Live subject statement

All experiments were performed in compliance with the relevant laws and institutional guidelines. Animal experiments were carried out in the approved EL13-BIOexp-04 facilities of the University of Patras and the protocol was approved by the local committee (Veterinary Department of Regional Units of Achaia, Region of Western Greece; Dr. Ariadni Giannouli was the responsible veterinarian for our institution). After informed written consent, skin biopsies were obtained from NS patients and healthy donors in Aghia Sofia Children's Hospital (Athens, Greece).

Methods

Materials

All chemical reagents and solvents were obtained from Sigma-Aldrich or Merck and used without further purification. Silica gel chromatography was performed using glass columns packed with silica gel 60 (70-230 mesh). Analytical thin-layer chromatography was performed on TLC silica gel 60 F_{254} (Merck) plates, and compounds were visualized under UV light at 254 nm and/or ninhydrin (Kaiser test). Nuclear magnetic resonance spectra (^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR) were recorded on a Bruker AVANCE III 600 Bruker Avance III 600 (600 MHz, ^{1}H; 151 MHz ^{13}C; 243 MHz ^{31}P) spectrometer. Chemical shifts are reported in parts per million (ppm) relative to a tetramethylsilane internal standard. Electrospray ionization mass spectra (ESI-MS) were recorded on an AmaZon SL instrument of Bruker Daltonics. The anti-KLK7 was obtained from R&D (AF2624) and was a goat polyclonal antibody or was provided by Professor Eleftherios P. Diamandis (Mount Sinai Hospital, Toronto, Canada) and was a rabbit polyclonal antibody. The anti-α-tubulin was obtained from Sigma (T9026) and was a mouse monoclonal antibody.

Chemical synthesis and representative NMR and ESI-MS spectra

The diphenyl amino-phosphonates **2a-c** were synthesized according to literature procedures and the spectra were in agreement with previously reported corresponding spectra.[1-4] Briefly, benzyl carbamate (1.0 mmol), triarylphosphite (1.0 mmol) and the appropriate aldehyde (1.5 mmol) were dissolved in acetic acid (3 ml) and the obtained mixture was refluxed for 2 hours. The reaction was monitored by TLC until benzyl carbamate was consumed. Then, the volatile products were removed in a rotary evaporator under reduced pressure. The oily residue was dissolved in acetone or hot chloroform (depending on product), several drops of hexane were added in the case of acetone or a 4-fold volume of methanol in the case of chloroform and left for crystallization at 4^{o}C overnight to give the phosphonates **1a-c** as racemic mixtures. The resulting solids **1a-c** were sufficiently pure for the following reaction. If necessary, the products were purified using silica gel column chromatography. Deprotection of the diphenyl (benzyloxy carbonylamino)phosphonates **1a-c** was achieved by catalytic transfer hydrogenation. The appropriate phosphonate (1.0 mmol) was dissolved in 4 ml of absolute ethanol under a nitrogen atmosphere. An equal weight of 10% palladium was added followed by the addition of 1,4-cyclohexadiene (0.94 ml, 10.0 mmol). The reaction proceeded for a minimum of 2 hours and the mixture was filtered (celite), washed with dichloromethane and evaporated under reduced pressure. The resulting crude amines **2a-c** were used directly in the next step without further purification.

Synthesis of tert-butyloxycarbonyl (Boc) protected peptide diphenyl phosphonate esters 3a-c

General procedure

To a stirred solution of crude **2a-c** (1.0 mmol) and *tert*-butyloxycarbonyl-L-phenylalanine (1.2 mmol) in dichloromethane (10 ml) were added *N,N'*-diisopropylethylamine (DIPEA, 3.0 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 1.2

mmol) and 1-hydroxybenzotriazole (HOBt, 1.2 mmol). The reaction was performed at room temperature overnight. DIPEA was added if necessary, to maintain the alkaline pH. The reaction was monitored by TLC until **2a-c** were consumed. Then, the mixture was diluted with dichloromethane (15 ml) and washed sequentially with aqueous citric acid (10%, 20 ml x 3), aqueous $NaHCO_3$ (5%, 20 ml x 3), H_2O (20 mL) and brine (20 ml), dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give. After purification by column chromatography (silica gel, hexanes/ethyl acetate 60:40), the desired Boc-protected phosphonic dipeptides **3a-c** were obtained.

***tert*-butyl 1-((diphenoxyphosphoryl)(phenyl)methylamino)-1-oxo-3-phenylpropan-2-yl carbamate (Probe 3a) (Boc-FBP)**. White solid, yield 85%. ^{1}H-NMR (600 MHz, 298 K, $CDCl_3$): δ 7.9-6.82 (m, 21H), 5.87 (m, 1H), 4.89 (m, 1H), 4.43 (m, 1H), 3.08-2.94 (m, 2H), 1.37 (s, 9H). ^{13}C-NMR (151 MHz, 298 K, $CDCl_3$): δ 170.70, 150.30, 150.04, 149.95, 149.87, 133.61, 129.67, 129.52, 129.23, 129.09, 128.77, 128.76, 128.70, 128.57, 128.44, 128.23, 126.82, 125.30, 125.20, 125.16, 120.46, 120.44, 120.41, 120.27, 51.08, 51.03, 50.03, 49.98, 28.09. ^{31}P-NMR (243 MHz, 298 K, $CDCl_3$): δ 13.68, 13.55. ESI-MS: *m/z* calcd for **3a** ($C_{33}H_{35}N_2O_6P$) $[M + Na]^+$ 609.21; found 609.05.

