Developing Novel Combinatorial Treatments for Tuberous Sclerosis Complex (TSC).

Submitted by Christopher Baxter, to the University of Exeter as a thesis for the degree Masters by Research in Medical studies, January 2021.

(Signature)…….

Acknowledgments

I would like to thank my supervisor, Dr Benjamin Housden for giving me the opportunity to undertake this project and for his invaluable help and support during this project.

Thank you to Professor Sebastian Oltean for his support and feedback during this project.

I would like to thank Dr Novia Wang and Katarzyna Sierzputowska for the training and help they provided me. And thank you to Misha Jackson for assisting with some of the laboratory work.

I would also like to thank Dr Michael Dillon, Dr Erin Reardon, Ruth Harrison and Tiffany Moore for their help and feedback during the writing process.

I would also like to acknowledge the technical services staff of the Living Systems Institute.

Abstract

The aim of this project is to identify new drug treatments for tuberous sclerosis complex (TSC). TSC is an autosomal dominant genetic disorder effecting 1 in 6000 births, it is characterised by the formation of hamartomas (benign tumours) throughout the body causing disfigurement, learning difficulties and organ failure. The development of new treatments is important because the current treatment, rapamycin, is severely limited, only showing a cytostatic effect on hamartoma development.

Several drug candidates have been identified as potential TSC treatments using a network of SS/L interactions between *Drosophila* and preapproved drugs (Housden *et al.*, 2017; Valvezan *et al.*, 2017). I assessed these candidates in *Drosophila* mutant cells to identify which would be most promising as the basis for a combinatorial treatment. Lithium chloride proved to be the most effective of the candidates tested, exhibiting a selective cytotoxic effect in *Drosophila* TSC cells. Lithium chloride was then screened against a library of one hundred and fifty-four FDA targets identified by Housden et al (2017) to identify possible synergistic combinations.

Fifteen possible candidates were identified in this screen. Three of the genes identified were related to purine synthesis, which has been identified as a potential candidate for TSC treatment before. Of these genes *ras (*analogous to *IMPDH)* has two approved drugs, Ribavirin and Mycophenolic acid (MPA), and one experimental drug, mizoribine. These drugs were tested in combination with lithium chloride in murine and human cells in order to identify possible synergistic interactions.

The preliminary results in both human and murine cells suggest that the synergy identified in the screen is conserved. However, preliminary results in human cells were inconclusive. Further testing is needed to properly validate these results and to develop new treatments for TSC.

Contents

List of figure and tables

List of supplemental material

Page 72 - Sierzputowska, K., Baxter, C. and Housden, B. (2018) ‘Variable Dose Analysis: A Novel High-throughput RNAi Screening Method for Drosophila Cells’, *BIO-PROTOCOL*. doi: 10.21769/bioprotoc.3112.

Background

Tuberous Sclerosis Complex:

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder affecting approximately 1 in 6,000 births (Gómez, 1995). It is highly inheritable, with a 50% chance of being inherited if one parent has the condition. In approximately two thirds of cases no parent exhibits signs of TSC (Sancak *et al.*, 2005). The condition is characterised by the formation of **hamartomas** (benign tumours) throughout the body. These hamartomas cause a wide range of symptoms; - skin markings such as ash-leaf spots (hypomelanotic macules), angiofibromas, ungual fibromas, shagreen patches, growths within the eyes (multiple retinal hamartomas), indications within the brain, for example, cortical dysplasias, subependymal nodules, subependymal giant cell astrocytoma and symptoms effecting other organs such as the heart (cardiac rhabdomyoma), lungs (Lymphangioleiomyomatosis (LAM)) and kidneys (Angiomyolipomas). Symptoms occur randomly in patients and are unpredictable.

TSC is caused by loss-of-function mutation of either the *TSC1* or *TSC2* genes (Osborne, Fryer and Webb, 1991). These genes form a protein complex (the tuberous sclerosis complex complex), which integrates signals from multiple upstream pathways and inhibits the **mammalian target of rapamycin (mTOR)**, a master regulator of cell growth (Tee, 2018). Mutations of the TSC complex affect the cellular growth mechanism, leading to uncontrolled cell growth and tumour formation in diseases such as TSC and a variety of sporadic cancers (Menon and Manning, 2008). *TSC1* and *TSC2* code for **hamartin** and **tuberin** respectively. Tuberin is a chaperone protein for hamartin; together they form a complex that activates the GTPases activity of ras homolog enriched in brain (*RHEB)*, preventing RHEB-GTP-dependent cell growth through mTOR (Garami *et al.*, 2003). Therefore, TSC1 and TSC2 are upstream regulators of RHEB (Inoki *et al.*, 2003) and the interruption of this pathway leads to unrestricted cell growth.

Mutations of the individual genes lead to one of two forms of the disease; TSC1 is the less common and generally less severe form, whilst TSC2 is more prevalent and severe (Gupta and Henske, 2018). Either form causes the formation of hamartomas;

these hamartomas are responsible for the damaging mechanisms of the disease. Due to the random nature of the hamartomas, any organ can be affected; there is, however, a prevalence for hamartomas forming in the brain, kidneys, heart and lungs (Rabito and Kaye, 2014). The issues caused depend on the location and size of the hamartomas. Most commonly the growth of the hamartomas leads to renal failure, epilepsy, learning difficulties, autism, pulmonary failure, kidney dysfunction and disfigurement (Gómez, 1995).

Treatment options for TSC currently include surgery, radiotherapy and chemical intervention with immunosuppressants, such as rapamycin, which inhibit the ability of mTOR to phosphorylate downstream substrates (Sarbassov *et al.*, 2006). Rapamycin binds to intracellular protein FKB12 to generate a drug receptor complex which then binds to and inhibits the kinase activity of mTOR (Sarbassov *et al.*, 2006). This causes dephosphorylation and inactivation of p70S6 kinase which stimulates the production of ribosomal components for protein synthesis and cell cycle progression.

None of the existing treatments are curative and those suffering with TSC require continuous monitoring and regular interventions to manage symptoms. In addition, treatment with rapamycin and its derivatives (rapalogs) only provide temporary benefits due to its cytostatic effect and when treatment is stopped, tumours rapidly grow back (Gómez, 1995). There is therefore a great need to identify new therapeutic drugs to effectively treat TSC.

Drug discovery

Drug discovery is a long process; the length of time to develop a new therapeutic agent is often 12 years or more from conception to implementation (Dimasi *et al.*, 2010). With scientific advances it is possible to screen drugs utilising *ex vivo* techniques to determine the mechanism of action and evaluate its therapeutic value. Even with these advances it is still a long process and candidates can fail at any point (Mohs and Greig, 2017).

Drug discovery often starts with identification of a viable **target**; - a gene, a protein, an enzyme etc. these can be found using phenotypic screening or by datamining current biomedical data to identify candidate molecules (Kurosawa *et al.*, 2008). Molecules

are then assessed against a variety of criteria, such as specificity, kinetics and potency. In order to treat tumorigenic diseases, drugs are developed which target specific gene products (targets). These targets will usually reduce cell viability in some way; - stopping growth or cell division by inhibiting genes required for growth or division or by activating cellular mechanisms such as apoptosis or autophagy. Most drugs will affect several genes that are not the target of that particular therapy (**off-target effects**) These can lead to unintended side effects (Berger and Iyengar, 2011). After the identification and testing of a drug its effectiveness in animal models is assessed and its potential for use in humans. The final stage of testing is clinical trials, significant numbers fail at the clinical stage due to the inherent differences between testing in a laboratory setting and clinical use. Only 15% of candidate drugs will eventually be approved for use in humans (Mohs and Greig, 2017).There is also a high chance of a drug being rejected due to toxicity or unintended side effects (Xue *et al.*, 2018). To be an effective therapeutic candidate; drugs must exhibit a degree of cytotoxicity towards target tumour cells whilst having as little impact on healthy cells as possible.

An **orphan disease**, is classified as a disease affecting ≤1 in 2000 people (Aronson, 2006). Due to the relative cost and investment required, drugs used to treat orphan conditions, like TSC, are usually considered financially inviable (McCabe, Claxton and Tsuchiya, 2005). In order to combat this, it is possible to repurpose **pre-approved** therapeutic candidates used to treat other conditions, which can increase clinical success and reduce development time and costs (Sertkaya, Aylin; Birkenbach, 2011). Benefits to using pre-approved drugs include - reduced development time, reduced costs, a greater understanding of clinically viable dosages and possible side effects. Many older drugs have wide ranging target effects and can be used to treat a multitude of conditions.

To identify possible therapeutic targets, a variety of tools are employed. The most common of those used is **high-throughput screening (HTS)**.

<u>High-throughput screening</u>

High-throughput screens (HTS) are experiments in which a test library is developed. in this case, the functions of many individual genes are systematically disrupted one

at a time and the resulting phenotypes measured. Screens are generally performed by treating cells in culture with genetic or pharmacological reagents targeting individual gene functions and measuring the effects of these treatments on a phenotype of interest (Mohr *et al.*, 2014). Using this approach, it is possible to identify genes and pathways that function in a specific biological process. Alternatively, screens can be performed in both healthy and disease model cell lines to study the mechanism of a disease or identify candidate targets (Zhan and Boutros, 2016). With this knowledge it is possible to identify treatments for diseases that are caused by genetic mutations e.g., cancer, TSC and amyotrophic lateral sclerosis (ALS).

In addition to 'single gene' screens, it is possible to perform **Genetic interaction (GI Screens)** in which pairs of genes are simultaneously disrupted. By analysing the resulting phenotype, it is possible to identify the functional relationships of those genes, what genes make up specific pathways and how these pathways function (Mani *et al.*, 2008). By mapping these genetic interactions and gaining insights into the structure and function of biological pathways and networks it is possible to take advantage of a type of genetic interaction called a **synthetic sick/lethal interaction (SS/L)**, whereby simultaneous **disruption** of two genes is lethal or results in a negative outcome for the target cell, but individual disruption of either has no effect (Hartwell *et al.*, 1997). PARP inhibitors are one such SS/L. When combined with a *BRCA2* mutation, PARP inhibitors resulted in significantly higher rates of toxicity (Chen, 2011). Cells with a *BRCA2* mutation are 90 times more susceptible to PARP inhibition compared to wild type cells; introduction of PARP inhibitors to patients undergoing chemotherapy for BRCA mutant cancers, significantly increased toxicity in mutant cells (Chen, 2011).

Silencing an essential gene, within either wild-type or mutant cells, will result in cell death, however, if only the double mutant is lethal this shows synthetic lethality and indicates a promising therapeutic candidate (Nijman, 2011). When one of these two genes are a tumour suppressor (such as TSC1 or TSC2), treatment to target the second gene is expected to selectively kill cells that carry the tumour suppressor mutation but leave healthy cells unaffected. Inhibiting specific genes that interact with the mutation, cellular processes can be disrupted Leading to a SS/L. This usage of multiple targets can also be applied to therapeutics themselves, by utilising

combinations of drugs more effective treatments can be created in the form of a **combinatorial treatment**.

Combinatorial treatments are often more effective than single treatments

Whilst single drugs alone can be therapeutically useful, there are limitations in using a single agent. There are multiple ways in which cells can become resistant to treatment: - adaption allows cells to reset themselves to counter the effects of a therapeutic, this is seen in BRAF-mutant melanoma resistance to RAF kinase inhibitors (Lito, Rosen and Solit, 2013). However, the addition of MAPK inhibitors counteracted the adaption, resulting in a more effective treatment (Chen and Lahav, 2016). The toxicity and dosage of a drug also presents challenges for example the bioaccumulation of lithium in the treatment of Bi-polar disorder. The addition of a second drug can reduce the dosage of the primary treatment whilst maintaining its effect (Chen and Lahav, 2016). The main aim of combinatorial treatments is to inhibit multiple pathways and create a **synergistic** effect that cytotoxically effects mutant cells (Lord, Tutt and Ashworth, 2015).

Identification of combinatorial treatments has its own challenges; there are over 200 million pairwise gene combinations in humans, each of which could be a possible candidate. The variables within a **combinatorial screen** introduce high **noise** levels; the more factors being screened for, the higher the signal to noise ratio. In this case, the target gene is screened with candidate 1 and candidate 2, introducing a higher level of noise and therefore a higher rate of false positives and off target effects. By using different methods some of these limitations are reduced. For example, utilising CRISPR/Cas9 to modify cells into TSC mutants reduces the variability inherent in natural cell lines. Testing drugs in cells is closer to clinical usage, a lot of drugs have multiple unknown targets so testing with a drug is more effective at demonstrating action than simply inhibiting a gene. Each individual method for inhibiting drug targets also has limitations and using the same methods multiple times compounds the effect.

In order to reduce the number of variables some GI screens make use of model organisms such as nematodes (*C. elegans*), flies (*D. Melanogaster*) or mice (*Mus Musculus*).

Use of *Drosophila* as an ortholog

Drosophila melanogaster has been used as a model organism for over 100 years (Beckingham *et al.*, 2005). *Drosophila* share approximately 60% of its genes with humans and 75% of known human disease-associated genes are conserved (Reiter *et al.*, 2001). The benefits of using the fly model are numerous; a short breeding time, a fully mapped genome and many genetic interactions are conserved from flies to humans (Pandey and Nichols, 2011). Using CRISPR/Cas9, it is possible to create a desired phenotype which can be used as a model for human diseases (Millburn *et al.*, 2016).

CRISPR/Cas9 is a system for gene editing adapted from a bacterial antiviral mechanism. This mechanism uses an enzyme, Cas9 and **guide RNA (gRNA)** to target viral DNA, once the gRNA identifies a complementary viral sequence it binds to the sequence and the Cas9 cuts the DNA, rendering the virus useless (Redman *et al.*, 2016). By creating synthetic gRNA complementary to a specific section of the genome, this method can be used for gene editing in *ex vivo* and *in vivo* environments and in multiple cell types, including embryonic stem cells. In gene editing, a gRNA is coded to a specific gene and incorporated into the cell, once the complex reaches the section the Cas9 cuts the DNA creating a double stranded break (DS break) this activates the DNA repair mechanism, which is largely error prone and can possibly lead to a loss of function mutation of that gene. Alternatively a target sequence of DNA can also be co-transfected, this DNA can then be incorporated into the genome using the cells own DNA repair pathway (Redman *et al.*, 2016).

CRISPR/Cas9 is a useful tool for silencing genes, however the GAL4/UAS system is also a valuable tool in Drosophila research, allowing a more targeted approach.

The GAL4/UAS system is used in *Drosophila* lines in order to highlight a specific area of interest (Brand and Perrimon, 1993). GAL4 is a transcription activator inserted downstream of a gene promoter and is transcribed when the gene activates. An upstream activating sequence (UAS) is attached to the target **short-hairpin RNA (shRNA**), GAL4 binds to the UAS activating expression of the target gene and fluorescent reporter. Only with the combination of GAL4 and the UAS will expression of the target gene and the fluorescent reporter take place (Brand and Perrimon, 1993).

In whole organism experiments, this can highlight functions within specific tissues, in cell lines, it allows the fluorescent reporter to act as an indirect measure of shRNA production. In many ways the approach is more nuanced than CRISPR/Cas9 and creates variability depending on the amount of shRNA production, rather than just completely silencing a gene.

In the case of *in vitro* testing the above methods also work for *Drosophila* cells. Housden et al (2017) previously created TSC mutant cells that provided results that were analogous to experiments performed in human cells. *Drosophila* cells are also easier to work with than mammalian cell lines, requiring 25°C as a growth temperature, they do not require a CO_2 incubator and do not harbour human pathogens (Cherbas and Gong, 2014). In mammalian cells many genes have paralogs, genes that share the same function. Inhibition of one of these targets will not create the desired effect as the other gene will compensate for the Inhibition. Fly cells do not have these parallels and so it is easier to identify interactions between target genes.

Drosophila have been used successfully in a variety of translational applications, including for TSC. Over 890 studies using *Drosophila* to investigate human diseases have been published from 2012-2020. These range from identifying the mechanism of action of specific diseases e.g. Charcot-Marie-tooth (Suda *et al.*, 2019) to possible therapeutics (Cunningham *et al.*, 2018). *Drosophila* has also been used to study TSC and its role in regulating synapse growth (Rajalaxmi Natarajan, Deepti Trivedi-Vyas, 2013). Work has previously been done in *Drosophila* to establish mechanistic factors of TSC (Tapon *et al.*, 2001).

Drosophila cells have also proven to be an effective model for human diseases and there are many benefits to screening in fly cells and then filtering into human cells. These benefits include reduced time until confluency, less stringent growing conditions and a reduction in human pathogen contamination.

There has been demonstrable success translating results from *Drosophila* to mice then to human cell models (Housden *et al*., 2017). Nicholson et al (2019) used *Drosophila* as a model to demonstrate an SS/L interaction between CDK4 and CDK6 which could be exploited as a treatment for Von-Hippel-Lindau (VHL) linked kidney

cancer. Valvezan *et al*., (2017) identified that inhibition of guanine nucleotide synthesis resulted in a cytotoxic effect on TSC mutant cells in *Drosophila* that was conserved to murine cells. More recently they further demonstrated that mizoribine has a significant anti-tumour affect in murine cell lines (Valvezan *et al.*, 2020)

RNAi for genetic interaction screens

RNA interference (RNAi) is a powerful method in molecular biology that allows for the manipulation of an organism's genes using RNAi molecules such as **short hairpin RNA (shRNA), short interfering RNA (siRNA)** or **double stranded RNA (dsRNA)** (Agrawal *et al.*, 2003). RNAi is a useful tool that allows us to inhibit specific genes by degrading specific gene products. In *Drosophila* RNAi can be triggered experimentally by exogenous introduction of dsRNA or constructs which express shRNA (Lehner, 2004). An enzyme called DICER digests dsRNA or shRNA into siRNA. These siRNA then attach to the target **messenger RNA (mRNA)** and cleave it preventing it from being replicated and thus silencing the gene (Agrawal *et al.*, 2003). The high degrees of efficiency and specificity are the main advantages of RNAi in Drosophila cells (Aagaard and Rossi, 2007), In mammalian cell lines CRISPR/Cas9 is commonly used as a gene editing tool.

