EXPERT OPINION





# Targeting kallikrein-related peptidases in prostate cancer

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***Introduction:*** Novel therapeutic compounds are needed for prostate cancer (CaP), given the limitations of already used drugs and the disease's mortality, often attributed to castrate resistance. Tissue kallikrein and kallikrein-related peptidases (KLKs) form a family of serine proteases aberrantly expressed and broadly implicated in human malignancies. In CaP, KLKs participate in the promotion of cell proliferation, extracellular matrix degradation, tumour cell invasion and metastasis.

***Areas covered:*** This review discusses the different ways of inhibiting, modulating and exploiting KLK activity and/or expression as emerging CaP therapeutics. KLKs are targeted by diverse naturally occurring substances, including proteinaceous inhibitors, low-molecular-weight peptides and $Zn^{2+}$. Synthetic KLK inhibitors include protein/peptide-based inhibitors and small molecules. A re-engineered serpin-based KLK inhibitor is under evaluation in first-in-human trials as a CaP therapeutic, whereas additional potent and selective KLK inhibitors with relevance to CaP have been synthesized. KLK3-activated pro-drugs have entered Phase I and Phase II clinical trials as therapeutics for prostate tumours. The KLK3-based PROSTVAC® vaccine is evaluated in Phase III clinical trials. Targeting *KLK* expression via RNA interference methods could represent another promising therapeutic approach for CaP.

***Expert opinion:*** Apart from their immense biomarker potential, KLKs also hold promise as the basis of novel CaP therapeutics.

**Keywords:** aptamers, gene expression targeting, imaging, immunotherapy, kallikrein-related peptidases, kallikreins, microRNAs, pro-drugs, prostate cancer, protease inhibitor, prostate-specific antigen, serine proteases, small-interfering RNA, tissue kallikrein

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## 1. Introduction

Tissue kallikrein (KLK1) and kallikrein-related peptidases (KLK2 – KLK15) form a multifaceted group of secreted serine proteases, referred to as KLKs. *KLK* genes form an uninterrupted ~ 300 kb long cluster on chromosome 19q13.3 – q13.4 (**Figure 1**), whereas plasma kallikrein (*KLKB1*) is located on 4q35 and is not closely related, in terms of structure and function, to *KLK*s [1-4]; for this reason KLKB1 will not be discussed further in this review.

KLKs form the largest group of serine proteases in the human genome and are characterised by common structural properties. The five coding exons of *KLK1* – *KLK15* genes encode for pre-pro-protein molecules bearing the signal peptide, in order to be secreted as pro-enzymes that will be ultimately cleaved to produce the active (chymo)trypsin-like protease (**Figure 1**) [2-5]. KLKs possess a typical tertiary structure consisting of β-sheets that give rise to two β-barrels with two solvent exposed α-helices. The common catalytic triad His57-Asp102-Ser195 (chymotrypsinogen numbering) is located on an active-site cleft, after the crossing

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**Article highlights.**

- Several members of the cancer biomarker family of tissue kallikrein and kallikrein-related peptidases (KLKs) are significantly deregulated in prostate cancer (CaP) and are implicated in the facilitation of prostate tumorigenesis and disease progression through the promotion of extracellular matrix degradation, tumour cell invasion, angiogenesis and metastasis. Aberrant KLK activity on other proteases, growth factors, cell surface receptors and hormones promotes these tumour promoting changes.
- KLKs are targeted by diverse naturally occurring substances, including proteinaceous inhibitors, low-molecular-weight peptides and $Zn^{2+}$. Engineered KLK inhibitors include protein/peptide-based inhibitors and small-molecule inhibitors. MDPK67b is a serpin-based inhibitor designed to block KLK2, KLK4 and KLK14 activity and is under evaluation in first-in-human trials as a CaP therapeutic. Sunflower trypsin inhibitor-FCQR-$Asn_{14}$ is another bioengineered potent, selective, stable and bioavailable KLK4 inhibitor which holds promise as a potential CaP therapeutic. Modified short peptides, activity-blocking antibodies, small-molecule inhibitors and aptamers that can target CaP-associated KLKs (KLK1 – 4, 7 and 14) have also been developed.
- The KLK3-activated pro-drugs L-377,202 and PRX302 have entered Phase I and Phase II clinical trials, respectively, as therapeutics for prostate tumours and have shown a favourable toxicity profile.
- The KLK3-based PROSTVAC® vaccine has already entered Phase III clinical trials for evaluation as a novel immunotherapeutic for CaP. Immunotherapeutic anti-KLK3 engineered antibodies have been developed. KLK4 is also a promising candidate for CaP immunotherapy.
- Targeting and restoring *KLK* expression via RNA interference methods could represent another promising therapeutic approach for CaP.
- KLK-based drugs have been developed for other pathological conditions, including skin diseases (KLK7 and multi-KLK inhibitors) and asthma (anti-KLK1 monoclonal antibody).

This box summarises key points contained in the article.

point of the two β-barrels. This cleft hosts distinct subsites that also interact with and align the substrate to the catalytic triad [5-10].

Despite the common structural characteristics shared by KLKs, these proteases act on an astoundingly diverse array of substrates (**Figure 1**). KLKs can process extracellular matrix (ECM) proteins, cell adhesion proteins, growth factors, cell surface receptors, hormones and other proteases, including pro-KLKs and matrix metalloproteinases (MMPs). The multifaceted activity of KLKs allows them to regulate central, yet dissimilar, physiological processes such as blood pressure regulation, skin desquamation, semen liquefaction and neuronal remodelling [4-7,11-13].

For such a broad biological function to be achieved, KLKs need to be firmly regulated at multiple levels. *KLK* transcription is regulated by steroid hormones and methylation status [5,14]. Post-transcriptionally, microRNAs (miRNAs) have been identified as an imperative way of controlling *KLK* expression [14]. At the post-translational level, pro-KLKs are activated by other KLKs via cross-cleavage, including even auto-cleavage (KLK activome) or by other peptidases [7,12,13]. Even after KLK activation, a final regulatory mechanism exists: a wide-ranging ensemble of endogenous KLK inhibitors [15].

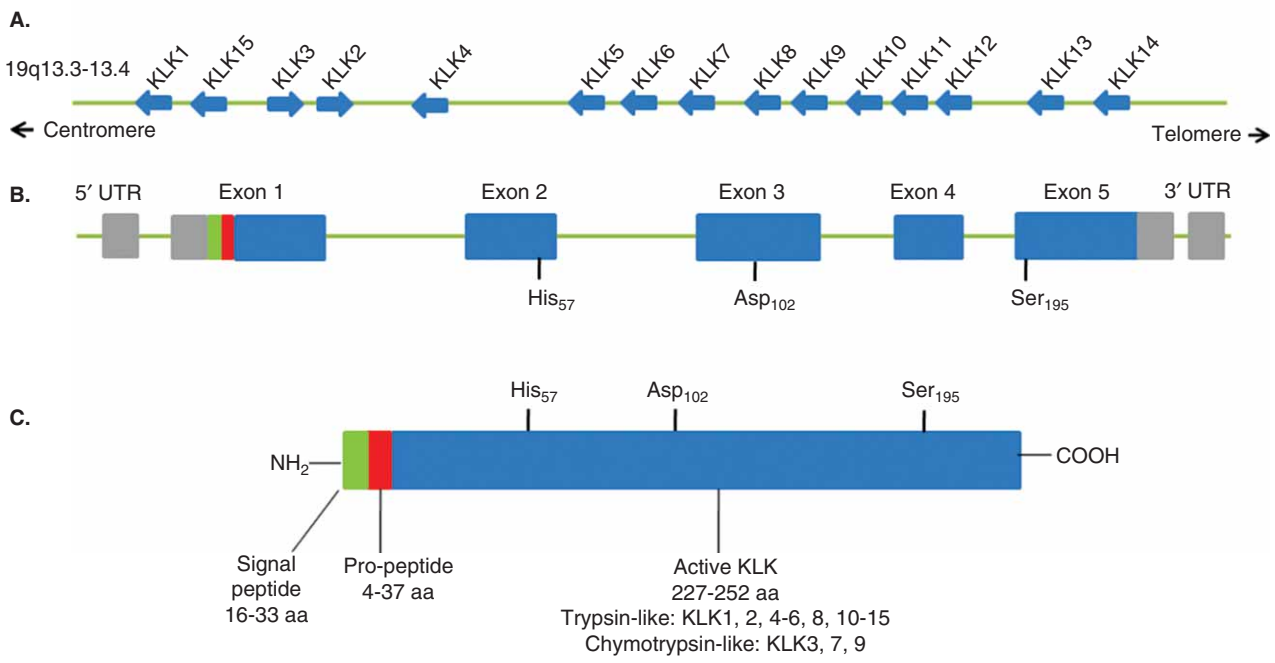
Nonetheless, the expression and/or activity of KLKs are found to be deregulated in many pathologies. Non-malignant conditions associated with KLK malfunction include, among others, skin diseases, neurodegenerative diseases and asthma [13,16]. KLKs have also become renowned for their continuously reported implication in processes considered as hallmarks of cancer, including tumour growth, angiogenesis, invasion and metastasis [7,12,17]. In prostate cancer (CaP), KLKs are involved in the promotion of cell invasiveness, induction of tumour growth, facilitation of epithelial-to-mesenchymal transition (EMT), degradation of ECM and bone metastasis [18]. Interestingly, every single kallikrein-related peptidase has been proposed as a biomarker for at least one human malignancy [17,19,20]. KLK3, or prostate-specific antigen (PSA), as it is largely known, is the most widely used biomarker in the clinic. Despite recent criticism, KLK3 testing is currently applied for the screening, diagnosis, prognosis and treatment monitoring of CaP [21].

