PirA- or PirB-binding nanobodies protect whiteleg shrimp from acute hepatopancreatic necrosis disease

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Abstract:

Acute hepatopancreatic necrosis disease (AHPND) is a devastating shrimp disease caused by a binary toxin, PirAB, produced by *Vibrio parahaemolyticus* and other closely related bacteria. To address AHPND, over three hundred unique single domain antibodies (also known as nanobodies) derived from the V_HH domains of *Lama glama* heavy chain only antibodies were raised against either PirA or PirB and characterized. Nanobodies were shortlisted based on their affinities for either PirA or PirB, their relative stability in intestinal fluids, and their ability to reduce PirAB-induced death in brine shrimp (*Artemia salina*). From these data, a subset of nanobodies were tested for their ability to reduce AHPND in whiteleg shrimp (*Litopenaeus vannamei*) and nanobodies targeting either PirA or PirB provided significant disease protection to whiteleg shrimp. These results show that nanobodies can be a new option for shrimp farmers to reduce or eliminate the impact of AHPND on their operations.

1. INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) is an infectious disease of penaeid shrimp characterized by atrophying hepatopancreas, empty guts, and high mortality (Tran et al. 2013). The disease has caused enormous losses to shrimp producers. As of 2021, the cumulative global shrimp production losses caused by AHPND were estimated to be $43 billion USD with production reduced to approximately 60% in affected areas (Kumar et al. 2021).

A plasmid-expressed binary toxin, PirAB, of *Vibrio parahaemolyticus* has been shown to be necessary and sufficient for the disease (Han et al. 2015, Lee et al. 2015). Although initially described in *V. parahaemolyticus*, *pirAB*-containing plasmids have since been shown to be spread to other *Vibrio* species (Hidehiro et al. 2015, Dong et al. 2017).

Current AHPND mitigation strategies have recently been reviewed (Kumar et al. 2021). Typical pond management strategies such as aeration control, feed quality assessment, disinfection of input materials, and antibiotic applications are often insufficient and, in many cases, the AHPND-causing bacteria are resistant to several antibiotics. Other mitigation strategies that can be applied include: (1) probiotic bacteria to improve overall shrimp or pond health and, in some cases, to produce antimicrobials; (2) lytic bacteriophage directed at AHPND-causing bacteria; and (3) phytochemicals that act as either immune stimulants or have antimicrobial properties. Notably, none of these strategies are directed at PirAB. As an alternative to these mitigation strategies, a new therapeutic option, targeted specifically to PirAB and amenable to inclusion in shrimp feed is desperately needed. Nanobodies, derived from the V_HH domain of camelid heavy chain only antibodies (Muyldermans 2013), offer one such option. These small proteins retain the high affinity antigen binding function of the larger antibody from which they are derived but can be recombinantly expressed in organisms such as

bacteria, yeast, algae, and plants (Barrera et al. 2015, de Marco 2020, Malaquias et al. 2021). Nanobodies have been successfully used in feed to treat chicken and swine diseases (Virdi et al. 2019, Lessard et al. 2020).

Furthermore, efficacy of antibody-based applications against PirA and PirB have been demonstrated previously in shrimp. Chicken egg yolk immunoglobulin IgY antibodies directed at PirA have previously been shown to reduce AHPND mortality in experimental shrimp infection models (Nakamura et al. 2019). IgY antibodies cannot easily be produced in fermentation processes using simple organisms, preventing scale-up; however, this experimental efficacy evidence further supports the development of nanobodies against PirA and PirB. Here, we describe the identification and characterization of nanobodies targeting either PirA or PirB, that can reduce AHPND mortality in whiteleg shrimp. The results indicate that nanobodies have the potential to be a new option for the reduction of AHPND in shrimp aquaculture.

2. MATERIALS & METHODS

2.1. Protein Purification

Vibrio parahaemolyticus pirA and pirB genes were synthesized by BioBasic (Markham, Ontario) and subcloned into pET26b between the NdeI and XhoI sites. The proteins were recombinantly expressed in and purified from *Escherichia coli* with C-terminal 6X-histidine tags as previously described (Lee et al. 2015).

