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Characterisation of Pharmacological Mechanisms and Potential Therapeutic uses of FK866

Ferguson, Niketa

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University of Plymouth

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Characterisation of the Pharmacological Mechanisms and Potential Therapeutic uses of FK866

Niketa Ferguson

A thesis submitted to Plymouth University in partial fulfilment for the degree of Masters of Philosophy

School of Biomedical and Healthcare Sciences

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Niketa Skye Ferguson

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The finding that NAD\(^+\) plays a role in a variety of signalling pathways, including gene expression, Ca\(^{2+}\) signalling and DNA repair mechanisms, has sparked interest in the proteins involved in these pathways as potential pharmacological targets for drug development. Recently, FK866, a potent inhibitor of nicotinamide phosphoribosyltransferase (Nampt) an important enzyme in the NAD\(^+\) rescue pathway, has been evaluated in clinical trials against cancer. The aim of this study is to further investigate the mechanisms and therapeutic characteristics of FK866 in different cancer cell lines and to determine if decreasing intracellular NAD\(^+\) levels can be used as a co-therapy strategy to improve the efficacy of current and new chemotherapy treatments.

Experiments measuring cell vitality showed that FK866 dose-dependently decreased cell vitality. To investigate NAD\(^+\) consumption during Nampt inhibition, NAD\(^+\) levels were measured in cells treated with FK866 and inhibition of each of the main NAD\(^+\) consuming enzymes (PARP, sirtuins or CD38). This revealed differential NAD\(^+\) consumption rates by the different NAD\(^+\) consuming enzymes in MDA-MB-231 cells, with sirtuins being the major NAD\(^+\) consuming enzyme. The glycolytic effects of Nampt inhibition was measured using SEAHORSE assays; which measured the oxygen consumption and extracellular acidification rates as well as measuring NAD\(^+\)/NADH ratios. In the
MCF-7 and MDA-MB-231 cell lines, FK866 had no effect on the oxygen consumption rates; however there was a general decrease in extracellular acidification rates indicating an effect on glycolytic activity. When measuring the NAD$^+/\text{NADH}$ ratio however, there was only a decrease in the MDA-MB-231 cells but no change in the MCF-7 cell line. Cell vitality and NAD$^+$ levels were measured after treatment with FK866 in addition to NAD$^+$ consuming enzyme inhibitors or the alkylating agent, Temozolomide to see if combination therapy would have more cytotoxic potential. This co-treatment indicated that there was no real positive effect on either the MCF-7 or MDA-MB-231 cells in either the cell vitality or NAD$^+$ levels. Finally the effects of FK866 and the oral PARP inhibitor, Olaparib, were investigated using 3D cell culture (spheroids) and compared with 2D monolayer cultures. The effects of FK866 showed little difference in spheroid or monolayer culture. However, when treating with Olaparib there was higher level of cell viability and NAD$^+$ levels with the cells grown in spheroid culture in comparison to cells grown in monolayer. In conclusion, this study has shown that FK866, as a single treatment decreases cell vitality, NAD$^+$ levels and glycolytic activity. However as a co-therapy with PARP or Sirtuin inhibitors there is an increase in the cell vitality and NAD$^+$ levels. Although similarities have been seen between spheroid culture and monolayers as a single treatment, FK866 does not seem to have the beneficial effects as a therapeutic.
Contents
Figures .......................................................................................................................... 6
Tables .......................................................................................................................... 8
Acknowledgements ....................................................................................................... 9
Abbreviations .............................................................................................................. 11
Chapter 1: Introduction ............................................................................................... 13
  1.1 Nicotinamide phosphoribosyltransferase (Nampt) and Nampt inhibition ......... 14
  1.2 Poly (ADP-ribose) polymerase (PARP) .............................................................. 16
  1.3 Sirtuins ............................................................................................................... 19
  1.4 CD38 ................................................................................................................... 21
  1.5 Breast Cancer .................................................................................................... 21
  1.6 Hypothesis ........................................................................................................ 22
  1.7 Aims ................................................................................................................... 23
Chapter 2: Methods ..................................................................................................... 24
  2.1 Cell Cultures ...................................................................................................... 24
  2.2 Treatments ......................................................................................................... 24
  2.3 MTT assay ......................................................................................................... 25
  2.4 NAD⁺ cycling assay ......................................................................................... 25
  2.5 NADH cycling assay ......................................................................................... 26
  2.6 Seahorse Experimental Procedure ..................................................................... 26
  2.7 Plate preparation for Spheroids ......................................................................... 27
  2.8 Spheroid formations ......................................................................................... 28
  2.9 Acid Phosphatase assay ................................................................................... 28
  2.10 Statistical Analysis ......................................................................................... 28
Chapter 3: The effect of FK866 on Glycolysis and the Mechanisms of NAD⁺ Consumption ......................................................................................................................... 29
Figures

Figure 1.1: Chemical structure of NAD$^+$ 13

Figure 1.2: NAD$^+$ synthesis pathway 14

Figure 1.3: Chemical structure FK866 15

Figure 1.4: Structure of PARP1 and PARP2 17

Figure 1.5: Mechanism of deacetylation of protein by sirtuins using NAD$^+$ 20

Figure 3.1: The *de novo* pathway and salvage pathway 30

Figure 3.2: The rate of oxygen consumption in MDA-MB-231 and MCF-7 cells when treated with FK866 33

Figure 3.3: Rate of extracellular acidification in MDA-MB-231 and MCF-7 cells when treated with FK866 36

Figure 3.4: The NAD$^+$:NADH ratio of MDA-MB-231 and MCF-7 cells when treated with FK866 39

Figure 3.5: NAD$^+$ levels of MDA-MB-231 cell line treated for 8 hours with FK866 and NPH 41

Figure 3.6: NAD$^+$ levels of MDA-MB-231 cell line treated for 8 hours with FK866 and Sirtinol 43

Figure 3.7: NAD$^+$ levels of MDA-MB-231 cell line treated for 8 hours with FK866 and Kuromanin 44
Figure 4.1: Cell vitality of MDA-MB-231 and MCF-7 cells when treating with FK866

Figure 4.2: Cell vitality of MDA-MB-231 and MCF-7 cells when treating with NPH, Olaparib, Sirtinol or Temozolomide

Figure 5.1: Images of MCF-7 and MDA-MB-231 cells grown in spheroid culture

Figure 5.2: The correlation between the viability of cells and the volume of the spheroid

Figure 5.3: Cell viability of MDA-MB-231 and MCF-7 cells grown in either monolayer or spheroid after treatment with FK866

Figure 5.4: Cell viability of MDA-MB-231 and MCF-7 cells grown in either monolayer or spheroid after treatment with Olaparib

Figure 5.5: NAD$^+$ levels of MDA-MB-231 and MCF-7 cells grown in either monolayer or spheroid after treatment with FK866

Figure 5.6: NAD$^+$ levels of MDA-MB-231 and MCF-7 cells grown in either monolayer or spheroid after treatment with FK866
Tables

Table 4.1: Cell vitality and NAD$^+$ of 48 and 72 hour co-treatments in MDA-MB-231 cells

Table 4.2: Cell vitality and NAD$^+$ of 48 and 72 hour co-treatments in MCF-7 cells
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AUTHOR'S DECLARATION

At no time during the registration for the degree of Master of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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Abbreviations

ADP – Adenosine diphosphate
ADPR – Adenosine diphosphate ribosylation
AIF – Apoptosis Inducing Factor
ARTs – mono (ADP-ribosyl) transferases
ATP – Adenosine triphosphate
BER – base excursion repair
BRCT – BRCA1 Terminus domain
cAMP – cyclic adenosine monophosphate
CNA – circulating nucleic acids
DBD – DNA binding domain
ECAR – Extracellular acidification rate
ERα – Oestrogen receptor
GPI – glycophaspatidylinositol
HER2 – Human epidermal growth factor receptors
HIC1 – Hypermethylated in cancer 1 gene
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA – nicotinic acid
NAAD – nicotinic acid adenine dinucleotide
NAD⁺ – nicotinamide adenine dinucleotide
NADH – nicotinamide adenine dinucleotide hydrogen
NAM/NMN – nicotinamide mononucleotide
NAMN – nicotinic acid mononucleotide
Nampt – Nicotinamide phosphoribosyltransferase
NMNAT – nicotinamide mononucleotide adenylyltransferase
npt – nicotinic acid phosphoribosyltransferase
NPH – 4-amino-1, 8-naphthalimide
NR – nicotinamide riboside
Nrk – nicotinamide riboside kinase
OCR – Oxygen consumption rates
OXPHOS – Oxidative phosphorylation
PAR – Poly (ADP-ribose)
PARP – Poly (ADP-ribose) polymerase
PBEF – pre B-cell factor
PFK – phosphofructokinase
p-HEMA – Poly-hydroxyethylmethacrylate
ROS – reactive oxygen species
Sirt1 – Sirtuin 1
TCA – Tricarboxylic Acid cycle
Chapter 1: Introduction

Nicotinamide adenine dinucleotide (NAD\(^+\)) is a co-enzyme which can be found in all living organisms (Figure 1.1). It is a dinucleotide where one nucleotide contains an adenine base and the other contains nicotinamide. It was originally discovered by Arthur Holden and William Youndin in 1906 (Manchester 2000), although Otto Heinrich Warburg identified nicotinamide at the active site of redox reactions as well as the function of NAD\(^+\) in hydride transferase in 1936 (Warburg 2010). Due to the connections that NAD\(^+\) has with most metabolic pathways such as adenine diphosphate (ADP)-ribosylation, the release of calcium from intracellular supplies of NAD\(^+\)(P) derivatives, the control of calcium ion influx by ADPR/TRPM2 as well as NAD\(^+\) dependent protein deacetylation, NAD\(^+\) is now considered a universal energy and signal carrying molecule (Berger et al., 2004). Metabolising enzymes and targets, both for extracellular NAD\(^+\) and its metabolites are constantly being identified.

![Chemical structure of Nicotinamide adenine dinucleotide (NAD\(^+\))](image)

**Figure 1.1:** Chemical structure of Nicotinamide adenine dinucleotide (NAD\(^+\))

The synthesis of NAD\(^+\) is essential for all living organisms, although the biosynthesis was thought to be of little importance for a long time because hydrogen transfer
reactions were not thought to involve the consumption of pyridine nucleotides. Signalling pathways that are dependent on NAD⁺ have the N-glycosidic bond which cleaves between the ADP-moiety and nicotinamide. The enzymatic processes of catalysing NAD⁺ synthesis have been known for decades however it has only been in the past 14 years that the eukaryotic enzymes, which are involved in the NAD⁺ synthesis pathway have been recognised (Emmanuelli et al., 1999). Tryptophan, nicotinate, nicotinamide and nicotinamide riboside are used to produce dinucleotides such as NAD⁺ or NAAD by nicotinamide mononucleotide adenyltransferase (NMNAT) where NAAD is converted to NAD⁺ by NAD⁺ synthase (Wei et al., 2008) (Figure 1.2). NMNATs are important because they catalyse the final step of the biosynthesis of NAD⁺. There have been three isoforms of this enzymes, the first was found to be localised to the nucleus (Emmanuelli et al., 2001), while the other two were found in the cytosol although the third is also located in the mitochondria (Zhang et al., 2003).

![Figure 1.2: NAD⁺ synthesis pathway (Wei et al., 2008)](image)

1.1 Nicotinamide phosphoribosyltransferase (Nampt) and Nampt inhibition

Nicotinamide phosphoribosyltransferase (Nampt) is a rate limiting enzyme which converts nicotinamide (NAM) to nicotinamide mononucleotide (NMN) during the
recycling of NAD\(^+\). Nampt is normally up-regulated in malignant tumour cells to preserve the levels of NAD\(^+\) (Hasmann and Schemainda 2003; Hufton \textit{et al}., 1999; Gehrke \textit{et al}., 2014; Sociali \textit{et al}., 2016). As it is involved in the NAD\(^+\) recycling pathway it means that by inhibiting Nampt, the levels of NAD\(^+\) will be affected therefore making them targets for new cancer therapies as there are increased levels of NAD\(^+\) in tumour cells (Khan \textit{et al}., 2007; Billington \textit{et al}., 2008; Sampath \textit{et al}., 2015). FK866 (Figure 1.3) is an anticancer agent that has been shown to disrupt NAD\(^+\) biosynthesis by inhibiting Nampt. This leads to the depletion of intracellular NAD\(^+\) which can result in cell death (Wosikowski \textit{et al}., 2002). There has also been a decrease in nicotinamide mononucleotide adenyltransferase (NMNAT) shown in cells after being treated with FK866 (Boulton \textit{et al}., 1997). Studies on cancer cell lines exposed to FK866 resulted in a slow progressing type of cell death due to the reduction of NAD\(^+\) (Bruzzone \textit{et al}., 2009; Hasmann and Scheimanda 2003; Nahimana \textit{et al}., 2009). FK866 is not cell cycle dependent so therefore is not subject to multi-drug resistance (Holen \textit{et al}., 2008). FK866 has successfully completed phase 1 of clinical trials and is currently going through phase 2 trials for cancer chemotherapy in T-cell and B-cell lymphoma (ClinicalTrials.gov Identifier: NCT00431912; ClinicalTrials.gov Identifier: NCT00435084) as well as advanced cutaneous melanoma (ClinicalTrials.gov Identifier: NCT00432107) (Pittelli \textit{et al}., 2010; Gehrke \textit{et al}., 2014).

![Figure 1.3: Chemical Structure of FK866](image)

FK866 can induce cell death by competitively binding to the nicotinamide binding site and inhibiting Nampt (Holen \textit{et al}., 2008; Gehrke \textit{et al}., 2014). During pre-clinical trials,
it showed to have a high anti-tumour activity on a variety of tumour cells in vitro and has been seen to cause delayed cell death which demonstrates characteristics of autophagy (Billington et al., 2008). Autophagy is suggested to be a last chance survival mechanism, which is brought about by cells being denied nutrients, therefore they begin to self-digest to gain energy and amino-acids to make up for metabolic inadequacies (Gozuacik and Kimchi 2007). However in cancer cells where apoptosis is unavailable because of the anti-proliferative control, autophagy can be seen as a second type of programmed cell death. When NAD+ levels are decreased, this energy saving response is expected due to NAD+ being an important part of glycolysis, the Kreb’s cycle and lipid-β-oxidation. FK866 can encourage a decrease in NAD+ levels without DNA damage and poly (ADP-ribose) polymerase (PARP) activation (Alano et al., 2004). The depletion of NAD+ can lead to PARP-induced cell death which could be linked to autophagy as this would be due to the reduction of NAD+ levels

1.2 Poly (ADP-ribose) polymerase (PARP)
Poly (ADP-ribose) polymerase (PARP) is a major factor in DNA repair in eukaryotic cells to deal with different environmental and endogenous genotoxic agents. The DNA damage can occur either directly, such as ionising radiation and alkylating agents, or indirectly after an enzymatic incision of a DNA lesion. This can set off an acute cellular response accompanied by the synthesis of poly(ADP-ribose) (PAR) by PARP. PARP-1 (Figure 1.4 a) is a nuclear zinc-finger binding protein which can detect and signal DNA breaks. At the site of a break, it can catalyse the transfer of ADP-ribose moiety from NAD⁺ to the nuclear protein acceptors in a DNA dependent way. Poly (ADP-ribosylated) proteins play a part in chromatin structure and in DNA metabolism. They can lose their affinity for DNA because of the negative charges of ADP-ribose polymers and therefore are inactivated following genotoxic stress (D-Amours et al., 1999). PARP and the proteins return to their normal state by poly (ADP-ribose) glycohydrolase. The nuclear proteins which are induced by DNA lesions during poly (ADP-ribosylation can be by base excision repair (BER) pathway (Shall and de Murcia 2000).
The main characteristics of the activity of PARP are the recognition and translations of the signals from the DNA as well as amplifying the formation of poly (ADP-ribose) (PAR). PARP-1 is a multi-functional enzyme which consists of four main regions; N-terminal DNA—binding domain (DBD) with 2 zinc fingers which behaves as a molecular nick sensor as well as an interface with protein partners; a nuclear location signal which can be interrupted by the caspase3 cleavage, which contains a BCRA1 Terminus domain (BRCT) motif which is involved in the protein to protein interactions. This region also contains sites involved in the negative regulation of PARP-DNA interaction; a C-terminal catalytic domain which includes a donor site (NAD⁺ binding) which is similar to the active site of mono-ADP ribosylating toxins. This domain is responsible for the nick-binding dependent PAR synthesis (Shall and de Murcia 2000). PARP-1 metabolises NAD⁺ to form branched ADP-ribose, which are then transferred to the nuclear proteins such as DNA polymerase 1 and 2. It has been suggested that poly-ADP-ribosylation plays a part in genome repair. This is because it modifies the architectural proteins close to DNA breaks which assist the opening of condensed structures of chromatins required for the repairing complex (D’Amours et al., 1999). However, by depleting the NAD⁺ levels, PARP-1 can also induce cell death.

