Sensitisation of cancer cells to TRAIL using a cFLIP inhibitor

Amy Ross

Master of Science (by Dissertation) in Molecular Biology

School of Life Sciences

University of Essex

Date of submission: May 9th 2021

Covid-19 impact statement

The Covid-19 pandemic caused the university labs to close limiting the access and experiments able to take place. The course of research then changed to analysing previously collected data. Due to the length of the project, it was decided that the data collected would be sufficient for this thesis considering the limitations. From March 2020 all thesis work was undertaken whilst working from home and conducting meetings online.

CONTENTS

Abstract

Cancer is often used as a broad term to describe over 277 variant types of the disease. The progress of which is thought to be due to genetic changes (Hasspour & Dehghani 2017). Research showed fundamental key characteristics that defined how cancer cells behave. These were condensed into eight key traits summarised as self-sufficiency from growth signals, insensitivity to growth signal factors, evading apoptosis, limitless replicative ability, continuous angiogenesis, metastatic activity, escaping the immune response and reprogramming metabolism. Apoptosis is a mechanism that triggers a cellular programme leading to cell death. There are two main modes of apoptosis, the extrinsic and the intrinsic signal transduction pathways. The extrinsic pathway, triggered by death ligands such as TRAIL, contains many proteins such as death receptors and eventually culminates in Caspase-8 and -10 initiating a downstream caspase cascade. There are however other proteins such as FLIP that can inhibit apoptosis triggered by death ligands. As FLIP is often overexpressed in cancer cells, compounds that can block its anti-apoptotic effect hold great promise. In this study a new inhibitor of FLIP was tested alone and in combination with TRAIL. Cell survival was measured with a Crystal Violet assay after treatment with FLIPi alone, recombinant TRAIL alone or a combination. Apoptosis was measured using a DNA hypodiploidy (Nicoletti) assay It was found that that cancer cells showed some cytotoxicity in response to the FLIPi, but the FLIP inhibitor did not induce apoptosis. Some cancer cells, such as colorectal cancer HCT116 cells and advanced prostate cancer cells C4-2B could be sensitised to TRAIL by the FLIP inhibitor, while

others could not. The molecular reasons for this heterogeneity must be elucidated before a combination of TRAIL and FLIP inhibitor can be progressed further.

Statement of originality

I declare that the work within this document is my own unless stated in the text.

Acknowledgements

I would like to say a sincere thank you to my family, you have taught me to be resilient in the face of hardship and determined in the pursuit of success. My mother has showed me the true meaning of strength, my brother the meaning of wisdom and my father the meaning of fortitude.

To close friends, I would like to say thank you, the support invaluable and never taken for granted.

Finally, I would like to thank Dr. Ralf Zwacka and Dr. Andrea Mohr for encouraging me to be the best scientist I could be. Thank you for the never-ending emails, questions and support.

Abbreviations

cIAP	Cellular inhibitors of apoptosis
CRC	Colorectal cancer
DED	Death effector domains
DISC	Death inducing signalling complex
DR	Death receptor
FdUDP	Fluorodeoxyuridine diphosphate
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FADD	Fas associated protein with death domain
FDA	Food and drug administration
cFLIP	Cellular FLICE-like interleukin protein

FUDP	Fluorouridine diphosphate
FUMP	Fluorouridine monophosphate
FUR	Fluorouridine
FUTP	Fluorouridine triphosphate
FLIPi	FLICE-like interleukin protein inhibitor
MAPK	Mitogen-activated protein kinase
OPRT	Orotate phosohoribosyltransferase

1.Introduction

The term cancer is often used as a broad term to articulate over 277 variant types of the disease. The evolution of cancer is thought to be down to genetic changes such as chromosomal translocations, point mutations, deletions, gene amplification and activation of gene insertions (Hasspour & Dehghani 2017).

Previous research ascertained fundamental processes that defined how cancer cells behaved. These were summarised into 8 key behaviours. Self-sufficiency from growth signals, insensitivity to growth signal factors, evading apoptosis, replicative ability becoming limitless, sustained angiogenesis, metastatic activity, tissue invasion, evading the immune response and reprogramming metabolism (Fouad and Aanei 2017).

Cancer cells oppose healthy functioning cells in the way homeostasis is regulated. Deviant signalling, mutations, gene amplification and faulty degradation machinery can all be implicated in growth signals and cell death evasion by cancer cells. Many issues however are seen -but require more research- within downstream signalling pathways for example when the RAS protein contains mutations a multitude of issues arises in the affected cells. Proteins involved with regulatory functions and other activated pathways then consequently produce effects that thoroughly encapsulate enhanced growth, enhanced proliferation and apoptotic activity suppression. Proliferation is also encouraged when check points in the cell cycle are interrupted. An infamous example of this is P53, a protein which responds and detects cell stress. The protein is in part

responsible for preventing any proliferation and then instigating repair for cells that can be salvaged, but cell death is the consequence for cells that are beyond repair.

Understanding the biological mechanisms that underpin different cancer types will allow more efficient treatment and consequently better patient prognosis. In this project, the hallmark of apoptosis, evasion was studied in several different types of cancer by examining the role of the anti-apoptotic protein cFLIP with the help of a chemical inhibitor.

1.2 Prostate cancer

1.2.1 Pathophysiology

Prostate cancer occurs in the prostate gland usually stirring in the outer gland cells of the prostate- referred to as acinar adenocarcinomas. This is the most common type, however there are 3 other types with significant occurrence. Ductal adenocarcinoma originates in the cells that line the prostate gland ducts, comparatively to acinar adenocarcinomas the spread is more rapid. Another type starts in the cells that line the urethra generally called transitional cell cancer but sometimes referred to as urothelial cancer. Most commonly transitional cell cancer migrates to the urethra from the bladder, but it can also do the opposite and migrate from the prostate into the bladder on rare occasions. The cells that cover the prostate can also become cancerous. These are the squamous cells and for that reason it is named squamous cell cancer. This type of prostate cancer spreads and proliferates more rapidly than adenocarcinomas. In order to characterise how abnormal, the cells are the Gleason

scale is used. Several biopsies of the tumour are taken and studied, each sample being graded between 1 and 5, with 5 being the cells are very abnormal and 1-2 being healthy 'normal' prostate cells these scores are then added together. Furthermore, the Gleason scale can elucidate glandular differentiation. Grade 1 describes carcinomas with a uniform, oval and medium sized glands but are seen to be well differentiated amongst normal tissue. Grade 2 is very similar the former but differs in the glandular presentation. In this grade the glands are less well defined than that of the previous grade and vary more in shape and size. Grade 3 starts to deviate from grades 1 and 2 more significantly, the glands take on a twisted, angular and irregular form. (Humphrey, 2004).

1.2.2 Diagnosis

The Tumour, Node, Metastasis method can also be used to characterise the tumour progression. Stage 1 within this method would suggest the tumour is contained to half of the prostate gland or less. Comparatively stage 2 is when the tumour has spread through more than one half of the prostate gland but is still encapsulated within the gland. When the cancer cells have ruptured through the capsule of the prostate gland it is said to be stage 3 once the tumour moves beyond this point it has usually metastasised in to surrounding areas such as the back passage and bladder; this is stage 4. Stage 4 can also include metastasis into the lymph nodes and/or other organs beyond the immediate area e.g. lungs. (Borley and Feneley, 2009). Within developed countries prostate cancer is the second biggest cause of deaths in men, with incidence on the increase. Although this increase could be attributed to more men getting tested

and so more men will have the disease. This will show as a lag in the data as it is collated and analysed. There are several risk factors that are strongly associated with prostate cancer, the foremost is age. Incidence is low under the age of 40 after which the likelihood increases exponentially. Other factors such as diet have been linked, with low levels of vitamin E, Vitamin D and selenium being taken in from the diet. High dietary intake of saturated fats has also been seen to increase the incidence. 95% of prostate cancers are not hereditary. The 5% that is hereditary can face a further increase in risk if it is the male in the family line has been affected. If the affected individual is the father the risk is increased by 1.3, if the individual is a brother the risk is increased by 2.5. Prostate cancer seems to arise when there are at least 8 mutational events. The initial event in the timeline is the loss of tumour suppressor genes, one of the most common is the p53 gene. Investigation showed the p53 gene to have been mutated in 64% of cancerous growths. The genes of p21 and p73 have also shown mutations when investigated in tumours, with the former having found to be mutated in 55% of tumours. The recently recognised p73 has been found have a high degree of homology to p53 and has some mutations when explored in prostate cancer. Tumour suppressor gene p10 has been characterised as one of the genes that is most mutated in prostate cancer and because of this is credited with the acquisition of the metastatic phenotype in individuals. Many of the different prostate tumour types evolve from the cells in the urethra previously mentioned called the transitional epithelium. These tumours also result from cells in ducts with both types leading to transitional cell carcinomas. These make up about 4% of the prostate cases. As already mentioned, adenocarcinomas are very common and make up 95% of prostate

cancer tumours (Mazhar and Waxman, 2002). Prostate cancer is characteristic for being initially asymptomatic. It is often diagnosed through a physical rectal examination and confirmed with a blood test. If the prostate is found to be cancerous it would feel rough at the edges opposed to a health gland being smooth, it would also usually have enlarged presentation with the latter giving rise to benign prostatic hyperplasia (BHP). Symptoms only start to occur at a late stage, BHP can cause symptoms such as frequent urination, a struggle to urinate and a small flow of urine. Very occasionally blood can also be found in the urine, which is a condition known as haematuria and is often caused when the gland is eroded or infected. If the individual goes untreated the cancer can enter advanced stages. This can cause pain to occur in the perineum and cause other symptoms to emerge due metastasis of the cancer to different parts of the body. The symptoms can range from weight loss to neurological complaints (Patel and Klein 2009).

There is one main problem associated with rectal examinations as a diagnostic method, this is the variation displayed by the medical professional assessing the patient. Prostate specific androgen testing (PSA) was then introduced with the first uses not for diagnosis but as tool to monitor prostate cancer progression. This method was popular as it allowed early detection. This was a massive advantage as the asymptomatic nature previously made early detection difficult. However, BHP can often present readings similar to that of an individual with prostate cancer leading to false positives (Hoffman, 2011).

1.2.3 Treatment

There are several treatment options available to patients, if the tumour has a low Gleason score (and is confined to the gland). ‘Watchful Waiting’ can be employed, the other strategy is radical surgery - removing the tumour by removing the prostate gland. The consequences of these treatment options result in an improved result for the individual. Comparatively if the Gleason score is high it corresponds with a later stage cancer. This often means the gland has ruptured and often metastasised into other areas. This consequently sees a poor outcome for the patient, however there are treatments that can be used to manage the cancer (Maitland and Collins, 2008).

Firstly, androgen modulation therapy – also known as androgen withdrawal therapy- is quite effective in cancer of later stages however positive responses only last for 1.5-2 years (Rocchi et al., 2004) These positive results include a decrease in PSA levels, a decrease in patient pain and a decrease in the size of the tumour. Cancerous tissue returning will give rise to symptoms returning and bone metastasis occurring, it also causes the androgen receptor mechanism to partially or completely breakdown. It allows the cells to survive and proliferate using mechanisms independent of the androgen signalling pathway (Rocchi et al., 2004). Initially the tumour recedes as a consequence of androgen ablation because androgens play a core role in cell proliferation and regulating apoptosis rates. When the anti-androgens are given as treatment it causes the levels of androgen to fall and thus lowering the rates of proliferation and increasing cell death.

1.3 Colorectal cancer

1.3.1 Pathophysiology

Colorectal cancer (CRC) is thought to be the second most common cause of death linked to cancer within the US and the third most common overall. It is thought that over an individual's lifespan the possibility of developing sporadic colorectal cancer is 5%. It occurs in the epithelial cells that line the large intestinal tract. Normal cell processes are disrupted, and so cancerous characteristics start to develop, one of the first is the ability of autonomous growth. In this stage the capability to metastasize and damage surrounding cells has not yet developed. These characteristics are caused by epigenetic factor modifications. This then results in the gene function being altered. Detecting CRC at this stage has the biggest influence on positive outcomes for the affected individual.

1.3.2 Diagnosis

At present there are four main methods used to screen for CRC: the faecal occult blood test, sigmoidoscopy, a virtual colonoscopy and the immunological faecal occult blood test (Jimenez et al., 2010). Similarly, to prostate cancer CRC can be categorised using the tumour-node-metastasis (TNM) method (Amin et al., 2017). This method was originally designed to allow a patient prognosis to be determined. It is now used to aid treatment plans and gain insight into how effectively a treatment is working. TNM described the primary tumour location (T), whether there is lymph node involvement (N) and whether the tumour has metastasised away from the primary site (M) (Bertero

et al., 2017). Faecal occult blood tests look for blood in the faecal matter, this occurs because adenomas often bleed. When adenomas progress to higher stages, the epigenetic factors such as mutational events that cause this can be detected. This is done by using molecular techniques to amplify the DNA found in the blood that has been shed into the faeces. The DNA ends up in the stool occurs because of bacteria and normal exfoliation by the mucus in the colon and by cancerous tumours and polyps. A massive advantage of this method is that it does not take the patient any amount of time to prepare for the procedure however is rather expensive comparatively to other methods.

An endoscope can be used to examine a part of the colon where 2/3 of CRC are found, this is the sigmoid portion and the rectum. Both methods have seen to have a positive impact on the mortality of patients, this is due to the time sensitive nature of the disease. The earlier the diagnosis the more effective treatment usually is. However, sigmoidoscopy allows very small polyps to be detected. It also involves less labour time and is cheaper than the faecal blood test. The biggest drawback to sigmoidoscopy is that the entire colon is not visualized and so some tumours could be missed. As a result of a virtual colonoscopy a three (or two) dimensional is created with a high resolution. Using radiography, a thin sample section is used in combination with computed tomography. This is beneficial comparatively to a traditional colonoscopy as antegrade and retrograde views can be seen throughout the colon (Huang et al., 2005).

1.3.3 Treatment

One of the most used treatments to combat CRC are fluoropyridines, more specifically fluorouracil. It acts through affecting the enzyme in pyrimidine nucleotide synthesis which effects the rate of synthesis. This affect occurs due to inhibition of thymidylate synthase. In order to augment the effect of fluorouracil it can be given in combination with leucovorin. It boosts the inhibition of DNA synthesis as the binding of fluorouracil to thymidylate synthase is stabilised.

The structure of fluorouracil is very similar to that of uracil, which is one of the four bases in RNA. The difference in the two structures is that 5FU has a fluorine atom in place of a hydrogen atom on the C-5 as seen in figure 1. 5FU uses the same mechanism to transport it into the cell; facilitated transport (Zhang et al., 2008)

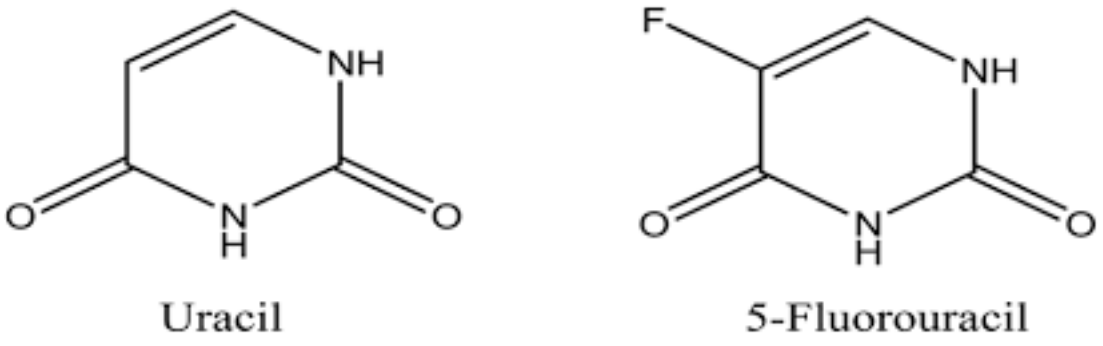

Figure 1 The structure of 5-Flurouracil comparatively to the structure of uracil. 5-Flurouracil is an analogue of uracil, only there is fluorine atom at the C-5 position instead of hydrogen (Zhang et al., 2008)

About 20% of Individuals with advanced stage disease that receive this treatment could see a reduction of up to 50% in the size of the tumour. Dependent on the delivery method the degree of adverse the side effects can vary. If the treatment is taken orally, digestive problems can arise, more specifically effects can be predicted based on a time dosing schedule. If patients receive doses continuously -usually for 4-5 days- toxic side effects such as Stomatitis and neutropenia can arise. This is inflammation of the mucous membranes in the mouth and a low level of white blood cells respectively.

However, if these doses are administered weekly the patient is more likely to endure diarrhoea. If the treatment is given intravenously, palmar plantar erythrodysestheia can develop. In palmar plantar erythrodysestheia the hands and feet may swell and become red and painful. With this administration route there is a lesser gastrointestinal and haematologic toxic effect associated. Current FDA (food and drug administration) approved drug are Fluorouracil, Capecitabine, Irinotecan, Oxaliplatin, Cetuximab and Bevacizumab (Meyerhardt and Mayer, 2005).

Once this occurs several active metabolites are produced intercellularly, fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate. These three metabolites disturb the process of thymidylate synthase (TS) and the synthesis of RNA. The enzyme dihydropyrimidine dehydrogenase catalyses the reaction and so is the limiting factor on the rate. It converts 5FU into dihydroflourouracil, when administered to a patient a majority (>80%) is catalysed in the liver. 5FU is activated once fluorouridiane monophosphate had been produced from 5FU. This can occur in one of two ways, firstly, through orotate

phosphoribosyltransferase. It does this using cofactor phosphoribosyl pyrophosphate. Secondly – which is a more indirect approach- it occurs through consecutive steps starting with flourouridine, phosphorylase with uridine kinase then being produced. Phosphorylation then converts flourouridiane monophosphate into fluorouridine diphosphate (Zhang et al., 2008). More metabolites are produced if flourouridiane diphosphate is phosphorylated further. More phosphorylation leads to the formation of flourouridine triphosphate, or fluorodeoxyuridine diphosphate can be formed by conversion using ribonucleotide reductase. Active metabolites can then be produced by the dephosphorylation or phosphorylation of fluorodeoxyuridine diphosphate. The metabolites produced are fluorodeoxyuridine monophosphate and fluorodeoxyuridine triphosphate correspondingly. Another activation pathway can occur which converts 5FU to fluorodeoxyuridine catalysed thymidine phosphorylase. Fluorodeoxyuridine then undergoes phosphorylation via thymidine kinase to produce fluorodeoxyuridine monophosphate. 5FU is converted into dihydroflourouracil, which is carried out by Dihydropyrimidine dehydrogenase to achieve this (Longley et al., 2003).

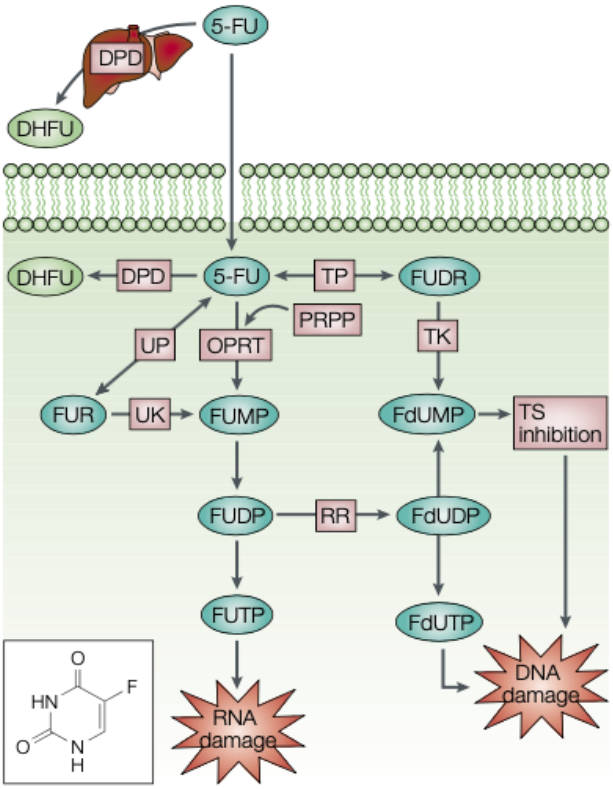

Figure 2: a schematic depicting the way 5FU is metabolised and the main metabolites that are active, FUMP, FUDP and FUTP. 5FU is predominantly converted to FUMP directly by OPRT with cofactor PRPP. It is indirectly converted by FUR through UP and UK sequential behaviour. FUMP is phosphorylated to FUDP and then phosphorylated again to FUTP which is the active metabolite. This metabolite can also be changed to FdUTP via RR. Dephosphorylation would convert FdUDP to generate active metabolite FdUMP and phosphorylated to create FdUTP. Also shown is the direct and indirect pathways with resultant RNA or DNA damage (Longley et al., 2003)

1.3.4 Thymidylate synthase summary

5,10-methylene tetrahydrofolate performs as methyl donor when deoxyuridine monophosphate is converted to deoxythymidine monophosphate. This produces fluorodeoxyuridine monophosphate, which is the active form of 5FU, which binds to the nucleotide site of TS. Produced from this reaction is a more stable ternary complex consisting of 5,10-methylene tetrahydrofolate and TS. This then prevents deoxythymidine monophosphate from contacting the nucleotide binding site on TS and consequently inhibiting the synthesis of deoxythymidine monophosphate. This subsequently causes an imbalance of deoxynuclotides and raises the levels of deoxyuridine triphosphate. The result of this is fatal DNA damage and the DNA repair and/or synthesis processes being impaired. The active form of 5FU and Deoxyuridine triphosphate get wrongly incorporated into the DNA. With these compounds being present in such high amounts, mechanisms to repair DNA, involving uracil-DNA-glycosy-lase fail. The continuous repair attempts and incorporation of the active 5FU and deoxyuridine triphosphate lead to breaks in DNA strands and cell death.

