Investigating Alterations in Macrophage Inflammatory Status and the effect on anti-CD47 Inhibitor Treatment for MDS

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Abstract

Myelodysplastic syndrome (MDS) is a rare form of blood cancer that can predispose individuals to acute myelodysplastic leukaemia. New treatment options aim to inhibit the action of CD47, an anti-phagocytic signal that increases in number as MDS progresses and prevents phagocytosis of leukemic cells. These treatments, however, have faced limited success in late stage MDS patients. It is theorised that the poor response rate is caused by a phenotypic shift in the macrophage population; becoming more anti-inflammatory because of a tumour-created micro-environment, as evidenced by Yang et al (2021). In this project, we aimed to use Venetoclax; a drug which could potentially shift the overall macrophage population to a pro-inflammatory M1 state, from an unpolarised M0 state or an anti-inflammatory M2 state to support anti-CD47 MDS treatment. We measured the impact of Venetoclax on the metabolic activity of the macrophages and utilised flow cytometry to measure specific cell markers to identify shifts in macrophage phenotype. Our results indicated that Venetoclax is minimally toxic to macrophages at concentrations around 1-2nM – when drugged with Venetoclax, the M0 macrophage population did shift, but not to an M1 phenotype, with the result of the transformation remaining unknown. However, Venetoclax does alter the M2 phenotype to an M1 state; ideal for a potential MDS co-treatment if further repeats and patient trials prove so.

Keywords: MDS, AML, CD47, CALR, Macrophage, Venetoclax

Introduction

Myelodysplastic syndrome (MDS) is a clonal blood disorder which leads to alterations in haematopoiesis, which can progress into acute myelodysplastic leukaemia (AML) and is increasingly prevalent with the aging population [Ogawa S, 2019; Maegawa S, et al, 2014].

This paper focuses on enhancing the treatment of MDS through anti-CD47 drugs, specifically, how we can increase their effectiveness in late-stage treatment using Venetoclax – a drug which can increase macrophage phagocytic activity [Rodriguez-Sevilla JJ, et al, 2023]. Whilst there has been interest in using CD47 inhibitors to treat MDS, clinical trials in late stage MDS have been less successful, which is theorised to be due to phenotypic shifts in the macrophage population towards the anti-inflammatory M2 state, caused by a tumour-altered microenvironment.

This study recorded and analysed how Venetoclax affects cell metabolism using MTT assays, and its potential to shift macrophages to a pro-inflammatory/anti-tumour M1 state using flow cytometry analysis to determine its potential as a drug.

Project Background

MDS arises when the stem cells responsible for producing myeloid blood cells operate abnormally and replicate without countermeasure [Dotson JL and Lebowicz Y, 2022]. As MDS progresses, transformed myeloid cells express more anti-phagocytic signals/CD47 and decrease the expression of pro-phagocytic signals/CALR to evade the immune system [Boasman K, et al, 2018]. New CD47 inhibitor treatment approaches have been developed to inhibit these anti-phagocytic signals in the affected cells either by themselves or in combination with standard therapies to enhance treatment and prevent progression into AML [Daver N, et al, 2025].

However, patients with late stage MDS treatment showed a decreased response to anti-CD47 drugs [Kapoor S, et al, 2021]; potentially caused by an alteration in the phenotype of surrounding macrophages due to a transformed microenvironment, increasing the proportion of less helpful anti-inflammatory/pro-tumour M2 macrophages to more helpful pro-inflammatory/anti-tumour M1 macrophages [Yang F, et al, 2021; Kouroukli O, et al, 2022].

Literature Review

MDS is group of myeloid neoplasms, classified by a decreased level of haematopoiesis, and commonly progresses into AML [Chen J, et al, 2019] because of shared mutations between the two diseases [Menssen AJ and Walter MJ, 2020]. Incidence rates of MDS are increasing, with the incidence being approximately 4 in 100,000 in 2019 [Zeidan AM, et al, 2019], and 4.6 per 100,000 the following year [Marsa A et al, 2020], MDS also has a sharp increase in incidence with age, with a median age of diagnosis at 71 years [Neukirchen et al, 2011], establishing the need for enhanced treatment strategies.

