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Review

Natural and synthetic inhibitors of kallikrein-related peptidases (KLKs)

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ABSTRACT

Including the true tissue kallikrein KLK1, kallikrein-related peptidases (KLKs) represent a family of fifteen mammalian serine proteases. While the physiological roles of several KLKs have been at least partially elucidated, their activation and regulation remain largely unclear. This obscurity may be related to the fact that a given KLK fulfills many different tasks in diverse fetal and adult tissues, and consequently, the timescale of some of their physiological actions varies significantly. To date, a variety of endogenous inhibitors that target distinct KLKs have been identified. Among them are the attenuating Zn^{2+} ions, active site-directed proteinaceous inhibitors, such as serpins and the Kazal-type inhibitors, or the huge, unspecific compartment forming α_2-macroglobulin. Failure of these inhibitory systems can lead to certain pathophysiological conditions. One of the most prominent examples is the Netherton syndrome, which is caused by dysfunctional domains of the Kazal-type inhibitor LEKTI-1 which fail to appropriately regulate KLKs in the skin. Small synthetic inhibitory compounds and natural polypeptidic exogenous inhibitors have been widely employed to characterize the activity and substrate specificity of KLKs and to further investigate their structures and biophysical properties. Overall, this knowledge leads not only to a better understanding of the physiological tasks of KLKs, but is also a strong fundament for the synthesis of small compound drugs and engineered biomolecules for pharmaceutical approaches. In several types of cancer, KLKs have been found to be overexpressed, which makes them clinically relevant biomarkers for prognosis and monitoring. Thus, down regulation of excessive KLK activity in cancer and in skin diseases by small inhibitor compounds may represent attractive therapeutical approaches.

1. Introduction

Regulation of protease activity in the living organism is a highly complex task that involves all levels of cellular organization. Control and timing of protease activity starts with gene expression, transcription and translation, and continues with protein targeting and zymogen activation. Once activated, the protease is often kept in check by endogenous inhibitors, while the last steps of protease regulation may be limited proteolysis and final degradation. The emerging research in the field of tissue kallikrein-related peptidases (KLKs) provides many diverse examples for nearly all aspects of protease regulation by inhibitors.

Tissue kallikrein (Kallikrein 1, KLK1) and the kallikrein-related peptidases are (chymo)trypsin-like serine proteases, belonging to family S1A of clan PA(S) according to the MEROPS classification [1]. Prior to the introduction of a new nomenclature in 2006, the KLKs were often referred to as hKs or rKs for human and rat proteins, respectively [2]. Fully sequenced genomes of placental mammals, such as primates or rodents, and even of marsupials (e.g. the opossum), exhibit at least eleven *KLK* genes, but usually lack the counterparts of human *KLK2* and *KLK3* [3,4]. However, the numbers of corresponding proteases vary from ten KLKs in cows, eleven in dogs, and 26 ones in mice. The latter possess a series of functional KLK1 paralogs [2,5,6]. Kallikrein 1 and the kallikrein-related peptidase genes are organized in a single cluster on chromosome locus 19q13.4 [7,8]. The 15 human KLK members are only distantly related to plasma kallikrein, which shares 38% identical residues with KLK1 in the catalytic domain, while KLK1 and trypsin share 46% identity [9,10].

One or more *KLK* genes are expressed in nearly all tissues and fluids of the human body. They fulfill a diverse range of tasks throughout one's lifetime from embryonic development to processes in adulthood [8,11–13]. KLKs are intracellulary synthesized as precursors with a signal peptide (15–34 amino acids) that is cleaved off upon secretion into the endoplasmatic reticulum. The proform or zymogen of the KLK protease is extracellularly activated by the removal of the propeptide (3–37 amino acids), resulting in active proteases of 223–238 residues (Fig. 1), and in some cases reaching molecular weights of up to 50 kDa due to heavy glycosylation [14]. The activation process of KLKs may involve autoactivation [15–17],

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KLK activation cascades [18–20], serine proteases from the thrombostasis axis, such as plasmin, plasma kallikrein, and factor Xa [21], or the proteolytic activity of other proteases, such as urokinase-type plasminogen activator (uPA), matrix metalloproteinases (MMPs), and dipeptidyl peptidase I [22–24]. However, the KLK activity is not restricted to regulation by steroid-dependent expression [25–27] or by fine-tuned zymogen activation. In the case of KLKs 6, 12, and 14, from example, regulation is likely, at least in part, achieved by autolysis [17,28–31]. Furthermore, in some cases an interplay of KLKs and their corresponding natural inhibitors has been established, even with pathophysiological significance [32]. However, many potential inhibitors of KLKs have not yet been unambiguously assigned to a given KLK. Another unusual feature of certain KLKs is the timescale of their activity, which can reach months, if not years, as seen with KLK4 in tooth development, which was also observed in a comparison of *Klk4/lacZ* knockin mice and the wild type [33,34].

Overall, the human KLKs can be subdivided into in several tissue-specific groups with distinct physiological substrates and functions. In the 1920s and 1930s, the first kallikrein (KLK1) was discovered and characterized as a proteolytic enzyme, mainly occurring in urine, kidney, and the pancreas, the latter being the inspiration for the protein's name which is derived from the Greek word for pancreas (καλλικρεας, Kallikreas) [35–37]. A major function of KLK1 is the reduction of blood pressure by releasing the peptide hormone Lys-bradykinin (kallidin) from low molecular weight kininogen, which effects muscle relaxation and inflammatory processes [38]. Knockout of the *Klk1* gene in mice causes both cardiovascular abnormalities and a defect of efficient renal tubular calcium absorption [39,40]. Intriguingly, administration of this protease can reduce cardiac and renal injuries, restenosis, and ischemic stroke and promotes angiogenesis and skin wound healing [41]. Similar to the other "classical" KLKs, KLK 2 and 3, which were discovered in the late 1980s, KLK1 features an extended "99-loop" (also called kallikrein loop) of 11 inserted amino residues with respect to chymotrypsin. Among the "new" KLKs, KLK 4–15 that were gradually characterized from the mid-1990s onwards, KLKs 8–13 possess 99-loop insertions from two to eight residues (see alignment in Fig. 1).

In the prostate, KLKs 2, 3, 4, 11, and to some extent KLKs 14 and 15, are produced for secretion into seminal plasma [8,11]. There, they most likely activate each other in a cascade-like manner resulting in the degradation of semenogelins and fibronectin, mainly by KLK3 and KLK14, for semen liquefaction [42,43]. Since KLK3 (PSA, "prostate specific antigen") blood plasma levels correlate with prostate cancer progression, an immunoassay for PSA has become a widespread medical application, despite its moderate reliability as prognostic biomarker for malignant processes. Thus, there still remains the need for additional specific KLK tumor markers [44–46]. One of these promising markers is KLK4, which is distinctly expressed in the early stages of prostate cancer [47]. Interestingly, KLK4 has the capacity to activate the proform of the urokinase-type plasminogen activator (pro-uPA) and to modulate the activity of its receptor uPAR, both of which play a significant role in prostate and ovarian cancer [48,49]. In addition, KLKs 2, 4, 5, 6, and 14 seem to be potential players in signal transduction via activation of G-coupled protease receptors, such as PARs 1, 2, and 4, resulting, e.g. in inflammation or in tumor cell proliferation and migration [50–56].

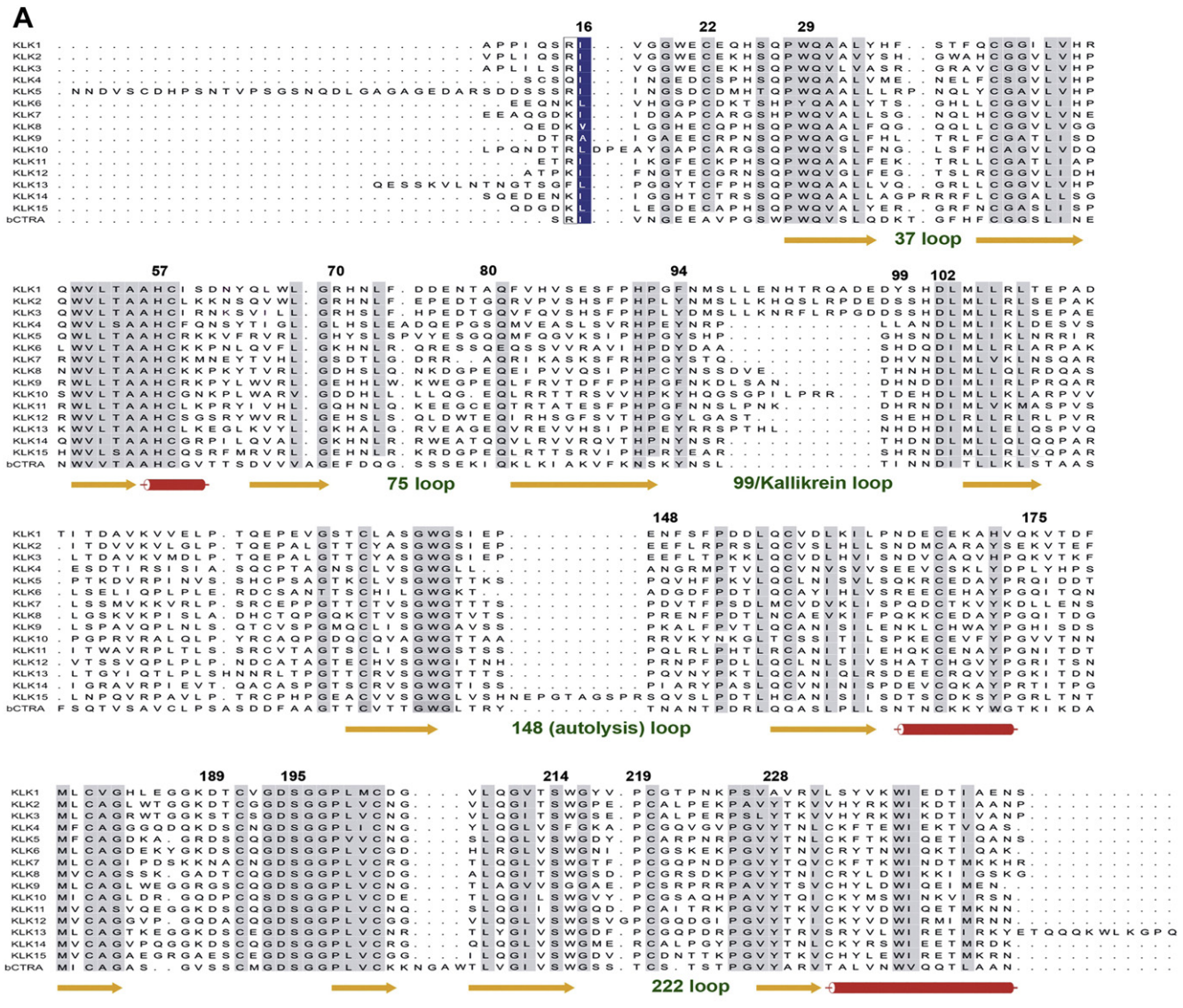
In addition to its role in the prostate, which is not yet fully understood, KLK4 is imperative for tooth development, particularly in formation of enamel, which also depends on MMP-20 [57]. Under normal circumstances, both proteases degrade the extracellular matrix required for the growth of dentin crystallites. Single mutations, however, result in either the malfunctioning of MMP-20 or KLK4, causing the hereditary disease *amelogenesis imperfecta*, which is characterized by very fragile teeth [58,59]. More specifically, KLK4 seems to be crucial for the formation of large coherent enamel crystallites, as seen in *Klk4* knockout mice [34].

A larger subset of kallikrein-related peptidases, namely KLKs 5, 7, 11, and 14, is highly expressed in human skin, mainly in the outermost layer, the *stratum corneum*, while KLKs 6, 8, 10, and 13 are found at medium expression levels [11,13,60]. KLKs 5, 7, and 14 are capable of degrading proteins of the corneodesmosomes, leading to desquamation, the shedding of cornified skin cells [61–63]. In contrast, KLK8 is involved in cellular differentiation and healing of the skin [64], similar to KLK6, which induced rapid wound healing by promoting keratinocyte proliferation and migration in a mouse model based on the shedding of E-cadherin by Klk6 [65]. Also, KLKs 4, 5, and 8 specifically activate the metalloproteinases meprin α and/or β, which are located in separate layers of the epidermis [66]. Tight activity regulation of these KLKs by several types of inhibitors is necessary, otherwise diverse skin diseases will develop [67].

Intriguingly, two KLKs, KLK6 and KLK8 (also termed neurosin and neuropsin, respectively), are expressed at higher levels in human brain [8,68]. KLK6 accumulates at brain lesions of humans and investigations in mice suggest that excessive KLK6 activity causes inflammation of the central nervous system and promotes multiple sclerosis through demyelinating activity [69,70]. The physiological role of KLK6 seems to be both de- and remyelination of glia cells, contributing to neurite and axon growth after injuries [71]. In contrast, KLK8, which mostly occurs in the hippocampus, is involved in long term potentiation (LTP) and memory acquisition by restructuring synapses, as shown by mouse models [72–75]. Furthermore, in human brains with Alzheimer's disease a more than 10-fold expression of KLK8 was observed [76]. On the other hand, *Klk8* knockout mice were shown to be susceptible to epileptic seizure [77]. Furthermore, single nucleotide polymorphisms in the human *KLK8* gene are associated with manic-depressive disorder and cognitive impairment [78].

