Bachelor Degree Project

Investigating the cause of reduction of lactate production in Panc-1 cell lines when subjected to digitoxin

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) yet remains one of the top 10 most prevalent and top five most common cause of cancer-related deaths solely in the United States. Its metastatic deviation from most cancer types rends it one of the most subtle yet perturbating disease. Hope lies within the traces of reproducibly mutated genes which are said to be found in nearly 95% of all PDAC. This study aimed to examine how digitoxin affects glycolysis and gene expression in Panc-1 cell lines. Panc-1 cells were cultured and treated with various concentrations of digitoxin for 24h-48h depending on the analysis and cell viability was then examined via the MTS assay. Lactate assay was conducted in order to investigate the degree to which panc-1 production of lactate was affected by digitoxin. Specific genes of interest (*LDHA*, *C-Myc*, *PFKM*, *PDP1*, *SLC2A* and *PMM1*) that take part in the glycolytic segment of the panc-1 metabolism were analyzed with qPCR in order to study their expression. Cell cycle assay was conducted in order to examine whether digitoxin also affected the panc-1 cell cycle and to what degree. Results from the MTS assay indicated that cell viability was indeed affected with increasing concentrations of digitoxin whereas; the lactate assay indicated that panc-1 cells were sensitive to 25nM digitoxin/higher due to how the decrease in lactate production became evident at that point. The gene expression of *PDP1* was significantly increased with high concentrations of digitoxin compared to other genes. The cell cycle assay indicated that most treatment groups did not make it to the G2 and M phase due to apoptosis. All in all, digitoxin showed to have apoptotic effects on panc-1 cells line although this effect was not evident in certain assays.

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List of abbreviations

MTS - CellTiter 96® AQueous One Solution Cell Proliferation Assay

LDHA - Lactate Dehydrogenase

PDP1 - Pyruvate Dehydrogenase Phosphatase 1

PMM1 - Phosphomannomutase 1

PFKM - 6-Phosphofruktokinase

PDAC - Pancreatic Ductal Adenocarcinoma

NADH - Nicotinamide Adenine Dinucleotide Hydride

TCA - Tricarboxylic acid cycle

ATP - Adenine Triphosphate

GLUT1 - Glucose Transporter 1

PDP1 - Pyruvate Dehydrogenase Phosphatase Catalytic Subunit 1

1. Introduction

The pancreas, located behind the stomach in the upper left abdomen, is an organ that is in charge of secreting digestive enzymes as well as synthesizing hormones that help regulate blood sugar (Talathi & Bhimji, 2018). The dual function is due to the two multiple cell types of which it is comprised; they both vary in composition, arrangement, route of secretion, mechanism and duration of action as well as half-life (Chan, Bruce & Siriwardena, 2016). The acinar cells; which are round-like secretory modules arranged in grape-like clusters, make up the exocrine segments of the pancreas which accounts for roughly 90% of the pancreatic tissues.
In certain cases, the exocrine segment of the pancreas develops pancreatic ductal adenocarcinoma (PDAC) which is a cancer that progressively causes scattering in regions of duct-like epithelium. This subsequently leads to epithelial cells which are rather less differentiated; accommodated within a pool of proliferative stroma (Rhim & Stanger). PDAC is said to exhibit a distinctively low density of vasculature along with a surrounding dense stroma, these characteristics facilitate the formation of several hypoxic regions and promote its overall aggressiveness (Neesse et al., 2011). Cancer refers to the uncontrolled cellular division which tends to develop exponentially over time, this phenomenon can occur in any tissues of the body and its type and name is often determined by the tissues in which it emerges. Although there are various types of cancers, they are all often governed by similar characteristics of growth and proliferation (Hausman, 2019).

With an estimated incidence of roughly 60,430 people in the US around 2021 (Anon), PDAC has made its way up into being the 10th most prevalent and one of the top five most common causes of death related to cancer solely in the United States (Rhim & Stanger). The reason why PDAC calls for an emergent need to comprehend has to do with the poor prognosis with which it arises, the survival rate of most individuals diagnosed with PDAC is rather low. Less than 5% of individuals tend to live up to 5 years postdiagnosis, this is because at that stage the cancer has already metastasized roughly 80% (Anon). Although most cancers from various origins tend to have rather similar hallmarks, PDAC partially differs in its ability to metastasize and create large tumors; eventually killing the host while circumventing the treatments which are shown to ameliorate other malignancies (Hausman, 2019).

