



The Antiviral Potential of Host Protease Inhibitors

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Torsten Steinmetzer and Kornelia Hardes

Abstract

The replication of numerous pathogenic viruses depends on host proteases, which therefore emerged as potential antiviral drug targets. In some cases, e.g., for influenza viruses, their function during the viral propagation cycle is relatively well understood, where they cleave and activate viral surface glycoproteins. For other viruses, e.g., Ebola virus, the function of host proteases during replication is still not clear. Host proteases may also contribute to the pathogenicity of virus infection by activating proinflammatory cytokines. For some coronaviruses, human proteases can also serve in a nonproteolytical fashion simply as receptors for virus entry. However, blocking of such protein-protein contacts is challenging, because receptor surfaces are often flat and difficult to address with small molecules. In contrast, many proteases possess well-defined binding pockets. Therefore, they can be considered as well-druggable targets, especially, if they are extracellularly active. The number of their experimental crystal structures is steadily increasing, which is an important prerequisite for a rational structure-based inhibitor design using computational chemistry tools in combination with classical medicinal chemistry approaches. Moreover, host proteases can be considered as stable targets, and their inhibition should prevent rapid resistance developments, which is often observed when addressing viral proteins. Otherwise, the inhibition of host proteases can also affect normal physiological processes leading to a higher probability of side effects and a narrow therapeutic window. Therefore, they should be preferably used in combination therapies with additional antiviral drugs. This strategy should provide a stronger antiviral efficacy, allow to use lower drug doses, and minimize side effects. Despite numerous experimental findings on their antiviral activity, no small-molecule inhibitors of host proteases have been approved for the treatment of virus infections, so far.

T. Steinmetzer (✉) · K. Hardes
Institute of Pharmaceutical Chemistry, Philipps University, Marburg, Germany
e-mail: steinmetzer@uni-marburg.de

Abbreviations

Agm	Agmatine
BBI	Bowman-Birk inhibitor
CCHFV	Crimean-Congo hemorrhagic fever virus
CMK	Chloromethyl ketone
Dec	Decanoyl
EboV	Ebola virus
GP	Glycoprotein
HA	Hemagglutinin
HAT	Human airway trypsin-like peptidase
HIV	Human immunodeficiency virus
HPAIV	Highly pathogenic avian influenza virus
HPIV	Human parainfluenza virus
hTyr	Homotyrosin
IAV	Influenza A virus
IBV	Influenza B virus
LASV	Lassa virus
LBS	Lysine binding site
LCMV	Lymphocytic choriomeningitis virus
LPAIV	Low pathogenic avian influenza virus
MERS-CoV	Middle East respiratory syndrome coronavirus
PAR-2	Protease-activated receptor 2
PC	Proprotein convertase
Phac	Phenylacetyl
PPI	Protein-protein interaction
SARS-CoV	Severe acute respiratory syndrome coronavirus
S-protein	Spike protein
TLSP	Trypsin-like serine protease
TTSP	Type II transmembrane serine protease

11.1 Introduction

At present, 588 human proteases are listed in the degradome database (Quesada et al. 2009), which can be further divided into five classes based on their catalytic mechanism. The majority belongs to the family of metallo (192 members), serine (184 enzymes), and cysteine proteases (164 members); in addition, 27 threonine and 21 aspartyl proteases are known, so far. Proteases are well-druggable targets and numerous small-molecule protease inhibitors have been approved in the past. The more than ten inhibitors of the angiotensin-converting enzyme (ACE) (Turk 2006), as well as the neprilysin (enkephalinase) inhibitor prodrugs sacubitril (Howell and Cameron 2016) and racecadotril (acetorphan) target Zn²⁺-dependent metalloproteases. The ACE blockers and sacubitril are suitable for long-term usage

as antihypertensive drugs, whereas racecadotril is approved as an antidiarrheal drug. Despite limited use, treatment of high blood pressure is also possible with aliskiren, a small-molecule inhibitor of the aspartate protease renin (Wood et al. 2003). Numerous synthetic inhibitors of the trypsin-like serine proteases (TLSP) thrombin (argatroban, dabigatran etexilate, bivalirudin) and factor Xa (rivaroxaban, apixaban, edoxaban, betrixaban) can be used as anticoagulants (Straub et al. 2011). Except argatroban and bivalirudin, all of these clotting protease inhibitors are orally available and suited for long-term use in the prevention of stroke, e.g., in patients suffering from atrial fibrillation. Meanwhile, more than ten gliptins have been approved in various countries. The gliptins are a class of oral hypoglycemic drugs for the treatment of diabetes mellitus type 2 targeting the serine protease dipeptidyl peptidase 4, thereby reducing the degradation of incretin hormones leading to an enhanced insulin secretion (Scheen 2015). The proteasome inhibitors bortezomib (Adams 2004) and carfilzomib (Kortuem and Stewart 2013) are used in patients with multiple myeloma and the first approved drugs targeting threonine proteases. Meanwhile, two additional proteasome inhibitors, ixazomib and oprozomib, obtained orphan drug status (Manasanch and Orłowski 2017). Besides addressing human proteases, numerous inhibitors of the aspartyl protease of HIV (Ghosh et al. 2016) and the NS3/4A serine protease of the hepatitis C virus (McCauley and Rudd 2016) are on the market. With few exceptions, most of these inhibitors are routinely used in combination with other drugs and not as single agents. Despite large efforts, no cysteine protease inhibitor has been approved, so far. One of the most advanced inhibitors of the papain-like bone-degrading protease cathepsin K is the nitrile derivative odanacatib, which was developed for the treatment of osteoporosis. Based on a clinical phase III trial, a high efficacy with increasing bone mineral density and reduced risk of fractures was initially reported, as well as a good safety profile (Chapurlat 2015). However, its further development was stopped at the end of 2016 due to a slightly increased risk of stroke (Mullard 2016). Other cathepsin K inhibitors, like the nitrile balicatib, failed in phase II due to complications with skin fibrosis (Brömme et al. 2016; Runger et al. 2012). Despite the lack of approved cysteine protease inhibitors, many other examples confirm the suitability of at least some proteases as excellent drug targets.

This should also apply to numerous host proteases, which are involved at various steps during the propagation cycle of certain viruses. The inhibition of host enzymes could be advantageous compared to the classical addressing of viral targets due to a low risk for rapid drug resistances. However, side effects may occur by targeting host enzymes, which are required for normal physiological processes. Moreover, most host proteases belong to families of structurally closely related enzymes, and therefore it might be challenging to address a single target without affecting other family members. In order to avoid side effects and a narrow therapeutic window, it is therefore advisable to develop such host protease inhibitors mainly for combination therapies, which should enable the use of lower drug doses.

In the following sections, numerous examples for the antiviral activity of inhibitors mainly addressing human serine proteases will be provided. In addition, structural aspects of the protease-inhibitor complexes will be discussed. Although first

approved inhibitors of ACE and thrombin have been discovered long before the target structures have been determined, all subsequent successful developments in the field of protease inhibitors were strongly supported by the availability of crystal structures, which is an important prerequisite for a rational structure-based drug design.

11.2 Serine Proteases as Antiviral Targets

11.2.1 Trypsin-Like Serine Proteases

The majority of the human serine proteases belongs to the subfamily S1A, possessing a chymotrypsin-like folding pattern, and to the family S8 with the two subfamilies S8A and S8B exhibiting a subtilisin- or kexin-like folding (Rawlings et al. 2014). First studies on the limited proteolysis and essential maturation of viral glycoproteins by host proteases were performed on influenza viruses. It could be demonstrated that the cleavage of the hemagglutinin (HA) precursor could be blocked by the broad-spectrum serine protease inhibitor diisopropylfluorophosphate (DFP) (Klenk and Rott 1973). It was found that the poor infectivity of influenza A viruses (IAV) grown in cultures of chick embryo cells could be strongly increased after treatment with exogenous trypsin, which cleaves substrates after basic residues like arginine or lysine (Klenk et al. 1975). At the same time, comparable results were described for the trypsin-catalyzed activation of HA from influenza B viruses (Lazarowitz and Choppin 1975). Although the digestive protease trypsin is not found in the respiratory tract, these initial studies suggested that other trypsin-like airway proteases should be involved in HA maturation. For instance, plasmin efficiently activated the HA of the A/WSN virions but failed to cleave the influenza B HA (Lazarowitz and Choppin 1975). A detailed analysis of the substrate sequences revealed that HAs of human and other mammalian influenza viruses as well as HAs of low pathogenic avian influenza viruses (LPAIV) are cleaved after a single arginine residue before a constant P1'-P3' Gly-Leu-Phe segment by trypsin-like serine proteases. In contrast, HAs of high pathogenic avian influenza viruses (HPAIV) are activated by furin-like proprotein convertases (PCs) at inserted multibasic sequences containing additional arginine or lysine residues in the adjacent non-primed positions (Garten and Klenk 2008). In addition to the HA activation of HPAIV, many other viruses depend on the correct cleavage of their surface glycoproteins by furin-like PCs (basic PCs) or by the neutral PC SKI-1 (Klenk and Garten 1994; Pasquato et al. 2013). Consequently, different inhibitor structures depending on the specific virus strains are required to target the appropriate activating protease.

The human genome encodes for approximately 70 different trypsin-like serine proteases, which cleave after a single basic residue, preferably after arginine in P1 position (Schechter and Berger 1967). Few of them, like matriptase or TMPSS13 (MSPL), strongly prefer substrates with additional basic residues in the non-primed region close to the cleavage site, e.g., in P4 and/or P3 position. The full-length enzymes can strongly differ in their molecular weights due to the presence of specific protein domains. However, all of them possess a relatively similar catalytic

domain of approximately 225–230 amino acids with a strong structural homology to chymotrypsin of the subfamily S1A of serine proteases. This facilitates a common numbering of the residues within the catalytic domain with respect to chymotrypsin(ogen) used throughout this chapter. So far, crystal structures are available for ~25 different trypsin-like serine proteases. In most cases, the structures have only been determined for their catalytic domains, and their knowledge is normally sufficient for a rational structure-based design of active-site-directed inhibitors. The protease domain of the trypsin-like serine proteases consists of two six-stranded barrel domains, held together by several transdomain straps. The residues Ser195, His57, and Asp102 of the catalytic triad are located at the junction between these two barrels. All trypsin-like serine proteases contain a negatively charged aspartate residue at the bottom of their S1 pocket responsible for accepting substrates and inhibitors with basic P1 residues.

Data from virus-infected cell cultures suggested that different secreted trypsin-like serine proteases are involved in the HA activation of human IAV and LPAIV depending on the host and specific virus strain. A protease named tryptase Clara was isolated from Clara cells of rat airway epithelium (Kido et al. 1992). Furthermore, miniplasmin purified from rat lungs was described as potential HA activator (Murakami et al. 2001). Miniplasmin is a degraded version of plasmin, only comprising the kringle 5 and protease domain and lacking the N-terminal kringle domains 1–4 (Al-Horani and Desai 2014). However, plasmin cannot efficiently activate the HAs with monobasic cleavage sites found in the presently circulating influenza strains, because it prefers substrates with bulky P2 residues like Phe, Tyr, and Trp (Swedberg and Harris 2011). In embryonated chicken eggs, a blood clotting factor Xa-like protease was found to activate HA (Gotoh et al. 1990). Again, it seems unlikely that human factor Xa could be involved, which is a very specific protease with a strong preference for substrates with glycine in P2 position due to the presence of a bulky Tyr99 on top of its S2 binding pocket. This special structural feature limits the access of substrates with larger P2 side chains. So far, only prothrombin and eventually the protease-activated receptor 2 (PAR-2) are known as well-cleavable natural factor Xa substrates (Oe et al. 2016); both possess a glycine in P2 position. Moreover, in rats and mice, cellular ectopic trypsins due to severe influenza infections and cytokine storms were detected as HA-activating proteases (Kido et al. 2012; Pan et al. 2011; Wang et al. 2010). An unusual extrapancreatic trypsin expression was also found in some human tumors (Paju et al. 2001; Sorsa et al. 1997), raising speculations that this could also happen as a consequence of excessive inflammation during viral infections. Porcine tryptases are additional candidates (Chen et al. 2000; Sato et al. 2003), although human lung tryptase has failed to activate the HA of the 1918 IAV (Stevens et al. 2004). Among the 15 kallikrein-related peptidases (KLKs) (Goettig et al. 2010), 12 of them possess a trypsin-like substrate specificity, and a potential HA activation of seasonal IAV was observed for KLK5 and KLK12 (Hamilton and Whittaker 2013).

Additional candidates have been found among the type II transmembrane serine proteases (TTSPs), which comprise 17 different enzymes (Antalis et al. 2011; Garten et al. 2015). Most of them show a restricted tissue-specific expression pattern; an exception is matriptase, which is ubiquitously found in epithelial layers of

most tissues. In 2006, the TTSPs TMPRSS2 (epitheliasin) and human airway trypsin-like peptidase (HAT or TMPRSS11D) were identified as activating enzymes of monobasic HAs of seasonal IAV when overexpressed in MDCK cells (Böttcher-Friebertshäuser et al. 2010; Böttcher et al. 2006). A potential HA cleavage of the 1918 IAV was also described by TMPRSS4 (Bertram et al. 2010; Chaipan et al. 2009). The virus spread is eventually promoted by additional TTSPs, like DESC1 (Zmora et al. 2014) or hepsin, whereas no HA processing was found for TMPRSS3 and TMPRSS6 (also named as matriptase-2) (Bertram et al. 2010). In contrast to these TTSPs involved in the HA activation of LPAIV, a strong substrate preference for HAs from HPAIV with Lys as P4 residue has been identified for TMPRSS13 (MSPL) (Okumura et al. 2010). This is a striking difference to the substrate profile of the PC furin, which strongly prefers arginine in P4 position (Rockwell et al. 2002). Independent studies with knockout mice revealed that the HA of the seasonal H1N1 virions including the 2009 pandemic virus is dominantly activated by TMPRSS2 (Hatesuer et al. 2013; Sakai et al. 2014; Tarnow et al. 2014), whereas TMPRSS2 alone is not sufficient for the maturation of the HA from H3N2 subtypes. A recent study showed that only the combined TMPRSS2^{-/-} and TMPRSS4^{-/-} knockout mice reduced H3N2 spread and signs of infection in lung, suggesting that both proteases are involved (Kuhn et al. 2016).

