THE METABOLIC PHENOTYPE OF PANCREATIC CANCER AND ITS LINK TO CYTOSOLIC CALCIUM HOMEOSTASIS AND SURVIVAL

A thesis submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Medical and Human Sciences

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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>3-BP</td>
<td>3-Bromopyruvate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic Acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible Factors</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetate</td>
</tr>
<tr>
<td>MOP</td>
<td>Mitochondrial Oxidative Phosphorylation</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium/Calcium Exchanger</td>
</tr>
<tr>
<td>NCKX</td>
<td>Sodium/Calcium-Potassium Exchanger</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>Oligo</td>
<td>Oligomycin</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate Kinase</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate Kinase M2 isoform</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma Membrane Calcium-ATPase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
</tr>
<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology and End Results Programme</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic Reticulum Calcium-ATPase</td>
</tr>
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ABSTRACT

The University of Manchester
Anthony Chan
Master of Philosophy

THE METABOLIC PHENOTYPE OF PANCREATIC CANCER AND ITS LINK TO CYTOSOLIC CALCIUM HOMEOSTASIS AND SURVIVAL
September 2012

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is an insidious and aggressive cancer characterised by poor survival rates. In cancer, there is a pathological switch in metabolism from mitochondrial oxidative phosphorylation to glycolysis, known as the Warburg effect. Cells depend on an ATP-driven plasma membrane Ca$^{2+}$ pump (PMCA) to maintain a low resting cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), high levels of which can produce cytotoxicity and cell death. The reliance of PDAC on glycolysis can be targeted by selective metabolic inhibitors. We hypothesize that disrupting the glycolytic ATP supply will impair PMCA, and its ability to maintain a low resting [Ca$^{2+}$]$_i$ that prevents cell death.

Methods & Materials
To measure the effect of glycolytic and mitochondrial inhibitors, we utilised an in situ PMCA activity Fura-2 assay to measure [Ca$^{2+}$]$_i$ clearance in the PDAC cell line, Panc-1. We also measured the effects of the inhibitors on intracellular ATP levels using bioluminescence assays, and cell necrosis rates using cell death assays.

Results
We firstly show that selective inhibition of glycolysis using 3-bromopyruvate (3-BP) induces cell necrosis in Panc-1 cells whereas inhibition of mitochondrial metabolism using carbonyl cyanide m-chlorophenyl hydrazone (CCCP) has no effect. Furthermore, we show that glycolytic inhibitors 3-BP and iodoacetate inhibits PMCA, and impairs the ability of the Panc-1 cells to maintain Ca$^{2+}$ homeostasis. Mitochondrial inhibitors CCCP and oligomycin has no effect on Ca$^{2+}$ clearance. Finally, we show that inhibition of glycolysis, but not mitochondrial metabolism, causes a significant reduction in intracellular ATP levels in Panc-1 cells.

Discussion
Our data suggests that an inhibition of the glycolytic ATP supply to PMCA in PDAC is an effective therapeutic target that could represent a new strategy for selectively killing PDAC cells and sparing normal, healthy cells.
Cancer of the pancreas is one of the most aggressive cancers responsible for over 250,000 deaths worldwide every year. It is usually diagnosed late, and as a result it is often too late to remove the tumour because either surgery is too dangerous or because it has already spread. As a result, only 1-3% of patients survive more than 5 years.

All cells in the body generate energy using one of two mechanisms – mitochondrial oxidative phosphorylation or glycolysis. Normally, mitochondria generate up to 90% of a normal cell’s energy requirements. In cancer however, there is a shift whereby glycolysis takes over and becomes the major source of energy. This is known as the Warburg effect. One of the uses of this energy is to maintain calcium at very low levels by using a calcium pump, which moves calcium across the cell membrane and out of the cell. If this pump is inhibited, the cell becomes overloaded with calcium, which is very toxic, and eventually leads to cell death.

The aim of this project is to test if inhibiting glycolysis in a pancreatic cancer cell has an effect on the calcium pump and the levels of calcium inside the cell. We also look at the effects of inhibiting glycolysis on cell death rates and on how it affects energy levels in the cell.

We found that inhibiting glycolysis profoundly affects the calcium pump, and the cell’s ability to maintain a low resting calcium level. Furthermore, by inhibiting mitochondrial oxidative phosphorylation, there was no effect on the pump. We also found that glycolytic inhibitors increased the rate of cell death, and significantly reduced the energy levels in the pancreatic cancer cells.

Our results suggest that inhibiting the main ATP supply in cancer (glycolysis) is an effective target that selectively kills pancreatic cancer cells, but at the same time sparing normal healthy cells that do not rely on glycolysis.
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INTRODUCTION

Background

Cancer is a disease defined by the World Health Organization as an 'uncontrolled growth and spread of cells', and is currently responsible for over 12% of all deaths worldwide. It carries a large disease burden for years of life lost due to death, and equivalent healthy years lost through living with disease.

Ductal adenocarcinoma of the pancreas (PDAC) is a particularly insidious cancer characterised by poor survival rates partly due to late presentation and subsequent poor staging at the time of diagnosis. It is the 13th most commonly occurring cancer with 250,000 deaths occurring worldwide, and accounts for 3.6% of all cancer deaths, and 0.5% of all deaths. The estimated DALYs (Disability Adjusted Life Years, defined as the number of productive years lost prematurely due to morbidity and mortality of a disease) lost due to pancreas cancer amounted to over 2 million years worldwide.

There are few large studies detailing pancreatic cancer epidemiology to the present date. We queried the Surveillance, Epidemiology and End Results (SEER) Programme – the largest cancer registry in the United States representing 15% of its population – for epidemiological data on pancreatic cancer in July 2011 using SEER*stat 7.0.4 software. The incidence of pancreatic cancer (Figure 1) between 1973 and 2008 averaged at 11.7 cases per 100,000, with no statistical increase over the time period (percentage change of 0.54%, annual percentage change of 0.04%, P = 0.51, weighted Least Squares method). The majority of cases occur in the head of the pancreas (48.6%), followed by the tail (8.4%) and body (7.7%). The prevailing histology as recorded by SEER is adenocarcinoma (77.6%), followed by mucinous (4.3%) and neuroendocrine (3.2%) tumours. This is similar to other previous epidemiology studies, who quote the majority of adenocarcinoma as being from a ductal origin.

The stage at which pancreatic cancer is diagnosed has worsened over time, with 9.95 cases per 100,000 people found with regional/distant spread in 2008 compared to just 7.35 cases per 100,000 in 1973. This is in contrast to other gastrointestinal cancers such as colorectal and gastric cancers which have decreased significantly over time, perhaps from the benefit of improved imaging and endoscopy, and screening programmes. We also show that survival from pancreatic cancer remains poor; the 5-year cancer-specific survival rate is 4.88% and this has not significantly changed over the last three decades.
Figure 1. Trend in Staging Incidence of Gastrointestinal Cancers between 1973 and 2008.
Pancreatic cancer is increasingly being diagnosed with late disease, despite advances in imaging technology and access to endoscopy. Oesophageal cancer also shows a similar trend, but gastric and colorectal cancer show declining rates of advanced disease at diagnosis (data from the SEER 17 Registry).

Pathophysiology of Cancer

The pathology of cancer involves normal cells changing to a premalignant state before turning into malignant cells that divide uncontrollably and prosper. The development of cancerous cells has been attributed to mutations in the genome, which subsequently lead to functional over-expression of ‘oncogenes’ that promote a cancer phenotype, or the under-expression of cancer-protective ‘tumour suppression genes’. These changes in gene expression ultimately affect the mechanisms of regulation for cell proliferation and apoptosis. It has been hypothesized that nearly all cancers establish 6 basic characteristics in order to be successfully viable: 1) self-sufficiency in growth signalling, 2) insensitivity to anti-growth signals and 3) an evasion to apoptosis, which in turn lead to 4) a limitless replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis.

Cancer cell function and physiology will therefore be in nature very different, and it has long been known that metabolism in cancer cells differs greatly from normal cells. Otto Warburg first published research detailing a fundamental difference in glucose utilisation where cancer cells exhibit high rates of aerobic glycolysis. The metabolic pathways are so altered as to predominantly favour division, growth and survival. Together with glutamine, alterations in glycolysis and the Krebs’ Cycle not only lead to a faster production of ATP, but also to the synthesis of lipids and amino acids essential for cancer progression. These pathological differences in cellular metabolism make the ‘Warburg Effect’ a target for developing an understanding and potential therapies against cancer.
The Metabolic Phenotype of Cancer & The Warburg Effect

All eukaryotic cells utilise glucose as the source from which it derives energy in the form of ATP for metabolism. In the process of glycolysis, 1 molecule of glucose is broken down over a series of 10 enzymatic steps to produce 2 molecules of pyruvate and 2 molecules net of ATP. Two molecules of the coenzyme NAD⁺ are also reduced to NADH in the process. Other intermediate monosaccharaides, such as fructose and galactose, can also enter (and drive) glycolysis at different points of the pathway.