Nanobodies were expressed with N-terminal TEV-cleavable 6X-histidine and Thioredoxin (TRX) tags in *E. coli* strain Rosetta-gami 2 grown overnight in autoinducing media (Formedium) at 30°C. For each nanobody, the *E. coli* cells were pelleted by centrifugation, resuspended in 10 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM imidazole, 50 mM $CaCl_2$, 12.5 μg/ml DnaseI, 1 mM PMSF, and lysed using sonication. The clarified soluble fraction was

passed over a HisTrap column (Cytiva) equilibrated with 10 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM imidazole, 50 mM $CaCl_2$, and the bound protein was eluted with an imidazole gradient (20 to 300 mM). TEV protease and EDTA were added to the eluted nanobody at concentrations of 40 μg/ml and 5 mM, respectively, and the nanobody was dialyzed overnight at 4°C into 10 mM HEPES, pH 7.5, 250 mM NaCl. After TEV cleavage, the nanobody was passed over a second HisTrap column equilibrated with 10 mM HEPES, pH 7.5, 250 mM NaCl and the nanobody protein was collected in the flowthrough. Depending on the pI of the nanobody, they are subsequently dialyzed to either 10 mM Tris-HCl, pH 8.8 and purified with anion exchange chromatography (HiTrapQ SP, Cytiva) or 10 mM HEPES, pH 7.0 and purified with cation exchange chromatography (HiTrap SP HP, Cytiva). In either case then are eluted from the ion exchange columns using NaCl gradients of 5 mM to 1 M. Nanobodies were concentrated to ~10 mg/mL in a final buffer of 20 mM HEPES, pH 7.4, 150 mM NaCl, aliquoted, flash frozen, and stored at -80°C.

2.2. Nanobody Discovery

The protocols used for the discovery of nanobodies are well-described (Baral et al. 2013) and were applied here to PirA and PirB. Briefly, llamas (Cedarlane Laboratories) were immunized with 100 μg of each antigen on days 1, 21, 42, and 63 and lymphocytes were collected from sera on days 28, 49, and 70. Lymphocyte RNA was converted to cDNA, which was subsequently used as a template for the PCR amplification of nanobody genes. The nanobody genes were used to created M13 phage libraries wherein each phage particle expresses a nanobody fused to the phage gIII tip protein and carries the corresponding nanobody DNA sequence internally. Phage were sorted into antigen-specific groups through three rounds of biopanning. Monoclonal phage were assessed by ELISA for binding to wells coated with either

10 μg/ml of antigen or phosphate buffered saline (PBS) and the nanobody genes from those phage with a four-fold greater binding to antigen-coated wells compared to PBS-coated wells were sequenced. Nanobodies with unique complementarity determining regions (CDRs) were selected for screening in *in vitro* experiments.

2.3. Nanobody ELISA

A 96-well plate was coated overnight at 4°C with 100 μL of either PirA (10 μg/ml) or PirB (20 μg/ml) in PBS. After the overnight incubation, antigen solutions were removed, wells were washed three times with 300 μL of PBS and blocked with 200 μL of 5% skim milk powder in PBS + 0.05% Tween-20 (PBST) for two hours at room temperature. Blocking solution was removed and wells were washed one time with 300 μL of PBST. Nanobodies were diluted in blocking solution, and 100 μL was added to wells. The final nanobody concentrations ranged from 4 pM to 400 nM. Plates were incubated with nanobody solutions for one hour at room temperature. Nanobody solutions were removed, wells were washed three times with 300 μL of PBST, and 100 μL of HRP-conjugated rabbit IgG anti-V_HH cocktail (GenScript) diluted 1:8000 in blocking solution was added to wells and incubated for one hour at room temperature. Anti-V_HH antibody solution was removed, wells were washed three times with 300 μL of PBST, and 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Abcam) was added to wells and incubated for 30 minutes at room temperature. Reactions were stopped with the addition 50 μL of 1 M HCl and absorbance at 450 nm was measured.

