2-DEOXYGLUCOSE PRETREATMENT SENSITIZES COLON CANCER CELLS TO CISPLATIN AND KILLER TRAIL INDUCED APOPTOSIS BY MCL-1 DOWNREGULATION

by

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Biological Sciences and Bioengineering, Master Thesis

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Key Words: Warburg effect, glycolysis, metabolism, apoptosis, 2-Deoxyglucose, Mcl-1, mTOR, AMPK.

Abstract

Altered metabolism of cancer cells provides new therapeutic strategies for anticancer treatment. Cancer cells preferentially select glycolysis and lactic acid fermentation for their metabolic requirements, even in the presence of sufficient oxygen. Targeting cancer cell metabolism gave promising results as the treatments are offering therapeutic selectivity in the name of killing cancer cells more effectively while sparing normal cells. Coupling glycolysis inhibition with chemotherapy is one of the new strategies against cancer.

In this thesis, we have studied with colon cancer cell lines HCT 116 WT, p53-/- and Bax-/-. Glycolysis inhibition was achieved with a glucose analog, 2-Deoxyglucose (2-DG) which is at the stage of clinical trials. Cisplatin and TNF-related apoptosis-inducing ligand (Killer TRAIL) were selected as anticancer agents. Pretreatment of 2-DG prior to cisplatin and Killer TRAIL treatment sensitized the cells to apoptotic cell death induced by these anticancer agents and increased cell death numbers. We observed several mechanisms to this sensitization effect. 2-DG sensitizes the cells to cisplatin and Killer TRAIL-induced apoptosis via downregulation of a crucial antiapoptotic protein Mcl-1, belonging to the Bcl-2 protein family. ATP depletion due to inhibition of glycolysis and cell cycle arrest are other contributing mechanisms. 2-DG also caused activation of energy biosensor AMPK and inhibition of cell growth and

protein synthesis regulator mTOR. For a detailed analysis of 2-DG's effects on the gene level, RNA microarray study was conducted. The results of this study harmonized with the data indicated.

KOLON KANSERİ HÜCRELERİNİN 2-DEOXYGLUCOSE İLE ÖN MUAMELESİ HÜCRELERİ SİSPLATİN VE TRAIL'İN SEBEBİYET VERDİĞİ APOPTOZA KARŞI MCL-1'İN PROTEİN SEVİYESİNDEKİ DÜŞÜŞ İLE HASSASLAŞTIRIR

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Anahtar kelimeler: Warburg etkisi, glikoliz, metabolizma, apoptoz, 2-Deoxyglucose, Mcl-1, mTOR, AMPK.

ÖZET

Değişen kanser metabolizması kanser tedavisinde yenilikçi bir terapötik tedavi stratejisi vaad etmektedir. Kanser hücreleri metabolik gereksinimlerini yeterli oksijen altında bile özellikle glikoliz ve laktik asit fermantasyonu yoluyla yaparlar. Kanser metabolizmasını hedeflemek kanser tedavilerinde terapötik bir seçicilik sunarak kanser hücrelerini daha fazla öldürürken normal hücreleri az oranda etkileme fırsatı sunmaktadır. Bu sebeple glikoliz inhibitörlerinin kemoterapi ile birlikte tedavi amaçlı sunulması kansere karşı yeni tedavi stretejilerindendir.

Bu tez çalışmasında kolon karsinoma hücre hatları HCT 116 WT, p53-/- ve Bax-/- kullanılmıştır. Glikolizin önlenmesi için glikoz benzeri bir molekül olan 2-Deoxyglucose kullanılırken, antikanser ajanları olarak sisplatin ve TNF-bağlantılı apoptozu indükleyen TRAIL molekülü seçilmiştir. Hücrelerin sisplatin ve TRAIL ile olan muamelesinden önce bir ön muamele olarak sunulan 2-Deoxyglucose hücreleri bu antikanser ajanlarının sebep olduğu apoptoza karşı hassaslaştırmış ve ölüm oranlarının artmasına sebep olmuştur. Bu duyarlı hale getirme durumu ile ilgili bir takım mekanizmalar belirlenmiştir. Buna göre, 2-Deoxyglucose hücreleri belirlenen antikanser ajanlarının sebep olduğu apoptoza karşı hassaslaştırırmayı Bcl-2 protein ailesinin apoptoz karşıtı önemli bir üyesi olan Mcl-1'in protein düzeyinde düşüşe sebep olarak

başarmaktadır. ATP üretiminde meydana gelen azalma ve hücre döngüsü içinde oluşan G1 fazındaki durma hassaslaştırma etkisine katkıda bulunan diğer mekanizmalardır. Ayrıca, 2-Deoxyglucose biyosensör AMPK'nin aktivasyonuna ve hücre büyümesi ve protein sentezi regülatörü mTOR'un inhibisyonuna sebep olmuştur. Daha detaylı bir bakış için, genlerin ekspresyonlarındaki değişimleri incelemek adına RNA mikroarray çalışması düzenlenmiş ve sonuçlarının deney verileri ile örtüştükleri görülmüştür.

To my dear family,

"While there is life, there is hope."

Cicero

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Last but never least, I want to scream out my love for my family. My mother Itır Kısakürek, my father Şükrü Kısakürek and my two brothers Alp and Arda Kısakürek, you will always be there in the center of my heart and I am doing what I am doing not only for myself but also for you all. I hope I will never disappoint.

Ali Fuat Kısakürek

June 2011, Istanbul

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HCT 116	Human Colon Carcinoma Cell Line
2-DG	2-Deoxyglucose
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
mTOR	Mammalian Target of Rapamycin
HIF-1	Hypoxia Inducible Factor 1
PI3K	Phosphatidylinositol-3 kinase
TRAIL	TNF-related apoptosis inducing ligand
DISC	Death Inducing Signaling Complex
PDK	Pyruvate Dehydrogenase Complex
HK2	Hexokinase 2
LDHA	Lactate Dehydrogenase A
PDH	Pyruvate Dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
SCO2	Synthesis of cytochrome c oxidase 2
TIGAR	TP53-induced glycolysis and apoptosis regulator

1. INTRODUCTION

1.1 The Warburg Effect: Cancer Cell Metabolism

The word ‘metabolism’ is of Greek origin and it basically means ‘a process of change’. In this context, its use is not different considering that we are dealing with the smallest living units, the cells of an organism. The cells in which happens enormous changes each and every second, from the continously active chemical processes to physical ones. The sum of it all keeps the cell or, in a broader sense, the organism alive.

Cellular metabolism has always been an interesting study of biochemical research. Especally if we are talking about cancer cells. In recent years cancer cell metabolism has become the hot topic it once was. Going back to the intial hypotheses on what a cancer cell’s metabolic needs are, metabolic alterations have proven to be crucial and important. Starting with Otto Warburg in 1920s and later with his contemporaries, cancer is linked to altered metabolism (1). However, the promising findings at the time were left in the shadow of the popular, new and complex oncogene revolution. It is ironic that may be it is time for oncogene revolution to take a back seat because the old and relatively easier study of cancer cell metabolism is about to take a hold.

Otto Heinrich Warburg, now remembered as one of the twentieth century's leading biochemists, found that cancer cells undergo a change in their energy metabolism, which is rather different from the usual condition of the normal cells. According to his studies, cancer cells show increased glucose consumption and they prefer fermentation over respiration for their metabolic needs (2, 3). This was very interesting considering these cells going for lactic acid fermentation even under sufficient oxygen and showing higher glucose uptake. As it is about to be explained in details, this crucial metabolic shift from the normal oxidative breakdown to the non-

oxidative breakdown of glucose gives benefits to cancer development and is widely accepted as a phenomenon underlying the most common phenotype of cancer cells (4).

Figure 1. 1 Otto Warburg won the Nobel Prize in 1931 for his studies on cellular metabolism.

This key molecular event, named after Otto Warburg as the 'Warburg Effect', benefits cancer in terms of the following (4): The glycolytic pathway serves as a rich source of carbon precursors for the biosynthesis of macromolecules that are needed as building blocks for a cancer cell dividing rapidly. It helps for a cancer cell's protection and rapid growth, providing an acidic surrounding(lactic acid fermentation) making it easier to invade neighbouring cells. And it eliminates a cancer cell's dependance on oxygen, meaning that the cell can continue to survive under oxygen deficient conditions.

But to realize the significance of the Warburg Effect, we should first understand cellular metabolism i.e. cellular needs such as energy generation and macromolecule biosynthesis. Then we will be able to see the complete picture of how cancer cells differ from normal cells in terms of their metabolic requirements.

1.1.1 Understanding Cellular Metabolism: What A Cell Needs?

Glucose is an important molecule for mammalian cells in terms of its being rich in potential energy thus an excellent fuel and of its serving as a versatile precursor providing metabolic intermediates for biosynthetic reactions (5):

In order to obtain energy from processing glucose, these cells have two options. Either going for aerobic respiration(oxidative phosphorylation) or choosing lactic acid fermentation considering the absence of oxygen. However glucose should first go through ‘glycolysis’, a crucial central pathway of glucose catabolism. Glycolysis can be explained as six-carbon glucose molecule undergoing a series of enzyme-catalyzed processes to give out two three-carbon products named pyruvate (FIG. 1.2). This process gives the cells a net gain of 2 molecules of ATP and the product(pyruvate) is ready for oxidative breakdown to yield more energy. From there on pyruvate gives out 2 molecules of Acetyl-CoA and they enter citric acid cycle (aka Krebs cycle, TCA cycle) to finally produce 4 molecules of both carbon-dioxide and water and overall generating up to 36 ATPs from one complete oxidation of a glucose molecule. That is basically what a mammalian cell does in order to satisfy its energy need in an aerobic condition. However there is also lactic acid fermantation standing as the other option but it is preferred mostly in anaerobic conditions other than some specific cell types such as contracting muscle cells and erythrocytes. But lactic acid fermentation is nowhere near oxidative phosphorylation in terms of providing energy as the only ATP gain comes from glycolysis.

Apart from both of these depending on the presence or absence of oxygen, there is another process called aerobic glycolysis which is like a mixture of both but relying on lactic acid fermentation much more. Therefore most of the pyruvate produced from glycolysis process into lactate leaving very little to enter mitochondria. The overall energy gain is a bit more than the usual lactic acid fermentation, giving out approximately 4 molecules of ATP(6) (FIG. 1.3).

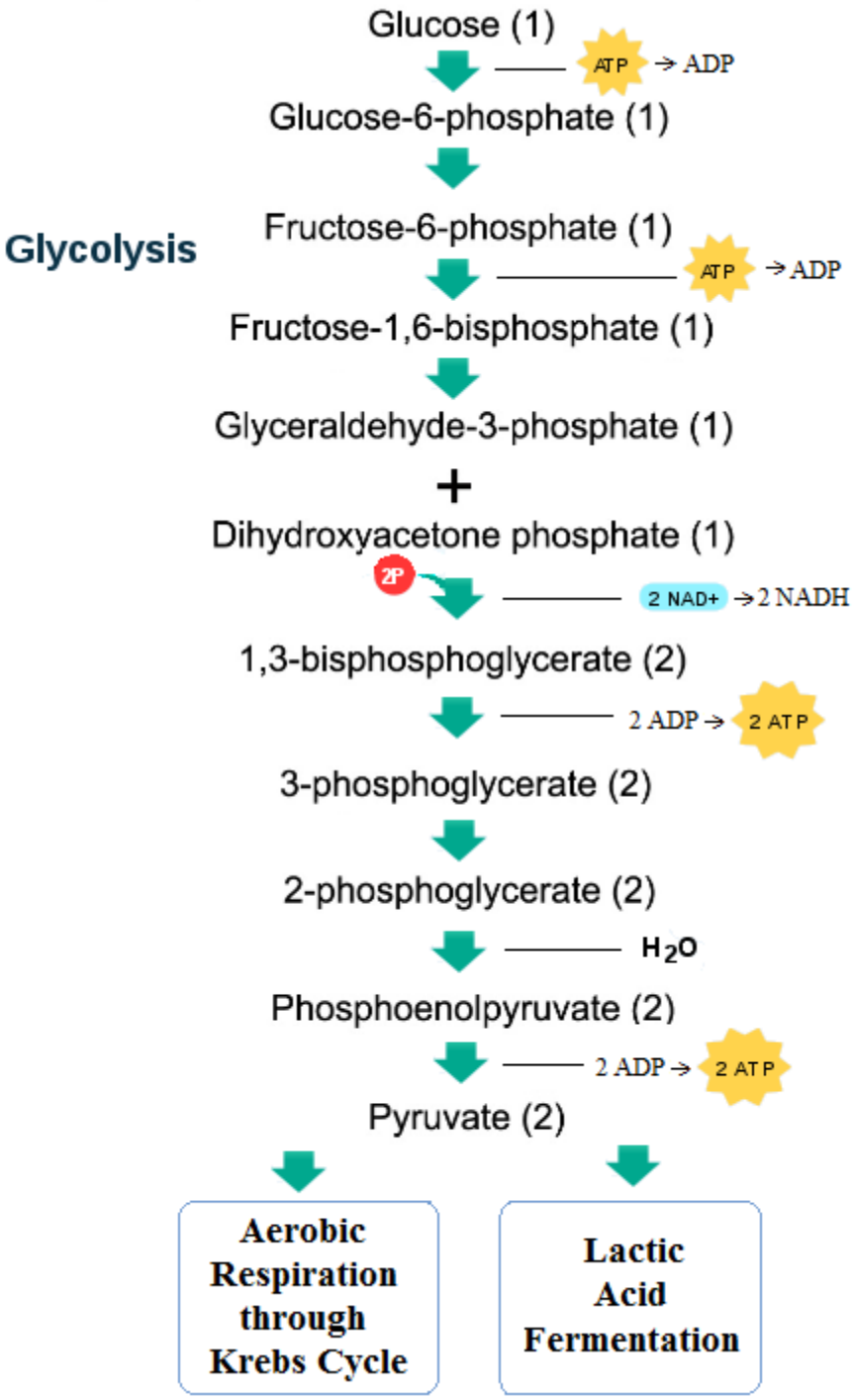


Figure 1. 2 Glycolysis followed by either Aerobic Respiration or Lactic Acid Fermentation

The important thing here is that with or without oxygen the cells undergoing aerobic glycolysis don't necessarily go for oxidative phosphorylation. Yes it gives out far less ATP but is that really a problem? Well, actually it is not. Because the cells choosing this aerobic glycolysis do choose it for some interesting benefits. The ATP

production is never a problem when you have a continuous availability of resources as glucose and nutrients in circulating blood.

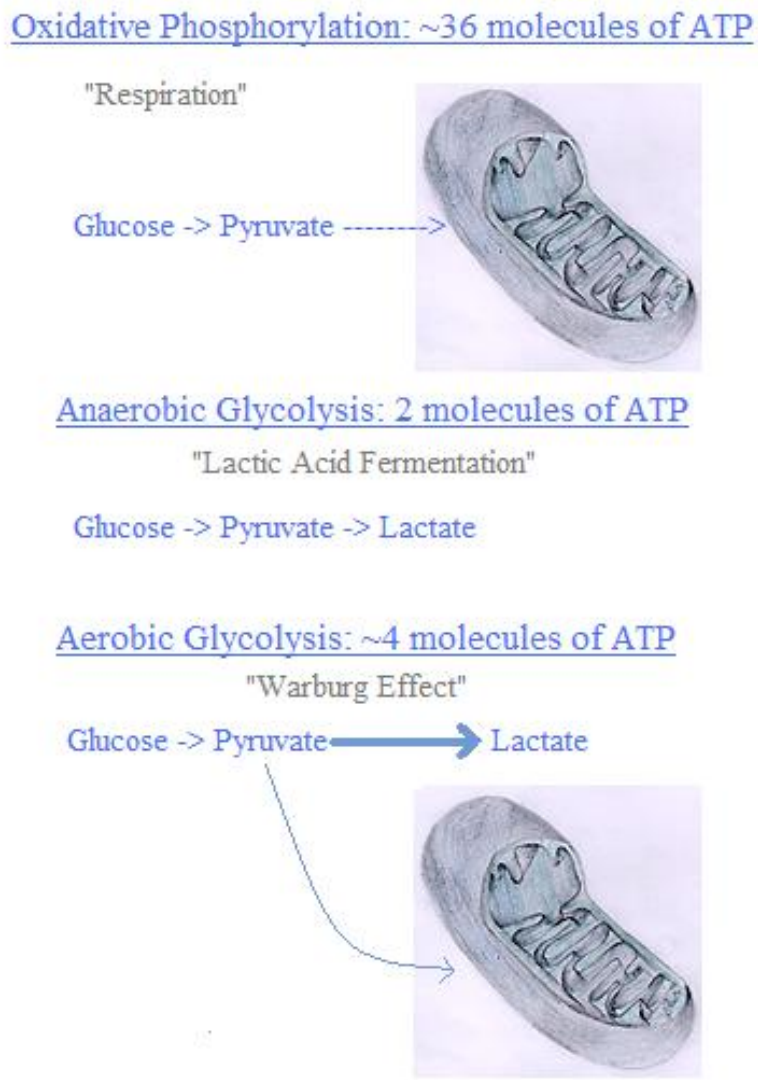


Figure 1. 3 Energy gaining processes: Oxidative Phosphorylation, Anaerobic Glycolysis and Aerobic Glycolysis

Higher glucose uptake and faster glycolysis remedy the deficiency of ATP production and thus the problem is solved. Think of a cell undergoing glycolysis successively in an aggressive manner in order to gain as much ATP as a normal cell easily gains from oxidative phosphorylation.

But what are the benefits of switching to aerobic glycolysis? Why cells choose it? Because they have some significant metabolic requirements i.e. macromolecule biosynthesis for the build up. What we should consider now is that we are now talking

about cells which are proliferating or cancerous as both show this switch and their requirements differ from that of differentiated(non-proliferating) cells. Proliferating cells and cancer cells need rapid cell division and with aerobic glycolysis they incorporate carbon into biomass faster providing useful intermediates for biosynthesis. For example, instead of going through oxidative phosphorylation acetyl-CoA, an important macromolecular precursor, is used for lipid synthesis while some other glycolytic intermediates serve for amino acid and nucleic acid synthesis(6, 7). These cells also use TCA cycle as a center for biosynthesis rather than using it for maximal ATP production, so it is actually the other way around as in TCA cycle they consume ATP(7).

