Testing a novel peptide drug towards a goal of reducing mortality in critically ill COVID19 patients

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Acknowledgements: We thank Rathi Srinivas and Madhavi Gavini from Novopyxis for providing the NP6A4 peptide and for technical guidance throughout the project

Background

COVID 19 has been devastating the globe over the past year, and although our knowledge of how the disease progresses is constantly evolving, there is a significant amount we now about the mechanisms underlying COVID19[1,2]. There is growing evidence that a 'cytokine storm' is a significant driver of mortality associated with COVID19, with studies showing high levels of hallmark inflammatory indicators in critically ill COVID19 patients[3-10]. This essentially consists of the patient's immune system going awry and causing white blood cells to constantly release a large number of small molecules called cytokines. The result of this is further activation of the immune system, which ends up attacking not only infected but also healthy patient tissues, resulting in organ failure.

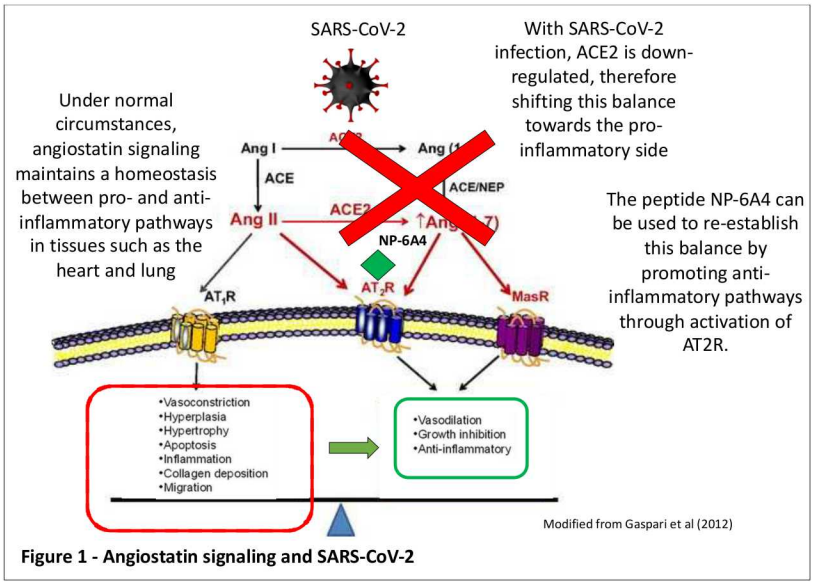


Figure 1 - Angiostatin signaling and SARS-CoV-2

Here, we tested a novel peptide drug, developed by our industry collaborators at Novopyxis, to mitigate the cytokine storm observed in critically ill COVID19 patients. The peptide, NP6A4, is an agonist of the angiotensin II type 2 receptor (AT2R) which is an anti-inflammatory, anti-fibrotic, and cardioprotective member of the renin-angiotensin (RAS) system[11]. This receptor is downregulated in heart disease and diabetes and has been a drug target for over 30 years[11-14]. NP6A4 is the first drug that can both activate it and upregulate it. NP6A4 has an FDA drug designation for pediatric cardiomyopathy, and recent work has shown that NP6A4 mitigates aortic stiffness and proteolytic activity in mouse model of aneurysm[15]. ACE2 cleaves Angiotensin II into Ang 1-7, a small anti-inflammatory peptide that activates the AT2R. When ACE2 is shed (as in SARS-CoV-2 infection), Angiotensin II is no longer converted to Ang 1-7, reducing the activity of AT2R. Since NP-6A4 increases both expression and signaling of AT2R, we hypothesize that NP-6A4-induced increase in anti-inflammatory signaling can fill in the gap in the anti-inflammatory defense system of heart caused the by loss of ACE2 due to SARS-CoV-2 binding and likely promote cell survival in lung tissue (and cardiomyocytes) (Figure 1). Indeed, a number of studies looking into using