Although the KLK9 protease is present in many tissues and dominates among all KLKs in fetal and adult heart [11], no physiological function has been defined so far, however, it may serve as an ovarian and breast cancer marker [79]. Similarly, KLKs 10, 12, 13 and 15 are associated with distinct cancers without established (patho)physiological roles [17,80,81]. Nevertheless, there are some indications of a tumor suppressor role for KLK10 in breast cancer [82], KLK12 may be involved in angiogenesis regulation [83], KLK13 in ovary tissue remodelling and interleukin processing [84,85], and KLK15 in KLK3/PSA activation [86]. Intriguingly, expression of KLKs in the female reproductive system appears to be complementary to the expression pattern of KLKs in prostate, suggesting an activation cascade that probably involves all KLKs during impregnation [87].

As we will see later for the KLKs, natural inhibitors of proteases often bind directly to the active site, exploiting some degree of complementarity at the interaction surface [88–90]. Expectedly, the more the components of the inhibitor bind to distinct specificity pockets of the protease, the more specificity and affinity can be gained for inhibition [91], which in some cases may be enhanced by additional binding of the inhibitor to so-called exosites [92–94]. Also, knowledge of the substrate specificity of the KLKs will be a guideline for the identification of endogenous (and perhaps exogenous) inhibitors, and for the design of synthetic substrates, as well as of highly specific inhibitors, which may eventually yield powerful pharmaceutical compounds. Numerous studies have investigated the specificity for all KLKs, using either individual chromogenic and fluorogenic substrates, such as in the case of KLKs 8, 12, 15 [17,95–97] or systematic positional scanning approaches for KLKs 3, 4, 5, 6, 7, 10, 11, 13, 14 [98–101], phage display for KLKs 1, 2, 3, 4, 6, 14 [102–107], or peptide libraries for KLKs 1, 2, and 3 [108–110]. In addition, KLK cleavage sites in natural substrates have also been analyzed to a large extent. Since these studies show many differences, sometimes stark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99/Kallikrein loop
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148 (autolysis) loop
189
195
214
219
228
222 loop
KLK1
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KLK3
KLK4
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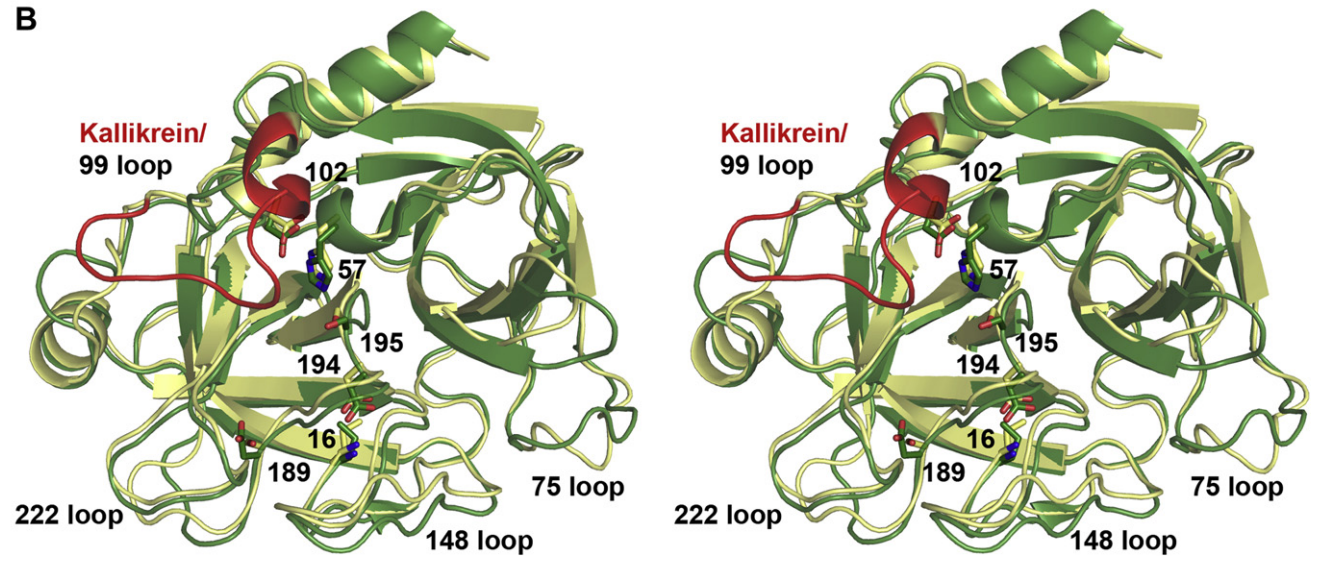
KLK14
KLK15
bCTRA


B
Kallikrein/
99 loop
102
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195
194
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75 loop
222 loop
148 loop
Kallikrein/
99 loop
102
57
195
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16
189
75 loop
222 loop
148 loop

Table 1
Statistical specificity patterns of human KLK peptidases cleavage sites, according to MEROPS specificity matrices for peptide residue positions P4 to P4′ that bind to protease subsites S4 to S4′ with the scissile bond between P1–P1′ (Schechter–Berger nomenclature [306]). In cases without established matrix, the information on natural and synthetic substrates was used to derive a score from the incidence of a residue, whereby the P1 value was always set to 100%. The font size corresponds to the frequency of a residue in published KLK substrates (an incidence cutoff was individually chosen, in order to limit the number of residues at a given position). Hydrophobic residues are depicted in black, polar ones in green, acidic ones in red, and basic ones in blue. For KLK9 only data of one substrate of the rat ortholog (rKLK9) were available [112], while for KLKs 10, 11, and 13, results from substrate profiling studies [99,100,307] have been treated as one substrate per study, employing only the top ranked residues in positions P4 to P1. For KLK12 published substrates from two studies were analyzed [17,96]. The primary tryptic specificity for Arg and Lys in P1 of many KLKs is conferred by an Asp189 at the bottom of the S1 pocket, which is replaced by a Glu189 in KLK15. The three chymotryptic KLKs with a preference for P1-Tyr, -Phe, or -Gln possess a Ser189 (KLK3), an Asn189 (KLK7), and a Gly189 (KLK9), but rat KLK that shares 40% identical residues with KLK9 (61% homology) exhibits an Asp189, although it has a tendency to cleave chymotryptic substrates. Apparently, KLK1 has a mixed tryptic/chymotryptic specificity, as was also observed for KLKs 10 and 11 [99].

KLK	P4	P3	P2	P1	P1′	P2′	P3′	P4′
KLK1	ASE	PAS	LFS	YR	SR	ATR	G	AR
KLK2	GTE	GSTQR	GLFSTR	R	LMS	GPAS	GS	GST
KLK3	ISQR	SQK	VLYQH	YQH	ST	GVS	SNQ	TE
KLK4	VIF	VYE	GPLFYS	YKR	GIS	GR	GD	G
KLK5	GPLD	GF	GPFS	GKR	GIS	LIS	GPS	GP
KLK6	PAS	GASK	GPVLFS	KR	GMSN	AVLSD	GLIS	GLWT
KLK7	G	GAKR	PLFD	FYR	L	R	SK	K
KLK8	AT	GVLDE	GPLR	KR	A	A	V	Y
rKLK9	C	I	P	Y	Y	L	D	C
KLK10	M	E	LFD	(M)R				
KLK11	M	PE	LFR	MR				
KLK12		PVFQD	PALFS	KR				
KLK13	VYE	AYR	ALR	KR	S	P	T	H
KLK14	PT	GVL	GPS	KR	GS	VLSD	T	GP
KLK15	I	PLE	GFS	R	I	V	G	G

discrepancies, for the single specificity subsites, a general overview is given in Table 1 based on the statistical approach of the MEROPS specificity matrices (http://merops.sanger.ac.uk/). The primary specificity of the kallikrein-related peptidases will allow a basic classification between inhibitors of the twelve tryptic KLKs (1, 2, 4–6, 8, 10–15) and of the three chymotryptic KLKs 3, 7, and presumably 9. The specificity of KLK9 remains still unclear, as it cleaved P1 Arg containing pro-KLKs at very slow rates [20], while enzymatic data are only available for the rat ortholog rKLK9 (Table 1) [111,112], which possesses as major determinant of P1 specificity an Asp189, in contrast to the unusual Gly189 of human KLK9 (Fig. 1). Furthermore, the preference of KLKs 1, 10, and 11 appears to be mixed tryptic and chymotryptic (Table 1) [99]. It should be noted that the proteolytic activity of recombinant KLK10 is rather slow and only observed *in vitro* with Leu16 as N-terminal residue, followed by three inserted residues (Fig. 1) [20]. In addition to a problematic KLK10 activation *in vivo* at Glu16C, if tryptic or chymotryptic proteases were the activators, KLK10 exhibits a Ser193 instead of the extremely conserved Gly193 (Fig. 1). A Ser193 might affect the oxyanion pocket function, similar to various factor XIa mutants of residue 193 with reduced activity and inhibitor binding capacity [113]. Nevertheless, in addition to the proof of enzymatic activity of recombinantly expressed KLK10, it was demonstrated that native KLK10 from human ovarian ascites fluid is associated with two endogenous protease inhibitors and reacts with an activity-based probe [114].

Fine tuning of any protease inhibitor interaction depends on characteristic structural features, including electrostatics, flexibility, and exosites on protein surfaces, which will be investigated

Fig. 1. Primary and tertiary structure of KLKs A: Sequence alignment of kallikrein 1 (KLK1) and the human kallikrein-related peptidases KLKs 2–15 with bovine chymotrypsin (bCTRA). Secondary structure is shown for KLK3/KLK5 for β-strands (arrows) and α-helices (cylinders). Propeptides are included and the numbering is derived from chymotrypsinogen [247]. The "classical" KLKs 1, 2, and 3 share 61–79% identical residues, while the conservation of the "new" KLKs 4–15 ranges from 38% to 57% [130,300]. Highly conserved residues are displayed with a grey background, while residue 16 that is located in the P1, position when the propeptide is cleaved off is shown with blue background. B: Overlay of KLK3 (beige) and KLK5 (green) as ribbon representation in stereo. The catalytic triad residues (His57, Asp102, Ser195), and Ser189 of KLK3 and Asp189 of KLK5, which determine chymotryptic or tryptic specificity, respectively, are depicted as stick models, as well as the N-terminal residue 16 with Asp194 that form a salt bridge, thereby stabilizing the active site [301]. The long 99 loop of the classical KLK3 is depicted in red.

in the following together with the most relevant biological, biochemical, and pharmaceutical aspects of KLK inhibition.

2. Natural endogenous KLK inhibitors

To date, a great variety of endogenous inhibitors with physiological significance in the activity regulation of mammalian kallikrein-related peptidases are known. They range from single metal ions to large protein complexes of more than 700 kDa. In fact, natural exogenous inhibitors are widely used for scientific and pharmaceutical studies (see Sections 3 and 4.). In general, nature employs the following basic principles of protease inhibition: attenuation by reversible binding of inhibitors, "KO" inhibition by irreversible binding to the inhibitory molecule, which often involves the formation of covalent bonds, and compartmentalization. The latter principle may be achieved by restricted tissue or organelle localization of the enzyme or inhibitor; by homo-oligomerization of the protease itself, which reduces the accessibility to the active site; or by compartment formation of large inhibitors that internalize proteases, for which some rare, but biologically significant examples exist.

2.1. Inhibition of KLKs by metal ions

The activity of several serine proteases is regulated by endogenous cations. Interestingly, alkali and earth alkali ions rather stimulate protease activity at distinct binding sites, as seen for the prominent examples of Na^+ with thrombin [115–117] or Ca^{2+} with trypsin [118,119]. Also, for KLKs 1, 3, 4, 6 and 8 activity stimulation by Ca^{2+}, Mg^{2+}, or Na^+ and K^+ ions has been observed [107,120–123]. Zn^{2+} is a metal ion with manifold functions in living organisms and is present in about 300 human enzymes [124,125]. It may play a dual role in activity regulation, as it can stimulate serine proteases, e.g. factor XII [126], and inhibit other ones, such as factor VIIa or uPA [127,128]. To date, Zn^{2+} inhibition as a significant regulatory mechanism for KLKs can only be excluded for the intensively studied KLK1 and KLK6, while KLKs 9–11, 13, and 15 still require further inhibition studies. Strikingly, for KLKs 2, 3, 4, 5, 7, 8, 12 and 14, inhibition by Zn^{2+} in the low μM range has been repeatedly reported (Table 2). Thus, Zn^{2+} should be considered as "attenuator" of KLK activity, which binds in a reversible manner to the targets for fine tuning of their proteolytic action.

2.1.1. Inhibition of prostatic KLKs 2, 3, and 4 by Zn^{2+}

Human kallikrein-related peptidase 2, also known as glandular kallikrein 2 (KLK2), is a tryptic protease. While recombinant KLK2 was inhibited by various serpins in the picomolar range, Zn^{2+} attenuated its activity against fluorogenic substrates, e.g. Pro-Phe-Arg-AMC, with a K_i of 3 μM [129]. Lovgren and coworkers describe the inhibition mechanism as a mixture of competitive and non-competitive inhibition, hinting to the binding of more than one Zn^{2+} per KLK2 molecule and interference with the substrate recognition region of KLK2. Based on the high sequence homology to KLK3 (79% identical residues [130]) and in particular the presence of the conserved His91, His101, and His233, one can assume that the Zn^{2+} inhibition mechanism of KLK2 may be, at least to some extent, similar to KLK3, which is described in the following.