It has been suggested that understanding the progressive manner of molecular events as well as the particularly unique biological features of pancreatic tumors would help intervene in this lethal cancer (Rhim & Stanger). While the survival rate of this malignancy is rather low, the genetic cause of its metastatic spread also varies from individual to individual. However, there happen to be some reproducibly mutated genes in nearly 95% of all PDAC which could signify that this malignancy depends on certain alterations at the molecular basis in order to develop (Bryant et al., 2014).

Oncogenic *KRAS* mutation is one of the few commonalities that is shared amongst PDAC cells, it is said to be the first oncogene found to have a crucial role in PDAC by chiefly feeding its proliferation, promoting differentiation and maintaining survival (Bryant et al., 2014). Most human cancers contain a version of Ras protein that has been mutationally activated; usually as a single point mutation in one of the three residues (G13, G12 and Q61) (Bryant et al., 2014). It has been suggested that when Ras is constitutively activated it causes a persevering stimulation of the downstream signaling pathways. This in turn subsequently leads to known phenotypic hallmarks of cancer such as metastasis, disruption in immune response, changes in the environment of tumors, changes in the metabolism, apoptosis suppression as well as elevated proliferation (Pylayeva-Gupta, Grabocka & Bar-Sagi, 2011).

There happens to be a metabolic difference between non-cancer cells and PDAC cells with regards to how they oxidize glucose into pyruvate, in non-cancer cells glucose is metabolized to pyruvate in the presence of oxygen (Hanahan & Weinberg, 2011). This subsequently leads to a minimum amount of lactate production and an utmost amount of ATP production during oxidative phosphorylation in the electron transport chain; whereas, the production of lactate via glucose was said to be tenfold more in tumor tissues (Warburg, 1956). It was first believed that these tumor tissues exhibited this phenomenon due to defective mitochondria; however, further researches suggested that most cancer cells had proper functioning mitochondria (Moreno-Sánchez et al., 2007). At the time, advantages of the increase in glycolysis of cancer cells yet remained unknown and rather controversial. However, hypothesis suggested that high levels of glycolytic intermediates were rapidly supplied in order to proliferate cells despite their inefficiency in net production of ATP. These intermediates were then said to be part of the biomass which facilitates the generation of new cells (Heiden, Cantley & Thompson, 2009). Studies suggest that there is a correlation between signaling molecules that take part in cell proliferation and the tendency of metabolic pathway regulations, particularly in *KRAS* (Heiden, Cantley & Thompson, 2009). This supports the aforementioned theory.

PDAC cells' ability to take up and utilize glucose is said to be altered by oncogenic *KRAS* (Racker, Resnick & Feldman, 1985). Previous studies suggest that this alteration subsequently leads to the upregulation of glycolysis. This was shown to be vital for PDAC given that cancer cells require sufficient energy and biosynthetic building blocks in order to proliferate (Racker, Resnick & Feldman, 1985). The increase in lactate production along with the augmentation of glycolytic activities is said to be facilitated by the increase in gene expression of glucose transporter *GLUT1*, which in turn is promoted by KRAS signaling. This *KRAS*-mediated metabolic alteration has been now shown to give PDAC cells several advantages including the ability to maintain survival in low glucose conditions such as low density of vasculature and dense stroma (Yun et al., 2009).

The gene expression of phosphofructokinase-1 (Pfk1), hexokinase 1&2 (Hk1, Hk2) and lactate dehydrogenase A (*LDHA*) - which are gene encoding rate-limiting glycolytic enzymes - are also said be altered by KRAS, all these changes combined contribute to the increase of glycolytic flux (Yun et al., 2009). It has been reported that there is a high likelihood that Myc transcription factors mediate the ability of mitogen-activated protein kinase (MAPK), a downstream effector of oncogenic KRAS, to alter the metabolism of glucose in PDAC, (Yun et al., 2009).

