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Investigating Metformin & Syrosingopine's synthetic lethality in PC3 & THP-1 cancer cell lines

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Abstract

The oral antidiabetic, metformin, has been documented to have antineoplastic activity in various cancer cell lines, associated with the inhibition of mitochondrial complex I. However, pre-clinical studies have struggled to demonstrate such antineoplastic activity utilising metformin concentrations attainable with standard antidiabetic dosing. Research has revealed that metformin combined with the anti-hypertensive drug, syrosingopine, lowers metformin's therapeutic threshold and sensitizes cancer cells to killing, by inhibiting monocarboxylate transporters (MCT)1/MCT4. The potent interaction between these drugs elicits a synthetic lethality, specific to transformed cells. This preliminary study aimed to investigate metformin's antineoplastic effects and its synergistic relationship with syrosingopine by measuring cell viability and extracellular lactate in PC3 (adherent) and THP-1 (suspension) cancer cell lines. Overall, the PC3 cell line responded better to treatment with combined doses of metformin and syrosingopine, or either drug alone; however, synthetic lethality was not observed in either cell line. In both cell lines, the interaction between metformin and syrosingopine was not statistically significant in the cell viability assays ($p>0.05$). Analysis of MCT1/MCT4 inhibition through the measurement of extracellular lactate was not statistically significant ($p>0.05$) and proved inconclusive. Additionally, the nature of the cell line, adherent or suspension, was statistically significant ($p<0.05$) in some treatment groups, suggesting that this may play a role in the efficacy of the drug treatments. Further research is necessary to better understand the underlying cellular mechanisms of metformin and syrosingopine's synthetic lethality, and syrosingopine's MCT1/MCT4 inhibition. Future research should focus on achieving metformin doses capable of exhibiting antineoplastic effects *in vivo*.

Keywords: metformin, syrosingopine, THP-1, PC3, monocarboxylate transporters, MCT1, MCT4, adherent cell lines, suspension cell lines, prostate cancer, acute myeloid leukaemia, antineoplastic, synthetic lethality.

Introduction

The global burden of cancer continues to rise, such that the World Health Organization predicts a 70% increase in cancer cases over the next twenty years (Arem & Loftfield, 2018). Globally, leukaemia represents 2.8% of all cancers, with the most common type being acute myeloid leukaemia (AML). Although there are high rates of complete remission following initial treatment, a substantial proportion of patients relapse, such that the prognosis of AML remains lamentable (Biondani & Peyron, 2018; Yuan *et al.,* 2020). Prostate cancer is the second most prevalent malignancy in males worldwide and one of the leading causes of cancer-related deaths. With concerning mortality rates, prostate cancer and AML (Biondani & Peyron, 2018; Gandhi *et al.,* 2018), and their potential treatments, remain a current and active area of research.

Despite the growing public health concern, cancer therapeutics currently achieve the lowest clinical trial success rate among all major diseases; in part, due to the paucity of effective anticancer drugs available. Cancer's complexity presents difficult challenges in the field of therapeutics. While targeting single cancer metabolic pathways has achieved clinical success, this has only modestly improved cancer survival in some cancers and is not considered a cure (Cagan & Meyer, 2017; Zugazagoitia *et al.,* 2016). Thus, drug combinations against several molecular abnormalities or cancer hallmarks are considered a promising alternative therapeutic strategy to treat cancer (Zugazagoitia *et al.,* 2016).

Metformin, an oral biguanide, is a widely prescribed drug for the management of type 2 diabetes mellitus (T2DM) (Noto *et al.,* 2012; Zakikhani *et al.,* 2006). Metformin has the ability to lower circulating levels of insulin by interacting with the principal metabolic organs in settings of insulin resistance and hyperinsulinemia (Benjamin *et al.,* 2016; Noto *et al.,* 2012). In addition to its antidiabetic properties, metformin reportedly has potential antineoplastic effects by inhibiting mitochondrial respiration in cancer cells, further reducing glucose and adenosine triphosphate (ATP) levels through the activation of adenosine 5'-mono-phosphate-activated protein kinase (AMPK) via the kinase, LKB1 (Benjamin *et al.,* 2016; Noto *et al.,* 2012; Tsilidis *et al.,* 2014; Zakikhani *et al.,* 2006). Metformin's antineoplastic effects can be categorised as direct (cellular) and indirect (systemic) (Figure 1).

Epidemiological studies have reported a significant reduction of cancer incidence in metformin-treated diabetic patients, supported by *in vitro* and *in vivo* studies in mouse xenograft models (Benjamin *et al.,* 2016), highlighting its potential as a cancer therapeutic. *In vitro* studies have demonstrated that metformin is capable of inhibiting growth and proliferation of tumour cell lines including thyroid and lung cancer cells (Bao *et al.,* 2012; Chen *et al.,* 2012; Wu *et al.,* 2011). Previous meta-analyses have supported metformin's association with decreased cancer risk in diabetic patients; however, these analyses had limited scope as they focussed solely on diabetic patients and were based on few observational studies (DeCensi *et al.,* 2010; Noto *et al.,* 2012; Stevens *et al.,* 2012). Although metformin has received mostly favourable reviews regarding its antineoplastic potential, there remain a multitude of studies disputing this, which suggest that metformin has limited or no effect as a cancer treatment, or no benefit in comparison to other antidiabetic drugs (Benjamin *et al.,* 2016; Nayan *et al.* 2017; Wu *et al.,* 2016). It is important to consider that the concentration of metformin used to obtain antineoplastic activity *in vitro*,

exceeds the *in vivo* serum concentration attained in an antidiabetic prescription, thus metformin's efficacy is likely to vary between different study samples (Benjamin *et al.,* 2016; Chong & Chabner, 2009). For metformin to function as an efficacious antineoplastic agent in a clinical setting, its therapeutic threshold must be lowered to an attainable range *in vivo*, potentially in combination with other compounds. Benjamin *et al.* (2016) identified syrosingopine as a compound that interacts with low, sublethal concentrations of metformin to induce cancer cell killing.

