A Suite of Activity-Based Probes To Dissect the KLK Activome in Drug-Resistant Prostate Cancer

Scott Lovell, Leren Zhang, Thomas Kryza, Anna Neodo, Nathalie Bock, Elena De Vita, Elizabeth D. Williams, Elisabeth Engelsberger, Congyi Xu, Alexander T. Bakker, Maria Maneiro, Reiko J. Tanaka, Charlotte L. Bevan, Judith A. Clements, and Edward W. Tate*

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ABSTRACT: Kallikrein-related peptidases (KLKs) are a family of secreted serine proteases, which form a network (the KLK activome) with an important role in proteolysis and signaling. In prostate cancer (PCa), increased KLK activity promotes tumor growth and metastasis through multiple biochemical pathways, and specific quantification and tracking of changes in the KLK activome could contribute to validation of KLKs as potential drug targets. Herein we report a technology platform based on novel activity-based probes (ABPs) and inhibitors enabling simultaneous orthogonal analysis of KLK2, KLK3, and KLK14 activity in hormone-responsive PCa cell lines and tumor homogenates. Importantly, we identified a significant decoupling of KLK activity and abundance and suggest that KLK proteolysis should be considered as an additional parameter, along with the PSA blood test, for accurate PCa diagnosis and monitoring. Using selective inhibitors and multiplexed fluorescent activity-based protein profiling (ABPP), we dissect the KLK activome in PCa cells and show that increased KLK14 activity leads to a migratory phenotype. Furthermore, using biotinylated ABPs, we show that active KLK molecules are secreted into the bone microenvironment by PCa cells following stimulation by osteoblasts suggesting KLK-mediated signaling mechanisms could contribute to PCa metastasis to bone. Together our findings show that ABPP is a powerful approach to dissect dysregulation of the KLK activome as a promising and previously underappreciated therapeutic target in advanced PCa.

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer among men in industrialized nations and remains a leading cause of cancer-related deaths.¹ Localized malignancies are treated with surgery and radiation therapy, and the 5-year survival rate of patients is close to 100%; however, post-operative recurrence often progresses to advanced PCa. Initial treatment for advanced tumors exploits the dependence of PCa cells on androgens by combining androgen-deprivation therapy (ADT) with direct targeting of the androgen receptor (AR),²,³ but many patients stop responding and develop castrate-resistant prostate cancer (CRPC). In CRPC, PCa cells evolve resistance to androgen-targeting therapies by restoring AR signaling through diverse mechanisms, and the majority of patients present with bone metastases with increased risk of morbidity and mortality due to alterations in skeletal integrity.⁴ Mapping critical pathways involved in establishing CRPC may enable identification of novel therapeutic targets which can ultimately reduce disease recurrence.⁵

AR is a transcription factor that dimerizes and translocates to the nucleus upon binding of androgens such as dihydrotestosterone (DHT), where it induces expression of a variety of genes important for PCa cell proliferation and survival (Figure 1A).⁶ Among these genes are specific serine proteases from the 15-member human kallikrein-related peptidase (KLK) family, which have versatile and crucial roles in extracellular proteolysis and signaling.⁷,⁸ For example, increased expression and subsequent leakage of KLK2 and KLK3, also known as prostate-specific antigen (PSA), into the vasculature are used in PCa diagnosis and progression monitoring.⁹,¹⁰ KLK2 and KLK3 also have functional roles in PCa and contribute to disease progression. KLK2 can activate protease-activated receptors (PARs) on the surface of neighboring fibroblasts, which in turn release cytokines that stimulate PCa cell proliferation,¹¹ while in the bone microenvironmen...
environment KLK3 promotes osteoprogenitor cell proliferation and osteoblast differentiation and establishment of bone metastases. More recently, KLK14 has also been implicated in CRPC development; while KLK14 is normally suppressed by AR signaling (Figure 1A), treatment with AR-targeted drugs may increase KLK14 expression to promote PCa cell migration and bone matrix colonization.