11.2.1.1 Inhibitors for the Treatment of Human Influenza Virus Infections

Proteinaceous Inhibitors

The concept of HA cleavage inhibition as realistic antiviral strategy was supported by studies with the broad-spectrum inhibitor aprotinin (**1**) in mice (Ovcharenko and Zhirnov 1994; Zhirnov et al. 1982a). The 58-mer Kunitz-type inhibitor, also known as BPTI or Trasylol[®], is isolated from bovine lungs and relatively well tolerated in animals and humans. It inhibits numerous trypsin-like serine proteases such as trypsin, plasmin, matriptase, and plasma kallikrein (Ascenzi et al. 2003); its complex with trypsin is shown in Fig. 11.1.

Trasylol was intravenously used for many years to reduce perioperative bleeding during open heart surgery but was withdrawn from market in 2008 when it became clear that it was associated with increased numbers of deaths as compared to treatment with standard antifibrinolytics (Fergusson et al. 2008). As nonhuman protein, it may cause hypersensitive reactions during cardiac surgery, although the risk for anaphylactic reactions at first-time exposure is very low (<0.1%) but might increase to ~2.7% after reexposure (Levy and Adkinson 2008). However, the application of aprotinin against influenza in form of an aerosol at significantly lower doses should minimize side effects (Zhirnov et al. 2011). In addition, an aprotinin variant with stronger kallikrein and similar plasmin inhibition has been developed, which exhibits a reduced immunogenicity in chimpanzees (Apeler et al. 2004). Antiviral potency against influenza was recently also demonstrated with the endogenous proteinaceous inhibitor hepatocyte growth factor activator inhibitor 2 (HAI-2) (Hamilton et al. 2014). HAI-2 and HAI-1 are type 1 transmembrane proteins both containing two Kunitz domains, which inhibit numerous trypsin-like serine proteases including

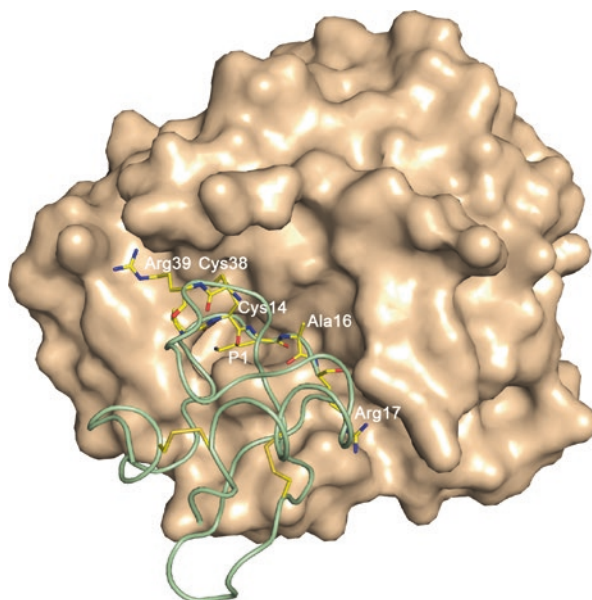


Fig. 11.1 Structure of aprotinin (1) in complex with trypsin (PDB, 3FP6). Trypsin is shown with its surface in beige, aprotinin as cartoon in green. Only the aprotinin residues in direct contact to the trypsin surface (Pro13-Cys-Lys-Ala-Arg17 and Cys38-Arg39) are shown as sticks with carbon atoms in yellow, as well as the three disulfide bridges. The P1 residue Lys15 binds into the S1 pocket of trypsin

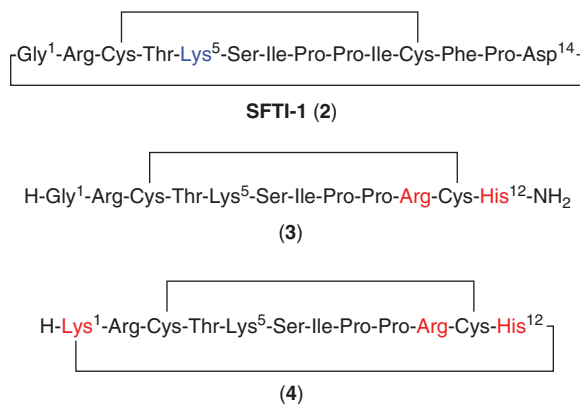


Fig. 11.2 Structure of the bicyclic 14-mer SFTI-1 (2) and of its optimized truncated monocyclic (3) and bicyclic (4) analogues

hepatocyte growth factor activator, matriptase (Szabo et al. 2008), hepsin, trypsin, and prostaticin (Liu et al. 2017). Sunflower trypsin inhibitor SFTI-1 (2), a bicyclic 14-mer, provides also an excellent scaffold for the design of serine protease inhibitors (Luckett et al. 1999) (Fig. 11.2).

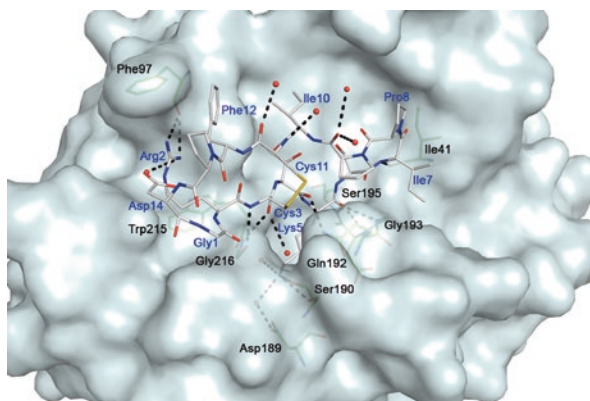


Fig. 11.3 Crystal structure of SFTI-1 (**2**) in complex with matriptase (PDB, 3P8F). Matriptase is shown with its transparent surface in cyan; important residues involved in H-bonds are shown as sticks with carbon atoms in green. SFTI-1 is presented as sticks with carbons in white, water molecules as red balls, and polar contacts as dashed lines in black. For better differentiation, matriptase residues are labeled in black and selected inhibitor residues in blue

It is one of the smallest naturally occurring peptidic protease inhibitors and comprises a typical Bowman-Birk motif of serine protease inhibitors (BBIs) with its characteristic 9-mer disulfide-bridged loop containing Lys5 as P1 residue (Li et al. 2007; McBride et al. 2002). The inhibitor is further stabilized by an additional head-to-tail backbone cyclization between the N-terminal Gly1 and C-terminal Asp14 residues. SFTI-1 inhibits trypsin and matriptase with K_i values of 100 pM (Lockett et al. 1999) and 100 nM (Long et al. 2001), respectively. After initial crystallization in complex with trypsin (Lockett et al. 1999), its binding mode has been determined also in the active site of matriptase (Fig. 11.3) (Yuan et al. 2011).

In contrast to the trypsin complex, the Lys5 amino group makes only a water-bridged interaction to Asp189 at the bottom of the S1 pocket of matriptase but forms no direct contact to its carboxyl group. It also interacts with the side chain and carbonyl oxygens of the adjacent Ser190 and with a second water molecule (Fig. 11.3). Numerous analogues of SFTI-1 have been made. For instance, its conversion to the more simple truncated monocyclic analogue **3** provided a K_i value of 6.2 nM against matriptase (Fittler et al. 2013). The bicyclic analogue **4**, containing a side-chain-to-tail cyclization, is even more potent with an inhibition constant of 2.6 nM (Fittler et al. 2014). However, despite this excellent potency, none of the SFTI analogues has been tested for antiviral activity.

Because of their high molecular weight aprotinin, HAI-1, HAI-2, or SFTI analogues should mainly inhibit extracellular HA processing, and it is rather unlikely that substantial amounts of these compounds can enter the cell and block the TTSPs in the secretory pathway. Despite this limitation, an aerosol formulation of aprotinin has been developed and approved in Russia for the treatment of mild-to-moderate influenza infections (Zhironov et al. 2011). It was described that in case of mammalian IAV and LPAIV with monobasic HAs, progeny particles are assembled, which still

contain some uncleaved HAs and therefore require further activation by extracellular enzymes sensitive to aprotinin. In contrast to these previous reports, more recent findings reveal that the monobasic HAs are processed in intracellular compartments without a further need for extracellular processing (Böttcher-Friebertshäuser et al. 2010, 2013). This suggests that small amounts of aprotinin can reach intracellular targets and achieve an antiviral efficacy (Zhirnov et al. 2011).

S1 Binders

Although the therapeutic use of peptide drugs (Vlieghe et al. 2010) and biologics including recombinant enzymes, hormones, and antibodies is steadily increasing and meanwhile well established, a treatment with synthetic, small-molecule drugs offers significant advantages. They can be produced at low costs in large quantities and offer the possibility for oral administration with a negligible risk of allergic reactions. In initial studies a weak antiviral effect after treatment with ECA (5, ϵ -aminocaproic acid, Fig. 11.4) was observed in mice infected by IAV including the plasmin-dependent WSN strain (Zhirnov et al. 1982a, b). Due to its small size, ECA (5) cannot bind efficiently to the active site of trypsin-like proteases and reduces mainly the activation of plasminogen by blocking the lysine binding site on the kringle domains of plasminogen (Al-Horani and Desai 2014). A pronounced protective effect was also observed, when IAV-infected mice were treated with a cocktail of 4-(2-aminoethyl)benzenesulfonylfluoride (6, AEBSF or Pefabloc®SC) and *p*-aminobenzamidine (7, *p*-AB) prior to infection. Both compounds are relatively unspecific protease inhibitors and can only occupy the S1 pocket of trypsin-like serine proteases. AEBSF 6 leads to a covalent sulfonylation of the active-site Ser195 side chain (second-order inactivation constant k_2/K_i for trypsin $30 \text{ M}^{-1} \text{ s}^{-1}$), while *p*-AB acts as a reversible competitive inhibitor with K_i values in the two- and three-digit micromolar range depending on the specific target (Stürzebecher et al. 2001).

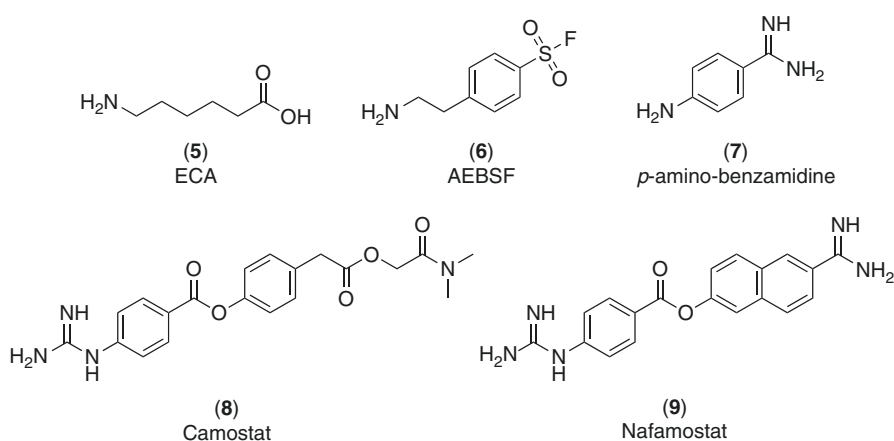


Fig. 11.4 Structures of the lysine analogue ECA and of numerous broad-spectrum inhibitors of trypsin-like serine proteases mainly targeting the S1-binding pocket

Although AEBSF **6** is more stable in buffer than the related phenylmethane sulfonylfluoride (PMSF), it is also susceptible to hydrolysis in aqueous media (Powers et al. 2002) and not suitable for further development. Derivatives of the classical active-site titrant *p*-nitrophenyl-*p*-guanidinobenzoate (Chase and Shaw 1967) including compounds like camostat **8** (FOY-305) and nafamostat **9** (FUT-175) (Fujii and Hitomi 1981) inhibit numerous trypsin-like serine proteases by covalent acylation of their active-site Ser195, providing relatively stable acyl-enzyme complexes. These compounds are active against numerous monobasic IAV and IBV strains (Hosoya et al. 1993; Lee et al. 1996; Someya et al. 1990) but suffer from instability of their ester bond in aqueous media and the circulation ($t_{1/2} < 1$ min after intravenous application of camostat in man (Midgley et al. 1994)). A very short half-life is also reported for nafamostat, although this could be an advantage for certain applications. Notably, nafamostat has been widely used as anticoagulant for hemodialysis patients with a tendency to bleed (Han et al. 2011).

Substrate Analogue Inhibitors

Although an antiviral efficacy was demonstrated for ECA **5** and the S1 ligands **6–9**, a stronger potency can be achieved with compounds also addressing adjacent binding sites. Trypsin-like host proteases possess relatively well-defined S2 and S3/S4 regions, often named proximal and distal binding pockets, respectively. Therefore, substrate analogue S3/S4-S2-segments were coupled with C-terminal decarboxylated P1 arginine mimetics or with stabilized non-cleavable P1-P1' scaffolds, e.g., arginyl-ketone moieties or other warheads targeting the active-site serine.

A well-suited P1 mimetic to target trypsin- and furin-like PCs is the 4-amidinobenzylamide group (4-Amba). The 4-Amba anchor makes numerous polar interactions to residues at the bottom and exit of the S1 pocket (Fig. 11.5) but exhibits no preference for special trypsin-like serine proteases. Within this inhibitor type, an improved selectivity for individual family members can partially be achieved only via their P2, P3, and/or P4 residues. All substrate analogue structures strictly require a P2 amino acid in L-configuration, whereas a D-configured P3-residue is often preferred. Its side chain can address the characteristic distal S3/4 binding pocket above Trp215, which is found and mostly well-defined in all trypsin-like serine proteases. In contrast, the side chain of a natural L-configured P3 residue is directed toward the solvent and makes fewer binding contributions. Interestingly, the backbone of the P3 residue is involved in a short antiparallel β -sheet interaction with a highly conserved glycine in position 216 of the protease domain, irrespective of an L- or D-configuration of the P3 amino acid. So far, no crystal structures of TTSPs involved in HA cleavage, like Tmprss2, Tmprss4, or HAT, are available. However, countless crystal structures of this inhibitor type in complex with numerous other trypsin-like serine proteases have been elucidated. As an example, Fig. 11.5 shows the binding mode of the substrate analogue inhibitor H-D-hTyr-Ala-4-Amba (**10**) (Fig. 11.6) in complex with trypsin, indicating the characteristic binding pockets (Maiwald et al. 2016). This inhibitor type could be refined following a well-established prodrug strategy that has been used for the development of the orally available thrombin inhibitor ximelagatran (Gustafsson et al. 2004).