In aerobic respiration, the pyruvate molecules enter the Krebs’ Cycle (also known as the Citric Acid Cycle, or the Tricarboxylic Acid Cycle) to be further oxidised to produce more ATP and NADH. Pyruvate is initially acetylated by pyruvate dehydrogenase to acetyl-CoA, and enters the Krebs Cycle by forming citrate with oxaloacetate. Several oxidative steps in the cycle convert three molecules of NAD⁺ to NADH, and also one molecule of guanosine triphosphate (GTP) and ubiquinol and two molecules of carbon dioxide. Oxidative phosphorylation then occurs in mitochondria to produce more ATP. The electron transport chain involves the potential energy stored in NADH from the Krebs’ Cycle to be released in a series of redox reactions. There are 4 protein complexes (I-IV) located on the inner mitochondrial membrane that oxidise NADH, and simultaneously translocating protons out of the mitochondrial matrix and into the inter-membrane space. The protons then move back into the matrix via the ATP synthase, and in doing so, each proton phosphorylates 1 molecule of ADP to ATP. Between 30 and 36 ATP molecules are generated by the process of oxidative phosphorylation from one molecule of glucose.

In the absence of an adequate oxygen supply required for glycolysis, anaerobic fermentation occurs whereby glycolysis is used to produce ATP quickly from glucose. The pyruvate is converted into lactic acid by reduction via the NADH, which is then converted into NAD⁺ for use in further glycolysis reactions. The total energy released per molecule of glucose in anaerobic respiration is 2 ATP molecules. Anaerobic respiration produces ATP at a much faster rate than aerobic respiration (by some 100 times), whereas respiration produces much more ATP per mole of glucose than fermentation (by about 18-fold)¹¹. These metabolic pathways are altered in cancer cells in order to meet the increasing demand (both in amount and pace) of ATP of uncontrollably dividing cells.

The Warburg Effect was first described in the 1920s by German physiologist Otto Warburg¹⁰, who postulated that the common cause of carcinogenesis was the “irreversible injuring of respiration”. Though experiments involving ascites cancer cells, Warburg observed that the ratio of mitochondrial respiration energy and anaerobic fermentation energy (from glycolysis) is much less in cancer cells compared with normal cells, with glycolysis generating 10% of ATP requirements in healthy cells to over 50% in cancer cells. In order for damaged cells to maintain their structure and function, the disrupted mitochondrial supply of ATP energy would be replaced by an anaerobic mechanism for energy. Warburg proposed that the “morphological inferiority” of this mechanism would change a highly differentiated cell into undifferentiated cells that can divide, grow and lead to cancer. Changes in carbohydrate metabolism have been found to be altered in many cancer cells¹²-¹⁴. Experiments by Reitzer et al. showed that 80% of glucose used by cells from the HeLa cell line undergoes glycolysis eventually gets fermented and is converted into lactic
acid; only about 5% enters the Krebs’ Cycle for oxidative phosphorylation. Glutamine has also shown to be metabolised to produce more than half the energy the cells require from oxidative phosphorylation. It has also been demonstrated by Rossignol et al that mitochondrial protein expressions are altered significantly for oxidative phosphorylation depending on the surrounding substrate (of either glucose or galactose) available to the cell. Warburg’s theoretical metabolic ‘switch’ occurs when a normal cell becomes cancerous.

The high concentration of glucose-6-phosphate seen in cancer cells is also not only used as a substrate for glycolysis and generate ATP, but can enter the pentose phosphate pathway to produce phosphoribosylpyrophosphate (a precursor for nucleic acids) and lipids. These can subsequently be used for cell growth and proliferation. This suggests that the Warburg Effect might not only be important during metabolism and ATP synthesis, but also in producing precursors for cell biosynthesis. Using glycolytic inhibitors would also target this mechanism.

**The Warburg Effect & Hypoxia**

Despite the Warburg effect occurring even in the presence of an adequate oxygen supply for aerobic respiration, it is recognised that hypoxia is one of the physiological stress factors in the tumour microenvironment that cause this shift. Hypoxia-inducible factor 1 (HIF-1), expressed in response to hypoxia, was demonstrated by Iyer et al. in 1997 as the main regulator of cellular oxygen homeostasis. When expressed, as well as up-regulating most glycolytic enzymes, HIF-1 up-regulates several genes including vascular endothelial growth factor (VEGF), a known promoter of tumour angiogenesis. It is currently a chemotherapeutical target by drugs such as bevacizumab (Avastin, Roche). HIF-1 has also been shown to induce glycolysis (by up-regulating hexokinase II, the first enzyme in the glycolysis pathway) and reduce mitochondrial aerobic metabolism by up-regulating pyruvate dehydrogenase kinase 1, responsible for inactivating the pyruvate dehydrogenase complex that subsequently stops the decarboxylation of pyruvate for entry into the Krebs cycle. The down-regulation of the MYC pathway is also thought to be partially responsible. The glucose transporter protein Glut1 has also been shown to be over-expressed via the HIF-1 pathway, facilitating the influx of glucose that can be used for glycolysis for rapid generation of ATP. HIF-1 has been found to be up-regulated in many cancers, including pancreatic, gastric, lung, breast and hepatic cancers. Several prognostic markers (in breast cancer) have been identified as a result.

**The Warburg Effect & Cell Death**

One of the characteristics of a cancer cell is to become more resistant to cell death, and this has been strongly linked to ATP levels. Eukaryotic cells die either from a controlled, self-mediated processed called ‘programmed cell death’ (apoptosis) or from irreparable damage which leads to necrosis. The process of apoptosis was first described in 1974 by Kerr et al from observations using an electron microscope, and occurs as a physiological process (such as during atrophy of the thymus gland in adults), or as a result of a pathological process. Apoptosis is ATP-dependent, and involves the separation from adjacent cells and the condensing and fragmenting of the cell into small ‘apoptotic bodies’. Both the nucleus and cytoplasm shrink with chromatin condensing within the nuclear envelop (pyknosis), with the nucleus breaking down and
fragmenting (karyorrhexis) to produce smaller ‘chromatin bodies’. Blebs form on the cell membrane, and eventually break off to form the apoptotic bodies. Surrounding phagocytes then engulf these bodies (efferocytosis), and degrade them enzymatically via lysosomal digestion.

The apoptotic process is orchestrated by a family of cysteine proteases called caspases, and begins with signalling (either extrinsically or intrinsically) to initiate the pathway. The mitochondrial apoptosis-induced channel (MAC) \(^{28}\) allows cytochrome C to be released into the cytoplasm, which binds with Apaf-1 (an apoptotic protease activating factor) and ATP, which then form a complex with pro-caspase-9, called apoptosome. This in turn cleaves pro-caspase-9 which activates caspase-9 \(^{39}\). The Bcl-2 (B-cell lymphoma 2) family of proteins act as regulators of apoptosis, exerting pro-apoptosis (such as Bax and Bak) and anti-apoptosis effects (such as Bcl-2 and Bcl-xl) \(^{30}\). An influx of Ca\(^{2+}\) is thought to be one stimulus that triggers apoptosis by inducing cytochrome C release \(^{31}\). Other direct initiators of apoptosis include the tumour necrosis factor (TNF) and Fas receptors which activate caspase-3 and -7 \(^{29}\). The CPP32/Yama(-like) proteases in apoptosis induced by the Fas pathway have been shown to be ATP-dependent \(^{32}\). Disruption of mitochondrial pathways has been linked to a suppression of apoptosis and resulting in unopposed and uncontrollable division \(^{33}\).

Necrosis is, by contrast, cell death that is not coordinated. A variety of noxious insults to the cell can cause necrosis, including hypoxia (coagulative necrosis) and bacteria / inflammatory processes (liquifactive necrosis). Lysozymes are released within cytoplasm of the cell, which disrupt its homeostatic processes, causing the cell to swell and burst, releasing its potentially toxic contents into surrounding healthy cells.