The continuing high incidence of CaP globally [22], the decreased survival of patients suffering from metastatic disease [23] and the limitations of currently used therapies transform the necessity of novel CaP drugs into a major clinical priority. Taking into account the multifactorial involvement of several KLK members in CaP pathobiology [18], it would be rational to consider KLK activity and/or expression as a promising target of personalised therapeutics.

## 2. Currently used therapeutics in CaP: limitations and unmet medical needs

Androgen deprivation therapy (ADT) remains the golden standard for the management of patients with advanced CaP not suitable for definite treatment. ADT is also used as an adjuvant therapy for high-risk localised CaP patients undergoing radical radiotherapy and for confronting disease progression following initial treatment [24].

Medical castration using long-acting luteinising-hormone-releasing hormone (LHRH) agonists (buserelin, goserelin, leuprorelin, triptorelin) and concomitant therapy with non-steroid anti-androgens (nilutamide, flutamide, bicalutamide) is used to generate castrate levels of serum testosterone. Additionally, LHRH antagonists are effective in rapidly decreasing the testosterone levels without flare; however, due to histamine-mediated side effects, they are currently FDA approved only for metastatic and symptomatic CaP (abarelix) [25].




**D.**

**KLK Substrates**

| ECM proteins | Seminal plasma | Blood | Growth factors & hormones | Proteases & enzymes | Cell adhesion & surface receptors |
|---|---|---|---|---|---|
| Collagen I-IV | Seminogelin I and II | Kininogen | IGFBP1-6 | proKLK1-15 | PAR1, 2, 4 |
| Fibronectin | Prostatic acid | Angiotensinogen | TGFβ | proMMP2,9 | uPAR |
| Vitronectin | Phosphatase | Fibrinogen | PTHrP | pro-uPA | E-cadherin |
| Laminin | | Plasminogen | GHRH | proHGF activator | Desmoglein 1 and 2 |
| Enamelin | | Prorenin | GH | | |
| | | Bradykinin B2 Receptor | Somatostatin | | |

**Figure 1. The common structural characteristics of KLKs at gene and protein levels, as well as a brief presentation of their most well-characterised substrates are shown. A.** The genomic localisation of *KLK* gene family on 19q13.3 – q13.4 is shown. The direction of transcription is indicated by the arrows. **B.** A schematic presentation of the common structural characteristic of *KLK* genes (five coding exons, four introns and location of the catalytic triad codons). **C.** An overview of the pre-pro-protein structure of KLKs (location of the signal peptide, pro-peptide, catalytic triad and mature enzyme) is shown. **D.** The most commonly reported groups of KLK substrates (ECM proteins, seminal plasma components, blood components, growth factors/hormones, proteases/enzymes, cell adhesion molecules/cell surface receptors) are shown.

ECM: Extracellular matrix; GH: Growth hormone; GHRH: Growth-hormone-releasing hormone; HGF: Hepatocyte growth factor; IGFBP: Insulin-like growth factor binding protein; KLK: Tissue kallikrein and kallikrein-related peptidase; MMP: Matrix metalloproteinases; PARs: Protease-activated receptors; PTHrP: Parathyroid hormone-related protein; TGFβ: Transforming growth factor β; uPA: Urokinase-type plasminogen activator; uPAR: uPA receptor.

Despite chemical castration, the majority of patients progress to lethal castrate-resistant prostate cancer (CRPC). Secondary hormonal therapy for disease progression after ADT includes anti-androgen withdrawal and/or switching to an alternative anti-androgen (initial flutamide to bicalutamide and *vice versa*) [24,25], novel therapeutic agents such as the CYP17 inhibitors ketoconazole [26] and abiraterone, approved for chemotherapy-naïve metastatic CRPC [27], as well as the novel androgen receptor (AR) antagonist enzalutamide (EDV3100) approved for docetaxel-treated metastatic CRPC [28]. First-line chemotherapy for CRPC includes mitoxantrone, estramustine and docetaxel; TAX-327 and SWOG 99-16 trials highlight docetaxel survival benefits compared to mitoxantrone [29,30]. Recently, cabazitaxel was approved for the treatment of patients with metastatic CRPC who progress despite docetaxel treatment [31]. Focusing on the palliative therapy of CRPC patients with bone metastasis, zoledronic acid [32], denosumab [33] and radium-223 [34] have been approved and used in clinical practice. Finally, clinical trials of immunotherapy-based treatments led to the approval of sipuleucel-T, an autologous cellular immunotherapy approach for asymptomatic or minimally symptomatic metastatic CRPC [35].

Although hormone therapy significantly improves the progression-free survival of patients before developing CRPC,

it is accompanied by harmful side effects and a downgrade in patients' quality of life. Malfunctions of long-term ADT include increased risk of diabetes mellitus, cardiovascular disease and myocardial infarction, metabolic syndrome, hyperlipidemia, non-metastatic bone fractures, hot flashes, loss of libido and erectile dysfunction. Additionally, chemotherapy can only benefit patients' survival on the order of months and with numerous side effects, such as nephro- and hepato-toxicity, low white blood cell counts and thus lower resistance to infections, thrombocytopenia, anaemia, fatigue, diarrhoea, nausea and hair loss. Finally, immunotherapy via sipuleucel-T is considered a complex and expensive procedure [24,25].

## 3. KLKs: a family of promising therapeutic targets for CaP

The expression of the majority of *KLK*s is significantly deregulated in prostate tumours. *KLK2* and *KLK4* are significantly overexpressed, at both the mRNA and protein levels, moving from normal or benign prostate epithelium to high-grade prostatic intraepithelial neoplasia (HGPIN) lesions and CaP [36-42]. Additionally, *KLK14* and *KLK15* expressions are also upregulated in CaP compared to benign prostate tissues, as well as in advanced disease [18]. Of significant interest, *KLK3* expression is downregulated in CaP and HGPIN lesions compared to benign and normal epithelium [40-43]. Although *KLK3* downregulation contradicts with its elevated serum levels in CaP patients, the disruption of prostate tissue architecture that take place during the progression of prostate tumours is responsible for its greater secretion into circulation. Moreover, *KLK5* and *KLK7* expression levels are reduced in CaP [18]. Finally, increased methylation of *KLK6* and *KLK10* promoters has been reported in CaP compared to benign epithelium [44].

The abovementioned deregulation of the majority of *KLK*s triggers an irreversible impact upon their substrates [18]. Several KLK members facilitate prostate tumorigenesis and disease progression through the development of an oncogenic microenvironment for prostate cells (**Figure 2**).

KLKs are able to activate the IGF-IGFR axis in prostate tissues. More precisely, KLK2 [45], KLK3 [45,46], KLK4 [47] and KLK11 [48] cleave IGF-binding proteins and increase IGFs availability in prostate microenvironment. This KLK-mediated overactivation of IGF-IGFR signalling induces mitogenic and anti-apoptotic stimuli in prostate cells which are essential for prostate tumorigenesis [49,50]. Moreover, KLK-related proteolytic activation of protease-activated receptors (PARs), a G-protein-coupled cell surface receptor family, launches intracellular cascades enhancing prostate cell proliferation and migration. More precisely, KLK2 and KLK4 have been documented to cleave PAR1 (KLK4) and PAR2 (KLK2 and KLK4) leading to enhanced proliferation of DU145 and PC3 cells through the activation of extracellular signal-regulated kinase (ERK) signalling [51,52]. Cleavage of PAR1 and activation of PAR1-induced intracellular cascades has also been attributed to KLK1. Treatment of DU145 cells with KLK1 resulted in a PAR1-related induction of their migration and invasiveness [53].