Cancer cells show high resemblance to proliferating cells in terms of metabolic requirements and this is basically because of their needs for rapid division. Cell proliferation is the increase in cell number due to cell growth and division. Mammals require cell proliferation for embryogenesis, growth, proper functioning of tissues and tumorigenesis(7). And for a cell to proliferate it must respond to the needs as the onset of proliferation brings serious changes. The cell enters the cell cycle, undergoing a heavy work of synthesis as they require to double their biomass in order to provide for the two daughter cells. The divergence between the normal proliferating cells and cancer cells comes with genetic alterations and some changes in cellular microenvironment(8). And also the ability of normal proliferating cells to come back to the resting state switching back to an oxidative metabolism unlike cancer cells(9). Therefore, to understand the reasons behind cancer cell metabolism and to think over the possible biological causes, it is best to go over the mechanistic perspectives of the Warburg effect.

1.2 The Mechanistic Perspectives of The Warburg Effect

At this point we know more or less what basically the Warburg Effect is. However, to go deeper in the understanding of it and to realize the exact reasons behind this phenomenon we need to elaborate on the mechanistic perspectives that give us information on the potential causes as to why cancer cells go for increased glycolysis. The perspectives can be explained under four different subtitles having crucial aspects

about their possible contributions to the Warburg effect as for each there are evidences sustaining them(10)(FIG. 1.4).

Figure 1. 4 Mechanistic perspectives of the Warburg effect.

1.2.1 Hypoxia

Hypoxia is a condition of having low levels of oxygen, often too low holding a threat against the proper functioning of cellular metabolism. Normally, cells rely on oxidative phosphorylation to fulfill their energy needs but when there is inadequate amounts of available oxygen they switch to the glycolytic phenotype that is basing their metabolic reqirements on glycolysis(11).

With studies like Warburg's research(2) on alteration of cellular metabolism independent of oxygen levels and Thomlinson and Gray's observation(12) of hypoxia in human lung cancer cells, hypoxia is mentioned among one of the special attributes of these cells. But what exactly is the reason behind the formation of hypoxic regions? Tumors' rapid expansion culminate in poorly formed tumor vasculature(13, 14). The vascular system of an organism is responsible for the transportation of oxygen and nutrients to the tissues, but in this context the blood vessels around tumor show aberrant

function(15, 16). This causes a selection among cells, distinguishing the ones that are able to respond and adapt to this condition. This is why tumor cells change their metabolism(Warburg Effect) to fit into the new environment, to survive in the new circumstances. This adaptation occurs with the activity of hypoxia-inducible-factor 1 (HIF1), a transcription factor controlling many other hypoxia related genes; stimulating the transcription of glycolytic enzymes, glucose transporters, survival and growth factors and so on(17).

1.2.2 Tumor Microenvironment

The metabolic microenvironment of tumors comes up with another characteristic along with hypoxia, which is lactic acidosis due to the upregulation of glycolysis. Lactic acid fermentation provides the acidic environment as these cells produce and transport it out causing an acidic extracellular condition(4). The low pH values contribute to Warburg effect in the sense that it gives cancer cells an immense growth advantage promoting their proliferation and invasion, and that might express why these cells alter their metabolism to benefit from this. Also lactate might play a role as a signaling molecule setting up a substructure for the alteration of metabolism(18). With the inductions of various cellular stress responses, adapting to these harsh environments further contributes to the distorted metabolic phenotype(1).

1.2.3 Defects in Mitochondria

Mitochondria are membrane enclosed organelles responsible for the production of power for eukaryotic cells. As the energy centers, the powerhouses, mitochondria are essential. The possible problems occuring in them can cause serious issues and major changes. In this sense, mitochondrial defects can play an important role in switching to glycolytic phenotype. It was Warburg again in the early 20th century proposing that cancer cells have defects in mitochondria, as he related that the giving up on oxidative phospshorylation might mean some kind of a complication(19, 20, 21). The probable

complications causing a defective mitochondria include mitochondrial DNA mutations due to free radicals; decreased efficiency of TCA cycle because of inappropriate intermediates; enzymatic dysfunctions and the decomposition of mitochondrial membrane(22).

This idea, overall, was rejected in the beginning and used against Warburg following the publications of his results(9): In 1956, Weinhouse rejected the possibility of a permanent respiratory problem(23) basing his arguments onto their finding of the ability to increase the OXPHOS activity of neoplasias when supplemented with NAD(24). Supporting Weinhouse results, in 2004 Russignol et al. showed that OXPHOS activity can be increased in cancer cells ruling out the discussions of irreversible impairment(25). However, some recent articles state mitochondrial defects(being not necessarily an irreversible impairment) as a potential reason behind cancer cells' increased glycolytic rate with the idea of mitochondrial dysfunction due to high levels of ROS production(26), mitochondrial DNA mutations(27) and uncoupling(28).

1.2.4 Oncogenic Signals

Another significant mechanistic perspective of the Warburg effect is the oncogenic signals that are driving the changes in metabolism, directly controlling it through signaling pathways involving known oncogenes and tumor suppressor genes(6) (FIG. 1.5).

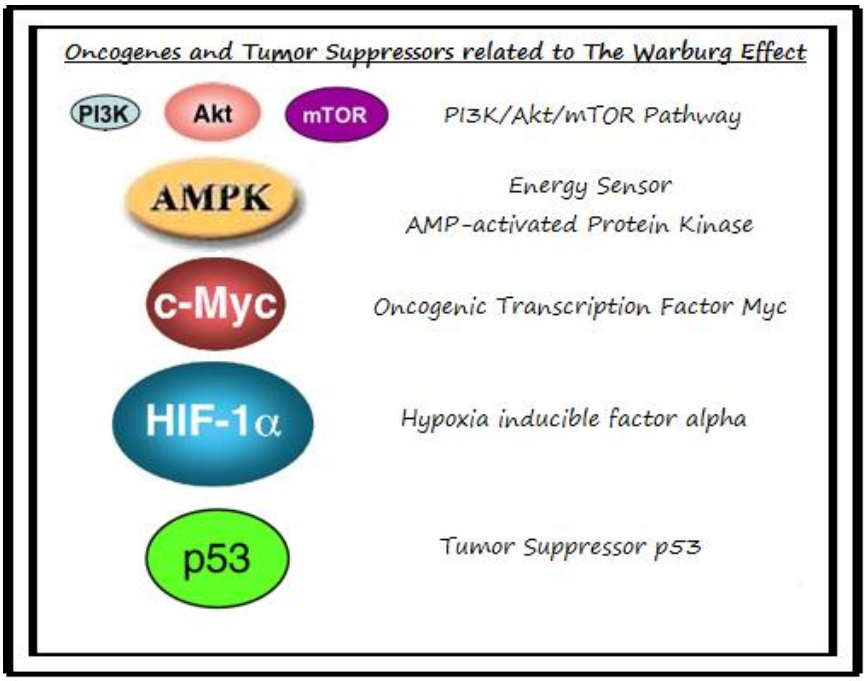


Figure 1. 5 List of some known oncogenes and tumor suppressor genes related to The Warburg Effect

Oncogenes of the PI3K/Akt/mTOR pathway, c-Myc and hypoxia inducible factors; tumor suppressors AMP-activated protein kinase and p53 are among the important genes having their names put on the map of the Warburg effect. These genes have significant effects over cellular metabolism, growth and proliferation. The next chapter, Molecular Mechanisms driving the Warburg Effect, will put the emphasis on three of these genes being mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and tumor suppressor p53.

1.3 Molecular Mechanisms driving The Warburg Effect

With the recent advances in genomics and proteomics, it is now easier to look into molecular mechanisms that contribute to the Warburg effect and tumorigenesis. The point being that the provided molecular insights enlighten us about the many aspects of the Warburg effect and how to use it against the cancer itself for cancer therapy(29).

1.3.1 AMPK: Adenosine monophosphate (AMP)-activated protein kinase

AMPK is a serine/threonine protein kinase getting activated by cellular stress condi-tions resulting in the depletion of ATP. Under the conditions of increased AMP/ATP ratio, AMPK responds to prevent ATP consuming pathways and activate the ones that are responsible for ATP generation(30). The importance of AMPK comes from the fact that it is found in all eukaryotes and it senses the cellular energy status regulating the energy balance. Being highly conserved as a heterodimer, it comprises a catalytic α subunit and regulatory β and γ subunits, apart from the structural information of the AMPK protein these also give us the understanding of its current model for activation(31, 32): with the decrease in ATP levels and increase in AMP, AMP goes and binds to nucleotide binding domains in the AMPK γ subunit eventuating in a conformational change in the heterodimer revealing the activation loop of the catalytic α subunit. Phosphorylation of a critical threonine in this loop does the job needed for the activation of AMPK.

The AMPK activity is linked to tumor progression with the understanding of its controlling some processes standing in connection to development of tumors, cell growth, proliferation and protein translation(33). As a low energy checkpoint, tumor cells have to overcome this to achieve proliferation in response to activated growth signaling pathways. Most of the time with oncogenic mutations and with other signaling pathways many cancer cells manage to hold back AMPK signaling. Alteration of metabolism is closely related to AMPK activity as the loss of it results in the activation of mTOR and HIF1, helping for the switch into the glycolytic phenotype and the proliferating status(1). AMPK itself appears to have a role also in protein synthesis as it phosphorylates and inactivates eEF2 kinase which in turn causes an inhibition of protein synthesis(34).

With the recent growing interest in AMPK signaling and the idea to use it against tumor progression, we have started to see the term ‘AMPK activation’ among cancer related articles dealing with Warburg effect and cancer cell metabolism(35, 36).

1.3.2 Mammalian Target of Rapamycin (mTOR) Pathway

Playing a key regulatory role in cell growth, proliferation and differentiation, mammalian target of rapamycin (mTOR) has been investigated intensely for its effects in tumor development and progression(37). As an evolutionarily conserved serine threonine kinase and a part of a crucial signaling ensemble involving insulin like growth factor-I receptor (IGFR), phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt/PKB) cons-tituting the IGFR-PI3K-Akt-mTOR signaling pathway, mTOR has been shown abnormally activated in various transformed cells and human tumors(38, 39). The dysregulation of mTOR signaling causes a favorable oncogenic environment for cancer and the activation of mTOR brings about a series of downstream consequences helping for the metabolic reprogramming.

mTOR's most distinguished functions are its controlling of protein synthesis at several levels and its enhancing of ribosome biogenesis. It phosphorylates and activates ribosomal kinase p70 S6K and this in turn activates some downstream targets to initiate translation. mTOR also phosphorylates eukaryotic initiation factor 4E binding proteins (4E-BPs) causing their release from eukaryotic initiation factor 4E (eIF4E) making space for translation initiation factors to bind and stimulate translation properly(37, 40) (FIG. 1.6). Thinking about a possible abnormal functioning such as a highly active mTOR, an increase in protein synthesis overall gives us a clue as to how mTOR pathway favor anabolic cell growth and proliferation in tumor cells(41).

Another feature of the mTOR pathway is that it promotes the expression of genes supporting the glycolytic phenotype, those genes being HIF1 and c-Myc mainly(6, 42, 43). This also gives an idea about mTOR's role in cancer development showing of decisive importance in many aspects.

mTOR, with all these significant activities, holds a great promise for anti-cancer mechanisms and cancer therapy. mTOR inhibitors have been used as therapeutic agents against cancer. Rapamycin is a well known mTOR inhibitor having the mechanism for cancer therapy as it's causing an inhibition of protein sythesis to an extent(~5% reduction) and resulting in G1 cell cycle arrest and leading cells to apoptosis(38). However, recent clinical updates state that rapamycin is effective over only a few cancers and the therapeutic response to it is highly variable(44).

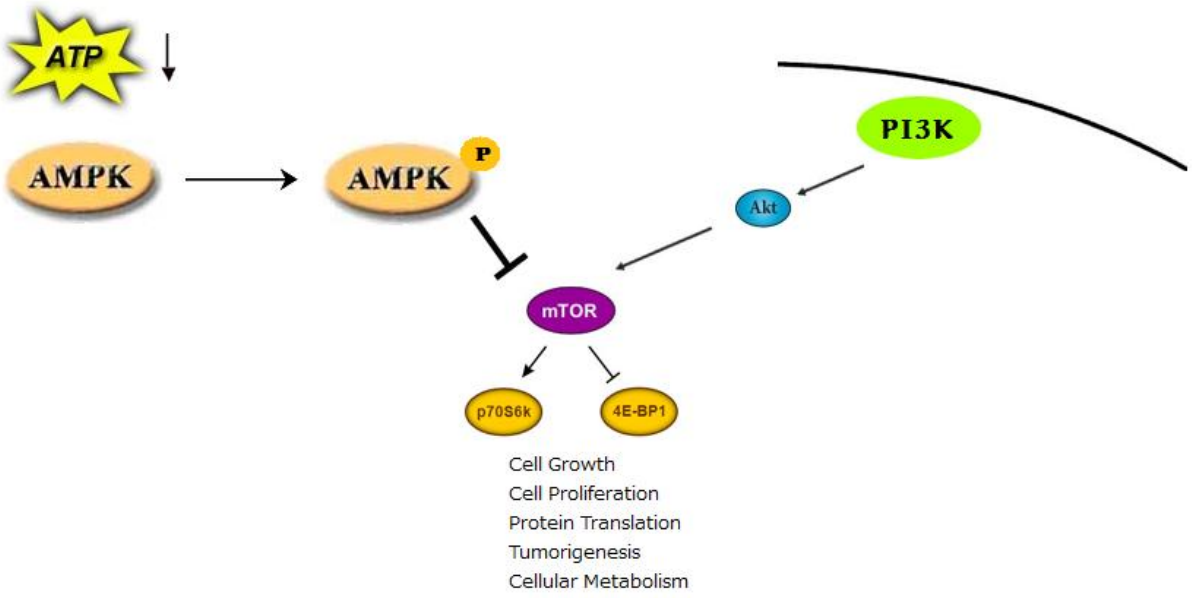


Figure 1. 6 AMPK-mTOR relation and mTOR Pathway (simplified)

1.3.3 Tumor Suppressor p53

Known as the 'Guardian of the Genome', p53 is a highly important tumor suppressor having critical roles in cellular metabolism, differentiation, cell cycle control and apoptosis. Inactivation of the p53 tumor suppressor pathway is one of the most common features detected in cancer considering the responses provoked by p53 such as inhibition of cell growth, cell cycle arrest and cell death in order to prevent tumor formation(45). However, recently its role in cell metabolism has gained a renewed interest and studies provide interesting findings about the energy generating metabolic pathways and their regulation by p53 (FIG. 1.7). This of course revealed p53's importance in terms of the Warburg effect(46).

TP53-induced glycolysis and apoptosis regulator (**TIGAR**) is one of the proteins involving in glycolysis and being regulated by p53(47, 48). In a 2006 dated paper, Vousden and his colleagues showed that p53-induced TIGAR expression causes a decrease in fructose-2,6-biphosphate levels inhibiting glycolysis and regulates apoptosis in a cell-type dependent manner. This is actually consistent with recent studies stating that the modulation of glycolytic rates can have a profound effect on the apoptotic sensitivity of cells, especially cancer cells(6).

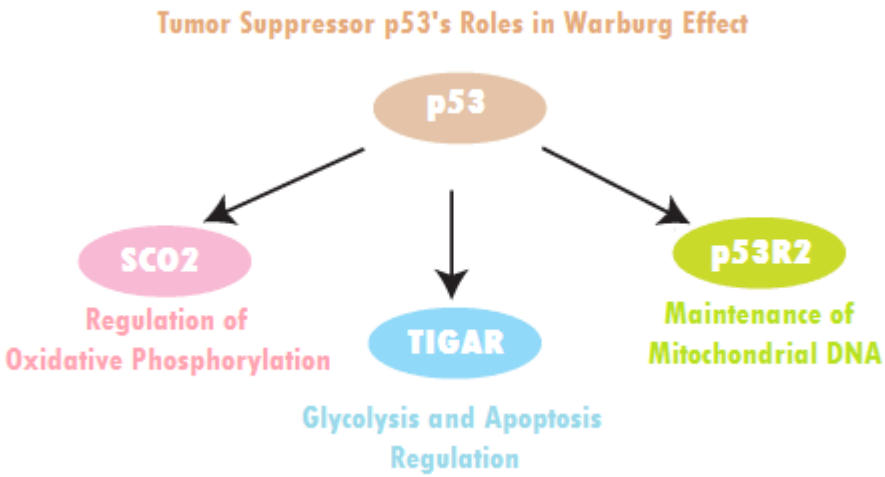


Figure 1. 7 p53 and Metabolism

Another insight into p53's effect on the mode of energy production comes with its regulation of an important assembly protein encoded by the 'synthesis of cytochrome c oxidase 2 (**SCO2**)' gene(49). SCO2 is required for the assembly of the cytochrome c oxidase (COX) complex inside the inner mitochondrial membrane that is the major site of oxygen use in mammalian cells. p53 regulates aerobic respiration through SCO2 with this controlling mechanism inside mitochondria(50, 51). Another mitochondrial control mechanism of p53 is the ribonucleotide reductase subunit **p53R2**. p53 regulates its expression whose responsibility is the maintenance of mitochondrial DNA and a possible loss causes a decrease in mitochondrial DNA and leads to dysfunction(52).

p53 also has a regulatory role along with HIF-1 over hexokinases, especially the isoform HK2 expressed in cancer cells. HK2 is an enzyme, a primary initiator of glycolysis phosphorylating glucose to glucose-6-phosphate thereby starting the glycolytic process(53).

Altogether p53 works as a tumor suppressor by all means, from its negative regulation of glycolysis to its induction of apoptosis for the elimination of cancer cells. However, dominating a huge network of various signaling pathways, p53 signaling is not yet clear. It has a contradicting nature giving out results like enhancing apoptosis in a condition and working against it in another, hindering glycolysis but also enhancing some of its steps and so on. p53 definitely deserves to be called the 'guardian'. For this matter, especially in cancer cells, loss of p53 signaling initially serves for the metabolic change driving the Warburg effect, however p53 is helping them for undergoing the

metabolic adaptation as well. Maddocks and Vousden described the situation as a 'double edged sword' meaning that p53 deficiency may also sensitise tumor cells to metabolic stress(54). A study in 2008 provided an interesting aspect as the wild-type p53 in breast cancer cells induces cell cycle arrest and therefore protecting tumor cells from cytotoxic damages which overall leads to reduced therapeutic response and poor prognosis(55).