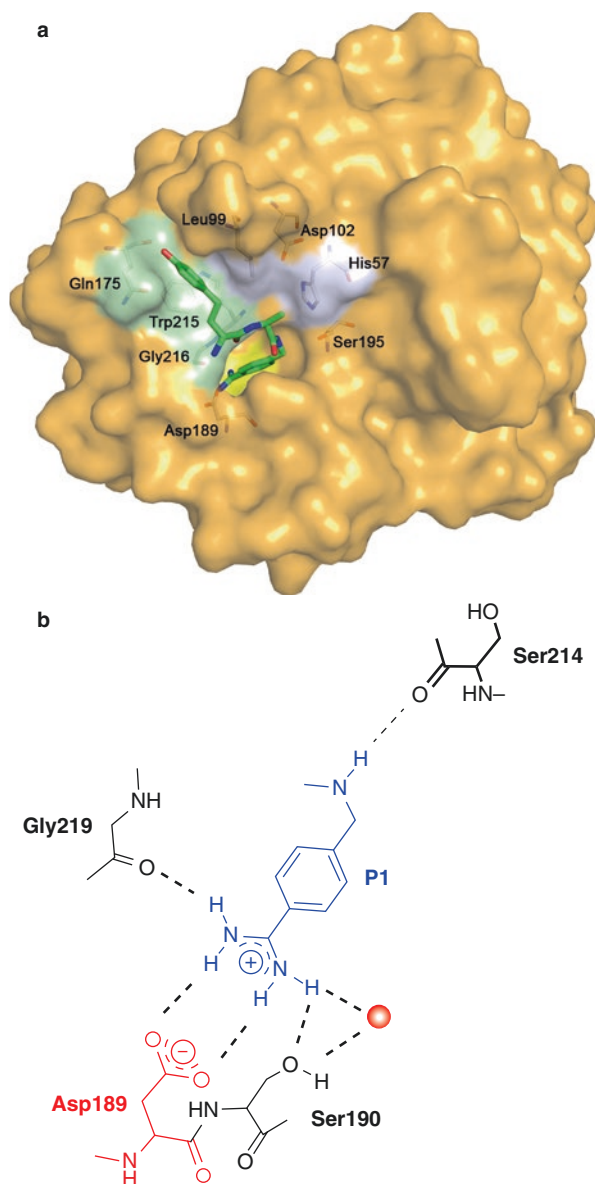


Fig. 11.5 Crystal structure of the substrate analogue inhibitor H-D-homoTyr-Ala-4-Amba (**10**) in complex with trypsin (PDB, 4MTB) (Maiwald et al. 2016). (a) Overall structure of the complex indicating the characteristic binding pockets similarly found in all trypsin-like serine proteases (S1 pocket with Asp189 at the bottom in yellow, S2 pocket below His57 and Leu99 in light blue, distal S3/4 pocket above Trp215 in light green). The trypsin residues of the catalytic triad (Ser195, His57, and Asp102) and some additional residues within the active site are labeled; water molecules are omitted. (b) Polar interactions of the 4-Amba residue in the S1 pocket of trypsin, a conserved water molecule found in nearly all crystal structures with arginine or benzamidines in P1 position, is shown as red ball. Most family members contain a Ser190 as shown for trypsin or an Ala190 (e.g., in HAT, thrombin, factor Xa) that makes an H-bond less

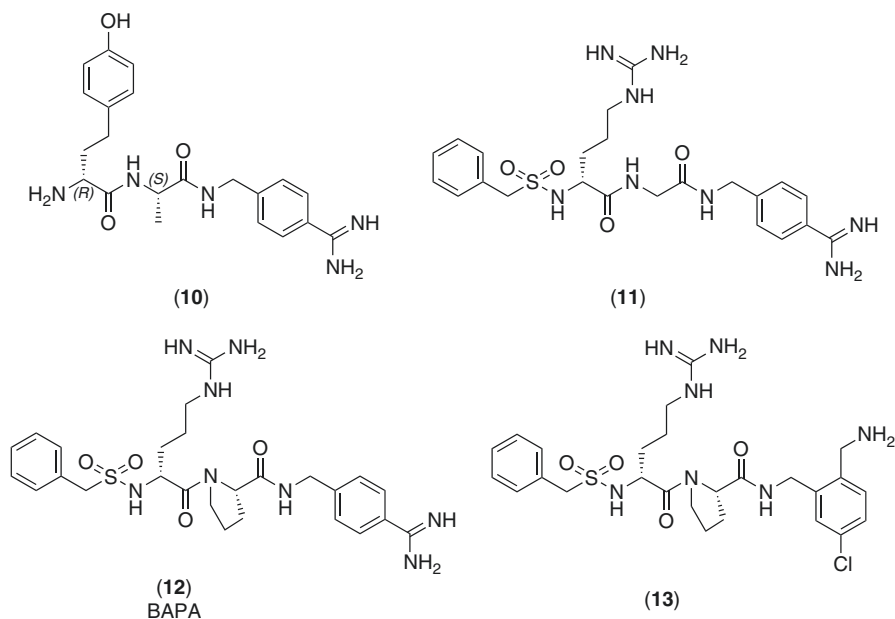


Fig. 11.6 Structures of substrate analogue inhibitors against trypsin-like serine proteases containing decarboxylated arginine mimetics like 4-amidinobenzylamide or 2-aminomethyl-5-chlorobenzylamide in P1 position (Maiwald et al. 2016; Schweinitz et al. 2006)

The conversion of the P1-amidine ($pK_a = 11.5$) into a hydroxyamidine ($pK_a = 5.2$) reduces the strongly basic character of the 4-Amba group and improves the bio-availability of the inhibitors. An enzyme system found in the liver and other organs (kidney, lung, brain, and intestine) consisting of cytochrome b5, a NADH-dependent cytochrome b5 reductase, and a P450 enzyme is involved in the reduction of the N-hydroxylated compounds (Clement 2002).

Due to a similar architecture of the S1 pocket in most trypsin-like serine proteases, it is challenging to achieve a pronounced selectivity for individual proteases within this inhibitor scaffold, although compound **10** is a relatively selective matriptase inhibitor ($K_i = 26$ nM) and shows reduced affinity against the clotting proteases thrombin ($K_i = 300$ nM) and factor Xa ($K_i = 570$ nM) (Maiwald et al. 2016). The potency of this inhibitor type can be further enhanced by elongation with a suitable P4 residue, preferably a sulfonyl group, whereas one of its oxygen atoms interacts with the amide NH of Gly219. For inhibitors containing a D-configured P3 residue, the N-terminal coupling of a benzylsulfonyl group induces a turn-like inhibitor backbone conformation when bound to the target, in which the N-terminal benzyl group binds in a shallow subpocket adjacent to the S1 site and comes in close van der Waals contact to the P1 phenyl ring (Fig. 11.7) above the Cys191-Cys220 disulfide bridge. Inhibitor **11** is a somewhat selective fXa inhibitor ($K_i = 3.5$ nM (Schweinitz et al. 2006)) due to the presence of glycine in P2 position but inhibits also trypsin and matriptase with inhibition constants of 10 nM and 130 nM, respectively.

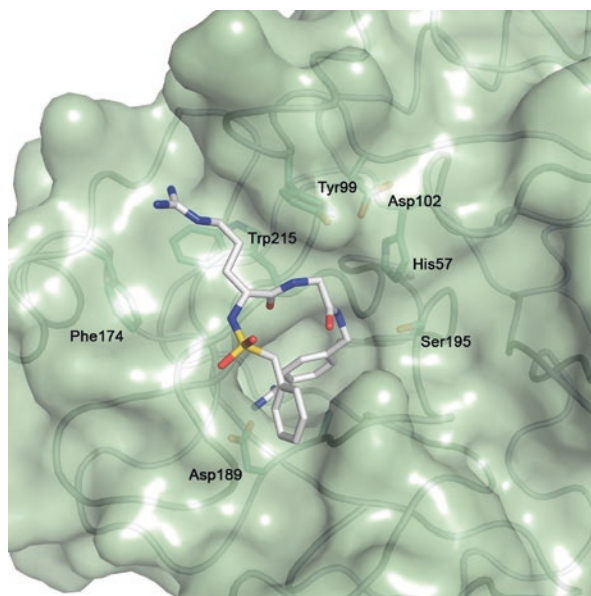


Fig. 11.7 Crystal structure of the substrate analogue inhibitor BzLs-D-Arg-Gly-4-Amba (**11**) in complex with a trypsin variant containing Leu99Tyr and Gly174Phe mutations (PDB, 3PMJ) (Tziridis et al. 2014). Only few side chains of characteristic residues within the active site of this trypsin mutant are shown and labeled. The inhibitor adopts a turn-like backbone conformation; the P3 D-Arg side chain binds into the distal S3/4 pocket above Trp215 and is probably involved in cation- π interactions to the aromatic residues Tyr99, Phe174, and Trp215

The replacement of glycine by proline in P2 position leads to the relatively non-selective inhibitor BAPA (**12**), which possesses one- or two-digit nanomolar potencies against the clotting proteases thrombin, fXa, plasma kallikrein (PK), fibrinolytic plasmin, and the TTSPs HAT, TMPRSS2, and matriptase (Hellstern et al. 2007; Maiwald et al. 2016; Sielaff et al. 2011a). A significant antiviral effect was found in BAPA-treated cell cultures infected with numerous monobasic IAV strains (Böttcher-Friebertshäuser et al. 2010, 2012; Böttcher et al. 2009; Sielaff et al. 2011a). Many analogues of this inhibitor scaffold with different P2 and/or P3 residues have been tested but were less effective than BAPA (data not published). It seems that compounds with a broader target spectrum exhibit an advantageous antiviral profile. This tendency confirms the promising results obtained for the relatively nonselective inhibitor aprotinin. Otherwise, less selective inhibitors that also target numerous clotting proteases might suffer from a narrow safety profile due to potential bleeding complications after i.v. treatment. Such side effects might be reduced, when the compounds will be inhaled, as described for aprotinin. Notably, BAPA (**12**) was well tolerated in a bleomycin-induced fibrosis model in mice when it was applied as aerosol via a microsyringe (data not published).

In principle, it should be possible to replace the C-terminal 4-Amba residue in these substrate analogue inhibitors. Numerous alternative P1 groups are known

from the field of thrombin and fXa inhibitors (Straub et al. 2011). Besides basic groups, many chloro-substituted aromatic P1 residues as used, for example, in inhibitor **13** have been developed (Sisay et al. 2010). The chlorine addresses a highly conserved Tyr228 side chain found at the back of the S1 pocket, whereas the aminomethyl group comes out of the S1 pocket and does not bind to Asp189. It provides an enhanced inhibitory potency against thrombin and fXa but leads to a reduced inhibition of HAT, matriptase, plasmin, and of several other trypsin-like serine proteases. However, only weak anti-influenza activity was found for compound **13** suggesting that the relevant HA-cleaving host proteases are not susceptible to such chloro-substituted aromatic P1 structures.

Covalent Substrate Analogue Inhibitors

Another strategy is the design of covalent inhibitors (Singh et al. 2011). Covalent bonds can be irreversibly formed, e.g., when serine proteases are inactivated by chloromethyl ketones (CMK) (Powers et al. 2002). Although such CMKs are useful tools for initial biochemical experiments and structure analysis, they are not suitable for drug development due to stability and selectivity problems. A more eligible approach is the incorporation of electrophilic warheads, which also form covalent but reversible bonds to their targets. This can be achieved by coupling of substrate-analogue P3-P2 or P4-P2 segments with P1 borarginine and arginal groups or with P1-P1'-arginyl ketones. These electrophilic groups can be attacked by the active-site serine providing covalent boronate ester-, hemiacetal-, or hemiketal-like complexes. The formed covalent bond significantly improves the affinity of the inhibitors. In the 1990s, a few arginal and arginyl-ketone-derived thrombin inhibitors reached preclinical and early clinical development (Steinmetzer et al. 2001), which was stopped due to the discovery of more suited non-covalent inhibitors, like melagatran containing the 4-Amba residue. The arginal derivative **14** acylated with the N-terminal Abz-fluorophore (Fig. 11.8) was described as an inhibitor of HAT ($K_i = 54$ nM) (Wysocka et al. 2010). CVS-3983 (**15**), a second aldehyde derivative containing an unusual dialkylated P3 residue, inhibits matriptase with an inhibition constant of 3.3 nM and reduced prostate cancer growth in a xenograft mice model (Galkin et al. 2004). However, none of these aldehydes was tested for antiviral potential. The reuse of reversible arginyl ketone inhibitors against TTSPs has started in 2012 with compound **16** (IN-1) (Fig. 11.8). Although it is a much stronger matriptase inhibitor ($K_i = 11$ pM), it potently inhibits additional trypsin-like serine proteases like matriptase-2, hepsin, HAT, and trypsin with K_i values <10 nM (Colombo et al. 2012). Compound **16** reduces breast cancer progression in mice (Zoratti et al. 2015) and inhibits H1N1 IAV propagation in human Calu-3 cells with an EC_{50} of 5.6 μ M (Beaulieu et al. 2013). It was postulated that the antiviral efficacy is due to matriptase inhibition. However, since the inhibitor concentrations used were well above the K_i values against related proteases, it is conceivable that the inhibition of additional targets contributes to the observed antiviral effects. Numerous analogues with modified P4-P2 peptide segments like compound **17** (K_i value for matriptase 0.92 nM) targeting also matriptase-2, hepsin, and hepatocyte growth factor activator (HGFA) have been prepared for tumor treatment or regulation of iron overload but were not further tested with influenza viruses, so far (Duchene et al. 2014; Han et al. 2014, 2016).