By utilising RNAi, phenotypes are created that are analogous to the disease being investigated, drug targets are then tested against these phenotypes to identify SS/L interactions, which can be exploited to explore novel treatment options for diseases with a genetic component (Nijman, 2011). In summary, these high-throughput GI screens utilise **knockdown** of multiple genes to determine SS/L interactions that can be exploited as therapeutic targets.

There are significant limits to GI screens; - RNAi results in 70–90% inhibition of gene expression(Nijman, 2011), therefore, partial knockdown or differential transfection efficiency within cells can result in false positives and negatives (Horn *et al.*, 2011). False negatives can be caused by high knockdown efficiency, an overly efficient knockdown can lead to a high proportion of cells dying, this gives a much smaller number of measurable cells, which are then assumed to be not significant (Nijman, 2010). Off target effects may also lead to incorrect conclusions regarding the

effectiveness of a particular target and produce unwanted side effects in the patient (Nijman, 2010).

Housden et al (2015) used a novel approach to overcome these issues to identify potential drug targets for TSC related diseases. By combining multiple techniques: Housden et al (2015) and Housden et al. (2017) used combinatorial screening to identify SS/L interactions between candidate genes and *TSC1* or *TSC2. Drosophila* wild-type cells were mutated using CRISPR/Cas9-assisted genome editing to knockout the *TSC1* or *TSC2* genes. DsRNA screens targeting all phosphates and kinases in the *Drosophila* genome were then performed on wild-type cells, *TSC1* and *TSC2* mutant cells. By combining *Drosophila* cells, and RNAi genetic interaction screens Housden *et al.* (2017) developed a novel combinatorial screen called **Variable dose analysis (VDA)** to identify potential synergistic treatments.

Variable dose analysis is an optimised assay for GI screens:

Variable Dose Analysis (VDA) is a novel GI screening method, developed by Housden et al, (2017) using shRNA to silence a target gene. VDA increases the sensitivity and reproducibility of screen results compared to previous methods, making this an ideal tool for this application (Housden *et al*., 2017). Cells are transfected with a complex made up of equal parts fluorescent protein (GFP), Gal4 and shRNA; the fluorescent protein is then measured and used as an indirect indicator of shRNA effectiveness. The cells are read on a flow cytometer and the fluorescence is used to calculate the relative knockdown efficiency of the target gene. Cells receiving a higher dose of shRNA will also receive a higher dose of GFP. Therefore, a higher level of GFP indicates a lower level of RNAi effectiveness, where effectiveness is considered a higher negative effect on cell viability. By measuring this over a population of cells rather than a single cell, we can extrapolate both the effectiveness of the RNAi and the possible mechanism of action. A high proportion of higher GFP positive cells indicates a lower effect, a low proportion of cells with a low GFP reading show a greater effect, a low proportion of cells with a higher GFP reading show an arresting rather than cytotoxic effect. VDA provides many benefits over traditional GI screens, by screening cells over a varied dosage of shRNA, phenotypes can be analysed at a sub-lethal level of knockdown efficiency. This method also reduces signal-to-noise ratio by 2.5-fold (Sierzputowska, Baxter and Housden, 2018). Two hundred potential

genes were identified that reduced viability of *TSC1* and *TSC2* cells, but not wild-type cells. These genes represent promising new therapeutic targets to treat TSC.

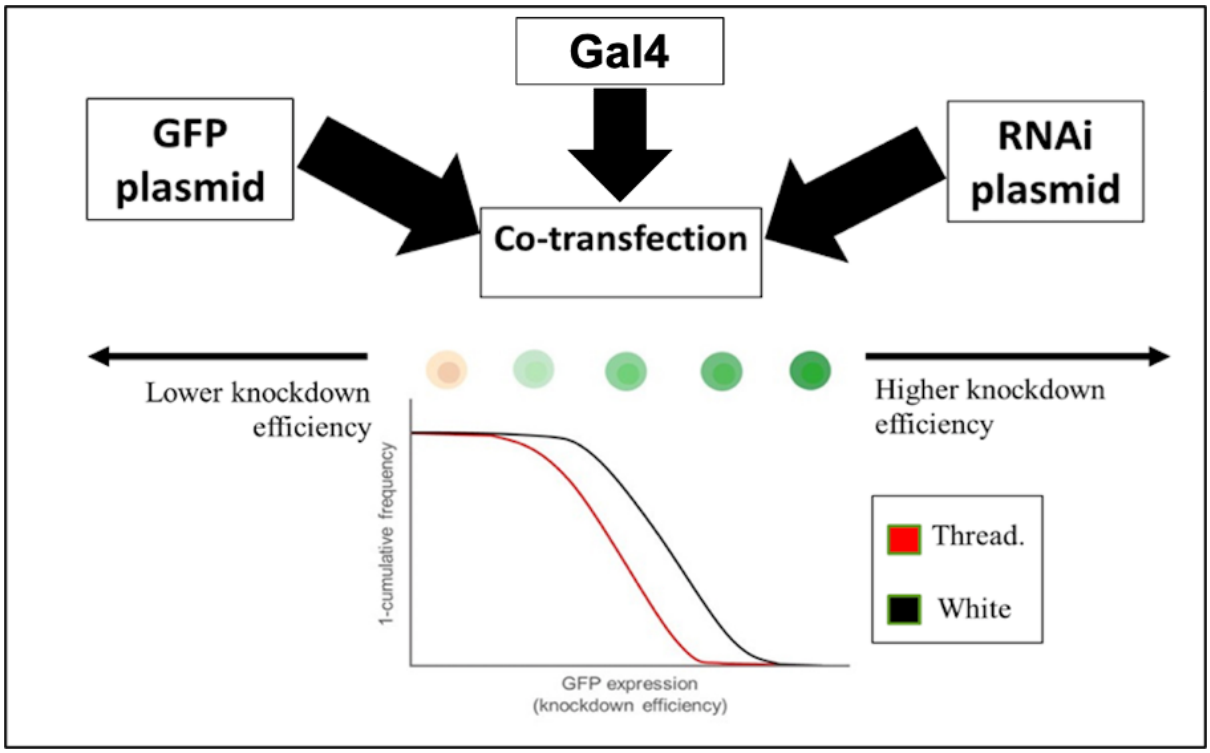

Figure 1: The VDA process. GFP, Gal4 and an RNAi are co-transfected into cells. These cells are measured on a flow cytometer and the data plotted on a graph. The area under the curve is measured and used to create a numerical value (adapted from Sierzputowska, Baxter and Housden, (2018). White and thread are used as negative and positive controls respectively.

Using VDA for the second candidate diminishes the characteristic noise in GI screens to present viable candidates for synergistic interactions. By combining these techniques issues of noise, false positives and variation will be reduced.

Project Summary

Building upon the original TSC screen (Housden *et al.*, 2017), which identified multiple possible targets for treatment, the aim of this project is to identify and characterise new combinations of drugs with the potential to effectively treat TSC. I achieved this by performing VDA screens in TSC1 mutant *Drosophila* cells in the presence of drugs that were previously identified by the Housden group as having a potential therapeutic benefit to TSC patients (Housden *et al.*, 2015, Housden *et al.*, 2017). By comparing screens performed in TSC mutant and wild-type cells with screens performed with and

without the candidate therapeutic drugs, I identified genes that synergise with the candidate drugs to produce powerful and specific killing of TSC cells.

I focused the screens on genes for which clinically approved drugs already exist. Therefore, any synergistic effects that I identified can be immediately tested using drug combinations. In addition, these drugs are already known to be safe for use in humans and so the resulting combinations can be rapidly translated into clinical use and patient gains.

Hypothesis

My hypothesis is that the application of newly developed combinatorial screening methods, developed in the Housden lab, will identify combinations of drugs that effectively and specifically kill TSC tumours, without significantly affecting healthy cells. There are three main parts to this project: -

Part 1:

First, I characterised seven candidate therapeutic drugs identified in previous studies and identified the most promising for development of combinatorial treatments. I tested the candidate drugs; - lithium chloride, chloroquine, mizoribine, rapamycin, SAHA, Vorinostat and Regorafenib in wild-type, *TSC1* cells. I used these results to determine which drugs, and at what doses, negatively affect the mutant *TSC1* cell lines, without affecting the wild-type cells. This determined which dose had the greatest potential benefit with minimal effect on healthy cells. In addition, the characterisation of the dose response for each of these drugs determined the optimal concentration for the screens to be performed in the second part of the project. Finally, I tested each of the seven drugs to determine which have cytotoxic versus cytostatic effects in TSC mutant cells by using cytometry assays to assess markers of cell viability and cell death. Drugs that have selective cytotoxic effects on TSC mutant cells at therapeutically viable concentrations were prioritised for screening using VDA. I determined lithium chloride was the most effective of the drugs.

Part 2:

In this aim, I used VDA to screen for candidate genes that synergistically interact with lithium chloride. I performed VDA screens targeting 154 genes, which are the

Drosophila orthologs of human genes that can be targeted with existing clinically approved drugs. By focusing on these genes, I identified candidates that can be immediately validated using existing drugs, thereby accelerating their translation into clinical use. Screens were performed in wild-type and *TSC1* cells in the presence and absence of Lithium chloride. Synergistic candidates were identified as genes that kill *TSC1* cells only in the presence of the drug. Hits from these screens were ranked based on the strength of their lethal effect on TSC cells in the presence of the relevant drug and combinations were further studied in part three. Fifteen genes were identified as possible targets and of these fifteen I focused on the purine synthesis pathway. It has been identified previously as a possible therapeutic target for TSC and research has been conducted on three purine synthesis targeting drugs.

Part 3:

Finally, I characterised drug combinations identified in part 2 that may be further developed for therapeutic use to treat TSC. To validate candidate synergistic effects identified in the screens, I tested the interactions pharmacologically by combining lithium chloride with **IMPDH** inhibitors and tested their effect in mammalian (murine and human) cells, compared to either drug alone. Previous studies have shown a high rate of validation between *Drosophila* and mammalian models of TSC (Valvezan *et al.*, 2017, 2020).

Chapter 1

Identifying promising drugs for the treatment of TSC tumours

Chapter summary:

The first objective of the project was to assess drugs already identified as candidate therapies for TSC to find the optimal candidate for further development as a combinatorial treatment. To identify candidate treatments for TSC, Housden et al (2017) performed a genome wide screen using *Drosophila* cells to determine SS/L interactions with *TSC1* and *TSC2*. Utilising dsRNA and VDA methods they found approximately 200 possible therapeutic targets (Housden *et al.*, 2017). Of the identified targets, five could be inhibited with existing clinically approved drugs (mizoribine, SAHA, orlistat, lithium and regorafenib), all of which showed selective viability effects on TSC mutant cells. A search of the literature also presented a further two possible candidates (chloroquine and rapamycin). These seven drugs were therefore used as a basis for the initial stages of the project.

Utilising *Drosophila* cells as a model system, candidate drugs were tested in wild-type and TSC1 mutant cell lines to determine their effects. Cell viability and cell death assays were used to determine whether the drugs showed selective effects on TSC mutant cells. An ideal candidate would be cytotoxic in TSC mutant cells and non-toxic in wild-type cells. Murine cells were also used to determine whether the effects were conserved across model systems and to ensure candidates that were ineffective in *Drosophila* cells were not missed. The results of these experiments were collated and the most successful of the drug, lithium chloride, progressed to VDA screening for combinatorial candidates.

Background

A successful treatment needs to be effective, safe and clinically viable; it must also meet a need that is not currently being met (Hughes *et al.*, 2011). Any prospective candidate therapeutic must meet all of these conditions. With this in mind, to identify the most promising candidate for further investigation for the treatment of TSC the following requirements were set:

1 – The drug must have selective viability effects in TSC mutant cells.
2 – The drug must be cytotoxic in TSC mutant cells.
3 – The drug must not be cytotoxic in wildtype cells
4 – The drug must be clinically viable for long term use in humans.

To evaluate these criteria for each of the seven candidates, three different experiments were performed: -

Viability dose curves

Firstly, to establish the most selective dose of each drug, viability dose curves were performed. Each drug was tested with between 4 and 8 concentrations to determine which exhibited the highest level of selectivity between wild-type and TSC cells. The aim was to find a strong viability effect on mutant cells and a minimal negative effect on wild-type cells. These experiments utilised CellTiter-glo (CTG) assays, which measure total adenosine triphosphate (ATP) in a cell population as an indicator of cell viability. ATP is able to store and transfer energy within cells and can be used as a measure of the cell viability; low levels of ATP indicate either lower numbers of cells, reduced cell size or lower metabolic activity of cells (Bonora *et al.*, 2012). The nature of the assay means it is not possible to differentiate between these effects.

Selectivity assays

During the project, I found that the viability dose curves were very noisy, and it was challenging to identify selective effects between cell types. To overcome this, I used selectivity assays, which are similar to the dose curve assays described above, except that only a single drug dose is assessed. These assays again use CTG as a measure of cell viability. Using fewer dosages allows additional replicate experiments to be performed and therefore enables more sensitive detection of selective viability effects.

Dosages for these experiments were determined from the viability dose curves and provided an enhanced view of the separation between the viability of wild-type and mutant cell lines.

Cell death assays

Neither the dose curve nor the selectivity assay is able to distinguish between cytotoxic and cytostatic effects. Therefore, cell death assays were performed using propidium iodide (PI) as a measure of cell viability. PI is a fluorescent intercalating agent that binds to exposed DNA within cells (Zhao *et al.*, 2010). PI is excluded from healthy cells but dead or dying cells have a more porous membrane and PI can pass through to bind to DNA (Rosenberg, Azevedo and Ivask, 2019). Therefore, a PI stain allows us to determine if the drug is having the desired cytotoxic effect.

Drugs

The original drugs to be tested were identified by Housden *et al.* (2017) as targeted therapies for TSC. These drugs were tested in CRISPR/Cas9 mutated *Drosophila* cells. The drugs identified were -

Lithium

Lithium has been used as a therapy for over 100 years. Primarily it is used to treat bipolar disorder and persistent depressive episodes that don't respond to other treatments. Despite its age, the mechanism of action is poorly understood (Wishart *et al.*, 2018) it has many different targets. Lithium inhibits *GSK-3β,* which activates mTOR. Lithium can bio-accumulate within the body, leading to lithium poisoning, however careful management of dosage can reduce the dangers (Wishart *et al.*, 2018). Current dosages of lithium range from 900 to 1200 mg/day for long term treatment of mania.

Orlistat

Orlistat, marketed as the diet drug Alli™ in the United Kingdom, is used primarily as a pancreatic lipase inhibitor. This enzyme breaks down triglycerides in the intestine for uptake. Inhibition of pancreatic lipase prevent hydrolysation of triglycerides into absorbable fatty acids. There is some research indicating that orlistat can be used in the treatment of pancreatic tumours by targeting fatty acid synthases (*FASN*)

(Sokolowska *et al.*, 2017). However, the poor bioavailability of orlistat, which is designed to work within the intestines rather than enter the bloodstream, creates issues around using it as a long-term treatment.

Regorafenib

Regorafenib, commercially referred to as stirvaga, is a multiple kinase inhibitor used in the treatment of colorectal cancer and advanced gastrointestinal stromal tumours (Wishart *et al.*, 2018). Regorafenib inhibits 18 different proteins making it more likely to exhibit cytotoxicity in humans. However, despite approval, it exhibits significant toxicity (Goel, 2018). It has caused fatal levels of liver toxicity in some patients (Béchade *et al.*, 2017). Due to its significant side effects and high cytotoxicity, there are significant questions over its suitability for the long-term usage required by TSC patients.

Vorinostat/SAHA

Vorinostat, or suberanilohydroxamic acid (SAHA), is a histone deacetylase inhibitor used in the treatment of cutaneous T cell lymphoma and currently in trials for persistent glioblastoma. In cancer treatments it disrupts cell differentiation, arrests growth and induces apoptosis (Bubna, 2015). By inhibiting histone deacetylase, SAHA causes the accumulation of acetylated histones and induces cell cycle arrest and/or apoptosis of some transformed cells (Richon, 2006). Vorinostat targets *HDAC1, HDAC2, HDAC3* and *HDAC6* (Lee and R, 2013) and the current dosage is 400mg daily.

