Unleashing the therapeutic potential of human kallikrein-related serine proteases

Ioannis Prassas[1,2], Azza Eissa[1,2*], Gennadiy Poda[3,4] and Eleftherios P. Diamandis[1,2]*

Abstract | Tissue kallikreins are a family of fifteen secreted serine proteases encoded by the largest protease gene cluster in the human genome. In the past decade, substantial progress has been made in characterizing the natural substrates, endogenous inhibitors and *in vivo* functions of kallikreins, and studies have delineated important pathophysiological roles for these proteases in a variety of tissues. Thus, kallikreins are now considered attractive targets for the development of novel therapeutics for airway, cardiovascular, tooth, brain, skin and neoplastic diseases. In this Review, we discuss recent advances in our understanding of the physiological functions and pathological implications of kallikrein proteases and highlight progress in the identification of kallikrein inhibitors, which together are bringing us closer to therapeutically targeting kallikreins in selected disease settings.

Proteases
Enzymes that break down the peptide bonds that link amino acids together in proteins and polypeptides in a process known as proteolysis; they are also known as peptidases, proteolytic enzymes or proteinases.

[1]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, M5G 1X5, Canada.
[2]Department of Clinical Biochemistry, University Health Network, Toronto, M5G 2C4, Canada.
[3]Drug Discovery Program, Ontario Institute for Cancer Research, Toronto, M5G 0A3, Canada.
[4]Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, M5S 3M2, Canada.
**These authors contributed equally to this work.*
Correspondence to E.P.D.
e-mail:
ediamandis@mtsinai.on.ca
[Au: E-mail address OK?]
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The majority of human serine proteases known to date remain poorly characterized with regard to their natural substrates, inhibitors and *in vivo* functions, and so only a few proteases — such as thrombin and factor Xa in the blood coagulation cascade — have entered the pharmaceutical drug development arena[1]. Among the relatively new, and not yet as extensively examined, serine proteases are members of the human tissue kallikrein (KLK) family[2]. This family encompasses human tissue kallikrein (KLK1) and 14 kallikrein-related peptidases (KLK2–KLK15). These fifteen homologous serine proteases are encoded by the largest protease gene cluster in the human genome[3]. Traditionally, the KLK family was known for the role of KLK1 in the kallikrein–kinin system, and for the clinical applicability of KLK3 as a prostate cancer screening biomarker[4,5]. Much less was understood about the remaining KLKs and their roles in normal physiology and disease.

However, over the past decade we have witnessed great advances in our understanding of the tissue and cellular localization, regulation and *in vivo* physiological (and pathophysiological) functions of most KLKs. Notable functional insights have emerged following the development of animal models with selectively modified KLKs (or endogenous inhibitors of KLKs) and with the identification of individuals with natural KLK deficiencies. Collectively, these studies have characterized KLKs as regulatory proteases with key signalling and innate immune-like properties, thus modifying the originally static view of them as redundant extracellular-matrix-degrading enzymes[6]. KLK proteases are now known to be involved in mechanistic pathways that regulate kidney function, skin desquamation, tooth enamel formation, seminal liquefaction, synaptic neural plasticity and brain function[7–9].

In parallel, the importance of maintaining tight regulation of KLK activity *in vivo* has also become apparent. Endogenous KLK activity is fine-tuned by several tissue-specific regulatory mechanisms and factors, including zymogen (also known as pro-enzyme) activation cascades; endogenous KLK inhibitors (such as serpins, macroglobulins and the serine protease inhibitor lympho-epithelial Kazal-type-related inhibitor 5 (LEKTI; encoded by the gene *SPINK5*)); **[Au:OK?]** micro-environmental pH; and single-metal-ion inhibitors of KLKs (such as Zn^{2+})[10], among others. Disruption of the balanced tissue-specific regulation of KLK activity has been linked to several pathologies, including respiratory diseases, neurodegeneration, anxiety, schizophrenia, skin-barrier dysfunction, pathological inflammation and cancer. These associations have triggered numerous pharmacological efforts towards the development of KLK-specific inhibitors as therapeutic agents[11,12]. As discussed below, these efforts have been further catalysed by the recent characterizations of the 3D crystal structures of KLKs and the elucidation of their preferred-substrate specificities.

In this Review, we summarize the progress that has been made in recent years in determining the biological roles and therapeutic implications of tissue KLKs in selected disease settings. We describe the current arsenal of KLK inhibitors and conclude with emerging opportunities and challenges associated with future development of KLK-based therapeutics.

Human tissue kallikreins: digesting the basics

The term 'kallikrein' was first introduced in the 1930s by Werle and colleagues to describe a kinin-generating substance in the human pancreas (which is known as 'kallikreas' in Greek)[13]. Since then, several KLK-like proteases have been identified and are now classified into two groups: plasma KLK (also known as KLK1B) and the tissue KLK family (KLK1–KLK15). Plasma KLK is a liver-derived protease with a genomic localization and structural conformation that is unrelated to those of the tissue KLKs[14–16]. Although there has been substantial pharmacological interest in plasma KLK (which was highlighted by the regulatory approval of ecallantide (Kalbitor; Dyax) as a plasma-KLK-inhibiting drug in hereditary angioedema in the United States)[17,18], this Review will focus solely on the emerging therapeutic potential of tissue KLKs (KLK1–KLK15). For more on the therapeutic aspects of plasma KLK, readers are referred to the reviews in REFS 4,14,19,20.

Tissue KLKs belong to the chymotrypsin- and trypsin-like serine endopeptidase family S1 (also known as clan PA **[Au:OK? Or 'part of the clan PA'?]**) branch of the human protease family tree[3,13] (FIG. 1a). Approximately 80% of the 178 known human serine proteases belong to the S1 family[21]. In addition to the KLKs, this family also encompasses major proteases such as thrombin, trypsin, chymotrypsin, elastase and matriptase (MEROPS Peptidases database)[22].

All 15 genes that encode the KLKs colocalize in a tandem cluster on the long arm of chromosome 19 (19q13.3–19q13.4) and share a high degree of genomic organization[3] (FIG. 1b). The shared nucleotide sequence identity between each of the 15 human KLKs ranges from 35% to 80%, with the highest shared identity observed among the classic KLKs (KLK1–KLK3) (Supplementary information S1 (table)). KLKs are initially synthesized as homologous single-chain pre-pro-KLK proteins, which are secreted following the removal of their pre-peptide secretion signal[8]. Inactive 'latent' pro-KLK zymogens are activated extracellularly by the trypsin-like cleavage of their pro-peptide after either arginine or lysine, with the exception being pro-KLK4, which is activated by metalloproteinase-mediated cleavage after glutamine[23]. The activation of pro-KLKs is a key mechanism in regulating KLK activity in tissues, and it is postulated to occur via a characteristic proteolytic activation cascade similar to the activation cascades involved in coagulation, fibrinolysis and the complement system[7,24].

Once activated, KLKs employ the classical catalytic mechanism of serine proteases. The peptidic substrate binds to the active-site surface of the enzyme, such that the carbonyl carbon of the scissile bond is positioned proximal to the nucleophilic serine (Ser195) of the catalytic triad. The hydroxyl group of this serine attacks the carbonyl carbon of the scissile peptide bond of the substrate, resulting in the activation, inactivation or degradation of the substrate. KLK proteases exhibit trypsin-like, chymotrysin-like or dual (tryptic and chymotryptic) activity. The interaction between the S1 site of a KLK and the P1 site of its substrate (using Schechter and Berger interaction nomenclature) is an important determinant of the potency and specificity of each KLK–substrate pair[16].

Several techniques have been applied to characterize the preferred prime-side and non-prime-side substrate specificities of individual KLKs, including phage display (which has been used to characterize KLK1–KLK3, KLK6 and KLK14)[25–29], positional scanning of synthetic combinatorial libraries (PS-SCL; used for KLK3–KLK7, KLK10, KLK11, KLK13 and KLK14)[30–33] and peptide screening (used for KLK1–KLK3, KLK6, KLK8 and KLK12–KLK14)[34–41]. As shown in Supplementary information S2 (table), trypsin-like KLKs (that is, KLK2, KLK4–KLK6, KLK8 and KLK11–KLK14) predominantly cleave the peptide bond when the carboxyl side of the amide bond at the P1 position of the substrate is a positively charged residue, such as in arginine or lysine. By contrast, chymotrypsin-like KLKs (namely, KLK3 and KLK7) cleave amide bonds that carry a large aromatic residue — such as that in tyrosine, tryptophan or phenylalanine — at the P1 position. This substrate specificity is rationalized by the level of shape- and charge-complementarity between the P1 side-chain of the substrate peptide and the S1 binding pocket of the enzyme.

Notably, a conserved aspartic acid (Asp189) is located deep inside the S1 pocket of most tryptic KLKs (although KLK15 contains a glutamic acid, Glu189, here instead). The carboxylate moiety of this Asp189 forms a strong ionic bond with either the positively-charged guanidine moiety of P1 arginine or the alkylamine moiety of the lysine side chain. By contrast, chymotrypsin-like KLKs contain Ser189 (in KLK3), Asn189 (in KLK7) or Gly189 (in KLK9) instead. This variation accounts for the P1-preference of these KLKs for tyrosine, phenylalanine, or glutamine, respectively. Interestingly, some KLKs (such as KLK1, KLK10, KLK11 and KLK14) have dual trypsin- and chymotrypsin-like activities. Based on their preferred-substrate specificities, several lists of putative KLK substrates have been suggested[8,42].

From a structural point of view, the 3D crystal structures of six human KLKs have been resolved to date (namely, KLK1 and KLK3–KLK7)[16,42–49]. As shown in FIG. 1c, the protein conformation of KLKs exhibits most characteristics of the archetypal (chymo)trypsin serine-protease fold, which consists of two adjacent six-stranded β-barrels that are interconnected by three transdomain segments — in between which lies the active-site domain. These KLKs possess a conserved catalytic triad in the following order: His57, Asp102 and Ser195, with the histidine residue acting as a proton donor, the aspartate residue ensuring proper orientation of the imidazolium ring of the histidine and the serine residue acting as a nucleophile. The oxyanion hole,

Skin desquamation
The physiological peeling or shedding of the outermost corneocytes of the skin. A typical cycle of skin desquamation (which takes ~14 days) involves the apical movement and terminal differentiation of skin keratinocytes into corneocytes and their eventual shedding after cleavage by skin-associated proteases.

Seminal liquefaction
The enzymatic breakdown of the seminal gel — formed by proteins from the seminal vesicles — to become more liquefied. A typical seminal liquefaction cycle is completed within 20 minutes following ejaculation.

Catalytic triad
The three conserved amino-acid residues that are at the centre of the active sites of many enzymes (for example, proteases, amidases, esterases and lipases) and synergistically account for their activity.

Prime side
According to Schechter and Berger's nomenclature of interactions between protease active sites and peptidic or protein substrates, enzyme binding subsites (S′) and their corresponding substrate peptide (P′) residues that are C-terminal to the scissile peptide bond are termed 'prime side' and designated as S1′, S2′ and P1′, P2′, respectively. **[Au:OK?]**

Phage display
A molecular laboratory technique for the production and screening of novel proteins or polypeptides. The desired gene fragment is expressed by bacteriophages, which display the resulting protein on their surface where it can be tested for biological activity or interactions with other proteins, peptides or DNA. **[Au:OK?]**

which stabilizes the tetrahedral transition-state intermediates that are formed during catalysis, is formed by the backbone amides of Ser195 and Gly193 (REF. 50). Six disulphide bridges stabilize the KLK serine-protease protein fold, which also participates in the formation of the S1 pocket **[Au:OK – the fold helps form S1?]** that contains the key anchoring Asp189 residue, as shown in FIG. 1c. Other sub-pockets that may provide additional binding affinity and could be used to improve selectivity are designated as S2, S3 and S4. Certain KLKs, such as KLK5, contain an additional S1′ **[Au: prime symbol? ok?]** sub-pocket and an exosite (FIG. 1d).