1.4 Targeting The Warburg Effect

Targeted therapeutics directed against cancer mostly rely on killing cancer cells with nonsurgical methods of cancer treatment such as radiation therapy and chemotherapy. The common problem in these treatments is that most of these anticancer agents do not have specificity for cancer cells as they also kill healthy and normal cells along the way, even in the radiation therapy where a degree of specificity is achieved through with localizing the radiation to the tumor(56).

With the recent studies on targeting cancer aiming for improved effectiveness, tumor physiology gained a lot of attention with the idea of exploiting the cellular and molecular differences between cancerous and normal cells and to use it for an alternative, selective cancer treatment(56, 50). Targeting the Warburg effect as an anticancer strategy suggest several potential therapeutic avenues. Glycolysis inhibition, especially, holds great promise as targeting the seventh hallmark of cancer(53), glycolysis, is also one of cancers' most vulnerable phenotypes.

1.4.1 Glycolysis Inhibition for Anticancer Treatment

Glycolysis inhibition is one important targeting mechanism against cancer metabolism. There are two mechanisms considering the glycolytic phenotype: Either targeting upstream regulators being HIF, PI3K, Akt, mTOR and AMPK or targeting the glycolytic pathway, the key metabolic enzymes critical for bioenergetic supply(57). The idea behind glycolysis inhibition is to use it against cancer while sparing normal tissues

because cancer cells show high glucose uptake and a huge increase in glycolysis. Here are some of the glycolytic enzymes as potential therapeutic targets(58, 59)(FIG. 1.8):

Hexokinase is a mitochondria associated enzyme of the first rate limiting step of glycolysis, phosphorylating glucose to glucose-6-phosphate. Among the four isoforms (Hexokinase I-IV), Hexokinase II is overexpressed in many cancer cells, playing the role in initiating and maintaining the high glycolytic rates(60, 61). HK2 might also be having a role in apoptosis regulation, along with its enzymatic activity(62). Robey and Hay, in their 2006 dated paper, claimed mitochondrial hexokinases as novel mediators of apoptosis stating their anti apoptotic properties(63). These features of HK2 make it an attractive target. Three known compounds used against HKs are 2-Deoxyglucose, 3-Bromopyruvate and Lonidamine: 2-Deoxyglucose (will be covered in details shortly) inhibits phosphorylation of glucose by HK,: 3-Bromopyruvate is an alkylating agent and it inhibits HK; Lonidamine works against glycolysis by dissociating HK from mitochondria thus inhibiting both glycolysis and mitochondrial respiration(59).

6-Phosphofructo-1-kinase (PFK) converts fructose-6-phosphate to fructose-1,6-bisphosphate. Four enzymes from PFKFB (1-4) family are responsible for this conversion. Especially PFKFB3 is a target of HIF-1, promoting glycolysis. One study showed that the inhibition of PFKFB3 resulted in suppression of glycolysis and tumor growth(64).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the sixth step of glycolysis. Arsenic is a known inhibitor. Recently GAPDH has been implicated in the initiation of apoptosis(65). This enzyme can be a crucial target like HK2, considering its roles in both glycolysis and apoptosis.

Pyruvate kinase is another important enzyme of the glycolytic pathway, producing pyruvate, the end product of glycolysis. This enzyme is known to be regulated by HIF-1 and Myc, especially the splicing variant PKM2 has been linked to cancer metabolism contributing to the Warburg effect(58).

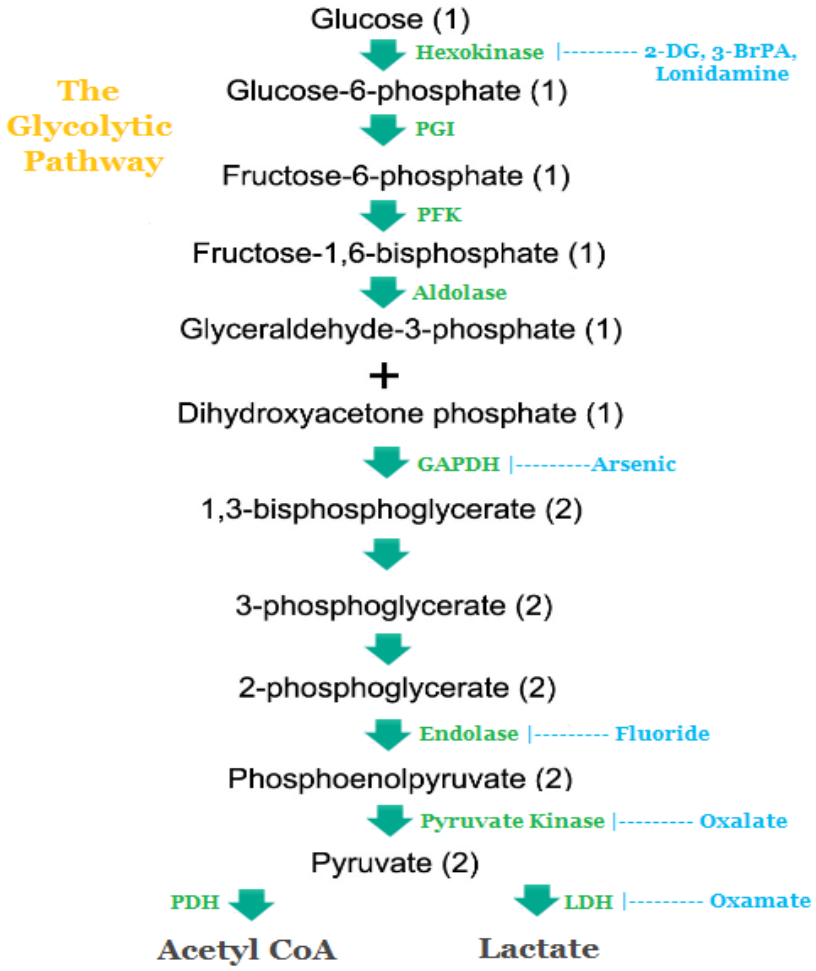


Figure 1. 8 Targeting the Glycolytic Pathway

Another target gene of HIF-1 and Myc is lactate dehydrogenase-A (LDHA) being the enzyme responsible for the conversion of pyruvate to lactate. Oxamate is used as a metabolic inhibitor against it. LDHA has been shown to be crucial for tumorigenesis with several studies, knowing its significance in lactic acid fermentation thus altered cancer metabolism(66). And two recent studies in 2006 and 2010 respectively stated that LDHA inhibiton causes oxidative stress and restrains tumor progression(67) and knockdown of LDHA severely diminishes tumorigenicity(68).

Pyruvate dehydrogenase (PDH) is the enzyme at the decision step whether the cell goes for oxidative phoshorylation or lactic acid fermentation because it serves for the conversion of pyruvate to acetyl CoA and therefore opening the way for oxidative phosphorylation. However, pyruvate dehydrogenase kinase (PDK) have the ability to inactivate PDH, empeding the progress of acetyl CoA production and supporting lactate formation. Inhibiting PDK can be effective for anticancer treatment(58).

1.4.1.1 Glycolysis inhibition by 2-Deoxyglucose (2-DG)

2-DG is an anti metabolic glucose with the difference of having a hydrogen instead of a hydroxyl group at the carbon in the second position. This rare, natural monosaccharide is known to inhibit glucose metabolism via acting as a competitive inhibitor. Once it gets inside the cells, it undergoes phosphorylation by hexokinase forming 2-DG-P which can not be metabolized in the next steps(59).

2-DG has come into prominence with every study from various prospects examining the physiological and pharmacological effects. Starting as a low glucose mimetic it has now been regarded as a promising anticancer and antiviral therapeutic. 2-DG earned its reputation as a possible anticancer compound with its inhibitory effect over glucose metabolism causing a mild depletion of ATP and its giving rise to problems in protein synthesis by disrupting protein glycosylation and ER quality control(69). 2-DG's effect on protein glycosylation is not well examined, only a couple of studies are there for this topic and they explain that 2-DG causes accumulation of misfolded proteins in ER and induces ER stress response(70, 71).

Glycolysis inhibition by 2-DG in tumor cells gave results such as decrease in ATP levels, cell cycle block and cell death according to Maher et al.(72). Zhu et al. contributes to this by indicating 2-DG induction of a dose- and time-dependent reduction in cell growth and energy levels *in vitro*(73). However, administration of 2-DG alone, even though high doses up to 250mg/kg appears safe for use(74), actually do not show significant anticancer activity *in vivo*. Its cytotoxic effects are varied among different tumor cell lines and there is also this fact that its effectiveness decreases in the presence of glucose leading to a partial inhibition (75, 59). Altogether, 2-DG does not lose any significance because its combination with chemotherapy and radiotherapy comes into the picture.

1.4.1.2 Combination of glycolytic inhibition and other anticancer agents

Even though 2-DG monotherapy is not that effective against tumor growth, its combination with chemotherapy and radiotherapy gave successful results in terms of

sensitization of cancer cells to anticancer agents(76). 2-DG treatment studies have given results announcing chemosensitizing and cytotoxic effects of this treatment as well as causing potentiation of chemotherapeutic drugs such as etoposide, ellipticine, cisplatin, carboplatin, 5-fluorouracil, doxorubicin, herceptin and cyclophosphamide(77, 78, 79). 2-DG also showed radiosensitization effect in a couple of studies(80, 81), and its combination with apoptosis inducers(82, 83) and with some other glycolytic inhibitors at once offer potential cancer treatments(84, 85). However, the actual network of this sensitization effect, and how exactly 2-DG potentiates these anticancer drugs is still not well known and in need of further examination and confirmation despite some recent studies(86, 87). 2-DG is now in clinical trials.

1.5 Apoptosis, as an Anticancer Strategy

In the context of cancer prevention and control, apoptosis is an essential cellular response happening constantly in mammals, killing and replacing damaged and harmful cells each second. It is vital to have a proper apoptotic signaling against aberrant cell proliferation and accumulation of genetic defects which undoubtedly provoke tumorigenesis(88).

Apoptosis occurs through three phases(89): First is the induction phase in which a signal is originated either extracellularly or intracellularly initiating the death of the cell. Extracellular signals can be toxins, growth factors, hormones or any other ligand binding to cell surface death receptors; whereas intracellular triggering of apoptosis is achieved when there is a stress condition such as DNA damage, hypoxia, nutrient deprivation, heat etc. Following the initiation, the effector phase takes place as the cell receives the signal and prepares to take action. In this phase, regulators (such as Bcl-2 proten family) and effectors (caspases) of apoptosis get to work to adjust the apoptotic signaling mechanisms. Then comes the third and final phase witnessing the degradation of the cell, undergoing shrinking, membrane blebbing, organelle relocalization and chromatin condensation ending up with the formation of apoptotic bodies, finishing the job.

As an anticancer stragetegy the majority of chemotherapeutic agents as well as radiation utilize the apoptotic pathway to kill cancer cells. To understand the basic

mechanisms, we will now look at the extrinsic and intrinsic apoptotic pathways and cover the regulators and effectors of apoptosis(FIG. 1.9).

1.5.1 Extrinsic and Intrinsic Apoptotic Pathways

The extrinsic apoptotic pathway begins with the activation of death receptors on the cell surface. Upon the binding of a death inducing ligand to its receptor such as TNF to TNF receptor 1, FAS ligand to FAS receptor, TNF-related apoptosis inducing ligand (TRAIL) to TRAIL receptors 1 and 2, there happens a conformational change in the intracellular domains of the receptors revealing the death domain which serves for the recruitment of a varity of apoptotic proteins to the receptor and the formation of a complex called Death Inducing Signaling Complex (DISC). DISC is responsible for the activation of caspase 8, initiating the caspase cascade(88).

The intrinsic apoptotic pathway, on the other hand, is induced by the integration and propagation of death signals originating from inside the cell such as DNA damage, hypoxia, oxidative stress, starvation and so on. Following the stress condition, mitochondria plays the leading role in the process as it causes the release of proapoptotic proteins into the cytosol. The electron carrier protein cytochrome c initiates the events once it gets into the cytosol upon the disruption of the mitochondrial outer membrane, then it goes and binds to an adaptor protein named Apaf-1, activating it. Activated Apaf-1 opens the way for procaspase activation by binding to procaspase 9 leading to the caspase cascade(90).

Both pathways use caspase family proteins as effectors and there are also intracellular regulators of these cell death programs, most importantly, Bcl-2 protein family members.

1.5.1.1 Caspases

The caspases are a group of enzymes known as cysteine proteases. They are the main executors of the apoptotic process. In the normal conditions they exist within the cell as inactive pro-forms, but upon apoptosis induction procaspases get cleaved to

bring out the active enzymes. Their active forms mediate apoptosis by an intracellular proteolytic cascade, cleaving several key proteins in the cell one by one so that the cell start to break down(88, 91).

Caspases divide into two classes: initiator caspases include procaspases 2, 8, 9, 10 while the executioner caspases consist of procaspases 3, 6 ,7. Initiator caspases are the ones that get activated first, then they cleave the executioner caspases to further promote apoptotic signaling(92).

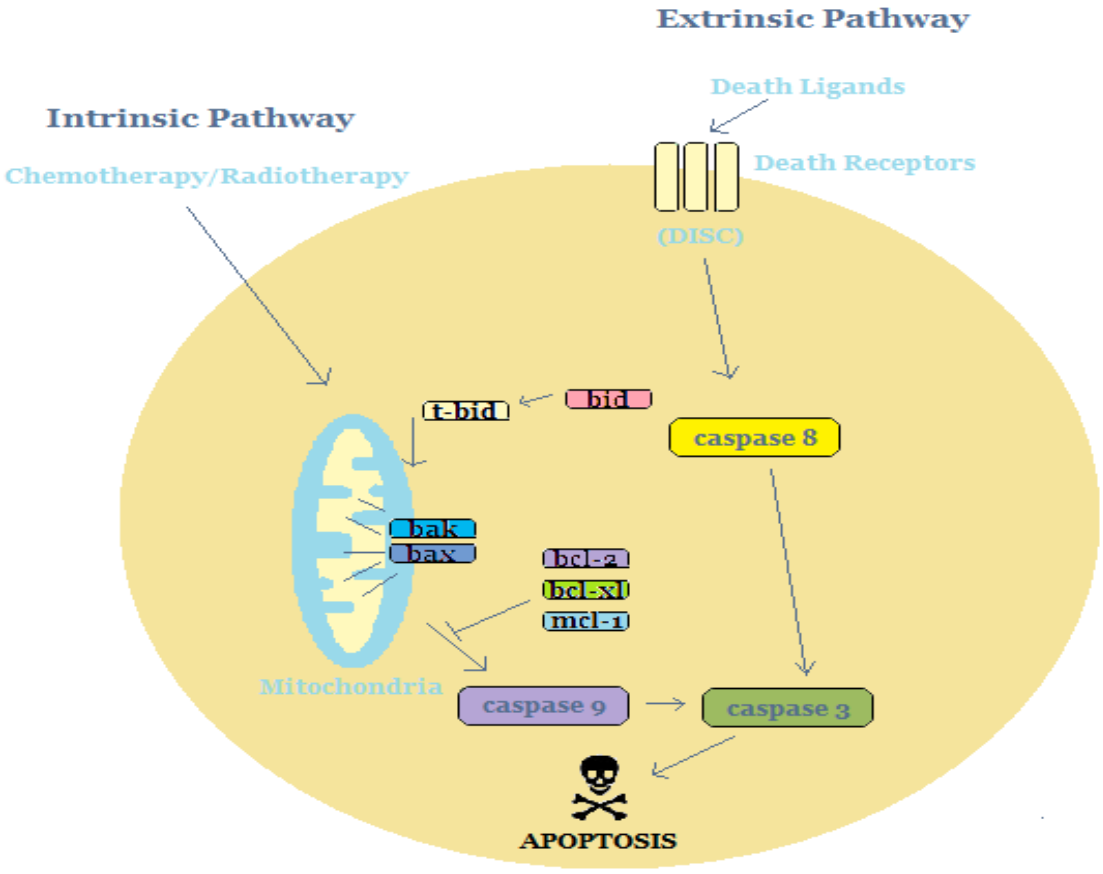


Figure 1. 9 Pathways of Apoptosis: Effector Caspases and Regulator Bcl-2 Family

1.5.1.2 Bcl-2 protein family

As the intracellular regulators of apoptosis, Bcl-2 protein family has always been approached with great interest. Having several members with different critical roles make this protein family an indespensable study in the understanding of apoptotic mechanisms. The family divides into two groups considering their contribution to

apoptosis: they are either antiapoptotic or proapoptotic. The antiapoptotic members are Bcl-2, Bcl-XL and Mcl-1, whereas Bax, Bak and Bok belong to the proapoptotic group along with Bid, Bim, Bad, Noxa, Puma and several others up to 30 known relatives(93, 94).

There are hundreds and thousands of articles on the members of Bcl-2 protein family, examining their roles and how they affect apoptotic signaling pathways alone or working together. They achieve their regulatory roles by controlling caspase activation and maintain mitochondrial integrity(95, 96). Proapoptotic members mostly rely on either (like Bad) binding to and inactivating antiapoptotic ones(97, 98) or (like Bax and Bak) by provoking the permeabilization of mitochondrial membrane and stimulating the cytochrome c release(99). Bid is another proapoptotic member of this family taking charge in between extrinsic and intrinsic pathways and it causes their overlapping as it gets truncated by caspase 8, translocates to the mitochondria and helps Bax/Bak for mitochondrial membrane permeabilization(94).

The prosurvival members Bcl-2, Bcl-XL and Mcl-1 inhibit apoptosis by blocking the release of apoptotic proteins from the mitochondria(100, 101). However, it is Mcl-1 having interesting features that outshine other antiapoptotic members, making it ‘an essential survival protein’ among them(102). Most importantly, it is capable of being induced upon proliferation and differentiation and it shares structural properties with some crucial cell cycle proteins such as cyclin D1, E, G2 and c-Fos unlike other Bcl-2 proteins. With these special attributes, Mcl-1 is able to regulate both apoptosis and cell cycle progression(103).