There is evidence that apoptosis and necrosis share (in parts) a common pathway in that inhibitors of Bcl-2 and Bcl-xl reduce the rate of both modes of cell death \(^{34}\). Both have been shown to be ATP-dependent but the mode of death is thought to be determined by intracellular ATP levels; in cancer cells, ATP levels are reduced, which cause increased rates of necrosis and an inhibition of apoptosis \(^{32}\). It has been shown that an influx of Ca\(^{2+}\) into the mitochondria (through the mitochondrial Ca\(^{2+}\) uniporter) releases cytochrome C, which diffuses across the cytoplasm and binds to the Ins\((1,2,5)P_3\) receptors \(^{31}\). Ca\(^{2+}\) readily diffuses through the outer mitochondrial membrane, and passes across the inner membrane through ion channels and protein transporters via highly selective Ca\(^{2+}\) channel (mitochondrial Ca\(^{2+}\) uniporter). Cytochrome C acts as an agonist, releasing the Ca\(^{2+}\) from the ER into the cytoplasm. This has a positive feedback effect, with all mitochondria in the cell releasing cytochrome C, which then act to produce the apoptosome and subsequent activation of the caspase pathway \(^{31}\). Bik, a member of the Bcl-2 family of apoptotic regulators, has been shown to enhance Ca\(^{2+}\) release through the Ins\((1,2,5)P_3\) receptor. An overload of cytosolic Ca\(^{2+}\) also leads to cell death mainly by the activation of calcineurin, a calmodulin-dependent phosphatase which promotes apoptosis \(^{35}\). Calcineurin dephosphorylates a member of the Bcl-2 family, BAD, which is known to be pro-apoptotic. Ca\(^{2+}\) is also a co-factor in dehydrogenase enzyme reactions in the Krebs’ Cycle which serve to produce ATP \(^{36}\).
Calcium & Cancer

Ca\(^{2+}\) is strongly involved in the regulation of cellular function, including cell death. Ca\(^{2+}\) signalling also plays an important role in the cell cycle, and it has been shown that [Ca\(^{2+}\)]\(_i\) is low during the G1 phase, and rises steadily prior to the S phase before falling again after reaching G2\(^{37}\). Ca\(^{2+}\) also regulates a diverse range of functions including, but not limited to, gene transcription, secretion, cellular contraction and muscular tone as well as neurotransmission and cell excitation. The importance of Ca\(^{2+}\) in cell signalling and action necessitates several mechanisms for Ca\(^{2+}\) homeostasis. A signalling ‘toolkit’ (comprising of several transporters and channels) tightly controls the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) moving Ca\(^{2+}\) into the cytoplasm of the cell during the ‘on’ reaction and out during the ‘off’ reaction (figure 2)\(^{38}\). The amount of Ca\(^{2+}\) that binds to Ca\(^{2+}\)-dependent effectors therefore depends on the cytosolic concentration controlled by the toolkit.

**Figure 2. Cellular Calcium Homeostasis (adapted from Berridge et al, 2007)**

Calcium homeostasis involves several mechanisms including ATPase pumps on the plasma membrane (PMCA) and on the sarcoplasmic reticulum (SERCA) to move calcium out of the cell cytosol against its concentration gradient if the intracellular concentration is too high. Sodium/calcium exchangers (NCX) also move calcium out of the cytosol and into the mitochondria. Calcium can be released into the cytosol from intracellular stores or from the extracellular compartment via voltage-operated channels on the cell membrane.

During the ‘off’ reaction, Ca\(^{2+}\) is removed from the cytoplasm by a variety of transporters located on the plasma membrane, its ER and the mitochondria within. Extracellular and ER Ca\(^{2+}\) is also constantly leaking into the cell cytoplasm along its electrochemical gradient. ATPase pumps located on the plasma membranes (Plasma Membrane Ca\(^{2+}\) ATPase, PMCA) actively moves 1 Ca\(^{2+}\) out of the cell hydrolysing 1 ATP in the process\(^{39}\). The PMCA is responsible for maintaining a low resting [Ca\(^{2+}\)]\(_i\). ATPase pumps located on the SR membrane (Sarcoplasmic Endoplasmic Reticulum Ca\(^{2+}\) ATPase, SERCA) actively moves 2 Ca\(^{2+}\) cations out of the cytoplasm of the cell into the ER per 1 ATP molecule. Ca\(^{2+}\) homeostasis by PMCA is therefore an ATP dependent process rather than one relying on ion stoichiometry. Other secondary exchangers also play a role in Ca\(^{2+}\) homeostasis, including the Sodium/Calcium exchanger (NCX) on the cell membrane that move 1 Ca\(^{2+}\) out for every 3 Na\(^{+}\) into the cytoplasm. NCX indirectly relies on the ATP dependent...
Na⁺/K⁺ ATPase pump. Uniporters on the mitochondria can also rapidly sequester Ca²⁺ which can later be slowly released back into the cytoplasm to be extruded by the PMCA or SERCA.

During the ‘on’ reaction, stimuli such as membrane depolarisations, agonists and intracellular messengers, increase extracellular Ca²⁺ entry and Ca²⁺ release from intracellular stores (ER and, muscles, SR). Ca²⁺ channels on the cell membrane, such as voltage-operated channels (found on excitable cells), receptor-operated channels (ROC), which open in response to certain stimuli (such as N-methyl-D-aspartate receptors sensitive to glutamate, and nicotinic acetylcholine receptors sensitive to ACh) and secondary-messenger-operated channels also open to increase [Ca²⁺]. Ca²⁺ is also released from intracellular stores such as the ER stimulated via inositol-1,4,5-triphosphate (Ins(1,4,5)P₃) receptors⁴⁰. Store-operated Ca²⁺ entry also plays a role in Ca²⁺ homeostasis. Once depleted of internal stores of Ca²⁺, there is an influx of extracellular Ca²⁺ into the cytoplasm by an as of yet unexplained messenger mechanism.

PMCA is present in every eukaryotic cell, and (together with NCX) forms the major regulator for cellular Ca²⁺ homeostasis which is dependent on ATP. The enzymes involved in glycolysis are bound to the plasma membrane of the cell itself and with the SR⁴¹, providing a ready supply of ATP to nearby transporters such as PMCA. It has been shown that PMCA preferentially utilises ATP produced from glycolysis occurring at these site at twice the rate compared to ATP in the cytoplasm⁴².

Four different isoforms of PMCA have been identified⁴³ with differences in distribution and expression, and in function with differing affinity to calmodulin. PMCA-1 and -4 are found in all cells, whereas PMCA-2 and -3 are found in the brain and heart. Different isoforms of PMCA have been found to be up-regulated in cancer cells, suggesting an alteration in Ca²⁺ homeostasis. A link between PMCA and cell death has been suggested, with caspases cleaving and inactivating PMCA in cells undergoing apoptosis, resulting in cellular overload of Ca²⁺; non-cleavable PMCA mutants slow down the process of cell apoptosis and leads instead to necrosis⁴⁴. PMCA-4 is up-regulated in differentiated colon cancer cells (HT-29)⁴⁵, and it has been shown that by inhibiting PMCA-4 receptors using small interfering RNA, there was a reduced rate of cell proliferation (although not cell death)⁴⁶. PMCA-2 has been shown to be over-expressed in breast cancer cells (more than 100-fold in some cell lines) as well as PMCA-4⁴⁷, the inhibition of which did not induce cell death⁴⁸.

The Present Study

The Warburg effect is a clear differentiation in the metabolic profile between normal and cancer cells, whereby glycolysis is the main ATP-producing pathway in cancer cells compared to normal cells that predominantly utilise mitochondrial oxidative phosphorylation. This presents a potential therapeutic target for selectively killing cancer cells and sparing normal cells. Furthermore, cells require a low resting intracellular Ca²⁺ concentration as to prevent cytotoxicity and cell death. PMCA is the main mechanism responsible for maintaining this low Ca²⁺ concentration and is itself dependent on ATP.
HYPOTHESIS

The Warburg effect implies that PDAC cells exhibit a switch from a mitochondrial to a glycolytic metabolic phenotype. This provides a rapid and ready source of ATP for PMCA to maintain a low $[\text{Ca}^{2+}]$, to prevent cell death. We hypothesize that inhibiting the glycolytic source of ATP in PDAC will inhibit PMCA activity and cause a reduction in $\text{Ca}^{2+}$ clearance leading to cell death.

The current study aims to measure the effects of metabolic inhibitors on PDAC cell viability, PMCA activity and the rate of $\text{Ca}^{2+}$ clearance, and the concentration of intracellular ATP.
MATERIAL AND METHODS

Preparation of Reagents

HEPES-buffered physiological saline solution (HEPES-PSS) (composition (mM): 10 HEPES, 137 Na⁺, 4.7 K⁺, 0.56 Mg²⁺, 1.28 Ca²⁺ and 5.5 glucose, with a pH of 7.4) was used in all the experiments performed. For the Ca²⁺ clearance assays, high concentration Ca²⁺ HEPES-PSS (HEPES-PSS-Ca²⁺) (composition (mM): 10 HEPES, 137 Na⁺, 4.7 K⁺, 0.56 Mg²⁺, 20 Ca²⁺ and 5.5 glucose, with a pH of 7.4) was used to increase [Ca²⁺]; prior to measuring the clearance rate. Reagents 3-BP, IAA and oligomycin were prepared using deionised water (Milli-Q Ultrapure Water System). CCCP (Sigma-Aldrich), cyclopiazonic acid (CPA, Tocris) and Fura-2-acetoxymethyl ester (Fura-2AM, Invitrogen Life Sciences) were dissolved and prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich Company) as per the manufacturer’s instructions. All reagents were brought up to room temperature prior to use in experiments.

Metabolic Inhibitors

Four mechanistically distinct metabolic inhibitors were used. Two inhibitors of glycolysis were chosen; 3-Bromopyruvate (3-BP) is a derivative of pyruvic acid that selectively inhibits hexokinase II to prevent the conversion of glucose to glucose-6-phosphate in the first step of glycolysis. Iodoacetate (IAA) is an alkylating agent that inhibits glyceraldehyde phosphate dehydrogenase to inhibit the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the proximal half of glycolysis. Two inhibitors of mitochondrial oxidative phosphorylation were also chosen; Oligomycin is a macrolide that inhibits ATP synthase, the enzyme between the mitochondrial membrane and its matrix that converts ADP and phosphate ions to ATP. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is a protonophore that dissipates the H⁺ concentration gradient of the mitochondrial membrane, and uncouples oxidative phosphorylation from ATP synthase.