Although there are 17 known PARP enzymes only PARP-1 and PARP-2 (Figure 1.4 b) are known to be activated by DNA strand breaks (Besson 2009, Audeh 2014). PARP-2 is DNA dependent and catalyses the formation of ADP-ribose polymers (Ame et al., 1999). Unlike PARP-1, PARP-2 does not contain a zinc finger although it does have a highly basic DNA binding domain as well as nuclear and nucleolar localisation signals. The binding domains of PARP-1 and PARP-2 are structurally different and PARP-2 does not bind as efficiently to DNA strand breaks as PARP-1 but recognises gap and flap structures (Yelamos et al., 2011).
Inhibitors of PARP-1 have therapeutic efficacy in experimental models of diabetes (Burkart et al., 1999), inflammation (Szabo 1998), shock (Liaubet et al., 2000) and breast tumours (Audeh 2014). There are three ways of inhibiting PARP-1 functions; 1) removing PARP-1 protein from the system, for example using knockout animals and gene silencing in cell culture models. This leads to the loss of PARP-1 and therefore DNA interactions, protein: protein interactions as well as the loss of catalytic activity. 2) By competitively inhibiting PARP-1 the polymer synthesis can be removed, however, PARP-1 can still be involved in the DNA binding and the protein to protein interactions although this can be more disruptive to the cell than the first method of inhibition. Neither of these two methods shows any effect on the NAD$^+$ levels. 3) The final method of inhibition is the depletion of NAD$^+$. This can be similar to competitive inhibition by making PARP-1 inactive, therefore inhibiting repair, replication and transcription processes. This also has effects on other NAD$^+$ utilising reactions such as glycolysis, apoptotic signalling and sirtuin function (Kirkland 2010).

PARP-1 inhibitors have been used for treatment of advanced cancers in clinical trials (Peralta-Leal et al., 2009). In cancer therapies, the mechanisms are different from other treatment models. The most common method, which seems to be the most effective, is targeting cells with deficiencies in BRCA1 and BRCA2 since this means that the cells have issues with double stand break repair. By inhibiting PARP-1, the repair of single strand DNA breaks is also inhibited, leading to persistent single strand breaks which can develop to double strand breaks through stalled replication forks. Double stranded breaks can become lethal to the cells due to the lack of BRCA3. This
treatment has shown the potential for a monotherapy as seen in clinical trials (Drew and Plummer 2009). During clinical trials, PARP-1 inhibitors and genotoxic drugs were combined when treating non-\textit{BRCA} cancers. It was found that this has a dose-limiting myelosuppression (Drew and Plummer 2009). It was seen in animal models that defected PARP-1 function and lower niacin levels can increase myelosuppression and the development of leukaemias (Kirkland 2009b). Previously nicotinamide has been used to inhibit PARP-1 during chemotherapy treatments; unfortunately this was converted to NAD\textsuperscript{+} particularly in the animal models (Kirkland 2009a). It could be possible for nicotinamide to increase PARP-1 activity and prevent NAD\textsuperscript{+} depletion which could make it an advantage to supplement with nicotinamide in many \textit{in vivo} models (Kirkland 2009a).

### 1.3 Sirtuins

Sirtuins are NAD\textsuperscript{+} dependent histone deacetylases (HDAC) which have begun to be acknowledged to be important in cancer cells (Moore \textit{et al}., 2012). The Sir2 protein was first found in \textit{Saccharomyces Cerevisae} and was identified by its role in transcriptional silencing of mating-type loci of the yeast (Frye 1999). Sirtuins roles in aging were originally found in yeast by a model of a similar lifespan. This model measured the number of times a yeast mother cell produced a daughter cell before senescing (Fry 2000). This discovery has encouraged studies into mammalian sirtuins. There are seven mammalian sirtuins, SIRT1-7, which are categorised by their ability to bind with NAD\textsuperscript{+} and their catalytic domain which is also known as the sirtuin core domain (Frye 2000). Although they can be conserved, the N and C termini are different, as well as differing in biological functions due to 1: enzymatic activities; 2: different binding partners as well as substrates and finally 3: the subcellular localisation as well as the pattern of expression. (Frye 2000; Moore \textit{et al}., 2012).

Most sirtuins catalyse NAD\textsuperscript{+}-dependent deacetylation although Sirt4 is an NAD\textsuperscript{+}-dependent mono-ADP-ribosyltransferase activity (Haigis \textit{et al}., 2006; Liszt \textit{et al}., 2005). Sirt1 and Sirt6 are both auto-ADP-ribosyltransferase as well as specific deacetylase activities (Michishita \textit{et al}., 2008). Sirtuins deacetylate modified lysine residues by
enzymatic processes that utilise NAD$^+$ cleavage with each reaction cycle (Suave et al., 2001). This makes sirtuin activity different from other histone deacetylases that hydrolyse acetyl-lysine residues due to sirtuins being intimately tied to the metabolism of the cells. The deacetylation by sirtuins begins with the cleavage of an amide from NAD$^+$ forming NAM as well as a covalent ADP-ribose peptide-imidate intermediate. This intermediate is then catalysed to form O-acetyl-ADP-ribose and deacetylated substrate (Figure 1.5) (Schmidt et al., 2004; Borra et al., 2004). The driving force of the sirtuin reaction relies on the hydrolysis of NAD$^+$. This reaction therefore has an effect on cellular processes such as DNA repair, metabolism, cell survival, cell proliferation and senescence.

![Diagram showing the mechanism of deacetylation of protein by sirtuins using NAD$^+$](image)

**Figure 1.5:** The mechanism of deacetylation of protein by sirtuins using NAD$^+$

Sirt1 is involved in both cell survival and cell death, which can be can be stimulated in different directions. One of the most effective ways to prevent cancer is energy restriction which has been seen in both rodents and primates. Sirt1 and Sirt3 exhibit survival functions, which could show that they promote tumourigenesis (Brooks and Gu 2009). Most evidence indicates that Sirt1 is an oncogene, which started with the identification of the Sirt1 substrate; tumour suppressor p53 (Luo et al., 2001; Vaziri et al., 2001). This is implied by the deacetylation of lysine on p53 by Sirt1, allowing the
cells to avoid p53 mediated apoptosis. Sirt1 also plays an important part in DNA repair as well as preventing tumourigenesis in mouse models. Sirt1 transgenic mice have been seen to live longer in leukaemia and colon cancer models (Deng 2009). When compared with normal tissues it has been seen that Sirt1 is overexpressed in different cancer types such as colon, skin, breast and prostate cancers (Zhang et al., 2009). However Wang et al., 2008 found that Sirt1 mRNA was reduced in cancers that have BRCA mutations compared with cancers with normal BRCA1. This indicates that there is a positive correlation between Sirt1 expression and BRCA1 which was confirmed via immunohistochemical staining in 45 human breast cancer samples (Wang et al., 2008).

1.4 CD38

CD38 is a multifunctional enzyme which generates a wide variety of biologically active compound using NAD⁺ as a substrate such as the synthesis and hydrolysis of cyclic ADPR as well as a Ca²⁺ mobilising agent (Billington et al., 2006). It seems to be more effective in degrading NAD⁺ than ADPR which has stimulated the theory that CD38 could also be a regulator of external NAD⁺ levels (Aksoy et al., 2006). A deficiency in CD38 has also been shown to accelerate an autoimmune disease in an ART2-dependent manner in NOD mouse model for type-1 diabetes. CD38 knockout mice have been seen to develop a more aggressive and rapid disease progression than the wild type (Chen et al., 2006).

1.5 Breast Cancer

According to the world health organisation 2014, breast cancer is the fifth leading cause of death in women as well as being the number one cause of death from cancer in women aged 20-59 years. The major problem is that although breast cancers can initially respond to therapy they develop resistance to chemotherapy, radiotherapy and biological therapies. There has been evidence to suggest that breast cancer is a group of diseases as opposed to a single disease which is encouraging scientists to rethink therapeutic strategies for breast cancer (Zhao et al., 2011). Individuals that have mutations in BRCA1 which is a breast cancer susceptibility gene, show an increased risk of developing breast cancer by 80% (Ford et al., 1998). Mutations in the breast cancer susceptibility genes (BRCA1 and BRCA2) account for an estimated 5-10%
of breast cancer cases (Schwartz et al. 2008). Although less than 1% of the general population seem to have these mutations, it does seem that it occurs more in certain ethnic groups for example Eastern European Jewish decent. The risks of women with these mutations to develop breast cancer can vary. BRCA1 mutations show between 44 and 78% of women with this mutation will develop breast cancer whereas BRCA2 see between 31 and 56% of women developing breast cancer (Antoniou et al., 2003; Chen and Parmigiani 2007). In terms of familial traits of breast cancer only 15 to 20% can be attributed to BRCA1 and BRCA2 mutations (American Cancer Society, 2013).

In this study, MDA-MB-231 and MCF-7 cell lines were selected as cell models. MDA-MB-231 is a triple negative cell line which means that there are no receptors of estrogen, progesterone or HER2. These cells are insensitive to some of the most effective therapies available for breast cancer treatment such as trastuzumab and endocrine therapies. MCF-7 is a human breast adenocarcinoma cell line which was developed from a pleural effusion from a patient with metastatic breast cancer (Levenson and Jordan 1997). It was originally taken in 1970 from a 69 year old Caucasian woman. MCF-7 cells are estrogen and progesterone positive unlike MDA-MB-231.

FK866 has been well documented and is currently in clinical trials for leukaemia, however very little research has been made in breast cancer cell lines. Due to the high level of BRCA mutations, PARP is one of the main enzymes for DNA repair. As PARP is an NAD⁺ consuming enzyme then by inhibition of Nampt by FK866 would therefore reduce the levels of NAD⁺ available to PARP for repair of DNA. FK866 has also been used as a single agent in cell cancer lines however it may have more of an effect on cell metabolism and cell viability if used as a co-therapy with other inhibitors of NAD⁺ consuming enzymes.

1.6 Hypothesis

NAD⁺ has long been thought of as a simple redox co-factor which is only involved in the fundamental reactions of basic metabolism such as glycolysis. However, it has been shown that NAD⁺ plays a more important role in a variety of signalling pathways such
as gene expression, Ca\(^{2+}\) signalling, post-translational modification of proteins and DNA repair (PARP, ADP-ribosyl transferases (ARTs)), CD38 metabolites or sirtuins. These findings have sparked an interest in the proteins involved in these signalling pathways as potential pharmacological targets for enzymes such as Nampt, PARP and Sirtuins; and number of drugs with diverse mechanisms of action is now in clinical trials. The hypothesis, to be tested here, is that decreasing intracellular NAD\(^+\) levels can be used as a co-therapy strategy to increase the efficacy of current and new chemotherapy treatments. The theory is that decreasing NAD\(^+\), and thus ATP, concentrations will increase the efficacy of the drugs that normally compete with ATP/NAD\(^+\) for binding sites on target enzymes (eg PARP or sirtuins). It is hypothesised that by inhibiting NAD\(^+\) levels and treating with current chemotherapeutics, there will be a higher level of cellular cytotoxicity.

1.7 Aims

The overall aim of this project was to investigate the mechanisms and therapeutic characteristics of FK866. This was achieved via the following objectives:

1. To investigate the effects of FK866 on respiration and metabolism in breast cancer cell lines, due to the role of NAD\(^+\) in glycolysis. This was achieved by measuring oxygen consumption rates, glycolytic activity via extracellular acidification rates and NAD\(^+\)/NADH ratios

2. To investigate NAD\(^+\) consumption in the cells treated with FK866 and identification of the major NAD\(^+\) consuming enzymes (CD38, PARP-1 and Sirt1) during Nampt inhibition.

3. To investigate cell vitality and NAD\(^+\) levels during co-therapy with pre-treatment of FK866 for 8 hours followed by treatment for either 2 or 3 days with a current or new chemotherapy treatments including PARP and Sirtuin inhibitors.

4. To assess if growing breast cancer cells as 3D cultures (spheroids) alters their response to treatment with FK866 or PARP inhibitors (Olaparib) as assessed in the objectives above.
Chapter 2: Methods

2.1 Cell Cultures

The two adherent cell lines used were MDA-MB-231 and MCF-7. MDA-MB-231 is a triple negative epithelial cell line from European Collection of Cell Cultures (ECACC). This cell line is cultured in Leboivitz L-15 medium (Lonza) with 10% heat-inactivated FBS (Lonza) and 2 mM L-glutamine (Lonza), incubated at 37°C for 3 days between subcultures. When subcultured, the cells are washed with PBS (Lonza) and trysinised with trypsin/versene (Lonza) before being counted and seeded at 2.5x10^5 cells/ ml as recommended by ECACC. MCF-7s are an epithelial cell line which was received from the University of West England. These were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Lonza) with 10% heat-inactivated FBS and 2 mM L-glutamine in the presence of 5% CO₂ and incubated at 37°C for 3 days between subcultures. When subcultured, the cells are washed with PBS and trysinised with trypsin/versene before being counted and seeded at 2.5x10^5 cells/ ml as recommended by ECACC. When running experimental procedures these cells were grown at 6x10^4 cells/ml in 24 well plates (Corning).

2.2 Treatments

The cells were treated with FK866 (Axon) at final concentrations varying from 1 nM to 300 nM. These were prepared in DMSO (Sigma Aldrich) and therefore the control was DMSO.

The other treatments used include PARP inhibitors 4-Amino-1,8-naphthalimide (NPH) (Sigma Aldrich) and Olaparib (Stratech); Sirtuin inhibitor Sirtinol (Sigma Aldrich); CD38 inhibitor Kuromanin (Sigma Aldrich); and methylating agent Temozolomide (Sigma Aldrich). All of these treatments were made up to varying concentrations from 100 nM to 100 µM. All were prepared in DMSO apart from Kuromanin which was prepared in PBS (Melford). The controls for each of these were the solution it was prepared in.
2.3 MTT assay

The MTT assay was used to test cell vitality in the presence of the cytotoxic drugs. It is a colourmetric assay which measures the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Melford) into an insoluble formazan produced by the mitochondria of viable cells. This assay is designed to measure cell vitality; this varies from viability by measuring the activity of the mitochondria which differs from viability which shows the total cells that are alive.

0.12M of MTT solution was added to 900 µl of PBS with cells adhered to the plate at a seeding density of 6x10⁴ cells per well. The plate was incubated for 2.5 hours at 37°C until a blue/brown precipitate was observed in the bottom of the wells. This converted dye was solubilised with 1 ml dimethyl sulfoxide (DMSO) (Sigma), and absorbance was measured at 540 nm using an Omega plate reader.

The blank was medium only with the MTT and DMSO, where the control was cells which had received no treatment. There were two repeats per plate of each drug concentration, control and blank. The cell vitality was then calculated as a percentage of the control.

2.4 NAD⁺ cycling assay

NAD⁺ cycling assay is used to determine the µM concentration of NAD⁺ per cell. This will essentially show whether the drugs which are being used have any effect on the levels of NAD⁺ produced. The protocol has been modified from Leonardo et al., 1996.

Cells were centrifuged at 1200 rpm for 5 minutes and the medium was removed. PBS was added to break each of the cell pellets. A cell count was performed using a haemocytometer and Trypan blue. The cell density was adjusted to 1x10⁶ cells/ml, before 1 ml of cell suspension was centrifuged at 15000 rpm for 2 minutes and the supernatant discarded. Pellets were immediately frozen in a dry-ice ethanol bath, dissolved in 250 µl 0.2 M HCl, and heated at 100°C for 10 minutes before being centrifuged again at 13000 rpm for 5 minutes to remove cell debris. This supernatant was stored at -20°C until use in the assay. The pellet was discarded.
The cycling mixture consisted of; 0.13 M Bicine, 2.16 mM Phenazine ethosulfate (PES), 2.04 mM Ethylenediaminetetraacetic acid (EDTA), 0.55 mM Thiazolyl blue (MTT) and 29.6% absolute ethanol, as well as 3 mM NaOH and 5 µl ADH per well. The standards were prepared at 0, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM and 60 µM of NAD⁺ by dissolving NAD⁺ (Melford) in PBS and then again in 0.2M HCl. Along with the cycling mixture, 49 µl of sample or standard was added to each well and the plate was incubated at room temperature in the dark for 30 minutes. The absorbance was measured in a 96 well plate reader at 565 nm. From the standard curve, the concentration of NAD⁺ per cell was then calculated.

2.5 NADH cycling assay

The NADH extraction was begun by the cells being washed and the 200 µl of trypsin to lift the cells from the plate. They were then centrifuged at 800 rpm for 5 minutes to remove the medium. The cells were resuspended in 1ml PBS to wash the cells. The cells were counted using a haemocytometer and then centrifuged again to remove the PBS before the pellets were frozen for one hour at -80°C. The pellets were broken up with 250 µl 0.2M NaOH and then heated at 100°C for 10 minutes. The samples were centrifuged at 1300 rpm for 5 minutes and the supernatant was saved for the NADH assay. The pellet was discarded.

The NADH standard curve was set up at concentrations of 5, 10, 20, 30, 40, 50, and 60 mM. They were prepared from synthesized NADH (Melford) and were dissolved in PBS. 20 µl of this was then taken and added to 250 µl 0.2 M NaOH (Sigma). The cyclic buffer consisted of 19.6 µl of 1M Bicine (pH 8.0), 16. mM PES (Sigma), 4.2 mM MTT (Melford), absolute ethanol and EDTA (pH 8.0) as well as 48 µl 0.1 M HCl and 5 µl ADH (400U/ml) dissolved in 0.1 M Bicine (pH 8.0). 151 µl of the cyclic mixture was added to 49 µl of either sample or standard curve and the plate was incubated for 30 minutes before being read in the plate reader at 565 nm. The results were then calculated using the standard curve to a value of nM per 1x10⁶ cells.