2.4. Gastrointestinal Tract Extract Stability Assay

Reactions containing 2.4 μL chicken jejunal extract, 5 μg nanobody in 0.8 μL PBS, and 4.8 μL of 150 mM NaCl were set up on ice. Control reactions without jejunal extract were also set up containing 5 μg nanobody in 3.2 μL PBS and 4.8 μL of 150 mM NaCl. The tubes were

incubated on ice for 5 minutes, followed by 42°C incubation for up to 24 hours. After incubation, 8 μL of preheated 2X SDS sample buffer was added to stop the reaction and nanobody stability was assessed using SDS-PAGE.

2.5. Artemia *in vivo* tests

Artemia salina cysts were purchased from Aquarium Direct (Saint-Charles-Borromée, QC, Canada) and rehydrated in 1.7 L of artificial seawater (pH 8.4-8.6; Alkalinity 3.2-3.8 mEq/L) prepared following manufacturer's instruction (Salinity™; Aquavitro®; Madison, GA, USA) in an Artemia hatchery blender (ZH-2000, Ziss Artemia Blender 2.0 L; 18cm×14cm×13cm). After 48 hours incubation between 26-28°C under constant aeration with a constant light source, the growth stage of Artemia was confirmed under a microscope. Only those that reached instar II larvae were used in the challenge test.

About 10 to 12 stage II Artemia were allocated into 0.5-mL artificial seawater per well in 24-well plates and acclimatized for 1 hour at 26-28°C. Diluted protein treatment solutions (0.5 mL) were added to each well to achieve final concentrations of 3 μM PirA, 3 μM PirB, and 15 μM nanobody in 10% PBS. The 24-well plates were placed on a shaking platform (90 rpm) for 5 minutes to ensure homogenous mixture of treatment solution with seawater. The plates were incubated in a non-shaking incubator between 26-28°C with a constant light source and mortality was measured at 42 hours post challenge. Each treatment group had 12 replicates wells. A negative control group that only received 10% PBS and a positive control group that received PirA and PirB but no nanobody were included in each experiment.

2.6. Whiteleg shrimp *in vivo* tests

Ten-day post-larval whiteleg shrimp (*Litopenaeus vannamei* PL10), that were 9-11 mm in length were used for assessing the efficacy of nanobodies in neutralizing the PirA/PirB toxin and

reducing mortality. One PL10 was put into 0.5-ml seawater (20 ppt of salinity, pH-7.8-8.2, 120-160 ppm of alkalinity) per well in 24-well plates and allowed to acclimatize for 1 to 2 hours. During acclimatization, PirA, PirB, nanobodies, and seawater were mixed as a 2X treatment solution and allowed to equilibrate for 30 minutes. After the PL10 acclimatization period, 0.5 mL of 2X treatment solutions were added to each well to start the test. The final nanobody concentration was 8 μM. The final PirA and PirB concentrations vary by batch and each batch required a titration curve to determine a concentration that produced a slowly increasing mortality curve over a 24-hour period that reaches 60-90% mortality by 24 hours. A typical PirA and PirB concentration used was 125 nM. Each treatment group had a total of 12 PL10s. Shrimp death was monitored every 2 hours for 24 hours. For each test, a negative control that contained only seawater and a positive control that contained PirA and PirB but no nanobody were included as additional groups. Whiteleg shrimp *in vivo* studies were conducted at ShrimpVet (Vietnam).

3. RESULTS

3.1. Identification of unique α-PirA and α-PirB nanobodies.

Nanobodies to PirA and PirB were obtained from four and seven llamas, respectively. In some cases, llamas were immunized with both PirA and PirB, while in other cases, they were immunized individually. In total, 127 and 205 unique nanobodies were raised to PirA and PirB, respectively. Of these, more than 90% could be successfully expressed in and purified from *E. coli* and moved into the *in vitro* screening stage described below.

3.2. Nanobody binding screen

All purifiable nanobodies were tested for the ability to bind either PirA or PirB in an ELISA-based assay. To exemplify these studies, the binding of nanobody NBX-PA1 to PirA and

nanobody NBX-PB1 to PirB in the ELISA-based assay are shown in Fig. 1A and 1B, respectively. Both nanobodies binding is detected in the sub-nM range, with midpoints of saturation at concentrations below 5 nM. The mean (and standard error of the mean) midpoints from five independent experiments for these nanobodies were: NBX-PA1, 2.1 ± 0.5 nM and NBX-PB1, 0.6 ± 0.1 nM. Although these are not equilibrium assays, this suggests a high inherent affinity.