Zn^{2+} inhibition of KLK3/PSA, which is mostly activated by KLK2 in seminal fluid, is clearly an important physiological regulatory

Table 2
KLK affinity matrix for the most relevant endogenous inhibitors. Data for KLKs 9, 10, and 15 were not available. The rating depended on given inhibition constants K_i or IC_{50} values that are roughly the doubled K_i (○ no inhibition, ● μM, ●● nM, ●●● pM range), or association constants that were also represented ($K_{ass} \geq 10^5\ M^{-1}\ s^{-1}$ ●●●, $\leq 10^3\ M^{-1}\ s^{-1}$ ●●), otherwise it was chosen according to the description of the respective publication ("strong inhibition" ●●●, etc., ● slow inhibition). * IC_{50} ** the protease remains active against small molecule substrates, while protein substrates cannot be cleaved anymore.

KLK	Zn^{2+}	α_1-AT	α_1-ACT	ATIII	α_2-AP	PCI	Kallistatin	Other serpins	LEKTI-1	a2M**
KLK1	○	● [171]	○ [196]	○ [196]			●● [308]	●●● [196]		●●● [229]
KLK2	● (3 μM)* [129]	○ [172, 309]	● [205], [172]	●●● (+heparin) [187, 309]	●●● [172, 309]	●●● < 25 pM [129]		●●● PI-6 [204] ●●● PAI-1 [205]		●●● [230] [231]
KLK3	● (24 μM) [134]	●● [175, 176]	●●● [232], [176]	● [187]		●● [192]		●● MNEI [203]		●●● [232]
KLK4	● (15 μM)* [137]	●●● [178]	○ [178]	● [178]	●● [178]			● PAI-1 [155]		● [178]
KLK5	● (4 μM) [15, 144]	○/● [173], [170]	○ [173]	○ [173], [170]	●●● [173], [170]	●●● [170]	○ [170]	●● C1I [170]	●● [218, 219]	● [173]
KLK6	○	● [174]	● [182]	●● [174]	● [174]				●● [63]	○ [174]
KLK7	● (10 μM) [147]	●●● [170]	●●● [170]	○ [170]	●● [170]	●●● [170]	●●● [170]		●● [218, 219]	
KLK8	● (3.3 μM) [123]	○ [170]	○ [170]	● [170]	●● [170]	●●● [170]	○ [170]	●●● PI-6 [206]		
KLK11		○ [307]	○ [307]	○ [307]	○ [307]	● [170]	○ [170]			
KLK12	● (10 μM)* [17]	●/●● [17], [170]	○ [170]	● [17], [170]	●●● [17]	●● [17]	○ [170]	●● C1I [17]		○ [17]
KLK13		○ [170]	○ [170]	● [170]	●● [170]	●●● [170]	● [170]	●● PAI-1 [170]	●● [63]	● [14]
KLK14	● (2 μM) [15, 31]	●● (162 nM) [63], [170]	● (5.6 μm) [63]	●● (198 nM) [63]	●● (130 nM) [63], [170]	●●● [170]	●● [170]	● PAI-1 [63]	●● [63]	

process, especially because it is here that the highest Zn^{2+} concentration in the human body is found, reaching up to 9 mM [131]. A first study on the activity of natural KLK3 against a chromogenic substrate, MeO-Suc-Arg-Pro-Tyr-pNA, demonstrated Zn^{2+} inhibition with an IC_{50} of 20 μM (K_i 6 μM), whereby a competitive inhibition type was found, involving the binding of at least two Zn^{2+} [132]. Whereas one group using recombinant KLK3 reported a K_{iapp} of 45 μM for Zn^{2+} with non-competitive inhibition, another group found for natural KLK3 an IC_{50} of 24 μM with competitive inhibition [133,134]. From a modelled homology structure of human KLK3, a Zn^{2+} binding site was proposed at His91, His101, and His233, and as a potential fourth ligand an Asp (95, 97, or 98). It was assumed that a Zn^{2+} bound His101 could sufficiently distort the catalytic triad via a shift of Asp102, resulting in decreased proteolytic activity [135]. Interestingly, in a horse kallikrein 3 (eKLK3) crystal structure an equivalent metal site was occupied by Zn^{2+}: the coordinating atoms were Asp91 Oδ2, His101 Nδ1, and His234 Nε2 (Fig. 2A) [136]. In human KLK3, that was crystallized with an antibody required for stabilization of the extended 99 loop but not containing Zn^{2+}, the region around the 75-loop exhibited promising ligands for Zn^{2+}: His25, His70, His75, Glu21, Glu77 and Asp78 [134]. How Zn^{2+} binding to these ligands could influence the protease activity will be elucidated by the example of KLK4.

Recombinant KLK4 tested with the chromogenic substrate Z-Phe-Val-Arg-pNA was inhibited by Zn^{2+} with an IC_{50} value of 16 μM, although a residual activity of about 25% was still observed at 1 mM Zn^{2+} concentration, hinting to a non-competitive or mixed inhibition type [137]. Intriguingly, in enamel which can only form properly by KLK4 activity, the zinc content is equivalent to 15 μM, which would correspond to the observed *in vitro* inhibition range [138]. As seen in the crystal structure, KLK4 exhibits a unique metal binding site in the 75 loop with a short helical segment from Glu74-Gln76, which allows the Glu77 Oε to adopt an optimal conformation for Zn^{2+} binding together with the His25 Nε2 (Fig. 2B) [137]. In contrast, the 75 loop of trypsin binds an activity enhancing Ca^{2+} through the Glu70 and Glu80 carboxylate groups [118], which are replaced by Leu70 and Ser80 in KLK4. Three external ligands from a second KLK4 molecule (Glu74 Oε, Asp75 Oδ, and Gln76 Oε) are additionally coordinating the Zn^{2+} [137]. Although these ligands could constitute a secondary Zn^{2+} inhibition site, the single mutations His25Ala or Glu77Ala abolish the Zn^{2+} inhibition of KLK4 completely. Thus, binding of Zn^{2+} at the 75 loop has a long-range inhibitory effect on the active site that is probably transmitted through His25 to the N-terminus, causing disruption of the salt bridge between Ile16 and Asp194, which is required for a functional active site conformation (Fig. 2B). This mechanism was confirmed by a strong increase in the accessibility of the Ile16 α-amino of KLK4 for an acetylating agent in the presence of 70 μM Zn^{2+} [137]. The same Zn^{2+}-dependent phenomenon was previously observed for the low activity form of uncomplexed coagulation factor VIIa (FVIIa) [139]. Interestingly, a Zn^{2+} mediated cross-talk between the 75 loop and the active site has been proposed for FVIIa as an explanation for the attenuatory effect on its activity, in particular because its S1 subsite displays an intrinsic tendency to disorder [127,140].

The physiological significance of Zn^{2+} inhibition of KLKs 2, 3, and 4 is most likely the finely balanced regulation of activation and protease activity [81,130,141]. Prostatic fluid contains Zn^{2+} concentrations up to 9 mM, which keeps the prostatic KLKs in an inactive state [131]. Upon ejaculation, the prostatic fluid mixes with epididymal fluid containing the spermatozoa and with seminal vesicles, which are rich in the structural proteins semenogelin I and II (SgI and II) being responsible for initial sperm immobilization in the seminal coagulum [142]. However, as both SgI and SgII harbor at least ten binding sites for Zn^{2+}, most of the seminal plasma Zn^{2+} will eventually be chelated leading to the activation the prostatic KLKs. Especially KLK3/PSA, as the major SgI and II degrading enzyme, will then rapidly process these proteins which results in semen liquefaction and allows the initiation of sperm movement [81,130,141,142].

2.1.2. Inhibition of epidermal KLKs 5, 7 and 14 by Zn^{2+}

It has been reported that the tryptic KLK5 activity against tripeptidyl-AMC substrates is inhibited around 12 nM Zn^{2+} with 25% residual activity [143], whereas comparable studies found an IC_{50} value of 4 μM and apparent inhibition constants K_i = 8 μM at pH 7.0 and K_i = 2.0 μM at pH 8.0 [15,144]. Considering the extracellular Zn^{2+} concentration of roughly 15 μM in skin, a low nanomolar inhibition constant would render its target protease virtually inactive. In contrast, a micromolar inhibitor or attenuator would allow the modulation of proteolytic activity in addition to stronger polypeptidic KLK inhibitors that are also present in the outer layers of the skin (see Section 2.2.2). The studies by Brattsand et al. and Debela et al. confirmed the reversibility of the Zn^{2+} inhibition by addition of either EDTA or Zn^{2+}-binding substrates, respectively. A comparison of a Zn^{2+}-free KLK5 crystal structure (Fig. 2C, left panel) with the Zn^{2+}-bound KLK5 and rat kallikrein 2 (rKLK2, tonin) structure can explain the inhibition mechanism to a large extent (Fig. 2C) [144,145]. In KLK5, Zn^{2+} is coordinated by the His96 Nδ1 and the His99 Nε2 and two water molecules. The His96 side chain has to rotate by more than 90° from the Zn^{2+}-free conformation to its position in the Zn^{2+} coordination sphere, while the rotation of His99 is less than 20°. In the KLK5 Zn^{2+} structure, the presence of the inhibitor leupeptin (not shown in Fig. 2C, middle panel) bound to the catalytic Ser195 and occupying the S2 pocket with a Leu side chain prevented most likely the formation of a coordination sphere for Zn^{2+} with a third ligand, namely His57. This conformation, representing the fully inhibited KLK, can be seen in the rKLK2 (tonin) structure, which exhibits the Zn^{2+} ligands His99, His97 (similarly positioned as the His96 of KLK5), and most significantly, His57, which is relocated from its position in the catalytic triad (Fig. 2C, right panel). The consequence of a disrupted catalytic triad must be inactivation of the enzyme. However, substrate binding should be possible in this inhibited state, due to the largely free S2 subsite, which only has to be unoccupied for the His57 side chain rotation. Also, the observed non-competitive Zn^{2+} inhibition of KLK5 is in good agreement with this mechanistic model, since Zn^{2+} does not compete with substrates for active site binding [144]. Interestingly, the substrate H-Gly-His-Arg-AMC yielded a K_i value of about 20 μM for Zn^{2+}, with a residual activity of 25% even at 100 μM [144] which can be explained by a replacement of His57 in the Zn^{2+} coordination sphere with the P2-His Nε2 or by a "substrate-assisted catalysis" mechanism. In the first case, the His57 of KLK5 would be pushed back into the catalytic triad, in the second case, the P2-His substrate would transiently reconstitute a functional catalytic triad and catalyze its own cleavage, as in the classic example of a subtilisin His64Ala mutant [146]. In any case, the employed Phe or Pro-P2 side chains of other fluorogenic or chromogenic substrates could not play the mechanistic role of His which has the capacity either to coordinate Zn^{2+} or to activate the Ser Oγ nucleophile.

Also, the chymotryptic counterpart of KLK5, KLK7, is inhibited by Zn^{2+} ($K_{i(app)}$ = 10 μM) when measured with tetrapeptidyl-AMC substrates, and reveals an even stronger inhibition with Cu^{2+} ($K_{i(app)}$ = 0.6 μM) [147]. Crystal structures of KLK7 without bound ions (Fig. 2D, left panel) and in complex with Cu^{2+} revealed direct metal binding by the His99 Nε2 and by the Thr96 carbonyl O via hydrogen bonds to a water molecule (Fig. 2D, middle panel). Since the mutation His99Ala abolished Zn^{2+} inhibition of KLK7 activity completely, this metal binding site must be the structural basis of the attenuating or inhibitory effect of Zn^{2+} and Cu^{2+} [147]. Similar to the coordination of Zn^{2+} in rKLK2 the catalytic His57 could constitute a more stable coordination sphere for the cation [145]. A striking parallel to the situation in KLK7 is found in a Cu^{2+} complex structure of the Arg96His mutant of rat trypsin which can also be inhibited by Cu^{2+} (21 μM) and

