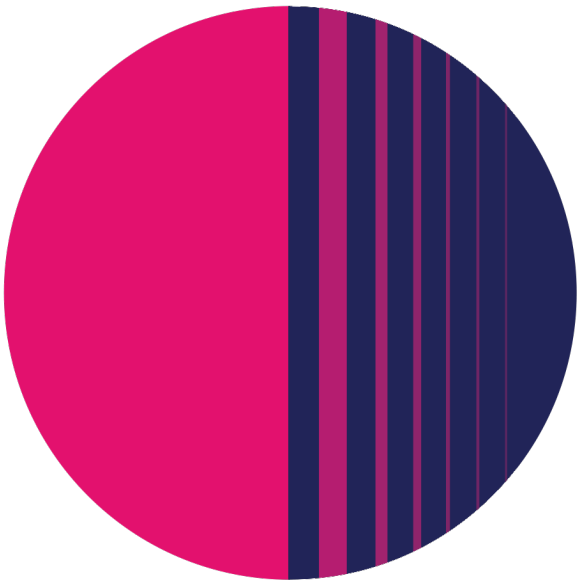

STUDENTS AS
CO-CREATORS

A Learning & Teaching Research Collaboration

Artesunate as a therapy for systemic inflammatory conditions: preventing neutrophil migration, cytokine, chemokine and NET release in response to Complement Protein 5 (C5a)

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1. Executive Summary/Abstract

Artesunate, a frontline anti-malarial drug derived from artemisinin has been shown within recent studies to be effective at reducing the effects of mouse neutrophil chemotaxis cytokine, chemokine and neutrophil extracellular trap (NET) release during systemic inflammatory conditions such as sepsis. This project aimed to evaluate the effect that artesunate has on human neutrophil chemotaxis cytokine, chemokine and NET release when exposed to the pro-inflammatory complement protein C5a. This was assessed through cell purification, ibidi chemotaxis assays and enzyme-linked immunosorbent assays (ELISA). Results from the experiments showed that artesunate could significantly decrease both the forward migration index (FMI) and velocity of human neutrophils towards C5a, as well as the lowering of NET release. In systemic inflammatory conditions, such as sepsis and CoVID-19, neutrophil overactivation by C5a can lead to organ damage and death. The results here show that artesunate has a potent anti-inflammatory effect on human neutrophils and suggest it may be of value as a novel therapeutic for systemic inflammatory conditions. Further research *in vitro* and *in vivo* should be conducted to fully understand the extent at which artesunate supress human neutrophil activation and at which therapeutically relevant concentrations.

2. Background and Aims

A key systemic problem is that of sepsis, this is the whole system immunological response of the body to an infection, which may ultimately lead to the dysfunction and death of multiple organs of the host. Using data collected from 2017 and published in PubMed, sepsis affects approximately 30 million people per year with over 8 million of these cases ending in death. It should be noted though that this data is considered to be an underestimate of the actual number of cases and deaths as statistics surrounding sepsis are poorly recorded due to a lack of sufficient and adequate healthcare (Dugani, Veillard and Kissoon, 2017). Sepsis is defined as the systemic immunological and inflammatory response to an infection throughout the body caused by the over migration of neutrophils and release of cytokines, chemokines and NETs, which can be initiated by activation of the complement protein C5a amongst others (Pop-Began *et al.*, 2014). An infection generally starts at one point within the body with a self-limited inflammatory response, though the dangers of sepsis start when this response exceeds the control mechanisms which compensate to keep the unaffected sections of the body within homeostasis. When these mechanisms are exceeded, microbes from the initial infection leave the site of infection and travel via the bloodstream to various other parts of the body resulting in a systemic infection (Guo and Ward, 2004). This induces body wide inflammation which will eventually, if left untreated lead to organ dysfunction and death. This study aims to test the hypothesis that the anti-malarial drug Artesunate will

prevent the over activation of neutrophils and release of cyto/chemokines and NETs caused by C5a in a controlled version of a systemic infection.

3. Methods

3.1 Blood samples and ethics

Blood samples were taken by a trained phlebotomist under University of Westminster ethics number ETH2324-0913. These vials of blood were drawn on the same day that the experiments were to be conducted and were used within 3 hours of collection.

