The Relationship Between Neurosteroid Structures and Inhibition of Pyroptotic Lysis

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Abstract

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Pyroptosis is a form of programmed cell death with inflammatory biological consequences. In moderation, pyroptosis helps to regulate and maintain a healthy immune response in the presence of invading pathogens. However, excessive, or inappropriate pyroptosis causes harmful consequences including inflammation and damage to tissues and organs. There are several steps within the pyroptotic cascade that result in lysis and release of cellular components implicated in harm to healthy cells and tissues. Previously identified small molecule inhibitors, like the amino acid glycine, have been shown to inhibit pyroptosis-mediated cell lysis, *in vitro*. Glycine is an ion channel modulator and examination of other ion channel modulators led to the discovery that pregnenolone sulfate also inhibits cell lysis in cells undergoing pyroptosis. Pregnenolone sulfate is a more potent inhibitor than glycine, but the mechanism of action for the protection is unknown. To begin to fill the gap in knowledge of how pregnenolone sulfate, and other small molecules, may be interacting with pyroptotic cells to prevent membrane lysis, we tested a rationally selected library of neurosteroids with similar structures to pregnenolone sulfate for inhibitive

activity. We found some neurosteroids retain the same level of activity in comparison to pregnenolone sulfate, but others exhibit a partial or total loss in activity. No single aspect of the structure was responsible for inhibitive activity; different parts of the molecule contribute to inhibition of pyroptosis. Together, the results of this thesis lay the foundation for a deeper knowledge of what mechanisms may provide protection and how we can design future inhibitors with these mechanisms in mind.

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Acknowledgments

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Chapter 1. Introduction

1.1 Recognition of Invading Pathogens by the Innate Immune System

Our immune system can maintain our body's health through a complicated network of pathways that relies on a diverse group of receptors to recognize different types of signals. The innate immune system is responsible for an expansive and rapid response to invading pathogens. This is our body's first line of attack against a diverse set of pathogens. Macrophages, neutrophils, and dendritic cells are all detector cells that patrol the body and utilize their pathogen recognition receptors (PRRs) to interact with pathogen associated molecular patterns (PAMPs). These PAMPs are conserved structural motifs of pathogens that are recognized by PRRs to then initialize innate immune responses (Zindel & Kubes, 2020). The PRRs consist of different types of sensor proteins like Toll-like receptors (TLRs) and Nod-like Receptors (NLRs). The various receptors can be located both throughout the cell, as well as along the membrane (Akira, Uematsu, & Takeuchi, 2006). There is a highly diverse group of PRRs within our bodies and they each differ in what signals they recognize, where they are located, the signaling cascades triggered, and the consequential host responses. There are some sensing receptors that can recognize "self" molecules and danger signals released from cells undergoing environmental stresses, necrosis, or even aging (Gong, Liu, Jiang, & Zhou, 2020). Host cells can monitor for both internal and external threats through the different locations of the PRRs.

1.2 Mechanism of Pyroptosis, Inflammatory Cell Death

Pyroptosis is an inflammatory form of cell death that is vital to a healthy immune system (Li, Dickinson, Coers, & Miao, 2023). Pyroptosis is initiated by pathogen recognition receptors detecting PAMPs to trigger formation of signaling platforms called inflammasomes (Figure A). The inflammasome is a multiprotein complex where ASC is an adaptor protein forming a large speck within the cell. The inflammasome complex activates

caspase-1, leading to the cleavage and activation of the pro-inflammatory cytokines IL-1ß and IL-18. Caspase-1 activation also results in the cleavage of the gasdermin D protein. The gasdermin D protein consists of two domains: the N-terminal pore-forming domain and the auto-inhibitory C-terminal domain. When the gasdermin D protein is in its full-length form, the C-terminal domain folds back over the N-terminal domain, blocking the surface responsible for lipid binding (Liu et al., 2019). Once the N-terminal domain of gasdermin D is freed, the protein translocates to the cellular membrane and oligomerize to form the gasdermin D pore (Huston, Anderson, & Fink, 2023). The gasdermin D pore is approximately 20 nm in diameter but can vary depending on the number of subunits that oligomerize into a single pore (Wang & Ruan, 2022; Zahid, Ismail, & Jin, 2021). The pore allows the passage of small cellular components, including the activated pro-inflammatory cytokines.

After pore formation, the plasma membrane protein ninjurin-1 also undergoes oligomerization. Ninjurin-1 oligomerization was recently discovered to play a role in pyroptotic membrane lysis because in ninjurin-1 knock out mice, there was no observed cell lysis leading to inhibition of release of large cellular components such as high mobility group box 1 (HMGB1) (Kayagaki et al., 2021). The final consequential event of pyroptosis is lysis. Membrane lysis then allows the release of cellular contents too large to pass through the gasdermin pore.

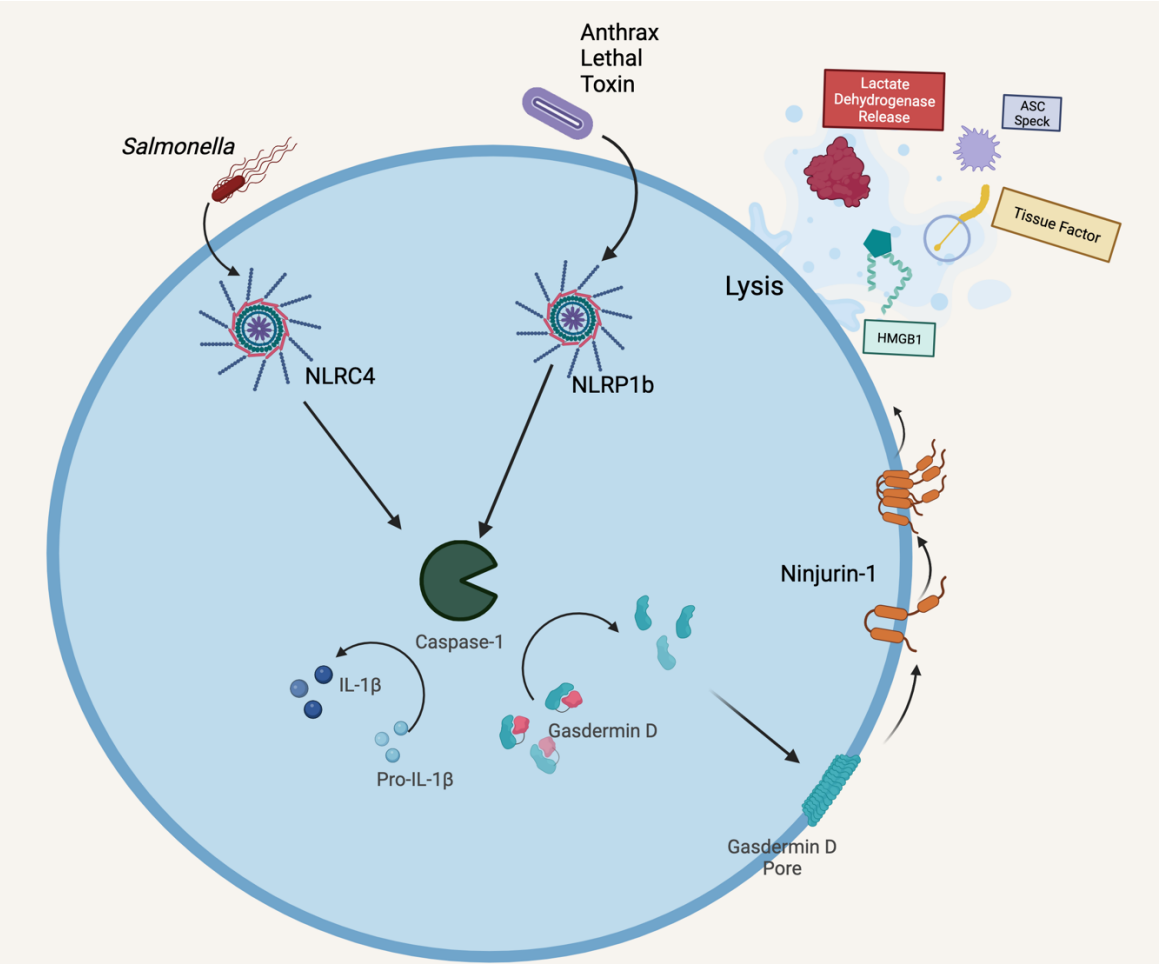


Figure A. Mechanism of Pyroptosis. Pyroptosis is triggered by many types of stimuli. Invading pathogens are recognized by nod-like receptors which consequently activate caspase-1. Caspase-1 cleaves pro-inflammatory cytokines and the pore-forming protein gasdermin D. The N-terminal domain of gasdermin D will translocate to the lipid bilayer and oligomerize to form the gasdermin D pore. After pore formation, the novel protein ninjurin-1 again oligomerizes and acts as a catalyst for cell lysis.

1.3 Biological Consequences of Cell Lysis During Pyroptosis

Pyroptosis has a pro-inflammatory effect beginning at the gasdermin D pore formation stage because of the activation and release of cytokines like IL-1β and IL-18. However, once the cell reaches the stage of membrane lysis, there are even more pro-inflammatory and damaging biological consequences. HMGB1 and ASC specks are cellular contents that are too large to pass through gasdermin pores but are released after

membrane lysis (Figure B). Tissue factor is released with parts of the plasma membrane during lysis. HMGB1 is a nuclear protein with various functions in healthy cells, but extracellular HMGB1 plays a role in the mediation of inflammatory and immune responses (Chen, Kang, & Tang, 2022). Tissue factor is a glycoprotein receptor released during lysis into the extracellular environment that forms a complex with coagulation factor VII leading to thrombosis (Ryan, Preston, & O'Neill, 2022). The ASC speck complex was recently discovered to be associated with seeding protein aggregates; contributing to the accumulation of misfolded proteins (Hulse & Bhaskar, 2022).