2. PURPOSE

Cancer is a class of diseases characterized by abnormal cell growth and division. It is a leading cause of death and according to World Health Organization it caused 7.6 million deaths in 2008 stating that it accounted for around 13% of all deaths(104).

Colon cancer is among the most commonly diagnosed cancers. We selected colon carcinoma cell lines HCT 116 WT, p53 -/- and Bax -/- for this study. For the effectively killing of these colon cancer cells, we aim to target their glycolytic metabolism and couple glycolysis inhibition to cisplatin and Killer TRAIL treatment. Our purpose is to sensitize the cells to apoptosis induced by these agents and potentiate their effects on colon cancer cells. Also we want to provide a mechanistic understanding of this sensitization effect: how it happens, which pathways it activates, what changes does it cause at protein and gene level, how does it affect apoptotic signaling, cell growth and proliferation signals and so on.

3. MATERIALS

3.1 Chemicals

Chemicals used are listed in Appendix A.

3.2 Antibodies

Antibodies used are listed in Appendix B.

3.3 Molecular Biology Kits and Cell Culture Materials

Molecular biology kits which are used for determination of cell proliferation, measurement of ATP levels, immunoblotting experiments, plasmid isolation and gene transfection are listed in Appendix C. Appendix C also includes specialty materials such as the used protein marker and plasmid.

3.4 Equipment

Equipment used for general procedures in the laboratory is listed in Appendix E.

3.5 Buffers and Solutions

3.5.1 Buffers and Solutions for FACS Analysis

Annexin V-FITC incubation buffer: 10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$ were dissolved in 500 ml of ddH_2O. The buffer is stored at 4°C.

3.5.2 Buffers and Solutions for Total Protein Isolation

Cell Lysis Buffer: 150 mM NaCl, 1% NP-40 and 50 mM Tris dissolved in ddH_2O; afterwards pH is adjusted to 8.0 by using 5M HCl solution. The buffer was stored at 40C.

Complete cell lysis buffer: Prior to protein isolation, complete lysis buffer is prepared by adding 1X protease inhibitors, 1X phosphatase inhibitors and 0.5 M PMSF freshly to cell lysis buffer.

10X PBS (Phosphate Buffered Saline): 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 were dissolved in 1L of ddH_2O and pH is adjusted to 7.4.

3.5.3 Buffers and Solutions for SDS polyacrylamide gel electrophoresis

1.5 M Tris-HCl pH 8.8: 1.5 M Tris was dissolved in ddH_2O and pH was adjusted at 8.8 with HCl.

0.5M Tris-HCl pH 6.8: 0.5M Tris was dissolved in ddH_2O and pH was adjusted at 6.8 with HCl.

3.5.4 Buffers and Solutions for Western Blotting

10X Running Buffer: 30.3 g Tris, 144.1 g Glycine, 10 g SDS were dissolved in 1L of ddH_2O and pH was adjusted at 8.3.

10X Transfer Buffer (TB): 30.3 g Tris and 144 g Glycine were dissolved in 1L of ddH2O.

1X Transfer Buffer: Before conducting transfer step, 1X TB is freshly prepared. 20% (v/v) methanol was added into 1X TB and the remaining volume was completed with ddH2O.

10X PBS (Phosphate Buffered Saline): 80 g NaCl, 2.0 g KCl, 14.4 g Na2HPO4 and 2.4 g KH2PO4 were dissolved in 1L of ddH2O and pH is adjusted to 7.4.

1X PBS-Tween20 (PBS-T): 10X PBS was diluted to 1X, 0.2% Tween20 was added to 1X PBS.

Blocking Solution: 0.05% (w/v) dried milk powder was dissolved in 1X PBS-T.

3.5.5 Buffers and Solutions for Cell Cycle Analysis

PI incubation buffer: 2 ml PBS and 12 µl TritonX100 were mixed. For ten samples, 1 ml was taken to which 10 µl RNase and 20 µl PI solution were added. The mixture is distributed equally (~100 µl to each) to the samples before incubation. No dye sample only has RNase in the incubation buffer.

4. METHODS

4.1 Cell Culture

HCT116 WT, p53 -/- and Bax -/- cells were cultured in McCoy's 5A Modified Medium (Modified) with L-glutamine added with %10 fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/ml). Cultures were kept in the incubator at 37°C and 5% CO_2. T-75 flasks, 6-well culture plates, 12-well culture plates and 96-well plates were used for the seeding of the cells. Cell splitting by trypsin was done after the cells' reaching to a considerable confluency (~80%). Cells are counted with hemacytometer according to the following formula:

$$Cell\frac{number}{ml} = Average\ count\ per\ quare \times Dilution\ factor \times 10^4$$

And the split cells were passaged to culture plates and flasks, taking the appropraite seeding densities(Table 4.1) of each into account.

Dimension	Seeding Density	Medium
6-Well	0.3×10^6	3 ml
12-Well	0.1×10^6	1 ml
96-Well	1×10^4	100 µl
T-75 Flask	2×10^6	15 ml

Figure 4. 1 Cell Seeding Density for Culture Plates and Flasks

For cryopreservation, cells were trypsinized and resuspended in complete medium containing 10% heat-inactivated FBS and 10% DMSO. The cell suspension in freezing medium was transferred into cryovials, frozen at -80 º C for 24 hours, and then stored in liquid nitrogen to remain until thawing.

4.2 Cell Death Analysis

Cells were seeded in 12-well culture plates considering the appropriate cell seeding numbers for flow cytometry analysis. After 24h of incubation, cells were attached to surface and they were ready for cisplatin and Killer TRAIL treatments. The dose dependence check for cisplatin treatment included the concentrations 0.1, 4, 8, 16, 32 μM, then we decided on the concentrations 20 μM and 30 μM for further experiments. For Killer TRAIL treatment we selected the concentration 100ng/ml. After the treatments, cells were harvested at 24 and 48 hours through trypsinization and washed twice with cold 1X PBS. The cells were centrifuged at 300g for 5 min, then the supernatant was discarded and the pellet was resuspended in 100μl 1X Annexin-V binding buffer then cell suspension was incubated with 2μl of FITC-conjugated Annexin V (Pharmingen) for 15 min at room temperature in the dark. 500 μl of 1X Annexin-V binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using FACS BD software.

For the potentiation of anticancer agents cisplatin and Killer TRAIL, glycolytic inhibitor 2-Deoxyglucose(2-DG) (Sigma) pretreatment was done. After the seeding and incubation steps, cells were treated with 10mM 2-DG for 24 hours. Next day, the medium wass discarded and renewed and cisplatin/Killer TRAIL treatments started. The next steps were followed as stated in the first paragraph.

4.3 Total Protein Isolation

HCT 116 cell lines WT, p53 -/- and Bax -/- were harvested for protein analysis having control and various treatment samples. They were washed in ice cold PBS and lysed on ice in 200 μl complete lysis buffer containing freshly added 1 mM PMSF,

protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Roche, Mannheim, Germany). It took 30 minutes on ice for the proper lysis of the cells, cell debris was removed by cold centrifugation (4°C) for 10 min at 13200 rpm. Supernatant containing the total protein extracts are transferred into eppendorf tubes and they were immediately put in storage at -80°C. Protein concentrations were determined with Bradford Protein assay.

4.4 Determining Protein Concentrations

Protein concentrations were determined with Bradford Protein assay. Bradford assay is a colorimetric protein assay that relies on the binding of the dye Coomassie Brilliant Blue G-250 to protein, giving out a blue color. Concentration measurement is based on this absorbance shift of the dye. The procedure we carried out is as follows: 1µl of protein sample was diluted with 4 µl distilled water in 95 µl of 1X Bradford reagent. With serial dilutions of BSA as 5, 2.5, 1.25, 0.625, 0.313 µg, we obtained a standart curve. By dividing the obtained optical density at 595 nm (OD595) to the slope of the standart curve, the protein concentration was calculated in µg/µl.

4.5 SDS-PAGE Gel Electrophoresis

The purpose of SDS-PAGE is to separate proteins according to their size. It is the most widely used technique for analyzing mixtures of proteins. The anionic detergent sodium dodecylsulfate (SDS) denatures all proteins to the same linear shape coating them with negative charges as it binds to the hydrophobic side chains of proteins and breaks non-covalent interactions. As the proteins are put into an electric field they move towards the positive pole through the polyacrylamide gel moving at different rates according to their size.

The overall procedure starts with the pouring of the gel. It consists of two parts, a stacking gel laying on top of a separation gel. As the proteins are passing from the stacking to the separating gel, they are confined to a narrow band due to the different

pH values. Bio-Rad mini protean gel apparatus were used for the preaparations of the gel.

Percentages of the separating gel can change according to the size of the proteins for better results. In our study, 12% separating gel was convenient for the proteins we checked out. Here are ingredients: for two gels in 1mm glasses 3.4ml distilled water, 2.5ml pH 8.8 1.5M Tris-HCl, 50µl 20% (w/v) SDS, 4ml 30% Acrylamide/ 0.8 bis-Acrylamide solution, 50 µl 10% APS and 5 µl TEMED in the given order. After the casting of the gel between the glasses, isopropanol was poured upon to prevent contact with oxygen and to eliminate bubbles. In about 40 minutes gel was ready, polymerized, we got rid of the isopropanol and stacking gel iwas prepared and casted: again for two gels in 1mm glasses 3.075ml distilled water, 1.25ml 0.5M Tris-HCl, 25µl 20% (w/v) SDS, 670 µl 30% Acrylamide/ 0.8 bis-Acrylamide solution, 25 µl 10% APS and 5µl TEMED in the given order. Combs were placed in order to get wells inside the gel. As the polymerization finished, the prepared protein samples(with loading dye) were loaded inside the wells along with a protein marker (Fermentas, Germany) and the gel was ready to run. In our condition, SDS-PAGE was run for one and a half hours at room temperature with a constant voltage of 100V.

4.6 Western Blotting Procedure

After the separation of the proteins on 12% SDS-PAGE, they were blotted onto PVDF membranes. The membranes were then blocked with blocking solution, 5% dried milk in PBS-Tween20, and incubated with appropriate primary and secondary antibodies(listed in Appendix B) diluted respectively to 1/2000 (v/v) and 1/5000 (v/v) in blocking solution. After the washes with PBS-Tween 20, detection of blots were performed by an enhanced chemiluminescence detecton system and the results were exposed to Hyperfilm-ECL.

4.7 Cell Cycle Analysis

Flowcytometric cell cycle analysis is a well-working method for the assessment

of cell cycle distribution. In this study, HCT 116 WT, p53-/- and Bax -/- cell cycle distribution were checked after 10mM 2-DG treatment for 24h via PI staining using flowcytometer.

The cells were seeded in 12-well plates in appropriate seeding densities. They were incubated at 37°C and 5% CO_2 for 24h in order to make them attach onto the surface. Next day, the treatment took place. 24 hours later, they were detached by trypsinization and washed with cold 1X PBS. Following the centrifugation for 5 minutes at 300g, the supernatant was discarded and the pellet was vortexed gently. We then added 5 ml 70% Ethanol into the tubes for fixation. At this step, the cells can be stored up to 4 weeks at 4°C in ethanol. After at least 2 hours of wait, the cells were ready for spin down (5 minutes at 300g), and another cold 1X PBS wash done, then another spin down. The supernatant was discarded and 100 µl PI solution was added to cells and we kept them in a dark storage for 45 minutes at room temperature. 500 µl of PBS was added to stop the reaction and they were ready for the FACS analysis.

4.8 Proliferation Assay

To confirm the antiproliferative effects of the anticancer agents, we performed proliferation assay by using CyQUANT Proliferation Assay Kit. We followed the procedure according to the given protocol. The cells were seeded in black microplates and after appropriate treatments they were given dye in lysis buffer. The fluorescence was measured by spectrofluorometer.

4.9 Transfection Procedure

Mcl-1 overexpression plasmid pCMV-Flag-hMcl-1 were bought from addgene. pCMV vector was used for mock transfections. The transient plasmid DNA transfection was done with the following procedure: HCT 116 WT, p53 -/- and Bax -/- cells were seeded in 12-well plates and transfected with the plasmid (1µg) using X-tremeGENE 9 DNA transfection reagent according to the manufacturer's protocol. The transfection

medium was removed next day and further treatments continued afterwards. The transfection efficiency was monitored via western blotting analysis.

4.10 RNA Isolation

RNAs from 2-DG treated HCT 116 WT and p53 -/- cells were isolated following this procedure: The cells had been seeded and treated in 6-well plates. After the treatment, medium was discarded and the cells were washed with cold 1X PBS. 500 µl TRIZOL was added and the cells started to detach from the surface. TRIZOL disrupts and breaks down the cells while maintaining RNA integrity. 5 minutes of wait at room temperature. Then, 100 µl Chloroform was added. We inverted the tubes 10 times after chloroform addition. Another 2-3 minutes of wait. The tubes were centrifuged at 12000 rcf for 15 minutes at 4°C. Three phases formed in the tubes as RNAs being in the upper phase. The upper phase was taken into a new tube and 250 µl of isopropanol was added. Tubes were inverted 10 times, and we waited for 10 minutes leaving them at room temperature. Another centrifugation step followed with the same condition as the previous one. The supernatant was discarded, 500 µl 80% EtOH was added, and the tubes were vortexed. The cells then underwent another centrifugation step, this time at 7500 rcf for 5 minutes at 4°C. The supernatant was discarded, we let the tubes dry for 10 minutes, to eliminate alcohol. After that, the RNA pellet was dissolved in 50 µl dH_2O-DEPC, the concentrations were checked via Nanodrop and they were ready for storage at -80°C.

4.11 Microarray

In the field of molecular biology, microarray technology provides crucial insights for the understanding of cellular function by measuring the expression levels of thousands of genes. In our study, we planned on to look for the change of gene expression levels of the colon cancer cell lines by examining the activity of the identified target genes of cancer pathways, metabolic pathways and apoptosis pathways.

Isolated RNAs from HCT 116 WT and p53 -/- cells with control and 2-DG treatment conditions sent to Dr. Pieter Faber from USA Cleveland Clinic, Genomic Medicine Institute. The RNAs were processed into cRNAs there, hybridized to arrays and scanned on Illumina BeadArray reader. The results were taken from Illumina's BeadStudio software which produced data files in excel-format. We wanted whole-genome gene expression analysis, and Illumina's gene expression arrays provided the most up-to-date expression content and high-throughput processing, promising the production of high-quality data for large gene expression studies, efficiently and economically. We selected the HumanHT-12 v4 Expression BeadChip for more biologically meaningful results through genome-wide transcriptional coverage of well-characterized genes, gene candidates, and splice variants.

4.12 Statistical Analysis of Cell Death Graphics

The results are expressed as mean ± SEM and the mean values were compared using Students *t*-tail test. Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

5. RESULTS

5.1 Results

5.1.1 Assessment of Apoptosis Induction Profile of Cisplatin and Killer TRAIL as selected anticancer agents:

Cisplatin and Killer TRAIL were selected as apoptosis inducers in this anticancer treatment. We did a literature reseach on Killer TRAIL's dose- and time-dependence. What we got out of this research is that the product data sheet suggests Killer TRAIL's induction of apoptosis in a concentration range of 10-100ng/ml. We have seen two different articles selecting doses 100ng/ml and 200ng/ml(82, 83). We decided on starting with 100ng/ml and going along with what the results would provide us. On the other hand, we wanted to do dose- and time- dependence experiments for cisplatin's induction of apoptosis, to decide on the concentration values for further experimental analysis.

The initial results for cisplatin concentrations varied in terms of apoptosis induction. HCT 116 p53 -/- cells were expectedly resistant in comparison to HCT 116 WT cells. But after 24 hours, we couldn't get sufficient cell death response up to 16μM cisplatin(FIG. 5.1). 32μM of cisplatin caused over 25% cell death for WT cells and near 15% for p53 -/- cell. This would be good enough for further studies. Still, as the 48-hour cell death analysis provided better and cleaner results, and 32μM this time causing up to 35% of cell death for WT cells and near 25% for p53 -/- cells made us select 48 hours as our time condition. For the dose, we chose 20μM and 32μM of cisplatin concentrations. The point of selecting 20μM was to stretch out the cell death percetage a little more than what 16μM has given us.

We also analyzed cell death after Killer TRAIL treatment, but in this set we had HCT 116 WT and Bax -/- cells. HCT 116 Bax -/- cells were highly resistant to Killer TRAIL, so they showed very little or no apoptosis induction, unlike WT cells. 100ng/ml of Killer TRAIL resulted in around 16% cell death in HCT 116 WT cells for both 24 and 48-hour conditions(FIG. 5.2). We selected to go for 48-hour treatment for the next experiments.