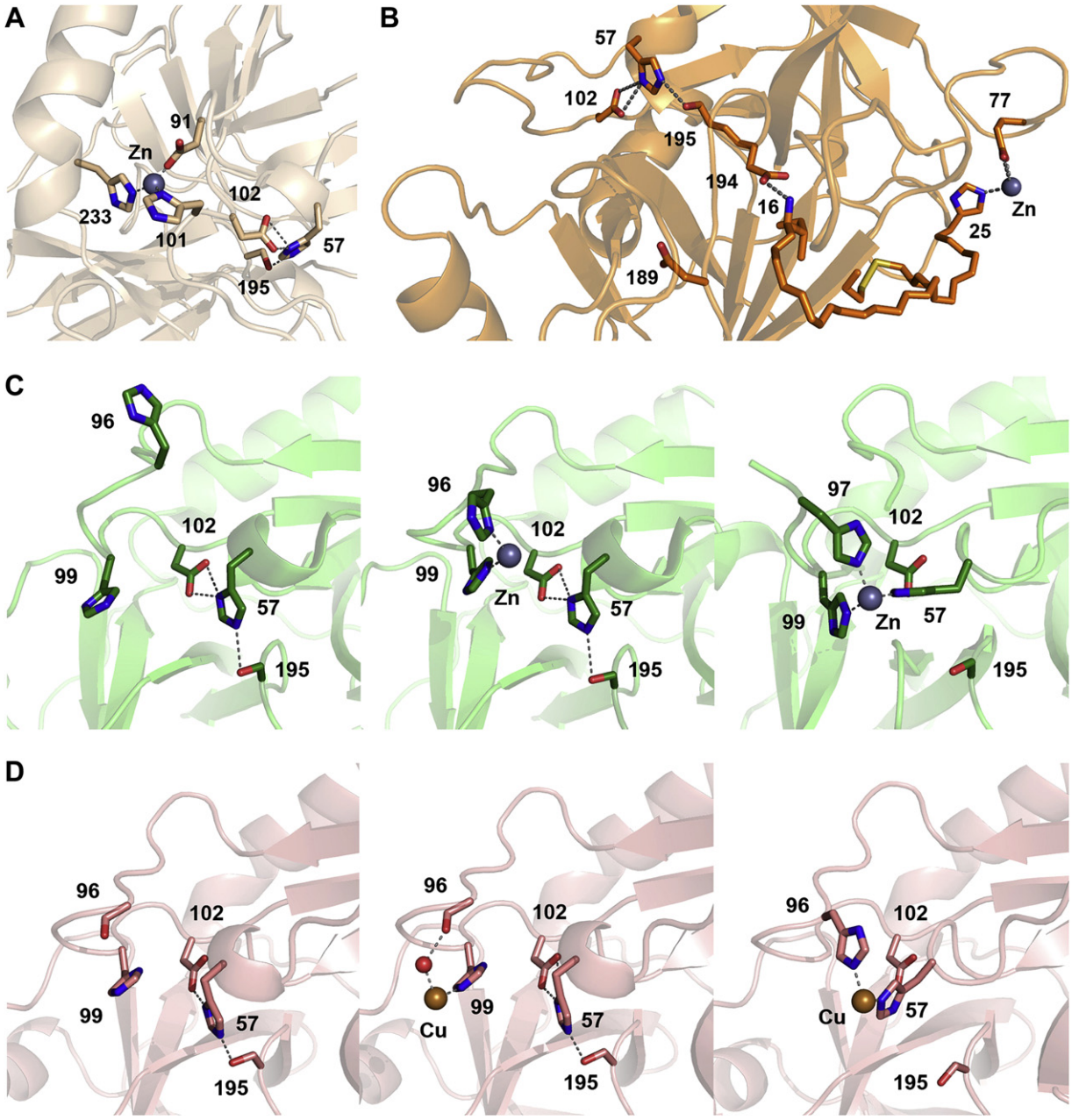

Fig. 2. Inhibitory metal binding sites in KLK crystal structures as ribbon representation with relevant residues as stick models. Dotted lines indicate hydrogen and coordination bonds, relevant residues are labelled with numbers. A: The Zn^{2+} ion (grey sphere) bound to horse KLK3 involves ligands Asp91, His101, and His234. The equivalent residues in human KLK3 are His91, His101, and His233. Probably, the Zn^{2+} binding to His101 causes a backbone shift of Asp102, concomitantly distorting the catalytic triad (Ser195, His57, and Asp102) and resulting in a reduced proteolytic activity. B: In KLK4, the inhibitory Zn^{2+} site is located far away from the active site, which was confirmed by the single mutations His25Ala and Glu77Ala. Zn^{2+} binding to KLK4 effects a disruption of the salt bridge Ile16-Asp194 via His25, thereby destabilizing the active site conformation and abolishing its functionality. C: Zn^{2+} binding to the active site of KLK5 and rKLK2 in standard orientation. On the left, the free active site of KLK5 is shown with both His96 and His99 rotated away from the S2 pocket. The catalytic triad, consisting of Ser195, His57, and Asp102 is connected by hydrogen bonds. In the middle, a Zn^{2+} ion (grey sphere) has bound to the ligands His96 and His99 with coordination bonds. On the right, the catalytic His57 of rKLK2 has relocated from the catalytic triad to become the third Zn^{2+} ligand, together with His99 and His97. Most likely, a similar conformation in KLK5 represents the reversible Zn^{2+} inhibited state. Since the S2 subsite is still accessible for most P2 side chains, the inhibited state allows binding, but not turnover of substrates, as long as the catalytic triad is disrupted. This mechanistic model is in agreement with the observed non-competitive Zn^{2+} inhibition of KLK5. D: Cu^{2+} binding to the active sites of KLK7 and the rat trypsin mutant Arg96His in standard orientation. On the left, the free active site of KLK7 is depicted with the catalytic triad connected by hydrogen bonds (dotted lines). In the middle, a Cu^{2+} ion (orange sphere) has bound to the ligands His99 and Thr96-C=O via a water molecule (red sphere) with coordination bonds (dotted lines). On the right, the catalytic His57 of rat trypsin mutant Arg96His has rotated out from the catalytic triad to become the second Cu^{2+} ligand, together with the mutated His96. A similar conformation in KLK7 with His99 instead of His96 may represent the reversible Zn^{2+}/Cu^{2+} inhibited state. As in KLK5 the S2 subsite is still accessible for P2 side chains, allowing binding, but not cleavage of substrates, as long as the catalytic triad is inactive, which agrees with the non-competitive Zn^{2+} inhibition of KLK7.

Zn^{2+} (128 μM) [148]. In this structure, the metal ion is liganded by His96 and His57 that has rotated out of the catalytic triad, which explains the inactivation of the enzyme [149]. Most likely, the disrupted catalytic triad with His57 bound to Zn^{2+} (or Cu^{2+}) represents the equivalent inhibited state for KLK7 (Fig. 2D, right panel). Again, the non-competitive Zn^{2+} inhibition of KLK7 agrees well with this structure based mechanistic model, in which substrate binding is still possible, as long as P2 side chains are not too large [147].

Recently, the group of Diamandis reported that a Zn^{2+} concentration in the low nanomolar range is sufficient for inhibition of KLK14 (IC_{50} = 12 nM) [31], similar to their findings on Zn^{2+} inhibition of KLK5 (IC_{50} < 12 nM) [143]. However, these values seem to be problematic in that both proteases would basically never be active in the tissues or fluids, e.g. the epidermis, where they are predominantly expressed. Moreover, an earlier study measured an IC_{50} of 2 μM for KLK14 [15].

Zn^{2+} modulated activity of epidermal KLKs may be highly relevant under physiological conditions. Because Zn^{2+} levels in mammalian epidermis are in the higher micromolar range (50–70 μg/g dry weight) with the Zn^{2+}/Ca^{2+} ratio playing a role in skin maturation [150,151], this metal ion could be important in the activity regulation of the KLKs 5, 7 and 14, in addition to their natural polypeptide inhibitors, the lympho-epithelial Kazal-type inhibitor (LEKTI) (see Section 2.2.2.). Notably, topical ZnO as contained in the widely used zinc ointments for skin improves wound healing and re-epithelialization [151], which may be based on reduction of an otherwise excessive KLK activity. Although copper regulation of proteases is not established, one should consider this alternative to Zn^{2+}, in particular for tissues that contain relatively high Cu^{2+} ion levels, such as the brain (0.5 μM), where KLK7 is also expressed [130,152].

2.1.3. Inhibition of KLKs 8, 10, and 12 by Zn^{2+}

The tissue distribution of the KLKs 8, 10, and 12 is more diverse than the aforementioned prostatic and epidermal KLK groups. Nevertheless, it has been demonstrated that KLKs 8 and 12 are inhibited by Zn^{2+} as well. For example KLK8, which is thought to have an important function in the hippocampus of the mammalian brain, is stimulated by 10 μM Ca^{2+}, but inhibited by 3 μM Zn^{2+} [123]. According to the sequence alignment (Fig. 1) it possesses a His99, which could coordinate the inhibiting metal ion together with His57 and a backbone carbonyl, as in KLK7. In the case of KLK10 little is known about activation, natural substrates and regulation, except for the non-prime specificity and its relatively slow cleavage of some pro-KLK peptides [20,99]. Since KLK10 possesses a Zn^{2+} binding site involving the catalytic triad with His57 and Asp102, as well as Asp99 in the S2 pocket, it is likely that bound Zn^{2+} would interfere with substrate binding and catalysis [107]. Similarly, knowledge of the physiological functions of KLK12 is scarce, although a systematic study on its inhibitors showed that its activity against fluorogenic substrates is attenuated by Zn^{2+} with an IC_{50} of 10 μM [17].

2.2. Proteinaceous endogenous KLK inhibitors

To date, the most important proteinaceous endogenous KLK inhibitors belong to the serpins (MEROPS class I.04), Kazal-type inhibitors (I.01) and the macroglobulins (I.39) [1]. Only two endogenous Kunitz-type inhibitors (I.02) acting on KLKs are known. The first one, bikunin, binds KLK1 in the picomolar range, which may also play an important role in lung function and diseases [153,154], while bikunin is also associated with KLKs 6 and 10 in ovarian cancer ascites fluid [114]. The second one is an inhibitor of hepatocyte growth factor activator (HGFA), which is involved in tumor progression and is itself activated by KLKs 4 and 5, which in turn are inhibited by the domains of HGFA inhibitory type 1 (HAI-1) [155]. One member of the whey acidic protein (WAP) type inhibitor family (I.17), the secretory leukocyte peptidase inhibitor (SLPI, also anti-leukoprotease, ALP) was shown to inhibit KLK7, which appears to be significant in protease regulation during desquamation [156].

2.2.1. Serpins – KLK inhibition in blood and prostate

Serpins (derived from *SER*ine *P*rotease *IN*hibitor) are proteins of 33–46 kDa that account for about 10% of human blood plasma [157–159]. They occur in all kingdoms of life, inhibit (chymo)trypsin-like and subtilisin-like serine proteases, as well as cysteine proteases, and play physiological roles even beyond protease inhibition [160–162]. In contrast to the substrate-like binding of canonical protease inhibitors, serpins act via a unique “springe suicide” mechanism on their target protease. After cleaving the P1–P1′-bond in the

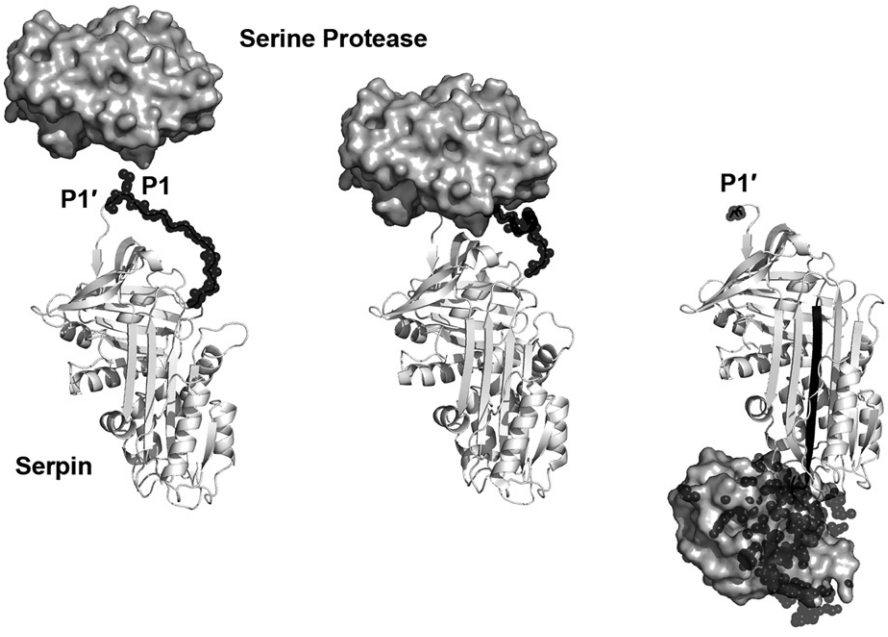

Fig. 3. Reaction of a serine protease shown with the molecular surface with a serpin as ribbon representation. Left: Protease and unreacted “virgin” serpin with central four-stranded β-sheet (PDB codes of trypsin from 1OPH and α_1-AT from 1QLP) [166,302]. The reactive loop is depicted as black spheres, the P1 and P1′ side chain are labelled. Middle: Michaelis–Menten complex of the protease with serpin corresponding to substrate-like canonical protease inhibitor interaction (PDB code 1OPH). Right: Covalent serpin–protease complex that exhibits now a cleaved reactive center loop with an N-terminal P1′ residue and an inserted fifth strand (black) in the central β-sheet that is covalently linked with the P1 residues to the catalytic Ser195. The activation domain of the protease (black spheres) including a large region of the active site is disordered as seen in several protease zymogen structures (trypsin and α_1-AT, PDB code 1EZX) [165].

reactive center loop by formation of a covalent bond from the P1 residue to the catalytic Ser195, it is then rapidly translocated by about 70 Å to the opposite part of the inhibitor [163,164]. Eventually, this reaction causes a massive structural disorder in the protease (up to 40%), mainly because all stabilizing interactions that were formed during enzyme activation are lost now, thus preparing the protease itself for final degradation by other proteases (Fig. 3) [165]. A wealth of mechanistic and structural studies, including several complex structures, have revealed fine details of various steps in serpin protease interaction (Fig. 4) [166–169]. Although no serpin-KLK complex structure is known to date and the physiological connection of KLK serpin interaction is often unclear, serpins are the best studied KLK inhibitors [12,25].