Cell Culture & Isolation

The human pancreas cancer cell lines Panc-1 and MIA PaCa-2 were chosen because they are both derived from pancreatic ductal adenocarcinomas. Panc-1 cells were originally cultured from a 56 year old Caucasian male who developed a regionally invasive adenocarcinoma of the head of the pancreas with spread to at least one lymph node. MIA PaCa-2 cells were originally cultured from a 65 year old Caucasian male who developed a regionally invasive adenocarcinoma of the body and tail of pancreas with spread to the periaortic area. MIA PaCa-2 cells have been found to grow and colonise more rapidly than Panc-1 cells, suggesting a more aggressive cancer phenotype.

Panc-1 and MIA PaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia USA) and in Dulbecco’s Modified Eagles Medium (DMEM 6429; composition: 4500 mg/L glucose, 30.8 mM L-glutamine, 40.9 mM sodium pyruvate and 53.6 mM sodium bicarbonate) supplemented with 10% foetal bovine serum and 10% penicillin/streptomycin (Sigma-Aldrich Company, Dorset, UK). Both cell lines were incubated in T-75 ventilated flasks (Corning, NY, USA) in a humidified environment of 37°C and 5% carbon dioxide. Cells were split at a
confluence of 70%, and a passage number of less than 30 were maintained. For the cell death assays, Panc-1 cells were seeded into black clear-bottom polystyrene 96-well microplates (Corning), and cultured in 50 µL of DMEM to achieve a confluence of 70%. For the Ca²⁺ imaging experiments, cells were seeded and allowed to adhere onto 16mm diameter borosilicate glass slides (VWR International Limited, UK) pre-sterilised with 70% ethanol and placed in a 6-well cell culture plate (Corning, NY, USA) and grown to achieve a cell confluence between 30 and 60%. For the ATP bioluminescence experiments, Panc-1 and Mia-PaCa-2 cultured cells were seeded into white luminescence-compatible clear-bottom polystyrene 96-well microplates (Corning) and cultured in 50 µL of DMEM to achieve a confluence of 70%.

**Cell Death Assays**

After seeding into compatible 96-well plates, Panc-1 cells were treated with 50µL of DMEM (control), 3-BP (100 µM, 300 µM and 1000 µM), CCCP (1 µM, 3 µM and 10 µM of CCCP) or a combination of 3-BP (300 µM) and CCCP (3 µM) for 30 minutes, 3 hours and 6 hours. Separate wells were also used measuring propidium iodide as a control to test for leakage. The cells were then stained with Hoechst (11µL of a 1 in 50 dilution of 10mg/ml, Invitrogen), and with propidium iodide (11µL, 1 in 100 dilution of 1mg/ml, Fluka). The plate was then incubated for 30 minutes before cell counting using an IN Cell Analyzer 2000 (General Electric Company, Fairfield, USA). Proprietary software was used to collect data, which was then analysed using Microsoft Excel 2010 (Microsoft, Richmond USA). Two-way ANOVA analysis was carried out with a Bonferroni post-test to compare each result with their respective control. A p-value of less than 0.05 was considered significant. The high-content assays in this part of the project were outsourced to an external company, Imagen Biotech (University of Manchester, UK).

**Ca²⁺ imaging with Fura-2**

Coverslips seeded with Panc-1 cells were removed from the 6-well cell culture plate, washed with HEPES-PSS, and placed into a 30mm diameter polystyrene petri dish containing 1 ml of HEPES-PSS with 5µM Fura-2AM (Invitrogen Life Technologies, NY, USA) in 50:50 DMSO (Sigma-Aldrich Company, Dorset, UK), with 20% pluronic acid (Invitrogen Life Technologies, NY, USA). The cells were allowed to load for 30 minutes at room temperature in zero-light conditions on an orbital shaker set to 30 rpm (Stuart Mini Orbital Shaker, Bibby Scientific Limited, UK). The cells were then washed with and immersed in HEPES-PSS for a further 30 minutes at room temperature to allow any remaining non-de-esterified Fura-2-AM to leak out of the cell and then equilibrate; the cells were then washed with HEPES-PSS again before imaging.

A gravity-fed perfusion chamber was set up with the coverslip mounted at the base, and continually perfused with HEPES-PSS from an automatic value system which allowed for fast switching and perfusion of test solutions (Harvard Apparatus, UK). A vacuum-assisted negative pressure circuit was set up using a vacuum pump (Vacuumrand ME2 vacuum system) to allow continual removal of the perfusion solution. The cells were imaged on a Nikon Eclipse TE2000-S inverted microscope (Nikon Instruments Inc, UK) using a 40X magnification 1.3 S Flour oil-immersion objective lens and images are captured with a CoolSNAP HQ charge-coupled device camera (Roper Scientific Photometrics, US). Cells were illuminated with a Cairn Optoscan
monochromater (Cairn Research, UK), and excitation light was separated from emission light using a fura-2 dichroic filter. MetaFluor Florescence Ratio Imaging Software v6.3r1 (Molecular Devices) was used to coordinate and capture background-subtracted regions of interest on the coverslip covering a manually-selected area representing a single cell at a rate of 0.2Hz. Excitation wavelengths (340 and 380 nm) were captured at a resolution of 232 x 173 pixels (6 by 6 binning), and the resulting 340/380 ratio calculated and recorded by MetaFluor.

The data from MetaFluor was exported to Microsoft Excel 2010 (Microsoft Corporation, Richmond USA) and graphed, before being analysed with Prism GraphPad 5.0.1 (California, USA). Statistical analysis comparing the secondary clearance phase to the time-matched controls was done using the Mann-Whitney U test. A p-value of less than 0.05 was deemed significant.

**ATP Bioluminescence Assays**

Total cellular ATP was measured using a firefly luciferase-based luminescence kit (Lonza ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit, Lonza Group Ltd, Basel, Switzerland). Bioluminescence is produced when luciferin is oxidised in the presence of ATP and luciferase to oxyluciferin:

\[
\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\text{Mg}^{2+}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \text{LIGHT}
\]

Each well was washed with and immersed in 50 µL of HEPES-PSS. Another 50µL of HEPES containing no inhibitor (control), 3-BP (500 µM), IAA (5 mM), CCCP (10 µM), oligomycin (10 µM) or a combination of 3-BP with CCCP, IAA with oligomycin or all four inhibitors (positive control), was added to each dish, and left to incubate for 30 minutes at 37°C. All reagents in the BioAssay Kit and the culture plate were brought up to room temperature, and 50µL of Cell Lysis Reagent (supplied) was added to each well and left at room temperature for 10 minutes. The ATP Monitoring Reagent Plus (supplied) was reconstituted with the Assay Buffer (supplied), and 100µL was added to each well and left to incubate for 2 minutes again at room temperature. A Synergy HT Multi-Mode Microplate Reader (BioTek, Vermont USA) was programmed to take a 1 second integrated reading of the bioluminescence level of each well. The data were collected using Gen5 Analysis (BioTek, Vermont USA) software before being exported to Microsoft Excel 2010 (Microsoft Corporation, Richmond USA) and analysed with Prism GraphPad 5.0.1 (California, USA).

The positive control levels were subtracted from each inhibitor/combination, and normalised to the negative control of that plate. An unpaired, two-tailed T-Test was used to compare the ATP-levels, with a P-value of less than 0.05 deemed as significant.
RESULTS

Inhibition of glycolysis induces necrotic cell death in Panc-1 cells

To test the effects of glycolytic and mitochondrial metabolic inhibitors on Panc-1 cell viability, high-throughput cell death assays were performed. Panc-1 cells were treated with 3-BP and CCCP at a variety of concentrations and time points as detailed in the Methods section (n = 3). The percentage of cells stained with propidium iodide after treatment represents the proportion of cell death by necrosis. DMEM media was used as a negative control, and a cocktail of 500 µM 3-BP and 3 µM CCCP was used to provide the positive control.

Low concentrations of 3-BP did not increase the rate of cell necrosis; treatment with 100 µM of 3-BP failed to produce a positive response even after 6 hours (Figure 3). When Panc-1 was treated with 300 µM of 3-BP, there is an increase in cell necrosis at 3 hours (p < 0.01) and 6 hours (p < 0.001). The same is seen with 1000 µM of 3-BP. This suggests both a time-dependent and dose-dependent response to 3-BP. On the other hand the mitochondrial inhibitor CCCP had no effect on cell viability at any concentration or time point, suggesting that CCCP does not induce necrosis.