3.2 Human neutrophil extraction

100% pure samples of human neutrophils were obtained from whole blood using MACSxpress® Whole Blood Neutrophil Isolation Kit as per manufacturer's instructions. Blood was mixed with MACSxpress Beads and negative magnetic isolation was used to separate neutrophil suspensions from other blood cells. Neutrophil suspensions were centrifuged for 10 min at 200 RCF. The supernatant was discarded and cells resuspended in DMEM + 10% FBS. Cells were identified through a fast-acting modified version of the May-Grünwald-Giemsa staining as described below.

3.3 Identification of isolated cells

Cell suspension was spun down onto glass slides using a cytocentrifuge at 400 RPM for 5 min and left to air-dry overnight. A modified version of the May-Grünwald-Giemsa staining was used to identify cell types (RAL DIFF-QUIK kit, RAL diagnostics). Slides were suspended in RAL Diff-Quick fixative solution (methanol based solution to stabilize cellular components) for 1 min, in RAL Diff-Quik solution I (Xanthene solution; a buffered solution of Eosin Y) for 1 min and in RAL Diff-Quik solution II (a buffered solution of thiazine dyes, consisting of methylene blue and Azure A) for 1 min. Nuclei were meta-chromatically stained red/purple and cytoplasm pink/yellow, as shown below.

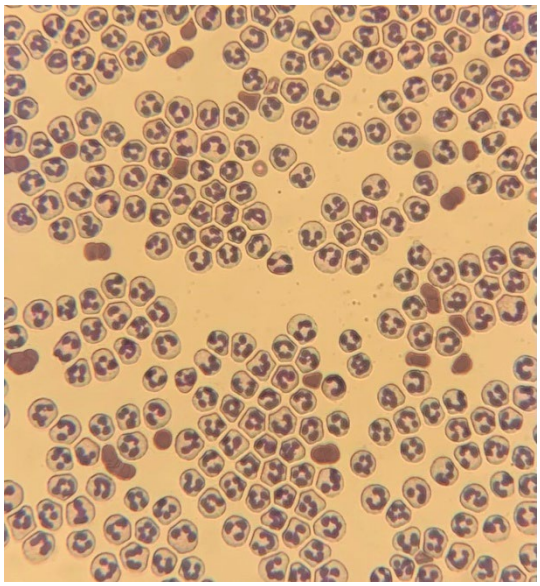

Figure 1. *Capture of RAL Diff-Quik (A modified Wright-Giemsa staining procedure) stained neutrophils from one of the extractions performed, with purple nuclei showing their characteristic shape and clear cytoplasm.*

3.4 ibidi μ-slide chemotaxis assays

ibidi μ-slide chemotaxis assay chambers, precoated with collagen IV along the central migration strip, were purchased from Thistle Scientific Ltd (Uddingston, Glasgow, UK). Neutrophils, isolated as above from human blood, were re-suspended within 30 min of collection in DMEM + 10% FBS at a concentration of 5 ×105 cells per ml and 6 μl was seeded along the central migration strip of an ibidi μ-slide chamber as per the manufacturer's instructions. Slides were incubated for 1 h at 37 °C in humidified 95% air/5% CO2, to allow neutrophil adherence to the central migration strip. DMEM (without added FBS) with and without added C5a (0.1-100nM) was then added to the wells on opposite sides of the central migration strip. For experiments in which effects of artesunate was to be tested, equal concentrations were added to both sides of the assays to create a linear concentration across the entire assay. Slides were incubated at 37 °C in 95% air/5% CO2 for 20 min to allow the generation of gradient of chemoattractant across the 1 mm wide × 70 μm deep central cell migration strip. Live-cell time-lapse microscopy was then conducted using a 10 × lens and dark-field illumination on a Nikon Eclipse Ti-E inverted microscope equipped with the Nikon Perfect Focus System (PFS). The microscope was housed in a temperature-controlled Perspex box (Solent Scientific) at 37 °C, with slides housed in a stage-mounted block in humidified 95% air/5% CO2. A maximum of 12 individual chambers (4 individual slides, 3 chambers per slide) could be imaged per experiment by using a motorized stage. Stage movement, lens focus and image acquisition were controlled by Nikon NIS Elements software. Experiments were conducted over 2 h, with images of each assay compartment taken every 2 min. The ImageJ Fiji TrackMate plug-in was employed to track individual neutrophils/macrophages. A chemotaxis and migration plug-in, provided by Ibidi, was used to calculate speed and forward migration index (FMI) data from the neutrophil tracks.