At first glance it seems surprising, that the serpin α_1-antitrypsin (AAT) fails to inhibit the definite tryptic KLKs 1, 2, 5, 6, 8, 11, and 13 *in vitro*, whereas strong inhibition is observed for the chymotryptic KLK7 with an association constant k_{ass} of 3.9×10^6 M^{-1} s^{-1} (Table 2) [170–175]. However, this inhibition pattern can be explained by the substrate-like reactive loop of AAT with the scissile bond between P1 Met and P1′ Ser that should be preferred by chymotryptic proteases, albeit with a low reaction rate of KLK3 [175]. The KLK3-AAT complex is found increased in benign forms of prostate hyperplasia from about 2% to 4% of total KLK3 in serum [176,177]. Recently, immunological analyses of ovarian cancer ascites fluid indicated that fractions of KLKs 5, 6, 8, and 10 form complexes with ATT [114]. Also, AAT strongly inhibits the tryptic KLKs 4 and 14 and to a lesser degree KLK12 [17,31,170,178].

Contrary to AAT, α_1-chymotrypsin (ACT) has been detected in complex with up to 85% of total KLK3 in blood plasma by immunoassays, whereby an increased ratio of free to ACT-complexed KLK3 is correlated with progression of prostate cancer [176,179]. Despite a significant concentration of ACT in seminal plasma, it is not bound to KLK3, which was explained by an interference of Zn^{2+} with complex formation [180]. The association constants k_{ass} for ACT with the chymotryptic KLK3, which seems to obey a slow inhibition kinetic, and KLK7 were 3.1×10^3 and 3.9×10^6 M^{-1} s^{-1}, respectively [133,170]. In line with the presence of a P1 Leu-P1′ Ser scissile bond, ACT does not or only moderately inhibit the tryptic KLKs 1, 2, 4, 5, 8, and 11–14 *in vitro* (Table 2) [31,170,172,173,178,181,182]. Interestingly, analyses of human milk and ascites showed that about 5% of the tryptic KLK6 are bound to ACT in an ACT-KLK6 complex, which remains stable after HPLC purification [182].

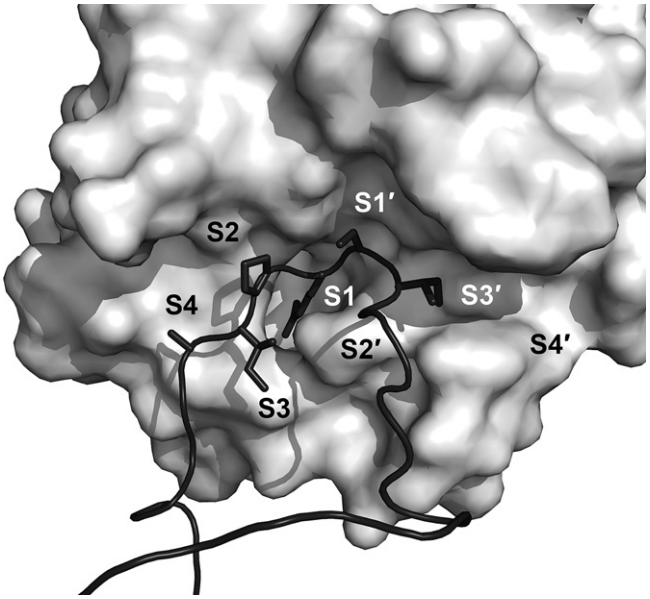

Fig. 4. Serpin reactive center loop (RCL) inserted into the active site of a serine protease represented as molecular surface (trypsin and α_1-AT, PDB code 1OPH). Canonical (substrate-like) binding before cleavage of the scissile bond between P1–P1′ is mediated by residues P4 to P1′, shown as black stick models. Various serpins exhibit canonical binding extended to P3′ and may overall interact with residues from P6 to P5′ [184].

Antithrombin III (AT) is the major inhibitor of fibrin clot generating thrombin and other coagulation factors in blood plasma, which is further enhanced by a ternary complex with heparin that links positively charged exosites of AT and its target protease for optimal binding [183,184]. The relatively slow AT inhibition seen for thrombin ($k_{ass} = 10^4$ M^{-1} s^{-1}) is accelerated up to 1000-fold by heparin [185,186]. A comparable effect of heparin has been observed for the inhibition of KLK2 by AT, which shows elevated expression and colocalization with KLK2 in tumor cells [187]. In accordance with a reactive center containing a P1 Arg-P1′ Ser, the chymotryptic KLK3 and KLK7 are not inhibited by AT [170,187]. Among the tryptic KLKs, only KLK6 and KLK14 are moderately inhibited by AT, although no heparin was added [31,174].

α_2-Antiplasmin (AP), the major inhibitor of the fibrinolytic plasmin, is unique in that it binds to the zymogen and the mature protease [188,189]. Whether AP is significant for the regulation of KLKs is an unsolved question. In agreement with the reactive bond P1-Arg-P1′-Met that seems to be designed for tryptic proteases, AP strongly inhibits KLKs 2, 4, 5, and 12 ($k_{ass} = 2.2 \times 10^5$ M^{-1} s^{-1}) and moderately inhibits KLKs 7, 8, and 13 [17,31,170,172].

The relatively unspecific heparin-dependent proteinase C inhibitor (PCI) inhibits various coagulation factors, and can probably switch between the P2 Phe-P1 Arg-P1′ Ser residues in the reactive center, so that either Phe or Arg inserts into the S1 pocket of the protease [190,191]. PCI is a fast binding inhibitor of KLKs 1, 2, 5, 7, 8, 13, 14, and a slow binding inhibitor for KLKs 3, and 12 [17,170,192]. Besides a fourfold increased association constant for PCI with KLK2 in the presence of heparin ($k_{ass} = 8.7 \times 10^5$ M^{-1} s^{-1}), an inhibition constant below 25 pM has been described [129]. Since PCI is present at high molar excess in seminal plasma, roughly 30% of total KLK2 and to a lower extent KLK3 are complexed with PCI, which are both implicated in the fertilization process [193–195].

Seemingly, one of the serpins, kallistatin, displays high specificity for KLKs, being a strong inhibitor of KLK1 and KLK7, while KLK14 is slowly inhibited [170,196]. In spite of the apparent dual preference for tryptic and chymotryptic KLKs, it cannot inhibit KLKs 5 and 11–13 [170]. The expression of kallistatin in pancreas and kidney matches that of KLK1 [197]. Interestingly, the specificity of kallistatin for KLK1 is derived from the reactive bond P1 Phe-P1′ Ser, since an exchange to P1-Arg results in a twofold higher inhibition of KLK, but also in similar inhibition of various other serine proteases that are not inhibited by wild type kallistatin [198,199]. Most likely, kallistatin regulates the KLK1 activity, influencing blood pressure and angiogenesis, which may also depend on the heparin binding capacity of kallistatin [200–202].

Inhibition of KLKs 5, 7, 8, 12–14 by the C1 inhibitor was only moderate *in vitro*, and may also not be significant *in vivo*, as well as the KLK3 inhibition by the monocyte/neutrophil elastase inhibitor (MNEI), which can switch at the reactive center P2 Phe-P1 Cys-P1′ Met to Phe as P1 residue for chymotrypsin-like proteases [170,203]. The complex of KLK2 with serpin PI-6 that is associated with tissue damage, necrosis or neoplasia and the complex with PAI-1 that seems to promote cancer by inactivation of the primary uPA inhibitor [204,205] are likely to be of physiological relevance in the prostate. However, serpins may interact with KLKs in other tissues, as exemplified by the human KLK8 (neuropsin) colocalization in keratinocytes with its strong inhibitor PI-6 [206] and by the inhibition of murine KLK8 by the tight binding serine proteinase inhibitor 3, which colocalize in mouse brain [207].

2.2.2. LEKTI-1 and 2 – multi-domain Kazal-type KLK inhibitors in skin

Soon after the discovery of the 15 domain, 120 kDa Kazal-type inhibitor, which was called lympho-epithelial Kazal-type inhibitor (LEKTI), it was found that mutations in its gene (*SPINK5*) cause Netherton syndrome. The syndrome is characterized by a severe autosomal recessive genodermatosis with altered desquamation, impaired keratinization, hair malformation and a skin barrier defect [208,209]. *SPINK5* knockout mice feature a Netherton syndrome-like condition, which is based on the hyperactivity of KLKs 5 and 7 that are not sufficiently controlled by their natural inhibitor, LEKTI-1 [210]. Human mutations in *SPINK5* generate LEKTI-variants that have lost different C-terminal domains, such as d1-4 or d1-8, which is accompanied by higher concentrations and activity levels of most epidermal KLKs [211]. Mutations in the *SPINK5* gene are also responsible for the widespread atopic dermatitis, which enhances the susceptibility of patients to food allergies and asthma [212–214]. In addition to the elevated degradation of corneodesmosomes in the stratum corneum, KLK5 causes atopic dermatitis-like lesions via PAR2-mediated thymic stromal lymphopoietin expression [215]. An NMR analysis of LEKTI-1 domains 1 and 6 demonstrated that their structures differ from the classical Kazal-type architecture, especially in the reactive center loops. This may be due to the lack of a third disulfide bond, which is present in the classical canonical serine protease inhibitor scheme. Only two of the 15 LEKTI-1 domains contain three disulfide bonds [216]. Nevertheless, several domains of LEKTI-1 are potent serine protease inhibitors and function according to the substrate-like canonical binding. Canonical Kazal-type inhibitors present the scissile bond between P1–P1′ to the protease, however, its cleavage is very slow compared with substrate turnover, as the hydrolysis constant K_{hydr} is in the range of unity ($K_{hydr} = [\text{Inhibitor}_{\text{cleaved}}]/[\text{Inhibitor}_{\text{uncleaved}}] = 1$) and the cleaved bond may be reconstituted again (Fig. 5) [89,217]. Full length LEKTI-1 contains mostly potential P1 Arg residues, but also a P1 Gln-P1′ Asp in the reactive center loop of the first domain that might target chymotryptic proteases, in particular KLK7. This suggestion is supported by a study on natural truncated versions of LEKTI-1 that usually comprise the first domain and did not fail to inhibit the chymotryptic activity in the outer layers of skin [211]. Further investigations revealed that domain 6 alone can inhibit both KLK5 and KLK7 [218]. Recombinant LEKTI-1 fragments of domains 6–8 and 9–12 inhibited KLK5 with K_i values between 1.2 and 5.5 nM, while fragment d6-9′ (a truncated domain 9) inhibited KLK7 with a K_i of 11 nM [219]. Additionally, LEKTI-1 d1-6, d6-9′, and d9-12 inhibited KLK5, KLK6, KLK13, and KLK14 with K_i values in the range of 2–416 nM, while only KLK5 was inhibited by d12-d15 ($K_i = 22$ nM) [63].

In cultured keratinocytes and in the epidermis it was demonstrated that the LEKTI-1 precursor is secreted as several fragments, of which d5, d6, d8-11, and d9-15 specifically inhibit KLK 5, 7, and 14, whereby the strongest inhibition was seen for KLK5 with d8-11, although a shift to acidic pH dissociated the otherwise nearly irreversible, tight binding complex [32].

Recently, a polypeptide of 85 amino acids, expressed in skin and encoded by the *SPINK9* gene with high homology to LEKTI-1 was discovered and termed LEKTI-2 [220]. This polypeptide was found colocalized with KLK5 in the epidermis, which possesses the unusual reactive bond P1-His-P1-Met and inhibits recombinant KLK5 ($K_i = 65$ nM), KLK8, but not KLK7 or 14 [221].

2.2.3. Macroglobulins – large compartments for unspecific protease removal

In a classical paper, Barret and Starkey elaborated a mechanism for α_2-macroglobulin (α_2M) inhibition, a huge protein of 720 kDa that was able to block the proteolytic activity of nearly all proteases. At that time it was also known that an α_2M inhibited protease was still active against small substrates, but no longer cleaved proteins. Thus, it was proposed that α_2M is proteolytically attacked by its target protease in a so-called bait region, which causes a conformational change in α_2M, resulting in an irreversibly trapped protease [222]. This mechanistic description has remained valid and has been supported and elaborated on by structural information from electron microscopy and X-ray crystallography. The structural arrangement of the protease free α_2M comprises two dimers with a large central cavity [223]. Upon protease cleavage in the bait sequence P^{692}QLQQYEMHGPEGLRVGFYESDV-MGRGHARLVHVEEPHT^{730} and subsequent thiol ester cleavage near the central region, two β-strands of α_2M untwist to expose the central cavity to proteases, e.g. to chymotrypsin, of which two molecules were covalently linked inside the central cavity [224,225]. Since the reactive internal β-cysteinyl-γ-glutamyl-ester of α_2M can react with any exposed Lys side chain on a protein surface, any protease can be trapped, and thus α_2M has been termed a "panproteinase inhibitor", which clears the blood from proteases destined for degradation [226,227]. Typically, the protease is not fixed to a large extent and retains a considerable flexibility inside the α_2M cavity after trapping [228].