Rapamycin

Rapamycin is the current treatment for TSC, as the drug Sirolimus (marketed as Rapamune by Pfizer) (Tee, 2018). As a treatment, it exhibits limitations; it arrests cell development rather than exhibiting a cytotoxic effect (Zheng, X. F. *et al*., 1995) Due to significant side effects caused by the usage of rapamycin and the regrowth of tumours once treatment is stopped, other interventions are required for TSC management (Cancer, 2016). In addition to TSC treatment, it is currently used as an immunosuppressant in cases of organ transplantation. It inhibits T and B cell activation through mTOR inhibition (Ye, 2017). Due to the importance of mTOR in cell growth and proliferation, rapamycin is currently being investigated as a possible treatment for a variety of immune conditions and cancers (Oaks, 2016 and Chan, 2004)

Chloroquine

Chloroquine has been used as an antimalarial agent for 70 years and is also used to treat lupus and rheumatoid arteritis (Verbaanderd *et al.*, 2017). It was briefly considered as a possible treatment for SARS-Cov-19 (Moore, 2020). Chloroquine is an autophagy inhibitor and has been identified as a possible therapeutic target for cancers where Hyperactivity of mTor leads to dysregulation of cell signalling (Johnson and Tee, 2017). Chloroquine is also theorised to reduce tumour growth by activating the P-53 pathway, responsible for growth suppression and apoptosis (Kim *et al.*, 2010).

Results

Drugs were first tested in viability dose curves; results were normalised to wells without a drug; Figure 1 shows the normalised results of these assays. Each graph is the result of twenty biological replicates. These are intended to show the optimal concentration for selectivity between loss if function mutated TSC1 and wild-type *Drosophila* cells, whilst also establishing no significant negative effect on wild-type cells. However, we found that these experiments resulted in high noise and selective effects were difficult to detect even for drugs with previously well-established selective effects (e.g. mizoribine (Valvezan *et al.*, 2017).

In the dose curves, the ideal result would show a strong separation between the wild-type and the TSC mutant cells. Lithium chloride shows a significant separation between wild-type and TSC cells at 2 mM and 4 mM. At higher concentrations, the separation was less apparent. Similarly, Regorafenib showed selectivity at very low concentrations (0.006 nM and 0.0006 nM). Mizoribine, SAHA and orlistat showed no significant selectivity at any dosage. Chloroquine showed a significant selective effect on the wild-type cells.

Dose curves are frequently noisy due to the high number of variables being tested and the difficulty associated with manually pipetting many different drug dilutions. In order to more accurately determine the selective effects of the drugs, we used selectivity assays using the lowest most selective concentration of the drug, for example, 2 mM lithium chloride. For drugs that showed no significant separation, the concentration with the smallest detectable effect on wild-type was chosen.

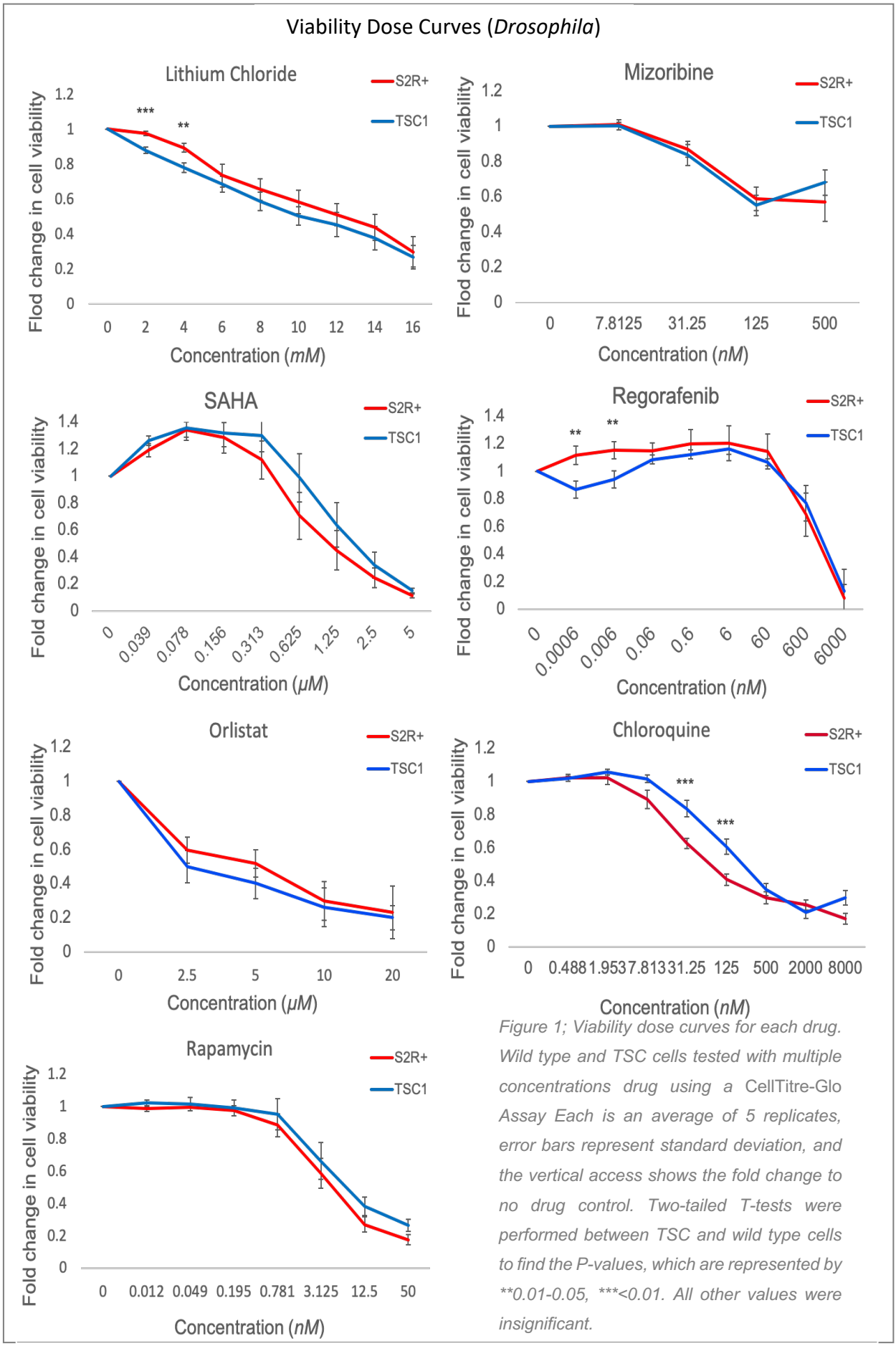

Figure 1; Viability dose curves for each drug. Wild type and TSC cells tested with multiple concentrations drug using a CellTitre-Glo *Assay Each is an average of 5 replicates, error bars represent standard deviation, and the vertical access shows the fold change to no drug control. Two-tailed T-tests were performed between TSC and wild type cells to find the P-values, which are represented by **0.01-0.05, ***<0.01. All other values were insignificant.*

Using the lowest effective concentrations from the dose curves, cells were screened for selectivity using CellTitre-Glo. Figure 2 shows the results of 16 biological replicates performed over two occasions. The selectivity assay showed a selective effect with lithium chloride, rapamycin and chloroquine. Regorafenib and mizoribine showed high levels of variability and no selective effect. Orlistat and SAHA showed no significant selective effect.

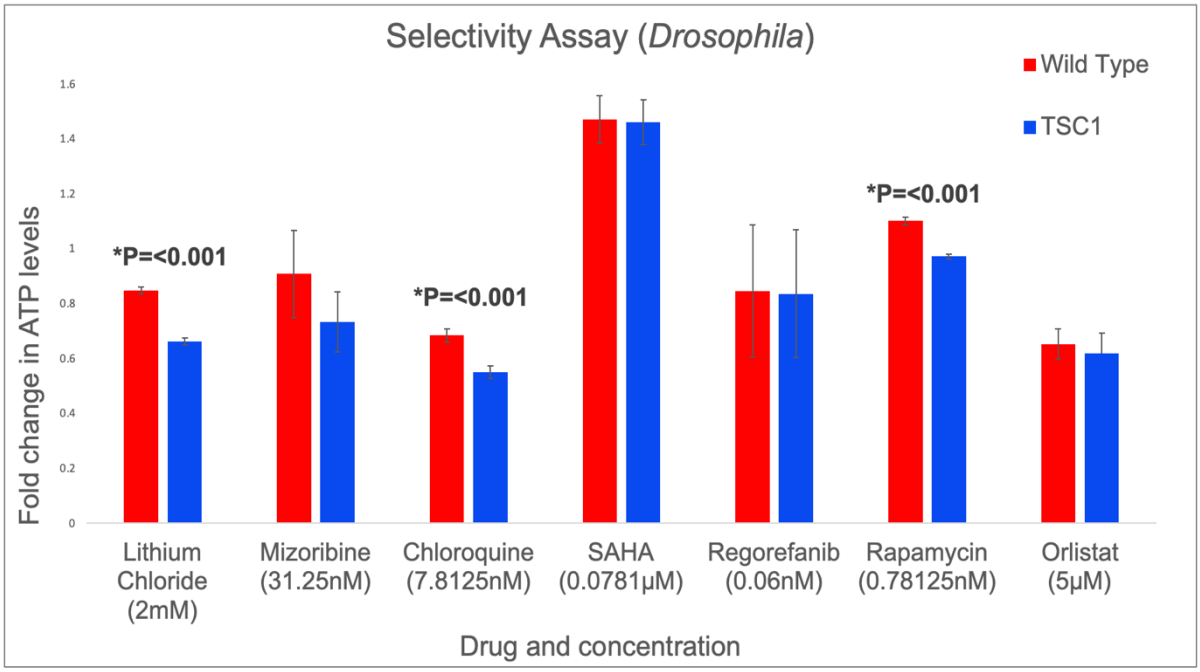

Figure 2; Selectivity assay in *Drosophila* cells. Cells were tested with one concentration of the drug using a CellTitre-Glo assay; results were normalised to no drug to measure the effect. 16 biological replicates were performed over 2 occasions and the results averaged. Error bars represent standard deviations. Two-tailed T-tests were performed between wild type and TSC cells and show significant effects in lithium chloride, chloroquine and rapamycin.

To determine whether the selective effects identified above are due to cytotoxic effects, cell death assays were performed. Two dosages were tested, the same dosage as that used for the selectivity assay and a lower dosage. Except in the case of lithium chloride, where 4 mM was used, as there was no lower dosage within the range initially tested.

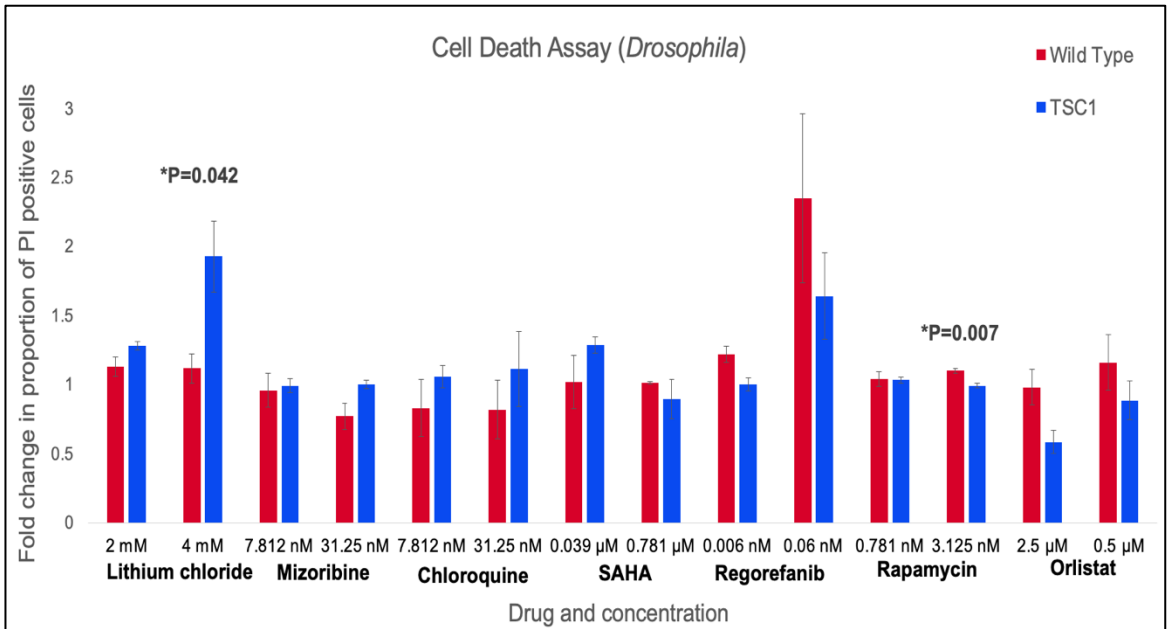

Figure 3: Cell death assays for *Drosophila*. 2 dosages of each drug were tested using a propidium iodide stain and results read on a flow cytometer 12 biological replicates, results were normalised to cells with no drug. Error bars show the variation in the means of each replicate. Two-Tailed T tests were performed to find the p-values. Rapamycin and lithium showed significant selective effects

Figure 3 shows the results from cell death assays, error bars represent standard error of the mean Lithium chloride was the only drug that showed a significant selectivity in TSC cells in the cell death assay. Mizoribine, chloroquine showed a trend towards greater cytotoxicity in TSC cells however they were not significant. SAHA and orlistat showed no significant selective cytotoxic effect in TSC cells at any concentration. Regorafenib showed a significant selective effect at 0.006 nM but was cytotoxic in wild-type cells. Rapamycin showed no selective effect, however as rapamycin is known to be cytostatic this is not unexpected.

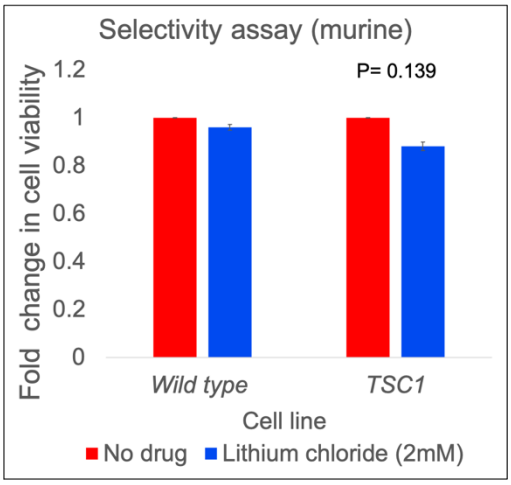

Figure 4: Lithium chloride murine selectivity results. A single dose of the drug tested using a CellTitre-Glo assay. Showing the average of 24 replicate, error bars represent the standard deviation of the mean. P-values were found using a two-tailed t-test. The murine cells show a trend to lower cell viability. Lithium chloride has been shown to be ineffective in murine cell lines.

Figure 4 shows the results of the murine selectivity assay for lithium chloride. There is no significant effect on TSC mutant cells, but a trend is evident. Housden et al., (2017) found that lithium was not as effective in murine cells.

Discussion

Drug / Criteria	Lithium Chloride	Rapamycin	Chloroquine	SAHA	Regorafenib	Orlistat	Mizoribine
Selective	√	√	√	X	√	X	X
WT Non Toxic	√	√	√	√	X	√	√
TSC Toxic	√	X	X	X	√	X	X
Clinically Viable	√	√	√	√	X	X	√

Table 1: Summary of the results of the drug tests. Green indicates it passed; red indicates a failure in that category. Regorafenib and orlistat failed on clinical viability, regorafenib due to its toxicity and orlistat due to poor bioavailability.

The intention is to find a drug that only selectively targets the mutant cell lines whilst leaving the wild-type healthy. Some cytotoxicity towards wild-type is acceptable but a larger amount is detrimental. Each of the drugs were scored against the criteria mentioned above to identify the most promising candidate for further analysis. Table 1 shows the collated results of the various assays. Selectivity is based on the selectivity assays, Non-toxic and toxic are based on the results from the cell death assays and clinical viability is based on a search of the literature. The dose curves showed significantly selective effects in three of the drugs, however due to the number of variables and the difficulty in pipetting small quantities (1 µl) the results cannot be considered conclusive. Because of this, results from the viability dose curves were not taken into account for the final characterisation of the drugs.

A major difficulty in testing candidate drugs is lack of reproducibility this has been shown repeatedly (Hunter, 2017). All the drugs tested in this chapter showed selective effects in other studies. Mizoribine especially has a well-documented effect on TSC cells (Valvezan *et al.*, 2017). This difference can be caused by a vast number of variables, for example, Environmental variability such as culture conditions, differences in the methodology, such as difference in supplier, as was seen with SAHA and vorinostat, culture conditions or variations in the health of the cell lines can all contribute to poor reproducibility in testing.

Rapamycin slows the growth of tumours until such time as they can be surgically removed, however, it requires further medical intervention rather than treating the condition at its source. It is an immuno-suppressant with several limitations. The most significant being it is not curative, only arresting cell proliferation of TSC positive cells (Cancer, 2016). This is shown in the results of the cell death assay, which showed no significant cytotoxic effect in TSC cells, however, the selectivity assay showed a drop in ATP levels in TSC cells which is most likely a cytostatic effect targeting the TSC cells.

Orlistat did not display selective cytotoxicity in *Drosophila* cells. Orlistat also presented clinical issues. It is not absorbed into the blood stream orally; it instead moves to the gut (Sokolowska *et al.*, 2017). So, for these reasons reason, it was not moved forward.

Regorafenib showed a significant selective effect in the dose curve. However, in the cell death assay it showed a significant cytotoxic effect in wild type cells. Regorafenib is known to be highly toxic, even at dramatically lower doses. It is used as a 'nuclear option' (an extreme option taken as a last resort due to its many side effects) in cases of extremely aggressive colorectal cancer where other treatments have failed (Goel, 2018), but shows significant side effects due to its many off-target affects (Béchade *et al.*, 2017). So, in addition to being nonselective, its clinical viability as a long-term treatment option is questionable.