The expression of prostate-cancer related KLKs depends on AR transcriptional activity and thus to the binding of AR to *KLK* promoters. Androgen response elements have been identified for *KLK2* – *KLK4* [5]. However, recent findings have highlighted that AR activity is also significantly enhanced by KLK2, KLK3 and KLK4 in prostate cells. More precisely, KLK2 interaction with ARA70, an AR co-activator, was found to be essential for AR transactivation in CaP cell lines as well as the maintenance of AR activity despite the presence of anti-androgens. Small-interfering RNA (siRNA)-mediated silencing of *KLK2* results in reduced cell growth and induces apoptosis in CaP cell lines [54].

Focusing on KLK4, its overexpression in CaP is essential for the maintenance of AR and mammalian target of rapamycin (mTOR) signalling in prostate cells. Protein–protein interactions between KLK4 and promyelocytic leukaemia zinc finger protein (PLFZ), an inhibitor of AR and mTOR1 pathways, suppress PLFZ stability and activity, facilitating in this way AR and mTOR1 mitogenic and anti-apoptotic signalling. Knockdown of *KLK4* expression in CaP cells leads to reduced AR mRNA levels and transcriptional activity, highlighting a positive feedback loop between KLK4 and AR in CaP [55]. Additionally, KLK4-mediated activation of PAR1 in the surface of prostate stroma cells leads to increased release of IL6 and the subsequent overactivation of AR transcriptional activity, through ERK and signal transducer and activator of transcription 3 intracellular pathways [52,56,57]. Similarly, positive regulation of AR expression levels has also been documented for KLK3. More precisely, stable transfection of CaP cells with small hairpin RNA (shRNA) against *KLK3* was found to reduce both mRNA and protein levels of AR, underlying the necessity of KLK3 for AR signalling [58]. Moreover, KLK3 interacts with ARA70 and stimulates ARA70-induced AR transactivation, which leads to the increased cell growth of AR-positive CaP cell lines [59]. The abovementioned positive feedback loop of KLK2, KLK3 and KLK4 with AR signalling highlights their significance for AR-related tumorigenic stimuli in prostate cells and the maintenance of AR signalling in CRPC.

In CaP, KLKs are also well documented to promote ECM protein degradation and thus to disrupt the physical barriers protecting from cancer invasion and metastasis. KLK2 and KLK3 have been found to directly cleave fibronectin and laminin, whereas KLK14 is also able to cleave collagens I – IV [18]. Moreover, KLK2 and KLK4 are able to stimulate ECM degradation via the activation of plasmin and MMPs. More precisely, overexpression of KLK2 and KLK4 in CaP promotes the accumulation of urokinase-type plasminogen activator (uPA), due to increased cleavage of pro-uPA, leading to plasmin and MMPs activation through uPA-uPA receptor (uPAR) axis [60,61]. Moreover, KLK2 can also facilitate uPA-uPAR system-related proteolysis by the degradation of plasminogen activator inhibitor-1 (PAI-1) [62], an inhibitor of

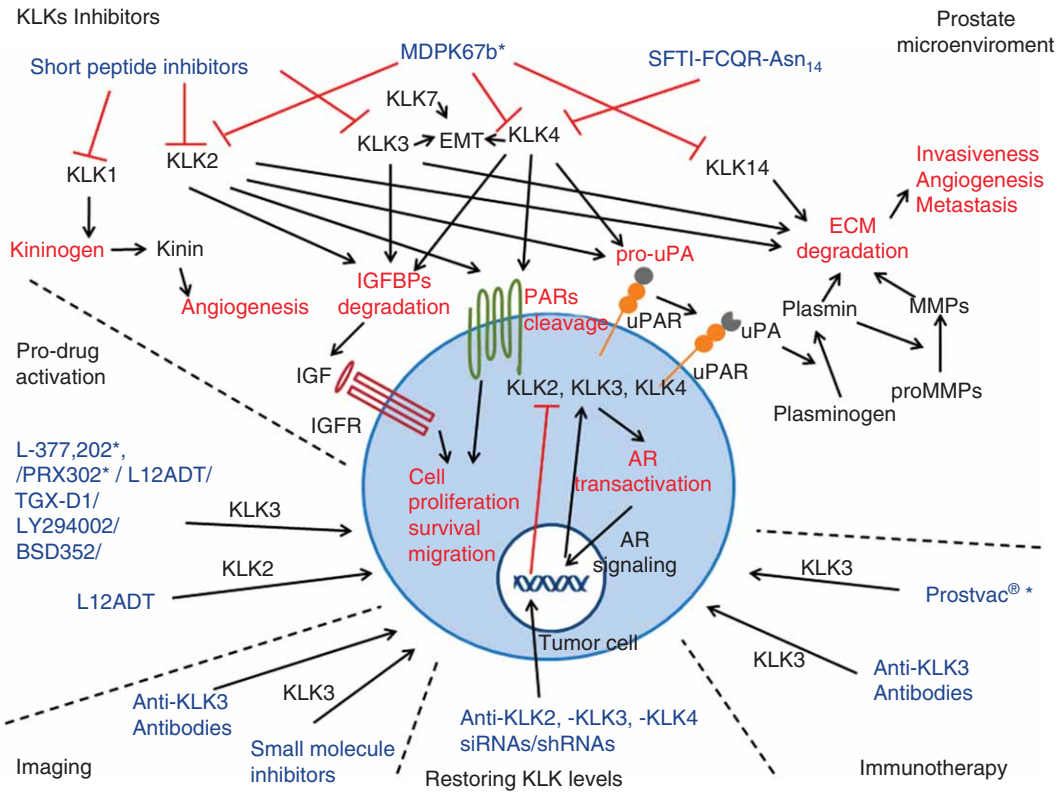



**Figure 2. The implication of KLKs in prostate cancer pathobiology and the various approaches of inhibiting and exploiting KLK activity and/or expression for the development of novel therapeutics are shown.** KLKs promote prostate cancer development and progression via AR transactivation, ECM degradation, stimulation of the uPA-uPAR-MMPs pathway, the IGF-IGFR axis, PARs activation and EMT facilitation. Approaches for exploiting KLKs as therapeutic targets include a variety of synthetic KLK inhibitors, activation of pro-drugs, KLK-related immunotherapy, KLK3-based imaging and reprogramming *KLK* expression trough RNA interference.

*Indicates that the therapeutic agent has entered clinical trials for prostate tumours.

AR: Androgen receptor; ECM: Extracellular matrix; EMT: Epithelial-to-mesenchymal transition; IGF: Insulin-like growth factor; IGFBP: IGF binding protein; IGFR: IGF receptor; KLK: Tissue kallikrein and kallikrein-related peptidase; MMPs: Matrix metalloproteinases; PARs: Protease-activated receptors; uPA: urokinase-type plasminogen activator; uPAR: uPA receptor.

uPA, whereas KLK3 can directly activate MMP2 from its precursor (pro-MMP2) [63]. Finally, the identification of uPAR as a direct substrate of KLK4 [64] highlights a possible key regulatory role of KLK4 on uPA-uPAR system-related proteolysis and cell adhesion interaction with vitronectin and integrins.

EMT represents a hallmark cellular process of the progression of epithelial solid cancers. Both KLK3 and KLK4 have been revealed to promote EMT-like changes in prostate tumours. More precisely, transformation of PC3 to overexpress KLK3 and KLK4 resulted in suppressed E-cadherin and enhanced vimentin expression, increased migration potential and spindle-shaped morphology [37,65]. Similar EMT-like changes are also triggered in CaP cells by the overexpression of KLK7 [66].

KLK1, which possesses kininogenase activity, promotes angiogenesis via the production of kinin and the subsequent activation of kinin B12 and B2 receptors [67,68], whereas an angiogenesis preventing role has been attributed to KLK3. Increased angiogenesis has been observed in CaP specimens with downregulated KLK3 levels [69]; KLK3 reduces endothelial cells proliferation and migration, attenuates their responses to the angiogenesis stimulators fibroblast growth factor-2 (FGF2) and VEGF and reduces metastatic disease in mouse models [70,71].

Finally, KLK3 has been documented to facilitate bone marrow metastasis of CaP [72,73]. Using antibody-mediated blockage of KLK3 or siRNA-mediated knockdown of *KLK3* expression, CaP cells adhesion to bone marrow endothelial cells was significantly diminished [74]. The bone metastasis promoting role of KLK3 is also supported by the parathyroid hormone-related protein and latent TGF-β cleavage. Similarly, the upregulation of *KLK4* expression in CaP cells during their co-culture with the osteoblastic-like SaOs cell line promotes their enhanced attachment to bone matrix proteins [75].