However colorectal cancer cells can develop a resistance to 5FU meaning that to re-sensitise cells to 5FU a combination treatment could be employed. When 5FU is administered to the patients with advanced colorectal cancer there is a low response rate of 15%. The resistance to 5FU is largely attributed to rapid DNA repair and increased metabolizing of the drug. Specific resistance revolves around the TS expression and inhibition involved in the 5FU mechanism. TS is coded for by the TYMS

gene and patients' prognosis seems to be linked with the level of this gene expression. Patients that have a higher expression in tumour cells exhibit a poorer prognosis.

More specifically 5FU is combined with platinum-based drugs such as oxaplatin and a folinic acid more specifically leucovorin. Oxaplatin is unique comparatively to other platinum-based drugs as it has a 1,2 – diaminocyclohexane ligand as part of its composition. The other platinum compounds combined with the previously mentioned ligand result in DNA repair processes to become heavily impeded. Although, resistance to oxaliplatin can occur and is linked the nucleotide repair pathway, a specific protein labelled WBSCR22 is also connected to resistance. The WBSCR22 protein can be used as a biomarker and furthermore may be a focus for development of new therapies to bypass the rising resistance (Van der Jeught et al., 2018)

1.4 Pancreatic cancer

Pancreatic cancer has been reported to be the 7th most common cancer globally. For example in 2008, out of 280,000 new cases of pancreatic cancer 265,000 lead to death, showing the aggressive nature of the disease (Bosetti et al., 2012).

This can be attributed to three main characteristics of pancreatic cancer 1) metastases in early stages comparatively to other cancers 2) the high morbidity rates are associated with both cachexia (muscle wastage), extreme weight loss, pain and low energy (asthenia) finally it is also resistant to most current treatments accommodating uncontrolled growth (Li et al., 2004). Worldwide it is the 14th most common cancer, with the highest incidence rates seen in Europe and North America. Patients have a 6%

five-year survival rate and only 25% survive one year. Most cases are diagnosed at later stages of disease. This is mostly due to the symptoms that present being ambiguous and generic to many other conditions (Kleeff et al., 2016).

1.4.1 Diagnosis

However, some symptoms that present can be linked to tumours in specific locations within the pancreas. Tumours located in the head of the pancreas while small are often only discovered when jaundice occurs without feeling pain. Some individuals also report some back or abdominal pain. Symptoms if they arise usually occur due to surrounding structures and organs being compressed. If the tumour is located in the tail of the pancreas, symptoms such as pain on the left side of the abdomen can occur initially, although more often symptoms are related to metastatic growth (Li et al, 2004).

Currently, there are few diagnostic tools and fewer which are very specific. Most treatment options revolve around palliative care with a small number of patients eligible for radical surgery, these eligible patients have localised tumours (Kleef at al., 2016).

There are a few genetic mutations that present themselves within pancreatic cancer, these are KRAS, CDKN2A, SMAD4 and TP53. It may be difficult to produce new therapeutic routes as at an epigenetic and metabolic level, pancreatic cancer is rather complex. Furthermore, there are several pathways and protein interactions that get activated complicating matters further. The most common type of malignancy in this type of cancer is adenocarcinomas categorised as malignant neoplasms. More specifically the most common form is ductal adenocarcinoma responsible for 367,000

new cases in 2015. Rarer forms are neuroendocrine tumours and acinar carcinomas with the former often secreting hormones. Acinar carcinomas sometimes release digestive enzymes into the circulatory system. The most seldom seen cases are colloid carcinomas, solid-pseudopapillary neoplasm and pancreatoblastomas (Vincent et al., 2011).

1.4.2 Pathophysiology

Suggested mechanisms producing cancers are the mutations and consequent inactivation of oncogenes and similar mechanisms with tumour suppressor genes. Growth factors also suffer from mutations that subsequently result in abnormal growth factors. Some growth factors and the relevant receptor have been found to effect signal transduction pathways for control and differentiation outcomes. Abnormalities that occur with mutations in these receptor pathways can lend a large advantage to the growth and survival of subsequent cancers. Many growth factors have been identified as being overexpressed -this included their receptors. cytokines, vascular endothelial growth factor and the family of epidermal growth factors are among those identified in this overexpression. Many hallmarks of cancer occur due to the copious amounts of factors promoting growth and the disruption of factors that inhibit growth. Commonly seen is the evasion of cell death, angiogenesis, metastasis and self-sufficiency from growth signals. These mechanisms have been elucidated quite well, for example hypoxia has been seen to manage the expression of vascular endothelial growth factor. Hypoxia is a common condition that arises when solid tumours occur, thus when cancerous tumours occur the function of the growth factor impeded. Furthermore,

tumour mass has been observed to cause changes in the extracellular pH leading to overexpression of vascular endothelial growth factor. Up-regulation of vascular growth factor and interleukin 8 operates through the acidosis and modulated by transcription factors NF-kB and AP-1 being transactivated. This deviant gene expression of proteins such as interleukin-1 that are involved when the cancer has metastasized, is the consequence of changes to the behaviour of transcriptional factors. There are two mechanisms put forward to explain this, of which the first is genetic alterations. These alterations occur on oncogenes genes such as K-ras and the outcome is continuous action in transcriptional factors. It is thought that this mechanism occurs in the initial stages of growth, with hypoxia and acidosis contributing in latter stages. Therefore, as growth continuous into advanced stages, tumour growth and development will become unrestrained and stress environments such as acidosis may increase seeing an increase in angiogenesis and metastasis. Being able to elucidate and target some of this gene expression may lead to more therapeutic avenues being produced in the future (Li et al, 2004).

1.5.3 Risk factors

There are several risk factors that are associated with pancreatic cancer. Age is seen to have a large impact on cases and consequently it is unusual to see a diagnosis of a patient under 30, with only 10% of individuals being under 55. It has been elucidated that reproductive factors do not impact incidence however more cases are seen in males over females. Different ethnicities seem to see different incidence rates. American populations find African Americans to have a much larger likelihood of

developing pancreatic cancer than Caucasian individuals. This could be attributed to other factors being linked more heavily to specific ethnicities. African- Americans may be found to be at a higher exposure to factors such a smoking and having higher incidence of diabetes. However, the biggest underlying cause of this variance could be attributed to other genetic interaction within the environments. Some studies have drawn a link between blood types and incidence. Some blood groups have a higher risk of adenocarcinoma of the pancreas developing. Blood groups O, A, B and AB carry the highest risk, this is thought to be a consequence of alterations in glycosyltransferase mechanisms. More specially those involved in the host inflammatory state across the mentioned groups (Rawla et al., 2019).

It has recently been suggested that the gut microbiota may influence the risk of developing pancreatic cancer. The most feasible mechanism put forward is that of immune activation inflammation is a consequence and propagates the same inflammation seen induced by tumour development. Research by Memba et al (2017) found an association between the development of pancreatic cancer and lower levels of Neisseria elongate and streptococcus mitis. Conversely when there were higher levels of porphyromonas gingivalis and Granulicatella adiacens the risk of cancer developing was increased.

Pancreatic cancer can only be regarded as having a familial cause if ≥2 first degree relatives have been diagnosed, this describes a relatively small percentage of 5-10% of cases. However, if there is a genetic association, the individual is up to 9 times more likely to develop pancreatic cancer comparatively to those with no familial risk. This

risk increases by just over 3.5-fold (32-fold increase comparatively to no risk) if there are ≥3 first degree relatives affected. Individuals that have type 2 diabetes have been found to have a higher risk (McGuigan et al., 2018).

Smoking is the factor that causes the greatest risk, rendering a positive correlation with the development of the disease. Analysis of many studies focused on the risk of smoking found that there was 74% higher risk for those that are current smokers and an increased risk of 20% for those individuals that no longer smoke. Once an individual has terminated smoking the risk doesn't decrease for at least 10 years with some studies suggesting that it takes 20 years for the risk to return to the same level as an individual who has never smoked. The length of time spent smoking and the volume smoked has also been found to increase the probability of developing this disease. The influence of e-cigarettes has yet to be elucidated but they thought to contain less harmful additives than those in traditional smoking. (Iodice et al., 2008). Diet contains a risk, more specifically the amount of alcohol consumed in one's diet. There is limited research related directly to the induction of pancreatic cancer by alcohol. However, the development of pancreatitis comes with an increased risk of pancreatic cancer evolving. Seen as the main cause of chronic pancreatitis is excessive alcohol intake alcohol can be loosely linked with the disease occurring (Samokhvalov et al., 2015). Pancreatitis is the resultant fibrosis and loss of both acinar and islet cells due to progressive inflammation of the pancreas. It is thought that in the US, between 1 and 14 individuals are affected per 100000 individuals. Individuals that experienced chronic

pancreatitis endured a 13-fold higher risk than those deemed as healthy or compared against a control group (McGuigan et al., 2018). Due to the amount of pancreatic cancer that arises from chronic pancreatitis this scenario could become a potential cancer screen. This is due to the low amount of chronic pancreatitis cases arising, however there is no current test, and a long period of time often elapses during which pancreatitis Is present, but cancer has not developed. Diet has been linked to cancer more specifically the consumption of red and processed meat. It is suggested that this is due to DNA damage caused. When this damage occurs carcinogens such as N-nitroso compounds are produced. However, specifically regarding pancreatic cancer there is tenuous evidence to suggested causation (McGuigan et al., 2018).

1.4.4 Diagnosis and staging

Although most cases only present symptoms at advanced stages if there is suspicion a physical examination can be carried out. Sometimes in individuals that are presenting jaundice, the abdomen can become distended but painful at the gall bladder location. Traditional computational tomography is the main method used for initial visualization. The favoured method is a dual phase helical CT, this is due to the high level of sensitivity associated along with the non-invasive nature. It has a successful identification rate of 98% for metastatic tumours and pancreatic adenocarcinomas. To provide a diagnosis for patients with tumours that are not viable for surgery, a fine needle aspiration biopsy can be completed using endoscopic ultrasonography to chaperon the procedure. This means that patients that are not suitable for surgical solutions can have a tissue diagnosis.

The previously mentioned -on page 2- Tumour, node, metastasis method can be applied to pancreatic cancer but when being assessed by clinician, tumours are categorised as resectable, metastatic and locally advanced. When described as unresectable it is due to the tumour metastasizing to other locations or arterial involvement e.g., the lymph nodes or invasion or inferior vena cava. In order to stage a patient accurately medical history and a physical examination are first considered. if this imaging is inconclusive the use of endoscopic ultrasonography can look for smaller tumours and elucidate vascular association.

1.4.5 Treatment

With respect to treatment the only possibility of somewhat curative action is surgery to remove the lesion, with the biggest limitation being very few cases being eligible. Surgery is performed according to the Whipple procedure, the head and uncinate parts of the pancreas, duodenum, proximinal 6 inches of the jejunum, gallbladder, common bile duct and distal stomach are removed. A surgical connection is then made between the common hepatic duct and the remainder of the pancreas and stomach joined to the jejunum. An alternative option to the is the pylorus-preserving pancreatic duodenostomy. The benefit of this procedure is that it offers the patient reduced blood loss and a shorter operating time. However, both carry similar risks: fistula of the pancreas, infection of the wound, delayed gastric emptying, anastomicleaking, haemorrhaging and endocrine insufficiency within the pancreas. If the cancer is in the body or the tail of the pancreas distal pancreatectomy is performed. As well as the affected areas being removed the spleen is also taken out as well. Due to the advanced

nature that surrounds body and tail lesions occurring the viability for surgery is less than a half of cases involving head lesions. Once the cancer has progressed to a metastatic nature surgery is no longer viable, causing chemotherapies to be considered. Gemcitabine is commonly used as an initial therapy with a 12-month survival rate for patients offering a reduction or stability in symptoms such as patient weight and pain comparatively with another option of treatment- fluorouracil monotherapy.

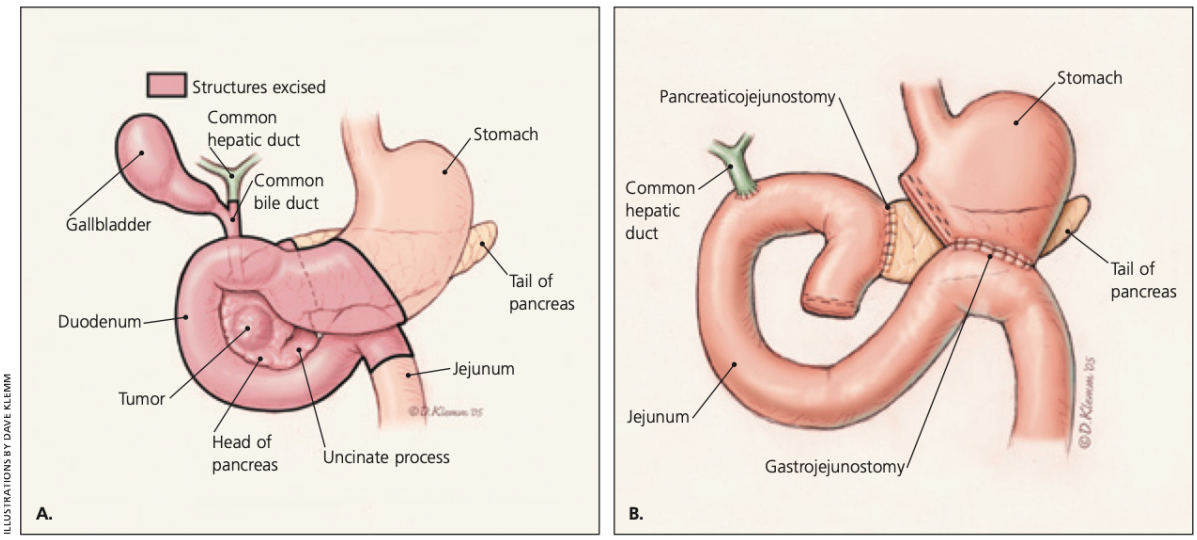

Figure 3: shows the areas affected by surgical treatment; the whipple procedure and the resultant system created. There is a connection of the remaining pancreas and stomach and hepatic duct to the jejunum (Freelove and Walling, 2006).

If the lesions are advanced in nature but are still localised external beam and intraoperative therapy can slow the progression for those patients not eligible for surgery. However, this method does not affect patient morbidity or the likelihood for metastasis to occur. Although there is the option of marrying the radiation therapy with fluorouracil-based treatment which comparatively with radiation alone, sees 40% of patients surviving 1 year. Another treatment option was pairing gemcitabine with radiation therapy, there is a rise in toxicity, yet it does not see a significant increase in survival when compared with a pairing with fluorouracil. After these options have been evaluated and exhausted, palliative care is applied (Freelove and Walling, 2006).

1.5 Breast cancer

Within the United Kingdom, breast cancer is the most commonly occurring malignancy is responsible for 18% of cancer found in women.

1.5.1 Risk factors

There are many risk factors associated with breast cancer and risk of disease development. As a woman ages the incidence will rise rather quickly, it is thought to be the most common cause of death for women between ages 40-50 (McPherson et al., 2000). Incidence is so connected with age, that for every decade a women risk will double until the menopause is reached. Most risk factors are connected to oestrogen, and it has been found when a female starts menstruating can affect the risk of developing cancer. The later in life menstruation starts the lower the risk of the disease developing, each year carries a 5% risk, i.e., for each year a female does not start

menarche there is a 5% lower risk. Similarly, menopause can add a higher risk to women that experience it at a later age with each subsequent year increasing the risk by 3% (Key et al., 2001). It is thought that enduring the menopause carries a protective mechanism by reducing the rate of risk, so much so that postmenopausal females with the same comparative factors are at a lower risk than premenopausal. Females that are perimenopausal suffer from immediate risk of development. After a woman has given birth, they are initially at an increased risk however this does then see a reduction. Compared with those that have not given birth those that have, have a 25% decrease of risk. Adding to this it seems that the more births experienced the more protected a female is from risk culminating in a 50% decrease in those with 5 births behind them. There is a secondary risk factor which affects woman and childbirth independently of the number of births. The age of the female for the first birth can affect risk, the younger the individual the more reduced the risk. The risk associated with endogenous hormones has been examined and associated with serum oestradiol levels. The risk for postmenopausal women with raised serum levels carries 2 times of that of women with lower levels. When women take oral contraceptives that contain both progesterone and oestrogen the risk of developing breast cancer increases up to 25%. This risk can be reduced once the pill is no longer taken although it takes up to 10 years after cessation for no significant risk to be apparent. Localised cancers of the breast occur in the largest number, comparatively with cancers that develop beyond the breast tissues, when combined oral contraceptives are taken. It is considered that in countries that see populations ingesting high fat diets, breast cancer rates are elevated. This is comparatively to countries like japan that are renowned for the lower

intake of fat. Interestingly a difference has not been observed between the risk seen in vegetarians and those that consume meat (Key et al., 2001). It has postulated that when diets contain more phyto-oestrogens – food such a soya- may reduce risk by blocking the effect of endogenous oestrogens which are more potent in nature (Key et al., 1999). There has also been a slight increase in risk associated with alcohol ingestion. It has also been seen that high levels of ionizing radiation are universally damaging to tissue; however, breast tissue has been found to be particularly susceptible to the damaging effects. The risk of cancer developing has been mostly explored in women up to the age of 40 and the risk has been calculated at 1 Gy ranging from 1.1 to 2.7. Information is still evolving however it is currently thought that there are at least five germ line mutations influencing the disease development. These are BRCA 1 & 2,P53, PTEN and ATM genes. BRCA 1 and 2 are infamous for causing high risk for breast cancer risk, ovarian cancer is also associated with these mutations. Li-Fraumeni cancer syndrome can be more likely to develop when there are mutations in the p53 gene. Other childhood cancers can develop such as sarcoma, brain tumours and early onset breast cancers. Mutations in PTEN can also be the cause of Cowden's disease, a cornerstone of this disease being the development of breast cancer. ATM is the fifth mutation that is thought of as high risk if there are mutated alleles. These germline mutations mentioned hold 20-25% of the risk among all breast cancers- that is within families that have had 4 or more cases of breast cancer these mutations would be involved. Furthermore, these mutations are thought to account for 5% of breast cancer cases throughout the population. A study in Iceland found that women that carried BRCA 1 mutations had a 37% development rate by the time 70 years of age

was reached. A different study of a population of Ashkenazi Jewish females in the US found that this rate was higher at 56% by the same age. (Key et al., 2001).

1.5.2 Sub-Types

The most common forms of breast cancer are ductal and lobular carcinomas, invasive in description and responsible for 75% and 15%, respectively (Honrado et al., 2005).

Traditionally, classification is based on histological type and grade. Now divided into in situ -comprised of ductal and lobular- and invasive types. All invasive carcinomas are histologically graded. A score is awarded (1-3) according to 3 categories. Proportion of tubule formation, degree of nuclear pleomorphism and mitotic count. This cumulates to a total score, 3-5 results in grade 1. 6-7 a grade 2 and 8-9 results in a grade 3 tumour. As with other types of tumours, staging is assigned according to the tumour-node-metastasis method. High grade (grade 3) lobular carcinomas are produced from lower grade tumours. This is due to random mutations in TP53, HER2 and MYC genes. Luminal, basal-like, HER2 -positive and HER -normal like are subtypes of intrinsic breast cancer types. Luminal subtypes can also be further split into A and B types (Vuong et al., 2014). It is estimated that 5-10% of all breast cancer cases are hereditary and are heavily associated with the BRCA1 and BRCA2 genes. Families that have high rates of ovarian and breast cancers have a high rate of mutations in these genes of roughly 45%. These genes are important as the normal functions are involved in tumour suppression through controls on cell processes such as division and death. They are also associated with metabolic functions such as cell cycle control, gene

expression regulation and repair in response to DNA damage. Another role is that of maintaining the structure of the chromosome. It is thought that cases of familial breast cancer differ to that of sporadic cases. The differences can be in morphological, molecular and immunophenotypic features. Invasive ductal carcinomas are more commonly seen in BRCA1 and 2 mutation carriers. BRCA1 mutations specifically, also carry a higher incidence of medullary carcinomas at 13%, whereas only 3% develop medullary carcinomas with BRCA2. The invasive ductal carcinomas carry a clear histotype, with projecting lymphocytic infiltrate, necrosis, pushing margins and numerous mitosis sites. An important note about tumours that arise from BRCA1 mutations is the high histological grade- grade 3- that the tumours often present at. Comparatively, grade 2/3 is the most common staging for BRCA2 tumours. Is has also been shown that cases with BRCA1 mutations also show a high amount (73-90%) of oestrogen receptor negativity and low expression of progesterone receptor (Honrado et al., 2005).

1.5.3 Diagnosis

Most woman usually raise a medical appointment due to a lump being found in the breast or breast pain. The first assessment is a physical examination of the patient, along with familial history being considered. If symptoms are not being presented a mammography of the breast tissue is performed. It has been seen to increase survival incidence of woman if screening is done between 50-70 years old. Women in the 20-39 age bracket will have a breast examination every 3 years, once 40 years old is reached a mammogram is carried out annually along with a physical examination.