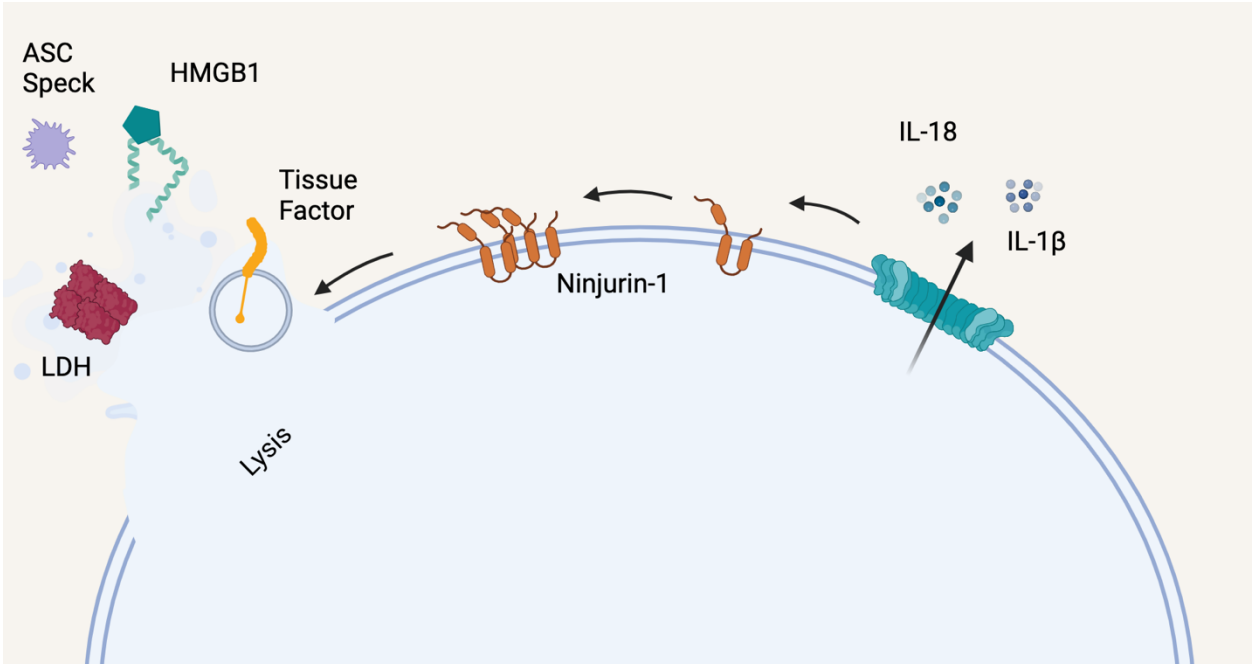


Figure B. Cellular Contents Released after Cell Lysis. ASC Speck protein, HMGB1, LDH, and Tissue Factor are released after membrane lysis in pyroptotic cells. These cellular components are only released after cellular lysis due to them being substantially larger than the diameter of the gasdermin D pore preventing passage.

1.4 The Clinical Relevance of Pyroptotic Cell Lysis

Pyroptosis is an essential and indispensable part of the immune system. Being a programmed cell death pathway, pyroptosis provides protection through clearance of unhealthy cells and healthy levels of pro-inflammatory effects (Jorgensen & Miao, 2015).

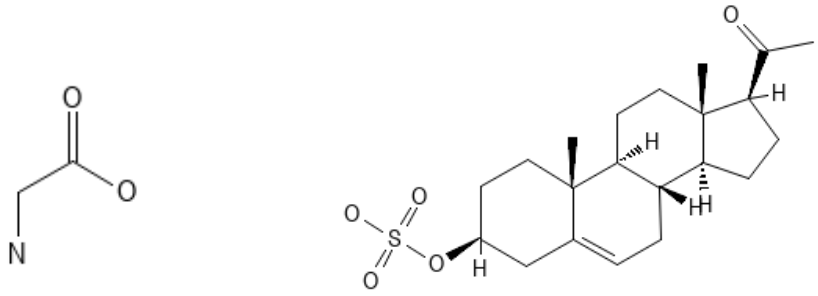
However, in cases with inappropriate or excessive pyroptosis there becomes a point where pyroptosis is no longer beneficial and becomes harmful. When pyroptosis begins to become harmful the dysfunction of the immune system and some organs are attributed to inflammatory levels that are out of control (Zheng, Chen, Gong, Chen, & Chen, 2021). Sepsis is one case where pyroptosis in moderation is influential in controlling an infection, but there is a threshold where there are excessive levels of pyroptosis that then contribute to a dysregulated immune response. Tissue factor is also released in pyroptotic cell lysis so when there are excessive levels of membrane lysis there is a correlation to sepsis disease progression through promoting blood coagulation and thrombosis development (Yang et al., 2019).

Sepsis is not the only disease where excessive levels of pyroptosis may heighten disease progression. The accumulation of misfolded protein aggregates has been shown to contribute to the progression of neurodegenerative diseases (Chiti & Dobson, 2017). Given the discovered crosstalk among ASC speck proteins and misfolded protein aggregates there is a possible parallel between excessive pyroptotic cell lysis resulting in an increased level of released ASC speck proteins and increased disease progression in neurodegenerative disorders (Chiti & Dobson, 2017; Hulse & Bhaskar, 2022).

1.5 Small Molecule Inhibition of Pyroptotic Cell Lysis

Glycine is an amino acid with the capability to block the final step of pyroptosis, cell lysis (Fink, Bergsbaken, & Cookson, 2008). While glycine inhibits membrane lysis, it does not block gasdermin D pore formation or caspase-1 activation further upstream (Loomis, den Hartigh, Cookson, & Fink, 2019). Glycine modulates ion channels and examination of other ion channel modulators led to the discovery that pregnenolone sulfate inhibits pyroptotic cell lysis and is more potent (Loomis et al., 2019). Pregnenolone sulfate is a

neurosteroid that is a noncompetitive antagonist for $GABA_A$ receptors (Pierce, Germann, Steinbach, & Akk, 2022). However, it is important to acknowledge that studies have shown a molecules ability to modulate $GABA_A$ receptor activity is not directly related to a molecules ability to inhibit cell lysis (den Hartigh, Loomis, Anderson, Frølund, & Fink, 2023; Loomis et al., 2019).



Glycine *Pregnenolone Sulfate*

Figure C. Chemical Structures of Glycine and Pregnenolone Sulfate. Glycine is a small molecule inhibitor of pyroptotic membrane lysis. Both glycine and pregnenolone sulfate act on ion channels; however, pregnenolone sulfate has a more complex chemical structure than glycine.

1.6 Pregnenolone Sulfate and Neurosteroid Characterization

Neurosteroids are steroids with the classical four ring carbon structure that interact with neuronal ion channels (Figure C). Pregnenolone sulfate is a lipophilic neurosteroid that modulates neuronal ion channels and inhibits membrane lysis in pyroptotic cells. While pregnenolone sulfate and many other molecules that share the steroid backbone structure have been characterized for their abilities to regulate neurological function, there is a gap in our understanding of whether structurally similar molecules may be able to inhibit pyroptotic lysis as seen with pregnenolone sulfate. Conversely, neurosteroids that have different molecular determinants for the $GABA_A$ receptor, whether they potentiate or inhibit, could share the ability to inhibit pyroptotic cell lysis.

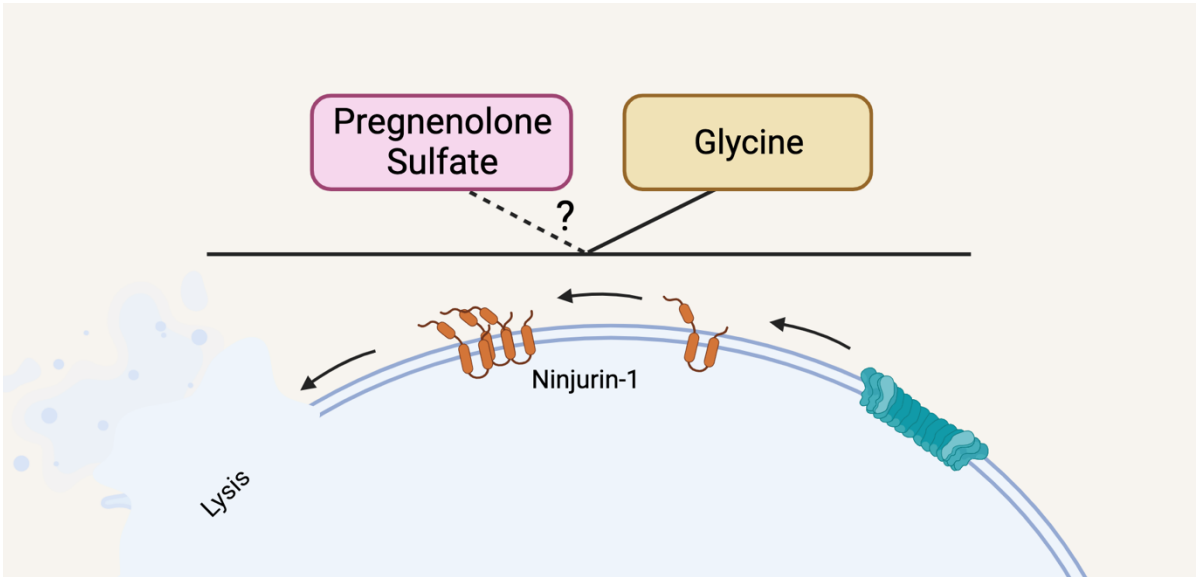


Figure D. Proposed Inhibition Point of Pregnenolone Sulfate. Glycine has been shown to block pyroptotic lysis through acting downstream of the gasdermin D pore. Pregnenolone sulfate also inhibits pyroptotic lysis and was shown to allow gasdermin D pore formation.