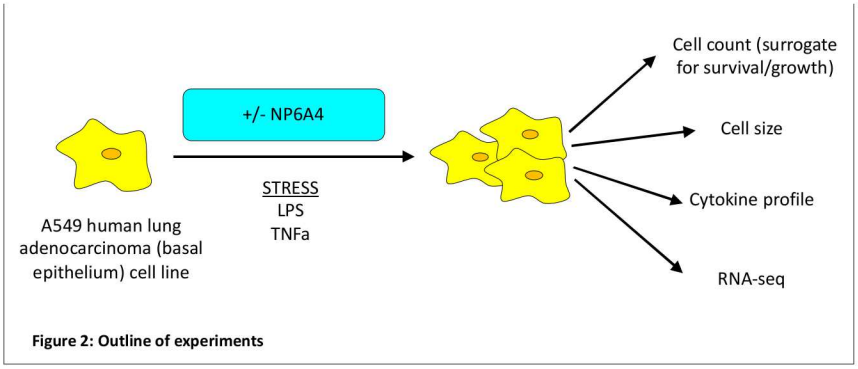


Figure 2: Outline of experiments

molecules that engage or inhibit RAS for treating COVID19 are currently being explored[16-18]. While there are multi-facted ways to approach the effects of RAS on COVID19, for this preliminary project, we sought to ask whether NP6A4 could serve as a treatment for lung epithelium cells as they are afflicted by the stress associated with a cytokine storm (which causes them to start secreting a number of 'alarm-bell' cytokines, and eventually become impaired and die as a result, in part, of overactive stress signaling)[19,20].

Experimental setup

All the experiments in this project had a similar setup, and we outline this setup in Figure 2. Our preliminary studies focused on the basal epithelium adenocarcinoma cell line A549 as a surrogate for lung epithelium. We also started studies in AC16 cardiomyocytes which we hope to peruse upon receipt of additional funding, but all the preliminary data we present here will be in A549. Briefly, we treated A549 with a stressor (LPS or TNFa as a surrogate for the cytokine storm-caused stress which occurs in COVID19) in the presence or absence of the peptide NP6A4. We then had four different read-outs for the result, as outlined in Figure 2:

(i) We counted cells as a surrogate for survival and growth using both a table-top Biorad cell counter as well as the Thermo Fisher CX7 high-content imager (for the latter, we stained cells with DAPI and used an automated protocol for counting).

(ii) We examined cell size using the DeepRed CellMask stain and an automated protocol on the CX7.

(iii) We performed a 48-plex cytokine profile on the cells using the company EveTechnologies

(iv) We performed RNA-sequencing (RNA-seq) to determine what pathways were responsible for observed phenotypes

Results

(i) LPS/TNFa stress reduces A549 cell treatment, and NP64A rescues this effect

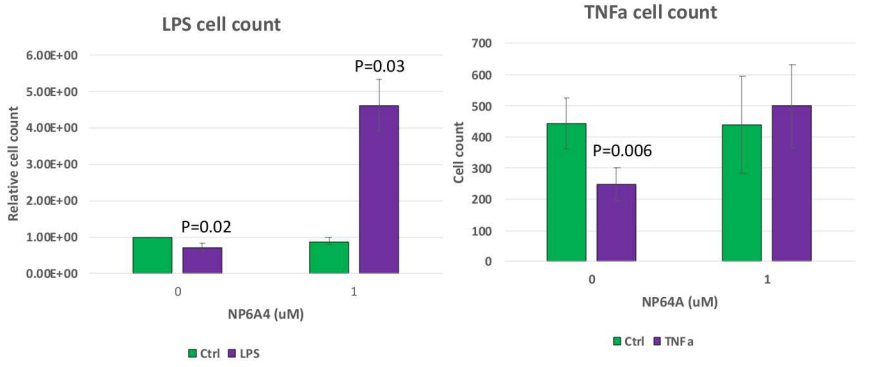


Figure 3: NP6A4 rescues stress-induced reduction in cell growth. Left – cells counted using the BioRad T20 table-top cell counter after LPS treatment for 24 hours, with our without 1uM of NP64A. Right – similar to the left graph, but TNFa treatment instead of LPS, and cells were counted using the CX7 microscope after being stained with DAPI. P-values are the result of a one-way ANOVA.

We began by using LPS on the A549 cells, and either co-incubating with NP6A4 or not, and counted the cells to determine survival/growth. For LPS, we plated 5,000 cells in a 24-well plate, and counted 24 hours later. LPS results are presented as relative cell counts, and TNFa results are presented as number of cells per image. We found that LPS and TNFa significantly reduced the cell number, and that this effect was reversed by the addition of NP64A in both cases. With the T20 cell counter experiment, we found that the peptide increased cell growth significantly, but this was not reproducible using the CX7, leading us to believe that perhaps the peptide in the presence of LPS altered the morphology of the cells, thus affecting the ability of the cell counter to identify individual cells. For this reason, we proceeded with additional experiments using only the CX7 imager. Also, since the effect using TNFa was greater than with LPS, we continued with just TNFa treatment as the stressor for subsequent experiments.