Kallikrein proteases in physiology and disease

Tissue-expression profiles of individual KLKs vary substantially throughout the human body. Some KLKs are exclusively expressed in a single tissue (for instance, KLK2 and KLK3 are only expressed in the prostate) or in a restricted number of tissues (such as KLK5–KLK8 and KLK13), whereas others are ubiquitously expressed (including KLK1, KLK9–KLK11 and KLK14)[51]. KLKs can function individually and/or participate in tissue-specific proteolytic cascades to mediate crucial physiological processes. As discussed below, abnormal KLK activity is associated with various tissue-specific pathologies.

KLK cascade in human skin epidermis. At least nine KLKs (KLK1, KLK5–KLK8, KLK10, KLK11, KLK13 and KLK14) are co-expressed in the stratum corneum and upper stratum granulosum of normal human epidermis, as well as in associated sebaceous glands, eccrine sweat glands, hair follicles and nerves[52–55]. KLK5 (formerly known as stratum corneum tryptic enzyme; SCTE) and KLK7 (previously known as stratum corneum chymotryptic enzyme; SCCE) were originally extracted from stratum corneum tissues[56,57]. To date, the total serine-protease activity in normal human skin is ascribed mostly to KLK5, KLK7, KLK8 and KLK14 (REFS 58–60). These KLKs are transported along with other skin-barrier proteins by lamellar granules in keratinocytes and secreted into stratum corneum interstices (FIG. 2). After secretion, these KLKs participate in a proteolytic activation cascade — which is initiated by KLK5 — in the stratum corneum interstitial milieu. Autoactivated KLK5 activates downstream pro-KLK7, pro-KLK8 and pro-KLK14 by cleaving their pro-peptides after the arginine or lysine residues. Activated KLK14, in turn, activates further pro-KLK5 in a positive-feedback loop[58,61].

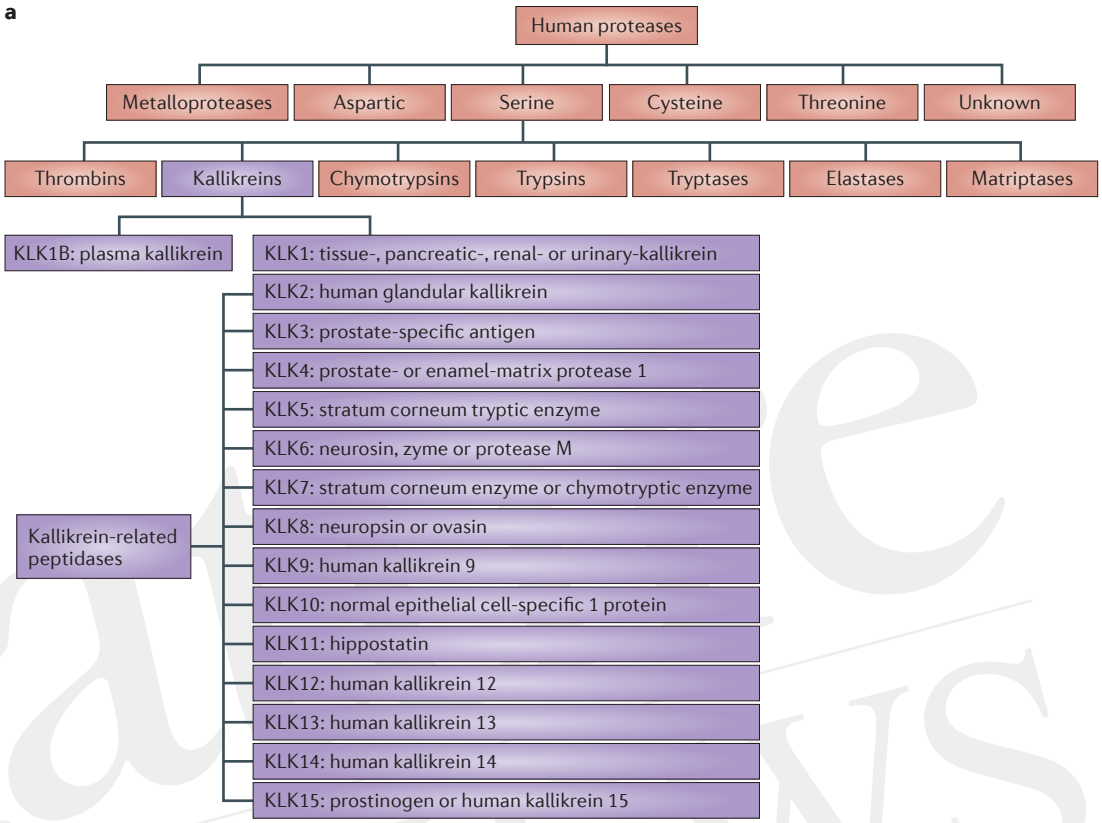
When they are physiologically required, pro-KLKs are activated to maintain healthy skin-barrier function by performing three main tasks: first, regulation of skin renewal and barrier thickness, by promoting desquamation and/or keratinocyte proliferation[58,62]; second, modulation of the lipid-rich permeability barrier by regulating lipid-processing enzymes and/or by the activation of keratinocyte-expressed proteinase-activated receptor 2 (PAR2)[63,64]; and third, induction of antimicrobial and innate immune responses by processing antimicrobial peptides and pro-cytokines[65–68]. Of these functions, the involvement of KLKs in skin desquamation is the best characterized thus far. Corneocyte cells in the uppermost stratum corneum are 'desquamated' (sloughed off the skin surface) every 2–4 weeks to ensure healthy skin-cell renewal. This desquamation process entails KLK-mediated proteolytic degradation of adhesion proteins, such as desmoglein 1 (DSG1), desmocollin 1 (DSC1) and corneodesmosin (CDSN), that connect corneocytes[52,69]. In addition to the skin-related protease inhibitors (such as LEKTI, **[Au:OK?]** shown in FIG. 2), several epidermal factors, such as transcription factor SP1 (REF. 70), vitamin D[71], retinoic acid[67] and the secretion of keratinocyte lamellar granules, maintain control of endogenous KLK expression and activity. In doing so, KLK-mediated desquamation and skin-barrier integrity can be maintained at physiologically healthy levels.

Abnormal and sustained KLK activity in the skin is detrimental, and this is best illustrated by the devastating and rare skin disease Netherton syndrome[72]. Netherton syndrome is characterized by severe desquamation, skin-barrier dysfunction and atopic skin-allergy-like symptoms. It is a genetic disease caused by mutations in *SPINK5* that lead to the abrogation of its gene product, the serine protease inhibitor LEKTI[69,73,74]. In-depth biochemical analyses of skin tissue from humans and mice with Netherton syndrome have identified KLK5 and KLK7 as key mediators of the pathogenesis of the disease[75–80]. Notably, although KLK5 is not the only KLK known to be inhibited by LEKTI, the LEKTI domains inhibit KLK5 more potently than they inhibit any of the other skin serine proteases. **[Au:OK?]** Research has also revealed that the pathogenic hyperactivity of KLK5 and KLK7 in LEKTI-deficient skin **[Au:OK?]** is further accentuated by a matriptase-dependent activation of these KLKs[81]. Hyperactive KLK5 induces inflammation and atopic-like lesions in the skin of humans and mice with Netherton syndrome via a pathway that is mediated by PAR2, thymic stromal lymphopoietin (TSLP) and nuclear factor-κB (NF-κB)[82,83]. Transgenic mice that overexpress KLK5, even in the presence of a functional LEKTI, exhibit hallmark symptoms of Netherton syndrome, including excessive corneodesmosomal degradation, stratum corneum detachment and upregulation of the same inflammatory cytokines and chemokines as in LEKTI-deficient mice[84] (TABLE 1).

The pathogenesis of atopic dermatitis has also been linked to aberrant activity of KLKs (particularly KLK7)[85–89]. Transgenic mice that overexpress KLK7 in the skin develop increased epidermal thickness and dermal inflammation, which is consistent with that seen in humans with atopic dermatitis[89]. PAR2 is also notably overexpressed in the epidermis of individuals with atopic dermatitis, suggesting that a KLK–PAR2 mechanism may be involved in the pathogenesis of this disease[90].

Psoriasis, a common multifactorial skin disease that affects around 2% of the population, is another skin pathology in which KLKs have been shown to be key factors. Psoriasis is characterized by autoimmune and epidermal aberrations that lead to epidermal scaling and hyperkeratosis[91]. The disease usually manifests with well-demarcated oval-shaped red 'lesional' plaques

Lamellar granules
Specialized secretory organelles typically found in keratinocytes and type 2 pneumocytes that are also known as membrane-coating granules, lamellar bodies, keratinosomes or Odland bodies. Keratinocyte trans-Golgi network and lamellar granules are part of the same continuous membrane structure, in which lamellar granules transport their cargo and fuse with the cell membrane at the border of the stratum granulosum and stratum corneum to release their contents into the extracellular milieu. **[Au:OK?]**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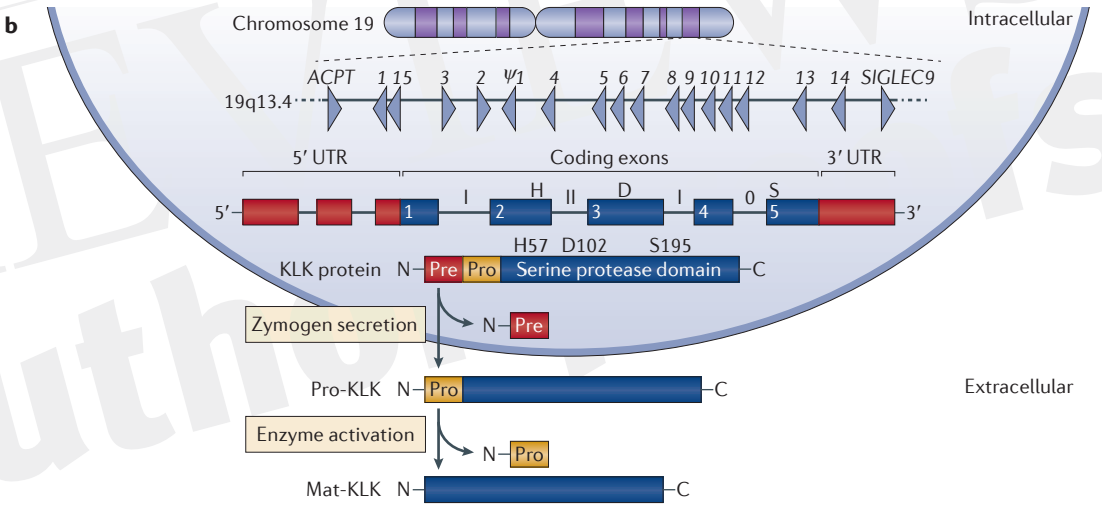
Elastases
Matriptases
KLK1B: plasma kallikrein
KLK1: tissue-, pancreatic-, renal- or urinary-kallikrein
KLK2: human glandular kallikrein
KLK3: prostate-specific antigen
KLK4: prostate- or enamel-matrix protease 1
KLK5: stratum corneum tryptic enzyme
KLK6: neurosin, zyme or protease M
KLK7: stratum corneum enzyme or chymotryptic enzyme
KLK8: neuropsin or ovasin
KLK9: human kallikrein 9
KLK10: normal epithelial cell-specific 1 protein
KLK11: hippostatin
KLK12: human kallikrein 12
KLK13: human kallikrein 13
KLK14: human kallikrein 14
KLK15: prostinogen or human kallikrein 15
Kallikrein-related peptidases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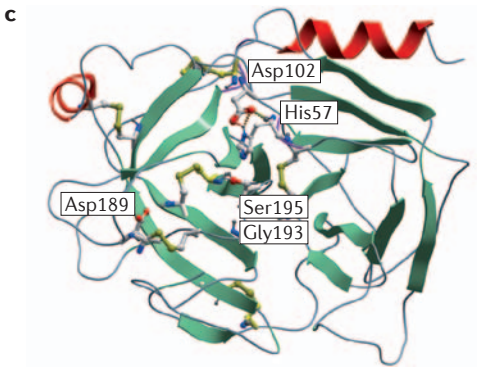
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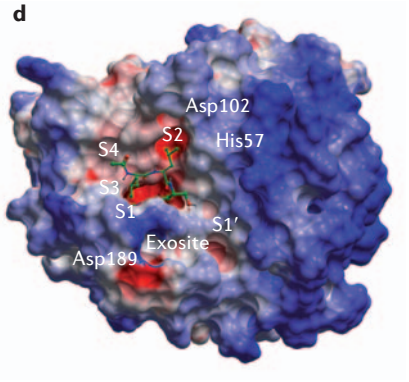
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Figure 1 | **The 101 of kallikrein serine proteases.** **a** | Kallikreins (KLKs) in the human protease family tree. In humans, over 690 proteases have been identified, the genes for which account for 2–4% of the genome[21]. These proteases are classified based on their catalytic mechanism into 200 metalloproteinases, 178 serine proteases, 160 cysteine proteases, 30 threonine proteases and 25 aspartic acid proteases; the remaining 97 are grouped in an 'unknown' category[1]. Of the 178 serine proteases, about 138 belong to the S1 family, which encompasses KLKs and other proteases, including thrombin, trypsin, chymotrypsin, elastase and matriptase[21]. KLKs are separated into human plasma kallikrein (KLK1B) and tissue kallikrein (KLK1) proteins, the genes for which are on chromosomes 4q35 and 19q13.4, respectively. The genes encoding the KLK-related peptidases colocalize with the tissue *KLK1* gene on chromosome 19q13.4 and are listed along with any alternative gene or protein names. **b** | Genomic and proteomic structure overview of KLK-related peptidases. KLK genes are situated on chromosome 19q13.4 and flanked by the testicular acid phosphatase gene (*ACPT*) and the sialic acid-binding immunoglobulin-like lectin 9 gene (*SIGLEC9*)[2]. Each arrow represents a KLK gene and denotes the direction of its transcription. The 5′ untranslated region (UTR) and 3′ UTR are shown in the primary mRNA transcript. The letters H, D and S indicate the locations of the catalytic histidine, aspartic acid and serine residues that make up the catalytic triad. In the KLK mRNA schematic, the boxes indicate exons and the line regions indicate introns. KLKs have five coding exons and an intron phase pattern of I–II–I–0 (REF. 3). KLK mRNAs are translated as inactive pre-pro-enzymes, which are directed to the endoplasmic reticulum for secretion after cleavage of their pre-peptide secretion signal. Extracellular cleavage of the pro-peptide after lysine or arginine by a trypsin-like protease is required for activation, except in the case of pro-KLK4, which is activated by a metalloproteinase after glutamine. **c** | The structure of human KLK5, a typical tryptic serine protease, in standard orientation. The structure of KLK5 was selected as a model structure for KLKs as it exhibits all five major subsites known to exist in serine proteases (namely, anchoring-S1, S2, S3, S4 and S1′ pockets) and a remote exosite[42]. KLK5 structure is depicted as secondary structural elements, with side chains shown for only the catalytic triad residues (Ser195, His57 and Asp102), residues that contribute to the oxyanion hole (the backbone amides of Gly193 and Ser195) and the six disulfide bridges (shown in yellow) that are conserved in serine proteases[50]. The structural elements are colour-coded as follows: α-helices are shown in red, β-sheets are shown in green, and connecting loops are shown in grey. The side chain atoms are depicted as coloured spheres: carbons are grey, oxygens are red, nitrogens are blue and sulfurs are yellow. **d** | The calculated electrostatic potential on the Connolly surface of KLK5. A cocrystallized molecule of the naturally occurring protease inhibitor leupeptin is shown in green. The active-site cleft consists of the anchoring S1 pocket (which contains the conserved Asp189) and the three selectivity subsites, S2, S3 and S4. An additional S1′ pocket, located to the right of the S1 pocket, and an exosite are also displayed. Mat-KLK, the mature active form of the protease; pro-KLK, the inactive form of the protease.

