JBC Papers in Press. Published on June 22, 2018 as Manuscript RA117.000871
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.RA117.000871

A potent, proteolysis-resistant inhibitor of kallikrein-related peptidase 6 (KLK6) for cancer therapy, developed by combinatorial engineering

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Short title: Combinatorial engineering of a proteolysis-resistant KLK6 inhibitor

Key words: protein engineering, directed evolution, selective binding, proteolysis, protease inhibitor, enzyme inhibition, X-ray structure

Abstract

Human tissue kallikrein (KLK) proteases are hormone-like signaling molecules with important functions in cancer pathophysiology. KLK-related peptidase 6 (KLK6), specifically, is highly up-regulated in several types of cancer, where its increased activity promotes cancer invasion and metastasis. This characteristic suggests KLK6 as an attractive target for therapeutic interventions. However, inhibitors that specifically target KLK6 have not yet been reported, possibly because KLK6 shares a high sequence homology and structural similarity with other serine proteases and resists inhibition by many polypeptide inhibitors. Here, we present an innovative combinatorial approach to engineering KLK6 inhibitors via flow cytometry–based screening of a yeast-displayed mutant library of the human amyloid precursor protein Kunitz protease inhibitor domain (APPI), an inhibitor of other serine proteases, such as anionic and cationic trypsins. On the basis of this screening, we generated $APPI_{M17L,I18F,S19F,F34V}$ (APPI-4M), an APPI variant with a KLK6 inhibition constant (*Ki*) of 160 pM and a turnover time of 10 days. To the best of our knowledge, APPI-4M is the most potent KLK6 inhibitor reported to date, displaying 146-fold improved affinity and 13-fold improved proteolytic stability compared with wild-type APPI ($APPI_{WT}$). We further demonstrate that APPI-4M acts as a functional inhibitor in a cell-based model of KLK6-dependent breast cancer invasion. Finally, the crystal structures of the $APPI_{WT}$/KLK6 and APPI-4M/KLK6 complexes revealed the structural and mechanistic bases for the improved KLK6 binding and proteolytic resistance of APPI-4M. We anticipate that APPI-4M will have substantial translational potential as both imaging agent and therapeutic.

Introduction

An important family of serine proteases, implicated in various proceses in both health and disease, is the family of human tissue kallikreins (KLKs) (1). The 15 serine proteases in this family are involved in the tissue-specific proteolysis of numerous endogenous substrates (1-3) and are considered major regulatory proteases with key signaling properties, acting either individually or as part of other proteolytic cascades (4). KLKs can also be employed clinically as markers for the diagnosis and prognosis of cancer (5) and as targets for therapeutic intervention; KLK6, specifically, has been shown to be a key factor in driving the invasion and migration of cancer cells (6-8).

Several independent studies reported that KLK6 expression is associated especially with a shorter overall survival and/or progression/disease-free survival in colon, gastric, lung, and ovarian cancers (9-18). In ovarian cancer, for example, accumulating data suggest that KLK6 is responsible for increased E-cadherin shedding, which, in turn, increases metastasis and ascites in epithelial ovarian cancer (7,19,20). Similarly, in colon cancer, in which KLK6 can be used for both prognosis and diagnosis, it induces the invasiveness and proliferation of cancer cells by down-regulating E-cadherin, thereby interrupting E-cadherin-mediated cell–cell adhesion – a prerequisite for tumor invasiveness and metastasis (14,16,21). Moreover, KLK6 appears to be relevant for breast cancer, as high KLK6 expression levels were recently shown to promote the invasiveness of breast cancer cells; KLK6 serum levels were found to be significantly higher in patients with invasive breast cancer, and patients with high KLK6 expression showed poorer survival rates (22-24).

Several studies have investigated how KLK6 is involved in the pathogenesis and metastasis of various types of cancer (21,24,25). For instance, KLK6 has been shown to be involved in the progression of melanoma, where it triggers tumor cells and tumor-associated parenchymal cells in the microenvironment of the tumor through the proteolysis and activation of the proteinase-activated receptor PAR1, which leads to the acquisition of a malignant phenotype by facilitating tumor cell invasion and metastasis (8,26,27). In addition, in a non-small cell lung cancer model system, KLK6 has been shown to promote the proliferation of lung tumor cells, and this effect was facilitated by KLK6 cleavage and activation of PAR2 (28). By degrading various proteins in the extracellular matrix (29), KLK6 has also been shown, *in vitro*, to increase the metastatic potential of tumor cells (29,30); concomitantly, both *in vitro* (6-8) and *in vivo* (7,31) studies have established a correlation between the ectopic expression of KLK6 and an increase in tumor proliferation and migration, while an RNA interference-induced KLK6 suppression reduces the cell growth, proliferation, and invasive potential of tumors (14,21). These data underlie KLK6 as an attractive target for novel molecules that could inhibit its proteolytic activity as a treatment for various cancers (32-34). Finally, numerous studies indicated that KLK6 may also be involved in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and others (35-41). For example, raising neutralizing KLK6 antibodies in mice exerted overall beneficial effects in models of multiple sclerosis and CNS inflammatory disease, suggesting that selectively inhibiting KLK6 is not expected to be considerably toxic.

Despite the numerous studies on human serine proteases, KLK inhibitors are still much sought-after (42-45). As the KLK family comprises 15 different members, many of which contain structurally similar binding pockets, the discovery of either natural or synthetic subtype-selective KLK inhibitors is a challenging task. KLK6 is structurally similar to other members of the KLK family and to other human trypsin-like proteases (46,47), rendering its specific targeting difficult to achieve. An additional challenge in developing KLK6 inhibitors is its resistance to inhibition by polypeptide serine protease inhibitors, according to several prior reports (48,49). Indeed, to the best of our knowledge, to date, highly potent, selective, and stable KLK6 inhibitors have not been reported.

Recently, we employed a combinatorial engineering approach that has enabled us to transform the human amyloid precursor protein Kunitz protease inhibitor domain (APPI) into a highly potent inhibitor of mesotrypsin – another member of the human serine protease family. Importantly, the engineered APPI showed a high selectivity toward mesotrypsin (as compared with other serine proteases), while the native APPI ($APPI_{WT}$) bound non-specifically to various serine proteases, including KLK6, and was susceptible to rapid cleavage and inactivation by mesotrypsin (50). We have previously found that the specificity of APPI toward serine proteases is largely directed by the sequence of its canonical binding loop, while its proteolytic stability is strongly influenced by residues buried within its scaffold (51,52). These findings suggest that both the affinity

and the proteolytic stability of APPI can be optimized by using combinatorial screening methods, so as to generate high affinity and stable KLK6 inhibitors based on the protein scaffold of the human APPI.

In the current study, we employed the powerful yeast surface display (YSD) technology (53,54) to screen combinatorial libraries of APPI variants containing mutations in both the active site and the scaffold. Using these libraries, we engineered an APPI that, as compared with its wild-type counterpart, demonstrates a 14-fold increase in proteolytic stability and a 146-fold higher binding affinity to KLK6, without increasing its binding affinity toward other KLK6. These optimized properties were the result of four mutations (two in the binding loop and two in the scaffold), which enabled the engineered APPI variant—termed here APPI-4M—to potently block the invasiveness of KLK6-dependent cancer cells in cell-based assays, in which a high binding affinity and an exceptional protease stability are crucial. Finally, by solving the crystal structure of both the $APPI_{WT}$/KLK6 and APPI-4M/KLK6 complexes, we elucidated the molecular basis of the improved binding affinity and proteolytic resistance of APPI-4M. This novel KLK6 inhibitor offers promising avenues for cancer therapy and imaging applications, as well as an extremely valuable laboratory reagent for deciphering the specific mechanisms by which KLK6 drives cancer progression.

Results

Constructing and screening the APPI library. To develop APPI variants with a strong affinity to the human KLK6, we used the YSD screening platform (53). We cloned the coding region of $APPI_{WT}$ into a YSD plasmid to present it on the surface of the *Saccharomyces cerevisiae* yeast fused with the Aga2p/Aga1p system (Fig. 1A-C). In the YSD plasmid, we placed $APPI_{WT}$ between the Aga2 protein and the C-terminus c-Myc epitope tag and verified the expression of $APPI_{WT}$ by detecting the c-Myc epitope using FACS (Fig. 1D). We first verified the ability of the presented $APPI_{WT}$ to bind a soluble, fluorescently labeled KLK6 (Fig. 1D). Next, we designed two APPI-based libraries, cloned these libraries into the YSD vector, and transformed them into yeast through homologous recombination (see the *Materials and Methods* for details); in one library, termed the $APPI_{WT}$ library (or 'random library'), we constructed a randomly mutated version of the $APPI_{WT}$ gene. Random mutagenesis in the entire APPI sequence resulted in an APPI library with 1–3 mutations per clone, throughout the entire APPI sequence, yielding a library size of about 9×10^6 clones. We constructed the second library based on our previously published sequence of $APPI_{M17G/I18F/F34V}$ (50) – a clone that demonstrates a superior proteolytic stability to that of $APPI_{WT}$. We generated this $APPI_{M17G/I18F/F34V}$ -based library using a PCR assembly of NNS-containing oligos combined with an error-prone PCR. The combination of these two mutagenesis strategies in the $APPI_{M17G/I18F/F34V}$ library yielded a library (termed 'focused library') with an average mutagenesis rate of 1–2 amino acid mutations per the overall 56 residues of APPI, with one mutation in the binding loop (positions 11–18, excluding Cys at position 14; Fig. 1C) and the other either within or outside of the binding loop, yielding an experimental library of about 3.5×10^6 different clones. A sequence analysis of the focused library revealed that, as designed, 79% of the sequences contained a single mutation within the canonical binding loop, 7% contained two mutations within this loop, 10% contained a single mutation within the loop and one mutation outside of the loop (a scaffold mutation), and only 1% did not contain any mutation.

As shown in Fig. 1D, the two yeast-displayed APPI libraries were well-expressed and bound KLK6 effectively. The flow cytometry results indicated that the binding distribution of the $APPI_{WT}$ clone to KLK6 was similar to that of clones in the $APPI_{WT}$-based library. In contrast to the $APPI_{WT}$-based random library, the $APPI_{M17G/I18F/F34V}$-based library (focused library) showed a more diverse binding pattern and displayed clones with various levels of binding to KLK6. Since the focused library showed greater diversity in KLK6 binding, we chose this library for further research.

As a first step toward isolating APPI variants with a high affinity to KLK6, we pre-sorted the focused library to isolate cells with a high expression signal (Fig. 2A, "post S_0"). Then, we propagated and re-sorted the isolated, high-expressing cells, this time in the presence of a fluorescently labeled KLK6. We used a diagonal sort gate to select the cell population with a high affinity to KLK6 relative to APPI expression (as assessed by detecting the c-Myc tag), which comprised 3.7% of the initial library (Fig. 2A). Such a selection protocol allowed us to decrease the bias for selecting variants with high expression levels but possibly a low binding affinity. To isolate clones with the highest intrinsic affinity to KLK6, we repeated this process such that, in each round of

sorting, the KLK6 concentration was reduced to increase the selective pressure in each subsequent round. After each sorting round, we analyzed the expression and KLK6 binding levels by using flow cytometry (Fig. 2A, "post S_{1-3}"). After the final sorting round (S_3), this process yielded the sequences of 17 clones, of which eight sequences (47%) contained the mutation G17L within the binding loop; of these, four contained only the G17L mutation and four contained an additional, scaffold mutation (S19F). The two most abundant clones in the sequence results, which we chose to use for further individual analyses, were a clone that comprised the single mutation G17L (termed $APPI_{M17L/I18F/F34V}$) and APPI-4M, a clone that comprised two mutations (G17L and S19F) relative to $APPI_{M17G/I18F/F34V}$. We used FACS to analyze the ability of these yeast-displayed clones to bind KLK6 and compared their binding with that of the yeast-displayed $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ clone (Fig. 2B). This analysis revealed that the binding affinity of both $APPI_{M17L/I18F/F34V}$ and APPI-4M to KLK6 was superior to that of $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ (Fig. 2B). Accordingly, we chose these clones for further analysis as purified soluble proteins.

APPI-4M confers a high affinity to KLK6 and resistance to KLK6-mediated hydrolysis. To further characterize the APPI variants $APPI_{M17L/I18F/F34V}$ and APPI-4M, we expressed and purified the soluble forms of both proteins, as well as the control $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ proteins (Fig. 3A). We expressed these proteins in *Pichia pastoris* as described previously (50), measured the dissociation constant (K_D) of the most potent variant, APPI-4M, and compared it with that of $APPI_{WT}$ by using surface plasmon resonance (SPR). The K_D for $APPI_{M17L/I18F/F34V}$ could not be measured because there was not a good fit for the SPR data. The K_D for APPI-4M was 36 pM, which was 145.7-fold lower (i.e., better) than that of $APPI_{WT}$ (5,290 pM) (Fig. 3B, Table 1). This improvement was due to both an increase in the association rate [k_{on} = $(3.17 \pm 0.01) \times 10^5$ M^{-1} sec^{-1} for APPI-4M, versus $(1.96 \pm 0.01) \times 10^5$ M^{-1} sec^{-1} for $APPI_{WT}$] and, more significantly, a 90-fold decrease in the dissociation rate [k_{off} = $(1.15 \pm 0.06) \times 10^{-5}$ sec^{-1} for APPI-4M versus $(104 \pm 0.20) \times 10^{-5}$ for $APPI_{WT}$].