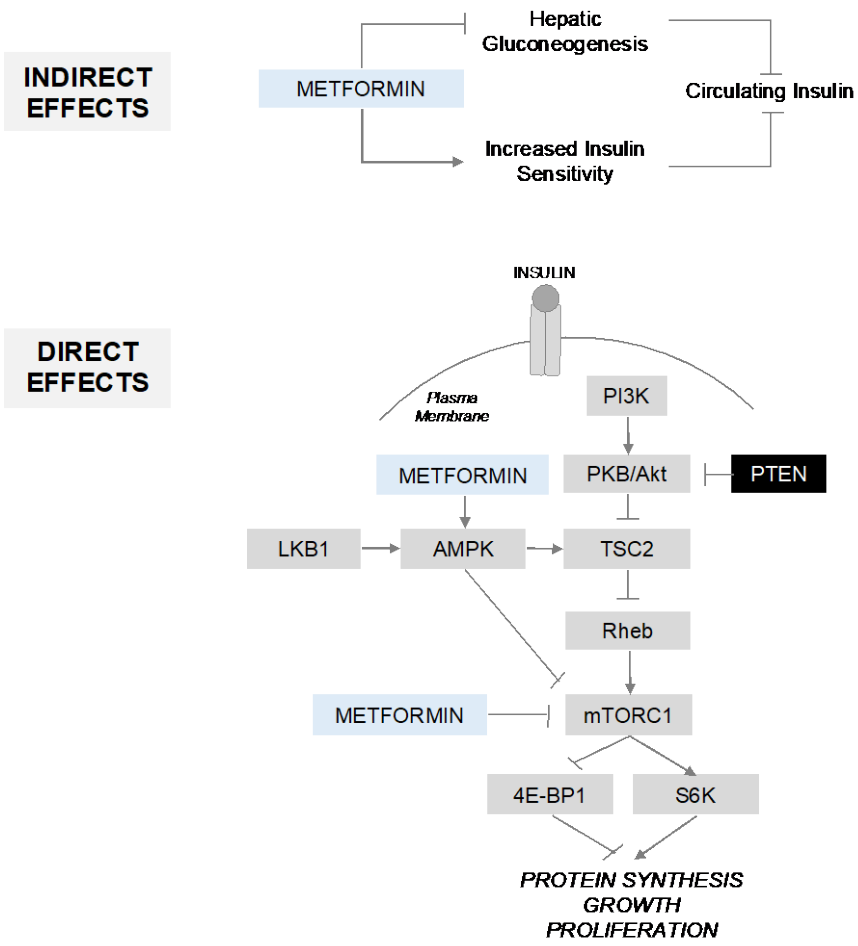

Figure 1: Direct and indirect antineoplastic effects of metformin. Metformin lowers blood glucose levels which reduces glucose availability to cancerous cells. Indirect and systemic sensitisation of tissues to insulin via metformin, reduces hepatic gluconeogenesis and circulating insulin levels, leading to the indirect reduction of receptor tyrosine kinase activation and PI3K signalling. At the cellular level, metformin's direct antineoplastic effects are mediated by AMPK's ability to inhibit transcription of crucial gluconeogenesis genes in the liver and trigger glucose uptake in the muscles, contributing to reduced levels of circulating insulin. AMPK's activation stabilises TSC2 and inhibits mTORC1, one of the major signalling hubs for cell growth, translation and metabolism. Metformin is also able to independently and directly target mTORC1 (adapted from Benjamin *et al.,* 2016; Dowling *et al.,* 2011; Noto *et al.,* 2012).

AMPK = AMP-activated protein kinase; 4E-BP1 = eukaryotic initiation factor 4E-binding protein-1; LKB1 = liver kinase B1; mTORC1 = mammalian target of rapamycin complex 1; PI3K = phosphatidylinositol-3-kinase; PKB/Akt = protein kinase B; PTEN = phosphatase and tensin homologue deleted on chromosome 10; Rheb = Ras homologue enriched in brain; S6K = ribosomal protein S6 kinase; TSC2 = tuberous sclerosis complex 2.

Syrosingopine, an antihypertensive, has been reported to elicit synthetic lethality with metformin, where the compounds interact synergistically and become capable of killing various cancer cell types without disturbing non-transformed cells (Benjamin *et al.,* 2016; Khammanivong *et al.,* 2020). While metformin acts as a mitochondrial complex I inhibitor, syrosingopine is responsible for the dual inhibition of MCT1/MCT4 (Figure 2) (Benjamin *et al.,* 2016; Saraei *et al.,* 2019). MCT1/MCT4 have been shown to be overexpressed in various cancers and their disruption in renal cell carcinoma, breast and pancreatic cancer has been reported to exert antitumour effects both *in vitro* and *in vivo*. This is believed to be due to the increased accumulation of toxic intracellular lactate, causing cell death by intracellular acidification (Benjamin *et al.,* 2018; Todenhöfer *et al.,* 2018).

Cancer cells are capable of rewiring their metabolism to promote cell proliferation, survival and tumour growth by processing pyruvate via glycolysis, termed aerobic glycolysis or the ‘Warburg effect’ (Khammanivong *et al.,* 2020; Potter *et al.,* 2016). This leads to the abovementioned accumulation of intracellular lactate, even in the presence of adequate oxygen, combatted by the overexpression of MCTs (Todenhöfer *et al.,* 2018). The ‘Warburg effect' is employed to compensate for transformed cells’ preferential ATP production via glycolysis, which is less efficient than oxidative phosphorylation (OXPHOS) for ATP generation. The resulting cytosolic acidification reduces glycolytic rate via inhibition of PFK1, a rate-limiting enzyme; thus, lactate and H^+ efflux is necessary to sustain continuously high rates of glycolysis (Benjamin *et al.,* 2018). Since the bi-directional MCTs are responsible for the H^+-linked efflux of L-lactate across the plasma membrane, syrosingopine’s reported inhibition of MCT1/MCT4 suggests MCT inhibition is a potential cancer therapeutic target (Halestrap, 2013). The activity of MCT1/MCT4 ablation, both pharmacologically and genetically, has been shown to reduce proliferation *in vivo* and *in vitro* (Le Floch *et al.,* 2011), and this anti-proliferative effect is supposedly augmented by biguanides, including metformin (Granja *et al.,* 2015).

While metformin alone may be insufficient to induce the killing of cancer cells, the reported synthetic lethality between metformin and syrosingopine may provide a novel option for both compounds in cancer therapy. Although studies have long reported metformin’s antineoplastic effects, few have discussed its synthetic lethality with syrosingopine in prostate cancer and acute myeloid leukaemia. It is hypothesised that the combination of metformin and syrosingopine induces a synergism capable of reducing cancer cell viability via dual inhibition of mitochondrial complex I and MCT1/MCT4, respectively. This study aimed to investigate the antineoplastic effects of metformin and its synthetic lethality with syrosingopine in the human monocytic cell line, THP-1 (suspension) and human prostate cancer cell line, PC3 (adherent) to determine the efficacy of different drug concentration combinations and compare their antineoplastic effects between adherent and suspension cell lines.