with silvery scales, which are surrounded by adjacent 'non-lesional' skin that has a normal appearance[92]. Of all the different KLKs, several lines of evidence implicate KLK8 in particular as an emerging candidate target for psoriasis. First, the total trypsin-like activity measured in psoriatic lesional skin is notably higher than that of both matched non-lesional skin from the same patients and normal skin from healthy volunteers[87]. Unlike the other skin-associated trypsin-like KLKs (such as KLK5, KLK6, KLK10 and KLK13), KLK8 is specifically upregulated in psoriatic skin lesions compared with matched non-lesional skin from the same patients and also with healthy skin from individuals without psoriasis[93,94]. Second, serum KLK8 levels correlate with 'psoriasis area and severity index' (PASI) scores[93]. Third, *KLK8* was recently identified as 1 of 130 differentially overexpressed genes that are associated specifically with psoriasis lesions[95]. Thus, KLK8 mRNA, protein and trypsin-like activity levels seem to correlate strongly with psoriasis severity. Interestingly, unlike the desquamatory KLKs (KLK5, KLK7 and KLK14), KLK8 does not cleave DSG1 (REFS 96,97) or activate PAR2 (REF. 90), and it is not inhibited by any of the epidermal LEKTI protease inhibitors encoded by *SPINK5*, *SPINK6* or *SPINK9* (REF. 98). Skin-inflammation-induced KLK8 expression and activity has been shown to stimulate *in vivo* keratinocyte hyperproliferation by inhibiting the transcription factor activator protein 2α (AP2α, encoded by *Ap2a*)[99]. *Ap2a*-knockout mice have thick skin, which is due to a skin-specific hyperproliferative defect[99]. Irritant-induced hyperkeratosis and acanthosis are largely inhibited in *Klk8*-knockout mice, further indicating the potential involvement of KLK8 in psoriasis[100] (TABLE 1).

Besides KLK8, crucial roles for other KLKs have also been described in the context of psoriasis. For instance, KLK7 has been associated with the pathogenesis of psoriasis, in which it is thought to exert its effects through a pro-interleukin-1β (pro-IL-1β)-dependent mechanism[101]. Interestingly, transgenic mice that overexpress human KLK7 in their skin exhibit increased epidermal thickness, hyperkeratosis and dermal inflammation, similar to that observed in inflammatory skin diseases in humans[89,102] (TABLE 1).

Moreover, KLKs are now considered major regulators of skin-resident immunity, as highlighted by their roles in the pathobiology of acne rosacea[65,103–105]. Individuals with rosacea exhibit facial inflammation in response to internal and external stimuli (such as emotional stress, sun exposure, alcohol consumption, hot beverages, spicy food or high temperatures) and have an exacerbated response to irritants and allergens as a result of skin-barrier dysfunction[106]. Compared with non-lesional areas, the facial skin of these patients has KLK5 overexpression, elevated KLK-mediated serine-protease activity and increased proteolytic processing of the cathelicidin antimicrobial peptide LL-37 into smaller fragments[65,107]. In mice, subcutaneous injection of active KLK5, in amounts mimicking those observed in rosacea skin, increases cathelicidin processing and induces leukocyte infiltration and inflammation, thereby confirming pathogenic KLK5 involvement in this disease[65] (TABLE 1). Although most of the KLK-related rosacea studies to date have focused on KLK5, other skin KLKs (including KLK7, KLK8 and KLK14) may be equally important in the pathogenesis of this disease[59,60,108].

KLK cascade in the prostate. Several KLKs (KLK2–KLK5, KLK11, KLK12, KLK14 and KLK15) are co-expressed in the prostate, although to varying degrees. For instance, KLK14 is expressed at 5 μg per l, whereas KLK3 is expressed at a staggering 10 g per l. KLK3 expression is restricted to the prostate and hence it is also known as the prostate-specific antigen (PSA). In normal prostate physiology, KLK3 cleaves fibronectin and the seminal-gel-forming proteins semenogelin 1 and semenogelin 2, leading to semen liquefaction and enhanced sperm motility[109–113] (FIG. 3a). Although other KLKs (such as KLK5 and KLK14) cleave fibronectin and semenogelins *in vitro* and are postulated to regulate KLK3 activity through a zymogen activation cascade, KLK3 remains the key executor of semenogelin hydrolysis during clot liquefaction *in vivo*. The prostate contains high concentrations of Zn^{2+} ions,

Acanthosis
An abnormal, diffuse hypertrophy of the stratum spinosum, as in eczema and psoriasis.

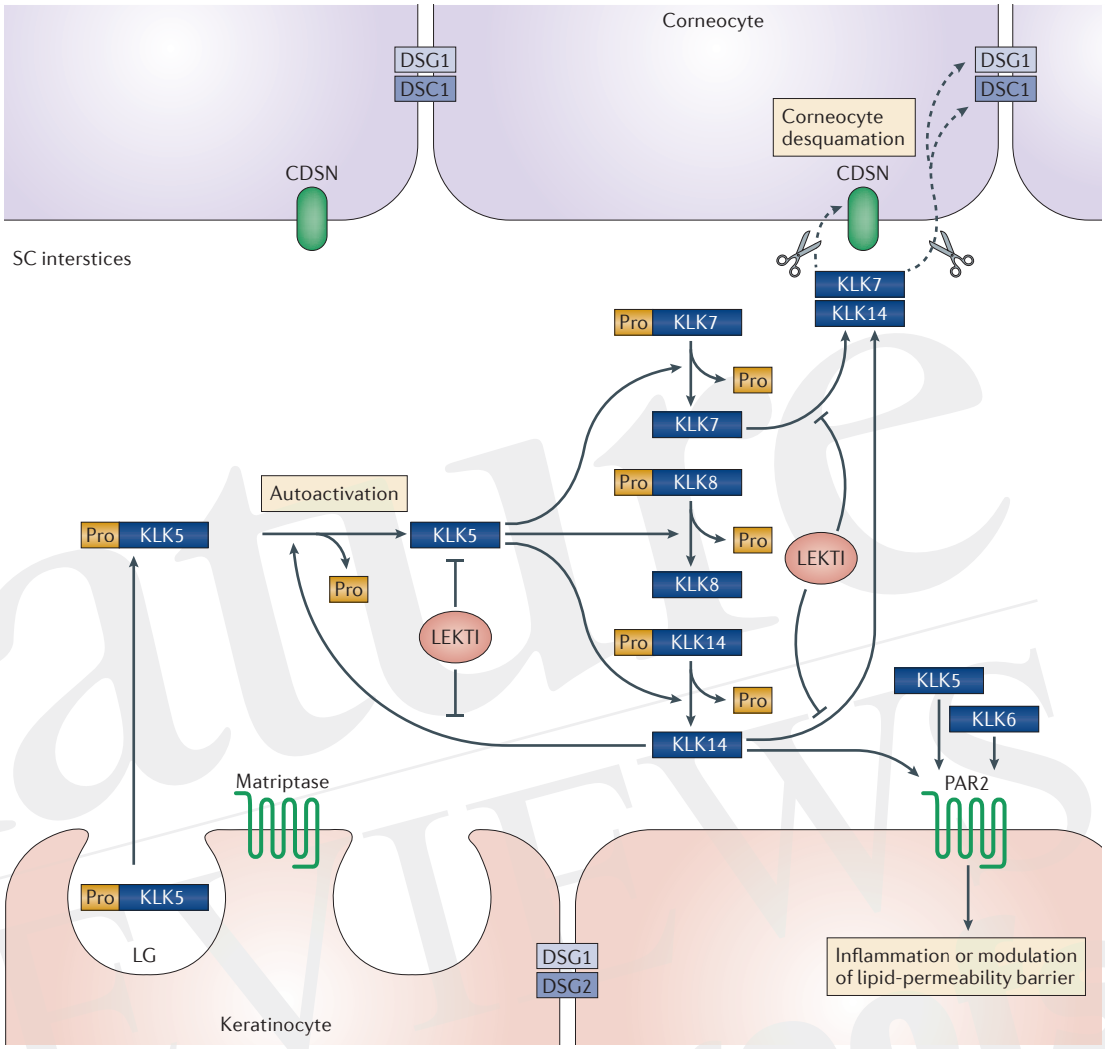


Figure 2 | **Kallikrein proteolytic cascade in skin epidermis.** Epidermal kallikreins (KLKs) and other barrier proteins are secreted by lamellar granules (LGs) of upper keratinocytes in the stratum granulosum **[Au: SG written out; not in fig.]** into stratum corneum (SC) interstices during terminal keratinocyte differentiation[79]. This process is regulated by an intrinsic increase in Ca^{2+} ions (from 0.06 mM to 2.0 mM) and a decrease in the pH gradient (from 7.0 to 5.0) from the lower stratum granulosum to the uppermost SC[61]. Secreted inactive pro-KLKs form an activation cascade whereby a pro-KLK is converted to an active KLK by removing the pro-peptide (yellow rectangle). A KLK may take on the role of the initiator, propagator and/or executor within the cascade, depending on its concentration, specificity and activity level. Pro-KLK5 is proposed to act as the cascade initiator as it autoactivates and then activates pro-KLK7, pro-KLK8 and pro-KLK14. In turn, activated KLK14 activates pro-KLK5 through a positive feedback loop[58]. Upon activation, KLK5 and KLK7 cleave corneodesmosomes (including desmoglein 1 (DSG1), desmocollin 1 (DSC1) and corneodesmosin (CDSN); indicated by the dashed arrows), leading to skin desquamation or shedding of SC corneocyte cells. Other epidermal serine-protease activators (such as matriptase) and protease inhibitors, such as lympho-epithelial Kazal-type-related inhibitor (LEKTI), regulate KLK activity in normal and diseased epidermis[76]. KLK5, KLK6 and KLK14 may target activation of keratinocyte-expressed proteinase-activated receptor 2 (PAR2), leading to inflammation or modulation of the lipid-permeability barrier. Absence of LEKTI-mediated inhibition of KLK5, KLK7 and KLK14 is implicated in Netherton syndrome[69]. The upregulated processing of antimicrobial cathelicidin peptides such as LL-37 by KLK5 and KLK7 is implicated in acne rosacea[65].

which regulate KLK activity by reversible and allosteric binding to the enzyme. Following ejaculation, Zn^{2+} ions relocate from KLKs to semenogelins, thus disinhibiting KLKs such that they liquefy the seminal clot[8].

