CHAPTER 9

Pharmacological Targeting of Human Tissue Kallikrein-Related Peptidases

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9.1 Introduction

9.1.1 Human Tissue Kallikrein-Related Peptidases

Traditionally, proteases were considered as blunt aggressors associated with protein demolition. As our knowledge on the roles of proteolytic pathways has largely expanded in recent years, it has become clear that proteases act as highly specific scissors to process specific protein substrates with various (patho)-physiological implications.\(^1\) Furthermore, proteolytic systems are organized in complex cascades that regulate important physiological processes such as food digestion, complement activation, blood coagulation, etc.\(^2-4\) Activation of an upstream protease initiates the cascade leading to the serial activation of downstream zymogens; thus, proteolytic cascades lead to rapid amplification of the overall proteolytic activity while they provide multiple regulatory points for fine tuning.

Human kallikreins are serine proteases that are divided into two categories: the plasma kallikreins and the tissue kallikreins (Figure 9.1A). Plasma kallikreins comprise only plasma kallikrein or Fletcher factor (KLKB1) encoded by a single gene mapped on human chromosome 4q35. This enzyme releases the
bioactive peptide bradykinin from high-molecular-weight kininogen (HMWK) (Figure 9.1B). Tissue kallikreins, renamed tissue kallikrein-related peptidases (KLKs), according to the most recent international nomenclature, constitute the largest family of serine proteases in the human genome and are tandemly arranged on human chromosome 19q13.4. All KLK genes encode for serine proteases with trypsin-, chymotrypsin-like, or mixed activity. KLK1, also referred to as tissue kallikrein, releases Lys-bradykinin from low-molecular-weight kininogen (LMWK) (Figure 9.1B). Human KLKs act alone or in complex proteolytic cascades named the 'KLK activome.' KLK cascades act in a tissue-specific manner and were implicated in various (patho)physiological processes, as summarized below.

### 9.1.2 Why Should We Target KLKs? Association of KLK with Disease

In recent years, a number of proteases have been selected as molecular targets for drug design and development, and several eventually entered the pharmaceutical market. Inhibitors of the following are widely known: (a) ACE metalloprotease *(e.g. captopril, enalapril)*, (b) thrombin serine protease *(e.g. argatroban)*, (c) HIV aspartic protease *(e.g. ritonavir)*, the proteasome inhibitor bortezomib, and many more. In December 2009, ecallantide—a drug that targets plasma kallikrein—received FDA approval for acute attacks of hereditary angioedema. Ecallantide is a 60-amino-acid residue recombinant peptide produced in *Pichia pastoris* that was identified and isolated by phage display. Ecallantide acts as a reversible high-affinity inhibitor of plasma kallikrein with a $K_i$ of 44 pM.

On the other hand, expression of specific KLKs has been associated with diverse physiological functions *(i.e. semen liquefaction, activation of antimicrobial peptides)*, and their aberrant regulation has been implicated in common human diseases, including hypertension, renal dysfunction, skin diseases, inflammation, allergies, neurodegeneration, and cancer. Therefore, KLK targeting is expected to provide new pharmaceutical compounds for the treatment of these pathologies.

### 9.1.2.1 Cancer

In addition to KLK3/PSA, which has found clinical applications in the diagnosis and monitoring of prostate cancer, several other KLKs are considered potential biomarkers, as they show deregulated expression in various types of cancer. The potential functions of KLKs in cancer growth and progression are currently under investigation. Certain KLKs have been shown to cleave components of the extracellular matrix *in vitro*, so they may promote cancer invasion and metastasis. A number of studies have implicated KLKs in the induction of epithelial-to-mesenchymal transition (EMT), which is a critical step in cancer metastasis. In prostate cancer, expression of KLK3 and KLK4
results in loss of E-cadherin and induction of vimentin, which is a hallmark of EMT.\textsuperscript{20} Additionally, KLK6 \textit{in vivo}\textsuperscript{21} and KLK7 \textit{in vitro}\textsuperscript{22} have been implicated in E-cadherin shedding. In breast-cancer cells, induction of KLK6 expression at physiological concentrations reduces growth of primary breast tumors with concomitant downregulation of vimentin expression.\textsuperscript{23} Furthermore, the aberrant overexpression of KLK6 observed in a small subset of breast tumors has been linked to hyperproliferation of breast\textsuperscript{23} and non-small-cell lung cancer.\textsuperscript{24} Cumulatively, KLKs seem to play dual roles in cancer depending on the concentration and amount of active enzyme and the tissue, type, and stage of cancer,\textsuperscript{8} as also illustrated from many studies on the functions of KLK3/PSA that can act either as a cancer fighter or as a cancer promoter.\textsuperscript{25}

9.1.2.2 Skin Diseases

9.1.2.2.1 Netherton Syndrome. Netherton syndrome (NS) is a severe type of ichthyosis characterized by generalized exfoliative erythroderma, a hair-shaft defect known as trichorrhexis invaginata, and severe atopic manifestations. NS is caused by mutations in the \textit{SPINK5} (serine protease inhibitor Kazal-type 5) gene that encodes for the serpin LEKTI (lympho-epithelial Kazal-type inhibitor) with a rare incidence of one case every 100 000 newborns.\textsuperscript{26} \textit{Spink5}\textsuperscript{−/−} mice recapitulate the clinical feature of NS, while they exhibit high activities of KLK5 and KLK7 proteases in epidermis.\textsuperscript{27} The fundamental role of KLK5 in the induction of inflammation associated with NS has been demonstrated in \textit{Spink5}\textsuperscript{−/−} mice, and in part, it is mediated through activation of PAR-2.\textsuperscript{28,29} LEKTI is a 15-domain (D1 to D15) type inhibitor of serine proteases.\textsuperscript{30} Each domain is separated by 14 spacing fragments. LEKTI requires proteolytic processing that releases the inhibitory domains.\textsuperscript{31} Only D2 and D15 match the Kazal motif (C-X\textsubscript{n}-C-X\textsubscript{7}-C-X\textsubscript{10}-C-X\textsubscript{2/3}-C-X\textsubscript{m}-C) perfectly; the rest exhibit a Kazal-type four-cysteine residue pattern.\textsuperscript{30} Interestingly, overactivation of KLKs in the absence of their endogenous LEKTI inhibitors leads to overactivation of cathelicidin and associated production of high levels of antimicrobial and proinflammatory peptides (LL-37 and derivatives).\textsuperscript{32} LL-37, which is a peptide of 37 amino acid residues derived from cathelicidin containing two Leu residues at the N-terminus, is a potent chemoattractant for neutrophils, monocytes and T-cells, and so can potentiate inflammation.\textsuperscript{33,34} In addition, LL-37 complexes with self-DNA are internalized by plasmacytoid dendritic cells, which become activated to further promote inflammation.\textsuperscript{35}