Once a lump has been found ultrasonography can be used to distinguish mass characteristics, solid or cystic breast mass. This method is beneficial if the mass cannot be found when first using a mammogram. Digital mammography can be used in place of standard mammography as the image can be find tuned for better visibility. Biopsies of masse can be performed, there are three techniques being used. Fine needle aspiration, core biopsy and excisional biopsy. Fine needle is used for cytology samples, core biopsies remove the centre of the mass and if the likelihood of malignancy is high excision biopsy methods are used (Apantaku, 2000).

1.5.4 Treatments

The main treatment selections are cytotoxic compounds, surgery, radiation and endocrine drugs. The treatments are based on whether the case is of a primary cancer or is systemic in nature. Radiation therapy is usually used in conjunction with surgery, to get rid of any lingering cancerous cells. Cytotoxic compounds are often used when the breast cancer is advanced, common drugs are docetaxel, 5FU and doxorubicin. Some HER-2 positive carcinomas are being treated with a monoclonal antibody that has been humanised for use called trastuzumab. A limitation of this therapy is the determining the patients genotype before application of treatment. Due to the evolving characteristics of breast cancer and the many different factors (such as age, receptors present and first line therapies used) new treatments must be elucidated (Radice and Redaelli, 2003).

1.6 Apoptotic pathways

1.6.1 Extrinsic pathway

Apoptosis and necrosis are two mechanisms of cell death within the body. Apoptosis is programmed cell death, often by external or internal stimulus. Necrosis is only initiated when there is external damage (Ghobrial et al., 2005). Apoptosis is an imperative process to maintain cell tissue populations. It can also occur if there is damage to the cells or if an immune response had been triggered and consequently cells need to be destroyed. Apoptosis can be instigated due to both physiological and pathological factors. Often stimulus such as cell damage activates the p53 dependant pathway, otherwise known as the intrinsic pathway. Other cells use the extrinsic pathway, this is activated by ligand binding. The cells often express a Fas or TNF receptor and binding leads to cross linkage of proteins (Elmore, 2007).

The extrinsic pathway contains many proteins such as death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases-8 and -10. Caspase-8 and 10 initiate a downstream caspase cascade. As previously introduced ligation of cell surface receptors is the starting point for this pathway. These receptors are referred to as death receptors. The TNF -tumour necrosis factor- receptor family is comprised of Fas, TRAIL (DR4), TNF R1, DR3 (Apo 2), DR5 (TRAIL R2), and DR6.

Immune surveillance of transformed or infected cells is heavily involved with Fas signalling pathways, faults in this pathway are associated with malignancies. The Fas

pathway is renowned for involvement in proliferative and inflammatory signalling as well as death related cell functions. Cell deaths activates the Fas ligand pathway, this causes Fas ligands that are membrane bound to associate with inactive Fas complexes consequently forming the death inducing signalling complex (DISC). This complex contains Fas-associated death domain protein along with caspase-8 and -10. Subsequently, activation of caspase-8 occurs this in turn causes a downstream caspase cascade. Caspase-8 activation can induce cell death without having previously activated the caspase cascade. Cell death can also be a consequence of interactions with the intrinsic pathway. Proapoptotic Bid is cleaved and brings about a release of cytochrome-c. There are many proteins and pathways that regulate the extrinsic pathway. When these mediators become abnormal, malignancies can result. This is evidenced by Landowski et al. (1997), mutations and deletions in Fas have been found in various hematologic malignancies. Transcription factors NFκB and activating protein 1 regulate the Fas ligand pathway. FAP-1, as-associated-death-domain-protein like interleukin-1β-converting enzyme-like inhibitory protein and decoy receptors (often TRAIL receptor 3 and 4) also inhibit this pathway through antagonistic stimulation of competitive binding with the Fas ligand (Ghobrial et al., 2005).

1.6.2 Intrinsic pathway

Key regulators of apoptosis have been found to be overexpressed in many cancers, this has been found to be caused without chromosomal translocation mutations. Bcl-2 proteins are key regulators of apoptosis, and this overexpression can lead to chemoresistance and even resistance to radiotherapy. It can also result in cells that

are in the G_0 cell division checkpoint thus adding to resistance. Conversely, this means if levels of Bcl-2 can be decreased, the apoptotic response of anticancer drugs can be stimulated.

Bcl-2 proteins are made up of two subgroups: proapoptotic and antiapoptotic constituents. The antiapoptotic proteins block the release of cytochrome-c and therefore supress apoptotic action. Post-translational alteration occurs on proapoptotic proteins after receiving a death signal. These alterations are those such as dephosphorylation and cleavage. The cleavage results in activation and translocation of the proteins into the mitochondria subsequently initiating apoptosis. Cytochrome-c and second mitochondria derived activator of caspases- often known as IAP-binding protein- are released due to the outer mitochondrial membrane becoming permeable. This cytochrome is then in the cytosol which then associates with apoptotic protease activating factor 1 (Apaf-1). This causes the caspase cascade to begin, starting with caspase-9 followed by caspase-3. Once activated the caspases cause the nuclear lamin to become cleaved and the nucleus to breakdown due to the influence of caspase-3 (Ghobrial et al., 2005).

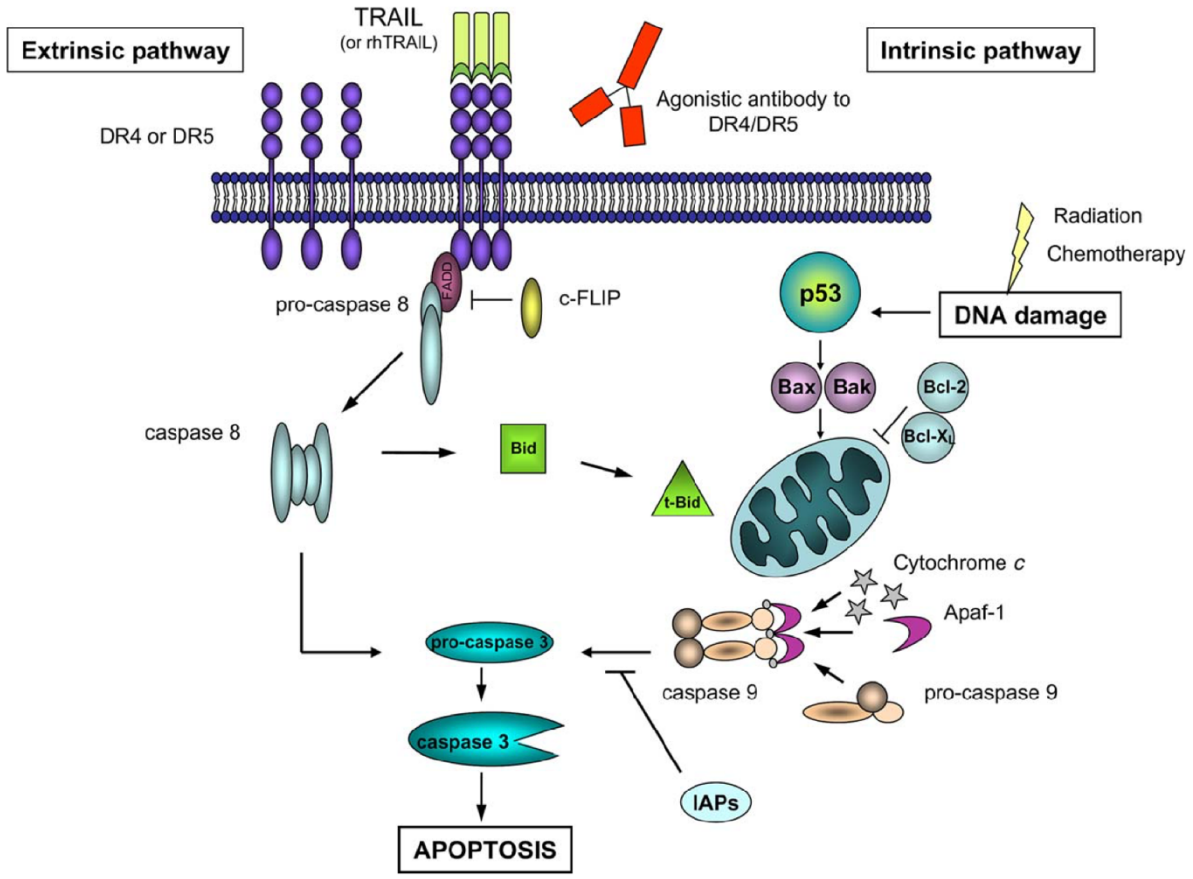

Figure 4 schematic of apoptotic pathways: intrinsic and extrinsic and the events that follow initiation. TRAIL binding to cell surface death receptors triggers the extrinsic pathway to be activated. These receptors are only found on cancer cells. Stresses such as damage DNA damage can activate the intrinsic pathway (Duiker et al., 2006).

1.7 Tumour necrosis factor related apoptosis inducing ligand (TRAIL)

1.7.1 TRAIL receptor activation

TNF-related apoptosis-inducing ligand, commonly referred to as TRAIL can induce cell death by apoptosis in many different types of cells. TRAIL interacts with the death receptors on cells resulting in cell death. Interestingly this only occurs with transformed and tumour cells. The lack of interaction with normal cells saw TRAIL rise to a position of a potential cancer therapy (Griffith et al 1998).

Many types of cancer have so called death receptors on the cell surface. TRAIL can activate the extrinsic pathway through ligation to the receptors, these are TRAIL-R1 and TRAIL- R2 also known as death receptors 4 and 5. Although it initiates through ligation interactions, there are also cornerstones of intrinsic pathways that have been observed (Mellier et al., 2010).

When TRAIL binds to the death receptors, clustering of the receptors occurs. This consequently causes the recruitment of Fas associated protein with death domain (FADD). The conscription of pro-caspase-8 follows and then forms the death inducing signalling complex due to the FADD adaptor protein. Pro-caspase 8 instigates DISC activation and causes caspase-3, -6 and -7 to become cleaved. This cleavage produces membrane blebbing, fragments of DNA being created and nuclear shrinkage. In some events the intrinsic pathway is engaged via caspase-8 (Refaat et al., 2014).

1.7.2 TRAIL resistance and cFLIP

TRAIL efficiency can be limited by the shortage of death receptors presenting on the cell surface.

Cellular FLICE inhibitory protein (FLIP) has very close homology to that of caspase-8. This means it is preferentially recruited into the DISC as it binds to FADD, thusly activation of caspase-8 is inhibited, and the extrinsic pathway is not activated. If cFLIP is inhibited it is thought that cells can be re-sensitised to TRAIL. It is thought that TRAIL induced apoptosis is induced by cycloheximide of actinomycin (a transcription inhibitor).

Increased cFLIP expression corelates with higher levels of TRAIL resistance, this can occur through post translational modifications and cFLIP degradation. There are two forms of cFLIP known and both can be engaged into the DISC complex. These are $cFLIP_s$ and $cFLIP_L$, both contain death effector domains (DED), the main difference is that of the caspase like domain. $cFLIP_L$ contains one in contrast to the absence of the domain in the $cFLIP_s$. However, $cFLIP_L$ lacks a cysteine residue which is responsible for catalytic activity. $cFLIP_L$ has been found in some cancers to be favourably drafted into the DISC (Wang et al., 2008). Resistance can also be increased by the cancer being driven towards a survival pathway. This happens when a secondary complex is formed after DISC formation. TNFR1-associated death domain (TRADD), IκB kinase, TNF receptor-associated factor 2 (TRAF2), receptor-interacting protein (RIP). Pro-caspase-8, FADD and cFLIP form this complex and is liable non-apoptotic signals being activated. These signals are activated through nuclear factor κB,

phosphoinositide 3-kinase (PI3K)/Akt), and mitogen-activated protein kinase (MAPK) pathways as seen in figure 4.

To circumvent this resistance combination treatments are being employed. The intent to create a synergistic effect re-sensitising the cancer cells to TRAIL. These combination approaches act through stress to the endoplasmic reticulum. The creates an upregulation of the DR4 and DR5, resulting in apoptosis being increased due to TRAIL (Refaat et al., 2014).

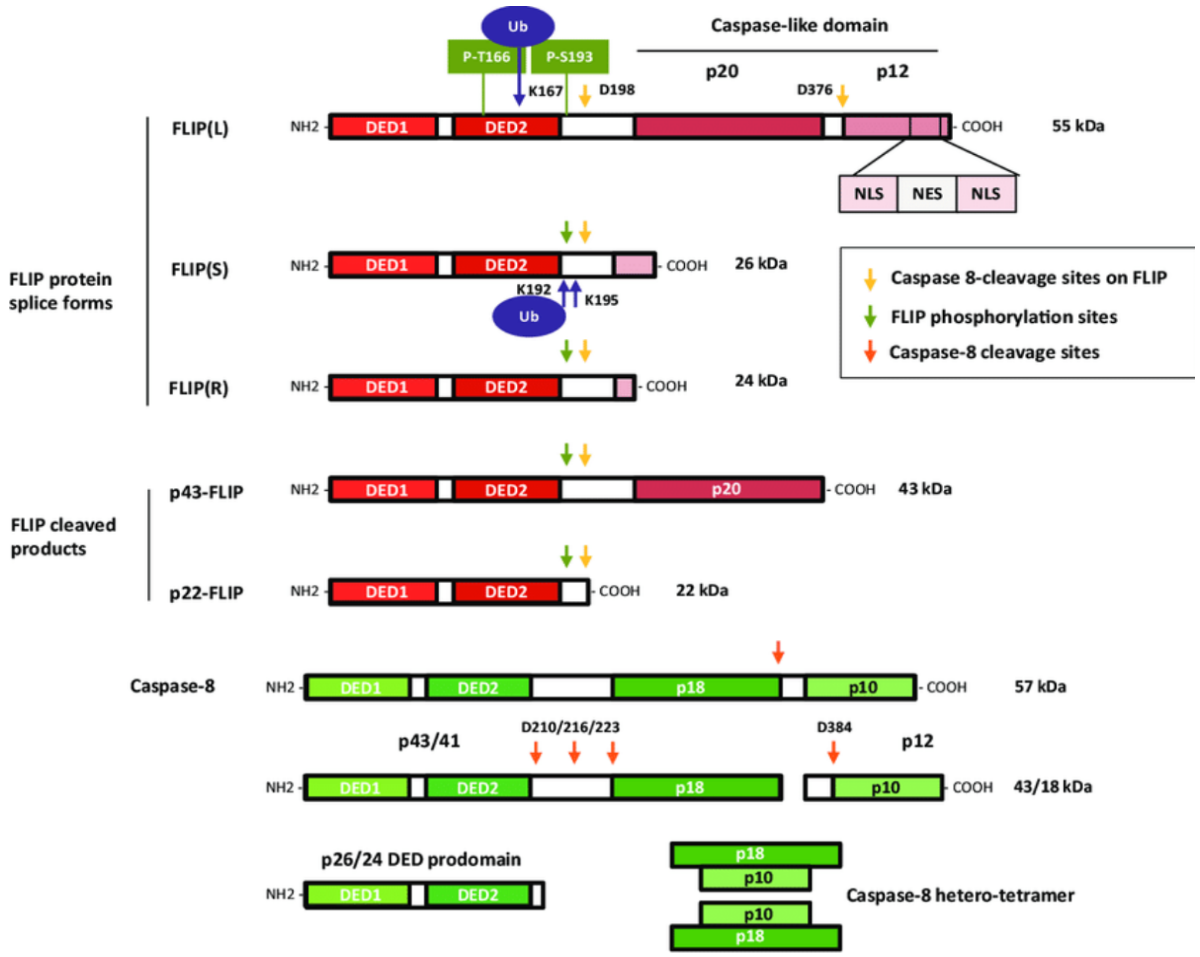

Figure 5 a schematic of the structure of the variants of cFLIP splice variants and caspase 8, DEDs, cleavage and phosphorylation sites are shown. All variants contain DEDs that allow recruitment into the DISC. FLIP(L) has a pseudo-kinase domain that can be cleaved by procaspase-8 – which is bound in the DISC- forming cleavage products p43- and p12. The FLIP(L) pseudo-kinase domain contains a nuclear localization signal and nuclear export signal also. FLIP (S) and (R) do not possess the pseudo-caspase domain that FLIP(L) has. They also have different C terminus. The green variants show procaspase-8 structure dimerisation of procaspase-8 leads which leads to a cleavage in the catalytic domain resulting in a p12 and p43/41 cleavage consequently (Humphreys et al., 2018).

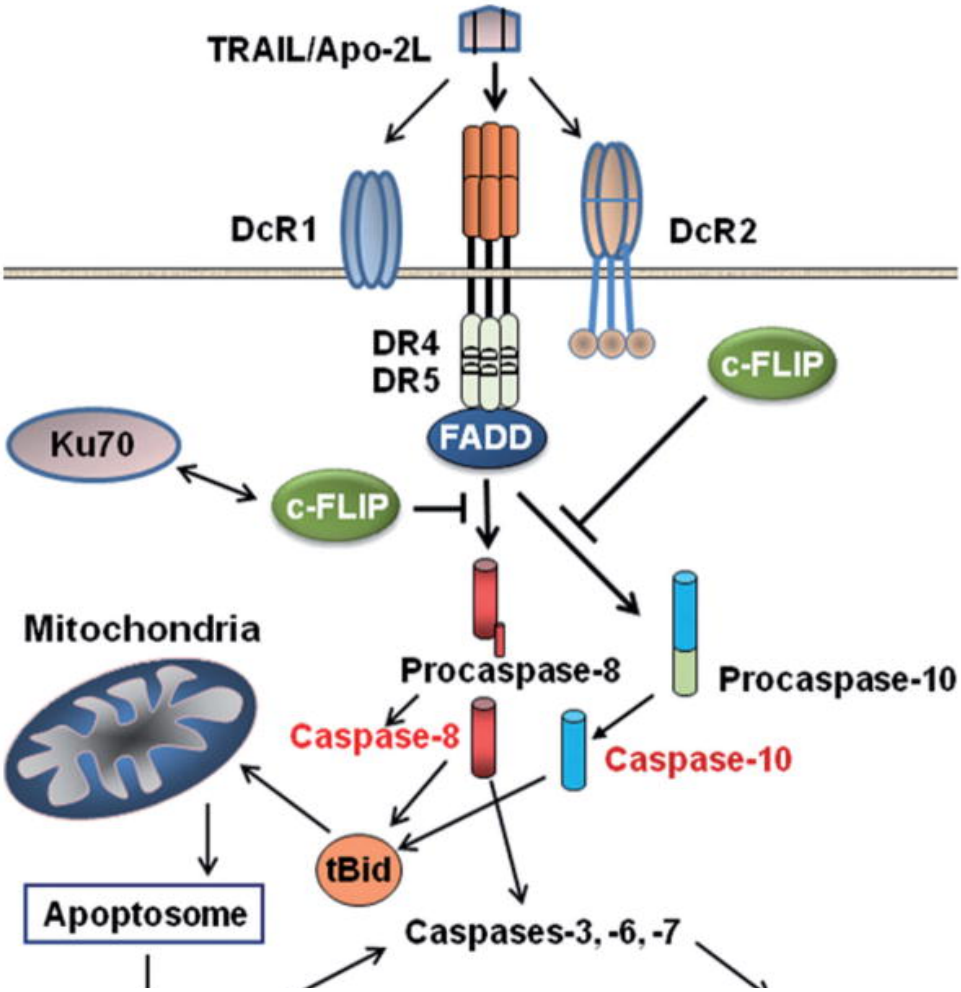

Figure 6 schematic of the role FLIP in the apoptosis pathway, including the inhibition of procaspase-8 and -10 activation consequently preventing the caspase cascade. *TRAIL can activate the extrinsic pathway through ligation to the receptors, these are TRAIL-R1 and TRAIL- R2 death receptors 4 and 5.* TRAIL binds to the death receptors causing clustering of the receptors. The recruitment of Fas associated protein with death domain (FADD) follows. The conscription of pro-caspase-8 follows and then forms the death inducing signalling complex due to the FADD adaptor protein. Pro-caspase 8 instigates DISC activation and causes caspase-3, -6 and -7 to become cleaved. (Safa, 2012)

1.8 Aims and objectives

A cFLIP inhibitor will be used followed by treatment with TRAIL. Apoptosis, proliferation and survival of the cancer cells during and after treatment will then need to be measured. Below are various methods that could be used to do this.

With the previous information in mind, the aim is to investigate the effect of clip inhibitors on apoptosis and the application to aid in overcoming TRAIL resistance.

1.9 Cell Lines used for the project

1.9.1 Prostate cancer PC3, LNCap AND DU145

Research showed that PC3 cells have some deleted segments on chromosome 1 overlapping LCE3Band LCE3C genes. These genes are often deleted in roughly 18% of prostate tumours, however their role in adenocarcinoma tumorigenesis have not been elucidated (Cerami et al., 2012). The gene PTEN is also deleted; this deletion renowned for being a motivator of prostate cancer (Carver et al., 2011).

When investigated by Seim et al. (2017) it was found that the LNCap cell line had a large number of stop gained events. These are changes that are usually resultant in nonsense mutations, non-functional proteins or sometimes reduced function in proteins. At amino acid location 318 of menin, a C into T mutation is found. This is a somatic inactivity mutation often found in endocrine cancers, hinting that menin acts as a tumour suppressor. This could explain why it is mutated in cancer cells.

