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Review

Phosphinic peptides as zinc metalloproteinase inhibitors

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Abstract. Solid-phase synthesis of phosphinic peptides was introduced 10 years ago. A major application of this chemistry has been the development of potent synthetic inhibitors of zinc metalloproteases. Specific properties of the inhibitors produced in recent years are reviewed, supporting the notion that phosphinic pseudo-peptides are useful tools for studying the structural and functional biology of zinc proteases.

Key words. Phosphinic; inhibitors; zinc proteases; transition-state; ACE; MMPs.

Introduction

Replacement in peptides of one peptide bond (CO-NH) by a phosphinic acid moiety, $-PO_2-CH_2-$, results in a new family of pseudo-peptides, termed phosphinic peptides. The chemical structure of phosphinic peptides shares some features with that of intermediates formed during the hydrolysis of peptides by proteolytic enzymes (fig. 1). Based on this analogy, phosphinic peptides have been proposed to represent a source of synthetic protease inhibitors [1–3]. Exploitation of this new family of pseudo-peptides has led to the identification of potent inhibitors, especially of zinc proteases [4, 5]. Potent phosphinic peptide inhibitors of acidic proteases, like the human immunodeficiency virus (HIV) protease, have been also reported [6]. However, given the high number of metalloproteases in comparison to acidic proteases, the former enzymes are the major targets for phosphinic peptides. Analysis of the human genome has led to the identification of 553 genes that encode proteases, the metalloprotease family being the largest, with 186 members (only 21 acidic proteases have been identified so far) [7]. Proteases are no longer viewed as nonspecific enzymes involved only in the degradation of dietary proteins, but are recognized as being involved in essential functions in all living animals [8]. The development of specific inhibitors, among other strategies, will be a valuable asset in probing the repertoire of functions mediated by these enzymes. Furthermore, these compounds can be the starting point in developing new therapeutic drugs, as illustrated by the success of protease inhibitors in the treatment of cardiovascular diseases and acquired immunode-

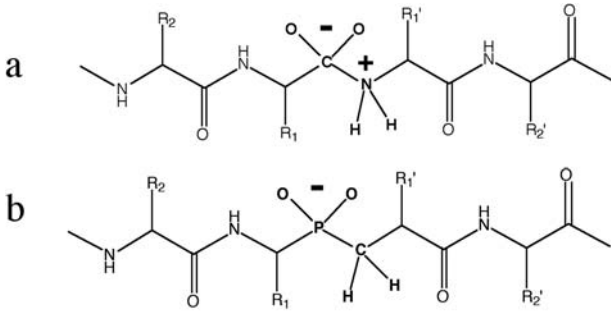

Figure 1. Analogy between the transient structure of a peptide substrate during hydrolysis (*a*) (transition state) and a phosphinic peptide (*b*).

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ficiency syndrome (AIDS). In the particular case of the zinc proteases, full exploitation of phosphinic peptide chemistry should yield potent and highly specific inhibitors for each member of this family. However, to help in the assignment of protease functions, beside the requirement for high selectivity, such inhibitors will also have to be stable enough to allow their use in cells, tissues or animals. This review focuses on the properties of phosphinic peptides that make this class of inhibitors suitable for studying zinc metalloproteases, both at the molecular and functional levels. Only studies on phosphinic peptides published after the year 2000 are considered, as surveys of the literature before this date are available [4, 5], and only results dealing with phosphinic peptides meeting the definition given above are discussed. The phosphinic moiety (PO_2CH_2) appears in various non-peptide structures, but this topic is outside the scope of the present review [9, 10].

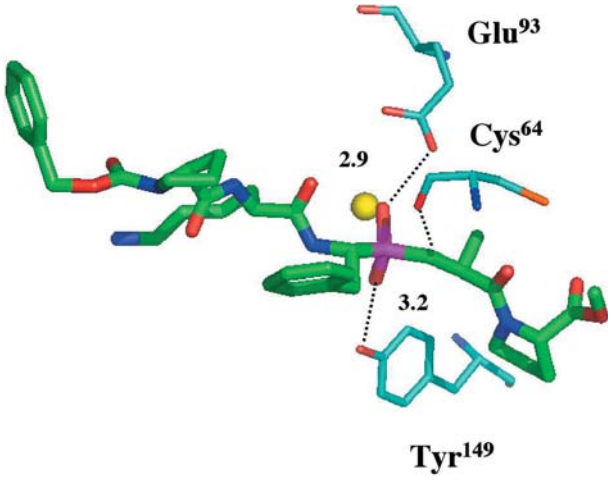

Figure 2. Detail of the interactions between the phosphinic moiety of a phosphinic peptide inhibitor (green) and three residues of the astacin active site (blue). Glu^{93} and $Tyr1^{49}$ contribute to transition-state binding through H-bonding interactions. Glu^{93} included in the HExxH motif is presumed to act as the general base during substrate hydrolysis. The zinc atom is in yellow.