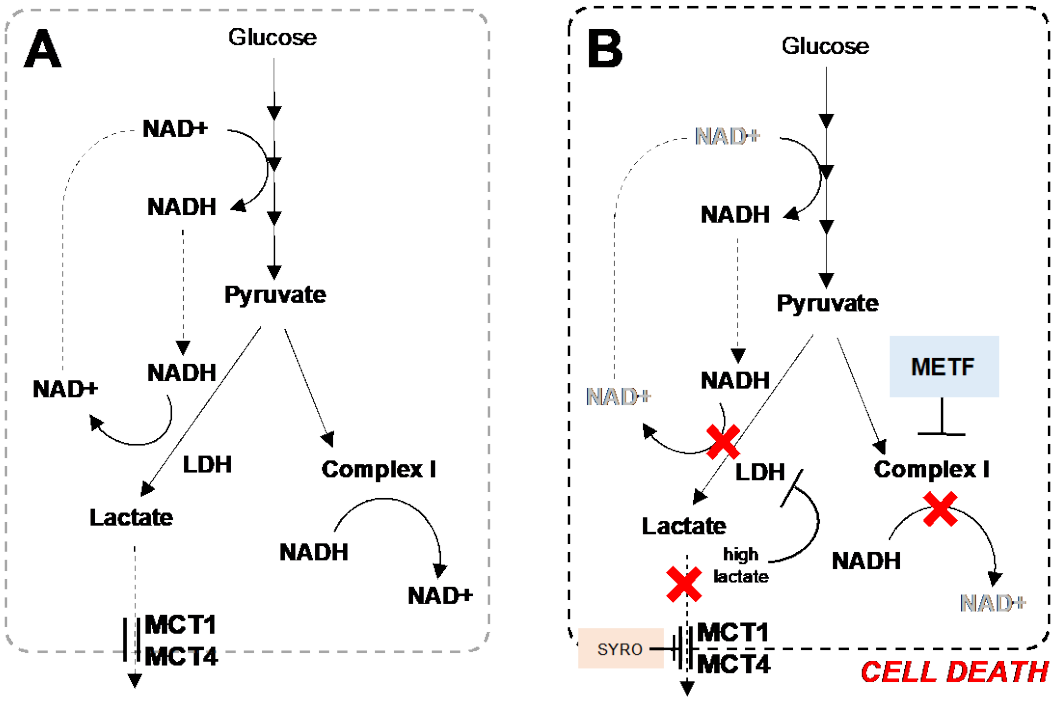

Figure 2: The role of metformin and syrosingopine in cancer metabolism. **(A)** Normal cancer cell metabolism. **(B)** Syrosingopine (SYRO) and metformin (METF) action on cancer cells' metabolic pathways. Mitochondrial complex I, an NADH (nicotinamide adenine dinucleotide + hydrogen) dehydrogenase, and lactate dehydrogenase (LDH) are responsible for regenerating NAD^+ required for glycolysis. Metformin's inhibition of mitochondrial NADH is combined with end-product LDH inhibition due to raised intracellular lactate levels as a result of syrosingopine's inhibition of lactate efflux, leading to a loss of NAD^+ regeneration, decreasing glycolysis. Lack of NAD^+ from both end-product LDH inhibition and simultaneous inhibition of complex I decreases NAD^+/NADH ratio, leading to loss of glycolytic ATP production, causing cell death (adapted from Benjamin *et al.,* 2018; Saraei *et al.,* 2019).

Methodology

Cell Culture & Plate Preparation

96-well plates were seeded with either PC3 (adherent) or THP-1 (suspension) cells at densities of 5×10^4 cells/well in 200 μL (adherent cells) and 100 μL (suspension cells) of phenol red and non-phenol red RPMI 1640 with 10% fetal bovine serum media, respectively, and incubated for 48 hours at 37°C and 5%CO_2. The difference in cell volumes was to allow for the addition of inhibitors, metformin and syrosingopine, in the wells. For adherent cells, the media could be removed prior to addition of the inhibitors whereas for the suspension cells, it could not as cells would be removed also. Following incubation, wells containing cells, dimethyl sulfoxide (Sigma-Aldrich, Birmingham, UK) controls and water controls were treated with 5 mM metformin (TOCRIS, Bristol, UK) and varying concentrations of syrosingopine (Sigma-Aldrich, Birmingham, UK); 2.5, 5, 7.5 and 10 μM (Benjamin *et al.,* 2016) and incubated once more for 48 hours at 37°C and 5%CO_2.

MTS Cell Proliferation Assay

Cell proliferation was measured by the addition of 20 μL MTS reagent (Promega, Southampton, UK) to the prepared plates according to the manufacturer's specifications (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) following the 48-hour incubation with inhibitors. Fluorescence, generated by the reduction of MTS tetrazolium compound by metabolically active cells to form a coloured formazan dye, was measured at 490 nm with a plate reader (VersaMax™ Microplate Reader) after a 1-hour incubation period at 37°C and 5%CO_2. Growth was normalized to untreated controls to calculate percentage cell viability, and each data point was performed in triplicate.

Lactate Measurement

Extracellular lactate levels were measured spectrophotometrically, directly from culture medium (adherent cells) or supernatant (suspension cells) as the exchange of lactate to pyruvate, captured by hydrazine, is proportional to the increase in absorbance as NAD^+ is reduced to NADH by LDH (protocol adapted from Grist *et al.,* 2018). Well contents from the prepared plates were transferred to individual cuvettes (following centrifugation for suspension cells) and the enzymatic reaction mixture of water, glycine (320 mmol/L), hydrazine (320 mmol/L), NAD^+ (2.4 mmol/L) and LDH (20 U/L) (all from Sigma-Aldrich, Birmingham, UK) was added to each cuvette ahead of measuring lactate concentration via the spectrophotometer (7310 Spectrophotometer, Jenway) at 340 nm. Absorbance values were then used to determine lactate concentration using the Beer-Lambert Law with 6.22 mM as the extinction coefficient for NADH. Each data point was performed in triplicate.

Quantification & Statistical Analysis

Quantification and analysis of raw data was performed using Microsoft Excel (version 16.16.19). Additionally, all graphs were produced in Microsoft Excel (version 16.16.19). Statistical analyses were performed using Minitab19 (version 19.2020.2.0). A two-way ANOVA was performed to determine whether interaction between the drugs, and their concentrations were significant in the cell viability assay. Additionally, 2-sample t-tests were performed to compare treatment efficacy between cell lines and to compare to controls in all assays. p-values <0.05 were considered statistically significant.

Results

Metformin & Syrosingopine's Synthetic Lethality

The PC3 cell line responded better to treatment than the THP-1 cell line compared to their respective controls as overall, all treatment groups displayed a lower cell viability in the PC3 cell line compared to the THP-1 cell line (Figure 3). When comparing to the relevant controls, the PC3 cell line's viability was statistically significant ($p<0.05$). Metformin and syrosingopine combined, and syrosingopine alone regardless of concentration, resulted in a lower cell viability compared to metformin alone in all PC3 treatment groups. In the THP-1 cell line, only two treatment groups with combined metformin and syrosingopine (7.5 and 10 μM) resulted in a lower cell viability compared to metformin alone. When compared to the relevant controls, the THP-1 cell line's viability was not statistically significant ($p>0.05$, $p=0.552$). Additionally, all concentrations of syrosingopine alone had the least impact on reduction of cell viability in the THP-1 cell line. Comparison of the cell

lines per treatment group via a 2-sample t-test determined that seven of the treatment groups had means that were statistically significant ($p<0.05$).