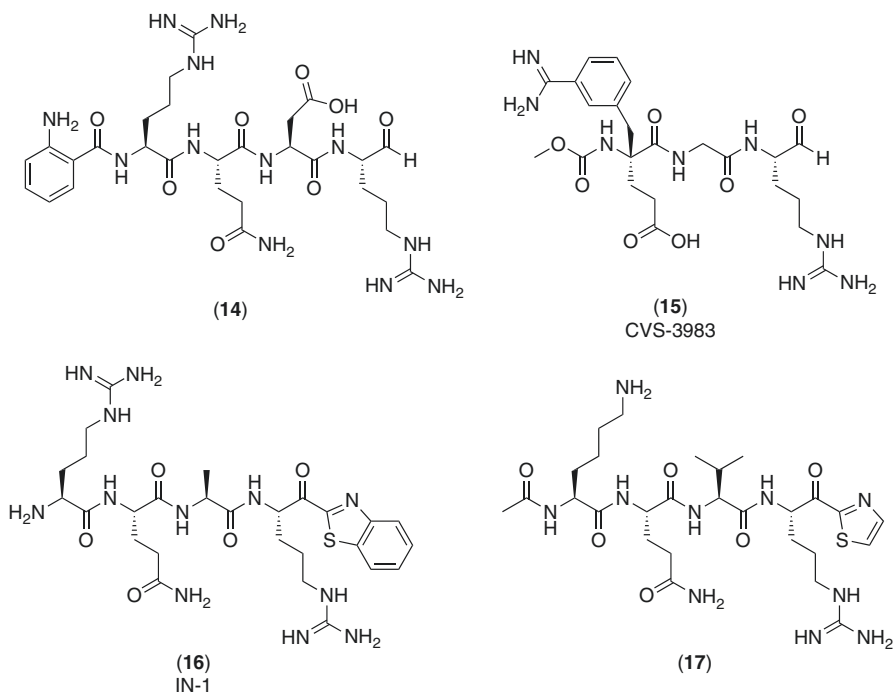


Fig. 11.8 Structures of peptidic arginal-, arginyl-ketobenzothiazole, and ketothiazole inhibitors (Colombo et al. 2012; Galkin et al. 2004; Han et al. 2016; Wysocka et al. 2010)

Sulfonylated 3-Amidinophenylalanine Derivatives

Suitable scaffolds for non-covalent inhibitors of trypsin-like serine proteases are the tertiary amides of sulfonylated 3-amidinophenylalanines, which were originally developed for the inhibition of trypsin, thrombin, or uPA and further used for the design of matriptase inhibitors (Steinmetzer et al. 2006; Stürzebecher et al. 1997). With some exception, this class of compounds provides relatively broad-spectrum inhibitors targeting numerous trypsin-like serine proteases. The uPA inhibitor **18** (Stürzebecher et al. 1999) (WX-UK1, Fig. 11.9) in combination with the 5-fluorouracil prodrug capecitabine reached clinical development for tumor therapy (Setyono-Han et al. 2005). Moreover, an orally available formulation in form of its hydroxyamidino prodrug mesupron (**19**) has also reached clinical phase II studies (indication pancreas and breast cancer). This suggests that this 3-amidinophenylalanine inhibitor type is suitable for drug development. Otherwise, compound **18** is only a moderate inhibitor of numerous trypsin-like serine proteases including matriptase with K_i values close to 0.5 μM . Its sterically demanding and rigid triisopropylphenylsulfonyl (Tips) group cannot fully occupy the distal binding pocket above Trp215 in the usual way (Setyono-Han et al. 2005; Stürzebecher et al. 1999). A significantly improved and selective matriptase inhibition ($K_i = 3.8$ nM) was achieved with inhibitor **20**. Its N-terminal β -alanyl-amide targets the distal S3/4 pocket and is most likely involved in cation- π interactions to Trp215 and Phe99 of matriptase (Steinmetzer et al. 2006). However, the tribasic and strongly polar

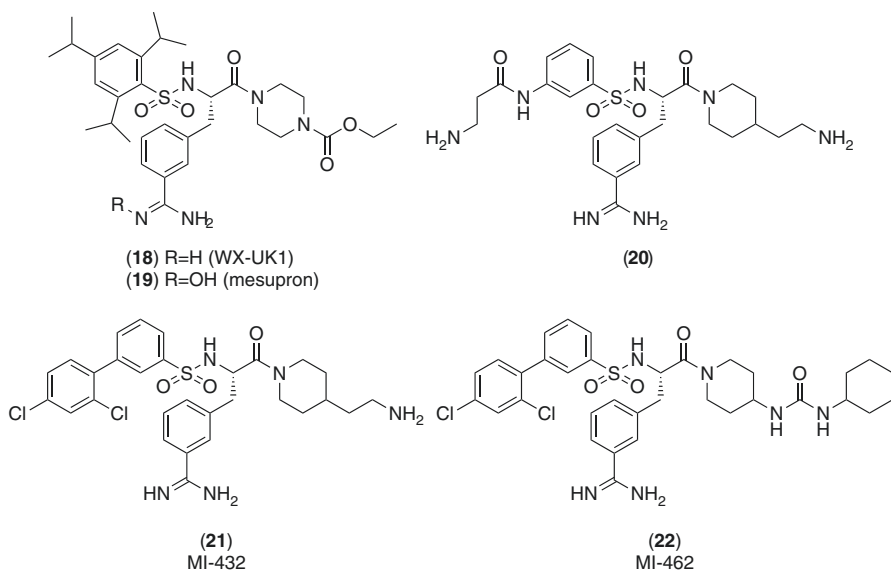


Fig. 11.9 Structures of tertiary amides of sulfonylated 3-amidinophenylalanines (Hammami et al. 2012; Steinmetzer et al. 2006; Stürzebecher et al. 1999). The hydroxyamidino prodrug strategy of mesupron (**19**) could also be adapted for other benzamidine inhibitors

character limits the membrane permeability and bioavailability of this compound. Therefore, the number of basic groups was stepwise reduced and provided compound **21** (MI-432) containing an N-terminal substituted biphenyl-3-sulfonyl group. This analogue inhibits matriptase and TMPRSS2 with similar inhibition constants of 2 nM and 0.9 nM, respectively (Hammami et al. 2012; Meyer et al. 2013). It is also a relatively potent thrombin inhibitor ($K_i = 20$ nM) but poor inhibitor of HAT ($K_i = 1.7$ μ M). Replacement of its C-terminal amino group by an urea portion provided the monobasic analogue **22** (MI-462) (Hammami et al. 2012) ($K_i = 5.1$ nM for matriptase and 630 nM for thrombin). Compounds **20–22** (Fig. 11.9) reduced the propagation of various LPAIV strains with monobasic HA cleavage sites in infected cells (Baron et al. 2013; Meyer et al. 2013). For instance, treatment with 50 μ M of compound **21** inhibited the multiple cycle replication of IAV H3N2 and H1N1 strains in Calu-3 cells (Meyer et al. 2013), in which TMPRSS2 was identified as the major HA-cleaving protease (Böttcher-Friebertshäuser et al. 2011).

So far, no crystal structure of TMPRSS2 is available in the protein data bank. Therefore, we have generated a homology model of TMPRSS2 in complex with inhibitor **21** using SWISS-MODEL (<http://swissmodel.expasy.org/>) (Biasini et al. 2014). The model was superimposed with the crystal structure of inhibitor **21** in complex with thrombin (PDB, 4E7R (Hammami et al. 2012)) and subsequent deletion of the thrombin structure. The inhibitor was energy minimized in the active site of TMPRSS2 using the software Molecular Operating Environment (MOE) (2016). The model shows the expected polar contacts known from numerous crystal structures of sulfonylated 3-amidinophenylalanines in complex with trypsin (Renatus et al. 1998), uPA (Zeslowska et al. 2000), and matriptase (Steinmetzer et al. 2006)

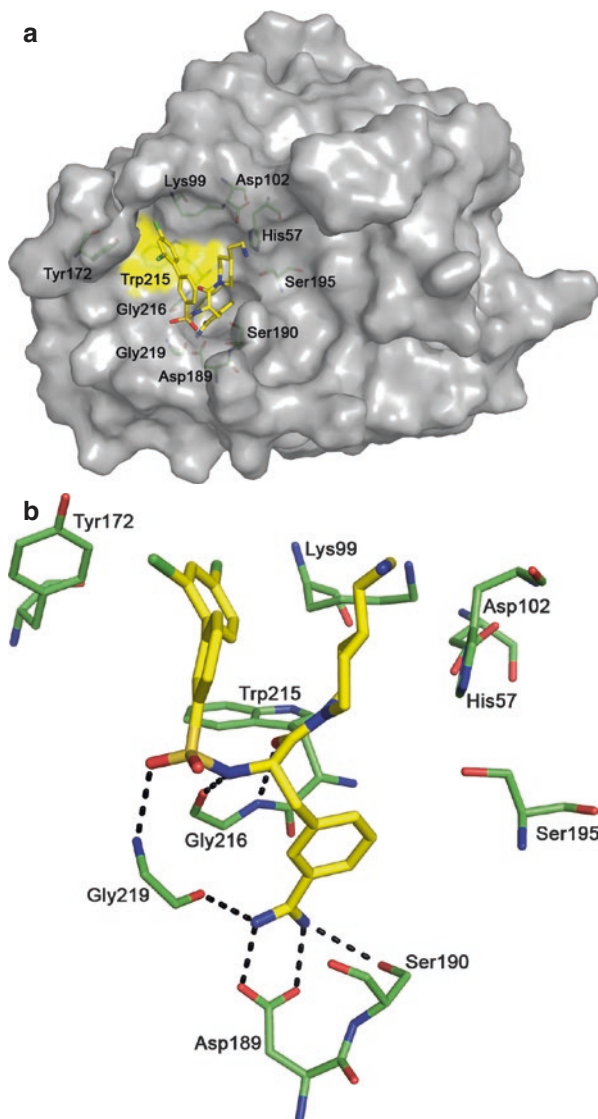


Fig. 11.10 Model of TMPRSS2 in complex with inhibitor **21**. (a) Structure of inhibitor **21** (stick model with yellow carbon atoms, nitrogen in blue, oxygen in red, chlorine in green) bound into the active site of TMPRSS2, shown with its surface in gray. The 2,4-dichloro-biphenyl-3-sulfonyl residue is directed toward the distal S3/4 pocket above Trp215 indicated by a yellow surface. (b) Polar interactions of inhibitor **21** in the active site of TMPRSS2 are shown as dashed lines in black; important TMPRSS2 residues are shown with carbon atoms in green

(Fig. 11.10). The benzamidine binds to Asp189, Ser190, and Gly219 at the bottom of the S1 pocket, whereas the backbone amide NH and carbonyl oxygen of the P1 3-amidinophenylalanine make a short antiparallel β -sheet-like interaction with Gly216, and one sulfonyl oxygen binds to the NH of Gly219. It should be noted that

in complexes with the substrate analogue inhibitors **10–17**, this Gly216 is addressed by the backbone NH and carbonyl of the P3 residue, which indicates a completely different binding mode of this inhibitor type.

Nonpeptidic Small-Molecule Inhibitors

Drug development is strongly focused on the design of nonpeptidic, small-molecule inhibitors. Usually, they are more stable than peptidic inhibitors, possess longer half-lives, and offer better chances to be orally bioavailable. Hundreds of highly potent nonpeptidic inhibitors targeting the clotting proteases are available, whereas only few TTSP inhibitors of this type have been described, so far. Most of the work was done in the field of matriptase inhibitors (Fig. 11.11). The bis-benzamidine **23** has a K_i value of 208 nM (Enyedy et al. 2001); and an enhanced potency ($K_i = 40$ nM) was described for analogue **24** (Goswami et al. 2013). The tribasic compounds **25** and **26** show improved matriptase inhibition with K_i values of 10 nM and 3 nM, respectively (Goswami et al. 2014). A less rigid core segment was used for the design of the tribasic inhibitor **27**, which inhibits matriptase and matriptase-2 with K_i values of 38 nM and 3.6 μ M (Furtmann et al. 2016). A group from Korea reported the discovery of the 2-hydroxydiarylamide derivatives **28** and **29** as TMPRSS4 inhibitors with IC_{50} values of 12 μ M and 6 μ M (Kang et al. 2013), respectively. Surprisingly, also the mucolytic cough suppressant bromhexine **30** was described as TMPRSS2 inhibitor ($IC_{50} = 0.75$ μ M).

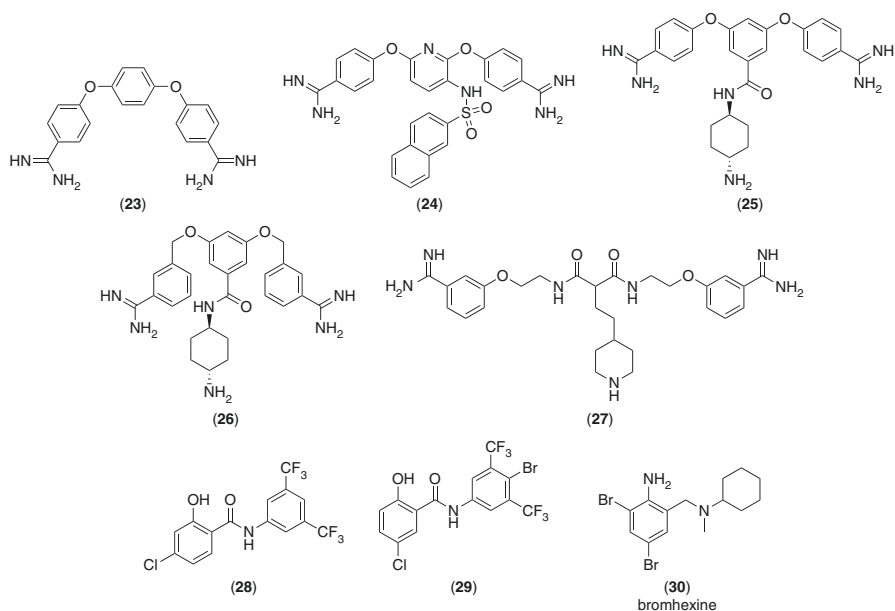


Fig. 11.11 Structures of nonpeptidic inhibitors of the TTSPs matriptase (**23–27**) (Enyedy et al. 2001; Furtmann et al. 2016; Goswami et al. 2013, 2014), TMPRSS4 (**28, 29**) (Kang et al. 2013), and TMPRSS2 (**30**) (Lucas et al. 2014)

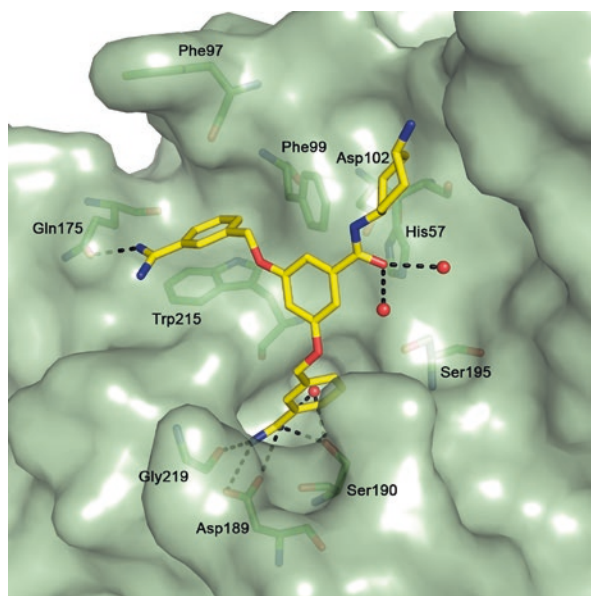


Fig. 11.12 Crystal structure of inhibitor **26** shown as sticks with carbon atoms in yellow (oxygen in red, nitrogen in blue) in complex with matriptase, presented with a transparent surface in green (PDB, 4O97) (Goswami et al. 2014). Important matriptase residues within the active site are shown with carbon atoms in green and are labeled. Water molecules in direct contact to the inhibitor are provided as red spheres, polar contacts as dashed lines in black

The binding mode of a few bis-benzamidine and several tribasic analogues in complex with matriptase was determined by crystal structure analysis. The complex with inhibitor **26** is shown in Fig. 11.12. One benzamidine occupies the S1 site, and the second targets the distal S3/4 pocket above Trp215, whereas the aminocyclohexyl group is directed toward the 60-loop of matriptase but not involved in direct polar contacts. Due to the lack of experimental structures of TMPRSS2 and TMPRSS4, the binding mode of compounds **28–30** cannot be trustfully predicted. Most of these nonpeptidic inhibitors were developed to address the TTSPs as targets for tumor treatment (Goswami et al. 2015; Kang et al. 2013; Lucas et al. 2014), although they might be also suitable for other indications.