We next determined the inhibition constants, K_i, using a method appropriate for quantifying tight, slow-binding inhibition in KLK6 assays versus the peptide substrate BOC-FSR-AMC (49), according to Equation 1 (see Experimental Procedures). Both the $APPI_{M17L/I18F/F34V}$ and the APPI-4M variants demonstrated a considerably improved inhibition activity compared with $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ (Fig. 3C), with K_i values of 326.1 ± 5.6 pM for $APPI_{M17L/I18F/F34V}$ and 160.1 ± 59.2 pM for APPI-4M, as compared with 2,240 ± 110 pM for $APPI_{WT}$ and 1,190 ± 120 pM for $APPI_{M17G/I18F/F34V}$. Thus, $APPI_{M17L/I18F/F34V}$ and APPI-4M showed a 7-fold and a 14-fold improvement in inhibiting KLK6, respectively, as compared with the $APPI_{WT}$ (Table 1).

To further evaluate the stability of the APPI-4M/KLK6 interaction, we employed an SDS-PAGE analysis. Surprisingly, we found that, unlike the $APPI_{WT}$/KLK6 complex, the APPI-4M/KLK6 complex is resistant to dissociation under reducing, denaturing conditions (Fig. 3D). The higher molecular weight band in APPI-4M/KLK6 incubations versus the $APPI_{WT}$/KLK6 incubations describes the formation of a stable covalent complex, which may indicate the formation of a more thermodynamically stable acyl-enzyme intermediate (see Discussion).

To test whether the APPI-4M variant is indeed more resistant to KLK6 proteolysis than $APPI_{WT}$, we incubated each protein with KLK6 (90 h and 300 h for $APPI_{WT}$ and APPI-4M, respectively) and used an HPLC-based time course hydrolysis assay to measure the concentration of intact APPI over time and, from that, we calculated the turnover time ($1/k_{cat}$) of each protein (Fig. 4, Table 1). This analysis revealed that APPI-4M is 13-fold more proteolytically stable than $APPI_{WT}$ (with turnover times of 230.07 ± 40.90 h and 16.62 ± 0.05 h, respectively, Table 1), such that, over the 90-h incubation period, $APPI_{WT}$ shifted from mostly intact to mostly cleaved, while APPI-4M showed only a negligible change.

APPI-4M is more potent than $APPI_{WT}$ toward KLK6, but not toward other serine proteases. To evaluate the potency of APPI-4M toward KLK6, we quantified the inhibition constants of both $APPI_{WT}$ and APPI-4M toward KLK6 and toward three other structurally and functionally similar serine proteases, including anionic trypsin, cationic trypsin, and coagulation factor XIa (FXIa) (Table 2). As compared with $APPI_{WT}$, APPI-4M demonstrated both a 14-fold increase in inhibition potency toward KLK6 and a weaker inhibition potency toward the other serine proteases (namely, a 40-fold, a 27-fold, and a 1.7-fold weaker inhibition potency toward anionic trypsin, cationic trypsin, and FXIa, respectively). Moreover, using SPR, we measured the binding affinities of the two APPI variants to KLK6, as compared with their binding to KLK1, KLK2, KLK3,

KLK4, and KLK5. This analysis revealed that the affinity of APPI-4M toward KLK6 was ~146-fold higher (i.e., a lower K_D) than that of $APPI_{WT}$, whereas its affinity toward the other KLKs was considerably lower and either did not significantly differ or was only marginally better than that of $APPI_{WT}$ (Table 3).

APPI-4M inhibits KLK6-mediated cell invasion and migration. To determine the ability of APPI-4M to inhibit the KLK6-dependent invasive behavior of cancer cells, we used human BT-20 cells – a metastatic breast cancer cell line that secretes high levels of KLK6 and low levels of other KLKs (29). Notably, BT-20 cells previously demonstrated a reduction in cell invasiveness in the presence of an anti-KLK6 antibody in a Boyden chamber assay (29), in which the cells migrate through a Matrigel-coated membrane and are visualized by light microscopy. Using this assay, we found that BT-20 cells treated with 10 nM, 50 nM, or 100 nM APPI-4M showed a significant decrease in invasiveness (namely, a 20%, 24%, and 37% decrease, respectively) in the number of cells that migrated through the Matrigel. In contrast, cells treated with $APPI_{WT}$ showed no significant decrease in invasiveness, as compared with untreated control. Cells transfected with a KLK6-specific siRNA showed a 50% decrease in the number of cells that migrated through the Matrigel (Fig. 5A,B,C) compared with a scrambled siRNA. Importantly, in an XTT viability assay (Fig. 5D), APPI-4M did not affect the viability of BT-20 cells, indicating that the observed decrease in the number of invading cells in the Boyden chamber assay was not due to cell death but, rather, due to the ability of APPI-4M to inhibit the invasiveness of the BT-20 cells.

To further examine the ability of APPI-4M to inhibit the migration of BT-20 cells, we used a wound healing assay, in which we estimated the percentage of recolonization of the scratched surface 24 h after cell wounding (Fig. 5E and F). Treating the cells with APPI-4M (100 nM) significantly inhibited their migration, as compared with untreated cells.

The crystal structure of the APPI-4M/KLK6 complex reveals optimized interactions. To elucidate the mechanism of binding and inhibition of APPI-4M to KLK6, and to compare it with that of $APPI_{WT}$, we crystallized each inhibitor in complex with KLK6 and solved the crystal structures. Data collection and refinement statistics are reported in Table 4. The crystal structure of KLK6/$APPI_{WT}$ was solved from a crystal that diffracted with an effective resolution of 1.95Å (see Experimental Procedures for more information). The crystal structure of KLK6/APPI-4M was solved from a crystal that diffracted to a maximum resolution of 2.3Å. The co-crystallization of KLK6 with $APPI_{WT}$ or with the APPI-4M variant produced a crystal form containing one molecule of KLK6 bound to a molecule of APPI, which occupied the active site in the classic inhibitory binding mode of canonical or Laskowski-mechanism inhibitors (55,56), in which the binding loop of the inhibitor occupies (roughly) the S3 – S3′ substrate-binding subsites in a substrate-like manner (Fig. 6A). The crystal lattice asymmetric unit also contained an additional molecule of APPI that had undergone proteolysis of the reactive site Arg-15 – Ala-16 bond (Fig. 6A); this cleaved form, designated APPI*, is comprised of two protein chains connected by two disulfide bonds. In the cleaved APPI* molecule, a nearly 180° rotation in the Cys-14 ψ angle (relative to the intact APPI molecule) allows the formation of a salt bridge between the Arg-15 side chain and the Asp-203 residue of KLK6, thus stabilizing the crystal lattice, while the Cys-14 carbonyl forms a hydrogen bond with the N-terminus of Ala-16 (Fig. 6B). Notably, crystals of the $APPI_{WT}$/KLK6 complex grew over the course of seven weeks, whereas crystals of the APPI-4M/KLK6 complex did not appear until 6 months after the initial setup of crystallization drops. Given the apparent requirement for a stoichiometric equivalent of cleaved APPI for formation of the crystal lattice, it is likely that the extended duration required to form the APPI-4M/KLK6 complex crystal reflects its much greater resistance to proteolysis.

Surprisingly, in the APPI-4M/KLK6 complex, the Arg-15 P1 residue of the inhibitory APPI molecule was present in two conformations: the expected “up” conformation, featuring a salt bridge with the KLK6 specificity pocket residue Asp-189, and a “down” conformation, which is not normally observed in complexes of enzymes with trypsin-like specificity (Fig. 6C). This “down” conformation was also the dominant species observed in the $APPI_{WT}$/KLK6 crystal. The “down” conformation of Arg-15 observed in these structures is similar to the conformation reported previously for the non-cognate binding of APPI to chymotrypsin (PDB:1CA0) (Fig. 6C). Unlike KLK6, chymotrypsin possesses a Ser-189 instead of Asp-189 and cannot form a salt bridge in the specificity pocket. It is likely that the “down” conformation of Arg-15 observed in the APPI/KLK6 complexes is an artifact of the acidic crystallization conditions, which may have resulted in a partial protonation of Asp-189. This perturbation of the P1-S1 subsite interaction is a localized phenomenon, which is not expected to impact the extended

interactions of the other contact residues at the interface.

The crystal structures reveal an optimization of the contacts between the residues of the APPI-4M binding loop and the primed side subsites of KLK6. In the $APPI_{WT}$/KLK6 complex, the $APPI_{WT}$ residues Met-17, Ile-18, and Ser-19 interact with the S2′, S3′, and S4′ subsites of KLK6, respectively (Fig. 7A). These subsites are largely shaped by His-39, Leu-40, and Leu-41 – residues that are unique to KLK6 and are poorly conserved between KLKs and among other trypsin-like proteases. In the APPI-4M/KLK6 complex, the hydrophobic interactions between APPI-4M and the primed side subsites of KLK6 are optimized (Fig. 7B). Phe-18 makes more extensive contacts with the S3′ subsite, while Phe-19 forms a ring-stacking interaction with His-39 of KLK6, thus locking this residue into a single side chain conformation. The crystal structures also reveal differences in the intramolecular packing within $APPI_{WT}$ and APPI-4M (Fig. 8A,B), which may further stabilize the conformation of the binding loop and maximize the interactions between the two proteins. Residues Leu-17, Phe-19, and Val-34 of APPI-4M form a hydrophobic cluster that fills the primed side of the KLK6 substrate-binding crevice (Fig. 8B), featuring more extensive hydrophobic interactions with KLK6 Phe-151 and Leu-40 than those observed in the $APPI_{WT}$/KLK6 complex. In the APPI-4M complex, Phe-19 also forms a ring-stacking interaction with His-39 of KLK6 (Fig. 8B,D). The mutated residues Phe-18 and Phe-19 of APPI-4M can wrap around the ridge formed by KLK6 residues 39-41 (Fig. 8D), thus forming a more extensive contact interface than that in the corresponding region of the $APPI_{WT}$/KLK6 complex (Fig. 8C).

Discussion

We developed a potent, 4-mutant KLK6 inhibitor by employing the powerful YSD technology to engineer an APPI scaffold that demonstrates an increased proteolytic stability and a higher binding affinity toward KLK6. To develop this unique inhibitor, we generated two libraries: one with multiple random mutations in the entire protein scaffold (termed 'random library'), and another more focused library, with multiple random mutations in the entire protein scaffold, having at least a single mutation in the APPI/KLK6 interface (termed 'focused library'). The focused library was constructed based on our previously published $APPI_{M17G/I18F/F34V}$ sequence. As expected, the focused library, when displayed on yeast, showed a superior affinity to KLK6 than the random library. This result is in agreement with previous data showing that the sequence of the canonical binding loop largely determines the affinity and specificity of APPI to its target serine proteases (50) and implies that generating diversity within the APPI binding loop, as opposed to randomizing the entire protein sequence, has more potential to improve the binding affinity. Sequencing results from three KLK6 affinity maturation screens of the focused library showed no consensus sequence for the selected clones; nevertheless, a repeating mutation at position 17, G17L, located in the inhibition loop, was identified in ~50% of the clones. The two most abundant YSD variants, namely $APPI_{G17L/I18F/F34V}$ and $APPI_{M17L,I18F,S19F,F34V}$ (APPI-4M), displayed the highest affinity towards KLK6, as measured by flow cytometry (data not shown).

To better characterize our most potent variant, APPI-4M, we purified this protein in its soluble form and tested its binding and inhibition potencies, as well as its stability towards cleavage by KLK6, and compared these parameters to those of $APPI_{WT}$. Our mutant outperformed $APPI_{WT}$ in all these parameters. When tested in binding assays using SPR, our mutant demonstrated a sub-nanomolar affinity with not only a higher k_{on} value, reflecting initial molecular recognition by KLK6, but also a lower k_{off} rate compared with $APPI_{WT}$, reflecting long-term complex stability. This finding has an important clinical implication, as a slow k_{off} rate may facilitate a durable pharmacological potency of a drug *in vivo* (57,58). Another important issue that we addressed was proteolytic stability. We show that KLK6 can cleave $APPI_{WT}$ within several hours, whereas APPI-4M proteolysis is observed only on a time scale of days. The turnover of APPI as a substrate of KLK6 is further emphasized by the presence of the product molecule APPI* in our crystal structures, cleaved at the Arg15-Ala16 reactive site bond. We have previously demonstrated that APPI* is severely compromised as a protease inhibitor, and thus this cleavage serves as a mechanism for inhibitor inactivation (59). As APPI-4M showed both a slow k_{off} rate and a high stability toward KLK6 cleavage, it may have prolonged on-target residence time and extended potency, leading to superior efficacy *in vivo* (60,61).