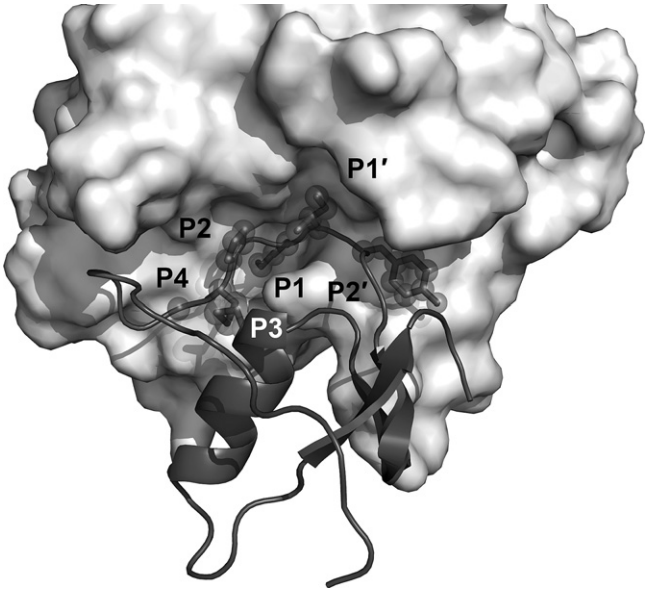

Fig. 5. Kazal-type inhibitor with reactive center loop (RCL) bound to the active site of a serine protease represented as molecular surface (trypsinogen with pancreatic secretory inhibitor, PDB code 1TGS). The 15 LEKTI-1 domains are homologous to the classical Kazal-type inhibitors, but 12 domains may be classified as subtypes. Canonical binding is achieved by residues P4 to P2′, shown as grey stick models with spheres. The bond between P1–P1′ is scissile, but cleavage is extremely slow, since the hydrolysis constant is close to unity and the cleaved bond may reconstituted again [89]. Specificity of LEKTI-1 domains for chymotryptic or tryptic KLK targets depends on the P1 residue, which is in most cases an Arg, but a Gln in the first domain.

The ability of α_2M to effectively internalize and trap KLK1 has been demonstrated *in vitro* and it was even used as a tool to remove it from samples, where the KLK1 activity was unwanted [222,229]. However, α_2M complex formation with KLKs is of physiological significance, too, as observed for KLK2 in serum of prostate cancer patients [230,231]. Also, KLK3 forms readily stable complexes with α_2M in blood serum, as well as with the α_2M homologue pregnancy zone protein (PZP), which exhibits some interesting mechanistic differences to α_2M [232,233]. Interestingly, KLKs 4, 5, 6, 12, and 13 were not significantly inhibited by α_2M [17,101,173,174,178], although in mouse brain KLK8 (neuropsin) was inhibited by the α_2M homologue murinoglobulin 1 and a novel inhibitor, phosphatidyl ethanolamine binding protein (PEBP) [207,234].

2.2.4. Autoinhibition in KLK4 oligomers – an exceptional case?

Compartmentalization within proteinaceous inhibitors as in the macroglobulins is quite rarely realized, whereas proteases provide

more examples of that kind. In the proteasome, bleomycin hydrolase, tricorn protease, or α- and β-tryptases the oligomerization leads to the formation of an internal cavity or blocking of the active sites that are not accessible for most potential protein substrates [235–240]. This compartmentalization represents some kind of self-inhibition, and can be overcome by activators, which induce a conformational change or transfer substrates to the active site as in the 26S proteasome [241].

In this context, recombinant, refolded KLK4 exhibited 700 kDa oligomers in solution, corresponding, to 24-mers, which were hardly active against chromogenic and fluorogenic substrates [137]. On the contrary, KLK4 that was refolded in the presence of 2 mM Ca^{2+} was only monomeric (ca. 25 kDa) and highly active, resulting even in autodegradation within a few hours [107]. Also, these studies revealed that in three known crystal forms KLK4 assembled to oligomers, consisting of stacked cyclic tetramers or octamers, respectively, in which the active sites are partially blocked (Fig. 6). Since binding and turnover of even small tripeptidyl-pNA substrates is sterically not possible, in particular due to a partially blocked S2 subsite, the tetramers and octamers appear to be self-inhibiting protease forms with regulatory potential [137].

3. Exogenous and synthetic inhibitors in KLK research

In biochemical and biophysical studies both exogenous natural inhibitors and synthetic small molecules have been used, mostly to analyze the primary specificity of the respective kallikrein-related peptidase and then for refined studies of extended substrate specificity. This approach can be exemplified by a study on mouse KLK1b27, in which the enzyme was characterized as chymotryptic serine protease, according to its inhibition by diisopropylfluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), and tosyl-phenyl-chloromethyl ketone (TPCK) and chymostatin, in contrast to the weaker inhibition by the tryptic enzyme inhibitors tosyllysyl-chloromethyl ketone (TLCK), leupeptin, antipain, soybean trypsin inhibitor (SBTI), and aprotinin (bovine pancreatic trypsin inhibitor, BPTI) [242]. Such knowledge often helps to improve the purification of a KLK by preventing autodegradation. Although the above mentioned inhibitor molecules are rather unspecific and bind to a great variety of proteases, they may serve as starting point for highly specific compounds, as in the recent example of an activity-based compound that was employed for immunofluorimetric measurements of active KLK6: the compound biotin-$(PEG)_4$-Pro-Lys-diphenylphosphonate formed a covalent bond to the nucleophilic Ser195 Oγ [243].

3.1. Small molecules

3.1.1. Sulfonyfluorides and diisopropylfluorophosphate as catalytic serine inhibitors

The unspecific irreversible, but toxic serine protease and esterase inhibitors PMSF (Pubchem CID 4784, http://pubchem.ncbi.nlm.nih.gov/) or DIFP (Pubchem CID 5936) and their derivatives

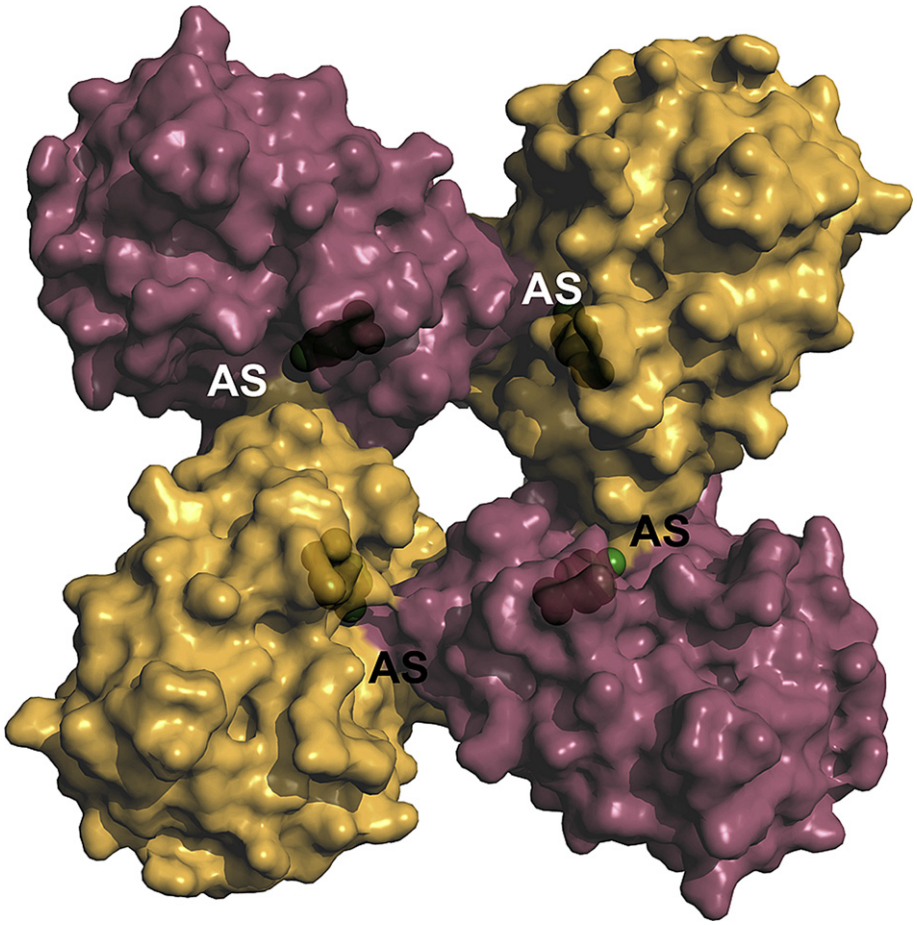

Fig. 6. Potential autoinhibitory homocomplex of KLK4 with monomers depicted as alternating purple and orange surface representations. In solution and in crystals KLK4 forms oligomers, consisting of cyclic tetramers that assemble to octamers, or even higher oligomers, such as dimers and tetramers of octamers. Due to intermolecular contacts, the active site clefts of KLK4 become very narrow at the catalytic centers (labelled AS) with Ser195 shown as green balls. In particular the S1 and S2 subsites are hardly accessible for small chromogenic and fluorogenic substrates, not to mention large protein substrates [137].

Table 3
Exogenous and non-natural inhibitor affinity matrix. The rating (○ no inhibition, etc.) is applied as for Table 2. *TPCK related compounds (see Table 4).

KLK	PMSF/AEBSF/DIPF	BEN/p-ABA	Leupeptin/antipain	Chymostatin	TLCK/PPACK	TPCK	SBTI/LBTI	BPTI
KLK1	● [245]	● [310]			● [245]		○ [245]	● [245]
KLK2		● [129]	● [129]		●● [129]			● [129]
KLK3	● [42, 175]	○ [42]	○ [42]	● [249]	○ [42]	○ [42]	○ [42]	○ [42]
KLK4		● [48, 137]			● [48]		● [48]	●● [48]
KLK5			● [15, 144]	○ [15]			● [15]	● [15]
KLK6		● [174]					●● [174]	
KLK7						● [147]*		
KLK8			● [123]	● [123]	● [123]	○ [123]		
KLK12		● [17]					○ [17]	● [17]
KLK14			● [15]	● [15]			● [15]	● [15]

react with nucleophilic serine and cysteine residues, as does the non-toxic trypsin-specific aminoethyl benzenesulfonyl fluoride (AEBSF, Pubchem CID 1701) [244]. For KLK1, inhibition by PMSF and for KLK3, reaction with PMSF and AEBSF has been reported [42,245]. Due to the high toxicity, the volatile DIFP is not used for purification purposes.

3.1.2. Benzamidines as tryptic S1 inhibitors

Both benzamidine (BEN) and its derivative para-amino-benzamidine (p-ABA) are specific for tryptic proteases, since they fill the S1 pocket and usually form a salt bridge between their positively charged amidino group and the negatively charged Asp189. Due to a lack of additional interaction with the protease, such as binding to the oxyanion pocket or to the S2–S4 subsites, inhibition constants of benzamidine range from high μM to mM. In case of KLK6 63 μM BEN was required for 20% inhibition, while the inhibitor was applied at 20 mM for crystallization and was well defined in the S1 site of the KLK6 structure [174,246]. Definitely, BEN and p-ABA are useful tools in protein purification and crystallization of tryptic KLKs, since they inhibit the proteolytic activity and rigidify the active site, as corroborated by more than 100 corresponding crystal structures (Pubchem CIDs 2332 and 1725, http://pubchem.ncbi.nlm.nih.gov/). Thus, crystal structures of pKLK1, KLKs 4 and 6 in complex with benzamidine or para-aminobenzamidine were solved, respectively, explaining subtle differences of the S1 specificity determinants (Tables 3, 4) (Fig. 7A, B, C) [137,246,247].

3.1.3. The aldehydes leupeptin, antipain, chymostatin interact with the S1 to S4 pockets

Remarkably, higher organisms utilize exclusively ions or polypeptides as protease activity attenuators or inhibitors, whereas microorganisms produce various small molecule inhibitors, which target host proteases [89]. For example, leupeptin (acetyl-Leu-Leu-Arginal) and antipain (carboxyphenylethyl-carbamoyl-Arg-Val-Argininal) are suitable competitive KLK inhibitors that are produced by Actinobacteria, such as *Streptomyces* [248]. The aldehyde function of leupeptin and antipain reacts with Ser and Cys nucleophiles to a transition state-like inhibited protease complex, which is reversible. Their P1 Arg is suited to inhibit tryptic KLKs, e.g. KLK2 around 100 μM, while antipain was a much stronger inhibitor of KLK8 (IC_{50} = 460 nM) than leupeptin (IC_{50} = 66 μM) [123,129]. Also, Leupeptin inhibited KLK5 at 1.7 to 10 μM and cocrystallized with this protease, whereby the N-terminal extension occupied the subsites S2–S4 [15,144] (Fig. 7D). The widely used chymostatin is the counterpart of

Table 4
Structures of KLK peptidases with PDB accession codes: pKLK, mKLK, rKLK, eKLK: porcine, murine, rat, equine kallikrein-related peptidase. Leupeptin = N-acetyl-L-leucyl-L-leucyl-L-argininal, Suc = succinyl, CMK = chloromethyl ketone, BPTI = bovine pancreatic trypsin inhibitor. *Acyl-intermediate, derived from morpholino-carbonyl-KGISSQY-7-amino-4-(trifluoromethyl)-coumarin. ** Structure not deposited. *** Un-published data.