9.1.2.2.2 Other Skin Diseases. X-linked ichthyosis is a disease characterized by hyperkeratosis and increased thickness of the stratum corneum. The disease is caused by mutations in the gene encoding steroid sulfatase resulting in increased levels of cholesterol sulfate.\textsuperscript{36} \textit{In vitro}, cholesterol sulfate acts as a competitive inhibitor of trypsin and chymotrypsin (\textit{K}\textsubscript{i} 5.5 \textmu M and 2.1 \textmu M, respectively),\textsuperscript{37} but it is currently unknown whether it can also inhibit KLK
proteases that represent major regulators in skin desquamation. Furthermore, KLKs are upregulated in psoriasis, a fact that may be linked to upregulation of the IL-20 subfamily in peeling-skin syndrome, rosacea, and atopic dermatitis. In addition, high levels of LL-37 have been shown in psoriasis and in rosacea.

### 9.1.2.3 Asthma

Plasma kallikrein and KLK1 are responsible for the generation of kinins as described (Figure 9.1B). Whereas plasma kallikrein is mainly expressed in the liver and circulates as a zymogen, it is KLK1 that is expressed in airways, where it mediates bronchoconstriction, induction of mucus hypersecretion, and vascular permeability, and triggers cholinergic and sensory nerve stimulation. Bradykinin inhalation by normal volunteers and asthmatic patients and in animal models for asthma has been shown to cause immediate bronchoconstriction, as well as indicating a major role of KLK1 in asthma.

![Diagram of Kallikreins](image)

**Figure 9.1** Schematic representation of human kallikrein gene and protein structure. (A) Structure of tissue and plasma kallikreins. (B) Crosstalk between KLK1 and plasma kallikrein (KLKB1). BK, bradykinin; HMWK, high-molecular-weight kininogen; LMWK, low-molecular-weight kininogen; Lys-BK, lysine-bradykinin.
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9.1.2.4 Neurodegeneration

Elevated levels of KLK1 and KLK6 have been found in plasma from multiple sclerosis (MS) patients, while reduced levels of KLK6 were measured in CSF of Alzheimer disease patients. In animal models of MS, KLK6 is secreted by infiltrating immune cells at sites of CNS inflammation and is considered to cleave myelin proteins. Blocking the activity of KLK6 reduces clinical symptoms and the onset of experimental autoimmune (allergic) encephalomyelitis (EAE) in rodents, which is an established experimental model for MS. On the other hand, it has been reported that KLK6 is secreted by oligodendrocytes and is necessary for the remyelination process and maintenance of normal myelination patterns. Consequently, it is speculated that also in the nervous system, KLKs may exert dual functions, as also described for thrombin. Although low concentrations of thrombin are neuroprotective, high concentrations, which may be caused by an increased blood–brain barrier permeability, exacerbate neuronal cell death. In addition, it has been found that KLK6 localizes in Lewy bodies, hallmark features of synucleinopathies, such as Parkinson’s disease, and is linked to degradation of α-synuclein in vitro.

9.1.3 Structure of KLKs

All KLK genes are composed of five coding exons with conserved intron phases (I-II-I-0). The three catalytic amino acid residues, namely His, Asp, and Ser, are encoded by exons II, III, and V, respectively (Figure 9.1). All KLK proteins are synthesized as preproenzymes with lengths varying between 244 and 293 amino acid residues. The secretion signal (pre) contains 16–33 residues and is necessary to direct KLK proteins to the secretory pathway. Activation of KLK proforms requires removal of the pro-peptide sequence, which is composed of three to 37 N-terminal residues. The mechanisms of pro-peptide removal include autoactivation, activation by other KLKs, or crossactivation by other proteases. The length of mature KLK enzymes varies between 223 residues (for KLK6) and 252 (for KLK13). The three-dimensional structure has been resolved by X-ray crystallography for KLK1, KLK3, KLK4, 5, and 7, KLK6, and pro-KLK6. Five or six disulfide bonds participate in KLK protein folding. Removal of the pro-peptide results in a salt bridge formation between the ammonium group of the N-terminal Ile/Leu (chymotrypsin numbering is followed throughout this review) and the side chain of Asp, a prerequisite for their enzymatic activity. In addition, the kallikrein loop at position 99 (or loop-99) is a characteristic feature for kallikreins, but it is present only in KLK1, 2, and 3. This loop is located prior to the active site Asp and is characteristic for kininogenase activity resulting in release of kinin (lys-bradykinin) from LMWK. Interestingly, however, KLK3 does not cleave kininogen, while KLK2 has 1000-fold lower activity than KLK1 in vitro. On the other hand, KLK5 and KLK14 can cleave LMWK in vitro, but they do not generate kinins, and KLK8 can cleave HMWK.
Generally, the amino acid residues that span the peptide scissile bond in a protease substrate are denoted $P_N\cdots-P_2-P_1\downarrow-P_1'-P_2'\cdots-P_N'$ (\downarrow marks the cleavage site between the acyl and leaving group, respectively) and the adjacent amino acid residues of the protease as $S_N\cdots-S_2-S_1\downarrow-S_1'-S_2'\cdots-S_N'$.\textsuperscript{63} The amino acid residue 189, which corresponds to the $S_1$ position, is essential for trypsin- or chymotrypsin-like activity. KLK1, 2, 4, 5, 6, 8, 10, 11, 12, 13, and 14 have Asp in the $S_1$ position, while KLK15 has Glu; therefore, they all exhibit trypsin-like activity. KLK3 and 7 have Ser and Asn in $S_1$, respectively, and are expected to exhibit chymotrypsin-like activity. KLK9 has Gly, which is rarely found in this position in serine proteases and probably has elastase-like activity, as Gly is also present in the same position in elastase.\textsuperscript{7,11} Exceptionally, although expected to have trypsin-like activity, KLK14 displays dual (mixed) activity as both a trypsin- and chymotrypsin-like enzyme.\textsuperscript{64,65}