3.3. Nanobody proteolytic stability screen.

All nanobodies were screened for proteolytic stability in a gastrointestinal (GI) tract extract. In early studies, a GI extract derived from shrimp was available; however, such samples were difficult to obtain in Canada due to import restrictions caused by infectious hypodermal and haematopoietic necrosis (IHHN) disease. Later studies were conducted with a chicken jejunal extract that could be more easily obtained. Some nanobodies were tested in both samples and although survival times in different extracts varied, the relative ranking of nanobody stability was consistent across extract types (data not shown). Fig. 2 shows the results for NBX-PA1 and NBX-PB1 in a chicken jejunal sample. NBX-PA1 was visible on the SDS-PAGE gel until the last measured time point (24 hours), while NBX-PB1 was visible until 6 hours. This places NBX-PA1 and NBX-PB1 in the top 1% and 12%, respectively, for proteolytic stability in this extract.

3.4. Nanobody protection of brine shrimp

Based on the combined results of nanobody binding and proteolytic stability studies a collection of 50 nanobodies from the original ~300 nanobodies were selected for PirAB neutralization studies in brine shrimp (*Artemia salina*). Working with brine shrimp allowed for an *in vivo* pre-screen that required less nanobody than tests in whiteleg shrimp and could be done inhouse. NBX-PA1 and NBX-PB2 were both able to reduce the mortality induced by PirAB by

approximately 50% (Fig. 3). From these studies, using a cut-off of 30% mortality reduction or better during nanobody treatment, the collection of 50 nanobodies was further reduced to 20 nanobodies for testing in whiteleg shrimp.

3.5. Nanobody protection of whiteleg shrimp

Nanobodies were tested for the ability to protect post-larval whiteleg shrimp (*Litopenaeus vannamei* PL10) from treatment with recombinantly expressed and purified PirAB in 24-hour immersion experiments. Both NBX-PA1 and NBX-PB1 significantly reduced mortality in whiteleg shrimp to disease induced by PirAB (Fig. 3). Any mortality seen in the nanobody protected shrimp was similar to the results seen in untreated control shrimp.

It was evident in these studies that the whiteleg shrimp PL10s were more susceptible to PirAB than the brine shrimp. The whiteleg shrimp were killed more quickly (average of ~80% mortality at 24 hours) than brine shrimp (average of ~60% mortality at 42 hours), despite the fact the whiteleg shrimp were treated with less PirAB (125 nM) than the brine shrimp were (3 μM). Similar concentrations of nanobodies were used with each species and we speculate that the reduced amount of PirAB used in the whiteleg shrimp experiments allowed for better protection by neutralizing nanobodies such as NBX-PA1 and NBX-PB1.

4. CONCLUSIONS

Despite the lack of an *in vitro* functional assay to identify nanobodies capable of neutralizing the activity of PirA or PirB, we were able to obtain several nanobodies that can protect whiteleg shrimp from PirAB-induced AHPND. This was accomplished by starting with several hundred nanobodies and filtering them through a series of protein-protein interaction, intestinal tract proteolytic stability, and brine shrimp *in vivo* test to shortlist nanobodies for whiteleg shrimp protection studies. These whiteleg shrimp protection studies show that nanobodies can reduce

shrimp mortality in the presence of PirAB to levels comparable to unchallenged control shrimp. The results presented here demonstrate the great potential nanobodies have for the protection of shrimp from acute hepatopancreatic necrosis disease. Nanobodies offer an excellent new opportunity to the shrimp aquaculture sector for the reduction of this disease which at this time causes billions in dollars of losses to producers every year (Kumar et al. 2021).

Works Cited

Baral TN, MacKenzie R, Ghahroudi MA (2013) Single-domain antibodies and their utility. Curr Protoc Immunol 103:2.17.1-2.17.57.

Barrera DJ, Rosenberg JN, Chiu JG, Chang Y-N, Debatis M, Ngoi S-M, Chang JT, Shoemaker CB, Oyler GA, Mayfield SP (2015) Algal chloroplast produced camelid VHH antitoxins are capable of neutralizing botulinum neurotoxin. Plant Biotechnol J 13:117–124.