(ii) TNFa causes hypertrophy in lung epithelium, which is reversed by NP6A4

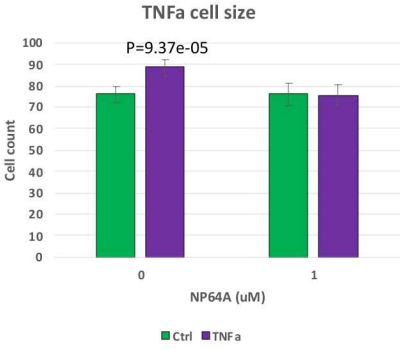


Figure 4: NP6A4 rescues stress-induced hypertrophy. Cell size measured using the CX7 imager after TNFa treatment for 24 hours, with our without 1uM of NP64A, tained with DeepRed Cell Mask. P-value is the result of a one-way ANOVA.

We wanted to follow up on the hypothesis that both the stressor and the peptide might be affecting the morphology of the lung epithelium. To test this we used the DeepRed Cell Mask from Thermo Fisher which stains cell membranes to examine cell size in the same conditions as we described in (i), only with just TNFa (not LPS). We found that TNFa induced hypertrophy (cell enlargement) in A549, and that this effect was again reversed by A549 (Figure 4). Taken together with the cell number data, this suggested that the physiological effects of cytokine stress in lung epithelial cells could be at least partially reversed by NP64A. We therefore wanted to determine the potential molecular mechanisms and consequences underlying these observations.

(iii) TNFa-stressed cells release a variety of cytokines, and a subset of these changes can be mitigated by NP64A treatment

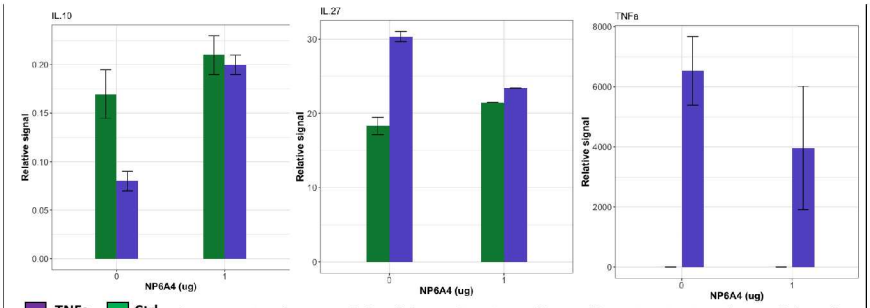


Figure 5: NP6A4 alters TNFa-induced changes in A594 cytokine profile. IL10, IL13, IL27 and TNFa all showed trends of NP6A4 reversing stress-induced changes.

We collected media from A549 that were exposed to TNFa, either with or without NP64A, and Eve Technologies (Canada) performed a 48-plex cytokine profile assay. We saw a number of cytokines being induced with TNFa treatment as expected (not shown). Specifically, however, a few cytokines stood out as being differentially affected by NP6A4, with the peptide reversing the effects of TNFa. While two replicates was not enough to obtain statistical significance, we present the preliminary data here, with the intent of following up with additional replicates (Figure 5). With the two pro-inflammatory cytokines (IL27 and TNFa), NP6A4 reverses the increase caused by TNFa, while with the anti-inflammatory cytokine IL-10, NP6A4 prevents the down-regulation by TNFa. This suggests that NP64A can alter the cytokine profile, which in turn could help tame the extent of a COVID19-induced cytokine storm[21]. With respect to TNFa, the high level of signal is likely due to the fact that we added exogenous TNFa as the stressor – however we still see that the presence of NP6A4 reduces the total levels of TNFa compared with cells treated just with TNFa, despite both samples getting the same amount of exogenous TNFa, suggesting an effect of NP6A4 on endogenous TNFa secretion.

(iv) TNFa-induced pathways in A549 are altered in the presence of NP6A4

Finally, wanted to determine the molecular effects that might be causing these differences. To this end, we performed RNA-seq on these samples[22,23]. When we compare the samples by principal component analysis (PCA), we see that the peptide alone does not have a significant effect on the global state of the population compared with the control (Figure 6). TNFa, on the other hand, significantly shifts the state of the cells as expected. When the cells are co-treated with TNFa and NP6A4, even though they do not fully