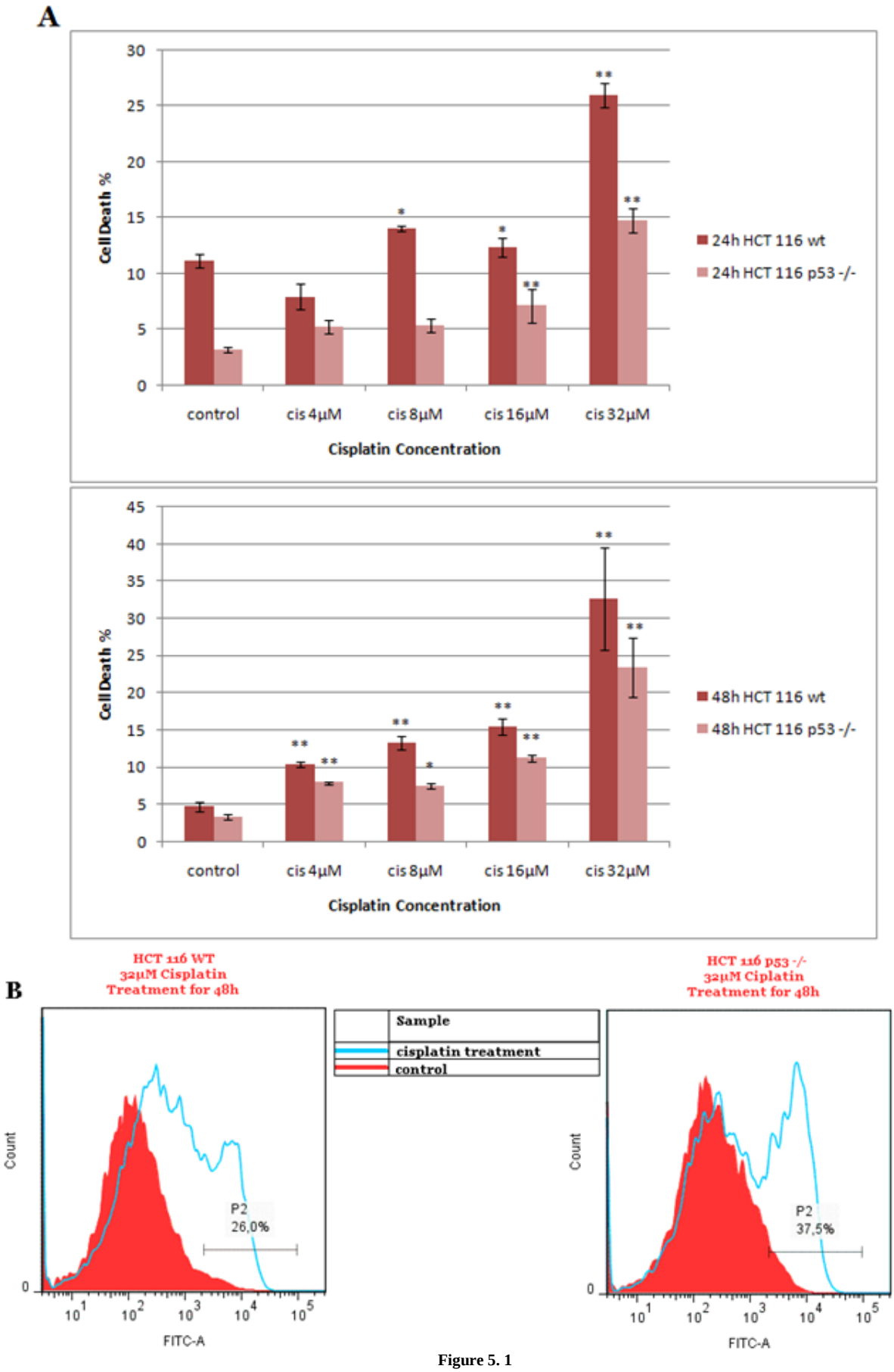

A
Cell Death %
30
25
20
15
10
5
0
control
cis 4µM
cis 8µM
cis 16µM
cis 32µM
Cisplatin Concentration
24h HCT 116 wt
24h HCT 116 p53 -/-
Cell Death %
45
40
35
30
25
20
15
10
5
0
control
cis 4µM
cis 8µM
cis 16µM
cis 32µM
Cisplatin Concentration
48h HCT 116 wt
48h HCT 116 p53 -/-
B
HCT 116 WT
32µM Cisplatin
Treatment for 48h
Sample
cisplatin treatment
control
Count
P2
26,0%
FITC-A
HCT 116 p53 -/-
32µM Ciplatin
Treatment for 48h
Count
P2
37,5%
FITC-A

Figure 5. 1

Figure 5.1 Apoptosis induction analysis of HCT116 WT and p53 -/- cells treated with increasing concentrations of cisplatin for 24 and 48 hours. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. **A-**The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at leat two experiments. ** $P < 0.01$, * $P < 0.05$ **B-** The histograms showing the shift in the intensity of fluorescence dye between control and 32μM cisplatin treated cells. (Appendix D has the detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.)

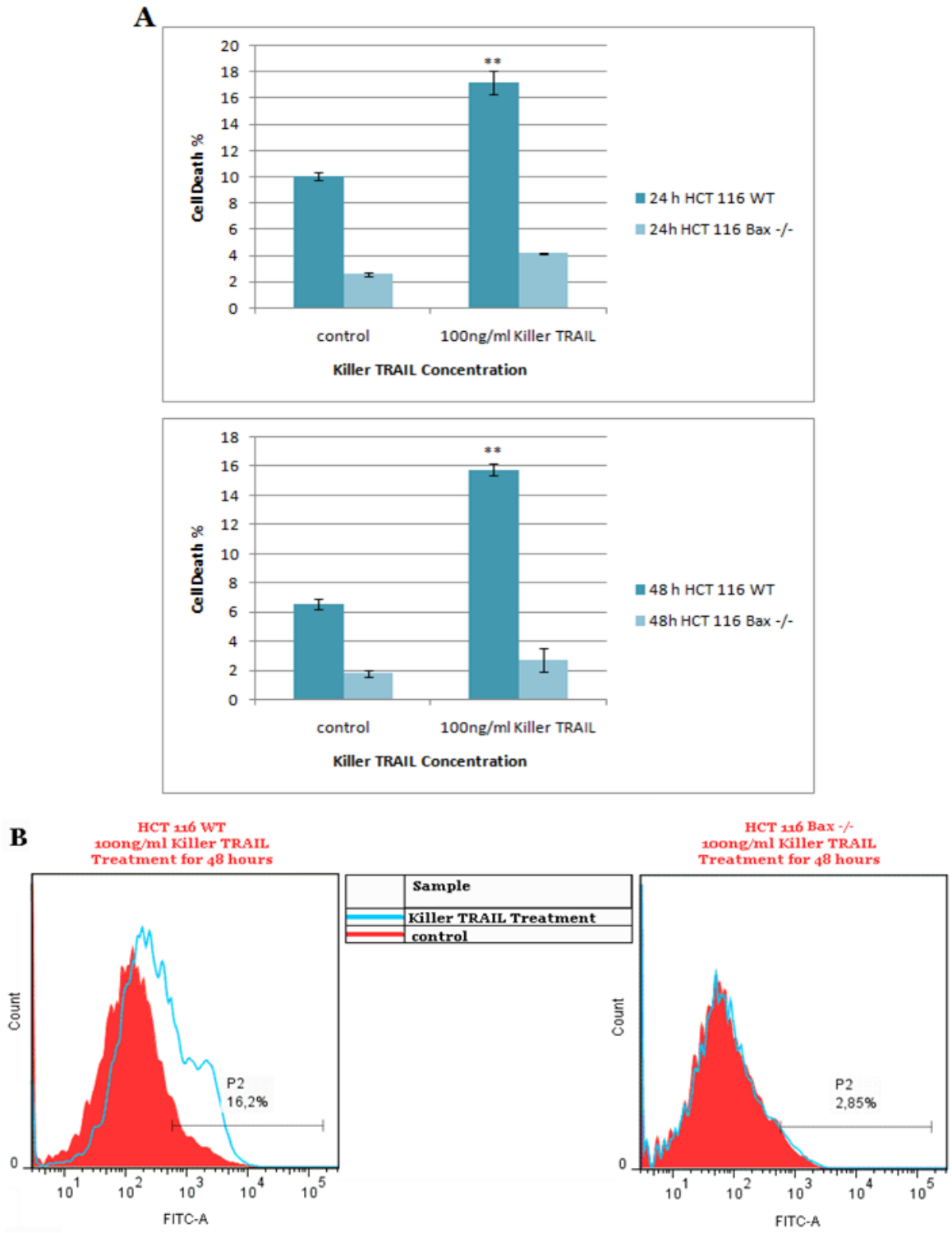


Figure 5. 2 Apoptosis induction analysis of HCT116 WT and Bax -/- cells treated with 100ng/ml Killer TRAIL for 24 and 48 hours. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. **A-** The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ **B-** The histograms showing the shift in the intensity of fluorescence dye between control and 100ng/ml Killer TRAIL treated cells. (Check Appendix D for whole data)

5.1.2 Sensitization of HCT 116 WT, p53 -/- and Bax -/- cell lines to Cisplatin and Killer TRAIL-induced apoptosis by 2-DG Pretreatment:

In dose- and time- dependence experiments on anticancer agents, our aim was to select a concentration that showed induction of apoptosis to an extent not so high not so low. Because in the next step it gave us the opportunity to clearly detect the potentiation of these anticancer agents when treated after the cells' pretreatment with 2-DG, a glycolytic inhibitor.

First HCT 116 WT and p53 -/- cells underwent 10mM 2-DG pretreatment for 24 hours. Then they were given cisplatin on several concentration levels for 48 hours. Potentiation of cisplatin by 2-DG pretreatment showed itself in 20 and 32 μM cisplatin concentration samples: Cell death percentages for WT sample rising around 10% and for p53 -/- sample around 13% (FIG. 5.3). The results were more than satisfying considering that 2-DG pretreatment clearly increased cell death percentages.

The other set was also ready to taste 2-DG before Killer TRAIL treatment. They were given 10mM 2-DG and the pretreatment lasted 24 hour long, exactly the same condition as the first set. Then Killer TRAIL treatment followed for 48 hours. This started off exciting because we had HCT 116 Bax -/- cells in this study set along with HCT 116 WT. And Bax -/- cells were highly resistant to Killer TRAIL-induced apoptosis. We were about to see whether or not 2-DG could sensitize Bax -/- cells and make them respond to the treatment by inducing apoptosis.

It came out, however, rather disappointing because nothing changed for Bax -/- cells as they were perfectly well. WT cells, on the other hand, showed increase in cell death. Apoptosis induction rose up 10% when Killer TRAIL was given to cells after 2-DG pretreatment(FIG. 5.4).

These results suggest that 2-DG can potentiate selected anticancer agents and increase cell death numbers by sensitizing the cells.

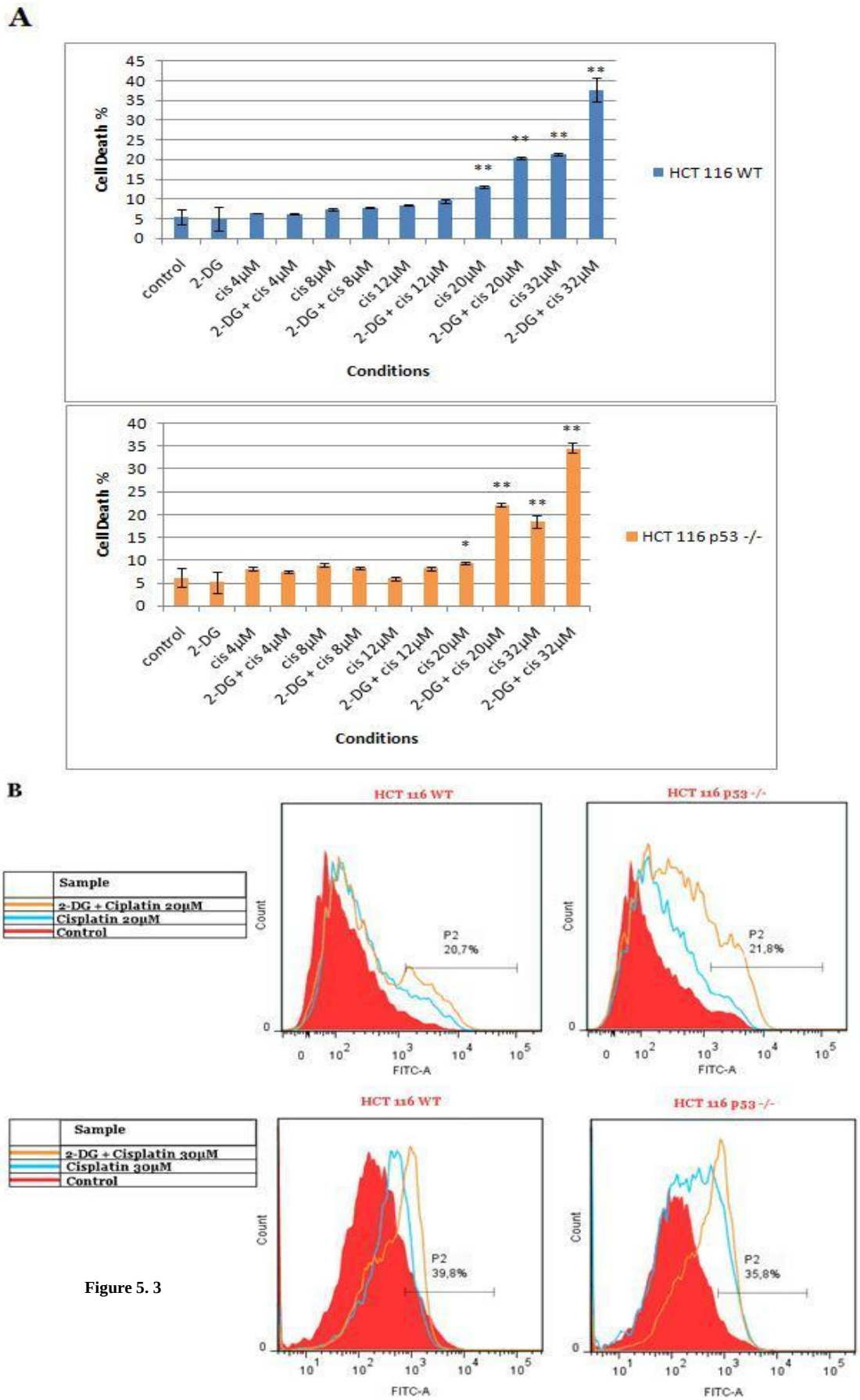

A
Cell Death %
HCT 116 WT
control
2-DG
cis 4µM
2-DG + cis 4µM
cis 8µM
2-DG + cis 8µM
cis 12µM
2-DG + cis 12µM
cis 20µM
2-DG + cis 20µM
cis 32µM
2-DG + cis 32µM
Conditions
HCT 116 p53 -/-
B
Sample
2-DG + Ciplatin 20µM
Cisplatin 20µM
Control
HCT 116 WT
HCT 116 p53 -/-
Count
P2
20,7%
21,8%
FITC-A
Sample
2-DG + Cisplatin 30µM
Cisplatin 30µM
Control
P2
39,8%
35,8%

Figure 5. 3

Figure 5.3 Sensitization of HCT116 WT and p53 -/- cells to cisplatin induced apoptosis. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. **A-** The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ **B-** The histograms showing the shift in the intensity of fluorescence dye between control, Cisplatin, 2-DG + Cisplatin samples. (Check Appendix D for whole data)

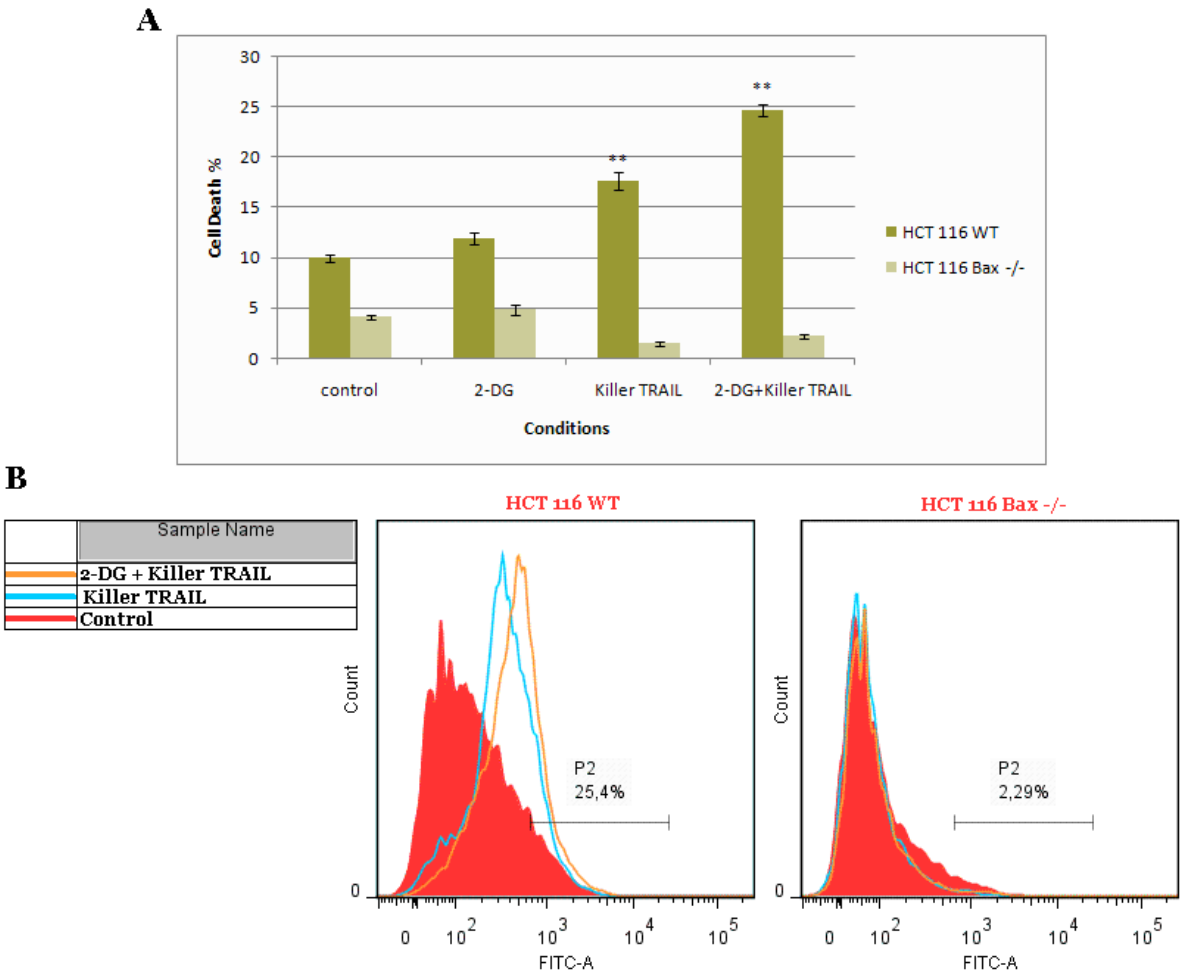


Figure 5. 4 Sensitization of HCT116 WT and Bax -/- cells to Killer TRAIL induced apoptosis. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. **A-** The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ **B-** The histograms showing the shift in the intensity of fluorescence dye between control, Killer TRAIL, 2-DG + Killer TRAIL samples. (Check Appendix D for whole data)

5.1.3 Checking antiproliferative properties of Cisplatin by Proliferation Assay

To further confirm the decrease in cell number, we performed proliferation assay for the first set, being HCT 116 WT and p53 -/- as the cell lines and cisplatin as the anticancer agent. We didn't perform this for the second set because we couldn't achieve Bax -/- sensitization and so performing the assay would be rather redundant.