***tert*-butyl 1-(1-(diphenoxyphosphoryl)-2-phenylethylamino)-1-oxo-3-phenylpropan-2-yl carbamate (Probe 3b) (Boc-FFP)**. White solid, yield 42%. ^{1}H-NMR (600 MHz, 298 K, $CDCl_3$): δ 7.32-7.01 (m, 20H), 6.58 (m, 1H), 5.11 (m, 1H), 4.62 (m, 1H), 4.26 (m, 1H), 3.39-2.74 (m, 4H), 1.37 (s, 9H). ^{13}C-NMR (151 MHz, 298 K, $CDCl_3$): δ 170.70, 150.30, 150.04, 149.95, 149.87, 133.61, 129.67, 129.52, 129.23, 129.09, 128.77, 128.76, 128.70, 128.57, 128.44, 128.23, 126.82, 125.30, 125.20, 125.16, 120.46, 120.44, 120.41, 120.27, 51.08, 51.03, 50.03, 49.98, 28.09. ^{31}P-NMR (243 MHz, 298 K, $CDCl_3$): δ 16.51, 16.24. ESI-MS: *m/z* calcd for **3b** ($C_{34}H_{37}N_2O_6P$) $[M + Na]^+$ 623.23; found 623.09.

***tert*-butyl 1-(1-(diphenoxyphosphoryl)-3-phenylpropylamino)-1-oxo-3-phenylpropan-2yl carbamate (Probe 3c) (Boc-FCP)**. Yellowish solid, yield 64%. ^{1}H-NMR (600 MHz, 298 K, $CDCl_3$): δ 7.36-7.45 (m, 18H), 6.77-6.76 (m, 2H), 5.90-5.83 (m, 1H), 4.97 (b, 1H), 4.23 (m, 1H), 3.39-3.29 (m, 1H), 3.23-3.19 (m, 2H), 3.03-2.98 (m, 2H), 2.45-2.24 (m, 2H), 1.41 (s, 9H). ^{31}P-NMR (243 MHz, 298 K, $CDCl_3$): δ 21.12, 21.06. ESI-MS: *m/z* calcd for **3c** ($C_{35}H_{39}N_2O_6P$) $[M + Na]^+$ 637.24; found 637.07.

Diphenyl 1-(2-(6-(6-(5-((3a*S*,4*R*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl) pentanamido)hexanamido)hexanamido)-3-phenylpropanamido)-2-phenylethyl phospho nate (biotin-FFP). The probe **3b** (0.017 mmol, 10 mg) was dissolved in dichloromethane (0.4 ml) and an equal volume of trifluoroacetic acid was added dropwise. The reaction was performed at room temperature and the progress was monitored with TLC. When reaction was completed (approx. 2 hours), the volatile components were removed under reduced pressure to give the unprotected phosphonic dipeptide **4b** as trifluoroacetate salt (10.25 mg, quant.). The product was checked with ESI-MS (*m/z* calcd for **4b** ($C_{29}H_{29}N_2O_4P$) $[M + H]^+$ 501.19; found 501.12) and was used directly in the next step without further purification. The biotin-FFP was synthesized by the coupling of biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (biotin-X-X-NHS) with **4b**. The biotin-X-X-NHS (10 μmol, 5.5 mg) was dissolved in dimethylsulfoxide (DMSO, 0.1 ml) and stirred on an ice bath. 1-hydroxybenzotriazole (HOBt, 0.68 mg) was added, followed by the addition of DIPEA (4 μl) after several minutes. The **4b** (5 mg, 8.1 μmol) was added as solution in DMSO (0.4 ml) and the reaction mixture left to warm at room temperature overnight and monitored with TLC. When the reaction was completed, the mixture was diluted with dichloromethane (15 ml) and washed with water. The aqueous phase was washed with dichloromethane (15 ml) and the combined organic phases washed sequentially with aqueous citric acid (10%, 20 ml x 3), aqueous $NaHCO_3$ (5%, 20 ml x 3), H_2O (20 ml) and brine (20 ml), dried (Na_2SO_4), filtered,

and concentrated *in vacuo*, to give, after purification by column chromatography (silica gel, chloroform/methanol 95:5 to 90:10), the desired biotinylated biotin-FFP (**5b**, expanded: biotin-X-X-FFP) as pale white solid (yield: 52%). ^{1}H-NMR (600 MHz, 298 K, CD_3OD): δ 7.39-7.05 (m, 20H), 5.05-4.97 (m, 1H), 4.70-4.65 (m, 1H), 4.48-4.55 (m, 1H), 4.29-4.25 (m, 1H), 3.46-3.43 (m, 2H), 3.18-2.91 (m, 8H), 2.71-2.68 (m, 2H), 2.19-2.13 (m, 4H), 2.07-2.04 (t, 2H), 1.74-1.56 (m, 7), 1.53-1.28 (m, 14H). ^{31}P-NMR (243 MHz, 298 K, $CDCl_3$): δ 21.12, 21.06. ESI-MS: *m/z* calcd for **3a** ($C_{51}H_{65}N_6O_8PS$) $[M + Na]^+$ 975.41; found 975.36.