Mizoribine was cytotoxic in all *Drosophila*, although at a much lower level than regorafenib. It also showed a selective effect between wild-type and mutant. Mizoribine is currently approved in Japan for use in treating renal transplant, however, is currently not approved by the FDA. Work previously done by (Valvezan, 2017) has shown that murine TSC cells are affected by treatment with mizoribine. My research however does not show the same results. Mizoribine is also not clinically approved worldwide and little is known regarding its long-term effects. Mizoribine showed little selectivity in either the dose curve or selectivity assay, however it has been shown to be selective by Valvezan et al,. (2017).

Vorinostat showed no selectivity between the wild-types and the mutant cells and exhibited a strong cytotoxic effect on both. However, vorinostat was not the same name or supplier as that tested previously, being an alternate name for SAHA. In order to maintain consistency, all experiments were performed again using SAHA. Preliminary results for SAHA showed very little selectivity between TSC and wild-type cells, however, in the cell death assays, SAHA showed a negative effect on wild-type cells rather than on TSC cells. SAHA has been shown previously to have some anti-tumour properties (Yang, 2015).

Chloroquine showed no selectivity in the viability dose curve, however, did show a significant effect in the selectivity assay. However, it failed to demonstrate a selective cytotoxic effect on TSC cells in the cell death assay. Despite showing significant selective effects in the selectivity assay chloroquine was rejected in favour of lithium chloride which showed a significant cytotoxic effect.

Lithium chloride was the only drug that met all criteria within the framework for these experiments. For this reason, it was determined Lithium chloride would be the most successful when moved onto combinatorial treatments.

Lithium chloride had a significantly selective effect in TSC1 in *Drosophila* cells, however the reaction was not conserved to murine cells (Figure 4). It is known that mice metabolise lithium chloride differently to humans and is less effective within murine cells (O'Donnell, 2007). Housden et al., (2017) also demonstrated that lithium was effective in human cells but not in murine cells. So, I will proceed with lithium despite its lack of effect in murine cells Lithium chloride also exhibited strong levels of cytotoxicity in mutant *Drosophila* cells, whilst showing a very low negative effect in wild-type cells. For these reasons, lithium chloride was chosen as the most effective drug to test with combinations. There was a significant selective effect between wild-type TSC1 mutant cells and there is a clear increase in cell death with the addition of lithium chloride (Figure 3).

Although lithium chloride is currently used as a therapeutic, there are several challenges to determine its viability as a treatment for TSC. I identified a selective effect at 2 mM. The current therapeutic range is between 0.6-1.2 mM, without toxicity.

The identified concentration is slightly above this range, so whether the lower concentration could be achieved while maintaining a selective cytotoxic effect will require further investigation. Lithium chloride is currently taken daily to manage bipolar and unipolar disorders, therefore, taking lithium at a higher dose rate over the long term could cause side effects. Lithium chloride is bio-accumulative, so regular blood tests are currently used to monitor lithium levels to maintain a non-toxic therapeutic level. However, using lithium as part of a combinatorial treatment may allow it to be used at a lower dose.

Lithium chloride was overall the most effective of the drugs. It's selectivity towards TSC1 in the *Drosophila* cells was clear and reproduceable and it showed a very low cytotoxic effect in wild-type cells. For these reasons, lithium chloride was selected for the next stages of the project.

Materials

Chemicals

Lithium Chloride in media – (Sigma-Aldrich 62476)

Mizoribine in media – (Sigma-Aldrich M3047)

Orlistat in ethanol – (Sigma-Aldrich O4139)

Vorinostat in DMSO – (LC Laboratories V8477)

SAHA in DMSO – (Sigma Aldrich SML006)

Chloroquine in media – (Sigma Aldrich C6628)

Rapamycin in ethanol – (LC Laboratories E4040)

Regorafenib in DMSO (LC Laboratories RS024)

Cells

Drosophila S2R+ (wild-type) – Schneider (1972)

Drosophila TSC1 - Housden *et al* (2015)

Drosophila TSC2 - Housden *et al* (2015)

Murine MEF BE2 TSC2 +/+ (wild-type) – Manning *et al* (2002)

Murine MEF BE2 TSC2 -/- Manning *et al* (2002)

Reagents

CellTitre-Glo – (Promega G7573)

Propidium Iodide – (Fisher scientific 11599296)

Schneider's *Drosophila* media – (Fisher Scientific 11520406)

One Shot™ Foetal Bovine Serum (Gibco A3382001)

Penicillin/Streptomycin – (Fisher Scientific 15140122)

Phosphate Buffered Saline (PBS) (Gibco, 20012019)

Mizoribine in media	0 µm	7.812 µm	7.812 µm	31.25 µm	31.25 µm	125 µm	125 µm	500 µm	500 µm
Rapamycin in ethanol	0 nM	0 nM	0.012nM	0.048 nM	0.195 nM	0.781 nM	3.125 nM	12.5 nM	50 nM
Regorafenib in DMSO	0 pM	0.0006 nM	0.006 nM	0.06 nM	0.6 nM	6 nM	60 nM	600 nM	6000 nM
Chloroquine in media	0 nM	0.488 nM	1.953 nM	7.812 nM	31.25 nM	125 nM	500 nM	2000 nM	8000 nM
LiCl in media	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	12 mM	14 mM	16 mM
SAHA in DMSO	0 µm	0.039 µm	0.078 µm	0.156 µm	0.312 µm	0.625 µm	1.25 µm	2.5 µm	5 µm
Orlistat in ethanol	0 µm	2.5 µm	2.5 µm	5 µm	5 µm	10 µm	10 µm	20 µm	20 µm

Table 2 – Showing the dosages used for the viability dose curves, blue indicates the dosages were used for cell death assays

Dose curves

A serial dilution of the drug being tested was added to cells and incubated over a five-day period. The total ATP level of the cells was measured to determine the viability of living cells.

- Cells were grown in Schneider's *Drosophila* media with 10% FBS and 1% penicillium/streptomycin until they reached 80% confluency.
- A 96-well plate was seeded with 5000 cells per well of the 2 cell types and inoculated with the drugs at the concentrations outlined in Table 1.
- Plates were incubated at 25°C for 5 days.
- After incubation, 50µl of CellTitre-Glo was added to each well and read on a Tecan plate reader.
- Results were normalised to controls (no drugs).
- External wells were ignored to avoid edge effect from differential temperatures and evaporation on the outside of the plates.

Selectivity assays

Each plate only used a single concentration of each drug and results are normalised to a well with no drug. 96 well plates were seeded with 5000 cells per well and inoculated with one concentration of the drug. This provided 16 biological replicates per plate and each plate was repeated twice. Similar to the dose curve experiments, selectivity assays were performed by testing total ATP levels after a five-day incubation with the relevant drug.

- A 96 well plate was seeded with 5000 cells per well and inoculated with one concentration of the drug.
- Plates were incubated at 25°C for 5 days.

- After incubation, 50μl of CellTitre-Glo was added to each well and read on a Tecan plate reader.
- Results were normalised to controls (no drugs).
- External wells were ignored to avoid edge effect from differential temperatures and evaporation on the outside of the plates.

Cell death assays

The PI assays used two dosages to determine if the drug was cytotoxic in wild-type or mutant cells. Dead or dying cells are stained with PI and analysed on a flow cytometer; this gives a readout of the percentage of dead or dying cells. Arrested cells are not stained because the PI cannot cross intact cell membranes. PI positive cells are measured as a percentage of the total cell population. Testing two concentrations in both mutant and wild-type cells gives a readout on whether an increased dosage causes a more cytotoxic effect in TSC cells compared to wild-type cells.

- 96-well plates were seeded with 5000 cells per well and inoculated with the drug dilutions outlined in Table 1.
- Plates were incubated at 25°C for 5 days.
- 100μl of 4% propidium iodide (in PBS) was added to each well and incubated for 1 min at room temperature.
- Plates were read on a flow cytometer.
- Results were normalised to wells with a positive and negative controls.

Chapter 2

Identification of synergistic drug combinations for TSC using VDA screens

Chapter summary:

Combinatorial treatments are often more efficacious than single drugs alone. Utilising combinations of drugs to treat a disease supresses multiple pathways within the cell and can result in a synergistic effect that leads to a better outcome for patients (Chen, 2016). Using multiple drugs also reduces the chance of resistance developing. additionally, each drug can be used at lower dose, thereby alleviating the potential for deleterious side-effects (Hartwell *et al.*, 1997).

In Chapter 1, I identified lithium chloride as the most promising of seven candidate drugs for the treatment of TSC. The next step was to identify candidate drugs that would synergise with lithium chloride, in order to find a possible combinatorial treatment to more effectively kill TSC tumour cells. To achieve this, I performed VDA screens in TSC1 mutant *Drosophila* cells, with and without lithium chloride. From these screens I identified fifteen genes that synergised with lithium chloride, identified their human orthologs and FDA-approved drugs that target the gene products.

Background

In order to identify genes that show a SS/L in the presence of lithium chloride I used a VDA RNAi screen. Using RNAi screens is a common way to identify drugs that target specific genes, however, finding strong synergistic interactions using traditional screening methods presents challenges, VDA nullifies some of these issues. It can also identify synergistic effects between lithium chloride and essential genes, which makes it more appropriate to identify strong SS/L interactions.

VDA – A novel high-throughput screening assay

In traditional screens, viability is measured using a cell count. This may be by directly counting cells using imaging or cytometry, or an indirect, relative counting method such as CellTiter-glo assays. However, these methods have several limitations; variation in target gene expression over a population of cells results in noise. Knockdown efficiency is only measurable over a limited range, weak knockdowns are ineffective, whilst strong knockdowns are lethal to both the wild-type and mutant cells (Housden *et al.*, 2017). This can lead to false positives and negatives, which in turn, can lead to highly effective therapeutic candidates being rejected (Nijman, 2011).

VDA tests over a range of knockdown efficiencies which mitigates this problem of noise (Sierzputowska, Baxter and Housden, 2018). The VDA method considers viability as a function of target gene knockdown efficiency. Because of this, VDA allows the detection of phenotypes and genetic interactions involving essential genes at sub-lethal knockdown efficiency (Sierzputowska, Baxter and Housden, 2018).

Figure 1 shows how the VDA method works. Plasmids encoding GFP, Gal4 and shRNA are formed together into transfection complexes. The shRNA will silence the target gene, the GFP provides a measurable reporter and the Gal4/UAS system ensures the expression of the shRNA. These are then transfected into cultured cells. Due to the inherent variability in transfections, the dose of plasmids received by each cell in the population will be different. However, each cell will receive the same ratio of GFP to shRNA. Therefore, the GFP levels can be used as an indirect indicator of shRNA levels and therefore, gene knockdown efficiency. shRNAs targeting essential genes will kill cells more effectively when expressed at high levels (high GFP cells)

and will have less effect when expressed at low levels (low GFP cells), leading to a change in GFP distribution over the population. Therefore, by analysing the GFP distribution of the cell population, shRNAs that affect cell viability can be identified.

I undertook a VDA screen to ascertain any synergistic interactions between lithium chloride and the target genes from the FDA library. All shRNAs in the FDA library correspond to a target for an FDA-approved drug; this would identify possible therapeutic candidates that would synergise with lithium chloride, to create a possible combinatorial treatment for TSC. An ideal candidate would show lower cell viability in TSC mutants with the addition of lithium chloride, whilst having no effect on wild-type cells.

Results

Preparation and normalisation of the RNAi library

To allow the proposed synergy screen, the RNAi library first needed to be amplified. The FDA library is an RNAi library consisting of 600 shRNA reagents targeting 154 genes whose functions can be inhibited with FDA-approved drugs. Using a library consisting of pre-approved drugs increases the likelihood of finding a suitable treatment.

The library is divided into ten 96-well plates. Each plate in the screen contained a random assortment of reagents as seen in Figure 2; furthermore, each plate had 5 negative (**White**) and 5 positive controls (**Thread**). White is a mutation that affects eye development in crossed flies and therefore in cells has no effect. Thread is a mutation that exhibits a significant increase in cell death leading to its use as a positive control. The majority of genes had three replicates over the 10 plates of the library; each reagent may have off-target effects, so by testing with multiple reagents, we can increase confidence that it is an on-target effect. Additionally, some reagents may not work, so we increase the chance of finding hits by testing each gene with 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Empty	positive control	sgg	PH4alphaEFB	Fak	whd	negative control	p38a	HDAC3	Prosbeta4	negative control	Empty
C	Empty	Tor	Atpalpha	Chd1	Amy-d	HDAC6	Aldh	positive control	Stat92E	htl	Rpn1	Empty
D	Empty	Dsor1	Cam	negative control	CG1640	for	CG6084	CG3011	CG8112	negative control	Pgd	Empty
E	Empty	Glo1	CG2543	CG30103	Prx5	CAH1	CG31871	Prosbeta5R1	src42A	Ts	GlcT-1	Empty
F	Empty	Prosbeta4R2	negative control	DNApol-epsilon58	positive control	Vha55	Nmdar2	Ide	GluRIA	Lip3	positive control	Empty
G	Empty	Nmdar1	CG7724	nAChRalpha5	Fpps	qm	positive control	Mes4	porin	Nep1	SdhD	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Figure 1: The typical set up of a plate. Green indicates a positive control well, purple a negative. The other wells contain a random assortment of shRNA. The edge wells contain cells but no shRNA in order to avoid edge effects.

independent reagents. Cells from the edge wells were checked prior to reading to ensure correct cell growth, but were left empty to avoid ‘edge effects’, whereby the temperature differential affects the results.

Library amplification

In order to amplify the library, each 96 well plate was transformed into chemically competent *E. coli* cells, cultured and then miniprepped. The resulting shRNA plasmids were then measured using a nanodrop to ensure the concentration was within acceptable parameters (over 45 ng/μl). If the concentration was below the threshold, the mini prep was repeated. The reagents were then normalised to 45 ng/μl using an EPI-motion robot with the addition of sterile water to each well. Five random wells from each plate, along with all control wells, were tested to check successful normalisation of concentrations. Finally, to ensure each shRNA was correct, five randomly chosen wells per plate were sequenced. Plates were then stored at -80°C until needed.

Plate set up

The plates were screened in pairs; one with (lithium chloride plate) and one without lithium chloride (no drug plate). These plates were screened using the same cells and reagents. To reduce variability as much as possible, each pair was treated as a single entity.

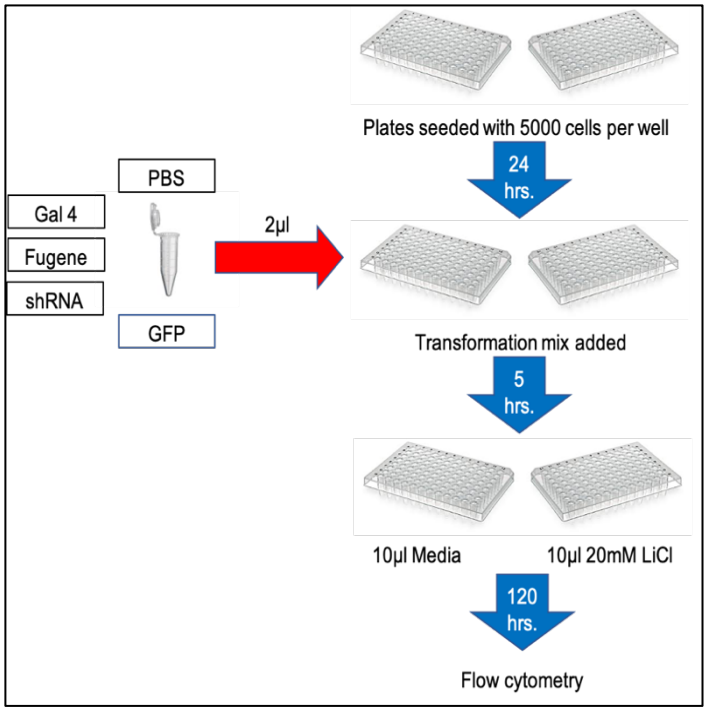

Figure 2: Schematic showing the steps taken to prepare the VDA plates. Blue arrows represent incubation times.

5000 cells in 100 μl of media were seeded into each well of two 96-well culture plates. They were then incubated at 25°C for 24 hours. After 24 hours, plates were transfected with a prepared mix of shRNA, Gal4 and GFP plasmid reagents. Plates were incubated for a further 5 hours before the addition of 10 μl 20 mM lithium chloride, for a final concentration of 2 mM; no drug plates had 10 μl of media added instead. As shown in Chapter 1, 2 mM lithium chloride showed a significant but small cytotoxic effect in TSC cells, while having little effect on wild-type

cells. Finally, plates were incubated for 120 hours at 25°C, before a being read on the flow cytometer.

Each library plate was screened as three biological replicates, both with and without lithium chloride, to allow identification of reproducible results. In addition, each plate contained five positive (targeting thread) controls and five negative (targeting white) controls, which were used for normalisation and to assess the quality of results from each individual plate.