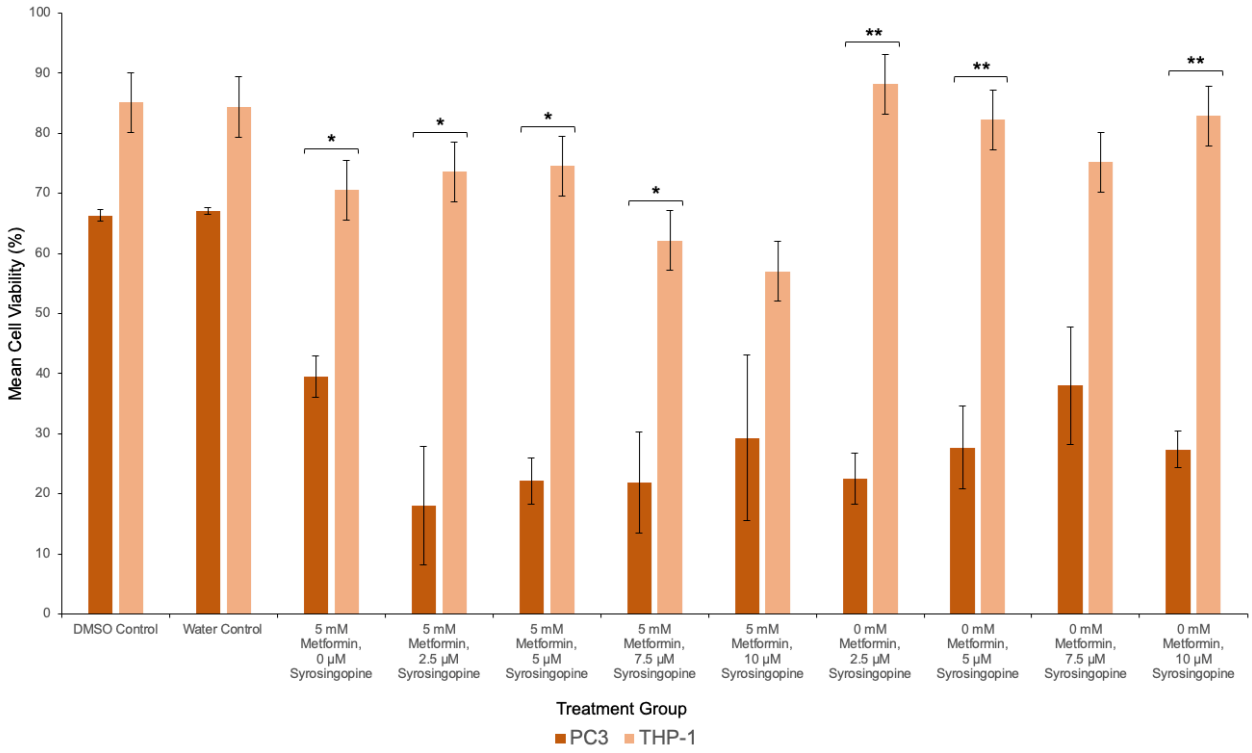

Figure 3: Efficacy of drug combinations to induce cancer cell killing.
An MTS proliferation assay was used to calculate and compare percentage cell viability following normalisation of growth to the appropriate untreated controls in PC3 (adherent) and THP1 (suspension) cancer cell lines, following treatment with increasing concentrations of syrosingopine (2.5, 5, 7.5 and 10 μM) alone and in the presence of 5 mM metformin. Absorbance was measured at 490 nm with a microplate reader. The data shown represents the mean of three replicates. Error bars were calculated from the standard error. Asterisks indicate statistical significance between means (*p <0.05, **p<0.01, ***p<0.001).

Potentiation of metformin by syrosingopine was observed in both cell lines (Figure 4), however, the two-way ANOVA determined that this drug interaction was not statistically significant ($p>0.05$) in either the PC3 ($p=0.746$) or THP-1 ($p=0.456$) cell line. Additionally, syrosingopine concentration was not statistically significant ($p>0.05$) in either the PC3 ($p=0.665$) or THP-1 ($p=0.117$) cell line, whereas metformin concentration was statistically significant ($p<0.05$), only in the THP-1 cell line (PC3; $p>0.05$, $p=0.313$). While potentiation was achieved, synthetic lethality was not, as neither the PC3 or THP-1 cell line reached 0% viability. Figures 3 and 4 show that the PC3 cell line responded better to treatment overall with a greater reduction in cell viability. In the PC3 cell line, a combination of 5 mM metformin and 2.5 μM syrosingopine, and for the THP-1 cell line, a combination of 5 mM metformin and 10 μM syrosingopine were the most effective cancer cell killing drug combinations.

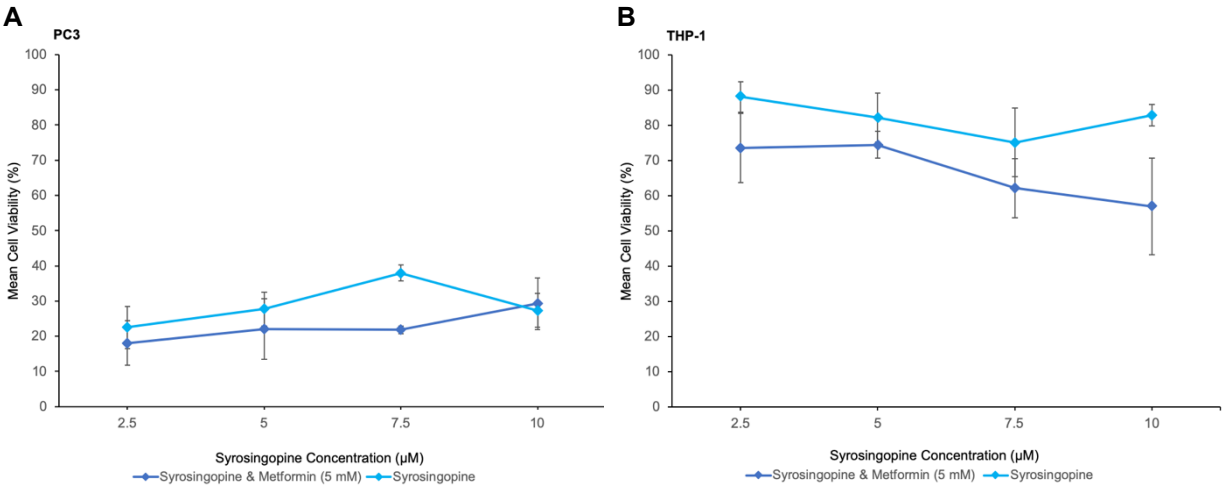

Figure 4: Synthetic lethality between syrosingopine and metformin**.**
An MTS proliferation assay was used to calculate percentage cell viability following normalisation of growth to the appropriate untreated controls. **(A)** PC3 (adherent) and **(B)** THP-1 (suspension) cells were treated with increasing concentrations of syrosingopine (2.5, 5, 7.5 and 10 μM) alone and in the presence of 5 mM metformin. Absorbance was measured at 490 nm with a microplate reader. The data shown represents the mean of three replicates. Error bars were calculated from the standard error. The PC3 cell line shows an overall positive trend with lower syrosingopine concentrations resulting in greater reduction of cell viability, irrespective of the presence of 5 mM metformin. The THP-1 cell line shows a negative trend in cell viability with increasing concentrations of syrosingopine. Overall, greater concentrations of syrosingopine in the THP-1 cell line resulted in a greater reduction in cell viability, irrespective of the presence of 5 mM metformin. For all treatment groups containing metformin and syrosingopine combined, cell viability was lower than in equal concentrations of syrosingopine alone, with the exception of 10 μM syrosingopine in the PC3 cell line.

