TUTORIAL REVIEW

CSR
www.rsc.org/csr

Extracellular proteases as targets for treatment of cancer metastases

Mijoon Lee,[a] Rafael Fridman[b] and Shahriar Mobashery*[a]

[a] *Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA. E-mail: mobashery@nd.edu; Fax: 574-631-2933; Tel: 574-631-6652*

[b] *Department of Pathology, Wayne State University, 8200 Scott Hall, Detroit, MI 48201, USA*

Received 7th January 2004
First published as an Advance Article on the web 13th August 2004

Metastasis, the dissemination of tumor cells to distant organs, is often associated with fatal outcome in cancer patients. Formation of metastasis requires degradation of extracellular matrices and several families of proteases have been implicated in this process, including matrix metalloproteinases (MMPs), serine and cysteine proteases. Inhibition of these enzymes in animal models of metastasis has shown impressive therapeutic effects. This report discusses the various approaches used for enzyme inhibition and describes new developments in drug design for inhibition of proteases in metastatic disease.

Introduction

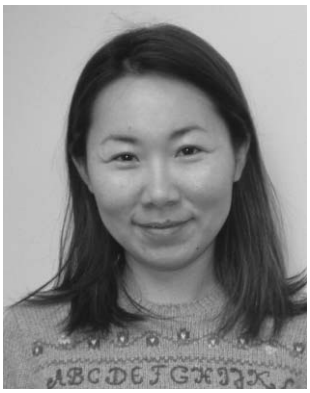
Cancer is a collection of over 100 devastating diseases that share a number of characteristics, a primary hallmark of which is out-of-control growth. However, in reality both from genotypic and phenotypic considerations, there are significant differences among these diseases, a fact that underlies the difficulties in the past few decades in their chemotherapeutic intervention. Whereas the scientific community aspires to understand the biochemical events that lead to the cancerous outcome in each case, understanding of the prospects for certain shared biochemical events for these diseases are emerging only recently.

It has been argued that tumors undergo a Darwinian evolution in a multistep process with dynamic changes in the genome. As it is becoming evident, there are multiple routes to development of cancer, in part because so many distinct metabolic and biochemical steps can be altered to give rise to

Mijoon Lee was educated at the Pohang University of Science and Technology, Korea, where she received her BSc in 1995 and PhD in 2002 under the supervision of Professor Dong Han Kim. She studied inhibitors of carboxypeptidase A, a model enzyme of zinc-dependent metalloproteases. She joined the Mobashery group as a postoctoral fellow in 2001. Her current research interest encompasses proteases (matrix metalloproteases and cathepsin B) and penicillin-binding proteins.

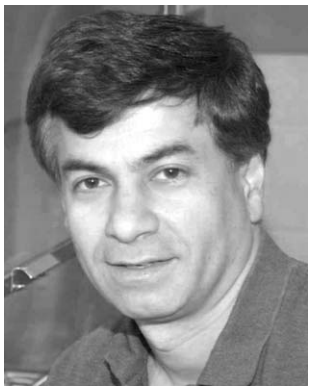
Rafael Fridman received his PhD (1986) from the Hebrew University in Jerusalem in Tumor Biology. Subsequently he served as a postdoctoral fellow at the National Institutes of Health (USA) in the area of tumor metastasis. After a short stint in the biotechnology world (1991 to 1992), he joined the Department of Pathology at Wayne State University, where he currently holds the position of Professor. His research focuses on understanding the regulation and function of matrix metalloproteinases and their role in cancer.

Shahriar Mobashery received his undergraduate (1981) and doctoral (1985) degrees from the University of Southern California and University of Chicago, respectively. After postdoctoral research (1986–1988) at the Rockefeller University, he joined the faculty at Wayne State University in 1989, where he rose through the ranks to Professor of Chemistry, Biochemistry and Pharmacology. Shahriar Mobashery and his research group relocated to the University of Notre Dame in 2003, where he holds the position of Navari Family Professor of Life Sciences in the Department of Chemistry and Biochemistry.



Mijoon Lee

Rafael Fridman



Shahriar Mobashery

DOI: 10.1039/b209224g

uncontrolled cell growth. Hanahan and Weinberg proposed six essential events as hallmarks of cancer.[1] These are self-sufficiency in growth signals (independence from the neighboring issues), insensitivity to growth inhibitory biochemical signals, evasion from programed cell death (apoptosis), ability to undergo limitless cycles of cell growth, sustained ability to be supplied by blood (angiogenesis), and tissue invasion and spread of cancer to other parts of the body (metastasis).

The authors have argued that it is difficult to have all these features present in the disease process, hence the relative rarity of cancer during lifetime of a typical human being. Mutations are relatively rare, as there are distinct repair mechanisms that monitor and repair damage to the genome routinely. Indeed, accumulation of multiple mutations, a necessary event for the onset of cancer, becomes progressively difficult within a human lifetime, leading to the assertion that an enhanced mutability must be present in the cells to result in cancer.

Regardless of what set of biochemical events leads to a given kind of cancer, the primary tumors are rarely lethal and often they are treatable by either surgery or by local irradiation treatment. However, when the tumor cells spread to distant organs, a process know as metastasis, the prognosis is very poor. A characteristic of malignant tumors is the ability of a small subpopulation of tumor cells to escape from the primary tumor (Fig. 1). These cells are released into the circulation in a

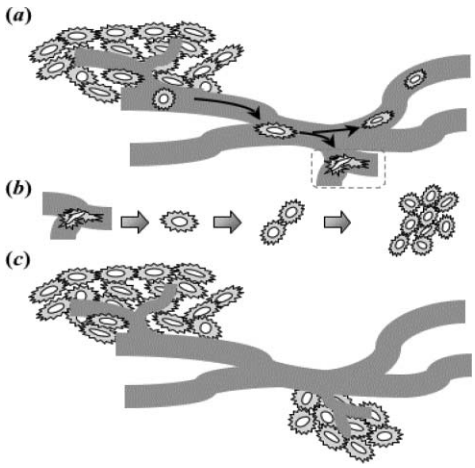


Fig. 1 Summary of events involved in tumor metastasis. (a) Detachment and escape of cancer cells from a primary tumor into the circulation, and arrest at a secondary site. (b) Possible fates of cancer cells in a secondary site: extravasation, initial growth and sustained growth. At each step, only a subset will proceed, and the remainder of cells might either go into a state of dormancy or die. (c) Cancer cells grow at the new location.

complex process involving a series of defined steps including detachment from the tumor mass, degradation of basement membranes, cell migration and invasion of adjacent capillaries (a process referred to as intravasation) that leads to entry into the circulation. The small number of cells that survive the voyage through the circulatory system arrive at new organ sites, where they attach to the endothelium. The presence of specific surface receptors in both the tumor cells and the endothelium together with organ-specific chemokines direct tumor cells to preferred sites for invasion. Adhesion to and recognition of those sites in the endothelium by the tumor cells is followed by the active process of extravasation, involving again degradation of extracellular marix (ECM)—a complex network of proteins that surrounds cells providing physical support—migration and tumor growth, resulting in the formation of a tumor colony. Thus, formation of metastases is a multistep process, all of which must take place successfully for the disease to progress. When metastases happen, the prospects for survival of patients become considerably worse, resulting in approximately 90% death in patients.[2]

As the primary tumor grows, its need for nutrients increases in proportion with its growth rate. The growth of the tumor results in hypoxic conditions due to poor vascularization. Consequently, hypoxia induces the expression of genes that promotes the formation of a new blood vessel from a pre-existing bed, a process known as angiogenesis. While this process supplies essential nutrients to the tumor cells, it also provides a route for the escape of metastatic cells out of the primary tumor site. Therefore, tumor angiogenesis contributes to metastasis formation.