Analogy with the transition state

The hypothesis that phosphinic peptides could mimic the structure of the substrate in the transition state was supported indirectly by observations showing that phosphinic peptides are extremely potent inhibitors of zinc proteases. For example, a Ki value of 70 pM was determined for the inhibition of the 24-15 zinc endopeptidase by the phosphinic peptide, $Z_{(R,S)}Phe\Psi(PO_2^-CH_2)_{(R,S)}Ala$-Arg-MetOH (a mixture of four diastereoisomers) [11]. Direct evidence supporting the transition-state analogy arose from the resolution of the crystal structure of zinc proteases in complex with phosphinic peptides. Thus far, only two crystal structures of mononuclear zinc proteases (astacin and MMP-11) in complex with a phosphinic peptide are available [12, 13]. These structures reveal that the phosphoryl group of the inhibitor mimics a substrate water-attacked carbonyl group and interacts with the electrophilic zinc atom of the enzyme active site (fig. 2). Moreover, the methylene group of the phosphinic moiety ($PO_2^-CH_2$) is within hydrogen-bonding distance and points toward the Cys^{64} carbonyl group from the protease. A similar interaction is assumed to take place between this protease carbonyl and the NH group of the cleaved peptide bond. In the astacin complex, the phosphoryl group is stabilized through two additional interactions involving glutamate and tyrosine side chains [12]. The glutamic acid (Glu^{93}) included in the HExxH motif is thought to act as the general base during catalysis, transferring a proton from the catalytic water to the amide of the scissile peptide bond. Interestingly, mutation of Glu^{93} and Tyr^{149} drastically reduces astacin activity, in agreement with the structure data, pointing to a role for these two residues in transition-state stabilization [14]. The crystal structure of astacin in complex with a hydroxamate inhibitor, a non-transition-state analogue, has also been solved [12]. Remarkably, it differs from the structure solved with the phosphinic peptide inhibitor. Few differences in structure are observed between astacin in the free state and bound to the hydroxamate inhibitor. This contrasts with the structure of astacin in complex with the phosphinic peptide inhibitor, which reveals an overall hinge-like movement, by which the distance between the lower and upper subdomain of astacin, defining the active-site cleft, is decreased by 1 Å, as compared to the free astacin structure. The conformational shift induced by phosphinic peptide binding, and the resulting structure of astacin, are crucial observations in unveiling the molecular events involved in astacin catalysis. Altogether, these results illustrate the unique value of phosphinic peptides, as transition-state analogues, in the study of the molecular machinery of zinc proteases.

Chemistry

Despite the development of phosphinic peptide chemistry on solid supports [15, 16], allowing either parallel or combinatorial synthesis of these compounds, few laboratories are actively exploiting this family of pseudopeptides [17–19]. Classical approaches to the synthesis of phosphinic peptides rely on the preparation of a pseudo-dipeptide phosphinic synthon, bearing suitable protecting groups, which can be used as a standard amino acid in a classical solid-phase peptide protocol (fig. 3a) [15, 16]. The tedious preparation of phosphinic synthons, which requires many steps of synthesis and