Extracellular Lactate

Extracellular lactate measurement was not statistically significant ($p>0.05$) in either the PC3 ($p=0.322$) or THP-1 ($p=0.363$) cell line compared to their respective controls (Figure 5). Comparison of the cell lines per treatment group via a 2-sample t-test determined that two of the treatment groups had means that were statistically significant ($p<0.05$). The lowest extracellular lactate concentration for both the PC3 and THP-1 cell lines was in the treatment group with 10 μM syrosingopine alone. In all treatment groups, extracellular lactate concentration was greater in the PC3 cell line than the THP-1 cell line, except the 5 mM metformin and 10 μM syrosingopine treatment group, though this was not statistically significant ($p>0.05$).

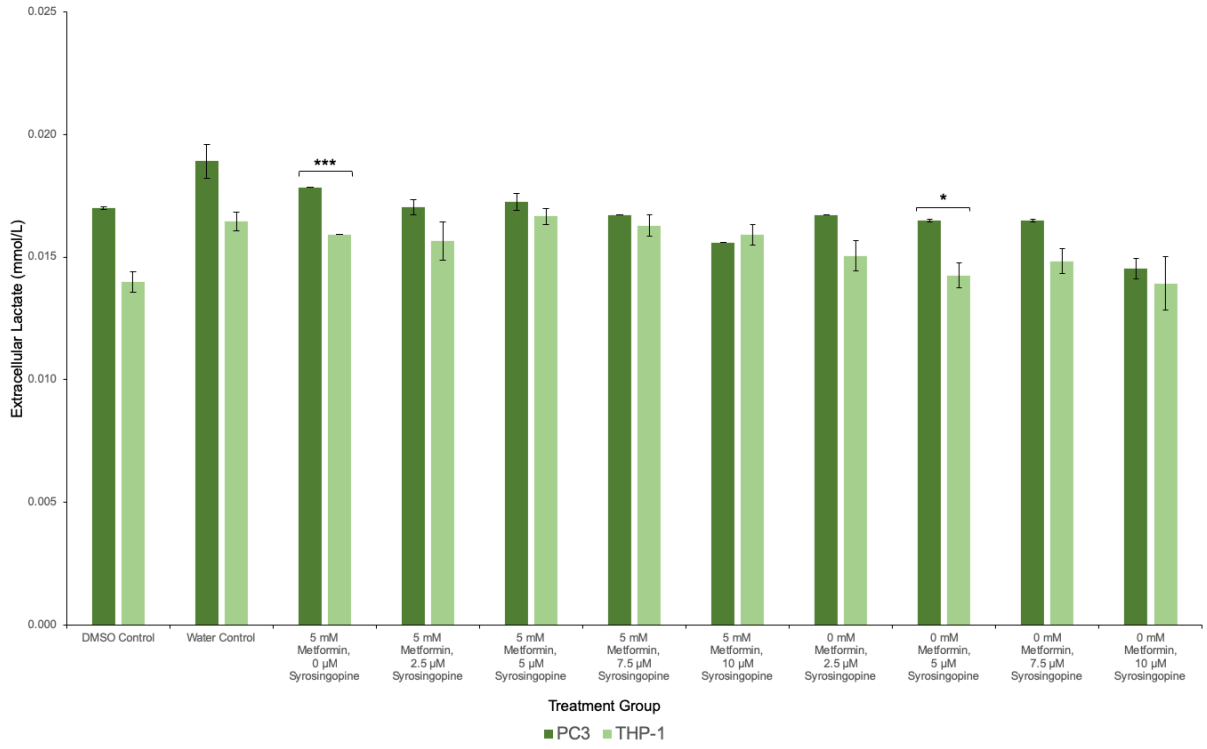

Figure 5: Extracellular lactate concentrations in adherent and suspension cancer cells. Extracellular lactate was measured spectrophotometrically and compared in PC3 (adherent) and THP-1 (suspension) cell lines following treatment with increasing concentrations of syrosingopine (2.5, 5, 7.5 and 10 μM) alone and in the presence of 5 mM metformin. Absorbance was measured at 340 nm in individual cuvettes. The data shown represents the mean of three replicates. Error bars were calculated from the standard error. Asterisks indicate statistical significance between means (*p<0.05, **p<0.01, ***p<0.001).

Discussion

Overall, the PC3 cell line responded better to treatment with combinations of metformin and syrosingopine, or single drug treatments, in all treatment groups compared to the THP-1 cell line. The decreased cell viability of both PC3 and THP-1 cancer cells was expected as metformin has been reported to inhibit complex I of the mitochondrial electron transport chain, consequently disrupting OXPHOS and mitochondrial ATP generation, resulting in cell death (Benjamin *et al.,* 2016; Wheaton *et al.,* 2014). Additionally, epidemiological research supporting metformin's antineoplastic effects has generated interest regarding the role of AMPK-mediated antineoplastic effects compared to those attributable to decreased insulin levels (Bodmer *et al.,* 2010; Landman *et al.,* 2010; Libby *et al.,* 2009). Research has shown that metformin's inhibition of complex I of the mitochondrial respiratory chain, leads to the generation of reactive nitrogen species and PKCξ activation, phosphorylating LKB1 which results in AMPK activation. Subsequently, the downstream suppression of mTORC1 signalling occurs, which interferes with cancer cell metabolism (Bodmer *et al.,* 2010; Noto *et al.,* 2012; Zakikhani *et al.,* 2006). Another explanation for metformin's antineoplastic effects, though less robust, is through the alteration of the host endocrine environment that consequently inhibits the growth of cancer cells, potentially by lowering insulin levels (Algire *et al.,* 2011; Chandel *et al.,* 2016).

However, it must be noted that this is not yet well understood, and although possible, the magnitude of these changes to the internal environment may not be sufficient to alter neoplastic behaviour. In this study, the PC3 cell line's cell viability was statistically significant compared to the relevant controls, implying that the drug treatments reduced cell viability. Current literature corroborates this, such that Chen *et al.* (2016) documented a decrease in PC3 cell proliferation following treatment with 2.5/10 mM metformin. Metformin-induced reduction of PC3 cell viability was also reported by Ben Sahra *et al.* (2008) who investigated the effects of metformin on human prostate cancer cell proliferation, showing that 5 mM metformin inhibited PC3 cancer cell proliferation with a 38% decrease in cell viability. However, they suggested that metformin's mechanism of action was a result of cyclin-D1 inhibition and pRb phosphorylation, independent of the AMPK pathway previously associated with metformin (Green *et al.,* 2010), emphasising how the molecular mechanisms underlying metformin's antineoplastic effects in prostate cancer remain unclear.