Tumour sensitive (TS) function via the P53 gene was seen in DU145. Expression of the P223L allele in PC3 confirmed the TS function of the gene. In DU145 cells methylation of p21Waf1 promoter is observed, and it has also been revealed that methylation of the p21Waf1 promoter occurs in PC3 and LNCaP cells as well. Activated p21Waf1 substantiated that P223L TS activity may have been influenced by cellular components/location. When cells have the temperature sensitive allele (at 37C) the p53 protein takes on the mutant conformation, resulting in loss or gain of function and a lack of ability to transactivate genes (Bajgelman and Strauss, 2006).

1.9.2 Colorectal cancer HCT116

HCT cells see two regions that have homozygous loss rather closely; there is a 50 kb separation of the 25 and 50 kb deletions in the WWOX gene. It was identified that among the 3 chromosome 16 containing material locations, one had a translocation breakpoint. One such heterozygous deletion was that of exons 6-8. This causes allele specific expression, which would be responsible for any biological changes, this can be suggested due to similar deletion transcripts being seen in other cancers (Alsop et al., 2008).

.

1.9.3 Pancreatic PancTu1

There are a few genes that are commonly associated with pancreatic cancer: KRAS, TP53, CDKN24 and SMAD4. These are oncogene and tumour suppressor genes respectively and are regarded as being the main drivers of this cancer type (Bazzichetto et al., 2020). The region of 8p11-21 is a location that often has alterations in it when pancreatic cancer arises and contains many complex rearrangements, oncogenes and tumour suppressor genes. Upon further research it was found that there is a translocation breakpoint focused on the NRG1 gene at the previously mentioned location. NRG1 is thought to code for various forms of epidermal growth factor like ligand, this could promote cell growth and differentiation, together with breakpoints seen in the gene there is a strong implication it is involved with pancreatic oncogenesis (Adelaide et al., 2003).

1.9.4 Breast cancer MDA-MB-231

This cell line is associated with both high levels of mutated P53 and phospholipase activity. When there is a lack of growth factors phospholipase initiates a survival signal allowing growth, also supressing apoptotic signals when undergoing serum withdrawal (Hui et al., 2006). Strano et al. (2000) suggest that theses mutated p53 subdues proapoptotic effects of p73 also.

2. Methods

2.1 Materials

All chemicals were purchased from sigma (St. Louis, MO), unless stated otherwise. Recombinant TRAIL (rTRAIL) was bought from R&D systems. The cFLIP inhibitor was generously provided by Professor Richard Clarkson, School of Biosciences, Cardiff University.

2.2 Cell Culture

The colorectal carcinoma cell line HCT116 was cultured in McCoy's 5A (Lonza). LNCaP, C4-2B, DU145 and PC3 prostate cancer cells were cultured in RPMI medium (Lonza). The breast cancer cell line MDA-MB-231 was cultured in DMEM (Lonza) and the pancreatic PancTu1 cancer cells were cultured in RPMI medium. All media were supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin.

2.3 Crystal Violet assay

Cell survival was measured after performing a crystal violet staining. Seven days after treatment with FLIPi alone, recombinant TRAIL alone or in combination with FLIPi, a Crystal Violet stain was added to paraform-aldehyde-fixed cells. The stain was then washed of after 1 h and the cells washed with water three times, before were left to

air-dry. Images were taken and for quantification the Crystal Violet was then extracted in 500 μl of methanol. The resulting samples were then analysed in a microplate reader to measure the absorbance at 490 nm to obtain quantitated cell survival values.

2.4 Nicoletti assay

Apoptosis was measured after carrying out a Nicoletti assay according to Riccardi and Nicoletti (2006). Treatments FLIPi alone, recombinant TRAIL alone or in combination with FLIPi. After 48 h after treatments with FLIPi alone, TRAIL alone or combinations of both agents, cell culture supernatants, trypsinised cells and PBS wash solutions were collected in one tube per sample and then centrifuged at 2,000 RPM for 5 minutes. Subsequently, the supernatants were discarded, and the pelted cells were lysed and resuspended in 200 ml of Nicoletti buffer (50 mg/ml propidium iodide (PI), 0.1% (w/v) sodium citrate, 0.1% (w/v) Triton X-100). The samples were measured in a BD Accuri C6 flow cytometer that detected the intensity of the PI fluorescence and analysed with the BD Accuri software. Each measurement was repeated three times.

Apoptotic fragmentation is a key characteristic of apoptosis, this assay uses fluorogenic reagents (Nicoletti buffer) that bind stoichiometrically to the nucleic content. When a sample is analysed using a flow cytometer if there are any apoptotic cells present there will be a broad hypodiploid sub G1 peak (Riccardi and Nicoletti (2006).

3. Results

3.1 Cell survival after cFLIP inhibitor treatment

cFLIP is involved in the apoptotic pathway and allows the cancer cells to avoid programmed cell death. This is a consequence of the inhibitory action of cFLIP on the caspase cascade initiation and thus allowing the cells to continue to survive and proliferate. cFLIP Therefore, the effects of a novel cFLIP inhibitor (FLIPi) on reducing cell growth in various cancer cell lines was investigated. First, the cytotoxic and apoptotic effects of the FLIPi on its own and in the second part in combination with TRAIL were examined.

HCT116 is a mammalian colorectal cancer cell line, that is a standard and frequently used cell line of this cancer type. It is one of three strains of malignant cells isolated from a male with colonic carcinoma. They harbour a mutation in codon 13 of the KRAS proto-oncogene but are p53 wild type. In response to TRAIL, they are considered to be moderately sensitive. First, we carried out survival assays on HCT116 cells after treatment with FLIPi. For this we stained the cells with Crystal Violet and quantified the stain in untreated controls and cells treated with various concentrations of FLIPi. Compared to the untreated control the FLIPi showed cytotoxic effects even at the lowest concentration of 1.6 μM that increased in a dose-dependent manner and was most pronounced at the concentrations of 50 and 100 μM (Figure 5). At 100 μM the survival of HCT116 cells was reduced by around 50% as compared to approximately

25% at 1.6 µM. There was a large error observed at a FLIPi concentration of 6.25 µM, which indicates that further repeats are required. However, this does not change the overall outcome of the experiment. In summary, the FLIPi inhibitor exerts cytotoxic effects even at low concentrations that increase with rising concentrations.

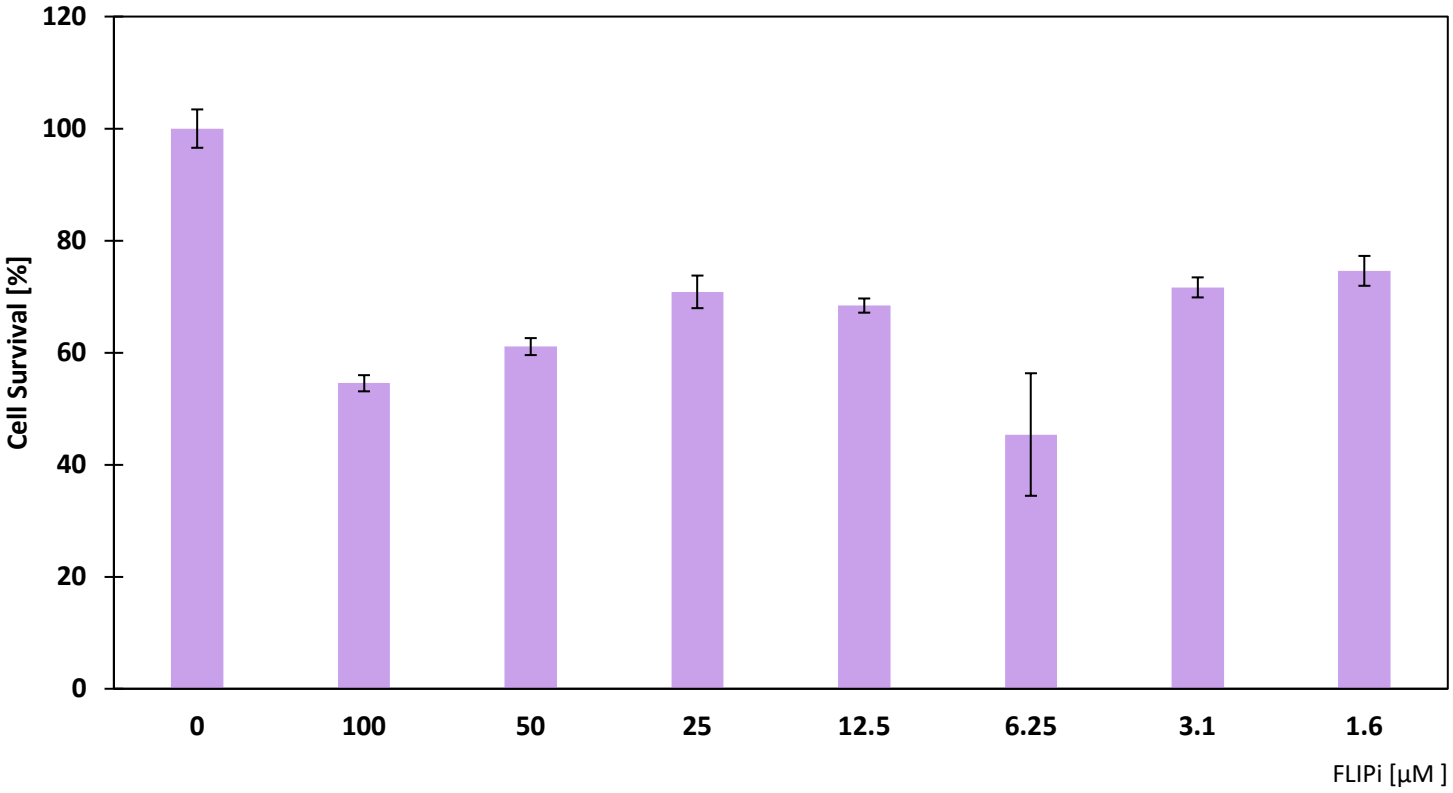

Figure 7 Cell survival measured in HCT116 cells pre-treated with decreasing concentrations of FLIPi for 7 days before harvesting. The control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Crystal violet stain was used to visualise viable cells, each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

LNCaP cells are isolated from a from a male patient with lymph node metastatic prostate carcinoma and are androgen dependent. It is one of several prostate cell lines often used and often used to model this cancer type. This cell line is responsive to 5-alpha-dihydrotestosterone but with respect to TRAIL is generally considered to be resistant. First, we carried out survival assays on LNCaP cells after treatment with FLIPi. This was done was done using crystal violet stain, using the control to quantify. Varying concentrations of FLIPi was then used to treat the cells. Cytotoxic effects were shown by FLIPi at 100 μM as a single treatment, with an approximate 80% reduction in cell survival compared with 0 μM (figure 8). Some lower effects were seen at 50 and 25 μM. In summary FLIPi exhibits cytotoxic effects at high concentrations of FLIPi but this effect decreases with lower concentrations.

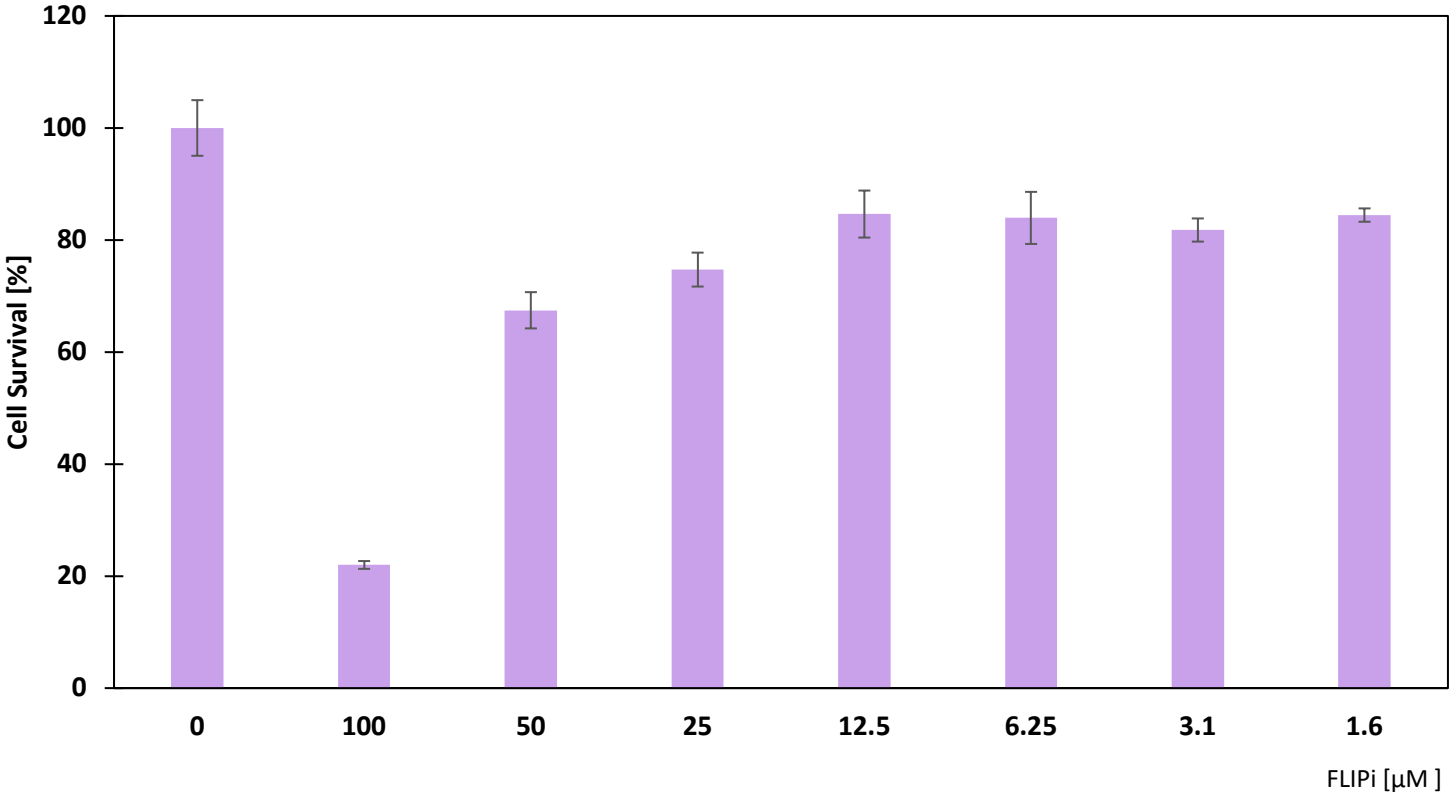

Figure 8 Cell survival measured in LNCap cells treated with decreasing FLIPi concentrations, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells stained with crystal violet to visualise each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

C4-2B is an androgen-independent subline of LNCaP cells developed by co-inoculation into an athymic male nude mouse with human fibroblasts which were derived from an osteosarcoma. There were no significant effects observed. However, some effect is seen with the 100 μM concentration comparatively with 0 μM with a 50% reduction in cell survival seen in 100 μM comparatively to that of the 0 μM (Figure 9). There is a decrease in cell growth with the 50 μM with approximately just over 50% in cell survival reduction comparatively to the 0 μM, an approximate 2% increase in cell survival reduction compared to with 100 μM. Figure 9 also demonstrates a dose curve for FLIPi in use with C4-2B cells, up to 6.25 μM. In summary this cell line has not been sensitised to TRAIL but does so cytotoxic effects at higher FLIPi concentrations.

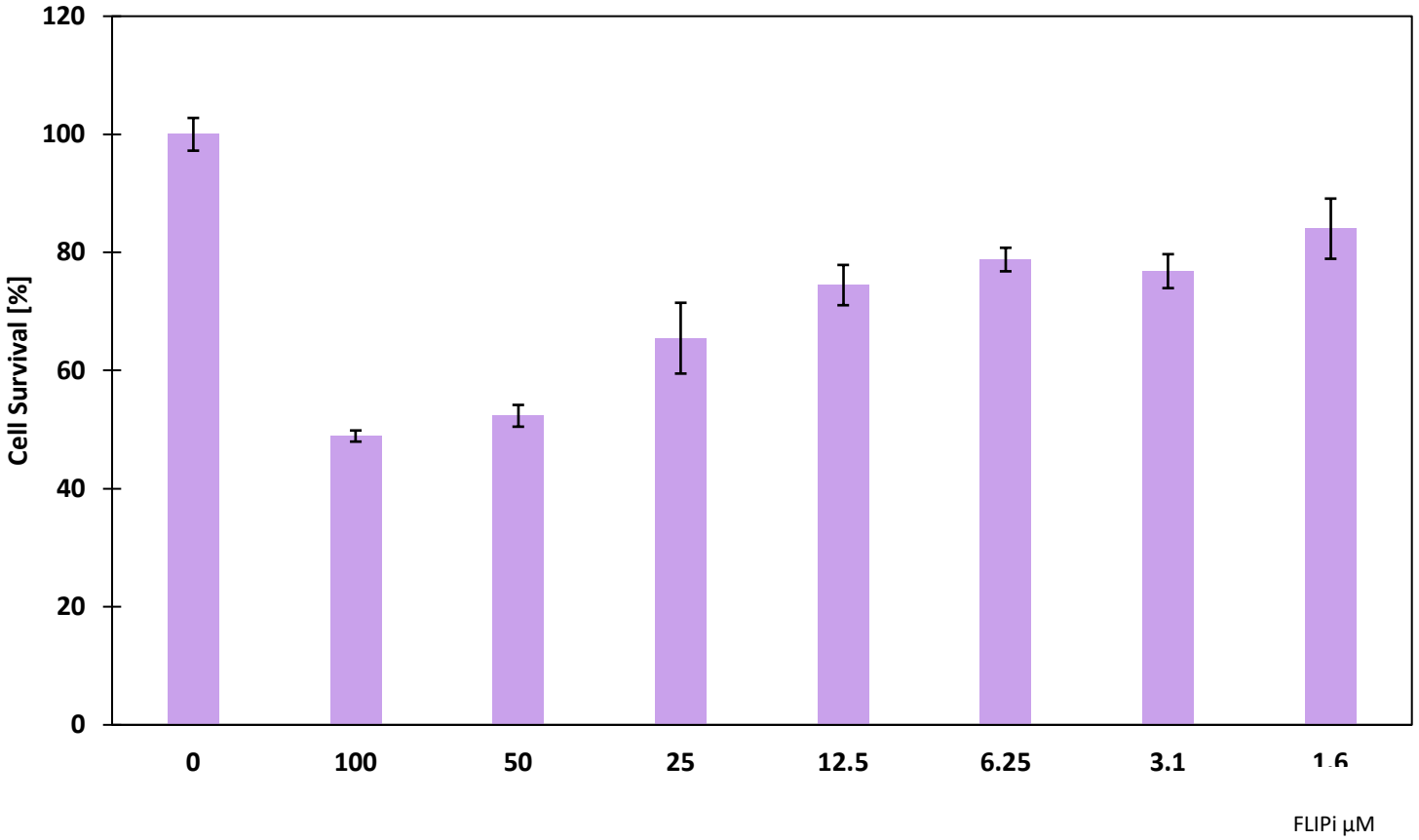

Figure 9 C4-2B cells with decreasing concentrations of FLIPI, the control for this assay is 0 µM of FLIPi with increasing concentrations, thereafter, initially starting at 100 µM. Crystal violet stain was used to visualise cell growth after 7 days of treatment. Each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

PC3 is a prostate cell line derived from bone metastases of a male diagnosed with grade IV prostatic adenocarcinoma. It is regarded as being highly metastatic and are androgen independent. It is regarded as being slightly sensitive to TRAIL. FLIPi 0 μM as expected had no effect on apoptosis. However, there were significant cytotoxic effects seen in FLIPi 100 μM, boasting an approximate cell survival reduction of an estimated 60% compared to the 0 μM. There were little cytotoxic effects seen in lower concentrations up of FLIPi. With very little cytotoxic effect seen in FLIPi 3.1 μM, comparatively to the 0 μM there was a small reduction of approximately 10%. Comparatively to the 0 μM control the FLIPi showed cytotoxic effects even at lower concentration of 3.12 μM that increased in a dose-dependent manner. In summary there are significant cytotoxic effects seen even at lower concentrations.