KLK regulation by zinc ions has also been implicated in prostate cancer, where Zn^{2+} concentrations are reduced owing to downregulation of Zn^{2+} ion transporters[114]. The reduction in Zn^{2+} levels in prostate cancer tissues is associated with enhanced prostatic KLK3 activity and concomitant over-degradation of tumour-promoting extracellular matrix components (such as fibronectin, laminin, galectin 3 and nidogen 1), insulin-like growth factor (IGF)-binding proteins (such as IGFBP3 and IGFBP5) and parathyroid-hormone-related protein (PTHRP)[114,115]. KLK3 activity mediates prostate cancer progression and metastasis, particularly to the bone[116].

Collectively, these data have rendered KLK3 an attractive target for the development of KLK3-based therapeutics, most of which are currently immunotherapies, for advanced prostate cancer (discussed in detail below).

Among the remaining prostatic KLKs, the androgen-regulated KLK4 has also attracted considerable attention in recent years as an emerging prostate cancer target. KLK4 is notably overexpressed in prostate cancer[117–119] and has been shown to initiate epithelial–mesenchymal transition through a PAR-dependent mechanism[120–123], mediate the metastasis of prostate cancer to bones[124] and regulate two of the most frequently-altered signalling pathways in prostate cancer progression: the androgen receptor (AR) pathway, and the phosphoinositide 3-kinase (PI3K), protein kinase B (PKB) and mammalian target of rapamycin (mTOR) pathway[125] (FIG. 3b). Interestingly, the delivery of nanoliposomes that contain *Klk4*-targeting small interfering RNA (siRNA) to mice bearing prostate cancer tumours results in a substantial reduction in tumour growth[125] (TABLE 1). Genetic association studies have highlighted variations in the *KLK4* locus that are associated with prostate cancer predisposition[126]. These findings have triggered many efforts for the development of small-molecule KLK4-specific inhibitors as candidate prostate cancer therapeutics (discussed in detail below).

KLK1 in airway, renal and cardiovascular systems. KLK1, the primary kininogenase in the airways, is known to activate the bradykinin B_2 receptor by cleaving the low-molecular-mass kininogen to generate the decapeptide Lys–bradykinin[127]. The early understanding that the pathophysiology of asthma is associated with a hyperactivation of B_2 receptors led to the suggestion that KLK1 may be a potential therapeutic target for airway diseases[128]. KLK1 is consistently elevated in the bronchial alveolar lavage fluid of individuals with asthma or chronic bronchitis, particularly after challenges with allergens or bronchoconstrictive stimuli. KLK1 is the primary kinin-generating enzyme in the lung, and KLK1-mediated production of kinin (which activates the bradykinin B_2 receptor) **[Au:OK?]** induces bronchoconstriction and hypersecretion of mucus. Several reports have confirmed a strong correlation between increased KLK1 activity and levels of the cytokine IL-8 and free bradykinin in bronchial alveolar lavage fluid, thus further reinforcing the possible pathological role of KLK1 in asthma[129]. Indeed, the level of KLK1 activity is increased in airway disease because KLK1 is also secreted by activated resident epithelial cells, such as alveolar macrophages, and recruited inflammatory cells, such as neutrophils[130]. In addition to its increased secretion, the spatial distribution and compartmentalization of KLK1 has an important role in regulating its activity. The glycosaminoglycan hyaluronic acid, which is involved in lung innate immunity, immobilizes KLK1 at the apical pole of airway epithelial cells **[Au:OK?]** and blocks its activity[131,132]. In airway diseases such as asthma, reactive-oxygen species (ROS) break down hyaluronic acid and liberate KLK1 kininogenase activity, which induces the activation of B_2 receptors[133,134] (FIG. 4).

Disinhibition of tissue KLK1 activates pro-epidermal growth factor (pro-EGF) in human airway goblet cells via a kinin-independent pathway[133,135]. Enhanced KLK1 activity in patients with chronic bronchitis, and other airway diseases that involve elevated oxidative stress, is associated with initial breakdown of hyaluronic acid, which leads to increased activation of KLK1. As shown in FIG. 4, KLK1 then processes pro-EGF to release mature EGF, which activates EGF receptor (EGFR). KLK1-mediated EGFR activation results in mucus hypersecretion and goblet-cell hyperplasia — two hallmark features of chronic bronchitis. Consistent with this pathway, *in vivo* inhalation or intravenous administration of a KLK1-specific antibody known as DX-2300 (Dyax Corp.) in a sheep model of allergic asthma resulted in a substantial inhibition of late-phase allergen-induced bronchoconstriction and airway hyperresponsiveness[136]. DX-2300 is a fully humanized antibody developed through phage-display technologies to specifically target KLK1 through a competitive inhibition mechanism, with an inhibitory constant (K_i) of 0.13 nM[136].

It is important to note that outside the airway system, KLK1 mediates crucial roles in other tissues, particularly in the renal and cardiovascular system. For instance, it is secreted into the lumen of the connecting renal tubules, where it has different regulatory effects on various apical transporters. Here, KLK1 activates epithelial sodium channels, inhibits cortical collecting duct H^+, K^+-ATPase and activates TRPV (transient receptor potential vanilloid) channels[137–139]. KLK1 is a unique kaliuretic factor that provides rapid and aldosterone-independent protection against hyperkalaemia after dietary K^+ ion overload[140]. Abrogation of *Klk1* in mice causes both cardiovascular abnormalities and defects in renal tubular absorption[141] (TABLE 1). In humans, the A1789G polymorphism in *KLK1* is associated with an increased risk of coronary artery stenosis[142]. A loss-of-function polymorphism in exon 3 of *KLK1* (which leads to Arg53 in the active site converting to His53) is detected in 5–7% of Caucasians **[Au:OK?]** and leads to a marked (50–60%) decrease in KLK1 activity in these individuals. The partial deficiency of KLK1 activity in these individuals has been linked to arterial dysfunction in the absence of hypertension or other haemodynamic changes[143,144]. Adenoviral delivery of human KLK1 has also been shown to exert a protective role against systemic lupus erythematosus **[Au:OK?]** and glomerular basement membrane (GBM)-specific-antibody-induced nephritis in mice and in humans[145,146]. Antagonism of the KLK pathway augmented GBM-specific-antibody-induced nephritis symptoms, whereas agonists dampened the severity of the disease. Combined, these studies suggest that, owing to its protective renal and cardiovascular role, enhanced KLK1 activity is probably beneficial in cardiac and renal injuries, whereas inhibition of KLK1 may prove beneficial in asthma and chronic bronchitis.

KLK4 in tooth-enamel development. The outermost part of the tooth, the enamel layer, is the hardest mineralized substance and the only epithelially derived

Table 1 | ***In vivo* animal model evidence supporting KLK associations with disease** [Au: Swapped order of tables to match text.]

KLK gene and related human diseases	Animal model findings [Au:OK?]	Refs
KLK1		
Cardiac and arterial abnormalities	The heart of a *Klk1*-knockout mice exhibits septum and posterior wall thinning and a tendency to dilatation, resulting in reduced left-ventricular mass	228
Myocardial ischaemia	*Klk1*-knockout mice have reduced arterial carotid dilation, making them more vulnerable to acute myocardial ischaemia	229
KLK4		
Amelogenesis imperfecta	*Klk4*-null mice display improper maturation of tooth enamel crystals owing to the premature termination of mineralization	150
	Mice lacking both KLK4 and MMP20 cannot complete the last maturation stages of enamel formation. KLK4 is still active in *Mmp20*-knockout mice, suggesting it may be activated by other unknown proteases	230
	Mutations in *KLK4* cause autosomal recessive hypomaturation amelogenesis imperfecta	151
Prostate cancer	Delivery of *Klk4*-specific siRNA in mice xenografted with prostate cancer cells conferred a reduction in overall tumour growth	125
KLK5		
Netherton syndrome and atopic dermatitis	Transgenic mice that express human KLK5 (Tg-*KLK5*) have severe scaling, detachment of stratum corneum, skin inflammation and flawed barrier function; contrary to *Spink5*-knockout mice, no lipid droplets were observed in Tg-*KLK5* mice	84
	KLK5 overexpression induced corneodesmosome degradation but did not alter terminal differentiation marker expression or induce lipid abnormalities	84
	KLK5 induces atopic-like lesions through PAR2-mediated TLSP expression in Netherton syndrome	82
Acne rosacea	Subcutaneous injection of human KLK5 into mice results in increased cathelicidin processing and skin inflammation, similar to acne rosacea	65
KLK6		
Multiple sclerosis	Mice lacking KLK6 have fewer mature oligodendrocytes and myelin-associated proteins in their spinal cord than do wild-type mice	165
Lewy body diseases	Adenoviral delivery of *KLK6* [Au:OK? Or did the mice receive mouse genes?] reduces the burden of toxic α-synuclein in mouse models of Lewy body dementia	174
CNS inflammation	Administration of KLK6-neutralizing antibodies in a mouse model of autoimmune encephalomyelitis reduced the severity of clinically relevant deficits	175
KLK7		
Skin inflammation and atopic dermatitis	Transgenic mice overexpressing human KLK7 exhibit severe skin inflammation with thickened epidermis, hyperkeratosis and pruritus	89
KLK8		
Schizophrenia, mood and anxiety disorders	*Klk8*-knockout mice display loss of neurogenesis and synaptogenesis in the hippocampus	157
	Klk8-knockout mice were unable to regulate physiological synaptic plasticity and suffered from LTP deficits and frail memory acquisition	158
	KLK8 activity induces EPHB2 receptor activation, which triggers a cascade of events that result in enhanced transcription of FKBP5, leading to anxiety-like behaviour in mice	161
	KLK8-mediated NRG1–ERBB4 receptor activation leads to impairment of GABAergic inhibitory transmission, and this pathway is implicated in schizophrenia pathogenesis	162
Psoriasis	*Klk8*-knockout mice exhibit reduced skin hyperkeratosis and acanthosis following skin stimulation with sodium lauryl sulfate, and the skin of *Klk8*-knockout mice has increased AP2α expression and decreased keratin 10 expression, consistent with having a role in inducing premature hyperkeratosis [Au:OK?]	100
SPINK5		
Netherton syndrome and atopic dermatitis-like allergy	*Spink5*-knockout mice display skin KLK hyperactivity (owing to abrogation of LEKTI) with overt signs of skin overdesquamation, TSLP-mediated inflammation and allergy onset	69,73