**Table 1. Summary of therapeutic approaches based on KLK inhibitors and KLK-targeting aptamers.**

| Type/agent | Target | Relevance | Description/therapeutic impact | Ref. |
|---|---|---|---|---|
| *Engineered KLK inhibitors* | | | | |
| Protein- and peptide-based inhibitors | | | | |
| SERPIN-type inhibitors | | | | |
| MDPK67b | KLK2, 4, 14 | Prostate cancer | Bio-scaffolding produced ACT-based inhibitor. Dose-dependent inhibition of prostate tumour growth in mouse xenografts. Evaluation in first-in-human trials. | [89,90] |
| DM107 | Multi-KLK | Skin diseases | Bio-scaffolding produced ACT-based inhibitor. Under investigation for lymphoepithelial Kazal-type-related inhibitor-associated skin diseases. | [89] |
| Naturally occurring proteinaceous-type inhibitors | | | | |
| SFTI-FCQR-$Asn_{14}$ | KLK4 | Prostate cancer | Bio-scaffolding produced SFTI-based inhibitor/ Stable in prostate cancer cell cultures. | [91] |
| SFTI-WCTF | KLK7 | NA | Bio-scaffolding produced SFTI-based inhibitor | [92] |
| Short peptides inhibitors | | | | |
| Benzyloxycarbonyl-Ser-Ser-Lys-Leu-(boro)Leu | KLK3 | Prostate cancer | Peptidyl boronic acid inhibitor/restricting the development of subcutaneous prostate cancer xenografts | [94] |
| Ahx-FSQn(boro)Bpg | KLK3 | Prostate cancer | Peptidyl boronic acid inhibitor/retardation of tumour growth and reduction of prostate-specific antigen serum levels in *in vivo* animal models | [95] |
| P3-D-Phe-conjugated synthetic peptides, L-4-aminomethylphenylalanine-conjugated synthetic peptides | KLK1 | NA | Synthetic peptide-based inhibitors | [15,96] |
| FE999024 | KLK1 | Various diseases | KLK1-specific peptide-based inhibitor. Inhibits the invasion of breast cancer cells (*ex vivo* model). Also showed promising results against allergic inflammation, virus-induced lung inflammation and acute pancreatitis | [15,16] |
| Pure peptides | KLK2 | NA | Pure peptide inhibitors optimised by cyclisation | [15,96] |
| Antibodies | | | | |
| DX-2300 | KLK1 | Asthma | Anti-KLK1 monoclonal antibody/under investigation for the treatment of asthma | [97] |
| Small-molecule inhibitors | | | | |
| Nitrogen-containing heterocyclic compounds | KLK7 | Skin diseases | Under investigation for potential application in skin diseases. Promising results in animal models. | [16] |
| 2-Azetidinone and triazole compounds | KLK3 | NA | Monocyclic β-lactam derivative (2-azetidinone). Triazole compounds identified by the screening of a chemical library of 50,000 compounds | [98,99] |
| 1,2,4-Triazole derivatives | KLK5, 7, 14 | Skin diseases | Potential usefulness for skin diseases, not cytotoxic to healthy human keratinocytes | [100] |
| *Nucleic acid aptamers* | | | | |
| RNA, DNA aptamers | KLK3, 6 | NA | Synthetic DNA or RNA molecules selected from pools of random-sequence oligonucleotides to specifically bind protein- and peptide-targets | [102-104] |

ACT: $\alpha_1$-antichymotrypsin; KLK: Tissue kallikrein and kallikrein-related peptidase; NA: Study not available yet; SFTI: Sunflower trypsin inhibitor.

## 4. Inhibiting and exploiting KLK activity: applications for CaP management

KLK inhibitors can be divided into naturally occurring/endogenous inhibitors and engineered synthetic inhibitors. Apart from the easily perceptible advantages of inhibiting KLKs centrally involved in pathophysiological conditions (**Table 1**, **Figure 2**), other ways of utilising KLK activity, such as KLK-mediated activation of pro-drugs, KLK-directed immunotherapy and KLK-based clinical imaging

(**Table 2**, **Figure 2**), can aid in confronting human pathologies and especially CaP.

In this part, we summarise the currently available approaches for inhibiting, enhancing and making use of KLK activity, centring the interest in CaP and therefore in KLKs1 – 4, 7 and 14.

### 4.1 Nature's repertoire for controlling KLK activity: metal ions, proteinaceous and other molecules acting as KLK inhibitors

Having in mind the broad physiological role of KLKs and the permanent nature of protein hydrolysis, it is logical to hypothesise that evolutionary pressure has led to a variety of mechanisms for restricting unnecessary proteolysis by KLKs. At the protein activity level, this is reflected by the expression of all KLKs as inactive precursors (zymogens) [13], as well as by the noticeable occurrence of diverse endogenous inhibitors, including large proteinaceous molecules and metal ions. Naturally occurring KLK inhibitors that are produced in plants or bacteria comprise proteinaceous substances, low-molecular-weight peptides and non-peptide agents.

#### 4.1.1 *Inhibition by $Zn^{2+}$*

It is well established that endogenous cations can regulate KLK activity. The most remarkable regulation comes from $Zn^{2+}$, which can inhibit KLKs 2 – 5, 7, 8, 12 and 14, and thus it is considered as a central, reversible regulator of KLK activity, especially for CaP-related KLKs [15]. Prostatic fluid contains the highest concentration of $Zn^{2+}$ in the human body [76], which inactivates KLKs; however, during ejaculation, it is mixed with epididymal fluid, containing high amounts of semenogelins that effectively bind $Zn^{2+}$. This leads to KLK activation, semenogelin cleavage and semen liquefaction [13,77]. A characteristic of malignant prostate is the significant decrease of $Zn^{2+}$ concentration [78]. It would be logical to hypothesise that one of the tumour-progression mechanisms in CaP could be that decreased $Zn^{2+}$ levels lead to aberrant activation of CaP-promoting KLKs. Given the already studied tumour-suppressor properties of $Zn^{2+}$ in CaP, restoration of $Zn^{2+}$ levels has already been considered as a potential CaP therapy [78]. Considering that the systemic administration of $Zn^{2+}$ can introduce toxicity to multiple tissues, the intratumoral injection of $Zn^{2+}$ comprises a more promising therapeutic strategy. This approach has been shown to reduce prostate tumour growth in mice models and to extend survival periods, without causing toxicity issues in other organs [79].

#### 4.1.2 *Proteinaceous inhibitors*

The most studied proteinaceous inhibitors are SERine Protease INhibitors (serpins), Kazal-type inhibitors and Kunitz-type inhibitors. These inhibitors can obstruct KLK activity by two main mechanisms: i) the canonical mechanism (Kazal-type, Kunitz-type inhibitors) that includes direct competition with the substrate for binding to the active site; and ii) the irreversible 'spring-suicide' mechanism (serpins), which includes insertion of the inhibitor's reactive loop in the active site, cleavage of the serpin–peptide bond, rearrangement of both the protease's and the inhibitor's structure and, ultimately, disruption of the catalytic triad [10,15,80].

Serpins occupy a notable portion of the human serum and can inhibit most of the KLK members. Focusing on CaP-related KLKs, KLK2 is inhibited by antithrombin III (AT), $\alpha_2$-antiplasmin (AP), proteinase C inhibitor (PCI), proteinase inhibitor 6 (PI-6) and PAI-1. Interestingly, KLK2–PI-6 complexes are indicative of tissue damage and necrosis in CaP, whereas the KLK2–PAI-1 complex hampers the inactivation of uPA by PAI-1, thus favouring CaP progression. KLK3 activity can be blocked by $\alpha_1$-antitrypsin (AAT), $\alpha_1$-antichymotrypsin (ACT), PCI and the monocyte/neutrophil elastase inhibitor. KLK4 serpin inhibitors are AAT and ACT. KLK7 and KLK14 are inhibited by AAT, ACT, AP, PCI and kallistatin; KLK14 is additionally inhibited by AT. Moreover, KLK1 is strongly inhibited by kallistatin [15,80,81]. Recently, the visceral adipose tissue-derived serpin (vaspin or serpinA12) was shown to target KLK7 [82].

The relationship between Kazal-type inhibitors and KLKs has been made apparent from studies in Netherton syndrome (NS), a genetic disease manifested by excessively disrupted skin homeostasis. NS is associated with mutations in the serine peptidase inhibitor Kazal-type 5 (*SPINK5*) gene, encoding for the lymphoepithelial Kazal-type-related inhibitor (LEKTI), that lead to insufficient KLK blocking and thus KLK hyperactivity [12,15]. Interestingly, there have been reported cases of NS patients who developed multiple skin malignancies [83]. LEKTI is processed to produce 15 individual subunit fragments that can differentially modulate KLKs 5 – 7, 13 and 14 [15]. Recent data show that the CaP-associated SPINK1 was found to inhibit KLK2 [84].