1.7 Aim of Thesis

In this thesis, we found pregnenolone sulfate is a powerful inhibitor of membrane lysis in cells undergoing pyroptosis. Inhibition of this lytic event is biologically significant when observing excessive or inappropriate pyroptosis because it inhibits the release of larger cellular components, like HMGB1. Reducing the number of cellular components released from pyroptosis would reduce the factors that continue to cause systemic harm through overactive immune responses and extreme inflammation. To build on this idea of inhibiting pyroptotic cell lysis, we wanted to test a range of pregnenolone sulfate analogs with similar structures. We were able to determine the inhibitory activity for a wide range of analogs that then can be connected to what structural components of pregnenolone sulfate are required for lysis inhibition. We found that there is not one sole structural aspect of pregnenolone sulfate that is responsible for activity, and there are other neurosteroids with similar structures to pregnenolone sulfate that are able to retain inhibitory activity. Additionally, we were able to support that pregnenolone sulfate and

other analogs act downstream of gasdermin D pore formation (Figure D). The findings of this thesis will contribute to a better overall understanding of the basic biological processes that lead up to cell lysis. The database of inhibitory neurosteroids collected in this thesis has developed a foundation for a deeper understanding in how these molecules regulate pyroptosis and begins to give us ideas of future therapeutic techniques surrounding inhibition of pyroptotic cell lysis.

Chapter 2. Materials & Methods

2.1 Cell Culture

Bone marrow-derived macrophages (BMDM) were cultured from BALB/c mice. The BMDMs were differentiated over seven days, incubated at 37°C, in 5% CO_2. The cells were grown in Dulbecco's Minimal Essential Medium (DMEM) including 30% L929 supernatant for differentiation, 10% Serum Plus medium supplement, 5 mM HEPES, and 100 U/mL of penicillin and streptomycin. On the seventh day of differentiation, BMDM were collected using phosphate-buffered saline (PBS) with 1 mM of EDTA then seeded into 96-well plates with phenol red free DMEM containing 5% Serum Plus medium supplement, 5 mM HEPES, and no antibiotics.

2.2 Pyroptosis Inducers

Two stimuli were used to induce pyroptosis in the BMDMs: anthrax lethal toxin and *Salmonella*. Anthrax Lethal Toxin was made with 1 ug/mL of protective antigen and 1 ug/mL of lethal factor, diluted into serum free DMEM. *Salmonella Typhimurium* (SL1344) was cultured in Luria Bertani (LB) Broth containing 0.3 M Sodium Chloride overnight, then diluted 1:15 into LB Broth with 0.3 M Sodium Chloride shaking and incubating at 37°C for three hours. The BMDM cells were infected with a multiplicity of infection (MOI) of 20:1. Macrophages were treated with either lethal toxin or *Salmonella* for 2 hours to induce pyroptosis.

2.3 Reagents

Pregnenolone sulfate and all other analogs were reconstituted in DMSO. Pregnenolone Methyl Ether was reconstituted in 100% ethanol after failing to reconstitute in DMSO. Each analog had a goal reconstitution of 2 mM but if it fell out of solution the concentration was gradually lowered until fully reconstituted and dissolved. Analogs were then aliquoted, stored frozen at –20 degrees Celsius, and thawed directly before use. Each neurosteroid was then diluted to multiple different concentrations into serum free DMEM,

and each concentration was standardized to 1% DMSO final. The neurosteroids were sourced from Cayman Chemical, Steraloids, and Santa Cruz Chemical.

2.4 LDH Release Assays

Cell lysis was measured by quantifying release of lactate dehydrogenase (LDH) in cell culture supernatants using the Cytotox96 assay kit (Promega). The maximum LDH release was determined by treating BMDM with detergent-based lysis buffer from the Cytotox96 kit for 2 hours. The background/spontaneous LDH release was measured by a negative control of no neurosteroids or stimuli, just DMEM media. Each condition for the LDH assay was tested in triplicate. The LDH release was calculated as follows: (experimental LDH – spontaneous LDH) / (maximum LDH – spontaneous LDH) * 100. The inhibition percentage was based upon the experimental conditions ability to inhibit LDH release in comparison to the average LDH release for the stimuli, both lethal toxin and *Salmonella* separately. For pregnenolone sulfate and each of the analogs, the BMDMs were treated at the full concentration range in the absence of additional stimuli to detect LDH release in the presence of the neurosteroid alone, allowing us to detect cytotoxicity. Additionally, the BMDMs were treated at the full concentration range for each neurosteroid in the presence of a detergent-based lysis buffer to detect any interference the neurosteroid may have with the LDH assay buffer.

2.5 Kinetic Microscopy

ToPro-3 is a membrane impermeant nuclear dye and was used to detect the formation of gasdermin D pore formation (den Hartigh & Fink, 2022). BMDM were imaged for the two-hour infection period with both stimuli, lysis buffer, and the PBS control conditions. Pore formation analysis was based on the percentage of conditional wells that showed pore formation in comparison to the total number of cells in wells. The PBS control

conditions, and lysis buffer conditions were used to test for cytotoxicity and interference with the fluorescent dye.

2.6 Quantification and Statistical Analysis

All statistical testing was performed on GraphPad Prism. Statistical tests used for each graph are indicated in figure legends. ImageJ cell counter plugin was used for pore formation total cell count.

Chapter 3. Results

3.1 Pregnenolone Sulfate Inhibits Pyroptotic Lysis Downstream of Pore Formation

Our first goal was to determine the dose response curve of pregnenolone sulfate protection against pyroptotic cell lysis (Figure 1A). To assess membrane lysis, we measured release of the large cytoplasmic enzyme, lactate dehydrogenase (LDH). BMDM were treated with pregnenolone sulfate at a twofold serial dilution, starting at 0.5 mM with constant final DMSO solvent at 1% in all conditions, including vehicle control. We used *Salmonella* infection and anthrax lethal toxin to trigger pyroptosis via the NLRC4 and NLRP1b inflammasomes, respectively. We assessed the membrane lysis by quantifying the percentage of LDH released into the supernatant (Figure 1B). At the maximum concentration of 0.5 mM, there is no cytotoxic effect when the cells are exposed to pregnenolone sulfate and PBS alone. We also determined there was no interference with the LDH detection assay shown through the lysis buffer condition remaining at 100% LDH release. There is a significant reduction in % LDH release for all three tested concentrations against *Salmonella* infection, inducing NLRC4-dependent pyroptosis, and lethal toxin exposure, inducing NLRP1b-dependent pyroptosis. Each concentration of pregnenolone sulfate tested caused a statistically significant reduction in LDH release compared to vehicle control for both *Salmonella* infection and lethal toxin treatment (Figure 1B). We calculated percent inhibition of LDH release by quantifying the LDH release in comparison to cells infected with either *Salmonella* or lethal toxin in the absence of pregnenolone sulfate (Figure 1C). At 0.5 mM, pregnenolone sulfate shows protection of around 58% inhibition for *Salmonella* infection and 91% inhibition for lethal toxin treated cells (Figure 1C). We observed a dose-dependent curve when quantifying LDH release inhibition and see that pregnenolone sulfate significantly loses protection when reducing concentration from 0.125 mM to 0.0675 mM; however there remains some residual

protection and not a total loss (Figure 1C). The inhibition curve trend is shown similarly for both lethal toxin and *Salmonella* infected cells. With support that pregnenolone sulfate inhibits membrane lysis against both *Salmonella* and lethal toxin infection, we next moved to determine whether pregnenolone sulfate was acting upstream or downstream of gasdermin D pore formation. We utilized live cell microscopy to detect uptake of the membrane impermeant fluorescent nuclear dye, ToPro-3, which is small enough to travel through gasdermin D pores when macrophages are undergoing pyroptosis. Pregnenolone sulfate at 0.25 mM robustly prevented LDH release but had no effect on uptake of ToPro-3 in cells treated with both *Salmonella* and lethal toxin, indicating there is no inhibition of gasdermin D pore formation (Figure 1D). Combining both the LDH data and ToPro-3 uptake data we show that pregnenolone sulfate protects against pyroptotic lysis and acts downstream of pore formation.