Docking. The docking process on KLK7 was performed on the crystal structure of the hydrolase (PDB code 5FAH), resolved at atomic resolution of 1.1 Å.[5] For the docking, compounds **3a-c** were docked into the binding site of the protein using AutoDock Vina and AutoDockTools.[6] for partial charges calculation and file format preparation. The protease was held rigid during the docking process, while ligands were allowed to be flexible. Docking calculations were performed using a grid box with dimensions of 45 x 45 x 45 Å, a search space of 10 binding modes and exhaustiveness was set to 20. The scoring algorithm implemented was using VinaSH,[7] an improved version of AutoDock Vina, which also considers sulfur and halogen bonding into the forcefield and scoring algorithm.

Mice

All mice were of the C57BL/6 background. *Spink5*$^{-/-}$ mice were kindly provided by Professor Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK).[8] Previously, we described the generation and genotyping of *Klk5*$^{-/-}$ mice.[9] All experiments with animals were conducted according to EU and National legislation.

Mice treatment with inhibitor

The genotype of the double knockout *Spink5*$^{-/-}$*Klk5*$^{-/-}$ mice was confirmed by PCR (*Klk5*: forward 5'-GCAGCTAGAGTTAAGAGCTC-3' and reverse 5'-GGTCAGAACTGTGTAGGC -3' for wt or 5'-GACCACCTCATCAGAAGCAG-3' for

knockout allele; *Spink5*: forward 5'-GAGTCTTGAGACAATAGT-3' and reverse 5'-GTAGGAGAGTTCTGTAAG-3') in independent duplicate experiments. On P3, 20 μl of 1 mM inhibitor in 45% isopropanol, 6% propylene glycol and 1.2% DMSO were applied on the right flank and allowed to evaporate. Then, 20 μl of solvent control were applied on the left flank and allowed to evaporate. Mice were treated in the same manner daily until P7 when they were photographed to assess the extent of macroscopic desquamation, then, mice were decapitated, and skin tissue was excised for RNA extraction.

Mice treatment with 12-O-tetradecanoylphorbol 13-acetate

Mice were shaved and 24 hours later 100 μl of 0.0002 M TPA in acetone was applied on their right flank. Acetone alone was applied on the left flank as negative control. TPA application was repeated once 24 hours later and mice were sacrificed 24 hours after the second application, skin biopsy was taken, embedded in OCT (optimal cutting temperature) and stored at -80^{o}C until sectioned with a cryotome.

Clinical specimens

Biopsies were taken from a healthy volunteer and two patients with Netherton syndrome at Aghia Sofia Children's Hospital (Athens, Greece), embedded in OCT (optimal cutting temperature) and stored at -80^{o}C until cryosectioned. All human subjects provided written consents.

RNA extraction and RT-QPCR

Mice tissues were pulverized with a pestle and mortar under liquid N_2. RNA was extracted with Nucleospin RNA (Macherey-Nagel). The quality of RNA was checked with agarose gel electrophoresis and the concentration was determined based on the absorbance at 260 nm. Total RNA (1 μg) was reversed-transcribed with the Superscript First Strand Synthesis System (Invitrogen). A total of 20 ng of cDNA were used for real-time PCR using SYBR

Green (Kapa SYBR FAST One-Step Universal) and primers described previously.[9] The housekeeping gene *Hprt1* was used for loading control.

Activography

Activography was conducted as described.[10,11] Briefly, cryosections of 5 μm were fixed in acetone for 10 min, re-hydrated in PBS for 5 min and the endogenous peroxidase was quenched with 3% H_2O_2 in PBS for 10 min. Then, the sections were blocked in PBS containing 0.3% BSA and 0.1% Triton X-100 for 5 min, washed with PBS and incubated for 2 hours at room temperature with 20 μM ABP in PBS. Finally, sections were washed with PBS and incubated with streptavidin-HRP polymer in PBS (1:500 dilution) for 1 hour at room temperature and developed with metal enhanced DAB substrate (Thermo Fisher) and counterstained with haematoxylin.

Western blotting

Tissue extracts were run on SDS-PAGE and protein were transferred on PDVF membranes. Membranes were blocked with 5% non-fat dry milk in PBS for 40 min at room temperature, washed twice with PBS and incubated with anti-KLK7 antibody (1:2,000 in PBS containing 1% non-fat dry milk and 0.05% Tween-20) for 16 hours at 4^{o}C. The membranes were washed three times in PBS containing 0.05% Tween-20 and incubated for 1 hour with secondary anti-rabbit antibody (1:2,000 in PBS containing 1% non-fat dry milk and 0.05% Tween-20). Finally, the membranes were washed four times with PBS containing 0.05% Tween-20 and specific bands were identified with ECL.