This study determined that the THP-1 cell line's viability was not statistically significant compared to the relevant controls, suggesting that the drug treatments did not reduce cell viability. However, recent literature has shown that metformin has antineoplastic effects in AML cell lines including THP-1, and is capable of reducing cell viability and proliferation. Yuan *et al.* (2020) showed that metformin has anti-leukemic effects; incremental concentrations of metformin (0.75-24 mM) increasingly inhibited THP-1 proliferation. Metformin is reported to target leukaemia through inhibition of mTORC1 activation, resulting from AMPK activation, which consequently prevents translation, notably of c-myc, cyclin D1 and Bcl-xL, crucial for cancer proliferation (Biodani & Peyron, 2018; Green *et al.,* 2010). Additionally, AMPK activity, which can be detected by the increased phosphorylation of AMPK's direct substrate, ACC, has been shown to be enhanced in metformin-treated AML cells (Castelli *et al.,* 2019; Green *et al.,* 2010). Green *et al.* (2010) reported that metformin's biochemical effects are blocked by the AMPK inhibitor, compound-C, which increases the phosphorylation of AMPK's substrate, ACC, and decreases mTOR activity. This shows the specificity of metformin towards the LKB1/AMPK tumour suppressor pathway which may be specifically activated by metformin in AML cell lines such as THP-1. Overall, metformin appears to be a potent inhibitor of AML cell proliferation via activation of the LKB1/AMPK pathway, markedly by the repression of mTOR-dependent oncogenic mRNA translation (Castelli *et al.,* 2019; Noto *et al.,* 2012); however, further research specifically utilising THP-1 cells is required. Nonetheless, there have been conflicting studies stating that AMPK-independent mechanisms, while important in metformin action, are secondary to the energetic stress caused by inhibition of OXPHOS (Chandel *et al.,* 2016).

Although metformin has apparent antineoplastic effects in both prostate cancer and AML, further research is necessary to better understand metformin's molecular mechanisms underlying its associated antineoplastic effects, as it cannot be assumed that these are identical to the more established antidiabetic effects. Furthermore, it is unclear whether clinically practical metformin doses are sufficient for the outlined mechanisms to operate. Metformin doses reported to be effective against cancer cell lines and models *in vitro*, are in the millimolar (mM) range, as was the concentration used in this study (5 mM). However, these are much greater than the doses prescribed for T2DM patients (6-30 μM) of ~30 mg/kg where plasma levels are in the 10 μM range (Biondani & Peyron, 2018; Graham *et al.,* 2011). Most

in vitro studies report that millimolar levels of metformin are necessary to induce antiproliferative effects, suggesting that antineoplastic activity in clinical settings is unlikely (Benjamin *et al.,* 2018; Graham *et al.,* 2011). Thus, the requirement for millimolar metformin concentrations *in vitro* may not be a useful predictor of the drug concentration required for activity *in vivo*, hence the need for mouse models to determine *in vivo* concentrations that demonstrate antineoplastic activity to reveal if this is achievable in human patients (Chandel *et al.,* 2016; Dowling *et al.,* 2016). Furthermore, cellular entry by the highly hydrophilic metformin is limited by expression of the organic cation transporter, thus studying the uptake of metformin by cells through encapsulation, nanocarriers or chemical modifications (Biondani & Peyron, 2018) presents an innovative area of research with the potential to improve clinical applications of metformin as an antineoplastic agent. Coupling mitochondrial vectors with metformin (MitoMet) has reportedly increased metformin's ability to interfere with OXPHOS, hinder proliferation and trigger apoptosis in pancreatic cancer cells, without affecting non-transformed cells (Boukalova *et al.,* 2016). Nevertheless, lowering the therapeutic threshold of biguanides appears to be the most promising way to implement their use as an anticancer therapy (Benjamin *et al.,* 2016). With this, syrosingopine's reported ability to elicit synthetic lethality with metformin in cancer cell lines to substantially lower the concentration of metformin required to induce cancer cell killing, shows great clinical potential.

Metformin & Syrosingopine's Synthetic Lethality

In this study, potentiation of metformin's antineoplastic effects was observed when in combination with syrosingopine in all PC3, and two THP-1 treatment groups compared to metformin alone. Additionally, combining the drugs resulted in reduced cell viability in all but one of the PC3 treatment groups and in all THP-1 treatment groups compared to syrosingopine alone. Overall, a synergistic relationship between metformin and syrosingopine was observed but synthetic lethality was not achieved in either cell line. Although the combining of syrosingopine and metformin did not induce the synthetic lethality that was expected, synergism between the drugs was observed and resulted in a reduced cell viability, observed in most treatment groups. However, in this study, the interaction between syrosingopine and metformin was not statistically significant in either cell line. Although the statistical significance indicates that the drug combination is not responsible for the potentiation and reduced cell viability in this study, syrosingopine is believed to sensitize cancer cells to killing by metformin. Syrosingopine and metformin combined are reported to elicit a synthetic lethality, such that this drug combination is believed to kill various cancer cell lines while leaving non-transformed cells undisturbed (Benjamin *et al.,* 2016; Khammanivong *et al*., 2020). Benjamin *et al.* (2016) observed that titration of syrosingopine in the presence of 5 mM metformin resulted in synergistic cell killing in various cancer cell lines. Although no prostate cancer cells were utilised in this study, leukemic cells from cell lines HL60, OPM2 and AML7991 were, suggesting that this synthetic lethality should have been observed in the THP-1 cell line, potentially even more so than in the PC3 cell line. This 2016 study acknowledges that some cancer cell lines are non-responsive to treatment, suggesting that this synthetic lethality is not a result of indiscriminate toxicity from the drug combination, but rather, selective (Benjamin *et al.,* 2016; Benjamin *et al.,* 2018). Additionally, in promyelocytic leukaemia cells, Benjamin *et al.* (2016) showed that 5 μM syrosingopine significantly increased the cells' sensitivity to metformin, reducing metformin's half maximal inhibitory concentration (IC_{50}) from 30 mM to ~2 mM.

Syrosingopine induces cell death with the electron transport chain inhibitor, metformin at concentrations below its cytotoxic threshold however, the mechanism by which syrosingopine elicits synthetic lethality with metformin is still unknown. Therefore, more research is necessary to better understand the underlying mechanisms of this synergism, specifically in the PC3 and THP-1 cell lines.