9.1.4 Identification of Substrates and Endogenous Inhibitors

The design of novel protease inhibitors that could be validated as drugs is aided by knowledge of the protease-specific substrate(s). The most common approaches for substrate identification include screening of chemical libraries\textsuperscript{66} or the substrate phage display technique.\textsuperscript{67} Positional scanning of synthetic combinatorial libraries was applied to finely map the substrate specificity and to identify novel substrates for KLK3–7 and KLK10–11.\textsuperscript{68} A library of FRET peptides was screened for KLK6 substrates.\textsuperscript{69} Substrate phage display was applied for substrate-specificity profiling of KLK2\textsuperscript{70} and KLK14 enzymes.\textsuperscript{65} Finally, synthetic tripeptide libraries were used to map the specificity of KLK15.\textsuperscript{71}

Results on the activity of KLK10 are currently contradictory, as Debela et al. reported trypsin-like activity for KLK10,\textsuperscript{68} while a later study failed to demonstrate any enzymatic activity for KLK10\textsuperscript{9} which is the only human KLK that has Ser instead of Gly at position 193. Gly193 is located in the oxyanion hole and functions to stabilize the oxyanion intermediate during hydrolysis of the peptide bond. The Gly193Ser substitution is extremely rare in serine proteases and was reported for mouse Klk13, which displays very restricted substrate specificity, namely, it only acts as a specific pro-renin converting enzyme.\textsuperscript{72} Similarly, KLK10 may have very restricted specificity.

Finally, mass-spectrometry-based methods can be used in order to identify complexes of KLKs with endogenous inhibitors in complex biological samples as was shown for the KLK6 complex with $\alpha_1$-antichymotrypsin.\textsuperscript{73} In this context, KLKs are immunoprecipitated from biological fluids or tissue extracts, and the immune-precipitants are analyzed by mass spectrometry, e.g. LC-ESI-MS/MS, in order to identify KLK-co-precipitated proteins. Hybrid ELISA assays can then be used for verification, where the capturing antibody is KLK-specific, and the detection antibody is specific for the identified inhibitor.
9.2 Peptide Modulators

9.2.1 Synthetic LEKTI Domains

Mapping of the LEKTI inhibitory domains in terms of KLK inhibition was performed in detail.\textsuperscript{74-76} KLK5, 7, and 14 were inhibited by LEKTI D8–11 and LEKTI D5,\textsuperscript{75} while KLK5 and KLK7 were also inhibited by LEKTI D6.\textsuperscript{74,76} Importantly, complexes of LEKTI with KLKs are reversible, dependent on pH. Lower pH values result in dissociation of complex formation and release of active protease, as shown in the epidermis where a pH gradient between the inner and outer layers regulates the activity of the KLK cascade that controls skin desquamation.\textsuperscript{11,75,77,78} Given the established importance of KLK5 and KLK7 in skin diseases, LEKTI D6 (domain 6) (compound 9.1) was designed and synthesized according to the fragment condensation method and regioselective disulfide bond formation.\textsuperscript{79} 9.1 inhibited KLK5 with an IC\textsubscript{50} of 125 nM; a recombinant form of LEKTI D6 produced in \textit{E. coli} showed equivalent activity (IC\textsubscript{50} = 134 nM).

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9.2.2 Cyclic Peptide Inhibitors

Generally, a cyclized peptide is less flexible and usually shows a higher affinity for its molecular target. In addition, cyclization increases peptide bioavailability, as it renders the peptide resistant to proteolytic degradation by exoproteases. Peptide inhibitors for KLK2 were designed based on linear inhibitory peptides isolated by phage display.\textsuperscript{80} The cyclized peptides (head-to-tail cyclization) were stable in plasma when compared to linear peptides and required more that 4 h for complete trypsin digestion (57% for 4 h) compared to 30 min for linear peptides.\textsuperscript{81} Attempts to produce cyclic peptide inhibitors against KLK1 based on the bradykinin sequence had limited success, and their \textit{K\textsubscript{i}} was about 10 \textmu M.\textsuperscript{82}

9.2.3 Peptide Activators of KLKs

It was found that an inactive form of mature KLK3/PSA is present in the serum of prostate cancer patients.\textsuperscript{83} Using X-ray crystallography, it was shown that the kallikrein loop can adapt two different conformations: the ‘open form,’ which is enzymatically active, and the ‘closed form,’ which is inactive.\textsuperscript{55} Antibodies that bind and stabilize the open conformation increase the activity of KLK3/PSA.\textsuperscript{55} Such a specific monoclonal antibody was developed and named 8G8F5,\textsuperscript{83} and its three-dimensional structure complexed with KLK3/PSA was resolved.\textsuperscript{55} The rational development of KLK3/PSA activators is based on the
tumor suppressor functions described for KLK3/PSA at several levels. Our better understanding of the dual roles of KLK3/PSA in prostate-cancer development and progression will greatly help dissect the stages and prostate-tumor subtypes that will benefit from compounds that activate KLK3/PSA from those that would benefit from the use of KLK3/PSA inhibitors.