Western blotting with ABP

Enzymes were reacted with the ABP for 2 hours, resolved on SDS-PAGE, then, the proteins were transferred on PVDF membranes. Membranes were blocked with 3% BSA in PBS for 40 min at room temperature, washed twice with PBS and incubated for 1 hour at room

temperature with streptavidin-HRP polymer (1:2,000) and subsequently washed four times with PBS containing 0.05% Tween-20. Specific bands were identified with ECL.

Gel zymography

The compounds were reacted with the enzymes at room temperature for 2 hours, then mixed with zymogram loading buffer without mercaptoethanol, incubated at 37^{o}C for 15 min and resolved on 12% SDS-PAGE containing 0.1% casein or gelatin substrates. Gels were washed twice with 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 2.5% Triton X-100 for 15 min, then 15 min with 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.1% Triton X-100, and finally incubated in the latter buffer for 24 hours and stained with Coomassie G-250. Proteolytic bands appear white against a blue background.

Enzyme-linked immunosorbent assay (ELISA) for active KLK7

Each well of a 96-well plate was incubated for 16 hours at 4^{o}C with 500 ng of anti-KLK7 antibody in 100 µl of phosphate buffered saline (PBS) as coating buffer (pH 7.4) Several dilutions of KLK7 and biotin-FFP (6.3 µM) were allowed to react for 16 hours at room temperature. The plate was washed two times with PBS (200 µl/well) and the blocking of remaining protein-binding sites in the coated wells was achieved by adding 200 µl/well blocking buffer [1% bovine serum albumin (BSA) in PBS] for 1 hour at room temperature. Then, the plate was washed four times with PBS (200 µl/well) followed by addition of the biotin-FFP/KLK7 reaction mixture incubated for 16 hours at room temperature. The plate was washed for 2 times with PBS and 100 µl of freshly prepared solution of streptavidin peroxidase polymer (1 ng/µl) were added in each well and incubated for 1 hour at room temperature followed by washing with PBS+0.005% Tween-20 (200 µl/well, 4 times). The plate was washed two times with 200 µl/well of 100 mM citrate buffer (pH 6.0) and color development was achieved in the dark with 100 µl/well solution of 2 mg/ml ortho-phenylenediamine and 1 µl/ml H_2O_2 30% in citrate buffer 100 mM for 4 min. 100 µl/well stop

solution (2 M H_2SO_4) was added and the absorbance at 492 nm was measured with the Infinite F50 Tecan instrument.

IC_{50} determination

The KLK7 inhibition study was carried out in assay buffer (100 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $CaCl_2$, 0.005% Triton X-100). KLK7 (6 nM) was incubated with the inhibitor (50 nM to 10 μM) for 16 hours at room temperature in the assay buffer. Then, the BODIPY-FL casein substrate (Molecular Probes) was added at 30 μg/ml and fluorescence was measured (λ_{exc}=485 nm, λ_{em}=530 nm). After 3 hours the fluorescence was measured again. Data were inserted into the online available program Very Simple IC50 Tool (http://www.ic50.tk/) for fitting and calculation of the IC_{50}.

Bioinformatic tools

Alignment was carried out with Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

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Table S1. Docking scores for R and S isomers of compounds 3a-c.

Compound	Score, kcal/mol
3a-R	-7.5
3a-S	-7.7
3b-R	-8.3
3b-S	-8.2
3c-R	-8
3c-S	-7.8