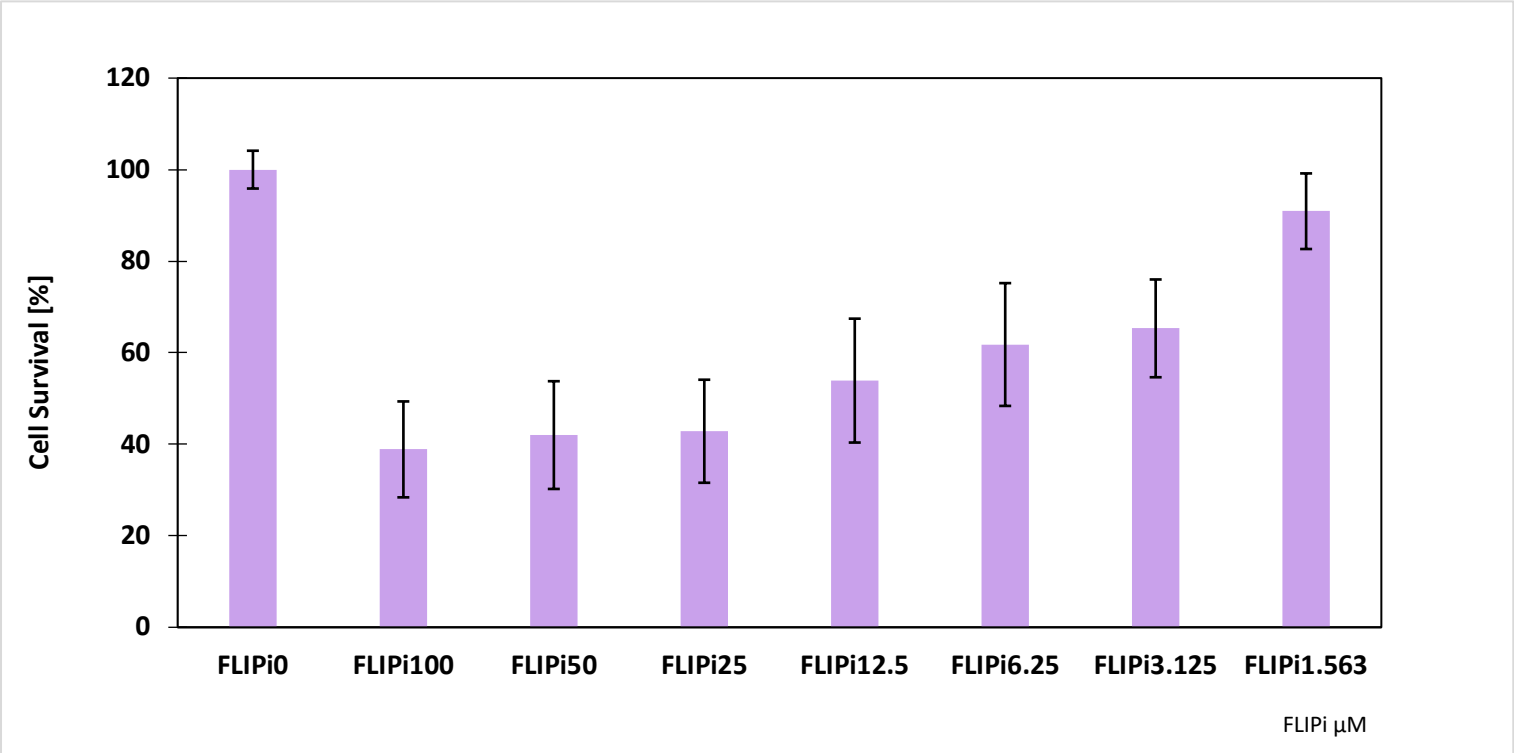

Figure 10 Cell survival measured in PC3 cells pre-treated with decreasing FLIPi concentrations, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells stained with crystal violet to visualise after 7 days of treatment. Each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

The DU145 cell line is a human prostate cancer cell line derived from a brain carcinoma in a male, it is described as not hormone sensitive and so is androgen independent. It is thought that it is moderately sensitive to TRAIL. However, there were cytotoxic effects seen in FLIPi 100 μM, boasting an approximate cell survival reduction of an estimated 30% compared to the 0 μM. There was very little cytotoxic effects seen in lower concentrations up of FLIPi. With no cytotoxic effect seen in FLIPi 3.1 μM, comparatively to the 0 μM there was actually a small increase of approximately 1% suggesting an error suggesting a repeat may be useful. This did not impact the outcome of the experiment. In summary, there is only cytotoxic effects seen at higher concentrations of FLIPi suggesting there has been no sensitisation.

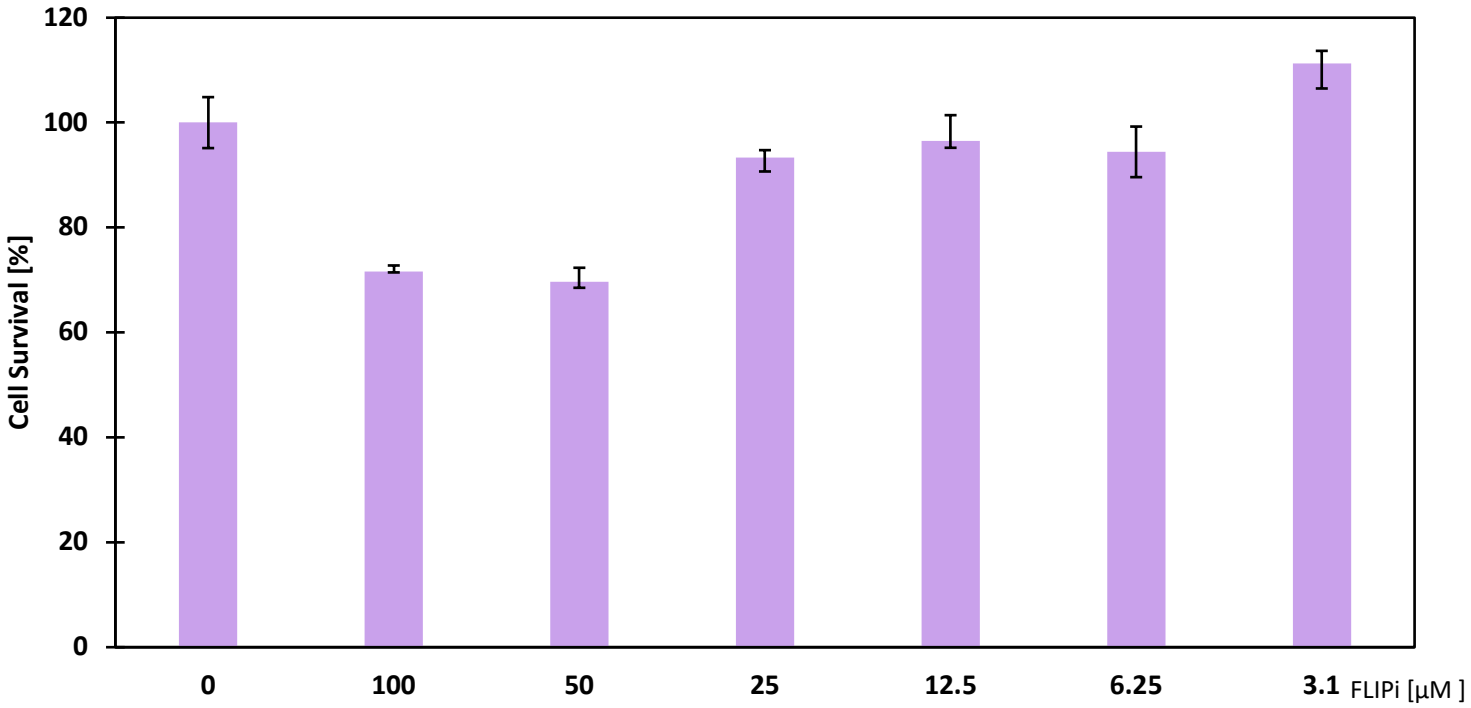

Figure 11 Cell survival measured in DU145 cells pre-treated with decreasing FLIPi concentrations, the control for this assay is 0 μM of FLIPi with increasing concentrations thereafter. Cells stained with crystal violet to visualise after 7 days of treatment. Each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

The breast cancer cell line MDA-MB-231 was used, as it is one of the most commonly diagnosed cancers. It was derived from a female metastatic mammary adenocarcinoma. It is a very aggressive cancer and is triple negative as it has an absence of lacking ER and PR expression and HER2 amplification. This cell line is regarded as very sensitive to TRAIL. Overall, there was no significant apoptosis seen with the FLIPi as a single treatment in the MDA-MB-231 cell line. There is a very slight increase in the apoptotic percentage with 100 μM of FLIPi in comparison to 0 μM, with an approximate 20% reduction cell survival. The biggest reduction on cell growth was observed at 50 μM FLIPi showing a cell survival rate of 63%. At lower concentrations there were much lower cytotoxic effects seen. In summary, there were some cytotoxic effects seen, but knowing this is a TRAIL sensitive cell line, repeats would be necessary, to confirm this lack of sensitization to TRAIL.

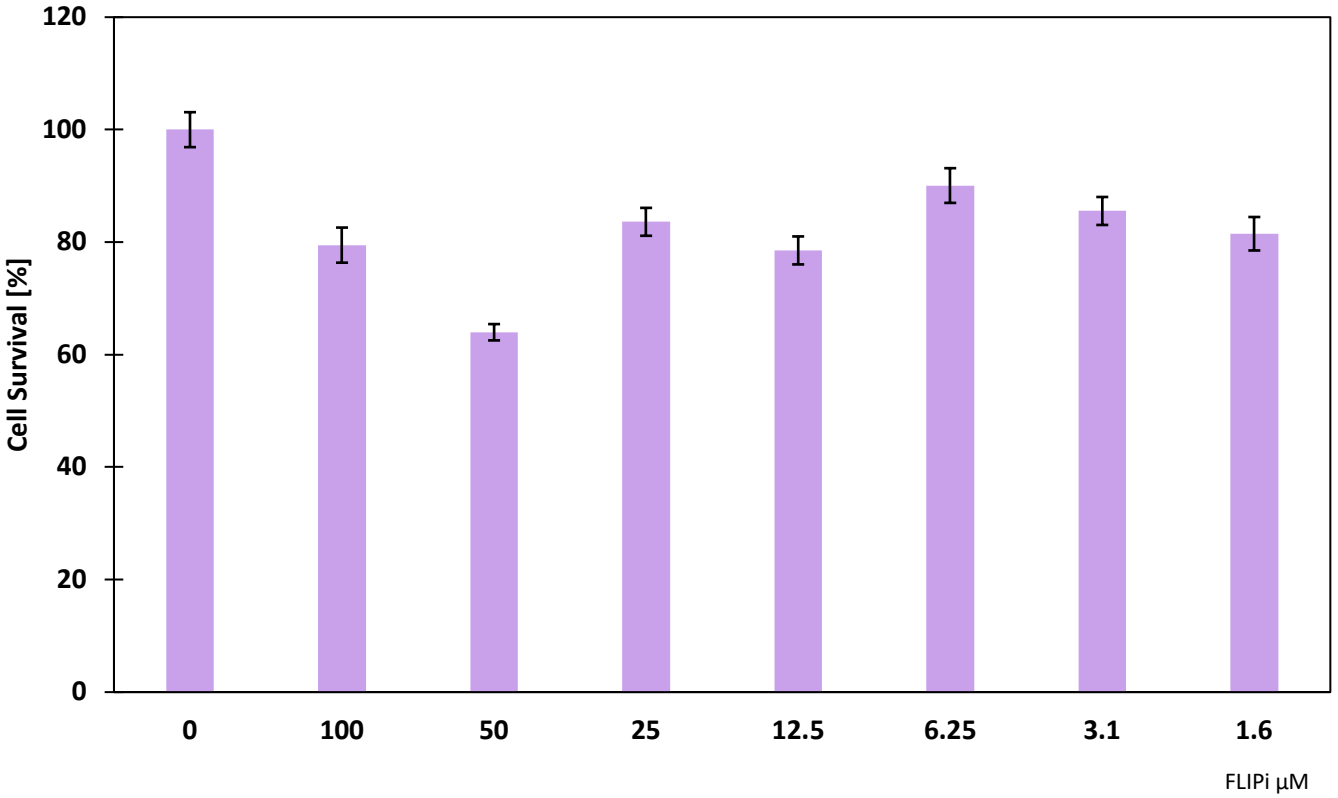

Figure 12 cell survival measured in MDA-MB-231 cells treated with decreasing FLIPi concentrations, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells stained with crystal violet to visualise after 7 days of treatment. Each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

Pancreatic cell line PancTu1 was used this was cancerous epithelial tissue from a male pancreas. This cell line is known to react poorly to TRAIL and so the expectation was that there would be limited cytotoxic effects seen. There were significant cytotoxic effects seen at 100 mM with an approximate 60% reduction in cell survival compared to 0 mM. Compared to the untreated control the FLIPi showed cytotoxic effects even at the lower concentrations of that increased in a dose-dependent manner starting to show little to no effect from 6.3 mM onwards. In summery there were some cytotoxic effects seen at higher concentrations of FLIPi.

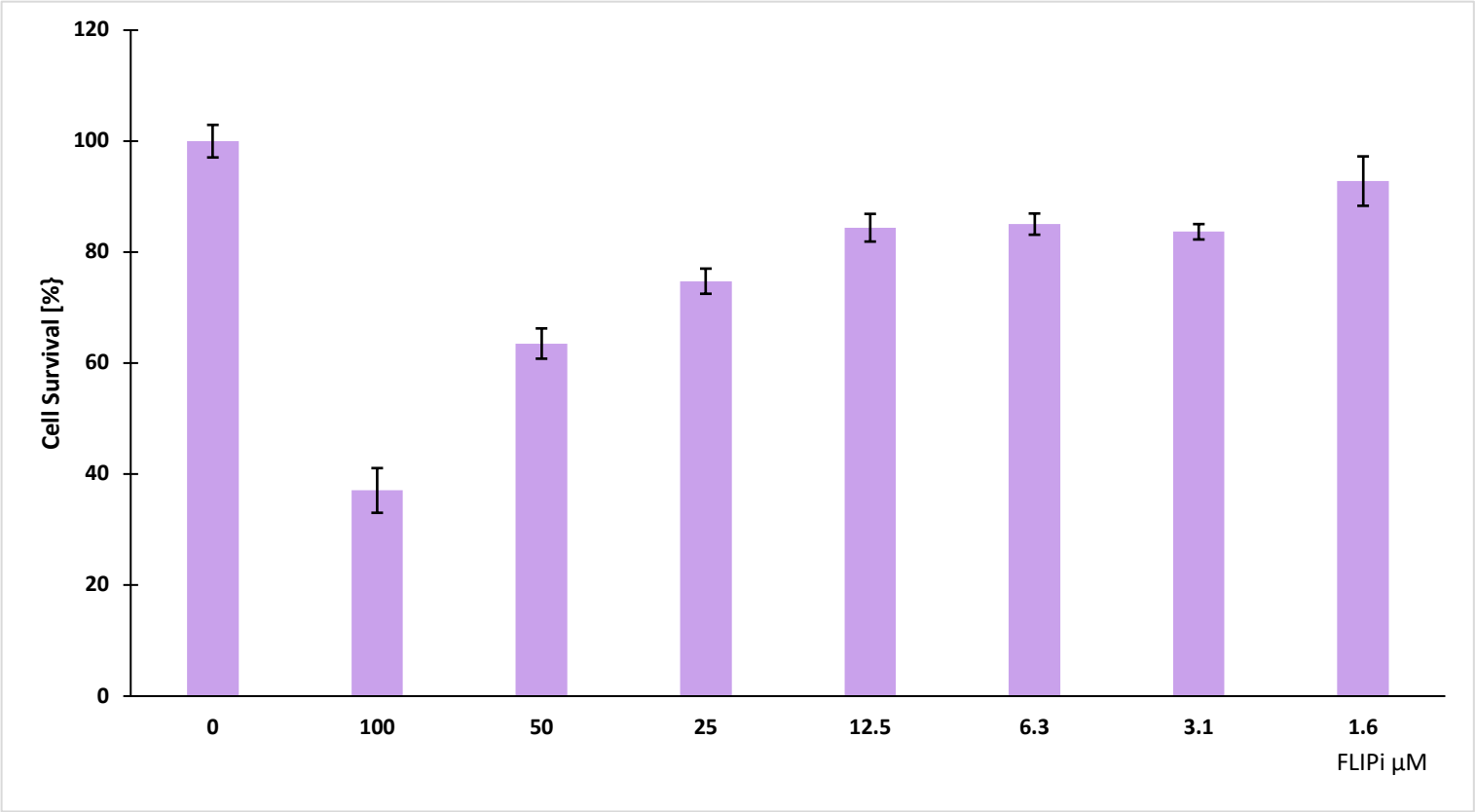

Figure 13 cell survival measured in PancTu1 cells treated with decreasing FLIPi concentrations, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells stained with crystal violet to visualise after 7 days of treatment. Each cell line was repeated 3 times, with each sample in each assay also being tested 3 times with an average being taken.

3.2 Apoptosis induction after cFLIP inhibitor treatment

Next, we measured apoptosis in cells treated with FLIPi. For this we used a DNA hypodiploidy assay also known as Nicoletti assay (Riccardi and Nicoletti, 2006). In this methods, harvested cells are made permanent for the DNA dye PI. The PI enters the cells and nucleus where it stains the DNA. The emitted fluorescent signal can be measured and quantified by flow cytometry. Apoptotic cells fragment their DNA, which leads to a reduced signal and the appearance of a sub-G1 peak in the flow cytometric histograms. The percentage of cells that appeared in the sub-G1 peak were quantified and defined as apoptotic.

When we tested PC3 cells for apoptosis induction, there was no significant apoptosis observed (Figure 14). All measured apoptosis levels were below 5% and can be considered as background and any fluctuations at and around this level as normal measuring variability. In conclusion, the FLIPi did not cause any apoptosis at the various levels.

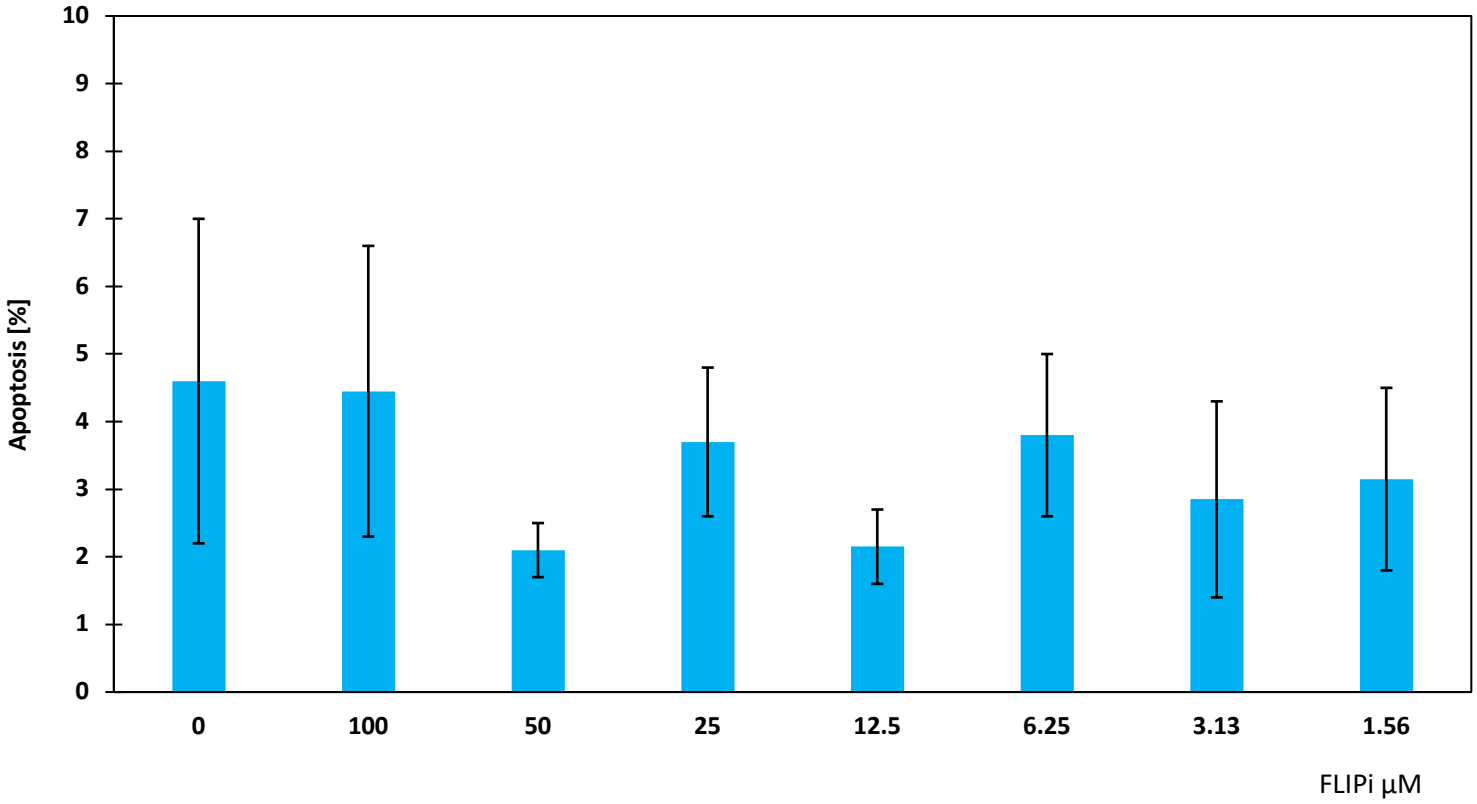

Figure 14 Apoptosis measured in PC3 cells treated with decreasing concentrations of FLIPi, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. This cell line was repeated 4 times with each concentration being tested 3 times with an average taken.

When tested apoptosis measurements in LNCaP cells did not reveal any significant apoptosis induction (Figure 15). There is possibly some increased apoptosis occurring at 100 µM of FLIPi but even that is at a very low level. All apoptosis rates were below 10% this could be considered as normally occurring apoptotic activity with variations between concentration being due to measuring viability. In conclusion FLIPi did not cause apoptosis to occur at either concentration.