In SDS-PAGE analyses of complexes formed between KLK6 and APPI-4M, but not between KLK6 and $APPI_{WT}$, we observed a shift of KLK6 to a higher molecular weight in the presence of the inhibitor, suggesting the formation of a covalent complex that

cannot be dissociated by reducing, denaturing conditions. This finding was surprising, because, while such covalent complexes are a hallmark of the unrelated serpin family of serine protease inhibitors, which form stable acyl-enzymes with their target proteases (49,62,63), the canonical inhibitors, including APPI, follow a different mechanism, in which the stable complex is a tightly but reversibly bound Michaelis complex (55,64). Previous studies of the chymotrypsin inhibitor 2, which belongs to a different family of canonical inhibitors, identified the rapid formation of a covalent acyl-enzyme by SDS-PAGE, but this species accumulated to represent only a small fraction of the inhibited enzyme due to the reversibility of acylation and the comparatively greater thermodynamic stability of the Michaelis complex (65,66). Thus, to the best of our knowledge, our observation represents the first detection of a covalent species formed by an inhibitor of the Kunitz family, as well as the only example of any canonical inhibitor forming a covalent complex with an inhibited enzyme, in which the covalent complex represented the major species. However, the crystal structure of the KLK6/APPI-4M complex did not reveal a stable covalent complex, most likely because the Michaelis complex/acyl-enzyme equilibrium was shifted by local electrostatic alterations in the active site due to the low pH of the crystallization process. Nevertheless, we speculate that the improved inhibitory potency and slower dissociation rate of APPI-4M relative to $APPI_{WT}$ may have been achieved, at least in part, through enhanced enzyme-inhibitor interactions that stabilize the conformation of the acyl-enzyme or the acylation transition state in the KLK6/APPI-4M complex, resulting in the thermodynamic stabilization of the acyl-enzyme relative to the Michaelis complex. The slow rate of formation of fully cleaved APPI-4M* indicates that the hydrolysis of the acyl-enzyme remains rate-limiting in APPI proteolysis, as is characteristic of canonical inhibitors (65), rendering APPI-4M a strong inhibitor – rather than a good substrate.

In addition to the clinical importance of the stability and affinity of an inhibitor, its lower potency toward other serine proteases is another highly significant factor for *in vivo* applications. Notably, APPI can inhibit several human proteases, including trypsin, chymotrypsin, plasmin, and FXIa (67,68); these proteases may act as modulators of *in vivo* APPI concentrations (i.e., KLK6 competitors) (69). We found that, as compared with $APPI_{WT}$, the APPI-4M mutant showed a substantially greater binding affinity toward KLK6, while its affinities toward cationic trypsin, anionic trypsin, and FXIa were decreased. In addition, our SPR results show that the binding affinity of the APPI-4M mutant toward KLK6 is stronger than toward the other KLKs, despite the high sequence and structure similarities among the KLKs (34). This enhanced affinity is compelling, especially considering that our YSD sorting strategy explicitly selected only for KLK6 binding and did not incorporate counterselection strategies.. Our crystal structures reveal altered interactions of APPI-4M at the enzyme interface, primarily involving the optimization of primed side interactions with the S2′, S3′, and S4′ subsites of KLK6. Notably, these subsites are largely shaped by KLK6 residues 39-41, representing a stretch of sequence that is highly unique to KLK6 and is poorly conserved between KLKs and other trypsin-like proteases. Indeed, the improved affinity of APPI-4M toward KLK6 versus other KLKs suggests that an optimization of the S2′-S4′ subsite contacts may represent a general strategy for developing selective KLK6 inhibitors (71-73).

Over the past decade, KLK6 has emerged as a significant player in various stages of cancer development, and it has been associated with cell malignancy in multiple cancers, including melanoma, ovarian, colon, gastric and breast cancers (8,19,21,23). Previous studies from the Mori laboratory demonstrated that siRNA silencing of KLK6 expression in gastric cancer cells can inhibit a variety of malignant phenotypes, implicating KLK6 as a therapeutic target in cancer (14). In breast cancer, Gosh *et al.* have identified BT-20 cells as a highly metastatic breast cancer cell line, which expresses high levels of KLK6 (29). It was found that efficient inhibition by a KLK6 antibody has led to striking suppression of BT-20 cellular invasion in a Matrigel transwell assays (29). Similarly, we found that treating BT-20 cells with APPI-4M, but not with $APPI_{WT}$, inhibits their invasion capabilities. We show, using siRNA transfection, that BT-20 cell invasiveness indeed depends on KLK6 expression. Importantly, APPI-4M did not inhibit the proliferation/viability of these cells, suggesting that, in these cells, KLK6 is responsible for invasion but not proliferation. Our study thus suggests that targeting KLK6 with the high-affinity, high-stability APPI-4M can offer therapeutic benefits in breast cancer, and it further identifies useful models for evaluating the efficacy of KLK6 inhibitors (e.g., in combination with cytotoxic or cytostatic chemotherapeutic agents).

Another important aspect of this study is that it provides the first proof-of-principle for an innovative

approach, developed here, to engineer protein-based, therapeutic-oriented inhibitors with improved target affinity and greater proteolytic stability. Despite the well-known advantages of protein-based drugs (e.g., their lower immunogenicity and toxicity relative to small-molecule drugs) (74), they often suffer from two main caveats: first, the naturally occurring protein on which the drug is based may be specific to several, structurally similar targets (indeed, in our case, the wild-type APPI similarly inhibits several target proteins) (75) – which may cause severe side effects in a therapeutic context. Second, they are typically susceptible to proteolytic degradation in the body – which limits their effectiveness. Our engineered APPI-4M protein showed a dramatic improvement in both these aspects relative to the wild-type APPI. Hence, the strategy developed here to engineer this potent stable inhibitor may have far-reaching implications, as it can potentially enable the production of other improved protein-based therapeutics. We also emphasize that, beyond the translational potential of this specific inhibitor, a significant advance of this work lies in the structural elucidation of key interactions responsible for modifying inhibitor affinity – insights that will be beneficial for future efforts toward KLK inhibitor development.

Experimental procedures

Reagents. Synthetic oligonucleotides were obtained from Integrated DNA Technologies (San Jose, CA). Restriction enzymes and polymerases were purchased from New England Biolabs (Ipswich, MA) and dNTPs were purchased from Jena Bioscience (Jena, Germany). The EBY100 yeast strain and yeast surface display (YSD) plasmid (pCTCON) were gifts from the laboratory of Dane Wittrup (MIT, MA). Bacterial plasmid extraction and purification kits were purchased from RBC Bioscience (New Taipei City, Taiwan) and yeast plasmid extraction kits were purchased from Zymo Research (Irvine, CA). The methylotrophic yeast *Pichia pastoris* strain GS115 and its expression vector (pPIC9K) were purchased from Invitrogen (Carlsbad, CA). The phycoerythrin (PE)-conjugated anti-mouse antibody was purchased from Sigma-Aldrich (St. Louis, MO). The mouse anti-c-Myc antibody (Ab-9E10) was obtained from Abcam (UK). Factor-XIa and its substrate S-2366 (Chromogenix) were obtained from Hematologic Technologies Inc. (Essex Junction, VT) and from Diapharma (West Chester Township, OH), respectively. The kallikrein-6 substrate BOC-Phe-Ser-Arg-AMC was obtained from Bachem (Bubendorf, Switzerland). Kallikrein-1 and Kallikrein-5 were obtained from ProSpec (Rehovot, Israel).

Generating the combinatorial APPI libraries. The synthesis and cloning of the DNA encoding the $APPI_{WT}$ gene and APPI random library are described elsewhere (50). Briefly, for the APPI random library, we constructed a randomly mutated version of the APPI gene by an error-prone PCR, using nucleotide analogues and a low-fidelity Taq polymerase, with the $APPI_{WT}$ gene as a template. The APPI focused library was constructed based on our previously published $APPI_{M17G/I18F/F34V}$ sequence (50). This library was generated by PCR assembly combined with error-prone PCR protocols, using a total of 10 overlapping oligonucleotides. In addition to generating random mutations in the entire APPI sequence (error-prone randomization) and to further incorporating random mutations in specific residues, seven of the 10 oligonucleotides were individually randomized by using NNS-degenerated codons at specific positions within the APPI inhibitory binding loop region (positions 11–18, excluding the Cys residue at position 14; Fig. 1D). The APPI random and focused DNA libraries were amplified and transformed into yeast through homologous recombination, sequenced at the DNA Microarray and Sequencing Unit [(DMSU), NIBN, BGU], and their yield was evaluated by dilution plating, as described previously (53).

Synthesis and cloning of the APPI library. The APPI focused library was constructed based on our previously published $APPI_{M17G/I18F/F34V}$ sequence (50) with a peptide linker (NH_3^+-APPI-LPDKPLAFQDPS-COO^-), using codons optimized for *Saccharomyces cerevisiae* and *P. pastoris* usage. The library was generated by PCR assembly using the following ten overlapping oligonucleotides (5'-3'):

(1) GGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTG
CTAGCGAAGTTTGTTCTGAACAAGCTG

(2) GAAGTTTGTTCTGAACAAGCTGAA**NNSGGTCCATGTAGAG**
CTGGTTTTTCTAGATGGTATTTCGATGTTACTG

(3) GAAGTTTGTTCTGAACAAGCTGAA**ACTNNSCCATGTAGAG**
CTGGTTTTTCTAGATGGTATTTCGATGTTACTG

(4) GAAGTTTGTTCTGAACAAGCTGAA**ACTGGTNNSTGTAGAG**
CTGGTTTTTCTAGATGGTATTTCGATGTTACTG

(5) GAAGTTTGTTCTGAACAAGCTGAA**ACTGGTCCATGTNNSG**
CTGGTTTTTCTAGATGGTATTTCGATGTTACTG

(6) GAAGTTTGTTCTGAACAAGCTGAA**ACTGGTCCATGTAGAN**
NSGGTTTTTCTAGATGGTATTTCGATGTTACTG

(7) GAAGTTTGTTCTGAACAAGCTGAA**ACTGGTCCATGTAGAG**
CTNNSTTTTCTAGATGGTATTTCGATGTTACTG

(8) GAAGTTTGTTCTGAACAAGCTGAA**ACTGGTCCATGTAGAG**
CTGGTNNSTCTAGATGGTATTTCGATGTTACTG

(9) GATGGTATTTCGATGTTACTGAAGGTAAATGTGCTCCATT CGTCTATGGTGGTTGTGGTGGTAATAGAAATAATT

TCGATACTGAAGAATATTGTATGGCTGTTTGTGGTTCTGCTAT TGGATCCTTGCCAGATAAACCATTGGCTTTCC

(10) GAGCTATTACAAGTCCTCTTCAGAAATAAGCTTTTGTTC AGATGGATCTTGGAAAGCCAATGGTTTATC

The synthetic library was assembled by a set of three PCRs, using low-fidelity Taq polymerase, as follows: in the first reaction, oligos number 2–9 were incorporated using error-prone PCR to generate both a site-specific saturation randomization of the binding loop residues (using NNS codons, where N = A/C/G/T and S = C/G) and an arbitrary randomization of their neighboring residues, ranging between E10 and G38 on the sequence, chosen for their involvement in the KLK6 hypothesized interaction interface. The error-prone PCR reaction was optimized to 15× PCR doublings using low-fidelity Taq polymerase, 1% nucleotide analogues, and 2 mM $MgCl_2$. Next, the assembled fragment was amplified by PCR using 5'-GAAGTTTGTTCTGAACAAGCTG-3' and 5'-GGAAAGCCAATGGTTTATC-3' primers, followed by a final PCR reaction using oligos number 1 and 10. The final PCR assembled fragment was gel-purified and cloned into a pCTCON vector via transformation by electroporation of EBY100 yeast cells and homologous recombination with the linearized vector (digested with *Nhe*I and *Bam*HI), as described previously (53). Random and site-specific mutagenesis using error-prone PCR and NNS degenerate codons in the APPI sequence yielded a library of approximately 3.5×10^6 variants, as estimated by dilution plating on selective SDCAA plates (15% agar, 2% dextrose, 1.47% sodium citrate, 0.429% citric acid monohydrate, 0.67% yeast nitrogen base, and 0.5% casamino acids). Sequencing results [obtained from the DNA Microarray and Sequencing Unit (DMSU) of the National Institute for Biotechnology in the Negev (NIBN), Ben-Gurion University of the Negev (BGU), Israel] revealed an average amino acid mutation rate of 1–2 mutations per 56 amino acids of the APPI sequence (data not shown).

Production of recombinant trypsins. The recombinant human anionic trypsinogen and the human cationic trypsinogen were expressed in *Escherichia coli*, extracted from inclusion bodies, refolded, purified, and activated with bovine enteropeptidase, as described previously (52,76).

