UNIVERSITY OF BIRMINGHAM

The identification of potential repurposed drug compounds and metabolic-altering factors that either inhibit or enhance the process of enclysis

[redacted]

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Abstract

Regulatory T cells (Tregs) play a major role in tempering inflammation in the liver and have become a novel tool in the treatment of autoimmunity and cancer. Through recent findings, a brand-new process of live T cell capture has been identified to toggle immunogenicity in the liver. Hepatocytes are able to capture live Treg cells in the hepatic microenvironment and eliminate them in acidic vesicles. This process, termed enclysis, carries huge potential as a therapeutic target against autoimmunity and cancer in the liver. As enclysis is a novel process, it has not yet been exploited therapeutically. In this project, 1200 FDA-approved drug compounds, where mechanism of action is already known, were investigated using high-throughput screens on their ability to modulate the process of enclysis. After three separate screens, using manual quantification of cell-in-cell structures, a shortlist of five inhibitors and five enhancers were identified to have their effect validated *in vitro*. Possible metabolic intervention was also investigated to determine any involvement of certain metabolites on the process. After in vitro validation, we identified four drug compounds (two inhibitors and two enhancers) as competent modulators of enclysis as well as a variety of metabolic conditions that could aid in inhibiting or enhancing the process. These drug compounds can now carry therapeutic potential in the possible treatment of autoimmunity and cancer in the liver.

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1. Introduction

1.1 Structure of the liver

As the liver is our largest internal organ, it naturally plays a vitally important role in detoxification of substances carried in the blood as well as acting on the front line of immunity [1]. 75% of the supply of blood to the liver is through the hepatic portal vein leading from the gastrointestinal tract. This allows the liver to perform its function of neutralising toxic compounds entering through the blood supply as well as maintaining a balanced equilibrium of immune tolerance to prevent an over exposure of immunogenicity [1]. The nutrients that are absorbed through the intestines during digestion will enter the liver through the portal vein, however with complex lipids such as chylomicrons these are transported through the lymphatic system [5]. The physical position of the liver within the human body allows it to be perfectly placed for metabolite gain from the blood, and this accessibility for blood allows for easier neutralisation of blood toxins [5]. The elimination of toxins occurs in the form of bile secreted from the liver which aids lipid digestion of the gut [5].

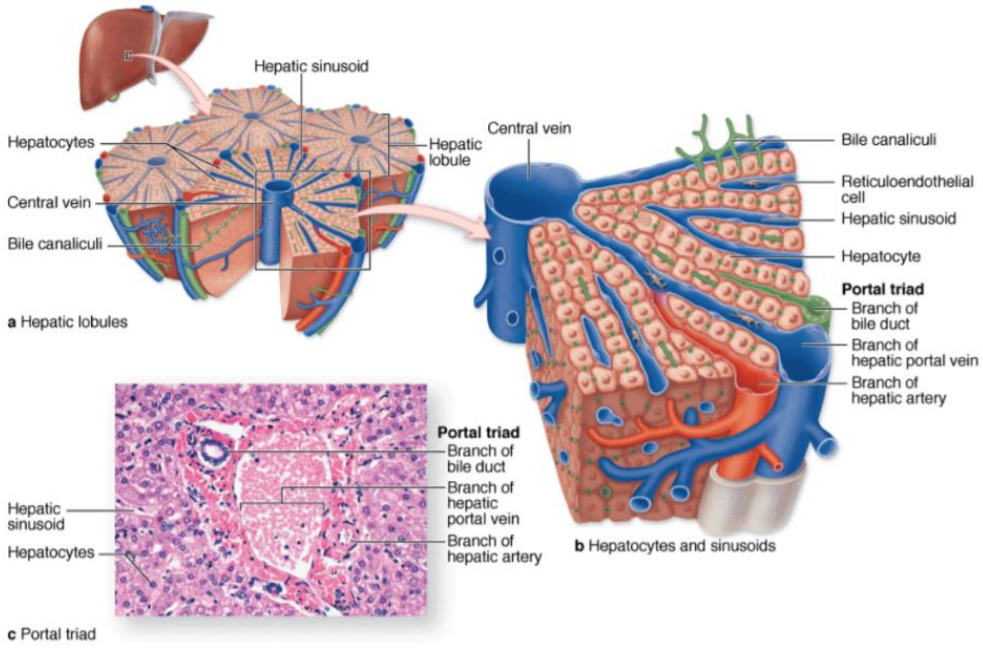


Figure 1. Structural layout of the liver a) Layout of the hepatic lobules is shown with the central vein protruding through the centre with peripheral vessels spanning to each edge of the lobule. **b)** the vessels projecting from the central vein group and form the portal triads made up of the portal vein, hepatic artery and the bile duct. Also seen is the extension of sinusoids running along each row of hepatocytes. **c)** Histological micrograph image illustrating the components of the portal triad [5].

The liver is made up of a multitude of hexagonal hepatic lobules, each surrounding a small central vein, that are characterised as the functioning unit of the liver [5]. As seen from figure 1b, the central vein is located in the middle of each hepatic lobule with a variety of blood vessels protruding across the periphery [5]. Each hepatic lobule contains up to six portal regions with a central vein in its centre [5].

The liver can be divided into three functioning sections: hepatocytes (parenchyma), the vascular system and the biliary system [2]. The hepatocytes make up 80% of the liver mass with the vascular and biliary system acting predominantly as a communication system throughout and adjacent to the liver [2]. Hepatocytes act as vital biochemical stalwarts within the parenchyma to selectively absorb and neutralise toxic substances that enter through the blood supply [3]. As a result of this process, a number of hepatocytes located around the local blood supply are damaged illustrating the importance of the regenerative properties of the liver [3]. The organisation of hepatocytes is key to performing their specific roles. This is shown through symmetrical alignment of two hepatocyte-thick segments that are enclosed by fenestrated endothelia [3]. Blood enters the liver through two differing ports depending on the nature of the blood. The portal vein will carry only nutrient-rich blood whereas oxygen-rich blood will enter via the hepatic artery [3]. Together, combined with the bile duct, they form the portal triad. Once the blood has passed through the portal triad it is combined within hepatic capillaries, called sinusoids,

where it eventually drains towards the central vein [3]. These sinusoids are flanked by specialised liver sinusoidal endothelial cells (LSEC) containing fenestrations, which allow important solutes and nutrients to reach circulation [3]. This allows rapid removal of immunogenic substances, including bacterial endotoxin. This removal maintains normal hepatic function [9]. These fenestrated sinusoids consist of a discontinuous layer of epithelial cells, which are only separated from hepatocytes by an extremely thin perisinusoidal space termed the space of Disse [5]. Within this space are projected microvilli from hepatocytes allowing the mutual exchange between blood plasma and the hepatocytes themselves [5]. The liver contains a thin layer of fibrous connective tissue, termed the stroma, which thickens as it moves towards the portal vein and hepatic arteries. This is used as a support network around the liver cells and sinusoids providing protection and structural support [5]

Hepatocytes show a preference of different metabolic processes depending on their zonation found along sinusoids [4]. Zonation of hepatocytes occurs in three zones spanning from the portal triad: Zone 1 (periportal zone), Zone 2 (Intermediate) and Zone 3 (perivenous) [4]. Since hepatocytes in zone 1 are the closest to the portal triads they are the most susceptible to damage from toxic infiltrates perfusing in from the blood [3]. Towards the hepatocytes in zone 3, however, the conditions become more hypoxic and are these have a reduced need for regeneration due to damage [3]. The zonation of hepatocytes also allows for a variation of metabolic processes along the sinusoidal region [4]. Hepatocytes are described as ‘highly metabolic parenchyma liver cells’ [4], and are proficient in a number of processes including, nitrogen metabolism, carbohydrate metabolism, drug conjugation and alcohol detoxification [4]. Zone 1 hepatocytes are seen to be excellent in the release of glucose from glycogen stores, and the formation of urea from ammonia and amino

acid hydrolysis [4]. Further away from the portal triad, in Zone 3, hepatocytes prove antithetical and allow for glucose uptake to be stored as glycogen, alcohol detoxification and drug conjugation [4].

1.2 Immune landscape of the liver

Due to the size and accessibility of the liver, it is no surprise that it holds a diverse immune landscape which is vitally important for the immunogenicity of the human body. Unlike other major organs, the liver acts as an immune-tolerising environment allowing transplantation, combined with external immunosuppression, without rejection [7]. Interestingly, the liver is a fundamental component in immune tolerance. For instance, hepatocytes will produce up to 90% of the circulating immune proteins found in the body [8]. The liver receives a continual barrage of antigenic substances from the blood with products from the gastrointestinal tract [6]. Therefore, the ability for the liver to form a layer of protection against this is paramount to prevent infection from unwanted pathogens [6]. In a healthy liver, the persistent need to remodel tissue and change metabolic activity, coupled with the constant exposure to harmful microbial substances will cause controlled inflammation [9]. The importance of regulating inflammation is clear, as this should normally only be activated whilst the liver attempts to remove harmful pathogens or toxic by-products of metabolic activity [9]. Controlled inflammation can show a variety of benefits including governing haemodynamic changes, migration of leukocytes, secretion of inflammatory mediators and permeability of surrounding capillaries [9]. Induction of inflammatory conditions is usually followed by strong mechanisms that resolve inflammation, through negative feedback, and drive regeneration of damaged tissue [9]. The role of inflammation is key within the liver and is paramount for maintaining tissue and general organ homeostasis [9]. Without regulation, prolonged inflammation can lead

to chronic infection, tumour progression and autoimmunity which will inevitably lead to cirrhosis and liver failure [9]. This can be a double-edged sword as there is a delicate balance between immunogenicity and immunotolerance and if liver tolerance is disrupted in chronic inflammation it can tilt the balance towards autoimmunity or viral infection, with cancer progression seen if the balance tilts the other way [6]. This illustrates the importance of the liver to maintain a perfectly neutral immune balance.

The hepatic blood supply is enriched with bacteria and general dietary antigens from the gut and, the liver has adapted to tolerate the foreign load as well as maintain immunosurveillance for any incoming pathogens [9]. The liver can also successfully provide antimicrobial resistance by arming inflammatory cytokines, chemokines and complement proteins; all vital in destroying foreign pathogens that invade the organ [6].

The liver has a vast armoury of key innate immune cells that hold residence within the organ. These include liver-resident macrophages, named Kupffer cells, dendritic cells and natural killer (NK) cells [6]. However, there is still work to be done on screening the full immune landscape of the liver in humans. Kupffer cells account for 90% of total macrophages found in the human body and one-third of all non-parenchymal cells [9]. These cells act as professional antigen presenting cells (APC) and express Pattern recognition receptors (PRRs), Fc receptors and complement receptors which induce phagocytosis and produce a variety of inflammatory cytokines [9]. PRRs bind to microbial associated molecular patterns (MAMPs) and damage associated molecular patterns (DAMPs) which are in abundance in the blood supply containing foreign antigens [9]. After binding to Kupffer cells, these are phagocytosed and degraded without the release of standard inflammatory mediators.

This allows for detoxification of hepatic blood whilst giving a reduced immune response and the maintenance of usual hepatic immunotolerance [9]. Despite having a limited population, Dendritic cells (DCs) play an important role in healthy livers by prompting a heavy T cell response when required [9]. Dendritic cells are found in two major subsets in the liver: myeloid DCs and plasmacytoid DCs. Immature myeloid DCs can differentiate into two forms, one that will promote immunity and ones that will favour immune tolerance [13]. These professional antigen presenting cells (APCs) strongly regulate innate and adaptive immunity and play a major role in organ transplantation depending on their tolerogenic or immunogenic tendencies [14]. DCs are shown to be essential in priming self-reactive T cells that have eluded tolerance, therefore inducing autoimmunity [15]. These properties have allowed DC to become key therapeutic targets in autoimmunity and transplantation rejection as well as antithetically being drafted as immune-stimulatory weapons in anti-cancer medication [14].

Myeloid-derived suppressor cells (MDSCs) are found in abundance in healthy livers and are bolstered in the presence of any chronic liver disease [10]. They are characterised by their action of suppressing T cell activation through immunosuppressive molecules such as IL-10 and TGFβ [11]. Conversely, neutrophils are notably missing from healthy liver with these only being produced following viral infection or inflammation [12].

Similarly, to the large myeloid cell population of the liver, there is also a varied population of lymphoid immune cells present [9]. This includes natural killer (NK) cells, NK T cells and ϒδT cells, which help induce cytokine production and contribute heavily towards regulating hepatic innate and adaptive immunity [9]. A strong abundance of adaptive lymphocytes is present in a healthy liver including a variety of

$CD4^+$ and $CD8^+$ T cells, described as classic major histocompatibility complex (MHC) – restricted, as well as B cells. More specifically, the liver holds a large enrichment of $CD8^+$ T cells, activated T cells and memory cells [9]. Due to the large population of these specific T cells, we see a myriad of T cell apoptosis, inciting the apt nickname for the liver of the 'graveyard' for T cells [9].

1.2.1 T regulatory cells

$CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory cells (Tregs) are produced in the thymus and vital in maintaining the perfect balance between immunogenicity and immunosuppression [21]. The importance of which is shown by knockout of Tregs in mouse models which prove fatal with the accumulation of autoimmune disorders and organ failure [22]. In humans, disruption in Treg development will lead to an abundance of immune-regulatory disorders usually stemmed from mutations in the FOXP3 gene. These include, polyendocrinopathy, X-linked IPEX syndrome and an array of autoimmune diseases, such as type 1 diabetes and inflammatory bowel disease [21]. To ensure proper development of Tregs, two specific processes must be implemented. The initial expression of FOXP3 and the formation of a CpG hypomethylation pattern specific to Tregs [23]. This induces Treg expression and allows for fully functioning suppression of lymphocyte populations including $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells [21].

Liver inflammation is described as the infiltration of lymphocytes into the hepatic environment [21]. Tregs are recruited to the site of inflammation, via sinusoids, through the work of the chemokine receptor CXCR3, which is especially present within the inflamed liver [21]. CXCR3 ligands are found within the endothelium of the sinusoidal projections within hepatic lobules [24].

As discussed, the enrichment of both effector and regulatory T cells will gauge the balance between tolerance and inflammatory-based autoimmunity in the liver. Usually, an influx of effector T cells causing acute hepatitis can be easily managed by regeneration of hepatocytes and removal of initial insult. However, if the inflammation persists and becomes unmanageable for Tregs, this will invite more chronic pathogenesis of hepatitis possibly resulting in cirrhosis and hepatocellular carcinoma (HCC) [21].

Hepatic Tregs occupy a partly anaerobic microenvironment enriched with an assortment of microbes, cytokines and hormones that will play a part in their function and survival [25]. For example, All-Trans retinoic acid (ATRA), found predominantly within stellate cells, will aid Treg stability within the inflammatory microenvironment of the liver through inducing the conversion of naïve T cells to $FOXP3^{+}$ Tregs via the action of TGF-β [31]. During inflammation, the hepatic microenvironment is embellished by several pro-inflammatory cytokines such as IL-12, IL-6 and TNFα, however, the Treg pro-survival cytokine IL-2 is diminished [32].

1.2.1.1 Mechanism of suppression for regulatory T cells

Tregs use a variety of mechanisms to fulfil their function of immune regulation. This may be via inhibition of the maturation of APCs, exploiting the adenosine pathway to disturb usual metabolic pathways, facilitating apoptosis and producing TGF-β and IL-10 [25]. T-lymphocyte-associated antigen 4 (CTLA-4) is vital in performing the suppressive action of Tregs [26]. CTLA-4 inhibits the stimulatory function of CD80 and CD86 on dendritic cells through trans-endocytosis [27]. Furthermore, CTLA-4 can suppress T cells through the action of essential amino acid-targeting enzymes such as histidine ammonia lyase which binds to CD80 on the surface of APCs [28].

TGF-β, a suppressive cytokine released by Tregs, allows for the continued maintenance of peripheral Tregs through supporting the ongoing expression of FOXP3 [29]. Similarly, to CTLA-4, TGF-β releases catabolizing enzymes to reduce essential amino acid level in APCs. Tregs can also inhibit effector T cells directly through the action of pericellular adenosine, catalysed by ectoenzymes expressed on the surface of Tregs named CD39 and CD73 [25]. Jurkat T cells have been used as a primary T cell substitute for a number of experiments, including this project. They are characterised as an immortalized human t lymphocyte cells, and are used as a substitute for primary T cells, and are perfect for high-content experiments.

1.2.2 Tolerogenic processes of the liver

Hepatic self-tolerance is a vitally important tool to maintain immune homeostasis as well as preventing any unwanted autoimmune takeover [21]. Tolerance is induced in two stages termed central and peripheral tolerance. These stages begin in the thymus and will promote negative selection of auto-reactive T cells and the production of Tregs [21].

1.2.2.1 Tolerogenic function of dendritic cells

Interestingly, dendritic cells have been proven to promote immune tolerance instead of immunogenicity [13]. DCs are pivotal in the regulation of both central and peripheral tolerance with any malfunction leading to severe autoimmune defects [14]. This is illustrated in a study by Ohnmacht et al. 2009, where fatal immunity occurred in all mice following dendritic cell depletion of all subsets [15]. Immature DC express major histocompatibility class II (MHC II) alongside several stimulatory molecules, such as CD40, and promote T cell anergy and Treg cell production [20]. This gives immature DC the name of tolerogenic DC [20]. Central tolerance is induced through

a thorough process, regulated by APCs and Tregs in the thymus, where self or foreign Ag-reactive thymocytes are negatively selected [14]. Proietto et al. proved the DCs that migrate from the periphery to the thymus will actively induce Treg production and, thereby, negative selection [14]. Furthermore, in Besin et al. 2008, non-obese diabetic mice were treated with thymic stromal lymphopoietin, secreted by Hassall's corpuscles in the thymus [16]. BM-derived DC were able to gain a tolerogenic phenotype and induce the conversion of naïve T cells into $CD4^+$ $CD25^+$ Tregs [16]. This illustrates the importance of DCs in the expansion of T cells in the central thymus [14].

Peripheral tolerance is regulated, by DCs, through a multitude of mechanisms, mostly involving T cell deletion, anergy and the induction of Tregs [14]. Production of immunosuppressive cytokines such as IL-10, TGF-β and indoleamine dioxygenase (IDO) play a key role in dampening immunogenicity [14]. The most potent of these is seen to be IDO, expressed by APCs, which illustrates vitally important tolerance functions [14]. In mouse models, IDO^+ $CD19^+$ DC will produce a large amount of IDO that will, in turn, dampen T cell function [17]. This is further supported by the use of siRNA to inhibit IDO expression which has shown to prevent DC suppression of T cell proliferation [18].

Dendritic cells have also been shown to illustrate tolerogenic mediation of Treg cells. DC that reside in the periphery are able to induce Treg production with the absence of DCs, in vivo, causing loss of Tregs as well as an increase of IFN-Ɣ and IL-17 which collectively promote T cell proliferation [19]. Furthermore, in a study performed by Worthington et al 2011. $CD103^+$ DCs increase hepatic tolerance by activation of TGF-β activated by integrin αvβ8 whereas DCs that lacked the integrin failed to do so [30].

1.2.3 Cytokine involvement in hepatic immunity

A number of different cytokines will affect the day-to-day action of Tregs, however, the survival and maintained function of Tregs is predominantly dependent on interleukin - 2 (IL-2) [33]. The mechanism of action used by IL-2 to support Tregs has been minimally explored in autoimmune diseases which illustrates the importance of further research. IL-2 will bind to a cell-surface a receptor (IL-2R) which is comprised of three subunits: alpha (IL-2RA,CD25), beta (IL-2RB,CD122) and gamma (IL-2RG,CD132) [34]. Whilst IL-2RG is expressed on each variety of leucocyte and IL-2RB on NK cells and memory $CD8^+$ T cells as the main subunit, IL-2RA is expressed on Treg cells constitutively which illustrates the importance of the alpha subunit [34]. For Tregs, IL-2RA is responsible for strong affinity binding of IL-2 with IL-2RB and IL-2RG important for the transduction of the signal to downstream pathways [33]. The downstream pathway activated is dependant on which receptor subunit is transduced. IL-2RB will activate the serine/threonine kinase/AKT pathway which will induce the production of anti-apoptotic proteins such as Bcl-2 that will promote T cell survival [35]. Conversely, signalling from IL-2RG will activate the JAK3 pathway and activate signal transducer and activator of transcription-5 (STAT-5) which is key for T cell differentiation and proliferation [35]. Tregs are able to competitively absorb IL-2 through the high concentration of CD-25 they have available on their surface. This allows for an increased level of survival and increased dampening of nearby effector T cells. In the inflamed liver, IL-2 availability decreases dramatically which illustrates how the action of Tregs is severely compromised within an inflamed hepatic microenvironment [36]. This accentuates the importance of potential IL-2 therapy in the treatment of diseases caused by inflammation. In a study performed by Jeffrey et al. 2017 [33], clinical grade IL-2 (Proleukin [VDLP]) was used to upregulate the

phenotype and function of Tregs in patients with autoimmune livers. It was proved that VLDP was able to promote STAT-5 phosphorylation, CTLA-4 function, CD25, FoxP3 and Bcl-2 in patients with autoimmune liver diseases. Additionally, VLDP was able to upregulate the action of CXCR3 ligands to act as a chemoattractant for Tregs to the inflamed liver [33].

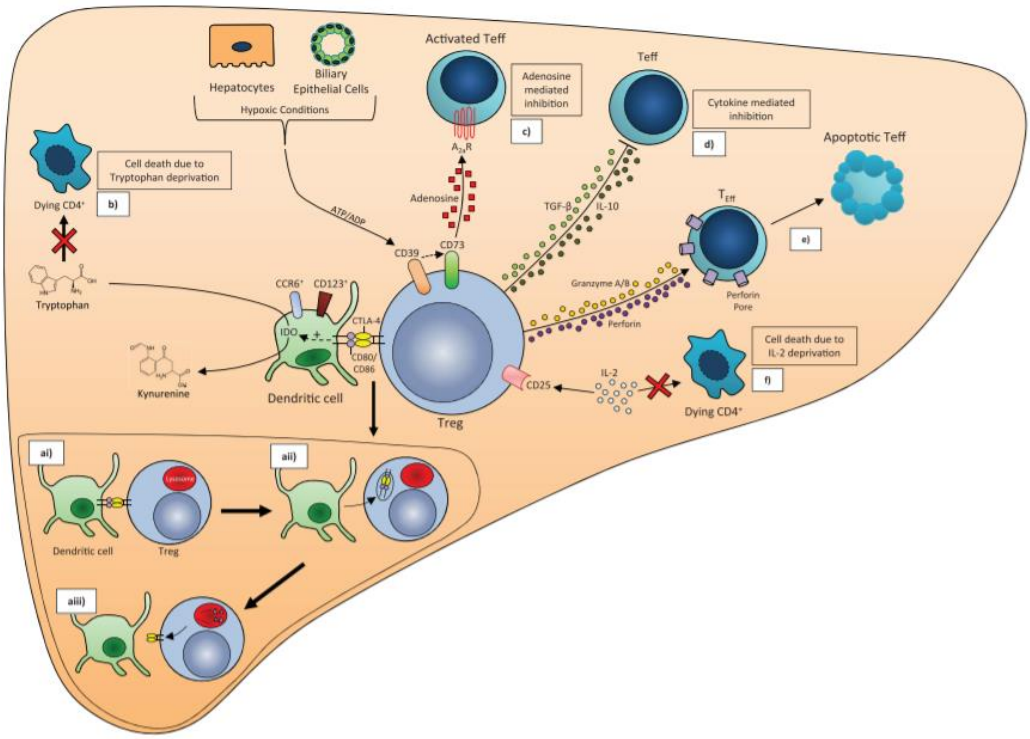


Figure 2. The mechanism of action of Tregs. The intracellular mechanisms associated with Treg function. Showing the interaction between Treg cells and effector T cells as well as dendritic cells to contribute towards immunotolerance of the liver. A) The suppressive function of Tregs acting on Dendritic cells, through CTLA-4, displaying antigen-presenting properties to inhibit future function of DCs. B) Starving of $CD4^+$ T cells of Trytophan leading to cell death, mediated through DCs. C) Regulation of activated effector T cell through adenosine production, mediated through CD73. D) Inhibition of effector T cells through cytokine release such as IL-10 and TGF-β. E) facilitating apoptosis in effector T cells through granzyme A/B and perforin action. F) the deprivation of IL-2 on effector T cells through increased Treg consumption through CD25.