3.5 ELISA Quantification of cytokines, chemokines and NETs

Neutrophils, isolated as above, were incubated with C5a for 4hrs and 37°C/5%CO_2 and the release of CXCL1, IL8, IL1b and IL6 and NETs were measured by ELISA using commercial kits (DuoSet; R&D Systems), with absorbance readings taken at 450 nm on FlexStation 3 Microplate Reader (Molecular Devices, CA, USA). The results are expressed as ng/mL or pg/mL of each cytokine/chemokine). In the same samples, the amount of cell free DNA (cf-DNA) was quantified as a measure of NET release using the Quant-IT™ PicoGreen® kit (Thermo Fisher) according to the manufacturer's instructions. The fluorescence intensity (excitation at 488 nm and emission at 525 nm wavelength), a measure of the amount of dye bound to DNA, was quantified by a fluorescence reader (FlexStation 3 Microplate Reader). The results are expressed as ng/mL of cf-DNA.

4. Results

4.1 C5a results in chemotaxis of human neutrophils

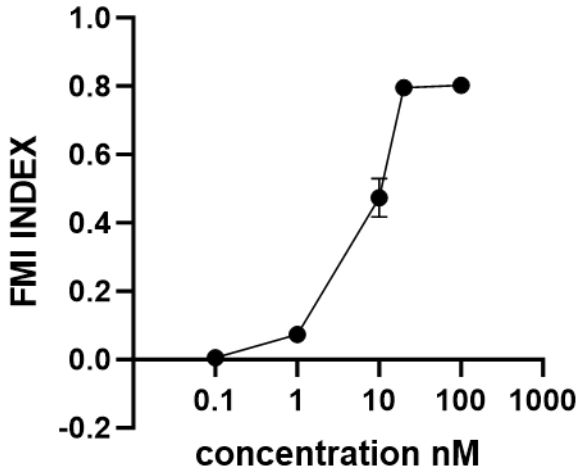

Figure 2.1 – Forward migration of neutrophils toward different concentrations of C5a in ibidi assays. The standard deviation was calculated and can be seen through deviation bars located on the plotted points (see point at 10nM).

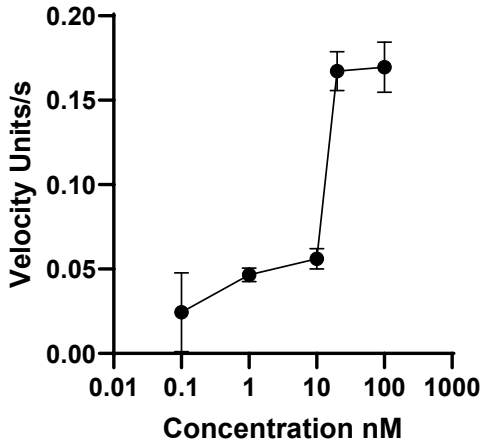

Figure 2.2 – Velocity of human neutrophils towards different concentration of the C5a in ibidi assays. The standard deviation was calculated and can be seen through deviation bars on each point except the point at 1nM.

Addition of C5a to an ibidi plate also containing human neutrophils led to significant forward migration of the neutrophils (Fig. 2.1). A relationship between the concentration of C5a and the forward migration of the neutrophils can be observed within Figure 2.1, as the FMI Index curve shows a positive correlation with the increasing concentration of C5a dosage. The lowest dose of C5a at 0.1 nM showed effectively 0.0 on the FMI index with a concentration of 1nM showing a 10% increase on the FMI index at 0.1. In addition, at 10nM of C 5a, the FMI index shows a reading of 0.5, an increase of 400% whilst at the highest concentration of C5a (100nM) the FMI recorded was 0.8 which is an increase in forward migration from 1nM by 700% though only a 60% increase from the result at 10 nM. The plateau shown by Figure 2.1 which forms at the concentration of 100 nM of C5a shows that 10nM would be the most ideal concentration to use in further experiments whilst achieving maximal effects without plateauing.

Higher concentrations of C5a significantly increase the velocity at which neutrophils move towards C5a. This is seen in Figure 2.2 with the velocity of these neutrophils increasing from 0.03 microns/second to 0.17

microns/second where the figure starts to plateau. This is an increase in velocity of 466.67%. As seen in Figure 2.2, low concentrations of C5a such as the lowest tested (0.1 nM) still cause the velocity of neutrophils to be higher than 0.00. Whilst not shown in the graph, Neutrophils are not seen to normally ever be completely still unless deceased. This is due to calcium signaling within the human neutrophils which causes the neutrophils to show small random movements.