Dong X, Bi D, Wang H, Zou P, Xie G, Wan X, Yang Q, Zhu Y, Chen M, Guo C, Liu Z, Wang W, Huang J (2017) PirABvp-Bearing Vibrio parahaemolyticus and Vibrio campbellii Pathogens Isolated from the Same AHPND-Affected Pond Possess Highly Similar Pathogenic Plasmids. Front Microbiol 8.

Han JE, Tang KFJ, Tran LH, Lightner D V (2015) Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of Vibrio parahaemolyticus, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Dis Aquat Organ 113:33–40.

Hidehiro K, Thi VP, T DL, Ikuo H (2015) Draft Genome Sequence of Non-Vibrio parahaemolyticus Acute Hepatopancreatic Necrosis Disease Strain KC13.17.5, Isolated from Diseased Shrimp in Vietnam. Genome Announc 3:10.1128/genomea.00978-15.

Kumar V, Roy S, Behera BK, Bossier P, Das BK (2021) Acute Hepatopancreatic Necrosis Disease (AHPND): Virulence, Pathogenesis and Mitigation Strategies in Shrimp Aquaculture. Toxins (Basel) 13.

Lee C-T, Chen I-T, Yang Y-T, Ko T-P, Huang Y-T, Huang J-Y, Huang M-F, Lin S-J, Chen C-Y, Lin S-S, Lightner D V, Wang H-C, Wang AH-J, Wang H-C, Hor L-I, Lo C-F (2015) The opportunistic marine pathogen Vibrio parahaemolyticus becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences 112:10798 LP – 10803.

Lessard PA, Parker M, Bougri O, Shen B, Samoylov V, Broomhead J, Li X, Raab RM (2020) Improved performance of Eimeria-infected chickens fed corn expressing a single-domain antibody against interleukin-10. Nat Food 1:119–126.

Malaquias ADM, Marques LEC, Pereira SS, de Freitas Fernandes C, Maranhão AQ, Stabeli RG, Florean EOPT, Guedes MIF, Fernandes CFC (2021) A review of plant-based expression systems as a platform for single-domain recombinant antibody production. Int J Biol Macromol 193:1130–1137.

de Marco A (2020) Recombinant expression of nanobodies and nanobody-derived immunoreagents. Protein Expr Purif 172:105645.

Muyldermans S (2013) Nanobodies: Natural Single-Domain Antibodies. Annu Rev Biochem 82:775–797.

Nakamura R, Pedrosa-Gerasmio IR, Alenton RRR, Nozaki R, Kondo H, Hirono I (2019) Anti-PirA-like toxin immunoglobulin (IgY) in feeds passively immunizes shrimp against acute hepatopancreatic necrosis disease. J Fish Dis 42:1125–1132.

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner D V (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Organ 105:45–55.

Virdi V, Palaci J, Laukens B, Ryckaert S, Cox E, Vanderbeke E, Depicker A, Callewaert N (2019) Yeast-secreted, dried and food-admixed monomeric IgA prevents gastrointestinal infection in a piglet model. Nat Biotechnol 37:527–530.

Figure Legends

Fig. 1: NBX-PA1 and NBX-PB1 bind to target antigens in an ELISA-based assay with low nM binding affinities. (A) NBX-PA1, but not NBX-PB2, is able to bind to PirA. (B) NBX-PB1, but not NBX-PA1, is able to bind to PirB. Data shown are from a representative experiment for each nanobody. Data points are the mean and standard deviation from triplicate wells.