1.3 Cell capture in the liver

The hepatic microenvironment is under constant threat of toxic substances and antigens entering through the blood stream [38]. The safe neutralisation of these substances is required for the liver to maintain a healthy homeostasis. Additionally, the clearance of dead and dying immune cells from the liver, termed efferocytosis, will allow for standard liver function to go uninterrupted and prevent build up of apoptotic debris [38]. This function has caused the liver to be termed the ‘graveyard’ for immune cells due to the level of cell turnover that occurs [38].

1.3.1 Efferocytosis

The clearance of dead and dying immune cells is crucial for immune function in the liver and plays a huge role in negating tissue damage and inflammation [38]. Cellular debris caused by apoptosis is cleared by circulating phagocytes (monocytes, dendritic cells, neutrophils), macrophages (Kupffer cells) and by epithelia found in the sinusoidal regions [39]. Within recent literature, the main efferocytes reported have been Kupffer cells which are regarded as the ‘liver-resident macrophages’, however hepatocytes, which make up of 80% of the total liver mass, have proven to be effective efferocytes [39]. Kupffer cells are shown to be activated through release of inflammatory signals and tissue injury, leading them to the site of cellular debris and resulting in the initiation of efferocytosis [39].

Similarly, stellate cells are proven to support resident macrophages well in the clearance of apoptotic cells [40]. Once activated, these will engulf apoptotic cells resulting in a release of tumour growth factor-β (TGF-β) [40]. Additionally, biliary epithelial cells (BECs) contribute towards hepatic grave yarding, through

efferocytosis, which is a key driving force in the deterioration of primary biliary cholangitis (PBC) [41].

Efferocytosis is non-specific in its type of dead cell targeted. Within the hepatic microenvironment exists a number of necrotic and apoptotic cells that need removal as part of the standard homeostasis [36]. If a population of necrotic cells becomes too evident, it could be an exacerbation of infection, alcoholism, drugs or ischemia-induced liver injury [36]. Furthermore, increased necrosis is associated with acute-on-chronic liver failure caused by the onset of hepatitis B infection [36]. Features specific to hepatocytes necrosis include pyknotic nuclei and eosinophilic degradation [36].

1.3.1.1 Clearance of Apoptotic cells

Cells that undergo programmed cell death, apoptosis, activate specific inducible pathways in order to die [42]. These pathways are split into two; the extrinsic pathway, and the intrinsic pathway. The extrinsic pathway, the more common of the two, involves the release of extracellular signals including, but not restricted to, Fas ligand (FasL) and a group of tumour necrosis factor cytokines (TNFs). These extracellular signals will reach their own respective death receptors on apoptotic cells and trigger an intracellular signalling cascade, leading to cell death [43]. Alternatively, apoptosis can be initiated intrinsically inside the cell. Through a variety of signals, or lack of, apoptosis can be triggered using the cells own intrinsic pathways [44]. The most common conditions triggering apoptosis in this way are the distinct lack of growth factors, endoplasmic reticulum stress or even orthodox DNA damage. After initial signalling, expression of the Bcl-2 mitochondrial protein is altered which, in turn, facilitates the downstream apoptotic cascade [44]. Increased

expression of Bcl-2 and, therefore, increased activity proapoptotic proteins will allow the secretion and activation of cytochrome C and caspase 9. The activation of caspase 9 will eventuate in further activation of degradation caspases 3,6 and 7 which will cause the proteolytic deterioration of the cell and the intracellular components [42]. Once the pathway of apoptosis has completed the apoptotic cells will present as morphologically different to live cells as they are smaller and form blebs on the cell surface [42].

1.3.1.2 Clearance of necrotic cells

Efferocytosis is not limited to apoptotic cells in the liver. Necrotic cells also make up a large portion of the cells that are targeted by the efferocytes in the liver. Necrosis is characterised as an unplanned, usually accidental, form of cell death which can also occur through active mechanisms [45]. The fundamental cause of necrotic cell death is usually either a breach in the plasma membrane of the cell, allowing invasion of foreign substances into the cell, or a loss of intracellular ATP [46]. Depletion of ATP levels within dying cells is seen as a link between apoptotic and necrotic cells [46]. Secondary necrosis is a process which sees cells that are undergoing apoptosis experience a distinct lack of ATP to complete the process which causes the unprecedented conversion to necrotic cells [47]. Morphological appearance of necrotic cells Is also the antithesis to that of apoptotic cells as instead of shrinking, necrotic cells will swell and illustrate broken down plasma membranes as well as a deterioration of the nucleus [48]. Necrotic cells will usually gather in tissue and, more specifically, form quickly into a group of necrotic cells. Due to the unstructured nature of necrotic cells, cells debris will be released quickly and, if not cleared from liver tissue, can induce chronic liver damage [48].

1.3.1.3 Recognition of apoptotic and necrotic cells for efferocytosis

Despite the nature of cell death, the dead cell and its debris must be cleared from the tissue to avoid damaging the surrounding microenvironment. Efferocytosis use the same phagocytes to engulf both apoptotic and necrotic cells, however, the process in which these cells are identified by efferocytes differs [49]. Apoptotic cells require a couple of different pathways to be recognised by efferocytes. The most common route is the presentation of phospholipid phosphatidylserine (PtdSer) on the extracellular surface of the plasma membrane which are recognised by phospholipid phosphatidylserine receptors (PSRs) [50]. The maintained integrity of an apoptotic cell allows for simple removal of the entire shrivelled body via phagocytes. This allows for a quicker, more beneficial, process as it will not incite any unwanted inflammatory response [51]. Conversely, necrotic cells will not keep the same level of cell integrity and will be more susceptible to releasing cell debris, allowing for a more difficult process of recognition and overall cell engulfment. Because of this, necrotic cell clearance is a more complex process. Due to the unstructured nature of necrotic cells, these are usually recognised using secretions of necrotic cell death [49]. Interestingly, these molecules are the same as the usual suspects used to detect pathogen invasion [36]. Following necrosis, opsonized necrotic cells are recognised by Fc and complement receptors which, in turn, trigger downstream signalling cascades that will activate local phagocytes [36]. Furthermore, necrotic cells are directly recognised through autoantigens activated by tissue damage caused by cell death. An overload of this platform of recognition, however, has implications for autoimmune disease in the liver [36]. Necrotic cells, as seen in apoptotic cells, can also undergo indirect recognition by inducing opsonin of intracellular items. Ficolin-

2/-3 can bind to DNA and induce indirect clearance of necrotic cells through its interaction with calreticulin protein [52].

The importance of swift apoptotic and necrotic cell clearance within the liver is clear, however what is also key is maintaining the balance between the separate process of recognition and removal of both apoptotic and necrotic cells. Removal of apoptotic cells will produce an anti-inflammatory environment and induce resolution signals for inflammation, for example, interleukin-10 (IL-10) and TGF-β [53]. Antithetically, the removal of necrotic cells will lead to the production of pro-inflammatory stimuli since most of the receptors involved are also linked to pathogen recognition [54].

1.3.2 Mechanism of efferocytosis

Professional phagocytes recognize both necrotic and apoptotic cells using characterized relevant receptors found in the liver [36]. As discussed, apoptotic cells are widely recognised through the expression of PtdSer on the outer surface of the plasma membrane of the apoptotic cell [50]. There are a multitude of receptors on the cell surface of phagocytes that recognise PtdSer. These include stabilin-1, stabilin-2, brain specific angiogenesis inhibitor-1 (BAI1), TIM-1/-3/-4 of the TIM family of transmembrane gylcoproteins and RAGE [36]. As important as the recognition of apoptotic cells is, the clearance via macrophage activation is what allows for the clearance of these cells from the microenvironment. Downstream intracellular pathways from the PtdSer receptors are key in initiating efferocytosis in this way. Through GTPase, Rac1 and ELMO1-DOCK180 interactions, TAM-family molecules, αvβ5 integrins and BA11 will act as facilitating proteins to drive downstream apoptotic signalling [36]. Through activation of this pathway, DOCK180 will be recruited by ELMO1 which allows for Rac1 activation which, in turn, will alter the

cytoskeletal structure of the macrophage and induce engulfment of the dead cell [55]. Engulfment of apoptotic cells is completed via the interaction of nuclear receptors. The nucleotides that are released through the compromised apoptotic cell are used as *'eat me'* signals which will increase the affinity of macrophages for efferocytosis through the activation of purinergic P2 receptors (P2X and P2Y) [56]. Interestingly, in recent findings, the liver X receptor (LXR) was shown to be vital in the recognition and engulfment of apoptotic cells by macrophages and dendritic cells [57].

1.3.3 Clinical impact of disrupted efferocytosis in liver tissue

The prolonging and exacerbation of certain diseases within the liver has been historically linked to the failure of removing dead or dying cells from the environment through efferocytosis. For example, the build up of dead cells in a microenvironment will invite a number of proimmunogenic molecules to the site of dying cells which increases the risk of autoimmunity in the liver [36]. This is performed through the attraction of scavengers, including neighbouring cells or phagocytes. Dead or dying cells produce 'find-me' signals to attract inflammatory effectors and 'eat-me' signals that initiate engulfment. This interaction between phagocytes and dying cells must be maintained at an equilibrium to avoid onset of autoimmunity [36].

Additionally, defects in efferocytosis can also benefit the survival of tumours within the liver microenvironment. For example, an overstimulation of CD47 *(don't eat me)* signal has been reported in myeloid leukaemia, aiding tumour survival [58,59]. Furthermore, downregulation of death receptors axl, MerTK and its ligand Gas6, caused by disrupted efferocytosis, plays a key role in the proliferation of certain colon cancers [60]. Efferocytosis is hypothesised to play a role in several liver

diseases, however these have not been investigated thoroughly. However, it is thought that increased efferocytosis is beneficial for preventing the onset of autoimmune hepatitis (AIH) and therefore reduce fibrosis build up in the liver [36]. Chronic exposure to alcohol, as well as causing widespread hepatocyte necrosis, can also reduce the clearance of dead and dying cells from the liver as macrophage activation is reduced due to the downregulation of MFG-E8 expression, which is expressed on the surface of apoptotic cells and binds to phosphatidylserine on phagocytic cells to initiate engulfment. The exploration of efferocytosis and the role is plays within the homeostasis of the liver can prove key in the discovery of possible therapeutics for liver disease and cancer [61].

1.4 Live cell capture

The work of the professional phagocyte does not end with efferocytosis. Cell in cell structures (CICs) have been reported with viable live cells found inside hosts, furthermore these host cells have been reported to not be solely phagocytes [1].

1.4.1 Entosis

The process of entosis will differ from orthodox efferocytosis through targeting the removal of solely live cells instead of dead or dying cells within the hepatic microenvironment. Additionally, efferocytosis will result in the lysosomal degradation of engulfed cells, however through entosis, cells that are captured remain viable inside the cell in structures termed vacuoles and can survive whilst the host cell determines its fate [1].

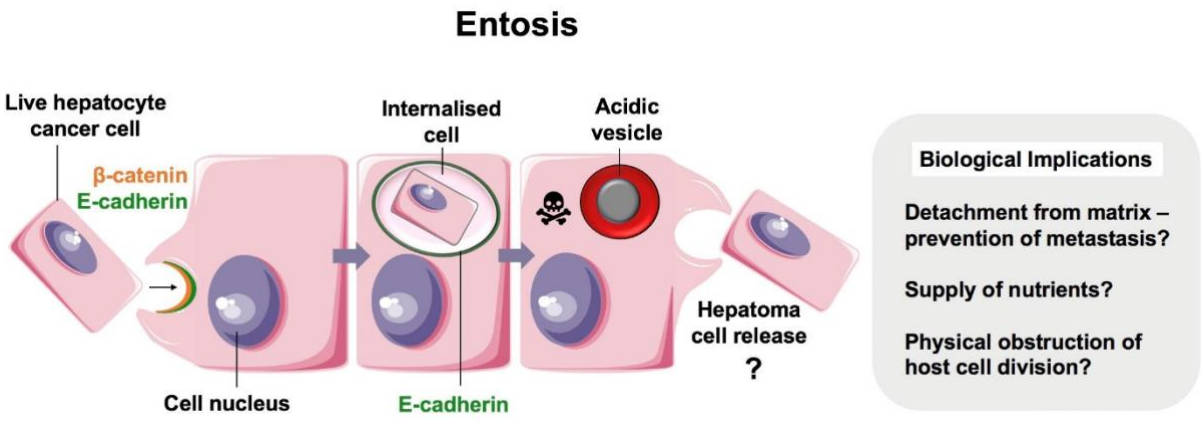


Figure 3. The process of entosis in neoplastic hepatocytes. HCC cells are shown to be engulfed by live neighbouring cells in a process that may involve β-catenin and E-cadherin. The HCC cells is internalised and its fate in then decided to whether it is degraded inside an acidic vesicle or It undergoes release unharmed [1].

From figure 3 the process of entosis in hepatocytes is described. The process was originally tested by Overholtzer et al 2007 [62]. In this report, it was described that through the process of entosis, the extracellular matrix of live cells would detach and cause CICs in tandem with adherens junctions. Entosis will also involves E-cadherin and β-catenin expression that will act as junctional proteins as well as the contraction of actomyosin induced by the activation of Rho-associated coiled-coil-containing protein kinase (ROCK) in the targeted cell [62].

Through further investigation of the process after the Overholtzer discovery in 2007, it has been described that entotic invasion will be initiated primarily through the plasma membrane acting as a binding site to facilitate CIC formation [63]. This acts through the plasma membrane undergoing blebbing and polarization [63]. The plasma membrane is seen to play such a key role in the activation of entosis, it has been described that the cellular composition of the plasma membrane itself can alter the affinity for the cell to undergo entosis. It is proven that liposomes and cholesterol

will inhibit entosis and the formation of CICs, which make up key components of the plasma membrane. It is hypothesised that this is due to both molecules inhibiting the contraction of actomyosin through preventing phosphorylation of myosin light chain kinases (MLCK) [64].

After internalisation, through entosis, the fate of the captured cell will vary based on the conditions. Most of the captured cells will succumb to lysosomal degradation within vacuoles, however, there are occasions of cells undergoing cell division within the host cell and, in turn, they are released unharmed. Destruction of target cells through the entotic process can be influential in possible tumour suppression [1]. Manipulating entosis to target cancer cells that have been detached from the extracellular matrix could prove beneficial in future potential HCC therapeutics. Additionally, Overholtzer et al. (2007) have demonstrated how engulfment of tumour cells can benefit host cell survival by siphoning key nutrients to cells that have a distinct lack of vasculature [62]. Interestingly, Overholtzer's group have illustrated that by starving adherent cells of glucose, such as hepatocytes, entosis can be induced through the activation of AMP-activated protein kinase (AMPK) [62]. Given this, if malignant cancer cells can perform entosis in conditions of glucose starvation, and provide key nutrients to vascular starved tumour cells, they can promote proliferation of more aggressive subtypes of cancer [62].

The clinical impact of entosis in Hepatocellular Carcinoma has not widely been investigated, however it is known that hepatomas are elite in the live engulfment of neighbouring cells, in a process which involved a rich concentration of E-cadherin, however it is unknown if hepatocytes perform actual entosis [7]. It is hypothesised how entosis would be a beneficial process for HCC as providing nutrients to cancer cells within the extremely hypoxic and isolated environment of HCC tumours can

push for survival and proliferation, illustrating the potential importance of targeting entosis in HCC future therapeutics.

1.4.2 Emperipolesis

Emperipolesis was first described in 1956 by Humble et al [65] and involves the movement and transfer of live cells after internalisation based on the Greek translation ‘inside-round-about-wandering’. Emperipolesis differs from both entosis and cell cannibalism as it solely focuses on the transition of the cell after internalisation, whereas the other types focus on the actual mechanism on cell capture [65]. Disruption in emperipolesis has been long linked to multiple types of chronic liver disease, including Autoimmune Hepatitis and viral infection. This process was first characterised by Bertilino et al 2001. In which a distinct variation of emperipolesis termed ‘suicidal emperipolesis’ was discovered. This specific process involves the engulfment and lysosomal degradation of autoreactive $CD8^+$ T Lymphocytes by hepatocytes. Bertilino et al. described the presence of Wortmannin as an inhibitor of emperipolesis. The inhibition of this process was shown to cause the accumulation of autoreactive T cells in the hepatic microenvironment which disturbs the immune balance of the liver [65].

1.4.3 Enclysis

Enclysis, first reported by the Stamataki lab in 2019, is a brand-new method of cell capture within the hepatic microenvironment directly translated from the Greek to enclose, to confine, to keep in captivity [1]. The process involves the preferential capture of $CD4^+$ T cells by hepatocytes and has been proven thus far using *in vitro* hepatoma cell lines Huh-7, HepG2 cells and primary hepatocytes [7]. It has been shown that intracellular adhesion molecule-1 (ICAM-1) plays an integral part in the

adhesion and preliminary capture of T cells, however, the ligands for ICAM-1 are not specific to $CD4^+$ T cells which seeds doubt into ICAM-1 acting as an enclysis-specific adhesion molecule [7]. Conversely, junctional protein β-catenin interacts with an enclytic vacuole selectively, which was not shown during efferocytosis as phagosomes containing dead cell matter were seen to show no localisation of β-catenin. Furthermore, enclysis differs from entotic cell capture in the lack of E-cadherin present during the enclytic cell capture, investigated using confocal imaging of specific antibody staining in co-cultures in Davies et al. 2019, which is present during entosis [7]. Additionally, enclysis requires no involvement of the RhoA/ROCK pathway, which is a key driving factor in entosis, proving further differences between the two processes. Despite this, both enclysis and entosis show the presence of membrane blebs [66].

Enclysis shares several similarities with micropinocytosis with the presence of distinct membrane alterations which aid the process of cell capture [67]. These alterations include ruffing and the formation of lamellipodia and membrane blebs, which shows enclysis specificity to emperipolesis due to these alterations being absent in the latter process. Furthermore, emperipolesis is sensitive to wortmannin, as described previously, however enclysis is not affected by this proving the mechanistic difference between the two processes [68].

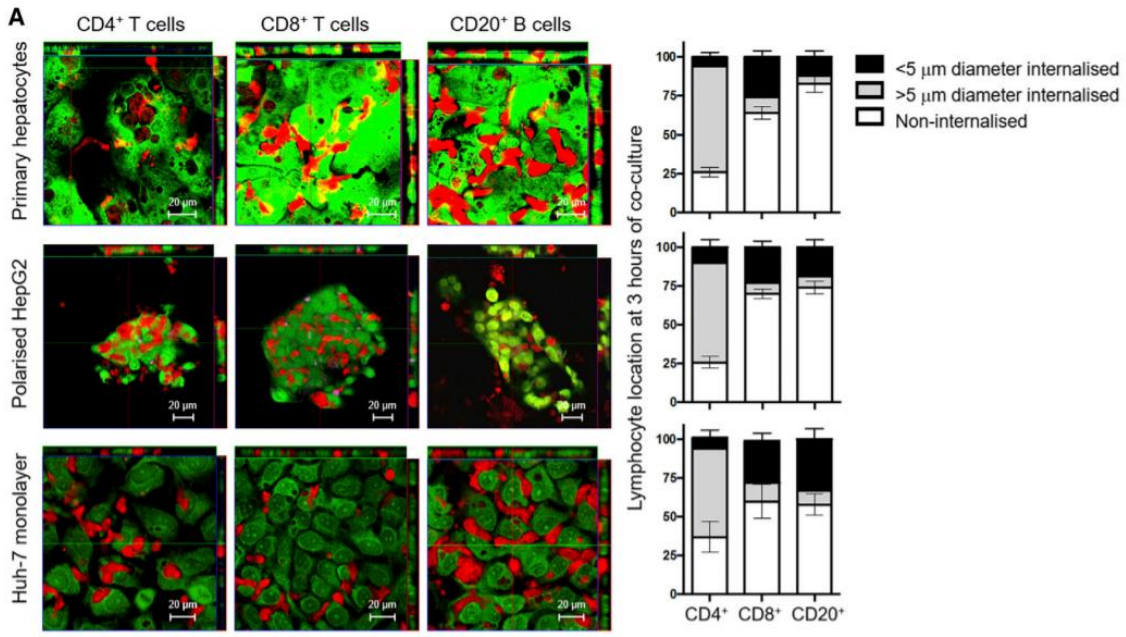


Figure 4. The preferential engulfment of CD4⁺ T cells. The ability for three different lymphocyte subsets to be engulfed during enclysis is tested. CD4⁺, CD8⁺ T cells and CD20⁺ B cells were incubated with Huh-7s, HepG2 cells and primary hepatocytes. The figure illustrates the preferential engulfment of CD4⁺ T cells based on the far larger amount of them showing >5µm of their diameter internalised [7].

As shown in figure 4 In 2019, Davies et al. were able to illustrate the specificity of enclysis for certain cell types. CD4⁺ T cells were preferentially engulfed over CD8⁺ T cells and CD20⁺ B cells, however, the most interesting finding was the selective targeting of T-regulatory cells (Tregs) which were three times more likely to be engulfed than any other subset of T cell [7]. As well as being targeted for initial engulfment, Tregs were also negatively impacted following entry into the host cell. Tregs inside acidic enclytic vesicles undergo lysosomal degradation and removal from the hepatic microenvironment. This, however, differs from non T-reg cells which are able to survive and pass through the host cell unharmed [1]. This is illustrated clearly in figure 5 [69].

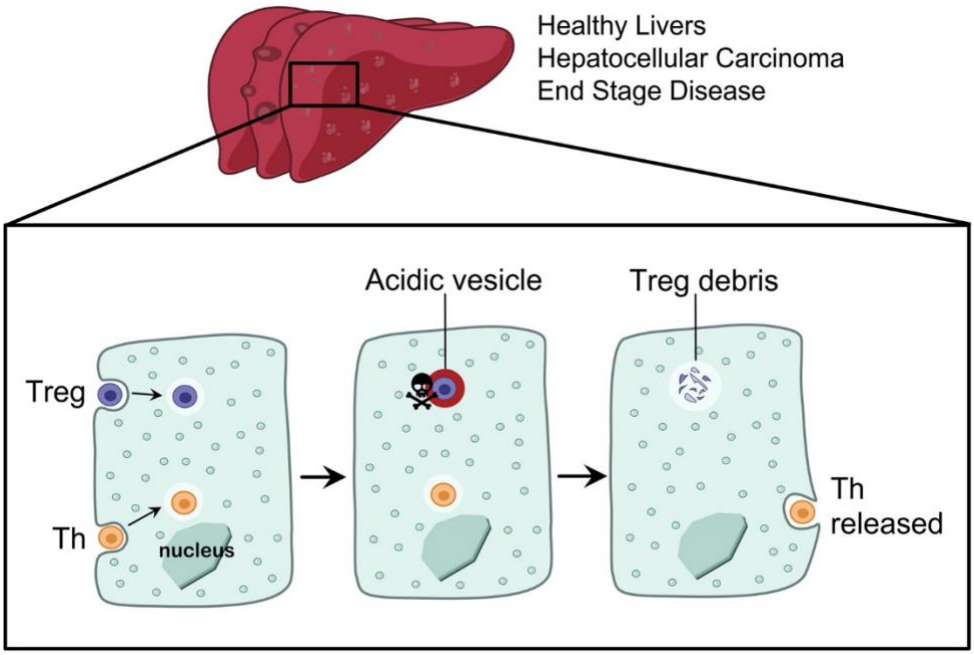


Figure 5. The process of enclysis. Encylsis is the preferential engulfment of $CD4^+$ T cells which culminates in the degradation and removal of Tregs from the hepatic microenvironment. Tregs and other subsets of T lymphocytes, such as T helper cells, were shown to experience differing cell fates after adhesion and engulfment through the enclytic vesicle. Tregs will undergo lysosomal degradation and removal where T helper cells will be released unharmed from the host cell through exocytosis [69].