Despite recent progress in determining the pathophysiological roles of individual KLKs in PCa, the potential of KLK inhibitors for therapeutic intervention remains to be determined. Once secreted by PCa cells, KLKs do not work in isolation but as components of a complex network called the "KLK activome" (Figure 1B) that is tightly regulated by proteolytic KLK autoactivation, cross-activation or deactivation, and inhibition by endogenous serine protease inhibitors. A method to quantify the activity levels of each KLK directly in a complex biological system would enable the dynamic response of the KLK activome to be determined in relevant PCa models and drug-resistant tumors and during drug/hormone-mediated perturbations and may lead to validation of specific active KLKs as drug targets or novel biomarkers.

Here we present a technology platform that enables simultaneous orthogonal readout of the activity of KLK2, KLK3, and KLK14 in androgen-responsive PCa cell lines and patient-derived xenografts (PDX), based on a chemical toolbox of first-in-class selective activity-based probes (ABPs) and inhibitors for activity-based protein profiling (ABPP, Figure
We use this platform to show that active KLK molecules are secreted into the bone microenvironment by PCa cells following stimulation by osteoblasts, supporting a double paracrine signaling mechanism that may contribute to the establishment of bone metastases mediated by KLK activity. Furthermore, using selective inhibitors and multiplexed fluorescent ABPP, we dissect the KLK activome directly in PCa cells and show that KLK14 drives PCa cell migration, a key step in the formation of distant metastases. Together these findings demonstrate that ABPP can provide a unique window on KLK activity and inhibition in PCa and suggest that KLK-activome dysregulation is a promising and previously under-appreciated therapeutic target in CRPC.

RESULTS

Development of a Selective Inhibitor and Activity-Based Probe for KLK3. Proof-of-concept for the first selective ABP targeting the PCa KLK activome started with KLK3, which exhibits chymotrypsin-like specity, in contrast
to KLK2 and KLK14 which are trypsin-like proteases. An optimized tetrapeptide designed to occupy the KLK S1–S4 subsites was envisaged for the ABP specificity element, grafted onto a peptidyl-diphenyl phosphonate (DPP) warhead which...
reacts specifically and exclusively with the Ser195 residue in the KLK3 active site.21 DPP 1 with a tyrosine-mimicking phenol in the first position (P1) was previously identified as a modestly potent inhibitor of KLK3,22 while peptidyl-boronic acid 2 is a potent and selective KLK3 inhibitor (Figure 2A).23 Both DPP and boronic acid inhibitors covalently modify Ser195, thereby mimicking the tetrahedral intermediate during amidolysis; however boronic acids form reversible covalent complexes and are generally less effective as ABPs. We therefore replaced the boronic acid in 2 with the DPP moiety in 1 and capped the N-terminus with a tetramethyl rhodamine ( TAMRA) fluorophore to afford 3, which served as a prototype ABP for KLK3. A mixed solid-phase and solution-phase approach was used for the synthesis of peptidyl-DPP compounds, as described previously (Figures S1 and S2).24 With this approach the peptidyl-DPP compounds are obtained as an equimolar mixture of two diastereoisomers with only the (R) configuration engaging the target KLK. KLKs are typically activated after their secretion from the cell, so we first examined the labeling profile of 3 in conditioned media (CM) obtained from LNCaP cells treated with androgen (10 nM DHT). LNCaP is an androgen-responsive human prostate adenocarcinoma cell line widely used to model key features of clinical disease, including secretion of KLK2 and KLK3 following androgen stimulation.6 LNCaP CM was treated with different concentrations of 3 for 1 h, and proteins were separated by SDS–PAGE. In-gel fluorescence revealed a single major target at ~32 kDa, labeled in a concentration-dependent manner (Figure 2B), and selective covalent modification of active KLK3 was confirmed by immunoprecipitation (Figure S3).

Despite exquisite selectivity, maximal KLK3 labeling was achieved only after treatment with 20 μM 3 for 1 h. We therefore optimized probe potency by systematically modifying each amino acid side chain in the P1–P4 positions of a morpholine-capped peptidyl-DPP inhibitor scaffold and determined the potency of each analogue using competitive-ABPP against KLK2 and KLK3 following androgen stimulation.6 LNCaP CM was treated with different concentrations of 3 for 1 h, and proteins were separated by SDS–PAGE. In-gel fluorescence revealed a single major target at ~32 kDa, labeled in a concentration-dependent manner (Figure 2B), and selective covalent modification of active KLK3 was confirmed by immunoprecipitation (Figure S3).