The most studied Kunitz-type inhibitor is aprotinin or bovine pancreatic trypsin inhibitor which was initially found to inhibit KLK1, but later was found to inhibit also KLKs 2, 4, 5, 12 and 14 [10,15]. Plants also produce potent Kunitz-type inhibitors; notably, the soybean trypsin inhibitor inhibits KLKs 4 – 6 and 14 [15]. Kunitz-type inhibitors are also produced by the human organism [80]. Other, canonical, non-selective, proteinaceous KLK inhibitors include elafin-like protease inhibitor antileucoprotease (inhibiting KLK7), the medical leech (*Hirudo medicinalis*)-isolated hirustasin (inhibiting KLK1) [15] and the sunflower trypsin inhibitor(SFTI) (blocking KLK4) [80].

Regarding lower-molecular-weight naturally occurring agents, cyclic depsipeptides, produced from the cyanobacteria *Chondromyces* represent the most promising agents and are under investigation by Novartis AG as potent KLK7 inhibitors [10,15].

### 4.2 Resynthesizing nature's regulatory molecules: engineered KLK inhibitors as emerging therapeutics

Naturally occurring KLK inhibitors described in Section 4.1 cannot be considered as ready-to-use therapeutics; despite

**Table 2. Summary of therapeutic approaches based on KLK activation of pro-drugs, KLK-driven immunotherapy and KLK-related imaging.**

| Type/agent | Target | Relevance | Description/therapeutic impact | Ref. |
|---|---|---|---|---|
| *Activation of pro-drugs* | | | | |
| L12ADT-based | KLK2 | Prostate Cancer | Chemically modified form of a thapsigargin analogue (L12ADT) conjugated with a KLK2-cleaved heptapeptide/inhibition of CaP cell lines *in vitro* growth and anti-tumour effect in corresponding *in vivo* models | [106] |
| L-377,202 | KLK3 | Prostate Cancer | Doxorubicin conjugated with KLK3-cleaved peptide/restrained prostate tumour growth in *in vivo* animal models and decreased toxicity compared to doxorubicin in KLK3-negative cells. Phase I clinical trials. | [107,110] |
| PRX302 | KLK3 | BPH | Aerolysin conjugated with a KLK3-cleaved peptide/intraprostatic administration in BPH patients. Phase II clinical trials. | [108,111] |
| TGX-D1-based | KLK3 | Prostate Cancer | Conjugated PI3K-β inhibitor TGX-D1 with a KLK3-cleaved peptide and the N terminus human epidermal growth factor receptor 2-binding domain | [112] |
| LY294002-based | KLK3 | Prostate Cancer | Chemically modified form of quercetin (PI3K inhibitor) with a KLK3-cleaved peptide/induction of apoptosis in prostate cancer cells | [113] |
| BSD352-based | KLK3 | Prostate Cancer | Anti-VEGF and anti-fibroblast growth factor peptides, conjugated with a KLK3-cleaved sequence/reduced tumour growth, induced apoptosis and anti-angiogenic properties in *in vitro* and *in vivo* studies | [114] |
| L12ADT-based | KLK3 | Prostate Cancer | Chemically modified form of a thapsigargin analogue (L12ADT) conjugated with a KLK3-cleaved peptide | [115] |
| D-arginine octamer protein-transduction domain-based | KLK3 | Prostate Cancer | D-arginine octamer protein-transduction domain conjugated with a KLK3-cleaved peptide/vector for the increase drug uptake by prostate cancer cells | [116] |
| *Immunotherapy* | | | | |
| PROSTVAC | KLK3 | Prostate Cancer | Pox viral-based vaccine expressing KLK3 and three major T-cell co-stimulatory molecules (TRICOM)/inhibition of prostate cancer cell proliferation and tumour growth, increased overall survival and reduction in the risk of death. Phase III clinical trials. | [120-124] |
| Anti-KLK3 engineered antibodies | KLK3 | Prostate Cancer | Anti-KLK3 antibodies/induced T-cell stimulation and anti-tumour response towards prostate cancer cells | [125-127] |
| 5-fluoro-2′-deoxyuridine anti-KLK3 IgG immunoconjugate | KLK3 | Prostate Cancer | Anti-KLK3 antibodies conjugated with the chemotherapeutic agent 5-fluoro-2′-deoxyuridine/enhanced KLK3 expressing tumour-specific cell death | [128] |
| *KLK3-based clinical imaging tools* | | | | |
| $^{125}$I-anti-KLK3 mAb | KLK3 | Prostate Cancer | $^{125}$I-labelled monoclonal antibody against free KLK3/effective targeting of free KLK3 in LNCaP mouse xenografts | [132] |
| Carbobenzyloxy-Ser-Ser-Gln-Nle-(boro)-Leu | KLK3 | Prostate Cancer | Carbobenzyloxy-Ser-Ser-Gln-Nle-(boro)-Leu conjugated with a bulky metal chelating group | [133] |

ADT: Androgen deprivation therapy; BPH: Benign prostatic hyperplasia; KLK: Tissue kallikrein and kallikrein-related peptidase; PI3K: Phosphoinositide 3-kinase inhibitor.

their great inhibitory capacity, they are characterised by reduced selectivity. Their application as therapeutic agents demands rational structure redesigning. Apart from improved selectivity, an ideal pharmaceutically relevant KLK inhibitor should encompass the following essential properties: i) a favourable Absorption, Distribution, Metabolism, Excretion, Toxicity (ADMET) profile [85]; ii) a low immunogenicity potential [86]; iii) a stable structure that will yet retain all necessary post-translational modifications [87], iv) high yield and low cost during production upscaling [88]; as well as v) a tissue-specific action.

Interestingly, the approach of bio-scaffolding, that is, the use of endogenous inhibitors as scaffolds for engineered drugs, has provided the first KLK inhibitor (MDPK67b, Med Discovery) ever to reach first-in-human trials for evaluation as a novel CaP drug [89,90].

#### 4.2.1 *Protein- and peptide-based inhibitors: bio-scaffolding, short peptides and antibodies*

MDPK67b is a restructured serpin-type inhibitor manufactured by Med Discovery as a potential CaP therapeutic agent. The basic bio-engineering principle that was followed was the replacement of the favourably accessible reactive site loop of the human serpin ACT with a cleavage site-bait region recognised by KLK2. MDPK67b emerged as the lead compound, inhibiting the CaP-related KLK2, KLK4 and KLK14. Mouse xenograft models bearing DU145-induced KLK2-overexpressing tumours were used as a relevant *in vivo* model to test the efficacy of MDPK67b; it has been reported that this animal model has KLK2 serum levels comparable to those measured in CaP patients. First, it was shown that *KLK2* overexpression led to the formation of more rapidly growing tumours, corroborating in this way the role of KLK2 in CaP progression *in vivo*. A MDPK67b dose-dependent inhibition of prostate tumour growth, reaching up to approximately 90%, was observed. Toxicity studies were carried out in appropriate animal models, including rodents and primates, and MDPK67b showed a favourable toxicity and immunogenicity profile [89,90]. These encouraging results led to the launch of first-in-human clinical trials by Med Discovery in order to investigate the safety, pharmacokinetic and pharmacodynamic profiles of MDPK67b; the ultimate objective is to evaluate this KLK inhibitor as a novel drug for asymptomatic CRPC patients [89]. The so far successful route of MDPK67b can be attributed to the plasticity of ACT's reactive loop and to its low immunogenicity, arising from the endogenous nature of ACT [80,89,90]. The biotech company Dermadis has also exploited ACT as a bio-scaffold for building a multi-KLK inhibitor (DM107) for LEKTI-associated skin diseases [89].

The bioavailable and cell-penetrating 14 amino acid SFTI has also been remodelled by substituting a selected tetrapeptide into the bio-scaffold. The finally selected variant SFTI-FCQR-$Asn_{14}$ is a very potent, selective, stable in CaP cell cultures and bioavailable KLK4 inhibitor [91]. Recently, a novel and potent SFTI-based KLK7 inhibitor, termed SFTI-WCTF, was also developed [92].

Short peptides that are themselves KLKs or mimic natural substrates of KLKs can also be chemically modified to achieve KLK inhibition. A series of synthetic peptide aldehydes, peptidyl boronic acids, β-lactam-based inhibitors and azapeptides have been evaluated as KLK3 inhibitors [93]. Of interest is the benzyloxycarbonyl-Ser-Ser-Lys-Leu-(boro)Leu inhibitor, which has an effect, although limited, in restricting the development of subcutaneous CaP xenografts [94]. Recently, Ahx-FSQn(boro)Bpg, a peptidyl boronic acid-based potent and selective KLK3 inhibitor, containing a bromopropylglycine group, was produced. This compound was shown to generate a significant alteration in KLK3 serum levels of *in vivo* animal models but had only a minimal effect in tumour growth [95]. Synthetic peptide-based inhibitors, containing a P3-D-Phe residue or the non-natural amino acid L-4-aminomethylphenylalanine, as well as the FE999024 peptidic inhibitor have been shown to block KLK1 activity. KLK2 pure peptide inhibitors have also been identified and were further optimised, in terms of inhibition capacity and stability, by peptide cyclisation [15,96].