1.4.3.1 Clinical implications of enclysis

It has been proposed that enclysis is a novel immune-altering pathway that can provide a new potential mechanism to toggle the level of hepatic inflammation. The ability to modulate the specific number of Tregs in the liver microenvironment will provide the opportunity for the liver to create a fine balance between maintaining immunotolerance and preventing a situation where it is immune ‘blind’ to the threat of proliferating cancers. This illustrates the clear therapeutic potential a process, such as enclysis, carries. If the process can be modulated and, for example, the Treg number in the liver is able to be enriched, due to dampened enclysis action, one would be able to significantly decrease the levels of effector T cells in the liver and

reduce inflammation. Since the stimuli for this process is currently unknown it is clear that the mechanism behind the function of enclysis is an area worth exploring.

1.4.3.2 Enclysis in Autoimmune Liver Disease (ALD)

Autoimmune liver diseases (ALD) cover a family of disorders that develop when loss of immune tolerance occurs in the liver. This family includes autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). For the majority, these disorders damage the liver through immune-mediated targeting of hepatocytes, in AIH, and the small and large bile ducts in PBC and PSC respectively [69]. Current therapies are not hugely viable with efficacy severely changing with each different patient. The ability to dampen effector T cell numbers through enclysis would illustrate a novel therapeutic target that could be explored with the modulation of the process [69].

1.4.3.2.1 Autoimmune hepatitis (AIH)

Autoimmune hepatitis (AIH) is characterised as a chronic liver disease that is caused by an overload of effector T cell activation and function [70]. 40% of cases are diagnosed as acute hepatitis before AIH diagnosis with 30% exhibiting previous history of liver cirrhosis and advanced fibrosis [69]. If diagnosed it its early stages, the 10-year survival rate can reach a peak of 93%, however, if no treatment is given, 40% of patients will die within six months of diagnosis [71]. There are several mechanisms that are activated to produce a variety of liver damage in AIH. Once self-antigens are presented to a T cell receptor on T helper cells, this will cause differentiation of T helper cells into a specific cell type that can initiate autoimmune damage on the liver [72]. Cytotoxicity in the liver is administrated through Th1 differentiation that incites IL-2 and IFN-γ release which activates cytotoxic $CD8^+$ T

cells. Furthermore, Th2 cells secrete cytokines IL-10, IL-4 and IL-13 which drive B cell maturation and, in turn, the eventual release of autoantibodies which leads to further cytotoxicity [73]. A surplus of IFN-Ɣ release will act negatively on hepatocytes by increasing the formation of MHC class 1 complexes which increase effector T cell action on hepatocytes [1].

AIH has been shown to be significantly affected by the number of Tregs found in the hepatic microenvironment. In a study by Lapierre et al. [74] AIH was shown to be more severe with the reduction of the number of Tregs in the microenvironment and the induction of intravenal Tregs was sufficient to restore immune homeostasis and send patients into remission [74].

Current therapies for AIH prove scarce with IL-2 treatment illustrating the most promising therapeutic to date [69]. Enclysis-targeting therapies could prove to fill the void in AIH treatments to maintain high Treg numbers after 28 days. Current therapies and the potential of enclysis-targeting are illustrated in table 1 [69].

Table 1. Potential current enclysis-targeting therapies

Type of therapy	Clinical/preclinical model	Outcomes
Corticosteroids	Immunosuppressive treatment with steroid and azathioprine diminishes intrahepatic Treg cells (66)	Life-long, does not restore liver homeostasis (25, 63)
Adoptive transfer (PolyTregs)	Xenoimmunized Type II AIH murine model (44)	Reduces the numbers of circulating autoreactive T cells and is sufficient to prevent AIH development in mice (44)
Adoptive transfer (arTregs)	Concanavalin-A-induced AIH murine model (60)	Selectively stimulates arTregs following adoptive transfer to alleviate injury and control AIH (60)
IL-2 Therapy	Murine AIH model (59) AIH patients clinical trials (57, 58)	In low dose IL-2-treated patients with refractory AIH, increases in Treg populations persisted until 28 days after treatment (57)
Retinoic acid and rapamycin agents	AIH patients clinical trial (63)	Enhances Treg function and reduces expression of Teff transcription factors (62, 63)
Enclysis Inhibitor	Enclysis inhibitors could be tested alone or in combination with existing Treg treatments	Enclysis inhibitors could potentiate Treg immunotherapy for AIH

PolyTreg, Polyclonally-expanded regulatory T cells; arTreg, Alloantigen-reactive regulatory T cells.

1.4.3.3 Targeting Enclysis in viral infection

The most common viruses that affect the function of the liver are Hepatitis B (HBV) and Hepatitis C (HCV) [69]. As hepatotropic viruses, these contribute towards more

transplantations than any other disease throughout the world, through ongoing liver damage leading to cirrhosis and creating a beneficial environment for tumorigenic growth [75]. Furthermore, HBV and HCV infection is behind 80% of the populations hepatocellular carcinoma (HCC) cases worldwide [76].

It is believed that enclysis can play a major role in the dampening of viral infection through its interaction with CD8+ T cells. Sprengers et al. (2006) [77] illustrated a link between the numbers of CD8+ T cells in the intrahepatic environment and the status of liver damage caused by HCV/HBV infection [77]. As Treg cells act to suppress the action of antiviral effector T cells, it is vital that the balance between the immunogenic and tolerogenic response is efficient enough to clear the viral infection. To prevent chronic infection of HCV and HBV and to quickly clear the virus from the host, it can be said that an environment with a low concentration of Tregs is beneficial in reaching antiviral immunity [69]. Hence, increasing enclysis may provide the specific boost needed to decrease Treg numbers and increase the immune control of hepatitis infection and allow for acute elimination of the virus.

1.4.3.4 Targeting enclysis in Hepatocellular Carcinoma (HCC)

Liver cancer is one of the world's biggest killers and the sixth most common type of cancer worldwide. HCC is the most common type of primary liver cancer and is heavily associated with poor prognosis and an extreme rate of recurrence due to a general late presentation and, in turn, late diagnosis. As mentioned previously, HCV and HBV are huge contributing risk factors towards HCC progression, with other contributing factors including alcoholic liver disease, alcoholic fatty liver disease, obesity, and steatohepatitis [69].

It is well documented that chronic inflammation is an important driver of HCC progression. Repeated and unresolved inflammation will induce further tumorigenesis through the activation of proinflammatory cytokines such as TNF-α and IL-6 [78]. Thus, creating a beneficial microenvironment for tumour growth and progression. Furthermore, the hypoxic environment these tumours exhibit, due to reduced blood flow to the liver, will create a more hostile environment and initiate apoptosis throughout the hepatocyte population. In turn, the production of reactive oxygen species is increased which drives mutagenesis of the tumour [79]. It will be key to explore enclysis in these conditions in the future to determine whether the cancer microenvironment manipulates cancer.

Additionally, HCC tumours are remarkable in their ability to evade the immune response targeted against them. The proinflammatory microenvironment present, as forementioned, allows for the recruitment of immunosuppressive Treg cells to the site of the tumour through the action of the higher density of chemokine CCR4 and its ligands CCL22 and CCL17 [80]. Once Tregs have reached the neoplastic environment, they undergo several immunosuppressive mechanisms to drive tumour survival. These include the ubiquitous expression of CTLA-4 which acts as an important partner to Tregs in immunosuppression. The interaction of CTLA-4 with CD80 and CD86 ligands, which are present on the surface of dendritic cells, has been proven to inhibit the activation and, therefore, the antigen-presenting function of these cells [81]. Furthermore, Tregs initiate the secretion of TGF-β and IL-10 which aid in the evasion of effector T cells, as well as preventing granzyme-dependant cytotoxicity [69]. These findings support the theory, found through several studies, that the increased number of Tregs found in the hepatic microenvironment correlates with a worse prognosis for patients and poor response to treatment. This

is further supported by a study performed by Yu et al. 2000 [82] in which Tregs had 3-times higher numbers in tumorigenic tissue compared with healthy tissue [82].

It has also been hypothesised that Tregs play a major role in, not just immune evasion, but effector T cell exhaustion, through the lack of activation of CD8+ T cells [83]. Effector T cells at the site of the tumour become exhaustive by overexpressing programmed death-ligand 1 (PD-L1) and, in turn, activating the programmed cell death pathway, inhibiting their function at the tumour site [84]. In late-stage HCC patients, increased infiltration of Tregs is seen to majorly disrupt the activation as well as the initial recruitment of Tregs to the site of the tumour. Additionally, Tregs associated with HCC significantly dampened effector T cell function with CD8+ T cells displaying decreased release of cytolytic enzymes such as perforin and granzyme A [85].

Currently, there are several therapeutic options available for targeting Treg function in patients with HCC, with the main goal of dampening their tumorigenic effect on CD8+ T cells. Langhans et al. (2019) [86] performed a study illustrating the role Tregs play in the intrahepatic tumour microenvironment. CD8+ T cells, isolated from HCC patients, were co-cultured with Tregs, treated with anti-PDL1 and anti-PD1, *in vitro* with resulted in the reversal of the programmed cell death pathway and increased IFN-Ɣ secretion and the general cytotoxicity of the T cells [86]. Thus, illustrating the importance of Treg involvement in the tumour microenvironment and the importance of modulating their number and function through enclysis.

Pharmacological intervention of enclysis could prove a major therapeutic milestone in the treatment of even later stage HCC. Locally targeting enclysis can dampen the effect of Treg cells and, in turn, boost the blockade of the programmed cell death

pathway as well as CTLA-4 involvement [69]. Thus, proving beneficial in acting as an anti-tumorigenic process.

1.4.3.5 Pharmacological targeting of enclysis

As mentioned previously, pharmacological intervention to either boost or inhibit the process of enclysis could prove key in the treatment of autoimmunity or HCC, depending on the desired function of Tregs required to clear the issue. This, however, remains somewhat unknown with the specific mechanism behind how enclysis preferentially targets Tregs remaining unknown. It is important when designing potential enclysis modulators, to consider the specificity of the process itself, for example, already proven FDA-approved endocytosis modulators may not be sufficient in modulating enclysis. Even though the process is mechanically similar, the adhesion molecules involved differ, illustrating now enclysis needs its own specific drug therapy. Furthermore, targeting enclysis will need to occur on a hepatocyte level with no direct targeting of T cells. This is to prevent a broad effect on effector T cells outside of the tumour environment which may leave the patient susceptible to future infection.

The exact mechanism behind each step in the process of enclysis is needed to be explored before definite FDA-approved treatments can reach potential patients. The Stamataki lab have proven that ICAM-1 plays a major role in the initial adhesion of T cells to hepatocytes, with ICAM-1 blockades inhibiting enclysis [69]. However, given ICAM-1 also plays a huge role in general adhesion of lymphocytic cells, we cannot confirm that targeting ICAM-1 ligands, such as β2 integrins, would target enclysis specifically and not perform a broad halt on all processes involving ICAM-1 [69]. Signalling cascades that occur post T-cell adhesion are also a potential therapeutic

target for modulating enclysis, however, similarly to the adhesion mechanism, this still needs to be explored further [69]. Initial understanding has shown the formation of enclytic vesicles to be heavily associated with β-catenin, thus the Wnt/β-catenin pathway can prove an ideal place to begin exploration of specific enclysis mechanisms [69].

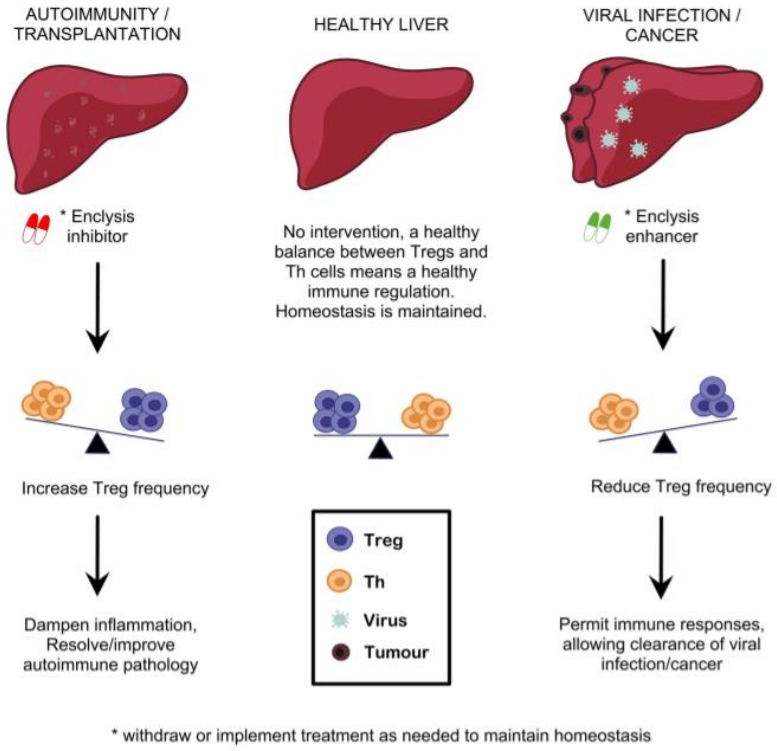


Figure 6. The balance between autoimmunity and cancer. The different potential diseases that could be targeted using the modulation of enclysis, including autoimmunity, cancer and viral infection. Enclysis is shown to potentially aid these issues by modulating the number of Tregs in the hepatic microenvironment, with the balance between too little Tregs and too many proving key in maintaining immune homeostasis [42].

1.5 Drug Repurposing

Drug repurposing, sometimes referred to as drug repositioning, is the process in which pre-existing FDA-approved drugs are re-investigated to determine whether they can be redefined in different treatments they were not originally designed for [87]. This process can provide a new method of quickly identifying therapeutic

agents to treat already known diseases. The importance of this process is the speed in which it can be performed, as once the drugs are already FDA-approved, many steps in the drug discovery pipeline, such as preclinical testing and safety assessments, are able to be skipped [88]. A huge advantage of drug repurposing over developing brand new drugs is the significant decrease in risk of failure. As the drug has already passed FDA approval, the risk of it being failed on safety grounds during clinical trials are extremely low as toxicity reports are all pre-existing and readily available for each drug [87]. Finally, and arguably most importantly in some cases, there will be a significantly reduced need for investment to get the desired drug to market due to saving costs in the preclinical phases I and II. To put this into context, to cost of bringing up a repurposed drug is estimated to be ~ $300 million compared to ~ $3 billion for a newly discovered drug [87]. Traditional drug development will usually consist of five stages, discovery, safety review, clinical research, FDA-approval and FDA post-market safety monitoring [88]. Drug repurposing, however, consists of only four steps, compound identification, compound acquisition, development and FDA post-market safety monitoring which cuts down the costs and a large part of the time [89].

To date, the process of drug repurposing has proven extremely hopeful, with a low success rate. A recent success story can be illustrated with the repurposing of Sildenafil citrate for the use of treating erectile dysfunction [90]. Sildenafil, originally used as an anti-hypertensive drug, was repositioned by Pfizer and remarketed as the market-leading Viagra which, in 2012, gained 47% of the market share of all erectile dysfunction treatments and gained a gigantic $2 billion worth of sales worldwide [90]. Additionally, Thalidomide, originally used as a sedative, gained a vast amount of negative press when it was discovered that the drug was causing severe skeletal

birth defects in children whose mother had taken the drug in the first trimester of the pregnancy [91]. Thalidomide has now been serendipitously repurposed for the treatment for multiple myeloma and now has been replaced by a much more successful derivative Lenalidomide receiving worldwide sales of $8 billion [92].

1.5.1 Computational approaches to drug repurposing

Drug repurposing is usually developed in three steps: identification of the desired drug compound, assessment of the mechanism of the drug in question and finally the assessment of the efficacy of the drug [87]. The first step, identification of the perfect compound, is a systematic process and can be divided into several sub-processes either computational or experimental [87]. Computational approaches are largely driven by data handling and involve a large-scale systematic review of the compound's chemical structure, genotypic and proteolytic data alongside the electronic health records [93].

Computational approaches can also be subdivided into 6 subcategories to further define the importance of this process. These include signature matching, molecular docking, genetic association, pathway mapping, retrospective clinical analysis and novel data sources [87]. *Signature matching* is a process which involves comparing the signature characteristics of the drug (structure and genetic profile) with another drug to determine if it could act similarly [94]. *Molecular docking* is a process which compares the shape and structure of specific molecular binding sites of drug compounds to determine whether a specific therapeutic protein target can be shared in similar binding sites [87]. *Genetic association* involves the analysis of the genetic makeup of a disease to determine whether it can be used as a potential drug target [87]. *Pathway mapping* involves analysing the genetic, protein or disease data that

can prove beneficial in finding targets for repurposed drugs [87]. *Retrospective clinical analysis* is the systematic analysis of electronic health records and data from clinical trials, this data could prove pivotal in determining whether it is worth a drug being moved forward through the drug repurposing pipeline [87]. *Novel data sources* allows for developers to look through large-scale high-throughput drug screens that already exist to use to explore possible methods of drug repurposing [87].

1.5.2 Experimental approaches to drug repurposing

Experimental approaches usually involve a first-hand, in lab, approach to determine whether repurposed drugs can be used for different therapeutic targets [87]. One experimental approach is the use of binding assays to identify target interactions. Techniques such as chromatography and mass spectrometry are used to determine appropriate binding partners for 'hit' repurposed drugs [95]. Finally, *phenotypic screening* is used to find drug compounds that illustrate beneficial effects that could be transferred to be used on relevant diseases that would be affected by the way the drug functions [96]. For example, in a study performed by Iljin et al. (2009) [97] a library containing 4,900 drug compounds underwent a high-throughput phenotypic screen in which they were exposed to four prostate cancer cell lines where disulfiram, a drug initially used for treating alcohol abuse, was determined to be a strong anti-neoplastic agent [97].

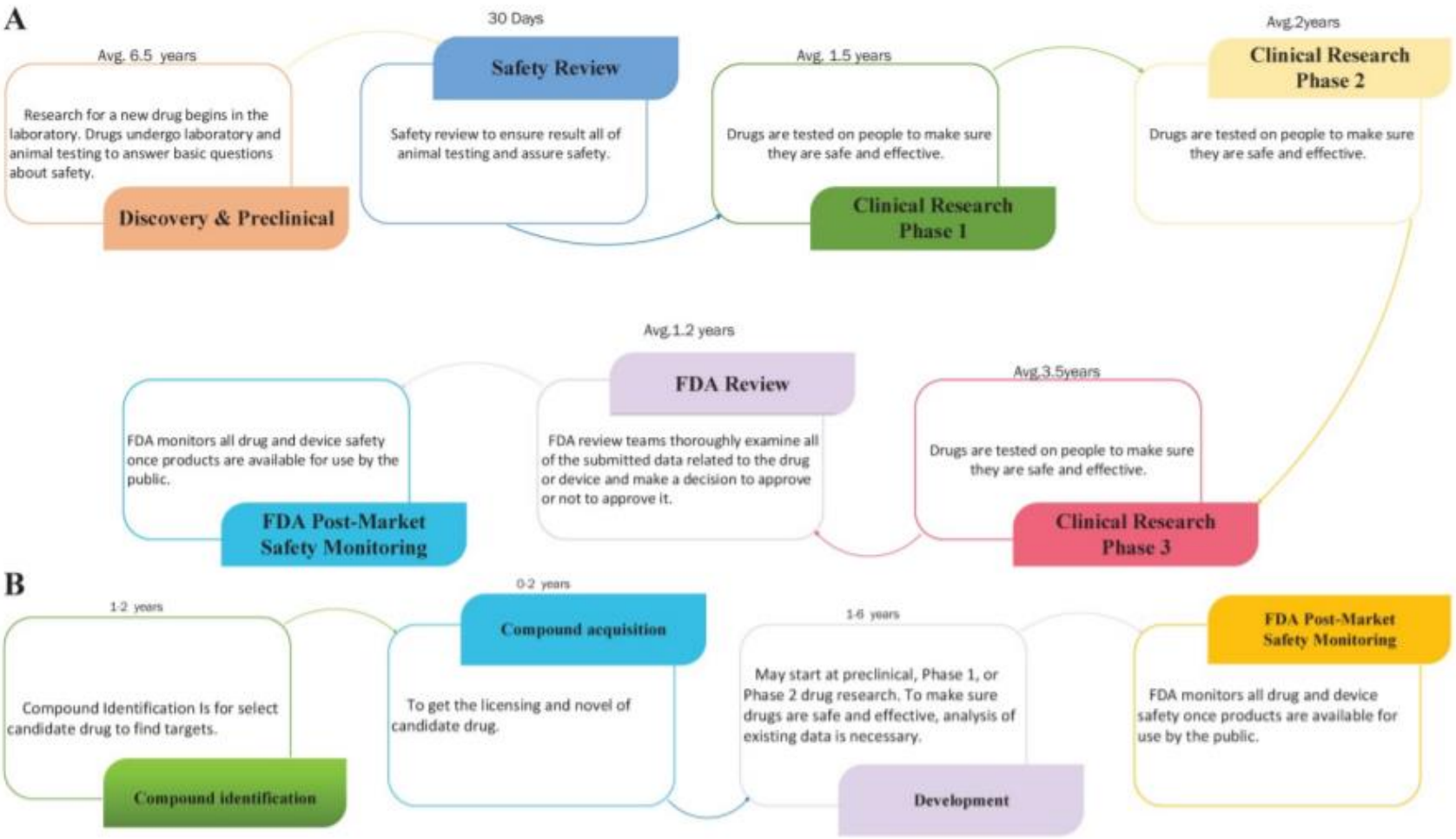

A
Avg. 6.5 years
Research for a new drug begins in the laboratory. Drugs undergo laboratory and animal testing to answer basic questions about safety.
Discovery & Preclinical
30 Days
Safety Review
Safety review to ensure result all of animal testing and assure safety.
Avg. 1.5 years
Drugs are tested on people to make sure they are safe and effective.
Clinical Research Phase 1
Avg.2years
Clinical Research Phase 2
Drugs are tested on people to make sure they are safe and effective.
Avg.3.5years
Drugs are tested on people to make sure they are safe and effective.
Clinical Research Phase 3
Avg.1.2 years
FDA Review
FDA review teams thoroughly examine all of the submitted data related to the drug or device and make a decision to approve or not to approve it.
FDA monitors all drug and device safety once products are available for use by the public.
FDA Post-Market Safety Monitoring
B
1-2 years
Compound Identification Is for select candidate drug to find targets.
Compound identification
0-2 years
Compound acquisition
To get the licensing and novel of candidate drug.
1-6 years
May start at preclinical, Phase 1, or Phase 2 drug research. To make sure drugs are safe and effective, analysis of existing data is necessary.
Development
FDA Post-Market Safety Monitoring
FDA monitors all drug and device safety once products are available for use by the public.

Figure 7. The drug repurposing pipeline. Illustrating the difference in number of steps between Drug repurposing and Drug discovery. **(A)** The steps for drug discovery of a brand-new possible drug with the timeline needed for each step to be completed. **(B)** The steps for Drug repurposing proving to be much shorter than drug discovery with far less steps that require a much shorter amount of time.

1.5.3 High-throughput screening

Drug repurposing is driven through the method of high-throughput screening [98]. Through this process,1000's of potential drug compounds can be analysed at once to determine their efficacy in performing the desired function. The Prestwick Chemical library is a bank of 1200 FDA-approved drug compounds that the Stamataki lab received access to, to perform a high-throughput screen on their ability to modulate enclysis.