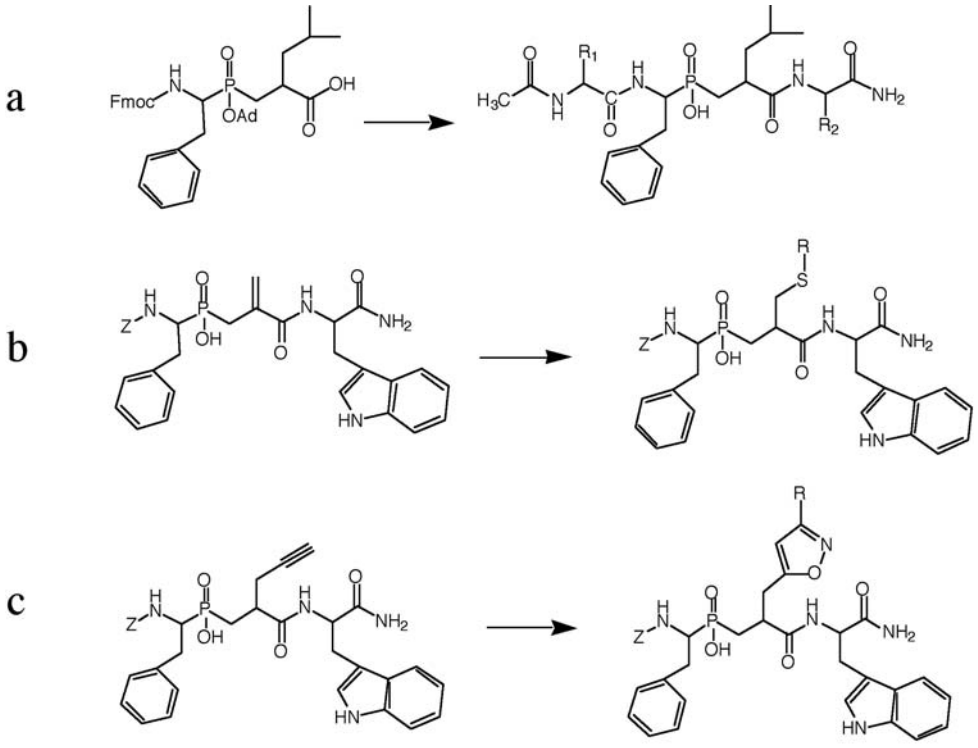

Figure 3. The use of phosphinic synthon leads to libraries containing a unique phosphinic moiety (*a*). In (*b*) and (*c*), a peptide template is diversified leading to phosphinic peptides containing various side chains in the P_1' position.

purification, limits the development of phosphinic peptides. Moreover, the use of this synthon strategy yields phosphinic peptides in which the nature of the residues framing the phosphinic moiety ($PO_2^-CH_2$) is the same for all compounds. As the nature of these residues, corresponding to the P_1 and P_1' positions of the inhibitors, is of critical importance for inhibitor potency and selectivity, this strategy may fail in the development of highly specific inhibitors. Despite this limitation, in some cases this strategy has yielded remarkable results [11, 20, 21]. To overcome these different hurdles, new chemical approaches have recently been developed. These approaches rely on diversification at the P_1 and P_1' positions of the inhibitors at the final step of the synthesis [22, 23]. Thus, phosphinic peptides harboring suitable functional groups, either in the P_1 or P_1' positions, are first synthesized by solid-phase synthesis (fig. 3b, c). Then, at the final step, the functional group can be modified by different chemical means to produce a variety of side chains at the P_1 or P_1' positions of the inhibitors. Development of the chemistry along these lines will in the near future enable the production of libraries from other libraries. In the first library, all the positions of the inhibitor, excepting the P_1 or P_1' position, could be diversified by combinatorial chemistry. From this library, new libraries can be prepared by introducing a particular side chain at the P_1 or P_1' position.

Selectivity of phosphinic peptide inhibitors

Highly potent inhibitors have been reported for the mononuclear zinc aminopeptidases, endopeptidases and proteases [4]. But as mentioned in the introduction, the main challenge in the development of synthetic inhibitors concerns their capacity to differentiate closely related families of targets. For instance, the family of the zinc matrix metalloproteinases (MMPs) contains 24 members (human genome) [24], all exhibiting extremely similar active sites, thus explaining the difficulty of identifying selective inhibitors for this family of proteases [25, 26]. Besides the similarity between the active site structure of MMPs, the class of inhibitors itself can play a central role in the selectivity, especially for the zinc protease inhibitors. In fact, most of the zinc protease inhibitors that have been developed to date exploit the presence of the zinc atom in the enzyme active site. Thus, strong zinc-chelating groups, like the thiolate (S^-) or hydroxamate ($NH\text{-}O^-$) moiety, have been utilized in the design of zinc protease inhibitors. Whereas this strategy has proven very effective in developing potent inhibitors of zinc proteases, one drawback has been the poor inhibitor selectivity of such compounds. Indeed, the binding of this class of inhibitors to their targets is mainly governed by the strength of the interaction between the zinc atom and the chelating group. This was illustrated by studies showing

that hydroxamate inhibitors, developed for MMPs [26], also turned out to be highly and sometimes more potent inhibitors of TACE (tumor necrosis factor-a converting enzyme), a zinc protease belonging to the ADAMs (membrane zinc metalloproteases containing a disintegrin and metalloprotease domain) family [27, 28]. Given the similarity between the active site of TACE and MMPs, this situation is to be expected. But even unrelated zinc proteases could be potently inhibited by such compounds. For instance, hydroxamate MMP inhibitors were observed to potently block the zinc metalloprotease domain of anthrax toxin, the so-called lethal factor [29]. As compared to hydroxamate compounds, phosphinic peptides were often observed to display lower potency, suggesting that the phosphinic group is a weaker zinc-chelating group than the corresponding hydroxamate group (fig. 4) [30, 31].