A newly emerging route of MDS treatment (outside of common medications like hypomethylating agent-based therapy which have success rates of roughly 50% in patients [Rodriguez-Sevilla JJ, et al, 2023]) has focused on CD47 inhibitor-based therapy. Most CD47 inhibitors are a category of immunotherapy drugs known as monoclonal antibodies which target the CD47 signal expressed on MDS transformed cells, ultimately resulting in phagocytosis of the transformed cell. CD47 acts to inhibit phagocytosis by communicating with SIRPalpha, a macrophage signalling molecule [Huang, C, et al, 2020] which triggers the “decision” to not phagocytose the mutated cell [figure 1]. Other targets include immune checkpoints and toll-like receptors [Gallazzi M, et al, 2022]. The effectiveness of CD47 inhibitors may decrease in late stage MDS because of a shift in macrophage phenotypes from pro-inflammatory/pro-tumour M1 to an anti-inflammatory/anti-tumour M2 phenotype.

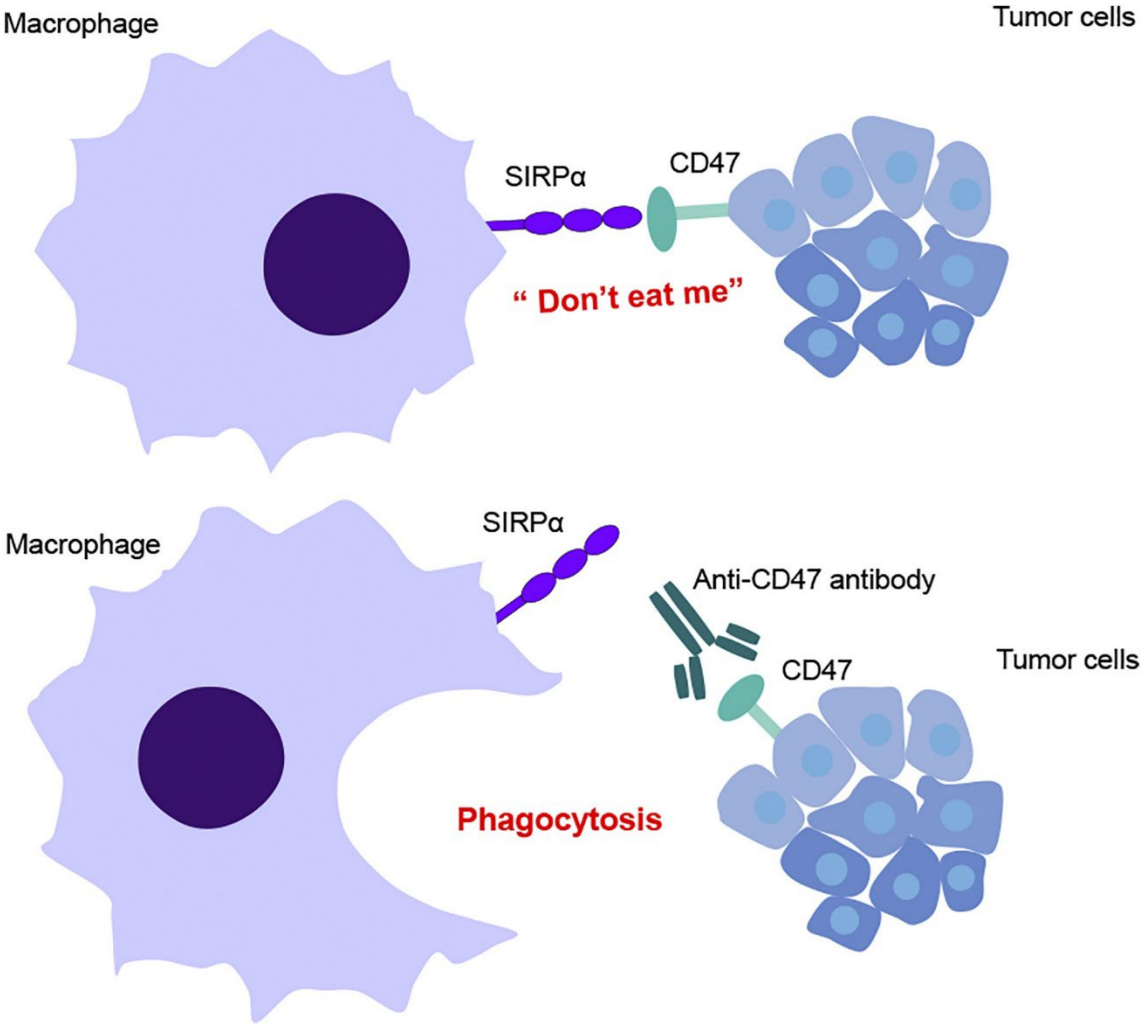

Figure 1: The result of monoclonal anti-CD47 antibodies on the macrophage-tumour cell interaction, dictating the phagocytic result. [Huang, C, et al, 2020].