Proteolysis and cancer metastasis

Throughout the metastatic cascade and formation of new blood vessels, there is an intense process of ECM degradation.[3] The ECM of all organs comprises the basement membrane and the connective tissue matrices. The interaction of cells with the ECM is mediated by specific cell surface receptors, which confer adhesive properties and transmit external stimuli that regulate cell behavior. A major component of ECMs is collagen, a large family of proteins composed of three polypeptide chains assembled into a triple helical conformation. All members of the collagen family form supramolecular structures in ECM although their sizes, functions and tissue distributions vary in different ECMs. A characteristic of most collagen molecules is their resistance to proteolytic degradation by many types of proteases, and therefore they represent a barrier for invasive tumor cells. In addition to collagens, the ECM contains multiple proteins including fibronectin and laminins, just to mention a few, various types of proteoglycans, growth factors, enzymes and protease inhibitors.

In order to modify and regulate the functions of the ECM, both normal and tumor cells produce a number of extracellular proteases, which are able to degrade ECM proteins. These enzymes include proteases that exist as membrane-anchored versions on the surface of the cells and as soluble forms excreted into the extracellular milieu. In some instances, they are also associated with specific proteins on the surface of the cell, in particular with cell adhesion receptors such as the integrins.[4–6] Therefore, the association of proteolytic enzymes with cell adhesion receptors facilitates proteolysis at the pericellular space. In cancer metastasis, proteolytic enzymes play a key role in degradation of ECM components, in particular the degradation of collagen, an event that is essential for tumor cell invasion. ECM degradation is also required for angiogenesis. Migrating endothelial cells must, like invasive tumor cells, penetrate the underlying subendothelial basement membrane and migrate through the connective tissue matrix during the formation of new capillaries. Also, ECM degradation releases bioactive molecules such as ECM-bound growth factors, which stimulate angiogenesis. Degradation of ECM components exposes cryptic sites that can promote tumor cell migration and influence angiogenesis. For example, generation of endostatin, a powerful inhibitor of angiogenesis, is the result of a specific proteolytic cleavage at the C-terminal region of collagen XVIII by matrix metalloproteinases. Thus, proteolysis of ECM may elicit opposite effects in cancer metastasis, underscoring the complexity of protease action in tumor tissues. As will be discussed in this report, there are several proteases that have been implicated in cancer metastasis, however, the full implications for the function(s) of each has not been yet completely elucidated. For invasiveness to be set in motion, the functions of these proteases must be turned on, with the loss of the control processes such as endogenous protease inhibitors, the main function of which is to prevent proteolytic havoc. Hence, intervention with synthetic protease

inhibitors represents an unexploited frontier in our attempt to control cancer metastasis. Currently, there is no antimetastatic agent that has become available for clinical use.

Enzymes of metastasis

Proteases are enzymes that hydrolytically degrade proteins. There are several that have been implicated in cancer metastasis. These enzymes belong to the families of serine proteases, cysteine proteases and metalloproteases.[7] The three different kinds of proteases have distinct origins and they follow different mechanisms in their hydrolytic reactions.[8] Serine proteases go through a two-step hydrolytic process, where a catalytic triad of active site residues (Asp–His–Ser) allows acylation of the serine residue by the protein substrate (Fig. 2).

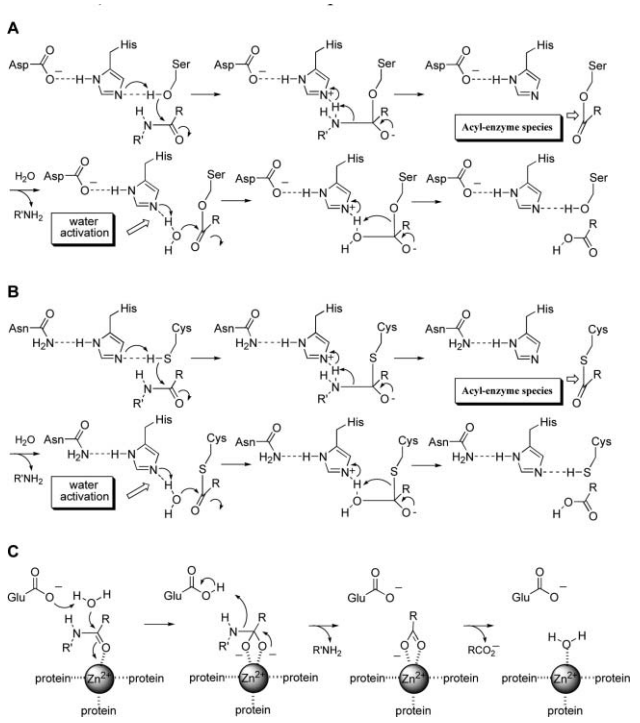


Fig. 2 Proposed mechanisms for (A) serine proteases, (B) cysteine proteases, and (C) zinc-dependent proteases (the peptidase activity). Several variations on these themes have also appeared in the literature.

The acylation event liberates a portion of the substrate as a product. The second enzymic step involves promotion of a water molecule for attack at the ester carbonyl to give rise to the second product of hydrolysis, in a process that regenerates the enzyme. A similar set of events operates in cysteine proteases, with the exception that their catalytic machinery involves a triad of Asn–His–Cys, and enzyme acylation proceeds at the cysteine residue. Despite their sharing of the strategy of catalytic triads in their mechanisms, cysteine and serine proteases have distinct protein folds and origins, so they are not the mere result of substitution of serine for cysteine, or *vice versa*.

Metalloproteases are dependent on a zinc ion within the active site for their catalytic functions. The mechanism by which the catalytic center of metalloproteases functions is currently the subject of debate. One group of authors argues for the stabilization of a tetrahedral species by attack of a promoted water molecule at the scissile amide bond of the protein substrate (Fig. 2). Others argue that an active site glutamate attacks the scissile carbonyl to give a mixed anhydride that undergoes hydrolysis in a second step. Also, it has been argued that the first mechanism may hold true for turnover of amides (as in protein substrates), whereas the latter is operative for ester substrates.

The discussion here underscores the difficulty in fighting tumor metastasis since there are at least three known classes of enzymes with distinct mechanisms involved in these events. It is not possible to inhibit all three by one type of enzyme inhibitor. Furthermore, it may not be desirable to inhibit all of these enzymes, as this may entail shutting down physiologic metabolic events that would require the functions of these enzymes. It may also be true that in different types of cancers distinct proteases may play influential roles at different stages of tumor progression. Hence, it is important to explore the individual functions of these enzymes in an effort to target them selectively in prevention of tumor metastasis.