The results gave countenance to the FACS analyses. Checking DNA content by this proliferation assay, 20μM Ciplatin treatment was selected as representative. And the results showed that when treated after 2-DG pretreatment, cisplatin gained a lot in terms of its antiproliferative properties causing further decrease in cell numbers.

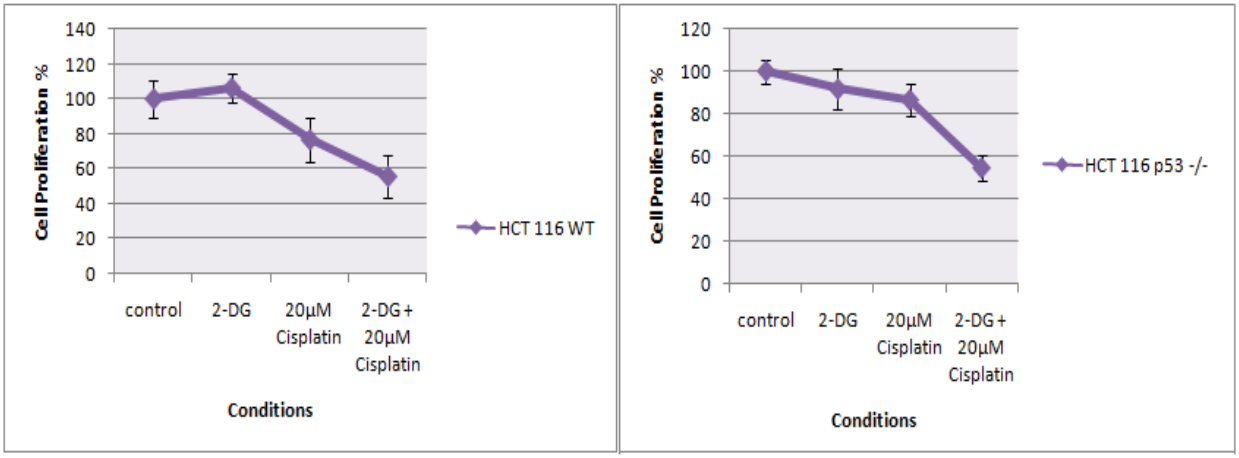


Figure 5. 5 Proliferation Assay results after HCT 116 WT and p53 -/- cells' treatment with Cisplatin and 2-DG. 2-DG pretreatment for 24 h prior to 48 h treatment of 20μM Cisplatin. Analysis was done on spectrofluorometer as the absorbances were measured. The graphs show the mean percentage of cell proliferation in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments.

5.1.4 Sensitization of HCT 116 Bax -/- Cells to Killer TRAIL-induced apoptosis

As the initial results came off unsuccessful when we tried sensitizing HCT 116 Bax -/- cells by 2-DG pretreatment to Killer TRAIL-induced cell death, we looked for other possible ways as to how we could do it. We had two other compounds that target

potential survival mechanisms that might help Bax -/- cells to resist against cell death signaling.

One of them was the BH3 mimetic, Abt-737, targeting Bcl-2 and Bcl-XL, two of the most important antiapoptotic Bcl-2 protein family members. The aim was to inhibit these antiapoptotic cell death regulators and therefore sensitize the cells if these proteins were really that important for Killer TRAIL induced apoptosis. But, as the results suggested, Abt-737 treatment couldn't work as we expected with no significant changes between 2-DG + Killer TRAIL and 2-DG + Abt-737 + Killer TRAIL sample (FIG. 5.6).

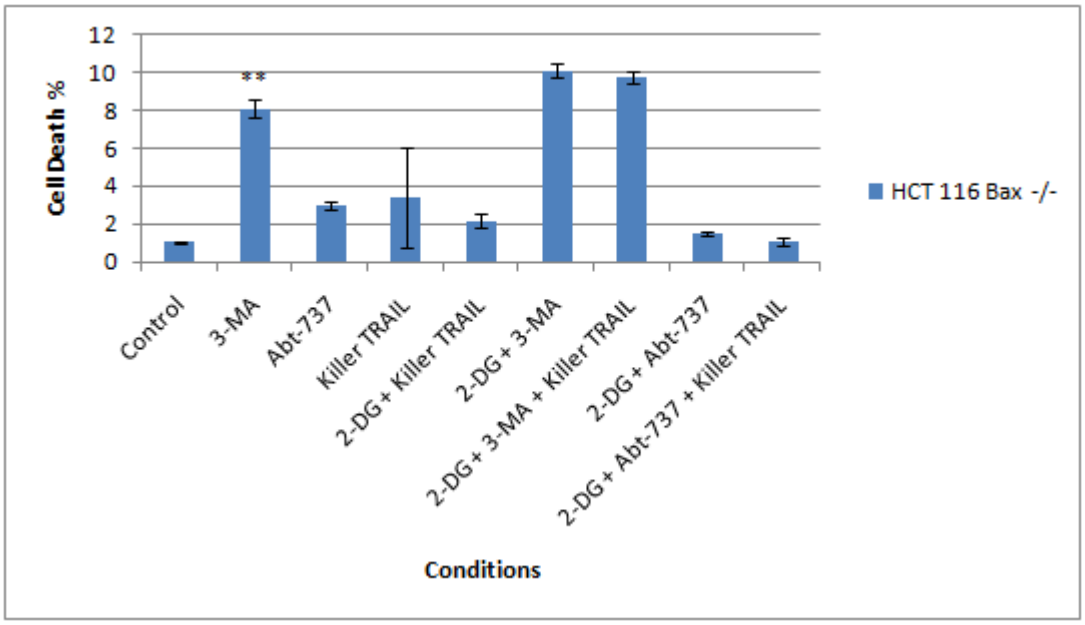


Figure 5. 6 Sensitization of HCT116 Bax -/- to Killer TRAIL-induced apoptosis. Treatment of HCT 116 Bax -/- cells with 400nM Abt-737 and 5mM 3-MA for 48h alone and concurrently with 100ng/ml Killer TRAIL after 2-DG pretreatment. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ (Check Appendix D for whole flow jo data)

As you may already seen by looking at figure 6, 3-Methyladenine (3-MA) was the other promising compound that ended up disappointing for our purposes. 3-MA is widely used as autophagy inhibitor. We thought autophagic cell survival might be responsible for this high resistance among Bax -/- cells. However, the results were confusing on this front, because we never thought 3-MA would increase cell death when treated alone. And 5mM was a widely used concentration level for this compound. This might mean two things: one is that this concentration was somehow

toxic to HCT 116 Bax -/- cells; two 48 hour long treatment caused some kind of an increase in autophagic flux resulting in autophagic cell death. However, both of these remarks went nowhere for our study considering that 3-MA treatment didn't help for potentiation of Killer TRAIL induced apoptosis when treated concurrently.

So both Abt-737 and 3-MA treatments couldn't sensitize Bax -/- cells, but was it due to not getting a proper induction of cell death because of their resistance? We checked their effect on HCT 116 WT cells to see what would happen when there was a proper apoptotic signaling (FIG. 5.7).

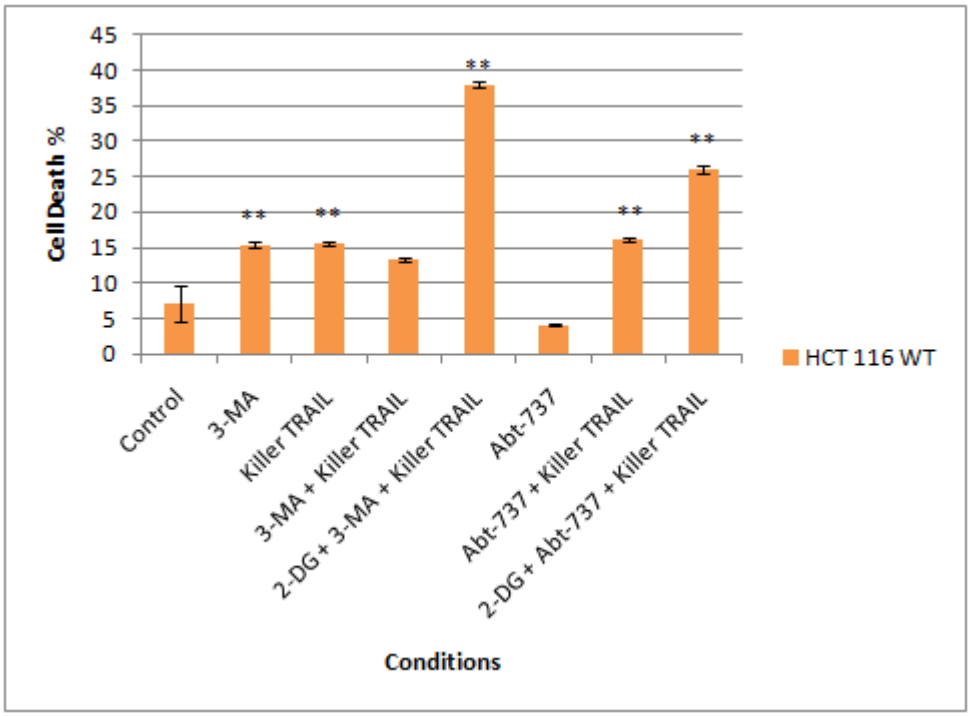


Figure 5. 7 Abt-737 and 3-MA effects on HCT 116 WT cells. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ (Check Appendix D for whole flow jo data)

These results confirmed their inability to potentiate Killer TRAIL induced apoptosis, because as you can see 3-MA caused 15% cell death when given alone and when it's given concurrently with Killer TRAIL the cell death percentage(~14%) didn't change. However, when it's given along with Killer TRAIL after 2-DG pretreatment the cell death percentage went higher in comparison to Figure 5.4's 2-DG + Killer TRAIL

sample. This might be a slight sensitization effect. But still it didn't help for anything, as it couldn't overcome Bax -/- cells' resistance.

Abt-737 treatment gave almost identical results as Figure 5.4, like it was not there at all. The percentages matched with control, Killer TRAIL and 2-DG + Killer TRAIL samples from figure 5.4. Nothing suggested a further potentiation of Killer TRAIL or sensitization of HCT 116 WT and Bax -/- cells.

5.1.5 Apoptotic Effects of Cisplatin and Killer TRAIL: Immunoblot Analysis

The anticancer agents cisplatin and killer TRAIL cause cell death by inducing apoptosis. After the intial flowcytometic analysis of HCT 116 cell lines following the treatments, Annexin V staining gave us the first impressions of apoptosis induction. The next step was to detect it on protein level by checking caspases, because caspase cleavage is the signature event of apoptotic signaling.

Caspase 3 and caspase 8 were checked for the first set, both HCT 116 WT and p53 -/- cells showed caspase 3 cleavage confirming the apoptotic response. Caspase 8 was not cleaved in this set (FIG. 5.8). And that was quite plausible considering that caspase 8 is important for extrinsic apoptotic pathway, getting cleaved by intracellular parts of death receptors. One thing we expected here was that the increase in cell death numbers could relate to increased caspase cleavage when cisplatin was given after 2-DG pretreatment. But cleaved caspase 3 western blot didn't come out that way. Even showing a slight decrease. However, this might be interpreted in a different way as that level of caspase 3 cleavage could be enough for an improved apoptotic response in sensitized cells.

We checked caspase 8 for the Killer TRAIL set and only for HCT 116 WT cells because Killer TRAIL couldn't induce an apoptotic response in HCT 116 Bax -/- cells. All cleaved products were clearly there in this immunoblot and this time we could even see a slight increase in cleaved caspase 8 products. However, interestingly, full lenght caspase 8 was also increased in 2-DG+Killer TRAIL sample.

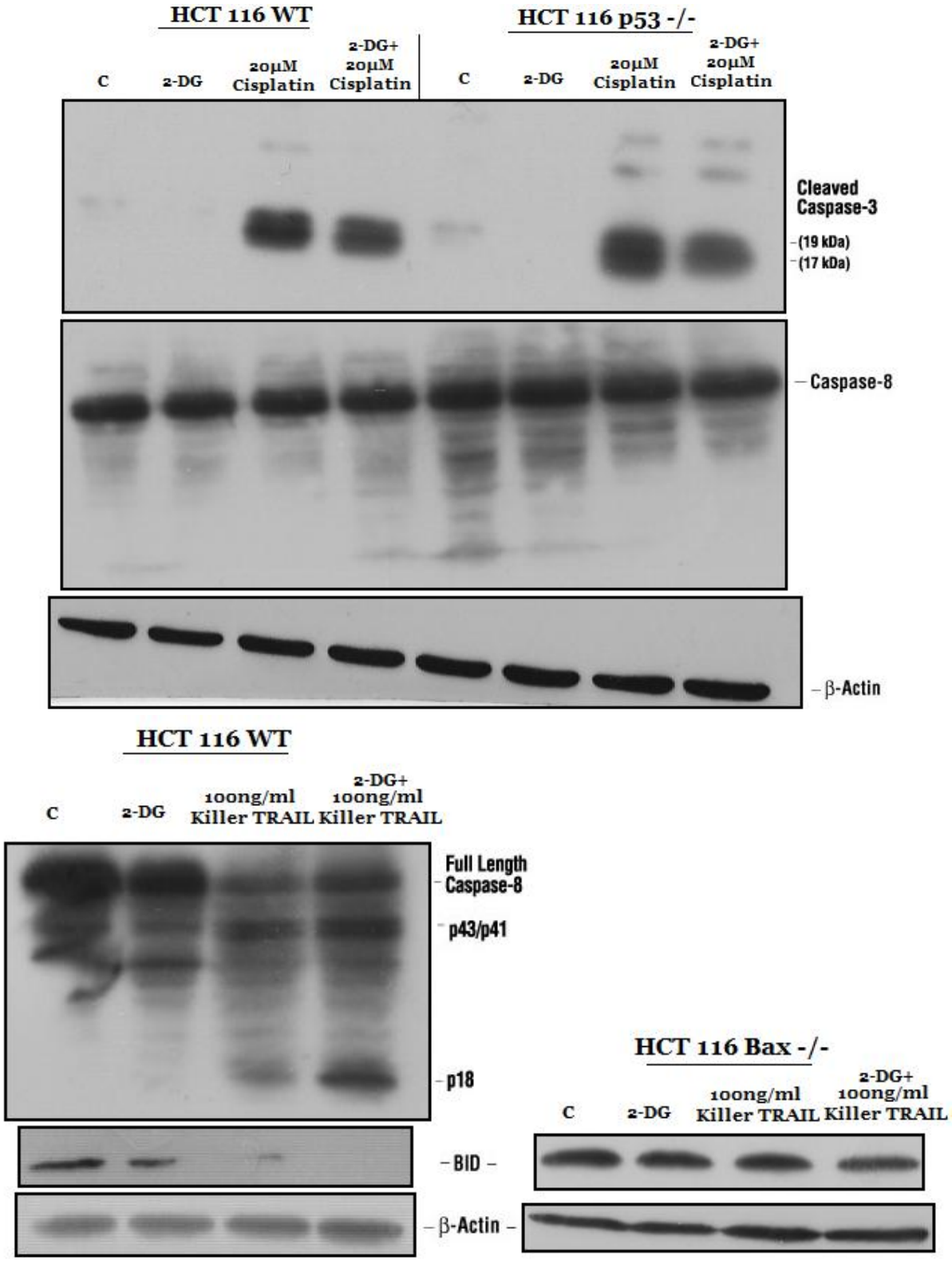


Figure 5. 8 Western Blot Analysis of Caspases and BID. Western Blot Analysis of HCT 116 WT, p53 -/- and Bax -/- cells following their treatment with 2-DG(10mM, 24h pretreatment) and Cisplatin||Killer TRAIL(20μM, 48h||100ng/ml, 48h). Total cytosolic proteins isolated, caspase protein levels were detected with antibodies. β- actin was used as loading control.

BID was another protein checked for this set. It is an important proapoptotic Bcl-2 family member overlapping the intrinsic and extrinsic pathways of apoptosis. The crucial feature is that it gets truncated by caspase 8. WT samples showed the decrease in BID protein levels and finally vanishing in killer TRAIL and 2-DG+Killer TRAIL samples, while Bax -/- cells expectedly did not.

The western blot results spotted effector caspases in action and so confirmed that our selected anticancer agents kill cancer cells by inducing apoptosis.

5.1.6 Role of Some Bcl-2 Protein Family Members in Sensitization of the Cells and Potentiation of Apoptotic Signaling

Bcl-2 protein family are crucial regulators of apoptotic signaling. Building the family upon two distinct classes named anti- and pro- apoptotic means so much in terms of their regulatory contributions. Therefore, after checking apoptosis markers in the previous western blotting experiments, we leaned over Bcl-2 protein family and looked for the changes on protein levels after 2-DG treatment. All three cell lines, HCT 116 WT, p53 -/- and Bax -/- provided samples for this study as they were treated with 10mM 2-DG for 24h. Our aim was to detect any meaningful change that might help us for the understanding of potentiation of apoptosis induction caused by cisplatin and Killer TRAIL.

First we checked antiapoptotic members of the family, being Bcl-2 and Mcl-1. While Bcl-2 protein levels didn't change, decrease of Mcl-1 protein levels in all cell lines (obviously in WT and Bax -/-, slightly in p53 -/-) after 2-DG treatment gave us a crucial result(FIG. 5.9). Being a highly important antiapoptotic protein regulating the apoptotic signaling in a preventive manner, Mcl-1's decreased protein levels means a hit out of the park in the name of getting the first clue to how 2-DG sensitizes the cells.

The next step was to check some of the proapoptotic members of the family, mostly the ones that have a connection to Mcl-1. However, the protein levels of both Bax and Bim didn't change(FIG. 5.9).