Antibodies have also been used to block KLK activity. The major advantage of this approach is that other protein areas, apart from the active site, can be targeted, thus enhancing in this way the inhibitor's selectivity. Anti-KLK deactivating antibodies have been developed for KLK1, KLK4, KLK6, KLK12 and KLK13 [15,16,80]. The anti-KLK1 human monoclonal antibody DX-2300, developed by Dyax, holds promise as a potential therapeutic agent for asthma [97].

#### 4.2.2 *Small-molecule inhibitors*

Several small-molecule KLK inhibitors that could prove to possess therapeutic properties have been identified. The monocyclic β-lactam derivative 2-azetidinone has been described as a potent KLK3 inhibitor [98]. Using high-throughput screening of chemical libraries, two compounds of the triazole family were identified as the most promising in terms of non-toxic KLK3 inhibition [99]. Recently, several promising 1,2,4-triazole derivatives that inhibit KLK5, KLK7 and KLK14 were identified [100]. KLK7 can be effectively targeted by nitrogen-containing heterocyclic compounds that show very encouraging results in alleviating key pathological events observed in skin diseases [16]. Finally, KLKs 1 and 6 can also be potently inhibited by recently developed aminopyridine derivatives and *N*-(4-aminomethylphenyl)-2-hydroxy-benzamides, respectively [16].

### 4.3 Nucleic acid aptamers: a new class of KLK targeting compounds

Aptamers are synthetic DNA or RNA molecules selected from pools of random sequence oligonucleotides to specifically bind protein or peptide targets. The binding affinities of aptamers are close to those of antibodies, whereas their small size, engineering plasticity and uncomplicated synthesis

make them attractive as novel protein-targeting therapeutics. Aptamers can be conjugated with polyethylene glycol, toxins or fluorescent moieties in order to get excluded by renal filtration, to act as targeted drugs, or to be used as detection reagents, respectively [101]. Active KLK3 can be selectively targeted by a selected RNA aptamer [102], whereas DNA aptamers can be used to quantify KLK3 levels through biosensors [103]. KLK6 can also be potently targeted by two recently developed highly stable DNA aptamers [104].

### 4.4 Activation of pro-drugs from KLKs: exploiting tissue-specific KLK activity to induce targeted CaP toxicity

Pro-drugs can be developed in order to achieve tissue-specific and, even better, cancer microenvironment-specific activity of toxic drugs and thus optimal tumour inhibition with minimal systemic toxicity [105]. In case of KLKs, the pro-drugs that have been developed can be generally described as non-toxic conjugates of peptide sequences, selectively recognised by KLK2 or KLK3, with commonly used cytotoxic agents. The relevant mechanism of action includes site-directed release of the cytotoxic substance specifically in the cancer milieu where KLK activity is evident. KLK activated pro-dugs hold promises as a novel targeted therapy for CaP.

Given the clear CaP-promoting role of KLK2 [18], this KLK may not only be viewed as a good candidate for targeted CaP therapy but it can also itself target pro-drugs to provide selective toxicity. A KLK2 heptapeptide substrate has been conjugated with an analogue of the toxic, non-specific, apoptosis-inducing ATPase pump inhibitor thapsigargin, named L12ADT. The synthesised pro-drug has been shown to inhibit the *in vitro* growth of CaP cell lines expressing KLK2 and also has been shown to retain a significant anti-tumour effect in corresponding *in vivo* models; nonetheless, prolonged intravenous administration caused local vein toxicity [106].

The arsenal of KLK3-activated pro-drugs against prostate tumours is far more thriving and advanced, given that some of these substances have already been successfully evaluated in Phase I and Phase II clinical trials [107,108]. Active KLK3 is found almost exclusively in prostate tissue and serum KLK3 activity is often blocked by serpins and other inhibitors [13]. Consequently, a highly targeted drug delivery is guaranteed via the pro-drugs approach. Parts of naturally occurring KLK3 substrates, such as semenogelin I and semenogelin II [13] have been optimised and conjugated with the anthracycline doxorubicin, which represents an extensively used chemotherapeutic agent [109]. The most promising of these engineered compounds is L-377,202 [107]. Initial experiments showed that L-377,202 had just the right properties to be considered as a promising CaP therapeutic agent: it exhibited decreased toxicity compared to doxorubicin in KLK3-negative cells, whereas at the same time it showed far more potency in restraining prostate tumour growth of *in vivo* animal models [110]. An initial Phase I clinical trial demonstrated that L-377,202 was well tolerated and a safe dose was established for Phase II studies [107]. Cyclophosphamide, vinblastine, 5-fluoro-2′-deoxyuridine and paclitaxel have already been used for the production of analogous KLK3-activated pro-dugs [109].

PRX302 is another KLK3-activated pro-drug that has been designed for non-systemic administration in patients suffering from benign prostatic hyperplasia (BPH). PRX302 can be described as an engineered aerolysin. Aerolysin is a natural cytolytic protein, synthesised by the bacterium *Aeromonas hydrophila* as proaerolysin, an inactive proform that binds to cell surface. Once bound, proaerolysin is activated via cleavage of its inhibitory domain by membrane-located proteases; the active aerolysin forms oligomers, which penetrate the cell membrane forming stable pores, thus inducing instant cell death. In PRX302, the inhibitory domain of proaerolysin has been replaced by a KLK3-cleavable peptidic sequence, leading to active KLK3 site-specific anti-tumour action [111]. Phase I and II clinical trials have already been performed and have provided evidence that show the safety and the efficacy of the intraprostatic administration of PRX302 in BPH patients [108].

Moving to the preclinical level, another pro-drug has been developed containing: i) a N terminus human epidermal growth factor receptor 2 (HER2)-binding domain; ii) a KLK3-cleavable peptide; and iii) the chemotherapeutic phosphoinositide 3-kinase (PI3K) β inhibitor TGX-D1. When this multi-component peptide–drug conjugate is administered to CaP cells *in vitro*, it binds to HER2 located on cell surface. After that, endogenous KLK3 cleaves the peptide sequence to release $NH_2$-S-L-TGX which is then transported into CaP cells, via peptide transporters, and a self-cyclisation process occurs to produce TGX-D1. Interestingly, this bioconstruct shows an enhanced uptake rate, compared to the parent drug TGX-D1 [112]. In an analogous approach, a CaP-specific PI3K inhibitor was produced via conjugation of the chemically modified form of the quercetin analogue LY294002 with a KLK3-cleavable peptide. The resulting pro-drug can effectively promote KLK3-dependent PI3K inhibition, accompanied by induction of apoptosis in CaP cells [113].

In another approach, BSD352, a complicated fusion peptide construct was built by incorporating the following parts: a cell-penetrating domain of the HIV transactivating regulatory protein, a BH3 domain of p53, an anti-VEGF peptide, and an anti-basic FGF peptide. The different parts of BSD352 have been conjugated with a KLK3 substrate in a pro-drug that was found to induce apoptosis in CaP cells, as well as to have anti-angiogenic properties and to inhibit tumour growth both *in vitro* and *in vivo* [114].

As in the case of KLK2, the thapsigargin analogue L12ADT was added to a KLK3-recognised substrate in order to produce a pro-drug for CaP with potent and highly selective *in vitro* and *in vivo* action without any apparent host

toxicity [115]. Another KLK3-based approach that could be used to increase targeted drug uptake by CaP cells has been described. The D-arginine octamer protein-transduction domain (positively charged) was conjugated with a peptide recognised by KLK3 and an octamer-polyanionic segment. Upon KLK3-mediated cleavage, the D-arginine octamer domain enters the cells. The desired drug could be attached to the protein transduction site and thus this system could serve as an effective transporter for the targeted molecular delivery of drugs into CaP cells [116].

Peptide-based activators, pseudopeptide analogues [96,117,118] and antibodies that stabilise KLK3 in its enzymatically active conformation [119] have also been developed. Given the Janus-like behaviour of KLK3 in CaP progression/suppression [18], it is difficult to safely conclude if patients would actually benefit from a KLK3 modifying treatment. Nonetheless, activators of KLK3 could be as well used, after optimisation, in order to enhance the pro-drug activator capacity of KLK3.

### 4.5 KLKs and immunotherapy: a novel approach for CaP treatment

Immunotherapeutic strategies have drawn the attention as a promising CaP treatment option, especially after the first-ever FDA approval for a therapeutic cancer vaccine, that is, sipuleucel-T [120].