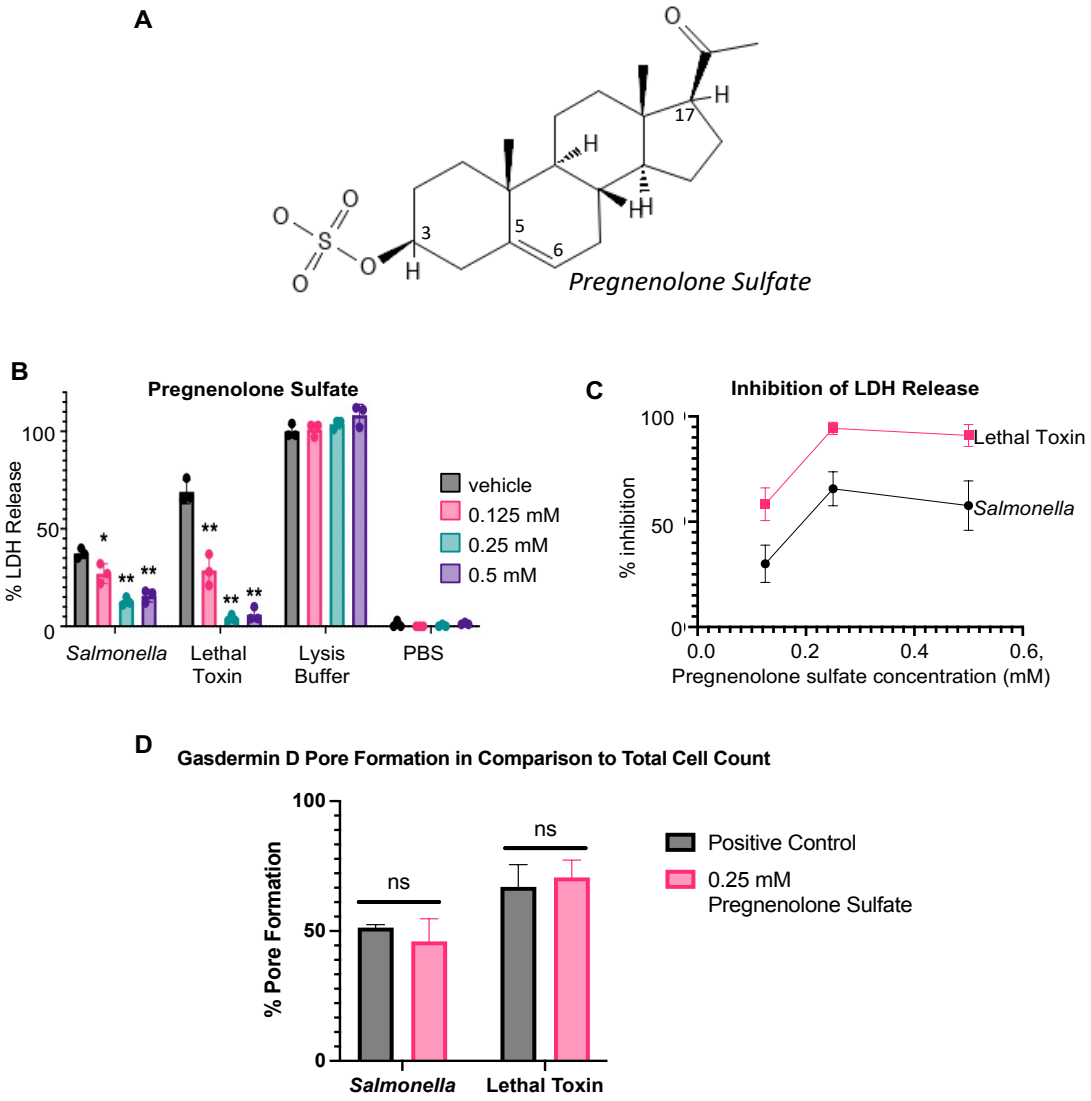


Figure 1. Pregnenolone sulfate inhibits pyroptotic lysis. A) Structure of pregnenolone sulfate with carbons three, five, six, and seventeen labeled. (B) Bone marrow-derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer or PBS in medium containing pregnenolone sulfate (titrated from 0.125 to 0.5 mM) or 1% DMSO vehicle control, and LDH released during lysis was measured. (C) LDH released during pyroptotic lysis in the presence of the indicated concentrations of pregnenolone sulfate was compared to LDH released in the absence of inhibitor (% inhibition). (D) ToPro-3 uptake in BMDM exposed to lethal toxin and *Salmonella* shows gasdermin D pore formation. Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons for % LDH release. A paired T-test was used for % pore formation between the positive control and pregnenolone sulfate.

3.2 Removing the Sulfate Group of Pregnenolone Sulfate Results in a Loss of Activity

Pregnenolone sulfate inhibits membrane lysis, but the mechanism of protection is not yet known. To begin understanding the relationship between pregnenolone sulfate's molecular structure and its ability to inhibit pyroptotic lysis, we decided to eliminate the sulfate group at carbon three and test the analog pregnenolone (Figure 2A). BMDMs were treated with the same stimuli and concentrations, except treated with pregnenolone for this experiment. The LDH release assay revealed pregnenolone has no protective activity against membrane lysis in response to *Salmonella* or lethal toxin at equivalent protective concentrations as pregnenolone sulfate (Figure 2B). We also found that pregnenolone does not interfere with the LDH assay or cause cytotoxicity in uninfected cells at any concentration. Together, these data suggest the carbon three position may be a critical molecular determinant for inhibitory activity of pregnenolone sulfate.

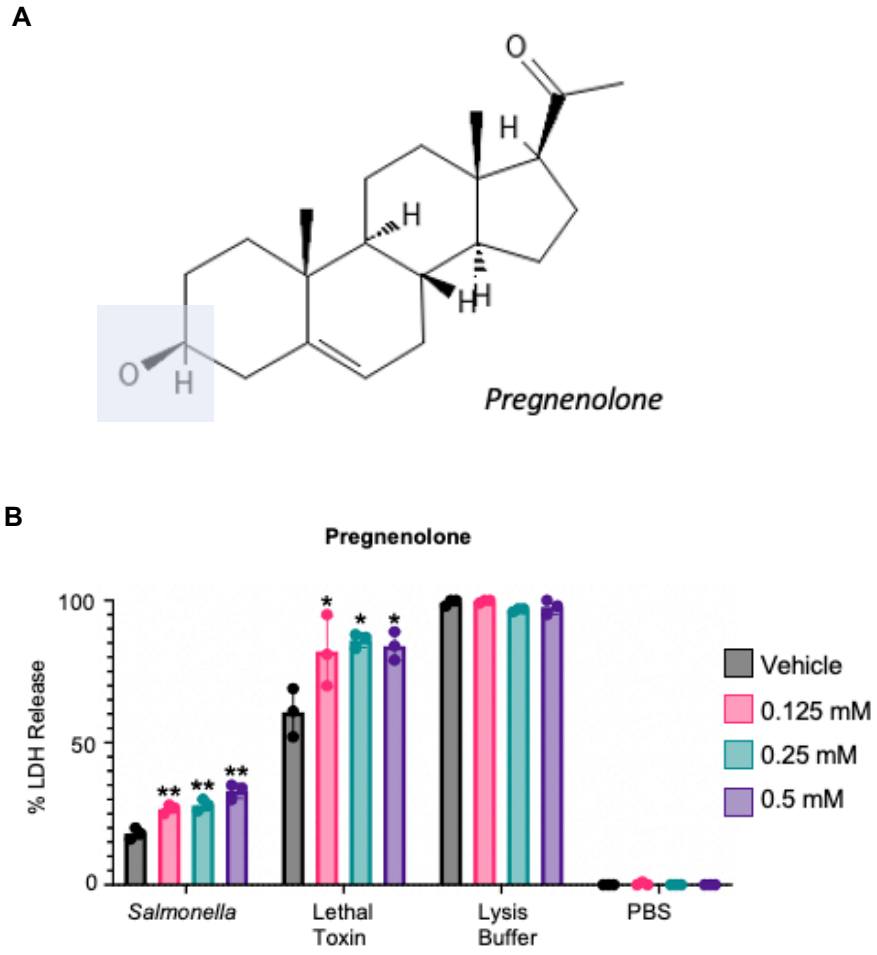


Figure 2. Pregnenolone does not inhibit pyroptotic lysis. (A) Bone marrow-derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer or PBS in medium containing pregnenolone sulfate (titrated from 0.125 to 0.5 mM) or DMSO vehicle control, and LDH released during lysis was measured. (B) The neurosteroid pregnenolone. Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

3.3 The Sulfate Group Replacement Impacts the Inhibition Activity of the Analogs Tested.

Given the dramatic loss in activity seen with pregnenolone, we next sought to determine whether a replacement of the sulfate group could restore the activity. We tested three different analogs with a replacement group at the carbon three position: pregnenolone hemisuccinate, pregnenolone methyl ether, and pregnenolone glucosiduronate (Figure 3A-D). For each analog, the same LDH release assay experimental layout was used. Pregnenolone hemisuccinate and pregnenolone methyl ether were tested at a twofold serial dilution starting at 0.5 mM, as used previously. Due to limited solubility, Pregnenolone glucosiduronate was tested at a twofold dilution starting at 0.25 mM. At equivalent concentrations, pregnenolone hemisuccinate displayed a similar level of protection as pregnenolone sulfate for both pyroptotic stimuli (Figure 3E), suggesting that the sulfate group is not absolutely required for activity. Pregnenolone methyl ether and pregnenolone glucosiduronate did not completely lose activity as seen with pregnenolone but were less potent level of inhibition against both stimuli tested at 0.25 mM (Figure 3E). None of the analogs tested interfered with the LDH assay or caused cytotoxicity in the uninfected treated cells at any concentration. While pregnenolone sulfate and pregnenolone hemisuccinate share the same level of activity at 0.25 mM, they also have similar trending dose response curves. The exception being at 0.125 mM pregnenolone hemisuccinate retains activity until 0.063 mM where pregnenolone sulfate loses activity at 0.125 mM (Figure 3F-G). Comparing those to the dose response curves pregnenolone methyl ether and pregnenolone glucosiduronate, the data shows a less potent but sustained protection at the full concentration range (Figure 3F-G).

After finding that pregnenolone hemisuccinate and pregnenolone sulfate share activity to inhibit pyroptotic lysis, the next step was to determine if they both act

downstream of gasdermin pore formation. We found both neurosteroids inhibit lysis without inhibiting ToPro-3 uptake as a marker of gasdermin D pore formation (Figure 4). This supports the hypothesis pregnenolone hemisuccinate and pregnenolone sulfate may be interacting with the same part of the pathway or a similar mechanism of protection. The surprising and significant finding of pregnenolone hemisuccinate's activity provides insight to the aspects of the molecular structure of the neurosteroid that have an impact on activity. There may be a structural quality that both the succinate group and the sulfate group share correlating to their activity. Also taking pregnenolone glucosiduronate and pregnenolone methyl ether into consideration, we find altering the molecular structure does not equate to either total retention or total loss of activity.