Syrosingopine has been documented to exert antitumour effects both *in vitro* and *in vivo* through increased accumulation of intracellular lactate via disruption of MCT1 and/or MCT4 (Todenhöfer *et al.,* 2018). Originally introduced as a well-tolerated anti-hypertensive drug in the late 1950s (Calesnick, 1959; Herrmann *et al.,* 1959), syrosingopine has since been reported as a dual inhibitor of MCT1/MCT4, critical regulators of intracellular lactate and pH. Treatment of cancer cells with syrosingopine therefore leads to elevated intracellular lactate levels and consequently, end-product inhibition of LDH. Thus, loss of NAD^+ regeneration capacity resulting from combined metformin and syrosingopine treatment, causes glycolytic blockade that leads to ATP depletion, crippling cell metabolism and consequently causing cell death (Benjamin *et al.,* 2018; Khammanivong *et al*., 2020). Ultimately, it is widely accepted that cancer cells' metabolic plasticity and ability to switch to alternative metabolic-fuelling pathways remains a substantial hurdle in developing cancer therapeutics. Uncovering these mechanisms has revealed the possibility of utilising the synthetic lethality approach demonstrated by metformin and syrosingopine. However, studies have shown that metformin can be potentiated or more effective in combination with other drugs and therapies, other than syrosingopine, and it may be that these combinations more efficaciously induce cancer cell killing (Li *et al.,* 2020; Zi *et al.,* 2018).

Colquhoun *et al.* (2012) demonstrated that metformin with bicalutamide anti-androgen therapy significantly reduced prostate cancer cell growth than either monotherapy; reducing proliferation rates in androgen receptor-positive cells and promoting apoptosis in androgen receptor-negative cells including PC3 cells. Furthermore, in glioma models, metformin therapy combined with repurposed drugs including itraconazole, naproxen, pirfenidone and rifampin is shown to inhibit glioblastoma cell proliferation, invasion and metastatic potential (Kamarudin *et al.,* 2019). Head *et al.* (2017) showed that itraconazole regulates AMPK and mTOR through direct targeting of the mitochondrial protein VDAC1, demonstrating itraconazole's potential to be used in combination with metformin to sensitize cancer cells to killing. Although more research is necessary to determine the efficacy of itraconazole and metformin combinations, particularly in PC3 and THP-1 cell lines, itraconazole treatment has already been reported to benefit patients with ovarian, pancreatic and other cancers, as well as improve remission rates in AML (Pounds *et al.,* 2017; Zhang *et al.,* 2020). Metformin and chemotherapeutic paclitaxel combinations have induced AMPK-level signalling to markedly inhibit cell viability in MCF-7 and A549 cancer cells, compared to either monotherapy (Rocha *et al.,* 2011; Zi *et al.,* 2018), further supporting that combination therapies can augment antitumour signals to provide an effective anticancer treatment. Combinational strategies involving metabolic inhibition with chemotherapy or repurposed drugs show promising advancements in clinical trials. Nevertheless, more research is required to determine the efficacy of these treatments in clinical settings, as well as in prostate cancer and AML, as most ongoing clinical trials present data only for multiple myeloma, chronic lymphocytic leukaemia and melanoma (Li *et al.,* 2020).

Suspension vs. Adherent Cell Lines

With the PC3 cell line responding better to treatment than the THP-1 cell line overall, statistical analysis of cell viability of eight treatment groups, between cell lines, revealed statistical significance. This indicated that the nature of the cell line, adherent or suspension, contributed to the cell killing potential of the drug combinations in these treatment groups. Suspension cells, including THP-1 cells, are three-dimensional cultures that are considered to more accurately mimic the *in vivo* conditions and environment of solid tumours compared to conventional monolayer cell culture, observed in adherent cell lines such as PC3 cells (Benjamin *et al.,* 2016). The three-dimensional nature of suspension cell culture suggests that treatment with metformin and syrosingopine should more effectively treat the THP-1 cells due to an increased volumetric cell density and surface area exposure compared to adherent PC3 cells. However, this was not observed in this study, perhaps as certain experimental steps are more efficient in adherent cell lines with changes in cell adhesion protein expression and oxygen availability (Malm *et al.,* 2020). Concentrations of metformin and syrosingopine, and the length of treatment required to kill cells of suspension or adherent cell lines may vary. Benjamin *et al.* (2016) found that the quantity of syrosingopine and length of treatment required to kill hepatospheres from hepatocellular carcinoma lines Huh7 and HpG2, known to respond to syrosingopine and metformin combinations in monolayer cell culture, was greater than that required for killing the same cell lines in two-dimensional cultures. Additionally, Tsuei & Martinus (2012) showed that 100/250 μM metformin concentrations lead to slightly reduced proliferation rates in THP-1 cells, but 10 mM metformin proved to be cytotoxic, resulting in unviable cells after 48 hours, suggesting greater concentrations of metformin are required to induce cancer cell killing in suspension cell lines.

Furthermore, media was not removed prior to addition of metformin and syrosingopine in the THP-1 cell line as this would remove the cells. Thus, consequent dilution of the treatment drugs could result in a less potent effect to reduce cell viability in the THP-1 wells compared to the PC3 wells where the media was removed. Suspension cells typically proliferate at a faster rate than adherent cells (Segeritz & Vallier, 2017), thus a potentially less potent drug combination, treating more cells, could explain the limited reduction in cell viability for the THP-1 cell line. However, this is not necessarily specific to cancer cell lines and contrasts the findings of Park *et al.* (2018) where breast cancer cell lines in suspension proliferated slower than adherent cell lines. In this 2018 study, cell cycle analysis was performed to determine the cause of slower proliferation of suspension cell lines and concluded that there was an increase in the G1 phase population compared to the adherent cells where more cells were in the G2/mitotic phase (Park *et al.,* 2018). Although this 2018 study utilised a cancer cell line, this may not be representative of all cancer cell lines including THP-1 and PC3. Another important consideration is that both the PC3 cell line's DMSO and water controls had a considerably lower viability compared to the THP-1 cell line's controls in this study, and neither cell line's control had 100% cell viability as expected. While the exact reason for this is unknown, microbial contamination in the cell culture procedure could explain the PC3 cell line's significantly reduced cell viability in all treatment groups, including the two controls.