Table 1 (cont.) | ***In vivo* animal model evidence supporting KLK associations with disease**

KLK gene and related human diseases	Animal model findings [Au:OK?]	Refs
Acne rosacea	Skin from *Spink5*-knockout mice exhibits altered cathelicidin peptide processing due to KLK hyperactivity	65
SPINK5 and matriptase		
Netherton syndrome	In wild-type mice, matriptase and LEKTI colocalize at the granular–transitional layer boundary where, in Netherton syndrome, premature corneodesmosome degradation causes epidermal separation	81
	KLK5 and KLK7 are overexpressed in the epidermis of *Spink5*-knockout mice and are activated in lower granular layers by membrane-bound matriptase	
	Mice lacking both *Spink5* and *St14* (the gene encoding matriptase) have lower levels of skin KLK activity, implicating a matriptase-dependent mechanism of skin KLK activation	

AP2α, transcription factor activator protein 2α; CNS, central nervous system; EPHB2, ephrin type-B receptor 2; FKBP5, FK506-binding protein 5; GABA, γ-aminobutyric acid; KLK, tissue kallikrein; LEKTI, lympho-epithelial Kazal-type inhibitor 5; LTP, long-term potentiation; MMP20, matrix metalloproteinase 20; NRG1, neuregulin 1; PAR2, proteinase-activated receptor 2; siRNA, small interfering RNA; SPINK5, serine-protease inhibitor Kazal-type 5; TSLP, thymic stromal lymphopoietin.

calcified tissue in the body[147]. Tooth-enamel formation (known as amelogenesis) is a highly orchestrated phenomenon that consists of multiple stages, including the pre-secretory, secretory, transition and maturation phases (as reviewed in REF. 148). Originally cloned as enamel-matrix protease 1, KLK4 is a key tooth protease that is secreted during the transition and maturation stages of enamel formation[147] (FIG. 5) and activated by matrix metalloproteinase 20 (MMP20). As KLK4 remains active in *Mmp20*-knockout mice, it is thought that KLK4 is activated by other, as yet unidentified, tooth proteases. Active KLK4 degrades enamel-matrix proteins (including amelogenin, enamelin and ameloblastin) and facilitates the lateral expansion of the hydroxylapatite crystal of the tooth via a transforming growth factor-β (TGFβ)-associated mechanism[149]. The importance of KLK4 in regulating enamel maturation is highlighted in *Klk4*-knockout mice and in humans carrying a single mutation in *KLK4* (REF. 150). Loss-of-function mutations in *KLK4* have been shown to cause defective tooth mineralization and crystal formation in the autosomal recessive condition known as hypomaturation amelogenesis imperfecta[151]. More recently, whole-genome-sequencing studies have identified another single nucleotide (frameshift) deletion in the *KLK4* locus as a major cause for amelogenesis imperfecta[152]. Future efforts for the restoration of normal KLK4 activity in teeth might therefore prove therapeutically useful for amelogenesis-related pathologies.

KLK8 in synaptic plasticity. KLK8, which was originally cloned as neuropsin in the mouse brain, has captured considerable attention in the neuroscience field. KLK8 is expressed in the hippocampus, the lateral nucleus of the amygdala and in other limbic system areas, all of which are involved in learning and memory[153,154]. KLK8 expression in neurons is induced by adjacent oligodendrocytes after spinal cord injury[155,156]. KLK8-deficient mice have marked impairment of early-phase long-term potentiation (LTP) in the Schaffer collateral pathway and in hippocampus-dependent memory[157,158] (TABLE 1). In the brain, KLK8 is believed to remain inactive until synaptic activation is triggered by a stimulus[159]. Upon stimulation, KLK8 is activated through a mechanism that is associated with NMDA (*N*-methyl-D-aspartate) and dual specificity mitogen-activated protein kinase kinase 1 (MEK1), and cleaves the presynaptic neural cell adhesion molecule L1 (L1CAM; also known as NCAML1), resulting in LTP enhancement[160] (FIG. 6a). Stress-induced KLK8 activity leads to increased cleavage of ephrin type B receptor 2 (EPHB2) on the surface of amygdala neurons, promoting anxiety-like behaviour in mice[161] (TABLE 1). Moreover, KLK8 was recently linked to schizophrenia as it directly targets the key schizophrenia-related molecule neuregulin 1 (NRG1)[162]. NRG1 and its receptor tyrosine-protein kinase, ERBB4, have important roles in regulating hippocampal and frontal cortical pyramidal neurons, and abnormal expression of NRG1 isoforms is associated with increased risk of developing schizophrenia[163]. KLK8-mediated cleavage of NRG1 results in the release of the NRG1 EGF-like domain, which, in turn, activates ERBB4 receptors and leads to an impairment of GABA (γ-aminobutyric acid) ergic inhibitory transmission and excessive postsynaptic excitation[162] (FIG. 6a). Single nucleotide polymorphisms of human *KLK8* have been associated with changes in attention or concentration, verbal intelligence quotient score and the incidence of bipolar disorder, further supporting the roles mentioned above for this protease in LTP, memory, emotional responses to stress and neuropsychological disorders[164].

KLK6 in neurodegenerative diseases. KLK6 was originally isolated from brain tissues as neurosin. It is abundant in the central and peripheral nervous systems and is mostly secreted by oligodendrocytes, pyramidal cells, astrocytes and glial cells[165,166]. As discussed below, the roles of KLK6 in the brain have been described in the context of several neurological diseases, including Alzheimer disease, Parkinson disease and multiple sclerosis.

The original finding that KLK6 expression was reduced in brain lesions associated with Alzheimer disease and Parkinson disease triggered further studies

Long-term potentiation
The biological process behind the persistent increase and long-lasting enhancement in synaptic strength following electrical or chemical stimulation of neurons. Long-term potentiation is a crucial cellular mechanism for both learning memory (also known as long-term memory) and working memory (also known as short-term memory).

on the involvement of this protease in neurodegeneration[167–169]. Alzheimer disease, the most common form of dementia, is characterized by pathological accumulation of amyloid-β, a proteolytic fragment of the amyloid precursor protein (APP). Not only has KLK6 been shown to efficiently cleave APP *in vitro*, but immunohistochemical examination of healthy brain tissue and brain tissue from individuals with Alzheimer disease has confirmed the proximal colocalization of these two molecules in the brain[41,168].

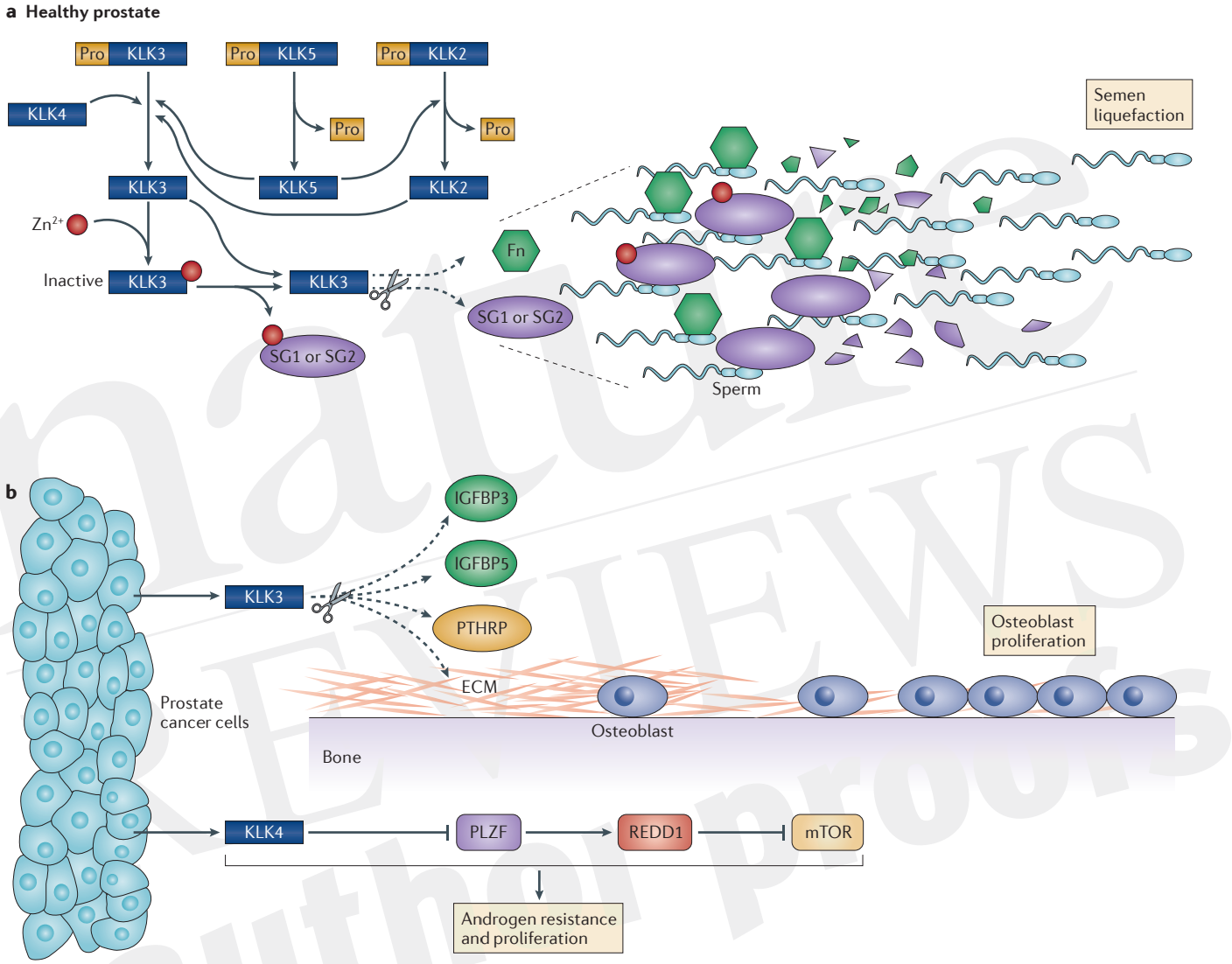


Figure 3 | **Kallikrein proteolytic cascade in the prostate.** **a** | Kallikrein 3 (KLK3; also known as prostate-specific antigen (PSA)) is present in several molecular forms in the prostate, including the zymogen pro-KLK3 and free active KLK3, or bound to Zn^{2+} ions, α_1-antichymotrypsin or antitrypsin in a KLK3–inhibitor complex[228]. In normal prostate physiology, KLKs are regulated by allosteric inhibition by Zn^{2+} ions[113]. At ejaculation, semenogelins sequester Zn^{2+} ions from KLKs, which leads to KLK disinhibition and initiation of a proteolytic activation cascade. KLK5 is postulated to autoactivate and then activate pro-KLK2 and pro-KLK3. Active KLK4, and to a lesser extent KLK2, are also able to activate pro-KLK3, unleashing KLK3 activity to hydrolyse fibronectin and semenogelin 1 (SG1) and SG2 to liquefy the seminal clot and release motile spermatozoa[113]. KLK activation is indicated by black arrows: pro-KLK is converted into an active KLK after removal of the pro-peptide (yellow rectangle). **b** | In prostate cancer tissues, Zn^{2+} levels are reduced owing to the downregulation of zinc transporters, and KLK levels and serine-protease activity are elevated. Prostate cancer cells can alter bone homeostasis by secreting KLK3, which influences bone formation by modifying the bone matrix or microenvironment. KLK3 targets tumour-promoting extracellular matrix (ECM) components (such as fibronectin (Fn), laminin, galectin 3 and nidogen 1), insulin-like-growth-factor-binding proteins (including IGFBP3 and IGFBP5) and parathyroid-hormone-related protein (PTHRP)[115,116].However, KLK4 mediates prostate cancer progression and androgen resistance by directly interacting with promyelocytic leukaemia zinc finger (PLZF) in prostate cancer tissues *in vivo* and inhibiting it from activating REDD1 (regulated in development and DNA damage response 1), which relieves inhibition of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), thereby keeping mTOR signalling (and thus proliferation) uncontrollably active[125]. The hyperactive androgen receptor pathway and mTOR pathway **[Au:OK?]** could be attenuated by targeting KLK4 in future novel drug development for prostate cancer.