Phage display was also applied to the identification of novel peptide activators of KLK3 activity. Initially, the B2 cyclic peptide (9.2) was selected. Based on B2, new peptides were produced, carrying replacements of the disulfide bond. Of these, 9.3 was found to stimulate the activity of KLK3/PSA. In designing such replacements, it is important to maintain the length of the S-S bridge (shaded region in Figure 9.2) to achieve maximum stimulation of the KLK3/PSA activity. The advantage of these replacements lies in their higher

![Figure 9.2 Peptide activators of KLKs.](image-url)
stability in plasma compared to the original compounds. Despite the fact that KLK3/PSA can cleave activating peptides when at high concentrations, the modified peptides exhibited a higher stability.\textsuperscript{85}

### 9.2.4 Depsipeptides

Depsipeptides are peptides that carry at least one ester linkage in place of amide.\textsuperscript{86} A variety of natural sources produce depsipeptides, including fungi, bacteria, and various marine organisms. A class of depsipeptides (General Formula 9.4) isolated from strains of \textit{chondromyces} bacteria or modified from the original peptides isolated by simple chemical procedures were found to specifically inhibit KLK7.\textsuperscript{87} Compounds 9.5, 9.6, 9.7, and 9.8 have an IC\textsubscript{50} of 1000, 400, 700, and 200 pM, respectively, while they are >10-fold more specific for KLK7 than for human neutrophile elastase, which is also a chymotrypsin-like protease. Compounds 9.5, 9.6, and 9.7 were extracted from \textit{Chondromyces crocatus}, while compound 9.8 was derived from a chemical reaction of 9.5 with 3-(chloromethyl)-1,5-dimethyl-1H-pyrazole.

Compound 9.5 has been assessed in experimental animals for the treatment of skin diseases. Specifically, in the mouse skin barrier disruption model (repeated skin stripping by S-Sqame skin sampling disks), it was found to accelerate barrier repair by 57%. Also, a single application of a solution of 9.5 in an oxazolone model of allergic contact dermatitis (ACD) in mice resulted in 40% inhibition of inflammatory ear swelling at 10 mM, while at 30 mM, it caused 46% inhibition of inflammation. Finally, in the 2,4-dinitrofluorobenzene (DNFB) model of ACD in swine, two applications of 1% solution of 9.5 inhibited inflammation by 30% and skin redness by 27%.\textsuperscript{87}
9.3 Inhibitors Designed Based on Bioscaffolds

Bioscaffolds are natural template structures that can be modified to become highly specific inhibitors. Modification is carried out either by recombinant DNA technology approaches or by conventional peptide chemical synthesis, in which case a small inhibitory fraction is selected for synthesis.

9.3.1 Engineered Sunflower Trypsin Inhibitor (SFTI)

Sunflower trypsin inhibitor (SFTI) is a member of the Bowman–Birk serine protease family. SFTI is a 14-amino-acid residue cyclic peptide (Gly-Arg-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp cyclized between the N-terminal Gly and the C-terminal Asp) isolated from sunflower seeds (*Helianthus annuus*) that was characterized and shown to exhibit a low $K_i$ of 150 pM for trypsin.\textsuperscript{88} This short peptide inhibitor can be produced in large quantities by standard chemical synthesis protocols. Chemical synthesis also facilitates the development of new variants that can be screened against novel protease targets for specific inhibition. In addition, Austin et al.\textsuperscript{89} reported the expression in *E. coli* of a library of cyclic SFTI variants based on the intramolecular native chemical ligation method. These procedures can be viewed as complementary to chemical synthesis protocols.

Recently, SFTI was used as a bioscaffold for the design and synthesis of a novel KLK4-specific inhibitor.\textsuperscript{90,91} Initially, a tetrapeptide phage display library was screened in order to identify the best substrate for KLK4 which had the sequence Phe-Val-Gln-Arg (FVQR). Then, a variant SFTI molecule was designed by replacing the P1 Lys residue of the substrate tetrapeptide with Arg, the P2 Thr with Gln, and the P4 Arg with Phe. The Cys at P3 position was not replaced by Val, as cysteine residues are important for structural integrity. The $K_i$ was calculated to be within the low-nanomolar range (3.6 nM) which is by 500-fold more selective for KLK4 than the closely related KLK14.\textsuperscript{90}

9.3.2 Mutant Serpins

Serpin bioscaffolds are amenable to engineering for the generation of highly specific inhibitors of certain serine proteases. Essential to serpins is the reactive center loop (RCL) that participates in the inhibition mechanism acting as a substrate for the targeted protease. Changes in the RCL sequence can modulate serpin specificity for specific proteases. Indeed, mutant serpins have been exploited for the development of KLK inhibitors.

9.3.2.1 Mutant Serpins Targeting KLK2

KLK2 reacts with $\alpha_1$-antichymotrypsin ($\alpha_1$-ACT) at a slow rate (12–16 h) due to the presence of Leu-Ser at P$_1$-P$_{1}'$ positions of the inhibitor, which
constitute an unfavorable peptide bond for cleavage by a trypsin-like enzyme. The substitution of the cleavable bond and neighboring amino acid residues is based on established KLK2 peptide substrates identified by phage display. A series of recombinant ACT molecules have been expressed and studied. The most selective was found to incorporate the Arg-Gly-Arg-\( \downarrow \)-Ser-Glu sequence. The new inhibitor has shown a rapid (within minutes) association with KLK2 compared to wild-type ACT (12–16 h). Currently, KLK2 ACT-derived inhibitors, such as the compound MDKP67b, are in development by Med Discovery (Switzerland). MDKP67b is a mutated form of ACT with four single amino acid substitutions that, at the time of writing, is about to undergo clinical trials as a targeted treatment for prostate cancer (http://med-discovery.webcreatif.net/Templates/Standard.aspx?id_page=43&flag=0).

9.3.2.2 Mutant Serpins Targeting KLK14

A similar approach to that described for KLK2 ACT-based inhibitors has been followed. Initially, a pentapeptide phage display library was screened to identify substrates specific for KLK14. Then, the identified peptidomimetics (E8: Leu-Gln-Arg-Ala-Ile and G9: Thr-Val-Asp-Tyr-Ala) were incorporated into the scaffold of either \( \alpha_1 \)-antitrypsin or \( \alpha_1 \)-antichymotrypsin. E8-engineered inhibitors showed relaxed specificity with various proteases with trypsin-like activity, while a G9-engineered ACT exhibited a very high specificity. The \( \text{ACT}_{E8} \) inhibitor showed a 1:1.2 (enzyme:inhibitor) stoichiometry of inhibition and a second-order rate constant \( K_a \) of 575 000. The \( \text{ACT}_{G9} \) has a 1:1.5 stoichiometry and a lower association constant \( K_a \) of 74 000 M\(^{-1}\) s\(^{-1}\). Finally, \( \text{ACT}_{G9} \) inhibited the liquefaction of human semen, a process that depends on the enzymatic activity of KLK14.