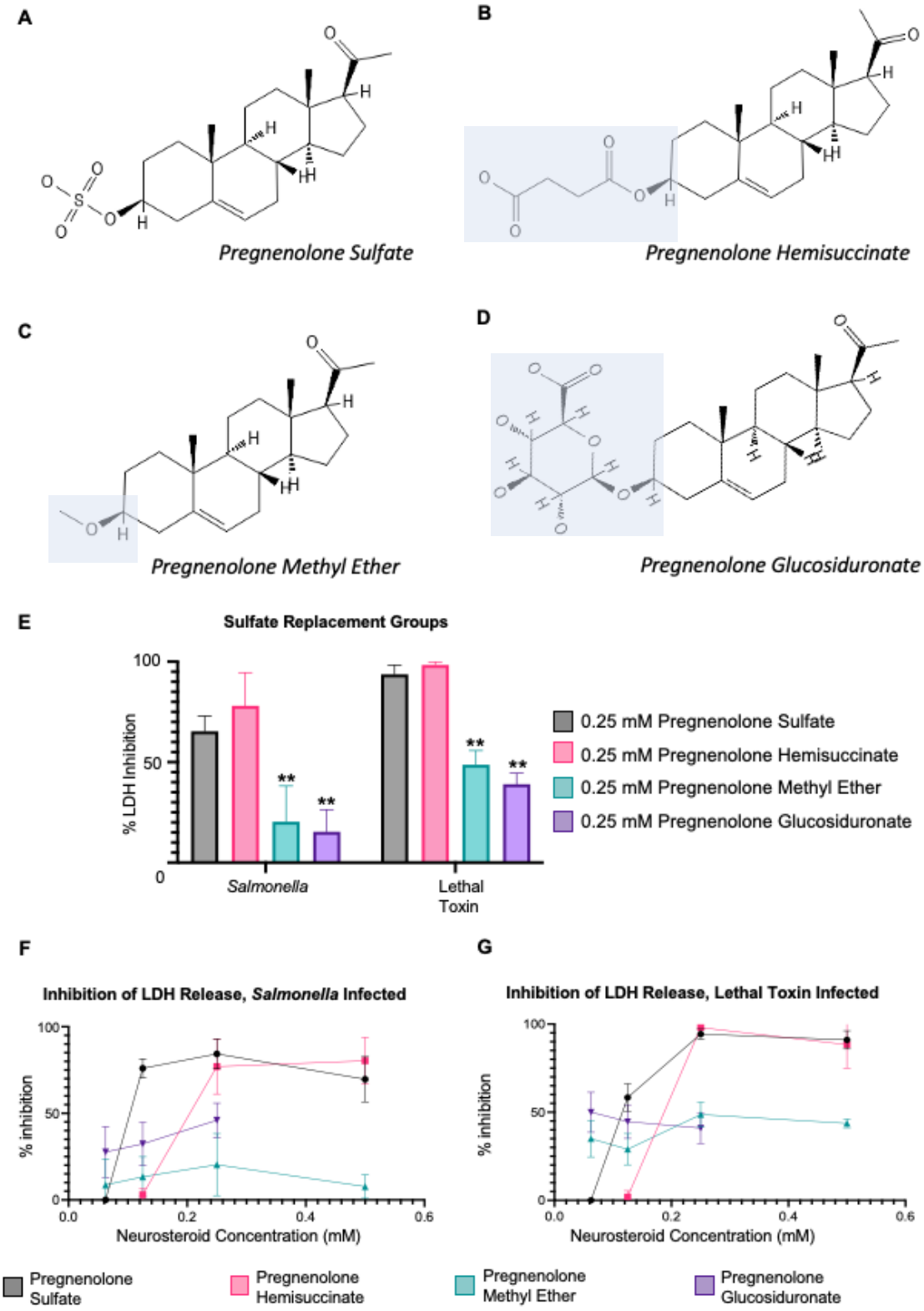


Figure 3. Pregnenolone Sulfate analogs with various replacement groups at the carbon three position have varying degrees of inhibition potency. (A) Chemical structure of

pregnenolone sulfate. (B) Chemical structure of pregnenolone hemisuccinate. (C) Chemical structure of pregnenolone methyl ether. (D) Chemical structure of pregnenolone glucosiduronate. (E) Bone marrow-marrow derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer, or PBS in medium. Containing either pregnenolone sulfate, pregnenolone hemisuccinate, pregnenolone methyl ether, pregnenolone glucosiduronate, or 1% DMSO vehicle control. LDH released during pyroptotic lysis in the presence of the indicated neurosteroids at 0.25 mM was compared to LDH released in the absence of inhibitor (% inhibition). (F) LDH released during pyroptotic lysis triggered by *Salmonella* in the presence of the indicated neurosteroids (titrated in the range from 0.063 mM to 0.5 mM dependent on solubility) was compared to LDH released in the absence of inhibitor (% inhibition). (G) LDH released during pyroptotic lysis triggered by lethal toxin in the presence of the indicated neurosteroids (titrated in the range from 0.063 mM to 0.5 mM dependent on solubility) was compared to LDH released in the absence of inhibitor (% inhibition). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *$P < 0.05$, **$P < 0.01$, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

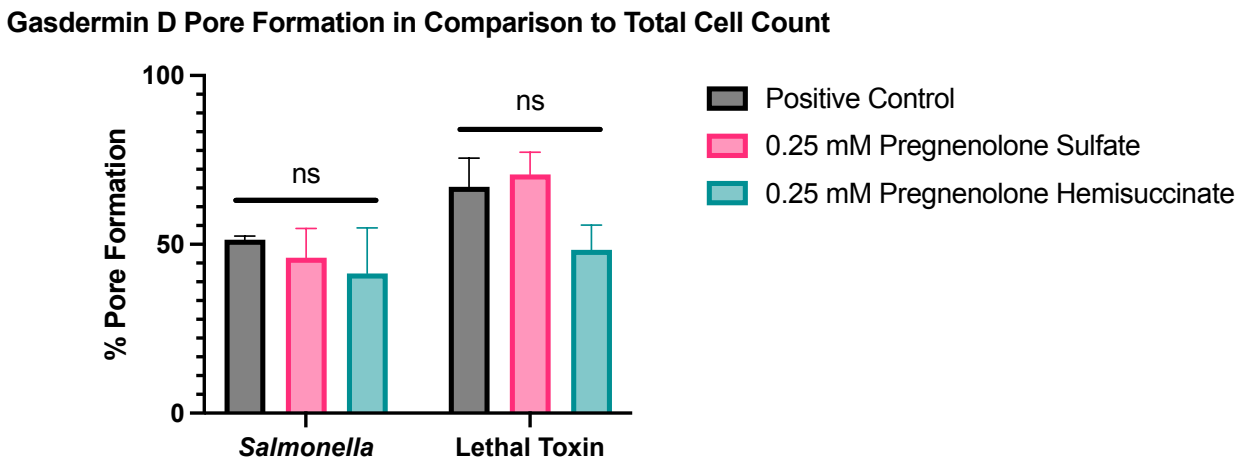


Figure 4. Pregnenolone sulfate and pregnenolone hemisuccinate allow Gasdermin D pore formation while still inhibiting cellular lysis. Bone marrow-derived macrophages were treated with *Salmonella* and lethal toxin containing pregnenolone sulfate, pregnenolone hemisuccinate, or DMSO vehicle positive control. Neurosteroids tested at 0.25 mM. Gasdermin D pore formation indicated by the uptake of ToPro-3 membrane impermeable nuclear dye compared to the total cell count in field of measurement (% pore formation). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

3.4 Non-Structure Characteristics, like Hydrogen Acceptor Sites, could be Playing a Role in Determining the Attributes Responsible for Activity.

As we observed that pregnenolone sulfate and pregnenolone hemisuccinate both have activity to prevent pyroptotic lysis, we next wanted to consider molecular characteristics they may share that could dictate activity. First, the two neurosteroids share the same number of hydrogen acceptor sites and hydrogen donor sites (Figure 5). Hydrogen acceptor sites are an important characteristic that impacts ligand binding and targeting affinity of a small molecule. Hydrogen donor sites on the other hand can increase off-target binding because of the molecule's ability to give away hydrogens too easily. Pregnenolone sulfate and pregnenolone hemisuccinate share the same potency in protection at 0.25 mM and the same number of hydrogen acceptor/donor sites. This finding leads to the possibility of these sites being impactful to the neurosteroids ability to prevent membrane lysis. Supporting this notion is pregnenolone methyl ether possessing fewer hydrogen acceptor sites and no hydrogen donor sites. However, pregnenolone glucosiduronate has a higher number of hydrogen acceptor sites and donor sites. Pregnenolone displayed a total loss in activity, while possessing the same number of hydrogen acceptor sites as pregnenolone methyl ether. It is plausible the locations of the hydrogen acceptor sites are partially responsible for activity or the increase in hydrogen donor sites negatively impacts the ability of hydrogen acceptor sites to interact with other binding sites.

Given that we are observing the integrity of the plasma membrane of the cells, a neurosteroids lipophilicity is an important consideration because it impacts a molecules ability to interact with the lipid bilayer of the plasma membrane. A higher XLogP3 value indicates a higher affinity to interact with highly lipid environments. Interestingly, pregnenolone, which is completely inactive, has the same XLogP3 as pregnenolone sulfate,

suggesting that there is not a direct correlation between lipophilicity and protection. In addition, pregnenolone methyl ether possesses a higher XLogP3 than pregnenolone sulfate but demonstrated reduced potency in activity (Figure 5). Pregnenolone glucosiduronate also had reduced potency, but has a lower XLogP3, together suggesting that lipophilicity does not directly predict the ability to inhibit pyroptotic lysis.

Neurosteroid	Activity	Hydrogen Acceptor Sites	Hydrogen Donor Sites	Lipophilicity (XLogP3)
Pregnenolone Sulfate	Protective	5	1	3.8
Pregnenolone Hemisuccinate	Protective	5	1	4.2
Pregnenolone Methyl Ether	Reduced Potency	2	0	4.8
Pregnenolone Glucosiduronate	Reduced Potency	8	4	2.9
Pregnenolone	Not Protective	2	1	4.2

Figure 5. Chemical characteristics may play a role in a neurosteroids inhibitory activity.

All data collected was sourced from PubChem. Pregnenolone sulfate and pregnenolone hemisuccinate possess the same number of hydrogen acceptor sites and hydrogen donor sites. Pregnenolone methyl ether has a loss in both hydrogen acceptor/donor sites. Pregnenolone glucosiduronate has an increase in hydrogen donor sites and an increase in hydrogen donor sites as well. Pregnenolone has a loss in hydrogen acceptor sites and the same number of hydrogen donor sites. There is no correlation between an increase in lipophilicity and activity.