Production of the human kallikrein 6. Recombinant pro-KLK6 was expressed and purified from a virus/insect cell line system Sf21 at the Protein Expression & Purification Core Facility, EMBL Heidelberg, Germany, as previously described (46). The pro-enzyme was activated with enterokinase, purified, and its concentration was estimated by UV-Vis absorbance at 280 nm with an extinction coefficient (ε_{280}) of 34.67×10^3 M^{-1} cm^{-1} in a DeNovix DS-11 spectrophotometer (DeNovix, Wilmington, DE). KLK6 was labeled with the DyLight-650 amine-reactive dye according to the manufacturer's protocol (Thermo Fisher Scientific, Waltman, MA), at a ratio of 1:5 (KLK6/dye).

Construction and cloning of the expression vector pPIC9K–APPI. The human cDNA of $APPI_{WT}$, based on a published sequence (PDB id 1ZJD), was amplified by PCR using a Phusion DNA polymerase with an upstream primer: 5′-AGCGTATACGTAGACTATAAGGATGACGAC GACAAAGAATTCG AAGTTTGTTCTGAACAAGCTG-3′ and a downstream primer: 5′-ATAGTTTAGCGGCCGC ATGATGGTGGTGATGGTGCCTAGGAATAGC AGAACCACAAACAGC-3′. The resulting construct included four restriction sites and two epitope tags (FLAG and HIS_6), as follows: *SnaBI*-FLAG-*EcoRI*-$APPI_{WT}$-*AvrII*-HIS6-*NotI*. The obtained DNA fragment was digested with *SnaBI* and *NotI* and sub-cloned by using the same restriction sites as for the *Pichia* expression vector pPIC9K. Next, the recombinant expression plasmid was used as a template for the construction of the APPI variants, as follows: the cDNA of each variant was amplified by PCR using an upstream primer: 5′-CGGAGCGAATTCGAAGTTTGTTCTGAACAAG CTG-3′ and a downstream primer: 5′-CGCTACCCTAGGAATAGCAGAACCACAAAC AGC-3′. The resulting construct included the restriction sites *EcoRI* and *AvrII*. The obtained DNA fragment was digested with *EcoRI* and *AvrII* and sub-cloned using the same restriction sites of the template vector. Finally, all sequences of the recombinant expression plasmids were confirmed by a DNA sequencing analysis (DMSU, NIBN, BGU). Expression vectors were linearized by *SacI* digestion and used to transform *P. pastoris* strain GS115 by electroporation. This process resulted in the insertion of the construct at the AOX1 (alcohol oxidase) locus of *P. pastoris*, thereby generating a His^+ Mut^+ phenotype. Transformants were selected for the His^+ phenotype on a 2% agar containing regeneration dextrose biotin (RDB; 18.6% sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 0.005% each of L-glutamic acid, L-methionine, L-

lysine, L-leucine, and L-isoleucine) and allowed to grow for 2 d at 30 °C. The cells were harvested from the plates and subjected to further selection for a high copy number by their ability to grow on 2% agar containing 1% yeast extract, 2% peptone, 2% dextrose medium, and the antibiotics G418 (Geneticin, 4 mg/ml, Invitrogen). To verify the direct insertion of the construct at the AOX1 locus of *P. pastoris*, the genomic DNA of the highest APPI-expressing colony from each APPI variant was extracted and amplified by PCR with an AOX1 upstream primer: 5´-GACTGGTTCCAATTGACAAGC-3´ and an AOX1 downstream primer: 5´-GCAAATGGCATTCTGACATCC-3´. The resulting linear DNA was gel-purified and its correct sequence was confirmed by DNA sequencing analysis (DMSU, NIBN, BGU).

Flow cytometry analysis and cell sorting. The yeast-displayed APPI library was grown in an SDCAA selective medium (2% dextrose, 0.67% yeast nitrogen base, 0.5% Bacto™ casamino acids, 1.47% sodium citrate, 0.43% citric acid monohydrate, pH 4.5) and induced for expression with an SGCAA selective medium (similar to SDCAA, but with 2% galactose instead of dextrose) according to an established protocol (53). The expression of the yeast-displayed proteins was detected by labeling the cells with the 9E10 mouse anti-c-Myc antibody (Abcam, Cambridge, UK) in a 1:50 ratio. Next, the cells were washed and resuspended in a goat anti-mouse secondary antibody, which was conjugated to phycoerythrin (Sigma) in a 1:50 ratio in a solution containing 50 M Tris-HCL, 100 mM NaCl, and 0.2% BSA (pH 7.3). The KLK6 binding signal and the APPI expression signal were analyzed by dual-color flow cytometry (Accuri C6; BD Biosciences, San Jose, CA).

Cell sorting of the APPI library was conducted with a BD FACS Aria III (Ilse Katz Institute for Nanoscale Science and Technology, BGU), as described in Fig. 1. Briefly, 30×10^6 yeast cells were sorted first, and cells demonstrating a high APPI expression signal were isolated to select for high-expressing clones (sort S_0). Following each successive sort, the number of yeast cells used was at least 10-fold in excess relative to the number of sorted cells from the previous sort. Several clones from each round of sorting were sequenced.

The same labeling procedure was used for the YSD-based titration curves of the individual clones, and the binding of different APPI clones to KLK6 was evaluated at KLK6 concentrations ranging from 100 fM to 10 nM. Each binding measurement was normalized to the APPI expression signal of the same clone.

Large-scale expression and purification of APPI proteins. *P. pastoris* GS115-APPI clones were first inoculated into 50 ml of BMGY (1% yeast extract, 2% peptone, 0.23% potassium phosphate monobasic, 1.18% potassium phosphate dibasic, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 1% glycerol) to an OD_{600} = 10.0 (10^8 cells/ml), followed by scaling-up to 500 ml of BMGY, until an OD_{600}=10.0 was reached (overnight growth at 30 °C with shaking at 300 rpm). The cells were harvested by centrifugation and resuspended in 1 l of BMMY (similar to BMGY, but with 0.5% methanol instead of glycerol) to an OD_{600} = 5.0 to induce expression, and were grown at 30 °C with shaking at 300 rpm. Methanol was added to a final concentration of 2% every 24 h to maintain induction. Following 5 d of induction, the culture was centrifuged again and the supernatant, containing the secreted recombinant inhibitors, was prepared for purification by nickel-immobilized metal affinity chromatography (IMAC). The supernatant containing the recombinant APPI was filtered through a 0.22-µm Steritop bottle-top filter (Millipore, Billerica, MA). The filtered supernatant was adjusted to 10 mM imidazole and 0.5 M NaCl at pH 8.0 and left to stand overnight at 4 °C. Then, a second filtration step was performed to remove any additional precipitation. The resulting supernatant was loaded on a HisTrap 5-ml column (GE Healthcare, UK) at a flow rate of 0.7 ml/min for 24 h, washed with a washing buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole; pH 8.0), and eluted with an elution buffer (similar to the washing buffer, but with 0.5 M imidazole) in an ÄKTA pure instrument (GE Healthcare). The eluted inhibitors were concentrated and the buffer was replaced with 50 mM Tris-HCl and 100 Mm NaCl, pH 7.3, in a 3-kDa molecular weight cutoff (MWCO) Vivaspin concentrator (GE Healthcare). Gel-filtration chromatography was performed with a Superdex 75 16/600 column (GE Healthcare) equilibrated with 50 mM Tris-HCl and 100 mM NaCl, pH 7.3, at a flow rate of 1 ml/min on an ÄKTA start instrument (GE Healthcare). An SDS-PAGE analysis was used to confirm the purity of the proteins. The correct mass of the pure proteins was validated by using a MALDI-TOF REFLEX-IV (Bruker, Billerica, MA) mass spectrometer (The Ilse Katz Institute for Nanoscale Science & Technology, BGU). Purification yields for all APPI clones were 0.8–50 mg per 1-l culture flask.

Surface plasmon resonance (SPR) studies. The affinity of binding between the APPI variants and KLKs (KLK1, KLK2, KLK3, KLK4, KLK5 and KLK6) was detected by using SPR spectroscopy on a ProteOn XPR36 system (Cytometry, Proteomic and Microscopy Unit, NIBN, BGU). $APPI_{WT}$ and APPI-4M were immobilized on the surface of a GLC chip by using the amine coupling reagents sulfo-NHS (0.1 M N-hydroxysuccinimide) and EDC (0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Bio-Rad). $APPI_{WT}$ and APPI-4M (2 µg) were each covalently immobilized on the chip in 10 mM sodium acetate buffer, pH 4.0, to give 237.4 and 439.9 response units (RU), respectively. A single flow channel immobilized with a BSA (3 µg) served as a background control. Each KLK was diluted in a phosphate-buffered saline with 0.05% Tween 20 to 0.625, 1.25, 2.5, 5, or 10 nM in 400 µl, and was circulated for 600 s at a flow rate of 25 µl/min, followed by 2400 s of dissociation at a flow rate of 25 µl/min. The response was monitored as a function of time at 25 °C. The kinetic constants, k_{on} and k_{off}, and the equilibrium affinity constant K_D between the APPI variants and KLKs were determined by using the 1:1 Langmuir model (77).

Inhibition studies. The inhibition constant K_i of $APPI_{WT}$ and its variants – $APPI_{M17G/I18F/F34V}$, $APPI_{M17L/I18F/F34V}$, and APPI-4M – in complex with KLK6 was determined according to a previously described methodology (50,76) with minor modifications. Briefly, tight binding experiments were conducted at a fixed concentration of the substrate BOC-Phe-Ser-Arg-AMC (t-Butyloxycarbonyl- L-phenylalanyl- L-seryl- L-arginine- 4-methylcoumaryl-7-amide) (1 mM). These assays were performed at 37 °C in the presence of various concentrations of inhibitor, using a Synergy2 microplate spectrophotometer (BioTek, Winooski, Vermont). KLK6 (50 µl) and APPI (50 µl) were mixed and equilibrated in a 96-well microplate (Greiner, Kremsmünster, Austria) prior to the addition of the substrate (50 µl). For the $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ reactions, the KLK6 concentration was 1 nM and the concentration range of APPI proteins was 0–50 nM. For the $APPI_{M17L/I18F/F34V}$ and APPI-4M reactions, no detectable activity of KLK6 was observed in the presence of the inhibitors, and, therefore, the concentration of KLK6 was changed to 100 nM and the concentration range of the APPIs was 419–1600 nM. The fluorescent signal of the reactions was monitored in an Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland), set at 355 nm for excitation and 460 nm for emission. Reactions including $APPI_{WT}$ and $APPI_{M17G/I18F/F34V}$ were followed for 4 h, and those including $APPI_{M17L/I18F/F34V}$ and APPI-4M were followed for 20 min to determine the initial steady-state rates of substrate hydrolysis. The inhibition constants were calculated by using Equation 1, as described previously (76):

$$\frac{(\boldsymbol{V_0 - V_i})}{\boldsymbol{V_i}} = \frac{[\boldsymbol{I}]_0}{\boldsymbol{K_i}(\mathbf{1} + [\boldsymbol{S}]_0/\boldsymbol{K_m})} \qquad \textit{Equation 1.}$$

where V_i and V_0 are the steady-state rates in the presence and absence of the inhibitor, respectively; K_m is the Michaelis constant for substrate cleavage; and $[S]_0$ and $[I]_0$ are the initial concentrations of the substrate and inhibitor, respectively. The affinity of the inhibitors was evaluated by conducting similar inhibition studies using cationic trypsin, anionic trypsin, and FXIa; briefly, tight binding experiments were conducted at a fixed concentration of 145 µM Z-GPRpNA as a substrate for cationic trypsin and anionic trypsin, or with 600 µM S-2366 as a substrate for FXIa. For the experiments with cationic and anionic trypsins, the concentrations of the inhibitors ranged between 0–80 nM and the concentration of the enzyme was 0.1 nM. For the experiment with FXIa, the concentrations of the inhibitor ranged between 2–10 nM and the concentration of the enzyme was 0.125 nM. The enzyme (8 µl), inhibitor (8 µl), and buffer (144 µl) were preincubated at room temperature for 60 min in a solution of 100 mM Tris-HCl and 1 mM $CaCl_2$ (pH 8.0) for cationic and anionic trypsins and 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl2 and 0.1% BSA for FXIa. The reactions were then initiated by diluting the mixture of enzyme and inhibitor into a pre-equilibrated microplate (non-binding, 96 well; Greiner, Kremsmünster, Austria) containing a buffer (152 µl) and a substrate (8 µl). The reactions were conducted at 25 °C and monitored spectroscopically for 2 h. The concentrations of cationic trypsin and anionic trypsin were quantified by active-site titration using pNPGB, which serves as both an irreversible trypsin inhibitor and a substrate. The concentrations of FXIa and KLK6 were determined by using UV-Vis absorbance at 280 nm, with extinction coefficients (ε_{280}) of 214.4×10^3 M^{-1} cm^{-1} for FXIa and 34.67×10^3 M^{-1} cm^{-1} for KLK6. The concentrations of the chromogenic substrates Z-GPR-pNA and S-2366 were determined in an end-point assay from the change in the absorbance (plateau after complete hydrolysis) that is obtained by the release of p-nitroaniline. The concentrations of the APPI

variants were determined by titration with pre-titrated bovine trypsin and the substrate L-BAPA, as previously described (76). The reaction buffers used in this study were as follows: KLK6 experiments: 50 mM Tris-HCl, 100 mM NaCl, and 0.2% BSA (pH 7.3); trypsin experiments: 100 mM Tris-HCl and 1 mM $CaCl_2$ (pH 8.0); FXIa experiments: 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.1% BSA (pH 7.6). Calculations were performed by using K_m values (mean ± standard deviation) of 329.33 ± 1.44 μM for KLK6, 22.8 ± 1.9 μM for cationic trypsin, 10.7 ± 0.7 μM for anionic trypsin, and 361.3 ± 12.1 μM for FXIa, as determined from at least three Michaelis–Menten kinetic experiments with triplicates (50). The results of the inhibition studies are reported as mean ± standard deviation.