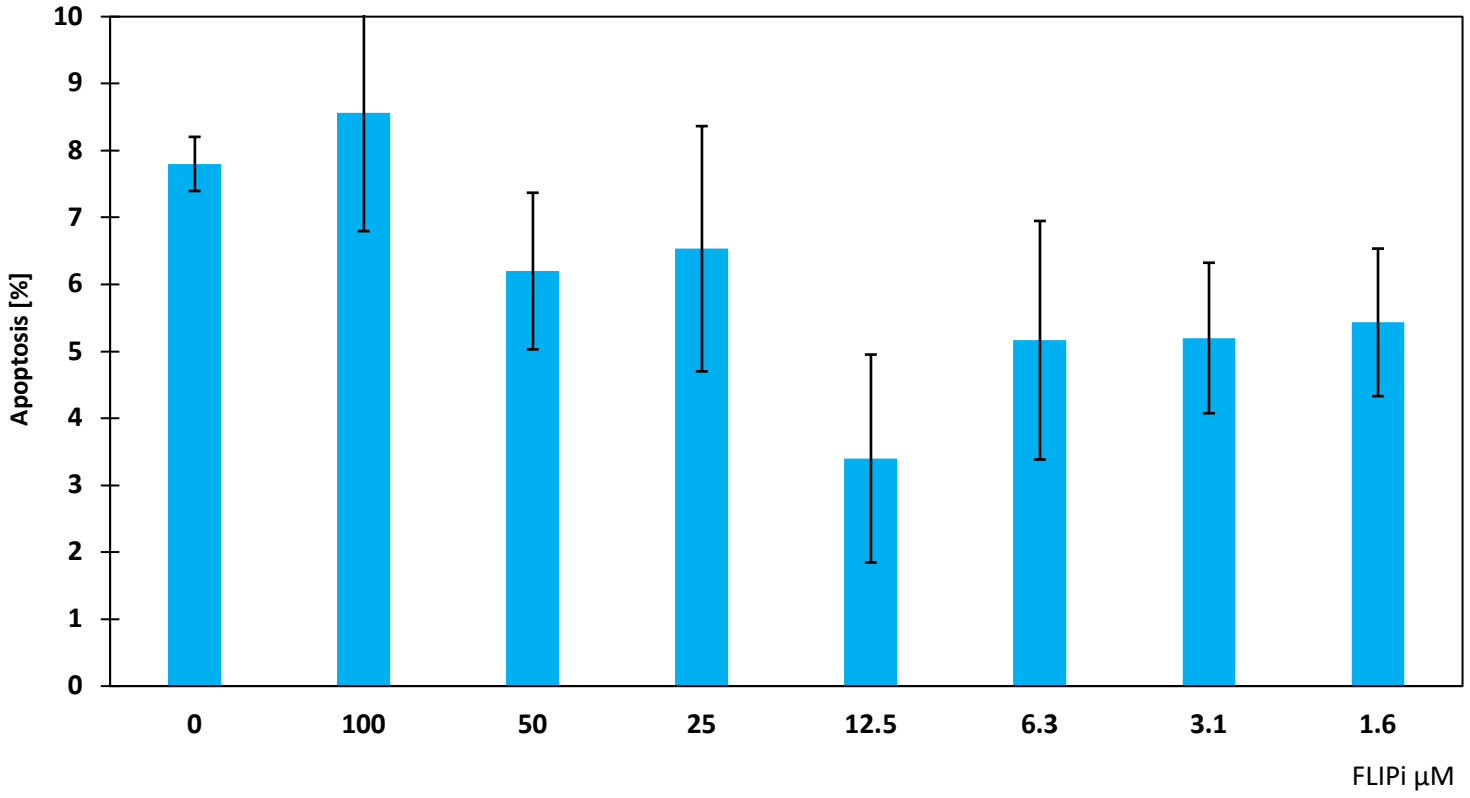

Figure 15 Apoptosis measured in LNCaP cells treated with decreasing concentrations of FLIPi, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. This cell line was repeated 4 times with each concentration being tested 3 times with an average taken.

HCT116 cells were tested for apoptotic induction and there was no significant apoptotic activity seen in HCT116 cells treated with FLIPi. All apoptosis levels were below 5% which can be credited to normally occurring apoptotic activity and variations that occur when measuring. In conclusion FLIPi did not induce apoptotic activity at either concentration.

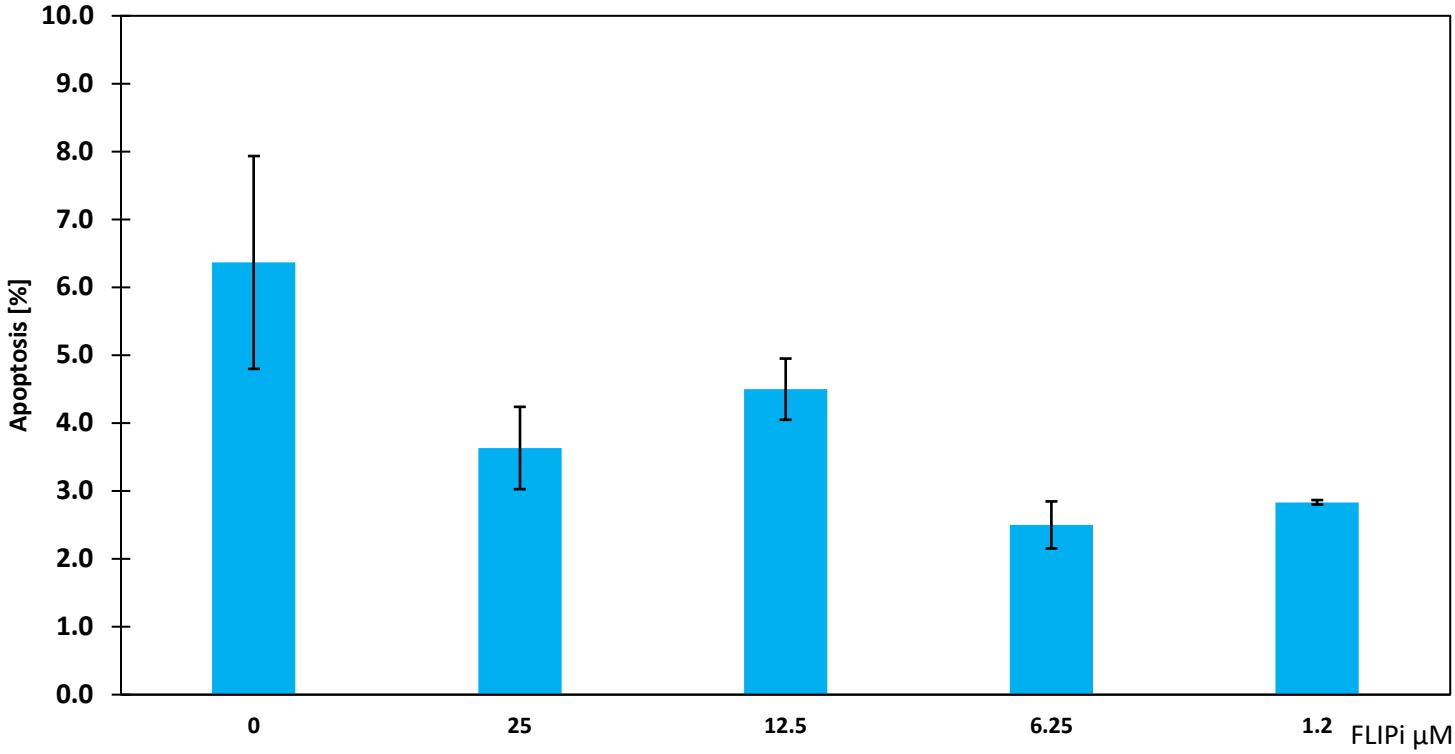

Figure 16 Apoptosis measured in HCT116 cells treated with decreasing concentrations of FLIPi, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. This cell line was repeated 3 times with each concentration being tested 3 times with an average taken

Similarly, in MDA-MB-231 cells there is no significant apoptosis induction after treatment with FLIPi of 50 µM. When tested for apoptotic induced activity, MDA-MB-231 showed no significant activity occurring (figure 17). All apoptosis levels were below 4% and can be considered as background/normal activity. Variations between the concentrations are due to normal variability in the measuring process. In summary FLIPi did not cause apoptotic activity at either concentration

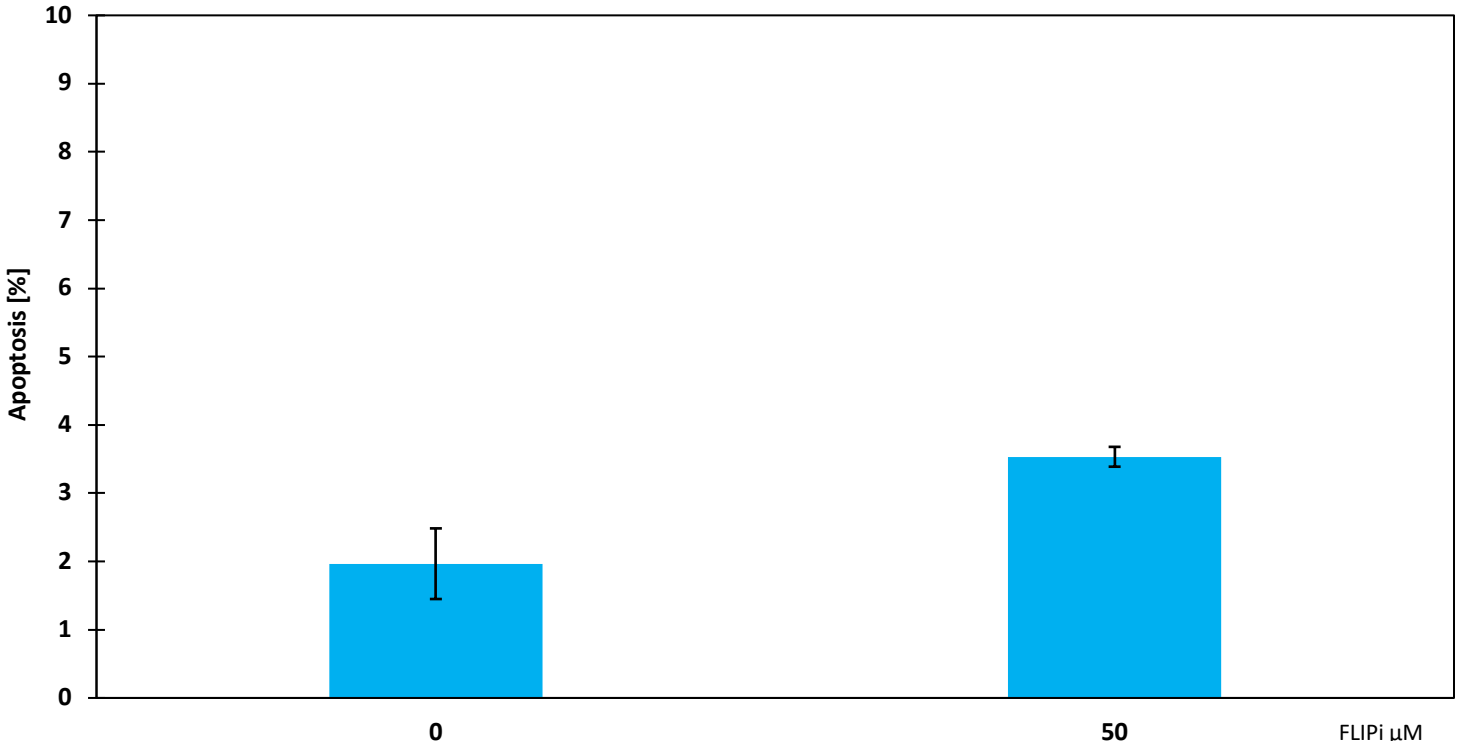

Figure 17 Apoptosis measured in MDA-MB-231 A cells treated with 0 (control) and 50 µM of FLIPi. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

When testing for apoptosis induction C2-4B showed no significant apoptosis observed at either concentration (Figure 18). Both concentrations have apoptotic levels of below 2%. This can be attributed to normal cell functions. In summary FLIPi did not induce apoptotic activity at either concentration.

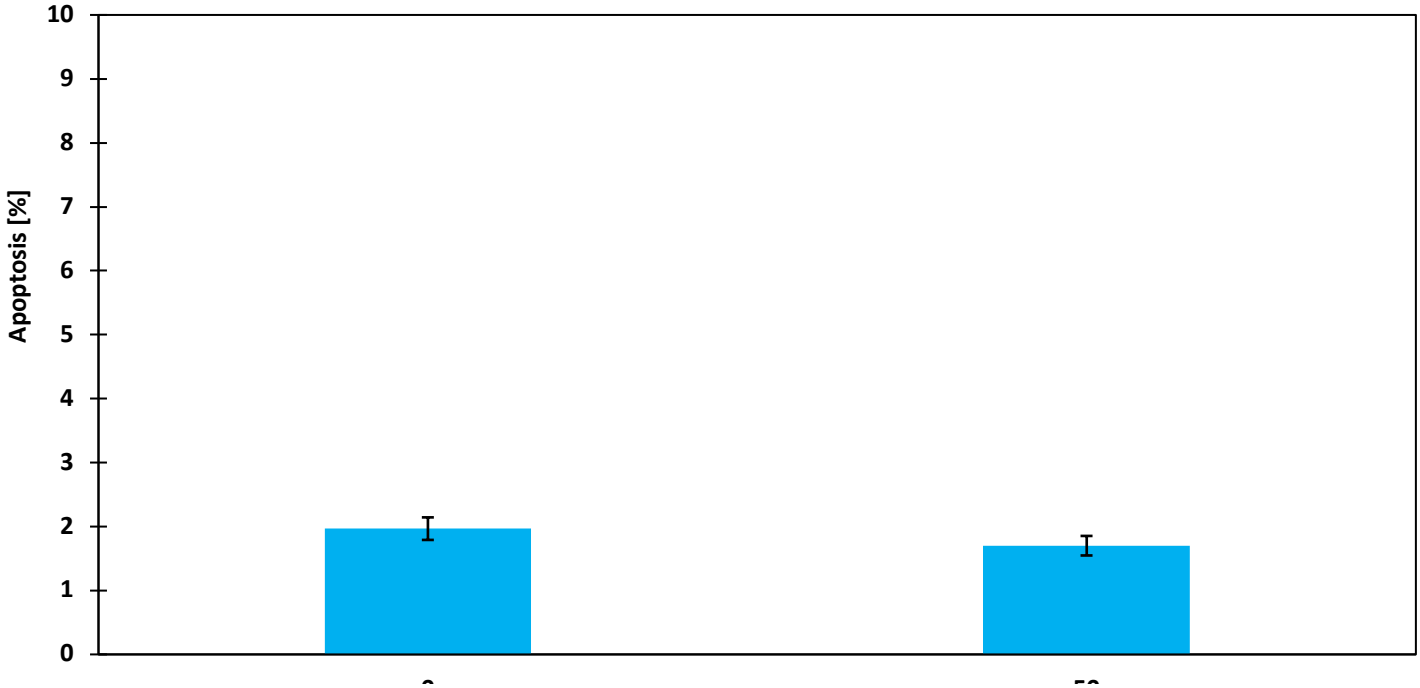

Figure 18 Apoptosis measured in C4-2B cells treated with 0 (control) and 50 μM of FLIPi. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

3.3 Combination treatments of FLIP inhibitor and TRAIL

Finally, we analysed the effects of the FLIPi and TRAIL on several different cell lines. As cFLIP is known to be a cellular inhibitor of caspase-8, the initiator caspase in extrinsic and TRAIL induced apoptosis, we hypothesised that the FLIPi could sensitise cells to the effects of TRAIL.

Firstly, prostate cancer cell line C4-2B was tested the in a combination approach. C4-2B cells shows relative r TRAIL resistance or in other words TRAIL only triggers a limited level of cell death (around 8%). However, when C4-2B cells were co-treated with FLIPi there is a sensitisation effect with more than double the apoptosis seen with the combined treatment compared to the single TRAIL treatment. Untreated and FLIPi treated cells showed no biologically significant levels of apoptosis. Thus, the FLIPi appeared to be able to block the apoptosis-inhibitory effects of cFLIP and sensitise C4-2B cells for TRAIL-induced apoptosis.

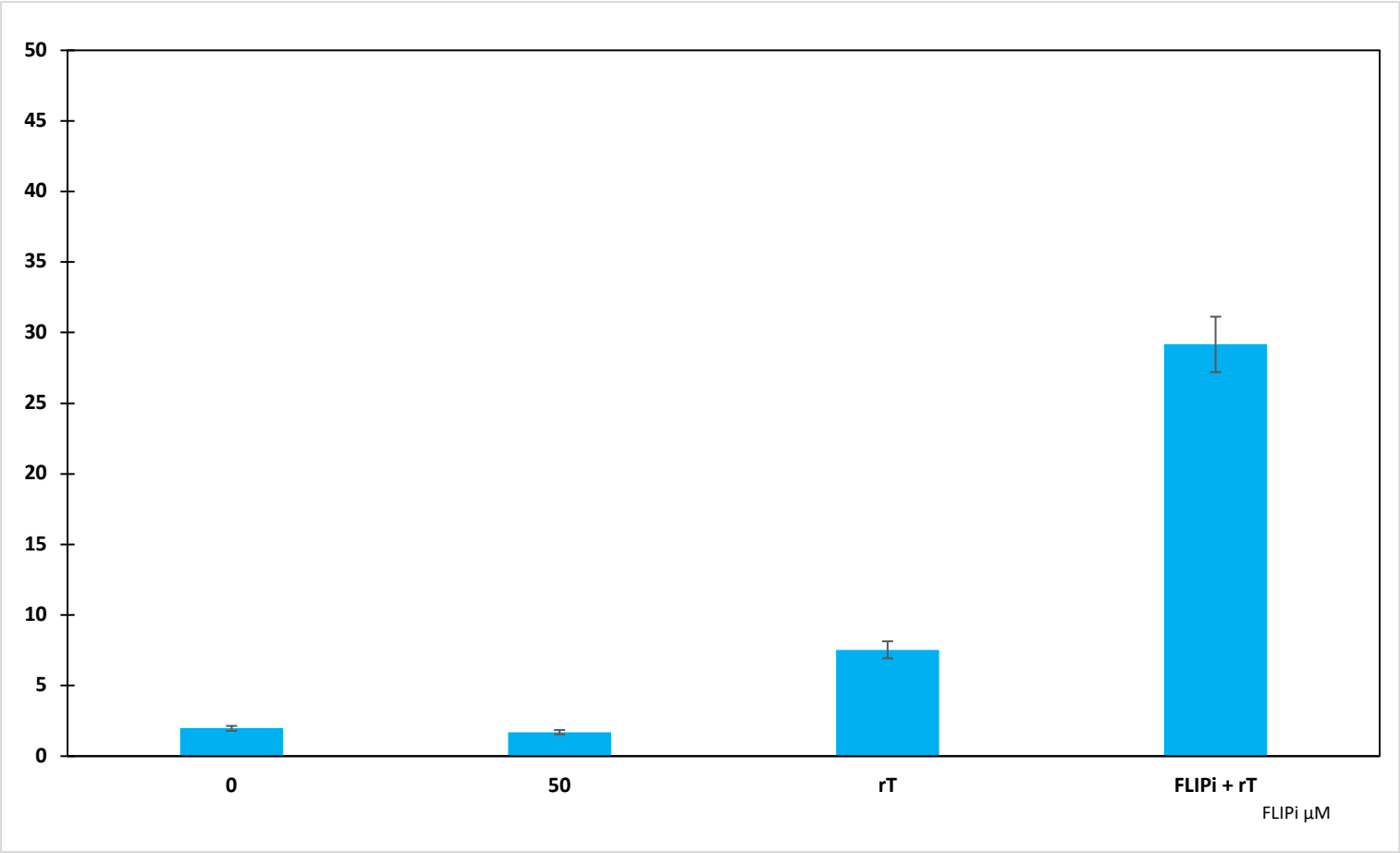

Figure 19 Apoptosis measured in C4-2B cells treated with 0 (the control) and 50 µM of FLIPi. Treated with recombinant TRAIL singularly and then co-treated with TRAIL 1 ng/mL and 50 µM FLIPi. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

.

Next LNCaP prostate cell line was tested, treated with a combination approach. There was a limited amount of apoptosis induced and shows a relative TRAIL resistance only inducing approximately 8.5% of apoptosis at most (figure 20). When co-treated with TRAIL there was a slightly increased amount of apoptosis triggered, at approximately 9% of apoptosis at the same concentration. Overall, there was no biologically significant apoptosis seen with FLIPi as a single treatment or when co-treated with TRAIL. Thus, appearing FLIPi does not sensitise cells to TRAIL.