Besides influenza viruses, other viral pathogens are also suitable targets for inhibitors of trypsin-like serine proteases (TLSPs). A few examples will be presented in the following paragraph.

11.2.1.2 TLSP Inhibitors for Treatment of Paramyxovirus- and Coronavirus Infections

Paramyxoviridae contain two surface glycoproteins, the receptor-binding protein (HN, H, G) and the fusion protein F. In contrast to the *Orthomyxoviridae*, where fusion of the viral membrane with host cell membrane occurs in the endosome, the activated F protein of many paramyxoviruses induces fusion of the virus envelope

and the plasma membrane. The F_0 precursor of human parainfluenza virus 1 (HPIV-1) is cleaved at a monobasic sequence typical for TLSPs (DNPQTR↓FFGAV) (Diederich and Maisner 2007). HPIV-1 causes respiratory infections, such as croup, especially among young children. In principle, some of the above-described inhibitors could also be suitable for the treatment of HPIV infections. With other paramyxoviruses, such as measles virus and mumps virus, F_0 is activated by furin, whereas F_0 of Nipah virus is cleaved by cathepsin L. Consequently, furin or cathepsin L inhibitors should be suitable for the treatment of these virus infections.

The spike (S) surface protein of respiratory coronaviruses (CoV) is also synthesized as inactive precursor protein that has to be activated by host proteases. The N-terminal S1 unit of the cleaved protein binds to receptors on host cells, and the C-terminal S2 unit enables the fusion of the viral membrane with host cell membranes. Both functions are essential for CoV propagation. Original studies suggested that the endosomal cysteine protease cathepsin L is solely required for spike activation and subsequent SARS-CoV infectivity (Simmons et al. 2005). Notably, a cathepsin dependency was also found for the enveloped Ebola virus of the *Filoviridae* family (Chandran et al. 2005) and Nipah virus (Diederich and Maisner 2007), as described above. Later, numerous groups have found that in the presence of cathepsin L inhibitors, the S-protein of SARS-CoV and MERS-CoV is activated by TMPRSS2 (Gierer et al. 2013; Matsuyama et al. 2010; Shulla et al. 2011; Zmora et al. 2014). Consequently, inhibition of SARS-CoV growth in Calu-3 airway epithelial cells was achieved by a combination treatment with the broad-spectrum serine protease inhibitor camostat (8) (Fig. 11.4) and the cathepsin inhibitor (23,25) trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester (EST) (Kawase et al. 2012). Based on these observations, it is suggested that SARS-CoV enters the host cells via two distinct pathways, one using TTSPs like TMPRSS2 and a second using the endosomal cathepsins L and/or B for spike activation. Interestingly, a recent study with a fresh clinical isolate of the human CoV 229E revealed a clear preference for host cell entry via TMPRSS2, whereas after 20 passages in HeLa cells the cathepsin L pathway became more important (Shirato et al. 2017). However, the cell culture virus showed a reduced ability for replication suggesting that the endosomal pathway is disadvantageous for HCoV-229E infection in humans. Based on these results, the authors suggested to target TMPRSS2 rather than endosomal cathepsins in CoV infections (Shirato et al. 2017). A similar tendency was previously found in an animal model of SARS-CoV infection, where viral spread and pathogenesis were only prevented by the TLSP inhibitor camostat and not by broad-spectrum vinylsulfonamide-type cysteine protease inhibitors targeting cathepsins L and B (Zhou et al. 2015). However, the authors argue that their new vinylsulfonamide inhibitors might be excellent lead structures for the development of inhibitors of Ebola virus entry.

11.2.2 Proprotein Convertases

The family of proprotein convertases comprises nine calcium-dependent serine endoproteases (furin, PC1, PC2, PC4, PC5, PACE4, PC7, SKI-1/S1P, and PCSK9). These enzymes play an important role in the maintenance of cell homeostasis by

activation or deactivation of proteins including prohormones as well as proforms of transcription factors, membrane receptors, and extracellular membrane proteins (Seidah and Prat 2012; Thomas 2002). Besides their physiological role, PCs are also involved in the activation of viral proteins and bacterial toxins. For example, several enveloped viruses depend on the cleavage of their surface glycoproteins by PCs to gain fusion capacity, which is required for virus propagation (Pasquato et al. 2013; Thomas 2002). All PCs possess a multidomain structure comprising a signal peptide, an N-terminal prodomain, followed by a catalytic domain, where the catalytic triad Ser-His-Asp is located, and a P-domain (Fig. 11.13) (Henrich et al. 2005).

Four PCs (furin, PC5B, PC7, and SKI-1/S1P) possess a transmembrane and C-terminal cytoplasmatic domain, which anchors them to cellular membranes. Furthermore, furin, PC5B, and SKI-1/S1P can be shed and released in a soluble

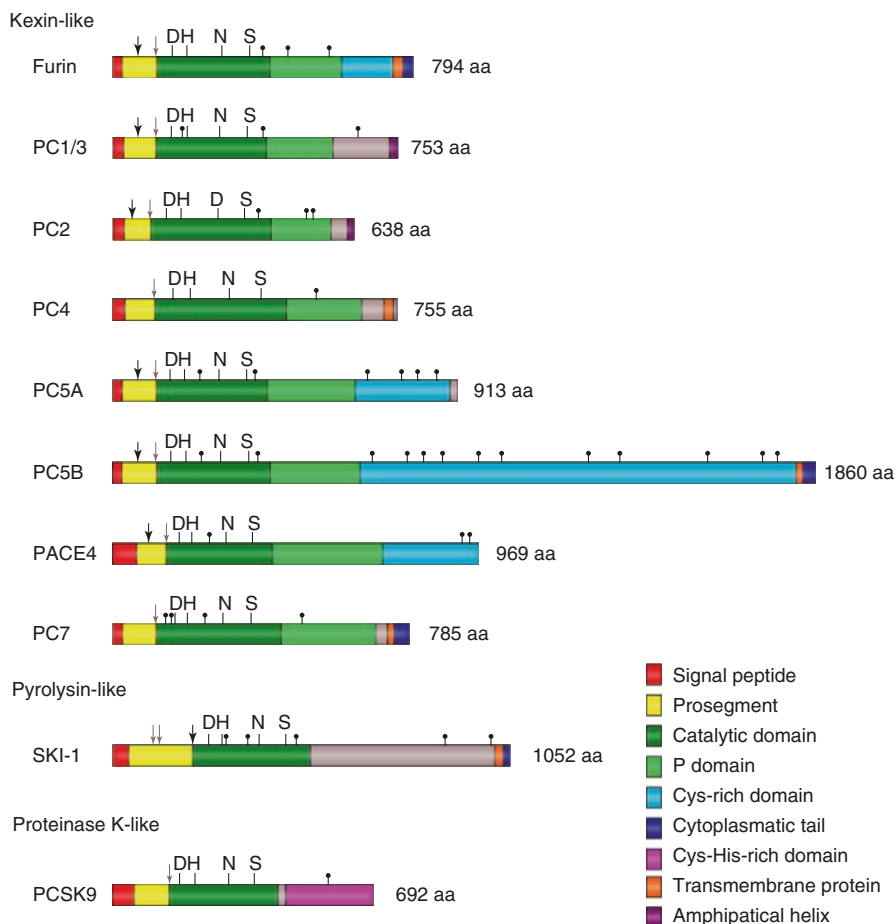


Fig. 11.13 Schematic structure of the human PCs. Residues belonging to the catalytic triad (S, H, and D) and oxyanion hole (N or in case of PC2 D) are indicated. N-glycosylation (†) and prodomain processing sites are highlighted with an arrow (primary cleavage site with a gray and secondary with a black arrow) (Seidah and Prat 2012)

form into the extracellular space. PC1 and PC2 are maintained in dense-core granules, whereas the remaining PC4, PC5A, PACE4, and PCSK9 are secreted. Furin and the related six PCs PC1, PC2, PC4, PC5, PACE4, and PC7 recognize multibasic cleavage sequences and, therefore, are also known as basic PCs or furin-like PCs. The catalytic domains of these enzymes show more than 50% identity (Thomas 2002). Due to the similar cleavage sites, a redundant behavior of these enzymes was found in overexpression experiments as well as in vitro studies. Despite overlapping consensus sequences and a high sequence homology, minor modifications in the recognition sequence as well as in their cellular localization lead to specific cleavages by the different PCs. In contrast, SKI-1/S1P cleaves after the consensus cleavage site (K/R)-X-(V/L/I)-Z↓, where Z is any amino acid except Val, Pro, Cys, Glu, or Asp and the spacer X is preferably a basic residue (Seidah 2013). After an autocatalytic cleavage at VFAQ↓S, PCSK9 forms a proteolytically inactive complex with its prosegment. So far, no other PCSK9 substrates are known.

Knockout of PCs in mice reveals their physiological significance especially during embryogenesis and contributes to the identification of specific PC substrates (Creemers and Khatib 2008; Seidah and Prat 2012; Taylor et al. 2003). Due to severe malformations, knockout of furin or SKI-1/S1P in mice leads to embryonic death, whereas PC5-deficient mice die at birth. For PACE4, a lethality of 25% was observed in knockout mice at embryonic day 14. In contrast, PC1- and PC2-deficient mice are viable but have several neuroendocrine peptide processing defects, and PC7 knockout mice show a loss of anxiety. The knockout of PC4 leads to infertility especially in male mice, whereas PCSK9 deficiency leads to lower plasma cholesterol levels.

The maturation of the PCs requires an autoproteolytic cleavage (Seidah and Prat 2012; Thomas 2002). In case of furin and furin-like PCs, two cleavages are needed to gain full enzymatic activity. After the removal of the signal peptide in the endoplasmic reticulum, a first cleavage leads to a conformational change of the enzyme, which is then the latent form. Enzymatic activity is obtained after a second cleavage in the prosegment, which leads to a release of the prosegment. The cleavages depend on the pH in the respective organelles. With exception of PC2 and SKI-1/S1P, the prosegments act as inhibitors of their respective enzyme. In case of SKI-1/S1P, three cleavages are required for full enzymatic activity. The subcellular localization of the PCs differs. Furin, PC5B, and PC7 have sorting signals in their cytosolic tails, which mediate recycling between the TGN and the cell surface. SKI-1/S1P shows also a broad distribution and is found in the ER, Golgi apparatus, endosomes, and lysosomes. In contrast, PC1 and PC2 are primarily found in dense-core vesicles of the secretory pathway, and PC4 is localized only in the plasma membrane of male and female germ cells. Furin, PC7, and SKI-1/S1P are ubiquitously distributed, and PC5 and PACE4 are widely distributed. In contrast, the expression of PC1 and PC2 is limited to neural and endocrine cells. PCSK9 can be found predominantly in the liver, intestine, and kidney.

The reported inhibitors against PCs can be categorized into various groups including macromolecular compounds, pure peptides, peptidomimetics, as well as nonpeptidic compounds, which will be described in the following sections.

11.2.2.1 Inhibitors of Basic Proprotein Convertases

Protein-Based Inhibitors

A common approach for protease inhibitor development is the optimization of natural inhibitors by mutation of their inhibitory recognition loops. The α 1-antitrypsin Portland (α 1-PDX) is a bioengineered serpin-type inhibitor containing the furin-adapted sequence R355-I-P-R358 instead of A355-I-P-M358 in its inhibitory loop. α 1-PDX inhibits furin with a K_i value of 0.6 nM in a slow tight-binding manner and is supposed to act as suicide substrate, yielding an inactive enzyme (Jean et al. 1998). It also inhibits PC1 ($K_i = 260$ nM) and PC5 ($K_i = 2.3$ nM) but has reduced potency against PC2, PC7, and PACE4 ($K_i > 1000$ nM). Its expression in cells blocked the processing of HIV gp160 as well as measles virus fusion protein and, thus, inhibited virus spread (Anderson et al. 1993; Watanabe et al. 1995). Based on the reactive loop of α 1-PDX, numerous mini-PDX peptides have been prepared. These acyclic- or disulfide-bridged cyclic 30-mers inhibit furin with IC_{50} values of 731 nM and 569 nM, respectively (Basak and Lotfipour 2005).