KLK	pro	apo	Inhibitor	Ion	Small molecule	Polypeptide
KLK1		1SPJ [311]	1HIA [263]			hirustasin
pKLK1			2KAI [312]			BPTI
			2PKA [247]		benzamidine	
rKLK2 (tonin)			1TON [145]	Zn^{2+}		
KLK3		2ZCH [134]	2ZCK [134]		Mu-KGISSQY- *	
eKLK		1GVZ [136]		Zn^{2+} [136]**		
KLK4			2BDH [137]	Zn^{2+}	p-aminobenzamidine	
KLK5			2PSX [144]		Ac-LLR- (leupeptin)	
			2PSY [144]	Zn^{2+}	Ac-LLR- (leupeptin)	
KLK6	1GVL [313]		1L2E [246]		benzamidine	
KLK7		3BSQ [314]	2QXG [147]		AAF-CMK	
			2QXH [147]		Suc-AAPF-CMK	
			2QXJ [147]	Cu^{2+}	Suc-AAPF-CMK	
KLK8			*** [315]	Zn^{2+}	Ac-LLR- (leupeptin)	
mKLK8		1NPM [316]				
mKLK13		1AO5 [317]				
KLK b3			1SGF [295]	Zn^{2+}		

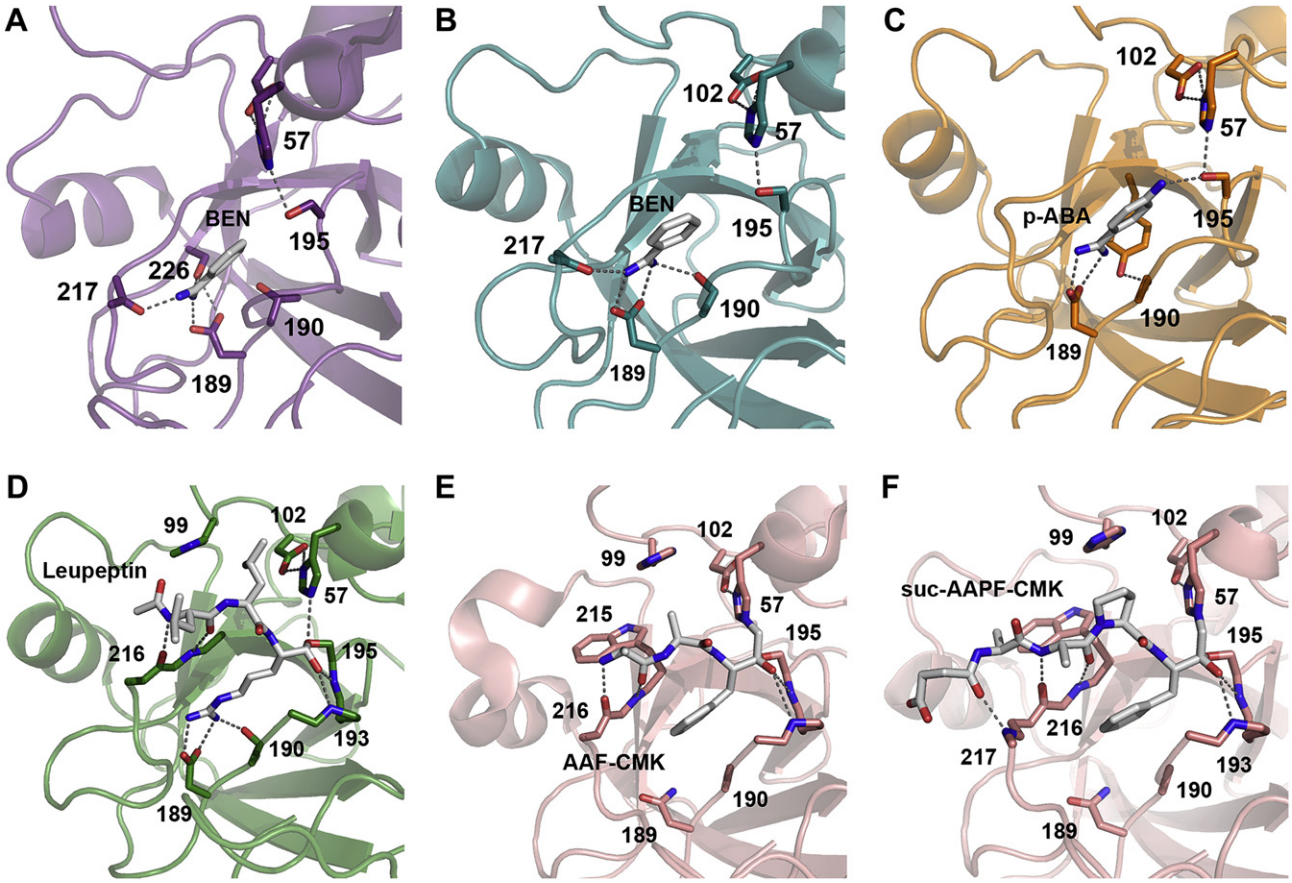

Fig. 7. Inhibitory small molecules bound to KLK active sites with important residues depicted as stick models. Hydrogen bonds with a distance shorter than 3.5 Å are shown as grey dotted lines and relevant residues are labelled with numbers. A: Porcine pKLK1 with benzamidine (BEN) occupying the S1 site. The BEN amidino group binds Asp189 only with one amino group, but involves the Ser226 Oγ and the backbone carbonyl O of His217. B: KLK6 binds the two amidino N atoms of benzamidine in the S1-pocket with both Oδ atoms and one with the Oγ of Ser190, as well as with the backbone carbonyl O of Asn217, similar to trypsin [303]. C: The binding mode of para-aminobenzamidine (PABA) to KLK4 is similar to KLK6, but the Ser190 does not participate in stabilizing the amidino groups of the inhibitor, which is additionally bound at the amino group by Ser195. In addition to Asp189, the Ser190 Oγ is enhancing the specificity of the S1 subsite for P1-Lys residues [304,305]. D: Leupeptin binds with the P1-Arg C covalently to the Ser195 Oγ of KLK5, while the former carbonyl O (now rather a $-O^-$) is accommodated in the oxyanion hole, formed by the backbone NHs of Ser195 and Gly193. The P1 Arg side chain is stabilized by Asp189 and Ser190. For clarity, additional hydrogen bonds of the P2-Leu carbonyl O and P1 Arg Nε via a water to Gln192 have been omitted. The relatively small hydrophobic S2 subsite is filled with the P2-Leu side chain. While the P3-Leu side chain extends to the solvent, its carbonyl O and amide NH tightly bound to the corresponding atoms of Gly216. The acetyl group of leupeptin partially occupies the hydrophobic S4 pocket. E: Ala-Ala-Phe-chloromethyl ketone (AAF-CMK) is covalently linked with the P1 methylene group to His57 Nε and via the C atom to the Ser195 Oγ of KLK7, whereby the former carbonyl O occupies the oxyanion hole. The P1-Phe side chain is located in the large S1 pocket, but does not interact with the Asn189 at the bottom of the pocket, which is more suited to bind residues with polar tips, such as Tyr or Gln, while Ala190 contributes to the hydrophobic character of this subsite [99]. Also, the S2 pocket is rather mixed hydrophobic polar and not completely filled by the P2 Ala, whereas the P3 Ala extends to the bulk solvent and forms hydrogen bonds from its backbone C=O and NH to the backbone of Gly216. F: Covalent bonds of Suc-AAPF-CMK to His57 and Ser195 of KLK7, as well as the orientation of the oxyanion and the P1-Phe side chain are the same as for AAF-CMK (Fig. 7E). The P2 Pro fits equally well as an Ala to the S2 subsite, bordered by His57 and His99 (Fig. 7E). Again, the P3 Ala makes only backbone interactions with Gly216, but contrary to the AAF-CMK, the P4 Ala occupies to some extent the hydrophobic S4 pocket, with the Trp215 side chain as base and His99 and Leu175 as borders, while the succinyl group in P5 position has no interaction partner on the KLK7 surface.

leupeptin and antipain and is directed against chymotryptic proteases. It corresponds largely to a tetrapeptide with a leucyl-phenylalaninal moiety and reacts with proteases as the above described aldehydes. Chymostatin inhibition was described for the chymotryptic KLK3, but also for the tryptic KLKs 8 and 14 (IC_{50} = 8 μM and 30 μM) [15,123,249].

3.1.4. Chloromethyl ketones

Contrary to reversible binding inhibitors, chloromethyl ketones (CKs, CMKs) are widely used irreversible cysteine- and serine protease inhibitors, which, similar to aldehydes, allow the design of specific inhibitors for an individual target. For example, such covalent inhibitors are suitable for co-crystallization with proteases, but often require an iterative process of optimization. For the analysis of the primary KLK specificity some standard compounds have been applied. In case of the tryptic KLKs 1, 4, 8 of tosyl-lysyl chloromethyl ketone (TLCK) was an efficient inhibitor, as well as D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK), which inhibited KLK2 at 5 μM [48,123,129,245]. Surprisingly, the chymotryptic counterpart tosyl-phenylalanyl chloromethyl ketone (TPCK) does not seem suitable to inhibit KLK3, whereas KLK7 was successfully crystallized with Ala-Ala-Phe-CMK and succinyl-Ala-Ala-Pro-Phe-CMK, resulting in structures with a resolution up to 1.0 Å (Fig. 7E, F) [147].

3.2. Polypeptidic inhibitors

3.2.1. Kunitz- type trypsin inhibitors

Several canonical substrate-like binding Kunitz-type inhibitors have been employed in research for the analysis of KLK enzymatic activity (Table 3) [89]. The most common ones are the soybean trypsin inhibitor (SBTI, I03.001 according to MEROPS), the lima bean trypsin/Bowman-Birk inhibitor (LBTI, I12.001, which occurs as

compound inhibitor LI12-001 consisting of I12.001 and I12.008) and aprotinin, the bovine pancreatic trypsin inhibitor (BPTI, I02.001), which is only found in the genus *Bos*. Interactions of proteases and the 181 amino acid SBTI are well understood on functional and structural level, not least because its reactive P1 Arg-P1′ Ile bond was an early target of bioengineering [250,251]. The much shorter LBTI comprises 71 residues with a P1 Lys-P1′ Ser reactive bond for trypsin-like and a P1 Leu-P1′ Ser for chymotrypsin-like proteases, but some related Bowman-Birk inhibitors possess a second reactive bond with P1 Arg [252,253]. Due to its anticarcinogenic effects this inhibitor type from plant nutrients gained much interest in medical sciences [254]. SBTI efficiently inhibited the tryptic KLKs 5 and 14 at an IC_{50} of 100 nM, while KLK6 required only 16 nM for 82% inhibition and KLK4 about 50 μM for 99% inhibition [15,48,174]. It was also demonstrated that SBTI is a good inhibitor of rKlk2 or tonin (K_i = 160 nM) and rKlk1-c9 (K_i = 70 nM) [111].

Mature BPTI is 58 residues long and presents a reactive loop with a scissile P1 Lys-P1′ Ala bond to tryptic proteases. The BPTI/bovine trypsin complex was one of the first refined crystal structures and revealed, in addition to details of canonical binding, that the Ser195 Oγ is covalently bound to the tetrahedral C atom of Lys15, representing the transition state of the cleavage reaction [255,256]. These findings were consistent with kinetic studies that found for BPTI with trypsin $K_{Hydrolysis} = [BPTI_{cleaved}]/[BPTI_{uncleaved}] = 0.33$, whereby equilibrium is reached in about one year [257]. Applications of BPTI are manifold in laboratories, such as the engineering of its specificity, or its use in cardiopulmonary surgery and organ transplantation [258,259].

Astonishingly, the pioneers of kallikrein protease research, Kraut, Werle, and Frey discovered as early as 1930 that certain extracts of bovine glands (containing BPTI) inhibited the enzymatic activity of kallikrein (KLK1) [260]. These seminal studies were later confirmed and evidence was found that other tryptic KLKs are well inhibited by BPTI, such as KLK 2 (IC_{50} = 30 μM), rKlk2 (K_i = 76 μM), KLK4 (100% inhibition with 4 μM BPTI), KLK5 (IC_{50} = 3 μM), KLK12 (IC_{50} = 2 μM), and KLK14 (IC_{50} = 3 μM) [15,17,48,129,245]. Despite its considerable specificity for tryptic KLKs, to date, only one kallikrein structure in complex with BPTI has been solved, demonstrating canonical inhibitor binding in a transition state-like manner for porcine pKLK1 (Fig. 8A, B) [247].