There is an increase in cell necrosis following treatment with a combination of 100 µM 3-BP and 3 µM CCCP only after 6 hours (P < 0.05). As expected, increasing the concentration of 3-BP to 500 µM together with 3 µM CCCP increases the rate of cell necrosis, with a statistical difference seen after 3 hours (p < 0.001). No increased rate of necrosis is seen, however, when adding 3 µM of CCCP to 100 µM of 3-BP (p < 0.05 even after 6 hours).

Figure 3. Cell Death Assays on Panc-1 Cells following Treatment with Glycolytic and Mitochondrial Inhibitors.
Panc-1 cells were treated with 3-BP (A), CCCP (B) and a combination of inhibitors (C) and stained with Hoechst (cell count) and PI (necrosis) at different time points (n = 3). Abbreviations: 3-BP, 3-Bromopyruvate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; PI, propidium iodide.
Validation of calcium clearance assays

The optimum loading conditions for the Panc-1 cells were found to be 5µM Fura-2AM in DMSO with 20% pluronic acid, loaded for 30 minutes at room temperature, and de-esterified for a further 30 minutes with HEPES-PSS. Under these conditions, good fluorescent signals were captured and measured using the methods described previously.

To test whether metabolic inhibitors had any effect on the PMCA and the rate of Ca\textsuperscript{2+} clearance, in situ [Ca\textsuperscript{2+}] clearance assays were utilised as previously described\textsuperscript{53}. Briefly, the cells were initially perfused with HEPES-PSS containing 0 mM Ca\textsuperscript{2+} (and 1mM of EGTA), and the ratio of 340/380 wavelengths at this stage represented the resting [Ca\textsuperscript{2+}]. The cells were then treated with 30 µM of CPA, an inhibitor of SERCA Ca\textsuperscript{2+}-ATPase, causing a transient rise in [Ca\textsuperscript{2+}], as Ca\textsuperscript{2+} leaked out of the ER. Eventually [Ca\textsuperscript{2+}] returned to normal homeostatic levels as the PMCA extruded the Ca\textsuperscript{2+} out of the cell. The rise in [Ca\textsuperscript{2+}], is shown as an increase in the 340/380 ratio, as more Ca\textsuperscript{2+} binds to the fura-2. All perfusion solutions from this point contain CPA. The cells were then perfused with HEPES-PSS-Ca\textsuperscript{2+} (containing 20 mM Ca\textsuperscript{2+}) to cause a rapid increase in [Ca\textsuperscript{2+}]; a sudden rise in 340/380 ratio is seen at this point. As the ratio reached a peak, the cells are then perfused with HEPES-PSS containing no Ca\textsuperscript{2+}, causing rapid clearance of Ca\textsuperscript{2+} that is presumably due to the PMCA and producing a fall in the 340/380 ratio. A paired experimental design was used whereby two clearance phases were performed concurrently. The first clearance phase acted as a control to which the effects of the inhibitor in the second phase can be compared to and normalised against. This was then compared with previous time-matched controls where no inhibitor is used in the second clearance phase.

A time-matched control is shown in Figure 4. The rate of Ca\textsuperscript{2+} clearance is calculated as a function of time and determined from the falling phase part of the curve. The rate can be calculated either by fitting a single exponential decay model to the curve, or by calculating the linear gradient at a single point in the curve. We utilised the linear gradient method previously described\textsuperscript{54} as it has been found that fitting an exponential decay model becomes unreliable at very slow clearance rates, or if Ca\textsuperscript{2+} does not recover to the same resting value\textsuperscript{53}. The maximum linear rate is measurable from the same standardised starting value and the second clearance rate (R2) is normalised to the first clearance rate (R1) and expressed as a percentage (\(\frac{R2}{R1} \times 100\%\)).
The ratio of 340 and 380nm wavelengths is measured and represents the relative concentration of intracellular calcium ([Ca\textsuperscript{2+}]). The assay begins with the addition of cyclopiazonic acid (1), which inhibits the SERCA pump and releases ER stores of Ca\textsuperscript{2+}. A small rise in [Ca\textsuperscript{2+}] is seen, but falls quickly as the PMCA functions to restore [Ca\textsuperscript{2+}]. Perfusion of high calcium HEPES (2) causes a rise in [Ca\textsuperscript{2+}] to a plateau level (3); at this point, zero calcium HEPES is perfused. PMCA then functions to reduce (4) and restore the [Ca\textsuperscript{2+}]. The metabolic inhibitor is added (5), and high calcium HEPES is perfused (6) again to see a sharp rise in [Ca\textsuperscript{2+}]. At the plateau level (7), zero calcium is perfused, and the PMCA then restores the [Ca\textsuperscript{2+}] (8). The effect of the metabolic inhibitor on the calcium clearance rate will be seen as a difference between the gradients at (4) and (8).

It has previously been shown that PMCA is the major Ca\textsuperscript{2+} clearance mechanism when SERCA is inhibited\textsuperscript{55}. NCX is particularly important in Ca\textsuperscript{2+} homeostasis in excitable cells, notably in muscle cells and neurons\textsuperscript{56}, but it has been reported to be expressed in pancreatic ductal cells from which PDAC cells are derived\textsuperscript{57}. It was therefore necessary to test whether NCX has any functional role in PANC cells and thus contribute to the Ca\textsuperscript{2+} clearance under the conditions of our Ca\textsuperscript{2+} clearance assay. An \textit{in situ} Ca\textsuperscript{2+} clearance assay were performed (n = 4) with all the Na\textsuperscript{+} in the perfusion solutions replaced in the second clearance phase with eqimolar NMDG which maintains the cellular osmotic and electrochemical gradient, but cannot be utilised in NCX (Figure 5). We found no significant effect on the Ca\textsuperscript{2+} clearance (Two-tailed Mann-Whitney U test, \(p = 0.8857\)), and the results of this assay implied that NCX does not play a significant part in Ca\textsuperscript{2+} homeostasis in Panc-1 cells.
Inhibition of glycolysis, but not mitochondrial oxidative phosphorylation, causes a reduction in functional PMCA activity in Panc-1 cells

The effect of the glycolytic inhibitors 3-BP and IAA were tested on the Ca^{2+} clearance rate of the Panc-1 cells (Figure 6B, 6C respectively). The glycolytic inhibitors 3-BP (500 µM) and IAA (5 mM) significantly reduced the relative clearance from 107.65% (time-matched control) to 72 ± 2.4% (p < 0.029) and 5mM IAA was 56 ± 4.5% (p = 0.029) respectively (n = 4).

The representative traces for both inhibitors show that the resting [Ca^{2+}] does not return to the baseline after treatment with the inhibitors, even after prolonged perfusion with zero Ca^{2+} solutions. A combination of 500 µM 3-BP and 10 µM CCCP also caused significant reduced Ca^{2+} clearance rate of the Panc-1 cells to 58.4 ± 8.4% (Mann-Whitney two tail test, p=0.0286).

Conversely, the mitochondrial inhibitors CCCP (10 µM) and oligomycin (10 µM) when applied alone did not have any effect on Ca^{2+} clearance in Panc-1 cells (Figure 6D, 6E). This suggests that PMCA activity is highly dependent on glycolytic metabolism for ATP for which PMCA is dependent on.
Figure 6. Inhibition of Glycolysis, but not Mitochondrial Metabolism, inhibits PMCA Activity
Inhibition of glycolysis, but not mitochondrial metabolism, inhibits PMCA activity to significantly reduce Ca\(^{2+}\) clearance rates in Panc-1 cells. In situ Ca\(^{2+}\) clearance assays were performed to test the effects of glycolytic inhibitors 500 µM 3-BP (B) and 5 mM IAA (C) and mitochondrial inhibitors 10 µM oligomycin (E) and 10 µM CCCP (D) on Ca\(^{2+}\) clearance, and a combination of 500 µM 3-BP and 10 µM CCCP (F) (n = 4). Panc-1 cells were initially perfused with HEPES-PSS with zero calcium before adding HEPES-PSS-Ca\(^{2+}\) to sharply increase [Ca\(^{2+}\)]. The inhibitor was added before the second clearance phase. The linear rate for the second clearance phase was normalised to the first phase. The clearance rates were then compared to previous time-matched controls (A) using the Mann-Whitney U Test. * indicates a statistically significant result (p < 0.05). Abbreviations: HEPES-PSS, HEPES-physiological saline solution; HEPES-PSS-Ca\(^{2+}\), HEPES-PSS with 20mM of Ca\(^{2+}\); 3-BP, 3-bromopyruvate; IAA, iodoacetate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone.

Inhibition of glycolysis, but not mitochondrial oxidative phosphorylation, causes a reduction in intracellular ATP levels in Panc-1 and MIA-PaCa-2 cells
We have shown that glycolytic inhibitors induce necrosis and reduce functional PMCA activity impairing intracellular Ca\(^{2+}\) clearance. As PMCA is dependent on ATP, we investigated the effect of the metabolic inhibitors on intracellular ATP levels. Panc-1 and MIA-PaCa-2 cells were treated with separately with 3-BP (500 µM), IAA (5 mM), CCCP (10 µM) or oligomycin (10 µM), and
with a combination of inhibitors (3-BP with CCCP, and oligomycin with IAA) (n = 3). For both Panc-1 (Figure 7A) and MIA-PaCa-2 (Figure 7B), 3-BP significantly reduced the intracellular ATP levels to 0.82% ± 0.39% and 0.48% ± 0.36% respectively (unpaired T-test, 2-tailed, p < 0.05). Using a combination of glycolytic and mitochondrial inhibitors also produced a significant fall in ATP levels.