However, there exists ample evidence to implicate specific proteases in metastasis. One of these enzymes is the serine protease urokinase plasminogen-activating (uPA) enzyme. This enzyme hydrolytically activates plasminogen (a biologically inactive protein) into plasmin, which is an important protease itself in the blood-clotting process.[9] uPA binds to a specific surface protein receptor known as urokinase-type plasminogen activator receptor (uPAR). The receptor uPAR was the first enzyme receptor identified on the cell surface. It is interesting to note that uPA- and uPAR-deficient mice can develop normally indicating that they are not critical for mouse development. uPA and uPAR are expressed in many different types of cells, however, the expressions are upregulated under disease conditions. A high level of activity is associated with the invasive areas of the tumor.

The cysteine protease cathepsin B is produced as a so-called "preproenzyme" containing a signal peptide in the rough endoplasmic reticulum. The signal peptide is cleaved in the endoplasmic reticulum to give rise to the inactive procathepsin B protein, which has to be processed proteolytically further to generate the active enzyme. Cathepsin B is found normally in both the lysosome, a cellular compartment involved in hydrolytic processing of biological macromolecules, and in the extracellular milieu. This enzyme is known to be overexpressed in a number of cancers. Secretion and relocalization of cathepsin B is believed to be important in tumor progression and clinical outcome for the patients. It is of interest that the activity of this enzyme is highest in the invasive edge of the tumor.[10] Whereas active cathepsin B is found both intracellularly (in association with lysosomal content) and extracellularly in soluble form, it has also been suggested that the active enzyme also associates with a certain receptor on the cell surface. The mechanism of this surface association remains to be elucidated. Also, it is not clear whether cathepsin B is directly involved in degradation of ECM or its role is primarily through activation of the other proteases implicated in cancer (discussed below).

The third class of proteases with known roles in tumor metastasis is the zinc-dependent matrix metalloproteinases (MMPs), of which 27 are known in humans. These proteins are often multidomain enzymes, with potentially many different physiological roles that are yet to be elucidated. It is remarkable that the active sites of these enzymes are very similar to one another, presenting a problem in selective inhibition. These enzymes are also expressed as zymogenic inactive proenzymes that have to be activated by other proteases. They are also inhibited by a family of protein inhibitors referred to as tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs are both soluble extracellular enzymes and also membrane anchored, the so-called membrane-type matrix metalloproteinases (MT-MMPs), of which six are known. The process of activation of the MMP zymogens is elaborate, at times requiring more than one protease for the function.[11,12] In a set of new discoveries, it is known now that MT1-MMP binds

to a requisite TIMP (TIMP-2) on the surface of the membrane. The non-covalent complex of MT1-MMP and TIMP-2 serves as a receptor for pro-MMP-2, which upon the formation of the ternary complex undergoes hydrolysis by an individual free-standing MT1-MMP to generate the active version of MMP-2. This example is given here to illustrate the intricate number of steps involved in some of these activation events and that each is highly regulated requiring an elaborate cascade of events that need to be set off-balance in the process of tumor metastasis, which requires an excess of proteolytic activity.

Many studies have shown a role for MMPs in cancer metastasis. Several members of the MMP family have been directly implicated in the ability of tumor cells to migrate and invade distant organs. Also, MMPs are known to be essential for angiogenesis. Although several MMPs have been described to be overexpressed in metastatic tumors, the evidence shows that in different cancer types MMP-2 and MMP-9 (known as gelatinases), MT1-MMP (MMP-14), MMP-1 (interstitial collagenase), MMP-13 (collagenase-3), MMP-7 (matrilysin) and MMP-11 (stromelysin 3) are generally associated with metastasis.[13,14]

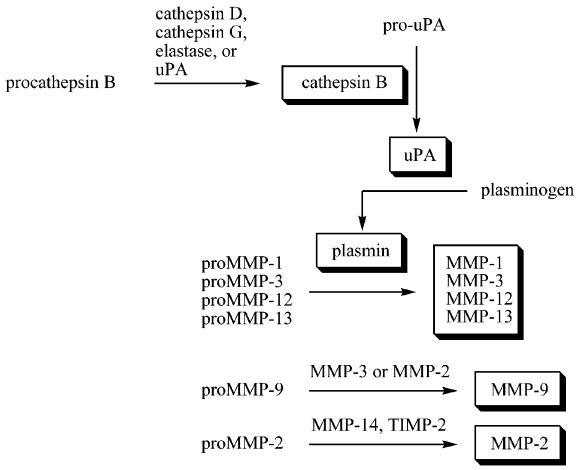


Fig. 3 The activation cascade of some of the proteolytic enzymes involved in tumor metastasis. The boxed activated enzymes degrade the extracellular matrix. Note the intricate interdependence of the enzymes in the activation events.

Fig. 3 shows some of these elaborate and intricately interwoven proteolytic events in zymogen activation, but it does not summarize all that is known of activation of the enzymes implicated in tumor metastasis. As is clear from the figure, some of the enzymes involved in tumor metastasis may exert their effect by activating others that may in turn play a more direct role in metastasis. For example, it is known that cathepsin B degrades the ECM, but is it this activity or that of its activation of pro-uPA to uPA or that of proMMP-3 to MMP-3 that might be important? Part of the problem is the fact that an assessment of these events in complex cellular system is extremely difficult. Even in simpler *in vitro* systems the answers cannot emerge readily, as evaluations of the degradation of the ECM in a quantitative manner with respect to the enzymic energetics and turnover parameters cannot be carried out effectively. This is in part due to the complexity of the ECM.

Inhibitors of the proteases implicated in cancer metastasis

Protease inhibitors can be classified in two groups, reversible or irreversible. Reversible inhibitors bind the active site of the targeted enzymes usually in a non-covalent manner, so they compete with substrates for the same binding site on the enzyme. Their inhibitory potency can be enhanced by hydrophobic interaction, or by hydrogen bonding and ionic interactions with charged residues in the active site of enzymes.

Irreversible inhibitors modify nucleophilic amino acids in active sites of enzymes, such as the carboxylate of Glu or Asp, thiolate of Cys, hydroxyl group in some Ser or Thr, or imidazole in His. Irreversible inhibitors often contain electrophilic moieties, which will be attacked by the active site nucleophile, resulting in enzyme-inhibitor complexes that are often stable.

To design enzyme inhibitors, the knowledge of the mechanism of the enzyme, its substrate specificity, and structure of the active site is useful. Moreover, computer-assisted modeling based on known three-dimensional structures of the target enzymes (X-ray or NMR structures) proved a powerful tool for inhibitor design.

The structures of the substrates are often used as starting points in design of enzyme inhibitors. As a general approach, the protease substrate and the active site of an enzyme are defined as depicted in Fig. 4.

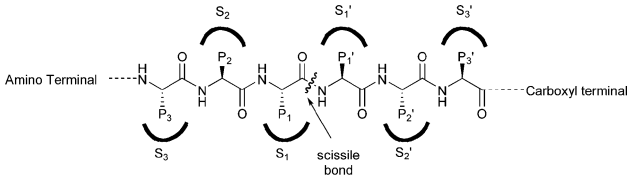


Fig. 4 Standard nomenclature for protease substrate cleavage. P_n, P_3, P_2, P_1, P_1', P_2', P_3', P_n', designate the amino acid side chains of a peptide substrate. Cleavage occurs between P_1 and P_1' residues. The corresponding binding sites in the protease active site are designated as S_n, S_3, S_2, S_1, S_1', S_2', S_3', S_n'.