PROSTVAC® is a KLK3-based immunotherapeutic plan currently under evaluation in Phase III clinical trials (NCT01322490) for asymptomatic or minimally symptomatic, chemotherapy-naïve, metastatic CRPC patients [121,122]. PROSTVAC is a pox viral-based vaccine which expresses KLK3 and three major T-cell co-stimulatory molecules, known as TRICOM, that is, B7.1 (CD80), lymphocyte function-associated antigen-3, and intracellular adhesion molecule-1.The general mechanism of action of PROSTVAC relies on the endowment of antigen-presenting cells with KLK3 epitopes and the subsequent activation of cytotoxic T cells ($CD8^+$) and helper T cells ($CD4^+$) that orchestrate an attack on KLK3-expressing CaP cells. As a result, both CaP cell proliferation and tumour growth rates are significantly reduced [120-124]. Phase II clinical studies have shown that despite there being no benefit with regard to progression-free survival, PROSTVAC resulted in an 8.5 months increase in overall survival and a 44% reduction in the risk of death. Moreover, it was well tolerated by patients [121-123]. Interestingly, in patients exhibiting longer survival intervals, a decrease in T-regulatory action and an increase in KLK3-mediated T-cell responses were manifested [121].

Immunotherapeutic anti-KLK3-engineered antibodies have also been described. A bispecific murine antibody targeting both human CD3 and KLK3 can arbitrate an anti-tumour response towards CaP cells both *in vitro* and *in vivo* [125]. Another murine anti-KLK3 (IgG1) antibody can induce significant antigen presentation by human dendritic cells and can mediate $CD4^+$ and $CD8^+$ T-cell activation [126]. Recently, an anti-KLK3 IgE antibody was constructed and shown to effectively induce T-cell stimulation and provoke anti-tumour and pro-survival effects when administered in *in vivo* mouse models [127].

Anti-KLK3 antibodies can also be conjugated with chemotherapeutic drugs and can enhance KLK3-expressing tumour-specific cell death *in vivo*, as it has already been described for a 5-fluoro-2′-deoxyuridine IgG immunoconjugate [128].

The study by Wilkinson *et al.* also introduces KLK4 as an immunogenic molecule capable of inducing specific $CD8^+$ cytotoxic T-cell responses *in vitro*, suggesting that KLK4-based immunotherapeutic vaccines should warrant further clinical investigation for CaP patients. Interestingly, potentially immunogenic peptide sequences, similar to that of KLK4, can be also found in other members of the KLK family [129].

### 4.6 KLK3 as a potential clinical imaging tool for prostate tumours

Optimised imaging techniques could be used to selectively detect the presence of malignant prostatic regions, extra prostatic growth and metastatic sites [130].

Monoclonal anti-KLK3 antibodies labelled for imaging purposes have been reported since 1987 [131], although they have showed limitations such as high liver uptake, high activity in blood and non-specific background signal [131,132]. A recently developed $^{125}$I-labelled monoclonal antibody against free KLK3 showed, using Digital Auto Radiography, effective targeting of free KLK3 in LNCaP tumour-bearing mice that was consistent with KLK3 expression sites. Targeting unbound KLK3 for imaging purposes could be more effective compared to targeting complexed KLK3, because free KLK3 is abundantly present proximal to its production sites, whereas KLK3 complexed forms are mainly found in blood circulation. Further evaluation of this technique is needed in order to make it exploitable via positron emission tomography (PET) or single-photon emission computed tomography (SPECT) [132].

Aside from antibodies, KLK3 inhibitors or KLK3-based pro-drug systems can be appropriately optimised to produce useful imaging tools. More precisely, the boronic acid-type KLK3 inhibitor carbobenzyloxy-Ser-Ser-Gln-Nle-(boro)-Leu has been modified by the addition of a bulky metal chelating group to the amino terminal end of this peptide. The fact that this adjustment did not alter the inhibitory capacity of the engineered compound suggests that it is highly promising for potential use in PET- or SPECT-based imaging strategies [133]. Additionally, the KLK3-dependent protein-transduction construct described previously (Section 4.4) could be modified by the conjugation of an appropriate imaging moiety, instead of an anticancer agent, as a cargo that could be selectively delivered inside CaP cells [116].

**Table 3. Summary of therapeutic approaches based on the restoration of *KLK* expression.**

| Type/agent | Target | Relevance | Description/therapeutic impact | Ref. |
|---|---|---|---|---|
| *Restoration of KLK expression* | | | | |
| siRNA-/shRNA-mediated silencing | | | | |
| *KLK2*-targeting | KLK2 | Prostate Cancer | Anti-*KLK2*-specific siRNAs/shRNAs/ suppressed cell growth, accumulation of the cells in G1 cell-cycle phase and activation of apoptosis in LNCaP cells. Detection of smaller tumours in LNCaP xenografts compared to controls | [54] |
| *KLK3*-targeting | KLK3 | Prostate Cancer | Anti-*KLK3*-specific siRNAs/shRNAs/ inhibition of LNCaP cells growth rate and reduced tumour weight and KLK3 secretion of LNCaP xenografts. Attenuation of C4-2B cells adhesion to bone endothelium. | [59,74,137] |
| *KLK4*-targeting | KLK4 | Prostate Cancer | Suppression of growth rate and anchorage-independent growth of LNCaP, LAPC4 and VCaP cell lines. Cell-cycle arrest in G1 phase and increased sensitivity to apoptosis in LNCaP cells. Lower growth rate of LNCaP xenografts. Suppression of androgen receptor signalling *in vitro* and *in vivo*. | [38,55] |
| miRNA-mediated targeting | | | | |
| miR-331-3p | KLK4 | Prostate Cancer | Experimentally validated miRNA able to target *KLK4*/reduced cell proliferation of DU-145. | [144] |
| miR-143 | KLK10 | Prostate Cancer | Experimentally validated miRNA able to target *KLK10*/reduced cell proliferation of DU-145. | [144] |

KLK: Tissue kallikrein and kallikrein-related peptidase; miRNA: microRNA; siRNA: Small-interfering RNA; shRNA: small hairpin RNAs.



## 5. Modulating *KLKs* expression: an overlooked therapeutic approach?

As previously described, the expression of the majority of *KLK*s is significantly deregulated in CaP and is thought to promote tumour progression. Consequently, the restoration of *KLK* expression, to a physiological state, represents an alternative and attractive KLK-targeting therapeutic approach for CaP (**Table 3**, **Figure 2**).

RNA interference (RNAi) using small non-coding RNAs represents the most essential cellular machinery for the post-transcriptional regulation of specific gene expression [134,135]. The crucial mediators of RNAi-related gene regulation are endogenous miRNAs, promoting gene silencing by partial complementarity with target mRNAs, and exogenous siRNAs or shRNAs requiring near-perfect base-pairing with target sequences. Exploitation of the cellular RNAi machinery could contribute to the targeted reprogramming of gene expression in CaP.

### 5.1 siRNA- and shRNA-mediated *KLKs* silencing

Exogenous delivery of siRNAs and shRNAs for gene silencing has been extremely used in cancer-related research in order to elucidate the role of specific genes in cancer scene and their potential therapeutic impact. Viral-based systems have been extensively used for the intracellular delivery of siRNAs. However, due to safety concerns, immunogenic and inflammatory responses and high production costs, non-viral approaches, such as liposomes, polymers, nanoparticles, carbon nanotubes, atelocollagen and chemical modifications of oligonucleotides for naked siRNAs delivery, have been successfully used instead of viral-based constructs [136].

Focusing on CaP and KLKs, siRNA-/shRNA-mediated knockout of *KLK2*, *KLK3* and *KLK4* restores their expression levels in the prostate microenvironment and produces encouraging therapeutic effects both *in vitro* and *in vivo*. Transfection of LNCaP cells with siRNA against endogenous *KLK2* leads to suppressed cell growth, accumulation of the cells in G1 cell-cycle phase and activation of apoptosis in androgen-independence disease stages. Additionally, significantly smaller tumours were detected in LNCaP xenografts upon stable expression of *KLK2*-targeting siRNA compared with control ones, thus highlighting the *in vivo* therapeutic dynamic for CRPC [54].

Similar benefits were also produced by a gene-specific shRNA lentiviral construct targeting endogenous *KLK3* expression in LNCaP cells. More precisely, transfection of

LNCaP cells in order to stably express shRNA constructs against *KLK3* significantly diminished their growth rate. An *in vivo* study of shRNA-mediated *KLK3* knockdown revealed the significantly reduced tumour weight and KLK3 secretion of LNCaP xenografts compared to control mice [137]. Moreover, *KLK3* silencing by siRNA delivery introduces cell-cycle arrest at the G1 phase and induction of apoptosis in CaP cell lines *in vitro*, as well as suppression of growth rate of *in vivo* xenografted tumours [59]. Based on the observation that antibodies against KLK3 inhibit the adhesion of CaP cells to bone marrow epithelial cells, siRNA-dependent silencing of *KLK3* in C4-2B androgen-independent cell line resulted in a similar attenuation of C4-2B cell adhesion to bone endothelium [74]. These data clearly highlight the beneficial therapeutic use of siRNA-mediated silencing of *KLK3* for the prevention of CRPC bone metastasis.

