**Supplementary Materials and Methods**

*S2.1 Materials and reagents*

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Phenol-free RPMI-1640, DMEM-High Glucose, 0.25% trypsin-EDTA, sodium pyruvate, penicillin-streptomycin (pen/strep), and L-glutamine were from Invitrogen (Grand Island, NY). Rooster hMSC High Performance Media is from RoosterBio (Frederick, MD). Fetal bovine serum (FBS) and Nunc Flat bottom MaxiSorp Immuno Plates were from Thermo Fisher Scientific (Waltham, MA). Charcoal-stripped FBS was purchased from Gemini Bioproducts (West Sacramento, CA). The anti-proaerolysin HG6 hybridoma (capture antibody) was previously isolated50 and MPC polyclonal (detection) antibody was generated in New Zealand White Rabbits using proaerolysin as the antigen 22 and purified using previously described protocols51. Mouse IgG isotype control antibody was from Vector Laboratories (Burlingame, CA). Human plasma and red blood cells (RBCs) were obtained from discarded clinical specimens in accordance with IRB-approved protocols. Mouse plasma was obtained from Innovative Research (Novi, MI). Recombinant human PSA was from BioRad (Hercules, CA).PC3 and DU145 prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC). LNCaP-95 (LN95) were generously provided by Dr. Alan Meeker (Johns Hopkins University, Baltimore, MD)31,32.

*S2.2* *Cell Culture*

All cell lines are routinely tested for mycoplasma contamination using the MycoSensor PCR Assay kit (Agilent Technologies, Santa Clara, CA) and authenticated using STR analysis by the Johns Hopkins Genetic Resources Core Facility. PC3 and DU145 cells were cultured in phenol-free RPMI-1640 supplemented with 10% FBS and 1% L-glutamine. LN95 were cultured in phenol-free RPMI-1640 supplemented with 10% charcoal-stripped FBS and 1% L-glutamine. All cultures maintained in a 5% CO2, 95% air humidified incubator at 37°C with regular media changes every 3-4 days and passaging following trypsinization according to standard protocols as needed.

*S2.3 Production and Purification of PRX302*

PRX302 was expressed in *Aeromonas salmonicida* as previously described8. The protein was purified using a method developed for native proaerolysin22 except that the clarified and concentrated culture supernatants were applied to a Bio-Scale CHT10-I column (Biorad) and eluted with a 0-300 mM sodium phosphate gradient at pH 6.5. The eluted fraction containing the PRX302 proaerolysin variant was further purified via filtration using an Acrodisc Mustang E membrane (Pall; Port Washington, NY) to remove endotoxin according to the manufacturer’s instructions.

*S2.4 Monoclonal Antibody Purification from HG6 Hybridoma*

The HG6 hybridoma was maintained in DMEM supplemented with 10% FBS, 1mM sodium pyruvate, 1% pen/strep, 1% L-glutamine, and 0.1% 2-mercaptoethanol at 37˚C in a humidified incubator with 5% CO2. Prior to media conditioning, cells were slowly adapted to 5% IgG-stripped FBS followed by expansion to the desired volume. Media was then conditioned for 1 wk prior to collection of supernatant, centrifuged at 2500 rpm for 5 min to remove cell debris, and passed through a 0.2 μm filter. Supernatants were concentrated to <1 mL using Amicon Ultra-15 Centrifugal Filter Units with a 10k MWCO according to manufacturer’s instructions. The concentrated conditioned media was then incubated with Protein G Agarose (Pierce Biotechnology, Waltham, MA), washed, and eluted according to manufacturer’s recommendations. IgG positive fractions were tracked using absorbance at 280 nm. Pooled fractions were then dialyzed against 1 L phosphate-buffered saline (PBS, pH = 7.4) for 2 hrs and 2 L PBS overnight at 4˚C. Final protein concentration was determined using absorbance at 280 nm with a molar extinction coefficient of 150,000 L mol-1 cm-1, aliquoted, and stored at -80˚C until use.

*S2.5 PA/PRX302 Sandwich ELISA*

Nunc-Immuno microwell plates were coated with the HG6 mouse monoclonal (i.e. capture) antibody in PBS (pH 7.4) and incubated overnight with gentle shaking at room temperature and protected from ambient light. Next, the capture antibody solution was removed, and the plate blocked with a 1% bovine serum albumin (BSA) solution in PBS, incubated at room temperature for 1 hr while shaking and protected from light. All PRX302 samples were diluted ≥1000-fold in blocking solution and incubated in coated plates for 2 hrs at room temperature while shaking and protected from light. Plates were washed 3 times with 0.05% Tween-20 in PBS. The MPC polyclonal rabbit (i.e. detection) antibody was diluted in blocking solution and incubated for 2 hrs at room temperature while shaking and protected from light followed by 3 rounds of washing with 0.05% Tween-20 in PBS. Anti-rabbit HRP-conjugated IgG (Cell Signaling, Danvers, MA) was diluted 1:5000 in blocking buffer prior to adding to each well and incubated for 1 hr at room temperature while shaking and protected from light. The plate was washed three additional times with 0.05% Tween-20 in PBS and incubated with a 1 mg/mL solution of ABTS substrate in peroxide buffer. The reaction was allowed to proceed for 1 hr at room temperature while shaking and protected from light. The reaction was stopped by addition of 100 μl of 5% sodium dodecyl sulfate (SDS). The plate was then read at 410 nm with a 570 nm wavelength correction. Varying concentrations of the capture and detection antibodies were used during the optimization process prior to selecting 4.0 and 5.5 μg/mL, respectively, for all further assays. All assays performed in duplicate with the exception of the intra- and inter-assay variation experiments, which included 12 replicates per concentration. A 4-parameteter non-linear regression of an 8-point standard curve was used to calculate PRX302 concentrations.