Digitoxin is a well-established cardiac glycoside that shows significant anti-cancer effects in various cancer types (Elbaz et al., 2012). It contains a specific ability to inhibit Na^+/K^+- ATPase pump when administered at concentrations between 0.5 - 5 μM (Rahimtoola & Tak, 1996). Lower digitoxin concentrations of 25-40 nM are also said to have Na^+/K^+- ATPase-pump inhibiting abilities (Anon). This inhibition triggers an increase in the activity of Na^+/Ca^{2+} pump which leads to an augmentation in the level of intracellular calcium; subsequently causing apoptosis in cancer cells (Elbaz et al., 2012).

1.2 Hypothesis

Given that the level of lactate production was reported to gradually decrease with increasing concentration of digitoxin, (Lindholm H, 2021, personal communication, 26 January). This draws specific attention genes that take part and/or interact with the glycolytic segment of panc-1 metabolism. Of the gens that will be investigated is lactate dehydrogenase (*LDHA*); the enzyme responsible for the conversion of glucose into lactate via pyruvate and vise-versa (de la Cruz-López et al., 2019). It is expected that its gene expression will decrease as the concentration of digitoxin increases due to its relationship with lactate production.
Another gene that will be monitored is c-*MYC*, given that it has been reported to be the prototypical cooperating oncogenic partner of *KRAS*, it is suggested that its transcription factors may be involved in the adjustment of glucose metabolism in Panc-1 (Sodir et al., 2020; Satoh et al., 2017). It is expected that its gene expression will decrease when subjected to higher concentrations of digitoxin. This is because in most cancers, *MYC* is said to be indirectly and relentlessly induced by “upstream” oncoproteins (e.x *WTN*, *Notch* and *RAS* itself). However, the *MYC* gene itself is seldomly the cause of its own aberrantly persistent activation (Sodir et al., 2020). Since digitoxin is said to reduce cell viability, it is therefore expected that the genes/pathway which will be inhibited will indirectly inhibit the indirect induction of *MYC*.
Pyruvate dehydrogenase phosphatase 1 (*PDP1*) also draws attention as it is one of the two significant effectors that connects glycolysis with further major metabolic pathways. Previous findings suggest that this gene is almost always overexpressed in PDAC, however, the exact cause and mechanism as to how this occurs yet remains

unknown (Li et al.). The *PDP1* gene is said to encode pyruvate dehydrogenase phosphatase, it is one of two isoforms of *PDP* in mammalian cells (Li et al., 2020). It acts as a chief regulator of pyruvate dehydrogenase complex (PDC); which is considered as its physiological function. It does this by removing phosphates from the E1α of the aforementioned complex, specifically on the serine sites, which in turn positively regulates the PDC catalytic activities (Stacpoole, 2017). Once the PDC becomes activated by the removal of phosphate, it irreversibly initiates the oxidative decarboxylation of pyruvate; giving rise to acetyl-CoA. Phosphomannomutase 1 (*PMM1*) gene will serve as a reference gene due to how *GAPDH* exhibited changes in gene expression when subjected to digitoxin, (Lindholm H, 2021, personal communication, 26 January). It permits the conversion of D-mannose 6-phosphate to D-mannose 1-phosphate (a substrate for GDP-mannose synthesis) by acting as a catalyst; leading to the secretion of multiple glycoproteins and the synthesis of glycosyl-phosphatidyl-inositol anchored proteins (Mardh & Westrom, 1976).
This study aimed to examine how digitoxin affects glycolysis and gene expression in Panc-1 cell lines.