9.4 Synthetic Inhibitors Based on Modified Peptide-Substrates or Small Molecules

Powers et al. outlined the principles underlying the design of micromolar inhibitors of serine proteases. In brief, a common approach involves the attachment of a reactive group, often referred to as the warhead (alkylating, acylating, phosphorylating or sulfonylating functional group, or others) to the appropriate peptide sequence that is specifically recognized by the enzyme of interest. The use of phosphonyllfluoridate derivatives leads to very potent inhibitors of serine proteases, but because of concomitant inhibition of acetylcholinesterase, they are highly toxic and, therefore, not the first choice for inhibitor design. However, recent developments have led to less toxic variants that have found numerous applications, e.g. variants based on the general formula \( \text{RCONHCH(R)-PO(OPh)}_2 \) that are modified peptides at the C-terminus. Another class of phosphonates includes the
peptidylphosphonate diphenyl esters derived from substitution of fluoride with the phenoxy group.\textsuperscript{95} Another difference between diphenyl esters and fluoridates is their differential stability, with the latter being unstable after prolonged incubation in aqueous solutions.\textsuperscript{95} Two of the most important examples of phosphonates include the FP-biotin (9.9)\textsuperscript{96} and FP-fluorescein (9.10)\textsuperscript{97} used as activity-based probes in chemical proteomics to tag serine proteases. In the case of 9.9, because of the long spacer between the electrophilic phosphorus atom and the biotin group, biotin that extends out of the active site is readily available to bind to streptavidin that can be exploited for detection. A type of phosphonate diphenyl ester was described and its ability to detect active KLK6 was demonstrated in an immunofluorimetric assay for KLK6 used as a prototype assay for quantification of active KLK6 in biological samples.\textsuperscript{98}

A modern approach exploits click chemistry to probe protease activity (Scheme 9.1). In this case, a fluorophosphonate alkyne (FP-alkyne, compound 9.11) was used to probe KLK7 activity \textit{in vitro}. The conjugate was allowed to react with an azide derivative of fluorescein (9.12) in the presence of copper ions, and the activity was reported by fluorescence.\textsuperscript{99} Addition of tris(triazolyl)amine is necessary in order to stabilize the Cu(I) formed by the reaction of Cu(II) and the reducing agent TCEP [tris(carboxyethyl) phosphine]. Cu(I) acts as a catalyst for this reaction.\textsuperscript{100} Although this is a two-step procedure compared to reaction with 9.10, the overall labeling efficiency is significantly higher. Further, the advantage of 9.11 is that a lack of the large hydrophobic fluorescein moiety significantly reduces background caused by unspecific hydrophobic protein binding. In the future, more
Scheme 9.1
specific KLK-phosphonate probes are expected to become available for probing KLK activity *in situ* and for *in vivo* imaging with implications for molecular diagnosis.

### 9.4.1 KLK1 Inhibitors

KLK1 was the first enzyme targeted for inhibition, probably because it was one of the oldest and best-studied members of the KLK family. The inhibitor, named CH-2856 (or FE999024 or VA999024) (9.13), was synthesized based on the principle for substrate recognition. Originally, it was known that the tetrapeptide amide H-DPro-Phe-Arg-Ser-NH₂, which is found in the natural substrate of KLK1 LWMK (Pro³⁸⁷-...-Ser³⁹⁰), was the minimum binding sequence. This sequence has been optimized through a series of chemical substitutions that involved: replacement of the C-terminal Arg-Ser-NH₂ with a series of (aminoalkyl)guanidines acting as synthetic residues; the best inhibitory constants were obtained with the alkyl group propyl, then replacement of Phe with the synthetic 2′-(1-naphthylalanine) and, finally, replacement of the N-terminal DPro with (4-Cl)-DPh. This inhibitor showed a high potency against KLK1 with a $K_i$ of 2.2 nM, while it was very specific compared to plasma kallikrein (454 times more potent), trypsin (454), thrombin (16,000), and plasmin (4,900). It has been shown to reduce eosinophilia in a guinea-pig model of allergic inflammation. Further, FE999024 has been found to inhibit the invasive potential of MDA-MB-231 breast-cancer cells in Matrigel assays and in an *ex vivo* lung invasion assay in rats. Also, FE999024 attenuates the effect of parainfluenza viral-induced lung inflammation and airway hyper-reactivity in guinea pigs. Finally, FE999024 limits the progression of oedematous to haemorrhagic pancreatitis in rats, indicating that it may also have potential applications in acute pancreatitis which implicates mainly the action of KLK1.

Another class of naphthamidine derivative compounds (compounds 9.14 and 9.15), developed as orally active serine protease inhibitors, show a very good inhibition against KLK1. Nafamostat (6-amidino-2-naphtyl p-guanidinobenzoate) (9.14), which is used in Japan in intravascular coagulation and acute pancreatitis, shows a $K_i$ of 320 nM for KLK1, but it can also inhibit plasma kallikrein ($K_i$ 12 nM), trypsin (15 nM), and other proteases with even higher affinity. Another naphthamidine derivative, sepimostat ([6-carbamimidoyl naphthalen-2-yl] 4-(4,5-dihydro-1H-imidazol-2-yl amino)benzoate) (9.15) has shown a better selectivity for KLK1 and a low $K_i$ of 29 nM, compared to plasma kallikrein (97 nM), thrombin (610 nM), and other enzymes. Based on their nanomolar range of $K_i$, these compounds may be used as lead compounds, in order to design KLK1 inhibitors with improved selectivity. Importantly, administration of sepimostat mesylate has not shown any serious side effects in mice and rats, and no adverse effects on the cardiovascular and respiratory systems in dogs.
Finally, derivatives of aminopyridine (General Structure 9.16, Figure 9.3), such as the (R)-2-amino-3-methyl-pentanoic acid [(S)-1-[(6-amino-pyridin-3-ylmethyl)-carbamoyl]-2-naphthalene-1-yl-ethyl]-amide (compound 9.17) and the (R)-3-methyl-2-methylamino-pentanoic acid [(S)-1-[(6-amino-pyridin-3-ylmethyl)-carbamoyl]-2-(3,4-dichloro-phenyl)-ethyl]-amide (compound 9.18), were developed recently as novel KLK1 inhibitors for the treatment of asthma, inflammatory conditions, cancer, etc. These compounds showed highly potent and selective activity against KLK1. Specifically, 9.17 and 9.18 have an IC$_{50}$ of 960 and 220 pM, respectively, while they are $>10 000$ times more selective when compared to plasma kallikrein, thrombin, trypsin, and plasmin.$^{107}$