APPI/KLK6 complex formation in SDS-PAGE. This assay was performed as described previously, with minor modifications (49). KLK6 (5 μM) was incubated for 1 h with $APPI_{WT}$ or APPI-4M, at 1:1, 1:5, or 1:10 molar ratios, in a solution of 50 mM Tris-HCl and 100 mM NaCl (pH 7.3) at 37 °C. Following this incubation, samples were prepared in a reducing sample buffer containing 1% SDS, 10% glycerol, 10 mM Tris-HCl, 0.2 mM EDTA, 80 mM DTT, 0.01% bromphenol blue, pH 6.8, ran on an SDS-PAGE, and stained with Coomassie blue.

APPI hydrolysis studies. The cleavage of intact APPI variants (between the residues Arg15 and Ala16) in time-course incubations with KLK6 was monitored by high-performance liquid chromatography (HPLC; Waters, Milford, MA), as described previously (50,59), with minor modifications. Briefly, KLK6 was incubated with $APPI_{WT}$ or APPI-4M in 30-μl aliquots in a solution of 50 mM Tris-HCl and 100 mM NaCl (pH 7.3) at 37 °C; the concentration of the inhibitor was 25 μM and the concentration of KLK6 was 3.28 μM. For analysis by HPLC, the aliquots were withdrawn at periodic intervals (over 90 h for $APPI_{WT}$ and 300 h for APPI-4M) and the samples were immediately quenched by acidification with 70 μl of 0.3 M HCl. The samples were resolved on a 50×2.0 mm Jupiter 4 μm 90 Å C_{12} column (Phenomenex, Torrance, California) with a gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.6 ml/min over 50 min. Intact inhibitors were quantified by peak integration of absorbance traces, monitored at 210 nm. The initial rates of hydrolysis over time were obtained by linear regression, using a minimum of seven data points within the initial linear phase of the reaction. The reported hydrolysis rates for each inhibitor represent the average (± standard deviation) of three independent experiments.

Cells. BT-20 cells were purchased from the American Type Culture Collection (ATCC). The cells were maintained in EMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% penicillin/streptomycin.

Small interfering RNA transfection. BT-20 breast cancer cells (also known as HTB-19 cells) were plated at 80% confluency 1 d before transfection and then transfected with KLK6-specific siRNA or scrambled non-specific siRNA using the TriFECTa RNAi kit (IDT, San-Jose, CA) with the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, dosing the cells twice with siRNA (45 picomoles) with a 24 h interval.

RNA extraction and RT-PCR analysis. Cells were lysed using Trizol (Invitrogen, Carlsbad, CA) and total RNA was isolated according to the manufacturer's instructions. After the RNA was quantified, 750 ng of RNA were used for cDNA synthesis by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Each cDNA sample was amplified using the Phusion Hot Start Flex polymerase (NEB, Beverly, MA). The following primers were used: *KLK6* (sense, 5′-GCA AGA CAG CAG ATG GTG AT-3′; antisense, 5′-CAC TTG GCC TGA ATG GTT TT-3′), and *GAPDH* (sense, 5′-GAG TCA ACG GAT TTG GTC GT-3′; antisense, 5′-TTG ATT TTG GAG GGA TCT CG-3′). GAPDH was used as a reaction control. The PCR-amplified cDNA was subjected to agarose gel electrophoresis. The DNA bands were imaged with the Vilber Fusion automatic gel imaging analysis system (Vilber, Marne La Vallée, France).

Cell invasion assay. The Boyden chamber invasion assay was performed as described previously (29) with the following modifications. The tumorigenic breast cancer cell line BT-20 (HTB19), which secretes KLK6, was used. Cells were cultured in complete culture medium, grown to 70% confluency, harvested, and suspended in a serum-free medium. Three hours prior to conducting the assay, inserts (ThinCert, 8 μm pore size; Greiner BioOne, Kremsmünster, Austria) were coated with 30 μg Matrigel (Corning, Corning, NY). Cells (1.5×10^4) were seeded in each insert. A solution containing either APPI-4M (10, 50 or 100 nM), $APPI_{WT}$ (10, 50 or 100 nM) or, as a control, 50 mM Tris-HCl and 100 mM NaCl (pH 7.3), was added to each insert to reach

a total volume of 200 µl. Each condition was performed in triplicate. Additional controls included KLK6-specific siRNA transfected cells and scrambled non-specific siRNA transfected cells, seeded in the same conditions. Culture media (600 µl) with 10% FBS was used as a chemoattractant in the lower chambers. The chambers were incubated in a CO_2 incubator at 37 °C for 36 h, and then the invasive cells were fixed and stained with the Dipp Kwik Differential Stain kit (American Mastertech Scientific, Lodi, CA). The migrated cells were counted (five fields per insert) with an EVOS FL cell imaging system (Thermo Fisher Scientific, Waltham, MA) at a ×10 magnification. The reported results are the mean (± standard error) of three biological repetitions. Statistical analysis was conducted with the Student's t-test.

Cell viability assay. Cell viability was evaluated by using the XTT Cell Proliferation Kit (Biological Industries, Israel), according to the manufacturer's instructions, in a 96-well plate. The cells were cultured in a culture medium, grown to 70% confluency, harvested, replaced in a serum-free medium, and 1.5×10^4 cells were seeded in each well. APPI-4M or $APPI_{WT}$ (10, 50 or 100 nM), 50 mM Tris-HCl and 100 mM NaCl (pH 7.3), as a control, was added to each well to reach a total volume of 200 µl, in triplicate. The plate was incubated in a CO_2 incubator at 37 °C for 36 h and cell viability was measured according to the manufacturer's instructions. The reported results are the mean (± standard error) of three biological repetitions. Statistical analysis was done using the Student's t-test.

Wound healing assay. BT-20 cells (1×10^5) were cultured as confluent monolayers in a 24-well plate. After reaching full confluency, a scratch was formed by removing a strip of cells across the well using a p200 pipette tip. The scratched monolayers were then washed twice to remove non-adherent cells, and then 500 µl of a 2% FBS medium, containing either APPI-4M (100 nM) or a 2% FBS medium (control), were added. The wells were photographed with an EVOS FL cell imaging system at a 4 × magnification, both immediately after cell wounding and again after 24 h. The experiment was performed in a triplicate and the images were analyzed using image J. Error bars in the figure represent the standard deviation (SD). Statistical significance was determined by Student's t-test, with $p < 0.05$ considered significant.

Protein crystallization, data collection, structure determination, and refinement. KLK6 was mixed with either $APPI_{WT}$ or APPI-4M in a 1:5 molar ratio and subjected to crystallization trials using the sitting drop vapor diffusion method. Initial crystallization conditions were screened by using the Index screening kit (Hampton Research, Aliso Viejo, CA) at 293 K. Each drop contained a mixture of a 0.3 µl crystallization solution and 0.3 µl of the $APPI_{WT}$/KLK6 or APPI-4M/KLK6 complex. For the $APPI_{WT}$/KLK6 complex, one crystal grew after 40 d in a drop containing 0.2 M ammonium sulfate, 0.1 M Bis-Tris (pH 5.5), and 25% polyethylene glycol 3350. The crystal was harvested and flash-cooled in liquid nitrogen prior to data collection. X-ray diffraction data were collected at 100 K in beamline ID30B at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The crystal diffracted to a maximum resolution of 1.85 Å with a low mean I/σI and multiplicity (1.08 and 2.0, respectively). However, the effective resolution of this structure was considered to be 1.95Å. The last shell (1.95-2.02) had a mean I/σI of 1.44 and a multiplicity of 12.8. The data has The crystal belongs to the P212121 space group, with unit cell dimensions of a 59.543 Å, b 77.702 Å, and c 92.207 Å, and it contains one molecule of KLK6 and two molecules of $APPI_{WT}$ in the asymmetric unit. For the APPI-4M/KLK6 complex, one crystal grew after 6 m in a drop containing 0.1 M tri-Na citrate (pH 5.6), 20% 2-propanol, and 20% polyethylene glycol 4000. The crystal was harvested and flash-cooled in liquid nitrogen prior to data collection. X-ray diffraction data were collected at 100 K in beamline ID23-1 at the ESRF to a maximum resolution of 2.3 Å. The crystal belongs to the P212121 space group, with unit cell dimensions of a 58.058 Å, b 77.752 Å, and c 91.130 Å, and it contains one molecule of KLK6 and two molecules of APPI-4M in the asymmetric unit. The X-ray data of both the $APPI_{WT}$/KLK6 and the APPI-4M-/KLK6 crystals were processed, merged, and scaled by XDS (78). Data collection statistics is provided in Table 3. Phase acquisitions and structure determination were performed by molecular replacement using Phaser (79) from the CCP4 Program Suite (80). Protein Data Bank (PDB) codes 1AAP and 1LO6 were used as the search models. Refinement was performed using Phenix.refine (81) and alternating rounds of model building and manual corrections were performed by COOT (82). The coordinates and structure factors were submitted to the PDB under the accession codes 5NX1 for $APPI_{WT}$/KLK6 and 5NX3 for APPI-4M/KLK6.

Acknowledgments
The authors thank Dr. Alon Zilka and Dr. Uzi Hadad for their technical assistance. FACS experiments were performed at the Ilse Katz Institute for Nanoscale Science and Technology, BGU. The structural studies were performed on beamlines ID23-1 & ID30-B at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. We are grateful to Christoph Mueller-Dieckmann for providing assistance in using those beamlines. We thank Eitan Rabinovich for providing KLK4. This work was supported by the European Research Council "Ideas program" ERC-2013-StG (contract grant number: 336041) and the Prostate Cancer Foundation (PCF) to N.P., the DKFZ-MOST (contract grant number: GR2495) to N.P and A.K.M. N.P. and E.S.R. acknowledge support from and US-Israel Binational Science Foundation (BSF). E.S.R. acknowledges support from the United Stated National Institutes of Health grant R01CA154387.

Conflict of interest
The authors declare that they have no conflict of interest with respect to the publication of this paper.

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Abbreviations: KLK, Human tissue kallikrein; APPI, amyloid precursor protein Kunitz protease inhibitor domain; BGU, Ben-Gurion University of the Negev; DMSU, DNA Microarray and Sequencing Unit; FACS, flow activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FXIa, factor XIa; SPR, surface plasmon resonance; NIBN, National Institute of Biotechnology in the Negev; PE, phycoerythrin; YSD, yeast surface display

Table 1. Inhibition, binding, and hydrolysis constants of $APPI_{WT}$ and APPI-4M.

APPI variant	K_i (nM) ‡	K_D (nM)†	k_{on} (M^{-1} s^{-1})†	k_{off} (s^{-1})†	Turnover time (h)*
$APPI_{WT}$	2.24 ± 0.11 §	5.29	$(1.96 \pm 0.01) \times 10^5$	$(10.40 \pm 0.02) \times 10^{-4}$	16.62 ± 0.05
APPI-4M	0.16 ± 0.06	0.04	$(3.17 \pm 0.01) \times 10^5$	$(1.15 \pm 0.06) \times 10^{-5}$	230.07 ± 40.90

‡ Determined by slow tight binding inhibition assays. † Determined by SPR.*Determined by HPLC. § Previously reported in (50).

Table 2. Inhibition (K_i) of $APPI_{WT}$ and APPI-4M toward various human serine proteases.

	KLK6 (pM)	Anionic trypsin (pM)	Cationic trypsin (pM)	FXIa (pM)
$APPI_{WT}$	**(2240 ± 110)**	**(1.74 ± 0.05)**	**(6.27 ± 1.01)**	**(410 ± 14)**
APPI-4M	**(160 ± 60)**	**(69.8 ± 8.0)**	**(168 ± 17.5)**	**(724.8 ± 33.7)**
Fold difference	**14**	**0.025**	**0.037**	**0.58**

Table 3. Binding affinity (measured by SPR) of $APPI_{WT}$ and APPI-4M toward Kallikreins.