4.3 Addition of the antimalarial compound artesunate, decreases the chemotaxis of human neutrophils towards C5a

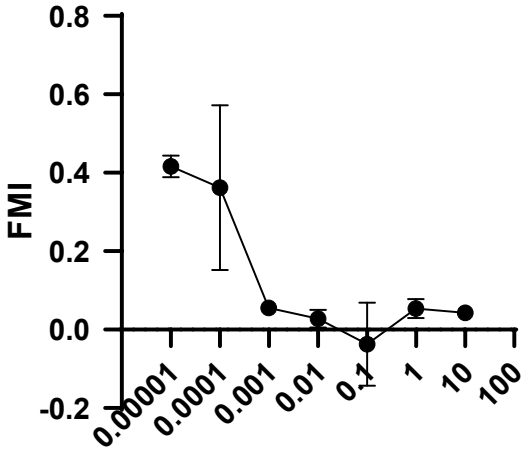

Figure 3.1 - Forward migration of neutrophils toward the chemokine C5a (10nM) with the addition various concentrations of artesunate. The standard deviation was calculated and can be seen through deviation bars located on the plotted points (see point at 0.0001nM).
This figure shows a significant drop in FMI when the concentration of artesunate is increased with the lowest level of FMI being at 0.1, though the error bar shown is the 2nd largest on the figure.

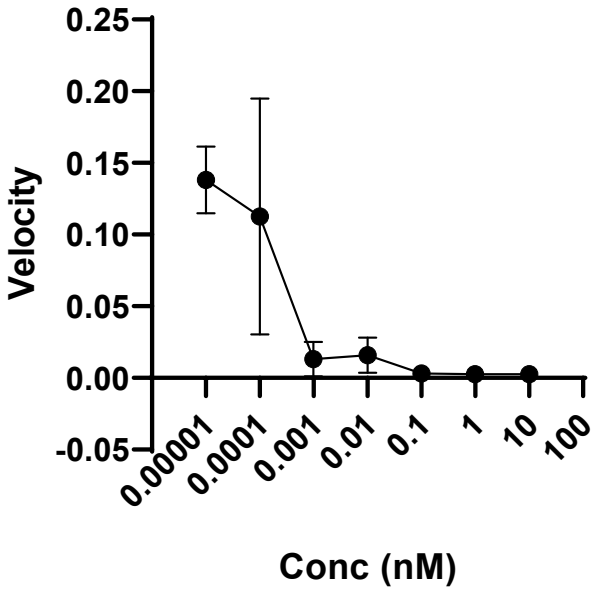

Figure 3.2 – Velocity of human neutrophils towards the chemokine C5a (10nM) in nanometres per second, with the addition of various concentrations of Artesunate. The standard deviation was calculated and can be seen through deviation bars on each point except the point at 0.0001nM. This figure shows the significant effect that artesunate has on the velocity of human neutrophils with practically no movement of neutrophils being observed between the addition of 0.1 and 10 nM of artesunate.

The addition of artesunate to the human neutrophils whilst in an ibidi slide along with C5a (10nM) causes a significant decrease in chemotaxis. This is seen in Figure 3.1. The inhibitory concentration 50 (IC50) value of artesunate can be equated to approximately 0.000307 nM through the use of Figure 3.1, though due to the large error bar at the point inline with 0.0001 nM of artesunate, this should be repeated to find a more accurate IC50 value. It is seen within figure 3.1 that artesunate has an effect at all concentrations tested, though the largest effect is seen at a concentration of 0.1 nM indicating that in future experiments, this may prove to be the most viable concentration to achieve maximum results.

At all concentrations of artesunate added seen within Figure 3.2, the velocity at which the human neutrophils move is decreased. Figure 3.2 can be compared to the results found in figure 2.2 and it can be concluded that artesunate causes a significant decrease in the velocity at which neutrophils travel towards C5a. It can be seen that that artesunate has an effect on neutrophils velocity at all concentrations tested, with the highest value recorded being approximately 0.14 nanometres/second and the lowest value recorded being equivalent to complete cessation of movement, these being at values exceeding 0.1 nM of artesunate.