There are two types of subsites to consider; primed sites and non-primed sites. The primed subsites are located at the carboxy-end of the scissile hydrolyzable amide bond. The non-primed subsites are on the amino-terminal side of the scissile bond.

The approach to development of specific enzyme inhibitors depends often on the kind of targeted enzyme. For example, urokinase inhibitors often contain a positively charged group that interacts with the negative charged Asp-189 within the enzyme active site. On the other hand, cathepsin B inhibitors usually bear electrophilic moieties, in part because the nucleophilicity of the active site thiolate makes it easier to pursue irreversible inhibition. MMP inhibitors often contain various zinc-chelating groups, because chelation of the zinc ion would make it unavailable for the catalytic events of the enzyme.

Inhibitors of urokinase

Urokinase is a serine protease and a member of the "trypsin-like" family. The urokinase activity is regulated by plasminogen activator inhibitor 1 and 2 (PAI1 and PAI2) in biological systems. Few synthetic urokinase inhibitors have been described, indicative of the fact that we are at the early stages of research on this enzyme.[15] Development of small molecule uPA inhibitors began with aryl guanidines, aryl amidines, or acyl guanidines. These contain positively charged guanidine, amidine, or simple amines as anchors, which interact with the negative charge (Asp-189) in the S_1 site of the enzyme. Compounds shown in Fig. 5 are the early examples of uPA inhibitors. Initial compounds in each class exhibited modest potency and poor selectivity, however, they were important in that they were studied with the use of X-ray analysis to explore

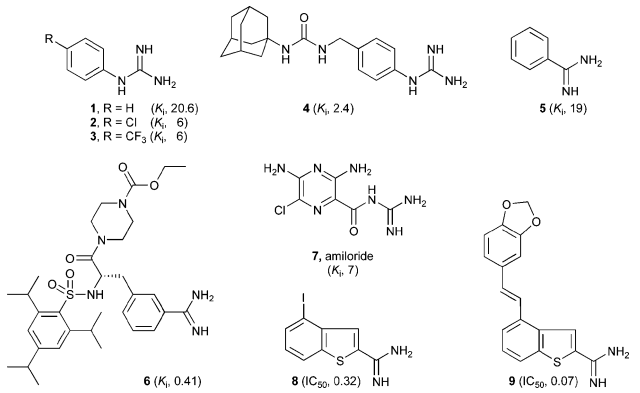


Fig. 5 Early examples of uPA inhibitors (K_i and IC_{50} values are given in μM).

the various modes of active site binding. Therefore, these relatively poor inhibitors paved the way for future developments in inhibitor design.

In uPA, the size of S_2 and S_3/S_4 is smaller than in other trypsin-like serine proteases. Investigation of the structures of subsites of uPA led to the description of a new subsite ($S_{1\beta}$), which is near the S_1 subsite.[16] The $S_{1\beta}$ pocket is a shallow subsite that has not been widely used for structure-based drug design of other serine protease inhibitors. Therefore, interactions with the $S_{1\beta}$ pocket afford the potential for creating selective uPA inhibitors. This subsite contains a number of polar amino acid residues and can be occupied by analogues of a naphthamidine ring. After screening of many hydrophilic groups, incorporation of an aminopyrimidyl group at the 8-position resulted in a 30-nM uPA inhibitor (**10**); Fig. 6, which

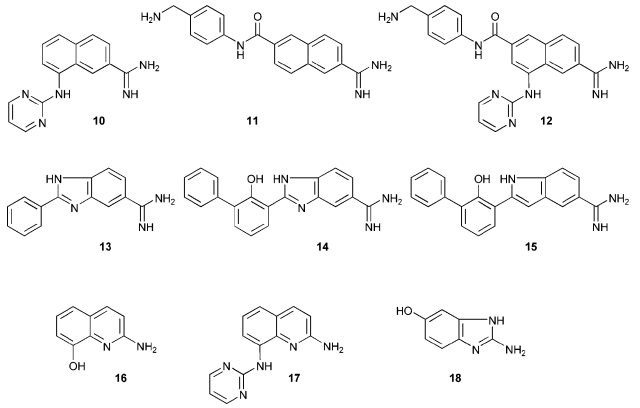


Fig. 6 Recently developed uPA inhibitors.

is an improvement of 200-fold in potency compared to naphthamidine (K_i, 5.9 μM). Compound **10** also showed selectivity toward uPA over other trypsin-like serine proteases (K_i values were 3.8, 23, 1, 1.6, 3.9 μM for plasminogen, t-PA, kallikrein, trypsin, thrombin, respectively). Combination of 6-substituent and 8-substituent on the naphthamidine template resulted in the most potent uPA inhibitor (**12**) reported to date (K_i = 0.64 nM).[17] Compound **12** exhibited excellent selectivity for uPA over related serine proteases, including plasmin (250-fold), tPA (1,100-fold), plasma kalikrein (67-fold), trypsin (33-fold) and thrombin (1,500-fold).

The improvement of potency and selectivity was achieved through modifications of the S_1 starting with benzimidazole **13**.[18] By extensive structure–activity relationship studies around the phenyl ring of compound **13**, compound **14** was arrived at (K_i of 400 nM). The X-ray structure revealed that the origin of potency of inhibition by this molecule is due to the formation of an unusual network of short hydrogen bonds between a water bound in the oxyanion hole, the inhibitor, and the catalytic serine, as well as S_1 and S_1' subsite bindings. Replacement of the benzimidazole by indole afforded 50-fold higher inhibition (**15**, K_i of 8 nM). Compound **15** exhibited selectivity in inhibition of uPA over other trypsin-like serine proteases (K_i values were 35, 78, 320, 100, 130 nM for t-PA, factor Xa, thrombin, plasmin, trypsin, respectively).

Although many amidine based compounds exist for uPA inhibition, a potent inhibitor with the requisite pharmacokinetic properties for a clinically useful agent has not been reported. New leads were found based on an X-ray screening method, which allowed for exposure of the enzyme crystals to mixtures of diversely shaped compound libraries.[19] The most potent ligand in the mixture bound at the active site of the crystalline macromolecules and X-ray analysis reveals 8-aminoquinoline (**16**). Incorporation of the aminopyrimidyl group at the 8 position increased the potency 100-fold (from K_i of 56 to 0.37 μM). The new compound, 8-aminopyrimidyl-2-aminoquinoline (**17**) is 38% orally available (*versus* none for the naphthyl compound). The search for a non-charged scaffold for uPA inhibitors was also tried by an NMR-based screening.[20] These efforts resulted in 2-amino-5-hydroxybenzimidazole (**18**), which was found to inhibit uPA with a K_i of 10 μM (pK_a = 7.4). This new scaffold could serve as a favorable starting point for development of a clinical agent against urokinase by additional structure-based optimization.

As a classical approach in discovery of uPA inhibitors, peptidyl based inhibitors have been developed (Fig. 7).

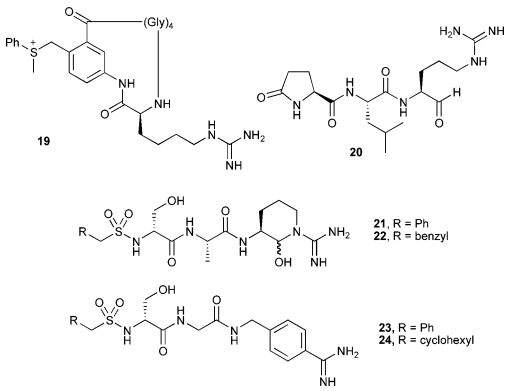


Fig. 7 Peptide-based uPA inhibitors.