New macrophage phenotype-altering therapies are emerging, such as Venetoclax; Venetoclax has a low objective response rate (19%) when used in monotherapy in treatment of AML [Konopleva M, et al., 2016] and so is most often used in combination with hypomethylating agents or other therapies when patients are ineligible for chemotherapy [Pollyea AD, et al., 2018]. Traditionally functioning as a B-cell Lymphoma-2 inhibitor, Venetoclax decreases the inhibition of apoptosis in transformed cells [figure 2] [Lasica and Anderson., 2021].

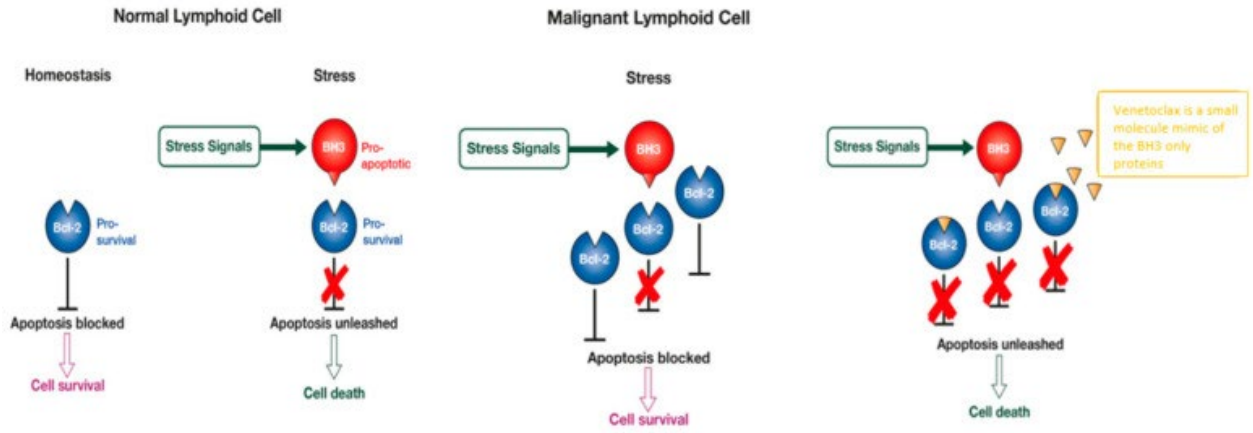

Figure 2: A simplified view of Venetoclax's effects on the complicated BCL pathway, other effects, such as the conversion of macrophage phenotype, may also be observed [Lasica and Anderson., 2021].

Methodology

Approved by the University of Lincoln, UK, Ethical committee; the THP-1 cell line (monocytes) was used as a seed to develop the M0 macrophages using Phorbol 12-myristate 13-acetate (PMA) differentiation, which causes cytokine production via Protein kinase C activation [Ai W, et al, 2013], initiating the monocyte's transformation into M0 macrophages. The cells were observed and photographed via a light microscope to visualise differentiation for three days of PMA treatment before being rested for a further two. When necessary, IL-4 and IL-6 were added for approximately 24 hours to develop M2 macrophages, and Venetoclax for 48 hours for drugged cells (using pre-established methods in the lab).

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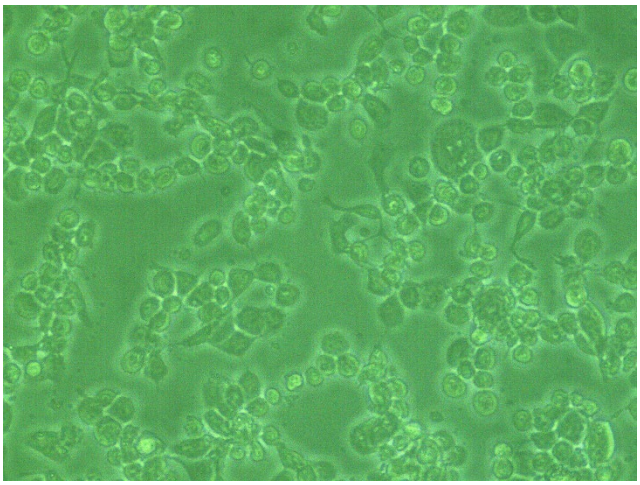