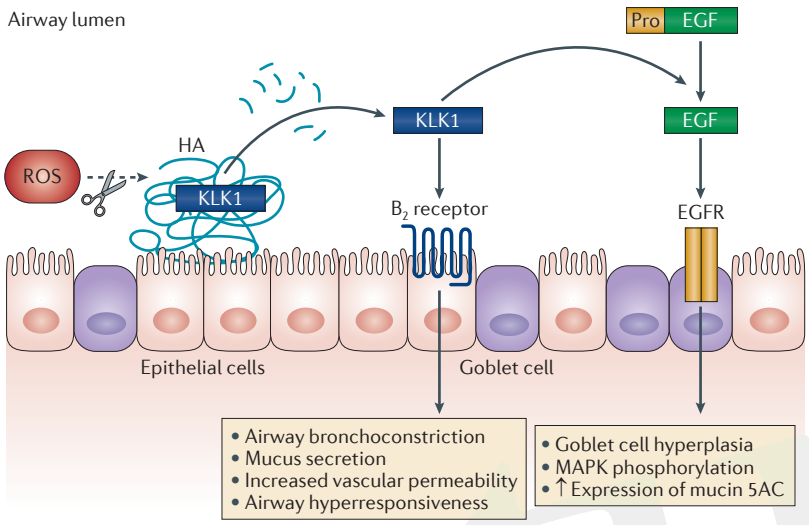


Figure 4 | **Kinin-dependent and kinin-independent roles of kallikrein 1 in the airway.** The glycosaminoglycan hyaluronic acid (HA) is present at the apical pole of airway epithelial cells, where it binds and inhibits kallikrein 1 (KLK1) in the airway lumen[131]. The tracheobronchial tree is exposed to increased levels of reactive oxygen species (ROS) by inhalation of exogenous sources (such as tobacco smoke) or by endogenous production in response to various inflammatory stimuli. ROS induce depolymerization and breakdown of HA and thus concomitant activation of KLK1 (REFS 132,133). Active tissue KLK1 plays a part in asthma pathophysiology by generating the Lys–bradykinin mediator that activates the B_2 bradykinin receptor, which induces airway bronchoconstriction, mucus hypersecretion, increased vascular permeability and airway hyperresponsiveness. Active KLK1 also has a key role in regulating oxidative stress-induced goblet-cell hyperplasia and mucus hypersecretion in human airways, by activating pro-epidermal growth factor (pro-EGF) and releasing mature EGF to activate EGF receptors (EGFRs) in neighbouring goblet cells[134]. KLK1-mediated EGFR activation is linked to goblet-cell metaplasia, mitogen-activated protein kinase (MAPK) phosphorylation and increased gene protein expression of mucin 5AC. Increases in KLK1 secretion and activity that are associated with the development of airway hyperresponsiveness are also dependent on the activation of resident and recruited inflammatory cells, including alveolar macrophages and neutrophils[130].

Parkinson disease is characterized by the abnormal aggregation of insoluble α-synuclein[170]. Lewy bodies, which contain α-synuclein, are predominant features of Parkinson disease and other synucleinopathies, such as dementia with Lewy bodies. Interestingly, KLK6 colocalizes with α-synuclein in the Lewy bodies of Parkinson disease[171]. *In vitro* and cell-based studies have shown that KLK6 can degrade α-synuclein extracellularly and therefore prevent its polymerization[172,173]. Notably, the phosphorylated form of α-synuclein — which is implicated in familial Parkinson disease — is more resistant to KLK6 degradation compared with normal α-synuclein. The decreased ability of KLK6 to degrade mutant α-synuclein has been associated with the pathogenesis of Parkinson disease, but this hypothesis awaits further *in vivo* verification[172]. Of note, lentivirus-mediated delivery of KLK6 in an *in vivo* α-synuclein transgenic mouse model of dementia with Lewy bodies **[Au:OK?]** results in non-toxic, wild-type-like α-synuclein clearance and reduced neuropathology[174] (TABLE 1).

Multiple sclerosis is a common immune-mediated neurodegenerative disorder that is characterized by damage of myelin sheaths, axonal loss and subsequent central nervous system (CNS) leukocyte infiltration. In contrast to the reduced levels observed in Alzheimer disease and Parkinson disease lesions, KLK6 is elevated in active multiple sclerosis lesions, as well as in the sera and cerebrospinal fluids of individuals with progressive multiple sclerosis[175,176]. The hypothesis that KLK6 participates in inflammatory CNS demyelination stemmed from findings of abundant KLK6 expression in inflammatory-cell subsets within CNS perivascular cuffs and at sites of CNS demyelination in animal models and individuals with multiple sclerosis[153,156,177,178]. In addition to cleaving myelin proteins such as myelin basic protein (MBP)[48], KLK6 can cleave components of the blood–brain barrier, such as laminin, fibronectin and collagens, and can induce inflammation via the activation of cell-surface PARs[48,176,179]. PARs are activated by proteolytic cleavage of their amino terminus, to release a 'tethered ligand' that binds intramolecularly to the rest of the receptor and activates downstream Ca^{2+}-associated signalling cascades. Studies have shown that PAR2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and in multiple sclerosis[180]. Indeed, antibody blockade of KLK6 function attenuates clinical and histological disease and reduces T helper type 1 (T_H1) cell responses in an experimental autoimmune encephalomyelitis model of multiple sclerosis[175]. A summary of the molecular pathways that underlie the pathobiological roles of KLK6 in neurodegenerative diseases is shown in FIG. 6b.

Kallikrein-targeted therapies

The potential of KLKs as therapeutic targets in a wide range of diseases has sparked considerable interest in the development of pharmacological KLK inhibitors. A growing list of KLK inhibitors has been reported by industrial and academic institutions during the past decade. These diverse inhibitors can be grouped in three general categories: small-molecule inhibitors, peptide-based inhibitors, and protein- or antibody-based inhibitors.

Small-molecule KLK inhibitors. Most of the early efforts for the discovery of small-molecule KLK inhibitors were based on high-throughput screening of large chemical libraries. For instance, high-throughput screening of a library of approximately 50,000 compounds revealed benzoxazinone derivatives and triazole derivatives as two classes of potent (but not especially selective) KLK3 inhibitors[181] (for example, compound 2 in TABLE 2). More recently, triazole derivatives have also been identified as putative inhibitors for several other KLKs, including KLK5, KLK7 and KLK14 (REF. 182) (for example, the KLK5 inhibitor compound 6 in TABLE 2). These triazole **[Au:OK?]** compounds lack selectivity, owing to their mechanism of action — covalent modification of the Ser195–OH group of the enzyme **[Au:OK?]**. Through extensive structure- and ligand-based mining of the ChemBridge Library (which contains approximately

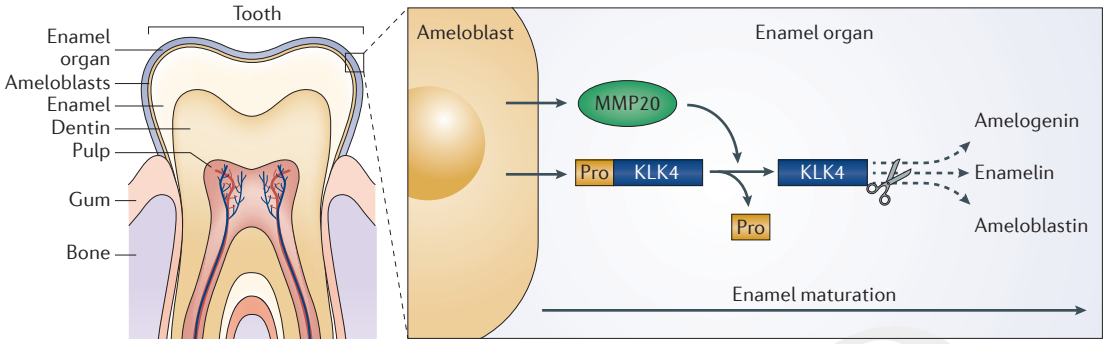


Figure 5 | **Kallikrein 4 in tooth development.** The soft protein-rich extracellular matrix secreted by epithelial ameloblasts during the secretory stage develops into hard enamel tissue. Before tooth eruption, enamel proteins are digested by kallikrein 4 (KLK4) and reabsorbed by ameloblasts to facilitate tooth hardening[148]. Pro-KLK4 is secreted by transition- and maturation-stage ameloblasts. Upon activation by matrix metalloproteinase 20 (MMP20), KLK4 degrades enamel-matrix proteins (namely, amelogenin, enamelin and ameloblastin) to provide space for lateral expansion of the tooth hydroxyapatite crystalline[229]. Reduction or absence of KLK4 activity has been shown to lead to amelogenesis-related pathologies, such as amelogenesis imperfecta[151].

600,000 compounds), additional small-molecule inhibitors for KLK5, KLK7 and KLK14 were identified[183]. Overall, the identified compounds (an example of which is compound 5 in TABLE 2) displayed weak potency (with a half-maximal inhibitory concentration (IC_{50}) greater than 50 μM) and low specificity (as they broadly inhibit several KLKs), limiting their future translational potential. Similarly, virtual screening against KLK6 revealed small molecules (for example, compound 7 in TABLE 2) that contained a para-amidobenzylamine P1 group and a 2-hydroxybenzamide scaffold as being potent KLK6 inhibitors; however, no data for selectivity were provided[184].

KLK7 inhibitors have been developed by tailored *de novo* chemical synthesis. Additionally identified KLK7 inhibitors include isomannide derivatives and nitrogen-containing heterocyclic compounds (such as compound 8 in TABLE 2)[185,186]. Using high-yield chemical synthesis, lactam-based enantiomers (for example, compound 3 in TABLE 2) have been generated that display potent covalent KLK3 inhibition[187]. An overview of all reported small-molecule KLK inhibitors is shown in TABLE 2, and further information regarding their properties is provided in Supplementary information S3 (figure).

Peptide-based KLK inhibitors. Traditionally, the development of peptide-based drugs has revolved around the investigation of three major sources: naturally occurring peptides, recombinant-peptide libraries and synthetic chemical libraries[188]. The main advantage of peptide-based drugs compared with smaller molecules is their larger surface interaction with the active pocket of the target enzyme. This thermodynamically strong interaction provides a starting point for the development of more selective protease inhibitors through the transformation of these peptides into non-hydrolysable analogues that bind to, and irreversibly block, the activity of serine proteases. In the KLK field, two early examples of this approach are the chemical modification of a low-molecular-weight kininogen peptide into a highly specific KLK1 inhibitor and of a semenogelin 2 peptide into a potent KLK3 inhibitor (compound 4 in TABLE 2)[189–191]. Phage-display peptide screening has been successfully used for the discovery of KLK2-inhibiting peptides as promising imaging probes for use in prostate cancer[192,193]. *De novo* solid-phase synthesis efforts for the development of peptide-based KLK inhibitors are exemplified by the synthesis of LEKTI-peptide domain 6 as a potent KLK5 and KLK7 inhibitor, the design of azapeptide-based inhibitors of KLK3 and the development of KLK1 inhibitors (for example, compound 1 in TABLE 2) from modified *N*-sulfonyltripeptides[194–196].