1.5.4 The shortlisted Prestwick Chemical Library drug compounds

1200 FDA-approved drug compounds from the Prestwick Chemical Library were analysed by past members of the Stamataki lab before the project began. These compounds were placed through three separate high-throughput screens where they were exposed to enclysis assays. Analysis of these screens was performed and their ability to modulate enclysis was determined on how they varied the number of cell-in-cell structures (enclytic vesicles) found in the hepatocytes. Once each drug compound was analysed, a shortlist was made of over 200 of the best compounds found by a past member of the lab. Once my project started, I reanalysed the shortlist of 200 compounds and picked out the best ten (five inhibitors and five enhancers) to investigate further *in vitro* as seen below in table 2.

Table 2. The shortlisted 'hit' drug compounds

Inhibitor	Enhancer
Clofibric Acid	Quinapril Hydrochloride
Amiloride Hydrochloride	Memantine Hydrochloride
Nifurtimox	Pralidoxime Chloride
Phentermine Hydrochloride	Acetylsalicylic Acid
Amprolium Hydrochloride	Alfuzosin Hydrochloride

1.5.4.1 Clofibric Acid

Clofibric acid (CA) is a clofibrate derivative and acts as an antilipidemic agent to treat hypertriglyceridemia and high cholesterol [99]. Its mechanism of action involves lowering serum lipid levels by reducing the number of the low-density lipoproteins. CA acts to increase the action of lipoprotein lipase (LL), in turn, causing an increase in the lipolysis of lipoprotein triglyceride. CA will also increase the removal of apolipoprotein B as well as reduce its synthesis. This, in turn, reduces the amount of VLDL carried through the hepatic environment. Finally, CA acts as PPAR-α receptor agonist in the liver [99]. This elucidates in increased beta-oxidation and lipoprotein lipase activity.

1.5.4.2 Amiloride Hydrochloride

Amiloride Hydrochloride (AM) is a diuretic used to conserve the homeostasis of potassium levels in the body with antihypertensive properties. It is used in conjunction with thiazide diuretics in the treatment of congestive heart failure. It acts to restore serum potassium levels to normal to avoid hypokalaemia [100]. The mechanism of action of AH acts through preventing sodium reabsorption in the

collecting ducts and distal convoluted tubes in the kidney [101]. This action decreases serum sodium levels and, therefore, the net negative potential of the tubular lumen allowing for increased retained potassium. These effects work harmoniously to decrease hypertension around the heart [100,101].

1.5.4.3 Nifurtimox

Nifurtimox is a nitro-anion metabolite acting as an antiparasitic drug in the treatment of Chagas disease, more specifically the parasite Trypanosoma cruzi [102]. It acts by reacting with nucleic acids of the parasite and inducing large-scale breakdown on the DNA sequence. Nifurtimox will form oxygen radicals, through the action of nitroreductase enzymes, that prove lethal to the parasite and ensures the cytotoxic levels result in parasite death. In 2020, Nifurtimox gained an accelerated FDA-approval for the use on paediatric patients due to very promising phase III clinical trials. The full mechanism of action of nifurtimox, however, is still yet to be elucidated [102].

1.5.4.4 Phentermine Hydrochloride

Phentermine Hydrochloride (PH) is a sympathomimetic anorectic agent used in the treatment of obesity to facilitate weight loss [103]. PH was introduced into market in 1959 as part of a weight loss campaign and is strongly chemically related to amphetamines [103]. PH shares a very similar mechanism of action to amphetamines as they both act as TAAR1 agonists [104]. Through TAAR1 activation, noradrenaline is released from the presynaptic cleft causing the stimulation of β-2-adrenergic receptors [105]. This causes the increase in noradrenaline, dopamine and indirectly serotonin [103].

1.5.4.5 Amprolium Hydrochloride

Amprolium Hydrochloride (AH) is a coccidiostat used in poultry to defend against proliferation of the Elmeria parasite. The mechanism of action occurs through blocking the thiamine transporter of the parasite to prevent carbohydrate synthesis. Little is still known about the mechanism of action of this drug due to it not being used in humans [106].

1.5.4.6 Quinapril Hydrochloride

Quinapril Hydrochloride (QH) is an angiotensin converting enzyme (ACE) inhibitor with antihypertensive qualities granted FDA-approval in 1991 [107]. Usually, angiotensin II acts as a vasoconstrictor which increase oxygen consumption and vascular pressure. An overload of angiotensin II causes a vast amount of myocyte hypertrophy and proliferation of vascular smooth muscle cells. Additionally, a side effect of angiotensin II action is the release of plasminogen activator inhibitor-1 (PAI-1), a main facilitator of thrombosis. QH acts through binding and inhibiting ACE, thus preventing the conversion of angiotensin I to angiotensin II, bradykinin is also spared from breakdown. This prevents the vasoconstricting properties of angiotensin II and allows for vasodilation [107].

1.5.4.7 Memantine Hydrochloride

Memantine Hydrochloride (MH) is an antagonist to the N-methyl-D-aspartate (NMDA) receptor used in the treatment of Alzheimer's disease [108]. Alzheimer's disease manifests as a disruption in the neurotransmission of glutamate causing neuronal excitotoxicity. MH prefers cation channels operated by NMDA receptors illustrating its importance in the treatment of Alzheimer's. MH shares similar mechanism of action to ketamine in targeting NMDA receptors, however MH will provide less deleterious effects [108,109].

1.5.4.8 Pralidoxime chloride

Pralidoxime chloride (PC) acts as a reactivator of cholinesterase's, outside of the central nervous system, and is used in the treatment of organophosphate poisoning [110]. Organophosphate poisoning phosphorylates cholinesterase and deactivates them causing a build up and overexpression of acetylcholine at the synaptic junction, thus causing the cholinergic fibres to continuously fire causing vast discomfort to the patient. PC must be administered 24 hours within exposure to organophosphates to reactivate acetylcholinesterase breaking the phosphate-ester bond between the organophosphate and acetylcholinesterase. Therefore, the accumulated acetylcholine is removed, and the synaptic junction can function normally [110].

1.5.4.9 Acetylsalicylic acid

Acetylsalicylic acid (ASA), more commonly known commercially as Aspirin, is an anti-inflammatory and anti-pyretic drug used for the treatment of fever or general pain which specifically acts as a non-selective cyclooxygenase (COX) inhibitor [65]. It has also been shown that ASA inhibits the aggregation of platelets, hence its involvement in preventing blood clots and myocardial infarction [111]. Interestingly, a study performed by Alfonso et al (2014) [112] proved taking aspirin every day can help prevent against the onset of several cancers including, liver, prostate and lung [112]. The mechanism of action of ASA works through blocking prostaglandin synthesis and acts upon both COX-1 and COX-2 enzymes. Once the acetyl group binds irreversibly to a serine residue on the surface of COX-1 enzyme, the production of inflammatory prostaglandins is halted, and pain is reduced [65]. Furthermore, ASA prevents the formation of thromboxane A2 from arachidonic acid, which is the key instigator in platelet formation [111].

1.5.4.10 Alfuzosin Hydrochloride

Alfuzosin hydrochloride (AFH) acts an alpha-1 adrenergic antagonist used in the treatment of benign prostatic hypertrophy (BPH) [113]. BPH is the benign growth of the prostate gland and results in urinary tract issues in men. Prevalence of BPH in men is extremely high for men over 70 with averages at ~80%. Once activated, alpha-1 adrenergic receptors found in the prostate, bladder and urinary tract lead to the contraction of smooth muscle. AFH acts by binding to alpha-1 receptors, decreasing their function, and allowing for the relaxation of smooth muscle in these areas to alleviate urinary symptoms [113].

1.6 Hypothesis and Aims

Since the process of enclysis is still a brand-new process, discovered only in 2019 by the Stamataki lab, there is a vast gap of knowledge that needs to be filled. For example, the role the Treg cell plays after being ingested by the hepatocyte is still unknown. Furthermore, the actual mechanism and intracellular pathways involved in the identification and degradation of Tregs by hepatocytes acts as the key missing link in fully understanding the process fully. The interaction between hepatocytes and T cells was validated *in vitro* by Davies et al. from this data, a drug repurposing approach was explored to determine whether a shortlist of FDA-approved drugs from the Prestwick Chemical library will enhance the formation of cell-in-cell structures caused by enclysis or conversely inhibit the process. Receiving this data will allow us to determine the efficacy of the shortlisted drugs and prove the ideal conditions for enclysis to take place *in vivo.* This will ideally provide several exciting new avenues to explore when using the process of enclysis as a potential future therapeutic target in the treatment of autoimmunity, cancer, and viral infection within the liver.

Hypothesis = ***We hypothesise the shortlisted FDA-approved drug compounds will either enhance or inhibit the number of live T cells ingested by hepatocytes and, therefore, increase or inhibit the process of enclysis enough that these can be explored therapeutically. Furthermore, we hypothesise that modulating the metabolic conditions that enclysis is performed under will further vary the number of cell-in-cell structures produced.***

1.6.1 Aims and objectives

1. To discover and characterise enclysis-modulating drugs from an FDA-approved library
2. To determine the effect of different metabolic conditions on the process of enclysis
3. To validate these drug compounds in vitro to determine any real potential to be pushed further in the drug repurposing pipeline

2. Methodology

2.1 Materials

Table 3: Laboratory equipment

Equipment	Manufacturer
Incubator	Sanyo
Heat Block	Boekel Scientific
JB Series Grant Water bath	Fisher Scientific
Axiovert Light microscope	Carl Zeiss
JuLi fluorescent cell microscope	NanoEnTek
CellInsight CX-5 High-content Screening Platform	ThermoFisher Scientific
Neubauer Haemocytometer	Marienfield
Cell tally manual counter	Fisherbrand
Pipetboy	Integra
Freezer (-20°C)	Appleton Woods
Freezer (-80°C)	Appleton Woods

Fridge (-4°C)	Appleton Woods
Centrifuge	VWR
Confocal Quantitative Image Cytometer CQ1	Yokogawa Electric Corporation
FACS Aria Fusion	BD Biosciences

Table 4: Chemicals

Chemical	**Manufacturer**	**Catalogue Number**
Lympholyte	Cedarlane	CL5020
RPMI	Gibco	31870-025
Trypsin	Gibco	25200-025
DMEM	Gibco	41965-039
PBS	Made in-lab	Made in-lab
CellTracker™ Green (CMFDA)	Invitrogen	C2925
CellTracker™ Violet (BMQC)	Invitrogen	C10094
CellTracker Blue™ (CMAC)	Invitrogen	C2110
Clofibric acid	Sigma	90323
Amiloride hydrochloride	Sigma	A4562
Nifurtimox	Sigma	N3451
Phentermine hydrochloride	Cambridge bioscience	CAY14184
Amprolium hydrochloride	TCI	5V6ME-BI
Quinapril hydrochloride	Clinisciences	A11736
Memantine hydrochloride	Cayman Chemical LTD	0446089-60
Pralidoxime chloride	Cambridge biosciences	CAY23666
Acetylsalicylic acid	Sigma	A5376
Alfuzosin hydrochloride	Sigma	A0232
Cytochalasin D	Stress marqq Biosciences LTD	130409
Non-essential amino acids	Gibco	11140-050
FBS	Gibco	
L-glutamine	Gibco	25030-024
Glucose-free media	Gibco	A24940-01
Sodium pyruvate solution	Gibco	11360-070
Glucose solution	Gibco	2318205
Methanol	Sigma	

Table 5. Consumables

Consumable	Manufacturer
Falcon T-175, T-75 Flask	Falcon
Falcon tube (25ml)	Falcon
Falcon tube (5ml)	Falcon
Stripette (5ml, 10ml, 25ml)	Costar, Cornering inc.
Pipette tips (10µl-100µl)	Starlab
96-well plate	Greiner Bio-One
Mr Frosty™ Freezing container	ThermoFisher Scientific
Cryovial tube	Fisherbrand™
Eppendorf tube	ThermoFisher Scientific
Pasteur pipette	Sigma

2.2 *In vitro* cell lines

Throughout the course of the project, two specific cell lines were used to act as *in vitro* models for an enclysis experiment. These cell lines were obtained from liquid nitrogen stocks in the laboratory.

2.2.1 Huh-7s

Human hepatoma-derived Huh-7 cells are characterised as a key experimental replacement for primary hepatocytes [114]. Huh-7s have been shown to be excellent at propagating the hepatitis C virus and are used predominantly in HCV work [68]. Huh-7s illustrate immortality as a cell line as they contain a number of tumorigenic cells, as well as being adherent. Huh-7s were cultured in a Falcon T-75/175 flask (depending on the cell number needed for the upcoming experiment) and grown at 37°C and 5% CO_2 in an IncuSAFE incubator. They were suspended in 10ml (T75) and 25ml (T175) of DMEM (Dulbecco's Modified Eagle Media) containing 1% L-glutamine, 1% non-essential amino acids, 1% penicillin and streptomycin and 10% FBS (Fetal Bovine Serum). To prevent overgrowth and death of the cell line, these would need to be split at a ratio of 1:10 every 3 days.

2.2.1.1 Maintaining Huh-7s

In order to maintain a healthy population of huh-7 cells, these require splitting every time they are used for an experiment or every 3 days if not being used. Since Huh-7s are adherent, they will grow in a confluent 2D monolayer on the surface of the flask. To remove the cells from the flask, all of the DMEM was removed and 10ml of Phosphate Buffer Solution (PBS) was added and let flow across the surface of the flask with the adhered Huh-7s. This was then removed and 5ml of trypsin-EDTA was added to the surface of the flask and left for five minutes in the incubator. After five minutes, the flask was checked under a 10x light microscope to ensure the Huh-7 cells are not clumped and are single-cell they are tapped vigorously. The trypsin was then quenched with 5ml of 'Complete DMEM' (CDMEM), containing FBS, to deactivate it and the suspension was placed in the centrifuge for five minutes at 1,500 RPM (423 x g). Once centrifugation has finished, the supernatant was discarded, and the cell pellet is resuspended in 10ml CDMEM. For a ratio of 1:10, 1ml was taken from the cell suspension and placed back inside a new T-75 flask. 9ml of CDMEM was then added to the flask to achieve a final volume of 10ml and placed back inside the incubator.

2.2.2 Jurkat cells

A Jurkat cell line is $CD4^+$ T lymphocyte cell line, originally extracted from the peripheral blood of a child with T cell leukaemia. This cell line is extremely popular and is usually used in experiments involving T cell signalling in HIV [115]. Jurkats were used throughout the project due to their immortality and availability compared to primary T cells. Primary T cells, despite their fantastic predisposition to enclysis, are difficult to isolate and only survive a couple weeks of incubation. Whereas a

Jurkat population can survive in an incubator for up to 30 passages, which makes them ideal for long-term assay work. Furthermore, Jurkat cells present as a homogenous cell line which prevents variation across different assays performed. Conversely to Huh-7 cells, Jurkat cells are non-adherent and grow in suspension. These are suspended in RPMI (Roswell Park Memorial Institute) media consisting of 1% L-glutamine, 1% non-essential amino acids, 1% penicillin and streptomycin and 10% FBS. These are also grown at 37°C and 5% CO_2 in an incuSAFE incubator. To prevent overgrowth and death of the cell line, these are also split every 3 days.

2.2.2.1 Maintaining Jurkats

Since Jurkat cells are non-adherent, there is no need for trypsin-EDTA in the process of splitting them. Jurkat cells are incubated in T-175 flasks to ensure a large cell number was maintained for larger experiments. To split Jurkats, all of the media was taken up and placed into a 50ml falcon tube and placed straight into the centrifuge for 5 minutes at 423 x g. After centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 25ml of ‘Complete RPMI’ (CRPMI) containing FBS. For a ratio of 1:10, 2.5ml of the cell suspension was placed back into a T-175 flask and then 22.5ml of RPMI was added to make a final volume of 25ml. The flask was then placed back into the incubator for growth.

2.3 Counting cells

To obtain the optimal volume of Huh-7 and Jurkat cells from their stock solution, the cells need to be counted to reach the desired concentration for the assay. The cells were split, as previously described, and before discarded the excess cells, 10µl was pipetted on a haemocytometer and placed under a 10x light microscope. Cells found in three four-by-four grids were counted using a tally counter and an average count

is taken and multiplied by 10^4 to determine the number of cells in the suspension/10µl. To calculate the optimal volume required to be taken from each cell suspension for each assay, the equation below is used:

Volume required = $\frac{Cell\ density\ of\ full\ cell\ suspension}{cell\ density\ required}$ x 0.1

= Volume required (mls)

2.4 Seeding Huh-7 cells onto a 96-well plate

Huh-7s are split and harvested as described in **2.2,** these are then counted as described in **2.3** and the required volume needed for the experiment is removed and resuspended in 25ml of DMEM. The optimal density for seeding Huh-7 cells was determined at $1.0x10^4$ cells/well based on Huh-7 density titrations. Once this density is achieved, 200µl of cell suspension was added to each well of the inside 60 wells of a 96-well plate. Seeding of the Huh-7 cells occurs the day before the experiment begins to allow them to fully adhere to the bottom of the wells and form a flat 2D monolayer.

2.5 Staining Huh-7 cells

To view the interaction between Huh-7 cells and Jurkats, both cell types must be stained with different immunofluorescent dye which can be picked up by the CX5 microscope, to visualise cell-in-cell structures formed. After the Huh-7 cells have successfully adhered to the 96-well plate and the 2D monolayer has been formed, the staining process begins. 200µl of DMEM was removed from each well and 100µl of PBS was added and removed to wash the cells of the CDMEM. A solution of 5ml of DMEM containing no FBS (SF-DMEM), and 2.5µl of CellTracker™ Green CMFDA dye at a dilution of 1/2000 (2.5uM) was prepared and 50µl of the solution was added

to the Huh-7s and left in the incubator for 30 minutes. After staining, to ensure the dye has successfully stained the hepatocytes, the cells were analysed using the JuLi^{TM} fluorescent microscope. The dye is then removed and 100µl of CDMEM is added to halt the action of the dye and to prepare for the Jurkats.

2.5.1 Staining Jurkats

Once the cells have been harvested as described in **2.2.2.1,** they are counted, as previously described, and the desired volume was removed to be used for the assay. The optimal density of Jurkats was determined to be $8.0x10^4$ following a Jurkat titration assay. The required volume of Jurkat cells was made up to 5ml using 'Complete RPMI' (CRPMI) containing FBS. This suspension was then slowly layered on top of 5ml of Lympholyte and centrifuged for 20 minutes at 1,200 RPM (340 x g) with a decreased deceleration. Layering with lympholyte will isolate only live cells in a cloudy layer to be extracted. After centrifugation, a cloudy layer of live cells appears in between the layer of lympholyte and RPMI. This cloudy layer was, in turn, extracted using a Pasteur pipette and placed into a 15ml falcon tube. These were centrifuged again for 5 minutes and resuspended in 5ml of RPMI containing no FBS (SF-RPMI). 2.5µl of CellTracker^{TM} Blue (CMAC) dye was added to the suspension of cells at a final concentration of 1/2000 (5uM). This was then placed in the incubator for 30 minutes. After incubation the suspension was centrifuged to remove all the excess dye and the Jurkats were resuspended in the required volume needed for the experiment. These were now ready to add on top of the Huh-7s for the enclysis assay. For metabolism assay, jurkats were resuspended in glucose-free media before co-cultured with huh-7s.

2.6 Designing the optimal conditions for an enclysis assay

To obtain the best results from future enclysis assays where the efficacy of drug compounds in modulating enclysis is tested, we must first identify the optimal conditions for enclysis to occur. It has been previously stated that the optimal percentage of cell-in-cell structures per 100 hepatocytes is 40%. This is due to this value being able to be inhibited and enhanced by drug compounds enough so that a difference can be identified. The plan was to design an assay that would achieve an enclysis coefficient closest to 40%. The negative control for each enclysis assay was Cytochalasin D. Cytochalasin D is used as a negative control as it prevents Huh-7s from producing any cell-in-cell structures by depolarising acting filaments which prevents any phagocytosis. Cytochalasin D was added at a concentration of 10µM to Jurkats. The huh-7's are pre-treated with cytochalasin D before jurkats are added.

2.6.1 Huh-7 number titration

The determine the optimal density of Huh-7 cells for an enclysis assay, a titration of three different densities was performed. Using the techniques described previously, Huh-7s are harvested and, using haemocytometer counts, were prepared in three differing densities. The densities used in this titration were decided at $0.8x10^4$, $1x10^4$ and $1.2x10^4$. These cells were then seeded in a 96-well plate, grouped in their separate densities. Huh7s were then stained with CMFDA and then 100µl of a stained solution of Jurkats were added on top of the Huh-7s to give a final well volume of 200µl. These were then left for 4 hours in the incubator for the Huh-7s to form cell-in-cell structures with the Jurkats.

2.6.2 Jurkat titration with BMQC/CMAC comparison

To determine the optimal density of Jurkats needed for an enclysis assay, another titration is run. Furthermore, this assay produced opportunity to find the best

fluorescent dye to be used on the Jurkats. Historically, CellTracker™ Violet BMQC was used to stain the Jurkats, however the fluorescent overlap was deemed to run too close to CMFDA which provided perfect opportunity to test the appearance of CMAC dye. Jurkat cells were harvested and counted as described previously and centrifuged at 423 x g for 20 minutes at an acceleration of 9 and deceleration of 1. Jurkat cells were calculated to a preliminary density of $4x10^4$/well. This gives a ratio of 4:1 to Huh-7s and is the initial concentration used to perform a serial dilution with. Once the live cells have been isolated, these were split in half where half are stained with BMQC, and the other half stained with CMAC dye. Once stained, 120µl of Jurkats were placed in one row, grouped by BMQC/CMAC staining, of a second clear 96-well plate at a density of $4x10^4$ cells/well. 60µl of RPMI was added to the following four rows and a serial dilution is performed where 60µl is taken from the wells containing Jurkats and diluted down the rows to create further densities of $2x10^4$, $1x10^4$, $0.5x10^4$ and $0.25x10^4$ in final ratios, compared to Huh-7s, of 4:1, 2:1, 1:1, 1:2, 1:4. 100µl of these concentrations of Jurkats were then transferred over to the plate containing the Huh-7s and incubated for 4 hours.

2.7 Shortlisting drugs from The Prestwick Library

As mentioned previously, a shortlist of over 200 drugs was created by a past member of the Stamataki lab, based on their ability to either inhibit or enhance the process of enclysis. 3 separate high-throughput experiments were conducted. The first using the Confocal Quantitative Image Cytometer CQ1 to image the enclysis assay, with the subsequent two using the CellInsight™ CX5 microscope. Manual counts of the 200 shortlisted compounds were performed using Image J software and once all drug compounds are analysed, the top 5 inhibitors and enhancers were identified using statistical analysis to be taken forward for further *in vitro* testing.

2.7.1 Cell-in-cell structure analysis by ImageJ

Images were obtained from each experiment and analysed to determine the number of cell-in-cell structures found by ImageJ software. Each drug compound had six fields of view that were analysed at x20 magnification. Each field of view was analysed manually using ImageJ software and the tally counting feature. Once the number of cell-in-cell structures found in each field of view was counted, this number was transferred to an excel spreadsheet were mean calculations of cell-in-cell structures for each drug were produced.