Artesunate causes a reduction in the release of cytokines, chemokines and Nets from C5a-stimulated neutrophils

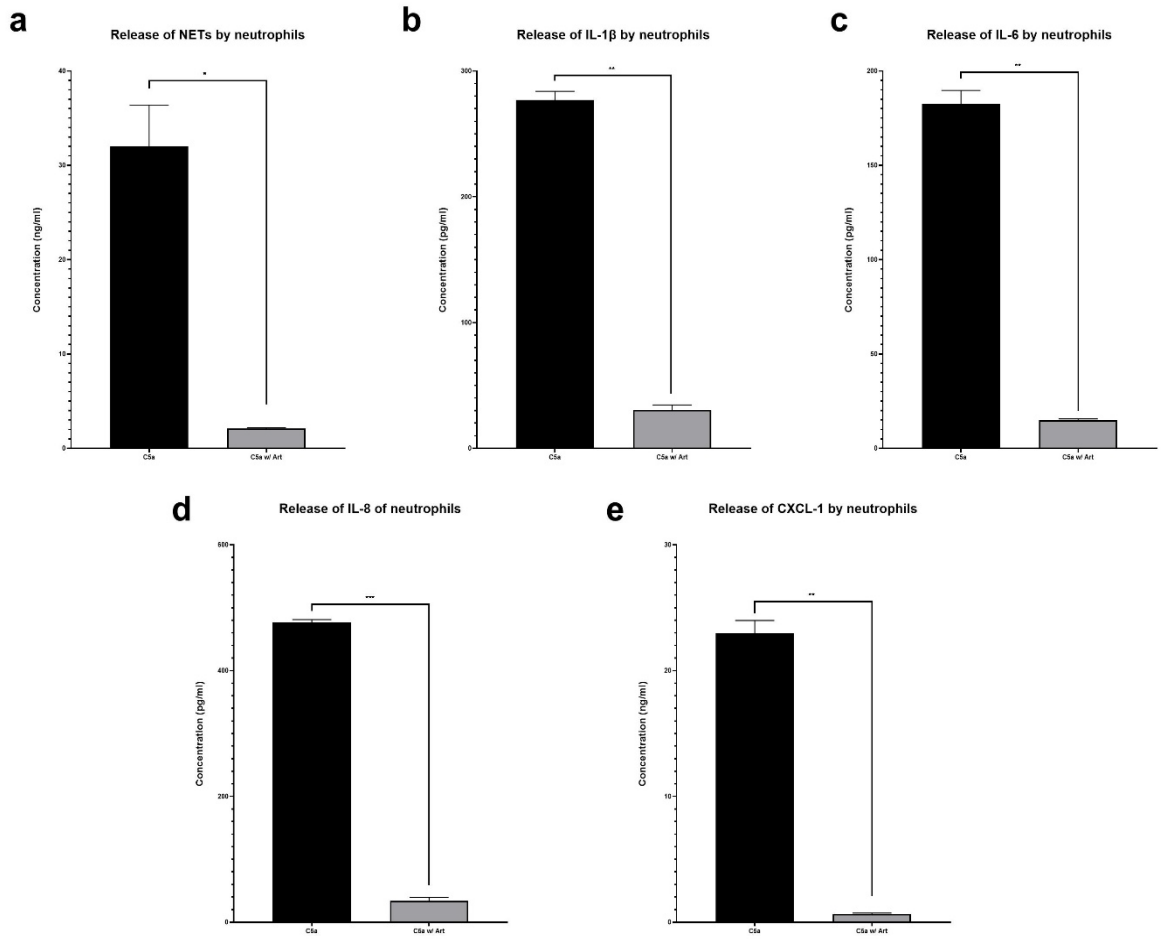

Figure 4. *Concentrations of* ***(a)****NETs,* ***(b)*** *IL-1β,* ***(c)*** *IL-6,* ***(d)*** *IL-8, and* ***(e)*** *CXCL-1 released by neutrophils incubated with 10 nM C5a. The black bars correspond to experiments only incubated with C5a, however the grey bars were incubated alongside 10 μM artesunate hence allowing comparison of the degree of inhibition. Each error bar shows mean ±SEM from n=2 trials. Two-tailed unpaired t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001 for artesunate trials compared to non-artesunate groups.*

The black bars in Figure 4 correspond to the samples from neutrophils incubated with only 10 nM C5a, while the grey bars had neutrophils incubated with both 10 nM C5a and 10 μM artesunate. This high value of artesunate was chosen so that maximal inhibition could be measured. The ELISA charts show that artesunate inhibited the release of all measured inflammatory substances from C5a-stimulated neutrophils, but specifically by: 93% for NETs (31.98 ng/mL ±4.411 to 2.097 ng/mL ±0.091); 89% for IL-1β (276.9 pg/mL ±6.93 to 30.35 pg/mL ±4.11); 92% for IL-6 (182.4 pg/mL ±7.318 to 14.87 pg/mL ±0.7145); 93% for IL-8 (477 pg/mL ±4.129 to 33.69 pg/mL ±5.588); and 97% for CXCL-1 (22.98 ng/mL ±1.011 to 0.645 ng/mL ±0.081). All the decreases are significant indicating that artesunate serves very well as an anti-inflammatory drug, in line with previous studies in mice.