2. Materials and methods

2.1 Chemicals and Reagents

All chemicals used throughout the experiment were purchased from Sigma Aldrich unless stated otherwise, this includes; Dulbecco's Modified Eagle's medium (DMEM) (catalog #30-2002) – high glucose, Trypsin, Phosphate-buffered Saline (PBS) and Digitoxin. The RNeasy® Mini Kit (50) (Cat #74104), High-Capacity cDNA Reverse Transcription Kit (Ref #4368814), Fast Advanced TaqMan Master Mix (Ref #4444554) and All probes (LDHA, C-Myc, PFKM, PMM1, PDP1 and SLC2A) were purchased from ThermoFisher, Sweden. The Cell cycle assay Kit was purchased from Biocolor. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (catalog #G3582) and Lactate-Glo TM Assay Kit were purchased from Promega Corporation, Sweden. The lactate assay was designed based on section 4.A and all lactate detection reagents were prepared according to section 3.A of the protocol (TM493).

2.2 Cell Line and Cell Culture

The Panc-1 cells is a commercial cell line purchased form ATCC (Anon) ; they possess an epithelial morphology with adherent growth properties. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (catalog #30-2002) – high glucose; along with fetal bovine serine (FBS) with a 10% final concentration, L-glutamine and PEST which were used as supplements in order to ensure adequate cell growth. Culturing took place in a 75cm2 culture-flask at 37°C; in a 5% CO_2 incubator

and the cells were passaged upon 80% of confluency, all chemicals were preheated to 37°C prior to usage. The cells were harvested by trypsinization upon each passage.

2.2.1 Cell count

A cell count was carried out in order to obtain an approximation of the number of cells in the resuspended cells media. This involved taking 40µl of the suspended cells and 40µl of trypan blue in a two-dilution factor and then gently mixing them via pipetting. A haemocytometer was used in order to facilitate a rapid estimation of the concentration of cells in the mixed sample, this process involved counting the cells in the known volume and later converting it to a number per millilitre. Each side of the chamber was then filled with 10µL of the mixed solution mentioned above, this was then viewed under a light microscope using 10x magnification and viable cells were counted.

2.2.2 Cell seeding

Panc-1 cells were seeded into 96-well plates at a density of 5000 cells/well (100µL media/well) and into 6-well plates at a density of 130.000 cells/well (2.4mL media/well). The 96-well plates were used for MTS, cell cycle assay and Lactate-Glo TM assays, whereas the 6-well plates were used for the extraction of mRNA.

2.3 MTS Assay (cell viability assay)

Panc-1 cells were seeded in 96-well plates at a density of 5000 cells/well (100µL media/ well), the plates were then treated with different concentrations of Digitoxin (1nM, 10nM, 25nM, 40nm and 100nM) diluted in DMEM. One column consisted of a blank and the other of control (plain media), whereas; 16 wells were treated per each concentration. The plate was then incubated for a period of 48h at 37°C; in a 5% CO_2 incubator. This was subsequently followed by the addition of 20µL of MTS to each well and a 60-minute incubation period. The absorbance was then measured at 490nm with Microplate Reader (Omega), this was done for all 3 replicates.
The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) is said to be bio-reduced to formazan dye (soluble in culture media) by viable cells in the presence of phenazine methosulfate (Kuete, Karaosmanoğlu & Sivas, 2017). Studies suggest that this conversion is carried out by NADPH-dependent dehydrogenase enzymes in cells that are metabolically active, the absorbance of the dye can be measured at 490-500nm for quantification (Kuete, Karaosmanoğlu & Sivas, 2017)

2.4 Lactate Assay

Panc-1 cells were seeded in 24-well plates at a density of 5000 cells/well (100µL) and incubated at 37°C in a 5% CO_2 incubator for a period of 24h, this was then followed by a digitoxin treatment in the various, aforementioned, concentrations per column of wells, this also included an untreated/control column. The plate was then incubated anew (37°C, 5% CO_2), 5µL of media was removed from each well of the different treatments including the control ensuing 24h, this was then diluted in 95µL of PBS and stored at -20°C, this process was repeated after 48h of incubation. Upon usage, the samples were thawed and 5µL of each sample was transferred to a 96-well assay plate; preloaded with 45preloaded with 45µL of PBS followed by the addition of 50µL of Lactate Detection Reagent (prepared according to section 3.A of the protocol) to each well. A standard curve of different concentrations [µM] of lactate (0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100) diluted in PBS was also prepared. This was then followed by a mild shaking of roughly 30-60 seconds including an incubation period of 60 minutes at room temperature. The luminescence was then measured using an Omega microplate reader.
The Lactate-Glo™ Assay is used to rapidly, sensitively an selectively detect L-Lactate in samples. The principal of this assay dictates that the oxidation of lactate is catalyzed by lactate dehydrogenase which reduces NAD+ to NADH. When NADH is present, pro-luciferin Reductase substrate is enzymatically reduced to luciferin by Reductase. Using Ultra-Glo™ rLuciferase along with ATP, luciferin can be detected in a luciferase reaction with regards to the light it produces. The amount of produced light is said to be proportional to that of the sample of lactate (Corporation)