Another major difference between thiolate or hydroxamate and phosphinic peptides is related to their chemical structures. Thiolate and hydroxamate chemistry only provides access to left- or right-hand-side inhibitors, thus allowing probing of either the primed or unprimed side of the active-site cleft (fig. 5). Phosphinic peptide chemistry offers the possibility of developing inhibitors able to interact with both the primed and unprimed side of the active-site cleft, allowing optimization of inhibitor selectivity by diversification of the P and P′ positions of the inhibitors. This strategy allowed the development of potent phosphinic inhibitors of MMPs that do not interfere with TACE (fig. 5) [32]. Exploitation of both the primed and unprimed sides of the active-site cleft proved also to be an efficient strategy in identifying inhibitors able to differentiate the two closely related active sites of somatic ACE (angiotensin converting enzyme). ACE inhibitors have been on the market for more than 20 years, with successful applications to treat various cardiovascular diseases. Most inhibitors currently used clinically interact with the two active sites of somatic ACE to a similar extent [33]. In order to unveil the functional roles of these two active sites, projects were developed to identify selective inhibitors of each ACE active site. Screening of libraries containing phosphinic peptides, designed to probe both the primed and unprimed side of the ACE active sites, resulted in the identification of the first selective inhibitor of the N-domain ACE active site (RXP407, fig. 5c) [21]. As compared to most conventional ACE inhibitors, RXP407 possesses an unusual structural feature, an aspartate in the P_2 position. The crystal structure of germi-

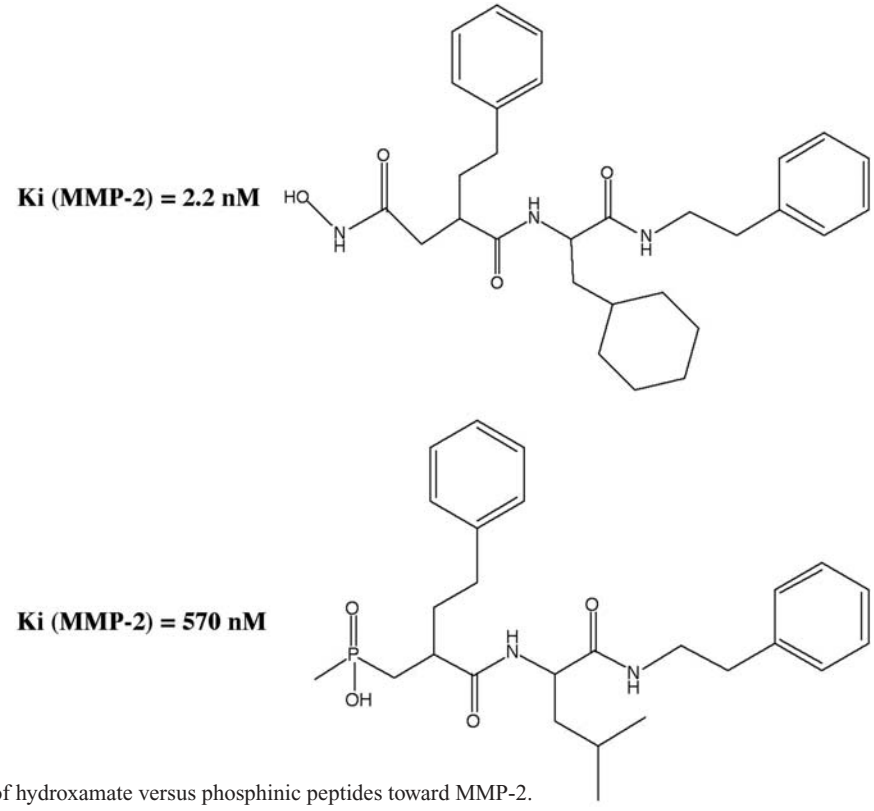

Figure 4. Potency of hydroxamate versus phosphinic peptides toward MMP-2.