There was no change in ATP levels in Panc-1 or MIA-PaCa-2 cells after treatment with either mitochondrial inhibitor. This provides further evidence that both cell lines are dependent on the glycolysis pathway for ATP generation. Interestingly, in these experiments, IAA caused no reduction in intracellular ATP levels in either Panc-1 or MIA-PaCa-2 cells. Subsequent repeat experiments in our lab, however, have shown a significant reduction (of 20 – 25%) in intracellular ATP using fresh IAA in the 1 mM range.

Figure 7. Glycolytic Inhibitors Reduce Intracellular ATP Levels in both Panc-1 and MIA-PaCa-2 cells
ATP levels are shown after 30 minutes of treatment with metabolic inhibitors (n = 3). Data were normalised by subtracting the raw counts obtained with a cocktail of inhibitors (positive control) and expressed as a percentage of the untreated control. Error bars represent the S.E.M. * indicates a statistically significant difference in ATP levels (unpaired T-test, 2-tailed).

Abbreviations: 3-BP, 3-bromopyruvate; IAA, iodoacetate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone.

We show that mitochondrial inhibitors may not act synergistically with glycolytic inhibitors to reduce intracellular ATP levels. Glycolytic inhibitors in isolation and in combination with mitochondrial inhibitors significantly reduce ATP levels in both Panc-1 and MIA-PaCa-2 cells. We also show that adding a mitochondrial inhibitor to a glycolytic inhibitor does not always produce a greater reduction in intracellular ATP. Adding a mitochondrial inhibitor CCCP to 3-BP does not cause any further reduction in ATP, suggesting that 3-BP alone causes maximal ATP depletion. This is in contrast to experiments involving IAA, where we show IAA on its own does not significantly reduce intracellular ATP levels in either Panc-1 or MIA-PaCa-2 cells, but the addition of the oligomycin (which on its own again does not significantly reduce ATP levels) does cause a statistically significant reduction. This provides further evidence that certain glycolytic inhibitors inhibit crucial parts of glycolysis whereas which cannot be compensated by other mechanisms. For those where these compensatory mechanisms are not impaired, adequate amounts of ATP can still be produced; parts of, or all, of these mechanisms may involve mitochondrial metabolism.
DISCUSSION

Pancreatic cancer accounts for 3.6% of all cancer deaths\textsuperscript{2}. Diagnosis is frequently delayed given the non-specificity of symptoms and late patient presentation, and hence pancreatic cancer is characterised by poor outcome\textsuperscript{58,59}. We show that although the stage of presentation is worsening, survival rates have not changed significantly over the last 4 decades. This suggests that although there is a higher recorded incidence of disease with regional/distant spread at the time of diagnosis, this may be attributed to better and more advanced diagnostic radiological techniques available. The static incidence rates and survival rates suggests that little progress has been made in diagnosing pancreatic cancer earlier or treating pancreatic cancer more effectively as to prolong survival. New strategies are therefore constantly sought in an effort to elucidate the mechanisms and develop new therapeutic strategies against cancer. The metabolic phenotype of cancer has resurfaced as an area of great interest despite the fundamental changes being described by Warburg over 9 decades ago\textsuperscript{60}. The current study shows that in PDAC, the Warburg effect is present and the disruption of the pathways used by these cells affects cell viability, PMCA function and Ca\textsuperscript{2+} clearance and intracellular ATP levels.

The Warburg effect can be seen from the results of the ATP assays performed in this study. We show that the ATP depletion in human PDAC cells caused by glycolytic inhibition is associated with reduced cell viability and necrosis within 1 and 3 hours of treatment. We also show that there is significant inhibition of PMCA and impairment of the cells ability to maintain Ca\textsuperscript{2+} homeostasis when glycolysis, rather than mitochondrial, metabolism is inhibited. Our data therefore suggests that ATP-dependent PMCA is critical to the survival of human PDAC, and a reduction in the ATP impairs the cancer cell from adequately clearing high [Ca\textsuperscript{2+}], and therefore reduces cell viability. Although NCX is expressed in some PDAC cell lines such as CAPAN-1 and BxPC3\textsuperscript{61}, we show that NCX plays no role in Ca\textsuperscript{2+} clearance. Other studies have also shown that PMCA is the dominant mechanism for Ca\textsuperscript{2+} extrusion in PDAC, and that its inhibition results in cell death by intracellular Ca\textsuperscript{2+} toxicity\textsuperscript{53,54}.

The Ca\textsuperscript{2+} clearance assays show that the Panc-1 cell line exhibits the Warburg effect, which becomes apparent when treating the cells with 3-BP and IAA, but not with mitochondrial inhibitors. In some cases, particularly when we abolish mitochondrial metabolism as well as glycolysis, the cell does not recover and [Ca\textsuperscript{2+}] never returns to homeostatic levels. It has been shown in previous studies that a depletion in intracellular ATP causes PMCA to fail and allows sustained cytotoxic increases in [Ca\textsuperscript{2+}]. Studies measuring intracellular ATP in mouse pancreatic acinar cells found that concentrations reduced to nearly zero following treatment with iodoacetate and oligomycin, and as a result, PMCA activity and Ca\textsuperscript{2+} extrusion was inhibited\textsuperscript{62}. Other studies have shown the importance of PMCA and the consequences of its inhibition through low intracellular ATP concentrations. Non-oxidative metabolites of alcohol such as fatty acid ethyl esters (FAEE) and fatty acids (FA) have been shown to increase [Ca\textsuperscript{2+}] in pancreatic acinar cells, with sustained increases inducing necrosis\textsuperscript{63} that can be prevented by increasing intracellular ATP levels\textsuperscript{64}.
It is also known that PMCA has two binding sites\textsuperscript{65}; a higher affinity catalytic binding site and a lower affinity regulatory site which needs to be occupied for PMCA to hydrolyse ATP at a maximal rate. Although a reduction in ATP would not profoundly affect PMCA (and Ca\textsuperscript{2+} extrusion) at the high affinity site, the PMCA would not function at an optimal as the low affinity site would not be occupied.

Glycolytic enzymes are also present on the cell membrane in close proximity and co-localising around the PMCA, producing localised ATP microdomains\textsuperscript{42}. Hardin et al\textsuperscript{66} have previously shown that PMCA preferentially uses ATP generated from these membrane-bound enzymes over mitochondria-generated ATP in the cytosol. Glycolytic inhibition is likely to affect these sites locally around the PMCA as well as on the whole cell. However, under conditions of metabolic stress whereby global ATP is reduced, then ATP produced in close proximity to the PMCA might be sufficient to keep the PMCA active, and giving the cell enough time to activate stress response pathways, apoptosis, autophagy and thus preventing necrosis.

As mentioned previously, different isoforms of PMCA are up-regulated in cancer cells, such as PMCA-4 in colon cancer\textsuperscript{45}. PMCA-1 has been shown to have a higher affinity for ATP, but is also much less stable and more prone to degradation by calpain, a proteolytic enzyme linked to necrosis\textsuperscript{67}. A pro-survival cancer phenotype may express more PMCA-4 to reduce its changes or necrosis. Reactive oxygen species (ROS) have also been implicated in cell necrosis, and there in evidence that increased levels of ROS impairs PMCA and Ca\textsuperscript{2+} homeostasis\textsuperscript{68}.

ATP depletion by glycolytic inhibition has previously been shown to reduce cell viability. Xu et al\textsuperscript{69} reported that 300 µM of 3-BP reduces ATP levels to less than 5% of normal after just 3 hours in the human leukaemia cells, with most cells undergoing cell death after 24 hours. Bhardwaj et al\textsuperscript{70} also showed that Panc-1 cell viability decreases when treated with glycolytic inhibitors in a dose-dependent manner, with cell survival reducing to less than 20% after 24 hours of treatment with 3-BP or IAA. They also found that 3-BP, and not IAA, affects the mitogenic pathways Akt and mTOR, suggesting that the two different inhibitors have a differing effect on cell signalling. Several ATP-dependent steps common to both necrosis and apoptosis have previously been identified, specifically involving Bcl-2 and Bcl-xl and Fas pro-apoptosis initiators\textsuperscript{34,71} and in the stages before nuclear collapse and DNA degeneration in apoptosis\textsuperscript{72}. In necrosis, several ATP-dependent kinase mediators have been identified downstream from Fas that lead to a ROS overload and ceramide-induced membrane disruption to trigger necrosis\textsuperscript{73}. Furthermore, it has been shown in human T-cells that depletion of more than 70% of intracellular ATP from normal induces necrosis (in a time-dependent fashion after 90 minutes) whereas higher ATP levels (50 – 70%) favour apoptosis\textsuperscript{72}. This provides further evidence that the increase in cell necrosis levels observed in the Panc-1 cells is in part due to the reduction in the concentration of intracellular ATP.