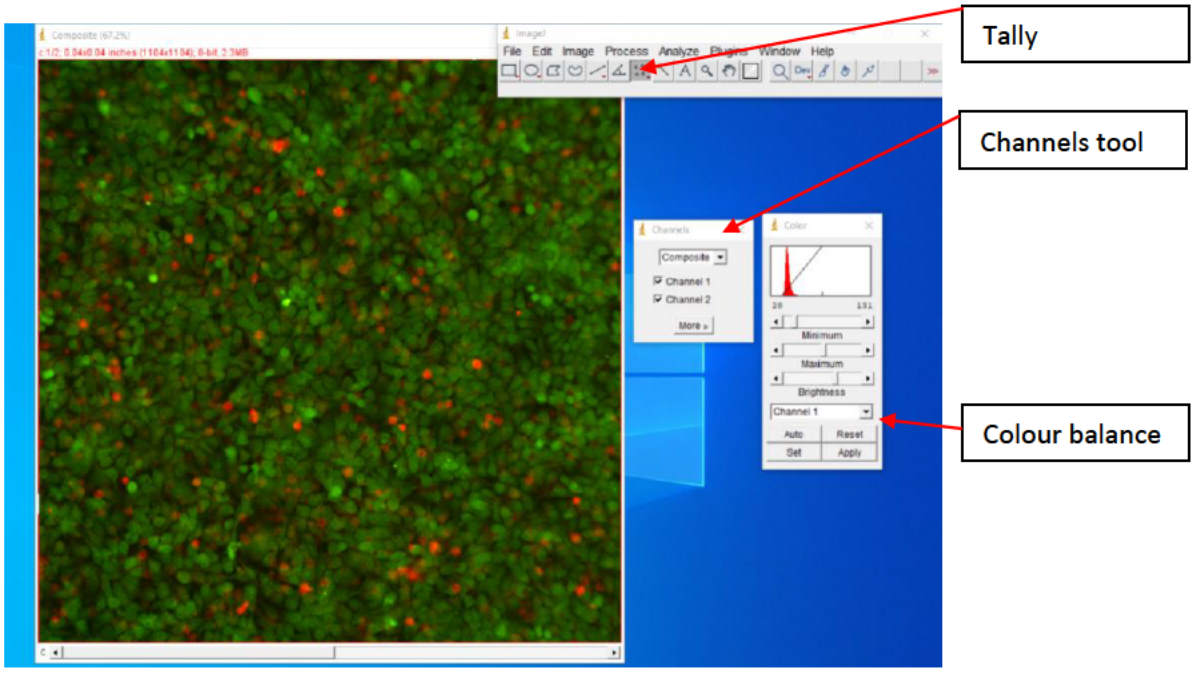


Figure 8. Image J software used to analyse images from the Prestwick Chemical Library as well as images taken using the CellInsight CX5 microscope. The software contains inputs to manage the colour balance of the two fluorescent dyes to ensure the clearest of images. The channels tool allows for each channel to be switched on and off to view each fluorescent channel separately. The tally setting allows for cell-in-cell structures to be manually counted and recorded.

Once the images are uploaded to ImageJ, the process of manually counting the cell-in-cell structures can begin. Since each cell type is stained with different fluorescent dye, the separate cell-in-cell events can be easily identified and analysed. The Huh-7s, stained with CMFDA, fluoresce in the ‘green’ channel at an excitation/emission wavelength of 492/517nm. The Jurkats, stained with CMAC, fluoresce through the ‘red’ channel at an excitation/emission wavelength of 353/466nm. Cell-in-cell structures are characterised by the presence of a vesicle-shaped hole in the cytoplasm of the hepatocyte. This can be seen clearly be switching off the ‘red’ channel and determining whether a clear hole is formed. This, in turn, is verified by switching the ‘red’ channel back on and determining whether a T cell is present within that hole. This process is seen below in figure 9.

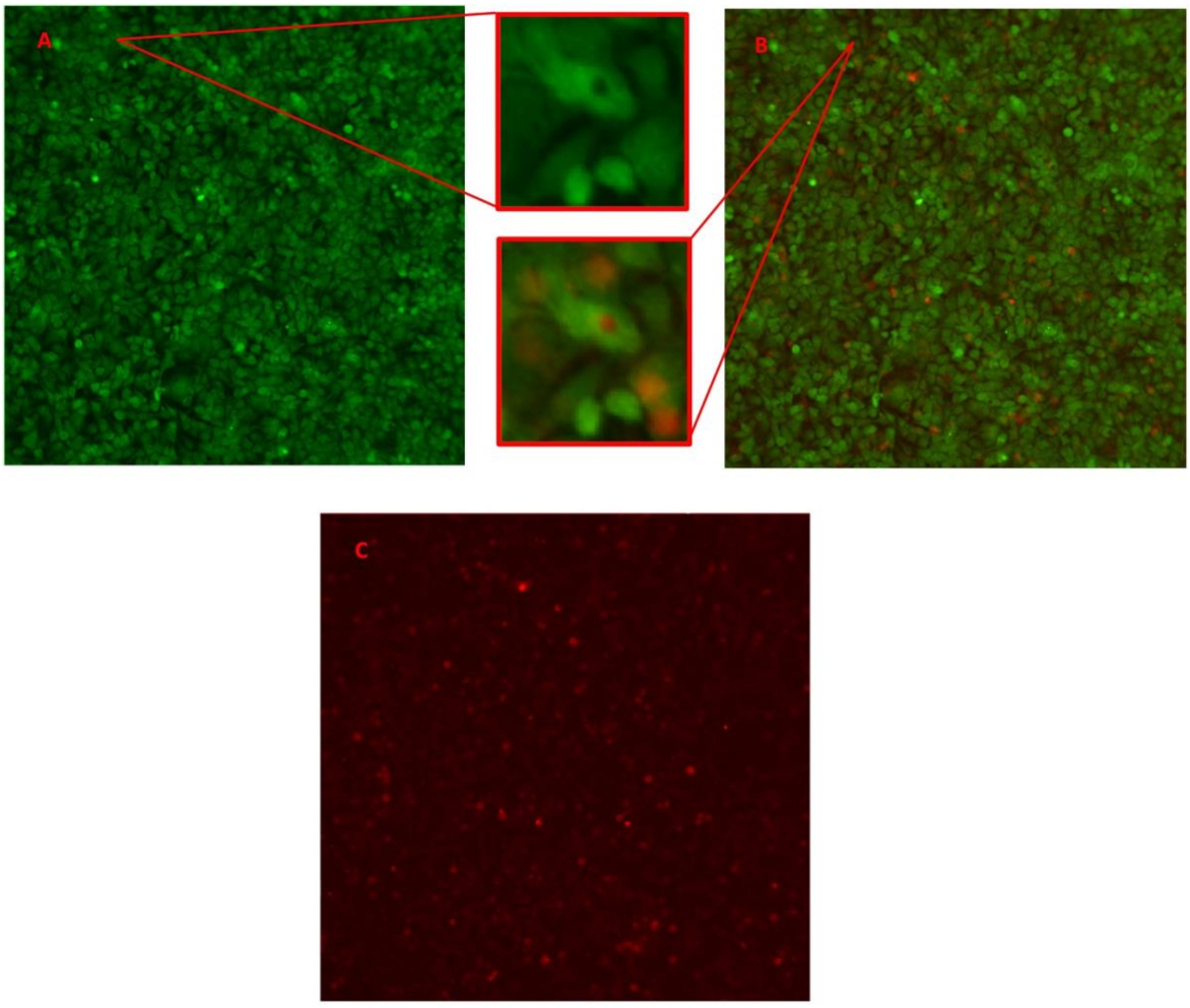


Figure 9. Distinguishing cell-in-cell structures found using ImageJ software. (A) hepatocytes shown using the 'green' fluorescent channel at a wavelength of 492/517nm. (B) a composite image of both the 'green' hepatocytes and 'red' T cells merged. (C) T cells shown using the 'red' channel at a wavelength of 353/466nm. Using the composite image, cell-in-cell structures can be identified by the presence of a 'hole' in the cytoplasm of the hepatocyte that is verified by the presence of the 'red' lit up by a T cell found in the hepatocyte, confirming the presence of enclysis.

2.8 Enriching for enclysis-competent Huh-7s

To ensure consistency throughout each enclysis assay performed, and to maintain enclysis levels at 40%, an enriched population of Huh-7 cells were produced, using flow cytometry, that were mechanistically superior in performing enclysis than wild-type Huh-7s. Wild-type Huh-7s were placed through eight separate flow sorts in which, each time, they were exposed to a large population of Jurkat cells in a process termed 'feeding'.

In preparation for the feed, Jurkat cells were harvested and stained as described previously, however, Huh-7 cells were left adhered in a T-175 flask where they were stained and prepared as the feed would occur in the flask itself. Once the Huh-7s were ready, the stained Jurkats were added into the flask in a final volume of 25ml and the flask was left for 24 hours in the incubator to allow enclysis to occur.

To successfully isolate enclysis-competent Huh-7s through flow cytometry, compensation samples must be prepared to set the control grids during analysis. Thus, four compensation samples were produced alongside the enclysis feed. This included, non-stained Huh-7s, CMFDA+ Huh7s, non-stained Jurkats and CMAC+ Jurkats. Once prepared, these are placed in labelled FACS tubes ready for analysis. Compensation tubes are important when more than one fluorochrome is being used.

If there is some spectral overlap, the compensation sample will ensure that certain fluorescence that is being detected is from the fluorophore that we want to measure. After 24 hours, the excess media, containing any un-engulfed Jurkats, is discarded, the whole flask is washed with PBS and treated with 5ml trypsin-EDTA and placed in the incubator for five minutes. After incubation, the Huh-7s (now combined with engulfed Jurkats) are quenched with 5ml of DMEM and centrifuged for five minutes. After centrifugation, the combined cells are resuspended in 1ml DMEM and placed in a FACS tube.

2.8.1 Analysis using FACS Aria Fusion

The FACS Aria Fusion works through focusing one cell at a time through a flow column, termed a ‘flow cell’. Each cell that passes through the flow cell is met with a laser which causes the absorption and scattering of light. Based on how the light is scattered the cells emit a certain fluorescent intensity. The machine will group all cells that scatter the light in the same way.

To isolate solely enriched Huh-7s (Huh-7e) that have managed to engulf a Jurkat cells and disregard any Huh-7s that haven’t managed to engulf a Jurkat, the ‘feed’ sample tube is run through the Aria Fusion. The sample tube is run alongside the compensation tubes. The compensation tubes containing Huh-7s will show up as FITC+/- (similar wavelength to CMFDA) depending on whether they are stained with CMFDA or not. This will allow comparison between FITC+ cells in the control sample and of those in the ‘feed’ sample to distinguish them from any other cell type. Similarly, compensation tubes containing Jurkats will show as BV421+/- (similar wavelength to CMAC) depending on whether they are stained with CMAC. Isolating Huh-7e means the identification and removal of FITC+/BV421+ cells during the flow

sort. This will produce a sample of only Huh-7 cells that have engulfed a Jurkat cell and are therefore proven to be competent at enclysis. Once this sample has been collected it is grown and expanded further.

2.9 Drug compound preparation

To perform enclysis assays in the presence of different drug compounds, the compounds retrieved must be prepared into a stock solution that can be used during *in vitro* assays. As the drugs arrive in solid form, they must be dissolved first in a medium they are soluble in to a concentration suitable that they can be successfully stored in a stock solution. To determine the mass needed for each drug to be stored at the correct concentration, the below formula is used.

Required concentration (mM) x relative formula mass x required volume (L) = mass required

The solutes and stock concentrations used for each drug can be seen below in Table 6.

Table 6. The drug preparation for the shortlisted drugs

Drug compound	Solute	Stock concentration
Clofibric acid	DMSO	30mM
Amiloride hydrochloride	DMSO	30mM
Nirfurtimox	DMSO	1mM
Phentermine hydrochloride	SF-DMEM	10mM
Amprolium hydrochloride	Methanol	10mM
Quinapril hydrochloride	DMSO	1mM
Memantine hydrochloride	SF-DMEM	10mM
Acetylsalicylic acid	DMSO	30mM
Pralidoxime chloride	SF-DMEM	10mM
Alfuzosin hydrochloride	DMSO	30mM

2.10 drug compound enclysis assay

To test the efficacy of each drug at modulating enclysis, they were tested in enclysis assays at two different concentrations, 10µM and 30µM. This was to determine the optimal concentration to use for further enclysis assays. To test these drugs, enclysis

assays were performed as usual, however each drug was diluted to 10 and 30µM using CDMEM. Once diluted, before Jurkat cells were added, stained Huh-7s were pre-treated in 100µl of the drug suspension for one hour before the Jurkats were added and the 4-hour incubation can begin.

The ‘hit’ drugs that modulated enclysis the most, from a certain concentration, were taken forward and investigated further. Dose-response assays were performed on the drugs that proved to be ‘hits’ in modulating enclysis. These drug compounds were tested at concentrations of 1µM, 10µM and 100µM to determine if their efficacy varied with concentration.

2.10.1 Methanol fixation

After four hours and the completion of the enclysis assay, the plate would undergo methanol fixation to prevent any further interaction between Huh-7s and Jurkats. As methanol is an alcohol, on interaction with cells, it acts to dehydrate them. This process involves the removal of lipids from phospholipid membranes and the precipitation of some proteins. After fixation, the cell membrane remains intact yet permeable and all intracellular mechanisms are halted. A further benefit to methanol fixation is these gives an ample opportunity to wash the Huh-7s of any ‘floating’ and unengulfed Jurkat cells that may disrupt the fluorescence during the imaging stage. Once the enclysis assay is complete, excess media is discarded and each well was washed with 100µl of PBS twice. The PBS was discarded and 50µl of ice-cold methanol is added and left for five minutes at room temperature. After five minutes, the methanol was removed and 100µl of PBS was added to the fixed cells to prevent them drying out. The plate was then wrapped in aluminium foil, to protect the fluorescence from the light, and is stored in the cold room before imaging.

2.11 Metabolic condition enclysis assay

Alongside testing the ability for different drug compounds to modulate the process of enclysis, we also tested how different metabolic factors would either inhibit or enhance the number of cell-in-cell events observed. To do this, we tested enclysis in the presence of different metabolites with three separate pre-treatment conditions. Firstly, Huh-7s were pre-treated with SF-DMEM for an hour and differing levels of FBS were added to the plate to determine how FBS affected enclysis. Samples of 0%, 3%, 10% and 20% FBS were prepared and added to Huh-7s. Secondly, another plate was pre-treated with glucose-free media + FBS for one hour. This plate was subsequently treated with different metabolites including, non-essential amino acids (NEAA), L-glutamine and NEAA + L-glutamine. Finally, a third plate was prepared with glucose-free – FBS pre-treatment for one hour. Similarly, to plate 2, this plate was thus treated with different metabolites including, NEAA, L-glutamine, and NEAA + L-glutamine.

Additionally, the action of sodium pyruvate on the process of enclysis was also tested further down the line.

2.12 CellInsight™ CX5 microscope

After enclysis assays are performed, the plates are fixed, as described previously, and taken down for imaging. Plates are imaged using the CellInsight™ high-throughput CX5 (hereby referred to as ‘CX5’) microscope and then analysed using ImageJ software. Once logged onto the computer connected to the CX5, HCS studios is opened, and the pre-written protocol saved onto the software is opened. The magnification is set at 20x and exposure time set at 0.01s. The fluorescence channels are set up with the ‘green’ channel detecting CMFDA set to 485nm, and

the ‘blue’ channel detecting CMAC set to 365nm. The area for scanning is selected and the plate is scanned. Nine separate fields of view are captured of each well and once completed, these are exported off the computer onto a hard drive. These are analysed using ImageJ software and all graphs are created using Graphpad™ Prism.

2.13 Statistical analysis

For statistical analysis, all experiments underwent three biological repeats as well as three technical repeats. This was to allow for the experiments to become relevant for statistical testing. Statistical tests were performed using GraphPad Prism software where nonparametric unpaired t-tests were performed.

3. Results

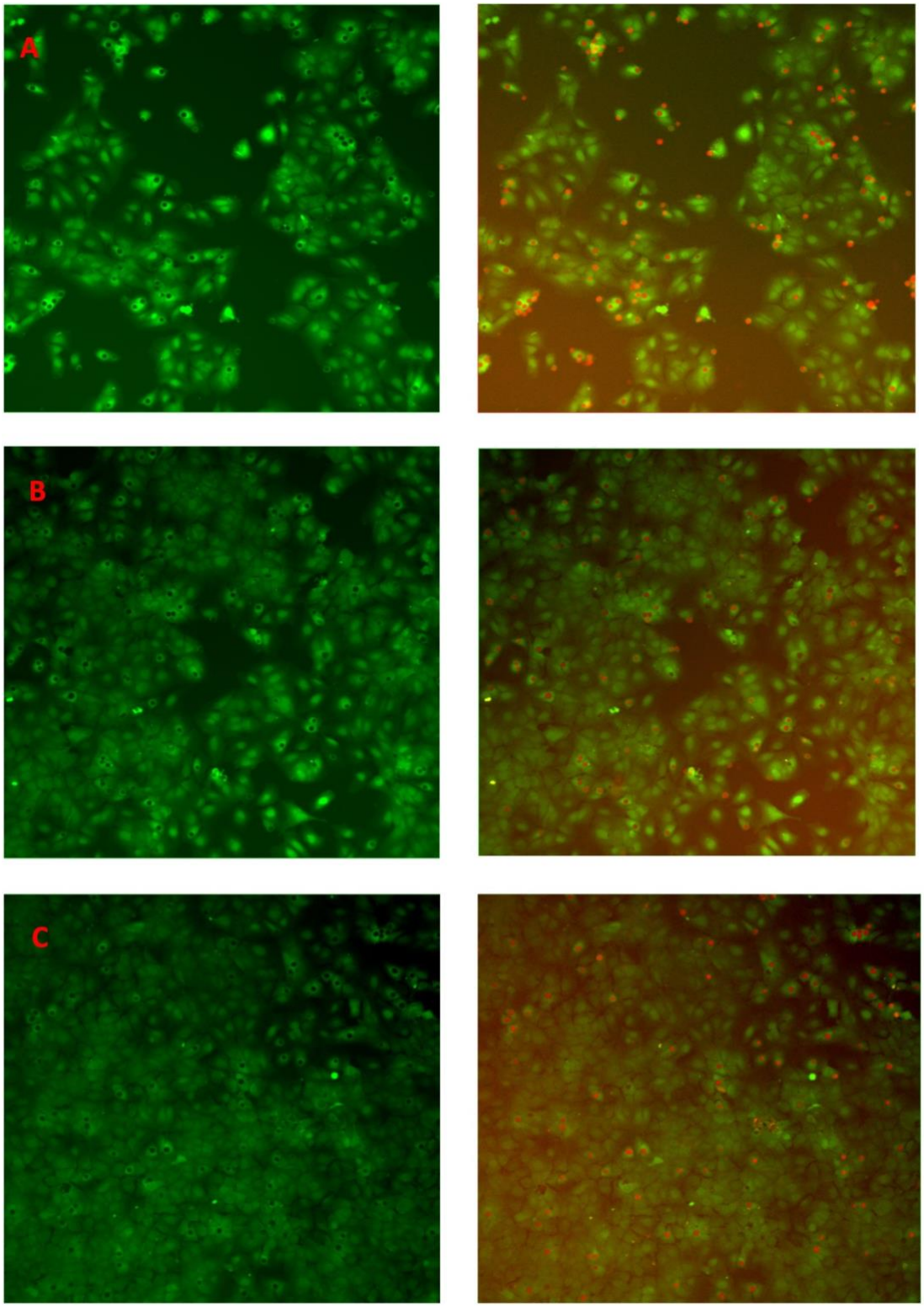
3.1 Creating optimal conditions for an enclysis assay

To determine the optimal conditions for an enclysis assay, a number of different titrations were tested to achieve the optimal ratio of Huh-7s to Jurkats to reach an enclysis coefficient of ~ 40% hepatocytes engulfing a T cell. 40% was previously chosen as the target for cell-in-cell structures per 100 hepatocytes because this level can be inhibited and enhanced enough that a difference could be noted.

3.1.1 Finding the optimal Huh-7 density

To maintain a consistent level of enclysis, the correct density of Huh-7s must be established to produce the optimal 2D monolayer for Jurkat to be captured by hepatocytes. This can largely be determined by viewing how confluent the monolayer appears on the CX5 microscope, however a titration was performed to

validate the number of cell-in-cell structures formed at each density. Huh-7s were seeded at a density of $0.8x10^4$, $1x10^4$, $1.2x10^4$ cells/well, as seen below in figure 10..



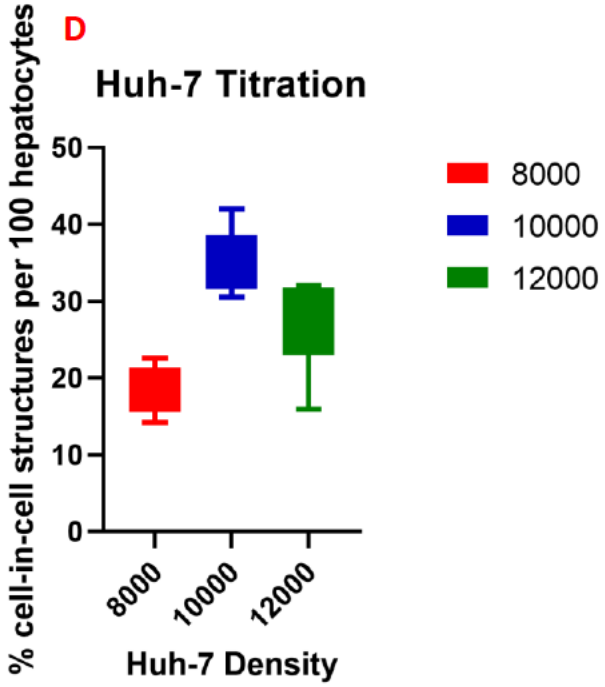


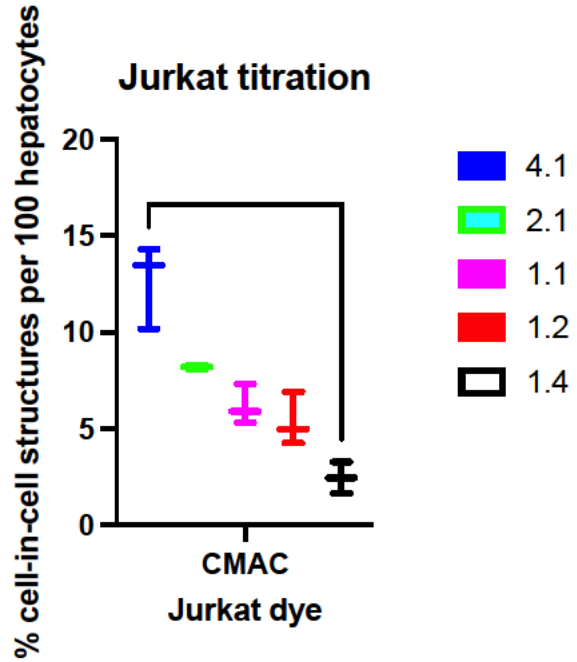
Figure 10. Titration of Huh-7 cells for optimal enclysis. Huh-7 cells were seeded at three different densities to determine the optimal density required to reach an enclysis coefficient of ~40%. Images are taken on the CX5 microscope, at magnification of 20x, where 9 separate fields of view are captured with a random image from each density chosen for the figure. **(A)** Huh-7s seeded at a density of 8000 cells/well where the 2D monolayer shows sub-confluency. Since Huh-7s are not fully confluent, gaps are produced in the well where T cells can fall through the gaps between hepatocytes. **(B)** Huh-7s seeded at a density of 10,000 cells/well where the 2D monolayer shows full confluency with discernible spacing for Huh-7 movement. **(C)** Huh-7s seeded at a density of 12,000 cells/well where the 2D monolayer exhibits full confluency, so much that there is no apparent spacing. Over-confluency causes the risk of contact inhibition with hepatocytes which slows down their intracellular mechanisms and, therefore, enclysis. **(D)** The percentage of cell-in-cell structures seen per 100 hepatocytes for each density, illustrating how 10,000 cells/well is the optimal density due to its reduced variation around 40%. Box and whisker plot illustrate median with whiskers showing interquartile range.