Cyclopeptide **19** inhibited urokinase selectively in an irreversible manner (k_{inact}/K_I = 2330 $M^{-1}s^{-1}$, K_i = 41 nM).[21] No inhibition is observed for plasmin, tPA or thrombin by compound **19**. Another peptide-based irreversible inhibitor is Pyr–Leu–Arg–CHO (**20**, IC_{50} = 5.8 μM), but this compound also inhibits plasmin (IC_{50} = 1.3 μM).[22] The development of increasingly potent and selective peptidyl based inhibitors was aided by the use of substrate phage display techniques.[23] This technique identified peptides that were hydrolyzed more efficiently by uPA than the plasminogen nonapeptide (a natural substrate of uPA). The minimum sequence identified by this method was Ser–Gly–Arg–Ser–Ala, which was utilized to design potent and selective peptide inhibitors of uPA. Gly or Ala are known to be good for S_2, Ser or Thr for S_3. Benzamidine or aminal were designed as Arg surrogates to bind the S_1 subsite. Inhibitory dissociation constants were 5.1, 3.1, 36, and 48 nM for compounds **21,22,23**, and **24**, respectively.[24,25]

Some of these small-molecule inhibitors of urokinase have been shown to inhibit tumor metastasis and also cancer growth in experimental animals.[15] One compound (**6**), a broad

spectrum inhibitor of serine proteases, has entered phase I of human clinical trials.[26]

Inhibitors of cathepsin B

Cathepsin B is a "papain-like" cysteine protease. The activity of papain-like cysteine proteases is regulated by a large number of endogenous protein-based inhibitors. Among them, Stefin B (also known as Cystatin B) and Cystatins C are known to be specific for cathepsin B.

The majority of synthetic cysteine protease inhibitors contain a peptide segment for recognition by the enzyme, which have been incorporated with a number of electrophilic functionalities that are able to react with the thiolate of active site cysteine.[27] The electrophilic entity could in principle allow for reaction with the active site serines of the serine proteases as well, which presents a potential problem in selectivity. To compound the problem, cathepsin B is a member of a closely related group of cysteine proteases. They have similar active sites and share the hydrolysis mechanism.

Reversible inhibitors usually contain an aldehyde, a methyl ketone, or a nitrile groups as the reactive electrophilic group. Representative examples of reversible selective cathepsin B inhibitors are given in Fig. 8. These functional groups react

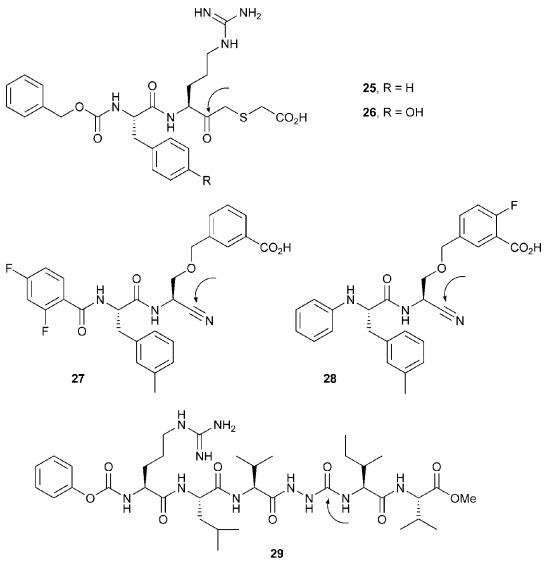


Fig. 8 Reversible cathepsin B selective inhibitors. The curved arrows indicate the sites of nucleophilic attack by the cysteine.

with the thiolate of the active site cysteine; however, this reaction is reversible. Recently, potent peptidyl-ketone inhibitors were prepared from a 2016-membered library of potential mercaptomethyl ketone inhibitors (**25** and **26**). Selectivity in inhibition of cathepsins was not shown.[28]

The dipeptidyl nitrile **27** (Fig. 8) was designed based on the X-ray crystallographic data and molecular modeling (K_i of 6.8 nM).[29] This compound showed 100-fold selectivity in favor of cathepsin B, compared to cathepsins S and L. Unfortunately, compound **27** and its analogues were found to have very poor pharmacokinetic properties and could not be used in *in vivo* testing, presumably due to the highly peptidic nature of this compound. A structure-guided approach to minimizing the peptide character of the compound resulted in the highly potent and selective cathepsin B inhibitor **28** (K_i = 12.2 nM) with much more favorable pharmacokinetic properties.[30] This compound is currently being profiled in animal models to further delineate the role of this enzyme in disease processes.

The azapeptide inhibitor **29** is another example of a reversible inhibitor. In this inhibitor, the peptide backbone was replaced by a hydrazide moiety, which resulted in a distinct geometric and electronic environment. Compound **29** is a highly potent (K_i = 88 pM) and selective inhibitor of cathepsin B.[31]

The most potent cysteine protease inhibitors to date have been irreversible inhibitors. Examples of this type of inhibitor include halomethyl ketones, acyloxymethyl ketones, acylhydroxamate, vinyl sulfone, or epoxysuccinates. Representative examples of irreversible selective cathepsin B inhibitors are depicted in Fig. 9.

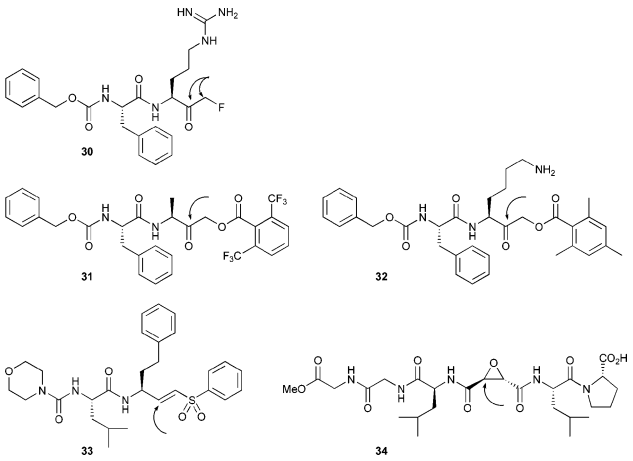


Fig. 9 Irreversible cathepsin B selective inhibitors. The curved arrows indicate the site of nucleophilic attack by the active site cysteine thiolate.

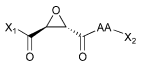
Halomethyl ketones were originally conceived as affinity labels for serine proteases for covalent modification of the active site of the target proteases. However, halomethyl ketones have limited clinical utility due to the inherent chemical reactivity of the moiety. This led to the development of the relatively weakly electrophilic groups, such as acyloxymethyl ketone, acylhydroxamate, *etc*.[27] An indication of the effectiveness of the inhibitor in inhibiting a given enzyme with these types of molecules is the evaluation of the second-order rate constant for the on-set of inhibition; the larger this number the better the inhibitor. For example, compound **31** inhibited cathepsin B with a second-order rate constant of 1.6 times; $10^6\ M^{-1}s^{-1}$. Unfortunately, this potent inhibitor was not active for oral dosing. Incorporation of polar or charged functional groups in the inhibitor structure afforded significantly enhanced oral dose availability. Compound **32** showed *in vivo* activity for inhibition of cathepsin B in the liver of rats, whose ED_{50} (effective dose for 50% inhibition) was 2.4–18 mg kg^{-1} by several different routes of administration (oral, intraperitoneal, and subcutaneous administration).