Extracellular Lactate

Measurement of extracellular lactate was conducted to identify whether syrosingopine could successfully inhibit MCT1/MCT4. Such that decreased extracellular lactate concentrations were expected in the treatment groups compared to the controls. This study found no statistical significance in extracellular lactate concentrations between treatment groups compared to their respective controls in both cell lines, implying that syrosingopine was not responsible for the extracellular lactate measured. Additionally, analysis between cell lines per treatment group identified statistical significance in two treatment groups, suggesting that the nature of the cell line contributed to the extracellular lactate measured for the above-mentioned reasons. It is reported that the majority of the lactate secreted by cancer cells accumulates in the extracellular space to create a tumour microenvironment that promotes cell invasion and metastasis (Brand *et al.,* 2016; Kato *et al.,* 2013). Lactate shuttling has been observed between cancer cells to form a metabolic symbiosis where highly glycolytic and hypoxic tumour cells efflux lactate that is imported and metabolised by more oxidative tumour cells, facilitating glucose delivery from the vasculature (Sonveaux *et al.,* 2008). Another lactate shuttling model has been described, termed the 'reverse Warburg effect' where cancer cells are thought to create a 'pseudo-hypoxic' environment, causing stromal fibroblasts to secrete hydrogen peroxide that then activates HIF-1α, glycolysis and MCT4 expression in stromal cells (Benjamin *et al.,* 2018; Doherty & Cleveland, 2013). The lactate produced by the stromal cells is exported by MCT4 and imported into cancer cells by MCT1, demonstrating that MCT1 can direct lactate uptake into cancer cells dependent on OXPHOS, highlighting that MCTs are critical for tumour growth (Doherty & Cleveland, 2013; Fantin *et al.,* 2006). Therefore, inhibition of these transporters leads to intracellular acidification, resulting in end-product inhibition of LDH and consequently ATP depletion and cell death (Benjamin *et al.,* 2018).

With this, low levels of extracellular lactate were expected to correlate with reduced cell viability in this study as a result of MCT1/MCT4 inhibition; however, this was not observed. Benjamin *et al.* (2018) reported that HeLa (cervical cancer) cells treated with syrosingopine and metformin showed a decrease in extracellular lactate levels. However, this study also reported that syrosingopine treatment alone and its role in intracellular lactate accumulation does not result in cell death as it is only cytostatic. They highlighted that a constant supply of NAD^+ is required to sustain a high rate of glycolysis which is possible not only by the conversion of pyruvate to lactate via LDH, but also via mitochondrial complex I, the target of metformin (Benjamin *et al.,* 2018; Fontaine, 2018; Vial *et al.,* 2019). Thus, it is the simultaneous inhibition of LDH and mitochondrial complex I that can lead to NAD^+ depletion, glycolytic blockade and consequently, cell death. Furthermore, Tsuei & Martinus (2012) observed an increased acidification in culture medium in cells treated with high concentrations of metformin, suggesting that metformin increases lactate levels, and without inhibition of MCT1/MCT4 by syrosingopine, this can be exported, further highlighting the significance of these transporters as targets in anticancer therapies.

MCT1 and MCT4 overexpression is a hallmark of several human malignancies and MCT4 expression is highly elevated in prostate cancer (Doherty & Cleveland, 2013; Todenhöfer *et al.,* 2018; Zhu *et al.,* 2014). Knockdown of MCT1/MCT4 has been shown to reduce cancer cell invasion activity after transfection with MCT1- and MCT4-specific small interfering RNAs (siRNA) (Izumi *et al.,* 2011). Zhu *et al.* (2014)

studied the silencing of MCT4 expression using siRNAs in oral squamous cell carcinoma *in vitro* and showed that knock-down of MCT4 decreased cell proliferation, migration and invasion. *MCT1* deletion using a CRIPR-Cas9 strategy has reportedly inhibited cancer cell migration and invasion in the SiHa (cervical carcinoma) cell line (Payen *et al.,* 2017). Knockdown and inhibition studies have demonstrated that selective inhibition of MCT1/MCT4 may reduce migration and invasion or increase chemo-sensitivity, but is insufficient to induce cancer cell death. This is due to cancer cells' metabolic plasticity and ability to metabolically shift to OXPHOS even in simultaneous inhibition of MCT1 and MCT4 (Doherty & Cleveland, 2013; Fisel *et al.,* 2018). Thus, once again supporting that drug combinations inhibiting both lactate transportation and OXPHOS such as syrosingopine and metformin, respectively, presents the most promising approach to induce cancer cell killing. Further work is necessary to determine whether these findings can be replicated in PC3 and THP-1 cells, but also to investigate whether MCT1 or MCT4 inhibition is of most significance from a therapeutic perspective. Nevertheless, thorough investigation and characterisation of MCT expression in cancer cells has the potential to identify new therapeutic approaches in cancer.

Limitations

The findings of this study are limited by a lack of biological replicates consisting of non-transformed cells. Ultimately, the metformin and syrosingopine drug combination must be able to elicit synthetic lethality and induce killing specific only to transformed cells to be considered a potential cancer therapeutic. Additionally, the PC3 and THP-1 controls were not 100% viable, limiting the comparison between the treatment groups and controls. Due to the mixing of metformin and syrosingopine per treatment group and in culture medium for suspension cells, the final concentration of the inhibitors used were likely diluted, thus it cannot be guaranteed that the specified concentrations were treating the cells. This study could have benefitted from a more extensive investigation of cell viability, potentially measuring viability kinetically over time, immediately after the cells were treated with inhibitors. While useful, the lactate aspect of the study had room for improvement as measuring extracellular lactate does not confirm that intracellular lactate levels are increasing, resulting in intracellular acidification from syrosingopine's MCT1/MCT4 inhibition. Thus, measuring intracellular and extracellular lactate simultaneously has the potential to more effectively investigate MCT inhibition and defective lactate transport in cancer cell lines. Most importantly, time constraints limited the ability to successfully optimise inhibitor concentrations, thus it was not possible to investigate optimal concentrations for reduced cell proliferation, viability and extracellular lactate measurement. This optimisation would have significantly benefitted the study due to the varying nature of the PC3 and THP-1 cell lines. Nevertheless, many of these limitations were due to time and accessibility restrictions, a consequence of the COVID-19 pandemic.

Conclusion

Individually, metformin and syrosingopine have well-established clinical applications outside of cancer therapy. Current literature suggests that the eradication of cancer will require simultaneous targeting of various cancer cell metabolic pathways to limit metabolic plasticity and adaptation. Studies support that the combination of these drugs targeting different pathways in cancer cell metabolism may be of potential clinical benefit in cancer therapeutics. This study investigated the potential of these

drugs combined to elicit a synthetic lethality to induce cancer cell killing in PC3 and THP-1 cell lines as prospective cancer therapies. The findings indicate that metformin and syrosingopine drug combinations have the potential to reduce cell viability, particularly in the PC3 cell line, but synthetic lethality was not achieved in either cell line. Furthermore, MCT1/MCT4 inhibition was investigated via the measurement of extracellular lactate levels, however these findings were inconclusive. Notably, this was a preliminary study due to a lack of biological replicates and optimisation of inhibitor concentrations. Further research is necessary to better understand the synthetic lethality induced by syrosingopine and metformin combinations, particularly the associated underlying cellular mechanisms. Future research should focus on identifying drug concentrations necessary to lower the therapeutic threshold of metformin and have the same antineoplastic effect *in vivo* as observed *in vitro*. Additionally, greater investigative measures such as gene-knockdown and simultaneous measurement of intracellular lactate levels should be conducted to analyse the significance of extracellular lactate levels associated with MCT1/MCT4 inhibition in cancer cell lines.

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