2.6 Seahorse Experimental Procedure

The sensor cartridge was hydrated overnight in PBS (Seahorse Bioscience) prior to the assay. The cells were treated for 24 hours before being was with PBS twice. They were
them treated with base medium (0.8 mM MgSO₄, 1.8 mM CaCl₂, 143 mM NaCl, 5.4 mM KCl and NaH₂PO₄). The sensor cartridge was loaded with 1 M Glucose, 5 mg/ml Oligomycin, which inhibits ATP synthase, 2 mM Antimycin A, which binds to the Qi site of cytochrome c reductase, and 3 mM Rotenone, which interferes with the electron transport chain in mitochondria, together, and 100 mM 2-deoxy-D-Glucose (2-DG), which inhibits glycolysis, into each of the 4 ports. The Seahorse XF24 was then calibrated and the assay was run taking 5 reading after each port was injected. Each loop consists of Mix for 1 minute, time delay for 2 minutes and then measure for 3 minutes.

The measurements were analysed by taking the averages of basal OCR, Glucose stimulated OCR, Oligomycin O and Rotenone/Antimycin A OCR. The coupling efficiency of glucose-stimulated respiration was calculated by 1-((oligomycin-rotenone/antimycin A)/(Glucose-rotenone/antimycin A)). The stimulation of basal OCR by glucose was calculated by (Glucose-Rotenone/Antimycin A)/(basal-rotenone/Antimycin A). Oligomycin insensitivity is normalised to glucose OCR and was calculated by (oligomycin-rotenone/antimycin A)/(glucose-rotenone/Antimycin A). Finally the final OCR calculation was to measure the non-mitochondrial OCR which is a fraction of basal which is calculated by rotenone/Antimycin A / basal. The ECAR parameters were calculated, the first calculation was glucose stimulated ECAR (glucose-2DG)/(basal-2DG). Stimulation of basal ECAR by oligomycin was calculated by (Oligomycin-2DG)/(basal-2DG). The spare glycolytic capacity was calculated by (oligomycin-2DG)/Glucose-2DG). Finally the non-glycolytic ECAR was measured by 2DG/basal.

2.7 Plate preparation for Spheroids

The protocol used was adapted from Nagelkerke et al., 2013 and Baron et al., 2012. Poly-hydroxyethylmethacrylate (P-HEMA) was prepared to 0.5% (wt/vol) in 95% ethanol. This was left on a shaking apparatus for 4 hours at room temperature to ensure all P-HEMA was dissolved. 96 well V-shaped plates were then treated with 50 µl of P-HEMA per well under sterile conditions. The p-HEMA treated plates were then left for 3 days in sterile conditions for the ethanol to evaporate leaving the plates evenly coated in P-HEMA. Once the ethanol was evaporated the plates were sealed with parafilm and wrapped in foil before being stored at 2°C until use.
2.8 Spheroid formations
Once the ethanol has evaporated from the plates, $1 \times 10^4$ cells were added to each well. The MCF-7 cells were added in DMEM alone and were centrifuged in the plates at 1000 x g for 10 minutes. They were then left in a 37°C, 5% CO₂, humidified incubator for 24 hours to allow spheroid cultures to form. The FK866 or Olaparib treatments were then added to these cells for 3 days.

MDA-MB-231 cells were added to the plates using L-15 treated with a matrix, Matrigel (Sigma Aldrich), at 2.5% to assist the cells to compact into a spheroid as these cells do not bind together without the matrix. They also then centrifuged at 1000 x g for 10 minutes before being placed on an orbital shaking platforms set at a constant rotation speed of 700 RPM in a 37°C incubator for 24 hours to allow the spheroids to form. Treatments were then added to these cells for 3 days.

2.9 Acid Phosphatase assay
The buffer for the assay used to assess acid phosphatase (APH) activity contains 0.1 M sodium acetate (3 M stock solution, pH 5.2) and 0.1% (vol/vol) Triton X-100 in deionised H₂O. This buffer can be stored at 4 °C for up to 4 weeks. The substrate solution is prepared by supplementing the assay buffer with 2 mg/ml of nitrophenyl phosphate (final pH 4.8) immediately before use. The substrate solution should always be freshly prepared and not be exposed to light.

The plates were centrifuged at 1000 x g and the medium was removed and the cells were washed twice with PBS before being centrifuged at 1000 x g. Once the cells have been washed 100 µl of substrate solution was added to each well before the plates were incubated for 90 minutes at 37 °C in the dark. After the incubation period 10 µl of 1 M NaOH was added to each well before the absorption was measured at 405 nm within 10 minutes.

2.10 Statistical Analysis
The statistical analysis performed on all the studies in this investigation was a one way analysis of variance (ANOVA). This was calculated using an online calculator, http://www.danielsoper.com/statcalc/calculator.aspx?id=43.
Chapter 3: The effect of FK866 on Glycolysis and the Mechanisms of NAD$^+$ Consumption

3.1: Introduction

NAD$^+$ is an essential co-factor for many enzymes involved in cellular and physiological processes. These include metabolism, apoptosis, DNA repair and inflammation (Imai., 2009; Khan et al., 2007; Luk et al., 2008). NAD$^+$ can be synthesised from tryptophan via the \textit{de novo} pathway (Figure 3.1); (Wei et al., 2008). However; the salvage pathway, represents the major route to NAD$^+$ biosynthesis in mammals (Suave., 2008; Olesen et al., 2010). Nampt is a rate limiting enzyme involved in the salvage pathway which converts Nicotinamide into NMN which is then converted to NAD$^+$ by nicotinamide mononucleotide adenyltransferase (Nmnat). FK866 specifically inhibits Nampt by competitively binding to the Nicotinamide binding site of Nampt.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure31.png}
\caption{The \textit{de novo} pathway and salvage pathway, showing the biosynthesis of NAD$^+$}
\end{figure}
Cancerous cells can overcome the normal need for growth factors when taking up nutrients by genetic mutations therefore increasing the nutrient uptake beyond the demands of cell growth and proliferation (Vander Heiden et al., 2009). Glycolysis is a metabolic pathway which converts glucose to pyruvate producing energy in the form of ATP. This process also involves NAD\(^+\) attaining a hydrogen ion when it is reduced via a redox reaction forming NADH continuing the electron transport chain. Intermediates which are necessary for proliferation such as ribose, glycerol and serine are produced in this process (Icard and Lincet, 2012). When healthy cells metabolise glucose to carbon dioxide via oxidation, a small amount of lactate is produced, however in most cancer cells, larger amounts of lactate are produced regardless of the availability of oxygen. It was hypothesised by Warburg that a defect in the mitochondria in cancer cells could result in impaired aerobic respiration and therefore the reliance on glycolytic metabolism (Vander Heiden et al., 2009).

Otto Warburg conducted a series of experiment in the 1920s, which compared the rate of oxygen consumption and the production of lactic acid in cancerous and healthy cells. He discovered that although both cancerous and healthy cells produce similar amounts of ATP, the oxygen consumption was higher in healthy cells whereas the lactic acid production was higher in cancerous cells (Warburg 1956; Zu and Guppy 2004). Therefore the Warburg effect (as it is now known) is the metabolic change observed in cancer cells from oxidative phosphorylation to glycolysis as the primary source of cellular energy. The centre of solid tumour masses tends to be hypoxic, which indicates that the cells present would rely on glycolysis (Icard and Lincet 2012). Increased levels of glycolysis have become the most commonly accepted and the most documented metabolic change for cancer cells (Moreno-Sanchez et al., 2014). This metabolic change also works to cause cellular response mechanisms that stem from the concentrations of intracellular compounds that are altered by the increased rate of glycolysis (Grimes et al., 2014).

PARP, sirtuins and CD38 are all NAD\(^+\) consuming enzymes. PARP cleaves NAD\(^+\) to form negatively charged polymers of poly (ADP-ribose) (PAR). PARP-1 and PARP-2 enzymes are crucial in DNA damage response as they use the PAR polymers produced from NAD\(^+\) as building blocks to repair damage to DNA (Yelamos et al., 2011). Inhibiting
PARP can be potentially therapeutic to cancerous cells; PARP inhibitors can be selective for gene defective tumour cells such as BRCA1 and BRCA2 mutations. Sirtuins are an important family of enzymes involved in eukaryotic metabolism. Sirtuins can be characterised by its NAD$^+$ dependence, as these enzymes couple the removal of acetyl modifications on lysine residues to the consumption of NAD$^+$ producing NAM and O-acetyl-ADP-ribose (Cui et al., 2015). 90% of all NAD$^+$ase activities in humans can be accounted for by the production of ADPR and nicotinamide by an NAD$^+$ glycohydrolase CD38 (Young et al., 2006; Vaisitti et al., 2011). It has been used as a negative prognostic marker for chronic lymphocytic lymphoma (CLL).

Whilst the mechanisms of FK866 are well established, the pathways that cause intracellular NAD$^+$ depletion have not been identified. Potential candidate pathways include: enzymes such as PARP involved in single strand DNA break repair, Sirt1, a NAD$^+$ dependent histone-deacetylase involved in post-translational protein modification and CD38, an NAD$^+$ glycohydrolase. In this chapter, the effect of FK866 on glycolytic activity, oxygen consumption and NAD$^+$/NADH ratio was measured in both MDA-MB-231 and MCF-7 cells to establish if there was any adverse effect whilst Nampt was inhibited. As NAD$^+$ plays an important role in glycolysis and requires Nampt which is involved in the salvage pathway to produce NAD$^+$ this would indicate how necessary the recycling of NAD$^+$ was to glycolysis and whether FK866 would have an effect on the oxidative stress and glycolytic activity. It would be hypothesised that as the NAD$^+$ levels decreased the glycolytic activity would also decrease. As well as this, the relative contribution of each pathway to FK866-induced NAD$^+$ depletion was defined. This knowledge will help to influence decisions about which pathways might be suitable for pharmacological targeting together with FK866 as a co-therapy agent.

### 3.2 Results

#### 3.2.1 Oxygen consumption rates (OCR)

In this chapter the Seahorse was used to measure the oxygen consumption and extracellular acidification rates whilst the cells were treated with FK866. The NAD$^+$ and NADH levels were measured using cyclic assays. MDA-MB-231 and MCF-7 cells were
used in this assay. The cells used for analysing the effect of FK866 on glycolysis were incubated with varying concentrations of FK866 for 24 hours prior to the assays being run.

OCR (oxygen consumption rate) is indicative of mitochondrial respiration and the changes in these rates are generally the result in changes in ATP synthesis rates that occur in response to the changes in ATP consumption. With the treatment of FK866 and the inhibition of Nampt; any effect in OCR could be due to reduced pyruvate production and therefore NADH production.
Figure 3.2: The rate of oxygen consumption in both MDA-MB-231 (a) and MCF-7 (b) cells when treated with FK866 for 24 hrs (number of independent experiments =3, number of repeats per experiment =2). This bar chart shows the effect of FK866 on the coupling efficiency, the effect of FK866 on glucose stimulation, the effect of FK866 on oligomycin insensitivity and the effect of FK866 on non-mitochondrial oxygen consumption.
MDA-MB-231 (Figure 3.2 a) and MCF-7 (Figure 3.2 b) cells were treated with varying concentrations of FK866 over 24 hours prior to OCR being measured. MDA-MB-231 shows a small change in the coupling efficiency of glucose stimulated respiration from 0.77 to 0.8 pMol/min at 10 nM FK866 however this change was not significant (p=>0.05). This was calculated by 1-((Oligomycin-rotenone/Antimycin A)/(Glucose-rotenone/Antimycin A)). The glucose stimulated oxygen consumption rates vary from 0.67 to 0.77 pMoles/min however these changes are also not significant. The MCF-7 cell line shows a coupling efficiency of glucose stimulated respiration of 0.67 pMoles/min at 30 nM and 0.7 pMoles/min at 10 nM and 100 nM therefore there is no significance in comparison to the control (p=>0.05). The glucose stimulation has a slight increase going from 0.67 pMoles/min in the control to 0.73 pMoles/min in the 100 nM which shows some significance between the control and 10, 30 and 100 nM FK866 (p=<0.05 ). Oligomycin insensitivity changes within both cell lines were insignificant with a change in MDA-MB-231 cells of 0.23 pMoles/min for the control to 0.2 pMoles/min for the 1 nM and MCF-7 shows rates varying from 0.3 pMoles/min at 10 nM and 0.33 pMoles/min at 30 nM (p=>0.05). From these results it can be seen that FK866 does not seem to affect the coupling efficiency, glucose stimulated oxygen consumption or the insensitivity to oligomycin. This indicates that there does not seem to be an effect on the oxygen consumption in these cells when treated with FK866.

MDA-MB-231 had some decrease in the non-mitochondrial oxygen consumption around 1 and 10 nM range, where the rate dropped from 0.2 pMoles/min in the control to 0.05 pMoles/min for 1 nM and 0.09 pMoles/min for 10 nM; however this was shown to not be significant (p=>0.05). The non-mitochondrial oxygen consumption rates is measured calculated by (Rotenone/Antimycin A)/(basal rate). MCF-7 cells also showed some variation in the non-mitochondrial oxygen consumption rates, varying between 0.17 pMoles/min at 10 nM and 25 pMoles/min at 3 nM however this has also been shown to not be significant (p=>0.05). Here the non-mitochondrial oxygen consumption can be seen to vary between cell lines. In MCF-7 there is an increase in oxygen consumption and in the MDA-MB-231 cells there is a decrease however these changes are not significant. Therefore it can be concluded from these results that FK866 does not have an effect of the oxygen consumption rate of the mitochondria in both MCF-7 and MDA-MB-231 cell lines.
3.2.2 Extracellular acidification rates (ECAR)

The rate of extracellular acidification (ECAR) reflects the rate of glycolysis. This pathway generates ATP and changes to the ECAR generally indicate changes in ATP synthesis rates which occur in response to the changes in ATP consumption. The ECAR is stimulated by glucose and oligomycin before the mitochondrial respiration is inhibited by rotenone and Antimycin A which gives the spare glycolytic activity. The non-glycolytic effect is measured after 2-deoxy-glucose (2-DG) is added to inhibit glycolysis. As ECAR is the measurement of acidification, it is measured in milli-pH units per minute (mpH/min). The extracellular acidification was measured in the MDA-MB-231 cell line with varying concentrations of FK866. This would indicate if inhibiting Nampt in these cells has any effect on glycolytic activity.
Figure 3.3: The rate of extracellular acidification of MDA-MB-231 (a) and MCF-7 (b) cell line after varying concentrations of FK866 after 24 hours (number of individual experiments = 3; number of repeats per experiment = 2). This bar chart indicates basal ECAR in both cell lines, is glucose stimulated ECAR, shows oligomycin stimulation, is spare glycolytic ECAR and finally indicates non-glycolytic ECAR.
MDA-MB-231 (Figure 3.3 a) and MCF-7 (Figure 3.3 b) cells were treated with FK866 at concentrations of 1 to 100 nM for 24 hours and ECAR was measured. The basal ECAR in both cell lines showed no significant change over the varying concentrations with measurements varying from 1.6 mpH/min at 100 nM and 3.97 mpH/min for 30 nM in MDA-MB-231 and 5.52 mpH/min for the 100 nM to 8 mpH/min for the control in MCF-7 cells (p=>0.05). These results indicate that there is some increase in the basal rates with increasing concentration of FK866. Glucose stimulated ECAR seemed to have similar results in both cell lines with an increase from 9.52 mpH/min for the control to 13.48 mpH/min for 100 nM in the MDA-MB-231 cell lines. The MCF-7 cells showed an increase from 6.18 mpH/min for the control to 12.4 mpH/min for 1 nM and 9 mpH/min for 10 nM however this then drops to 4.6 mpH/min for the 100 nM. Neither of these results were significant (p=>0.05). When stimulating with glucose it can be seen to have different responses between the cell lines. MCF-7 shows a result that would be expected with a gradual decrease over the higher concentrations of FK866 however the MDA-MB-231 cell line shows a gradual increase over the increase in concentrations. This could be due to the difference between cell lines. Oligomycin inhibits ATP synthase by blocking the proton channel which is necessary for oxidative phosphorylation of ADP to ATP. The stimulation of basal ECAR with oligomycin in MDA-MB-231 shows a similar response as the stimulation of glucose where the control rate was 18.79 mpH/min increasing to 28.2 mpH/min for 30 nM. This indicates that there is an effect on the glycolytic activity with FK866 (p=>0.05). The MCF-7 cells shows an increase in the ECAR rate with oligomycin stimulation for 6.2 mpH/min for control to 12.4 mpH/min for 1 nM, dropping to 4.6 mpH/min for 100 nM which is significant (p=<0.05) it also shows that as the concentrations of FK866 increase there is a slight decrease in the ECAR rates. This again indicates that the inhibition of Nampt needs to be greater to have a lowering effect on stimulation of glycolysis. Spare glycolytic capacity was calculated with the (oligomycin-2-DG)/(glucose-2-DG). It is expected that FK866 would show a decrease in glycolytic activity which is what is seen in the MCF-7 cells. It can be seen from the results that there is little change between treatments in the MDA-MB-231 cells, varying from 1.93 mpH/min in the control to 2.26 mpH/min at 30 nM. MCF-7 cells shows a difference between the spare glycolytic capacity with rates varying from 1.7 mpH/min for 1 nM to 5.2 mpH/min for 100 nM however there does
not seem to be any significance in either of these results \((p=>0.05)\). The non-glycolytic ECAR shows some change over concentrations. In MDA-MB-231 cells there seems to be an increase over the varying concentrations of FK866 with 0.26 mpH/min for the control to 0.62 mpH/min for 1 nM \((p=>0.05)\). This indicates that FK866 also has some effect on the ATP production after inhibiting glycolysis. By having an effect on the ATP production, it therefore would have an effect on not only the metabolism in cells but also cellular processes such as cell division. The variation shows between treatments with 0.17 mpH/min for 3 nM to 1.57 mpH/min of the control in MCF-7 cells \((p=>0.05)\). Although these results do not reach significance, it might suggest that FK866 has some effect on glycolysis. It can be seen that although the stimulation of ECAR by glucose and stimulation of basal ECAR with oligomycin show some increase in mpH/min therefore indicating there is an increase in glycolytic activity when treating with lower concentrations of FK866; the higher concentration of 100 nM shows some decrease meaning inhibition of Nampt and therefore the reduction of NAD\(^+\) has an effect on glycolysis but this is only with higher concentration of inhibitor it could indicate high levels of inhibition are necessary for the effect.