We show that the addition of a MOP inhibitor with a glycolytic inhibitor does not always significantly reduce ATP further than with the glycolytic inhibitor alone. It has been reported by Xu et al\textsuperscript{69} that ρ\textsuperscript{0} cells, which contain no mitochondrial DNA and therefore incapable of undergoing MOP, show a greater reduction in ATP levels and cell viability when treated with 3-BP. This suggests that MOP does play some significant role in ATP synthesis in cancer cells. One of the reasons why mitochondrial inhibitors may not act synergistically with 3-BP is because it is thought 3-BP has an effect on the mitochondria itself. It is known that hexokinase II is up-regulated in
cancer cells, and is particularly overexpressed and bound to voltage-dependent anion channels on the outer membrane of mitochondria. It has also been shown by Pastorini et al that in HeLa cells, hexokinase II inhibits apoptosis by interfering with BAX binding to mitochondria and preventing the release of cytochrome C. Xu also found that the concentration of dephosphorylated BAD increases after treatment with 3-BP, and hypothesizes that the dephosphorylated BAD forms a complex with and inactivates anti-apoptotic Bcl-2 and Bcl-xl proteins, allowing the pro-apoptotic protein BAX to accumulate in the mitochondria to cause the release of cytochrome c and initiate apoptosis.

The mode of cell death has also been found to vary following treatment with 3-BP. Vali et al reported an increase in both apoptosis and necrosis in rabbits transfected with VX-2 tumour cells into the liver and treated with 3-BP. High concentrations caused peripheral liver necrosis, but when reduced, the ratio of tumour necrosis to apoptosis reduced, and eventually a concentration was identified whereby complete tumour apoptosis was obtained. Other studies have also shown a pro-apoptotic effect of 3-BP in hepatocellular carcinoma and multiple myeloma cells. Quin et al has also shown that 3-BP induces predominantly necrotic cell death in melanoma cell lines, again with different rates of cytotoxicity depending on the concentration of 3-BP and the length of treatment. ATP levels fell to within 5% of normal. Furthermore, reactive oxygen species (ROS) levels (in this case superoxide anions) increased and correlated well with the rate of necrosis. Studies by Kim et al have also shown a role of ROS in mitochondrial disruption and cell death. A panel of human HCC cell lines treated with 100 µM 3-BP was shown to reduce ATP levels by up to 60% after 24 hours. This lead to cell death rapidly after just one hour by both ATP-depletion dependent necrosis and apoptosis that was independent of caspase. It was also found to induce the production of ROS, leading to mitochondrial dysregulation (as determined by a loss of the mitochondrial membrane potential) and apoptosis. This was further confirmed by the observation that the addition of an antioxidant (N-acetyl-L-cysteine) prevented the increase in ROS as well as protecting the mitochondrial membrane potential and ultimately against cell death. Apoptosis was not found to be related to the level of hexokinase II expression in this study.

**Targeting Glycolysis**

Glucose is transported into cells down its concentration gradient via a glucose transporter (GLUT) on the cell membrane. There are 12 isoforms of GLUT currently identified, with Class 1 GLUTs (GLUT1-4) identified as having a high affinity for glucose and being responsible for basal glucose transport. It has long been known, however, that an over-expression of glucose transporters (such as GLUT-1, which is normally typically expressed on erythrocytes) has been associated with cancer progression and poor tumour prognosis. Initial experiments by Yamamoto et al measuring mRNA levels from a range of surgically excised normal and cancerous tissue showed high levels of GLUT expression, particularly GLUT-1 and GLUT-3 in pancreas cancer. From immunohistochemical analysis of GLUT-1 expression in pancreas neoplasia, Basturk et al reported a positive correlation between GLUT-1 expression and tumour histological grading (from low grade PanIN 1A dysplastic lesions which were almost all negative to ductal adenocarcinoma where 74% of cases showed some degree of GLUT-1 expression) and
ductal adenocarcinoma size. All patients with serous cystadenomas had either moderate or significant tumour GLUT-1 expression.

With such differences in expression between normal and cancerous tissue, the GLUT family has since been used as a therapeutic target in treating cancer. A study by Noguchi et al showed that suppressing GLUT-1 expression in a moderately-differentiated gastric cancer cell line was successful in significantly reducing glucose transport, and also affected the cell cycle with more cells moving into the S-phase of the cell cycle. In vivo studies involving nude mice showed slower tumour growth in the early stages of treatment. Young et al produced a mouse mammary tumour cells with reduced expression of GLUT-1, showing an altered glycolysis rate, lipid synthesis and cell proliferation. In vivo experiments involving nude mice (in which the altered cells were transplanted into) showed reduced tumour growth in cells where GLUT-1 expression was decreased.

A recent study by Liu et al showed that inhibiting GLUT-1 using a compound (WZB117) derived from pentagalloyl glucose (PGG) has the effect of down-regulating glycolysis which ultimately induced cell-cycle arrest in human NSCLC (H1299) and breast ductal carcinoma (A549) cell lines. ATP assays show significant reductions in intracellular ATP after treatment with WZB117 at 6 (89% of normal) and 12 hours (85% of normal), suggesting that glucose is the main (and irreplaceable) source of energy utilised by cancer cells. This finding correlates with our results of intracellular ATP reduction when glycolysis is disrupted. The key glycolytic enzymes hexokinase II and PKM2 were found to be reduced at 6 hours, but up-regulated after 24 hours. WZB117 also reduced cell proliferation rates by up to 50% after 48 hours of treatment. Animal studies using nude mice xenografted with NSCLC subcutaneously in the flank showed the tumour size reduced by more than 70% following treatment with WZB117, with 20% of tumours reportedly eradicated completely.

The other family of proteins responsible for glucose transport other than GLUT is the sodium/glucose co-transporter (SGLT), found predominantly in the small intestine and the proximal tubule of the nephron. The role of SGLT in cancer and its role as a therapeutic target are not as clear as the GLUT transporters. Ishikawa et al looked at expression levels of SGLT-1 and -2 in primary lung cancers and found no significant difference between normal and cancerous tissue. There was also no difference in expression of SGLT-1 between primary and metastatic lung lesions, but there was an increase in SGLT-2 expression in metastatic liver and lymph node lesions compared to the primary. Real-time PCR and immunohistochemical analysis of head and neck squamous cell carcinomas (HNSCC) by Helmke et al showed an increase in SGLT-1 expression, but not SGLT-2. GLUT-1 was found to be strongly expressed in HNSCC by Reisser et al, but not GLUT2, 3 or 4. Recently, SGLT-1 was implicated for its role in the epidermal growth factor receptor (EGFR) pathway. Weihua et al reported reduced intracellular glucose levels in the prostate cancer cell line PC3-MM2 with reduced EGFR expression (transfected with EGFR siRNA). The expression of SGLT-1 was also reduced in those cells (whereas expression of GLUT-1 was normal), and a correlation was seen between the reduction in EGFR protein levels, and SGLT-1 protein levels and intracellular glucose concentrations. The development of SGLT inhibitors for treatment of type 2 diabetes is well documented but, so far, there is little research into its role as a cancer chemotherapeutic agent.
**Glycolytic Metabolic Inhibitors as Novel Treatments for Cancer**

Several glycolytic inhibitors targeting different enzymes in glycolysis have been tested as potential anti-cancer agents. It is known that the M2 isoform of PK is overly expressed in cancer cells, and there is evidence that PKM2 is a regulator of glycolysis\(^9\). PKM2 can exist in two allosteric forms; a tetramer, which has a high affinity for PEP and favours pyruvate and ATP production, and a monomer that has a low affinity for PEP, which allows glycolytic metabolites to accumulate and become available for biosynthesis functions\(^9\). The tetramer of PKM2 is thought to be come activated by fructose-1,6-phosphate\(^9\). PKM2 also translocates to the nucleus and acts as a transcriptional activator for HIF-1, which increases the expression of several genes such as SLC2A1 (which encodes GLUT-1) and VEGFA (which encodes VEGF which stimulates angiogenesis)\(^9\). PKM2 is therefore regarded as a key pro-oncological enzyme and a strong potential therapeutic target.

Ko et al. has published extensively on the use of 3-BP as an anticancer drug\(^9\); 3-BP was tested on the highly glycolytic hepatocellular carcinoma cell line AS-30D, and was shown to rapidly and completely deplete ATP levels at concentrations above 0.02mM whilst not affecting levels in normal hepatocytes until above 0.03mM. Cell viability was ultimately affected, dropping to 10% with the ATP depletion. These are in concordance with our results on the pancreatic cancer cell lines as we show a substantial fall in ATP levels at 0.5mM. Ko also showed the *in vivo* effects of 3-BP in an animal model, whereby cancer was induced in rats in the abdominal cavity and in the upper back with proliferating HCC cells and then treated with 3-BP. Impressively, regression was seen within a week, with tumours disappearing after a month and all animals remaining tumour free. More recently, Ko et al published a case study\(^9\) on a 16 year old male diagnosed with a primary fibrolamellar liver cancer treated with 3-BP. The patient responded to treatment with tumour destruction (confirmed by computed tomography) and went on to survive 2 years before succumbing to liver failure.