2.5 RNA-extraction & cDNA synthesis

Panc-1 cells were seeded in a 6-well plate, treated with digitoxin and incubated overnight (37°C, 5% CO_2) and RNA was extracted using a RNeasy® Mini Kit, the RNA concentration was then measured by Nanodrop. cDNA was then synthesized using an RNA concentration of 34ng/µL and 39.2ng/µL from different passages. This was carried out using a High-Capacity cDNA Reverse Transcription Kit, the samples were further diluted to a working of concentration of 5ng/µL.

2.6 Gene Expression Assay

The gene expression of *LDHA*, *C-Myc*, *PFKM*, *PDP1*, *SLC2A* and *PMM1* (used as a refence gene) were analyzed with qPCR. 2µL of each probe was loaded in a 96-well mini plate and dried at 40°C for roughly an hour. 2µL of the diluted cDNA samples were then mixed with TaqMan™ fast advanced Master Mix (ref# 444554) at a 1:1 ratio and loaded onto the plate in duplicates for every treatment including control which consisted of untreated cells and the no-template-control (NTC) which consisted

of RNase-free water and probe; used for detection of DNA contaminations. The fold change was calculated using the Delta CT method.

2.7 Detection and Measurement of Cell Cycle Phases (Cell-clock Assay)

Panc-1 cells were seeded in 96-well plates at a density of 5000 cells/well (100µL) and incubated at 37°C in a 5% CO_2 incubator overnight, they were then treated as previously stated. Ensuing overnight incubation, all culture media was then removed from each well and replaced with 200µL of fresh-colorless culture medium; followed by the addition of 39µL of Cell-Clock Dye Reagent and an incubation period of 1h. The dye reagent and culture medium were then removed ensuing the 60 minutes incubation period and each well was washed with 200µL of warm. This subsequently removed and replaced with 200µL DMEM, the plate was then placed under a digital microscope and three images were taken per well for image processing using ImageJ softwear. The Cell-Clock assay functions by supplying a dye to the cells which, in turn, upon uptake; express it in different colors depending on the cycle in which they were prior to incubation. This permitted a color-change based analysis which helped distinguish between percentage of each cell with regards to the different cell cycle phases in which they were (Anon).

2.8 Statistical Analysis

A student T-test was carried out for in order to investigate whether there was a significant difference between the treated samples and the controls, differences were considered significant if $P<0.05$, this was represented with one star (*), P-value differences <0.01 are represented with two stars (**) whereas; P-value differences higher <0.001 are represented with three stars (***). This analysis was carried out for all method where applicable,.

3. Results

3.1 Cell viability Assay (MTS)

The results from the cell viability assay illustrated somewhat of a clear trend of a decline in cell viability with increasing digitoxin concentration. Figure 1 below was generated from the average of three different cell passages (n3) which were treated as biological replicates, the values obtained from each treated sample were normalized to the average of the control. There was a significant difference between the control samples and samples treated with 25, 40 and 100nM of digitoxin as illustrated with the stars in figure 1. The P-value at 25nM was 0.090, at 40nM 0.014 and at 100nM

0.011, this indicates that at the digitoxin concentration approaching 100nM, there were less cells remaining to bio-reduce MTS to formazan dye.

