Soluble Epoxide Hydrolase Inhibitors as an Anti-Inflammatory Treatment for SARS-CoV-2 Infection and Coronavirus Disease 2019 (COVID-19)

Principal Investigators:
Harrison W. Kuhn* and Riggs Sanchez*

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**Augustana University -- 2001 S. Summit Ave. Sioux Falls, SD 57197 USA*

SPECIFIC AIMS

This proposal's *objective* is to determine the effectiveness of synthetic and natural product selective epoxide hydrolase inhibitors for reduction of inflammatory signaling agonists in SARS-CoV-2 (SC-2) infected cell lines.

As of May 2021, COVID-19 is an ongoing worldwide pandemic caused by the SARS-CoV-2 coronavirus variant, believed to have originated in Wuhan, China. An inflammatory response characterized by cytokine and eicosanoid overproduction accompanies infection and results in severe symptoms leading to pneumonia and death in immunocompromised patients. Soluble epoxide hydrolase inhibitors (sEHIs) have shown to be effective in treating cases of neurologic inflammation and are proposed to reduce the hyperinflammatory response of COVID-19 infection. The sEH inhibitor EC5026 developed by EicOsis Human Health, Inc., has been granted fast track designation for treatment of inflammatory neuropathic pain. Two other sEHIs, AR-9281 and AUDA have also been shown to have therapeutic effects *in vitro* on inflammatory response. The current leading treatment for severe SC-2 infection is the glucocorticoid dexamethasone which will be used to compare with sEH inhibitors. Completion of this study will elucidate the potential for use of sEHIs as anti-inflammatory drugs in clinical treatment of severe inflammation response requiring hospitalization.

The *central hypothesis* is that sEHIs reduce the severity of infection via inhibition of downstream cytokine and eicosanoid storms and inflammatory enzyme production. The rationale is that a designed and vetted inhibitor will elicit increased pharmacological response in comparison to known broad sEHIs and generic anti-inflammatories. This work with coronavirus disease can also translate to inflammatory responses from other airborne pathogens.

Specific Aim: Determine the effectiveness of synthetic selective epoxide hydrolase inhibitors on inflammatory response associated with SARS-CoV-2 infection.

For this, we will culture commercial Calu-3 cell lines *in vitro* with SARS-CoV-2 infection and treat them with EC5026, AR-9281, and AUDA sEHIs. The current SARS-CoV-2 drug treatment dexamethasone will be used for comparison to current treatment. The resultant inflammatory response reduction will be quantified utilizing commercial cytokine assays and EET/DHET assays. Viral load will be evaluated with RT-qPCR. *Working hypothesis:* The commercial sEHIs will show a significant reduction in inflammatory signaling molecules in comparison to current treatments for viral infection by SARS-CoV-2. The *expected outcomes* of this work will be a better understanding of the anti-inflammatory capabilities of a selective epoxide hydrolase inhibitor on virally infected cells. Analysis should show a decrease in proinflammatory cytokines, eicosanoid products, and viral mRNA which will indicate successful treatment. The results of this study will *positively impact* the options available to mediate severe COVID-19 symptoms as the use of selective soluble epoxide hydrolase inhibitors can potentially be implemented in the clinical treatment of symptoms associated with SARS-CoV-2 infection.

RESEARCH STRATEGY

Significance:

COVID-19 is an ongoing pandemic as of May 2021. To date, over 140 million cases have been confirmed worldwide, where over 3 million patients have lost their lives to this illness.[1] National responses varied around the world, with some nations banning recreational travel, mandating the use of face coverings in public spaces, and implementing social distancing procedures. In previous coronavirus outbreaks, SARS-CoV emerged from Asia in early 2003. Around 8,000 patients were infected worldwide, where 774 died.[2] Mutated variants of SC-2 continue to emerge throughout the world and threaten additional national lockdowns and stresses on healthcare providers and systems. New therapies can reduce the number of hospitalizations as well as reduce severity of lung-tissue scarring, cardiovascular strain, and other side effects, currently known to affect patients long after infection and treatment. SC-2 infection has become clinically problematic due to high fever and pneumonia leading to acute respiratory distress syndrome (ARDS), which has proven fatal. From clinical data, it has been determined that elevated levels of serum interleukin-6 (IL-6), IL-8, tumor necrosis factor (TNF)-α, IL-1β, and other cytokines are characteristic of ARDS, respiratory failure, and other adverse clinical outcomes.[3] The likely mechanism of clinical pathologies is alluded to as infiltration of immune cells in the lung which overproduce pro-inflammatory cytokines resulting in lung damage and multi-organ dysfunction. The inflammatory response from SC-2 infection has been characterized as a cytokine storm due to the rapid, hyper-elevation of inflammatory markers.[4] The underlying mechanisms of the cytokine storm provides a viable target for anti-inflammatory agents, as shown in Figure 1, from Hammock et al.[5] Inflammation is the physiological response to injury, infection, and/or toxins that damage cells in the body. Damaged cells release cytokines, which are molecules that stimulate the immune system to fight off foreign compounds and organisms and to clean up damaged tissue. An important precursor in the inflammatory pathway is arachidonic acid (AA) that is metabolized by three different pathways: cyclooxygenase (COX) pathway that yields prostaglandins, lipoxygenase (LOX) that produces the leukotrienes, and cytochrome P450s (CYP)-dependent AA derivatives notably gives rise to epoxye-icosatrienoic acids (EET) and other epoxy fatty acids (EpFA).[6] The EETs and EpFAs are important autocrine and paracrine signaling molecules for inflammatory response in tissues. One pitfall of the EET inflammatory signaling is that EETs are converted into 1,2-dihydroxy-fatty acids (DHETs) in the presence of sEH which results in a loss of inflammatory control and some proinflammatory effects. First discovered in the context of insect developmental biology for epoxidation of metamorphosis hormones, the sEH enzyme was identified in mammalian tissues in 1972.[7] Years of further investigation determined the central role of sEH is a conversion of precursor inflammatory molecules into active signaling forms.[7] Small molecule inhibitors of sEH have been developed to increase EET and EpFA levels with the goal of regulating the inflammatory response.[8] Because inflammation is a common symptom associated with many diseases, sEHIs have been explored in treatment of hypertension, cardiac hypertrophy, arteriosclerosis, brain and heart ischemia/reperfusion injury, cancer and pain.[6,8] In the proposed study, sEHIs will evaluated as a treatment option for the reduction of severe symptoms of SC2 infection by transfecting Calu-3 cells with SC2 *in vitro* and treating the cultures with sEHIs AR9281, AUDA, and EC5026 and current inflammatory treatment, dexamethasone. (Fig. 2) The response to treatment will be quantified with an inflammatory markers assay, EET/DHET assays, and RT-qPCR for evaluation of relative viral load. An overall reduction in inflammatory markers, DHET's and viral load will indicate a reduction in inflammation and a more positive clinical outcome.

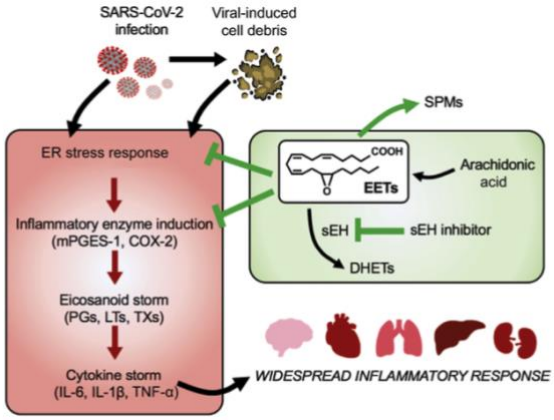

Figure 1: Inflammatory pathway involving role of sEH inhibitors on signaling cascade and cytokine storm.[5]

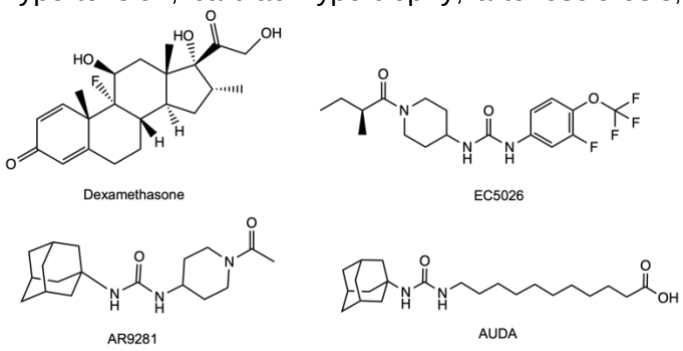

Figure 2: Anti-inflammatory and sEHIs used to evaluate inflammatory response mediation in COVID-19 infected cells.