The peptidyl vinyl sulfone inhibitor **33** is a highly potent cathepsin inhibitor with a broad spectrum. The second-order rate constants for inhibition of cathepsins B, S, K and L by **33** were 4.3, 26, 0.77, and 0.39 $\times$ $10^6\ M^{-1}s^{-1}$, respectively. This inhibitor was tested in several animal models, but not specifically for inhibition of the cathepsin B activity.

Epoxysuccinate derivatives were synthesized as cathepsin inhibitors since E-64, a natural product, was isolated and shown to be a potent cysteine protease inhibitor. Some of the structurally related cathepsin B selective inhibitors are shown in Table 1.

The highly cathepsin B selective and potent inhibitor **34** was designed based on CA-074 as a lead structure, which was elaborated with the peptide Leu–Gly–Gly from the cathepsin B propeptide.[32] It inhibits cathepsin B with a k_2/K_i value of 1 520 000 $M^{-1}s^{-1}$, and it is 1260-fold more selective for cathepsin B, compared to cathepsin L

Table 1 Cathepsin B selective epoxysuccinate derivatives



Derivative	X_1	AA	X_2
E-64	HO	Leu	$NH(CH_2)_4NHC(NH_2)_2$
E-64c	HO	Leu	$NH(CH_2)_2CH(CH_3)_2$
E-64d	EtO	Leu	$NH(CH_2)_2CH(CH_3)_2$
CA-074	*n*Pr-NH	Ile-Pro	OH
CA-074-OMe	*n*Pr-NH	Ile-Pro	OMe

Both extracellular and intracellular cathepsin B have been targeted for inhibition. Attachment of β-cyclodextrin, rhodamine, and heptapeptides derived from penetratin, a peptide widely used for the intracellular delivery of peptides and oligonucleotides, have been achieved.[33] These moieties replace the methyl ester of glycine, and the resultant compounds did not affect the inhibitory potency nor selectivity. The inhibitor **34** attached to β-cyclodextrin and rhodamine were not cell permeable, giving only inhibition of extracellular cathepsin B (second-order constants, 1 050 000 and 1 530 000 $M^{-1}s^{-1}$). On the other hand, incorporation of penetratin to compound **34** was cell permeable and showed *in vivo* cathepsin B inhibition (second-order constants, 6 100 000 $M^{-1}s^{-1}$) and tumor cell invasion inhibition.

Epoxysuccinates are useful in *in vivo* studies due to their potent inhibitory activity, stability and permeability into cells and tissues. Some of the recent studies have showed that E-64c, E-64d, CA-074, CA-074-OMe inhibited cathepsin B *in vivo* and inhibited cell invasion in murine[34] and human breast cancer cell line.[35] None of the cathepsin B inhibitors have entered clinical trial to date.

Inhibitors of matrix metalloproteinases

Of the 27 known MMPs, MMP-2, -9 (gelatinases), and MT1-MMP (MMP14) have received special attention in cancer research. The issue of selectivity in inhibition is also relevant in the context of MMP inhibitors because of the multitude of the functions that these enzymes play *in vivo*. However, the vast majority of the known inhibitors for MMPs are broad-spectrum inhibitors, essentially each exhibiting potent inhibition of many, if not all MMPs.[36] However, a handful of selective inhibitors for some of these enzymes has emerged recently.[37]

MMP inhibitors were initially designed based on the amino acid sequence of the collagen cleavage site by MMP1. Inhibitors which contain moieties for binding at the primed subsites are generally known to be more effective than those with moieties for binding at the non-primed subsites. The strategy pursued in the vast majority of these inhibitors is to retain a zinc-binding entity such as the hydroxamate, formyl hydroxamate (reverse hydroxamate), sulfhydryl, phosphonate, or carboxylate in the inhibitor; the typical order for potency of the binding of the ligands is the same as given here as well. Hydroxamates bind the zinc ion in a bidentate manner, a chelation, which may be at the root of enhanced binding to the metal ion.

Batimastat and marimastat (**35** and **36**; Fig. 10) are broad-spectrum inhibitors. Marimastat contains a combination of α-hydroxy and a P_2' *tert*-butyl group to afford oral activity, in contrast to the more lipophilic batimastat, which has to be administered parentrally.

Based on the X-ray analysis, P_1 and P_2' residues are directed away from the active site into the solvent, presenting the possibility of linking the two groups covalently. Cyclization of

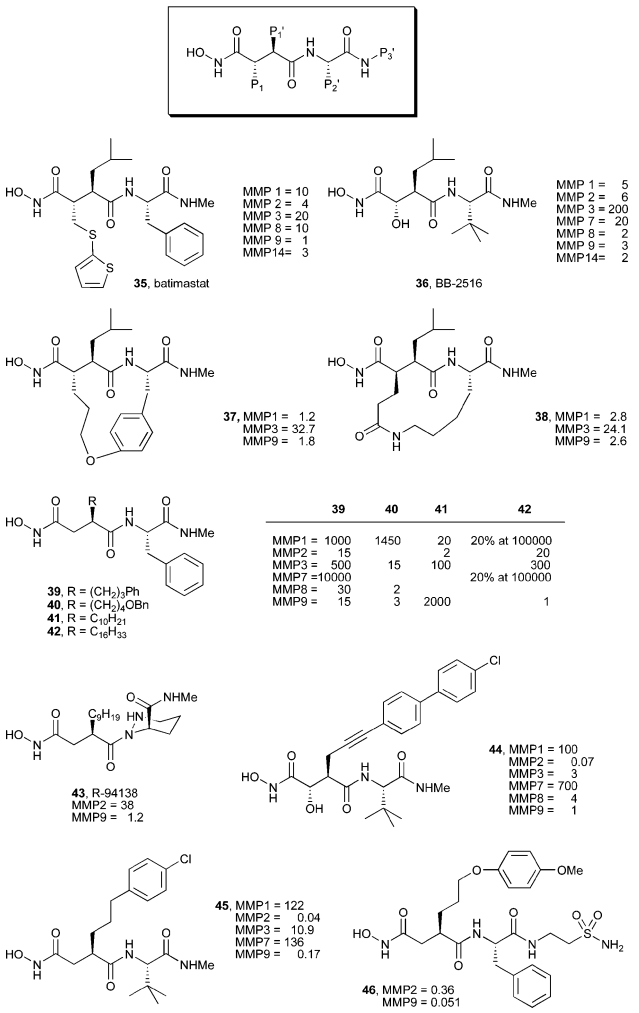


Fig. 10 MMP inhibitors based on the succinyl hydroxamate template (IC_{50} values are given in nM).

P_1 and P_2' resulted in a similar potency, but increased aqueous solubility (**37** and **38**).

Introduction of large alkyl groups at S_1', such as 3-phenylpropyl, C9–C16 alkyl chains, 4-butylbenzyl ether, and the rigid biphenylalkynylmethylene group, increased selectivity toward MMP-2 and MMP-9 over MMP-1 (**39–46**).