### 3.2.3 NAD\(^+\)/NADH

NAD\(^+\) is involved in glycolysis, where it is reduced to NADH, and FK866 has been involved in inhibiting Nampt which recycles NAD\(^+\). Therefore the NAD\(^+\):NADH ratio was measured to see what the effect would be on the ratio after treatment of FK866 after 24 hours.
Figure 3.4: The NAD\textsuperscript{+}:NADH ratio measured using NAD\textsuperscript{+} and NADH cycling assay to see whether the ratio alters with the varying treatments of FK866 in MDA-MB-231 and MCF-7 cells (number of experiments = 3; number of repeats per experiment = 5).

The NAD\textsuperscript{+}:NADH ratio (Figure 3.4) was measured first by the NAD\textsuperscript{+} and NADH levels in each cell line 24 hours after treating with varying concentrations of FK866. Both cell lines in general show a decrease in NAD\textsuperscript{+}:NADH ratio which is to be expected due to the reduction in NAD\textsuperscript{+} levels whilst treating with FK866. With MDA-MB-231 cells there is some decrease in the NAD\textsuperscript{+}:NADH ratio from the control at 2.1 to 1.6 for the 100 nM treatment where this is statistically significant (p=<0.05). However for the MCF-7 cell line the NAD\textsuperscript{+}:NADH ratio seems to stay relatively the same varying from 2.4 for the control to 3.5 for 1 nM back to about 2.7 for the 100 nM concentration, and these results are not significant (p=>0.05).

From these results, it can be deduced that there is no effect on oxygen consumption rate in the mitochondria in MDA-MB-231 and MCF-7 cells with varying treatments of
FK866 after 24 hours. It is also seen there is a change in glycolytic activity in both cell lines, where both seem to have an increase in glucose stimulated ECAR and stimulation by oligomycin, however there was some change in the MCF-7 cells. There is a difference between the non-glycolytic. It was increased in the MDA-MB-231 cell line when treated with FK866 however; the MCF-7 doesn’t seem show any change. The NAD\(^+\):NADH ratio also does not seem to alter with varying concentrations in the MCF-7 cells whereas with the MDA-MB-231 cells this does decrease. The non-glycolytic ECAR shows only an effect on the MDA-MB-231 cells, which could be a connection to the NAD\(^+\):NADH ratio. Therefore from these results we can see that by decreasing NAD\(^+\) via inhibiting Nampt with FK866 there is an effect on both cells when stimulating glycolysis either with glucose or oligomycin however this is only the case with higher concentrations of FK866 which means that glycolysis is not sensitive to low levels of inhibition of NAD\(^+\) over 24 hours.

3.2.4 The Mechanisms of NAD\(^+\) consumption whilst treating with FK866

The effects of FK866 has been well documented (Hassmann and Schemainda 2003; Holen et al., 2008 Tolstikov et al., 2014), however this is not the case for NAD\(^+\) dependent enzymes when inhibiting Nampt, therefore this was investigated using the MDA-MB-231 cell line. In this study the cells were treated with either FK866, an inhibitor of NAD\(^+\) consuming enzymes or a combination of the FK866 and NAD\(^+\) inhibitor. NAD\(^+\) levels were then measured every 2 hours between 4 and 8 hours. From these results, it will be clear which enzyme is the main consuming enzyme of NAD\(^+\) in each of this cell line.

MDA-MB-231 is a triple negative cell line so it is assumed that there would be higher concentration of PARP-1. It was hypothesized that PARP-1 would therefore be the main consumer of NAD\(^+\) within this cell line.
4-Amino-1,8-naphthalimide (NPH)

NPH is a specific PARP-1 inhibitor. MDA-MB-231 cells were treated for an 8 hour period with 3 separate treatments, FK866, NPH or a combination of FK866 and NPH. NAD\(^+\) was then extracted and measured using the NAD\(^+\) cyclic assay and was calculated in comparison to the control.

![Graph showing NAD\(^+\) levels over time](image)

**Figure 3.5:** MDA-MB-231 cell line was treated over 8 hours with FK866, NPH, or a combination of both FK866 and NPH. The results show the percentage of NAD\(^+\) compared to 0 hour time point to indicate what percentage of the consumption of NAD\(^+\) can be attributed to PARP-1 whilst inhibiting Nampt. (number of experiments = 4; number of repeats per experiment = 5).

PARP-1 is a major consumer of NAD\(^+\). However from **Figure 3.5** it can be seen that PARP-1 does not seem to play a significant role in consuming NAD\(^+\) whilst inhibiting Nampt with FK866, which is indicated by the 3.61% decrease at the 8 hour time point in NAD\(^+\) levels from the combination of the two inhibitors compared to treating with FK866 itself. This result does not seem to be significant (p>=0.05) and due to the small
value could be due to natural variation within the cells. It was originally suspected that PARP-1 was a main consumer of NAD$^+$ however these results indicate that this is in fact not the case. However as there is only a 5% difference in NAD$^+$ levels when comparing NPH treated cells to the control vehicle cells this could indicate that the results may differ with a longer incubation period. Also as NPH is a specific PARP-1 inhibitor it may be possible that if treated with PARP inhibitor Olaparib the results may differ as Olaparib inhibits PARP-1 and PARP-2 as there is some evidence to suggest that PARP-2 replaces PARP-1 when PARP-1 is inhibited.

**Sirtinol**

Sirtinol inhibits Sirt1 in mammalian cells. The cells were treated over an 8 hour period with 3 separate treatments, FK866, Sirtinol or a combination of both FK866 and Sirtinol. The NAD$^+$ was then measured using the NAD$^+$ cyclic assay and then calculated in comparison to the control.
Figure 3.6: MDA-MB-231 cells were treated with FK866, Sirtinol, or a combination of both FK866 and Sirtinol over an 8 hour period. The NAD⁺ levels were then calculated to a percentage of 0 hour time point to show the consumption of NAD⁺ with Sirt1 whilst inhibiting Nampt (number of experiments = 4; number of repeats per experiment = 5).

Sirt1 is an NAD⁺ dependent histone deacetylase (Cui et al., 2015). The combination of FK866 and Sirtinol has shown some change in the NAD⁺ levels (Figure 3.6) in comparison to the FK866 alone which has a value of 53.45% when treated for 8 hours (p=<0.05) in comparison to 0 hours whereas the combination of the two treatments have a value of 103.8% in comparison to 0 hours (p=>0.05). Sirtinol alone showed a drop in NAD⁺ levels to 69.12% (p=>0.05). It was predicted that PARP-1 would be the main consumer of NAD⁺ in MDA-MB-231 cells however from these results it can be seen that Sirt1 seems to consume more NAD⁺ in this cell line than PARP-1.

Kuromanin

Kuromanin is a flavonoid which inhibits CD38 (Kellenberger et al., 2011). The cells were treated over an 8 hour period with FK866, Kuromanin or a combination of both. The
NAD⁺ was extracted and measured using NAD⁺ cyclic assay and was calculated in comparison to the control.

![Graph showing NAD⁺ levels over time for different treatments.](image)

**Figure 3.7**: MDA-MB-231 cells were treated over 8 hour period with FK866, Kuromanin, or a combination of both treatments before the NAD⁺ levels were measured and calculated to a percentage of the 0 hour time point, which shows the consumption of NAD⁺ that can be attributed to CD38 whilst inhibiting Nampt (number of experiments = 4; number of repeats per experiment = 5).

It has never been reported whether MDA-MB-231 expresses CD38 or not, so is unclear about what results would occur with this treatment; however the single treatment with Kuromanin seems to show very little change in the NAD⁺ levels dropping to 86% which is the same as the natural drop seen in the control samples (p=>0.05) (Figure 3.7). When combining the treatments the decrease in NAD⁺ levels were at 43.9% which was 9.56% increase in NAD⁺ levels when treated with FK866 alone (p=>0.05). From the single treatment results, it could be assumed that CD38 is not expressed in MDA-MB-231 cells however from the combination treatments it can be seen that the NAD⁺ levels are increased after 8 hours of treatment and therefore would indicate the presence of CD38. The presence or absence of CD38 in MDA-MB-231 cells will need to
be verified via PCR as from this experiment it would seem to be inconclusive regarding the expression of CD38 in MDA-MB-231 cells.

3.3 Discussion
This study shows the effect of FK866 on glycolytic mechanisms, as well as which NAD\(^+\) consuming enzymes are the main contributors to NAD\(^+\) consumption when inhibiting Nampt. The knowledge from this study could help influence decisions about which pathways may be suitable for pharmacological targeting with FK866 as a co-therapy agent. NAD\(^+\) is a major component in cellular biochemistry. NAD\(^+\) is involved in glycolysis as well as DNA repair (PARP) and histone deacetylation (sirtuins) (Berger et al., 2004). The salvage pathway for the biosynthesis of NAD\(^+\) involves Nampt which converts NAM into NMN before it is converted to NAD\(^+\) using NMNAT (Figure 3.1). Nampt is targeted by FK866 which binds to the NAM binding site. It has been suggested that inhibition of this and limiting NAD\(^+\) availability, can inhibit major processes in cancer cells such as DNA repair and glycolysis, and induce the cells into an autophagic style cell death (Billington et al., 2008). Cancer cells have high metabolism and high levels of glycolysis (Raghaven et al., 2016). The Warburg effect is the metabolic change observed in cancer cells from oxidative phosphorylation to glycolysis as the primary source of cellular energy (Warburg 1956). NAD\(^+\) and NADH play a major role as co-enzymes in accepting and donating hydride ions in the glycolysis pathway, the respiratory chain and the redistribution of electron equivalents generated from these catabolic pathways in the \textit{de novo} biosynthesis of macromolecules (Houtkooper et al., 2010). Generally in a healthy cell, NADH, which is produced from the glyceraldehyde-3-phosphate dehydrogenase reaction, can be reoxidised to NAD\(^+\) by the lactate dehydrogenase reaction, the citric acid cycle or the mitochondrial respiratory chain. The NAD(H)-reoxidation occurs in conjunction with the rate at which uncoupled respiration takes place, for instance under conditions of high uncoupling protein (Houtkooper et al., 2010). During this study it was theorised that FK866 would have an inhibitory effect on glycolysis due to its effect on Nampt and NAD\(^+\) recycling. This would be because Nampt is a rate limiting enzyme involved in NAD\(^+\) biosynthesis,
so by inhibiting this enzyme the biosynthesis rate of NAD$^+$ would be decreased. The glycolytic pathway would therefore be slowed and the cell metabolism would be affected. The SEAHORSE XF24 measures oxygen consumption and extracellular acidification which can be used to calculate the metabolic rate of cells and was therefore used to identify if FK866 played a part in disrupting this process.

Both cell lines were treated for 24 hours with varying treatments of FK866 and then were run on a XF24 seahorse to measure the OCR and ECAR, as well as running NAD$^+$ and NADH cyclic assays to measure NAD$^+$ : NADH ratio. Changes to cellular metabolism capacity can be a component in causing cellular dysfunction caused by changes in oxidative stress (Rovito and Oblong 2013). Nicotinamide has been shown to partially protect oxidative phosphorylation however this is not in a dose dependent manner. It is also not able to restore to control levels after oxidative damage occurs, which suggests protection of glycolysis (Rovito and Oblong 2013). From the OCR results, it can be determined that there does not appear to be any change between the control and the varying concentrations of FK866 in both MDA-MB-231 and MCF-7 cell lines. The FK866 does not appear to have any effect on the oxygen consumption rates which is indicative of no effect on oxidative stress. This correlated with Warburg’s hypothesis which states that cancer cells develop a defect in the mitochondria that leads to impaired aerobic respiration which therefore means that in these cells, FK866 would not have an effect on oxidative stress (Vander Heiden et al., 2009). Cells will increase glycolysis to meet the energy demand whilst being treated with oligomycin (Nickens et al., 2013). This can be seen in all these samples with an increase from the basal line on the ECAR. Over varying concentrations, the ECAR results show a general increase in glucose stimulated ECAR and stimulation by oligomycin until 100 nM FK866 which shows a decrease. This indicates that the lower concentrations of this drug do not inhibit NAD$^+$ biosynthesis enough to have any effect on these cells and particularly with 1 nM treatment it seems to increase glycolytic activity when stimulated by glucose and oligomycin. There is no change in the spare glycolytic capacity and non-glycolytic ECAR in MDA-MB-231 cells however there is some change in the spare glycolytic capacity in the MCF-7 cells. This would indicate that FK866 is still having some effect on MCF-7 cells without stimulation to glycolysis and seems to increase the
spare glycolytic capacity where which therefore indicates that FK866 has a negative effect on the electron transport chain in the mitochondria. The spare glycolytic capacity is measured after the interference of the electron transport chain by Antimycin A and Rotenone. Therefore FK866 does appear to have some effect on the glycolysis in both cell lines; however MDA-MB-231 cells appear to need more stimulation from glucose and oligomycin. It does not appear to have any effect on oxygen consumption therefore meaning the cells are still respiring as normal over increasing concentrations of FK866.

NAD⁺ plays an important role in energy production and cellular resistance to stress. FK866 mediates NAD⁺ depletion through competitive inhibition of Nampt, a key mitochondrial enzyme involved in the regulation of NAD⁺ biosynthesis from nicotinamide (Hasmann and Schemainda 2003; Muruganandham et al., 2005; Gehrke et al., 2014). In the MDA-MB-231 cell line there appears to be a decrease in the NAD⁺:NADH ratio, whereas MCF-7 cells, there is no change. This indicates that in MDA-MB-231, FK866 does exhibit an effect on the NAD⁺:NADH ratio levels, which can be seen when looking at the results from the PARP, Sirtinol and CD38 inhibition with Nampt inhibition. The MCF-7 does not appear to exhibit any alteration in the NAD⁺:NADH ratio; however FK866 does seem to have some effect on the glycolysis. In both cell lines FK866 does appear to have some impact on the glycolysis. In both cell lines the NAD⁺ and NADH levels decreased; however the MCF-7 cells appeared to decrease at a similar rate keeping the ratio the same between the two for all concentrations, whereas the rate of decrease of NAD⁺ was higher than the rate of decrease in NADH, therefore altering the ratio in the MDA-MB-231 cells. Tumour cells can produce high levels of NAD⁺ and NADH which can alter metabolic activities and can be linked to mitochondrial dysfunction that inhibits OXPHOS and increase reactive oxygen species (ROS). This promotes uncontrolled cell growth can cause further DNA damage (Santirdrian et al., 2013). The ratio of lactate and pyruvate is connected with the NAD⁺:NADH ratio within the cells. This means that if the metabolic activity increases and the oxygen availability decrease, the result will be increased glycolysis rates and therefore an increase in the local lactate production (Wilhelm and Hirrlinger 2012).
PARP, sirtuins and CD38 are all NAD\(^+\) consuming enzymes. PARP-1 forms negatively charged PAR on the target protein by cleaving NAD\(^+\). By inhibiting PARP-1, the depletion of NAD\(^+\) and ATP can be protected (Bakondi et al., 2004; Erdelyi et al., 2009; Canto et al., 2013). PARP-1 is important to DNA damage response which has stimulated the development of small molecule PARP inhibitors as potential therapeutic agents for cancer. When treating MDA-MB-231 cells with NPH and FK866 together there was very little change in NAD\(^+\) levels. This shows that in the triple negative cell line, which is expected to have high levels of PARP, the NAD\(^+\) consumption is not significantly affected by the inhibition of PARP-1. This could be due to the fact that PARP-1 and PARP-2 have similar uses and when one is inhibited the other replaces its use (Steffen et al., 2013). To investigate this it would be advised to repeat using a PARP-1 and PARP-2 inhibitor for example Olaparib.

Sirtuins are a group of NAD\(^+\) dependent deacetylases which regulate eukaryotic metabolism (Cui et al., 2015), however unlike most deacetylases, Sirt1 uses NAD\(^+\) to produce NAM and O-acetyl-ADP-ribose when removing acetyl modification on lysine residues. From the results, we have seen that Sirtinol has a reversing effect on NAD\(^+\) levels over an 8 hour period in MDA-MB-231 cells. Sirt1 expression has been seen in most human breast cancer samples and its expression has been connected to distant metastasis and poor prognosis (Chung et al., 2015). During this study, MDA-MB-231 showed that there was no change in consumption of NAD\(^+\) when treating with both FK866 and Sirtinol when compared to the control. This indicates that the main consumer of NAD\(^+\) when inhibiting Nampt is Sirt1 in MDA-MB-231 cells.