3.5 Eliminating the Carbon 5-6 Double Bond on Pregnenolone Sulfate Reduces Inhibitory Activity.

We have previously found that the carbon three position impacts the activity of the neurosteroid. We next chose to examine whether altering the carbon ring structure may impact activity. Pregnenolone sulfate and pregnanolone sulfate are structurally similar except for the loss of the carbon 5-6 double bond in replacement of a single hydrogen covalently bonded to carbon five (Figure 6A-B). To determine whether this steroid backbone alteration affects activity, we treated cells with pregnanolone sulfate and induced pyroptosis. We found that pregnanolone sulfate did not inhibit the LDH assay or cause cytotoxicity in uninfected cells at any concentration (data not shown). The concentrations of pregnanolone sulfate were tested at a twofold dilution starting at 0.25 mM. Pregnanolone sulfate demonstrated similar inhibition of *Salmonella*-induced lysis at 0.25 mM in comparison to pregnenolone sulfate (Figure 6C). The dose-response inhibition curves showed a gradual loss in inhibitory activity for both *Salmonella* and lethal toxin-induced pyroptosis (Figure 6D-E). We next measured the uptake of the ToPro-3 as a measure of gasdermin D pore formation and found that the loss of the double bond while retaining the sulfate group on pregnanolone sulfate does not block gasdermin D pore formation (Figure 7). This finding suggests that pregnanolone sulfate also acts downstream of gasdermin pore formation when inhibiting membrane lysis.

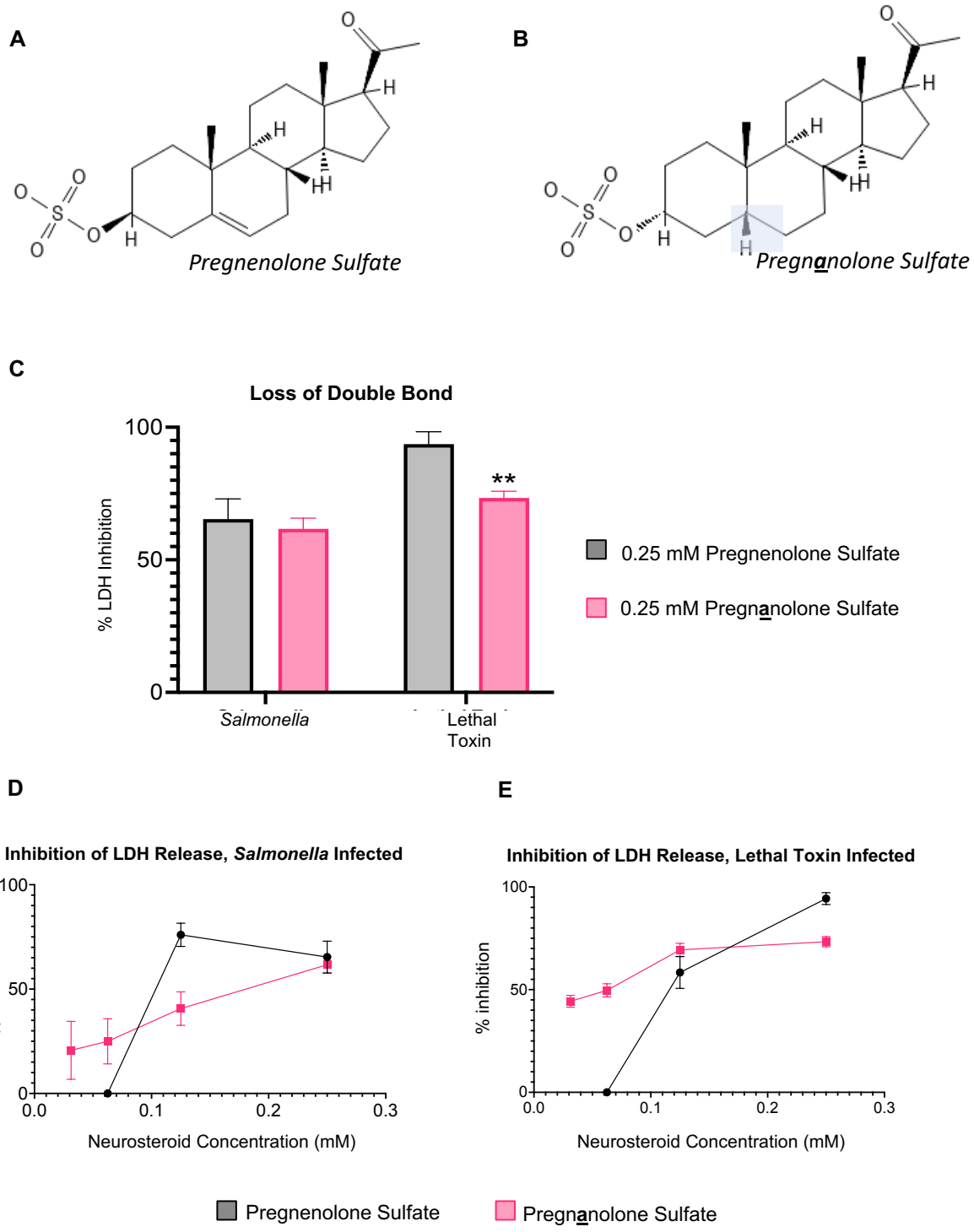


Figure 6. Pregnanolone sulfate is a less potent inhibitor of pyroptotic lysis. (A) Chemical structure of pregnenolone sulfate (B) Chemical structure of pregnanolone sulfate. (C) Bone marrow-marrow derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer, or PBS in medium. Containing either pregnenolone sulfate, pregnanolone sulfate, or 1% DMSO vehicle control. LDH released during pyroptotic lysis in the presence of the indicated neurosteroids at 0.25 mM was compared to LDH released in

the absence of inhibitor (% inhibition). (D) LDH released during pyroptotic lysis triggered by *Salmonella* in the presence of the indicated neurosteroids (titrated in the range from 0.031 mM to 0.25 mM dependent on solubility) was compared to LDH released in the absence of inhibitor (% inhibition). (E) LDH released during pyroptotic lysis triggered by lethal toxin in the presence of the indicated neurosteroids (titrated in the range from 0.031 mM to 0.25 mM dependent on solubility) was compared to LDH released in the absence of inhibitor (% inhibition). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using paired T-test.

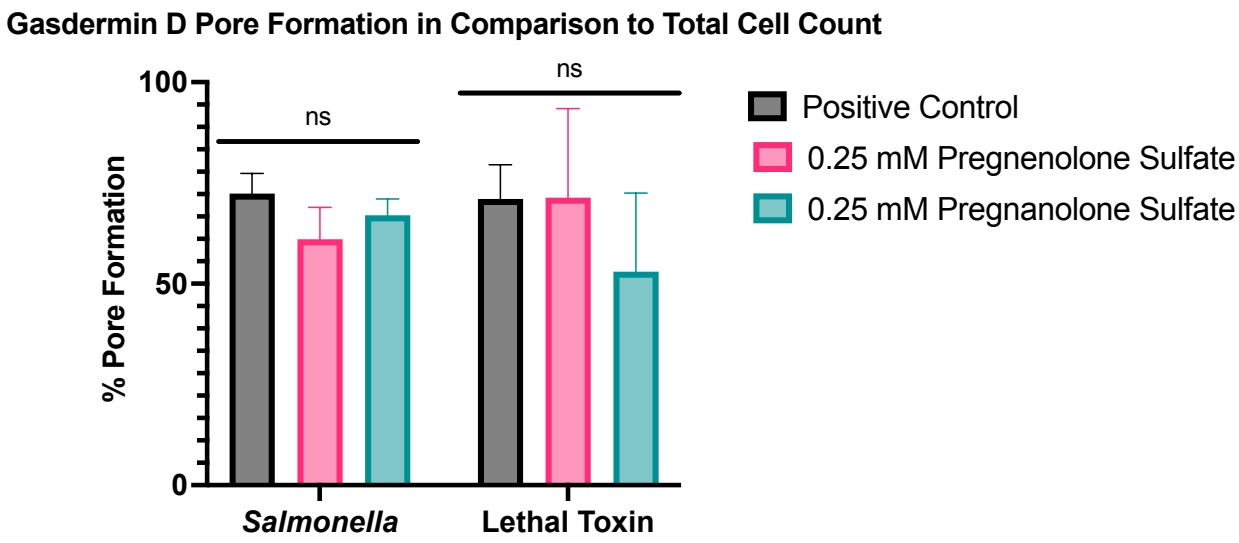


Figure 7. Pregnenolone sulfate and pregnanolone sulfate both allow gasdermin D pore formation. Bone marrow-derived macrophages were treated with *Salmonella* and lethal toxin containing pregnenolone sulfate, pregnanolone sulfate, or DMSO vehicle positive control. Neurosteroids tested at 0.25 mM. Gasdermin D pore formation indicated by the uptake of ToPro-3 membrane impermeable nuclear dye compared to the total cell count in field of measurement (% pore formation). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

3.6 Plane Shifting Structural Components of the Neurosteroid May Increase Ability to Inhibit Lysis.

We next wished to examine whether further alterations to the steroid backbone would impact activity. Epipregnanolone sulfate and epiallopregnanolone sulfate are two commercially available neurosteroids that are analogs of pregnanolone sulfate. They exhibit plane shifting of both the carbon three position and the carbon 5-6 position (Figure 8A-C). Epipregnanolone sulfate and epiallopregnanolone sulfate were tested at a twofold dilution starting at 0.25 mM. We found an increase in potency to inhibit pyroptotic LDH release at 0.25 mM for both analogs in comparison to pregnanolone sulfate at 0.25 mM (Figure 8D). Neither epipregnanolone sulfate or epiallopregnanolone sulfate exhibit interference with the LDH assay or cytotoxicity in uninfected BMDMs (data not shown). Both analogs maintain a slightly higher potency than pregnenolone sulfate over the range of concentrations tested for both stimuli and show a similar inhibition curve trend (Figure 8E-F). Our preliminary data suggest that ToPro-3 uptake still occurs in the presence of epipregnanolone sulfate and epiallopregnanolone sulfate (data not shown). Together, these data suggest that plane shifting may slightly improve potency to inhibit pyroptotic lysis, without affecting gasdermin D pore formation.