### 9.4.2 KLK3/PSA Inhibitors

KLK3/PSA has long been a target for inhibitor development, and several groups have made various attempts to design novel inhibitors. An early approach aimed to develop new inhibitors was based on 2-azetidinone analogs (General Formula 9.19) and led to the identification of 9.20 that exhibited a relatively high IC$_{50}$ of 8.98 μM.$^{108}$ Later studies used 9.20 as a lead compound and synthesized 9.21 with potent inhibitory activity against KLK3/PSA and an IC$_{50}$ of 226 nM [for the (S, S) isomer].$^{109}$ 9.21 in racemic mixture has a low IC$_{50}$ of 340 nM.$^{110}$ These
Figure 9.3  KLK1 inhibitors. $R_1$ and $R_2 = H$, OH, ($C_{1-10}$)alkyl, ($C_{1-6}$)alkoxy, ($C_{2-6}$) alkenyl, ($C_{3-10}$)cycloalkyl, heterocycloalkyl, aryl, heteroaryl, aryl($C_{1-4}$)alkyl and heteroaryl($C_{1-4}$)alkyl. $R_3 = H$, ($C_{1-10}$)alkyl, ($C_{2-6}$)alkenyl. $R_4$, $R_5 = H$, ($C_{1-10}$)alkyl, ($C_{2-6}$)alkenyl, ($C_{3-10}$)cycloalkyl, heterocycloalkyl, aryl, heteroaryl, aryl($C_{1-4}$)alkyl and heteroaryl($C_{1-4}$)alkyl. $R_6$, $R_7 = H$, ($C_{1-10}$)alkyl, ($C_{2-6}$)alkenyl, ($C_{3-10}$)cycloalkyl, heterocycloalkyl, aryl, heteroaryl, aryl($C_{1-4}$)alkyl, aryl($C_{2-4}$)alkenyl, heteroaryl($C_{1-4}$)alkyl, -SO$_2$($C_{1-4}$)alkyl, -SO$_2$aryl, and -SO$_2$aryl($C_{1-4}$)alkyl. $R_8$, $R_9$, $R_{10} = H$, ($C_{1-10}$)alkyl, halogen, HO, ($C_{1-6}$) alkoxy, $R_{11}$, $R_{12} = H$, ($C_{1-6}$)alkyl. In addition, $R_6$ and $R_7$ together with the nitrogen atom to which they are attached, could form a four- to seven-membered nitrogen-containing ring that may also contain N, O, and S, and may carry a substitution with one or two substituents that could be ($C_{1-6}$)alkyl, ($C_{1-6}$)alkoxy, halogen, CN, or HO, or the N-containing ring may be fused to an aryl group. $R_4$ and $R_6$ together with the atoms to which they are attached may form a saturated or unsaturated four- to seven-membered N-containing ring which may also contain N, O, and S, and may carry a substitution with one or two substituents that could be ($C_{1-6}$)alkyl, ($C_{1-6}$)alkoxy, halogen, CN, and HO. $R_5$ may be absent, and $R_4$ and $R_6$ together with the atoms to which they are attached may form a five-, six-, nine-, or 10-membered mono- or bi-cyclic N-containing aromatic ring that may also contain N, O, and S, and may carry a substitution with one, two, or three substituents that could be ($C_{1-6}$)alkyl, ($C_{1-6}$)alkoxy, halogen, CN, HO, aryl, and COOR.

Compounds inhibit KLK3/PSA in an irreversible manner through the generation of a covalent acyl-enzyme complex with the active-site Ser.$^{110}$

Methodologies based on high-throughput screening (HTS) are currently available and can be applied to screen for inhibitors against multiple enzymes or the opposite. Recently, such methods were employed for screening libraries containing approximately 50 000 different molecules for KLK3/PSA inhibition.$^{111}$ During this HTS, a high-affinity inhibitor 9.22 with IC$_{50}$ 300 nM was identified.$^{111}$
HTS can yield novel lead compounds that require single-step optimization by molecular modeling and simple group replacements to increase affinity.

Recently, modified peptides carrying either aldehyde or borate groups were synthesized based on the recognition sequence of KLK3/PSA. The Cbz-Ser-Ser-Lys-Leu-(boro)Leu (Cbz, benzylxoycarbonyl) (Compound 9.23) has a $K_i$ of 65 nM for KLK3/PSA which is 60 times lower than that for chymotrypsin. Its
development was designed based on the classical KLK3/PSA substrate: His-Ser-Ser-Lys-Leu-Gln, selected form semenogelin II, which is a KLK3/PSA natural protein substrate.\textsuperscript{113} Glu at the C-terminus is not recommended for aldehyde modification due to hemiaminal formation (Scheme 9.2)\textsuperscript{114} and also Glu boronic acid modifications are not feasible to produce.\textsuperscript{112}

Successful replacement of Gln by Leu or nor-Leu was carried out. Leu led to the production of 9.23.\textsuperscript{112} Despite the fact that 9.23 had a marginal effect in subcutaneous prostate-tumor xenografts,\textsuperscript{112} it may find applications in diagnosis as described below. Boronic acid led to powerful inhibitors, since the aldehyde-modified peptide Cbz-Ser-Ser-Lys-Leu-Leu-CHO exhibited a $K_i$ of 6.51 $\mu$M which is 100-fold higher than the boronic acid analog 9.23.\textsuperscript{115} In addition, nor-Leu replacement led to development of the Cbz-Ser-Ser-Lys-Leu-n(boro)-L inhibitor that exhibited an even lower $K_i$ of 25 nM.\textsuperscript{116}