APPI-KLK	K_D (nM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
$APPI_{WT}$ – KLK6	5.29	$(1.96 \pm 0.01) \times 10^5$	$(104 \pm 0.20) \times 10^{-5}$
APPI-4M – KLK6	0.04	$(3.17 \pm 0.01) \times 10^5$	$(1.15 \pm 0.06) \times 10^{-5}$
$APPI_{WT}$ – KLK1	ND	NA	NA
APPI-4M – KLK1	ND	NA	NA
$APPI_{WT}$ – KLK2	0.55	$(2.59 \pm 0.05) \times 10^4$	$(1.41 \pm 0.02) \times 10^{-5}$
APPI-4M – KLK2	0.44	$(2.53 \pm 0.04) \times 10^4$	$(1.10 \pm 0.02) \times 10^{-5}$
$APPI_{WT}$ – KLK3	ND	NA	NA
APPI-4M – KLK3	ND	NA	NA
$APPI_{WT}$ – KLK4	10.08	$(3.81 \pm 0.05) \times 10^4$	$(4.13 \pm 0.02) \times 10^{-4}$
APPI-4M – KLK4	2.34	$(7.39 \pm 0.04) \times 10^4$	$(1.73 \pm 0.02) \times 10^{-4}$
$APPI_{WT}$ – KLK5	3.24	$(1.31 \pm 0.02) \times 10^4$	$(4.26 \pm 0.01) \times 10^{-5}$
APPI-4M – KLK5	1.97	$(3.46 \pm 0.05) \times 10^4$	$(6.80 \pm 0.12) \times 10^{-5}$

ND, binding not detected. NA, not available.

Table 4. Crystallographic and refinement statistics. Values in parentheses correspond to the highest resolution shell of the data.

	KLK6/APPI$_{WT}$	KLK6/ APPI-4M
Data collection		
X-ray source	ESRF ID30B	ESRF ID23-1
Wavelength (Å)	0.969	0.976
D_{min} (Å)	46.10–1.85 (1.92–1.85)	46.5–2.3 (2.4–2.3)
Space group	P212121	P212121
Cell dimensions		
a,b,c (Å)	59.543, 77.702, 92.207	58.058, 77.752, 91.130
α,β,γ (º)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Total reflections	73,717	37,715
Unique reflections	36,867	18,865
Rpim (%)	3.12 (64.19)	5.26 (35.21)
Completeness (%)	99.52 (96.37)	99.14 (95.6)
I/σI	13.48 (1.08)	8.51 (2.13)
Multiplicity	2.0 (2.0)	2.0 (2.0)
$CC_{1/2}$	0.999 (0.574)	0.996 (0.760)
Refinement		
Refinement resolution (Å)	46.10–1.85	46.520–2.296
Total reflections used	36,851	18,856
RMSD bond lengths (Å)	0.006	0.007
RMSD bond angles (º)	0.87	0.91
Ramachandran plot		
Favored (%)	97.83	98.14
Outliers (%)	0.00	0.00
R_{work}/R_{free} (%)	18.48/22.60	17.47/22.56
Protein atoms	2565	2555
Solvent molecules	195	142
Average B-factor (Å^2)	42.03	34.16
Protein	41.81	34.1
Solvent	44.82	35.25
Wilson B factor (Å^2)	28.97	28.31
PDB ID	**5NX1**	**5NX3**

Imposing a resolution cut-off of 1.95Å for the KLK6/APPI$_{WT}$ data, results in 1.44 I/σI, multiplicity of 12.8 completeness of 99.07% and $CC_{1/2}$ of 0.747 in the highest resolution shell.

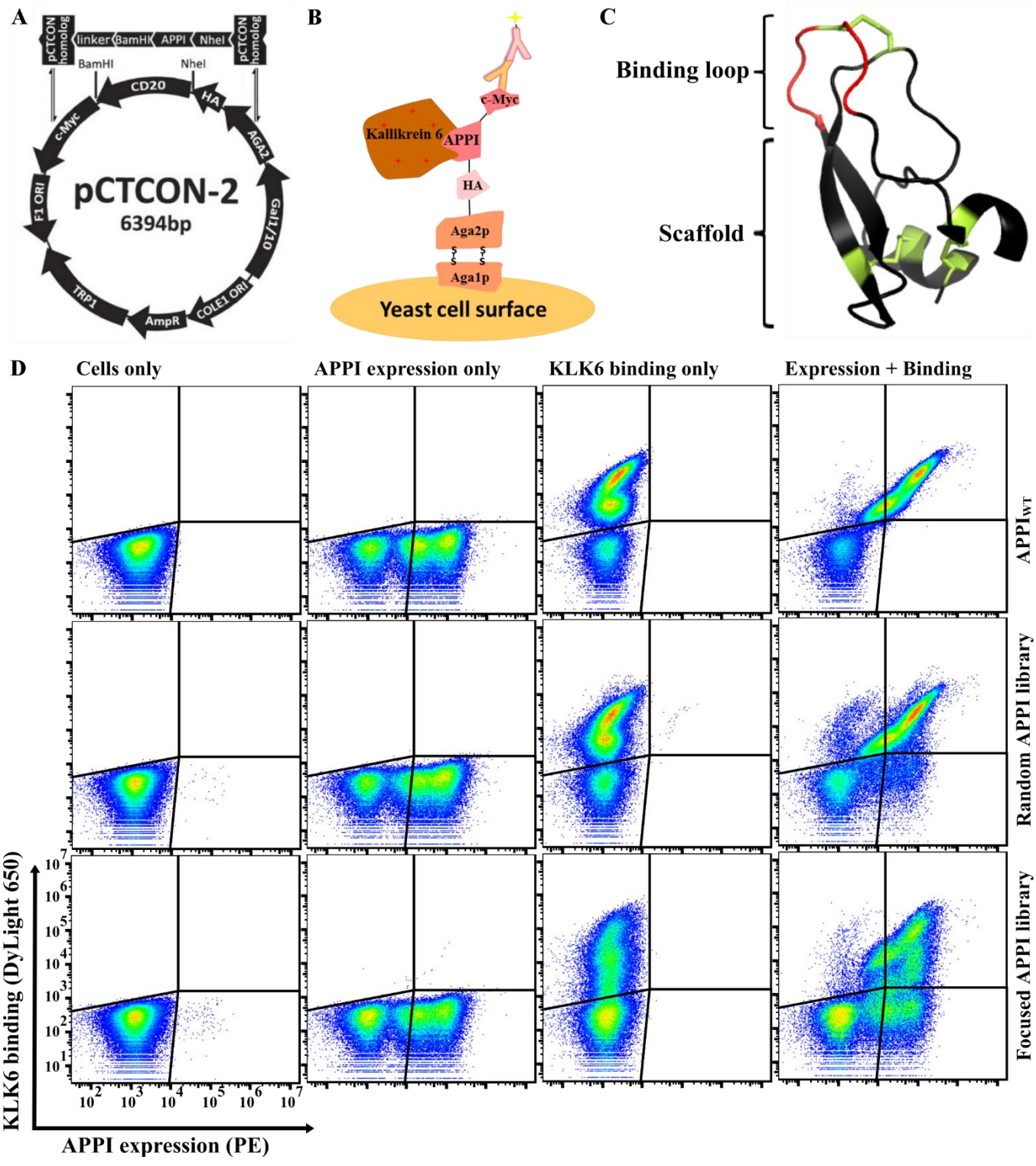

Figure 1. FACS analysis of the different YSD APPI libraries, showing expression and KLK6 binding. (**A**) The YSD vector (pCTCON-2) is aligned with the general scheme of the insert: the insert gene consists of the APPI gene, which is flanked by two restriction sites (BamHI and NheI), followed by a linker sequence (LPDKPLAFQDPS) on the 3' end, along with two pCTCON homologous sequences. (**B**) APPI is displayed on the yeast cell surface as a translational fusion to Aga2p, which is linked to Aga1p by two disulfide bonds. Surface expression is detected by using fluorescence-labeled antibodies binding to the C-terminal of the c-Myc epitope tag, while target binding is detected using fluorescence-labeled KLK6, via FACS. (**C**) 3-D structure of APPI (PDB 1ZJD). (**D**) The expression of APPI and the binding of 10 nM of labeled KLK6 were determined in a YSD system for an $APPI_{WT}$ clone, for a library of mutants based on $APPI_{WT}$, and for a library of mutants based on $APPI_{G17M,I18F,F34V}$.

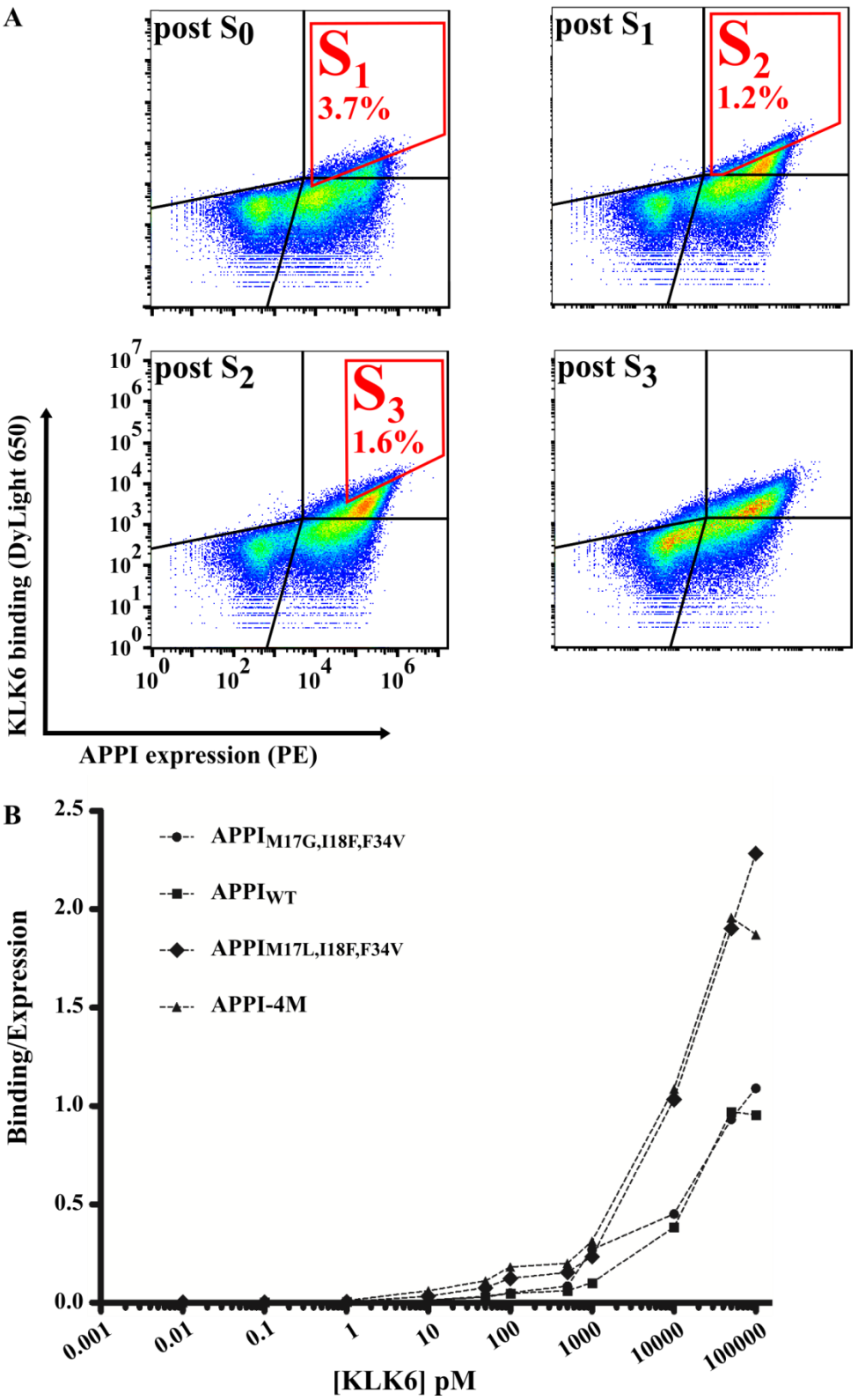

Figure 2. Affinity maturation of the APPI library. (**A**) To enrich the APPI library for high-affinity KLK6 binders, four sequential rounds of FACS sorting were conducted (S_1–S_3 marked in red). The results of the analysis after each sorting round are presented. S_0 represents an initial sorting round intended only for selecting a cell population with high APPI expression, regardless of KLK6 binding. In S_1, 500 pM of

DyLight-650–labeled KLK6 were used to select cells with a high APPI expression and a high KLK6 binding, which comprised 3.7% of the entire cell population. In S_2, 50 pM of DyLight-650–labeled KLK6 were used to select cells with a high APPI expression and a high KLK6 binding (gated in red), which comprised 1.2% of the entire cell population. In S_3, 50 pM of DyLight-650–labeled KLK6 were used to select cells with a high APPI expression and a high KLK6 binding, which comprised 1.6% of the entire cell population. (**B**) FACS binding titration curve of KLK6 to yeast cells expressing $APPI_{WT}$ or its three variants. A leftward shift in the curve indicates a higher affinity. The binding signal of KLK6 is normalized to the expression signal of the APPIs.