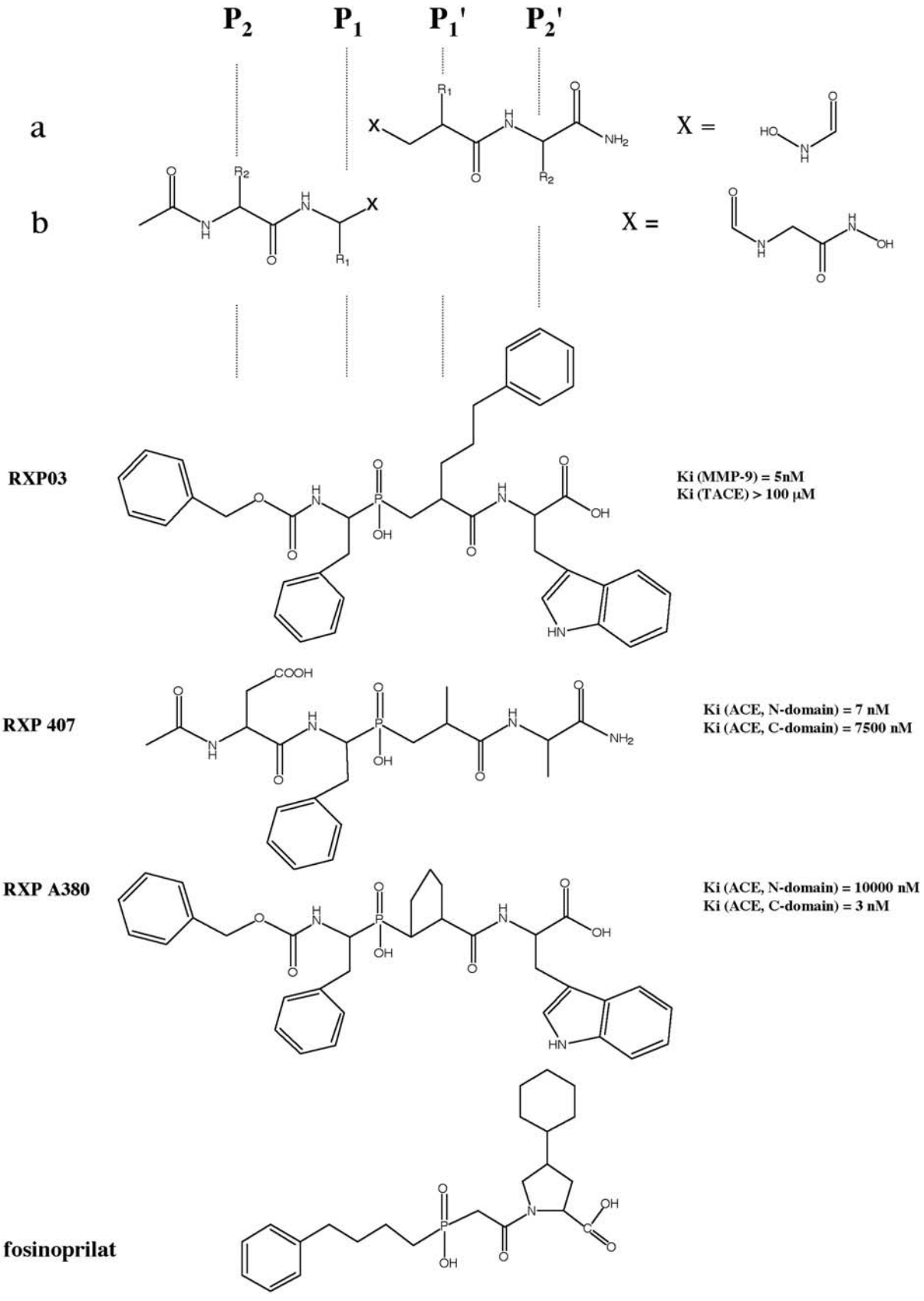

Figure 5. Hydroxamate chemistry allows development of (*a*) right-hand-side or (*b*) left-hand-side inhibitors interacting, respectively, with the primed or unprimed side of the active site cleft. Phosphinic chemistry leads to inhibitors interacting with both sides of the active site.

nal ACE has recently been reported [34]. This form of ACE contains only one active site, corresponding to the C-domain of somatic ACE. Based on this structure, the authors developed a model of the N-domain, in which the RXP407 was docked. This study indicates the presence in the N-domain S_2 subsite of an arginine near the aspartate of RXP407 [33]. Interestingly, this arginine is replaced by a glutamate in the C-domain, in the corresponding S_2 subsite, an observation that may explain why the C-domain binds RXP407 with a much lower potency. An extremely selective inhibitor of the C-domain was also developed [35]. This phosphinic peptide (RXPA380, fig. 5d) interacts with the S_1 to S_2' subsites of the enzyme active cleft. As with astacin, it is expected that these selective inhibitors will be useful probes in disclosing the molecular determinants responsible for the selectivity of the N- and C-domain ACE active sites.