Figure 10 illustrates the beginning of the identification of the optimal conditions for future enclysis assays. Three separate densities of Huh-7s were seeded (8000,10,000,12,000 cells/well) where three very different 2D monolayers are produced. Images are taken using the CX5 microscope at a magnification of 20x where nine fields of view are captured. Figure 10 A illustrates Huh-7s seeded at a density of 8000 cells/well. The 2D monolayer formed accentuates sub-confluency, which decreases levels of enclysis. This could prove that whatever drives enclysis is possibly upregulated in confluent cells under close contact.

Figure 10 B illustrates Huh-7s seeded at a density of 10,000 cells/well, the 2D monolayer formed illustrates optimal confluency with hepatocytes producing a tight monolayer yet with clear discernible spacing between cells. Figure 10 C shows Huh-7s seeded at a density of 12,000 cells/well where the 2D monolayer formed proves extremely overconfluent where very little gaps are seen between each hepatocyte. These results are shown quantitatively in figure 10 D, where an enclysis assay was performed using the three separate densities of hepatocytes. The number of cell-in-cell structures found in each density was analysed using ImageJ to determine which density was optimal in producing enclysis at a percentage nearest to 40%. Figure 10 D proves Huh-7s seeded at a density of 10,000 cells/well were optimal as the percentage of enclysis observed was nearest to the 40% mark and had the least amount of variation, compared to 8000 and 12,000 cells/well where the percentage of enclysis averages around 20% and 30% respectively. This allowed us to conclude that 10,000 cells/well was seen as the optimal density for Huh-7s to be seeded at for future enclysis assays.

3.1.2 Finding the optimal Jurkat density and appropriate fluorescent dye

Once an optimal density of Huh-7s is found, the appropriate ratio of Jurkats must be found to again, produce an enclysis assay that reaches percentages of enclysis ~ 40%. Once the plate is seeded at 10,000 cells/well, Jurkats are added at different concentrations in a serial dilution. Jurkats are added in the following ratio to hepatocytes: 4:1, 2:1, 1:1, 1:2, 1:4. The percentage of enclysis at the separate concentrations is measured using ImageJ as seen below in figure 11.



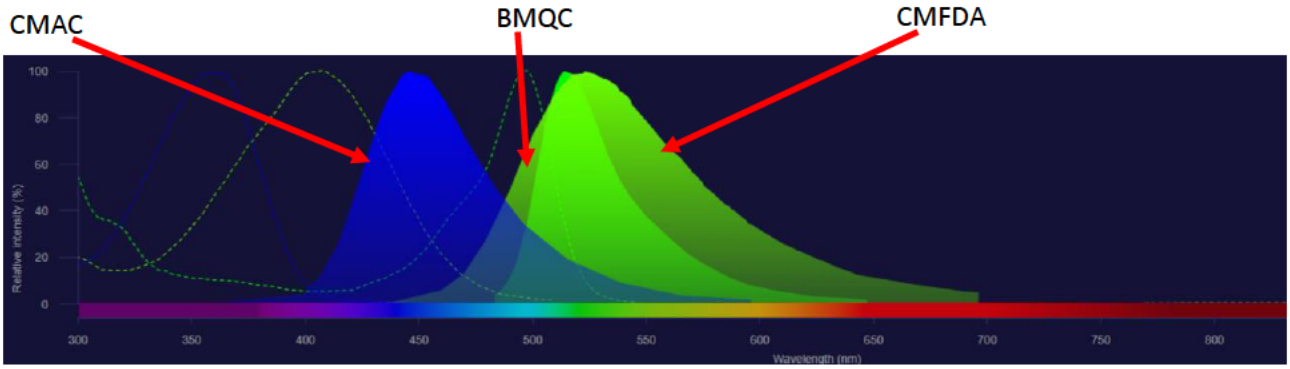


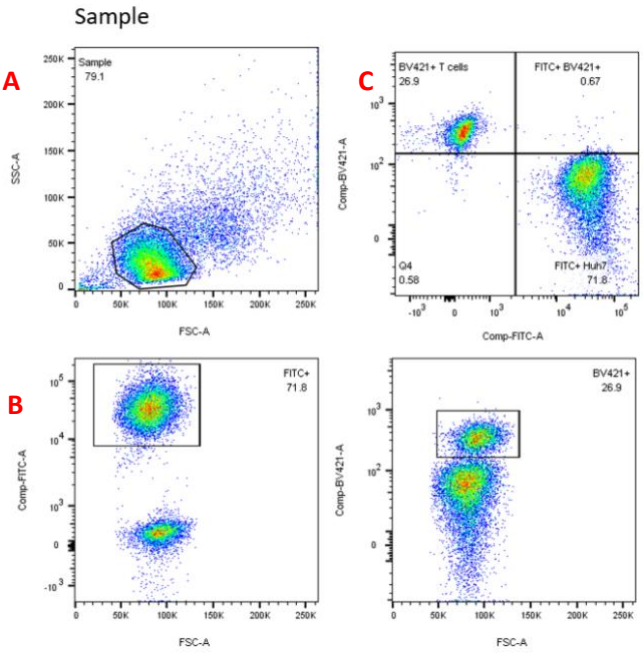
Figure 11. The optimal frequency of Jurkat cells to be used in enclysis assays. (A) To determine the optimal density of Jurkats to be used in future enclysis assays, a titration was performed in the shape of a serial dilution with Jurkats added to Huh-7s at five separate ratios: 4:1, 2:1, 1:1, 1:2, 1:4. The percentage of enclysis observed at these five separate ratios was analysed using ImageJ and graphs were plotted using GraphPad Prism. A paired parametric test was performed which illustrates a ratio of 4.1 jurkats to Huh-7s proved a significant increase in enclysis compared to a ratio of 1.4, with a p value = 0.0272 **(B)** Alongside the titration, two separate fluorescent dyes (CMAC and BMQC) were tested to determine the optimal dye to be used for Jurkats in future enclysis assays to avoid any spectral overlap with CMFDA to ensure the clearest images are produced by the CX5 and specific instances of cell-in-cell structures are easily identified. The three separate dyes are compared using ThermoFisher Spectraviewer online, where spectral overlap can be analysed.

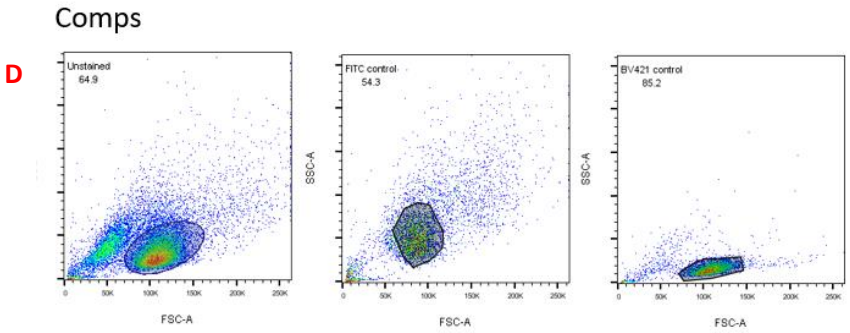
Figure 11 illustrates the optimal density of Jurkats needed to produce an enclysis assay that creates an enclysis coefficient closest to 40%. The Jurkats are added in five separate ratios compared to Huh-7s as follows: 4:1, 2:1, 1:1, 1:2, 1:4. Since the Huh-7s are seeded at a density of 10,000 cells/well, the highest concentration of Jurkats added was 40,000 cells/well. From figure 11 (A) it is shown that as the density of Jurkats increase, the percentage of cell-in-cell structures produced also increases. The density of Jurkats that reached closest to 40% was the ratio of 4:1 which peaked at near 20%, with the lowest percentage of enclysis seen to be 1:4. This illustrates a direct correlation between the increased number of Jurkats and the increased percentage of cell-in-cell structures formed. This was confirmed with a paired parametric t test in which the ratio of 4.1 proved significantly higher than 1.4 with a p value = 0.0272. Since, the highest ratio of Jurkats did not reach 40%, this leaves potential to increase the concentration of Jurkats added further to see the concentration in which the percentage of enclysis plateaus and enclysis cannot be enhanced further.

Furthermore, figure 11 also illustrates the optimal fluorescent dye used to stain the Jurkats to ensure no spectral overlap is seen with CMFDA and the images created to distinguish cell-in-cell structures are clear enough to analyse. Figure 11 (B) accentuates the spectral overlap of CMAC, BMQC and CMFDA. To determine the optimal fluorescent dye to stain Jurkat cells, the emission of said dye must not overlap with the emission of the CMFDA that stains hepatocytes. As seen in figure 11 (B) BMQC dye shares a large portion of spectral overlap with CMFDA which causes overlap of fluorescence in images produced through the CX5 as fluorescence isn't as the emission values are too close. CMAC, however, shows little-to-none overlap with CMFDA allowing for cell-in-cell structures to be viewed distinctly and, in turn, CMAC to be used to stain Jurkats in future experiments.

3.1.3 Enclysis-competent Huh-7 cell enrichment

The final aspect in creating the optimal conditions for an enclysis assay was to produce a population of enriched Huh-7 cells that are more competent in performing enclysis than wild-type hepatocytes. After a 'feed' with Jurkats, the Huh-7s that contain a Jurkat through enclysis are isolated, using flow cytometry, from those who have not been able to engulf a Jurkat. This became the mark of a hepatocyte that is deemed 'competent' in enclysis.


Sample
A
Sample
79.1
SSC-A
FSC-A
C
BV421+ T cells
26.9
FITC+ BV421+
0.67
Q4
0.58
FITC+ Huh7
71.8
Comp-BV421-A
Comp-FITC-A
B
FITC+
71.8
BV421+
26.9


Comps
D
Unstained
64.9
FITC control
54.3
BV421 control
85.2
SSC-A
FSC-A

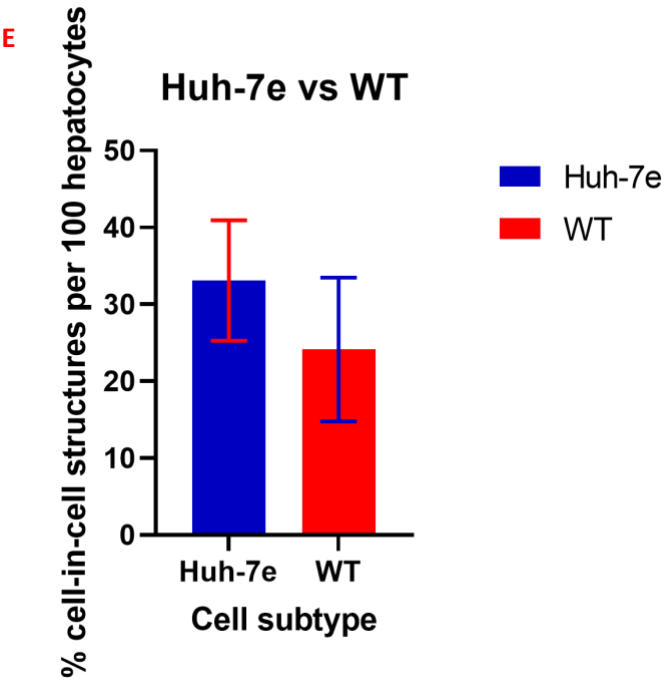

E
Huh-7e vs WT
% cell-in-cell structures per 100 hepatocytes
50
40
30
20
10
0
Huh-7e
WT
Cell subtype

Figure 12. Enrichment of enclysis-competent Huh-7 cells by flow cytometry. (A) Forward and side scatter of the 'feed' sample containing both Huh-7s and Jurkats, with gating shown around the live subsets of both cells, eliminating any cell debris or dead cells. **(B)** Forward scatter against both FITC+ and BV421+ fluorescence intensity plotted for the compensation samples with gating around the fluorescent positive cells to determine a benchmark for cells isolated from the 'feed' sample. **(C)** fluorescent intensity of FITC+ cells and BV421+ cells compared with a FITC+/BV421+ quadrant used to gate for enclysis competent cells. **(D)** Forward scatter and side scatter of all compensation samples, gating for the cells that exhibit either BV421+ or FITC+ staining. **(E)** An enclysis assay performed in the presence of either enriched Huh-7s or wild-type Huh-7s, illustrating Huh-7e's could be more competent in performing enclysis than wild-type cells. All flow sorting taken place using the Aria Fusion.

Figure 12 illustrates the process taken by flow cytometry in producing a population of enriched Huh-7s from wild-type cells. The flow sort was performed eight times where each population became more enriched with enclysis competency than the last. Figure 12 (A) illustrates the forward scatter vs the side scatter of the 'feed' sample. Gating is seen in the central bulk of the cells where only live Huh-7s and Jurkats are isolated. Dead cells or any cells debris is eliminated using this gating system. The forward scatter vs fluorescent intensity is shown in (B) for both BV421+ Jurkats and FITC+ Huh-7s. The live cells that are positive for fluorescent staining are located and gates are drawn around those cells, so only cells with fluorescent intensity high enough to be gated are isolated in the sort. The sorting of the enriched Huh-7s is seen in figure 12 (C). The plot compares the fluorescence intensity of BV421+ Jurkats and FITC+ Huh-7s. Four separate quadrants are drawn around the sample data and cells that are positive for either BV421 or FITC are sorted into Q1 and Q3 respectively. Q3 (top right) contains the Huh-7s that have engulfed a T cell and, in turn, prove to be enclysis competent, and therefore show as FITC+/BV421+. These cells are then able to be isolated from the rest using the AriaFusion. (D) illustrates

the forward scatter vs side scatter of the compensation samples used to gate for the live Huh-7s and Jurkats.

Once the sort is performed and the Huh-7e's are isolated from the rest of the sample, they are expanded in a culture flask. The efficacy of their enclysis competency is then tested against wild-type Huh-7s to determine whether there is a difference in cell-in-cell structures formed. From figure 12 (E) Huh-7e's are shown to possibly be more competent in performing enclysis than wild-type cells. This is because the mean enclysis coefficient stands out ~ 30% for Huh-7e's compared to ~20% for wild-type cells. However, as the error bars are seen to share some overlap, we cannot confidently confirm that enriched cells are significantly more competent than wild-type cells.

3.2 Prestwick library drug compound shortlisting

In order to provide a final shortlist of enclysis-modulating drug compounds that can be tested *in vitro,* a major screen of the preliminary Prestwick Chemical Library shortlist was undertaken. Three independent experiments were analysed of 300 drug compounds that were previously determined as 'hit' drugs by a former member of the lab. These enclysis assays were analysed using manual counts of cell-in-cell structures using ImageJ software where nine fields of view were analysed to create an average cell-in-cell structure count for each drug. These were plotted against the entire cohort of drugs to determine any stand outs.

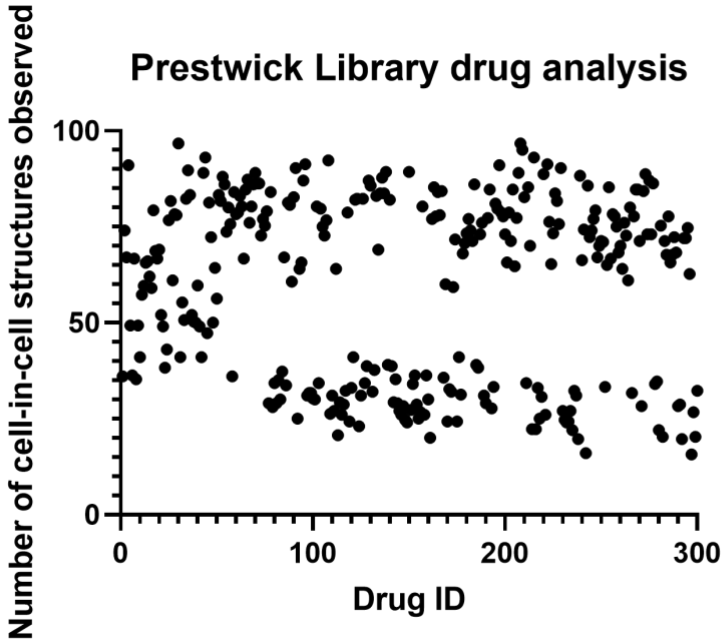


Figure 13. Huh-7 engulfment of jurkats analysis of the preliminary Prestwick Library drug compound shortlist. A shortlist of 300 drugs, produced by former members of the lab, was analysed to create a final shortlist of the best five enhancers and inhibitors of enclysis. Three individual experiments were analysed through counting cell-in-cell structures for each drug compound using ImageJ software. These were subsequently plotted using Prism software. The top five enhancers and inhibitors of enclysis were identified using standard deviation calculations to find the compounds that differ the furthest from the mean value of the control wells. Control wells contained a co-culture of huh-7s and jurkats with 0.1% DMSO.

Figure 13 illustrates the analysis of the entire 300-drug preliminary Prestwick Library shortlist. The compounds were spread over 15 individual 96-well plates with 15 separate control samples. It is clear from figure 13 the difference in cell-in-cell structure counts between enhancer compounds and inhibitor compounds given a large gap between the two populations. The greatest enhancer reaching ~100 cell-in-cell structures counted with the lowest lying at ~20. Each plate contained a 0.1% DMSO control which had the number of cell-in-cell structures found analysed and

was used in standard deviation calculations to identify compounds that were ‘hits’ and fell far enough away from the control mean values to be deemed genuine modulators of enclysis.

To determine if a drug was a modulator, standard deviation calculations were performed on each plate using 15 separate control mean values. Each plate was treated as a separate experiment and once analysed the raw count of cell-in-cell structures was plotted in figure. A compound was deemed a ‘hit’ inhibitor or enhancer if the number of cell-in-cell structures surpassed ±2 standard deviations away from the control mean of their own plate in an equation seen below.

Potential enhancer of enclysis = Average count for control wells + 2 standard deviations

Potential inhibitor of enclysis = Average count for control wells – 2 standard deviations

Once a shortlist of drugs that surpassed the threshold for ‘hit’ inhibitors and enhancers was created, the top 5 that had the most cell-in-cell structures found for enhancers and the least for inhibitors were initially chosen. Each drug was subsequently researched for toxicity in the liver to determine whether, if suitable, would be able to be used clinically as a drug that effects the hepatic environment. If a drug on the shortlist produced any form of liver toxicity in prior reports, it was cleared off the shortlist and the next best drug that caused no liver toxicity was added. The drugs were compared to a 0.1% DMSO vehicle control on each plate.

3.3 *In vitro* drug compound testing on enclysis

The five ‘hit’ inhibitors and enhancers of enclysis purchased to test if their effect on enclysis in *in vitro* assays was consistent. The drugs were prepared as described in

2.7 and stored in the freezer at -20°C in their stock solution. The enclysis assay was performed and the drugs were used at a concentration of 10µM which was seen as the optimal concentration used for the drugs based on previous work with Prestwick Library drug compounds in the lab. This is seen as the optimal concentration as it is a high enough concentration to modulate the process of enclysis without causing any toxicity.

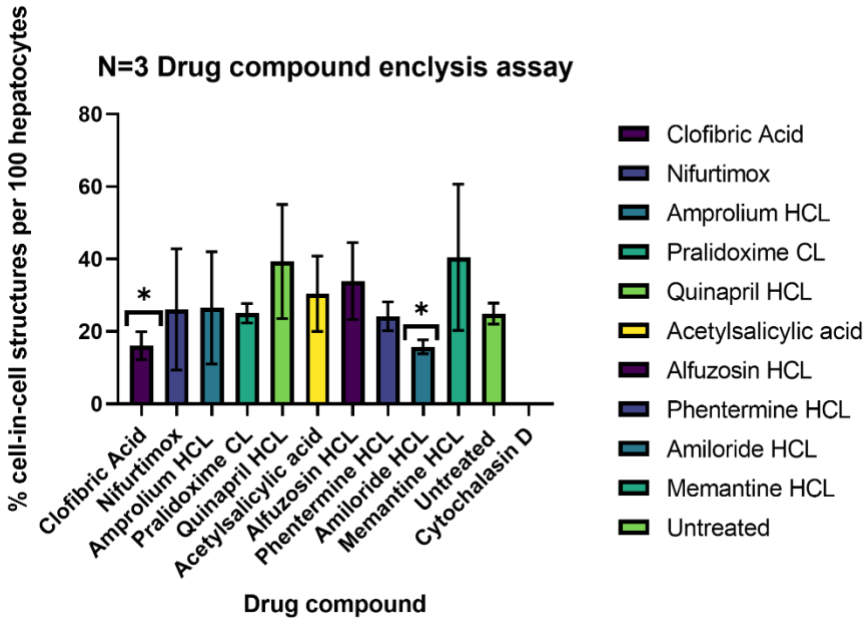


Figure 14. N=3 analysis of the five 'hit' inhibitors and enhancers of enclysis. The top five inhibitors and enhancers of enclysis, shortlisted from the Prestwick Chemical Library 300-drug shortlist were tested to determine their effect on enclysis *in vitro.* After pre-treatment of the drugs with Huh-7e's for an hour at 10µM, Jurkats are added, and the number of cell-in-cell structures found is analysed using ImageJ. The percentage of enclysis is directly compared to the untreated control to determine whether the modulation is significant or not. Cytochalasin D was used a positive control for inhibition of enclysis due to its ability to complete deplete the ability to form cell-in-cell structures. Unpaired T-tests were performed on the data with all calculations performed on GraphPad Prism. An unpaired t-test was chosen due to the data from the drug compounds being independent of each other.

Figure 14 illustrates the effect each ‘hit’ drug compound has on the process of enclysis. Each drug undergoes pre-treatment with Huh-7e’s for an hour at a concentration of 10μM before Jurkats are added and enclysis can take place. The ability of each drug to modulate enclysis is directly compared to the untreated control which consists of Huh-7e’s and Jurkats with no pre-treatment of any drugs. It is clear from figure 14 that several ‘hit’ compounds are able to modulate the process of enclysis with, however, a large amount of variation. Quinapril hydrochloride stood out especially due to their mean percentage of cell-in-cell structures formed reaching as high as 40% with the highest value for each reaching 58% and 60% respectively. Alfusozin hydrochloride was also worth a mention due to a considerable increase in mean percentage of enclysis compared to the control of 37%. Due to the large variation between biological repeats, error bars for each enhancer is seen to overlap with the error bars found for the untreated control which prevents a concrete decision made on these compounds that they are significant enhancers of enclysis. However, since these compounds produced a lot of promise in an n=3 drug compound assay experiment, we decided to take Quinapril hydrochloride, Memantine hydrochloride and Alfusozin hydrochloride forward to perform dose-response assays to attempt to gain results that would label them possible enhancers of enclysis. Statistical analysis was performed on the data using GraphPad Prism software to determine whether any enhancers were deemed statistically significantly different compared to the untreated control. An unpaired T-test was performed on each compound in comparison to the untreated control. However, given the large variation each enhancer gained a p value > 0.05 and was deemed, therefore, not significantly different to the untreated control.

Conversely, Clofibric acid and Amiloride hydrochloride, from the inhibitors shortlist were able to modulate enclysis enough that the mean value for percentage of cell-in-cell structures identified was much lower than the untreated control. Clofibric acid illustrates a mean percentage of cell-in-cell structures formed at 17% with Amiloride hydrochloride going as low as 16%. Statistical analysis performed through an unpaired T-test was able to identify Clofibric acid and Amiloride hydrochloride as significant inhibitors of enclysis with values of 0.0345 and 0.0104 respectively which allows them to be labelled statistically different to the untreated control given $p < 0.05$. Clofibric acid and Amiloride hydrochloride were subsequently brought forward to perform dose-response assays to validate their effect.