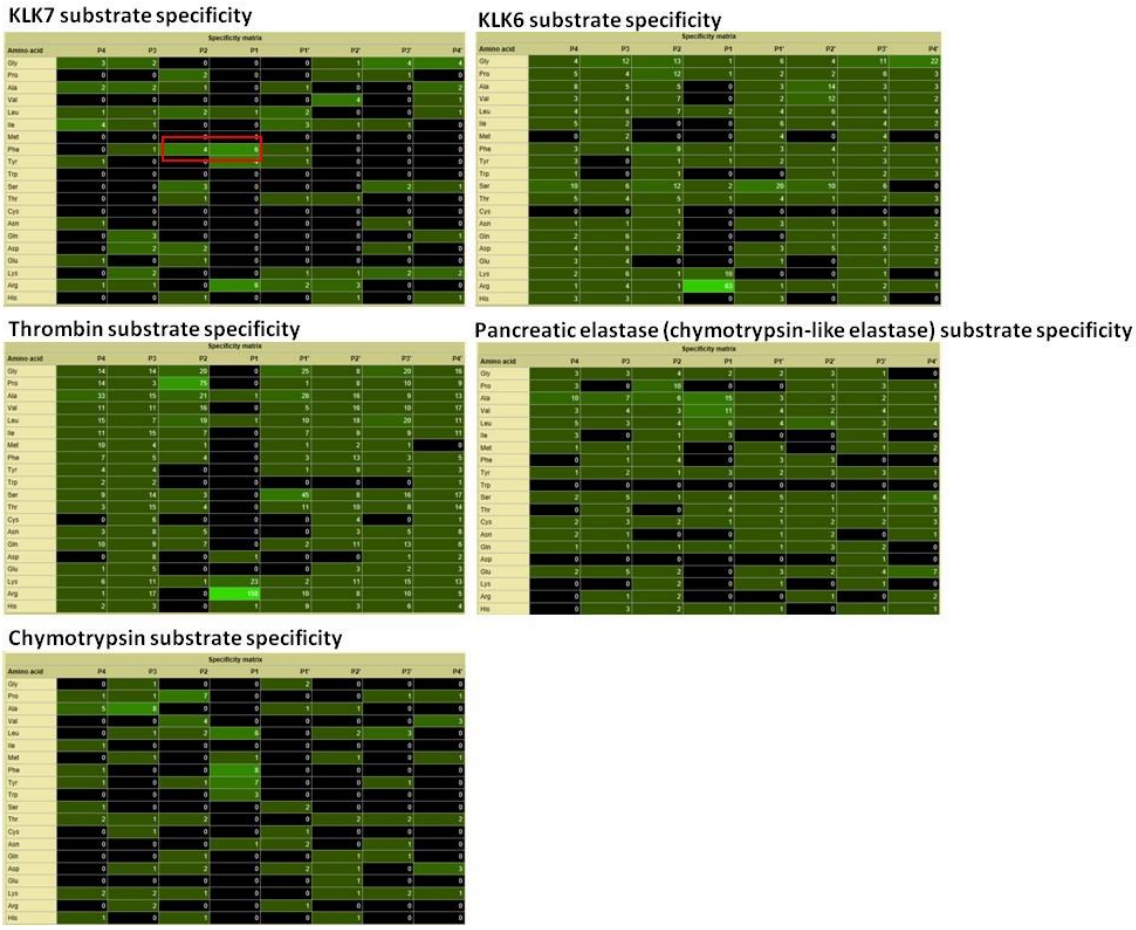


Figure S1. Representative results of substrate specificity of the KLK7 serine protease and other (KLK6, thrombin, pancreatic elastase, chymotrypsin) serine proteases extracted from the MEROPS database. The Phe-Phe P2-P1 motif specific for KLK7 is marked with a red box.

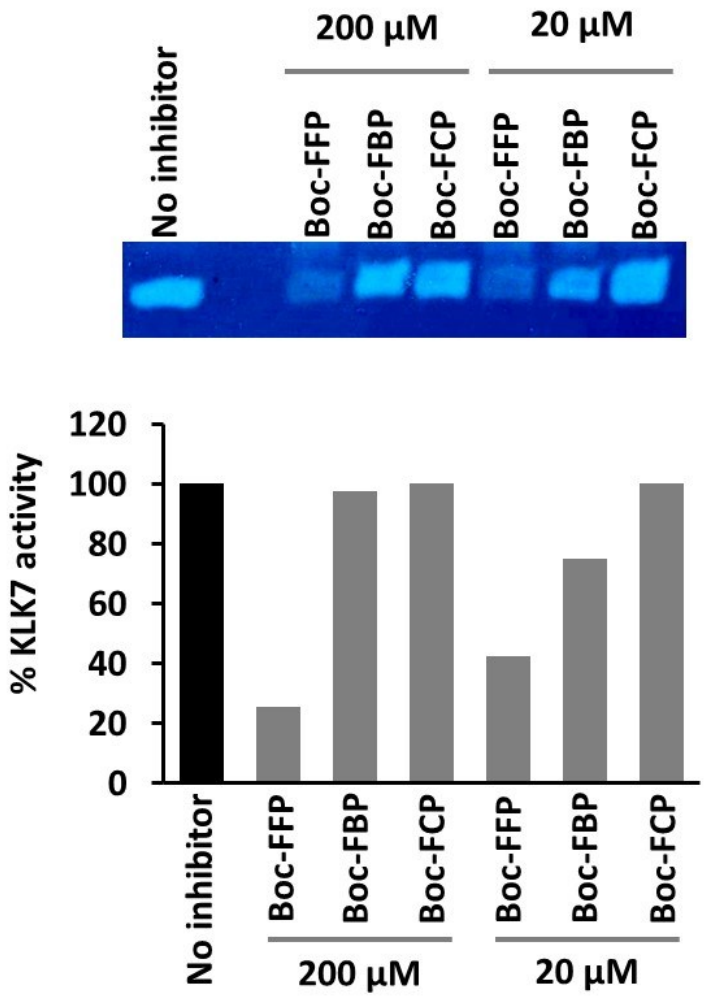


Figure S2. Casein gel zymography demonstrating efficient inhibition of KLK7 proteolytic activity by the Boc-FFP inhibitor. The Boc-FBP and Boc-FCP could not inhibit KLK7. In each lane 65 ng of active KLK7 were loaded (*upper*). Quantification of data with ImageJ (*lower*).