Development of rather long phosphinic peptides covering the whole enzyme active-site cleft might be useful in developing MMP inhibitors able to differentiate one particular MMP among others [18]. To date, most of the inhibitors that have been developed bind to the S_1 to S_2' subsites of these enzymes. Identification of more selective compounds might require the design of compounds able to interact with distal subsites of MMPs, a strategy that could be easily developed with phosphinic peptide chemistry. Developing inhibitors with molecular weight higher than 600 will strongly limit the use of such inhibitors for in vivo experiments. However, if highly selective, such inhibitors can be useful in demonstrating the correctness of some hypotheses on the function of the targeted proteases. Functional tests with such inhibitors will be contingent on important requirements, such as their relative in vivo stability and their ability to interact with their targets under these conditions.

In vivo studies

As far as the targeting of zinc proteases in vivo is concerned, it is important to underline that most of the known zinc proteases are expressed outside the cell, either in a membrane-bound form located at the membrane surface, or as a soluble form that can interact with the different proteins of the extracellular matrix or with a receptor. Thus, as compared to targets located inside the cell, zinc proteases are rather accessible, as their targeting does not require crossing of the cell membrane. Absorption through the intestinal barrier can be overcome by intraperitoneal (i.p.) or intravenous (i.v.) administration of inhibitor to animals. Radioactive labeling of phosphinic peptide inhibitors allowed the study of their pharmacokinetics in animal models. Despite the presence of peptide bonds in their structure, RXP407 and RXPA380 were found to be fairly stable in vivo, showing that these pseudo-peptides are not efficiently hydrolyzed by nonspecific peptidases [21, 35]. As no major metabolites were detected, this also suggests that these compounds are protected from modifications by cytochrome P450 enzymes of the liver. This remark holds particularly for RXPA380, which is mainly eliminated by hepato-biliary excretion. Indeed, 24 h post-injection, RXPA380 was recovered intact in the animal feces (fig. 6) [35]. Possible explanations for the unexpected absence of rapid metabolism of phosphinic peptides under physiological conditions could be the strong negative charge of the phosphinic group. This negative charge may prevent both the interaction of these pseudo-peptides with nonspecific peptidases and their uptake by hepatocytes. The in vivo stability of RXP407 and RXPA380 made possible their use in probing the function of the two ACE active sites in animal models. This study allows a functional role to be proposed for the two ACE domains and demonstrates that selective ACE inhibitors can lead to the development of a novel class of therapeutic drugs [36]. The phosphinic peptide inhibitor of MMPs, depicted in figure 4c (RXP03), was also proven to be fairly stable in vivo. In vivo RXP03 activity was first evaluated in a rat model of liver ischemia. Hepatic ischemia, occurring during liver transplantation or cardiogenic shock, induces the expression of several MMPs, an event suspected to be associated with increased necrosis of hepatic cells. In agreement with this proposal, RXP03 treatment was observed to reduce in rat liver injury triggered by ischemia [37]. In a rat model of abdominal aortic aneurysm, radiolabeled RXP03 was used to visualize aneurysm lesion areas on aortic transverse sections. Indeed, aneurysm lesions are associated with overexpression of MMPs [38]. Animals bearing aneurysms were treated with radiolabeled RXP03 (i.v. injection); 4 h after the inhibitor injection animals were sacrificed and abdominal aortic sections were prepared. Radioactivity distribution on these frozen sections were analyzed by radioimaging. As shown in figure 7, radiolabeled RXP03 distributes mostly in the aneurysm lesions (7b) and not in the healthy adjacent parts of the same aorta (7a). Recently, RXP03 was used to image tumors in mice, a tissue also characterized by overexpression of MMPs. These studies are representative of the applications that can be developed with phosphinic peptides. They demonstrate that phosphinic peptides are accessible useful tools for performing pharmacological studies and testing functional hypotheses. Actually, only one phosphinic peptide (fosinopril, an ACE inhibitor) is used in the clinic to treat hypertension.

Other targets

The main targets of phosphinic peptide inhibitors reported in the literature are mononuclear zinc proteases.