Flow cytometry

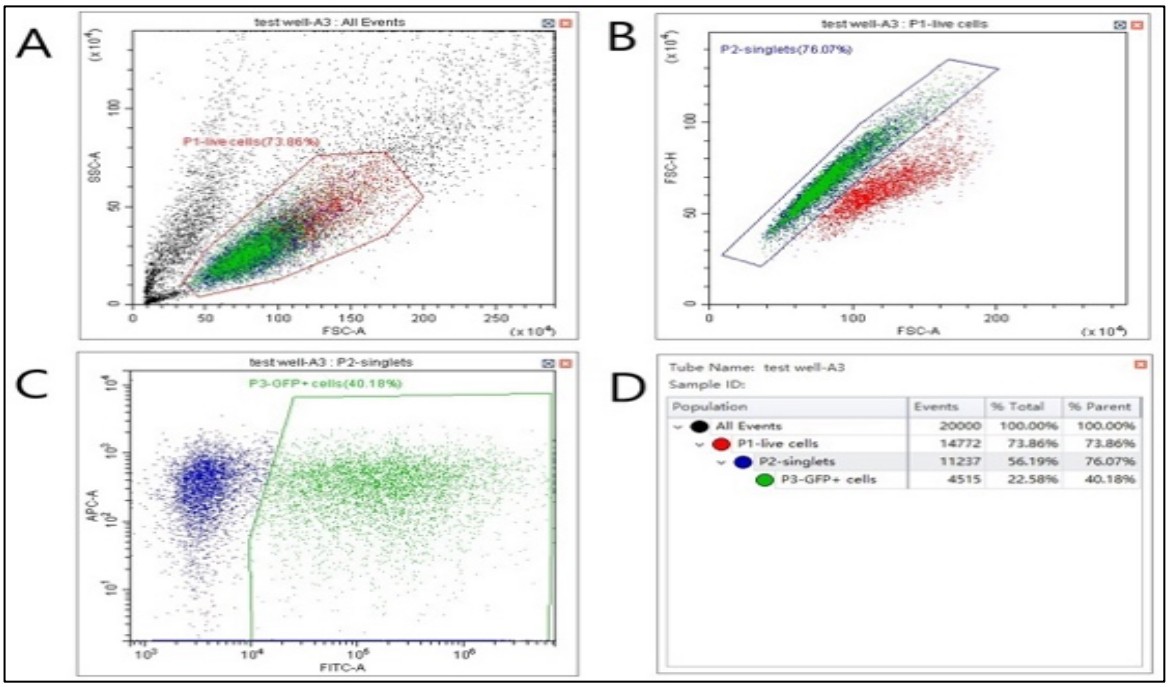

Figure 3: Example of the gating for flow cytometry. From Sierzputowska, Baxter and Housden, (2018). Gate A is gated by cell size to remove debris, Gate B for cell height to remove doubles and finally Gate C for flourescence.

After incubation, 50 µl of PBS was added to each well and the plates were read on a flow cytometer (Beckman Coulter - CytoFlex S). Cells were first measured using side scatter area (SSC-A) and forward scatter area (FSC-A) to remove cell debris from the results. Then, cells were gated using FSC-A and forward scatter height (FSC-H) to remove doublet cells. Finally, the remaining population was gated with the optimal filter for GFP; fluorescein isothiocyanate (Fit-C) and allophycocyanin (APC-A) which accounts for autofluorescence in the cells (Figure 4).

Analysis of screen data

To convert the flow cytometry data to VDA data, the flow cytometry data is run through a MATLAB script. The MATLAB script divides GFP signal by FSC in order to normalise to variation in cell size. Cells are then divided over the distribution of GFP intensity and finally, the area under the distribution curve is normalised to remove variability of cell numbers across samples. Finally, the cumulative distribution is calculated and then the area under it measured. This gives a numerical readout (arb) which can then be normalised and compared (Sierzputowska, Baxter and Housden, 2018).

In order to process the screen results, the negative and positive controls (white and thread) were checked to determine if the plate worked. White wells needed a result higher than 200 arbitrary units (arb); thread wells needed to be lower than 180 arb as determined by Housden *et al.* (2017). Any results not meeting these thresholds were removed prior to normalisation so as to not skew the results. Any plate with three or more control failures was considered unusable and repeated.

Normalisation of the results

Firstly, to neutralize position effects within the plate, each well was normalised to the median values from each column the results of which, were then normalised to the median of each row. Samples were then normalised to white and thread samples. The median thread (MTh) was subtracted from the plate normalised result (Equation 1). TNR denotes the Thread Normalised Result.

$$TNR = PNR - MTh \qquad (1)$$

Median thread was subtracted from median white (MWh) and the thread normalised results were normalised to white minus thread to give the final normalised result (Equation 2). NR denotes the Normalised Result -

$$NR = \frac{TNR}{(MWh - MTh)} \qquad (2)$$

Finally, after normalisation of the results, a correlation analysis was performed to compare each replicate of the plates. This showed whether the results were consistent across the screens.

Correlation coefficients

To determine whether plates were accurate replicates, a correlation analysis was performed between the three no drug and three lithium chloride plates to ensure the results were within acceptable parameters. The results of these co-efficient are shown in Table 1. The overall quality of the results was high.

	No drug 1 and 2	No drug 1 and 3	No drug 2 and 3	Licl 1 and 2	LiCl 2 and 3	LiCl 1 and 3
Plate 1	0.921568	0.532187	0.499229	0.852986	0.629588	0.610242
Plate 2	0.533045	0.550389	0.678057	0.531435	0.620392	0.656265
Plate 3	0.689965	0.682343	0.652457	0.687698	0.619448	0.639738
Plate 4	0.480110	0.577321	0.371524	0.712729	XXXXXXX	XXXXXXX
Plate 5	0.816909	0.833238	0.910367	0.708761	0.759276	0.824750
Plate 6	0.836025	0.808255	0.800566	0.890308	0.829430	0.830633
Plate 7	0.571190	0.702540	0.578319	0.477377	0.628323	0.690011
Plate 8	0.687687	0.689792	0.826141	0.671373	0.762700	0.731196
Plate 9	0.701318	0.724753	0.632533	0.750123	0.612620	0.517450
Plate 10	0.416404	0.650856	0.822888	0.308304	0.382329	0.571251

Table 1: The correlation coefficients of the plates, a higher correlation indicates a stronger relationship between the two variables. Correlations above 0.5 were considered to show a strong enough relationship between the two plates. XXXXXXX indicates a plate that repeatedly failed and were discounted. The correlation is shown on a scale. Green indicates a high level of correlation shading to red for a lower correlation between replicates.

Identifying hits

In order to establish a synergistic interaction with lithium chloride, normalised lithium chloride results were subtracted from the normalised no drug results; if the no drug plate showed a difference greater than 0.1 arb than the lithium chloride plate, it was considered a hit.

Originally, over 30 hits were found. To make the list more manageable, I focused on the strongest hits (difference between lithium and no drug result > 0.2 arb). This increase in threshold gave a total of 15 hits (Figure 2).

Two of these hits, *GSKT* and *CG9192*, are both gene targets of lithium chloride (Wishart *et al.*, 2018). These genes are essentially secondary positive controls. It is expected that lithium chloride treatment would inhibit lithium chloride targets, and

therefore, make them more sensitive to further inhibition by shRNA. These genes interact in such a way that it is similar to a higher dosage of lithium chloride and in this case, we would expect a higher rate of cell death. This indicates the high quality of the screen results.

To further narrow down the hit list, I compared the screen results to a database of known gene-gene interactions (String-DB.org, (2019)) (Figure 5). Any known or predicted protein-protein interactions were mapped, these are shown in Figure 5. Any genes showing known interactions with other hits were considered to be more viable as targets; a connection between two hits could signal a pathway is being affected rather than an individual gene. This indicates a stronger likelihood the result is not a false positive. The hits were found over multiple functions within the cell, for example, DNA repair (*POLA* and *Pole2*), purine synthesis (*CG11089* and ***ras***) and ATP binding (*VHA55* and *Vha68-2*).

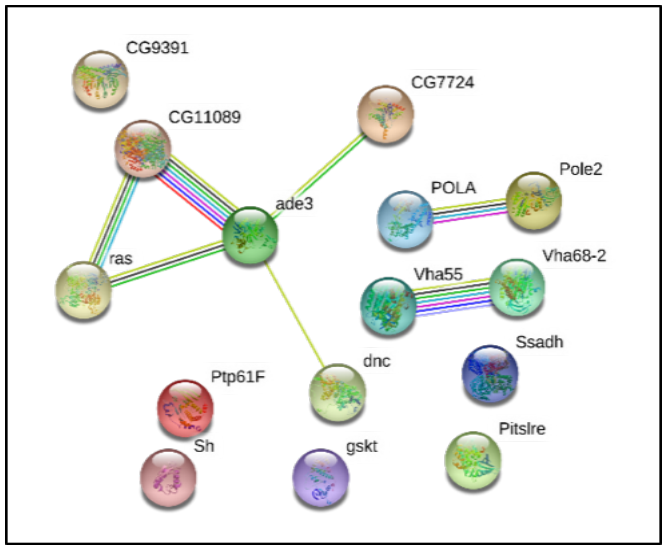

Figure 4: The 15 hits found with VDA (string-db.org, 2019). The connections show known or theorised interactions between each gene. The connections represent Green, gene neighbourhood, Red, gene fusion, Dark blue, co-occurrence, Yellow, text mining, Black, co-expression, Blue, Protein homology, Light blue, from curated database, or Purple, experimentally determined.

In order to find therapeutic candidates, the hits were converted to their human orthologs (Table 2).

Drosophila Gene	Human Ortholog	Drugs
CG11089	ATIC	AICA ribonucleotide, Beta-Dadf, Msa, Multisubstrate Adduct Inhibitor, Tetrahydrofolic acid, Pemetrexed, Methotrexate
Ssadh	ALDH5A1	Chlormerodrin, NADH, Succinic acid, Valproic Acid, Sodium oxybate
CG9391	IMPA1	Lithium cation, L-Myo-Inositol-1-Phosphate, Lithium citrate, Lithium succinate, Lithium carbonate
gskt	gskt	Lithium cation
dnc	PDE4D	Adenosine monophosphate, Rolipram, Cilomilast, Dyphylline, Roflumilast, Crisaborole, Tetomilast, (S)-Rolipram, (R)-Rolipram
Ptp61F	PTPN2	Dodecane-Trimethylamine
CG7724	HSD3B2	NADH, Trilostane, Medroxyprogesterone acetate, Corticotropin, Tibolone
Vha55	ATP6V1B2	4-(2-Aminoethyl)Benzenesulfonyl Fluoride, Gallium nitrate
ras	IMPDH1	,NADH, Mycophenolate mofetil, Ribavirin, MPA, 6-Chloropurine Riboside, 5'-Monophosphate, VX-148, Taribavirin
ade3	GART	Glycinamide Ribonucleotide, (10R)-10-Formyl-5,8,10-Trideazafolic Acid
POLA	POLA1	Clofarabine, Fludarabine, Cladribine, Nelarabine
Pitslire	SLC6A1	Tiagabine, Clobazam, Guvacine
Pole2	POLE2	Cladribine
Sh	KCNA3	Dalfampridine
Vha68-2	Hsap\ATP6V1A	Alendronic acid, Etidronic acid, Bafilomycin A1, Bafilomycin B1

Table 2: The fifteen hits found in *Drosophila* along with their human orthologs. Orthologs were found using Millburn et al., (2016) and drugs using (Wishart *et al.*, 2018). The yellow highlighted genes show the genes targeted by lithium chloride.

Table 2 shows the original *Drosophila* genes, the human orthologs and drugs that target those genes. These drugs were reviewed for availability, effectiveness and possible side-effects. *CG9192* and *GSKT* as lithium chloride targets would not show any benefit and consequently were disallowed. It was decided the best possible targets were those that showed a known connection in the interaction map (Figure 5). Six of the drugs had no connections in the interaction map and were consequently not followed through as possible therapeutic targets. *ras* showed both a synergistic interaction with lithium chloride and interacted with two other genes, *ade3* and *CG11089.* These genes are part of the purine synthesis pathway. *IMPDH* is the human ortholog of *ras*. Purine synthesis that has both been indicated as a possible target for TSC treatment previously (Housden, *et al*. (2017) and Valvezan, *et al.* (2017)).

Discussion

In summation, first the shRNA library was amplified, then transfected into cells. Plates were screened with and without lithium chloride and the results analysed in MATLAB. A larger difference between the plates inoculated with the drug and plates with no drug indicated a stronger interaction between the target gene and lithium chloride, likely indicative of a synergistic interaction and a higher rate of cytotoxicity. Results were compared across the library and from this data, synergistic interactions targeting the purine synthesis pathway were identified.

CG9391. *CG11089*, *Ade3*, *Ssadh* and *sh* were found across multiple reagents. The rest were identified from a single reagent that scored particularly highly in a single plate. The correlation coefficient of the plates was also taken into account when choosing the hits. A higher coefficient indicated a reproducible result, so in some cases, genes were particularly strong in one plate.

The fifteen genes that showed a synergistic interaction with lithium chloride were within the same biological pathways. Due to the stronger likelihood of finding a therapeutic target using those genes that show a connection, it was decided to focus on those. *Vha55* and *Vha68-2* mediate acidification in organelles to allow for ATP binding. *POLA* and *Pole2* are part of the DNA repair pathway. *CG11089, Ade3* and *ras* are part of the purine synthesis pathway. *dnc* is also related in its role in adenine and guanine catalysis. Because of the number of genes found in the purine synthesis pathway and because Valvezan (2017) had previously identified the purine synthesis pathway as a possible therapeutic target for TSC, it was decided to focus on that particular pathway.

Purine synthesis

Purines can be synthesised either by *de novo* purine synthesis or harvested from free nucleotides. Inosine monophosphate dehydrogenase (*IMPDH*) controls the process by which guanine nucleotides, required for DNA and RNA synthesis, are formed. *De novo* purine nucleotide synthesis creates IMP, the precursor to guanine and adenine nucleotides. By disrupting the production of IMP, cell growth can also be disrupted (Yin *et al.*, 2018). *IMPDH*, analogous to *ras* in *Drosophila,* is conserved across many different species, however, most organisms have multiple genes which encode for

IMPDH (Hedstrom, 2009). It's relative importance within cancer and viral infection, and alternate pathways for nucleotide acquisition, make it an excellent candidate target for treatment. Reduction of available nucleotides decreases DNA synthesis and limits cellular replication.

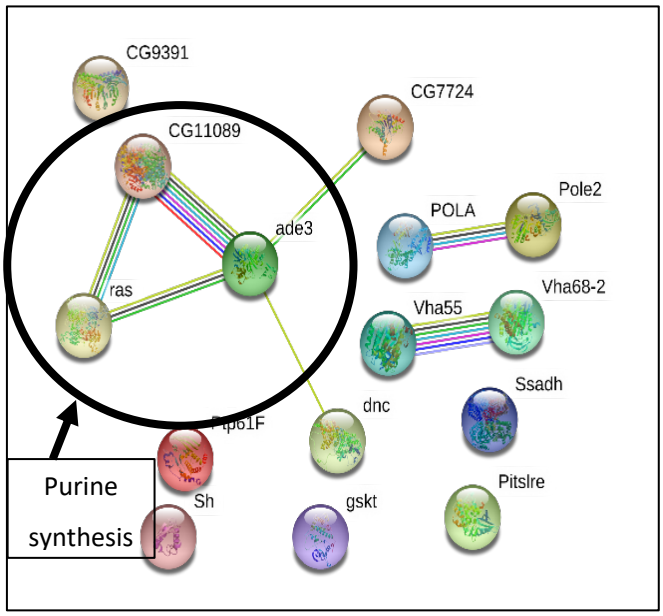

Figure 5:- The 15 genes from the VDA screen with the purine synthesis pathway highlighted (string-db.org, (2019).

IMPDH catalysis the conversion of IMP to XMP which is subsequently converted into GMP by GMP synthetase. Inhibition of *IMPDH* also creates an increase in adenine availability the mis-regulation of metabolic pathways may be more significant than the simple lack of guanine nucleotides (Hedstrom, 2009). Purine synthesis blockers block a cells access to the main pathway for nucleotides, specifically guanine. This forces the cells to use an alternative method of guanine acquisition, many cancers can't maintain cellular growth with the salvage pathways and thus reduces the viability of the cells. In some cancers, inhibition of purine synthesis induces apoptosis (Hedstrom (2009). The rapid growth of cells leads to a high demand for nucleotides, more than can be sustained by alternative salvage pathways.

Both adenine and guanine production are regulated by purine synthesis and these then form DNA and RNA. Valvezan, *et al*. (2017), showed that blocking purine synthesis forces cells to undergo autophagy, and as the mTor pathway is de-regulated in TSC mutant cells, cells continue to grow until they die. Wild-type cells still have access to mTor, so their growth can be regulated, leading to a lower rate of cell death. The purine synthesis pathway has previously been implicated in regulation of mTor (Hoxhaj *et al.*, 2017).

Valvezan, *et al*. (2017) also identified mizoribine as a possible therapeutic candidate, and a variety of other pre-approved drugs also exist that target purine synthesis. For

these reasons it was decided to target purine synthesis as the second stage of combinatorial treatment.

Of the drugs that target *IMPDH,* Ribavirin, mycophenolic acid (MPA) and Mycophenolate mofetil are the only approved drugs (Wishart *et al.*, 2018). Mycophenolate mofetil is a prodrug of MPA. Therefore, it was decided to focus on MPA, ribavirin and mizoribine; -

Mizoribine

Mizoribine is an experimental immunosuppressant used in renal transplants. It was identified by Housden et al., (2017) as a possible therapeutic for TSC. It was also tested by Valvezan et al., (2017) and shown to be cytotoxic in TSC cells. Mizoribine is primarily used as an immunosuppressant in renal transplants and steroid-resistant nephrotic syndrome, but also as a therapeutic for rheumatoid arthritis, lupus nephritis and other rheumatic disease. Mizoribine inhibits guanine synthesis without being incorporated into nucleotides (Yokota, 2002). It selectively inhibits inosine monophosphate synthetase and guanosine monophosphate synthetase, resulting in the complete inhibition of guanine nucleotide synthesis without incorporation into nucleotides (Yokota, 2002). There is very little metabolization of mizoribine in the body and its maintenance dosage is set at 50 mg (Nakamura *et al.*, 2013).