5. Discussion

As it has been established in the 2022 study by Morad *et al.,* artesunate is a potent inhibitor of chemotaxis and inflammatory action in mice neutrophils during systemic inflammation. By initiating systemic inflammation using LPS/Covid-19 spike protein into the lung, they could cause neutrophilic invasion simulating that which happens during systemic infections. By providing a prophylactic injection of

artesunate, the researchers were able to effectively neutralise neutrophilic infiltration into mouse tissues, highlighting the potential of artesunate in the field of systemic inflammations (Morad, *et al.,* 2022). This leads into the trials using human neutrophils, at the present *in vitro*. Firstly, it was necessary to acquire a pure sample of human neutrophils to investigate which was obtained via the extraction steps detailed previously. Centrifugation and staining would ensure that the assays on different tests would be as consistent as possible by ensuring neutrophil purity (>98%) and concentration as these could all impact in the end, the neutrophils' sensitivity to chemoattractants.

Part i: C5a concentration µ-slide chemotaxis assays

In the ibidi assays, the human neutrophils display explosive positive chemotaxis towards C5a showing up to a 47-fold increase from baseline chemotaxis to chemotaxis at 20 nM (0.017 ±0.001 to 0.795 ±0.005), highlighting the importance of C5a as an inhibitory target. This data is further backed up with current knowledge of the role of C5a and its interactions with neutrophils, such as with alveolar neutrophils who cleave C5 into C5a and serves to recruit further granulocytes, such as neutrophils to the site of insult while also reducing neutrophil apoptosis and contributing to vasodilation (Guo, Riedemann, and Ward, 2004).

These factors combine to explain how sepsis arises as the initial recruitment due to the infection serves as the initial surge of neutrophilic bombardment and when the infection is not cleared, neutrophilic processing of complement proteins into C5a cause persistent neutrophilic recruitment, creating a sink in the affected tissue with this neutrophilic presence being highly damaging to the tissue due to them causing oxidative stress (via ROS release), and coupled with degranulation/NET release which releases their cytotoxic products into the tissues (Yang, *et al.,* 2021). Furthermore, the vasodilatory effect of C5a causes the small vessels of the lungs to become 'leaky' causing oedema, another characteristic of sepsis induced ARDS (Yang, *et al.,* 2021), and eventually these factors lead to organ dysfunction via both the loss of the pulmonary microenvironment essential for proper ventilation (oedema closes up alveoli and increases pressure within thoracic cavity, preventing the proper filling of lungs with air.) and also simply due to overwhelming tissue damage (damage to alveolar cells/capillaries which reduces gas exchange.).

Part ii: Artesunate concentration µ-slide chemotaxis assays

Armed with this knowledge, the implications of artesunate as an anti-inflammatory agent become clear. Via further trials with the µ-slides, artesunate inhibits human neutrophils similarly as observed in murine experiments with a 94% decrease in chemotaxis observed with 0.01 µM of artesunate even when exposed to 10 nM of C5a (The EC_{50} value carried forward from the previous tests) which normally elicited chemotaxis of almost 30 times the control value. It instead brings the xFMI value at 0.01 µM of artesunate down to 0.028 ±0.013, equivalent to the control xFMI value at 0 nM of C5a of 0.017 ±0.001 (Two-tailed unpaired t testing showed the difference between these two values as non-significant, $p = 0.4519$). Essentially, meaning that a dose of artesunate merely of 0.01 µM was enough to inhibit neutrophil chemotaxis back down to non-activated levels, a major discovery as because the cycle of neutrophilic recruitment and subsequent accumulation described before could be halted, a wedge driven in to halt or lessen the symptoms of sepsis/ARDS allowing it to be more clinically manageable.