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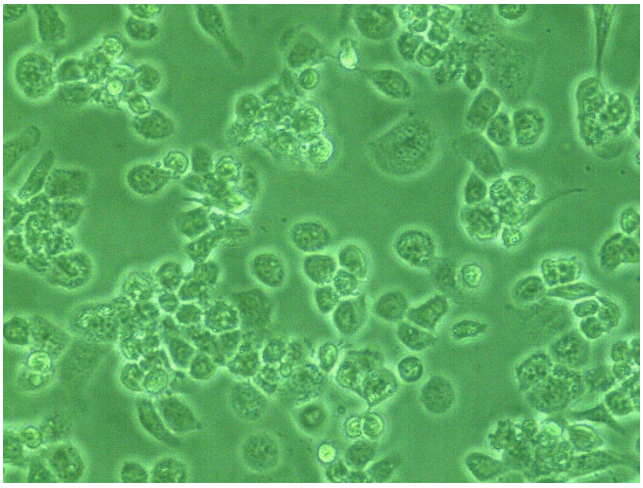

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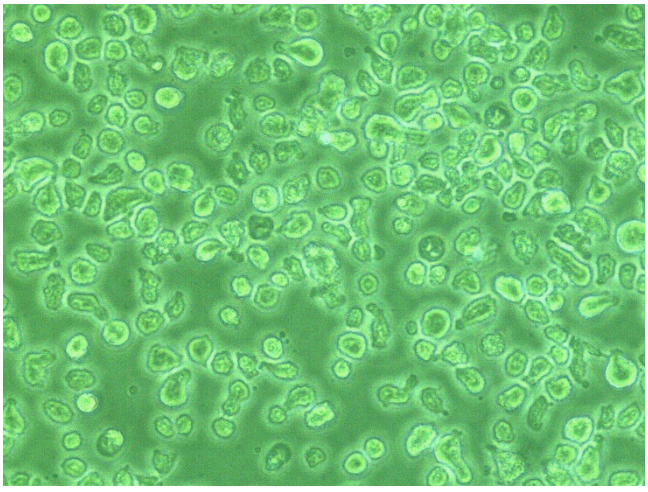

Figure 3: a: Day one of THP-1 cells with PMA. b: Day three of THP-1 cells with PMA. c: Day two of rest (reseeded with no PMA) with visible differences, including a more rounded, slightly larger shape, showing macrophages creation.

Rested THP-1 cells were used for MTT assays to measure the presence of metabolic activity [Meerloo JV et al, 2011] and compare how drugging with Venetoclax affects metabolic activity. However, phenol red contained in the FBS media interfered with spectrometer readings for the control, which required further optimization. In brief, the final MTT protocol used seeded cell numbers over one-hundred-and-thirty-thousand and went as follows: Aspiration of the supernatant, and resuspension of the cells with fresh media and an equal volume (50ul) of MTT solution; the plates were then incubated for 2-4 hours in a dark environment at 37^{o}c. Once a black precipitate appeared, the aspiration of the media-MTT solution was conducted, with the addition of 100 µl DMSO to act as a medium, and HCL to neutralize the phenol red; and finally, quantification of the absorbance using a spectrophotometer at 570nm with a 630nm reference wavelength.

Utilizing flow cytometry allowed for the effects, toxicity, and therefore, potential viability of the drug to be analyzed; different macrophage inflammatory states were measured by using dual-channel flow cytometry techniques. To lift the macrophages from the adhesion plate, 1ml acutase was added (after the aspiration of the media). Centrifugation of the macrophage-acutase solution was then undertaken before several washing steps with PBS. Once completed, the supernatant was removed, and the macrophages were resuspended in PBS – the addition of an Fc blocker was integral to avoid false positives via non-specific binding and proceeded the addition of the specific antibodies to indicate macrophage phenotype [figure 4]. The cells were incubated for 30 minutes in darkness, diluted in PBS and the underwent centrifugation before the removal of the supernatant. Finally, the macrophages were resuspended in the flow buffer and added to the Miltenyi Biotec MACSQuant Analyzer 16 Flow Cytometer. Using the MACSQuantify software, the cells were gated after a control was analysed, and the data was produced (figure 6a; 6b; 7). The procedure was conducted with one million M0, and M2 macrophages.

Macrophage Phenotype	Antibody Markers
M0	CD14 SIRPalpha
M1	CD80 CD64
M2	CD163 CD206

Figure 4: Macrophage phenotypes and relative antibody markers.