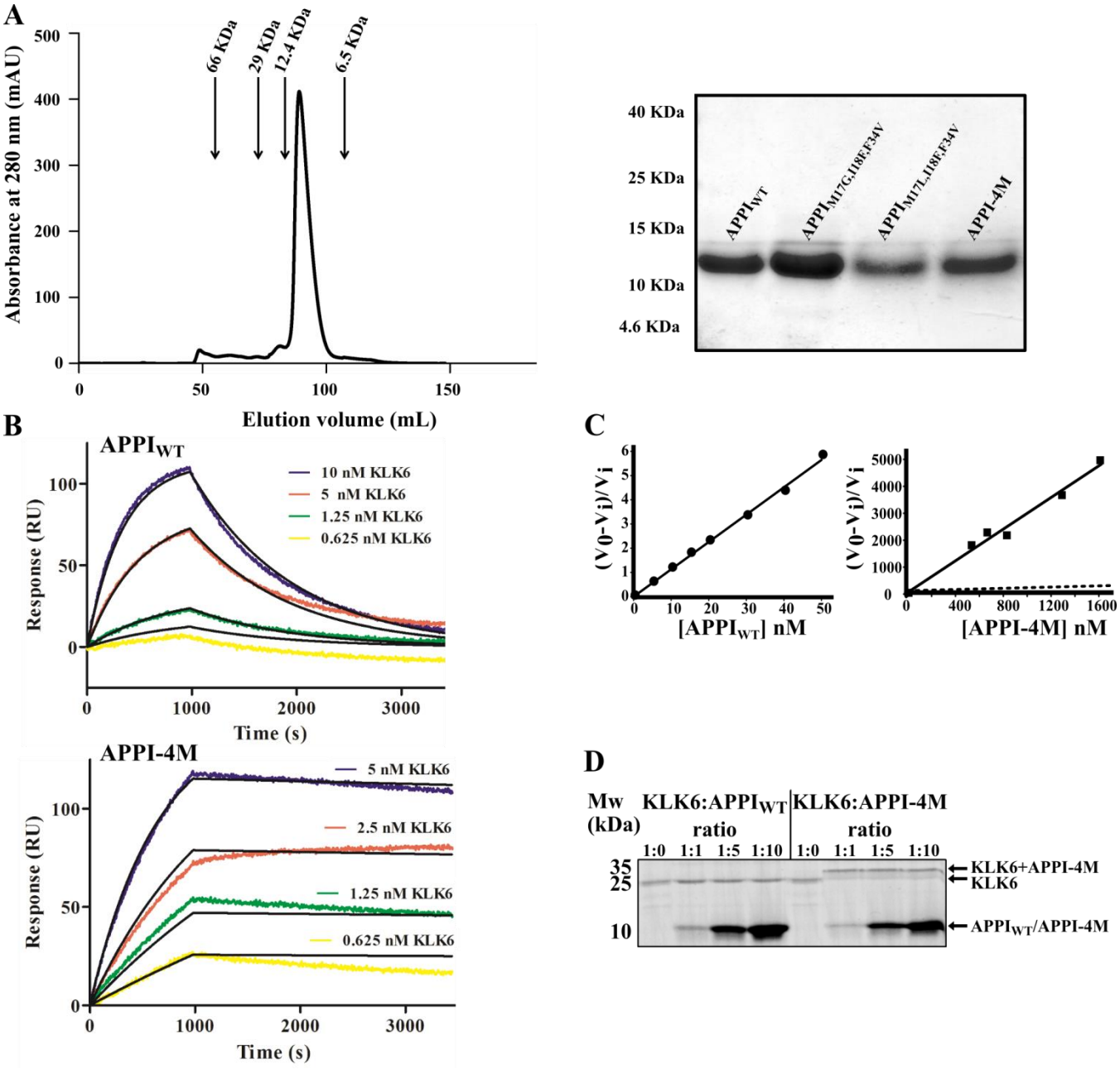

Figure 3. Binding and inhibition kinetics of KLK6 by $APPI_{WT}$ and APPI-4M. **(A)** Purification of soluble APPI variants. Left: Size-exclusion chromatography for APPI-4M. Arrows indicate correlations between the elution volume and size, according to known standards. –Right: An SDS-PAGE analysis of the purified APPI variants on a 15% polyacrylamide gel under reducing conditions. (B) A surface plasmon resonance binding experiment, in which 2 µg of either $APPI_{WT}$ (top panel) or APPI-4M (lower panel) were mounted as the ligands on a GLC chip. Six KLK6 concentrations (0–10 nM; represented by different colors) were used as the analytes. **(C)** Slow tight-binding inhibition of KLK6 catalytic activity by $APPI_{WT}$ (left; 1 nM KLK6) and APPI-4M (right; 100 nM KLK6). The K_i of the reaction was calculated by using Equation 1 (see Experimental Procedures). V_0 represents the uninhibited rate and V_i represents the rate in the presence of APPI. In the APPI-4M plot, the dashed regression line indicates the slope of the inhibition by $APPI_{WT}$. **(D)** Formation of complexes between KLK6 and APPI-4M. The SDS-PAGE was performed under reducing conditions

and with KLK6/APPI molar ratios of 1:0, 1:1, 1:5 and 1:10. Note the formation of stable, higher molecular weight complexes in lanes loaded with KLK6/ APPI-4M, but not with KLK6/APPI$_{WT}$ complexes.

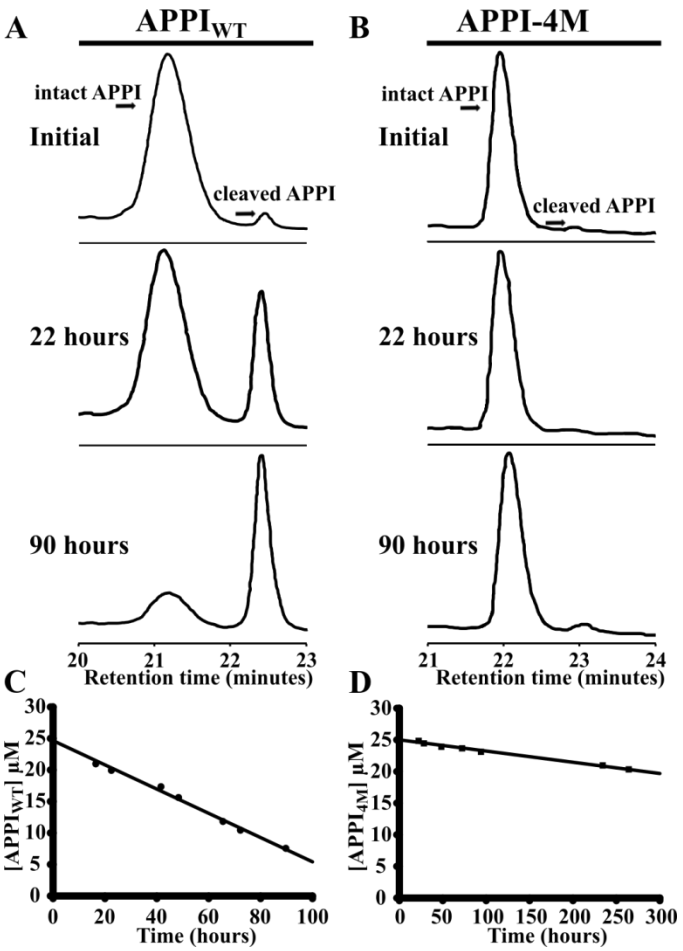

Figure 4. Hydrolysis of APPI$_{WT}$ (**A, C**) and APPI-4M (**B, D**) by KLK6, as determined by HPLC. The depletion of intact APPI over time was quantified by integrating the HPLC peak, which represents the intact APPI variant, at eight different time points (examples shown in A,B), and then determining the rate of depletion by linear regression (C,D). APPI concentrations were 25 µM for each variant, and the molar ratio of KLK6 to APPI was 1:8.

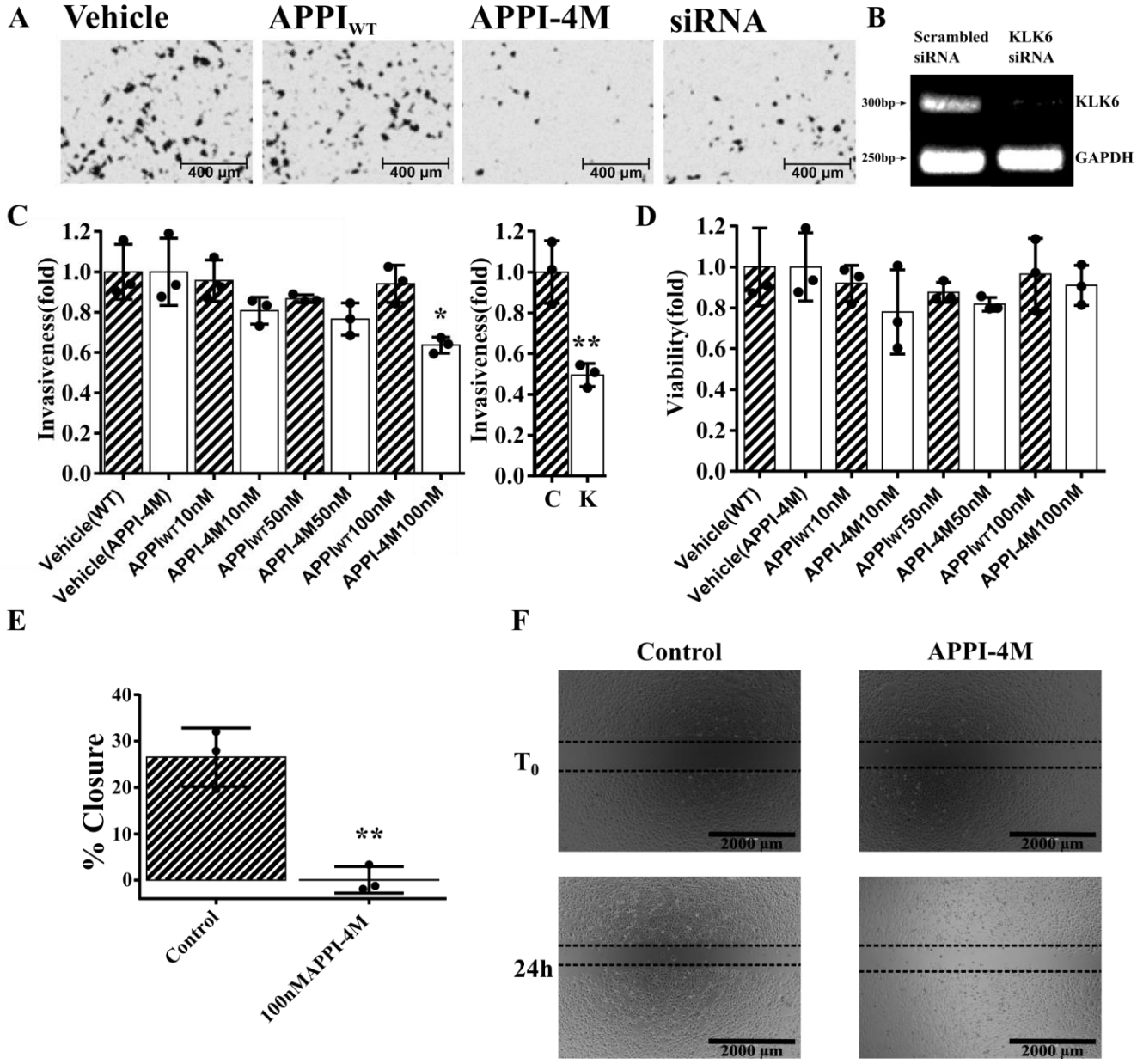

Figure 5. APPI-4M decreases the invasiveness of BT-20 breast cancer cells, but not their viability. The invasive ability of BT-20 cells in the presence of APPI-4M was evaluated by using a Matrigel-coated Boyden chamber. The cells that successfully invaded the Matrigel 36 h after plating were stained and quantified. **(A)** Representative images of the invading cells treated with either buffer (vehicle), APPI$_{WT}$, or APPI-4M (100 nM). siRNA-transfected cells were used as a control. **(B)** Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was conducted in BT-20 breast cancer cells that were transfected with KLK6 small-interfering RNA (siRNA). After the cells were transfected with siKLK6 or with a small-interfering control (scrambled siRNA) for 48 h, whole RNA was extracted and the RT-PCR was performed. GAPDH: glyceraldehyde 3-phosphate dehydrogenase. **(C)** Quantification of invading cells. C – Control (scrambled siRNA); K – KLK6-siRNA. Bars represent means ± standard deviation (SD) of three biological repetitions. **(D)** Quantification of cell viability using the XTT assay, 36 h after incubation with APPI$_{WT}$ or APPI-4M. Bars represent means ± SD of three biological repetitions. **(E)** Quantification of migrating cells (see panel F), normalized to untreated cells (control). Bars represents the mean (± SD) of triplicate experiments. * $p < 0.05$. ** $p < 0.01$ (Student's t-test, as compared with untreated control). **(F)** BT-20 cells were scratched by

removing a strip across the well. Then, the cells were treated with APPI-4M for 24 h. The area free from cells was counted.