*S2.6 Intra- and Inter-Assay Variation, Specificity, Sensitivity, Linearity of Dilution, Parallelism and Spike/Recovery Assays*

Intra- and Inter-assay variation was determined by performing 3 independent assays on different days with 12 replicates per concentration per assay. Coefficients of Variation (CV%) <10% within each assay and <15% across assays were considered acceptable. Specificity was determined by omitting the HG6 capture antibody or substituting a non-specific mouse IgG isotype control (Vector Laboratories). Sensitivity (i.e. limit of detection) defined as three standard deviations above the average blank (i.e. zero standard) reading determined across each of these assays. Linearity of dilution was documented by calculating the Pearson’s correlation coefficient (cc) of the percent expected vs. the percent measured based on the standard curve with a cc >0.98 considered acceptable. To validate the effects of different biological matrices (i.e. Saline + 1% BSA, RPMI + 10% FBS, Rooster High Performance Media, mouse plasma, or human plasma), dilution series in the indicated matrix were prepared and assayed as described above to determine PRX302 concentrations. Parallelism determined based on guidelines proposed by Plikaytis et al.52. Recovery was determined by calculation of the percent measured vs. the percent expected with values between 80-120% of the expected concentration considered acceptable. In matrices that did not meet these parameters, serial dilutions into assay buffer (PBS + 1% BSA) from the stock concentration in the respective matrix were performed, and the assays were repeated at the identified dilution to eliminate matrix effects. If no value was reported in any experiment, it was below the limit of detection.

*2.7 Microparticle (MP) Size and Charge*

Dynamic light scattering was used to determine mean particle size and size polydispersity index (PDI). MPs (1 mg) were suspended in ultrapure water (2 mL) and placed into plastic cuvettes. The suspension was then sonicated and size measurements were performed using Nano ZS Zetasizer (Malvern Instruments). Another 1 mL of the same solution was then used in a zeta potential cuvette to determine the charge of MPs via Zetasizer.

*2.8 Drug Loading and Encapsulation Efficiency for PRX302-loaded PLGA Microparticles*

MPs (1 mg) were lysed overnight using 2 mL of NaOH-SDS. The solution was then subjected to a microBCA assay according to manufacturer instructions (Thermo Scientific). Plates were read using a Tecan microplate spectrophotometer. Using the standard curve of the free PRX302 prepared in the lysis buffer, the concentration of PRX302 in the lysed MPs was determined and used to calculate the drug loading in the PRX302-loaded MPs (mass of drug out of total MP mass) and the encapsulation efficiency (amount of drug successfully encapsulated out of initial amount of drug used in the fabrication process).

*S2.8 Statistical Analysis*

Statistical analysis was performed using GraphPad Prism 8.0.0 for Windows, GraphPad Software (San Diego, CA). Two-way Analysis of Variance (ANOVA) was used for comparisons across multiple groups and 2-sided T-tests for comparisons between individual groups when appropriate. A p-value < 0.05 was considered statistically significant.

**Supplemental Figure Legends:**

**Figure S1**. **Nucleotide and amino acid sequence of proaerolysin and PRX302, a PSA-activated proaerolysin variant.** Wild type furin activation sequence (i.e. KVRRAR) in proaerolysin, the parental toxin, and PSA-dependent activation sequence (i.e. HSSKLQ) in PRX302 that was generated via site-directed mutagenesis highlighted in the gray box.

**Figure S2: Optimization of PRX302 sandwich ELISA. (A)** Serial dilutions of the HG6 monoclonal capture antibody to determine optimal concentration. Performed in duplicate. **(B)** Serial dilutions of MPC polyclonal detection antibody to determine optimal concentration. Performed in duplicate **(C)** PRX302 standard curve using optimal CAb (i.e. 4 μg/mL) and DAb (i.e. 5.5 μg/mL) concentrations. Standard curve represents average of 3 independent runs with 12 replicates per concentration per assay. Error bars represent +/- standard deviation (SD).

**Figure S3: PRX302 sandwich ELISA validation in different biological matrices. (A)** Parallelism in different matrices. **(B)** Linearity of dilutions in different matrices [Pearson’s correlation coefficient (cc) ≥0.999]. **(C)** Recovery following serial dilutions into assay buffer of spiked samples in different matrices. **(D)** Parallelism in different matrices post-dilution. All assays run in duplicate. Error bars represent +/- standard deviation (SD).