Turkey ovomucoid third domain (OMTKY3) belongs to the family of Kazal-type inhibitors and normally inactivates serine proteases of the S1A fold that prefer a neutral P1 residue. Exchange of A15-C-T-L18 to R15-C-L-R18 in its reactive site loop leads to a moderate furin inhibitor with an association constant K_a of 1.1×10^7 M⁻¹ (Lu et al. 1993), which roughly corresponds to a reciprocal dissociation equilibrium constant of ~90 nM.

Like OMTKY3, inter-alpha-inhibitor protein (I α Ip) is known to be a potent serine protease inhibitor, e.g., against trypsin, chymotrypsin, or acrosin. I α Ip was first isolated from human plasma and is a multicomponent complex, consisting of two heavy and one light chain, called bikunin, which are linked via a chondroitin linker. Bikunin possesses two protease domains of the Kunitz-type that are likely to inhibit furin, because treatment with I α Ip provided a significant protection against anthrax toxin in cell culture studies and in mice (Opal et al. 2005). Eglin C was originally isolated from the leech *Hirudo medicinalis* and belongs to the potato I inhibitor family. It inhibits several serine proteases, e.g., subtilisin, human leukocyte elastase, or cathepsin G. Insertion of a multibasic recognition site by mutation of P42-V-T-L45 to R42-V-K-R45 resulted in a strong furin inhibitor with a K_i value of 1.6 nM (Komiya and Fuller 2000; Liu et al. 2004).

Additional furin inhibitors were designed by mutation of the homotetrameric glycoprotein α_2 -macroglobulin (α_2 -M), which is found in high concentrations in human blood. It is a potent broad-spectrum protease inhibitor with a unique inhibition mechanism. After protease mediated cleavage in the so-called bait region, the internal S-esters hydrolyze and trigger a conformational change of α_2 -M. The protease is enclosed by α_2 -M and sterically shielded from its substrates (Barrett and Starkey 1973). Replacement of its original G683-F-Y-E-S-D688 sequence by R683-S-K-R-S-L688 yielded a potent furin inhibitor, which blocked the processing of von Willebrand factor, TGF- β 1, and HIV-1 gp160 (Van Rompaey et al. 1997).

The 45-kDa proteinase inhibitor 8 (PI8) was the first reported furin inhibitor, which is not a serpin reactive-site mutant. PI8 belongs to the group of ovalbumin-type

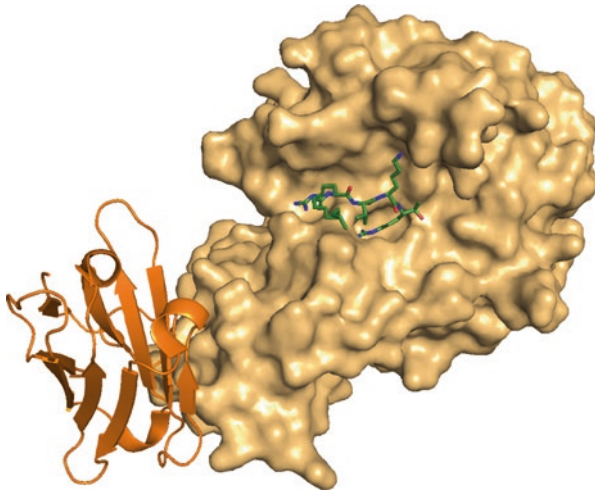


Fig. 11.14 Structure of human furin (shown with its surface in beige) in complex with the irreversible active-site inhibitor Dec-Arg-Val-Lys-Arg-CMK (stick model with carbons in green, nitrogen in blue, and oxygen in red) and with the furin inhibitory nanobody Nb14, shown in cartoon style in orange (PDB, 5JMO) (Dahms et al. 2016b). Although the nanobody binds far away from the active site, it blocks the binding of larger proteinaceous substrates

serpins, containing two furin recognition sequences within its R336-N-S-R-C-S-R342 segment. The inhibition of furin by PI8 consists of two steps, starting with the rapid formation of a loose complex and followed by the slow isomerization to a stable complex. The overall K_i value for recombinant and soluble furin in vitro is 53.8 pM (Dahlen et al. 1998).

Recently, several specific nanobodies against furin have been described (Zhu et al. 2012). These nanobodies are dromedary-derived single-domain antigen-binding fragments. A crystal structure of one of these nanobodies in complex with furin and an additional active-site inhibitor reveals that they do not directly bind to the active site (Fig. 11.14) (Dahms et al. 2016b). Nevertheless, some of these nanobodies inhibit furin-mediated cleavage of diphtheria toxin by a noncompetitive mechanism (K_i values of approximately 25 μ M) and the activation of anthrax toxin (Zhu et al. 2012). Obviously, they block the cleavage of larger proteinaceous substrates by steric hindrance.

Peptide-Based Inhibitors

The second group of inhibitors comprises various peptides and peptidomimetics. Due to their relatively high molecular weight, negligible oral bioavailability, and stability problems, these compounds are not the first choice for drug development. Nevertheless, numerous injectable peptides are used in therapeutic applications (Vlieghe et al. 2010). The first potent PC inhibitors were the irreversible substrate analogue chloromethyl ketones (CMK), which covalently bind to the enzyme (Garten et al. 1989; Hallenberger et al. 1992). The most widely used basic PC

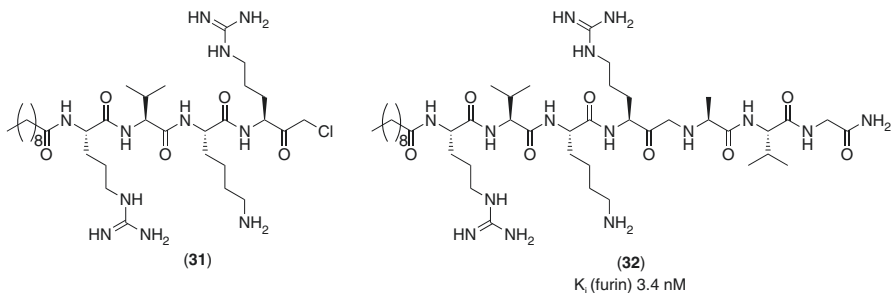


Fig. 11.15 Structures of substrate analogue arginyl-ketone-derived furin inhibitors. The CMK derivative **31** is a covalent irreversible inhibitor, whereas the ketomethylene compound **32** binds by a covalent reversible mechanism (Anglikler 1995; Garten et al. 1994)

inhibitor is the commercially available derivative Dec-Arg-Val-Lys-Arg-CMK (**31**, Fig. 11.15), which targets all of the known furin-like PCs in the nanomolar range (Garten et al. 1994; Jean et al. 1998). In cell culture, it blocks the furin-catalyzed cleavage of HIV gp160 into gp120 and gp41. Furthermore, the first crystal structure of furin was obtained when it was complexed with this inhibitor (Henrich et al. 2003). However, CMKs are not suited for further development. In vivo studies with the thrombin inhibitor D-Phe-Pro-Arg-CMK revealed a very short half-life <5 min (Hanson and Harker 1988). The same P4-P1 segment of the CMK **31** served for the design of analogues containing pseudo peptide bonds, like the ketomethylene inhibitor **32** (K_i value of 3.4 nM against furin, Fig. 11.15) (Anglikler 1995).

So far, only few endogenous PC inhibitors are known. A prominent role plays autoinhibition by prodomains (Zhong et al. 1999). Furin-like PCs are synthesized as zymogen and are activated by autocatalytic cleavage within the prodomain. The prosegment acts as an intramolecular chaperon, needed for the correct folding, regulation of enzymatic activity, and transport within the secretory pathway. A moderate K_i value of 156 nM was determined for the complete 83-mer prodomain of furin. Furthermore, this inhibitor reduced the proliferation, migration, and invasion of cancer cells (Basak et al. 2010). Several truncated derivatives have been synthesized. The most potent one, the 24-mer DYYHFWHRGVTKRSLSPHRPRHSR, inhibits furin with an inhibition constant of 0.9 μ M. Moreover, some peptides derived from the prodomain of PC1 inhibit furin in the same range (Basak and Lazure 2003).

A combinatorial peptide library containing approximately 52 million hexapeptides was scanned to identify PC1 and PC2 inhibitors. For instance, Ac-Leu-Leu-Arg-Val-Lys-Arg-NH₂ inhibits PC1 and PC2 with K_i values of 3.2 and 360 nM, respectively, whereas it is only a moderate furin inhibitor with an inhibition constant of 1.4 μ M. On the other hand, the unprotected analogue H-Leu-Leu-Arg-Val-Lys-Arg-OH has a stronger furin affinity (0.42 μ M) but reduced potency against PC2 (3.4 μ M) (Cameron et al. 2000).

We have developed a new peptidomimetic lead structure for basic PCs by incorporation of decarboxylated arginine derivatives in P1 position. Starting from the

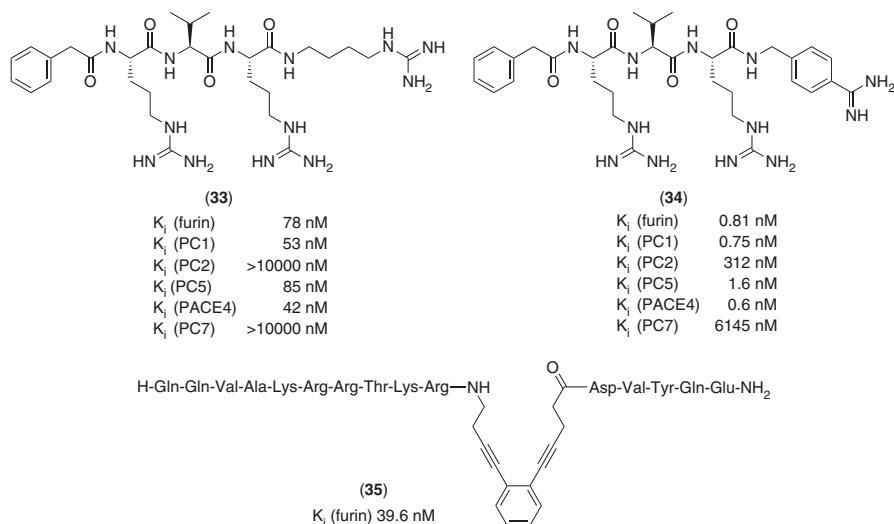


Fig. 11.16 Peptidomimetic PC inhibitors (Basak et al. 2009; Becker et al. 2010; Cameron 2000)

agmatine compound Phac-Arg-Val-Arg-4-Agm (**33**, $K_i = 78$ nM, Fig. 11.16) or the equipotent noragmatine analogue, the approximately 100-fold more potent 4-Amba inhibitor **34** has been prepared (Fig. 11.16). It inhibits furin with a K_i value of 0.81 nM and PC1, PACE4, and PC5 with similar efficacy but has reduced potency against PC2 and PC7 (Becker et al. 2010) and negligible affinity against the trypsin-like serine proteases thrombin, factor Xa, and plasmin. Further modification of the P3 position by *tert*-leucine or penicillamine provided inhibitors with enhanced potencies (Becker et al. 2011; Hardes et al. 2015). A considerably improved affinity was achieved by incorporation of basic P5 residues (Becker et al. 2012). The most potent analogue **36** (MI-1148, Fig. 11.17) contains a *para*-guanidinomethyl substitution at the P5 phenyl ring and inhibits furin with a K_i value of 5.5 pM (Hardes et al. 2015). These inhibitors have a similar selectivity profile, as described for analogue **34**, and also inhibit PC4 in the low picomolar range. To the best of our knowledge, these compounds are the most potent synthetic inhibitors of furin-like PCs. Some of these compounds enabled the determination of crystal structures in complex with human furin (Fig. 11.17) (Dahms et al. 2014, 2016a; Hardes et al. 2015). A nearly identical binding mode was found for an analogous inhibitor containing the P5 guanidinomethyl substitution in meta-position (Dahms et al. 2014). The antiviral efficacy of inhibitor **36** and/or of its P3 Val analogue MI-701 (Becker et al. 2012; Hardes et al. 2015) was extensively studied in cell culture infected with numerous furin-dependent viruses. A significant antiviral effect was found with HPAIV H5N1 and H7N1 strains (Hardes et al. 2015; Lu et al. 2015) and with canine distemper virus (CDV), which belongs to the *Paramyxoviridae* and is closely related to measles virus (Hardes et al. 2015). Moreover, both inhibitors reduced the replication of Semliki Forest and chikungunya virus, both belonging to the alphaviruses,

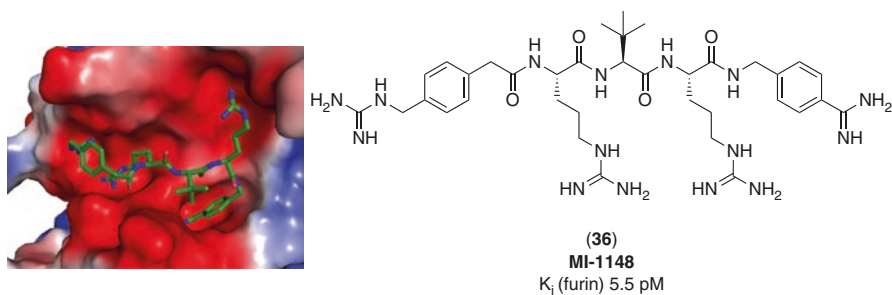


Fig. 11.17 Structure of inhibitor MI-1148 (stick model with carbons in green, nitrogen in blue, and oxygen in red) in complex with human furin (PDB, 4RYD) (Hardes et al. 2015). Furin is shown in surface representation colored by its electrostatic potential (negatively charged regions are shown in red and positive areas in blue)

where furin cleaves the precursor protein p62 in the TGN (Hardes et al. 2017). An antiviral effect was also found against flaviviruses like West Nile and dengue-2 virus (Kouretova et al. 2017) and, for MI-701, also against Borna disease virus (Lennartz et al. 2016). Furthermore, both compounds strongly protected cells against anthrax, Shiga, and diphtheria toxin (Becker et al. 2012; Hardes et al. 2015).

Furthermore, inhibitor **35** (Fig. 11.16) containing a β -turn inducing enediynyl amino acid moiety was prepared. The amino and carboxyl groups of this unusual amino acid were coupled to peptide sequences around the cleavage sites within the prodomain of furin. This compound inhibits furin with a K_i value of 40 nM and blocks the cleavage of a fluorogenic peptide derived from the spike protein of human SARS coronavirus with an IC_{50} value of 193 nM (Basak et al. 2009).