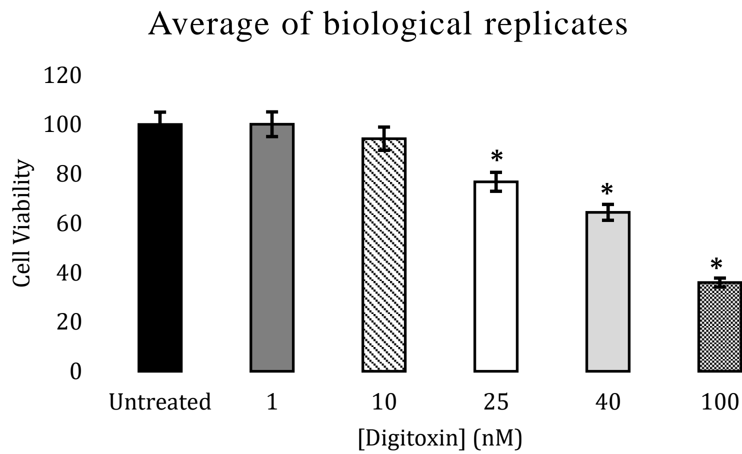

Figure1. Changes in cell viability of PANC-1 cells ensuing a 48h of digitoxin treatment period, each bar represents (N3), the errors bars indicate the SME of the samples. The mean was calculated in order to obtain the overall change in cell viability and the standard deviation examined how scattered the data were from the mean. The stars represent the significant difference in P-value between the control and treated samples.

3.2 Lactate Assay

The effect that different concentrations of digitoxin have on panc-1, with regards to the rate of aerobic glycolysis, is represented in figure 2. The analysis measured extracellularly-produced lactate at different time intervals (24h; grey line and 48h; black line) ensuing digitoxin treatment. It is evident that the production of lactate begins to reduce at 10nM as this was the concentration to which panc-1 were most sensitive. The initial lactate concentration at 48h was slightly higher than at 24h, however, the decrease trend seemed to be somewhat similar. A student T-test indicates that there was a significant difference at only 100nM which exhibited a P-value of 0,187 compared to the control which had a p-value of 0.971.

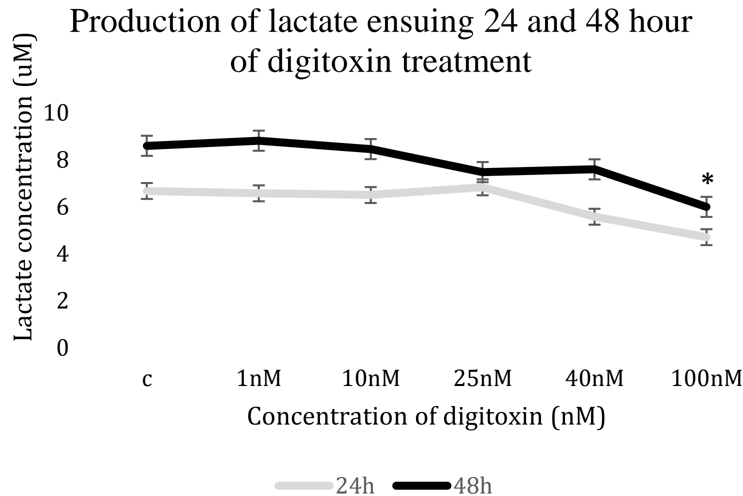

Figure 2. The amount of lactate (µM) produced by each treated and control samples with regards to the concentration of digitoxin (nM) with which they were subjected, is represented on the Y-axis. The Lactate production is measured in intervals of 24h and 48h ensuing digitoxin treatment. The error bars represent standard errors, the X-axis illustrates the different treatment groups including the control.

3.3 Gene expression

PDP1 was predominantly overexpressed in high concentrations of digitoxin compared to other genes, it exhibited a tremendous upregulation. However, this difference became evident around 10nM, at 100nM the difference was rather significant compared to other genes. Overall, most genes seemed to have been sensitive to 10nM of digitoxin except for *SLC2A*; as it illustrated a slight upregulation as the concentration increased compared to *LDHA* where the gene expression quickly stagnated directly after 10nM. *MYC* exhibited an upregulation from 1-10nM and a downregulation from 25nM and higher. *PFKM* exhibited a significant upregulation at 10nM compared to other concentration as illustrated in figure 3.