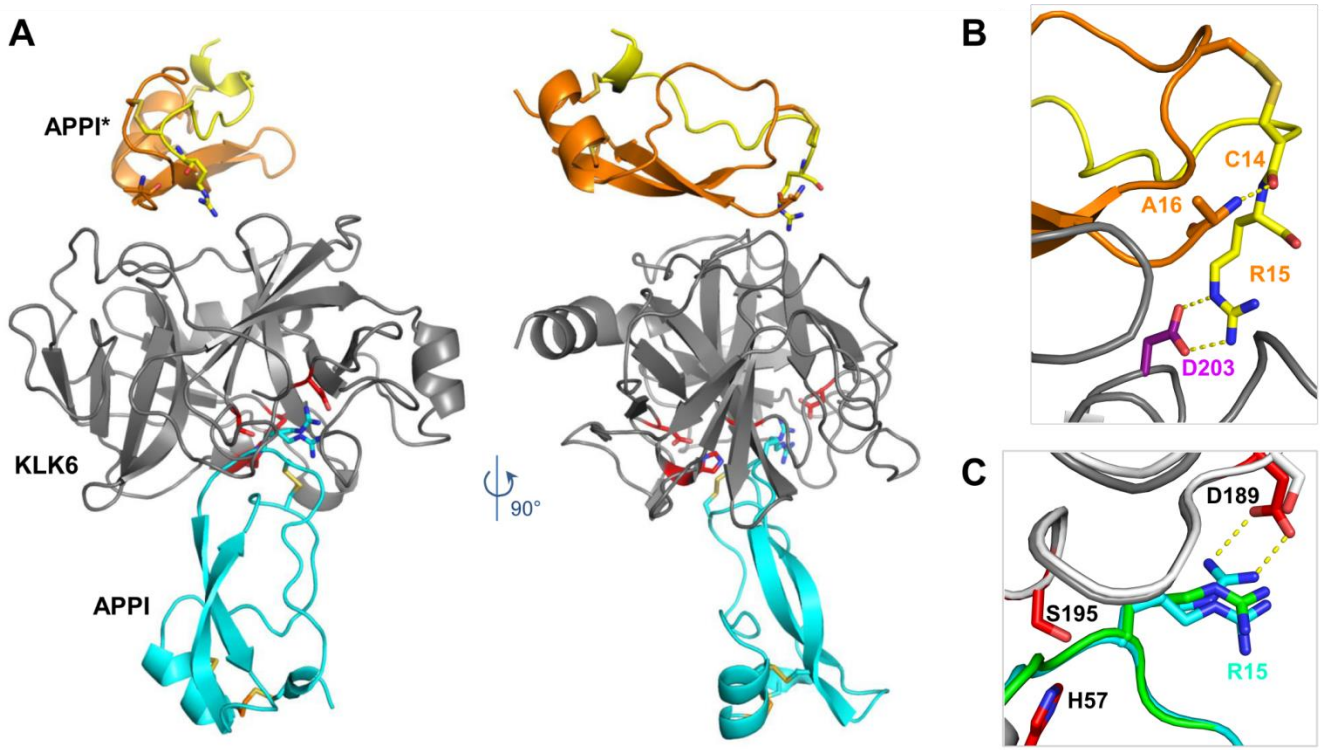

Figure 6. Crystal structure reveals an inhibited KLK6/APPI-4M complex and a cleaved product APPI-4M*. **(A)** The co-crystal structure of KLK6 with the APPI-4M variant shows one molecule of KLK6 (gray) bound to a molecule of APPI-4M occupying the active site in the inhibitory mode (cyan). The crystal structure also reveals an additional APPI-4M molecule that has undergone proteolysis at Arg-15 – Ala-16 to yield the product APPI-4M*, which is comprised of two protein chains (yellow and orange) connected by two disulfide bonds. **(B)** The cleaved APPI-4M* molecule reveals a large rotation of the Cys-14 ψ angle (relative to the intact APPI-4M), allowing the formation of a salt bridge between the Arg-15 side chain and the Asp-203 residue of KLK6, thus stabilizing the crystal lattice, while the Cys-14 carbonyl forms a hydrogen bond with the Ala-16 N-terminus. **(C)** Arg-15 of the intact APPI-4M was observed in both the expected "up" conformation, in which it forms a salt bridge with KLK6 Asp-189 (dotted yellow lines), and a "down" conformation, which is similar to that previously reported in the superposed structure of APPI (green) bound to chymotrypsin (white) (PDB:1CA0).

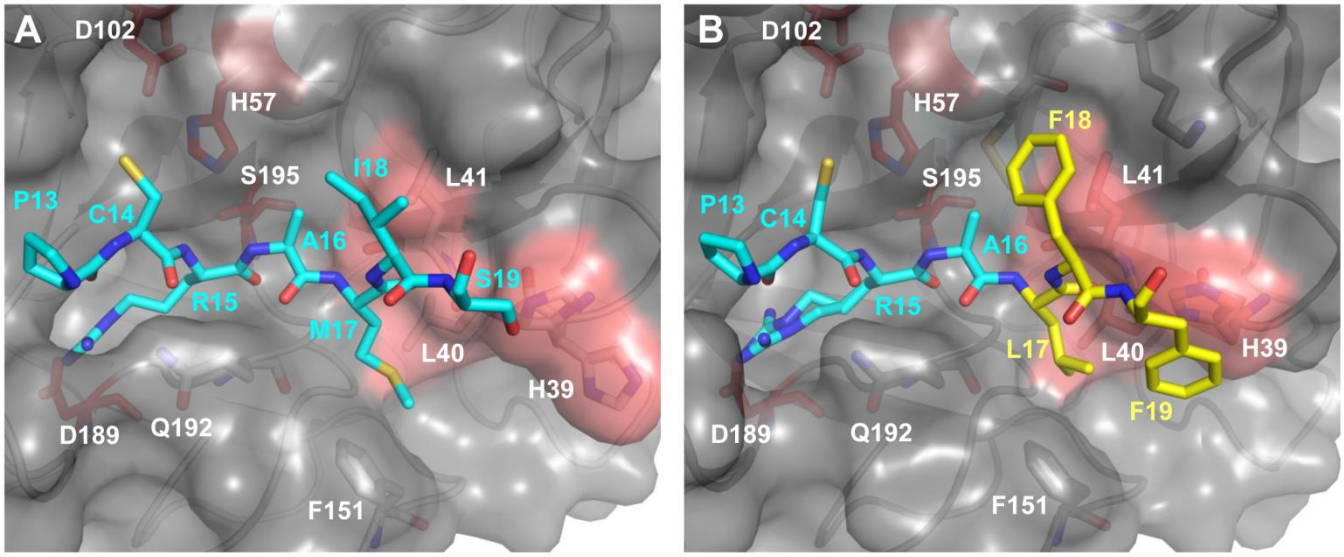

Figure 7. Binding loop mutations of APPI-4M optimize interactions with the primed side subsites of KLK6. **(A)** In the complex of KLK6 (gray surface) with $APPI_{WT}$ (cyan sticks), APPI residues Met-17, Ile-18, and Ser-19 interact with the S2′, S3′, and S4′ subsites of KLK6, respectively, which are shaped by His-39, Leu-40, and Leu-41 (salmon surface patch). **(B)** In the APPI-4M/KLK6 complex, Phe-18 of APPI-4M makes more extensive contacts with the S3′ subsite of KLK6, while Phe-19 forms a ring-stacking interaction with His-39 of KLK6, thus locking this residue into a single side chain conformation. Leu-17 and Phe-19 residues of APPI-4M also form intramolecular hydrophobic contacts, which may help to stabilize the conformation of the binding loop.

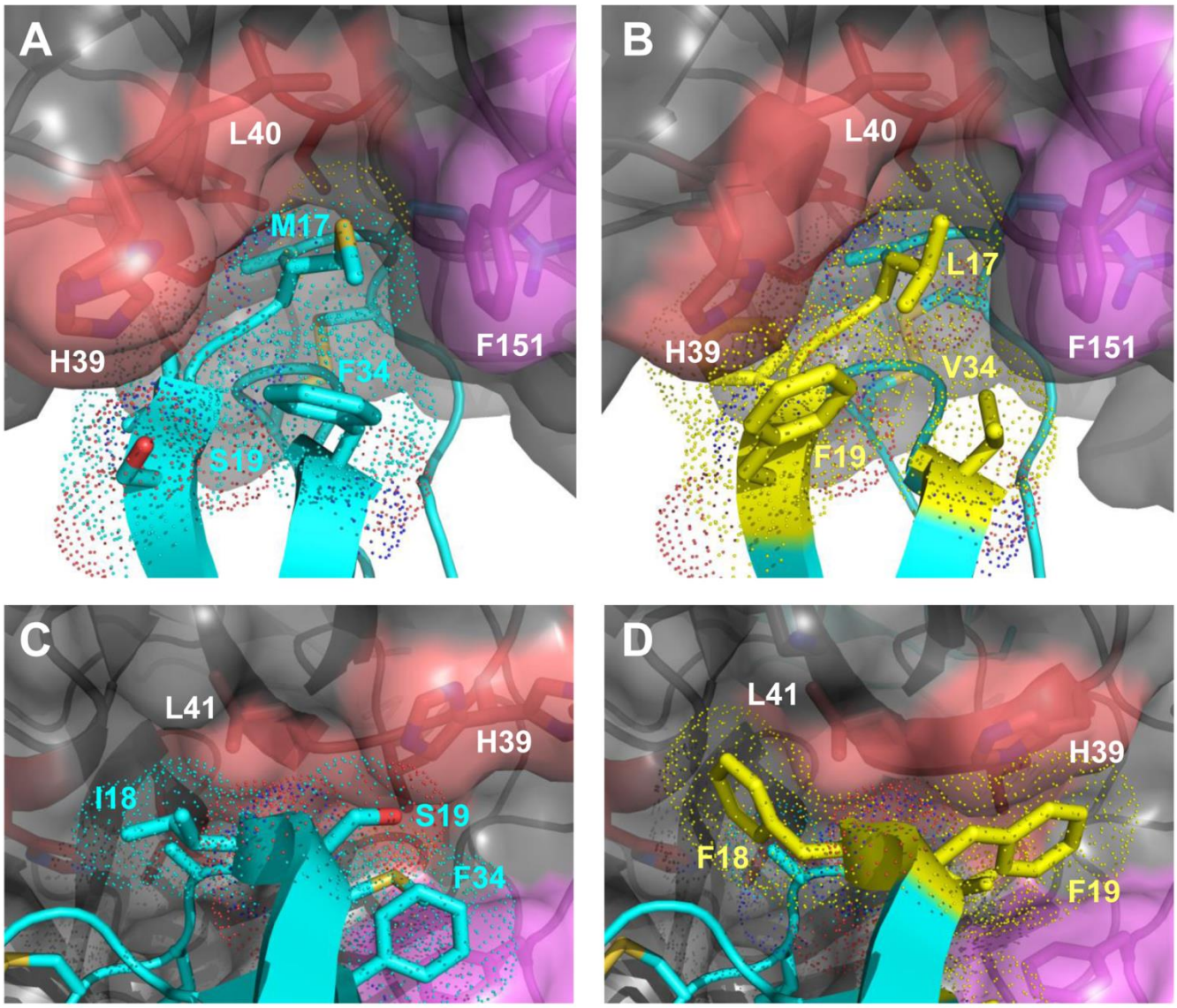

Figure 8. Four APPI mutations optimize intra- and intermolecular packing interactions with KLK6. **(A, B)** Side view of the primed side of the substrate binding cleft between KLK6 residues 39-41 (salmon surfaces) and Phe-151 (lavender) portrays van der Waals packing interactions in complexes with **(A)** $APPI_{WT}$ (cyan) and **(B)** APPI-4M (mutated residues shown in yellow). The mutated residues Leu-17, Phe-19, and Val-34 of APPI-4M form a hydrophobic cluster that fills the crevice and form hydrophobic interactions with KLK6 residues Phe-151 and Leu-40, while Phe-19 also forms a ring-stacking interaction with KLK6 His-39. **(C, D)** Rotated view, revealing how the mutated residues Phe-18 and Phe-19 of APPI-4M wrap around the ridge formed by KLK6 residues 39-41 **(D)**, thus forming a more extensive contact interface than in the $APPI_{WT}$/KLK6 complex **(C)**.

A potent, proteolysis-resistant inhibitor of kallikrein-related peptidase 6 (KLK6) for cancer therapy, developed by combinatorial engineering

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J. Biol. Chem. published online June 22, 2018