Results

The MTT assay showed that a 1nM concentration of Venetoclax resulted in a mean absorbance 1.59x greater than the 2nM concentration, signifying that a 1nM concentration was less/minimally toxic, but the flow cytometry showed 2nM concentrations held more pronounced results [Figure 5], Other concentrations (10nM and 20nM) of Venetoclax proved to be too toxic to the cells (data not shown), as such, both 1nM and 2nM were chosen for the following experiments.

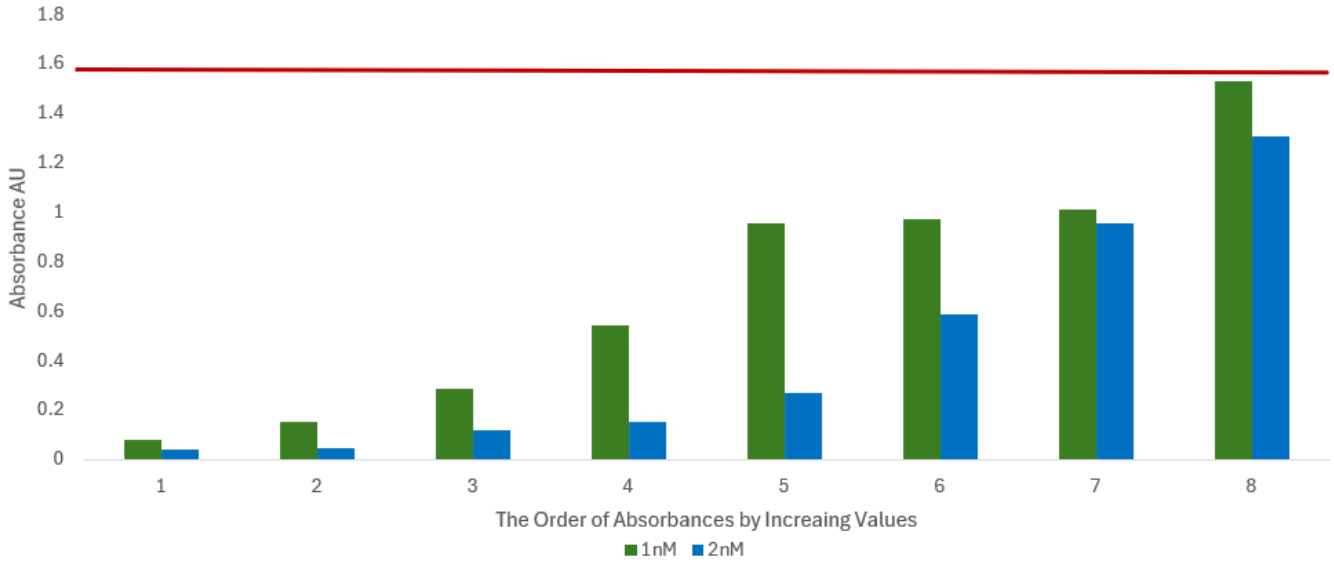

Figure 5: The Metabolic rate-proportional absorbance (AU) of 1nM and 2nM concentrations of Venetoclax on M0 macrophages in order of Increasing Values in an N=1 experiment, a control sample gave a top value 1.585 (red).

As we had theorized, applying Venetoclax to the M0 cells would see less expression of M0 markers, this correlates to less M0 macrophages, consistent with the theory that they had differentiated into M1 macrophages.