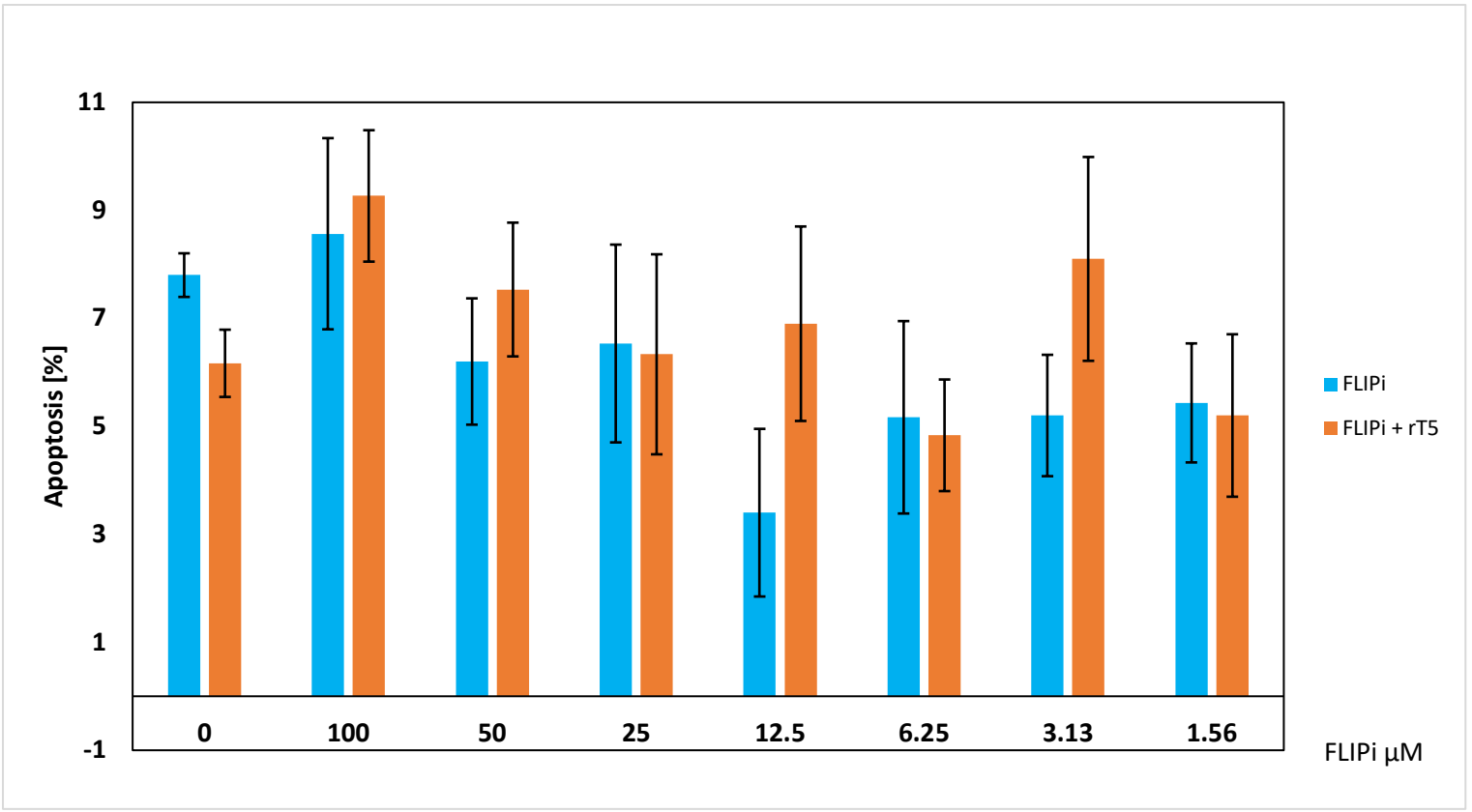

Figure 20 Apoptosis measured in LNCaP cells treated with varying concentrations of FLIPi, the control for this assay is 0 µM of FLIPi with increasing concentrations thereafter. Then treated with recombinant TRAIL and FLIPi as a co-treatment, with recombinant TRAIL at 5 ng/ml. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

Then HCT116 cells were tested. When using a combined treatment approach a high amount of apoptosis is triggered; approximately 67%. Comparatively, FLIPi as a singular treatment shows no significant apoptosis triggered and shows this cell line as being relatively TRAIL resistant. TRAIL as a singular treatment induces an estimated half of the apoptosis compared to that of the combined treatment. Overall FLIPi appeared to be able to sensitise cells to TRAIL by blocking the effects of cFLIP for TRAIL induced apoptosis to occur.

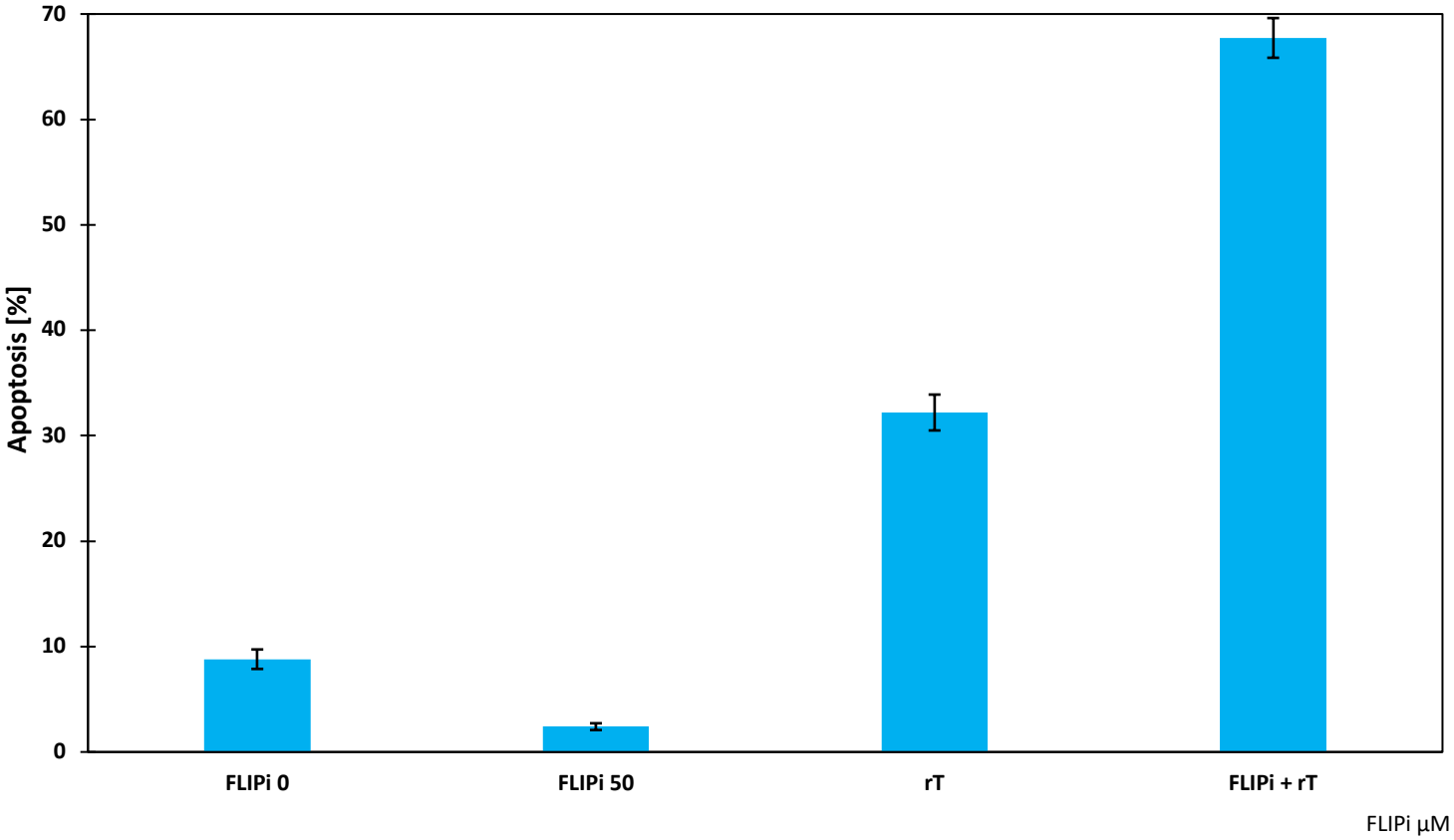

Figure 21 Apoptosis measured in HCT116 cells treated with 0 (control) and 50 µM of FLIPi. Treated with recombinant TRAIL singularly and then co-treated with TRAIL 50 µM and FLIPi, with recombinant TRAIL 5 ng/ml. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

Lastly MDA-MB-231 breast cancer cell line was tested using a combination treatment approach. MDA-MB-231 shows a relative sensitivity to TRAIL with approximately 40% cell death being triggered (figure 22). However, when MDA-MB-231 cells were co-treated with FLIPi there was sensitisation seen at an estimated 30%. It appears FLIPi can block the inhibitory effects of cFLIP to sensitise the cells for TRAIL induced apoptosis. Overall, there was sensitisation seen when using FLIPi and TRAIL together. However, as MDA-MB-231 is known to already be sensitive to TRAIL induced apoptosis, more apoptosis would be expected to be induced. Due to this more repeats would be beneficial.

In conclusion MDA-MB-231, HCT116 and C4-2B cell lines are sensitised to TRAIL using a FLIP inhibitor in combination with TRAIL.

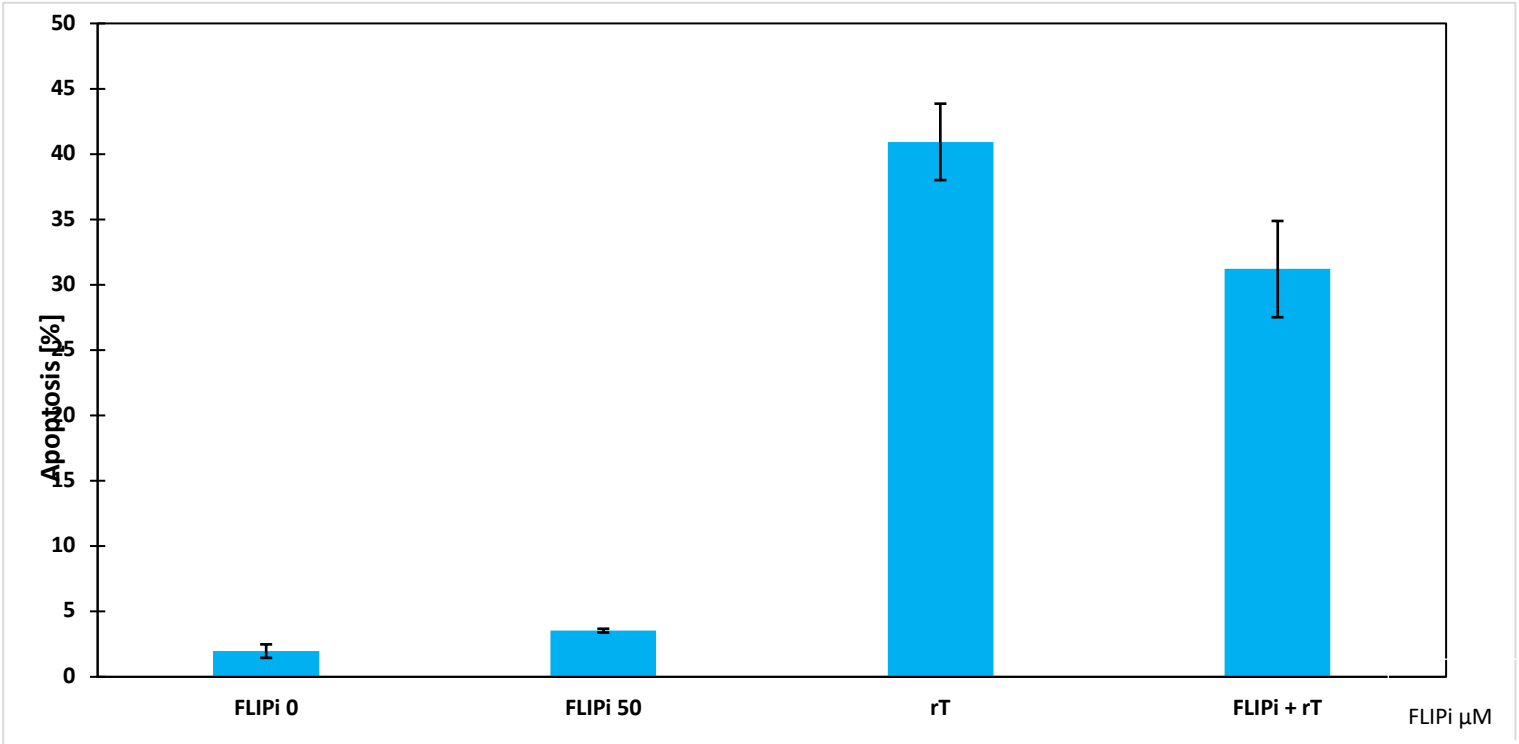

Figure 22 Apoptosis measured in MDA-MB-231 cells treated with 0 (control) and 50 µM of FLIPi. Treated with recombinant TRAIL singularly and then co-treated with TRAIL and FLIPi, recombinant TRAIL 5 ng/ml. Cells treated with Nicoletti buffer and measured using flow cytometry, measured 48 hours after treatment with FLIPi. Each concentration was tested 3 times each and an average taken for use in analysis.

4. Discussion

This project was undertaken to ascertain the ability and efficiency of a novel small chemical FLIPi to sensitise cancer cells to TRAIL. In order to do this, FLIPi was used singularly and in combination with TRAIL.

Apoptosis and survival of the cancer cells after treatment was measured using crystal violet and DNA hypodiploidy (Nicoletti) assays. Overall, the effects of the FLIPi were mixed, with some cancer cells responding to inhibitor/TRAIL combinations while others did not. Understanding the reasons will be a question to be addressed in the future.

The HCT116 cell line is known to be sensitive to TRAIL. Crystal violet assays show no significant effect of cell survival after treatment with varying concentrations of FLIPi. However, there was an approximate 50% reduction in cell survival at 100 μM compared to that of 0 μM. Preliminary Nicoletti results also support this conclusion, although there is some apoptotic activity seen and the most seen at 6.25 μM. There is very little apoptosis seen at 0 μM and 50 μM. There is a small amount of apoptotic activity seen when treated with 5 ng/ml of recombinant TRAIL, however the largest and notable amount of apoptosis occurred with a combination treatment of TRAIL and FLIPi. Suggesting the addition of FLIPi aids in sensitising the cells and enabling the action of TRAIL.

C4-2B cell line showed no significant change in cell survival observed within the crystal violet assay. Nicoletti assays showed no increased apoptosis when treated with 50 μM of FLIPi. However, this supports previous evidence suggesting no sensitisation as

when treated singularly with FLIPi as there was no significant increase in apoptosis. Similarly, there was only a small increase when cells were treated with 5 ng/ml of recombinant TRAIL. However. When treated with a combination there was a significant increase apoptotic percentage.

LNCaP cells showed no significant effects, although there was a low cell survival seen with the highest single concentration of FLIPi used – 100 μM. Nicoletti results also corroborate the limited effects seen with FLIPi. When treated with TRAIL and FLIPi in combination there was also no significant effects observed. This suggests a resistance to TRAIL and no sensitisation being achieved.

Prostate cancer cell line PC3 saw a slight reduction in cell survival at concentrations of 100 μM and 50 μM however overall, there is no sensitisation evident. Similarly, there was no significant effect seen with prostate cancer cell line DU145.

Deletions in this cell line such as PTEN- a known driver of tumorigenesis- could suggest these mutations would lend an advantage of survival to cancer cells. This could help evade cell death resulting in a resistance and/or seeing treatments be ineffective.

The MDA-MB-231 cell line was treated singularly with varying concentrations of FLIPI shows no significant effect on cell survival. However, there was significant apoptosis seen with initial Nicoletti experiments. There was a reduction in the amount of apoptosis observed when a combination of FLIPi and recombinant TRAIL was administered alluding to a resistance to TRAIL with this cell line.

Little sensitisation is seen in the DU145 cells line. Previous research suggests that when DU145 cells lose the wild type p53 allele, tumour progression, resistance to traditional therapies and a worsened prognosis are the consequences. This could suggest that mutation in this gene could lead to a lack tumour suppression and reaction to treatment.

4.1 cFLIP action in the apoptosis pathway

4.1.1 FLIP action summary

FLIP is most known for the inhibitory characteristics is possesses on the death receptor-mediated apoptotic cascade. Once the ligand has bound to the receptor, with respect to TRAIL this would be death receptor 4 or 5 the death inducing signalling complex is formed (Wajant, 2019) . This complex enlists FADD which is an adaptor protein and also engages caspase-8 at the cytosolic domain of the receptor (Roth and Reed, 2004). Clustering of pro caspase-8 zymogen occurs around aggravated receptor complexes which forms the bases of the induced proximity mechanism (Sprick et al., 2000). This mechanism causes caspase-8 to be cleaved into p43 and p10 fragments, subsequently p43 is broken down into p18 which is the active caspase unit. However, when FLIP is present death effector homotypic interactions are formed between FADD and FLIP molecules. This causes proteolytic cleavage of FLIP and recruitment into the DISC. The p43 fragment remains in the DISC bound to the previously formed complex. This means that FLIP proteins are dominant negative inhibitors of caspase-8 within the signalling cascade

4.1.2 Functions of FLIP in forming the Ripoptosome and the role in Necroptosis

Cells can initiate caspase-8 activation independent of extrinsic death ligand to death receptor interactions, in very specific conditions. A protein - Receptor interacting protein kinase 1 (RIPK1) – was found to interact with tumour necrosis factor receptor 1 signaling complex. This protein is imperative in cell survival, apoptosis and necroptosis regulation. It is regulated through ubiquitylation and phosphorylation processes, with cellular inhibitors of apoptosis (cIAP) are found to ubiquitylate RIPK1. When cellular inhibitors of apoptosis were examined in order to ascertain whether elimination would affect cell death processes. It was found that spontaneous death receptor independent formation of ripoptosome, a complex containing RIPK1, FADD, caspase-8 and cFLIP. Procaspase-8 is integrated into the ripoptosome and processed via heterodimerisation which results in the cleavage and activation of RIPK1 thus degrading the ripoptosome. This results in caspase-8 mediated apoptosis being implemented exclusively. However, cFLIP can play a key role in this process. When cFLIP in integrated into the ripoptosome, heterodimerisation activates procaspase-8. This can cleave substrates and the activate RIPK1, although once the ripoptosome is disassembled procaspase-8 become inactivate again. Consequently, this means cellular survival is achieved by blocking apoptosis (Tsuchiya et al., 2015).

Necroptosis is initiated when cFLIP promotes the assembly of the ripoptosome, however cannot activate procaspase-8. This is physiologically regulated by RIPK1, receptor-interacting protein kinase 3 and mixed lineage kinase-like protein,

distinguishing this process from unintentional necrosis (Pasparakis and Vandenabeele, 2015). Other death-inducing protein complexes are formed with the involvement of cFLIP such as apoptotic inhibitory complex. The complex is made up of DR5-, FADD-, Caspase-8, and FLIPL and has currently been found in MCF-7 breast cancer cells (Day et al., 2008).

4.2 Functions of FLIP

It has been suggested overexpression of cFLIP can lead to apoptosis rather than inhibit it. This was thought to be due to clustering of large- non-physiologic- amounts of FLIP protein, resulting in activation of caspase-8. However, at lower (physiologic) expression, cFLIP actually performs a protective role, and death receptor induced apoptosis is inhibited (Chang et al., 2002). FLIP binds to FADD which interferes with TNF-α, Fas-L, and TRAIL-induced signalling pathways and current research suggests that overall cFLIP has anti-apoptotic characteristics in cancer cells. It is thought that the discord surrounding cFLIP function is due to the varying forms/structure cFLIP can have. $cFLIP_s$ has been implicated in TRAIL induced DISC formation and consequent apoptosis and have been seen to inhibit this pathway. $cFLIP_L$ is also involved in these processes but also when expressed in higher levels it inhibits Fas-induced activation of caspase-8. Oppositely when the expression is low it can also mean caspase-8 activation is boosted. It is thought that the long form of cFLIP forms heterodimeric enzyme molecules with key features of substrate specificity and having extremely similar catalytic activity to that of caspase-8 homodimers. This is despite cFLIP being protease dead. It has also been seen that cFLIP could contribute to survival signalling

via AKT, ERK, and NF- κB signalling pathways through acting as an effector. NF-κB and ERK signalling pathways can be activated by binding to the specific adaptor proteins specific to each pathway due to a high level of expression of $FLIP_L$ (Safa et al., 2008).

4.3 Levels of cytoplasmic FLIP and TRAIL sensitivity

Evidence would suggest that MDA-MB-231 breast cancer cell should be sensitive to TRAIL (Di et al., 2013). This cell line presents a mesenchymal like morphology, and research suggests that this leaves the cells vulnerable to TRAIL and the pro-apoptotic effects. (Huang et al., 2020).

Investigating the extrinsic pathway shows that for cFLIP to cause inhibition it must be able to impede the caspase-8 recruitment. cFLIP would have to be present in the cytoplasmic compartment of the cell so that there can be interactions with the DISC. Wnt signalling has been associated with mesenchymal-like cells and consequent increased signalling. Due to this it could be suggested that sub-cellular compartmentalisation of cFLIP would be the biggest influencing factor in shaping TRAIL sensitivity (French et al., 2015). Levels of cFLIP in the cytoplasm was found to be reduced in this specific cell line and in that of similar tumours. Thus, supporting the theory of reduced cFLIP levels leading to increased sensitivity it is thought that this cell line sees cFLIP situated in the nuclear and peri-nuclear compartment.

4.4 FLIP protective properties evidenced by MCF7 cell line

Upon investigation over high levels cFLIP had a protective action. It was seen that within MCF-7 cell line which is cancer stem/progenitor line is protected from TRAIL due to overexpression of cytoplasmic cFLIP expression. Conversely it was found that nuclear FLIP did not possess this protective effect, and if fact both types have been implicated in breast cancer stem cell maintenance mechanisms. This is because raised levels of both types of cFLIP were found to lead to a significant increase in tumourspheres forming but have differing functions in relation to apoptosis inhibition (French et al., 2015).

4.5 FLIP and HCT116 cell line

It has been well established that over-expression of cFLIP is linked to resistance in colorectal cancer. This resistance applies to standard treatments such as 5FU and oxaliplatin, once again highlighting why ongoing research into cFLIP inhibitors is so vital. It also extends to therapies that target apoptotic pathways. cFLIP prevents cell death when involved with both monovalent and bivalent inhibitor of apoptosis protein (IAP) (Humphreys et al., 2018).