Development of Selective Inhibitors and Activity-Based Probes for KLK2 and KLK14. Both KLK2 and KLK14 display a strong preference for Arg in P1,20 and we anticipated that development of selective ABPs would require optimization of the P2–P4 positions to differentiate between unique preferences of the S2–S4 subsites. Building on prior work,29–32 we generated a positional scanning substrate library derived from 19 natural amino acids (excluding Cys) and 86 structurally diverse non-natural amino acids to provide a detailed analysis of active site preferences at each protease subsite. Three sublibraries consisting of 105 mixtures of 361 fluorogenic peptidyl coumarins were generated and screened against KLK2 and KLK14 (Figure 3A; see Figures S9 and S10 for library synthesis and structures of non-natural amino acids), and scatter plots of relative initial rates of hydrolysis for each amino acid revealed specificity preferences at the P2, P3, and P4 positions (Figure 3B–D and Figures S11–S14). Despite a general preference for aromatic residues at P2, KLK2 processed substrates with a P2 cyclohexyl alanine (Cha) at the highest rate, whereas KLK14 demonstrated dual specificity at P2, processing both aliphatic residues such as aminobutyric acid (Abu) and aromatic residues such as benzyl histidine (His(Bzl)) (Figure 3B). Both proteases favored basic amino acids at P3, with KLK14 preferring Lys while KLK2 preferred the shorter chain dianinobutyric acid (Dab) (Figure 3C). At P4, KLK2 processed aliphatic (e.g., Abu) and aromatic residues with a flexible linker such as benzyl-serine (Ser(Bzl)), and KLK14 preferred medium and large aromatic residues such as 4-bromophenylalanine (Phe(+Br)) and benzothiazol-2-yl alanine (Ala(Bth)) (Figure 3D).

On the basis of these data, we hypothesized that the divergent specificity preferences of KLK2 and KLK14 could be exploited to develop selective chemical probes. The preferred amino acid for each subsite was incorporated into a peptidyl-DPP scaffold to afford prototype probes 22 and 32 directed toward KLK2 and KLK14, respectively (Figure 3E), using a phenylguanidine Arg mimic in P1 to ease synthesis.33 Kinetic analyses (Figure S15) revealed that although 22 is a potent inhibitor of KLK2 (k_inact/Ki = 3274 ± 89 M−1 s−1), it has significant cross-reactivity with KLK14 (k_inact/Ki = 511 ± 18 M−1 s−1), and we therefore further optimized the KLK2 inhibitor scaffold by systematically altering P2, P3, and P4 residues with other hits from the substrate library screen. From nine peptidyl-DPP analogues, compound 31 was most optimal (Table 1, Figure S16) with similar potency toward KLK2 (k_inact/Ki = 3076 ± 87 M−1 s−1) delivered by a P2 Tyr substitution and with >30-fold selectivity over KLK14 (k_inact/Ki = 94 ± 7 M−1 s−1) because of increased steric bulk at P4 (Tip in place of Abu). We took a similar approach to optimize compound 32 against KLK14 (k_inact/Ki = 523 ± 46 M−1 s−1), identifying compound 39 (Table 1, Figure S17) with 80-fold higher potency toward KLK14 (4474 ± 1976 M−1 s−1) and 220-fold selectivity over KLK2 (k_inact/Ki = 204 ± 13 M−1 s−1).