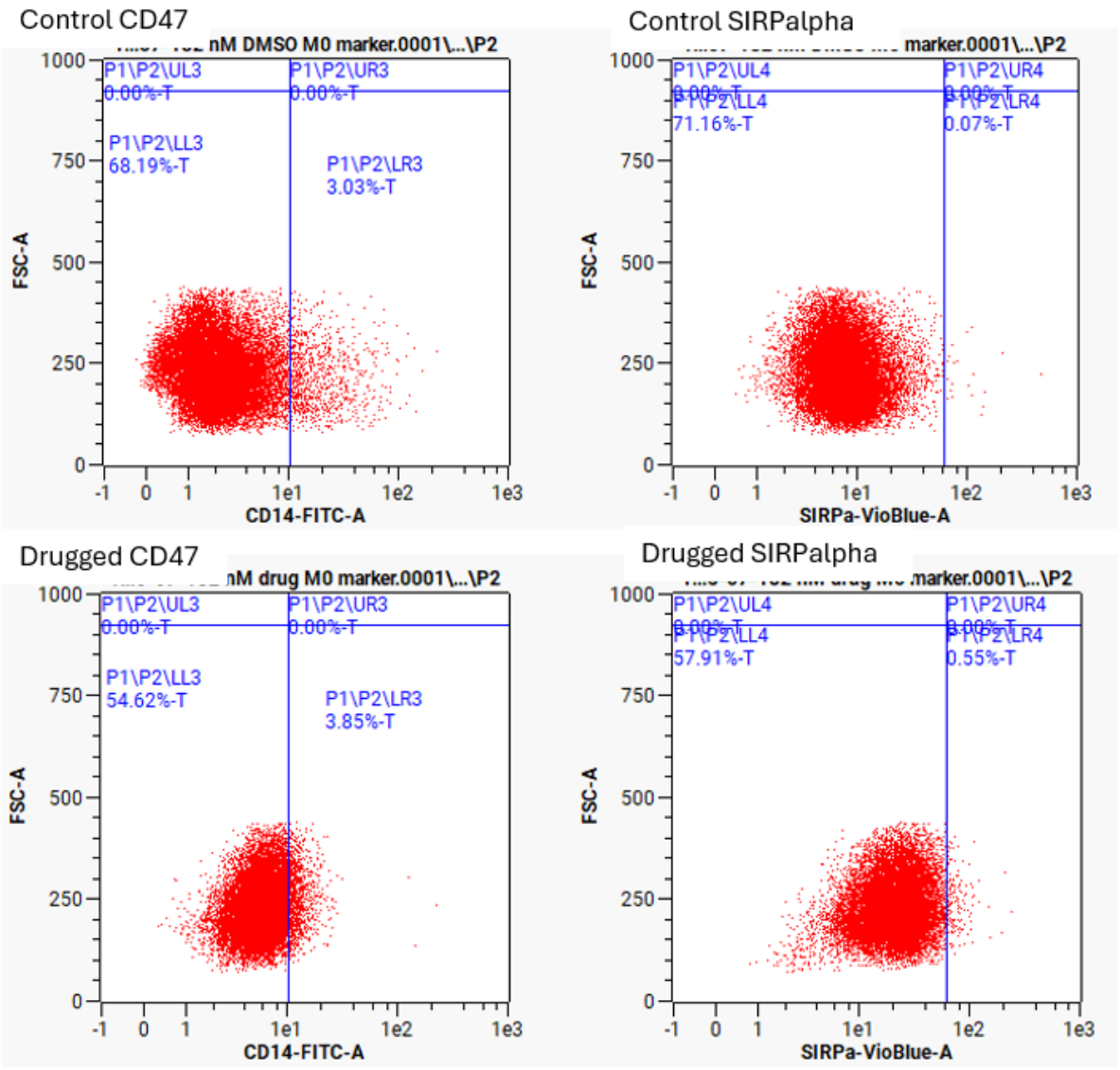

Figure 6a: The control (top) and drugged (bottom) M0 markers (CD14 (left), SIRPalpha (right)) on M0 cells at 2nM DMSO and Venetoclax respectively.

However, this theory was proved wrong, when the M1 markers were measured, only a small increase (3.3%) in fluorescence was found, suggesting the macrophages had not yet been converted to the M1 phenotype, despite not remaining in the M0 state [Figure 6a]. The result of this is unknown, further optimization and multiple (n=3) repeats are required to confidently show they do not transform into M1 cells, with research required to explore other avenues.