Wilson et al. (2007) also showed HCT116 cells as being sensitive to TRAIL, and it is thought that relative sensitivity levels is concurrent to the amount of death receptors on the cell surface. It was found that that $cFLIP_L$ was a promoter for the first cleavage step of caspase-8 activation. However, it prevents any more cleavage to the active p10

and p18 subunits, resulting in obstructing caspase-8–mediated apoptosis (Micheau et al., 2002).

It has also been found that when cFLIP is downregulated, cell lines can be sensitised to TRAIL, with results showing a synergy; more apoptosis was induced with down regulation of cFLIP and cell lines being treated with traditional chemotherapies (Fulda et al., 2000). It is theorised that colorectal cancer cells form DISCs that contain cFLIP and that these regulate the activity of antiapoptotic signalling pathways where there is no ligand binding activation. However, when cFLIP is down-regulated or silenced, it results in these DISCs activating caspase-8 and consequently inducing apoptosis. $cFLIP_L$ was found to interact with DR5 in a TRAIL and FADD-independent manner and if this interaction was interrupted it caused in TRAIL independent apoptosis to occur (Jin et al., 2004). $FLIP_{L\ is}$ thought to - in respect to D5 - inhibit ligand-independent recruitment of FADD and caspase-8. This would suggest that $cFLIP_L$ inhibits ligand-independent recruitment of FADD and caspase-8 in to the DR5 DISC. therefore down-regulating $cFLIP_{L\ can}$ induces apoptosis by this process (Wilson et al., 2007). Therefore, it could be suggested that inhibiting cFLIP could also have this apoptosis induing effect.

4.6 cFLIP and LNCaP cell line

The results of this research did not show sensitisation to TRAIL with the LNCaP cell line. However, research showed that high levels of cFLIP expression in this cell line lead to apoptotic inhibition via acceleration of androgen independence (Valnet-Rabier et al., 2005).

Wilson et al. (2008) and Zhang et al. (2004) also found that LNCaP cell line was poorly sensitive to TRAIL. It was also found that when cFLIP was over expressed the pro-apoptotic initiation by activation on caspase -8, was inhibited. Down regulation of cFLIP was also found to sensitise cells when TRAIL was administered (White et al., 2006).

FLIP was found to be higher in LNCaP cells than cells that are known to be TRAIL sensitive even after 24 hours of TRAIL treatment.

Comparatively, in PC3 cells FLIP and mRNA levels were found to be lower in cells that are TRAIL-sensitive after TRAIL treatment. This suggests a strong connection between TRAIL sensitivity of cFLIP expression (Zhang et al., 2004). This could infer a higher level of cFLIP in this cell line and thus why it is not a TRAIL sensitive.

Also as previously mentioned, tumour suppressor genes could be mutated in this cell line. However, due to the lack of knowledge on the functional regions of some of these specific genes such as menin the full effect on cancer behaviour is hard to determine (Seim et al., 2017).

4.7 Further strategies to use FLIP as a cancer therapy

Due to FLIP being both proapoptotic and antiapoptotic in nature, these properties can be manipulated in order to sensitise cells to TRAIL overcoming the current obstacle of resistance (Mathas et al., 2004). Small interfering RNAs (siRNA) have been seen to knockdown the expression of cFLIP in several cell lines such as H460 lung carcinoma cells (Sharp et al., 2005). This research showed that the knockdown in expression of cFLIP synergised TRAIL action and therefore chemotherapy induced apoptotic activity.

New therapies are used in order to reach apoptosis to be triggered at a lower threshold and therefore be a more effective treatment. Recent research used small interfering RNAs (siRNAs) that downregulated the expression of cFLIP. As previously discussed, this could lead to more sensitisation to TRAIL and therefore overcoming the resistance problem.

Due to the significant similarities in caspase-8 and cFLIP structure, small molecules that prevent cFLIP from being recruited into the DISC will also prevent caspase-8 from moving into the DISC. This would result in apoptosis being inhibited, so research into this avenue of exploration could be useful for expanding treatments (Safa, 2012).

Inhibiting cFLIP is thought to be difficult as there is no structure where small molecule ligands can bind. This is due to the highly conserved properties of the DED which predominately use homotypic binding. However, in the last few years a couple of FLIPi have been developed, one of which was tested here. If such inhibitors would not have been feasible the inhibitor tested would not have worked at all as the DEDs would be similar in all cell lines. (Safa et al., 2008).

4.8 Summarising remarks

There were some promising results, HCT116 cell line was known to be sensitive to TRAIL. With some suggestions this is due to the role cFLIP plays in regulating ligand binding induced apoptosis and the amount of receptors present. When downregulated or silenced enhanced apoptotic induction by TRAIL is seen suggesting great promise in the use of inhibiting this cFLIP protein.

MDA-MB-231 saw some apoptosis with Nicoletti assays, with a reduction in the amount of apoptosis observed when treated with a combination of FLIPi and recombinant TRAIL. This suggests a TRAIL resistance evidenced by an increase in apoptosis- compared to a single treatment of FLIPi- when cFLIP was administered. Overall, there is a promising future of future research with this inhibitor. Previously mentioned association with mutations with p53 could aid in understanding why this is such a hard to treat and aggressive cell line.

Some sensitisation was seen in the pancreatic cell line; however, this would need more repeats due to a lack of time. Due to the breakpoints seen within chromosome 8 with pancreatic cancers, it could be worth exploring the NRG1 gene that is mutated as it could be an oncogene/tumour suppressor and targeting it could be a good therapeutic target.

However, there was not sensitisation seen in all cell lines. PC3 cell line is thought to be moderately sensitive TRAIL. However, cytotoxic effects were seen during cell survival assays, but no further evidence was seen as a result of measuring apoptosis after treatment. DU145 cells are thought to be moderately sensitive to TRAIL however, there was no sensitisation seen in the results. C4-2B and LNCaP cell lines also showed no sensitisation to TRAIL.

4.9 Conclusion

This vein of research -finding a novel therapeutic agent- is important as it could overcome the necessity to use traditional chemotherapies that are not as damaging to healthy cells without compromising on the quality of cancer cell destruction. This would greatly improve patient prognosis and reduce the trauma of the disease.

Bibliography

ADELAIDE, J., HUANG, H. E., MURATI, A., ALSOP, A. E., ORSETTI, B., MOZZICONACCI, M. J., POPOVICI, C., GINESTIER, C., LETESSIER, A., BASSET, C., COURTAY-CAHEN, C., JACQUEMIER, J., THEILLET, C., BIRNBAUM, D., EDWARDS, P. A. & CHAFFANET, M. 2003. A recurrent chromosome translocation breakpoint in breast and pancreatic cancer cell lines targets the neuregulin/NRG1 gene. *Genes Chromosomes Cancer,* 37**,** 333-45.

ALSOP, A. E., TAYLOR, K., ZHANG, J., GABRA, H., PAIGE, A. J. & EDWARDS, P. A. 2008. Homozygous deletions may be markers of nearby heterozygous mutations: The complex deletion at FRA16D in the HCT116 colon cancer cell line removes exons of WWOX. *Genes Chromosomes Cancer,* 47**,** 437-47.

AMIN, M. B., GREENE, F. L., EDGE, S. B., COMPTON, C. C., GERSHENWALD, J. E., BROOKLAND, R. K., MEYER, L., GRESS, D. M., BYRD, D. R. & WINCHESTER, D. P. 2017. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin,* 67**,** 93-99.

APANTAKU, L. M. 2000. Breast cancer diagnosis and screening. *Am Fam Physician,* 62**,** 596-602, 605-6.

BAJGELMAN, M. C. & STRAUSS, B. E. 2006. The DU145 human prostate carcinoma cell line harbors a temperature-sensitive allele of p53. *Prostate,* 66**,** 1455-62.

BAZZICHETTO, C., CONCIATORI, F., LUCHINI, C., SIMIONATO, F., SANTORO, R., VACCARO, V., CORBO, V., FALCONE, I., FERRETTI, G., COGNETTI, F., MELISI, D., SCARPA, A., CIUFFREDA, L. & MILELLA, M. 2020. From Genetic Alterations to Tumor Microenvironment: The Ariadne's String in Pancreatic Cancer. *Cells,* 9.

BORLEY, N. & FENELEY, M. R. 2009. Prostate cancer: diagnosis and staging. *Asian J Androl,* 11**,** 74-80.

BOSETTI, C., BERTUCCIO, P., NEGRI, E., LA VECCHIA, C., ZEEGERS, M. P. & BOFFETTA, P. 2012. Pancreatic cancer: Overview of descriptive epidemiology. *Molecular Carcinogenesis,* 51**,** 3-13.

CARVER, B. S., CHAPINSKI, C., WONGVIPAT, J., HIERONYMUS, H., CHEN, Y., CHANDARLAPATY, S., ARORA, V. K., LE, C., KOUTCHER, J., SCHER, H., SCARDINO, P. T., ROSEN, N. & SAWYERS, C. L. 2011. Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. *Cancer Cell,* 19**,** 575-86.

CERAMI, E., GAO, J., DOGRUSOZ, U., GROSS, B. E., SUMER, S. O., AKSOY, B. A., JACOBSEN, A., BYRNE, C. J., HEUER, M. L., LARSSON, E., ANTIPIN, Y., REVA, B., GOLDBERG, A. P., SANDER, C. & SCHULTZ, N. 2012. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov,* 2**,** 401-4.

CHANG, D. W., XING, Z., PAN, Y., ALGECIRAS-SCHIMNICH, A., BARNHART, B. C., YAISH-OHAD, S., PETER, M. E. & YANG, X. 2002. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J,* 21**,** 3704-14.

DAY, T. W., HUANG, S. & SAFA, A. R. 2008. c-FLIP knockdown induces ligand-independent DR5-, FADD-, caspase-8-, and caspase-9-dependent apoptosis in breast cancer cells. *Biochem Pharmacol,* 76**,** 1694-704.

DI, X., ZHANG, G., ZHANG, Y., TAKEDA, K., RIVERA ROSADO, L. A. & ZHANG, B. 2013. Accumulation of autophagosomes in breast cancer cells induces TRAIL resistance through downregulation of surface expression of death receptors 4 and 5. *Oncotarget,* 4**,** 1349-64.

DUIKER, E. W., MOM, C. H., DE JONG, S., WILLEMSE, P. H., GIETEMA, J. A., VAN DER ZEE, A. G. & DE VRIES, E. G. 2006. The clinical trail of TRAIL. *Eur J Cancer,* 42**,** 2233-40.

ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol,* 35**,** 495-516.

FREELOVE, R. & WALLING, A. D. 2006. Pancreatic cancer: diagnosis and management. *Am Fam Physician,* 73**,** 485-92.

FRENCH, R., HAYWARD, O., JONES, S., YANG, W. & CLARKSON, R. 2015. Cytoplasmic levels of cFLIP determine a broad susceptibility of breast cancer stem/progenitor-like cells to TRAIL. *Mol Cancer,* 14**,** 209.

FULDA, S., MEYER, E. & DEBATIN, K. M. 2000. Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer Res,* 60**,** 3947-56.

GHOBRIAL, I. M., WITZIG, T. E. & ADJEI, A. A. 2005. Targeting Apoptosis Pathways in Cancer Therapy. *CA: A Cancer Journal for Clinicians,* 55**,** 178-194.

HOFFMAN, R. M. 2011. Clinical practice. Screening for prostate cancer. *N Engl J Med,* 365**,** 2013-9.

HONRADO, E., BENÍTEZ, J. & PALACIOS, J. 2005. The molecular pathology of hereditary breast cancer: genetic testing and therapeutic implications. *Mod Pathol,* 18**,** 1305-20.

HUANG, Z., YU, P. & TANG, J. 2020. Characterization of Triple-Negative Breast Cancer MDA-MB-231 Cell Spheroid Model. *Onco Targets Ther,* 13**,** 5395-5405.

HUI, L., ZHENG, Y., YAN, Y., BARGONETTI, J. & FOSTER, D. A. 2006. Mutant p53 in MDA-MB-231 breast cancer cells is stabilized by elevated phospholipase D activity and contributes to survival signals generated by phospholipase D. *Oncogene,* 25**,** 7305-10.

HUMPHREY, P. A. 2004. Gleason grading and prognostic factors in carcinoma of the prostate. *Mod Pathol,* 17**,** 292-306.

HUMPHREYS, L., ESPONA-FIEDLER, M. & LONGLEY, D. B. 2018. FLIP as a therapeutic target in cancer. *FEBS J,* 285**,** 4104-4123.

IODICE, S., GANDINI, S., MAISONNEUVE, P. & LOWENFELS, A. B. 2008. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. *Langenbecks Arch Surg,* 393**,** 535-45.

JIN, T. G., KURAKIN, A., BENHAGA, N., ABE, K., MOHSENI, M., SANDRA, F., SONG, K., KAY, B. K. & KHOSRAVI-FAR, R. 2004. Fas-associated protein with death domain (FADD)-independent recruitment of c-FLIPL to death receptor 5. *J Biol Chem,* 279**,** 55594-601.

KEY, T. J., SHARP, G. B., APPLEBY, P. N., BERAL, V., GOODMAN, M. T., SODA, M. & MABUCHI, K. 1999. Soya foods and breast cancer risk: a prospective study in Hiroshima and Nagasaki, Japan. *Br J Cancer,* 81**,** 1248-56.

KEY, T. J., VERKASALO, P. K. & BANKS, E. 2001. Epidemiology of breast cancer. *Lancet Oncol,* 2**,** 133-40.

KLEEFF, J., KORC, M., APTE, M., LA VECCHIA, C., JOHNSON, C. D., BIANKIN, A. V., NEALE, R. E., TEMPERO, M., TUVESON, D. A., HRUBAN, R. H. & NEOPTOLEMOS, J. P. 2016. Pancreatic cancer. *Nat Rev Dis Primers,* 2**,** 16022.

LANDOWSKI, T. H., QU, N., BUYUKSAL, I., PAINTER, J. S. & DALTON, W. S. 1997. Mutations in the Fas antigen in patients with multiple myeloma. *Blood,* 90**,** 4266-70.

LI, D., XIE, K., WOLFF, R. & ABBRUZZESE, J. L. 2004. Pancreatic cancer. *Lancet,* 363**,** 1049-57.

LONGLEY, D. B., HARKIN, D. P. & JOHNSTON, P. G. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer,* 3**,** 330-8.

MAITLAND, N. J. & COLLINS, A. T. 2008. Prostate cancer stem cells: a new target for therapy. *J Clin Oncol,* 26**,** 2862-70.

MATHAS, S., LIETZ, A., ANAGNOSTOPOULOS, I., HUMMEL, F., WIESNER, B., JANZ, M., JUNDT, F., HIRSCH, B., JOHRENS-LEDER, K., VORNLOCHER, H. P., BOMMERT, K., STEIN, H. & DORKEN, B. 2004. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med,* 199**,** 1041-52.

MAZHAR, D. & WAXMAN, J. 2002. Prostate cancer. *Postgraduate Medical Journal,* 78**,** 590-595.

MCGUIGAN, A., KELLY, P., TURKINGTON, R. C., JONES, C., COLEMAN, H. G. & MCCAIN, R. S. 2018. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol,* 24**,** 4846-4861.

MCPHERSON, K., STEEL, C. M. & DIXON, J. M. 2000. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ,* 321**,** 624-8.

MELLIER, G., HUANG, S., SHENOY, K. & PERVAIZ, S. 2010. TRAILing death in cancer. *Mol Aspects Med,* 31**,** 93-112.

MEYERHARDT, J. A. & MAYER, R. J. 2005. Systemic therapy for colorectal cancer. *N Engl J Med,* 352**,** 476-87.

MICHEAU, O., THOME, M., SCHNEIDER, P., HOLLER, N., TSCHOPP, J., NICHOLSON, D. W., BRIAND, C. & GRUTTER, M. G. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem,* 277**,** 45162-71.

PASPARAKIS, M. & VANDENABEELE, P. 2015. Necroptosis and its role in inflammation. *Nature,* 517**,** 311-20.

RADICE, D. & REDAELLI, A. 2003. Breast cancer management: quality-of-life and cost considerations. *Pharmacoeconomics,* 21**,** 383-96.

RAWLA, P., SUNKARA, T. & GADUPUTI, V. 2019. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J Oncol,* 10**,** 10-27.

REFAAT, A., ABD-RABOU, A. & REDA, A. 2014. TRAIL combinations: The new 'trail' for cancer therapy (Review). *Oncol Lett,* 7**,** 1327-1332.

RICCARDI, C. & NICOLETTI, I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc,* 1**,** 1458-61.

ROCCHI, P., SO, A., KOJIMA, S., SIGNAEVSKY, M., BERALDI, E., FAZLI, L., HURTADO-COLL, A., YAMANAKA, K. & GLEAVE, M. 2004. Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. *Cancer Res,* 64**,** 6595-602.

ROTH, W. & REED, J. C. 2004. FLIP protein and TRAIL-induced apoptosis. *Vitam Horm,* 67**,** 189-206.

SAFA, A. R. 2012. c-FLIP, a master anti-apoptotic regulator. *Exp Oncol,* 34**,** 176-84.

SAFA, A. R., DAY, T. W. & WU, C. H. 2008. Cellular FLICE-like inhibitory protein (C-FLIP): a novel target for cancer therapy. *Curr Cancer Drug Targets,* 8**,** 37-46.

SAMOKHVALOV, A. V., REHM, J. & ROERECKE, M. 2015. Alcohol Consumption as a Risk Factor for Acute and Chronic Pancreatitis: A Systematic Review and a Series of Meta-analyses. *EBioMedicine,* 2**,** 1996-2002.

SEIM, I., JEFFERY, P. L., THOMAS, P. B., NELSON, C. C. & CHOPIN, L. K. 2017. Whole-Genome Sequence of the Metastatic PC3 and LNCaP Human Prostate Cancer Cell Lines. *G3 (Bethesda),* 7**,** 1731-1741.

SHARP, D. A., LAWRENCE, D. A. & ASHKENAZI, A. 2005. Selective knockdown of the long variant of cellular FLICE inhibitory protein augments death receptor-mediated caspase-8 activation and apoptosis. *J Biol Chem,* 280**,** 19401-9.

SPRICK, M. R., WEIGAND, M. A., RIESER, E., RAUCH, C. T., JUO, P., BLENIS, J., KRAMMER, P. H. & WALCZAK, H. 2000. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity,* 12**,** 599-609.

STRANO, S., MUNARRIZ, E., ROSSI, M., CRISTOFANELLI, B., SHAUL, Y., CASTAGNOLI, L., LEVINE, A. J., SACCHI, A., CESARENI, G., OREN, M. & BLANDINO, G. 2000. Physical and functional interaction between p53 mutants and different isoforms of p73. *J Biol Chem,* 275**,** 29503-12.

TSUCHIYA, Y., NAKABAYASHI, O. & NAKANO, H. 2015. FLIP the Switch: Regulation of Apoptosis and Necroptosis by cFLIP. *Int J Mol Sci,* 16**,** 30321-41.

VALNET-RABIER, M. B., CHALLIER, B., THIEBAULT, S., ANGONIN, R., MARGUERITTE, G., MOUGIN, C., KANTELIP, B., DECONINCK, E., CAHN, J. Y. & FEST, T. 2005. c-Flip protein expression in Burkitt's lymphomas is associated with a poor clinical outcome. *Br J Haematol,* 128**,** 767-73.

VAN DER JEUGHT, K., XU, H. C., LI, Y. J., LU, X. B. & JI, G. 2018. Drug resistance and new therapies in colorectal cancer. *World J Gastroenterol,* 24**,** 3834-3848.

VUONG, D., SIMPSON, P. T., GREEN, B., CUMMINGS, M. C. & LAKHANI, S. R. 2014. Molecular classification of breast cancer. *Virchows Arch,* 465**,** 1-14.

WAJANT, H. 2019. Molecular Mode of Action of TRAIL Receptor Agonists-Common Principles and Their Translational Exploitation. *Cancers (Basel),* 11.

WANG, X., CHEN, W., ZENG, W., BAI, L., TESFAIGZI, Y., BELINSKY, S. A. & LIN, Y. 2008. Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. *Mol Cancer Ther,* 7**,** 1156-63.

WHITE, S. J., LU, P., KELLER, G. M. & VOELKEL-JOHNSON, C. 2006. Targeting the short form of cFLIP by RNA interference is sufficient to enhance TRAIL sensitivity in PC3 prostate carcinoma cells. *Cancer Biol Ther,* 5**,** 1618-23.

WILSON, C., WILSON, T., JOHNSTON, P. G., LONGLEY, D. B. & WAUGH, D. J. 2008. Interleukin-8 signaling attenuates TRAIL- and chemotherapy-induced apoptosis through transcriptional regulation of c-FLIP in prostate cancer cells. *Mol Cancer Ther,* 7**,** 2649-61.

WILSON, T. R., MCLAUGHLIN, K. M., MCEWAN, M., SAKAI, H., ROGERS, K. M., REDMOND, K. M., JOHNSTON, P. G. & LONGLEY, D. B. 2007. c-FLIP: a key regulator of colorectal cancer cell death. *Cancer Res,* 67**,** 5754-62.

ZHANG, N., YIN, Y., XU, S. J. & CHEN, W. S. 2008. 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules,* 13**,** 1551-69.

ZHANG, X., JIN, T. G., YANG, H., DEWOLF, W. C., KHOSRAVI-FAR, R. & OLUMI, A. F. 2004. Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res,* 64**,** 7086-91.