In previous research, this inhibitive action was not shown at a similar level in other anti-malarials, suggesting a unique mechanism of action owned by artemisinin analogues (Morad, *et al.,* 2022). The underlying structure for inhibition is uniquely linked to artemisinin analogues, which is the endoperoxide 1,2,4-trioxane ring, as every analogue of artemisinin showed similar levels of inhibition apart from deoxyartemisinin, which lacks this crucial structure. This inhibition has been shown to not be through direct antagonism but instead modification of the protein through cysteine residues catalysed by Fe^{2+} as by subjecting the serum to chelation to remove the iron ions, this completely mitigated artemisinin's inhibition capabilities (Morad, *et al.,* 2022).

It was deduced that artesunate likely exhausts intracellular calcium stores by targeting sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA) 3. In experiments with neutrophils, artesunate invoked a spike in cytoplasmic calcium levels despite no external calcium present, thus ruling out passive or active

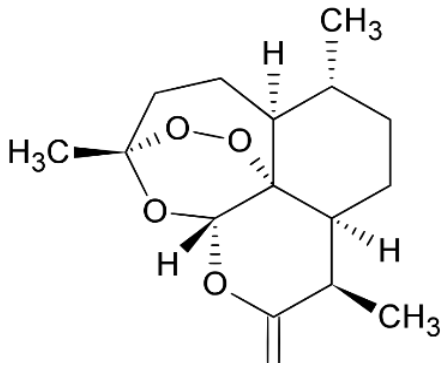

Figure 8. *Chemical structure of artesunate showing the endoperoxide 1,2,4-trioxane ring. (Morad, et al., 2022)*

calcium influx as the cause of this increase. This observation is consistent with a similar event seen with a known SERCA inhibitor, thapsigargin; hence, no leading calcium pulses can be generated which leads to the inhibition of chemotaxis (Morad *et al.,* 2022). This was demonstrated with the direct effect artesunate has on chemotaxis towards H_2O_2, which potently inhibited chemotaxis towards it via direct interference with the generation of leading calcium pulses, unlike other studied inhibitors which were antioxidants and hence affected the H_2O_2 gradient, inhibiting the generation of ADPR, the ligand interacting with the TRPM2 channel and causing chemotaxis (Morad, *et al.,* 2022). As such when ADPR was supplied, chemotaxis was no longer inhibited with these other inhibitors unlike with artesunate. SERCA3 was specifically ruled as the targeted isoform as other SERCA inhibitors also target SERCA1 and SERCA2, critical isoforms in muscle contraction or expressed in many organs respectively meaning these inhibitors are very toxic whereas artesunate is not. One such inhibitor is the previously mentioned thapsigargin, which displays the Ca^{2+} exhaustion seen with artesunate hence linking the activity of artesunate as a SERCA3 inhibitor (Morad, *et al.,* 2022).

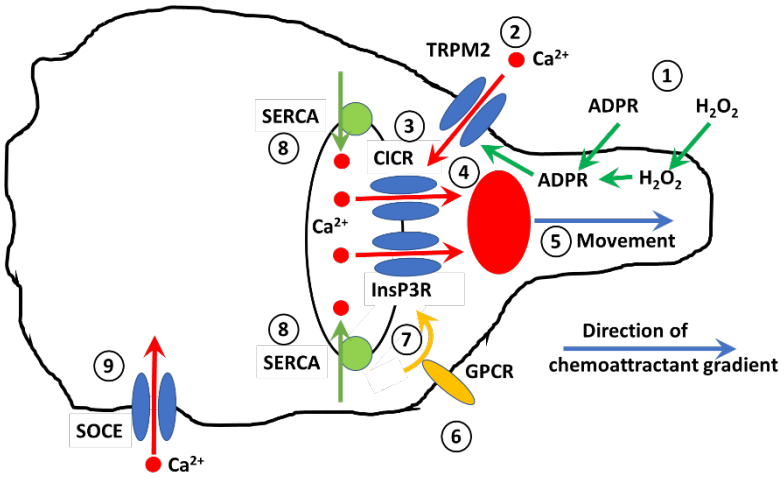

Figure 9. *Schematic diagram of intracellular pathways dictating neutrophil chemotaxis (Hassan et al., 2022).*

With the use of artesunate to prevent neutrophilic infiltration during systemic inflammation, cytotoxic shock to the pulmonary tissues would be lessened as there would be less neutrophils to cause damage meaning it would be easier for the patient to recover and reduce the severity of the condition, however the finding thus far only prevents future accumulation but does not deal with the already present army of neutrophils causing damage. However, a limitation of these assays was their sensitivity, old reagents such as an old artesunate stock produced crystals which interfered with reading the cells during the ImageJ processing, and lead to wide error bars. Further repeats should've been carried out to narrow the bars and eliminate any outliers or erroneous results arising from poor reagents and further strengthen the observations seen.