To gain further insights into site preference, we performed molecular modeling studies using previously determined KLK2 and KLK3 crystal structures34–36 in the Molecular Operating Environment (MOE) software package36 to propose binding determinants of 31 and 20. For the purpose of modeling the specificity elements of each probe class, the biotin in 31 was replaced with an acetyl group, and morpholine-capped derivative 15 was used to model 20. We found that 31 and 20 likely bind to their cognate KLK active sites in a conventional extended conformation, with the amino acid side chains of P1–P4 occupying the S1–S4 pockets (Figure S18A,B). In both poses the phosphonate warhead is located in close proximity to the catalytic serine, with the P=O moiety...
establishing an H-bonding interaction with the NH group on the backbone of Gly193, which would activate the phosphonate for nucleophilic attack (Figure S18C,D). Our model highlighted the differing properties of the S1 and S4 pockets of KLK2 and KLK3 as key determinants of probe specificity. For example, the P1 phenylguanidine of 31 forms a salt bridge with the carboxylate group of Asp189 at the bottom of the S1 pocket of KLK2. In contrast, for KLK3 the residue at the bottom of the S1 pocket is serine, which is also flanked by the polar side chains of Ser227 and Thr190. Consequently, the KLK3 S1 pocket is polar at the bottom and hydrophobic on the sides and has a preference for medium hydrophobic side chains with polar neutral head groups. In agreement with this, the P1 benzamide group of 20 forms key hydrogen bonds with Ser227 and Thr190. Furthermore, our model suggests that the bulky P4 Tle residue of 31 is accommodated by the S4 pocket of KLK2, which has significant hydrophobic character imparted from several residues of the kallikrein loop.37 A crystal structure for KLK14 is yet to be solved, but a homology model by de Veer et al. provides a potential explanation for the selectivity of our KLK2 and KLK14 probes.38 The S2 pocket of KLK14 is narrow owing to the flanking side chain of His99, and thus small aliphatic residues, such as the P2 Abu of 39, are preferred. Conversely, the S4 pocket of KLK14 is large with Trp215 positioned at the base, which is predicted to form π-stacking interactions with large aromatic residues such as the P4 2-Nal of 39.38 The selectivity for 31 is likely derived due to the combination of suboptimal interactions of the S2 and S4 pockets of KLK14 with P2 Tyr and P4 Tle, respectively. Conversely, the selectivity of 39 is likely derived from the fact that the S4 pocket of KLK2 cannot accept the large aromatic 2-Nal side chain present at P4.

With ABPs for KLK2, KLK3, and KLK14 in hand, we next addressed selective readouts of KLK activities in a complex biological system. Compounds 20, 31, and 39 were incubated with CM obtained from LNCaP-K14 cells, in which KLK14 expression has been placed under the control of a doxycycline-inducible promoter, stimulated with 10 nM DHT and 200 nM doxycycline (Dox).15 Streptavidin blotting revealed a single target between 25 and 32 kDa for each compound, labeled in a concentration-dependent manner (Figure 4A), showing that the only detectable targets of these probes are their cognate targets between 25 and 32 kDa for each compound, labeled in a concentration-dependent manner (Figure 4A), showing that the only detectable targets of these probes are their cognate targets.

**Table 1.** \(k_{\text{act}}/K_c\) Values of Peptidyl-DPP Analogues for KLK2 (Left) and KLK14 (Right) (a)

| Scaffold | KLK2 | Selectivity (fold-change) | KLK14 | Selectivity (fold-change) |
|---------|------|---------------------------|------|---------------------------|
| ABP analogue | \(k_{\text{act}}/K_c\) (M\(^{-1}\) s\(^{-1}\)) | | \(k_{\text{act}}/K_c\) (M\(^{-1}\) s\(^{-1}\)) | |
| Bio-Phe-Abu-Arg | 523 ± 46 | 1 | 18 | 11 |
| Trp(Bz)-Lys-His(Bz)-Arg | 1121 ± 54 | 39 | 6 | 46 |
| Thr(Bz)-Lys-Abu-Arg | 15187 ± 16896 | 25 | 1190 | |
| Thr(Bz)-Lys-Abu-Dab-Tyr-Arg | 24172 ± 7022 | 151 | 184 | 218 |
| Thr(Bz)-Lys-Abu-Dab-Phe(3,4-F\(_2\))-Arg | 4474 ± 8756 | 40 | 680 | 1668 |
| Thr(Bz)-Lys-Abu-Dab-Abu-Arg | 5231 ± 704 | 5 | 2374 | 602 |
| Thr(Bz)-Lys-Abu-Dab-Tyr-Arg | 12846 ± 2314 | 107 | 8 | 33 |
| Thr(Bz)-Lys-Abu-Dab-Phe(3,4-F\(_2\))-Arg | 15268 ± 2374 | 1162 | 570 | 116 |
| Thr(Bz)-Lys-Abu-Dab-Abu-Arg | 20215 ± 496 | 1162 | 570 | 116 |
| Thr(Bz)-Lys-Abu-Dab-Tyr-Arg | 28112 ± 1976 | 97 | 2374 | 4 |
| Thr(Bz)-Lys-Abu-Dab-Phe(3,4-F\(_2\))-Arg | 31174 ± 204 | 1162 | 570 | 116 |
| Thr(Bz)-Lys-Abu-Dab-Abu-Arg | 34286 ± 115 | 184 | 218 | 57 |
| Thr(Bz)-Lys-Abu-Dab-Tyr-Arg | 38404 ± 40 | 1162 | 570 | 116 |
| Thr(Bz)-Lys-Abu-Dab-Phe(3,4-F\(_2\))-Arg | 44635 ± 496 | 1162 | 570 | 116 |