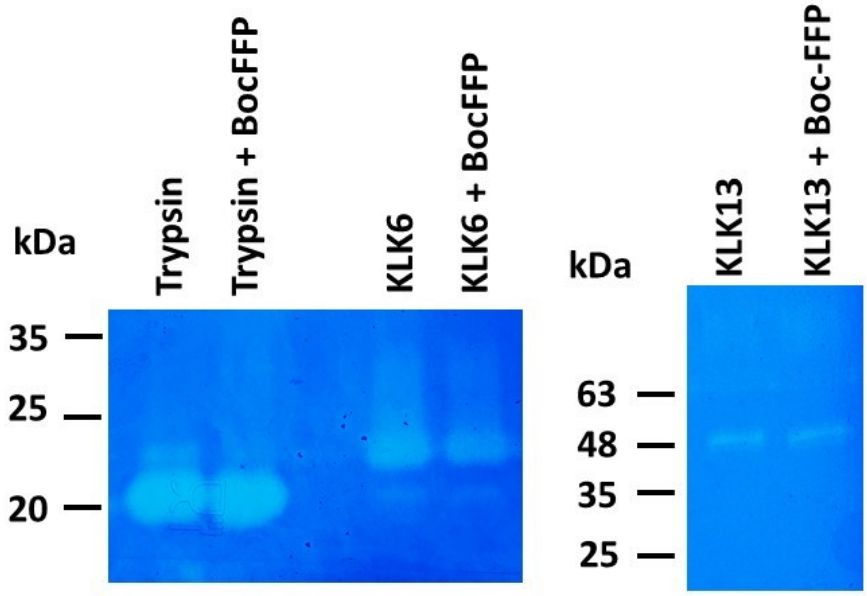


Figure S3. Casein gel zymography shows that Boc-FFP cannot inhibit the activity of KLK6, KLK13, and trypsin.

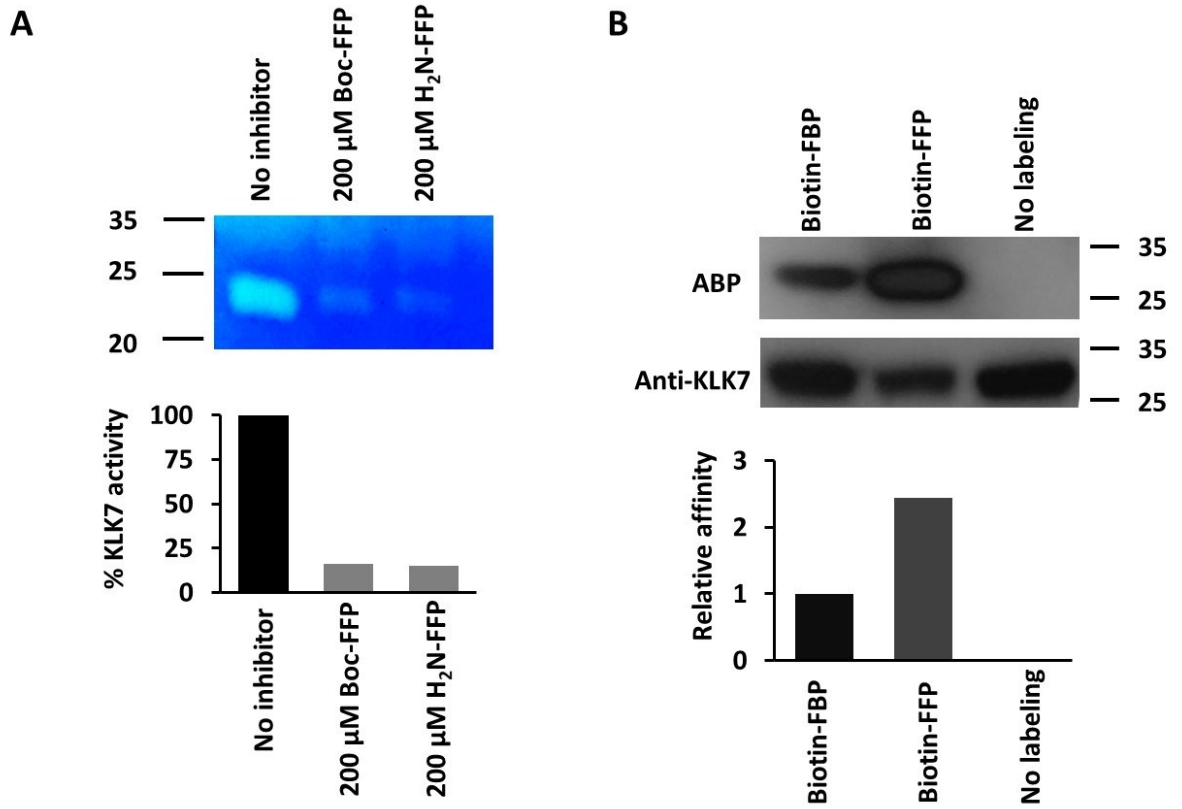


Figure S4. Binding of inhibitors and ABPs to KLK7. (A) Casein gel zymography shows that both the Boc-FFP and the free amino-FFP (compound **2**) inhibit the activity of KLK7. The concentration of inhibitors was 200 µM. (B) Comparison of detection efficiency of the active rKLK7 with the biotin-FFP (**5a**) and biotin-FBP (**5b**) ABPs by Western blotting, shows that biotin-FFP exhibits significantly higher signal intensity (*upper gel*). Detection of total KLK7 protein by conventional Western blot using a KLK7-specific antibody (AF2624, R&D) (*lower gel)*. The concentration of ABPs was 50 µM. In all experiments 65 ng of KLK7 were used. Protein sizes are shown in kDa. Quantification was carried out with ImageJ.