CD38 is a NAD\(^+\) glycohydrolase that produces ADPR and nicotinamide which accounts for more than 90% of all NAD\(^+\)ase activities in humans (Young et al., 2006; Vaisitti et al., 2011). It is a widely used as a negative prognostic marker by clinicians in chronic lymphocytic lymphoma (CLL), as it is expressed on the surface of CLL cells (Vaisitti et al., 2011). It was unclear as to whether MDA-MB-231 expresses CD38. However when combining the Kuromanin and FK866 treatments there was some increase in the NAD\(^+\) levels when comparing with FK866 alone. This would indicate that either there is some CD38 present or that Kuromanin has an effect on another NAD\(^+\) consuming enzymes. However this cannot be proven without completing PCR analysis.
The results from the SEAHORSE have shown that FK866 does not have an effect on oxygen consumption in either of the breast cancer cell lines; however it does have an effect through the stimulation of glucose and oligomycin which inhibits the ATP synthase in glycolysis. This reduces the electron flow through the electron transport chain but does not stop the process completely due to a proton leak or mitochondrial uncoupling. This therefore, indicates some increase in glycolysis, however there is no significant difference. The NAD$^+$:NADH ratio shows a decrease in the MDA-MB-231 cell line after varying treatments of FK866 but not in the MCF-7 cells, this ratio decrease may be connected to the non-glycolytic activity increase. Inhibition of CD38 did cause some attenuation in the MDA-MB-231 cells. Inhibition of PARP-1 caused a slight attenuation suggesting that PARP-1 is partially responsible for the NAD$^+$ consumption in this cell line as well. Treatment with Sirtinol to inhibit Sirt1 completely reversed the FK866-induced NAD$^+$ activity in MDA-MB-231 cells. This suggests that sirtuin activity is the major mechanism responsible for NAD$^+$ consumption whilst inhibiting Nampt in MDA-MB-231 cells. This data will help identify cells that may be particularly responsive to FK866 and helps to identify possible co-therapies for these cells.
Chapter 4: Co-treatment of FK866 and anti-cancer agents in MDA-MB-231 and MCF-7 cells

4.1 Introduction

Nampt is a multifunctional protein which has potential immunoregulatory properties. Originally it was assumed to be involved in B cell development as a cytokine (Rongvaux et al., 2002), however it is now known to be a rate limiting enzyme which is involved in the first step of NAD\(^+\) biosynthesis. Nampt is over expressed in cancers such as breast, colorectal, gastric, lung and prostate, suggesting that it plays a crucial role in the tumour physiology (Hufton, 1999; Gehrke et al., 2014; Sociali et al., 2016). Nampt has been proven to be present in high concentrations in the cytosol as well as the nucleus; therefore the down regulation of Nampt can control tumour growth both in vitro and in vivo as well as sensitising cells to DNA damaging agents (Hasmann and Schemainda 2003). This can lead to a decrease in tumour growth and an induction of apoptosis caused by NAD\(^+\) depletion which is due to NAD\(^+\) being a substrate for PARP and sirtuins.

Originally FK866 was thought of as a non-competitive inhibitor of Nampt however recently it has been seen to directly compete with nicotinamide for the Nampt active site which induces apoptosis (Hasmann and Schemainda 2003; Gehrke et al., 2014). As FK866 is not cell cycle dependent it is therefore not involved in multi-drug resistance (Holen et al., 2008). It reduces intracellular NAD\(^+\) which can result in selective deaths in solid tumours (Hasmann and Schemainda 2003; Billington et al., 2008; Travelli et al., 2011; Gehrke et al., 2014). Nampt inhibitors such as FK866 have shown anti-cancer activities in pre-clinical models of both solid and hematologic cancers (Galli et al., 2013; Sociali et al., 2016). This is seen by the depletion of NAD\(^+\) and ATP levels which led to tumour growth inhibition (Hasmann and Schemainda 2003; Galli et al., 2013, Sociali et al., 2016). FK866 has been shown to cause delayed cell death which demonstrates characteristics of autophagy which is brought about by cell nutrient denial (Billington...
et al., 2008). With a lack of nutrients, the cells must gain energy and amino acids through self-digestion (Gozuacik and Kimchi 2007). In cancer cells, where apoptosis is unavailable because of the anti-proliferative control, autophagy is thought of as the second type of programmed cell death. A decrease in NAD$^+$ levels leads to an energy saving response which is expected as NAD$^+$ is important for glycolysis, the Kreb’s cycle and lipid-oxidation.

PARP is a major enzyme involved in DNA repair, as well as being involved in apoptosis, which is activated by DNA damage in tumour cells. Damage to DNA which could have occurred either directly, such as ionising radiation or mono-functional alkylating agents, or indirectly after an enzymatic cut in the DNA strand, can set off an acute cellular response as well as the synthesis of poly(ADP-ribose) by PARP. It may be possible that autophagy is associated to the background of PARP-induced cell death (Xu et al., 2006) which would be due to the reduction of NAD$^+$ levels. Sirtuins are a family of enzymes which have progressively been acknowledged to be important to cancer (Moore et al., 2012), but their involvement in breast cancer genesis and evolution remains to be explained. Sirt1 is a NAD$^+$-dependent histone deacetylase (HDAC) that has been linked to longevity, gene silencing, control of the cell cycle and energy homeostasis (Landry et al., 2000; Borra et al., 2005; Haigis and Guarente 2006; Dai et al., 2007; Byles et al., 2010). It also has roles in neuro-degeneration and inflammation (Yamamoto et al., 2007). Sirt1 is also involved with the tumour suppressor protein itself (Motta et al., 2004; Pruitt et al., 2006; Dai et al., 2007).

As seen in Chapter 3, over an 8 hour period FK866 has an effect on the NAD$^+$ levels within different cell lines. It can also be seen that by inhibiting with a combination of NAD$^+$ consuming enzyme inhibitors that this can be reversed. The effect of pre-treating MDA-MB-231 and MCF-7 cells with FK866 for 8 hours, reducing the NAD$^+$ levels, before treating with a secondary treatment will be investigated in this chapter by assessing the cell vitality as well as the NAD$^+$ levels. The secondary treatments include 4-amino-1,8-naphthalimide (NPH) (a PARP-1 inhibitor), Olaparib (a PARP-1 and 2 inhibitor), Sirtinol (a Sirtuin inhibitor) or Temozolomide (a methylating agent). It is hypothesised that there will be a synergistic effect when the cells are treated with a co-treatment as the NAD$^+$ levels will have been significantly reduced after the 8 hours prior to
secondary treatment. Therefore it should be observed which of these treatments would be viable for co-therapy with the breast cancer cell lines MDA-MB-231 and MCF-7.

4.2 Results
The cell vitality and NAD$^{+}$ levels were measured in both cell lines over both 48 and 72 hour periods to analyse whether pre-treating cells with FK866 for 8 hours before a second treatment would have an effect on the cells, as a possible co-therapy. The cell vitality was initially measured with treatments only to allow for comparison to the treatments with FK866.

Initially the MDA-MB-231 and MCF-7 cell lines were treated with FK866 alone as and the cell vitality was measured after 3 days. It was seen previously that FK866 has an effect on NAD$^{+}$ levels after 8 hours so this experiment aims to show if after 72 hours as a single treatment, that the cells are viable or not.
Figure 4.1: Cell vitality measured by MTT assay in MDA-MB-231 and MCF-7 cell lines treated with varying concentrations of FK866 (number of individual experiments = 4; number of repeats per experiment = 2). It can be seen that over these concentrations there is a significant decrease in cell vitality in both cell lines.

As seen in the previous chapter, FK866 decreases the NAD⁺ levels after 8 hours and also has some effect, although not significant, on the glycolytic activity therefore it can be assumed that there would be some effect on the cell vitality after the cells were treated with varying concentrations of FK866 over 72 hours, as shown in Figure 4.1. Both cell lines show a decrease in cell vitality over an increase in concentration of FK866. The MDA-MB-231 cells show an initial decrease from 3 nM (66.60% of the control) to 300 nM (49.53% of the control) (p=<0.05). The MCF-7 cells show an initial decrease from 10 nM (82.08% to 49.65% of control treatment cell vitality) to 100 nM FK866. In both cell lines these results are seen to be significant (p=<0.05).

The cell vitality was measured after 72 hours when treating with individual treatments of NPH, Olaparib, Sirtinol and Temozolomide. This will indicate how MCF-7 and MDA-MB-231 cells will behave with single treatment.
Figure 4.2: MDA-MB-231 (a) and MCF-7 (b) were treated with one of four treatments including NPH, Temozolomide, Sirtinol or Olaparib for 3 days. The cell vitality was then measured using the MTT assay and was calculated as a percentage of the control (number of individual experiments = 3; number of repeats per experiment = 2)
Before the co-treatment of the cells with FK866 and a secondary treatment could be carried out, the cells were treated with only the secondary treatments to analyse any effects on the cell vitality. The cells were treated for 72 hours with NPH, Olaparib, Sirtinol or Temozolomide before the cell vitality was measured using the MTT assay (Figure 4.2). From the results it can be seen that the MDA-MB-231 cells (Figure 4.2a) showed very little change in cell vitality when treated with NPH, with the cell vitality decreasing to 89.31% when treating with 10 µM (p=>0.05). Conversely the MCF-7 cell line (Figure 4.2b) exhibited an apparent increase in cell vitality to 137.43% when treated with 30 µM, these results however are not significant (p=>0.05). When treating with Olaparib there seems to be a slight dip in the cell vitality at lower concentrations of treatment before the vitality increases. The MDA-MB-231 cells show a dip in the cell vitality to 86.16% when treated with 1 µM Olaparib increasing to 118.47% when treated with 30 µM Olaparib (p=>0.05). The MCF-7 cells show a drop in cell vitality to 78.79% of the control when treated with 300 nM Olaparib before increasing to 95.62% of control when treated with 30 µM Olaparib, again these results are not significant (p=>0.05). Both cell lines, when treated with Sirtinol also show a slight dip in cell vitality over lower concentrations before recovering at higher concentrations although this seems to be more prominent in the MCF-7 cells as opposed to the MDA-MB-231 cells. In the MDA-MB-231 cells the dip is seen at 1 µM with a cell vitality of 73.19% of the control, with higher concentrations of treatment this increases to 104.96% of the control at 30 µM Sirtinol (p=>0.05). In the MCF-7 cell line the dip is also seen at 1 µM however the cell vitality drops further than in the MDA-MB-231 cell line showing a percentage of 67.44% of the control (p=>0.05). At the higher concentrations the cell vitality increases to 118.27% when treating with 100 µM Sirtinol (p=>0.05). When treating with TMZ however the cell lines behave quite differently, where the MDA-MB-231 cells show a drop in cell vitality the MCF-7 cells show an increase in cell vitality. The MDA-MB-231 cells show a drop in the cell vitality to 51.52% of the control when treating with 30 µM TMZ however the MCF-7 cells show an increase in cell vitality to 129.40% of the control when treating with 30 µM TMZ (p=>0.05). These results indicate that although with some treatments the cell lines can behave similar the treatments can be different depending on the type of cell used.
4.2.1 Co-therapy

MDA-MB-231 and MCF-7 cells were treated with 10 nM FK866 for 8 hours prior to treatment with NPH, Olaparib, Sirtinol or TMZ for 48 or 72 hours. After the 48 or 72 hours has passed the cell vitality and NAD$^+$ levels were measured. As it was seen in Chapter 3 the NAD$^+$ levels decrease after 8 hours of treatment with FK866 therefore the pre-treatment with the FK866 will reduce NAD$^+$ levels which could add to the effect that these treatments may have on both cell vitality and NAD$^+$ levels.

<table>
<thead>
<tr>
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<th>Cell Vitality</th>
<th>NAD$^+$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>72 h</td>
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<tr>
<td>Control</td>
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Table 4.1: Cell vitality and NAD⁺ of 48 and 72 hour co-treatments in MDA-MB-231 cells when treated with a) NPH, b) Olaparib, c) Sirtinol and d) Temozolomide after 8 hour pre-treatment with 10 µM FK866. These were compared to cells without treatment to calculate percentage of control (n=3).

<table>
<thead>
<tr>
<th>Temozolomide</th>
<th>Cell Vitality 48 h</th>
<th>Cell Vitality 72 h</th>
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<th>NAD⁺ 72 h</th>
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<tbody>
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<td>127.13</td>
<td>169.75</td>
<td>401.08</td>
<td>194.84</td>
</tr>
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</table>

After 48 hours of treatment the cell vitality was measured using the MTT assay to assess whether treating with FK866 for 8 hours prior to treatment would have an effect on the cell vitality. Generally with each treatment there is not much change in the cell vitality after 48 hours when comparing to the control (Table 4.1). When the cells were treated with a secondary treatment of NPH for 48 hours both cell lines seem to generally show some increase in cell vitality (Table 4.1a). MDA-MB-231 cells show an increase in cell vitality to 146.95% of the control when treated with 3 µM NPH (p<0.05), this does begin to decrease back towards the control rates with higher concentrations with a cell vitality of 130.47% when treating with 100 µM NPH (p=>0.05), although not as much as in the MCF-7 cell line (Table 4.2a). When treating with the second PARP inhibitor, Olaparib, we get similar results where, over the lower concentrations the cell vitality increases but then begins to decrease at the higher concentrations in both cell lines (Table 4.1b). When treated with 10 µM Olaparib the MDA-MB-231 cells increased cell vitality to 141.41% of the control before decreasing to 124.12% when treating with 100 µM Olaparib. When MDA-MB-231 cells were treated with the Sirt1 inhibitor, Sirtinol, there seems to be a slight increase in cell vitality (p=>0.05) (Table 4.1c). MDA-MB-231 cells show an increase in cell vitality to 127.73% of the control when treated with 10µM Sirtinol before decreasing to 101.94% when treating with 100 µM Sirtinol however none of these show significance (p=>0.05). Finally, Temozolomide shows a similar result to previous treatments after 48 hours.
where there is some increase in cell vitality although it is not significant (Table 4.1 d). The MDA-MB-231 cells show an increase in cell vitality to 138.06% of the control when treating with 10 µM before decreasing to 127.13% of the control when treating with 100 µM (p=>0.05).

The cell vitality was also measured after 72 hours incubation with NPH, Olaparib, Sirtinol or Temozolomide after an 8 hour pre-treatment of 10 nM FK866 (Table 4.1). When the cells were treated with NPH it was seen to have some decrease in the cell vitality at the lower concentrations however this appears to increase when treating with higher concentrations of NPH (Table 4.1 a). MDA-MB-231 cells show a sharp decrease to 63.90% when treated with 100 nM NPH which then increases to 102.24% when treated with 10 µM NPH (p=>0.05). When the MDA-MB-231 cell line was treated with varying concentrations of Olaparib (Table 4.1 b) it showed an increase to 136.87% when treated with 3 µM decreasing to 105.56% when treating with 100 µM (p=>0.05). The MDA-MB-231 shows an increase in cell vitality over increasing concentrations of Sirtinol (Table 4.1 c) to 158.07% of the control when treating with 10 µM (p=>0.05). When treating with Temozolomide there seems to be an increase in the cell vitality in the MDA-MB-231 cells (Table 4.1 d). This is seen with an increase in cell vitality to 174.33% when treating with 30 µM Temozolomide (p=<0.05).

The NAD⁺ levels were then measured in samples taken after 48 and 72 hours of treatment (Table 4.1). When the cells were treated with NPH after 48 hours there is a decrease in NAD⁺ to 34.46% when treating with 10 µM FK866 (p=<0.05). This could be due to MDA-MB-231 cells being a triple negative cell line so therefore more dependent of PARP-1. During the Olaparib treatments it was seen that the there was a general increase in NAD⁺ levels after 48 hours (Table 4.1 b). The results show an increase in MDA-MB-231 cells to 191.95% NAD⁺ when compared to the control whilst treating with 30 µM Olaparib (p=>0.05). It seems that this is the opposite result that was seen in the cells treated with NPH; this could be due to the inhibition of PARP-2 as well as PARP-1. When cells were treated with Sirtinol it was seen that MDA-MB-231 cells showed no real change in NAD⁺ concentration when compared to the control (Table 4.1 c). In MDA-MB-231 the concentration varied between 64.85% of control when
treating with 3 µM and 106.56% of control when treated with 1 µM Sirtinol although these are not significant when comparing with the control (p=>0.05). Temozolomide is a DNA methylating agent which is not known to have any effect of NAD⁺ levels. From the results of the 48 hour treatment shows a large increase in NAD⁺ levels (Table 4.1 d). The MDA-MB-231 cells show an increase to 401.08% NAD⁺ when compared to the control when treated with 100 µM Temozolomide (p=>0.05).

When the NAD⁺ levels were measured after 72 hours with NPH (Table 4.1a), the MDA-MB-231 cells shows some increase at lower concentrations raising to 188.25% when treating with 300 nM before decrease back to 98.47% of the control when treating with 100 µM NPH (p=>0.05). When treated with Olaparib the cells showed a general decrease in NAD⁺ levels with a decrease to 66.20% when treated with 3 µM Olaparib (p=>0.05) (Table 4.1 b). When the cells were treated with Sirtinol they showed an initial decrease during the lower concentrations of Sirtinol, dropping to 30.65% when treated with 300 nM however this increases again to 128.16% when treated with 100 µM Sirtinol (p=>0.05). After the cell lines were treated with Temozolomide the NAD⁺ levels were varying however there is a general increase in the NAD⁺ levels (Table 4.1 d). MDA-MB-231 cells where they generally increase to 194.84% when treating with 100 µM Temozolomide (p=>0.05). When comparing these results to the 72 hour cell vitality it can be seen that in general with a change in the NAD⁺ levels correlate with the changes in cell vitality.
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Table 4.2: MCF-7 cells were pre-treated for 8 hours with 10 nM FK866 before being treated with either a) NPH; b) Olaparib, c) Sirtinol or d) Temozolomide for 48 or 72 hours. The cell vitality and NAD\(^+\) levels were measured and calculated as a percent of the control treatment (n=3).