Mycophenolic acid

Mycophenolic acid (MPA) is an IMPDH inhibitor. It is primarily used as an immunosuppressant in cases of organ transplantation to stop rejection. It is also currently used in the treatment of autoimmune conditions such as lupus nephritis and Behçet's disease (Shugaiv *et al.*, 2011). MPA blocks *de novo* purine synthesis. By inhibiting IMPDH, DNA synthesis is decreased, which in turn limits cellular replication (Allison and Eugui, 2000). MPA indirectly impacts the immune system by reducing leukocyte adhesion to endothelial cells (Allison and Eugui, 2000). Current dosages are up to 1,440 mg a day.

Ribavirin

Ribavirin is a broad-spectrum antiviral drug used to treat hepatitis C and viral haemorrhagic fevers. It is also under investigation for use in acute myeloid leukaemia

(Borden and Culjkovic-Kraljacic, 2010). It is a guanine analog and can be incorporated into RNA in place of either guanine or adenine (Te, Randall and Jensen, 2007). Ribavirin targets IMPDH, a rate limiting step in *de novo* purine synthesis (Borden and Culjkovic-Kraljacic, 2010). Current dosages of ribavirin are between 600-800 mg daily.

Materials

Cells

Drosophila S2R^{+} (wild-type) (DGRC: 150) – Schneider (1972)

Drosophila TSC1 - Housden *et al* (2015)

General

Nunc^{TM} Cell Culture Treated flasks with filter caps (Thermo Scientific 136196)

Propidium Iodide – (Fisher scientific 11599296)

Schneider's *Drosophila* Medium (Gibco N21720024)

One Shot^{TM} Fetal Bovine Serum (Gibco A3382001)

Penicillin-Streptomycin (Gibco 15070063)

Lithium Chloride– (Sigma-Aldrich 62476)

Phosphate Buffered Saline (PBS) (Gibco, 20012019)

96-well tissue culture plate (JET Biofil, TCP011096)

96-well PCR plate (Sigma-Aldrich 781375)

Mini prep

Mini prep kit (Promega) (Qiagen 27106)

Lennox broth 5g NaCl (Melford L24060)

96 deep well plates (Fisher scientific 278743)

Competent cells

Transformation

FuGENE® HD transfection reagent (Promega, E2311)

pActin-GFP

pActin-GAl4

shRNA library

Methods

Mini prep and normalisation

- Inoculate 1.5mL of Luria broth with 2µl of shRNA
- Incubate for 18hrs at 37°C
- Centrifuge plates at 400rpm for 5 mins
- resuspend in 250µl buffer.
- Add 250µl lysis Buffer and mix
- Add 350µl neutralisation buffer and mix
- Centrifuge for 10 min at 13,000 rpm (~17,900 x g)
- Add 800µl of the supernatant and Centrifuge for 30–60 s
- adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
- Discard the flow-through, and centrifuge for 1 minute
- Place the column in a clean 1.5 ml microcentrifuge tube., add 50µl H_2O
- let stand for 1 min and centrifuge for 1 min.

Plate set up (Library)

An EPImotion liquid handling Robot was used to normalise the library to a final concentration of 45ng/µl

Plate set up (transfection)

- Seed a 96 well plate with 5000 cells per well
- Incubate at 25°C for 24hrs

Transfection

90ng shRNA
90ng GAL4
5ng GFP
0.6µl fugene
1.4µl PBS

- Prep the transfection reagent and incubate at 25°C for 15 mins,

- Add 2μl of the transfection reagent to each well in the 96 well plate.
- Incubate for 5 hrs at 25°C.
- Add 10μl of 20mM Lithium chloride suspended in media for a final concentration of 2mM.
- Incubate for 120hrs at 25°C.
- Add 50μl of PBS
- Read on a flow cytometer.

Flow cytometry

For settings and gating see Sierzputowska, Baxter and Housden, (2018).

Data analysis

For data analysis see Sierzputowska, Baxter and Housden, (2018).

Preliminary results

Fifteen candidate targets were identified using the variable dose analysis in Chapter 2. One of these targets, *ras (*human ortholog *IMPDH),* can be targeted using three clinically approved drugs: mizoribine, mycophenolic acid (MPA) and ribavirin. These drugs have been validated by Valvezan et al. (2020). Preliminary tests were performed in murine and human cells to attempt to reproduce those results and establish a synergistic effect with lithium chloride.

Selectivity assays and dose curves were performed in murine and human cell lines using the same methodologies as chapter one. In addition, they were also performed with the addition of 2 mM lithium chloride , as determined in chapter one to half the wells in order to test for synergistic effects.

Figure 1. shows the results of the murine selectivity assay. Lithium chloride and mizoribine were not selective, this reflects the results from chapter one that showed mizoribine not being selective and also further supports the findings from Housden et al. (2017) that lithium is not selective in murine cells.

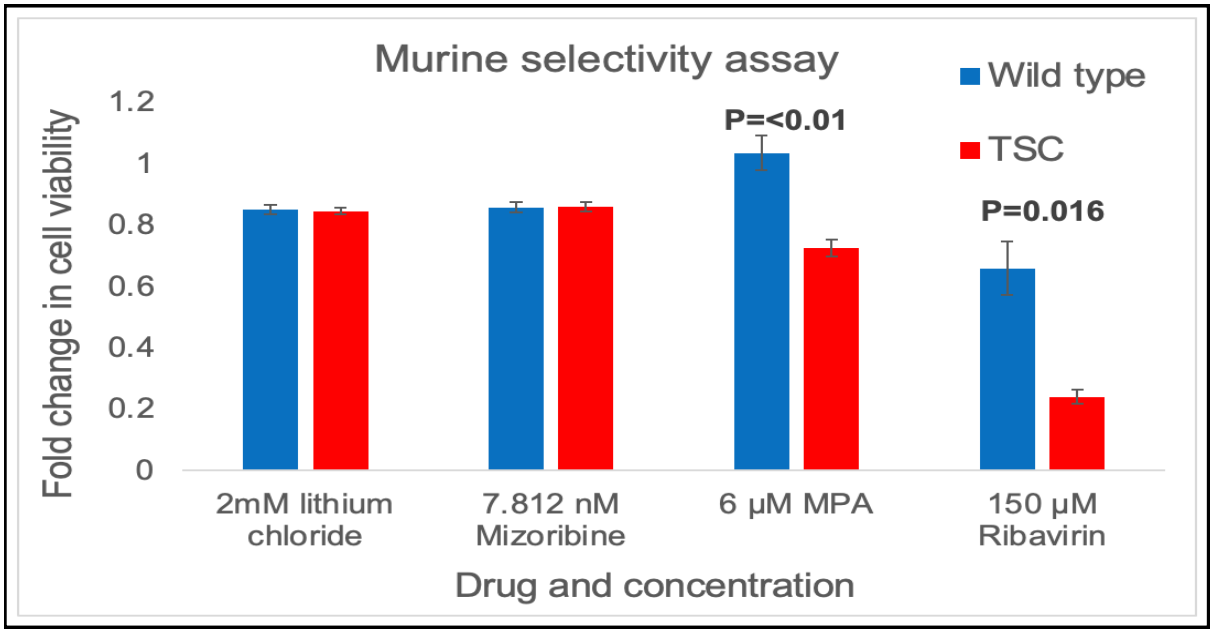

Figure 1. Selectivity assay in murine cells. A single dosage of each drug was tested using a CellTitre-Glo assay, plates were read using a plate reader. The results show average of 8 biological replicates. Error bars represent the standard deviation of the mean. P-values were obtained using a two-tailed t-test between wild type and TSC cells. Both MPA and ribavirin showed significant statistical differences lithium and mizoribine showed no

Both ribavirin and MPA showed a significant selective effect in the murine cells, consistent with previously published results (Valvezan *et al.*, 2020). Ribavirin showed the greatest selective effect on TSC cells. I therefore focused on Ribavirin for further experiments.

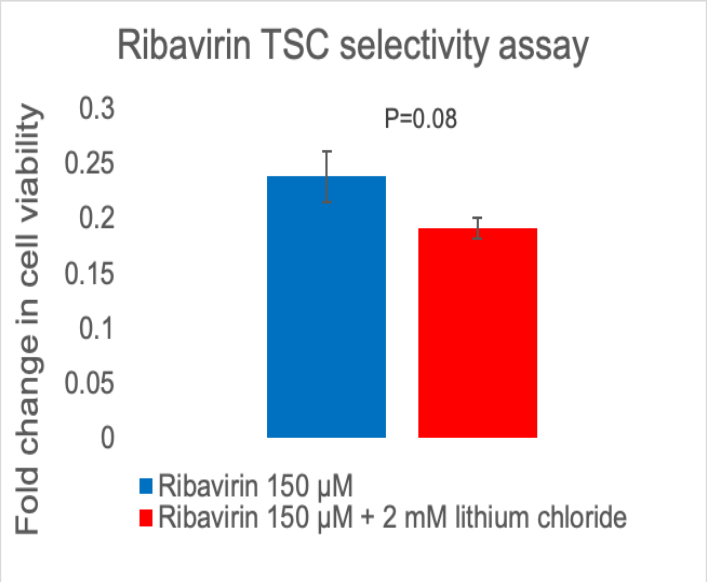

Figure 2. Selectivity assay showing the synergistic interaction between lithium chloride and ribavirin. A single dosage of ribavirin was tested with and without the prescence of 2 mM lithium using a CellTitre-Glo assay, plates were read using a plate reader. A two-tailed t-test was used between lithium and no lithium to demonstrate significance Each bar is an average of 8 biological replicates, error bars represent the standard deviation of the mean.

To assess whether ribavirin exhibited any synergistic effect with lithium it was tested in TSC cells with the addition of 2 mM lithium chloride which is shown in Figure 2. There is a clear selective effect in TSC with the addition of lithium chloride, which matches that seen in the screen results (Chapter 2). The results do not pass the threshold to be considered significant, however, there is a trend towards greater selectivity and the weak affect could be caused by lithium chlorides weak effect in murine cells or the dosage of ribavirin being too high; if the ribavirin dosage is too high it could have a stronger cytotoxic effect on the wild type cells reducing the perceived effect of lithium as lithium is known to be selective towards TSC.

To test whether the ribavirin dose was too high I performed a dose curve in wild type and TSC cells, with and without lithium chloride, Figure 3. shows this dose curve. Like the selectivity assay it shows a clear selective effect between wild type and TSC.

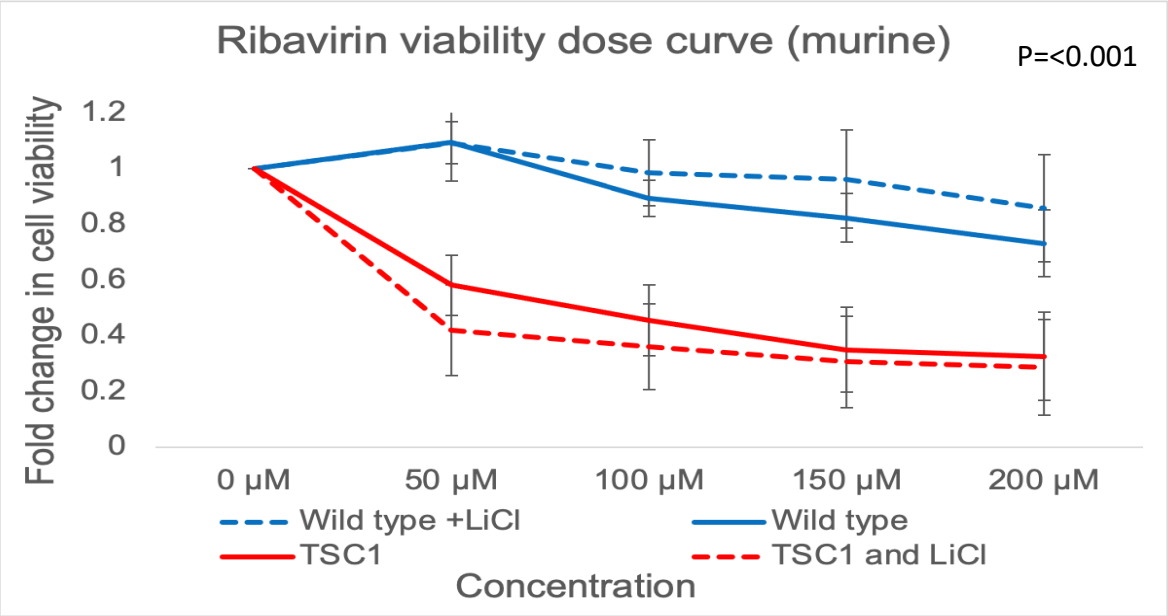

Figure 3 - Ribavirin dose curve. Murine cells were tested with varying doses of ribavirin, with and without the addition of 2 mM lithium, using a CellTitre-Glo assay, plates were read using a plate reader. Each point is the average of 8 replicates. Results were normalised to cells with no drug. Error bars show the standard deviation in the means of each replicate. The P value is gained using a two-tailed t-test between all TSC averages and wild type averages.

As seen in the selectivity assay there is a significant selective effect between wild type and TSC with ribavirin. There is no significant synergy between lithium chloride and ribavirin in either wild type or TSC1 cells at any single concentration. However, the selective effect in TSC shows as significant across the entire curve.

Finally, Figure 4. Shows the same dose curve repeated in human cells. The selective effect of ribavirin is also shown again in human cells. However, the synergistic effect shows a rescue effect in TSC cells.

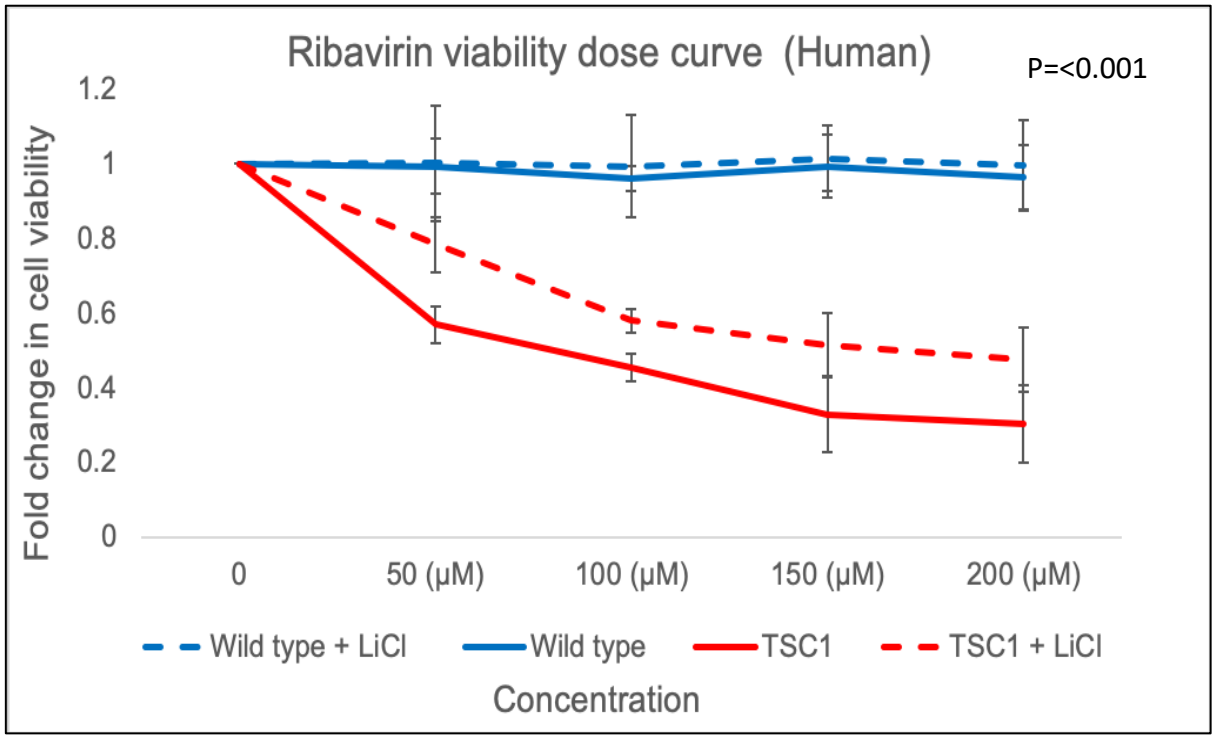

Figure 4 – Human selectivity dose curve comparing wild type and TSC cells, cells were tested with varying doses of ribavirin, with and without the addition of 2 mM lithium, using a CellTitre-Glo assay, plates were read using a plate reader.. Each point is an average of 12 biological replicates. Error bars show the standard deviation in the means of each replicate. The P value is gained using a two-tailed t-test between all

These are preliminary results and more replicates would need to be performed. Further testing would establish both the true validity of the results and also the effect (cytostatic or cytotoxic) of the drugs and to establish a synergistic interaction between lithium chloride and the IMPDH targeting drugs.