Part iii: ELISA tests

The ELISA tests provided evidence of artesunate's additional inhibitory actions, its effect on the release of NETs, and some interleukins to name a few. Artesunate inhibited the release of the cyto/chemokines by on average 93%, a staggering decrease which would directly correlate to a decrease in inflammation namely with the interleukins IL-1β, IL-6 and IL-8 who are heavily associated with the onset of COVID-19 cytokine release syndrome (CRS) (Darif, *et al.,* 2021). When IL-6 inhibited (via the monoclonal antibody, tocilizumab), it was associated with an increase in survival, the improvement of ARDS in patients and a decrease in inflammatory markers with similar improvements being observed when inhibiting IL-1β with the drug Anakinra (Darif, *et al.,* 2021). On the other hand, IL-8 is correlated with netosis (the release of NETs) as shown in the 2005 study which noted that inhibition of IL-8 via anti-IL-8 antibodies caused a decrease of netosis associated with neutrophil activation (Gupta, *et al,*. 2005). The detection of netosis can be done via the measurement of 2 highly specific biomarkers: MPO-DNA, and citrullinated histone H3 (Cit-H3) which were previously shown to be elevated in many COVID-19 patients (Zuo, *et al.,* 2020; Darif, *et al.,* 2021) indicating a large amount of NET release, meaning that IL-8 inhibition would directly reduce NET release which is associated with reduced lung damage (Castanheira, and Kubes, 2019). Similarly, blocking CXCL-1 also showed promising results in supressing inflammatory lung damage while also reversing neutrophilic influx in mice (Komolafe, and Pacurari, 2022) which could indicate that a similar result may occur in humans.

Overall, the inhibition of these various key markers of inflammation through artesunate usage would lead to an improvement in sepsis/ARDS severity even after neutrophil inflammation, however these results could be more conclusive by including within the ELISA assays a double negative control, excluding both

artesunate and C5a from the solution, in order to gain a value for the baseline level of release so that the artesunate assay could be compared to it to gauge the difference between cyto/chemokine release of the two tests. Furthermore, the relationship between IL-8 and NETs should be investigated to highlight if artesunate is directly inhibiting NET release or via the means of inhibiting IL-8 with a potential experimental procedure being incubating the neutrophils with artesunate and IL-8 to see if the IL-8 bypasses the drugs inhibition to cause netosis.

6. Conclusion and future work recommendations

The findings presented here have important clinical implications for the development of novel anti-inflammatory therapies. Artesunate, with its demonstrated efficacy in preventing C5a-induced chemotaxis and cyto/chemokine and NET release in the inflammatory response, holds promise for the treatment of not only sepsis/ARDS, but various inflammatory conditions with which neutrophilic invasion is associated such as atherosclerosis or acute pancreatitis (Rosales, 2018). Moreover, further investigation is warranted to elucidate the underlying mechanisms of artesunate's inhibitory effects on pro-inflammatory substance release as although the mechanism through which artesunate interferes with SERCA3, disabling the Ca^{2+} mediated process of locomotion in neutrophils, has been elucidated, conversely the secondary target which blocks the synthesis and/or release of pro-inflammatory substances remains obscured (Morad, *et al.,* 2022). Hence, further research is justifiably warranted to fully elucidate its therapeutic potential and pave the way for its transition into clinical practice.

7. Lessons learned

We learned the importance of thorough planning of experiments before beginning them, as often there are many complicated stages to each experiment, each of which must be understood before commencement. We learnt that even with good experimental technique errors can occur, due to human error and inherent errors in analysis software. We learnt to work as a team as find practical solutions to problems in the lab.

8. Research Group Reflection

As a team, everyone worked well together with only a few minor issues throughout the way. These issues were mainly down to scheduling issues and traveling to university at certain times may have caused some issues, mostly in part due to public transportation strikes or incidents. The problems that were in our control could easily be corrected in the way that we did correct them, by scheduling better and learning each other's schedules though the issues out of our control such as public transport union strikes, we unfortunately have no way of avoiding. Overall, this was a successful project and everyone, both staff and students alike worked well together and built a good staff-student relationship, something key to making projects run smoothly.

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