The MCF-7 cells were treated in the same method as the MDA-MB-231 cells with the data shown in Table 4.2. After 48 hours the cell vitality and NAD\(^+\) levels were measured.

When treated with NPH (Table 4.2 a), the cell vitality increases to 142.64\% when treating with 10 µM NPH, however this decreases again to 110.25\% when treating with 100 µM NPH (p=>0.05). When treated with the Olaparib (Table 4.2 b) the cells showed a general increase in cell vitality with an increase to 131.66\% when treating with 10 µM Olaparib which decreased to 119.37\% when treating with 100 µM cell vitality (p=>0.05). The increase and decrease in cell vitality with the MCF-7 is not as high as the MDA-MB-231 which could be due to the differences in cell line. Both the NPH and Olaparib treatments show similar results in both cell lines although the Olaparib does not show quite as high a rise in cell vitality, this could be due to NPH being a PARP-1 inhibitor and Olaparib being a PARP-1 and 2 inhibitor. When these cells were treated with Sirtinol (Table 4.2 c) for 48 hours there was an increase to 128.05\% with a concentration of 30 µM (p=>0.05) which is a similar pattern that is seen in the MDA-MB-231. The MCF-7 cells show a similar pattern to the MDA-MB-231 cell line when treating with Temozolomide (Table 4.2 d) with an increase to 132.60\% when treating

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Table 4.2: MCF-7 cells were pre-treated for 8 hours with 10 nM FK866 before being treated with either a) NPH; b) Olaparib, c) Sirtinol or d) Temozolomide for 48 or 72 hours. The cell vitality and NAD\(^+\) levels were measured and calculated as a percent of the control treatment (n=3).

The MCF-7 cells were treated in the same method as the MDA-MB-231 cells with the data shown in Table 4.2. After 48 hours the cell vitality and NAD\(^+\) levels were measured.

When treated with NPH (Table 4.2 a), the cell vitality increases to 142.64\% when treating with 10 µM NPH, however this decreases again to 110.25\% when treating with 100 µM NPH (p=>0.05). When treated with the Olaparib (Table 4.2 b) the cells showed a general increase in cell vitality with an increase to 131.66\% when treating with 10 µM Olaparib which decreased to 119.37\% when treating with 100 µM cell vitality (p=>0.05). The increase and decrease in cell vitality with the MCF-7 is not as high as the MDA-MB-231 which could be due to the differences in cell line. Both the NPH and Olaparib treatments show similar results in both cell lines although the Olaparib does not show quite as high a rise in cell vitality, this could be due to NPH being a PARP-1 inhibitor and Olaparib being a PARP-1 and 2 inhibitor. When these cells were treated with Sirtinol (Table 4.2 c) for 48 hours there was an increase to 128.05\% with a concentration of 30 µM (p=>0.05) which is a similar pattern that is seen in the MDA-MB-231. The MCF-7 cells show a similar pattern to the MDA-MB-231 cell line when treating with Temozolomide (Table 4.2 d) with an increase to 132.60\% when treating
with 10 µM Temozolomide, before decreasing to 126.03% when treating with 100 µM Temozolomide (p=>0.05). These increases in cell vitality, although not significant, could indicate an increase in cell proliferation.

When measuring the NAD$^+$ levels over 48 hours MCF-7 cell line was treated with NPH it showed an increase in NAD$^+$ levels to 223.87% when treating with 100 µM NPH (p=>0.05). The increase in cell vitality in MCF-7 cells corresponds to the cells having more available NAD$^+$ however the MDA-MB-231 cells also show an increase in cell vitality with a decrease in NAD$^+$ although this could be due to the cells not responding to the decrease in NAD$^+$ levels and would possibly need a longer period of time with the treatment to see an effect. The MCF-7 cells show a decrease to 23.66% NAD$^+$ when comparing to the control whilst treating with 100 µM Olaparib (p=>0.05). The MCF-7 cells show a decrease in NAD$^+$ levels at lower concentrations of Sirtinol where there is a decrease in the NAD$^+$ levels to 50.61% of the control when treating with 30 µM Sirtinol however at the highest concentration it increases again to 103.61% of the control (p=>0.05). When the cells were treated with Temozolomide the NAD$^+$ levels fluctuated between 60.99% when treated with 100 µM Temozolomide and 118.68% when treating with 1 µM Temozolomide in MCF-7 cells (p=>0.05). When comparing the cell vitality results with the NAD$^+$ results when cells have been treated for 48 hours it indicates that in some cases, for example the NPH in MCF-7 cells there was a correlation between increase in NAD$^+$ and an increase in cell vitality which was also seen in the MDA-MB-231 cells when treated with Temozolomide. However in some cases such as the Sirtinol treatment in MCF-7 cells where the NAD$^+$ levels were decreased, the cell vitality was still showing some increase. This could be due to a delayed response between lowering of the NAD$^+$ levels and its effect on cell vitality.

The cells were also treated for 72 hours to see if a longer exposure to the treatment would have a greater effect on the cells. The MCF-7 cell line shows a decrease in cell vitality to 75.72% of the control when treating with 3 nM NPH (p=>0.05). When treating with Olaparib the MCF-7 cells do not show a significant change in the cell vitality with an increase to 117.25% when treating with 3 µM Olaparib and a decrease to 91.58% when treating with 100 µM Olaparib (p=>0.05). This behaviour is different to the inhibition with NPH which could be due to the inhibition of both PARP-1 and
PARP-2. The MCF-7 cells however show very little change in the cell vitality when treated with Sirtinol except when treating with the lowest concentration which shows an increase to 142.26% and begins to show an increase again when treating with 100 µM with an increase to 113.30% of cell vitality (p=>0.05). This difference in cell lines shows similar results as seen in the 48 hour treatment however it seems that the cell vitality has increased with the increased treatment time therefore this treatment appears more effective than the 48 hours treatment, particularly in MCF-7 cells. The MCF-7 cells only appear to show an increase in cell vitality when treating with the highest concentration of Temozolomide which has an increase to 134.51% of the control (p=>0.05). The cell vitality in both cell lines show similar patterns to the 48 hour treatment, but depending on the exact treatment, there are some benefits to the increased treatment time, particularly when treating with lower concentrations of NPH.

NAD$^+$ levels were also measured after treatment for 72 hours to determine if there was any effect on the NAD$^+$ levels in comparison to the control. NPH has been shown to have decreased levels of NAD$^+$ in the MCF-7 cells whereas the MDA-MB-231 cells do not exhibit a significant change in NAD$^+$ levels. The MCF-7 cells show a decrease in NAD$^+$ levels decreasing to 64.88% when treated with 30 µM NPH (p=>0.05). However when treating with Olaparib, the MCF-7 cells show a decrease to 32.07% when treated with 100 µM Olaparib however when treating with 300 nM Olaparib there was an increase in the NAD$^+$ levels to 108.08% of the control (p=>0.05). When the cells were treated with Sirtinol there was a general decrease in cellular NAD$^+$ levels with a decrease to 58.44% when treated with 100 µM (p=>0.05). There was a general increase in NAD$^+$ levels to 248.71% when the cells were treated with 30 µM Temozolomide (p=>0.05). The MCF-7 cells show a higher increase in NAD$^+$ levels than when treated with Temozolomide than the MDA-MB-231 cell line.

From these results it seems to show that there is a general increase in cell vitality which could be due to the increase in cell proliferation when cells are pre-treated with 10 nM FK866. It was hypothesised that the NAD$^+$ results would correlate with these results which are seen in both 48 and 72 hour treatments.
4.3 Discussion

Tumour cells show an increase in glycolysis and a high concentration of NAD\(^+\), therefore treatments are being developed to target the recycling enzymes. One of these treatments is FK866 which targets Nampt, an NAD\(^+\) recycling enzyme. It was hypothesised that when treating breast cancer cells with FK866 8 hours prior to a secondary treatment of with NPH, Olaparib, Sirtinol or Temozolomide for 48 or 72 hours, there would be a synergistic effect by reducing cell vitality and NAD\(^+\) levels. This was investigated by measuring the cell vitality using the MTT assay and the NAD\(^+\) levels using the NAD\(^+\) cycling assay.

FK866 inhibits Nampt therefore decreasing the NAD\(^+\) levels which would illicit some effect on glycolysis (see Chapter 3). This would therefore have an effect on cell proliferation. It has been shown in various cell lines that there is an effect on cell vitality when treated with FK866 and it has shown that this is the case with both MCF-7 and MDA-MB-231 cell lines as well. It was seen in Chapter 3 that there is an inhibitory effect on NAD\(^+\) levels after 8 hours of treatment by approximately 50%. PARP-1 and PARP-2 uses NAD\(^+\) as building blocks for repair of DNA so therefore treatment with Olaparib or NPH would block this process. The MCF-7 cells when treated for 48 hours generally show an increase in cell vitality and NAD\(^+\) levels but when treating for 72 hours the vitality and NAD\(^+\) decreased showing that with a longer treatment there is an effect when pre-treating with FK866 for 8 hours prior to treatment. However when the MCF-7 cells were treated with Olaparib the cell vitality increased although there was still a decrease in the NAD\(^+\) levels. The MDA-MB-231 shows an increase in cell vitality but a decrease in NAD\(^+\) levels when treated with 48 hours with NPH however when treated for 72 hours it shows an initial decrease before restoring the cell vitality whereas the NAD\(^+\) shows an initial increase before returning to control levels. When treated with Olaparib the cell vitality in the MDA-MB-231 cells were increased, the NAD\(^+\) levels were increased over 48 hours however after 72 hours the NAD\(^+\) levels began to decrease. Both treatments seem to have an effect on the cell lines however it seems that NPH has more of an effect on the cell vitality although at higher concentrations of the treatments there is not a significant difference between cell vitality results. This is due to both NPH and Olaparib being PARP inhibitors however
NPH only inhibits PARP-1 as opposed to Olaparib which inhibits both PARP-1 and PARP-2. As there seems to be a decrease in NAD$^+$ when the cells were treated with Olaparib but not when treated with NPH this could be due to the inhibition of both PARP-1 and PARP-2. Interestingly, studies have shown that increasing concentrations of FK866 with a set concentration of Olaparib does seem to show some sensitivity (Bajrami et al., 2012), however the study continued with media and drug being replaced every 72 hours for 15 days whereas this study was only conducted for 48-72 hours.

Sirtinol is a Sirt1 inhibitor. Sirtuins are a major NAD$^+$ utilising enzyme, as seen in Chapter 3. They are a family of deacetylases which reverse the acetyl modifications of lysine residues on histones using NAD$^+$, producing nicotinamide which is recycled by Nampt to form NAD$^+$. From the cell vitality results, there is an increase in the vitality of both cell lines although the MCF-7 cells do begin to decrease with higher concentrations. The NAD$^+$ levels show a general decrease in both cell lines for both time points although the MCF-7 cells also show a recovery in NAD$^+$ levels. There is a significant association between the expression of Sirt1 and distant metastasis as well as poor prognosis in breast cancer cells (Hiraike et al., 2011; Yuan et al., 2013). It can also promote cell survival through the p53 pathway. Oncogenic signalling of estrogen receptor α requires Sirt1 (Yuan et al., 2013), which could explain the lack of effect on the cell vitality in MDA-MB-231 cells which could be due to MDA-MB-231 cells being triple negative as they have no estrogen receptor α.

Temozolomide is a DNA methylating agent. A methylating agent binds to the DNA, and in the case of Temozolomide it binds primarily to guanine, which results in single-stranded DNA breaks, growth arrest and apoptosis (Horton et al., 2009). As it is able to pass the blood brain barrier it is mainly used for malignant glioma tumours. In the MDA-MB-231 cell line the treatment showed a general increase in cell vitality when treatment over 48 and 72 hours when the cells were pre-treated with FK866. There does seem to be an increase in the NAD$^+$ levels however Temozolomide has not been shown to have any effect on NAD$^+$ utilising or synthesising enzymes. This is a similar to the result seen in MCF-7. The change in NAD$^+$ levels could possibly be due to the single strand breaks caused by Temozolomide and therefore inducing a DNA repair response from PARP although this has not been proven.
From all the results presented in this study, MCF-7 cells show an increase in cell vitality with all treatments apart from when treated with NPH where there was a decrease in the cell vitality. The MDA-MB-231 shows an increase in cell vitality or very little change from the control with all treatments investigated in this study. Although there seems to be an adverse effect on the cell vitality the NAD⁺ results seem to show some decrease in NAD⁺ levels. This could indicate that a decrease in NAD⁺ and the inhibition of Nampt has a protective quality to the cells. It has been reported in another study that when inhibiting Nampt in mice the MDA-MB-231 cells appear to metastasise when treated with PARP inhibitors (Santidrian et al., 2013). This is another indication that Nampt inhibition and therefore the decrease in NAD⁺ levels and the increase in cell vitality could result in metastatic growth. This contradicts the hypothesis however from the NAD⁺ levels it can be seen that even with the inhibition of Nampt at the beginning of the incubation and then treating with NAD⁺ utilising enzyme inhibitors, the NAD⁺ levels do increase which could lead to an increase in glycolytic activity of the cells. Potentially a follow on from this experiment would be to pre-treat with the NAD⁺ utilising enzyme inhibitors before treating with FK866 similar to that of Bajrami et al., 2012; which did indicate MDA-MB-231 cells had some sensitivity to Olaparib after 15 days when treated with a secondary treatment of FK866. However although there does seem to be some effect on these cells when they are pre-treated with FK866 this is not significant and therefore from these results it would indicate that FK866 does not show a therapeutic benefit after these timepoints however there is the potential that there could be some therapeutic benefits when incubating for a longer period of time.
Chapter 5: Treatment of Cancer Cell Spheroid Cultures with FK866

5.1 Introduction
Monolayer (2D) cell cultures have been used in cancer research for several decades however, they have certain limitations. For example, due to often being grown in a non-biological environment such as treated plastics, they develop a flat and stretched morphology, and as a result do not experience the same cell to cell interactions as seen in in vivo tissues, resulting in them being more susceptible to cytotoxic treatments (Nagelkerke et al., 2013; Raghavan et al., 2016). These limitations have resulted in a demand for the development of 3D cultures which could provide a more viable model as they are able to act more like cells typical to those grown in vivo and this could lead to a better understanding of how tumours may respond to therapeutic treatments (Dufau et al., 2012). Cells can adapt to their surroundings and respond to signalling regarding cell proliferation, differentiation and function leading to various cell morphologies dependent on the environment and other factors (Knight and Przyborski 2015). The differences in morphology of monolayer and spheroid cells can alter the effect of cellular processes such as apoptosis, proliferation and drug sensitivities (Baron et al., 2012; Knight and Przyborski 2015). The differences between monolayer and spheroid morphologies also extend to growth patterns, protein expression and the relationship with the extracellular matrix (Kim, 2005; Baron et al., 2012). With the development of spheroid culture, the requirement to use time consuming and expensive in vivo studies is reduced. This is because spheroid cultures show favourable similarities to in vivo tissues, such as the expression of antigens, pH and oxygen gradients within the microenvironment, as well as the rate at which growth factors are produced and the distribution of the proliferating cells within the spheroid (Dufau et al., 2012; Kim 2005; Baron et al., 2012).

Investigations into the use of breast cancer cells to produce 3D cell cultures began 35 years ago (reviewed in Nelson and Bissell 2005). The early investigations into culturing
breast cancer spheroids showed that although they should have been able to generate relevant physiological behaviours in response to differentiating signals, attempts to culture rodent mammary glands failed in the ability to maintain the cells that could form acini and produce milk in response to the lactogenic hormone (Nelson and Bissell 2005). Gene expression in 3D cell culture can produce clinical expression profiles better than those generated in 2D cultures. 3D culture can therefore provide an improved method for assessing therapeutic methods as well as improving the clinical efficacy (Raghavan et al., 2016). Due to this it is now becoming more common to use 3D cultures to develop new anti-cancer therapies which should therefore improve pre-animal and pre-clinical studies for selection of treatment candidates (Kunz-Schughart et al., 2004; Hirschaeuser et al., 2010). 3D cultures from human cell lines such as breast and ovarian cancers, have been seen to have uses in studies regarding the regulation of physiology and therapeutic issues (Rodriguez-Enriquez et al., 2008). This makes the study of 3D culture important to understanding cell to cell and cell to matrix interactions (Shield et al., 2009).

Nampt mediated NAD$^+$ biosynthesis in cancer cell lines plays an important role in many physiological processes such as metabolism, energy generation, apoptosis, DNA repair and inflammation. Nampt activity can be over-expressed in several types of tumour including breast cancers. The Nampt inhibitor, FK866 has been the subject of extensive studies due to its potential importance in the control of tumours through its inhibition of Nampt. The FK866 molecule competitively inhibits Nampt by binding to the nicotinamide binding pocket and is specific to Nampt. FK866 also exhibits anti-tumour activity in tumour cells (Socialli et al., 2016).

