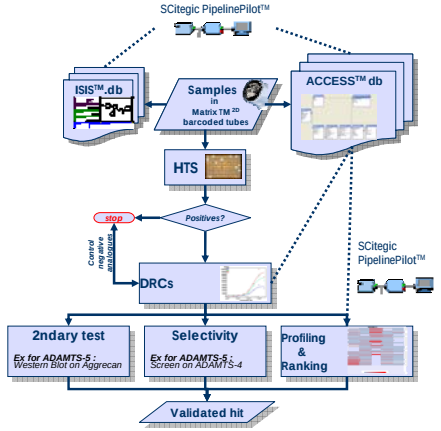

Figure 6: Decision tree for hit validation

Screening results & Validataion

Table 3 and Figure 5 summarize the screening campaigns results. First, hit-rates obtained for the targeted library are good. They are slightly higher for the target ADAMTS-5. This result is partially due to lower Z' factor, a consequence of the Elisa assay's lower reproducibility. Nevertheless, lower attrition rate in this assay resulted with 18 validated hits. Interestingly, these screenings allowed the identification of low micromolar or submicromolar (100nM-1µM) hits for the targets ADAMTS-5, NEP and IDE. These compounds are under Hit-to-Lead optimization. Secundary tests were as often as possible developed to confirm activity and finalize characterization of primary hits (Figure 6).

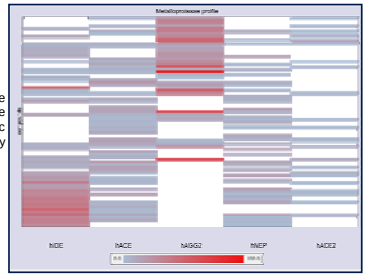

Figure 7: Excerpt of the metalloprotease profile of our potential Zinc binding library

Conclusion

We developed a screening tool for metalloproteases of therapeutic interest that is composed of a well-designed library and a series of assays. This tool allows the profiling of a library using a chemical biology approach. Finally as can be seen from our Metalloprotease Profile (Figure 7) done with PipelinePilot from Scitegic™ (a division of Accelrys™), our library is generally targeted towards metalloproteases without being focused, thus allowing to reach selectivity towards a specific target. This further justifies the exclusion of more promiscuous hydroxamates as Zinc Binding Group.

Notes
1. MDL® Comprehensive Medicinal Chemistry (CMC-3D) is the electronic version of the 6th volume of « Comprehensive Medicinal Chemistry » Pergamon Press 1990. Note that the left-hand side group of molecules includes weak bases that are non-protonated at pH 7.4 (like pyridine) and that the right-hand side group includes weak acids non-ionized at pH 7.4 (like hydroxamates).
2. Commercial libraries analyzed are AsinexTM(Gold) et ChembridgeTM.
3. The assay was performed as previously described with minor modifications (Miller et al, Analytical Biochemistry, 2003). Briefly, an immobilized 41-residue peptide was used as a substrate for ADAMTS-5. Aggrecanase-mediated hydrolysis at the Glu/Ala site resulted in an immobilized product that reveals an N-terminal epitope (NH3+-Ala-Arg-Gly-Ser-Val), recognized by the specific antibody BC-3. The HRP-conjugated secondary antibody (goat anti-mouse IgG peroxidase conjugated is used with the peroxidase substrate TMB for revelation at 450nm of the activity.

References

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