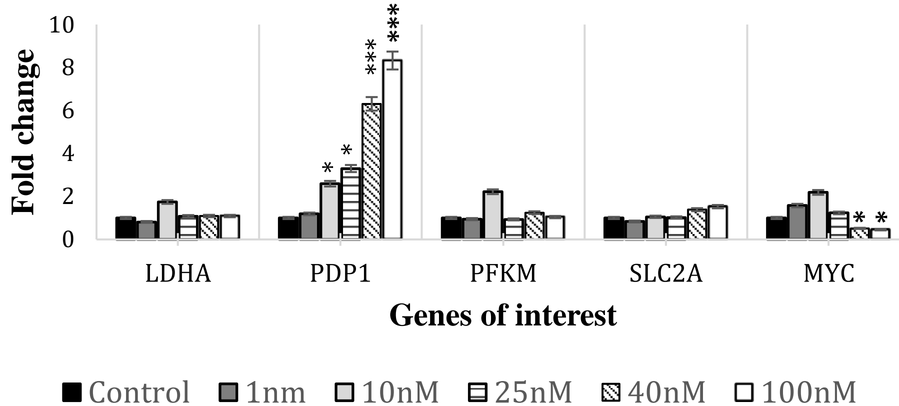

Figure 3. The expression of genes in treated and control samples with regards to the concentration of digitoxin with which they were subjected. The colored bars represent the different treatments for each gene and the expression of genes is illustrated on the X-axis; where each color/pattern represent the treated and control samples. The fold change is represented on the Y-axis which shows whether there have been an upregulation or downregulation in gene expression. The graph was plotted based on sample values which were obtained by normalizing the CT values of the aforementioned genes to that of the reference gene (PMM1) following the Delta CT method.

3.4 Cell cycle assay

At 100nM of dogitoxin, there was a tremendous decrease in cell viability which made it somewhat challenging to analyse, however, it is evident that there were more cells at the G0/G1 phase compared to the G2 and M phase. The control samples also illustrated that there were more cells in the G0/G1 phase of the cell cycle compared to the G2 and M phase, in fact, the number cells seemed to get less and less further down the cycle. From 1- 40nM there seemed to be roughly an equal number of cells at the G0/G1 and S phase, however, the number of cells at the G2 & M phase seemed to decrease with increasing digitoxin concentrations.

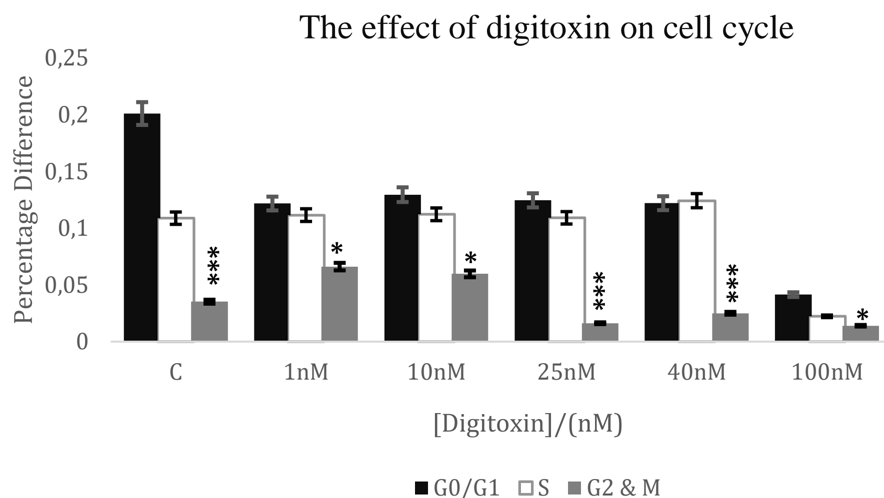

Figure 4. The percentage difference of cells within different cell cycles is represented on the Y-axis, whereas the different treatment groups are represented on the X-axis. The error bars represent the percentage errors whereas the colors represent the cell cycle phases in which the cells were upon assay completion. The graph represents the average percentage change of various digitalized images taken from different wells.