In addition, the HA cleavage site of a H5 influenza virus was used as scaffold for the development of peptidic inhibitors (Shiryaev et al. 2007). The best compound TPRARRRKKRT-NH₂ (**37**, Table 11.1) inhibits furin with a K_i value of 23 nM, whereas other PCs are less affected. Further optimization was achieved with peptide (**38**), which inhibits furin, PC5, and PACE4 in the low nanomolar range, but has reduced potency against PC7 ($K_i = 490$ nM) (Remacle et al. 2010).

The inhibitory potency against furin could be further enhanced with poly-arginine derivatives, found by a positional scanning of combinatorial L- and D-hexapeptide libraries (Cameron et al. 2000). Hexa-D-arginine (**39**, Table 11.1) inhibits furin and PC5 with K_i values around 200 nM in the same range, whereas it is less active against PC1 and PC7. The elongated analogue nona-D-arginine-amide (D9R-amide, compound **40**, Table 11.1) possesses a significantly improved inhibition constant of 1.3 nM. In contrast to the analogous L-peptide, which was cleaved by furin, the D-configured D9R-amide was found to be fully stable (Cameron et al. 2000; Kacprzak et al. 2004).

Another attempt to synthesize potent PC inhibitors is based on the monocyclic sunflower trypsin inhibitor SFTI-1 (Fig. 11.2) (Fittler et al. 2015). Therefore, the SFTI-1 backbone was used as a starting point for several modifications like the implementation of a furin cleavage motif and truncation of the inhibitor. The most

Table 11.1 Peptidic inhibitors of basic PCs (Cameron et al. 2000; Fittler et al. 2015; Fugere et al. 2007; Kwiatkowska et al. 2016; Remacle et al. 2010; Shiryayev et al. 2007)

No.	Compound	K_i value (nM)	PC1	PC4	PC5	PACE4	PC7
		Furin					
37	TPRARRRKKRT-amide	23	441		232	162	152
38	Acetyl-RARRRKKRT-amide	6.5			2.6	2.6	490
39	H-(DArg) ₆ -OH	106 ^a 265 ^b	13 000		206	580	1875
40	H-(DArg) ₆ -amide	1.3			19		81
41	KRCKKSIPPICF-amide	0.49					
42	Acetyl-LLLLRVKKR-amide	400				18	

^aCameron et al. (Cameron et al. 2000)

^bFugere et al. (Fugere et al. 2007)

potent compound (**41**) (Table 11.1) of this series inhibits furin in the low nanomolar range ($K_i = 0.49$ nM), whereas matriptase-1 is only poorly ($K_i = 560$ nM) and trypsin not affected.

Moreover, peptidomimetic compounds containing a multi-Leu motif have been described (Levesque et al. 2012). The aim of this modification was the discrimination between furin and PACE4. The most selective derivative of this series (**42**) inhibits PACE4 with a K_i value of 18 nM, whereas furin was 22-fold less affected. Further modification with polyethylene glycols of different length could improve the selectivity profile, whereas incorporation of 4-aminobenzylamide or 2,3-dehydroagmatine as P1 residue resulted in less selective PACE4 inhibitors compared to furin (Kwiatkowska et al. 2016).

Small-Molecule Inhibitors

Small molecules are the most promising candidates for drug development, because they have a better chance for oral bioavailability and should be better suited to address intracellular targets. Nevertheless, only a few nonpeptidic PC inhibitors have been reported; most of them possess moderate potency. The only efficacious derivatives were obtained within a series of guanidinylated 2,5-dideoxystreptamine derivatives; the most potent analogue **43** (Fig. 11.19) inhibits furin and PC5 with inhibition constants of 6 and 4 nM, respectively, whereas its affinity against PACE4 and PC7 is reduced (Jiao et al. 2006). Derivative **44** (**1n**) enabled the determination of a crystal structure in complex with human furin (Fig. 11.18) (Dahms et al. 2017). Surprisingly, two inhibitor molecules bind close to the active-site cleft. The first molecule (with carbon atoms in green) binds to the S4 and S2 sites of furin, without addressing its S1 pocket. The binding of the second molecule (shown with carbon atoms in yellow), which does not form strong polar contacts to furin, could be an artifact caused by the unusually high inhibitor concentrations used during crystallization.

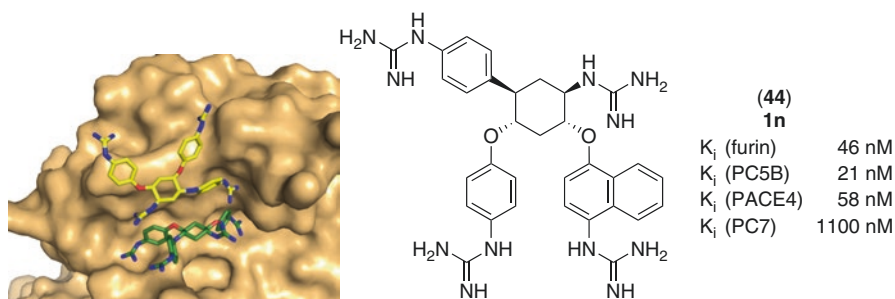


Fig. 11.18 Crystal structure of the simultaneous binding of two 2,5-dideoxystreptamine derivatives **44** (**1n**, shown as stick model) in complex with human furin (PDB, 5MIM) (Dahms et al. 2017). Surprisingly, the S1 binding pocket is not addressed

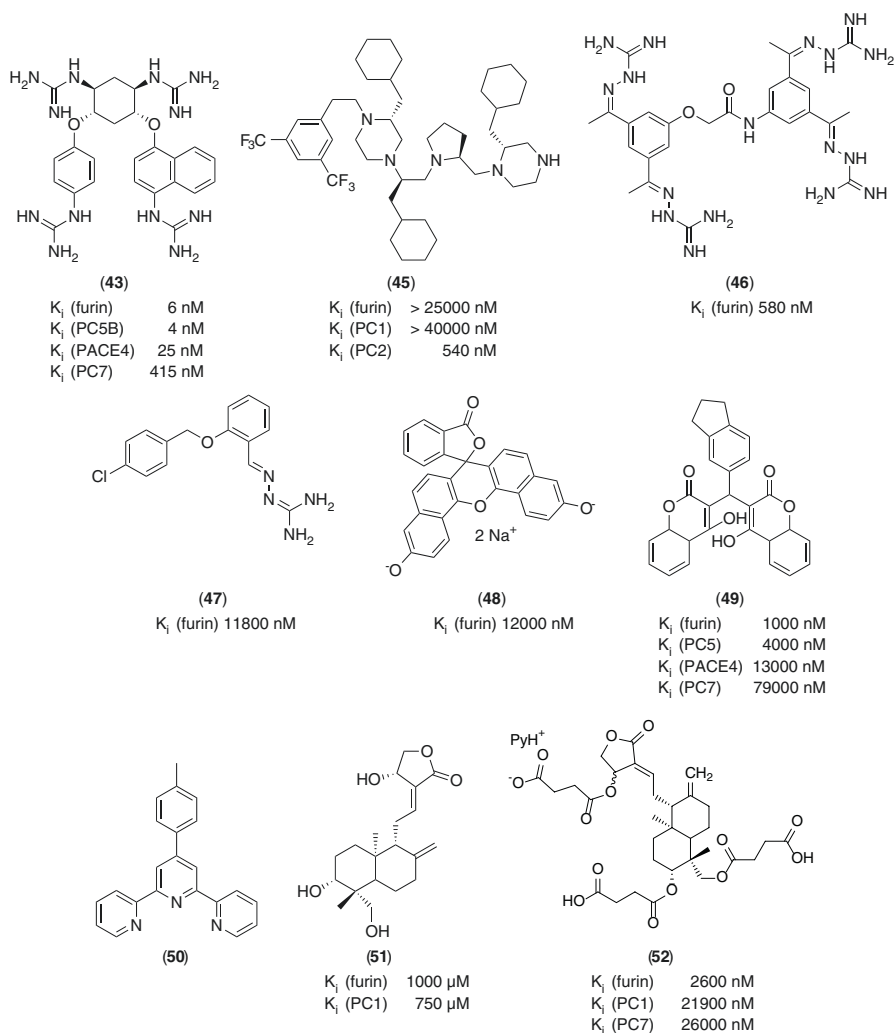


Fig. 11.19 Known small-molecule inhibitors of PCs (Basak et al. 1999; Coppola et al. 2008; Jiao et al. 2006; Komiyama et al. 2009; Kowalska et al. 2009; Podsiadlo et al. 2004; Sielaff et al. 2011b)

Reduction of peptide bonds by treatment of their amide precursors with borane-tetrahydrofuran provided a series of multibasic piperazine and pyrrolidine containing derivatives, like **45** (Fig. 11.19) with submicromolar affinity against PC2 and weak affinity against PC1 and furin (Kowalska et al. 2009). Among a series of weakly basic guanylylhydrazones, several compounds like derivative **46** were prepared, which inhibit furin with inhibition constants close to 0.5 μ M (Sielaff et al. 2011b). Even, the mono-guanylylhydrazone derivative (**47**) still showed a significant furin inhibition with a K_i value of 11.8 μ M (Komiyama et al. 2009). Compound **47** also inhibits PC5 and PACE4 in the same range, whereas a reduced potency was observed against PC7. Despite the poor in vitro potency of the cell-permeable

naphthofluorescein derivative B3 (**48**) with a K_i value of 12 μM against furin, it was able to inhibit the furin-catalyzed activation of the remodeling protease proMT1-MMP, leading to decreased MMP-2 activation and cell motility of CHO cells. Treatment with B3 also reduced the invasiveness of human fibrosarcoma cells (HT1080) (Coppola et al. 2008).

Furthermore, furin-inhibiting dicoumarol derivatives could be identified by HTS. Some of these compounds protected cells against furin-activated anthrax toxin and inhibited proMT1-MMP processing. Compound **49** noncompetitively binds to furin with an inhibition constant of 1 μM (Komiya et al. 2009). Recently, a series of zinc and copper ion chelate complexes was described to inhibit furin in the micromolar range; the structure of the most suitable chelate ligand TTP (**50**) is shown in Fig. 11.19. The authors speculated that the active-site histidine might be coordinated by the zinc or copper ions. Interestingly, the solvated Zn^{2+} was less potent than its chelated form, whereby the free chelate ligands did not affect furin (Podsiadlo et al. 2004).

One approach to find new nonpeptidic furin inhibitors is the testing of compounds from natural sources. An example of this attempt is the screening of the chemical constituents of the medicinally used plant *Andrographis paniculata*. Derived from the major component andrographolide **51**, several semisynthetic compounds have been tested. The most potent derivative is the andrographolide-trisuccinate pyridinium salt **52** (Fig. 11.19) with a K_i value of 2.6 μM (Basak et al. 1999).

11.2.2.2 SKI-1/S1P Inhibitors

Peptide-Based Inhibitors

In analogy to the CMK inhibitors against furin-related PCs, irreversible inhibitors for SKI-1/S1P were prepared (Pasquato et al. 2006). The substrate analogue backbone was derived from the cleavage site of the Lassa virus (LASV) glycoprotein IYISRRLL. Based on this sequence, a 4-mer (Dec-RRLC-CMK; **53**, Fig. 11.20), a 6-mer (Dec-ISRRLC-CMK), and a 7-mer (Dec-YISRRLL-CMK) were synthesized. Interestingly, the 4-mer shows in vitro a 250-fold higher potency compared to the longer derivatives, whereas in cell culture experiments, all inhibitors are almost equipotent. The CMK derivatives were able to block the infection of lymphocytic choriomeningitis virus (LCMV) and chimeras of LCMV containing the LASV glycoprotein.

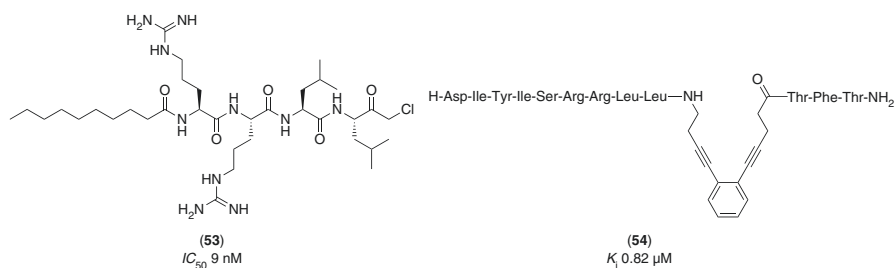


Fig. 11.20 Known peptidic inhibitors of SKI-1/S1P (Basak et al. 2015; Pasquato et al. 2006)

Furthermore, enediynyl peptides were synthesized based on the amino acid sequence of cleavage sites of known substrate sequences like the LASV glycoprotein and the prodomain of SKI-1/S1P (Basak et al. 2015). The most potent compound (**54**, Fig. 11.20) inhibits SKI-1/S1P with a K_i value of 0.82 μM .

Protein-Based Inhibitors

The concept of mutation and thereby optimization of natural serine protease inhibitors for a new target was also applied for the development of SKI-1/S1P inhibitors (Maisa et al. 2009; Pullikotil et al. 2004). Instead of the multibasic furin cleavage site, the SKI-1/S1P-specific motif (R/K)-X-X-(L/T) \downarrow (Seidah and Chretien 1999) was introduced. The overexpression of the $\alpha 1$ -AT RRVL variant was found to inhibit both CCHFV (Crimean-Congo hemorrhagic fever virus) and LASV glycoprotein maturation. Blocking of arenavirus GPC processing had a strong antiviral effect in suppressing cell-to-cell spread and formation of viral particles. Micromolar concentrations of the wild-type prosegment of SKI-1/S1P were needed to inhibit the enzyme in vitro. A prosegment with the amino acid exchange R134E was the most potent mutant inhibiting the cleavage of CCHFV preGC (Pullikotil et al. 2004).