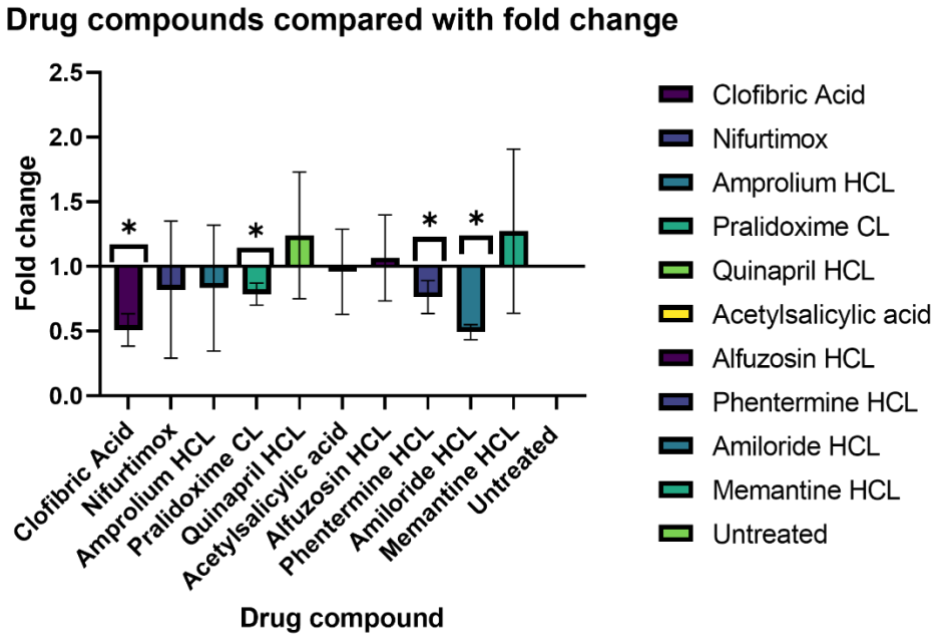


Figure 15. Fold change analysis of shortlisted drug compounds. The N=3 data can be expressed using fold change analysis compared to the untreated control. This form of displaying data can allow a varied approach of viewing potential inhibitors and enhancers as the average counts for each drug is directly compared to the average counts for the untreated control. Fold change is calculated by dividing the average count for each drug by the average of the control wells. Statistics performed using unpaired t tests using GraphPad Prism software.

Figure 15 allows for a different approach in viewing how each compound varies enclysis in comparison to the untreated control. Fold change was calculated for each compound by dividing the average cell-in-cell count by the average for the control wells. We could hypothesise that Quinapril hydrochloride, Memantine hydrochloride and Alfuzosin hydrochloride were possibly enhancers of enclysis as they had a fold change value >1 which illustrates a higher value to the untreated control, with values of 1.24, 1.27 and 1.07 respectively. However, these drugs did not provide significantly different results when statistics were performed as the had a P value of >0.05. Furthermore, Clofibric acid, Amiloride hydrochloride, Pralidoxime chloride and Phentermine hydrochloride proved to the best inhibitors with the greatest fold change difference in comparison to the untreated wells. These drugs proved to be significantly different to the untreated control when unpaired t tests were performed on the sample. These drugs illustrated a p value of 0.0024, 0.0001, 0.0128 and 0.0321 respectively.

From this data, we are still unable to confidently propose an enhancer of enclysis, however Clofibric acid, Amiloride hydrochloride, Pralidoxime chloride and Phentermine hydrochloride showed potential to be taken forward to validate their inhibitory qualities. However, given Clofibric acid and Amiloride hydrochloride proved statistically concrete in both experiments, these will be taken forward as potential inhibitors.

3.4 Dose-response assays on ‘hit’ drugs

After the first in vitro drug analysis, a secondary shortlist was formed to prove the enhancing capabilities of Quinapril HCl, Memantine HCl and Alfuzosin HCl and also validate the already statistically significant inhibitory qualities of Clofibric acid and

Amiloride HCl. In order to test this, a dose response assay was performed to determine the effect of each drug at three different concentrations 1µM, 10µM and 100µM, to experiment whether an increased concentration would enhance enclysis without causing toxicity as well as determining whether the drugs can have the same effect but at a smaller concentration which would reap a number of benefits.

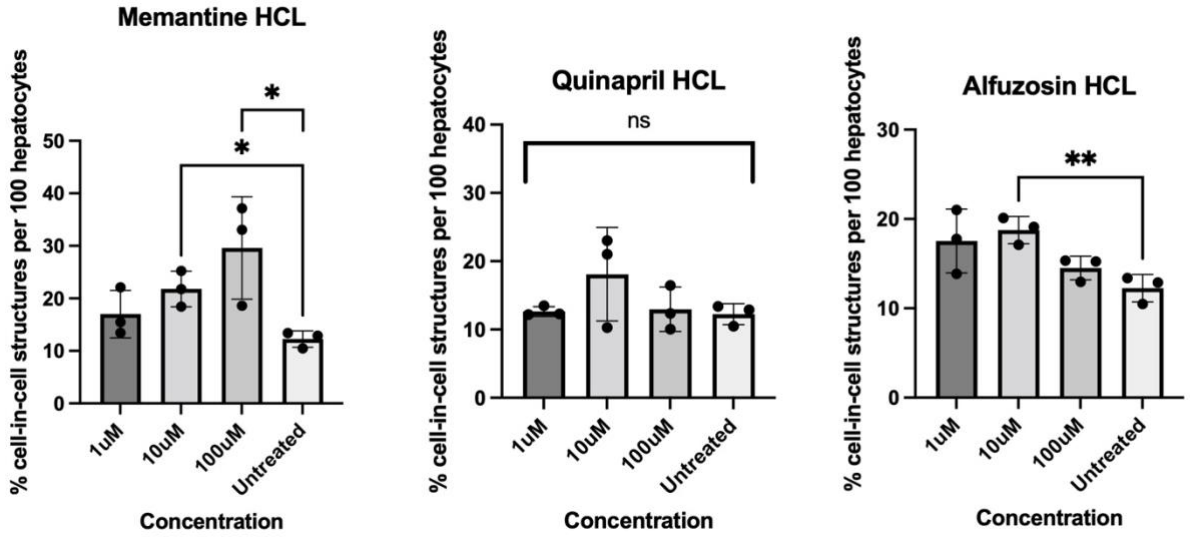


Figure 16. Dose response assays for 'hit' enhancers of enclysis. The three shortlisted enhancers of enclysis are tested at three concentrations in a dose response assay to determine whether their enhancing effect is repeated and whether it is either improved at concentrations away from the 10µM used in the initial experiment. Alfuzosin HCL, Memantine HCL and Quinapril HCL are used to pre-treat Huh-7e's at concentrations of 1µM, 10µM and 100µM. After the enclysis assay, cell-in-cell structures are analysed at each concentration, and the average count is plotted against an untreated control average value. Unpaired nonparametric T-tests were performed comparing each concentration for each drug. Memantine HCl was shown to significantly enhance enclysis at concentrations of 10uM and 100uM with p values = 0.0115 and 0.0382 respectively. Quinapril HCl proved to not significantly enhance enclysis with no concentrations showing any significant increase. Alfuzosin HCl only showed a significant increase at 10uM with a p value = 0.0065.

To validate the initial efficacy of potential enhancers, that have not yet proved statistically concrete, Alfuzosin HCL, Memantine HCL and Quinapril HCL shown in the first N=3 drug assay, a dose-response assay is performed to validate the effect

of the enhancing drugs away from the concentration used in the first experiment. From figure 16 we can conclude that each ‘hit’ drug is able to enhance the percentage of cell-in-cell structures formed the best at 10µM for Quinapril HCL and Alfuzosin HCL as the mean percentage of enclysis is highest with each drug at 10µM with values showing 18% for both. However, Quinapril HCl proved no concentration had a significant enhancement of enclysis compared to the pre-treatment, so we cannot conclude this a concrete enhancer of enclysis. Alfuzosin HCl proved the best enhancer at 10uM with a p value = 0.0065. Memantine HCL is shown to be the best enhancer in this experiment by producing the highest percentage of cell-in-cell structures formed compared to the others, with a value of 20% at 10µM, which was significantly increased compared to the untreated with a p value = 0.0115. Interestingly, Memantine HCL was also shown to perform the best at the higher concentration of 100µM, with a mean percentage reaching 30%, again a significant increase compared to the untreated with a p value =0.0382. This is antithetical to the other enhancers who show a more decreased percentage of enclysis at the higher concentration of 14% and 12%. These findings allow us to confidently state that the enhancing capabilities of Alfuzosin HCL and Memantine HCL are validated using the dose-response assay with statistical analysis confirming this. Quinapril HCL is still unable to be deemed a significant enhancer as the large variation shown by the drug means and no significant increase at any concentrations once statistically analysed. Furthermore, we can hypothesise that, if used therapeutically, Memantine HCL would need to be used at a higher concentration compared to Alfuzosin HCL, given its increased efficacy at 100µM.

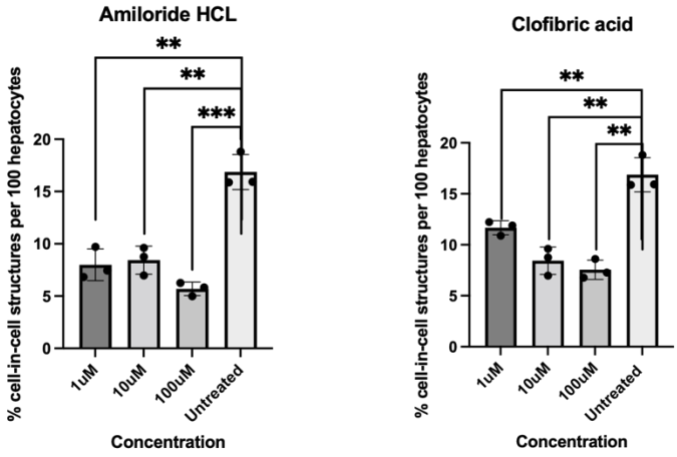


Figure 17. Dose-response assay for 'hit' inhibitors of enclysis. The two shortlisted inhibitors of enclysis were examined further with their efficacy investigate at three concentrations. Clofibric acid and Amiloride hydrochloride were pre-treated with Huh-7e's at 1μM, 10μM and 100μM and an enclysis assay was performed. After the assay, the cell-in-cell structures formed are counted using ImageJ software and graphs are plotted using GraphPad Prism software. Unpaired nonparametric t tests were performed on Prism where each concentration was compared to the untreated control. Amiloride HCl proved a significant decrease in enclysis for all three concentrations, 1uM, 10uM and 100uM with p values = 0.0024, 0.0024 and 0.0004 respectively. The same can be said with clofibric acid that proved a significant inhibitor of enclysis with p values = 0.0077, 0.0024 and 0.0011 respectively.

Figure 17 illustrated how Clofibric acid and Amiloride hydrochloride were both significantly able to inhibit the process of enclysis. The dose-response assay was used to validate these findings and determine whether the inhibitory qualities can be further improved by modulating the concentration of drug used and whether increasing will cause any toxicity. Both Clofibric acid and Amiloride hydrochloride prove to validate the initial N=3 drug assay results by maintaining a percentage of cell-in-cell structures formed much lower than the untreated control. At 10μM, both Clofibric acid and Amiloride hydrochloride have mean percentage of enclysis of ~7% compared to ~17% for the untreated control, illustrating their ability to significantly inhibit encysis at this concentration with p values = 0.0024 and 0.0024 respectively

Interestingly, both Clofibric acid and Amiloride hydrochloride acted as better inhibitors when the concentration was increased to 100µM, as both show the lowest percentage of enclysis at this concentration, again illustrating a significant reduce in enclysis at this concentration with p values = 0.0011 and 0.0004 respectively. This may prove that increasing the concentration further than 100µM may cause even larger inhibitory effects. However, it is uncertain whether the decrease in enclysis may be caused by slight toxicity with the increased dosage of drug. This would have to be explored further in future work.

These results allowed us to confirm that Amiloride hydrochloride and Clofibric acid and are sufficient inhibitors of enclysis all with huge potential to be investigated further. Alfuzosin hydrochloride and Memantine hydrochloride are have shown promise in a second round of experiments but cannot yet be confirmed as enhancers of enclysis.

3.5 Drug combination experiments

After determining Alfuzosin hydrochloride, Memantine hydrochloride, Clofibric acid and Amiloride hydrochloride as concrete modulators of enclysis on their own, it was discussed that these initial shortlisted drugs can be used to test the effect of combining potential inhibitors and enhancers to illustrate whether drugs that did not make the final shortlist can be used in conjunction to make a difference.

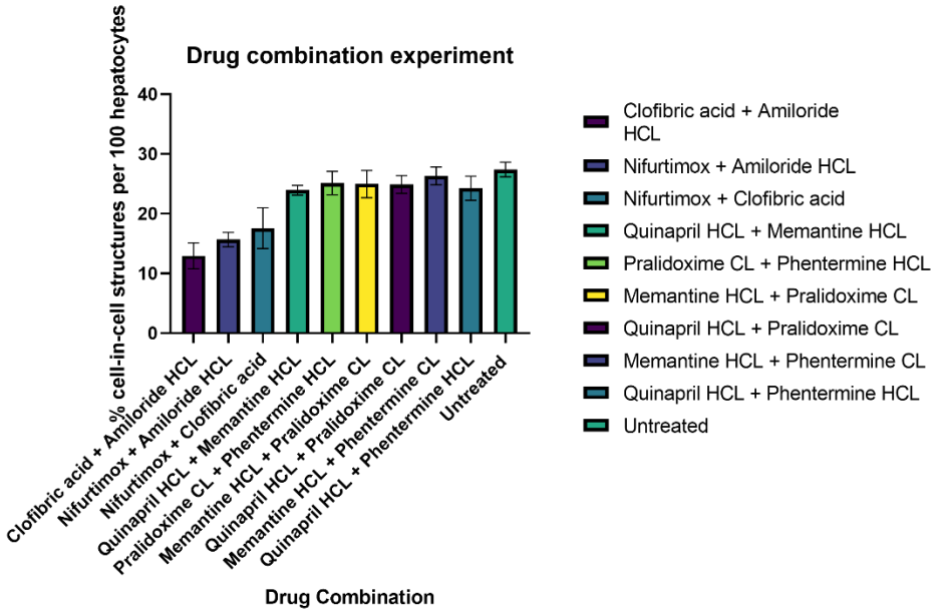


Figure 18. A drug combination experiment combining the effects of the shortlisted drugs. A selection of drugs were chosen to be combined to be tested in enclysis assays to determine whether combining inhibitors and enhancers can increase modulation of enclysis, compared to just using drugs on their own. This attempted to open new doors for possible therapeutics since combination therapies are such common place in treatments nowadays. Huh-7e's were pre-treated with each combination drug for one hour before the addition of Jurkats. Cell-in-cell structures were counted for each condition using ImageJ and all graphs were plotted using GraphPad Prism software.

Figure 18 illustrates the effect of different inhibitors and enhancers taken from the first Prestwick Library shortlist to determine whether, if they are used in conjunction with other drugs, they provide a larger effect on enclysis. From figure 18 we see a untreated control value at ~30% with potential enhancing conjugates such as Quinapril HCL and Phentermine HCL not illustrating higher levels of enclysis than the untreated control values, as the largest percentage of cell-in-cell structures seen for the enhancing conjugate drugs is using Memantine HCL and Phentermine HCL combined which gave an average cell-in-cell structure count of ~25%. Furthermore, for each enhancer combination there is some error bar overlap which would provide no statistical power to be different from the untreated control. This illustrates,

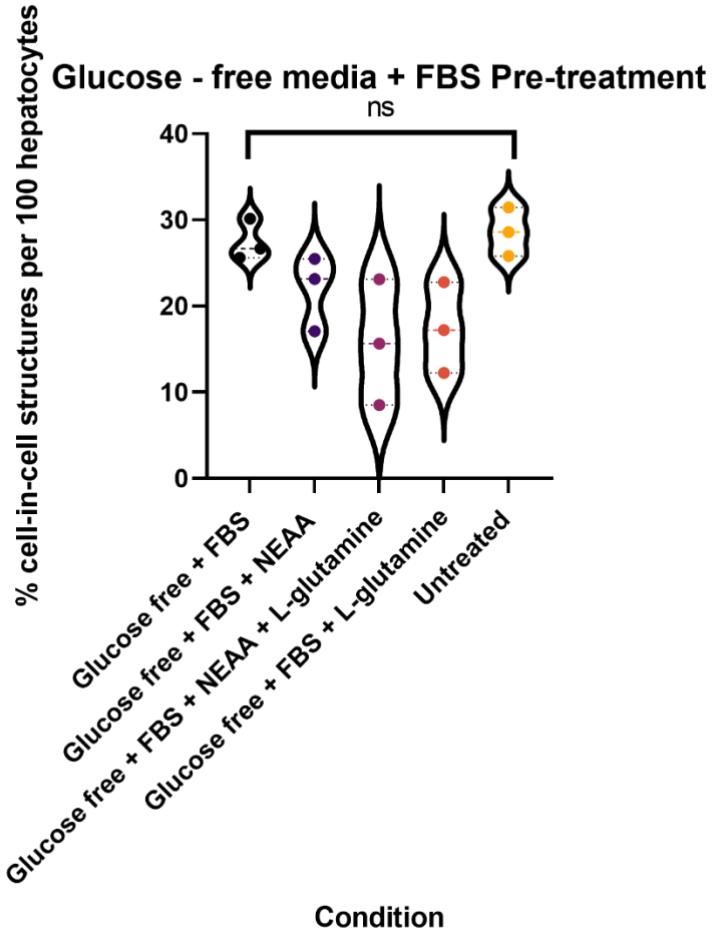
combining enhancing compounds made no difference in the amount of enclysis seen during the enclysis assays. This could also prove that these compounds are not enhancers of enclysis as these results back up data from the first drug assay.

On the other hand, it is clear from figure 18 that combining the effects of enclysis inhibitors made far more of a difference than enhancers. For example, Clofibric acid + Amiloride HCL, Nifurtimox + Amiloride HCL and Nifurtimox + Clofibric acid all created an assay which had a much-decreased average count of cell-in-cell structures compared to the untreated control. These combinations recorded average counts of 12%, 15% and 17% respectively compared to 30% for the untreated control. To determine whether this effect is greater than just using a drug alone, we must compare to a single-drug dose in the same assay.

3.6 Metabolic modulation of enclysis

The liver encounters a wide variety of metabolic factors during normal homeostasis and disease. An unbalance of these factors can contribute to liver disease such as nonalcoholic steatohepatitis (NASH) and since the liver has such a mutualistic relationship with the gut, I chose to investigate whether metabolic factors have an effect of enclysis in the liver. Once the final shortlist of drugs was made to take on for further therapeutic investigation in future work, it was decided to experiment whether tweaking the metabolic environment of Huh-7s would, in fact, aid in enhancing or inhibiting enclysis *in vitro*. Huh-7e's were seeded as usual, but before Jurkats were added, the cells were pre-treated with three different conditions. These conditions included SF-DMEM, Glucose-free media + FBS and Glucose-free media – FBS pre-treatment. The cells were pre-treated for one hour before a number of different metabolic-activity-altering reagents were added.

A


Glucose - free media + FBS Pre-treatment
ns
% cell-in-cell structures per 100 hepatocytes
40
30
20
10
0
Glucose free + FBS
Glucose free + FBS + NEAA
Glucose free + FBS + NEAA + L-glutamine
Glucose free + FBS + L-glutamine
Untreated
Condition

B

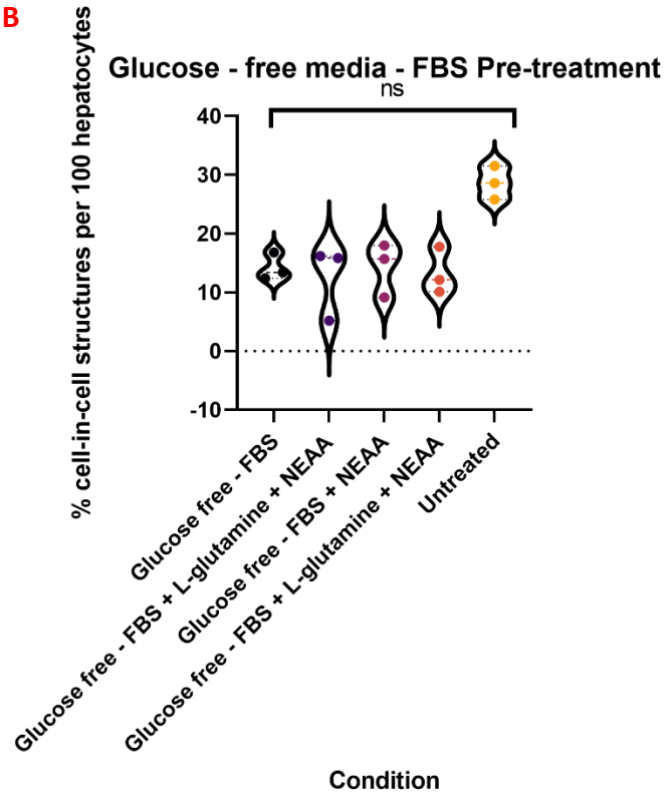

Glucose - free media - FBS Pre-treatment
ns
% cell-in-cell structures per 100 hepatocytes
40
30
20
10
0
-10
Glucose free - FBS
Glucose free - FBS + L-glutamine + NEAA
Glucose free - FBS + NEAA
Glucose free - FBS + L-glutamine + NEAA
Untreated
Condition

Figure 19. Glucose-free media ± FBS pre-treatment of Huh-7e's with the addition of different metabolic reagents. (A) Following pre-treatment of Huh-7e's with glucose-free media with FBS for one hour, different combinations of metabolites are added to investigate whether metabolic pathways used in enclysis can be manipulated to modulate the efficacy of the process. Non-essential amino acids (NEAA) and L-glutamine are added in different combinations, at a concentration of 10% diluted in glucose-free media. Statistics performed using paired non-parametric t tests. **(B)** Huh-7e's undergo pre-treatment with glucose-free media without FBS for one hour. Again, NEAA and L-glutamine are added in different combinations at a concentration of 100% diluted in glucose-free media. The number of cell-in-cell structures found is then analysed using ImageJ. Statistics performed using paired non-parametric t tests.

Figure 19 illustrates the ability of different metabolites to affect metabolic pathways within the process of enclysis and decrease the number of cell-in-cell structures formed. From figure 19 (A) Glucose-free media + FBS treatment showed similar ability to effect enclysis in comparison to the untreated control which had an average percentage of cell-in-cell structures formed at ~30%. This is also similar to the mean value given by glucose-free media + NEAA, which shows a slightly lower percentage to the untreated control, however two of the value do lie close to the values on the untreated control and glucose-free media + FBS, with one outlier decreasing the average. Interestingly, once L-glutamine is added, on its own and in conjunction with NEAA, the percentage of enclysis is decreased thoroughly with the lowest values dipping to 12% and 8% respectively. This provides initial evidence that the addition of L-glutamine may prove to be an inhibitor of enclysis. However, once paired non-parametric t tests were performed each condition was proven to be not significantly different to the untreated control so no concrete conclusion can be made.

This theory can be validated using figure 19 (B) where Huh-7e's are pre-treated with glucose-free media without the addition of FBS. Once again, the untreated control was shown to have a percentage of enclysis at ~30%, yet the addition of NEAA and glucose brings each condition down to a level of enclysis at ~10%. The removal of FBS from the glucose-free media has a larger inhibitory effect compared to the glucose-free media containing FBS. Without the extra additives, glucose-free media + FBS has an enclysis percentage of ~30%, however, glucose-free media – FBS decreases the level of enclysis to ~15%. This is the repeated with the addition of NEAA and L-glutamine as enclysis levels are also decreased with glucose-free media – FBS pre-treatment compared to glucose-free media + FBS. This illustrates the importance of FBS treatment in enclysis assays, to ensure enclysis levels stay high. As seen in figures 19, 20 and 21, the effect of FBS on enclysis is clear. Each figure has illustrated that when the assays were performed without FBS treatment, enclysis decreased dramatically, this was consistent over the three experiments. This could determine that FBS is a vital component in the mechanisms of enclysis. Furthermore, this validates the theory that the addition of L-glutamine will decrease the levels of enclysis as they match the values seen in the glucose-free media + FBS treatment. However, it is more difficult to determine the effect of L-glutamine as the effect of the missing FBS is already decreasing the value of enclysis. Furthermore, once paired non-parametric t tests were performed, each condition proved to be not

significantly different to the untreated control so no concrete conclusions can be made.