(a) Each data point is a mean value ± SEM (N = 3).
changes in response to specific AR signaling perturbations. In the LNCaP-K14 cells used in previous experiments the expression of KLK14 is increased by activation of a doxycycline-inducible promoter, and to assess AR-mediated changes in KLK14 activity, we reverted to WT LNCaP cells, which are known to have low basal KLK14 expression. To enable detection of each KLK in WT LNCaP cells, we incubated LNCaP conditioned media with 1 μM of each KLK ABP, enriched labeled proteins on streptavidin beads, and visualized activity levels by immunoblotting with the appropriate KLK antibody (see Figure S19 for workflow). As expected, the expression and activity of KLK2 and KLK3 in LNCaP cells were upregulated in response to treatment with DHT, and this increase was nullified by co-treatment with enzalutamide (Enz), a competitive AR inhibitor employed in the clinic to treat CRPC. In contrast, KLK14 expression and activity was decreased upon treatment with DHT but restored by co-incubation with Enz (Figure 4B and Figure S21 for blot quantification).

To assess KLK activity levels at different disease stages, we profiled six PCa patient-derived xenograft (PDX) tumors isolated from diverse tissues including prostate and metastases in lymph node, liver, and bone. Fresh-frozen PDX samples were homogenized in 1% Triton in PBS and incubated with 1 μM KLK ABP, followed by enrichment and immunoblotting. KLK2 was expressed in all samples, and KLK3 was expressed in all but one; however, expression and activity were substantially decoupled, with KLK2 and KLK3 activity seen only in PDXs

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**Figure 4.** KLK activity profiling in PCa cells and PDX homogenates. (A) Streptavidin blotting shows potent and selective labeling of KLK2, KLK3, and KLK14 by 31, 20, and 39, respectively, after treatment of LNCap-K14 conditioned media for 1 h. (B) KLK activity and expression in the LNCaP cell line after treatment with 10 nM DHT ± 10 μM Enz. KLK activity was assessed by streptavidin enrichment of probe-labeled KLK molecules following treatment of conditioned media with 1 μM of either 31, 20, or 39 for 1 h. See Figure S21 for blot quantification. (C) KLK activity and expression in six different PDX homogenates. KLK activity was assessed using the same method as in (B). The tissue origin and AR expression levels of the six PDX samples are shown. The + sign indicates the staining intensity of PDX samples by an AR antibody. Abbreviation: IHC = immunohistochemistry, TURP = transurethral resection of the prostate. See Figure S22 for blot quantification.)
generated from metastases including lymph node, liver, and bone. LuCaP 35 PDX, which lacked KLK3 expression and had decreased KLK2 expression, has also previously been shown to have low AR expression. KLK14 activity decoupled from expression was evident in all samples, with higher KLK14 activity in LuCaP 35, BM18, and LuCaP 105 (see Figure 4C and Figure S22 for blot quantification).

Strikingly, we found that simultaneous activation of all three KLKs was observed only in PDX cells from bone metastases (BM18 and LuCaP 105). Advanced PCa has a propensity to metastasize to bone and dysregulate bone resorption and bone formation through complex paracrine signaling events with osteoblasts and osteoclasts (cells that mediate bone generation or absorption, respectively). Treatment options for PCa bone metastases remain inadequate and generally palliative, and there is an urgent need to identify novel therapeutic targets.

Osteoblasts secrete growth factors including IL-6 which can induce AR signaling in PCa cells even in the absence of androgens, as seen during ADT, and we hypothesized that osteoblasts might therefore also induce PCa cell secretion of active KLK molecules into the bone microenvironment under androgen-deprived conditions. To test this hypothesis, primary human osteoprogenitor cells were isolated from bone tissue and cultured in osteogenic media (1 M \(\beta\)-glycerophosphate, 200 mM ascorbate-2-phosphate, and 0.1 M dexamethasone) for 6 weeks as described previously, forming a dense mineralized collagen-rich bone-like matrix (Figure S23).