Protein- and antibody-based KLK inhibitors. In addition to peptides, large naturally occurring protease inhibitors such as serpins have been used as templates for the engineering of targeted KLK inhibitors. The basic concept underlying this approach is the use of recombinant DNA techniques to augment and selectively redirect the intrinsic KLK-inhibiting activity of naturally occurring inhibitors. A characteristic example of this approach is the successful bio-engineering of the sunflower trypsin inhibitor 1 (SFTI1) **[Au:OK?]** into a potent KLK4-inhibiting protein. By minimally modifying its natural amino-acid sequence, both the potency and the specificity of KLK4 inhibition of SFTI1 **[Au:OK? or 'SFTI1 inhibition by KLK4'? Please confirm meaning]** were greatly improved (by 100-fold and 500-fold, respectively)[197,198]. Through a similar re-engineering approach, depsipeptides, a class of macrocyclic peptides produced by the *Chondromyces crocatus* bacterial strain, have been successfully modified into potent and selective KLK7 inhibitors[11] (for example, compound 9 in TABLE 2). In a similar manner, serpins have been used as starting scaffolds for the development of more specific KLK inhibitors. On the basis of their preferred substrate

Chondromyces crocatus
A Gram-negative bacteria strain that belongs to the myxobacteria family. They live predominantly in the soil and feed on insoluble organic substances. Although they are poorly distributed ecologically, numerous secondary metabolite products of *Chondromyces crocatus* have been recently found. **[Au:OK?]**

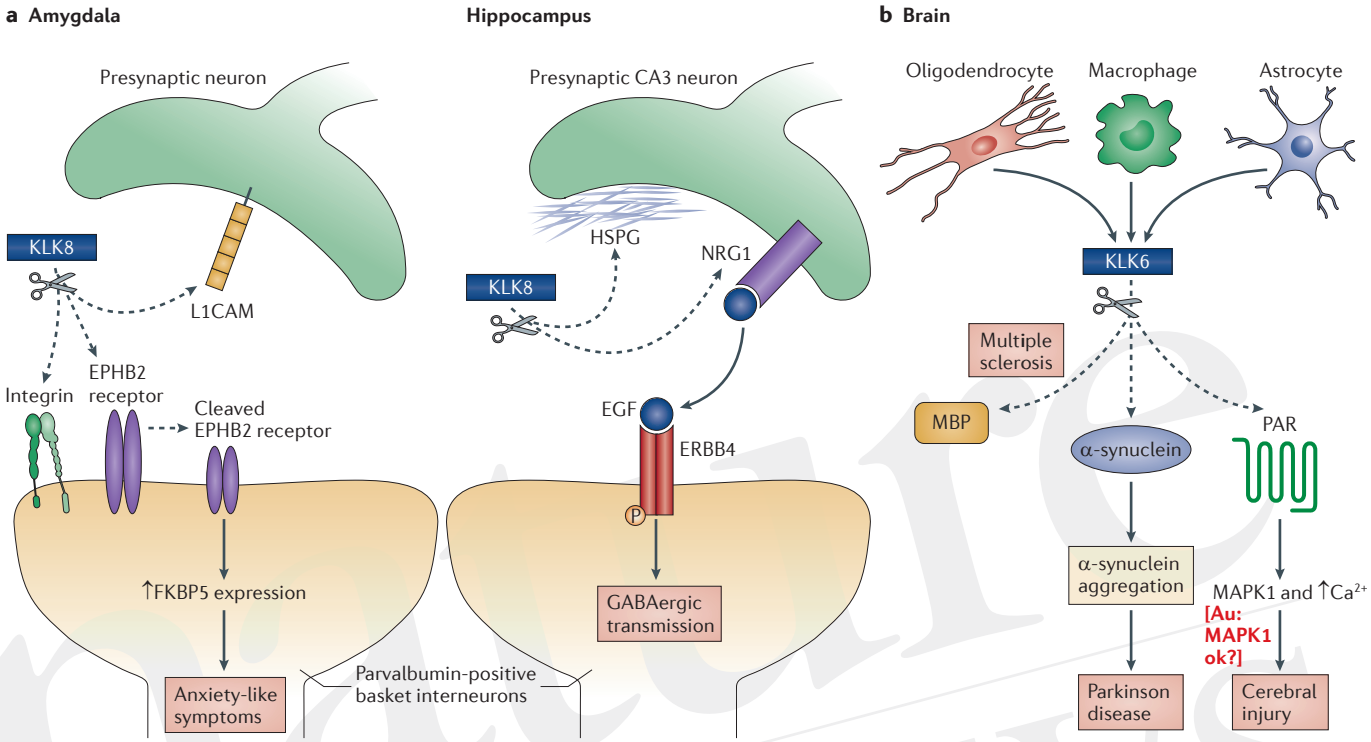


Figure 6 | **Kallikreins in brain biology and pathobiology.** **a** | Kallikrein 8 (KLK8) has numerous important roles in neuronal synapses in the amygdala and hippocampus[154]. KLK8 activity modifies the presynaptic neural cell adhesion molecule L1 (L1CAM) in the amygdala, inducing long-term potentiation[157,160]. Stress-induced KLK8 activity results in cleavage of ephrin type B receptor 2 (EPHB2) on amygdala neurons, which in turn triggers a cascade of events that result in enhanced transcription of FK506-binding protein 5 (FKBP5), leading to anxiety-like behaviour in mice[161]. EPHB2 is a receptor tyrosine-protein kinase that associates with NMDA (*N*-methyl-D-aspartate) receptors, the function of which may be compromised in anxiety. In the hippocampus, KLK8 has an important role in regulating GABA (γ-aminobutyric acid)ergic transmission. In wild-type mice, KLK8 cleaves the heparin-binding domain of neuregulin 1 (NRG1) and releases a functional epidermal growth factor (EGF)-like domain of NRG1 from the matrix component heparan sulfate proteoglycan (HSPG), which acts as a ligand moiety that binds and activates tyrosine-protein kinase ERBB4 receptors on parvalbumin-positive basket interneurons[162]. Basket interneurons form inhibitory synapses on the soma of pyramidal cells and control their output via GABAergic transmission. Loss of KLK8 (for example, in knockout mice) causes a decrease in inhibitory input to pyramidal cells owing to impaired cleavage of NRG1, leading to an attenuation of GABAergic inhibitory transmission. KLK8-mediated NRG1 cleavage and ERBB4 receptor activation and phosphorylation lead to impaired GABAergic transmission and excessive postsynaptic excitation, which is a novel pathway implicated in schizophrenia[163]. **b** | Simplified illustration of KLK6-regulated pathways in the brain. KLK6 is secreted by oligodendrocytes, macrophages and astrocytes. The KLK6-mediated degradation of myelin basic proteins (MBPs), α-synuclein and proteinase-activated receptors (PARs) have been described as important steps in the pathogenesis of multiple sclerosis, Parkinson disease and central nervous system (CNS) injury, respectively[165,173,176,178]. KLK6 targeting of amyloid precursor protein in Alzheimer disease is not shown. MAPK1, mitogen-activated protein kinase 1 (also known as ERK2); **[Au:OK?]** P, phosphate group.

specificities, α1-antitrypsin and α1-antichymotrypsin were modified through adjustment of their reactive loops to generate potent new inhibitors of KLK14 and KLK2, respectively[25,34,199,200].

The therapeutic usefulness of KLK-based antibodies has also been reported. Examples include the successful use of the fully humanized KLK1-specific antibody DX-2300 in reducing late-phase allergen-induced bronchoconstriction and airway hypersensitivity in a sheep model of asthma, and the use of KLK6-neutralizing antibodies in reducing the severity of symptoms in experimental autoimmune encephalomyelitis and in a viral mouse model of multiple sclerosis[136,175,176].

KLK-based immunotherapies. The highly tissue-specific expression of KLK3 has been exploited for the development of KLK-based immunotherapies (also described as therapeutic vaccines) for prostate cancer[201–204]. For instance, PROSTVAC, a viral vector-based vaccine that consists of the transgenes for both KLK3 and three T-cell co-stimulatory molecules (TRICOM), has been used as a targeted immune enhancer against prostate cancer[205]. Early results of randomized controlled trials of PROSTVAC revealed substantial improvements in the overall survival of patients who received the vaccine **[Au:OK?]**, with milder side effects compared with conventional chemotherapies[201]. A randomized

Table 2 | **Summary of the current pharmacological arsenal of KLK inhibitors**

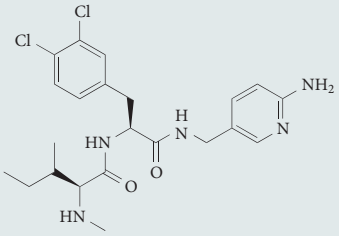
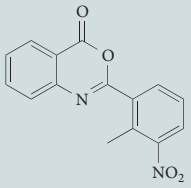
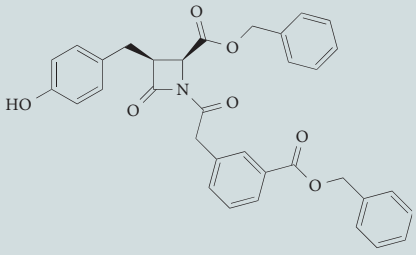
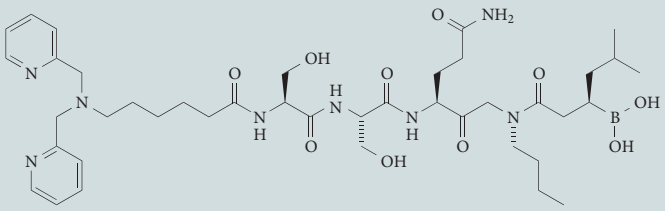
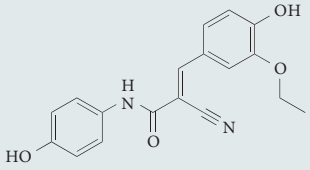
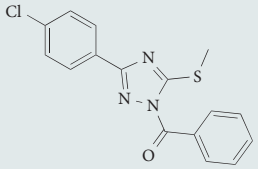
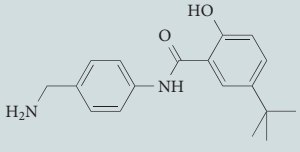
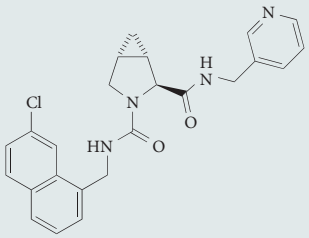
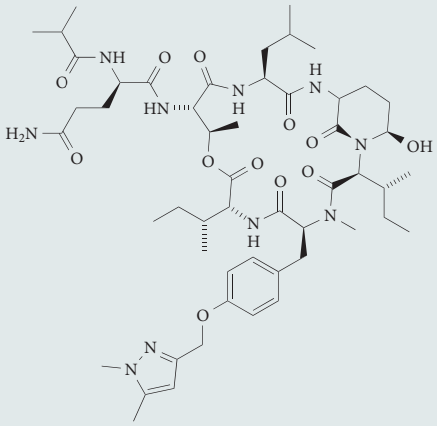
Target KLK	Inhibitor structure	Compound number	IC_{50} (nM)	Refs
KLK1		1	0.22	195
KLK3		2	300	181
		3	340	187
		4	30	189
KLK5		5	117,000	183
		6	140	182
KLK6		7	300	184

Table 2 (cont.) | **Summary of the current pharmacological arsenal of KLK inhibitors**

Target KLK	Inhibitor structure	Compound number	IC_{50} (nM)	Refs
KLK7		8	3	185
		9	0.2	11

IC_{50}, half-maximal inhibitory concentration; KLK, tissue kallikrein.

blinded Phase II trial revealed minimal differences of PROSTAVAC in overall time to progression; however, it was associated with a notable improvement in overall survival (25.1 months for PROSTVAC versus 16.6 months for placebo)[206]. Based on these findings, an international Phase III trial is ongoing in patients with asymptomatic castration-resistant prostate cancer to validate the effects of PROSTVAC on overall survival in larger cohorts (ClinicalTrials.gov identifier: NCT01322490). The FDA approval of sipuleucel-T (Provenge; Dendreon Corporation), a prostatic-acid-phosphatase-based vaccine for the treatment of asymptomatic androgen-resistant prostate cancer, demonstrated the therapeutic potential of immunotherapy in treating prostate cancer[207,208]. The identification of the patient groups that are most likely to benefit from such regimens, and the optimization of the timing of immunotherapy with regard to existing treatment regimens (for example, before or after androgen deprivation) are areas of intense ongoing research[209–213].

Perspectives and conclusion

Despite the substantial progress in determining the pathophysiological roles of individual KLKs in specific tissues and selected disease settings, the full therapeutic potential of targeting these KLKs has not yet been unleashed. Pharmacological efforts to develop KLK-targeting drugs have begun; however, important questions regarding the preclinical development of these compounds still need to be addressed. Is targeting one KLK better than targeting several KLKs simultaneously? How much inhibitor promiscuity can be clinically afforded? How can we account for compensatory *in vivo* mechanisms? Are there antagonizing or synergistic activities among different KLKs?