PARP is a highly conserved enzyme which assists in the repair of DNA. Olaparib is an inhibitor of both PARP-1 and PARP-2. When inhibiting PARP-1 and 2 with Olaparib it can result in single strand DNA breaks which are of no consequence to normal cells as they can efficiently repair this type of DNA damage (Shaw, Hall 2013). However, when cells have deficiencies in homologous repair, then single strand annealing would be the only mechanism for repairing DNA. By inhibiting PARP, it is unable to assist in annealing the single strand breaks which would result in double strand breaks which cannot be repaired in cells with BRCA mutations. This can ultimately lead to apoptosis.
or senescence (Iglehart, Silver 2009; Helleday 2011). This results in cells with the BRCA mutation have a higher sensitivity to PARP inhibitors.

FK866 has only been studied using monolayer and suspension cell cultures, it is unknown if its effect on NAD\(^+\) levels and cell viability will be the same in 3D cell models. NAD\(^+\) levels and cell viability were measured in 3D cell cultures treated with FK866 and Olaparib and the results were compared to cells grown in monolayer cultures. It is hypothesised that cells grown in monolayer culture will be more susceptible to treatments of FK866 and Olaparib than 3D cultures due to the lack of cell to cell interactions that are present in monolayers.

5.2 Results

Due to the physiological differences between monolayer and spheroids it has been suggested that monolayers are not entirely valid for testing the effect of drugs (Hirshchhaeuser et al., 2010). So far all research into FK866 has been carried out in monolayer cultures. Therefore in this chapter the effect of FK866 and Olaparib on cell viability and NAD\(^+\) levels were compared between spheroid and monolayer cultures of breast cancer cells.

Firstly it was important to investigate how the spheroids would be grown. A recent publication by Nagelkerke et al., 2013 tested various methods for forming spheroids and from their results it was indicated that growing the cells in a v-bottom plate that has be pre-treated with poly-HEMA was the best method for spheroid growth. The cells were added to the plates and centrifuged for 5 minutes at 800 rpm before being left in an incubator for 24 hours for the spheroids to form. This method was therefore used in this study.
The MCF-7 cells form tight spheroid cultures within 24 hours with no assistance of a matrix, which would normally help the cells adhere to one another as well as assisting in proliferation and differentiation (Figure 5.1 a). However the MDA-MB-231 cells did not form spheroids at all when following this protocol so ECM gel (Sigma Aldrich) was used as a matrix to assist with the formation. These cells were also incubated on a rotating plate to help the spheroids form. These spheroids formed (Figure 5.1 b) however they are loosely formed when compared to the MCF-7 cells. These results confirmed the method of spheroid preparation, and allowed assays to be run with these cells. To measure the NAD$^+$ levels in the spheroids, the calculations needed to take a different approach, therefore the volume of spheroid vs viability was compared to determine if there was any correlation between spheroid volume and number of viable cells, which would then indicate whether the volume of spheroid could be used to calculate NAD$^+$ levels.
Figure 5.2: The correlation between the viability of cells and the volume of the spheroid. This indicates that as the viability decreases the volume increases although this is not significant (p=>0.05). (number of individual experiments = 3; number of repeated per experiment = 5)

The graph (Figure 5.2) indicates that as the viability of the cells decrease the volume of the spheroid increases. However this could be due to the fact that as the viability decreases the spheroids appear to be looser. After seeing that there is in fact some connection with the volume of the spheroid and the cell viability, the NAD$^+$ levels could be calculated using the volume of spheroid as opposed to cell number.

5.2.1 Cell Viability

Cell viability was measured in both MCF-7 and MDA-MB-231 cell lines in monolayer treated with FK866. Due to MTT being unable to penetrate spheroids, the APH assay was used to measure cell viability as this method has been used in other studies to measure cell viability. The APH assay is based on the quantification of cytosolic acid phosphatase activity which determines cell viability in both monolayer and spheroid cultures. This works when the intracellular acid phosphatase in viable cells hydrolyses $p$-nitrophenyl phosphate to $p$-nitrophenol which gives a colour change dependent on viable cells.
Figure 5.3: MDA-MB-231 and MCF-7 cells were treated with varying concentrations of FK866 from 1 nM to 300 nM over 72 hours before the cell viability was measured using the APH assay. A) show the results of these cells grown under monolayer (number of individual experiments = 3; number of repeats per experiment = 2), b) shows the results of these cells grown in spheroid cultures (number of individual experiments = 4; number of repeats per experiment = 5)
The APH assay was used to measure the cell viability in MDA-MB-231 and MCF-7 cell lines grown in monolayer cultures when treated for 72 hours with FK866 (Figure 5.3 a). It can be seen that the viability decreases to 78.18% when treated with 300 nM FK866 (p= <0.05) in MCF-7 cell lines however the MDA-MB-231 cell line shows a decrease to 62.92% when treating with 100 nM FK866 (p= <0.05). Although this shows slightly different results to the MTT assay (Chapter 4), where there was a decrease in effect from 10 nM, there is an effect on cell viability. The difference could be due to MTT measuring vitality as opposed to viability. The vitality measured the activity of the mitochondria within the cells whereas viability is the determination of living cells based on a total cell sample. Therefore there is an effect on the activity of the mitochondria within the cells from 10 nM FK866 however there is not an effect on the number of living cells until 100 nM FK866.

The cell viability in spheroid cultures was measured and calculated as percentage of the control (Figure 5.3 b). Although the MCF-7 cells seem to show some increase when treated with lower concentrations of FK866, increasing to 202.95% when treated with 1 nM FK866 (p=<0.05) it decreases to 66.72% when treating with 300 nM FK866 (p=>0.05). The MDA-MB-231 cells also show a decrease in cell viability with a gradual decrease to 39.35% when treating with 300 nM FK866 (p=<0.05). When comparing this to the monolayer culture there does appear to be an increased effect on cell viability when treating spheroids than monolayers. The effects of FK866 in spheroids decrease by 11.46% compared to the monolayers and MDA-MB-231 see a decrease in cell viability by 23.57% when compared to monolayer cells. This shows that the cells grown in spheroid are more susceptible to the Nampt inhibitor which could be due to increases in cell to cell interactions in spheroid cultures.
Figure 5.4: The effect on cell viability of MDA-MB-231 and MCF-7 cell lines when treating with Olaparib using the APH assay. A) shows the results of this in MDA-MB-231 and MCF-7 cells grown in monolayer (number of individual experiments = 3; number of repeated per experiment =2) and b) shows the results of these cells grown in spheroid culture (number of individual experiments = 4; number of repeats = 5).
Both breast cancer cell lines were grown in monolayers and treated with varying concentrations of Olaparib, a PARP-1 and 2 inhibitor, for 72 hours before the cell viability was measured (Figure 5.4 a). The cell viability in MDA-MB-231 cells show an increase in cell viability when treating with lower concentrations of FK866 however the cell viability decreases to 31.82% when treating with 100 µM Olaparib (p=<0.05). The MCF-7 cells show very little change in cell viability until treated with higher concentrations where the cell viability decreases to 43.43% of the control when treated with 100 µM Olaparib (p=<0.05). This indicates that only higher doses of Olaparib have an effect on decreasing the cell viability of cells grown in monolayer.

The spheroids were treated with varying concentrations of Olaparib from 100 nM to 100 µM for 72 hours before the cell viability was measured as a percentage of the control (Figure 5.4 b). When treating with lower concentrations, the MCF-7 cell line shows some increase in cell viability. This decreases to 92.14% of the control when treating with 30 µM of Olaparib (p=>0.05). The MDA-MB-231 also shows an increase in cell viability when treating with the lower concentrations of Olaparib which begins to decrease at higher concentrations to 104.38% when treating with 100 µM Olaparib (p=>0.05). When comparing the cell viability of cells grown in spheroid culture to the cell viability in monolayers there is an increase in cell viability in both cell lines with an increase of 72.56% in MDA-MB-231 cells and an increase of 48.71% in MCF-7 cells. This indicates that although Olaparib has an effect on the cells grown in monolayer at higher concentrations of Olaparib, the spheroids do not show an effect. This could be due to the increase in cell to cell interactions that are seen when cells are grown in 3D culture (Grimes et al., 2014; Knight and Przyborski 2015). From the trends seen in these results it could be possible that if the cells were treated with higher doses than were investigated here, then there could be some effect on the cell viability.

5.2.2 NAD⁺ Concentrations
After measuring the cell viability in both monolayer and spheroids for both cell lines, the NAD⁺ levels were measured to analyse whether there is any difference in how the treatments affect the NAD⁺ levels between the different methods of cell culture.
Figure 5.5: The NAD\(^+\) levels were measured in FK866 treated MD-MB-231 and MCF-7 cells. A) shows the effects of FK866 on NAD\(^+\) in the cells grown in monolayer and b) shows the effects of FK866 on NAD\(^+\) on the cells grown in spheroid cultures (number of individual experiments = 3; number of repeats per experiment = 5)
The NAD\(^+\) levels were measured in both MDA-MB-231 and MCF-7 cell lines which were treated with varying concentrations of FK866 and grown in monolayer after 72 hours (Figure 5.5 a). The MDA-MB-231 cell line shows an increase in the NAD\(^+\) levels to 98.49% when treated with 3 nM before decreasing to 69.60% when treating with 300 nM FK866 (p=<0.05). The MCF-7 cell line shows a greater decrease to 52.41% of the control when treating with 300 nM FK866 (p=<0.05). It can be seen that over the 72 hours the NAD\(^+\) levels decrease which corresponds to the results seen in previous chapters (Chapter 3). This correlates to the decrease in the cell viability, as when the NAD\(^+\) levels decrease, the cell viability also seems to decrease which is to be expected with this treatment.

The volumes of the spheroids were calculated in \(\mu m^3\) before the NAD\(^+\) concentrations were calculated as a percentage of the control (Figure 5.5 b). The MDA-MB-231 cells treated with lower concentrations of FK866 whilst grown in spheroids show an increase in NAD\(^+\) levels however; this begins to decrease at the higher concentrations to 70.95% when treating with 300 nM FK866 (p=>0.05). When compared to the monolayer the NAD\(^+\) levels decrease to a similar percentage of control with the 300 nM however; when treated with lower concentrations there seems to be more of an effect on depletion of NAD\(^+\) levels in the monolayer than in the spheroid cultures. This indicates that the lower concentrations have an effect on NAD\(^+\) levels in monolayer whereas the spheroid cultures require the higher concentration of FK866. The MCF-7 cells show a decrease in NAD\(^+\) levels to 68.27% when treating with 100 nM FK866 (p=<0.05). The monolayer shows a greater decrease in the NAD\(^+\) levels when compared to spheroid cultures which indicates that the treatment has less of an effect on spheroid culture until higher concentrations. Both cell lines seem to show similar effects in both the cell viability and the NAD\(^+\) levels. This correlates with the hypothesis that when growing cells in spheroid culture, the lower concentrations of FK866 seem to have little effect on the viability and NAD\(^+\) levels which could be due to the cell to cell interactions which could interfere with the uptake of the treatments in the cells whereas the monolayer is more susceptible to this treatment as it has a different morphology and lacks some cell to cell interactions. The NAD\(^+\) levels were then measured in spheroid and monolayer cultures which were treated with the PARP
inhibitor Olaparib to determine what the results of inhibition with an NAD$^+$ utilising enzyme.

**Figure 5.6:** MDA-MB-231 and MCF-7 cell lines grown in monolayer (a) and spheroid (b) culture which have been treated with varying concentrations of Olaparib and the NAD$^+$ levels were measured (number of individual experiments = 3; number of repeats per experiment = 5).
MDA-MB-231 and MCF-7 cells were grown in monolayer before being treated with varying concentrations of Olaparib from 100 nM to 100 µM to show how the NAD$^+$ levels vary in this range (Figure 5.6 a). The MCF-7 cells show some increase in NAD$^+$ levels to 180.09% when treating with 30 µM Olaparib (p=<0.05). The MDA-MB-231 cells also show an increase in NAD$^+$ levels to 362.76% when treating with 100 µM Olaparib (p=<0.05). Therefore the NAD$^+$ levels increase alongside increasing concentrations of Olaparib. This could be due to the inhibition of the NAD$^+$ consuming enzymes PARP-1 and 2 which therefore increases the NAD$^+$ concentration within the cells. When comparing this to the cell viability it seems that as the NAD$^+$ levels increase the cell viability decreases, this seems to be a different trend to what has been seen in the FK866 treatments however; as Olaparib inhibits PARP-1 and 2, which are NAD$^+$ consuming enzymes, this could explain the reason for the increase in NAD$^+$ levels. By inhibiting PARP this prevents the repair of DNA causing the cells to go through apoptosis and therefore decreasing the cell viability.

MDA-MB-231 and MCF-7 cells where grown in spheroids and treated with Olaparib for 72 hours (Figure 5.6 b). When MDA-MB-231 cells were treated with the lower concentrations of Olaparib the NAD$^+$ levels begin to show some decrease to 63.86% of the control when treated with 1 µM (p=<0.05) however with the higher concentrations the NAD$^+$ levels increase to 123.38% of the control when treated with 100 µM Olaparib (p=>0.05). The MCF-7 cells also show a decrease in NAD$^+$ levels when treated with lower concentrations of Olaparib to 74.10% when treated with 1 µM increasing to 130.06% when treating with 100 µM Olaparib (p=>0.05). When looking at the trends in the monolayer culture data, this correlates with the cell viability results whereby the cell viability increases at lower concentrations of treatment however it begins to decrease after being treated with the higher concentrations. When compared to the levels seen in the monolayer again it correlates with the cell viability showing that the increase in NAD$^+$ levels is not as high as the increase in monolayer which indicates that the cells grown in spheroid culture are not responding as well to the treatment as the cells grown in monolayer.
5.3 Discussion

Traditional methods of cell culture are centred on growing cells in a monolayer for their ability to adhere to treated plastics. These cells are easy to grow and maintain as well as straightforward to manipulate however; they lack the complexity of a solid tumour (Nagelkerke et al., 2013). Some of the environmental conditions observed in solid tumours can be artificially replicated into cultures, such as hypoxia and nutrient deficiency (Nagelkerke et al., 2013). 3D cultures are now being used to study different areas of cellular biology such as metabolism, apoptosis, proliferation and metastasis (Mullen-Klieser 1997; Kunz-Schughart 2004; Hirschhaeuser et al., 2010). The cells at the centre of the spheroid suffer from a lack of oxygen therefore making them hypoxic similar to solid tumour cells in vivo. In this chapter, 3D cell culture models were compared to monolayers and treated with FK866 (Nampt inhibitor) or Olaparib (PARP-1 and 2 inhibitor). The effects of these treatments were investigated by measuring the cell viability and NAD$^+$ levels to assess if there is a difference between the different methods of cell culture and their response to these treatments.

FK866 is a Nampt inhibitor which decreases the NAD$^+$ levels and therefore should reduce glycolysis (see Chapter 3) as well as other mechanisms such as DNA repair. During the investigations presented in this chapter, the monolayer and spheroid cultures seemed to have display similar responses when treated with FK866, although no response was seen in the spheroid culture until the higher concentration treatments. This is because as a single layer of cells, such as the monolayer, the treatments can easily affect the cells biochemical processes, however once the cells are in spheroid form they display more characteristics similar to that of in vivo tumour cells. Therefore a treatment which is effective on a monolayer culture may not exhibit the same effect on spheroid and animal models (Knight and Przyborski 2015). From the results presented in this chapter, it can be concluded that FK866 does have an effect on cells in spheroid although it requires a higher concentration of FK866 to achieve this result. This could be due to lower concentrations being unable to penetrate the hypoxic cells in the centre of the spheroid (Grimes et al., 2014) however at this stage this is as hypothetical explanation of the results.
Olaparib is a PARP-1 and 2 inhibitor which shows inhibition in micromolar concentrations. By inhibiting PARP, the repair of single strand breaks is also inhibited. Due to this, DNA forks are produced which then increases the likelihood of the development of double stranded breaks therefore leading to cell death via apoptosis (Shaw and Hall 2013). The results of this study show that there was some effect on both cell lines, however the effects of this treatment were more prevalent in the monolayer culture as opposed to the spheroid culture. As with the FK866 treatments this indicates that the lower concentrations of Olaparib are assumed to be unable to penetrate the hypoxic cells at the centre of the spheroid, potentially be due to the increased cell to cell interactions which are not seen in monolayer. Therefore it is likely to be necessary to treat the spheroid cultures with higher doses and/or longer treatment times, of Olaparib to achieve similar results to those seen at lower concentrations in monolayers.