Small-Molecule Inhibitors

With the role of SKI-1/S1P in the cholesterol and fatty acid synthesis in mind, a high-throughput screen was conducted to identify lead structures lowering plasma cholesterol and triglycerides (Hay et al. 2007). Based on screening results, improved derivatives were prepared. The most potent compound from this study (**55**, Fig. 11.21) inhibits SKI-1/S1P with an IC_{50} value of 8 nM. However, the less potent analogue PF-429242 (**56**) was used for further evaluation because of its lower molecular weight and lipophilicity. PF-429242 is a reversible, competitive inhibitor of SKI-1/S1P with an IC_{50} value of 170 nM. Furthermore, it is highly selective for SKI-1/S1P; other serine proteases like trypsin, plasmin, kallikrein, or furin are not inhibited by this compound. However, PF-429242 suffers from rapid clearance and poor oral bioavailability in rats. Inhibition of SKI-1/S1P by PF-429242 blocks the cleavage of the glycoprotein of the old-world arenaviruses LASV and LCMV, resulting in reduced virus replication in infected cell cultures (Urata et al. 2011). Interestingly, no escape variants could be detected during or after the treatment with this SKI-1/S1P inhibitor. These studies were extended to several new-world arenaviruses like Junin or Guanarito virus, where PF-429242 also efficiently blocked glycoprotein processing and virus production (Pasquato et al. 2012).

A set of nonpeptidic isocoumarinyl sulfone derivatives was tested against SKI-1/S1P. However, only one compound (**57**, Fig. 11.21) showed a weak inhibition of SKI-1/S1P ($K_i = 255 \mu\text{M}$) (Basak et al. 2015).

Several known serine protease inhibitors, among them AEBSF (4-(2-aminoethyl)-benzene sulfonylfluoride) and *p*-aminobenzamidine (Fig. 11.4) as well as inhibitors of furin or furin-related PCs like Dec-RVKR-CMK, were tested against SKI-1/S1P. A significant inhibition was only found for DCI (3,4-dichloroisocoumarin, compound **58**, Fig. 11.21), which exhibited a slow irreversible binding mode with an apparent inhibition constant of 6.8 μM (Bodvard et al. 2007).

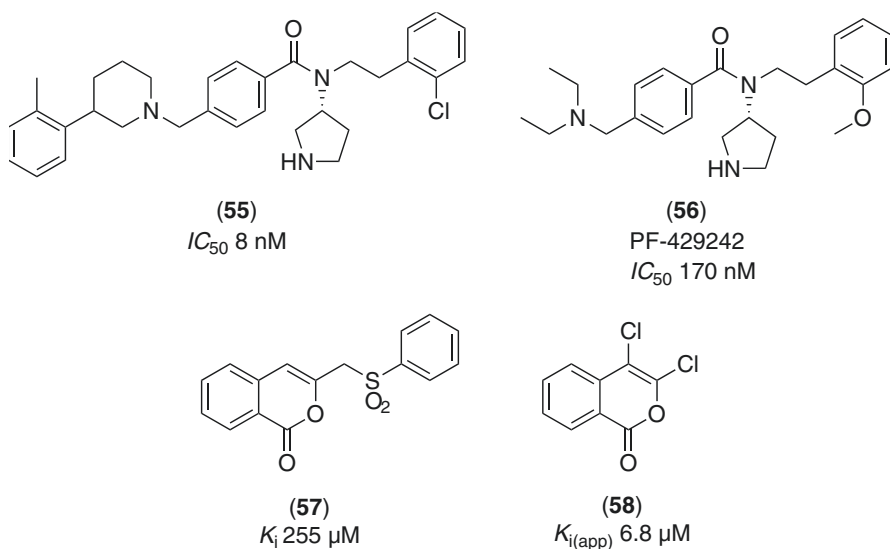


Fig. 11.21 Known small-molecule inhibitors of SKI-1/S1P (Basak et al. 2015; Bodvard et al. 2007; Hay et al. 2007)

11.3 Miscellaneous Host Proteases as Antiviral Targets

Numerous viral surface proteins are cleaved by endosomal cysteine proteases. The papain-like cathepsin L activates the Ebola virus (EboV) glycoprotein (GP) (Chandran et al. 2005) or Nipah virus F protein (Diederich et al. 2008), and despite contradictory reports, cathepsins might be also involved in the GP activation of some coronaviruses (Zhou et al. 2015). In contrast to the majority of serine protease inhibitors, most cysteine protease inhibitors achieve potency by a covalent modification of their target enzymes, which can be reversible or irreversible (Siklos et al. 2015). Only a few basic examples of typical cysteine protease inhibitors will be provided in this paragraph. Classical warheads leading to a relatively specific irreversible inhibition of cysteine proteases are the epoxysuccinates, vinylsulfones, and allylsulfones, whereas chloromethyl and diazomethyl ketones react with both the cysteine and serine proteases. The archetypal epoxysuccinate E-64 (59) (Fig. 11.22) was isolated from *Aspergillus japonicus* and forms a thioether bond with the active-site cysteine (Hanada et al. 1978). It is a nonselective inhibitor of nearly all cysteine cathepsins, with the exception of cathepsin C (Turk et al. 2012). Many analogues of E-64 have been prepared by replacement of its leucyl-agmatine segment. The more hydrophobic ethyl ester prodrug E-64d (60) reached clinical phase III trials (Satoyoshi 1992). Peptidic vinylsulfones represent an additional class of widely used cysteine protease inhibitors, including numerous cathepsins. The active-site cysteine attacks the Michael acceptor like vinylsulfone and becomes irreversibly

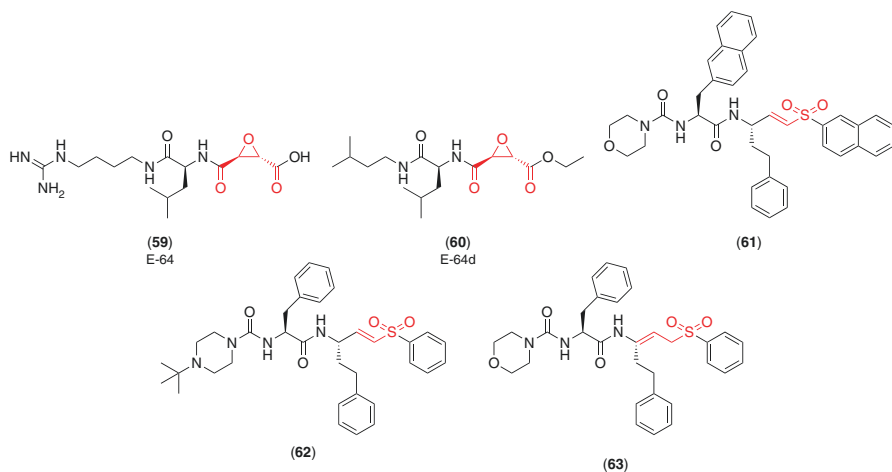


Fig. 11.22 Structures of covalent irreversible cysteine protease inhibitors, which make an alkylation of the active-site cysteine. The reactive epoxy-succinate, vinylsulfone, and allylsulfone segments are colored in red (Fennell et al. 2013; Palmer et al. 1995; Siklos et al. 2015; Zhou et al. 2015)

alkylated. For instance, cathepsins L and B are very rapidly inactivated by inhibitor **61** with second-order rate constants (k_{inact}/K_i) of $9.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $4.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, respectively (Palmer et al. 1995). A considerable inhibition of EboV GP and SARS-CoV S-protein cleavage was observed for close analogues in cell culture, like compound **62**. However, this compound was completely inactive in a lethal SARS-CoV mouse model (Zhou et al. 2015). A considerably slower inactivation of cathepsin B ($k_{\text{obs}}/[I] = 9.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) and other cysteine proteases was found by the allylsulfone **63** and numerous analogues, indicating a reduced electrophilicity of the allylsulfone segment (Fennell et al. 2013). Peptidic chloromethyl or diazomethyl ketones (Powers et al. 2002) are also very effective in vitro cysteine protease inhibitors. However, they suffer from instability in vivo and often from low selectivity.

Similar as done in the field of serine protease inhibitors, α -keto-derived cysteine protease inhibitors have been prepared, including α -ketoacids (**64**), α -ketoesters (**65**), and α -ketoamides (**66**) (Fig. 11.23). The ketone moiety of these analogues is attacked by the active-site cysteine leading to a covalent but reversible thiohemiketal complex. Additional examples are summarized in a recently published review (Siklos et al. 2015). Probably the most promising warhead for the development of covalent reversible cysteine protease inhibitors is the nitrile group. Nitriles react with the active-site cysteine forming a thioimidate adduct. A myriad of peptidic nitrile inhibitors have been prepared (Frizler et al. 2010), for instance, compound **67** inhibits cathepsins L and B with IC_{50} values of 20 nM and 1.8 nM (Greenspan et al. 2001). A potency considerably enhanced by four to five orders of magnitude could be achieved by the replacement of the C α carbon in P1 position with nitrogen. For instance, the resulting azanitrile **68** binds to cathepsin L with a K_i value of 74 pM and possesses a similar picomolar potency against cathepsins S and L

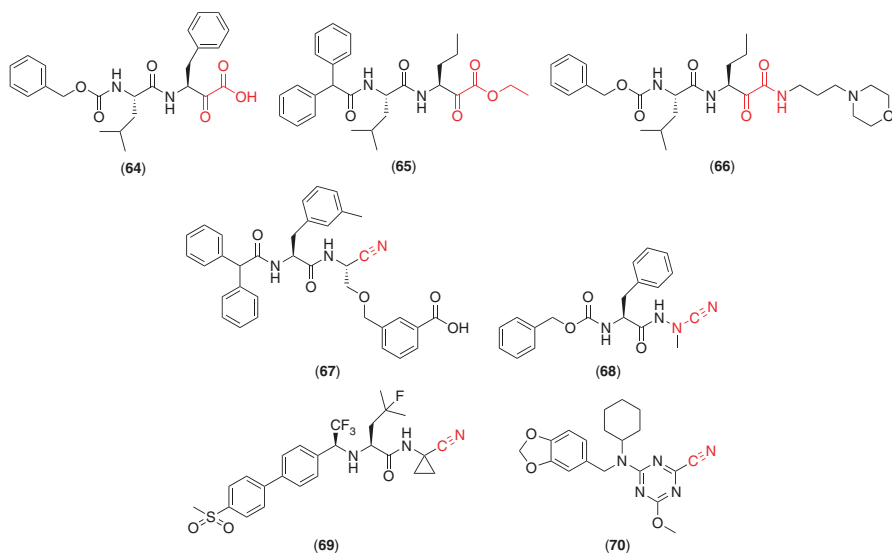


Fig. 11.23 Structures of covalent reversible cysteine protease inhibitors containing α -ketoacids, α -ketoesters, α -ketoamides, nitrile and azanitrile warheads, which are colored in red (Siklos et al. 2015)

(Löser et al. 2008). The cathepsin K inhibitor odanacatib **69** reached clinical phase III development, but its development was recently cancelled due to side effects. Moreover, completely nonpeptidic nitrile derivatives have been developed, such as compound **70** that inhibits cathepsin L with a K_i value of 2 nM (Fig. 11.23).

Numerous cathepsin inhibitors have been prepared in the past. However, despite a few proof of concept studies, their deeper characterization and optimization as antivirals are limited, so far. They have been mainly tested in nonviral applications, e.g., for cancer therapy, osteoporosis, and rheumatoid arthritis or in neurodegenerative diseases (Siklos et al. 2015; Turk et al. 2012).

Zinc-dependent host metalloproteases from the MMP (matrix metalloproteases) or the ADAM (a disintegrin and metalloprotease) families might also be involved in the entry and fusion of certain viruses, as recently described for a neurovirulent murine CoV strain (Phillips et al. 2017). Moreover, a considerable amount of the EboV GP is shed by the metalloprotease TACE (ADAM17) (Dolnik et al. 2004). The soluble GP activates dendritic cells and macrophages and causes the release of pro- and anti-inflammatory cytokines and affects vascular permeability. The dys-regulated inflammatory host response seems to contribute to the high virus pathogenicity (Escudero-Perez et al. 2014). These results suggest that inhibitors of metalloproteases may have antiviral activity. Although more than 50 clinical trials with metalloprotease inhibitors for the treatment of various cancers failed, their broad anti-inflammatory potential has aroused new interest (Vandenbroucke and Libert 2014).

11.4 Host Proteases as Receptors for Virus Entry

A few respiratory viruses use membrane-bound host proteases independent from their proteolytic activity as entry receptors. A surface region far away from the active site of the ubiquitously expressed serine protease dipeptidyl peptidase 4 (DPP4, also called CD26) serves as human cellular receptor of MERS-CoV (Raj et al. 2013). Consequently, no antiviral effect could be observed after treatment of MERS-CoV-infected cells with active-site-directed DPP4 inhibitors. Moreover, the SARS-CoV and HCoV-NL63 use angiotensin-converting enzyme 2 (ACE2) (Li et al. 2003; Wu et al. 2009) and aminopeptidase N (Yeager et al. 1992) as human receptors. Both proteases do not show any sequence or structural similarity with DPP4 (Wang et al. 2013). The crystal structure of the receptor-binding domain of the MERS- and SARS-CoV S-protein in complex with DPP4 (Lu et al. 2013; Wang et al. 2013) and ACE2 (Li et al. 2005) has been determined, respectively. The complexes reveal typical protein-protein interactions (PPI). The receptor region on DPP4 and ACE2 are relatively flat missing deep binding pockets normally found in the active site of proteases. Although no examples are known so far, it should be possible to inhibit the entry of these virions by blocking the described PPIs with suitable ligands.

Conclusion

So far, only inhibitors addressing viral proteases have been approved for the treatment of certain virus infections. A huge arsenal of excellent inhibitors against host proteases has been developed in the past for treatment of chronic diseases, such as hypertension, diabetes, risk of thrombosis, inflammatory ailments, and cancer, but only few of them reached the clinic. Despite loss of patent protection, many of these failed inhibitors or their analogues could still be suitable for short-term treatment of acute life-threatening infectious diseases, without being hampered by side effects that might develop after long-term application. One of the most important prerequisites for successful drug development is the identification of a valid target. For some virus infections, the relevant host proteases have been identified, in other cases there are still uncertainties, and further basic research on target identification is needed. Since proteases usually belong to families of similar enzymes which substitute each other, a broad-spectrum inhibitor could be tolerable or even advantageous for the special treatment of infectious disease, although selective drugs are usually preferred for most applications to minimize side effects. Ideally, host protease inhibitors should be used in combination with additional drugs. This strategy should improve the antiviral efficacy and allow the use of reduced concentrations, thereby minimizing side effects. The development of effective and tolerable host protease inhibitors will hopefully expand the arsenal of antiviral drugs in the future.

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