4. Discussion

PDAC yet remains one of the top 10 most prevalent and top five most common cause of cancer-related deaths solely in the United States, (Rhim & Stanger). Factors such as the poor prognosis with which it arises, low survival still rate, the manner in which it differs in its ability to metastasize, the variance in metastatic spread within patients and its ability to circumvent the treatments which are shown to ameliorate other malignancies rends into one of the most subtle yet perturbating disease (Bryant et al., 2014). Hope lies within the traces of reproducibly mutated genes which are said to be found in nearly 95% of all PDAC, this signifies that this malignancy depends on certain alterations at the molecular basis in order to develop (Bryant et al., 2014). Different analytic methods were applied in this study in order to examine how digitoxin affects glycolysis and gene expression in Panc-1 cell lines.
This included monitoring the viability of the aforementioned cells ensuing a subjection to different concentrations of digitoxin.

Specific genes of interest which take part in the glycolytic segment of panc-1 metabolism were analyzed in order to investigate whether the variance in digitoxin concentration had an impact in the expression of the selected genes; with hope to somewhat pinpoint the portion of metabolism which causes a reduction in lactate production. In order to analyze whether digitoxin also affects the Panc-1 cell cycles.

4.1 Cell Viability Assay (MTS)

As illustrated in figure 1, cell viability seemed to have been affected by digitoxin as predicted in the hypothesis. The reduction of viable cells had an impact on the amount of formazan produced, this reduction was an indirect indication that different concentrations of digitoxin lead to cellular apoptosis. In the current study, cell viability was less sensitive to 1nM of digitoxin, however, the color change and the recoded fluorescence indicated that the effect became somewhat evident around the concentration of 10nM and higher as illustrated in figure 1.
Previous findings suggest that MTS assays have several advantages including being water-soluble as well as its ability to produce less toxic compared to other assays such as MTT. However, factors such as incubation period, cell type and number are rather disadvantageous to the absorbance measurements at 490nm. To pile on, factors such as the ratio of MTS detection reagents to seeded cells are somewhat vital as they could influence the absorbance measurements, this leaves little to no room for pipetting errors (Wang, Henning & Heber)

4.2 Lactate Assay

Given that elevated concentrations of digitoxin was said to impair the production of lactate in panc-1 cells (Lindholm H, 2021, personal communication, 26 January), a lactate assay was conducted in order to examine to what degree this reduction was evident. In the current study, the concentration of lactate decreased with elevated concentrations of digitoxin, however, the significance of this decrease was only noticeable around 10nM. This somewhat indicates that, in this study, the cells were sensitive to 10nM with regards the amount of lactate they produced. Although this difference was apparent for both time intervals (24h & 48h), the results were not as expected. Figure 3 indicates that, although there was a decrease in lactate production ensuing the 48h digitoxin treatment, the overall production of lactate was significantly higher compared to the samples removed after 24h.

4.3 Gene expression

In previous studies, it was found that invitro induced overexpression of PDP1 in different cell lines (Panc2 and KP3) by transfection ORF encoding plasmid of the genes lead to a significant promotion of the growth rate in both cell lines; whereas the knockdown of PDP1 had an opposite effect (Li et al., 2020). In the current study, the findings seem to deviate from the expected function of digitoxin with regards to its ability to trigger cellular apoptosis. PDP1 seemed to continuously upregulate with high concentrations of digitoxin; signifying that the effect of digitoxin induces the growth of panc-1 cells. However, it is evident from the MTS assay (figure 1) that the increase in digitoxin concentrations reduces cell viability. Most genes remained unaffected, *MYC* which was expected to downregulate did indeed showed signs of downregulation after 10nand *LDHA* stagnated shortly after 10nM.

4.4 Cell-clock assay

The cell cycle assay indicated that most treatment groups did not make it to the G2 and M phase due to apoptosis. This can be linked with the MTS assay which illustrated a decrease in cell viability. Higher concentration of digitoxin seemed to have caused apoptosis before the cells to progress to later stages of the cycle.
All in all, digitoxin showed signs of apoptotic effect in most assays, however, in the gene expression PDP1 expressed unexpectedly.

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