During drug trials, many treatments seem to fail when they progress from monolayer cell culture to animal models (Grimes et al., 2014; Raghaven et al., 2016). By using 3D culture as an intermediate step between these phases, there is more accurate information on how the treatments are likely to work in solid tumours. This is because their metabolic profiles as well as signalling and cell to cell interactions are similar to that of in vivo cells (Hirschhaeuser et al., 2010; Grimes et al., 2014). FK866 treatment of spheroids showed similar results to the monolayer albeit requiring a higher concentration to achieve the same level of effect. It would therefore be useful to continue this line of investigation with a combination of treatments similar to that of Chapter 4 to investigate whether there would be a similar effect with the cells grown in spheroid culture opposed to monolayer. Olaparib shows that the effectiveness of the treatment decreases as the model is changed from monolayer to spheroid. This could indicate that this treatment is not as effective as first thought, however the next step of this study would be to either increase concentrations, as examination of the data shows that higher concentrations than initially investigated could be more effective, or increase the treatment over 5 days or possibly even 15 days similar to the co-therapy work conducted by Bajrami et al., 2013.
Chapter 6: General Discussion and conclusions

NAD⁺ is a co-enzyme which is found in all living cells as a carrier of electrons during redox reactions. It is most commonly known for the role it plays in glycolysis, however it is also plays a part in other pathways such as DNA repair and the deacetylation of histones. The recycling of NAD⁺ involves the enzyme Nampt which converts NAM to NMN before being converted to NAD⁺ via the NMNAT enzyme. Nampt has been seen to be up-regulated in some cancerous cells (Hassmann and Schemainda 2003). Nampt has become a target enzyme for chemotherapy treatments as it has been shown to be a pre-B-cell colony-enhancing factor (PBEF), as well as a poor prognostic marker when overexpressed in patients receiving doxorubicin-based chemotherapy for breast cancer (Hufton et al., 1999; van Beijnum et al., 2002; Folgueira et al., 2005). One treatment that has been developed to target Nampt is FK866. The overarching aim of this project was to investigate the mechanisms and therapeutic characteristics of FK866 as these remain poorly understood at this time. In addition to this, it was hypothesised that by decreasing the NAD⁺ levels (and thus ATP concentrations), FK866 will increase the efficacy of drugs that normally compete with ATP/NAD⁺ for binding sites on targeting enzymes (e.g. PARP and Sirtuins) and thus may have a role as a co-therapy strategy to increase the efficacy of many current and new chemotherapy agents.

Chemotherapy treatments aim to incite a range of processes such as DNA damage, ATP depletion and apoptosis via the apoptotic biochemical cascade, all with the intention of causing cell death in cancerous cells. FK866 was first synthesised in 2003 (Hassmann and Schmeinda 2003) and fits into all the requirements of a chemotherapy treatment since it has a low molecular weight and decreases intracellular NAD⁺, which should theoretically should lead to cell death. FK866 interacts with the active site of Nampt and directly competes with nicotinamide for the binding site. It could therefore be used as a chemotherapy agent as published research suggests tumour cells are more susceptible to the inhibition of NAD⁺ biosynthesis than healthy cells (Hassmann and Schmeinda 2003; Wosikowski et al., 2002; Holen et al., 2008). FK866 is not known to
be subject to common mechanisms involved in multidrug resistance, as its activity is not cell cycle dependent (Holen et al., 2008). It has been shown to have high anti-tumour activity in a wide variety of tumour cells, from solid tumours and leukaemias in vitro to a large number of human xenografts in nude mice and rats (Holen et al., 2008). FK866 has been shown to cause delayed cell death which indicate autophagy (Billington et al., 2008).

FK866 has been shown to cause delayed cell death which indicate autophagy (Billington et al., 2008). It was found that as a single agent FK866 decreases the cell vitality, NAD⁺ levels and glycolytic activity. NAD⁺ is consumed by 3 enzymes, PARP, CD38 and Sirtuins. It was found in this project that when inhibiting Nampt, all three enzymes are involved in NAD⁺ consumption, however in the breast cancer cell line, MDA-MB-231; Sirtuins were the main consuming enzyme when Nampt was inhibited. When pre-treating the cells with FK866 before treatments with NAD⁺ consuming enzyme inhibitors, it was shown that there was a non-significant increase in the cell vitality in the cells after pre-treatment with FK866 prior to treatment with an NAD⁺ consuming enzyme inhibitor or methylating agent however there was a decrease in the NAD⁺ levels.

Otto Warburg conducted experiments into the connection between the production of lactic acid and the rate at which oxygen was consumed in healthy and tumour cells. He found that although both cell types produced a similar amount of ATP, healthy cells consumed higher levels of oxygen in cancer cells as well as showing an increase in lactic acid (Warburg 1956). This is now commonly known as the Warburg effect. With FK866 inhibiting the biosynthesis of NAD⁺, the oxygen consumption rates were measured in both MDA-MB-231 and MCF-7 cell lines, it was found that FK866 does not have any significant effect on oxygen consumption. Nicotinamide has been previously shown to partially protect oxidative phosphorylation however this was found to not be dose-dependent. The cells treated were also unable to recover after the oxidative damage which indicates some protection of glycolysis (Rovito and Oblong 2013).

It is believed that an increase in glycolytic activity can be caused by mitochondria in tumour cells, which show signs of having impaired respiration (Moreno-Sanchez et al., 2014). Cells that are found on the outer layer of a tumour divide faster than those found in the centre of the tumour, therefore they are more likely to produce ATP via
oxidative phosphorylation whereas the hypoxic cells at the centre of the tumour rely more on glycolysis to produce ATP (Wallace 2005; Wu et al., 2007). Whilst cells are treated with oligomycin they the need to tap into glycolysis to achieve the ATP generation required (Nickens et al., 2013). This can be seen in all the samples during this study where there was an increase in glycolytic activity from basal levels after treatment with oligomycin. Over the varying concentrations of FK866 used, it was seen that there was a general increase in glucose stimulated extracellular acidification rate (ECAR), and stimulation of oligomycin was up to 100 nM FK866. Even with this there was no significant change in the spare glycolytic capacity and non-glycolytic ECAR. This indicates that the lower concentrations of FK866 seem to increase the activity of glycolysis however; at the higher concentrations a decrease in activity of glycolysis was observed.

The production of ATP and the catabolic metabolism can be connected via the NAD^{+}:NADH redox state, which indicates that low levels of NAD^{+} can limit the energy production within cells. During this study the NAD^{+}:NADH ratio was measured using the NAD^{+} cycling and NADH cycling assay. The NAD^{+} and NADH were measured with varying concentrations of FK866 over 24 hours. In the MDA-MB-231 cells, FK866 does exhibit some effect on the NAD^{+}:NADH ratio, whereas there was no observed change in the MCF-7 cells. The depletion of the NAD^{+}:NADH ratio by FK866 may indicate some cytotoxicity in the MDA-MB-231; however the increase in glycolysis at lower concentrations may indicate an increase in cell vitality. The NAD^{+}:NADH ratio is linked to the ratio of lactate and pyruvate within cells due to the local lactate production. The lactate production results from the decrease in oxygen availability and increase in glycolysis (Wilhelm and Hirrlinger 2012).

Although NAD^{+} is used in glycolysis it is also utilised by other enzymes in different biochemical processes within the cell such as DNA repair as well as NAD^{+} dependent protein deacetylation. These enzymes include PARP, Sirtuins and CD38. PARP-1 cleaves NAD^{+} and forms large, negatively charged PAR molecules. NAD^{+} and ATP depletion by PARP-1 can be protected by inhibiting PARP-1 (Bakondi et al., 2004; Erdelyi et al., 2009; Canto et al., 2013). Clinically PARP inhibitors could have potential as therapeutic agents and there is evidence to suggest that they are selective for tumours with
recombination gene defects like \textit{BRCA} 1 and \textit{BRCA} 2 mutations. This means that some of these inhibitors could be useful as single agents. When measuring NAD$^+$ levels after 8 hours of treatment with PARP-1 inhibitor, NPH, and FK866 MDA-MB-231 cells show a small decrease in the NAD$^+$ levels when compared to FK866 alone. This indicates that in the MDA-MB-231, although it was originally thought to be a main consumer of NAD$^+$, it appears that PARP-1 is not the main consumer in this cell line. The reason that the MDA-MB-231 show little change could be due to the fact that PARP-1 and PARP-2 have similar uses and when one is inhibited the other replaces it (Steffen \textit{et al.}, 2013).

Sirtuins are enzymes which are involved in the regulation of metabolism (Cui \textit{et al.}, 2015). Sirt1 uses NAD$^+$ to produce NAM as well as O-acetyl-ADP-ribose when the acetyl molecules are removed from lysine residues. It was hypothesised that sirtuins would be an NAD$^+$ consuming enzymes however were not thought to be the main consumer in MDA-MB-231 cells. It was seen that there was no change in the NAD$^+$ consumption when the cells were treated with FK866 and Sirtinol when compared to the control which indicates that Sirt1 is the main consumer of NAD$^+$ whilst inhibiting Nampt. It has been seen in most breast cancer cell lines that Sirt1 expression can be linked to metastasis and poor prognosis (Chung \textit{et al.}, 2015), this would indicate that Sirt1 plays a more important role in breast cancer than originally thought and therefore would explain why when inhibiting Nampt and Sirt1 seems to be completely reverse the effects of Nampt inhibition with FK866, returning to the control levels. CD38 is a NAD$^+$ glycohydrolase and is a widely used negative prognostic marker by clinicians in chronic lymphocytic lymphoma (CLL), as it is expressed on the surface of CLL cells (Vaissitti \textit{et al.}, 2011). It was unclear what to expect in terms of the results regarding MDA-MB-231 whilst inhibiting CD38 as it is unknown whether this cell line expresses CD38. However when combining the Kuromanin and FK866 treatments there was some increase in NAD$^+$ levels when compared to FK866 alone. This gives some indication that there is CD38 present although it could be that Kuromanin is having some other effect on these cells. To prove the presence of CD38 it may by useful to complete further analysis for example PCR.

As FK866 has an effect on NAD$^+$ levels after 8 hours, it was hypothesised that there would be a synergistic effect when pre-treating cells with FK866 prior to treatment
with NPH, Olaparib, Sirtinol or Temozolomide. MCF-7 cells showed a general increase in cell vitality after treating with NPH and Olaparib after 48 hours whereas the MDA-MB-231 cells showed an increase with these treatments of this time point. When treated for 72 hours the cell vitality decrease compared to the 48 hour treatment however the Olaparib treatment shows an initial increase but at higher concentrations seem to decrease to similar levels compared to the control in both MDA-MB-231 and MCF-7 cells. After 72 hours the NAD\(^+\) seems to decrease over varying concentrations of both Olaparib and NPH although the MAD-MB-231 seem to show some increase after treating with higher concentrations of NPH. This data would suggest that both treatments seem to have an effect on the cell lines; however NPH has more of an effect on the cell vitality. This is due to both NPH and Olaparib being inhibitors of PARP. As there seems to be more of a decrease in NAD\(^+\) levels when treating with Olaparib than with NPH this could be due to the inhibition of both PARP-1 and PARP-2. This is similar results to that published by Bajrami et al, 2012 where they report sensitivity to Olaparib with increasing concentrations of FK866. MDA-MB-231 showed that Sirt1 was a main consumer of NAD\(^+\) whilst inhibiting Nampt with FK866 for 8 hours however when treating the cells for 48 and 72 hours the cell vitality increased as the NAD\(^+\) levels decreased. As there is a connection with Sirt1 and poor prognosis in breast cancer cells, this confirms the results seen with the increase in cell vitality (Hiraike et al., 2011; Yuan et al., 2013; Chung et al., 2015). The lack of effect on the cell vitality in MDA-MB-231 cells could be due to the cells being triple negative as they have no estrogen receptor \(\alpha\) which requires Sirt1 (Yuan et al., 2013). Temozolomide is a DNA methylating agent which binds to the DNA, primarily guanine, and results in the single stranded DNA breaks (Horton et al., 2009). The treatment of Temozolomide with a pre-treatment of FK866 showed a general increase in cell vitality in both cell lines over 48 and 72 hours. There is also an increase in NAD\(^+\) levels although Temozolomide has not been shown to have an effect on NAD\(^+\) utilising or synthesising enzymes. This change in NAD\(^+\) levels could be due to PARP levels being increased in response to DNA damage although this has not been proven, performing a western blot may help in this aspect. Although some results from this study would indicate a positive response overall it can be seen that this is not a viable method of treatment with these cells. Potentially a different
approach or longer incubations would be a more viable approach to analysis this further.

Both the studies presented in Chapters 3 and 4 made use of monolayer cell cultures. A new method has been developed in recent years for the use of 3D cell culture, whereby the cells are grown in spheroid as seen in Figure 5.1 as opposed to the single layer of cells characteristic of monolayer cultures. Monolayers lack the functionality seen in tumour cells in vivo due to their flat and stretched morphology, which is physically dissimilar to tumour cells, and their lack of cell to cell interactions which makes them more susceptible to cytotoxic effects (Nagelkerke et al., 2013). Spheroid culture is therefore a valuable substitute which will provide a more accurate insight into the tumour response when treated with therapeutic agents, giving a similar response to that seen in vivo. (Dufau et al., 2012). When both monolayer and spheroid cultures were treated with FK866, they showed similar effects in both cell lines, although the spheroid culture required a higher concentration of FK866. It was expected that the spheroid cultures would be slightly less responsive than the monolayers because as mentioned, monolayer morphology means it is easier for the treatment to have an effect on the biochemical processes, however when in spheroid form they share more characteristics, typically seen in in vivo tumour cells (Knight and Przyborski 2015). When the cell lines were grown both in monolayer and spheroid cultures before being treated with Olaparib it was seen that although there was some effects on both cell lines there was more pronounced effect seen in the monolayer. Both treatments require higher concentrations when treating spheroid culture which would indicate that the morphology of the spheroid are harder to penetrate, resulting in higher treatment concentrations to incite a response in particular this could be due to the hypoxic cells at the centre of the spheroid, as well as the increase in cell to cell interactions which are not seen in monolayer cultures (Hirschaeuser et al., 2010; Grimes et al., 2014).

6.1 Conclusions

This project has investigated the characterisation of the pharmacological mechanisms and potential therapeutic uses of FK866. It was hypothesised that the decreasing intracellular NAD$^+$ levels can be used as a co-therapy strategy to increase the efficacy
of current and new chemotherapy treatments. By decreasing NAD\(^+\), and thus ATP production and availability, the concentrations will increase the efficacy of the treatments that would normally compete for ATP/NAD\(^+\) binding sites on targeting enzymes (e.g. PARP and sirtuins). Therefore by inhibiting NAD\(^+\) levels and treating with current chemotherapeutics, there will be a higher level of cellular cytotoxicity. FK866 as a single treatment has been shown in this study to have an effect on cell viability, NAD\(^+\) levels and glycolytic activity in breast cancer cell lines, although the effects on glycolytic activity were not significant. The effects seen on the cell viability and NAD\(^+\) levels were seen in both monolayer and spheroid cultures which indicates that this treatment, although requiring higher concentrations in the spheroid cultures, could illicit beneficial responses in in vivo investigations. When combining the treatments it was found that Sirt1 is the main consumer of NAD\(^+\) whilst inhibiting Nampt in MDA-MB-231 cell line although CD38 and PARP-1 also play a smaller role in NAD\(^+\) consumption. When the MCF-7 and MDA-MB-231 cells were pre-treated with FK866 before being treated with current chemotherapeutics such as Temozolomide or NAD\(^+\) consuming enzyme inhibitors such as NPH, Olaparib and Sirtinol it was seen that after 72 hours of treatment there was a small increase in cell vitality in most treatments with the exceptions of MCF-7 showing a decrease when treating with NPH. The NAD\(^+\) levels however seemed to show a general decrease excluding the cells treated with Temozolomide which is expected as Temozolomide has not been known to have an effect on NAD\(^+\) levels. The trends that were seen between the 48 and 72 hours indicate that potentially longer exposure to the treatments may result in a decrease in cell vitality From the results of each chapter seen in this investigation it can be concluded that FK866, as a single agent exhibits some degree of therapeutic effect as well as having an effect on cellular mechanisms. However as a pre-treatment this is not a viable treatment in terms of these cell lines. Therefore FK866 does not have a use in therapeutic treatments.

**6.2 Future Work**

Results from the present study have highlighted a number of areas for further investigation. For example, the Seahorse measurements in spheroid and with co-therapy would be useful as this would give a greater insight into the oxygen
consumption dynamics and glycolytic activities under these conditions. So far there has not been a significant amount of studies using the SEAHORSE assay in spheroid cultures however methods are being developed for this in different areas of research for example diabetic research. It would also be of interest to determine the effect on cell vitality if co-treatment times were increased beyond those utilised in this study, akin to the work of Bajrami et al., 2012. That study, showed that after 15 days there was a decrease in cell viability. By default I feel it would also be necessary to repeat this with a pre-treatment with NAD⁺ to see if increasing the concentration of extracellular NAD⁺ has any effect on the cells with PARP and sirtuin inhibitors. When analysing the results presented in Chapter 3, MDA-MB-231 cells showed some inhibition of NAD⁺ consumption when treating with FK866 and Kuromanin, a CD38 inhibitor, which could indicate that there is CD38 present in these cells. It is normally associated with leukaemia cells however it could potentially be possible for it to be present in these cells, therefore by performing PCR on these cells it will determine whether or not there is any presence of this enzyme. If this is not the case then potentially Kuromanin is reacting with another enzyme within these cells and it could be of interest to determine what this is. Spheroid cell culture would appear to offer an effective new method for drug analysis in a cell culture relevant to the ultimately intended target (in vivo tumour cells) and would be interesting to take this FK866 research further with cell lines grown in spheroid culture. This would include repeating the experiments conducted in Chapter 4 but with the cell lines growing in spheroid culture. Measuring the glycolysis in spheroids would be advantageous although the Seahorse is a relatively new machine and the methods used to measure oxidative stress and glycolytic activity have so far been with mainly adherent cells. Recent development work by Seahorse Biosciences has resulted in plates for islet capture however they have not guaranteed that these would work with spheroid culture and therefore would be difficult to get a working protocol. They are however making huge advances in this technology so hopefully in the future this will be possible. Although this procedure would be ideal in the future it can also be expensive to run as at the moment the islet capture plates are more expensive than monolayer plates. It would also be useful to take this research into more cell lines of breast cancer and also into
ovarian cancer as the BRCA gene and PARP activity are important in both types of cell and the PARP activity with BRCA mutations rely on NAD$^+$ for DNA repair.
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