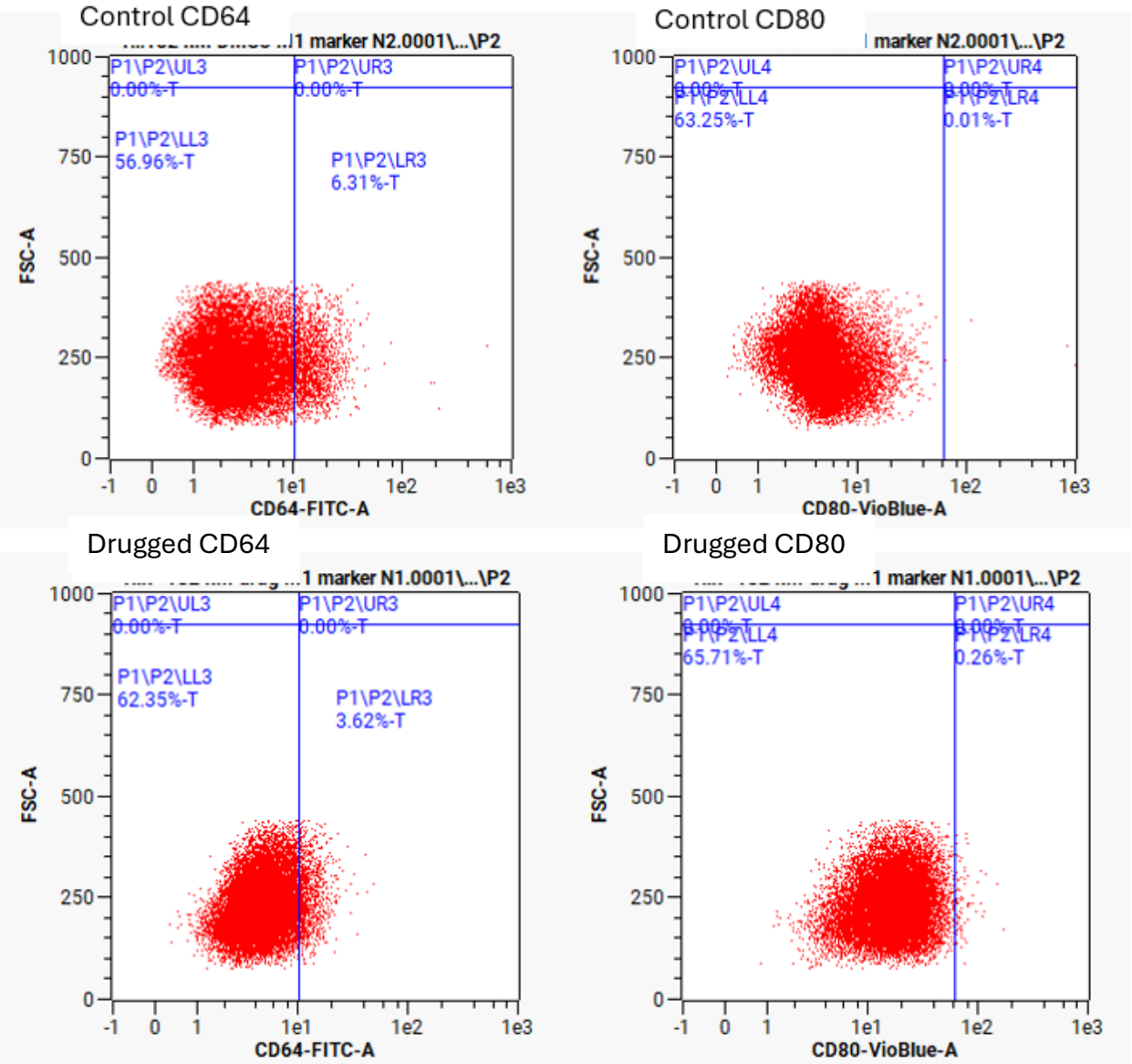

Figure 6b: The control (top) and drugged (bottom) M1 markers (CD64 (left), (CD80) (right)) on M0 cells at 2nM DMSO and Venetoclax respectively.

When M2 macrophages were drugged with Venetoclax, it appeared that a portion of them had developed into M1 macrophages, indicated by an increased fluorescence when exposed to M1 markers (Figure 7), as was theorized, allowing for possible viability for Venetoclax to act as a co-treatment for MDS.