Innovation:

sEHIs have been previously investigated for use in a myriad of maladies but have not been utilized for treatment of symptoms associated with highly transmissible, fast-mutating, coronavirus diseases. Currently in the clinic, monoclonal antibody treatments, antiviral Remdesivir and corticosteroid anti-inflammatory, dexamethasone, are readily utilized; however, antibody treatments require healthcare supervision and a wealth of local donors/supply. Given limited treatment options available, adding additional vectors to mitigate symptoms is beneficial to clinicians and patients affected by diseases conferring a severe inflammatory response. In light of the current COVID-19 pandemic, focus on anti-inflammatory small molecules has increased due to the cytokine storm associated with SC-2 infection. In other research, sEHIs have been successfully used to treat neuropathic pain by stabilization of EpFA levels in cells.[7] EpFAs naturally reduce pain, resolve inflammation, and maintain normal blood pressure.[7] The sEHI, EC5026, developed by EicOsis, LLC of Davis, CA has shown significant pain reduction and is orally available for administration.[7] EC5026 has entered into Phase 1 clinical trials for neuropathic pain treatment. Given the successes of EC5026's ability to treat pain caused by inflammation, it has been selected as a potential treatment for virally-induced inflammation associated with SARS-CoV-2 infection. The theory behind mitigating the inflammatory response is elevation of EpFAs to promote natural anti-inflammatory action.

APPROACH

A research study will be undertaken to explore therapeutic effects of sEHIs on SC-2 infections. The anti-inflammatory properties of sEHIs provide a potential mechanism for reduction of infection-related cytokine storms that result in disease pathologies. The use of biochemical methods of analysis will provide a quantifiable measurement of changes to the inflammatory response. Using the methods of analysis, a comprehensive comparison of synthetic sEHIs to current anti-inflammatory treatment, dexamethasone, will be performed to determine effectiveness of sEHIs for treatment of SC-2 infections.

Aim 1: Determine the effectiveness of synthetic selective epoxide hydrolase inhibitors on inflammatory response associated with SARS-CoV-2 infection.

Strategy: Investigation of sEH Inhibitors

The *objective* of this aim is to determine the anti-inflammatory ability of sEHIs on the inflammatory response that results during SC-2 infection and the changes in DHETs (the product of sEH catalysis). The *working hypothesis* for this aim is that sEHIs will reduce inflammatory response in cells responding to viral infection by reduced formation of DHETs. Our *approach* is to culture and infect Calu-3 human cells with SC-2, determine the effectiveness of sEHIs on reducing cytokine release during SC-2 infection and compare the effectiveness to a known SC-2 treatment. The sEHIs EC5026, AR-9281, and AUDA will be used as experimental compounds and dexamethasone as a comparison. The cytokine response will be measured using a commercially available inflammatory assay for quantitative comparison. An assay measuring DHET levels present in cells will also be measured to determine effectiveness of sEH inhibition. The *rationale* for this aim is to expand the understanding of sEHIs and their ability to decrease inflammation in the COVID-19 disease state.