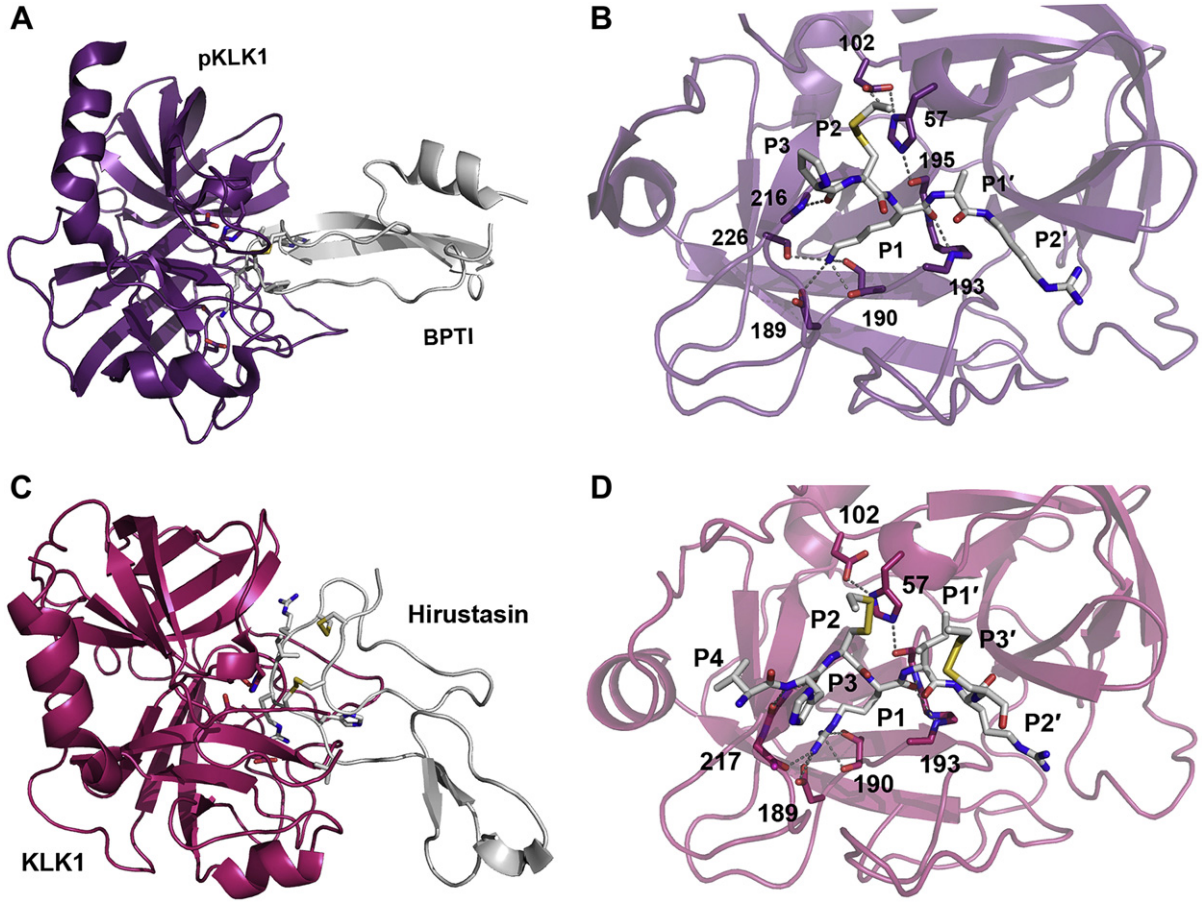

Fig. 8. Two complexes of porcine and human KLK1 with the exogenous inhibitors BPTI (Kunitz-type) and hirustasin (antistasin-type), respectively. Proteins are shown as ribbon representation, relevant residues as sticks labelled with numbers, and hydrogen bonds as grey dotted lines. A: pKLK1-BPTI complex displayed with the protease in purple and the inhibitor in white. B: Closer view of the active pKLK1 site, where only the interacting reactive loop residues of BPTI are shown. The P1 Lys is bound with its carbonyl O to the oxyanion hole, while its Nζ-atom is hydrogen bonded to the Thr190 Oγ and carbonyl O, the Ser226 Oγ and virtually not to the Oδ1 of Asp189. The P2 Cys14I-Cys38I (I for inhibitor chain) disulfide bridge occupies the S2 pocket, while the P3 Pro only forms one hydrogen bond with its carbonyl O to the NH of Gly216. In contrast to many other canonical binding inhibitors, no interaction of the P4 residue Gly is observed. On the prime side, P1′ Ala fits to the S1, pocket, but the P2′ Arg exhibits a non-canonical binding around the S2, and S3, subsites. C: KLK1-hirustasin complex depicted with the protease in magenta and the inhibitor in white. D: Closer view of the active KLK1 site, where the inhibitor residues P4 to P3′ are displayed, although canonical binding ends with P1′. The P1 Arg of hirustasin is bound the oxyanion hole and its side chain fills the S1 pocket largely, whereby the Nη1 atom is hydrogen bonded to the Thr190 Oγ and carbonyl O, the Nη1 binds the carbonyl O of His217, whereas the interaction distances with the Oδ atoms of Asp189 are relatively long. Similar to BPTI, the P2 Cys29-Cys48 disulfide bridge is located in the S2 pocket and rigidifies the reactive loop region together with the P3′ Cys33I-Cys50I disulfide, which adopts as the P2′ Arg a not-substrate-like position, in contrast to the P1′ Ile. Typical for canonical P3 and P4 residues, the side chain of His28I extends to the solvent and Val27I occupies the S4 subsite.

3.2.2. Hirustasin

Although produced by the leech *Hirudo medicinalis*, the canonical 55 residue inhibitor hirustasin with a reactive P1 Arg-P1′ Ile bond apparently inhibits not blood coagulation, as the thrombin inhibitor hirudin or other protease inhibitors from leech [261,262]. Moreover, hirustasin discriminates strongly between KLK1 (K_i = 13 nM) and plasma kallikrein, which is not inhibited at all [261]. A P2 Cys forming a disulfide characterizes this unusual inhibitor type (I15.001) from the antistasin family, of which a crystal structure was solved in complex with KLK1, exhibiting the typical transition state geometry at the reactive P1-Arg and tight interactions from P4 to P2 with the corresponding specificity pockets of the protease (Fig. 8C, D) [263].

4. Pharmaceutical approaches

Most of the small molecule inhibitors that have been employed in biochemical studies of KLKs are often of insufficient specificity, or cannot be administered to patients. Thus, the synthesis of biotolerable drugs or the engineering of large biomolecules from natural sources is required. The function of distinct KLKs in certain diseases is not always well defined, as shown in an overview disease matrix (Table 5). However, KLKs are attractive diagnostic and therapeutic targets, particularly the members of the family that are involved in prostate cancer and skin diseases [264].

4.1. Synthetic small compounds directed against KLK3 and KLK7

In order to maximize bioavailability and efficiency some basic principles should hold true for the compound to be designed. A famous and successful guideline for small molecule drugs is to obey Lipinski's "rule of five" and its recent modifications [265–267]; nonetheless, a significant part of pharmaceuticals is derived from natural compounds which tend to exceed the initial rule's suggested molecular weight of 500 Da [268]. It is also of high importance to consider the exact time and tissue localization in an organism for the administration of a given drug, in order to avoid undesirable side effects [269]. Strategies to obtain optimal drugs usually employ combinations of 1) massive screening campaigns; 2) "directed evolution"; 3) "rational design", often supported by structural data of biomolecules as well as a more sophisticated multiparameter design, yielding extremely tight binding serine protease inhibitors [270–273]. Common features of small molecule serine protease inhibitors are peptidomimetic groups that bind some of the specificity pockets and an altered "scissile" bond, which may react with the catalytic serine, such as those present in phosphonyl, boronic acid, inverse substrate esters, α-keto heterocycles, isocoumarins, and succinimide derivatives [274].

The major target among the KLKs for medical and pharmaceutical research is definitely KLK3/PSA. Thus, small molecules have been designed by pharmaceutical researchers and companies, stimulated by the recent advancements of structural data on several KLKs (Table 4). Due to the required mimicking of a natural active site binding molecule, such inhibitory compounds often contain amino acid derived groups, as the β-lactam derived 2-azetidinone with a Tyr side chain that inhibited KLK3 with an IC_{50} of 226 nM and was apparently designed obeying the "rule of five" [275]. In particular, for KLK3 heterocyclic compounds in a structure–activity relationship approach reached an IC_{50} of 300 nM [276].

The pharmaceutical relevance of KLK7 inhibitors is evidenced by patents for a small molecule KLK7 inhibitor [277] ("Use of cyclic depsipeptides to inhibit Kallikrein 7", WO2009024528 (A1). Novartis) that was analyzed by X-ray crystallography with the purpose of applying such compounds in treatments for human skin diseases, in particular atopic dermatitis, psoriasis or Netherton's syndrome [278].

4.2. Peptide-based inhibitors of KLKs

Already in 1981, a very efficient inhibitor of KLK1 was generated, which contained a P3-D-Phe residue and reached an IC_{50} of 800 nM [279]. Potent analgesic and anti-inflammatory peptidic compounds that contain the non-natural amino acid L-4-aminomethylphenylalanine inhibited KLK1 with a K_i of 1.2 μM [280]. Another synthetic peptide-based inhibitor specific for KLK1 (FE999024) was

Table 5
Disease matrix for human kallikrein-related peptidases. The double dots indicate confirmed and the single dots putative roles in the respective disease, also in cases when the KLK is used as a disease marker or acts as a tumor suppressor.

KLK	Cancer			Skin disease		Teeth	Lung	Brain		
	Prostate	Ovarian	Breast	Netherton syndrome	Atopic dermatitis	Amylo-genesis Imperfecta	Asthma	Multiple sclerosis	Alzheimer	Epilepsy
KLK1							●● [318]			
KLK2	● [25]									
KLK3	●● [25]		● [25]							
KLK4	●● [47,319]					●● [58,59]				
KLK5		●● [25,114]	● [25]	●● [211]	● [60]					
KLK6		●● [25,114]	● [26]	● [211]				● [70]	● [320]	
KLK7				●● [211]	● [60]					
KLK8		●● [25,114]	● [26]	● [211]	● [60]				● [76]	● [77]
KLK10		●● [25,114]	● [25]							
KLK11	● [25]	● [25]	● [26]	● [211]	● [60]					
KLK13		● [25]		● [211]						
KLK14	●● [31]		● [25]	● [211]						

capable to attenuate breast cancer cell invasion in a Matrigel assay [281]. Pure peptide based-inhibitors of KLK2 for targeting prostate cancer were found by screening phage display peptide libraries, resulting in 10–11 amino acid long linear peptides with IC_{50} values reaching 1.4 μM [282]. This basic approach was extended and improved by introducing cyclic peptides as efficient KLK2 inhibitors [283,284]. Also, so-called azapeptides that contain one N-atom instead of a Cα, e.g. tBOC-Ser-Phe-aza-Tyr-phenoxy, resulted in KLK3 inhibition with a K_i of 500 nM [285]. Non-hydrolyzable boronic acid inhibitors, such as carbobenzyloxy-Ser-Ser-Lys-P1-Leu-(boro)-P1′-Leu, turned out to be much more specific for the chymotryptic KLK3 with a K_i of 65 nM than for chymotrypsin itself (K_i = 3.9 μM) [286]. This inhibitor type was improved by introducing the non-natural amino acid nor-leucine (Nle) into CBZ-Ser-Ser-Gln-Nle-(boro)-Leu to result for KLK3 in a K_i of 25 nM, which could be employed as imaging agent for prostate cancer by attachment of a metal chelating group to the compound [287]. A detailed overview of peptide based and other small molecule inhibitors of KLK3 by LeBeau and coworkers is focussed on their usage in selective imaging and in targetted treatment of prostate cancer [288]. Recently, the naturally occurring 14 residue long cyclic sunflower trypsin inhibitor (SFTI of the Bowman-Birk family) was re-engineered by a combination of molecular modeling and sparse matrix substrate screening, to block the proteolytic activity of KLK4 (K_i = 3.6 nM) and potentially block stimulation of PAR activity [289]. Fully synthetic domain 6 of LEKTI, which was generated by fragment condensation of residues 1–22 and 23–68, inhibited KLK5 with an IC_{50} of 120 nM, which is slightly lower than measured for the recombinantly produced peptide (IC_{50} = 135 nM) [290].

4.3. Endogenous human inhibitors serve as scaffold for therapeutic agents

After determination of the substrate specificity of KLK2 [106], selected substrates sequences were transplanted into the reactive site loop of α1-antichymotrypsin (ACT), yielding a highly specific KLK2 inhibitor with high reactivity, which could be a useful tool to detect and target prostate cancer progression [291]. In a related approach, based on determination of the KLK14 specificity by phage display [104], selected substrates were incorporated as modified reactive site loop into the scaffold of serpins α1-antitrypsin (AAT) or ACT, which formed covalent complexes with KLK14, especially the engineered ACT inhibitors [292].

4.4. Engineered antibodies inhibit the proteolytic activity of KLKs

The antibody scaffold for the design of novel protease inhibitors offers some advantages over common inhibitors that bind in a substrate-like manner, since antibodies can in addition to binding of the often conserved protease active sites exploit binding to more variable individual exosites on the protease surface, which may enhance the selectivity of inhibition extraordinarily [293]. In case of KLK1 an engineered antibody bound specifically to the active site of the tryptic protease, resulting in inhibition of the peptidase activity with an inhibition constant around 130 pM, paving the way for a novel cure of asthma [294]. Also, a monoclonal antibody against KLK4 was capable of reducing its activity *in vitro*, although this engineered molecule needs further optimization [178]. Similarly, for KLK13, which is overexpressed in ovarian cancer patients, a monoclonal antibody inhibited its activity, leading possibly to a therapeutic application [14].

5. Conclusions and outlook

Kallikrein-related peptidases are a family of serine proteases that has diversified through evolution for physiological tasks that have arisen with the special developments of mammals compared with their ancestors. These novel functions comprise proteolytic processes in fertilization, desquamation, or a sophisticated neuronal plasticity. However, the activity of KLK proteases requires tight regulation by modulators, as activating or attenuating ions or reversible and irreversible proteinaceous inhibitors. In some cases, the failure of endogenous inhibitory systems in humans leads to excessive activity of KLKs with pathophysiological consequences.

Apart from the current scientific and pharmacological key interest, KLK functions may go far beyond the normal protease activity, as seen in some impressive examples depicting the regulatory potential of KLKs. First, in mouse nerve growth factor 7S, the two active mouse KLK b3 proteases or γ subunits are inhibited by Zn^{2+} mediated complex formation with an inactive β subunit and two inactive α subunits (KLK homologs), whereby the γ active sites are shielded from potential substrates [295–297]. Secondly, the mysterious localization of KLK4 in the cytoplasm as full length protein and in the nucleus of transfected prostate cancer cells (PC-3) as N-terminally truncated KLK4-205 awaits new functional models for KLKs, in particular due to a likely involvement in cancer [298,299]. Built according to the rather simple scaffold of (chymo)trypsin-like serine proteases, the kallikrein-related peptidases represent exciting biomolecules with astonishing variations in their structure, function, and physiological regulation. Eventually, our increasing knowledge of the underlying mechanisms of KLK activity control, will help to design very specific compounds directed to individual KLKs in order to cure diseases in which they play an important role.

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