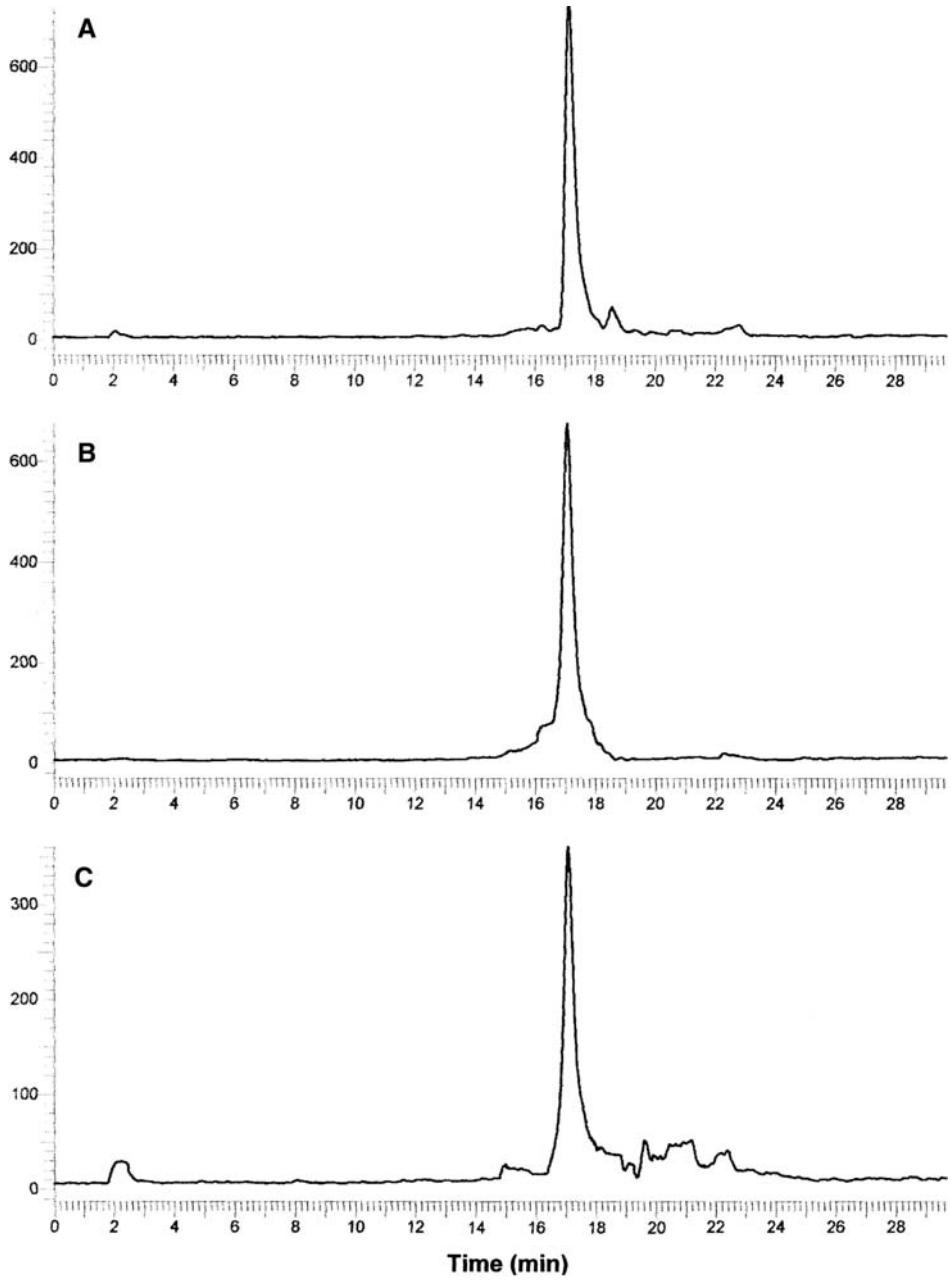

Figure 6. Radio-HPLC (high pressure liquid chromatography) profiles corresponding respectively to (*A*) pure radiolabeled RXPA380 in water after 24 h at room temperature, (*B*) feces extract obtained 24 h after the injection of RXPA380 in mice and (*C*) plasma extract obtained 30 min after the RXPA380 injection in mice.

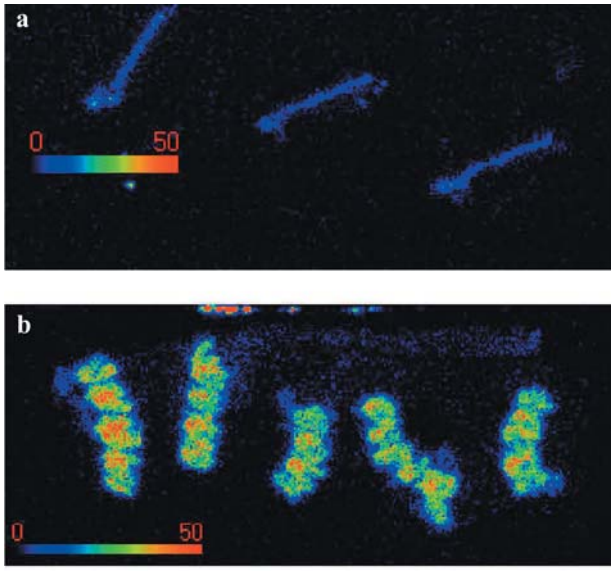

Figure 7. Radioimaging of tritiated RXPO3 binding in normal aortic sections (*a*) and aortic sections bearing aneurysms (*b*).