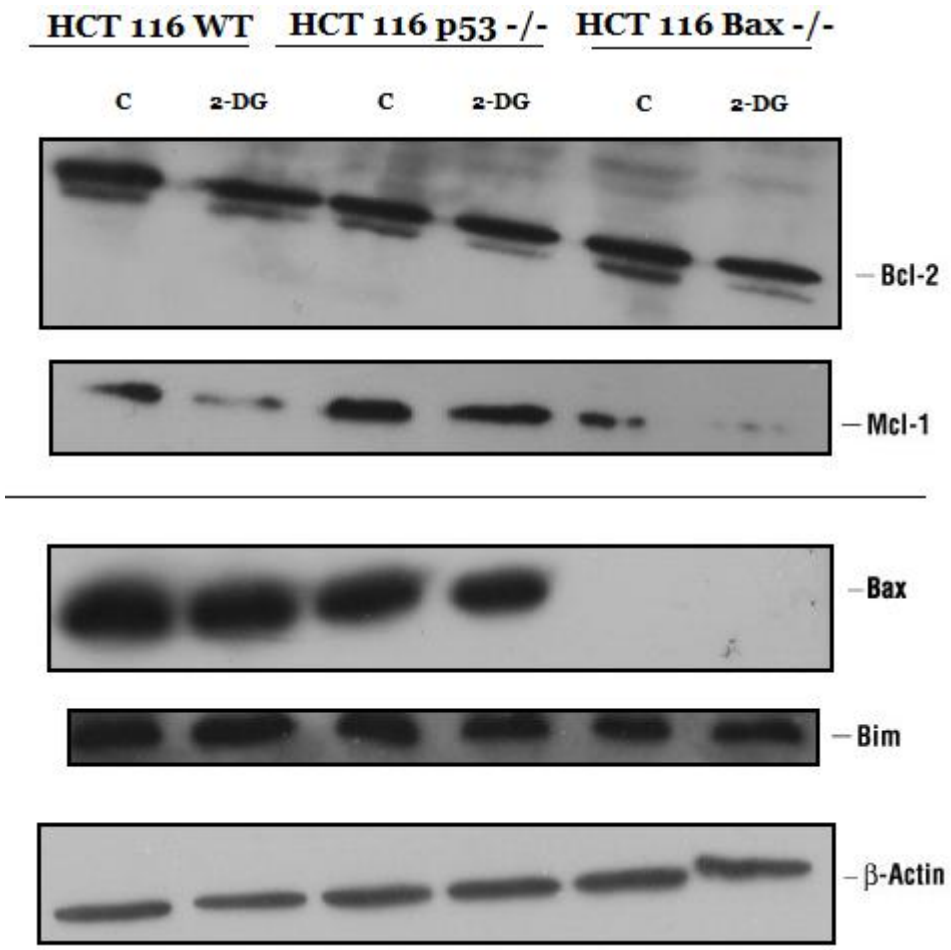


Figure 5. 9 Western Blot Analysis of Bcl-2 Portein Family. Western Blot Analysis of HCT 116 WT, p53 -/- and Bax -/- cells following their treatment with 2-DG (10mM, 24h). Total cytosolic proteins isolated, Bcl-2 family protein levels were detected with antibodies. β- actin was used as loading control.

5.1.7 Assessment of ATP Levels: 2-DG and Energy Metabolism

To look at the event from a different perspective, finally in the light of the Warburg effect we measured ATP levels after the cells' treatment with 10mM 2-DG. Our expectation was to detect a difference after 24h treatment. However, the cells showed little or no decrease in ATP levels after that time period. We then changed the treatment time to 5h in order to see an early response from the cells and the plan served our purpose by giving us a result of around 20% decrease in ATP levels of all three cell lines(FIG. 5.10 A).

To further confirm the ATP decrease, we checked the levels of phoshorylated AMPK alpha. AMPK is a conserved enzyme working as an energy sensor. When ATP levels decrease, it gets activated via phosphorylation and signals for energy need to activate energy generating pathways. We checked for the protein levels of its phoshorylated form after 5 hours of 10mM 2-DG treatment. And the results confirmed the decrease in ATP levels as all three cell lines showed AMPK activation(FIG. 5.10 B). The decrease in ATP levels after 2-DG treatment may very well be one of the effective mechanisms of 2-DG's ssensitization effect over HCT 116 cell lines.

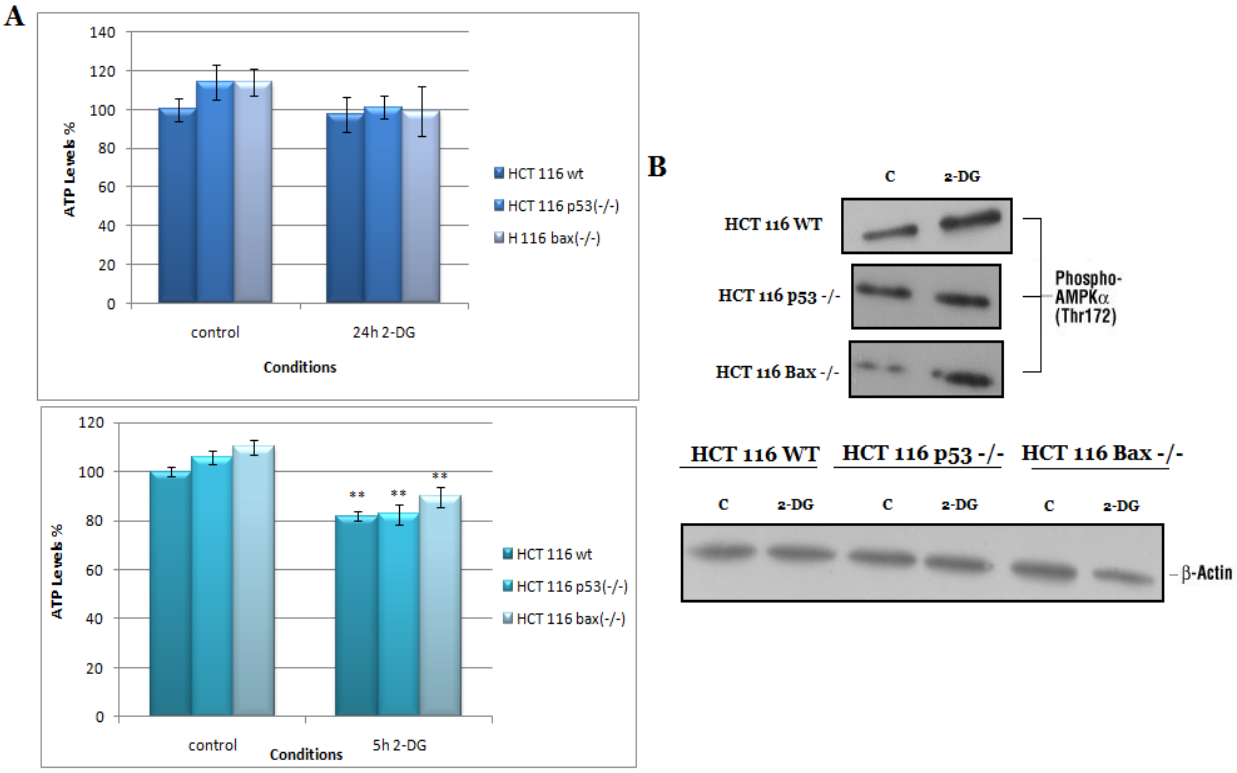


Figure 5. 10 2-DG treatment effects on cellular ATP levels. **A-** ATP levels measured in HCT 116 WT, p53 -/- and Bax -/- cell lines after 10mM 2-DG treatment for 5h and 24h. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ **B-** Total cytosolic proteins isolated from all three cell lines after 10mM 2-DG treatment for 5 hours. Phospho-AMPK protein levels were determined by western blotting. β- actin was used as loading control.

5.1.8 Regulation of mTOR Pathway

AMPK activation is shown to affect mTOR activity(36, 79).Therefore, with the increase in activation of AMPK, we wanted to look at one of the significant cancer

related pathways, mTOR signaling. mTOR is a highly important protein kinase having regulatory roles in cell growth, proliferation and survival as well as protein synthesis. To assess mTOR activity and in order to do it in a clear way, we checked the levels of the phosphorylated form of p70 S6 kinase, which is a downstream protein kinase working as translation regulator and it is the main target of mTOR. Hence, a potential change in mTOR's activity would reflect itself upon the activation levels of p70 S6 kinase. The results suggested so as phospho-p70 S6 kinase protein levels were decreased after 10mM 2-DG treatment for 24h(FIG. 5.11). We can say that 2-DG has an inhibitory effect on mTOR and thereby it opens a giant pool of various possibilities as to how mTOR inhibition serve for the cell's sensitization considering its roles in growth, proliferation, protein synthesis and so on.

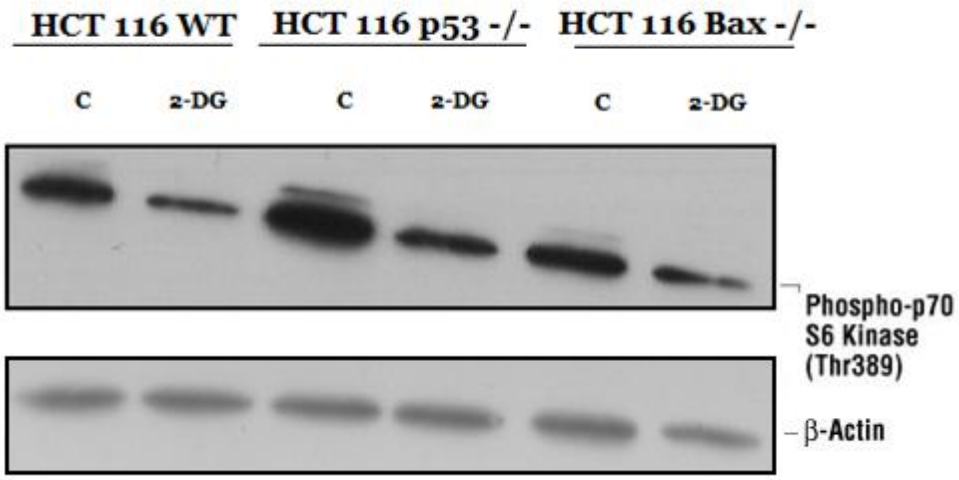


Figure 5. 11 Western Blot Analysis of Phospho-p70 S6K. Western Blot Analysis of HCT 116 WT, p53 -/- and Bax -/- cells following their treatment with 2-DG (10mM, 24h). Total cytosolic proteins isolated, phospho-p70 S6 kinase protein levels were detected. β- actin was used as loading control.

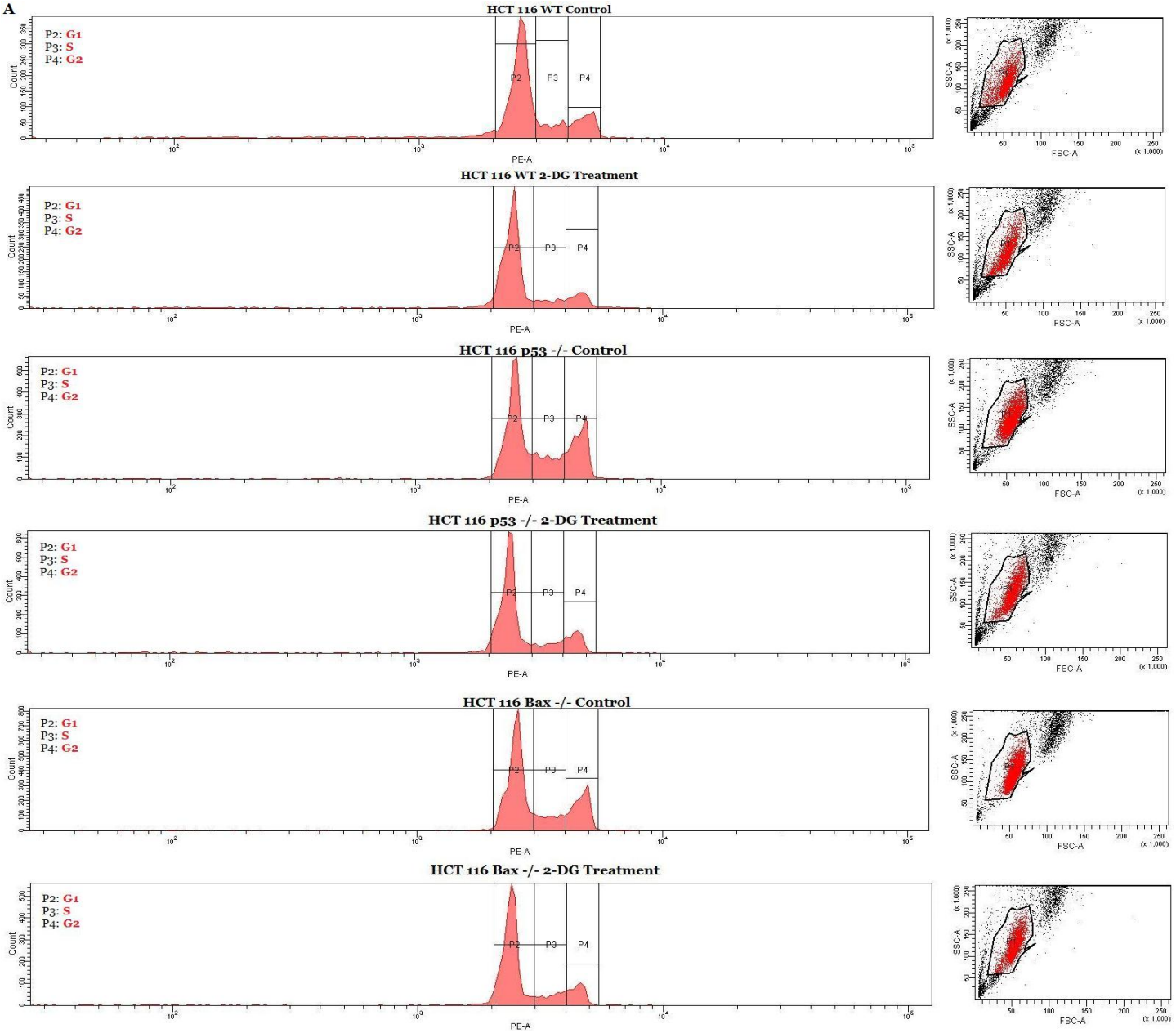
5.1.9 Effect of 2-DG Treatment on Cell Cycle Distribution

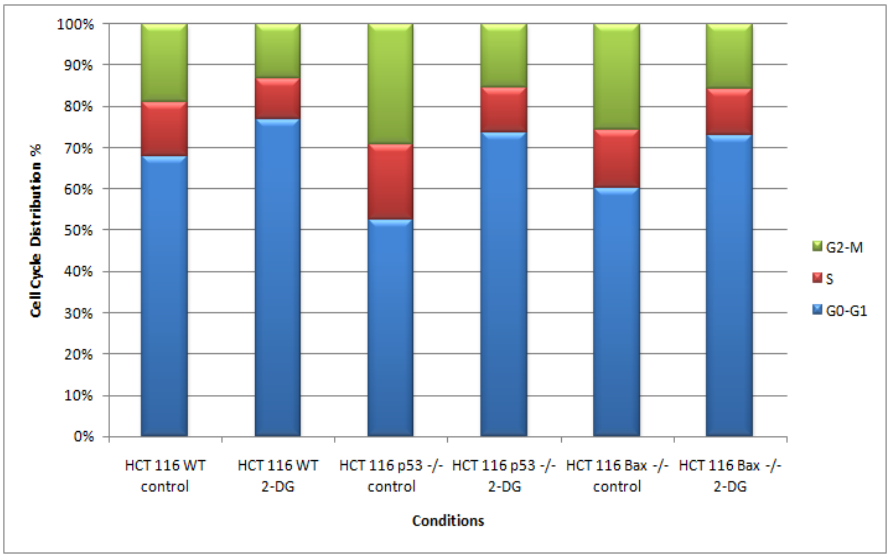
Performing cell cycle analysis was an idea thrown out for the consideration of another possible sensitization mechanism. We wanted to show if 2-DG would cause a difference in cell cycle distribution, maybe even cell cycle arrest in comparison to control samples. And with the results, we found out that 2-DG caused cell cycle arrest at G1 phase, bringing down the cell numbers of S and G2 phase(FIG: 5.12 A). It is the

point where a cell decides between dividing and resting. Apparently 2-DG treatment prevented cells from going into the phase of synthesis.

For this matter, p53 protein levels were detected by western blotting because of it's utmost importance as a cell cycle regulator among its many other specifications. 2-DG caused a decrease in p53 protein levels(FIG: 5.12 B), giving us another interesting result to discuss and interpret as p53 signaling has connection to cell cycle, Mcl-1, AMPK and mTOR.

A


HCT 116 WT Control
HCT 116 WT 2-DG Treatment
HCT 116 p53 -/- Control
HCT 116 p53 -/- 2-DG Treatment
HCT 116 Bax -/- Control
HCT 116 Bax -/- 2-DG Treatment
P2: G1
P3: S
P4: G2
Count
PE-A
SSC-A
FSC-A


Cell Cycle Distribution %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
HCT 116 WT control
HCT 116 WT 2-DG
HCT 116 p53 -/- control
HCT 116 p53 -/- 2-DG
HCT 116 Bax -/- control
HCT 116 Bax -/- 2-DG
Conditions
G2-M
S
G0-G1

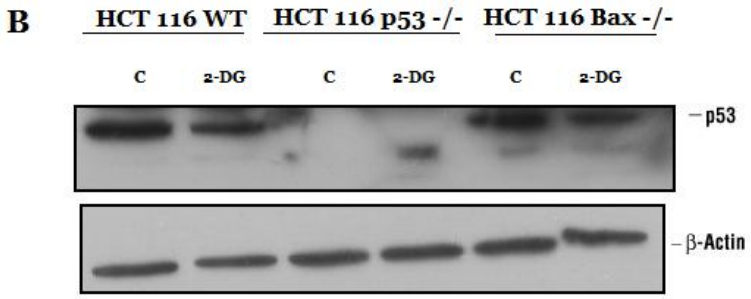


Figure 5. 12 Cell Cycle Analysis. **A-** Flowcytometric analysis of cell cycle distribution of HCT 116 WT, p53 -/- and Bax -/- cells following their treatment with 2-DG (10mM, 24h). **B-** p53 protein levels were detected via western blotting. β-actin was used as loading control.