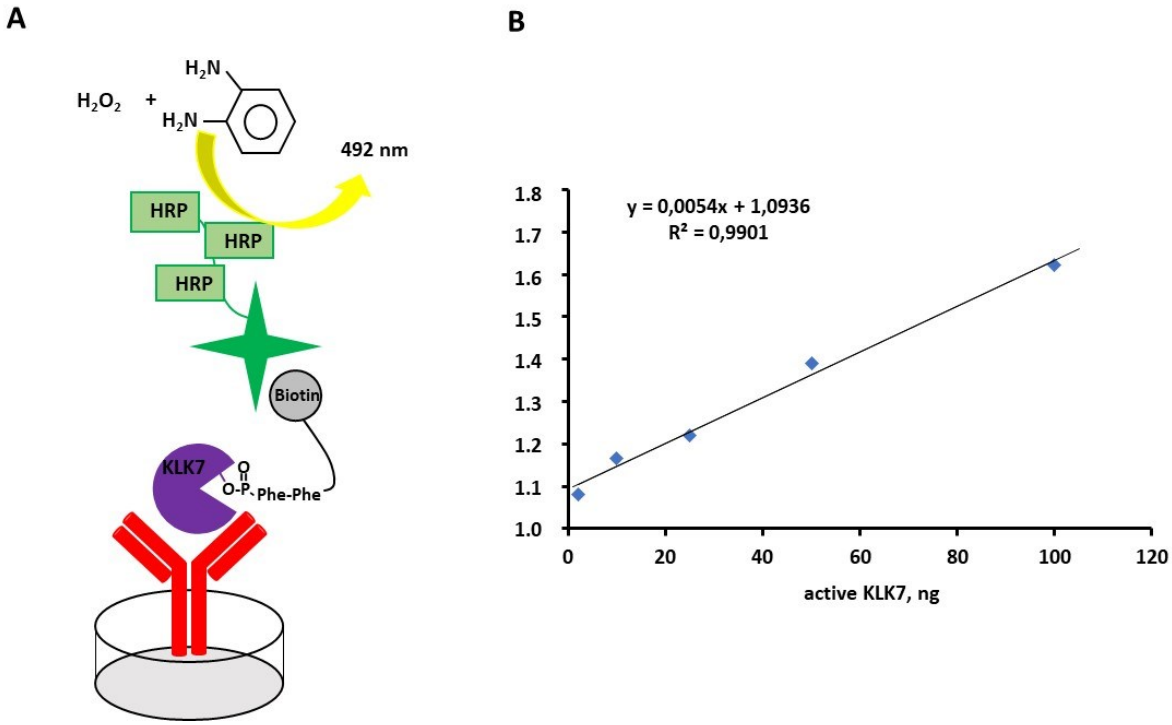


Figure S5. Development of a specific ELISA for the detection of active KLK7. (A) Schematic diagram of the ELISA. Briefly, the sample containing KLK7 was incubated with the biotin-FFP to form the KLK7-ABP adduct. A rabbit polyclonal anti-KLK7 antibody was immobilized in 96-microtiter plates and used as a capturing antibody to capture the KLK7-ABP adduct. The reaction was developed with streptavidin-HRP polymer assisted oxidation of ortho-phenylenediamine by H_2O_2. (B) Representative quantification of active KLK7 protease using the ELISA described in A, shows linear correlation of KLK7 concentration with the determined activity.

Figure S6. Sequence alignment of human KLK7 and mouse Klk7.