9.4.3 Imaging Based on KLK3/PSA

The highly specific expression of KLK3/PSA in the prostate gland makes it ideal for imaging purposes. \textit{In vivo} imaging is based on designing specific
inhibitors that carry a group (e.g., a radioactive or fluorescent tag) which can be observed live. In this context, LeBeau et al. synthesized the modified peptide-boronic acid inhibitor 9.24 of KLK3/PSA (Ser-Ser-Gln-n(boro)-L) in such a way that the N-terminus carries a group that chelates rhenium (Re) ions. Re was used as a 99mTc analog due to its similar size and polarity. Indeed, the bulky metal ion along with the ligands did not change the affinity of the inhibitor for KLK3/PSA that could be exploited for the diagnosis of prostate cancer by PET or SPECT-based imaging methods.

Another very recent approach that can be applied for in vivo imaging based on KLK3/PSA exploits gold nanoparticles (AuNPs). In this case, thiol-terminated (through Cys) KLK3/PSA recognition peptides (His-Ser-Ser-Lys-Leu-Gln-Cys) were synthesized and conjugated at the N-terminus with either a near-infrared dye (Quasar 670), to enable detection in vivo, or a dark quencher (BHQ2). Both peptides containing either the dye or the quencher were used to decorate 20 nm AuNPs through their free thiol group at their C-terminus. Both the AuNP and the dark quencher quenched fluorescence emitted by the Quasar 670 dye. Upon protease action, the dye is released and monitored as fluorescence emission. This type of nanoparticle was tested only with trypsin and uPA, but similar approaches are expected to find more and more applications in the future.

### 9.4.4 KLK7 Inhibitors

KLK7 is considered to play a role in deregulated epidermal desquamation and inflammation, characteristic features of NS, atopic dermatitis, rosacea, psoriasis, and other skin diseases. As a result, development of KLK7-specific inhibitors has been an intense field of study. The KLK7-targeting compounds may be the active ingredients of future pharmaceutical products or cosmetics. At Novartis Institutes for Biomedical Research, a class of organic molecule with the general structure 9.25 shown in Figure 9.4 was developed as KLK7 inhibitors exhibiting IC$_{50}$ between 1 nM and 10 μM. For example, 9.26 \{(1S,2S,5R)-3-aza-bicyclo[3.1.0]hexane-2,3-dicarboxylic acid 3-[(7-chloro-naphthalen-1-ylmethyl)-amide] 2-[(pyridin-3-ylmethyl)-amide] has an IC$_{50}$ of 3 nM. Compound 9.27 has been used to test the recovery of skin-barrier disruption after stripping mouse skin with S-Sqame skin sampling disks where it exhibited enhanced recovery. In addition, it displayed anti-inflammatory activity in the TPA (phorbol 12-myristat-13-acetate)-induced irritant dermatitis model in mice. Also, compound 9.28 showed anti-inflammatory activity in the DNFB model of allergic contact dermatitis in swine.

The heterocyclic compounds 9.29, 9.30, and 9.31 are also KLK7-specific inhibitors with an IC$_{50}$ of 20, 50, and 100 nM, respectively. Compound 9.31 was used in KLK7-transgenic mice suffering from increased skin inflammation, hyperkeratosis, itching that becomes more frequent with age, and increased epidermal thickness. The external application of 9.31 in KLK7-transgenic mice resulted in an improvement in TEWL (transepidermal water loss) with approximately half the efficiency of betamethasone (Betnovat, Glaxo
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Figure 9.4 KLK7 inhibitors. In the general formula $R_1 = H, CN, (C_{1-8})$alkyl, $(C_{2-8})$alkenyl, $(C_{2-8})$alkynyl, halogen, $(C_{1-8})$alkylamino, $(C_{1-8})$alkyloamino$(C_{1-8})$ alkyl, $(C_{1-8})$alkoxy, halo$(C_{1-8})$alkyl, $R_2 = (C_{1-8})$alkyl, $(C_{1-8})$alkylamino, $(C_{1-8})$alkylamino$(C_{1-8})$alkyl, di$(C_{1-8})$alkylamino$(C_{1-8})$alkyl, halo$(C_{1-8})$ alkyl, $(C_{1-8})$alkoxy, $(C_{1-8})$alkoxy$(C_{1-8})$alkyl or $(CH_2)_m Z$ where $m = 1, 2$ or $3$ and $Z =$ unsubstituted or substituted $(C_{3-8})$cycloalkyl, $(C_{6-18})$aryl or heterocyclic having five- to six-ring members and one to four heteroatoms from N, O, and S. $R_3 = H, (C_{1-8})$alkyl, $(C_{1-8})$alkoxy, $(C_{6-18})$aryl or heterocyclic having five- to six-ring members and one to four heteroatoms from N, O, and S, $n = 1, 2$ or $3$, $X = (CH = CH)$, -NH-, $(N = CH)$, O or S.

SmithKline AB), while it improved skin morphology without the side effects of betamethasone.¹²⁰

9.4.5 General Modulators of KLKs

Cardiac glycosides were identified by HTS as novel transcriptional regulators of KLKs that could be exploited therapeutically, as they affect KLK activities, although they do not directly inhibit KLK enzymatic activity.¹²¹ Modulation of KLK transcription is also conferred by epigenetic drugs,¹²² vitamin D₃ analogs,¹²²,¹²³ 9-cis and 13-cis retinoic acid,¹²⁴ and steroid hormones.¹⁶ However, these classes of compounds do not act specifically on KLK genes but act on a wide range of other genes. Azelaic acid (nonanedioic acid, product of ozonolysis of oleic acid) reduces KLK5 expression in skin with significant implications for the
treatment of rosacea. This is probably the basis for the observed reduction in inflammation in rosacea after application of azelaic acid-containing creams.