LNCaP cells were then either directly cocultured with osteoblasts or treated with conditioned media from osteoblasts (here termed “indirect coculture”) in androgen-depleted media (Figure 5A), and CM was obtained from indirect or direct coculture treated with 1 \(\mu\)M of each KLK ABP, followed by enrichment and immunoblotting. In agreement with our hypothesis, both direct and indirect coculture of LNCaP cells with osteoblasts resulted in an increase of total and active KLK2 and KLK3. In line with the AR-dependent and prostate-restricted expression of these proteases, neither KLK was detected in osteoblast CM alone, and very low expression was seen in CM obtained from androgen deprived LNCaP cells (Figure 5B and Figure S24 for blot quantification). The concentration of active and total KLK14 also increased in coculture compared to LNCaP cells alone, but in contrast to KLK2 and KLK3 this was due to secretion by osteoblasts, which suggests a role for osteoblasts in enhancing AR activation and promoting PCa growth in the bone microenvironment.
suggesting that osteoblasts may contribute to KLK14 activity in bone metastases.

**Dissecting the Role of the KLK Activome in Prostate Cancer Progression.** The data presented above provide evidence that KLK activity in PCa is regulated by the AR at a level beyond simple changes in expression and raise the possibility that KLK activities may be actionable targets for therapeutic intervention at different disease stages, in particular during PCa metastasis to bone. However, KLKs are predicted to form complex proteolytic networks and it is often not clear which protease(s) should be inhibited for maximum phenotypic response in a particular disease setting. Experiments with purified proteins show that KLK14 can activate pro-KLK2 and pro-KLK3 by proteolytic cleavage of their N-terminal propeptide sequence, and it has been suggested that KLK2 can also activate pro-KLK3. To enable dissection of the PCa KLK activome directly in LNCaP cells, we further expanded the KLK probe toolbox by synthesizing KLK2_fABP (42) and KLK14_fABP (43) (Figure 6A and Figure S2), fluorescent ABP (fABP) analogues of KLK2_bABP and KLK14_bABP bearing dyes fluorescing at discrete wavelengths, which retain excellent potency and selectivity for

![Figure 6](https://doi.org/10.1021/jacs.1c03950)
KLK2 and KLK14, respectively (Figure S25). KLK2_fABP and KLK14_fABP were combined with KLK3-selective fABP 21 (KLK3_fABP) in an “fABP cocktail” to a final concentration of 1 μM each and incubated for 20 min with CM obtained from LNCaP-WT, LNCaP-mK14, or LNCaP-K14 cells following 48 h treatment with 10 nM DHT to enable simultaneous multicolor readout of KLK2, KLK3, and KLK14 activity. Both LNCaP-WT and Dox-treated LNCaP-mK14 cells expressing catalytically inactive mutant KLK14[S195A] under the control of a Dox-inducible promoter had low levels of active KLK3, while KLK2 and KLK14 activity was below the detection limit (Figure 6B). However, upon Dox-induced KLK14 expression in DHT-treated LNCaP-K14 cells a significant increase in the activity of all three KLKs was evident (see Figure 6B and Figure S26 for gel quantification). Importantly, the total abundance of secreted KLK2 and KLK3 remained constant upon treatment with Dox, demonstrating an increase in the ratio of active to inactive KLK independent from protein expression. To enable selective inhibition of active KLK molecules, LNCaP-K14 cells were co-incubated with 1 μM KLK2_bABP, KLK3_bABP, or KLK14_bABP, 10 nM DHT, and 200 nM Dox for 48 h. Residual KLK activity in CM was then assessed using the fABP cocktail (Figure 6B). These data demonstrate that inhibition of either KLK2 or KLK3 activity does not affect the activity of other KLKs, suggesting that KLK2 and KLK3 do not cross-activate the other PCa KLKs. However, inhibition of KLK14 resulted in a significant decrease in the activity levels of both KLK2 and KLK3, suggesting that KLK14 cross-activates the proforms of both these proteases.