A

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness)	4

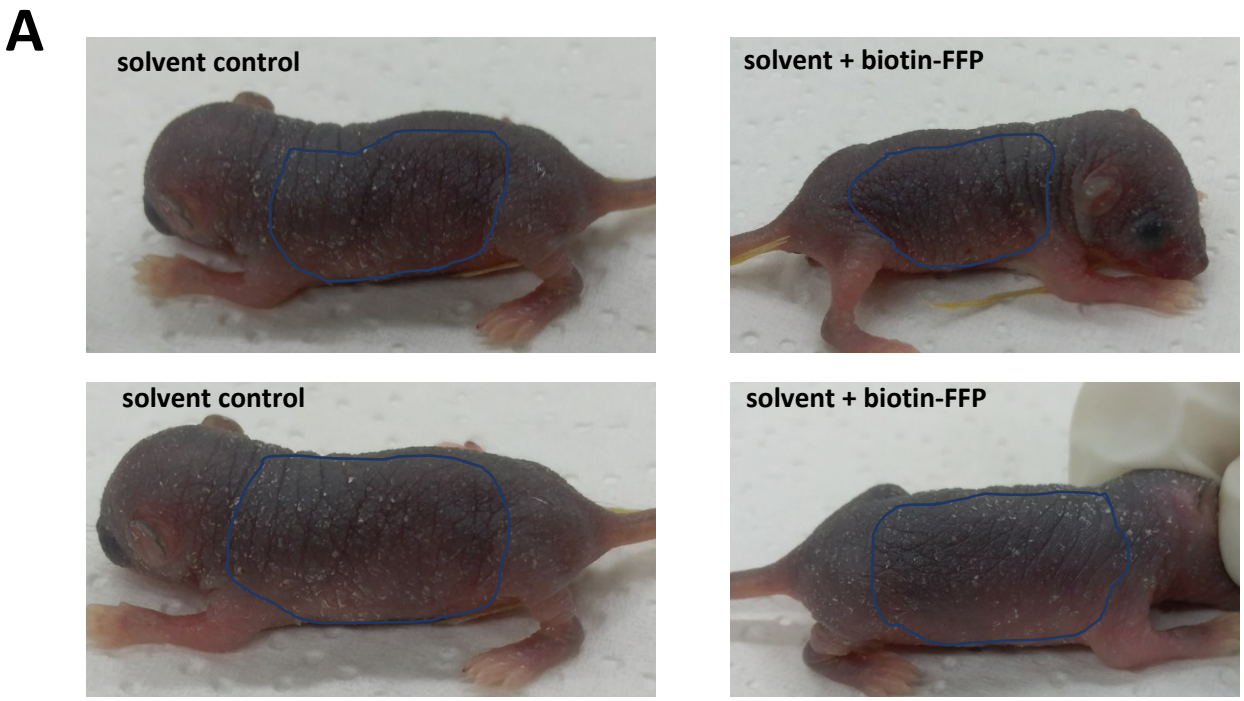
Oedema Formation	Score
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe erythema (raised more than 1mm and extending beyond area of exposure)	4

B

		Erythema and Eschar Score after			
Genotype	**Substance**	**4**	**24**	**48**	**72**
wt	Boc-FFP	0	0	0	0
	Biotin-FFP	0	0	0	0
Klk5$^{-/-}$	Boc-FFP	0	0	0	0
	Biotin-FFP	0	0	0	0

		Oedema Score after			
Genotype	**Substance**	**4**	**24**	**48**	**72**
wt	Boc-FFP	0	0	0	0
	Biotin-FFP	0	0	0	0
Klk5$^{-/-}$	Boc-FFP	0	0	0	0
	Biotin-FFP	0	0	0	0

Figure S7. Acute skin irritation and corrosion. (A) Tables with scores for erythema and oedema phenotype as obtained from https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecdtg404.pdf. (B) Both wt and *Klk5*$^{-/-}$ mice do not display acute skin irritation and corrosion after application of Boc-FFP and biotin-FFP indicating no toxicity of these compounds. Thus, they can be used for pharmacological assessment of their effectiveness in preclinical models.



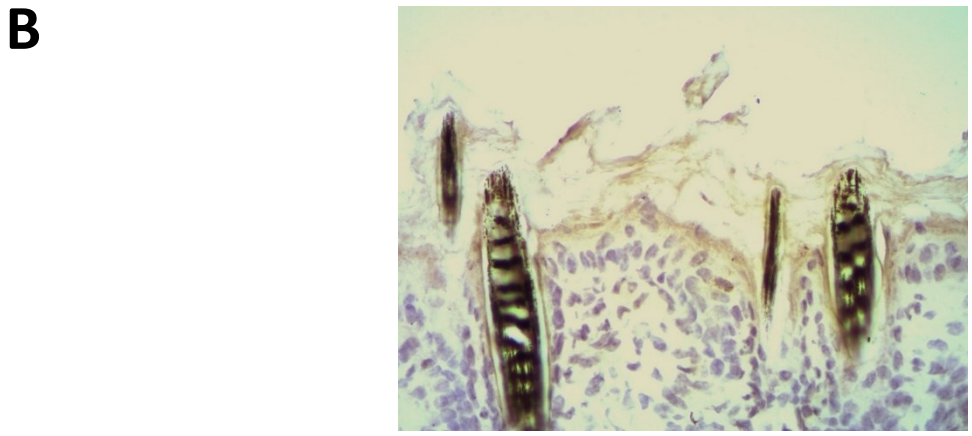


Figure S8. (A) Macroscopic images of *Spink5*$^{-/-}$*Klk5*$^{-/-}$ mice treated with biotin-FFP. The area treated with the biotin-FFP is defined by the blue line box on the mice right side. As control, solvent alone (see Methods) was applied on the left side of each mouse. (B) Activography in *Spink5*$^{-/-}$*Klk5*$^{-/-}$ skin biopsy section shows localization of active Klk7 in the stratum granulosum and stratum corneum.