9.5 Antibody-Derived Inhibitors

Monoclonal antibodies can be specifically designed and engineered to become powerful and highly specific inhibitors (or activators). For therapeutic purposes, human antibodies isolated by antibody phage display methodology should be used ideally, as they do not elicit immune responses.

9.5.1 Potential of Antibody Phage Display Strategy

Antibody phage display involves the expression and display of a fragment of an antibody molecule on the surface of a filamentous phage, usually M13 or the fd. RNAs are extracted from human volunteer B-cells and reverse-transcribed to cDNA. Then, cDNAs are converted to DNAs and cloned into the genome of the filamentous phage. Each phage will carry only one copy of genetic material and its representative antibody. Phages can be engineered to express only a single copy of the antibody (monovalent) or multiple copies (polyvalent). Monovalent methodology is more common, since it is not expected to interfere with the ability of phages to infect bacteria. Phages with displayed antibodies are then screened over an immobilized target. Figure 9.5 summarizes the antibody phage display method. Each phage carries only one distinct antibody. At the end of the experiment that includes multiple rounds of selection, the obtained phages displaying high-affinity antibodies are amplified in E. coli and sequenced. The antibody-encoding gene is transferred into bacteria to produce large quantities of the antibody.

9.5.2 Antibodies Directed Against KLKs

Dyax has developed a series of human monoclonal antibodies based on highly diverse phage display library and a high-throughput surface plasmon resonance (SPR) affinity screening method. One of the KLK1-specific antibodies (DX-2300) exhibited competitive inhibition with a very low $K_i$ of 130 pM. Since DX-2300 shows competitive inhibition and lack of binding to KLK1-complexed with active site inhibitors, it is concluded that it likely binds to the active site or next to it. KLK1 was reported as the primary kininogenase in airways, and its activity is upregulated in bronchoalveolar lavage fluid from patients with asthma and allergic rhinitis, indicating that this antibody may become a valuable tool for the therapy of asthma. In an allergic sheep model of asthma, DX-2300 inhibited the allergen-induced late-phase bronchoconstriction and the airway hyper-responsiveness to carbachol.

Another interesting approach for the production of antibodies involves active immunization. It was mentioned earlier that in EAE, a mouse model of multiple sclerosis, administration of rat Klk6 resulted in the production of
Figure 9.5  Schematic representation of antibody phage display methodology. (A) Antibody structures and their major fragments. (B) Structure of a filamentous phage with monovalent and polyvalent antibody display. (C) General scheme for the selection of human monoclonal antibodies.
anti-Klk6 that attenuated disease symptoms.\textsuperscript{48} In the same context, administration of anti-Klk6 antibodies (\textit{i.e.} passive immunization) also resulted in the attenuation of symptoms.\textsuperscript{48}

### 9.6 Future Directions

Human KLKs represent a major proteolytic system that participates in many tissues with additional functions yet to be identified. Despite the fact that KLKs constitute a relatively new family of serine proteases, certain KLKs were targeted for therapeutic applications in various disorders mainly in skin diseases such as NS, psoriasis, \textit{etc.} and in cancer. Development of animal models will facilitate the study of KLKs and identification of their as-yet unknown physiological functions, as well as the assessment of novel pharmaceutical compounds targeting KLKs. Currently, the \textit{KLK1}\textsuperscript{−/−},\textsuperscript{131,132} \textit{KLK4}\textsuperscript{−/−},\textsuperscript{133} and \textit{KLK8}\textsuperscript{−/−} mice\textsuperscript{134} have been generated and phenotypically described. \textit{KLK1}\textsuperscript{−/−} displays defects in calcium renal reabsorption\textsuperscript{131} and altered renal potassium balance.\textsuperscript{135} \textit{KLK4}\textsuperscript{−/−} mice suffered from hypomaturation amelogenesis imperfecta and showed enamel defects in their teeth,\textsuperscript{133} as also observed in humans carrying homozygous \textit{KLK4}-inactivating mutations.\textsuperscript{136} \textit{KLK8}\textsuperscript{−/−} displayed mild hyperkeratosis with no other symptoms. Generally, all the aforementioned phenotypes of general KLK knockout mice are not severe or life-threatening and point to the safety of KLK targeting. Safety issues in terms of KLK targeting can initially be interrogated in experimental animal models. In addition, transgenic mice overexpressing KLK7,\textsuperscript{137} KLK6,\textsuperscript{21} and KLK5\textsuperscript{138} have been studied, as well as double transgenic KLK2/KLK3 mice to prove activation of KLK3 by KLK2 \textit{in vivo}, and again these mice did not shown any adverse effects.\textsuperscript{139}

The search for new KLK inhibitors is ongoing. Aryl lactones, which were initially developed as chymotrypsin inhibitors,\textsuperscript{140} represent another potential class of organic inhibitors of KLK proteases. Indeed, the \textalpha-aryl lactone 9.32 inhibited KLK13, indicating that this may provide a new class of lead compounds for the development of novel inhibitors with high specificity against KLKs.\textsuperscript{73}
was generated by in vitro selection (SELEX).\textsuperscript{141} In addition, peptides that bind specifically to target proteins can be isolated by polysome selection, which provides peptides with higher affinities. Peptide ligands with a high affinity for KLK3/PSA were identified by polysome selection, such as the Met-Glu-Arg-Cys-Pro-Ile-Lys-Met-Phe-Tyr-Asn-Leu-Gly-Ser-Pro-Tyr-Met-Asn-Ile peptide that displayed a dissociation constant, $K_d$, of 800 pM.\textsuperscript{142} Finally, KLKs are expected to find applications in gene-therapy protocols. It was shown recently that adenoviral delivery of the Klk1 gene into mice suffering from anti-glomerular basement membrane-induced nephritis elicited protective effects.\textsuperscript{143} Further, in transgenic rats, KLK1 was shown to prevent cardiac fibrosis in diabetic cardiomyopathy,\textsuperscript{144} while adenoviral delivery of KLK1 was applied in mouse models for the induction of vessel formation and maturation.\textsuperscript{145}

References


42. P. J. Barnes, *Agents Actions Suppl.*, 1992, **38**, 432.