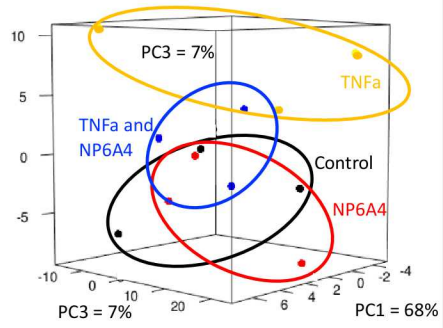
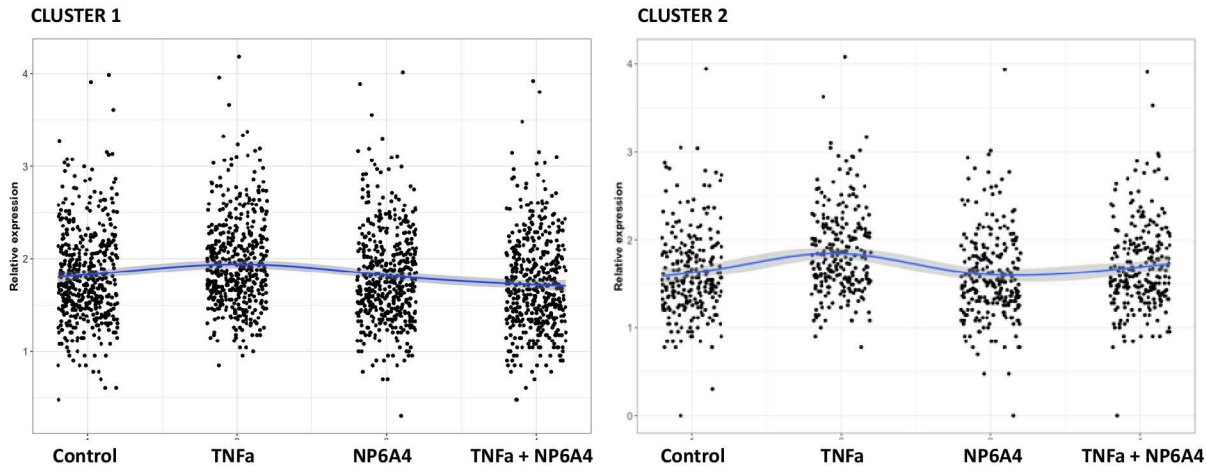


Figure 6: Principal component analysis (PCA) of RNA-seq data. The first 3 dimension were plotted. Numbers in brackets represent the contribution of each principal component.

return to their ground state, the population of cells are notably shifted compared with just TNFa, suggesting that while the peptide may not affect the cells in their ground state, it has a significant effect on the molecular profile of the cells (Figure 6). To further probe this effect, we used a program called DP_GP Cluster, which clusters genes based on their behavior across a range of samples using a Dirilecht process Gaussian process model[24]. We identified a number of clusters (not shown), and focused specifically on two of them where NP6A4 appeared to reverse the effects of TNFa (Figure 7). We looked more closely at what pathways the genes in these clusters came from using gene ontology analysis[25], to get an idea of which processes were likely causing the protective effects of NP64A. We found that processes such as apoptosis, cytoskeletal organization and protein transport were prominently featured in cluster 1, with cell surface proteins, adhesion and signaling peptides found in cluster 2. These results provide us with intriguing candidate genes and pathways to further explore in order to understand how NP6A4 is regulating cellular outcomes.



Category	Enrichment score
Membrane-enclsoed organelle/nuclear lumen	5.12
Apoptosis	4.36
Ubiquitylation	3.45
Cytoskeleton	2.64
Protein dimerization	2.5
Cytoskeleton organization (actin)	2.32
Protein transport	2.27
Kinase/phosphorylation	2.11

Category	Enrichment score
Signal peptides/disulfide bonds	2.26
Cell adhesion	1.96
Cell surface	1.82
Cell motility	1.79
Lysosome/degredation	1.68
Blood vessel angiogenesis	1.62
Extracellular matrix	1.55
Integrins/ECM	1.54

Figure 7: Clusters of TNFa-induced genes are affected by NP6A4. TOP– gene expression patterns in two clusters that show some reversal of TNFa-induced gene expression (Cluster 1 shows a slightly greater TNFa reversal in the presence of NP6A4 than cluster 2). Bottom – Gene Ontology enrichment categories denoting relevant pathways targeted by NP6A4.

Conclusions and future directions

We have shown that NP64A can reverse the effects of TNFa on lung epithelial cells both at the cellular phenotypic level as well as the molecular level. These results suggest that NP6A4 is a promising therapy for diseases such as COVID19 that are a systemic multi-organ problem, affecting the delicate balance of the immune system to fight infection by not become overactive (as is the case in a cytokine storm). In addition, immunomodulatory drugs such as NP6A4 can have broad applications across many infectious diseases, as well as other conditions relevant to public health such as autoimmune disease and cancer, which makes this therapeutic of broad interest to the scientific and clinical community. We will explore

this therapeutic further by publishing our results in a peer-reviewed journal and applying for follow-on funding.

This work was funded by the Sandia Bioscience LDRD program (FY20).

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