2-Deoxyglucose (a competitive metabolic inhibitor for GLUT glucose transporters and hexokinase, and an inhibitor of phosphoglycose isomerase) was found by Maher et al.\(^9\) to inhibit growth in pancreatic cancer cell lines by as much as 59% in Panc-1 cells and over 95% in MIAPaCa-2 after 2 days of treatment. A similar but less profound result was seen when the cells were treated with oxamate, a competitive metabolic inhibitor of lactate dehydrogenase at the final step of glycolysis. It was also noted that the effect on proliferation was more profound when the cell lines were grown under hypoxic conditions where it was found that GLUT-1 expression levels increased and correlated with the increased sensitivity.

**Oxidative Phosphorylation Inhibitors as Novel Treatments for Cancer**

Although our results report fewer significant effects of MOP on pancreas cancer intracellular ATP levels, cell function and cell proliferation, there is evidence in the literature that the MOP pathway can be used as a therapeutic target\(^9\).\(^9\).

In glucose-limiting conditions, MOP inhibitors have been shown to be cytotoxic to Panc-1 cells. Momose et al.\(^9\) reported that treatment with efrapeptin F (a fungal toxin that acts as a potent ATPase inhibitor) was cytotoxic to Panc-1 cells cultured in glucose limiting conditions. Panc-1 cells grown in nutrient-deficient media were found to have reduced levels of ATP, and they were shown
to be sensitive to MOP inhibitors, suggesting that MOP contributes to intracellular ATP productio. CCCP has also been to reduce intracellular ATP concentrations in rat basophilic leukaemia cells, but only in zero-glucose conditions\(^{100}\); this suggests that MOP plays a role in maintaining ATP concentrations in the absence of glycolysis. In keeping with our results, Mohr et al reported that CCCP does not significantly reduce ATP levels if the cancer cells are grown in glucose-containing media. Cheng et al\(^{101}\) also reported that using a glycolytic inhibitor (2-deoxy-D-glucose) together with MOP inhibitors (Mito-CP and Mito-Q) produced a decrease in ATP levels in the human breast cancer cell lines.

CCCP has also been shown to have an inhibitory effect on cell growth in human pulmonary adenocarcinoma Calu-6 cells\(^{102}\). Han et al reported a reduction to less than 10% of normal in Calu-6 cell growth and producing cell arrest at the G1 phase. Caspase-dependent apoptosis was also detected. Other MOP inhibitors including 2,4-dinitrophenol and antimycin A, have also been shown to induce apoptosis in Calu-6 cells via G1 phase arrest \(^{103,104}\). The Warburg effect is also accompanied by a metabolic remodelling of the mitochondrial membrane in which it becomes hyperpolarised\(^{105}\), and it has been hypothesised that this hyperpolarisation is partly responsible for supressing apoptosis\(^{106}\). Some agents can reverse this hyperpolarisation and restore MOP. Dichloroacetate (an inhibitor of pyruvate dehydrogenase kinase) is one such therapeutic agent that has been found to induce apoptosis and inhibit cell proliferation and tumour growth\(^{105}\). It is hypothesized that increasing the amount of mitochondrial pyruvate promotes glucose oxidative phosphorylation rather than glycolysis which has the effect of restoring apoptosis\(^{106}\). Treatment with dichloroacetate has also been shown to induce apoptosis and cell-cycle arrest in the colorectal cancer cell line LoVo\(^{107}\), the human endometrial cancer cell lines Ishikawa, RL95-2, KLE, AN3CA, and SKUT1B\(^{108}\) and on HeLa cells when treated with cystplatin\(^{109}\). Mitochondrial hyperpolarisation, when reversed by dichloroacetate, has also been shown to be a therapeutic target in the treatment of gliobastomas.

**Summary**

The Warburg Effect is an observation whereby cancer cells use glycolysis for the production of ATP, compared to oxidative phosphorylation as seen in normal cells. This fundamental difference presents a potential therapeutic target. We showed firstly show that glycolytic inhibitors, but not MOP inhibitors, reduce Panc-1 cell viability and induce necrosis. We also show that the glycolytic inhibitors impair PMCA function and the ability of Panc-1 to maintain calcium clearance. MOP inhibitors, however, do not affect calcium clearance. Finally, we show that glycolytic inhibitors significantly reduce ATP levels in Panc-1 cells.

Our data suggests that an inhibition of the glycolytic ATP supply to PMCA in PDAC is an effective therapeutic target that could represent a new strategy for selectively killing PDAC cells and sparing normal, healthy cells.
LIMITATIONS OF STUDY

The current study was limited by many factors; the ones of importance are listed below. Steps have been made to address these issues for future research.

Cell Lines

One of the obvious limitations of the study was the usage of only 2 immortalised cell lines in the experiments, and whether they truly represents the behaviour of \textit{in situ} pancreatic cancer. Although Panc-1 and MIA-PaCa 2 are well characterised and widely used in research, they represent only a small subset of pancreatic adenocarcinomas. The results (and conclusions) from our experiments may be different if performed on other pancreas cancer cell lines. Control data for comparison is also difficult to generate, as there are no immortalised cell lines of normal pancreas lineage.

The advantages of using cell lines are clear. A wide variety of cancer cell types is currently available for use at a relatively low budget and, once obtained, can be grown indefinitely in a laboratory. The accessibility of cell lines for research is superior to primary tissue, where the process of obtaining primary tissue for experimental use is both lengthy and subject to a higher ethical standard. Protocols for the preparation and use of cell lines are more established and validated than for primary samples which have to be meticulously prepared. However, cell lines are grown in artificial environments and respond and develop differently than \textit{in situ} tumours to produce a different morphology. There is much debate as to whether using cell lines rather than primary tissue directly from a patient for genomic studies is clinically accurate\textsuperscript{110}. Some cell lines, such as those immortalised by glioma tumours, bear so little resemblance to their primary equivalent that clinical correlation is difficult. Some mutations discovered in cell lines have also been subsequently found to be insignificant when comparing primary tumour samples with the corresponding normal tissue. This is of concern if the study were to progress onto high throughput analysis techniques such as GeneChip microarray or metabolomics studies.

In order to address these limitations, future experiments will be done concordantly using a wider variety of pancreas cancer cell lines. The use of primary pancreatic adenocarcinoma cells and normal pancreas and pancreatic ductal tissue will also produce a more accurate model with better clinical correlation, and can be used to validate data from cell line studies. A better comparison of pancreatic cancer tissue and normal pancreatic tissue can also be made given the absence of a normal pancreas cell line.

Ethics approval for using both cancerous and normal tissue from surgical resections from patients undergoing a Whipple’s Operation for pancreatic adenocarcinoma has already been successfully obtained during the course of the MPhil from the NHS National Research Ethics Service (reference number 10/H1010/57).

Metabolic Inhibitors

The metabolic inhibitors used during these experiments were very limited to those affecting the glycolytic and oxidative phosphorylation pathways. Furthermore, only 2 enzymes were inhibited
per pathway. This limits the analysis of the metabolic pathways and how they are affected at each step. Cancer cell show great metabolic plasticity and the inhibition of one pathway will lead to alterations and compensations in other pathways to maintain essential cell metabolites. It is known that different pathways, such as the pentose 5 pathway and glutaminolysis play a part in maintaining cellular ATP levels and provide anabolic pathways for the cancer cell.

**High-throughput Experiments**

The experiments performed rely on the measurement of one metabolite (mainly ATP) and indirect assessment of cellular function. Although we assess the end result of treatment with metabolic inhibitors, we are unable to fully explain the mechanisms in between. High throughput experiments, such as GeneChip microarray analysis and metabolomics would provide detailed information on cell gene and metabolite expression respectively. Furthermore, how these expression levels change depending on which metabolic pathways are blocked would help further explain the mechanism of change in cancer cells.
SUMMARY OF FUTURE WORK

Future work as a result of this study will focus more deeply on elucidating the metabolic pathways and mechanisms of the cancer phenotype. The limitations of the study will be addressed in that more cell lines will be used to provide a wider profile of pancreatic cancer. In addition, future experiments will also involve the use of freshly surgically resected pancreatic tissue (both with normal tissue and cancer tissue) so that differences between the normal metabolic profiles can be compared to the cancerous profile that has undergone Warburg changes. Studies looking at the wider cellular function will also be performed and expanded outside that of calcium homeostasis. A wider range of metabolic inhibitors (including glycolytic and mitochondrial, but also extending to glutamine inhibitors and the pentose pathway as detailed in Appendix One) will also be used and their effects tested on the metabolic profile. Combinations of inhibitors will also be looked at in an attempt to curb the metabolic plasticity that cancer cells exhibit.

Finally, high-throughput metabolomics studies will be performed to construct a complete metabolic profile, and their responses to the different metabolic inhibitors.
APPENDIX ONE: METABOLIC MAP
REFERENCES


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