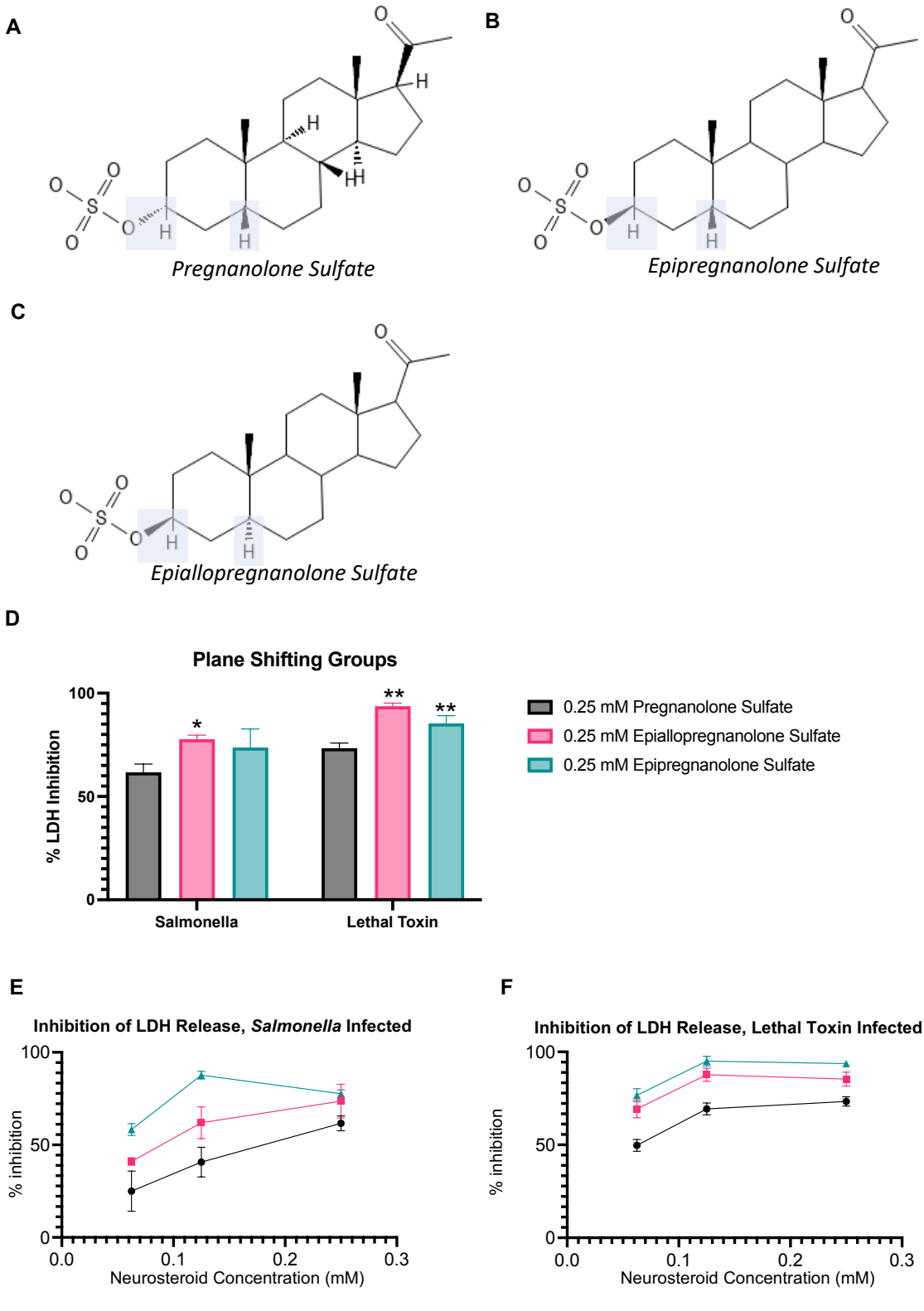


Figure 8. Plane shifting in the chemical structure of pregnanolone sulfate does not reduce inhibitory activity. (A) Chemical structure of pregnanolone sulfate. (B) Chemical

structure of epipregnanolone sulfate. (C) Chemical structure of epiallopregnanolone sulfate. (D) Bone marrow-derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer, or PBS in medium. Containing either pregnanolone sulfate, epipregnanolone sulfate, epiallopregnanolone sulfate, or 1% DMSO vehicle control. LDH released during pyroptotic lysis in the presence of the indicated neurosteroids at 0.25 mM was compared to LDH released in the absence of inhibitor (% inhibition). (E) LDH released during pyroptotic lysis triggered by *Salmonella* in the presence of the indicated neurosteroids (titrated from 0.063 mM to 0.25 mM) was compared to LDH released in the absence of inhibitor (% inhibition). (F) LDH released during pyroptotic lysis triggered by lethal toxin in the presence of the indicated neurosteroids (titrated from 0.063 to 0.25 mM) was compared to LDH released in the absence of inhibitor (% inhibition). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *$P < 0.05$, **$P < 0.01$, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

3.7 The Carbon 17 Position Changing Impacts the Activity of the Pregnenolone Sulfate Analogs Leading to the Understanding of Activity being Dependent on Multiple Structural Positions.

Our results thus far indicate that the carbon three, five, and six positions on pregnenolone sulfate impact the activity level of the analogs tested to varying degrees. The carbon 17 position is on the opposite end of the molecule without being a part of the steroid back bone (Figure 9A-C). We tested dehydroepiandrosterone sulfate (DHEA Sulfate) and cholesterol sulfate at concentrations of 0.25 mM, 0.125 mM, and 0.063 mM. At the 0.25 mM, DHEA Sulfate shows reduced protection against lysis induced by both stimuli; however, Cholesterol Sulfate retains protection in comparison to Pregnenolone Sulfate (Figure 9D). However, both analogs show an unexpected level of consistency when looking at the complete dose response curve. The inhibition curves for treated cells infected with *Salmonella* show slight reduction in activity at decreasing concentrations for the analogs (Figure 9E). When treated cells are exposed to lethal toxin the potency of activity shows a highly consistent level of protection for DHEA Sulfate and cholesterol sulfate (Figure 9F). Cholesterol sulfate and DHEA sulfate support there are multiple different characteristics and structural attributes that can impact a neurosteroid's ability to prevent membrane lysis in cells undergoing pyroptosis.

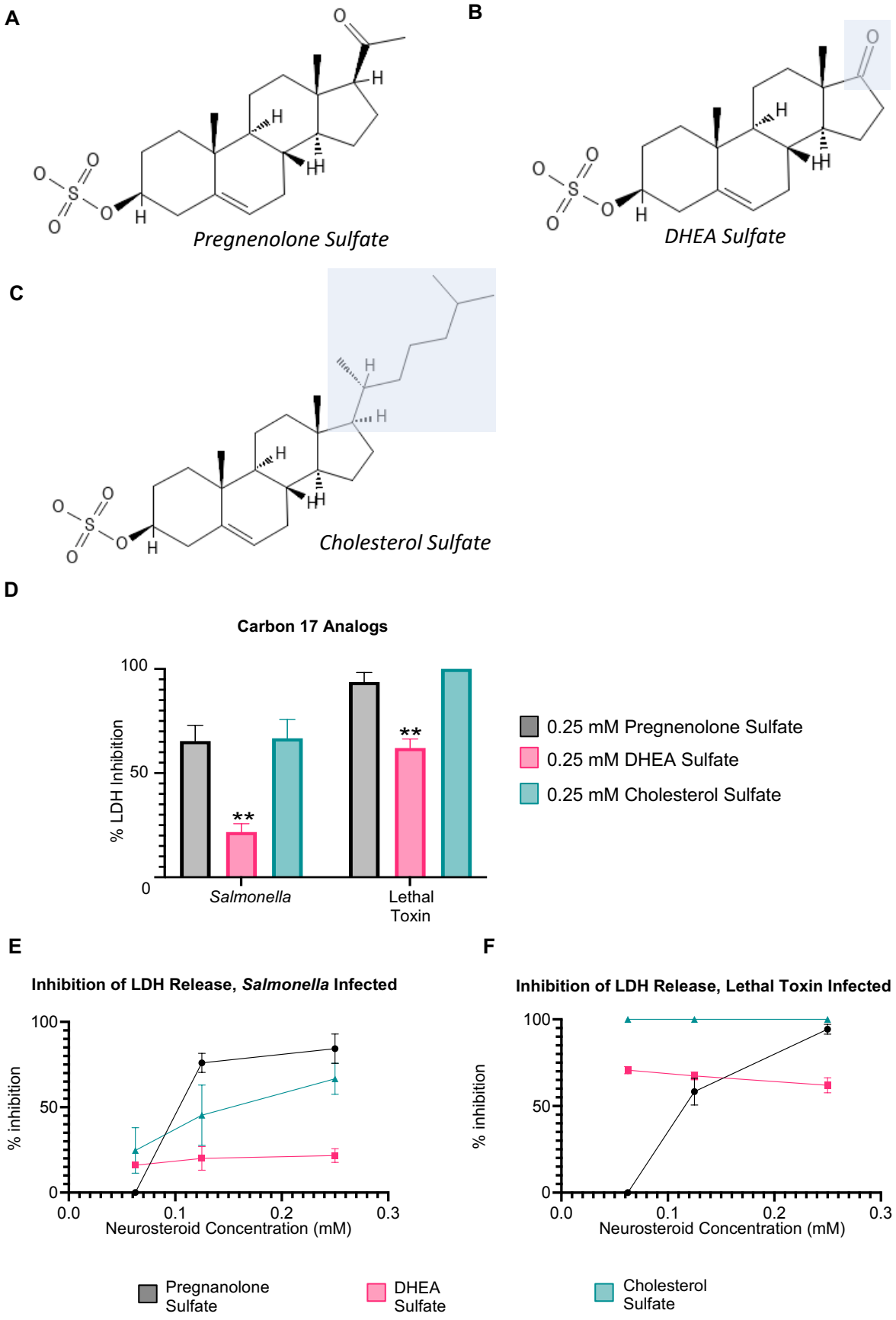

A
Pregnenolone Sulfate
B
DHEA Sulfate
C
Cholesterol Sulfate
D
Carbon 17 Analogs
% LDH Inhibition
100
50
0
**
**
Salmonella
Lethal Toxin
0.25 mM Pregnenolone Sulfate
0.25 mM DHEA Sulfate
0.25 mM Cholesterol Sulfate
E
Inhibition of LDH Release, Salmonella Infected
% inhibition
100
50
0
0.0
0.1
0.2
0.3
Neurosteroid Concentration (mM)
F
Inhibition of LDH Release, Lethal Toxin Infected
% inhibition
100
50
0
0.0
0.1
0.2
0.3
Neurosteroid Concentration (mM)
Pregnanolone Sulfate
DHEA Sulfate
Cholesterol Sulfate