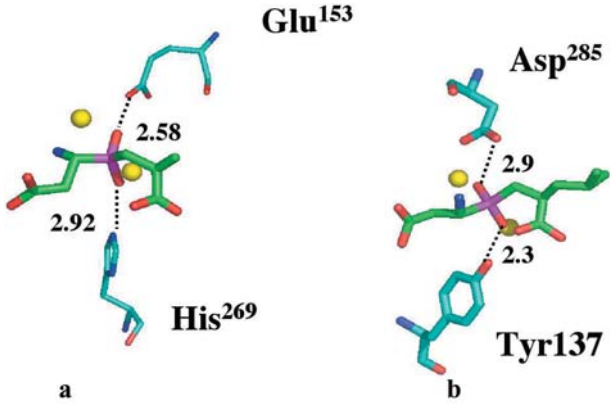

Figure 8. Comparison of the binding mode of phosphinic dipeptides AspY ($PO_2^-CH_2$)AlaOH (*a*) and AspY ($PO_2^-CH_2$)LeuOH (*b*), respectively, in the active site of aminopeptidase PepV from *Lactobacillus delbrueckii* and the isoaspartyl dipeptidase from *E. coli*.

However, a few reports have shown that potent phosphinic inhibitors of dinuclear zinc aminopeptidases could also be developed. A Ki value of 66 nM was reported for the inhibition of leucine amino peptidase (LAP) by homoPheΨ($PO_2^-CH_2$)PheOH (mixture of four diastereoisomers) [39]. For this family of dinuclear zinc aminopeptidases, phosphinic peptides also turned out to be useful for structural studies. The roles of the two zinc ions in stabilizing in the transition-state intermediate have been delineated by solving the crystal structure of one of these aminopeptidases in complex with a phosphinic dipeptide (fig. 8a) [40]. The same approach was used to study another dinuclear zinc peptidase, the isoaspartyl dipepidase from *Escherichia coli* (fig. 8b) [41]. Comparison of these two structures reveals the different structural strategy that has emerged during evolution to stabilize transition-state structures (fig. 8). In these structures, the glutamic acid (Glu^{153} in aminopeptidase pepV) and the aspartic acid (Asp^{285} in isoaspartyl dipeptidase) are presumed to act as the general base during substrate hydrolysis.

Recently, we suggested that phosphinic peptides can be viewed as a general class of ligands able to interact specifically with accessible metal binding sites of proteins. The number of proteins bearing such metal binding sites exceeds by far the number of metalloproteases and thus constitutes a rich source of unexploited targets. To test this hypothesis, libraries of phosphinic peptide have been immobilized on solid support to create polyfunctional affinity columns [42]. The ability of such columns to specifically capture proteins was evaluated by passing crude liver homogenates through them. Interestingly, some proteins were shown to interact specifically with particular phosphinic peptide sequences. Remarkably, most of the captured proteins were enzymes containing a zinc atom. Potent inhibitors of betaine homocysteine S-methyltransferase (BHMT) were identified using this strategy (Ki values in the micromolar range), showing that the identified compounds in this case bind to the enzyme active site. Grafting one of these inhibitors to a Sepharose support provides an affinity column, allowing one-step purification of BHMT enzyme from crude liver homogenates [42]. Although the potency of these phosphinic peptides toward such zinc enzymes is moderate, this strategy allows rapid identification of new leads that can be optimized by classical procedures. The metalloenzymes or metalloproteins identified by this strategy are located inside the cell. As mentioned above, the presence of the negatively charged phosphoryl group on these pseudo-peptides prevents their transfer inside the cell. Thus, the use of phosphinic peptides to probe the function of these intracellular proteins will require masking of the negative charge by the design of a prodrug. Examples of phosphinic prodrugs have been reported, in which the phosphoryl group is masked by ester groups [43]. Active compounds can be generated from these prodrugs by esterases cleavage of the ester group.

Conclusion

Completion of the genome sequences of many organisms has led to the identification of new proteases that belong to the zinc metalloprotease family. Because these proteases are involved in the control of many different functions, there is an urgent need to develop synthetic inhibitors able to interfere specifically with these various enzymes, in different contexts, to unveil their particular functions. Phosphinic peptides display many properties

that allow the study of this family of enzymes, from structural biology to function. The future of this family of particular peptides will depend on the chemical progress that is achieved. Easier synthetic pathways to diversify their structures, supporting parallel or combinatorial chemistry, are needed to offer biologists a wide range of selective ligands.

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