Moreover, knockdown of *KLK4* in LNCaP prostate cells following siRNA transfection resulted in the significant suppression of cell proliferation rate *in vitro* [38]. More recently, the lentiviral-mediated stable expression of shRNA construct against *KLK4* in LNCaP, LAPC4 and VCaP prostate cell lines was shown to reduce their growth rates and their anchorage-independent growth. The *in vitro* therapeutic potential of *KLK4* silencing was, thereafter, confirmed *in vivo* by the lower growth rate of xenografted prostate tumours. The impact of *KLK4* knockdown in CaP cells homeostasis revealed cell-cycle arrest in G1 phase, as well as promotion of apoptosis, indicating the role of *KLK4* silencing in sensitising CaP cells to apoptosis-related therapies [55].

### 5.2 miRNA-mediated targeting of *KLKs*

The miRNAs have attracted great attention recently due to their ability to regulate the expression of the vast majority of human genes, their involvement in cell proliferation, differentiation, signal transduction and apoptosis, their ability to control multiple targets and their deregulated expression in human diseases and mainly cancer [138]. In CaP, microarray-based studies have highlighted the deregulation of a great number of miRNAs in tumour tissues compared to normal or benign epithelium [139,140].

Several preclinical studies in CaP have pointed out the therapeutic utility of miRNAs. Liposome-mediated delivery of miR-34a, leads to the inhibition of tumour growth and lung metastasis of CaP in mice, as well as to prolonged survival periods following treatment. Delivery of miR-15a and miR-16 to CaP xenografts was documented to reduce tumour growth, whereas delivery of miR-16 or miR-203 in metastatic CaP mouse models was able to inhibit the growth of tumours in the bone marrow [141].

Using algorithm-based analyses, the majority of *KLK*s has been predicted to be targeted by multiple miRNAs [142]. Among them, the regulation of *KLK10* by let-7f, miR-224, miR-516a and miR-143 [142-144], of *KLK6* by let-7f [142], of *KLK1* by miR-224 [145], of *KLK5* by miR-382 [146] and the control of *KLK4* by miR-331-3p [144] have already been experimentally validated in human malignancies. Focusing on CaP, transfection of DU-145 with miR-331-3p or miR-143, which both are significantly downregulated in CaP, resulted in decreased *KLK4* and *KLK10* expressions, respectively, and in consequent reduction in cell proliferation [144]. These results clearly point out the tumour suppressor role of the abovementioned miRNAs in CaP; in the case of miR-331-3p, this is also supported by the prediction of *KLK2* targeting. Additionally, miR-224, which is predicted to target *KLK15*, is found significantly downregulated in CaP tissues, compared to benign ones, and in more advanced CaP tumours; miR-224 downregulation is also associated with biochemical relapse [147].

The benefits of restoring CaP-related *KLK* expression via miRNAs are their small, and thus less antigenic, size and their ability to concurrently target several genes, which amplifies their therapeutic impact. Several systems, such as liposomes, miRNA conjugation to peptides able to bypass plasma membrane and lentiviral vectors, have been successfully used for the delivery of miRNAs to treatment sites. Nonetheless, the fact that miRNAs target multiple and possibly unrelated genes might be a disadvantage compared to siRNA-based approaches.

## 6. Conclusion

KLKs have been broadly recognised, through continuously reported clinically oriented and mechanistic research studies, as a group of molecules that play a crucial role in the pathobiology of human malignancies [11,12,17-19]. This holds true especially for CaP, where KLK members 1 – 4, 7 or 14 are involved in tumour growth, cell invasiveness, angiogenesis or metastasis [18]. The important limitations of currently used CaP therapeutics, for example, the inevitable development of castrate resistance despite ADT, the systemic toxicity and the limited survival benefit of chemotherapy, as well as the high cost of immunotherapy [25], have driven researchers to identify emerging drugs that could overcome these deficiencies. The broad inhibitory dynamics of KLKs and the rational exploitation/modification of their activity and expression can be considered as the basis of novel strategies for confronting prostate malignancies.

## 7. Expert opinion

Aberrant proteolytic function has been identified as a crucial event in numerous malignant and non-malignant human diseases. Consequently, the therapeutic use of protease inhibitors has been considered, studied and exploited extensively throughout the years [148].

Protease inhibition has already been applied as a key therapeutic modality for several pathophysiological conditions, with remarkable clinical and commercial success. The most thriving examples include the utilisation of proteasome inhibitors for the treatment multiple myeloma and mantle cell

lymphoma, angiotensin-converting enzyme inhibitors for the treatment of hypertension and congestive heart failure, HIV protease inhibitors for treatment of HIV infections [148-150] and ecallantide (trade name Kalbitor®, Dyax Corp., investigational name DX-88), which is a re-engineered Kunitz-type inhibitor against KLKB1, currently being used as a medication for acute hereditary angioedema attacks [148,151].

The well-reported therapeutic potential of KLK inhibitors may also be translated into a clinical reality. Skin disorders represent an ideal setting for using KLK inhibitors, as their targets can be easily accessed during local drug application. In fact, several KLK inhibitory compounds have shown promising results regarding skin diseases [10,16,89] and are expected to enter clinical trials.

Regarding CaP, MDPK67b, an engineered ACT-based multi-KLK inhibitor [89,90] (blocking KLK2, KLK4 and KLK14 activity), has shown encouraging results in restraining prostate tumour growth in mouse models. Toxicity studies in rodent and primate models, as well as cynomolgus monkeys, showed a favourable safety profile. These data led to the launch of first-in-human studies for MDPK67b [89]. Results are highly anticipated in the near future in order to find out whether MDPK67b will be the first KLK inhibitor to be used for CaP treatment. The bio-scaffold approach has also been used for the production of a SFTI-based KLK4 inhibitor which shows great promise in terms of inhibitory potency, selectivity and stability [91], whereas further work is needed to evaluate its potential as a CaP therapeutic. Modified short peptides, antibodies, small molecule inhibitors and aptamers that can block the activity of CaP-associated KLKs (i.e., KLK1 – 4, 7 and 14) and are described throughout this review hold promise as novel CaP therapeutics.

Despite the fact that the abovementioned results are more than encouraging for the future use of KLK inhibitory molecules, we should always keep in mind the recent Phase III clinical trial failures of several MMPs inhibitors, which were initially regarded as promising cancer therapeutics. Possible reasons for these abysmal failures included the inefficiency of preclinical and target validation studies in predicting the wide biological spectrum of MMPs inhibitors that also blocked tumour-suppressor proteases and/or activated tumorigenic molecular pathways [152-154].

KLKs have long passed the era when they were considered as one-dimensional proteolytic molecules and have entered the world of cell signalling and protease crosstalk networks [13]. Consequently, a multidisciplinary approach might be more relevant for the production of a new generation of KLK inhibitors applicable for oncology, including CaP treatment. The further elucidation of the physiological roles of KLKs as well as the identification of new ones, combined with data from crystallographic, three-dimensional structuring and bioinformatics studies can lead to even more optimised KLK inhibitors. Besides selectivity issues, which can be overcome by exploiting the significant substrate specificities of KLKs and re-engineering the already available repertoire of inhibitory molecules, potential toxicity, industrialisation and commercial strategies should also be considered.

Apart from KLK inhibition, KLK activation of pro-drugs and KLK-based immunotherapy represent two very promising therapeutic strategies. Indeed, the KLK3-activated, doxorubicin pro-drug L-377,202 [107] and aerolysin pro-drug PRX302 [108] have gone through Phase I and Phase II clinical trials, respectively: L-377,202 for the treatment of CaP and for BPH [107,108]. What is more, the KLK3-based PROSTVAC vaccine has already entered Phase III clinical trials for evaluation as a novel immunotherapeutic for CaP [121,122].

In a more theoretical yet exciting approach, targeting the gene expression of heavily upregulated *KLK* genes in CaP (e.g., *KLK4* [36], *KLK15* [155] and *KLK14* [156]) seems an alternative way to regulate KLKs. The recent clinical success of modified antisense oligonucleotides that target the expression of the cytoprotective protein clusterin (OGX-011, OncoGeneX Technologies) in Phase II trials and the commencement of relevant Phase III trials pave the way for gene expression modifying therapeutics for CaP [157]. Overexpressed *KLK* genes could also be considered as targets for CaP treatment via the use of analogous constructs, including modified antisense oligonucleotides, siRNA, shRNA and miRNA constructs.

We believe that the already suggested therapeutic modalities and the ongoing both basic and clinical researches on KLKs provide grounds for predicting that the first therapeutic application of a KLK-based molecule will be a reality for CaP treatment in the coming years.

## Declaration of interest

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