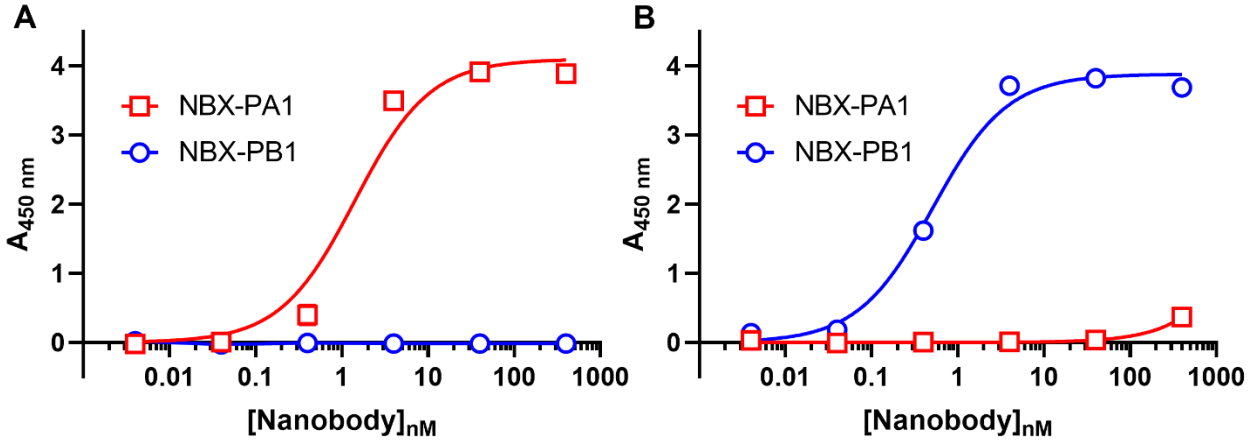
Fig. 2: NBX-PA1 and NBX-PB1 (both have predicted molecular weights of 12.5 kDa) survive for 24 and 6 hours, respectively, in the presence of a chicken jejunal extract. For each nanobody, the SDS-PAGE gel shows the following from left to right: 11 and 17 kDa molecular weight markers; the nanobody, incubated at 42^{o}C in the absence of extract at the start and endpoints of the experiment to show that any observed degradation is extract dependent; the extract without nanobody at the start of the experiment to aid in the identification of the nanobody on the gel; a time course of the nanobody incubated at 42^{o}C in the presence of the extract; and the extract without nanobody at the end of the experiment to aid in the identification of the nanobody on the gel.

Fig. 3: NBX-PA1 and NBX-PB1 reduce PirAB-induced mortality in brine shrimp. Data shown are the means and standard deviations from three to seven independent experiments. All other groups are statistically different then the PirAB treated group by unpaired student's t-test (*$p < 0.05$, **$p < 0.0001$).

Fig. 4: NBX-PA1 and NBX-PB1 protect whiteleg shrimp from PirAB. Data shown are the means and standard deviations from three or four independent experiments for untreated (green, triangle), PirAB treated (black, inverted triangle), PirAB + NBX-PA1 treated (red, square), and PirAB + NBX-PB1 treated (blue, circle) whiteleg shrimp. At 16 hours and all following timepoints (*) the difference in percent mortality between PirAB treated and either PirAB +

277 NBX-PA1 treated or PirAB + NBX-PB2 treated is statistically different ($p < 0.01$) by unpaired
278 student's t-test.
279

Fig. 1


A
B
NBX-PA1
NBX-PB1
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2
3
4
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0.1
1
10
100
1000
$[Nanobody]_{nM}$