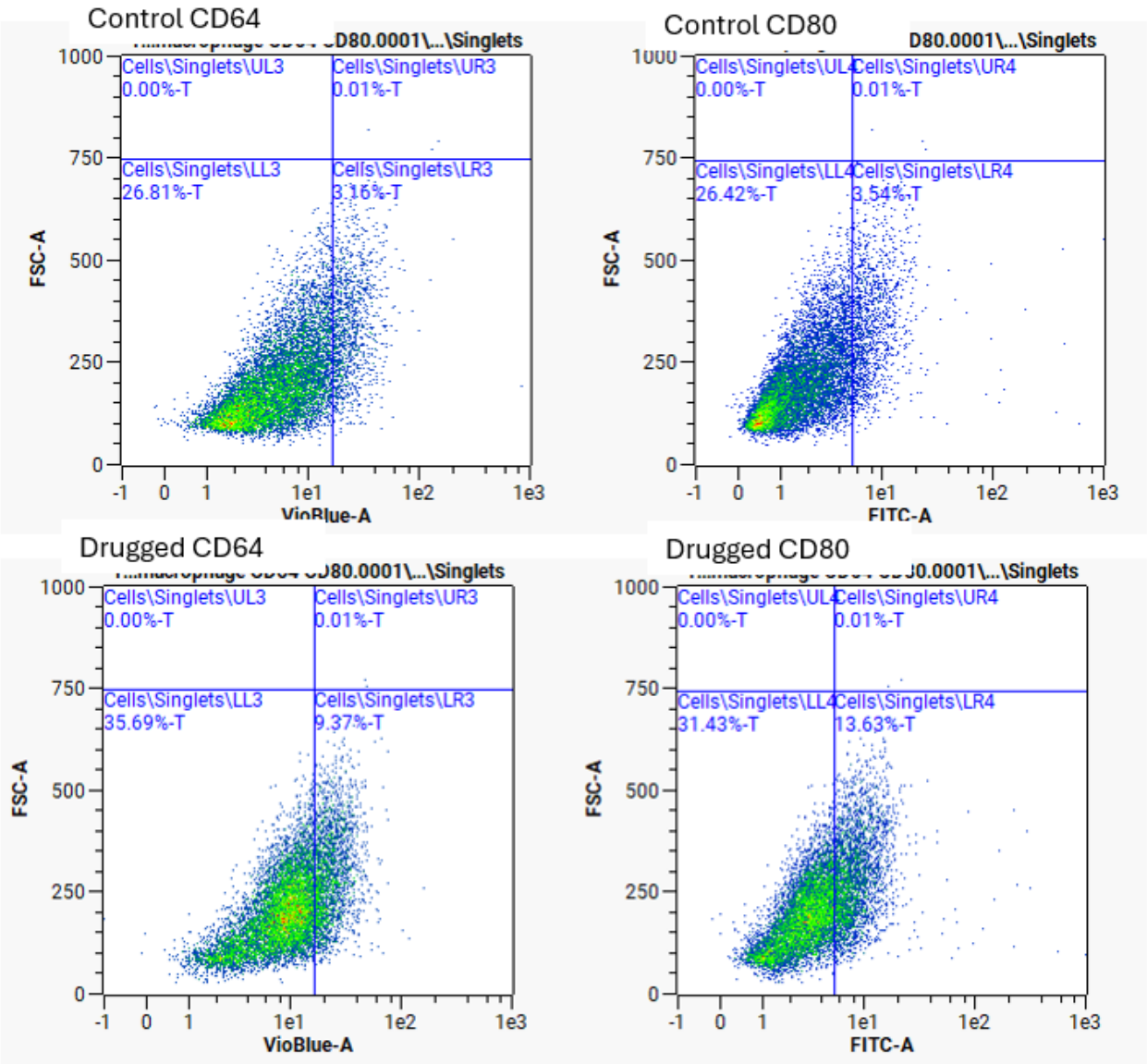

Figure 7: The control (top) and drugged (bottom) M2 macrophages with M1 markers (CD64 (left), CD80 (right)) at 2nM of DMSO and Venetoclax respectively, showing an increase in response rate to CD64 of 5.81% (3.16% to 9.37%) and to CD80 of 10.09% (3.54% to 13.63%) when given Venetoclax.

UROS Experience

UROS as a scheme developed under Student as Producer allowed me to engage in practical research experience I otherwise would not have had the opportunity to. Such a task involved using the Flow Cytometer, a flexible machine capable of reading the fluorescence, size, and complexity of individual cells. This was a machine I had studied

in my second year Immunology module but had only had experience using in this research study.

I appreciated the independency and autonomy I was given when undertaking laboratory procedures. I felt I played an instrumental role in the project, which I value as the least experienced on the team. Consequently, I felt more prepared to engage in academic and practical opportunities, such as the NHS placement year in Immunology and Blood Sciences from September 2025.

With more time I would have increased the repeats for the experiments and addressed the systematic error that appeared on the MTT assays. I would recommend the UROS scheme to anyone looking to gain practical experience and understand the relevant professional environment.

Conclusion

The results for the M2 macrophages allow us to suggest that Venetoclax could be a suitable candidate for the co-treatment of late stage MDS with CD47 inhibitors, due to its low toxicity and ability to alter anti-inflammatory M2 macrophages into pro-inflammatory M1 macrophages. However, this is limited by its failure to transform M0 macrophages in the same way, possibly reducing the effectiveness of the drug in lower-risk patients (with different macrophage phenotype ratios), in either scenario, further testing would be required to quantify the effectiveness of the drug in a laboratory setting before moving into a patient-based setting, as well as to research the transformative result of the M0 macrophages when combined with Venetoclax.

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