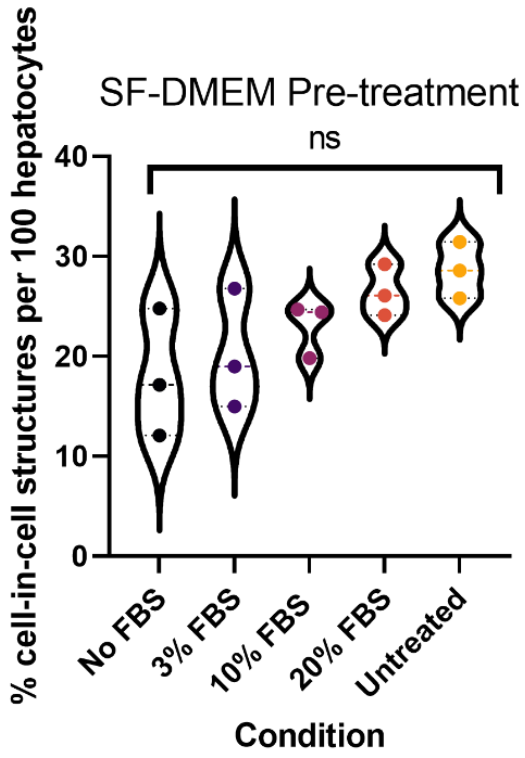


Figure 20. SF-DMEM pre-treatment of Huh-7e's with the addition of different concentration of FBS. Huh-7e's are pre-treated with SF-DMEM for an hour until FBS is diluted in in SF-DMEM at concentrations of 3%, 10% and 20%. These concentrations were used as they provide a balanced range to determine the best concentration of FBS to determine a difference in enclysis between no FBS and 20% FBS. Once Jurkats are added and enclysis is performed, cell-in-cell structures are counted and analysed using ImageJ. Statistics performed using paired non-parametric t tests.

To further validate the effect of FBS on enclysis, Huh-7e's were pre-treated with SF-DMEM for one hour before different concentrations of FBS is added at concentrations of 3%, 10% and 20% to create a range of results that would

determine the difference FBS makes on the level of enclysis. From Figure 20 it is illustrated that as FBS increases, the percentage of cell-in-cell structures formed also increases. For example, the wells containing no FBS has an average cell-in-cell structure count as low as 12%, whereas at 20% FBS the percentage of enclysis shows considerably less variation and has an average count of ~30%. This supports the conclusion that increased FBS will increase the percentage of enclysis which Is clearly illustrated in figure 18 where all conditions containing FBS increases enclysis levels substantially. However, once an paired non-parametric t test is performed on the data, the increase in FBS shows no significant difference between each condition and the untreated control with each condition showing a p value <0.05 at 0.25.

3.6.1 Further metabolite combination experiments

To investigate the effect of further metabolites including the effect sodium pyruvate has on enclysis, a metabolic ‘knockout’ experiment is performed where Huh-7e’s are, again, pre-treated with glucose-free media and NEAA, FBS, L-glutamine and sodium pyruvate are all added together in conjunction, with one metabolite knocked out at each step to determine which metabolite makes the biggest difference to enclysis.

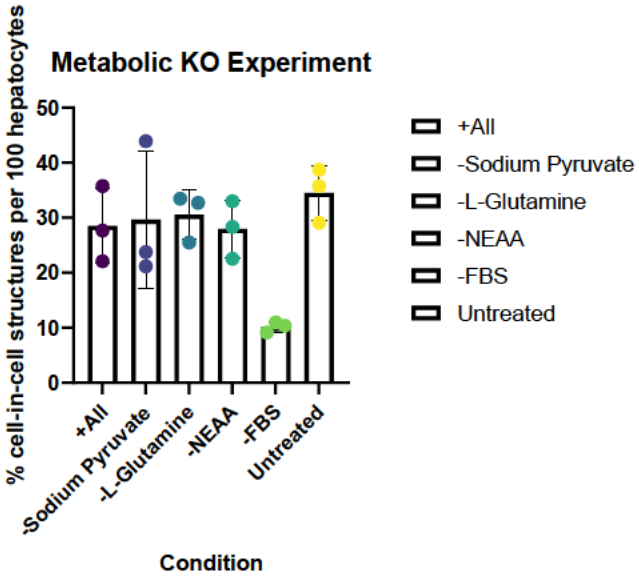


Figure 21. A metabolic knockout experiment to determine which metabolite is the optimal enclysis modulator. Huh-7e's are pre-treated with glucose-free media for one hour and five separate solutions are prepared, one containing all four metabolites labelled 'all' and the rest containing all but one with each metabolite being knocked out once. The level of enclysis observed is analysed to determine which metabolites are essential for keeping enclysis at a standard level and which cause it to decrease. The untreated sample contained CDMEM containing 5% L-glutamine, NEAA, penicillin and streptomycin and 10% FBS.

Figure 21 illustrates the effect each metabolite used in this project has on the process of enclysis. Each metabolite was added at a 5% concentration to glucose-free media after pre-treatment. Firstly, from figure 21. we are unable to validate the previously described inhibitory effect that L-glutamine has had on enclysis as in conjunction with each metabolite there seems to be no effect as each condition, excluding the FBS knockout, shows an average level of enclysis ~30% with very little variation, with each condition illustrating error bar overlap with the untreated control, proving them presence and knockout of those metabolites are unable to modulate enclysis enough to be relevant. However, it is clear from figure 21 that once again the importance FBS plays in producing a strong coefficient of enclysis is extremely

apparent with the average count seen as low as 10% with very small amounts of variation between repeats. This proves that FBS may augment enclysis, however with previous experiments p values have been >0.05 which proves further investigation is needed.

3.6.2 L-glutamine titration validation

The final experiment of the project was to investigate the true effect that L-glutamine has on the process of enclysis by eliminating any other metabolite and focusing on L-glutamine alone. Huh-7e's are seeded as usual and pre-treated with glucose-free media for one hour. Before Jurkats are added, solutions of differing concentrations of L-glutamine are prepared as follows: 3%, 5%, 10% and 20%. These concentrations were used as it provides a broad enough range to determine whether enclysis is truly modulated with the increased density of L-glutamine.

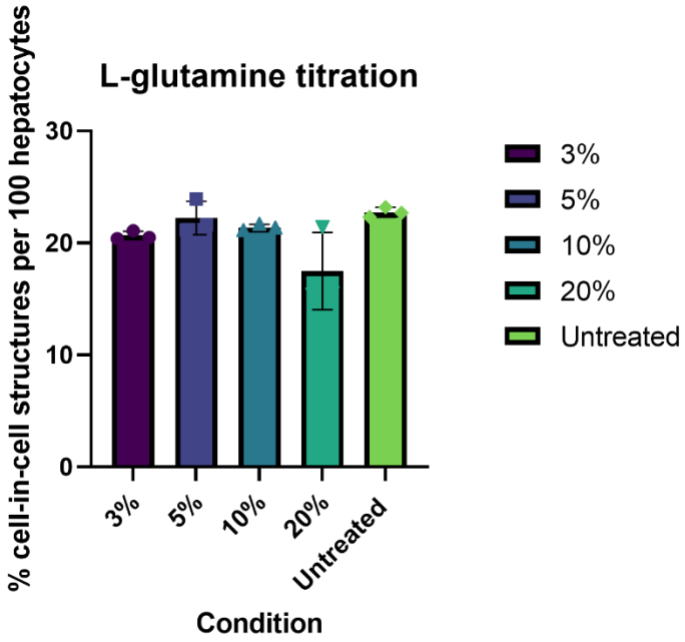


Figure 22. L-glutamine titration. Huh-7e cells are pre-treated with glucose-free media for one hour before L-glutamine is added in four separate concentrations: 3%, 5%, 10% and 20%. L-glutamine is added to Huh-7e's for the first time on its own without the presence of any other metabolites to determine whether L-glutamine does modulate enclysis. The Jurkats are added and the enclysis assay is performed. Cell-in-cell structures formed are counted using ImageJ and graphs are plotted using GraphPad Prism.

Figure 22 illustrates the effect L-glutamine has alone on the process of enclysis. Previously, the effects of L-glutamine have been tested in conjunction with other factors, such as FBS or NEAA as well as always being tested at 5%. We deemed this was not enough investigation to determine whether L-glutamine is the true cause of the decrease In enclysis seen from the previous metabolic experiments. It was decided to perform the titration and increase the concentration of L-glutamine used to 20% to illustrate how much of a modulating factor it could be. From figure 22 we can see at 3%, 5% and 10%, L-glutamine produces a percentage of enclysis that is extremely similar to the untreated control with very little variation through 3 biological repeats. All three concentrations, as well as the untreated control, have consistent

percentages of enclysis ~20%. This rules against the initial findings that glucose inhibits the process of enclysis. However, at 20% L-glutamine, the mean percentage of enclysis decreases to ~16% compared to the untreated control which is illustrated by the considerable drop in figure. Unfortunately, there is a good amount of variation between the repeats illustrated at 20% with error bars overlapping with those from the untreated control.

3.7 Key findings

From the results present in this project I can state the following conclusions:

- $1x10^4$ is the optimal density for seeding Huh-7s
- $4x10^4$ is the optimal density for seeding Jurkats
- CMAC is the optimal dye for staining Jurkat cells
- Clofibric acid and Amiloride hydrochloride are competent inhibitors of enclysis.
- The present of L-glutamine has no effect on inhibiting enclysis.

4. Discussion

After three individual screenings of the Prestwick Chemical Library, manual validation of a 300-drug shortlist and in vitro testing of the top five inhibitors and enhancers of enclysis, a final shortlist of drugs has been produced. This chapter will review the results found on each ‘hit’ drug and discuss how their mechanisms of action could be linked to modulating cell capture in the liver. Furthermore, we will theorise possible metabolic pathways that are manipulated by metabolites during enclysis and why they cause an increase or decrease.

4.1 Generation of Huh-7e

Figure 12 illustrates the cell sort performed by flow cytometry that allowed a specific population of huh-7s, that are competent in performing enclysis, to be isolated. After 8 sorts, we believed we had a population of enclysis-competent huh-7 cells. However, the population sorted was extremely small which questions how competent the isolated sample is. When comparing these cells to the wild-type, there was no significant increase in the levels of enclysis, which unfortunately illustrates how these enriched cells have no significant difference to the wild-type. After more successful sorts, however, we may produce an enriched population in the future.

4.1 Mechanism of action behind enclysis modulating drugs

After vigorous testing *in vitro*, a final shortlist of drugs that passed validation to be moved on through the drug repurposing pipeline is produced. The mechanism of action of these drugs could prove key in discovering which aspect of enclysis they alter and, in turn, could unlock more secrets in determining the full intracellular mechanism of enclysis which is still yet to be fully understood.

4.1.1 Clofibric acid

As discussed, Clofibric acid is used therapeutically as an antilipemic agent which serves as a metabolite to clofibrate. CA is predominantly used in the treatment type III hyperlipoproteinemia and hypertriglyceridemia. Since Clofibric acid already acts as an agonist of the PPAR-α receptor in the liver, resulting in increased beta-oxidation and lipoprotein lipase activity, we can theorise that this drug has a known high efficacy in the hepatic microenvironment. It Is also known that CA is metabolised in the liver through rapid de-esterification illustrating its ability to be quickly cleared, which is beneficial for targeting liver diseases. Furthermore, CA falls under a heterogenous class of drugs termed peroxisome proliferators (PPs). PPs

have shown evidence to act as hepatocarcinogens in rodents and in a study performed by Penna et al [116] treatment with CA induced wholescale apoptosis in a hepatoma cell line. CA was also seen to induce apoptosis in Jurkat cell lines through activation of caspases 2 and 3, most likely caused by endoplasmic reticulum stress. Interestingly, these findings were not repeated in vitro for our experiments as no apoptosis was reported, yet this could cause issues when moving forward in testing CA on hepatoma and lymphoma cell lines [116].

PPAR receptors play a significant number of roles in the hepatic microenvironment, with the most significant being the role in regulating inflammation. PPAR-Ɣ has key interactions with clofibrates and their derivatives, and once activated enhances phagocytosis through macrophages. This acts through increased expression of CD36 which is a major scavenger receptor, key in initiating phagocytosis, as well as increasing expression of IL-13 to increase phagocytosis [117]. These findings illustrate CA could play a major role in the modulating enclysis in the liver. Despite the literature detailing how increased clofibrates increasing the rate of phagocytosis, our findings found it to be a very competent enclysis inhibitor yet given how it plays a significant role in PPAR activation, it may also act on cell capture receptors used in enclysis which represents a significant therapeutic potential for this drug [117].

4.1.2 Amiloride hydrochloride

As described previously, Amiloride hydrochloride (AH) acts a pyrizine used in the treatment of hypertension. AH is not metabolised but excreted to the kidneys for removal. Unfortunately, there is not a large amount of literature that links AH to any sort of cell capture in the body. However, in a study performed by Vandivier et al. (2009) it was reported that AH was able to impair efferocytosis through mediation of

amiloride-sensitive ion channels. Furthermore, in research performed by Hermetet et al. (2016) on work investigating efferocytosis of apoptotic HPV cervical cancer cells, it was shown that clearence of late-apoptotic cells was inhibited by the presence of amiloride hydrochloride. This validates the findings in the project and provide a significant basis for future work with AH in inhibiting other cell-capture mechanisms within the liver. Finally, since AH shows no toxicity in the liver, it currently proves one of the most exciting drugs found in this compound and illustrates huge therapeutic potential in the treatment of autoimmunity and cancer in the liver.

4.1.3 Alfuzosin hydrochloride

Alfuzosin hydrochloride (AFH) acts as an alpha-1 adrenergic antagonist predominantly used in the treatment of benign prostatic hypertrophy. AFH undergoes extensive hepatic metabolism with only 11% of the dosage given detected in urine unchanged. This proves beneficial when thinking therapeutically as the liver has no issue metabolising this drug illustrating its potential uses on the liver. Unfortunately, there is no current evidence in literature that links AFH to any sort of cell capture in the liver, however from our findings there are strong grounds for future experiments to determine which aspects of enclysis it targets. However, given its easy metabolism and lack of toxicity in the liver, there are no current red flags in passing it down the next steps of the drug repurposing pipeline. Due to the lack of statistical significance in the first drug assay, this drug cannot be stated as a possible enhancer.

4.1.4 Memantine hydrochloride

Memantine hydrochloride (MH) is traditionally used as an NMDA receptor antagonist in the treatment of Alzheimer’s. MH is partially metabolised in the liver where 48% is

shown to excrete as urine unchanged, illustrating minimal NMDA receptor antagonist activity. MH acts solely on NMDA receptors to prevent glutamate action, but there is no current evidence that links MH to any sort of cell capture within the body as its effects are solely neurological in nature. However, the evidence found in this project set the groundwork to be used for future experiments to link the *in vitro* effects of MH to any stage of the process of enclysis. Furthermore, the evidence gathered in this project would give some means to push MH down the drug repurposing pipeline and possibly test this drug using primary cells, to boost more significant results. Due to the lack of statistical significance, this drug cannot be stated as a possible enhancer of enclysis, worth further investigation.

4.2 FBS enhancing the process of enclysis

Whilst modulating FBS levels, it was shown that an increased concentration of FBS can possibly increase the amount of cell-in-cell structures formed and, in turn, the process of enclysis. Fetal Bovine Serum (FBS) is a fixture used in CDMEM and CRPMI for culturing both Huh-7s and Jurkat cells. FBS contains a mixture of components, with over 1000 substances found in its make-up. FBS contains several growth factors including proteins, electrolytes, carbohydrates and enzymes, all to support growth and function of the cell line it is suspending. FBS is also important to protect cell lines against sudden pH shifts, toxic agents and shear forces. Interestingly, FBS is a major source of attachment factors for cells to allow for confluent strong monolayers to be formed in culture flasks. It could be hypothesised that the increase in FBS, causing increased enclysis is caused by the increased concentration of these attachment factors improving the efficacy of initial binding of Jurkats to Huh-7s. Furthermore, increased growth factors would increase general metabolism pathways of hepatocytes and provide an environment more favourable

for enclysis as Huh-7s have all the nutrients needed for any intracellular process. Again, this interaction will need to be investigated further in future experiments to determine which aspect of enclysis FBS acts upon or whether it solely makes Huh-7s more ready to perform the process. Due to the lack of statistical significance, FBS cannot be deemed an enhancer of enclysis, however this provides another possible avenue to explore whilst attempting to determine ways to modulate enclysis for therapeutic value, even if it is to increase enclysis levels in *in vitro* assays.

4.3 L-glutamine inhibits the process of enclysis

From three independent experiments, we were able to determine that treating Huh-7s with L-glutamine with other metabolites as well as on its own, L-glutamine could possibly have an inhibitory effect on the process of enclysis, due to the lack of statistical analysis we are unable to confirm this and investigate it further. L-glutamine and its derivatives play a large role in Huh-7 culture with it being added at 5% to all our culture mediums. Therefore, it is already known that L-glutamine aids in cell growth and proliferation and could make Huh-7s more readily active to perform enclysis. On topic with this, L-glutamine has been shown to prevent apoptosis of cells which could benefit in performing enclysis of live cells. In a study performed by Jiang et al (2017) [120] it was shown that L-glutamine reduced levels of TUNI-induced apoptosis, tested through measuring cell viability using CCK-8 cell counting kit illustrating the survival-promoting effects of L-glutamine [121]. This could allow for all cell types to maintain high survival rates and be more readily available to perform live cell capture. Furthermore, L-glutamine has been shown to affect lymphocytes and macrophages in performing phagocytosis. A study performed by Wallace et al. (1992) [121] illustrated a steady increase in phagocytosis performed by macrophages directly correlated with an increase in L-glutamine. This was described

as a possible immune suppressor due to increased phagocytotic tendencies, which shows antithetical results to what was produced in this project [121]. All further correspondence concerning L-glutamine's involvement in any sort of cell-capture points towards increased viability of cells to perform phagocytosis. This, again, acts against the hypothesis in this project, however, the mechanism of enclysis is a lot more complex than standard phagocytosis as it involves the selective capture of Treg cells in the liver. Interestingly, in a review written by Philip Newsholme (2021) [122] it is described that L-glutamine plays a major role in the function of immune cells. The concentration of L-glutamine found can modulate the proliferation of T-lymphocytes by increasing the rate of IL-2 production [122]. Furthermore, supporting previous statements, when L-glutamine was reduced from 2mM to 0.2mM = MHC class II expression was reduced by 40%, which supports previous conclusions that increased L-glutamine increases phagocytosis [122]. Thus, it can be concluded that further experimental work is required to discover any link between L-glutamine and the inhibitory effect of enclysis. First, I believe it is a requirement that any future work involving enclysis requires the use of primary T cells instead of a jurkat cell line, and possibly primary hepatocytes, instead of the huh-7 cell line, to recapitulate the environment that enclysis would occur in naturally. Furthermore, since after eight rounds of sorting, there was no significant difference in the enriched population of cells compared to wildtype, this did not aid in any of the non-statistical findings of the project.

4.4 Future work

There is several steps that still must be taken before any of the 'hit' drugs can reach clinical trials and go to market. In future experiments, the effect of the 'hit' drugs will be tested using primary hepatocytes and primary T cells isolated from patients. The

use of primary cells will produce more accurate findings as the effect of the drugs on cells from donor patients can be validated. If hepatocytes and T lymphocytes are isolated from donor patients suffering from HCC or AIH, for example, the effect of these drugs can be determined on a patient-by-patient basis which allows for some personalisation on the treatment plan.

Once the drugs have been validated using primary cell lines, their effect must be validated *in vivo* using mouse models. The mouse model will recapitulate the closest environment found in human patients. If enclysis can be reproduced in animal models to the same extent as what was seen *in vitro*, drugs can push forward into clinical trials and their toxicity can be investigated. However, with testing on animal models, comes several challenges. Even though, mouse models are the closest usable model to humans, they are still poor predictors of drug safety in humans. They also carry a large financial burden on research and can delay drug approval. Furthermore, attaining the correct ethics to use mouse models can prove difficult and lengthy to acquire. The use of animal models in this project would have allowed us to visualise live cell-in-cell capture on a large scale. Hypothetically, liver sections would be cut from mice and kept alive in media. These would then be treated with each drug compound, stained, and imaged live using multi-photon microscopy to determine live cell-in-cell structures.

It would also prove interesting to investigate if enclysis is liver-specific. If preferential live cell capture is identified in other organs or tissues in the body, enclysis can be redefined and possibly used to treat other cancers and areas of autoimmunity around the body. This can be tested by determining whether other organs have functional cell types that undergo cell capture. If so, these cells can be isolated and co-cultured with different immune cells. If cell-capture occurs, to determine whether

this is enclysis, cell surface proteins involved in this process will be determined to compare to other cell capture mechanisms involved in the organ.

Furthermore, it is vitally important that the mechanisms behind each step of enclysis are explored to determine future therapeutic targets to modulate the process. If all adherence proteins are identified, they can be used as targets to prevent initial adherence to Jurkats. This would be done via knock-down experiments on all known adherence proteins expressed on hepatocytes, from then we can determine which proteins are important in maintaining enclysis numbers. Furthermore, hepatocytes that have been co-cultured with T cells can be washed and lysed for qPCR and western blot analysis to determine which genes and proteins are upregulated during enclysis. Additionally, to further understand how enclysis affects the hepatic microenvironment, the intracellular mechanisms involved with the process must be investigated to provide further therapeutic targets to modulate the process. This involves identifying specific pathways involved in the formation of the enclytic vesicles and the preferential degradation of Treg cells. Using specific biomarkers for intracellular proteins, the increased interaction of these can be determined during the process of enclysis. Furthermore, entire RNAseq can be performed on the hepatocytes to determine what proteins are upregulated during disease and cancer to determine whether adherence proteins, involved in enclysis, are modulated in disease. Once every aspect of enclysis is determined and each component that carries out the process is identified, more work can begin on targeting every step to either inhibit or enhance enclysis in the liver.

It is also vitally important that further investigation is carried out on the interaction between L-glutamine and enclysis. The findings produced in this project are antithetical to what is described in literature regarding the involvement of L-glutamine

on cell capture [120]. If there is a link determine between L-glutamine preventing uptake of T cells, on a hepatocyte level of T cell level, we can begin to validate our findings.

4.5 Limitations

Throughout this project there were a number of limitations and set-backs that delayed results which resulted in some incomplete experiments having to be omitted from the project results. The largest limitation I experienced was the number of bacterial infections that developed during the summer months of the project. This delayed a significant number of enclysis assays whilst the source of the infection was determined. The delay was further exacerbated by the break-down of the CX5 microscope for two months. In an attempt to remedy this, I undertook training on a separate high-content microscope named Zeiss CellDiscoverer7. Unfortunately, this microscope failed to give images that we had no other option other than to wait for the CX5 to be repaired before we could continue.

On a biological note, a limitation to the results given is that these assays are performed *in vitro* using cancer cell lines. It is unknown whether these results will repeat using primary hepatocytes and primary T cells, or *in vivo* using animal models. Thus, conclusions on shortlisted drugs cannot be fully confident until these experiments have been performed. Finally, the highest level of enclysis seen would differ from assay to assay. This would mean that one assay may have an untreated control that would reach an enclysis percentage as high as 40%, however another type of assay may only reach 20%. This was totally dependant on the conditions on the day, some members of the lab may have opened the incubator door more often than other days that would decrease the level of enclysis as the heat would escape.

4.6 Conclusions

To conclude, we can state that from 1200 FDA-approved drug compounds in the Prestwick Chemical Library, we identified two potential inhibitors of the process of enclysis. We can state, with confidence, Clofibric acid and Amiloride hydrochloride are competent inhibitors of enclysis. Finally, we can hypothesise a role for FBS and L-glutamine affecting enclysis *in vitro*, however this must be investigated further.

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