Methodology: Culture, Infection, and Drug Therapy of Calu-3 Cell Lines

A viral sample available from the American Type Culture Collection (ATCC) will be used for creation of a viral stock. Obtained on March 16, 2020, the NR-53514 sample was collected using a nasopharyngeal swab from a 63-year-old male with a fatal outcome. The *Cercopithecus aethiops* liver cell line, Vero E6, from ATCC will be cultured per provider guidelines.[9,10] Vero E6 cells will be infected with the viral sample to create a working viral stock for the experimental cell lines. Use of SC-2 active virus will involve biosafety level 3 precautions to ensure safety of personnel.[11] The supernatant containing SC-2 will be harvested to transfect Calu-3 cells, a lung epithelial cell model, which will serve as the experimental cell line.[12,13] Cultures will be performed per provider guidelines prior to addition of 20 µl of the Vero E6 culture supernatant in 96-well tissue culture plates with 178 µL of culture media. A control Calu-3 cell line will be treated with Vero E6 media lacking viral infection. Infected cell cultures will be incubated for 36 hours prior to treatment.[12] For the treatment with EC5026, 10 mM EC5026 in 1 mL DMSO will be obtained from MedChem Express. A serial dilution in DMSO to concentrations of 1000 µM, 300 µM, and 100 µM (100x) for administration are each added at volumes of 2 µL into the 200 µL wells to give dosing concentrations in media of 1 µM, 3 µM, and 10 µM (1x), respectively.[7] Stock solutions of AR-9281, AUDU, and dexamethasone at 10 mM concentrations in 1 mL DMSO will be obtained from MedChem Express and undergo the same dilution methods as EC5026. Cells will be dosed once daily for three days for the given compound and concentration.[7,14] Following dosing regimens for the three day period, cells will be exposed to

ultraviolet (UV) light for inactivation of SC-2 virus prior to analysis.[12] Cells will be transferred to 1.5 mL Eppendorf tubes and centrifuged. The supernatant will be separated from the pellet and transferred to another tube. The pellet will be resuspended, exposed to the cell lysis buffer, and homogenized for DHET analysis. The supernatant will be collected and undergo analysis of inflammatory cytokine levels and viral mRNA will be quantified.

Analysis:

To quantitatively evaluate the amount of extracellular cytokine storm inflammatory markers, the RayBioTech (Peachtree Corners, GA) Quantibody Human Inflammation Array 1 kit will be utilized. The array kit allows for direct analysis of cell culture supernatants, mitigating extensive purification steps. A standard concentration curve dilution strategy is provided with the kit. The array evaluates levels of IL-1 α, IL-1beta, IL-10, IL-13, IL-4, IL-6, IL-8, MCP-1, and TNF-α using a traditional sandwich-based EILSA and microarray laser scanner. A usage procedure supplied with the kit will be utilized. All treatments of cells will be evaluated in duplicate. Each plate contains 16 wells, 8 for the standard, and 8 available for samples. For each evaluation, 2 plates will be used for each set of 15 samples. A standard regression line will be generated from relative absorbances and known standard concentration to determine the concentration of inflammatory markers. From control cells (SC-2 infected, only DMSO), levels of disease state inflammatory markers will be measured and compared to levels of treated cells to evaluate effects. An overall reduction in inflammatory markers will imply that sEHIs serve to reduce the inflammatory response triggered by infection. An overall increase in inflammatory markers will imply that sEHIs have no effect on inflammatory marker reduction and thus do not reduce the inflammatory response triggered by infection.

As further indication of the performance of sEHIs, cytoplasmic levels of DHET 11,12 and DHET 14,15 will be evaluated with the Eagle Biosciences (Amherst, NH) EET/DHET ELISA kit for 11,12 and 14,15. During analysis, a sample of cells after the treatment course will be lysed, homogenized, and then centrifuged to pellet cellular debris. Triphenylphosphine (TPP) will be added to the sample to achieve a concentration of 0.1 mM, per kit instructions. The ELISA will be read on a 96-well microplate reader at 450 nm. From provided internal standards, a concentration curve will be generated in statistical analysis software and plotted. Experimental absorbance readings will be used to determine the relative concentration of DHET and EET using the regression equation. EET will be determined from the subtraction of sample concentration in non-hydrolyzed wells from samples in wells that underwent hydrolysis in the procedure. DHET determination will be from samples that underwent the hydrolysis step. Should the ratio of DHET to EET be lower than 1 and smaller than the viral-positive control, it is inferred that the inflammatory response is reduced with sEHI treatment. If the ratio is greater than 1 and the value larger than the positive-viral control, the inflammatory response is increased upon sEHI treatment. Should the ratio be relatively equivalent, as determined by Grubb's test for outliers, between treated samples and positive-viral control, it is inferred that the sEHIs make no difference in changing the inflammatory response. Results will be reported in graphical and tabular format utilizing a 95% confidence interval for statistical significance.