Prior to the establishment of bone metastases circulating PCa cells must first enter the bone microenvironment by migrating across the sinusoidal wall. We have previously shown that increased KLK14 activity drives PCa cell migration and colonization of mineralized bone matrices. However, the specific contributions of the KLK activome (KLK2, KLK3, and KLK14) toward PCa cell migration are yet to be elucidated. We first confirmed that treatment of LNCaP-K14 cells with 10 μM KLK2_bABP, KLK3_bABP, and KLK14_bABP resulted in no cytotoxicity over a 96 h time period (Figure S27). We then assessed the migratory capacity of LNCaP-K14 cells using a transwell assay (see Figure S28 for standard curve). Induction of KLK14 expression in LNCaP-K14 cells (200 nM Dox) resulted in increased migration, as expected, which was nullified by co-treatment with 1 μM KLK14_bABP (Figure 6C). Conversely, treatment with 1 μM KLK2_bABP or KLK3_bABP had no significant effect on the number of migrating LNCaP-K14 cells. Simultaneous treatment of LNCaP-K14 cells with 1 μM each KLK2_bABP, KLK3_bABP, and KLK14_bABP resulted in a significant decrease in cell migration, suggesting that KLK14 activity is a key driver of PCa cell migration, while KLK2 and KLK3 activities individually make minor contributions.

Overall, these data dissect the KLK activome in LNCaP cells and show that KLK14 activity drives PCa cell migration.

# DISCUSSION

Despite recent progress in determining the pathophysiological roles of individual KLKs at different stages of PCa, the potential of selectively inhibiting these proteases for therapeutic intervention is yet to be realized. It has become clear that KLKs do not work alone but instead are individual components of complex networks that when deregulated drive disease progression through amplified proteolysis. However, delineating the overlapping, synergistic and opposing activities of individual prostatic KLKs in complex environments remains very challenging. Inspired by multiplexed ABP toolkits previously reported for the proteasome, neutrophil serine proteases, and caspases, we developed the first probe set enabling simultaneous assessment of the activity of each PCa-relevant KLK in PCa samples. Peptidyl-DPP ABPs were established for each protease, and potency and specificity were further optimized in PCa supernatants by competitive ABPP, an approach that enables assessment of the properties of each covalent inhibitor directly in a complex biological environment. Development of selective ABPs for KLK2 and KLK14 was complicated by their similar trypsin-like activity; indeed, proteases with the same primary (P1) specificity are often coexpressed in tissues, and to generate selective tools, it is necessary to explore more complex specificity determinants. Here we applied positional scanning libraries to identify mutually exclusive preferences in the S2–S4 pockets of KLK2 and KLK14; however this approach does not account for potential cooperativity between protease subsites in driving selectivity. Further optimization across ABPs incorporating different combinations of preferred amino acids illustrated the importance of exploiting cooperativity and in the case of KLK14 identified an ABP with optimal selectivity which features none of the top amino acid hits identified for each individual subunit in the library screen. Interestingly, both KLK2 and KLK14 demonstrated dual specificity in certain subpockets, suggesting a role for these subunits in tuning substrate profiles. We note that while the present set of probes has been optimized for PCa, each tissue type expresses a different set of secreted proteases, and thus the specificity sequence required to obtain probe selectivity may depend on the physiological context.

Recent development of KLK-knockout and transgenic mice and selective inhibitors have revealed key associations between individual KLK activities and the onset or progression of diverse diseases. However, whether these associations are due to the activity of an individual KLK or because of cross-activation of other proteases, including other KLKs, is poorly understood. The KLK activome has to date been modeled only using purified proteins, and it remains very challenging to deconvolute the biological relevance of a specific cross-activation event in more complex systems.

We leveraged our chemical toolkit to dissect the KLK activome in PCa cells by inhibiting one KLK and assessing the change in activity of the remaining KLKs, demonstrating that KLK14 cross-activates KLK2 and KLK3. We suggest that our approach could be used in principle to dissect any disease-related or tissue KLK activome and reveal which KLKs represent potential targets for therapeutic intervention in a specific context.