Fig. 2

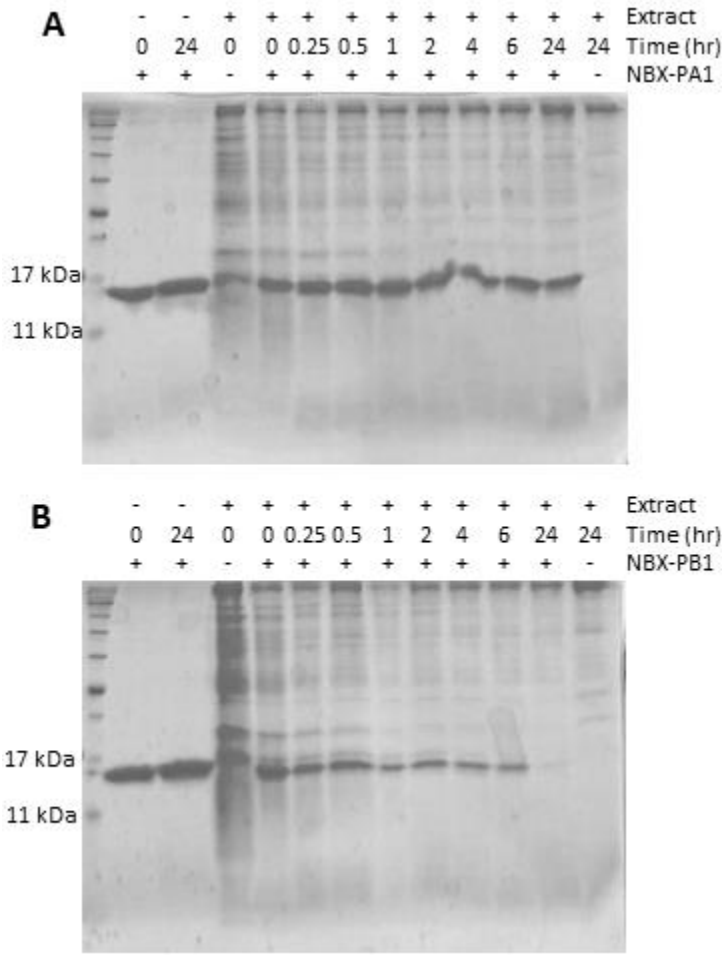

A
Extract
Time (hr)
NBX-PA1
0 24 0 0 0.25 0.5 1 2 4 6 24 24
17 kDa
11 kDa
B
Extract
Time (hr)
NBX-PB1
0 24 0 0 0.25 0.5 1 2 4 6 24 24
17 kDa
11 kDa

Fig. 3

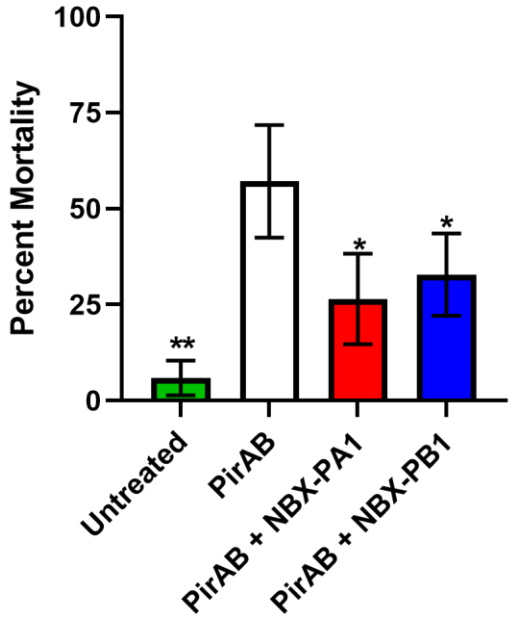
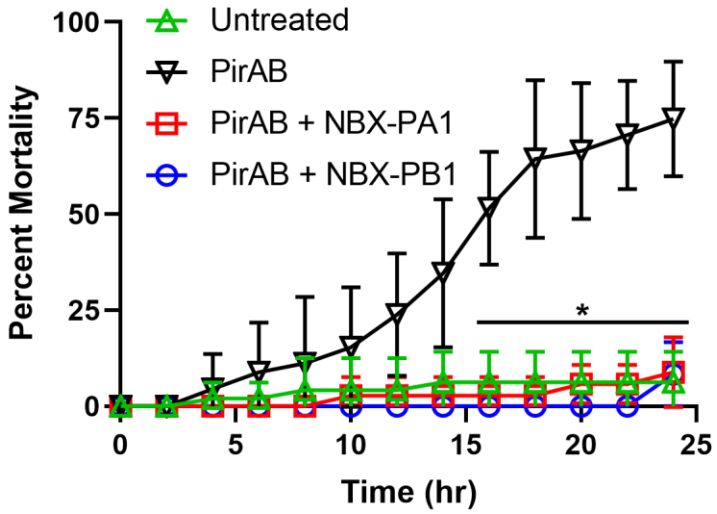

100
75
50
25
0
Percent Mortality
**
*
*
Untreated
PirAB
PirAB + NBX-PA1
PirAB + NBX-PB1

Fig. 4


Untreated
PirAB
PirAB + NBX-PA1
PirAB + NBX-PB1
100
75
50
25
0
Percent Mortality
*
0
5
10
15
20
25
Time (hr)