The sulfoamide-hydroxamate compound **47** (Fig. 11) was the first non-peptidic MMP inhibitor to enter clinical trials in cancer treatment. The isopropyl substituent in compound **47** slows down metabolism of the adjacent hydroxamic acid and the basic pyridyl group. The broad-spectrum inhibitor AG-3340 (**48**, prinomastat) was incorporated with a six-membered ring to provide a ligand pre-organization and a P_1' substituent that was ensconced deeply within the S_1' pocket.

The hydroxamate group is often unstable *in vivo*, providing the impetus for exploration of MMP inhibitors with a carboxylate as the zinc ion ligand. Bay12–9566 (**49**) is as an orally active, non-peptidic, non-hydroxamate MMP inhibitor. This inhibitor entered clinical trials, however, it was subsequently withdrawn. Compounds **50–53** have large P_1' residues such as 1,3-pyrrole, dibenzothiophene, aromatic amide and disubstituted tetrazole and preferentially inhibited MMP-2 or MMP-9. The large group preference for P_1' for MMP-2 or -9 was shown in hydroxamate inhibitors as well.

Compounds **54** and **55** contain a thiolate as a zinc-chelating group and showed nanomolar inhibition of MMPs.

Compound **56** is known to selectively inhibit gelatinases (MMP-2 and MMP-9) in an irreversible manner (Fig. 12).[38] The phenoxyphenyl group binds to P_1' and the sulfonyl group

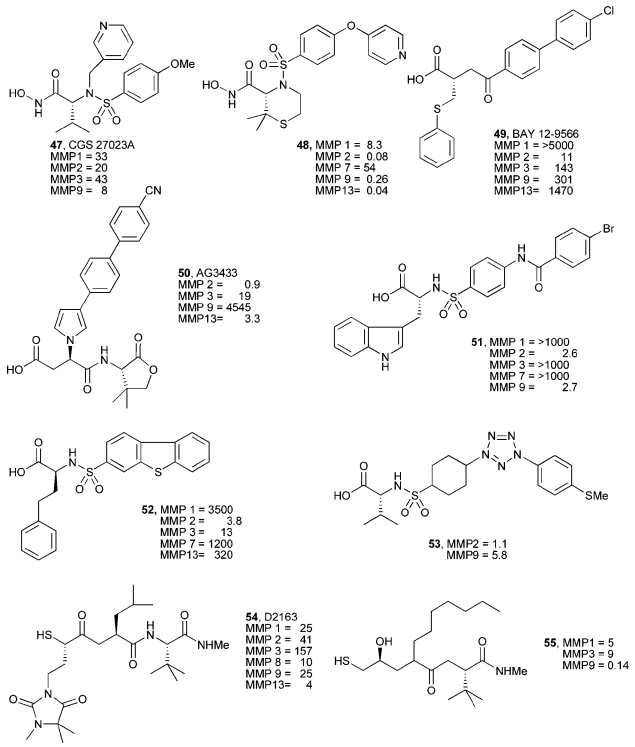


Fig. 11 Additional MMP inhibitors. IC_{50} values are given in nM.

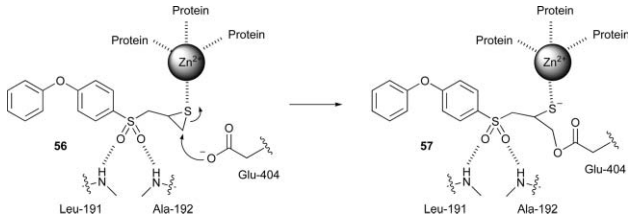


Fig. 12 Schematic of compound **56** in the active site of MMP-2. The coordination of the thiirane to the active site zinc ion and the subsequent ring opening as a result of nucleophilic attack by a glutamate residue are depicted.

provides hydrogen bonding with the enzyme backbone (Leu-191 and Ala-192). The thiirane group was expected to coordinate with the zinc ion, promoting it for nucleophilic attack by the active site Glu-404. The resultant species **57** gives irreversible inhibition of gelatinases. This compound is the first mechanism-based inhibitor (''suicide substrate'') disclosed for MMPs.

No specific MT1-MMP (also known as MMP14) inhibitor are known to date. Some of the broad-spectrum inhibitors inhibit MT1-MMP with nanomolar IC_{50}.

Recently, certain sulfate-containing natural products (**58–60**)[39,40] have been discovered as MT1-MMP inhibitors (Fig. 13). The origin of the activity of these compounds is

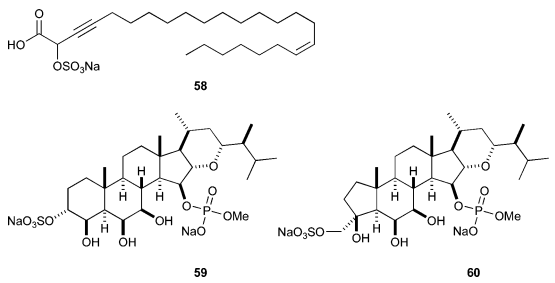


Fig. 13 MT1-MMP inhibitors.

presumably the presence of the sulfate moiety, since that is shared among them. It is important to point out that the desulfated analogue of **58** did not show MT1-MMP inhibition.

The success of several synthetic MMP inhibitors in animal models for cancer metastasis and angiogenesis prompted the human clinical trials for compounds such as **35,36,43,45,47–50**, and **54**. Although some studies showed encouraging results, others have been disappointing. Several reasons were postulated to explain the poor performance of broad spectrum MMP inhibitors in clinical trials, including the advanced disease stage of the patient population, side effects due to inhibition of closely related enzymes such as the ADAMs, lack of specificity, toxicity and inability to assess inhibitory efficacy, just to mention a few.[13,14] It is hoped that by addressing these issues we would be able to improve the effectiveness of inhibitors of enzymes associated with metastasis. First, it is possible that administration of protease inhibitors at early stages of the disease will have a better impact on tumor invasion and on angiogenesis. Alternatively, continuous administration of selective inhibitors with acceptable toxicity may permit targeting proteolytic activity throughout the span of the disease. Second, determination of the protease profile for each cancer type will help to target specific proteases on an individual basis with specific inhibitors, avoiding unwanted side effects. Third, development of sensitive assays such as imaging to monitor inhibitor targeting and efficacy in tissues will help to validate drug effectiveness.

Metastasis remains a bottleneck in effective intervention of cancer. What we have learned so far in inhibition of these important proteases and how we render this knowledge into therapeutic strategies in the near future should make an important impact in how cancer is approached in clinical settings.