Our data using LNCaP cells demonstrate that KLK activity in the tumor microenvironment is likely regulated by AR signaling (Figure 4B). However, while KLK2 and KLK3 activity is upregulated by AR activation, KLK14 activity increases upon AR inhibition. The latter observation suggests a role for active KLK14 in the development of resistance to AR competitive inhibitors. Despite the divergent response to AR signaling, we show that KLK2, KLK3, and KLK14 are coexpressed and active in hormone responsive cells from different sites of PCa metastasis, including lymph nodes, liver, and bone. An explanation for this paradox lies in the
heterogeneity of prostate tumors, which develop resistance to AR inhibition through diverse mechanisms including upregulation of AR-V7 (an AR splice-variant with constitutive transcriptional activity that lacks a ligand-binding domain) and glucocorticoid receptor (GR). 63−65 Both AR-V7 and GR signaling drive increased KLK2 and KLK3 expression even in the face of Enz treatment. Similarly, a subset of AR-null PCa cells have high KLK14 expression, which is not perturbed by DHT treatment. 66 We hypothesize that these diverse PCa cell populations contribute to a buildup of KLK activities in the tumor microenvironment. In this study we found that androgen-independent activation of AR in PCa cells by osteoblasts results in an increase of KLK2 and KLK3 activity in the bone microenvironment. Previous studies have shown that KLK3 increases bone volume and osteoblast numbers in vivo via a TGFβ-dependent mechanism. 67−69 It has also been demonstrated in vitro that KLK2 and KLK14 can activate TGFβ-1 and TGFβ-2.66 Furthermore, our data show that osteoblasts, as well as PCa cells, secrete active KLK14 into the bone microenvironment and that KLK14 can cross-activate KLK2 and KLK3, thus providing a proteolytic cascade that may amplify osteoblast proliferation and differentiation and result in a double paracrine signaling event that drives both tumor growth and woven bone formation (Figure 5C). 51,52 We propose that the KLK activome warrants further study as an actionable therapeutic target in bone metastatic PCa, either alone or in combination with enzalutamide.

Finally, the chemical tools developed in this study may allow KLK activity to be explored as a novel biomarker in PCa. Population-based screening with the PSA test has decreased PCa mortality; however, due to the test’s relatively poor specificity, it has also increased the detection of indolent cancers and resulted in overtreatment of patients. Identification of novel biomarkers with increased specificity for aggressive PCa detection may aid in risk stratification and the appropriate identification of men for prostate biopsy. 9 An early hallmark of PCa is the downregulation of zinc transporter proteins, which results in a decrease in the concentration of Zn2+ ions in the prostate. 67 Zinc ions allosterically regulate the activity of KLKs, and thus there is an increase in KLK activity in prostatic fluid obtained from patients with PCa. 19 Measurement of KLK activity in first void urine samples, which contain prostatic fluid, may be worth exploring in the quest for a more accurate diagnostic tool. 68−70 To this end, we note that biotinylated ABPs have previously been integrated into ELISA platforms to enable highly sensitive and high throughput assessment of protease activity. 71 Similarly, as KLK activity is higher in PCa tissue compared to neighboring healthy tissue, KLK probes could in future be used as fluorescent guides for surgeons striving to obtain negative margins during tumor resection, 72 potentially facilitated by the development of quenched-fluorescent derivatives.

■ CONCLUSION

This study describes the development of a versatile chemical probe platform to enable dissection of KLK activome activity in PCa, leading support for the hypothesis that the KLK activome drives PCa progression and holds promise as an actionable therapeutic target in CRPC.
Alexander T. Bakker — Department of Chemistry, Molecular Sciences Research Hub, Imperial College London, London W12 0BZ, U.K.

Maria Maneiro — Department of Chemistry, Molecular Sciences Research Hub, Imperial College London, London W12 0BZ, U.K.; orcid.org/0000-0001-6229-9157

Reiko J. Tanaka — Department of Bioengineering, Imperial College London, London SW7 2AZ, U.K.

Charlotte L. Bevan — Department of Surgery and Cancer, Imperial Centre for Translational and Experimental Medicine, Imperial College London, Hammersmith Hospital, London W12 0NN, U.K.; orcid.org/0000-0002-7533-0552

Judith A. Clements — Australian Prostate Research Centre-Queensland (APCRC-Q), Institute of Health & Biomedical Innovation and School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Translational Research Institute, Woolloongabba, QLD 4102, Australia

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.1c03950

Notes

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