Depletion of cellular purines inhibits mTor which would explain why IMPDH targeting drugs are effective in regard to treating TSC, Rheb responds to guanine availability so a reduction in available nucleotides would lead to a reduction in cell viability. It is possible that the increase in guanine nucleotides activates Rheb leading to enhanced cellular growth. In healthy cells this results in increased growth while the cell is still regulated by mTOR which reacts to adenine, however in cells with a TSC mutation there is no inhibition of Rheb leading to increased cellular growth with a reduction of available nucleotides, leading to cell death, while healthy cells maintain their growth.

Conclusions and future work

Summary

TSC is an autosomal genetic disorder effecting 1 in 6000 births. Current treatment is Rapamycin, an immunosuppressant that induces a cytostatic effect on TSC mutant cells. The aim of this project was to identify and characterise possible alternative synergistic therapeutic candidates. Using a variety of cell viability and cell death assays in *Drosophila* cells, lithium chloride was identified as the most promising of the original seven candidates when used alone. It showed a significant selective cytotoxic effect in TSC mutant cells and no significant effect in wild type cells (Chapter 1).

A VDA genetic interaction screen was performed in TSC mutant *Drosophila* cells to identify genes that synergised with lithium chloride to kill TSC cells. 154 genes were tested and fifteen were determined to have a synergistic effect with lithium. One of these, *ras analogous to IMPDH*, had also been identified by Valvezan *et al.*, (2020) as a possible therapeutic target for TSC. Three drugs targeting IMPDH were found, MPA, ribavirin and mizoribine (Chapter 2).

Finally, preliminary tests were performed in murine and human cells to assess the validity of the VDA screens. Two of the drugs, MPA and ribavirin exhibited a significant selective effect in murine cells and preliminary data shows a trend towards a promising synergistic interaction between ribavirin and lithium chloride.

Lack of reproducibility in screens is a challenge.

Identification and characterisation of candidate drugs is a difficult process. Poor reproducibility of drug test results is a well-documented limitation in studies of this kind. The advent of HTS has led to an increase in testable combinations however many reviews have found inconsistencies between studies and poor correlation of results despite similar criteria for testing (Haibe-Kains *et al.*, 2013), a reproducibility rate of 50% is considered successful, but many have even lower levels of reproducibility (Haibe-Kains *et al.*, 2013). This is especially evident here, with mizoribine, which has a shown a significant selective effect on TSC cells within the literature (Valvezan *et*

al., 2020). However, in my experiments no selective effect has been shown even though the GI screen showed a strong result for mizoribine.

There are many possible reasons for lack of reproducibility; Variation in cell lines, drug characteristics, environmental issues and Human error can all lead to radically different results across studies.

Incorrect Identification of the mechanism of action of drugs can severely impact the identification of therapeutic candidates, in some cases, such as lithium the sheer number of targets make identification difficult. However other research has shown that many cancer drugs do not target the gene products originally identified, but instead work via off-target effects (Lin *et al.*, 2019).

Cell lines can significantly affect the outcome of experiments, number of cells added to wells and growth rate of cells may affect cell metabolism and response to the drugs (Hatzis *et al.*, 2014). Cells cultured for extended periods of time may become contaminated or otherwise change their genetic profile. The genetic background of the cells and the genes expressed can differ also between cell lines, the heterogeneity present in most tumorigenic diseases mean that the genetic profile of cells can vary significantly (Wang *et al.*, 2017). Differences in growth medium and conditions can also impact the health and viability of the cells.

The characteristics of the drugs added can lead to variations; storage conditions, evaporation and solubility can lead to a change in the dosage being added (Hatzis *et al.*, 2014). The supplier of the drug can also impact on results. In this study we found widely different results between vorinostat from two different suppliers for unknown reasons. Characterisation of drug targets is also important, many older drugs have multiple targets that may not be completely characterised, for example lithium chloride which has over 300 targets, but the mechanism of action is poorly understood. In this case drugs may hit a different target to that which was originally thought or may hit multiple targets (Lin *et al.*, 2019).

Human error can be responsible for variation, pipetting accuracy and calibration can lead to variations in drug dosages, many of the quantities used are very small leading

to a greater variation between individuals testing. Even the use of standard techniques and robots where possible will still lead to variation.

Environment

Storage conditions of drugs and growth conditions of cells can have a significant impact on the outcome of experiments, as can ambient temperature and laboratory environment. The tissue culture tools (plates, flasks and media) can have substantial effects on both the cells health and their reaction to compounds added to the cells.

There are ways to mitigate some of these issues; Scientific journals have been created purely to attempt to solve this issue (i.e. bio-protocol). suggestions have been made for greater information on laboratory conditions to be included in methodologies. Using the same equipment and reagents as those specified by previous research, Maintaining the health of cell lines and preforming regular testing to check the integrity. Ensuring timings are consistent across experiments, the interactions between drugs and cells may vary dependent on incubation time and at what stage a drug is added.

Drug testing is noisy, the number of variables present in any given experiment can lead to huge differences in results, in order to mitigate these problems, I used multiple methods and tests (selectivity assays, cell death assays and VDA) to reduce some of this noise. By discounting edge wells due to levels of evaporation and normalising all wells to the plates to remove variations in temperature. Using machines to reduce human variability in pipetting was also used to increase the replicability.

Conclusions

The main conclusion from my work is that lithium and purine synthesis inhibitors synergise to selectively kill TSC deficient cells, and that this combination of drugs has potential as a new clinical therapy. The results from my experiments are generally consistent with those shown by others in the field (Housden *et al.*, 2017; Valvezan *et al.*, 2020). The high noise levels in testing a drug's efficacy are a significant and well documented issue and show the need for new approaches to target identification that provide more consistent results. VDA is a novel method that mitigates some of these problems; it reduces noise level within the screens, and greatly reduces the incidence of false positives and negatives caused by overly efficient knockdowns or differential transfection efficiencies.

My results show that lithium synergises with purine synthesis inhibitors to kill TSC cells, but the mechanism of this inhibition is unknown. I theorise that lithium may act though a mechanism whereby ATP hydrolysis is inhibited. ATP is required to provide chemical energy to most cellular processes, the interruption of this supply causes interruptions in critical metabolic and signalling pathways and eventually can lead to cell death. ATP releases energy from the second and third Phosphate groups (Dunn and Grider, 2020). Magnesium is a vital part of this process and is required for phosphorylation. It has also been shown that a reduction in available ATP slows cell growth (Gout *et al.*, 2014). Lithium binds to magnesium, forming an ATP-magnesium-lithium complex (Briggs *et al.*, 2016), which prevents the release of energy from the ATP molecule. Because mTOR responds to environmental factors (Choo *et al.*, 2010), healthy cells will respond to a reduction in ATP by regulating growth. However, cells which exhibit the TSC mutation cannot regulate cell growth due to the dysregulation of *RHEB;* this, coupled with the lack of energy provided by ATP, leads the TSC mutant cells to undergo apoptosis. In this manner, lithium exhibits a selective effect on TSC mutant cells (Figure 1). In support of this hypothesis, unpublished data from the Housden lab has shown a five-fold enrichment in ATP dependent enzymes among genes that have pharmacogenetic interactions with lithium (Wang et al., unpublished).

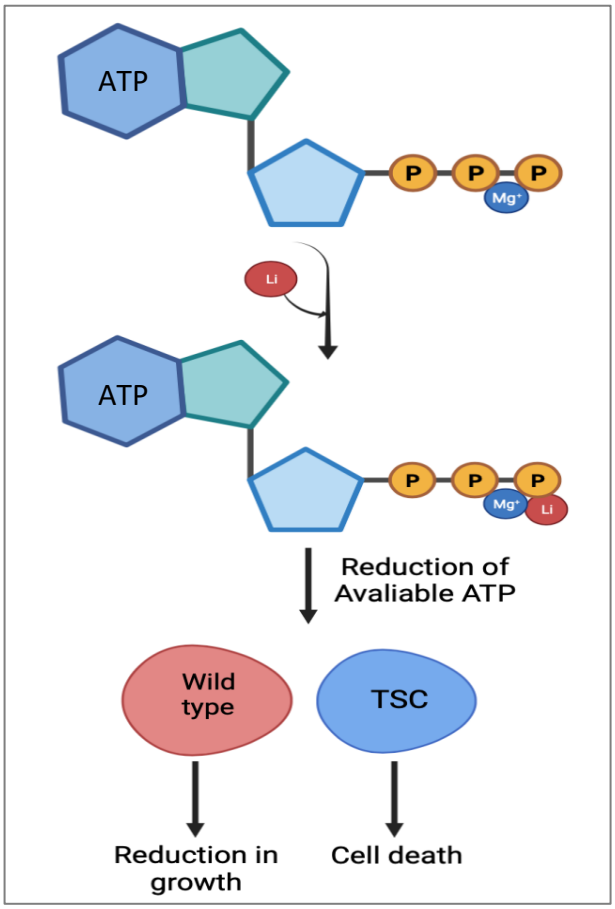

Figure 1a – Showing the theorised action of lithium, Lithium binds to the magnesium required for ATP creating an ATP-magnesium-lithium complex leading to a reduction in available ATP. Wild type cells respond to the change by slowing growth, whereas TSC cells, unable to regulate growth, die. Created with Biorender.com

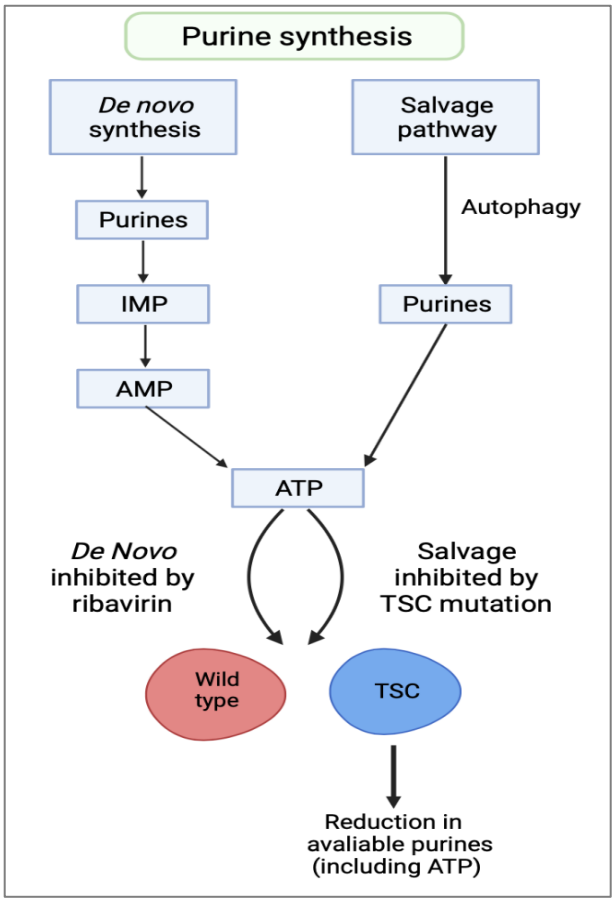

Figure 1b – Showing the theorised action of ribavirin, ribavirin inhibits de novo purine synthesis leading to a reduction in available purines. Wild type cells can use the salvage pathway, however, the TSC mutation inhibits autophagy. This coupled with the reduction in available ATP from lithium causes the cells to die. Created with Biorender.com

Ribavirin's use as an antiviral medication is predicated on its ability to disrupt RNA synthesis by replicating guanine nucleotides (Te, Randall and Jensen, 2007). In the TSC mutant cells we hypothesis that it has a similar effect. Replacing guanine purines disrupts a cell's ability to synthesize DNA and RNA which then disrupts a cells growth. Wild type cells are capable of utilising the salvage pathway to acquire purines through autophagy. However, the TSC mutation inhibits autophagy, limiting the availability of purines, including ATP. Along with the mutant cells inability to adapt to a reduction in available purines or regulate its growth, this leads to apoptosis.

In summary the increased growth in TSC positive cells leads to a requirement for greater amounts of purines, including adenine, which is a key component of ATP, however lithium and ribavirin block the availability of these to cells through different mechanisms. Ribavirin reduces the ability of the cell to generate purines, presumably leading to a reduction in ATP levels. Lithium reduces the ability of the cell to use ATP. I hypothesise that these effects synergise to kill cells due to a lack of available ATP. Healthy cells are able to regulate their growth based on the amount of available energy. In addition, healthy cells can downregulate Tor activity (which TSC cells cannot), leading to increased autophagy and therefore purine salvage to maintain purine supply. This likely explains the selective effect of the drug combination in TSC deficient cells. Further work will be required in order to prove or disprove this hypothesis.

These results show that both lithium and ribavirin are drugs worth further investigation in the treatment of TSC. Both have shown significant selective effects on TSC cells and limited negative effects on wild type cells. Both have also shown synergistic interactions across several types of cells. They have also shown cytotoxic effects in the mutant cells, a more effective treatment than the arrest effect exhibited by ribavirin.

In clinical terms their short and long-term effects on humans are well understood due to their long usage as treatments as are the usable dosages and general safety profiles. This makes them excellent candidates for further investigation. It should be noted, however, that there are still some hurdles to overcome. For example, lithium has a narrow therapeutic window, a small difference between effective concentrations and minimum toxic concentration, which will require further investigation to determine if a safe, effective dose is possible.

Future work

Preliminary testing in human cells showed a selective effect with ribavirin but no synergistic effect between lithium chloride and ribavirin. This could be due to many factors; the health of the cells and the combined dosages of both ribavirin and lithium may not have been optimal. In order to establish a cytotoxic effect with combined lithium and ribavirin, cell death assays should be performed. Despite both showing individual cytotoxic effects the combined effect is not yet established. The combination should also be tested in other cell lines as there is often variation between models.

Further work is also needed to characterise the correct dosages of lithium chloride and ribavirin in human cells for TSC. Dosages used in this study were equivalent to clinically viable dosages, however, no clinical data exists on combined dosages. Further work should be undertaken to establish a minimum effective dosage of lithium when used in conjunction with TSC. Subsequent work is also needed to truly validate the selective effect within human cells and further establish a synergistic interaction between lithium chloride and IMPDH inhibiting drugs, however preliminary results are promising that a combination of lithium chloride and ribavirin could be repurposed in order to treat TSC.

In order to determine if the reduction in ATP is relevant to the cytotoxic effect shown by lithium, ATP levels in lithium treated cells should be measured to confirm the proposed mechanism. Also, experiments using alternative methods that reduce available ATP, should be undertaken in both wild type and TSC cells in order to confirm the hypothesis that lithium reduces ATP availability for TSC cells.

As previously mentioned off-target effects are common during drug discovery and testing. To conclusively determine whether the cytotoxic effect seen in TSC cells is caused by a reduction in purine availability as hypothesised, alternative purine synthesis inhibitors such as MPA or mizoribine should be tested. Trialling purine synthesis inhibitors that inhibit different nucleotides would show whether the inhibition of purine synthesis or the inhibition of guanine and adenine synthesis specifically, is responsible for the cytotoxic effect on TSC cells, or whether it is caused by an undetermined off target effect.

Finally, lithium and ribavirin should be tested in other cell lines with similar tumour suppressant mutations i.e., PTEN, NF1 or LKB1 mutant cell lines. In order to determine if the drugs are TSC specific or if they have applications in a wider range of diseases.

Once these investigations have been performed, the potential new therapy would be poised to enter pre-clinical development using *in vivo* mouse models before continuing to clinical trials.

Glossary of terms

Adenosine triphosphate (ATP) – a molecule which provides energy for most cellular processes.

Combinatorial screen – a screen using multiple simultaneous perturbations.

Combinatorial treatment – Using two or more therapeutics to treat a disease.

Disruption – Replacement of a functional gene with an inactivated one.

Genetic interaction – Where the effects of one gene are modified by the disruption of another.

Genetic interaction screen (GI screen) – A high-throughput screen using genetic interactions.

Guide RNA (gRNA) – A short RNA sequence which binds to a target DNA sequence

Hamartin – a protein produced by *TSC1.*

Hamartomas – Benign tumours.

High-throughput screening (HTS) – a method of rapidly testing biochemical or cellular events.

IMPDH – A human gene, the ortholog of *ras*.

Knockdown – Replacement of a functional gene with an inactivated one.

Mammalian target of rapamycin (mTOR) -

Noise – measured level of variance in a population of cells.

Off-target effect – When a gene that is not the target is affected.

Orphan disease – A disease affecting less than 1 in 6000 people (EU).

Pre-approved drug – Drug approved by the FDA for use in humans.

ras – A *Drosophila* gene, the ortholog of *IMPDH.*

RNA interference (RNAi) – using RNA molecules to inhibit gene expression or translation there are four main types: - **Double stranded (dsRNA), Short hairpin (shRNA)** and **Short interfering (siRNA).**

Synergistic – An increased effect greater than that seen from either compound alone

Synthetic sick/ lethal interaction (SS/L) – Simultaneous disruption of multiple genes leading to a reduction in viability.

Target – The gene of interest that is inhibited by a drug.

Thread – A *drosophila* gene, used as a positive control.

Tuberous sclerosis complex (TSC) – A mendelian disorder characterised by the formation of benign tumours.

Tuberin – a protein produced by *TSC2*

Variable dose analysis (VDA) – A novel high-throughput screen using RNAi

White - A *drosophila* gene, used as a negative control

Bibliography

Aagaard, L. and Rossi, J. J. (2007) 'RNAi therapeutics: principles, prospects and challenges', *Advanced drug delivery reviews*. 2007/03/16, 59(2–3), pp. 75–86. doi: 10.1016/j.addr.2007.03.005.

Agrawal, N. *et al.* (2003) 'RNA interference: biology, mechanism, and applications', *Microbiology and molecular biology reviews : MMBR*. American Society for Microbiology, 67(4), pp. 657–685. doi: 10.1128/mmbr.67.4.657-685.2003.