References

1 D. Hanahan and R. A. Weinberg, *Cell*, 2000, **100**, 57.
2 M. B. Sporn, *Lancet*, 1996, **347**, 1377.
3 R. A. Cairns, R. Khokha and R. P. Hill, *Curr. Mol. Med.*, 2003, **3**, 653.
4 L. M. Coussens and Z. Werb, *Chem. Biol.*, 1996, **3**, 895.
5 A. F. Chambers and L. M. Matrisian, *J. Natl. Cancer Inst.*, 1997, **89**, 1260.
6 R. Kalluri, *Nat. Rev. Cancer*, 2003, **3**, 422.
7 J. E. Koblinski, M. Ahram and B. F. Sloane, *Clin. Chim. Acta*, 2000, **291**, 113.
8 D. Leung, G. Abbenante and D. P. Fairlie, *J. Med. Chem.*, 2000, **43**, 305.
9 P. A. Anderson, L. Kjøller, L. Christensen and M. J. Duffy, *Int. J. Cancer*, 1007, **72**, 1.
10 B. F. Sloane and E. Elliott, *Perspect. Drug Discovery Des.*, 1996, **6**, 12.
11 M. Seiki, *Cancer Lett.*, 2003, **194**, 1.
12 I. Massova, L. P. Kortra and S. Mobashery, *FASEB J.*, 1998, **12**, 1075.
13 C. M. Overall and C. López-Otín, *Nat. Rev. Cancer*, 2002, **2**, 657.
14 M. Egeblad and Z. Werb, *Nat. Rev. Cancer*, 2002, **2**, 161.
15 T. W. Rockway, V. L. Nienaber and V. L. Giranda, *Curr. Pharm. Des.*, 2002, **8**, 2541; T. W. Rockway and V. L. Giranda, *Curr. Pharm. Des.*, 2003, **9**, 1483; T. Steinmetzer, *IDrugs*, 2003, **6**, 138.
16 V. L. Nienaber, D. Davidson, R. Edalji, V. L. Giranda, V. Klinghofer, J. Henkin, P. Magdalinos, R. Mantei, S. Merrick, J. M. Severin, R. A. Smith, K. Stewart, K. Walter, J. Wang, M. Wendt, M. Weitzber, X. Zhao and T. Rockway, *Structure*, 2000, **8**, 553.
17 V. Klinghofer, K. Stewart, T. McGonigal, R. Smith, A. Sarthy, V. Nienaber, C. Butler, S. Dorwin, P. Richardson, M. Weitzberg, M. Wendt, T. Rockway, X. Zhao, K. I. Hulkower and V. L. Giranda, *Biochemistry*, 2001, **40**, 9125.
18 E. Verner, B. A. Katz, J. R. Spencer, D. Allen, J. Hataye, W. Hruzewicz, H. C. Hui, A. Kolesnikov, Y. Li, C. Luong, A. Martelli, K. Radika, R. Rai, M. She, W. Shrader, P. A. Sprengeler, S. Trapp, J. Wang, W. B. Yong and R. L. Mackman, *J. Med. Chem.*, 2001, **44**, 2753.

19 V. L. Nienaber, P. L. Richardson, V. Klighofer, J. J. Bouska, V. L. Giranda and J. Greer, *Nat. Biotechnol.*, 2000, **18**, 1105.
20 P. J. Hajduk, S. Boyd, D. Nettesheim, V. Nienaber, J. Severin, R. Smith, D. Davidson, T. Rockway and S. W. Fesik, *J. Med. Chem.*, 2000, **43**, 3862.
21 M. Wakselman, J. Xie and J.-P. Mazaleyrat, *J. Med. Chem.*, 1993, **36**, 1539.
22 M. Kawada and K. Umezawa, *Biochem. Biophys. Res. Commun.*, 1995, **209**, 25.
23 S. H. Ke, G. S. Coombs, K. Tachias, D. R. Corey and E. L. Madison, *J. Biol. Chem.*, 1997, **272**, 20456.
24 S. Künzel, A. Schweinitz, S. Reißmann, J. Stürzebecher and T. Steinmetzer, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 645.
25 S. Y. Tamura, M. I. Weinhouse, C. A. Roberts, E. A. Goldman, K. Masukawa, S. M. Anderson, C. R. Cohen, A. E. Bradbury, V. T. Bernardino, S. A. Dixon, M. G. Ma, T. G. Nolan and T. K. Brunck, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 983.
26 S. Sperl, M. M. Mueller, O. G. Wilhelm, M. Schmitt, V. Magdolen and L. Moroder, *Drug News Perspect.*, 2001, **14**, 401.
27 F. Lecaille, J. Kaleta and D. Brömme, *Chem. Rev.*, 2002, **102**, 4459; J. C. Powers, J. L. Asgian, Ö. D. Ekici and K. E. James, *Chem. Rev.*, 2002, **102**, 4639; H.-H. Otto and T. Schirmeister, *Chem. Rev.*, 1997, **97**, 133.
28 W. J. L. Wood, L. Huang and J. A. Ellman, *J. Comb. Chem.*, 2003, **5**, 869.
29 P. D. Greenspan, K. L. Clark, R. A. Tommasi, S. D. Cowen, L. W. McQuire, D. L. Farley, J. H. van Duzer, Ro. L. Godlberg, H. Zhou, Z. Du, J. J. Fitt, D. E. Coppa, Z. Feng, W. Macchia, L. Zhu, M. P. Capparelli, Ro Goldstein, A. M. Wigg, J. R. Doughty, R. S. Bohacek and A. K. Knap, *J. Med. Chem.*, 2001, **44**, 4524.
30 P. D. Greenspan, K. L. Clark, S. D. Cowen, L. W. McQuire, R. A. Tommasi, D. L. Farley, E. Quadros, D. E. Coppa, Z. Du, Z. Fang, H. Zhou, J. Doughty, K. T. Toscano, A. M. Wigg and S. Zhou, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4121.
31 E. Wieczerzak, P. Drabik, L. Lankiewicz, S. Oldziej, Z. Grzonka, M. Abrahamson, A. Grubb and D. Brömme, *J. Med. Chem.*, 2002, **45**, 4202.
32 N. Schaschke, I. Assfalg-Machleidt, W. Machleidt and L. Moroder, *FEBS Lett.*, 1998, **421**, 80.
33 N. Schaschke, I. Assfalg-Machleidt, W. Machleidt, T. Laßleben, C. P. Sommerhoff and L. Moroder, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 677; N. Schaschke, D. Deluca, I. Assfalg-Machleidt, C. Höhneke, C. P. Sommerhoff and W. Machleidt, *Biol. Chem.*, 2002, **383**, 849.
34 N. Sever, M. Filipic, J. Brzin and T. T. Lah, *Biol. Chem.*, 2002, **383**, 839.
35 A. Bervar, I. Zajc, N. Server, N. Katunuma, B. F. Sloane and T. T. Lah, *Biol. Chem.*, 2003, **384**, 447.
36 M. Whittaker, C. D. Floyd, P. Brown and A. J. H. Gearing, *Chem. Rev.*, 1999, **99**, 2735; J. W. Skiles, N. C. Gonnella and A. Y. Jeng, *Curr. Med. Chem.*, 2001, **8**, 425.
37 S. Brown, S. O. Meroueh, R. Fridman and S. Mobashery, *Curr. Top. Med. Chem.,* in press.
38 S. Brown, M. M. Bernardo, Z. Li, L. P. Kotra, Y. Tanaka, R. Fridman and S. Mobashery, *J. Am. Chem. Soc.*, 2000, **122**, 6799.
39 M. Fujita, Y. Nakao, S. Matsunaga, R. W. M. van Soest, Y. Itoh, M. Seiki and N. Fusetani, *J. Nat. Prod.*, 2003, **66**, 569.
40 M. Fujita, Y. Nakao, S. Matsunaga, Y. Itoh, R. W. M. van Soest, M. Heubes, D. J. Faulkner and N. Fusetani, *Tetrahedron*, 2001, **57**, 3885.