Our understanding of the complex proteolytic networks that govern KLK activity *in vivo* is far from complete. KLKs do not work in isolation, but rather as individual parts of a complicated network of proteases, substrates and endogenous inhibitors[214–216]. In such a complex context, delineating the overlapping, synergistic and opposing activities of an individual KLK is challenging. Recent successes with the development of *Klk*-knockout and transgenic mice have revealed key associations between individual KLKs and the pathophysiology of several diseases (as summarized in TABLE 1). Moreover, the emergence of proteome-based degradomics technologies has brought ample new opportunities for a deeper understanding of individual protease contributions to proteome homeostasis in health and disease[217–221]. Together, these advances are expected to enhance our understanding of the true therapeutic potential of targeting certain KLKs in specific disease settings.

A second challenge, common to all multi-membered enzymatic families, is the difficulty of designing KLK inhibitors with exquisite specificity[222,223]. The active sites of KLKs are well conserved, meaning that inhibitors that target the active site of any given KLK are prone to inhibit other KLKs. This problem is accentuated in the

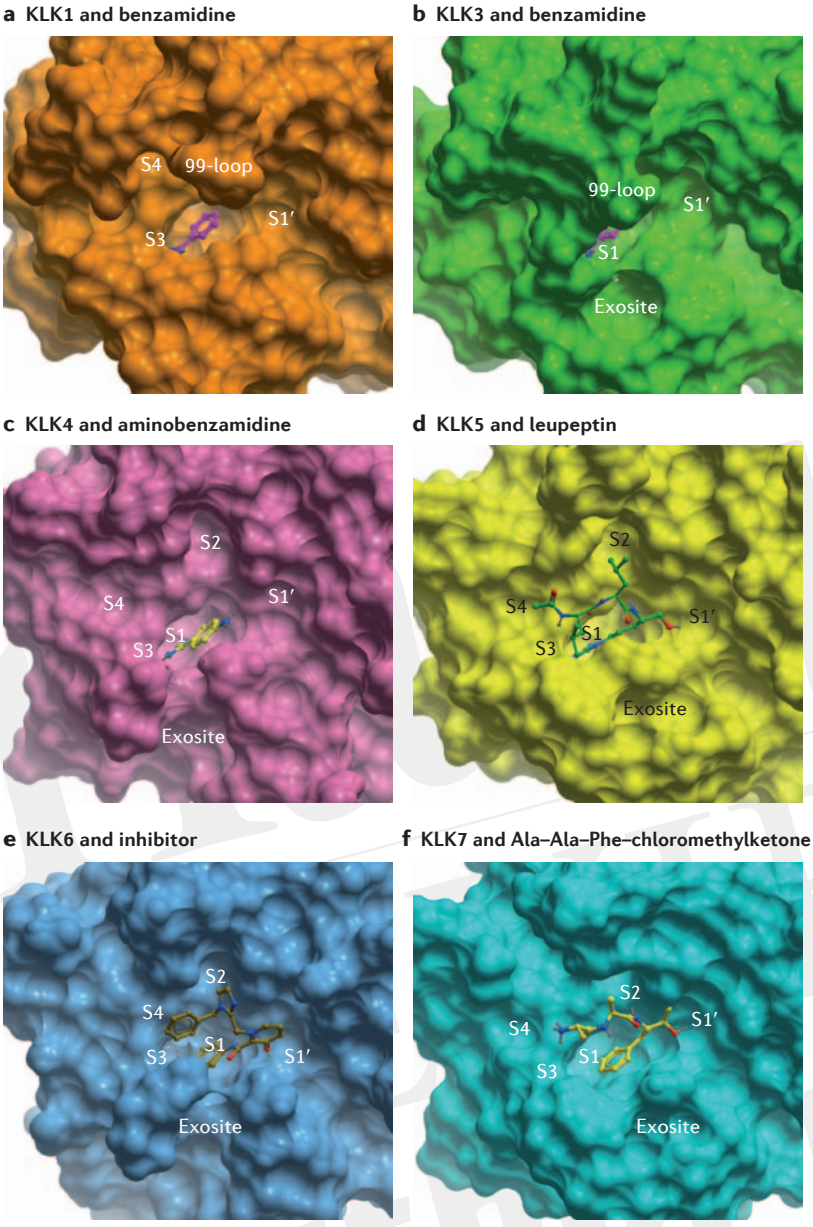


Figure 7 | **Side-by-side comparison of six human kallikrein structures.** The Connolly surfaces of the active sites of six kallikreins (KLKs) are depicted. Sub-pockets that are common to all serine proteases are labelled S1–S4 and S1′. The exosite (allosteric to S1) is labelled where present. The long so-called 99-loop that covers the S2 site is labelled in KLK1 and KLK3. In the cases of KLK1 and KLK3, we overlaid the benzamidine from pig KLK1 to indicate the location of the S1 pocket of these enzymes because only the apoprotein structures of the human ligand-free enzymes are publicly available. The longer (up to 11 residues long) 99-loop is more obvious in the classical KLKs (namely, KLK1–KLK3) than in the other KLKs. This longer loop covers a part of the S2 and S4 pockets in KLK1 (part **a**) and KLK3 (part **b**). Human KLK1 (Protein Data Bank (PDB) ID: 1SPJ) is shown with benzamidine (part **a**). KLK1 has a characteristically small S2 pocket owing to the presence of the bulkier Tyr99 (compared with, for instance, His99 in KLK5). KLK3 (PDB ID: 2ZCH) is shown with benzamidine (part **b**); KLK4 (PDB ID: 2BDH) with aminobenzamidine (part **c**). KLK5 is shown bound to leupeptin, a covalent peptide inhibitor (part **d**). KLK5 has a markedly smaller S3 pocket owing to the bulky Tyr218 (as compared with the smaller Val218 in KLK1, which has a larger S3 pocket). KLK6 (PDB: 4D8N) is shown with a drug-like small-molecule inhibitor (PubChem ID: 57404341) (part **e**). The structure of KLK7 (PDB ID: 2QXG) is shown with Ala–Ala–Phe–chloromethylketone, a covalent peptide inhibitor (part **f**). The S4 pocket of KLK7 is notably different owing to the presence of its polar Thr217 residue (compared with, for instance, the less polar Asp217 in KLK5).

case of small-molecule inhibitors owing to their small surface of interaction with their target enzyme. Thus, most of the reported small-molecule KLK inhibitors (described in TABLE 2) exhibit low or medium selectivity against their target. Future efforts in the development of KLK-specific compounds should benefit from the recent resolution of the crystal structures of several KLKs. Despite their general conformational homology, individual KLKs have unique structural sites that could be exploited in the design of targeted inhibitors. FIGURE 7 provides a side-by-side comparison of all the currently available human KLK structures, and highlights signature areas in the S1, S2, S3, S4 and S1′ pockets of individual KLKs. These differences are even more prominent when KLK structures are compared with the structures of other key serine proteases, including trypsin, chymotrypsin and blood coagulation factors (such as thrombin, factor Xa and factor VIIa) (see Supplementary information S3 (figure)). Careful consideration of these structural differences may lead to the development of highly selective serine-protease inhibitors, as exemplified by the recently approved anticoagulant drug apixaban (Eliquis; Pfizer), a highly specific inhibitor of factor Xa[224,225]. The high specificity of this drug is based on a uniquely snug fit between its non-basic P1 needle (a methoxyphenyl group) and functional groups in the uniquely shaped S3 and S4 pockets of this enzyme (see Supplementary information S3 (figure)).

A third major obstacle to targeting KLKs pharmaceutically is related to the limited bioavailability of peptide-based KLK inhibitors[226]. Generally, peptide inhibitors that are composed solely of natural amino acids are considered poor drug candidates owing to their intrinsically low plasma stability and limited serum-clearance time. Several strategies have recently been developed to circumvent these problems, including novel peptide-cyclization methods, the use of non-natural amino acids, blocking of N- and C-terminal ends, amide-bond replacement and N-terminal esterification[188]. Such approaches, which have already been successfully implemented for the pharmacokinetic enhancement of several peptides, may facilitate the preclinical development of other peptide-based KLK inhibitors in the near future.

The pathophysiology and potential therapeutic benefits of targeting specific KLKs in selected diseases are becoming increasingly appreciated. Although the picture is not yet complete, it is evident that tissue KLKs are attractive and promising therapeutic targets. The lessons learned from previous failures of drugs against other protease families (such as the MMPs) should be kept in mind to avoid common pitfalls along the transition of the first preclinical KLK-targeting therapeutics into the clinic[227]. Now, more than ever, all the necessary KLK research tools are coming together. We hope that the topics discussed here spark more basic-research investigations and trigger further pharmaceutical efforts towards the development of druggable KLK inhibitors that could prove effective in the treatment of KLK-associated pathologies.

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Competing interests statement
The authors declare no competing interests.

DATABASES

ClinicalTrials.gov: https://clinicaltrials.gov/
PubChem: https://pubchem.ncbi.nlm.nih.gov/
RCSB Protein Data Bank: http://www.rcsb.org/pdb/home/home.do

SUPPLEMENTARY INFORMATION

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Author Biographies

Ioannis Prassas obtained his Ph.D. from the Department of Laboratory Medicine and Pathobiology at the University of Toronto, Ontario, Canada. Currently, he is a postdoctoral fellow at the Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada. His main research activities focus on the use of proteomic technologies for the delineation of the mechanism of action of existing drugs and the development of novel serine protease inhibitors as skin therapeutic agents.

Azza Eissa completed her Ph.D. on kallikrein (KLK) serine proteases as an Alexander Graham Bell Canada Graduate Scholar at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada **[Au:OK?]**. As a Ph.D. student with Eletherios Diamandis she elucidated a role for KLK8 in normal human skin-barrier function and in psoriasis. Currently, she is pursuing a medical degree at the University of Toronto, Ontario, Canada, to fulfil her ambition of becoming a clinical scientist.

Gennadiy Poda received his M.Sc. from the Moscow Institute of Physics and Technology (MIPT), Russia, and his Ph.D. in computational chemistry from the Institute of Bioorganic Chemistry at the National Ukrainian Academy of Sciences, Kiev, **[Au:OK?]** Ukraine. He has contributed to a number of drug discovery projects at Pharmacia Corporation and Pfizer Inc., both in St. Louis, Missouri, USA. Currently, he is responsible for chemoinformatics and computational chemistry at the Ontario Institute for Cancer Research, Toronto, Canada.

Eleftherios P. Diamandis currently serves as Division Head of Clinical Biochemistry at Mount Sinai Hospital, Toronto, Ontario, Canada, as Biochemist-in-Chief at the University Health Network and as Professor and Head of Clinical Biochemistry at the Department of Laboratory Medicine and Pathobiology at the University of Toronto, Ontario, Canada. His research activities focus on the discovery and validation of cancer biomarkers, proteomics, mass spectrometry and translational research. **[Au:Would you like to include a laboratory homepage?]**

Key points

- Tissue kallikreins (KLKs) constitute a family of 15 secreted serine proteases that are encoded by the largest protease gene cluster in the human genome.
- KLKs were traditionally known for their clinical applicability as cancer markers — for instance, KLK3 (also known as prostate-specific antigen) is a marker for prostate cancer.
- The field of KLK research has recently blossomed with the development of KLK-knockout models and the elucidation of the 3D structures of several KLKs.
- Novel pathophysiological roles for these proteases have recently been assigned in various tissues, such as the airway, cardiovascular system, tooth, skin and brain tissues.
- The promise of KLKs as therapeutic targets in various pathologies — including skin diseases, hereditary angioedema, neurodegeneration, inflammation and cancer — is emerging.
- Systematic efforts for the development of the first generation of KLK-based inhibitors as candidate therapeutics have already been initiated.

TOC blurb

000 **Unleashing the therapeutic potential of human kallikrein-related serine proteases**

Ioannis Prassas, Azza Eissa, Gennadiy Poda and Eleftherios P. Diamandis

Members of the family of kallikrein (KLK)-related proteases are found in various tissues — including the airway, prostate and brain — and have a wide range of functions. The authors describe the roles of KLKs in health and disease, and outline the small-molecule, peptide-based, protein-based, antibody-based and immunotherapeutic strategies that are being used to target KLKs in certain diseases. **[Au:OK? Blurb should be 65 words max.]**