Prior research has shown that SC-2 progression results in increased production of viral mRNAs and viral proliferation. In order to determine whether treatment compounds have an effect on disease progression, a comparative analysis of the level of viral mRNA following the three-day treatment period will be performed. Viral progression of SC-2 will be measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR), a technique that uses fluorescence to quantify mRNA levels;[12,10] Using the Gold Biotechnology, Inc. One Step RT-qPCR kit for SARSCoV-2 (COVID-19) Detection kit, the viral load present in each sample will be quantified. RT-qPCR will be performed per manufacturer protocol for the samples. The fluorescent tag 6-carboxyfluorescein (FAM) included in the kit will serve to quantify the relative level of mRNA per sample. FAM has an absorption wavelength of 495 nm and an emission wavelength of 517 nm. Absorption spectra will be obtained from a plate reader and a calibration curve will be generated using the ΔC_q calibration method.[15] Comparison of data between experimental samples and the virally-infected control will provide insight into the potential changes to infection progression in the presence of sEHIs. The relative viral mRNA is expected to be highest in the placebo group as compared to treatment samples. A reduction of viral load in sEH inhibitor-treated cells as compared to the dexamethasone-treated cells would indicate a potential improvement in the disease treatment, but *in vivo* comparison would be necessary.

Expected Outcomes:

The overall outcomes of this aim will be expanded knowledge of the use of sEHIs on acute inflammatory responses in human cells. This aim will show the effective ability of sEHIs to regulate inflammation in a disease pathology *in vitro* for the current COVID-19 pandemic. The known use of sEHIs in reduction of inflammation for other disease states provides justification for the potential benefits in SC-2 infection. Successful treatment will

be described by reduction in inflammatory and elevation in anti-inflammatory cytokines, reduction in DHET synthesis, and reduction of apoptotic caspases. Although this study will not find a cure, reduction of symptoms that would lower the number of patients requiring hospitalization would be considered a success. Further studies will investigate *in vivo* effects and reduction of symptoms caused by cytokine storms.

Potential Pitfalls, Alternative Strategies, and Safety Concerns:

A potential problem with this research is that EC5026 has completed Phase 1 of clinical trials and is pending published results, which may result in delay of experimentation in humans. Because this is an *in vitro* study, animal models will need to be utilized and must demonstrate effective treatment capacity and low toxicity/severe side effects prior to application for clinical use.

In the event of failure to produce viral stock in the Vero E6 cell line, other known cell lines including hepatic Vero CCL-81 and HuH7 cells will be employed.[9,13] For experimental cultures, the cell lines intestinal Caco2, hepatic Huh7, and neuronal U251 can also be used for SC-2 treatments as described in Cagno.[13] Dosing of cells may need to be altered to observe therapeutic effects. The duration of treatment could potentially be too short to show significant reduction in inflammatory markers and viral mRNA. Analytical methods such as Liquid Chromatography Mass Spectrometry (LC-MS) may need to be utilized in order to more accurately identify levels of various eicosanoids (DHETs, EpFAs, and EETs). For viral load analysis, measurements may be performed throughout the dosing regimen to observe duration of treatment required for viral load reduction.

The use of active respiratory virus poses an added risk to experiments. Per CDC regulations, working with SC-2 infected cell cultures requires use of biosafety level 3 (BSL-3) facilities and personal protective equipment. Monitoring of the current guidelines on use of SC-2 in the laboratory will be performed to maintain compliance on the current protocols for handling. Restrictive use of cell cultures and viral samples will need to be established per CDC guidelines. The facilities used in this research will need verification of biosafety levels and researchers will be required to comply with all protocols. While working with SC-2 cells, the use of personal protective equipment, respiratory protection, decontamination, storage, and containment of disease will be strictly followed. The use of UV inactivation will be used to limit the duration of biosafety concerns during the analysis portion of the experiments. For experiments not involving live SC-2 virus, BSL-2 precautions should be taken to ensure safety.

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