Allison, A. C. and Eugui, E. M. (2000) 'Mycophenolate mofetil and its mechanisms of action', *Immunopharmacology*. doi: 10.1016/S0162-3109(00)00188-0.

Aronson, J. K. (2006) 'Rare diseases and orphan drugs', *British journal of clinical pharmacology*. Blackwell Science Inc, 61(3), pp. 243–245. doi: 10.1111/j.1365-2125.2006.02617.x.

Béchade, D. *et al.* (2017) 'Fatal Acute Liver Failure as a Consequence of Regorafenib Treatment in a Metastatic Colon Cancer', *Case Reports in Oncology*. doi: 10.1159/000479732.

Beckingham, K. M. *et al.* (2005) 'Drosophila melanogaster--the model organism of choice for the complex biology of multi-cellular organisms.', *Gravitational and space biology bulletin : publication of the American Society for Gravitational and Space Biology*. Volume 55, Pages 105-145,

Berger, S. I. and Iyengar, R. (2011) 'Role of systems pharmacology in understanding drug adverse events', *Wiley interdisciplinary reviews. Systems biology and medicine*. 2010/08/27, 3(2), pp. 129–135. doi: 10.1002/wsbm.114.

Bonora, M. *et al.* (2012) 'ATP synthesis and storage', *Purinergic signalling*. 2012/04/12. Springer Netherlands, 8(3), pp. 343–357. doi: 10.1007/s11302-012-9305-8.

Borden, K. L. B. and Culjkovic-Kraljacic, B. (2010) 'Ribavirin as an anti-cancer therapy: acute myeloid leukemia and beyond?', *Leukemia & lymphoma*, 51(10), pp. 1805–1815. doi: 10.3109/10428194.2010.496506.

Brand, A. H. and Perrimon, N. (1993) 'Targeted gene expression as a means of altering cell fates and generating dominant phenotypes', *Development*, 118(2), pp. 401 LP – 415. Available at: http://dev.biologists.org/content/118/2/401.abstract.

Briggs, K. T. *et al.* (2016) 'A Molecular Model for Lithium's Bioactive Form', *Biophysical Journal*. doi: 10.1016/j.bpj.2016.06.015.

Bubna, A. K. (2015) 'Vorinostat-An Overview', *Indian journal of dermatology*.

Medknow Publications & Media Pvt Ltd, 60(4), p. 419. doi: 10.4103/0019-5154.160511.
Chen, S.-H. and Lahav, G. (2016) 'Two is better than one; toward a rational design of combinatorial therapy', *Current opinion in structural biology*. 2016/08/10, 41, pp. 145–150. doi: 10.1016/j.sbi.2016.07.020.
Cherbas, L. and Gong, L. (2014) 'Cell lines', *Methods (San Diego, Calif.)*. 2014/01/13, 68(1), pp. 74–81. doi: 10.1016/j.ymeth.2014.01.006.
Choo, A. Y. *et al.* (2010) 'Glucose Addiction of TSC Null Cells Is Caused by Failed mTORC1-Dependent Balancing of Metabolic Demand with Supply', 38,4 (2010): 487 99. *Molecular Cell*. doi: 10.1016/j.molcel.2010.05.007.
Cunningham, P. C. *et al.* (2018) 'Neurodegeneration and locomotor dysfunction in Drosophila scarlet mutants', *Journal of Cell Science*, 131(18), p. jcs216697. doi: 10.1242/jcs.216697.
Dimasi, J. A. *et al.* (2010) 'Trends in risks associated with new drug development: Success rates for investigational drugs', *Clinical Pharmacology and Therapeutics*. doi: 10.1038/clpt.2009.295.
Dunn, J. and Grider, M. H. (2020) *Physiology, Adenosine Triphosphate (ATP)*, *StatPearls*. Updated 2021 Feb 27]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan.
Garami, A. *et al.* (2003) 'Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2', *Molecular Cell*. doi: 10.1016/S1097-2765(03)00220-X.
Gómez, M. R. (1995) 'History of the tuberous sclerosis complex', *Brain and Development*. doi: 10.1016/0387-7604(94)00130-8.
Gout, E. *et al.* (2014) 'Interplay of Mg2+, ADP, and ATP in the cytosol and mitochondria: Unravelling the role of Mg2+ in cell respiration', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1406251111.
Gupta, N. and Henske, E. P. (2018) 'Pulmonary manifestations in tuberous sclerosis complex', *American journal of medical genetics. Part C, Seminars in medical genetics*. 2018/07/28, 178(3), pp. 326–337. doi: 10.1002/ajmg.c.31638.
Haibe-Kains, B. *et al.* (2013) 'Inconsistency in large pharmacogenomic studies', *Nature*. 2013/11/27, 504(7480), pp. 389–393. doi: 10.1038/nature12831.
Hartwell, L. H. *et al.* (1997) 'Integrating genetic approaches into the discovery of

anticancer drugs', *Science*. doi: 10.1126/science.278.5340.1064.
Hatzis, C. *et al.* (2014) 'Enhancing reproducibility in cancer drug screening: how do we move forward?', *Cancer research*. 2014/07/11, 74(15), pp. 4016–4023. doi: 10.1158/0008-5472.CAN-14-0725.
Hedstrom, L. (2009) 'IMP dehydrogenase: structure, mechanism, and inhibition', *Chemical reviews*, 109(7), pp. 2903–2928. doi: 10.1021/cr900021w.
Housden, B. E. *et al.* (2017) 'Improved detection of synthetic lethal interactions in Drosophila cells using variable dose analysis (VDA)', *Proceedings of the National Academy of Sciences of the United States of America vol. 114,50 (2017): E10755-E10762. doi:10.1073/pnas.1713362114*
Hoxhaj, G. *et al.* (2017) 'The mTORC1 Signaling Network Senses Changes in Cellular Purine Nucleotide Levels', *Cell reports*, 21(5), pp. 1331–1346. doi: 10.1016/j.celrep.2017.10.029.
Hughes, J. P. *et al.* (2011) 'Principles of early drug discovery', *British journal of pharmacology*. Blackwell Science Inc, 162(6), pp. 1239–1249. doi: 10.1111/j.1476-5381.2010.01127.x.
Hunter, P. (2017) 'The reproducibility "crisis": Reaction to replication crisis should not stifle innovation', *EMBO reports*. 2017/08/09. John Wiley and Sons Inc., 18(9), pp. 1493–1496. doi: 10.15252/embr.201744876.
Inoki, K. *et al.* (2003) 'Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling', *Genes & development*. 2003/07/17. Cold Spring Harbor Laboratory Press, 17(15), pp. 1829–1834. doi: 10.1101/gad.1110003.
Johnson, C. E. and Tee, A. R. (2017) 'Exploiting cancer vulnerabilities: MTOR, autophagy, and homeostatic imbalance', vol. 61,6 699-710. 12 Dec. 2017 *Essays in Biochemistry*. doi: 10.1042/EBC20170056.
Kim, E. L. *et al.* (2010) 'Chloroquine activates the p53 pathway and induces apoptosis in human glioma cells', *Neuro-oncology*. 2010/01/27. Oxford University Press, 12(4), pp. 389–400. doi: 10.1093/neuonc/nop046.
Kurosawa, G. *et al.* (2008) 'Comprehensive screening for antigens overexpressed on carcinomas via isolation of human mAbs that may be therapeutic', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0712202105.
Lee, J. and R, S. H. (2013) 'Cancer Epigenetics: Mechanisms and Crosstalk of a HDAC Inhibitor, Vorinostat', *Chemotherapy*, 2(111), p. 14934. doi: 10.4172/2167-

7700.1000111.
Lehner, B. (2004) 'How to use RNA interference', *Briefings in Functional Genomics and Proteomics*. doi: 10.1093/bfgp/3.1.68.
Lin, A. *et al.* (2019) 'Off-target toxicity is a common mechanism of action of cancer drugs undergoing clinical trials', *Science Translational Medicine*. doi: 10.1126/scitranslmed.aaw8412.
Lito, P., Rosen, N. and Solit, D. B. (2013) 'Tumor adaptation and resistance to RAF inhibitors', *Nature Medicine*, 19(11), pp. 1401–1409. doi: 10.1038/nm.3392.
Lord, C. J., Tutt, A. N. J. and Ashworth, A. (2015) 'Synthetic Lethality and Cancer Therapy: Lessons Learned from the Development of PARP Inhibitors', *Annual Review of Medicine*. doi: 10.1146/annurev-med-050913-022545.
Mani, R. *et al.* (2008) 'Defining genetic interaction', *Proceedings of the National Academy of Sciences*, 105(9), pp. 3461 LP – 3466. doi: 10.1073/pnas.0712255105.
McCabe, C., Claxton, K. and Tsuchiya, A. (2005) 'Orphan drugs and the NHS: should we value rarity?', *BMJ (Clinical research ed.)*. BMJ Publishing Group Ltd., 331(7523), pp. 1016–1019. doi: 10.1136/bmj.331.7523.1016.
Menon, S. and Manning, B. D. (2008) 'Common corruption of the mTOR signaling network in human tumors', *Oncogene*. vol. 27 Suppl 2,0 2 (2008): S43-51 doi: 10.1038/onc.2009.352.
Millburn, G. H. *et al.* (2016) 'FlyBase portals to human disease research using Drosophila models', *Disease models & mechanisms*. The Company of Biologists Ltd, 9(3), pp. 245–252. doi: 10.1242/dmm.023317.
Mohr, S. E. *et al.* (2014) 'RNAi screening comes of age: Improved techniques and complementary approaches', *Nature Reviews Molecular Cell Biology*. vol. 15,9 (2014): 591-600. doi: 10.1038/nrm3860.
Mohs, R. C. and Greig, N. H. (2017) 'Drug discovery and development: Role of basic biological research', *Alzheimer's and Dementia: Translational Research and Clinical Interventions*. vol. 3,4 651-657. 11 Nov. 2017 doi: 10.1016/j.trci.2017.10.005.
Moore, N. (2020) 'Chloroquine for COVID-19 Infection', *Drug safety*. Springer International Publishing, 43(5), pp. 393–394. doi: 10.1007/s40264-020-00933-4.
Nakamura, G. *et al.* (2013) 'Mizoribine as a safe and effective combined maintenance therapy with prednisolone for anti-neutrophil cytoplasmic antibody-associated vasculitis in a hemodialysis patient', *CEN case reports*. 2013/01/30.

Springer Japan, 2(2), pp. 139–143. doi: 10.1007/s13730-012-0050-1.
Nijman, S. M. B. (2011) 'Synthetic lethality: general principles, utility and detection using genetic screens in human cells', *FEBS letters*. 2010/11/19. Elsevier Science B.V, 585(1), pp. 1–6. doi: 10.1016/j.febslet.2010.11.024.
Osborne, J. P., Fryer, A. and Webb, D. (1991) 'Epidemiology of Tuberous Sclerosis', *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd, 615(1), pp. 125–127. doi: 10.1111/j.1749-6632.1991.tb37754.x.
Pandey, U. B. and Nichols, C. D. (2011) 'Human disease models in drosophila melanogaster and the role of the fly in therapeutic drug discovery', *Pharmacological Reviews* vol. 63,2 (2011): 411-36. doi: 10.1124/pr.110.003293.
Rabito, M. J. and Kaye, A. D. (2014) 'Tuberous sclerosis complex: perioperative considerations', *The Ochsner journal*. the Academic Division of Ochsner Clinic Foundation, 14(2), pp. 229–239. Available at: https://pubmed.ncbi.nlm.nih.gov/24940133.
Rajalaxmi Natarajan, Deepti Trivedi-Vyas, Y. P. W. (2013) 'Tuberous sclerosis complex regulates Drosophila neuromuscular junction growth via the TORC2/Akt pathway.', *Human molecular genetics*, 22,10, pp. 2010–2023.
Redman, M. *et al.* (2016) 'What is CRISPR/Cas9?', *Archives of disease in childhood - Education & practice edition*, 101(4), pp. 213 LP – 215. doi: 10.1136/archdischild-2016-310459.
Reiter, L. T. *et al.* (2001) 'A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster', *Genome Research*. doi: 10.1101/gr.169101.
Richon, V. M. (2006) 'Cancer biology: mechanism of antitumour action of vorinostat (suberoylanilide hydroxamic acid), a novel histone deacetylase inhibitor', *British Journal of Cancer*, 95(1), pp. S2–S6. doi: 10.1038/sj.bjc.6603463.
Rosenberg, M., Azevedo, N. F. and Ivask, A. (2019) 'Propidium iodide staining underestimates viability of adherent bacterial cells', *Scientific reports*. Nature Publishing Group UK, 9(1), p. 6483. doi: 10.1038/s41598-019-42906-3.
Sancak, O. *et al.* (2005) 'Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype – phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex', *European Journal of Human Genetics*, 13(6), pp. 731–741. doi: 10.1038/sj.ejhg.5201402.
Sarbassov, D. D. *et al.* (2006) 'Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB', *Molecular Cell*. doi: 10.1016/j.molcel.2006.03.029.

Sertkaya, Aylin; Birkenbach, A. (2011) 'Examination of clinical trial costs and barriers for drug development', *Erg*.
Shugaiv, E. *et al.* (2011) 'Mycophenolate mofetil as a novel immunosuppressant in the treatment of neuro-Behçet's disease with parenchymal involvement: Presentation of four cases', *Clinical and Experimental Rheumatology*. vol. 29,4 Suppl 67 (2011): S64-7.
Sierzputowska, K., Baxter, C. and Housden, B. (2018) 'Variable Dose Analysis: A Novel High-throughput RNAi Screening Method for Drosophila Cells', *BIO-PROTOCOL*. doi: 10.21769/bioprotoc.3112.
Sokolowska, E. *et al.* (2017) 'Orlistat reduces proliferation and enhances apoptosis in human pancreatic cancer cells (PANC-1)', *Anticancer Research* vol. 37,11 (2017): 6321-6327. doi: 10.21873/anticanres.12083.
Suda, K. *et al.* (2019) 'Reduction of Rpd3 suppresses defects in locomotive ability and neuronal morphology induced by the knockdown of Drosophila SLC25A46 via an epigenetic pathway', *Experimental Cell Research vol.* 385,2 (2019): 111673. doi: 10.1016/j.yexcr.2019.111673.
Tapon, N. *et al.* (2001) 'The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation', *Cell* vol. 105,3 (2001): 345-55. doi: 10.1016/S0092-8674(01)00332-4.
Te, H. S., Randall, G. and Jensen, D. M. (2007) 'Mechanism of action of ribavirin in the treatment of chronic hepatitis C', *Gastroenterology & hepatology*. Millennium Medical Publishing, 3(3), pp. 218–225. Available at: https://pubmed.ncbi.nlm.nih.gov/21960835.
Tee, A. R. (2018) 'The Target of Rapamycin and Mechanisms of Cell Growth', *International journal of molecular sciences*. MDPI, 19(3), p. 880. doi: 10.3390/ijms19030880.
Valvezan, A. J. *et al.* (2017) 'mTORC1 Couples Nucleotide Synthesis to Nucleotide Demand Resulting in a Targetable Metabolic Vulnerability', *Cancer Cell*. doi: 10.1016/j.ccell.2017.09.013.
Valvezan, A. J. *et al.* (2020) 'IMPDH inhibitors for antitumor therapy in tuberous sclerosis complex', *JCI Insight* vol. 5,7 e135071. 9 Apr. 2020. doi: 10.1172/JCI.INSIGHT.135071.
Verbaanderd, C. *et al.* (2017) 'Repurposing Drugs in Oncology (ReDO)-chloroquine and hydroxychloroquine as anti-cancer agents', *Ecancermedicalscience*. Cancer

Intelligence, 11, p. 781. doi: 10.3332/ecancer.2017.781.
Wang, T. *et al.* (2017) 'Gene Essentiality Profiling Reveals Gene Networks and Synthetic Lethal Interactions with Oncogenic Ras', *Cell*. 2017/02/02, 168(5), pp. 890-903.e15. doi: 10.1016/j.cell.2017.01.013.
Wishart, D. S. *et al.* (2018) 'DrugBank 5.0: a major update to the DrugBank database for 2018', *Nucleic acids research*. Oxford University Press, 46(D1), pp. D1074–D1082. doi: 10.1093/nar/gkx1037.
Xue, H. *et al.* (2018) 'Review of Drug Repositioning Approaches and Resources', *International journal of biological sciences*. Ivyspring International Publisher, 14(10), pp. 1232–1244. doi: 10.7150/ijbs.24612.
Yin, J. *et al.* (2018) 'Potential Mechanisms Connecting Purine Metabolism and Cancer Therapy', *Frontiers in immunology*. Frontiers Media S.A., 9, p. 1697. doi: 10.3389/fimmu.2018.01697.
Yokota, S. (2002) 'Mizoribine: Mode of action and effects in clinical use', *Pediatrics International*. John Wiley & Sons, Ltd, 44(2), pp. 196–198. doi: 10.1046/j.1328-8067.2002.01536.x.
Zhao, H. *et al.* (2010) 'Rationale for the real-time and dynamic cell death assays using propidium iodide', *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 77(4), pp. 399–405. doi: 10.1002/cyto.a.20867.
Zheng, X. F., Florentino, D., Chen, J., Crabtree, G. R., & Schreiber, S. L. (1995) 'TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin.', *Cell*, 2(1), pp. 121–130.

Supplemental materials