Figure 9. The carbon 17 position of pregnenolone sulfate impacts inhibitory activity based on molecular alteration. (A) Chemical structure of pregnenolone sulfate (B) Chemical structure of DHEA sulfate. (C) Chemical structure of cholesterol sulfate. (D) Bone marrow-marrow derived macrophages were treated with *Salmonella*, lethal toxin, detergent-based lysis buffer, or PBS in medium. Containing either pregnenolone sulfate, DHEA sulfate, cholesterol sulfate, or 1% DMSO vehicle control. LDH released during pyroptotic lysis in the presence of the indicated neurosteroids at 0.25 mM was compared to LDH released in the absence of inhibitor (% inhibition). (E) LDH released during pyroptotic lysis triggered by *Salmonella* in the presence of the indicated neurosteroids (titrated from 0.063 mM to 0.25 mM) was compared to LDH released in the absence of inhibitor (% inhibition). (F) LDH released during pyroptotic lysis triggered by lethal toxin in the presence of the indicated neurosteroids (titrated from 0.063 mM to 0.25 mM) was compared to LDH released in the absence of inhibitor (% inhibition). Representative data (mean +/- SD, n=3) from two or more independent experiments are shown. *P < 0.05, **P < 0.01, compared to vehicle control using ordinary one-way ANOVA with Dunnett's Post-hoc test and multiple comparisons.

Chapter 4. Discussion

4.1 Summary of Findings

This thesis project has given us valuable insight into how altering the molecular structure of pregnenolone sulfate impacts its ability to inhibit pyroptotic cell lysis. First, we demonstrated that pregnenolone sulfate was protective against membrane lysis in both *Salmonella* infected and lethal toxin treated cells, at concentrations of 0.063 mM, 0.125 mM, and 0.250 mM. Including both a lysis buffer condition and a PBS with 1% DMSO condition, the data shows pregnenolone sulfate does not interfere with the LDH assay or cause cytotoxicity in uninfected treated bone marrow-derived macrophages. Testing the LDH release in cells treated with pregnenolone revealed that without the sulfate group there was a total loss of activity. However, the sulfate group is not solely responsible for the inhibitive activity because we see pregnenolone hemisuccinate retains the same level of inhibitive activity as pregnenolone sulfate. The retention of activity indicates there may be similarities in certain characteristics the two neurosteroids share that contribute to the mechanism of action.

Pregnenolone sulfate and pregnenolone hemisuccinate share a different group on the carbon three position, but the rest of their structures are the same. The two neurosteroids also share the same number of hydrogen acceptor sites and hydrogen donor sites; these sites are important when developing chemicals because it effects ligand targeting and binding affinity (Steiner, 2002; Wade & Goodford, 1989). The ability to observe which hydrogen acceptor sites are active during inhibition is out of the scope of this project but would be an interesting future step in understanding how the neurosteroids are working mechanistically. Looking at other analogs with alterations at the carbon three position, we see an increase in hydrogen acceptor sites does not mean the neurosteroid will retain the same potency for inhibition as pregnenolone sulfate, as seen with pregnenolone

glucosiduronate. This could be because of the increase in hydrogen donor sites which is linked to off target binding, or the location of the hydrogen acceptor sites plays a larger role in activity rather than the overall number of hydrogen acceptor sites. Pregnenolone showing a complete loss in activity whereas pregnenolone methyl ether retaining inhibitive activity supports the hypothesis that the location of the hydrogen acceptor sites is influential in activity of the neurosteroid.

Lipophilicity was another characteristic taken into consideration since the XlogP3 number of a neurosteroid impacts the molecule's interactions with lipid membranes (Naylor et al., 2018). There was no correlation between an increase in lipophilicity and an increase, or retention, in inhibitive activity. Replacing the sulfate group with other groups also resulted in a loss of potency in protection in some cases. Pregnenolone methyl ether and pregnenolone glucosiduronate show a significant loss in protection but unlike pregnenolone, were not completely inactive. This may indicate there are multiple factors at play that impact activity or they may act through a different mechanism that is not as effective in blocking pyroptotic lysis.

Carbon three is not the molecular determinant of activity. Cholesterol sulfate and dehydroepiandrosterone sulfate (DHEA sulfate) retain the sulfate group at carbon three but have vastly different levels of activity in comparison to each other and to pregnenolone sulfate. DHEA sulfate shows a significant reduction in activity whereas cholesterol sulfate shows the same level of lysis inhibition as pregnenolone sulfate. Replacing chemical groups on the other side of the neurosteroids can change the activity of the neurosteroid even when retaining other groups, like the sulfate. Like the carbon three replacement groups, the carbon 17 replacement group impacts the level of inhibition and activity retention. Cholesterol sulfate is known to heavily interact with lipid membranes as a stabilizing

molecule so we cannot rule out that it is inhibiting lysis through a different mechanism than pregnenolone sulfate (Bleau, Bodley, Longpré, Chapdelaine, & Roberts, 1974; Bleau, Lalumiùre, Chapdelaine, & Roberts, 1975).

Eliminating the double bond between carbon five and six on pregnenolone sulfate slightly reduces the inhibitive activity. The change in activity when manipulating the four-carbon ring structure supports the concept that multiple aspects of pregnenolone sulfate may be responsible for its activity. It is still unknown if the change in structure allows pregnanolone sulfate to act at the same mechanism at a lower potency, or if it has a different mechanism of action. Other smaller components, like plane shifting, were found to increase activity of epiallopregnanolone sulfate and epipregnanolone sulfate in comparison to pregnanolone sulfate. Plane shifting was the only alteration to the neurosteroid that showed a statistically significant increase in activity when tested at the same concentration. Data showing a way to possibly increase an inhibitors potency could be beneficial when looking at potential therapeutics.

Looking at the thesis project, we find strong evidence that there is not one sole part of the structure of pregnenolone sulfate that is responsible for activity. We see a wide array of affects depending on the type of change that is made to the pregnenolone sulfate molecule. Both structure and chemical characteristics may play a role in neurosteroids' ability to inhibit membrane lysis after pyroptosis is induced. Whether characteristics, like hydrogen acceptor/donor sites, are responsible is yet to be determined but we see evidence that the location of the sites may be more important than the number of hydrogen acceptor or donor sites. For the neurosteroids tested, we saw gasdermin D pore formation was allowed, suggesting that they all act downstream of gasdermin D pore formation.

4.2 Future Directions

The data collected in this thesis project lay the groundwork for several next steps. We have collected data supporting gasdermin pore formation for many of the analogs tested but would like to determine whether they all act downstream of gasdermin D pore formation. We have yet to see a molecular structure alteration correspond with inhibition of gasdermin D pore formation, but this may change as more analogs are tested. Gasdermin D pore formation is followed by ninjurin-1 oligomerization. Ninjurin-1 oligomerization is a recently identified, but poorly understood, prerequisite for pyroptotic lysis. For pregnenolone sulfate and any other neurosteroids that act downstream of pore formation, we can next test if ninjurin-1 oligomerization is inhibited using a native page western blot as in our prior studies (den Hartigh et al., 2023). This step helps narrow down even further a possible mechanism of action for the neurosteroids. We currently do not understand what triggers the oligomerization of ninjurin-1 and the neurosteroids inhibiting lysis may lead us to understanding what molecules can modulate this process. There is also the question of whether these molecules are protein or lipid binding, which is critical to understanding the molecular requirements for inhibition of cell lysis. If we observe inhibition of ninjurin-1 oligomerization, future experiments will seek to determine if the neurosteroid is acting directly on the ninjurin-1 protein or if it is acting on the membrane preventing the oligomerization of ninjurin-1.

The recent structural characterization of the $GABA_A$ receptor has enabled computational modeling of interactions with pregnenolone sulfate and analogs (Mortensen et al., 2023). If we find that pregnenolone sulfate and other analogs interact directly with ninjurin-1, computational modeling could be used to look at potential binding sites or how other analogs may interact given specific modeled changes. The LDH data collected in this thesis project provides experimental evidence for molecular changes that affect activity and

could support predicted binding sites revealed with computational modeling. The pregnenolone sulfate analogs tested within this thesis are all commercially available. Computational modeling could allow us to predict new compounds with increased potency, which could be custom synthesized to further evaluate different relationships between structure and activity. Testing compounds that combine structural alterations at multiple sites may also reveal novel insights into the structure-activity relationship. In addition, testing combinations of active and inactive molecules, for example, pregnenolone combined with pregnenolone sulfate could reveal unexpected antagonists of activity.

Within this project, there has been a small group of neurosteroids tested. Small molecule libraries are also available, some of which include neurosteroids, and utilizing these libraries would help expand our knowledge of how diverse chemicals impact LDH release. Testing such a large and diverse group of small molecules would give us the opportunity to look for more potent inhibitors in comparison to pregnenolone sulfate and a larger dataset to find common characteristics that may impact activity.

Pregnenolone sulfate is shown to be inhibitive against pyroptotic lysis and when analogs with structure changes are tested some retain protective activity against cell lysis. Understanding there are multiple components and characteristics that impact inhibitive activity to varying degrees gives us insight for future development of chemical compounds. We now know the inhibitory abilities of several different types of analogs and can utilize this data to expand on future questions surrounding the correlation between structure and activity while also developing a deeper knowledge of the mechanisms in which neurosteroids may inhibit membrane lysis. For the purpose of this thesis, all neurosteroids were tested at concentrations higher than what could be used for actual therapeutic

techniques. However, with further development, the findings in this thesis project lay the foundation for future small molecule design and therapeutic techniques.

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