5.1.10 Finding out Mcl-1's Importance: Mcl-1 Overexpression and Apoptosis Induction

In order to place further emphasis on Mcl-1 and to label it 'critical' for this study, we analyzed the effects of Mcl-1 overexpression. We studied it with the first set, HCT 116 WT and p53 -/- cell lines were transfected with the overexpresion plasmid, pCMV-Flag-hMcl-1. Following the transfection, after 24 hours of incubation, the cells underwent 10mM 2-DG pretreatment for 24 hours and then 20μM and 32μM cisplatin treatment took place for 48 hours.

Mcl-1 overexpression was highly effective in the name of its antiapoptotic properties, as cell death percentages went down at least 10% in each treated sample(FIG. 5.13 A). Even reducing the cell death numbers of 20μM cisplatin treated samples to the control levels.

Alongside flowcytometric analysis of apoptosis induction, western blotting results in Figure 5.13 B confirmed the overexpression of Mcl-1 protein.

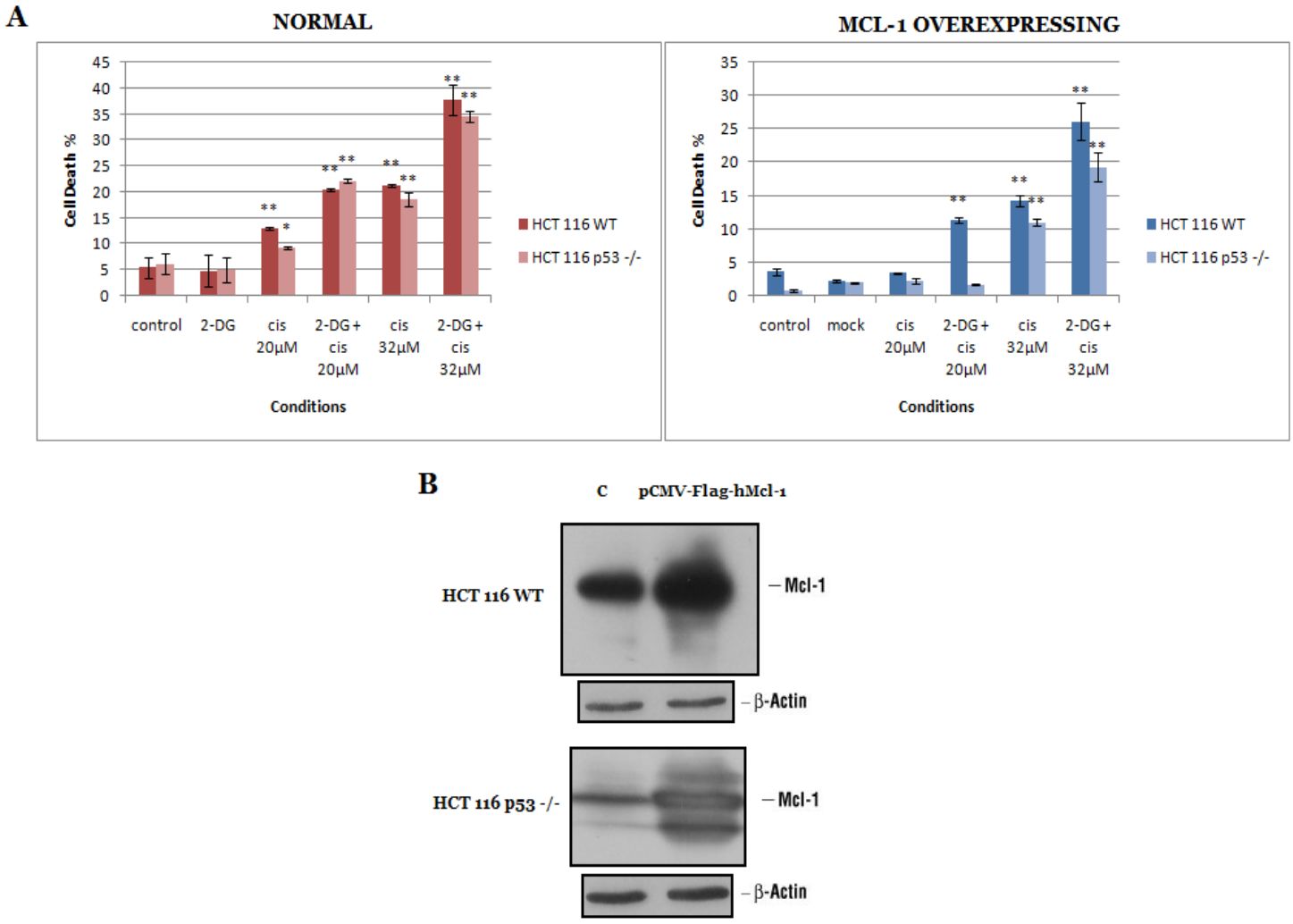


Figure 5. 13 Overexpression of Mcl-1 in HCT 116 WT and p53 -/- cells to see its regulatory effect over cisplatin-induced apoptosis. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. **A-** The graphs show the mean percentage of cell death in the control cells and the drug treated cells. Data are shown as mean ± SEM representative of at least two experiments. ** $P < 0.01$, * $P < 0.05$ (Check Appendix D for whole flow jo data) **B-** Confirmation of Mcl-1 overexpression in HCT 116 WT and p53 -/- cells by western blotting, protein isolation after transfection (48h).

5.1.11 2-DG Effects on Gene Level: Microarray Study

In order to determine the identity of the genes that exhibit changes in their expression levels after 2-DG treatment, we went for microarray analysis. Isolated RNA samples from HCT 116 WT and p53 -/- cells (control and 10mM 2-DG treated -24h-) were sent as triplicates to Genomic Medicine Institute in Cleveland, where the analysis was done. We were informed at every level from the beginning with concentration checks and integrity tests. HumanHT-12 v4 Expression BeadChip was used as the

RNAs were processed into cRNAs and hybridized to it. After the scanning on Illumina BeadArray reader, the results were taken from Illumina's BeadStudio software which produced data files in excel-format.

After a heavy work of analyzing the results, we selected 29 genes related to the study and divided them in four groups. The list was created with the following criteria: a p-value for differential gene expression of 0.05 or better and changes that happened in both WT and p53 -/- samples.

The results were interesting and very promising for the understanding of the effects of 2-DG on expression of genes that are related to cellular metabolism, apoptosis, cell cycle, cell growth, proliferation and tumorigenesis. It will also help for the future of this study in the name of selecting targets and determining signaling networks.

5.1.11.1 Genes related to apoptosis

TRIAP1 (aka p53CSV) gene expression was increased in both WT and p53 -/- samples after 2-DG treatment. Interestingly, this protein is stated as inhibitory to apoptosis and the gene is a target of p53. A study found that its induction happens when the cells have low levels of genotoxic stress but not when it is severe(105). Therefore we might say that 2-DG treatment alone causes a stress response in HCT 116 cells lines, and TRIAP1 expression increases.

Caspase 2 and Caspase 3 are members of cysteine proteases and their sequential activation plays a central role in apoptosis. As the western blotting results didn't show cleavage for caspase 3 after 2-DG treatment, its induction might mean some kind of a preparation for apoptosis. Unlike caspase 3, however, caspase 2 is not a well studied caspase and its role is not well defined. A recent article came up with an interesting analysis on its versatility, suggeting multiple functions in apoptosis, DNA repair and tumor suppresion(106).

PDCD2, 5 and 6 are genes for programmed cell death proteins. PDCD2 is shown to induce apoptosis in human cells through activation of the caspase cascade(107). PDCD6 downregulation is associated with inhibition of apoptosis in ovarian cancer cells(108). PDCD5 is as well related to apoptosis induction. Its nuclear translocation is stated as an early and universal signal for apoptosis(109). Especially this protein held

our attention because there are some publications relating its low expression levels to downregulation of apoptosis(110, 111), and interestingly there are studies about its sensitization effects on cisplatin chemotherapy(112, 113, 114, 115).

APOPTOSIS			
		HCT 116 WT	HCT 116 p53 -/-
Gene	Definition	Fold(2-DG/C)	Fold(2-DG/C)
TRIAP1	Homo sapiens TP53 regulated inhibitor of apoptosis 1 (TRIAP1), mRNA.	1,43	1,69
Casp2	Homo sapiens caspase 2, apoptosis-related cysteine peptidase (CASP2), transcript variant 1, mRNA.	1,34	1,76
Casp3	Homo sapiens caspase 3, apoptosis-related cysteine peptidase (CASP3), transcript variant beta, mRNA.	1,79	1,4
PDCD2	Homo sapiens programmed cell death 2 (PDCD2), transcript variant 1, mRNA.	1,57	1,25
PDCD5	Homo sapiens programmed cell death 5 (PDCD5), mRNA.	1,68	1,77
PDCD6	Homo sapiens programmed cell death 6 (PDCD6), mRNA.	1,45	1,67
DDIT4	Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.	0,65	0,06
BCL-AF1	Homo sapiens BCL2-associated transcription factor 1 (BCLAF1), transcript variant 2, mRNA.	1,41	1,71
BID	Homo sapiens BH3 interacting domain death agonist (BID), transcript variant 1, mRNA.	2,22	1,94

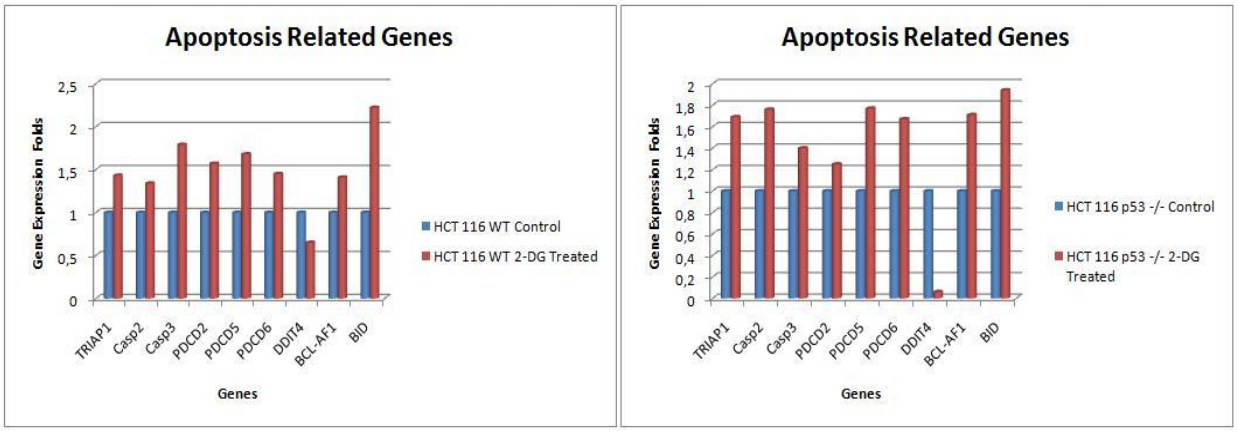


Figure 5. 14 Microarray Results: Expresion folds of the genes related to Apoptosis.

BCL-AF1 and BID belong to Bcl-2 protein family and these have regulatory roles in apoptosis just like the other members. BID is a well known proapoptotic member that has enhancing effects over apoptosis. BCL-AF1 (aka BTF) is a relatively less known member. A 2010 dated review gave an overview of findings from past studies of this protein and they leaned over its role as an inducer of apoptosis and repressor of transcription(116). Also it has been shown to interact with other Bcl-2 protein family members and its overexpression induces apoptosis(117).

5.1.11.2 Genes related to cellular metabolism

LDHA codes for lactate dehydrogenase A enzyme. This enzyme is responsible for the conversion of pyruvate into lactate. The decrease in expression is probably the

result of glycolysis inhibition upon 2-DG treatment, as you will see mot of the enzymes of the glycolytic pathway got downregulated.

COASY, coenzyme A synthase catalyze the last two steps in CoA synthesis. Coenzyme A derivatives play important roles in energy metabolism, especially Acetyl CoA. This enzyme gets in interaction with S6K(118) and so it might be another link between mTOR pathway and energy metabolism, apart from AMPK.

PDK3 code for an isozyme of pyruvate dehydrogenase kinase. It phosphorylates and inactivates pyruvate dehydrogenase complex which transform pyruvate into Acetyl-CoA linking glycolysis to citric acid cycle. It is decreased in both WT and p53 -/- HCT 116 cells. Interetingly it has been stated that the activation of pyruvate dehydrogenase complex(in this context, PDK downregulation helps for it) triggers apoptosis in cancer cells that selectively convert glucose to lactate(119).

METABOLISM			
		HCT 116 WT	HCT 116 p53 -/-
Gene	Definition	Fold(2-DG/C)	Fold(2-DG/C)
LDHA	Homo sapiens lactate dehydrogenase A (LDHA), transcript variant 1, mRNA.	0,73	0,45
COASY	Homo sapiens Coenzyme A synthase (COASY), transcript variant 4, mRNA.	1,34	1,37
PDK3	Homo sapiens pyruvate dehydrogenase kinase, isozyme 3 (PDK3), mRNA.	0,37	0,32
HK2	Homo sapiens hexokinase 2 (HK2), mRNA.	0,44	0,24
C12orf5 (TIGAR)	Homo sapiens chromosome 12 open reading frame 52 (C12orf52), mRNA.	1,79	1,43
C12orf5 (TIGAR)	Homo sapiens chromosome 12 open reading frame 5 (C12orf5), mRNA.	1,9	2,9
SCO2	Homo sapiens SCO cytochrome oxidase deficient homolog 2 (yeast) (SCO2),	2,21	1,79
	nuclear gene encoding mitochondrial protein, mRNA.		
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.	0,77	0,6
ACADM	Homo sapiens acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain (ACADM),	2,48	1,8
	nuclear gene encoding mitochondrial protein, mRNA.		

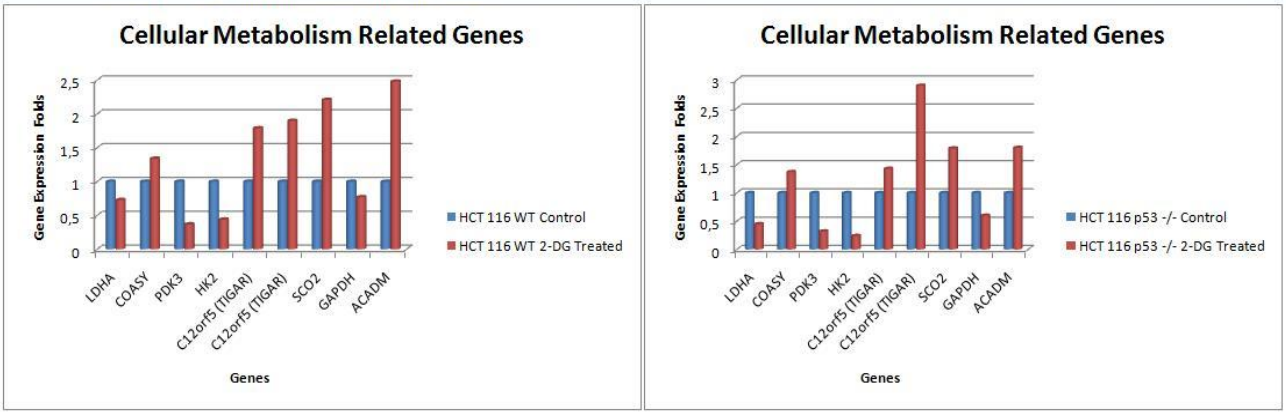


Figure 5. 15 Microarray Results: Expresion folds of the genes related to metabolism.

HK2 is the important enzyme here, as it interacts with 2-DG once it enters the cells. It commits glucose to the glycolytic pathway converting it to glucose 6-phosphate at the very beginning. HK2 gets downregulated after 2-DG treatment. One recent publication about its downregulation came up with the idea of a sensitization effect over cancer cells to chemotherapy(120).

C12orf5 codes for TIGAR, TP53-induced glycolysis and apoptosis regulator. A detailed analysis over its functions suggested that its effects are cell and context dependent and it gets activated by low levels of stress(47).

SCO2 is a mitochondrial protein acting as a copper chaperone to cytochrome c oxidase (COX) which is crucial for aerobic ATP production(121). We did not elaborate on the reason why it gets upregulated after 2-DG treatment.

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase catalyzes the sixth step of glycolysis. It is another important glycolysis enzyme showing decrease in expression levels after 2-DG treatment.

ACADM gene produces acyl-coenzyme A dehydrogenase enzyme that is crucial for fatty acid oxidation. During periods of fasting, the cells satisfy their energy need from fatty acids. It fits appropriately to the context as we inhibit glycolytic pathway by 2-DG treatment so that the cells look for alternative energy pathways.

5.1.11.3 Genes related to cell cycle

CDKN1B gives out the protein cyclin-dependent kinase inhibitor 1B(aka p27/kip1). Belonging to the Cip/Kip family of cyclin dependent kinase (CDK) inhibitor proteins, it controls cell cycle progression at G1(122).

CCNE1 and CCNB2 code for the cyclin family members E1 and B2, respectively. Cyclin E1 regulates G1-S transition but it is also shown to be having roles in cell proliferation and apoptosis regulation. It overexpression resulted in sensitization of hematopoietic cells to radiation(123). Cyclin B2 is also another cell cycle regulator which has been found to be upregulated in human cancers. In a 2010 dated study, it is observed that its expression levels decreased significantly after therapeutic treatments of cancer patients(124). In this study, after 2-DG treatment its expression levels decreased.

GADD45- A and B are growth arrest and DNA damage genes that are in charge of modulating cell cycle regulation and stress responses(125, 126).

CELL CYCLE			
		HCT 116 WT	HCT 116 p53 -/-
Gene	Definition	Fold(2-DG/C)	Fold(2-DG/C)
CDKN1B	Homo sapiens cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B), mRNA.	0,76	0,6
CCNE1	Homo sapiens cyclin E1 (CCNE1), transcript variant 1, mRNA.	1,61	1,44
CCNB2	Homo sapiens cyclin B2 (CCNB2), mRNA.	0,59	0,19
GADD45B	Homo sapiens growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA.	0,12	0,72
GADD45A	Homo sapiens growth arrest and DNA-damage-inducible, alpha (GADD45A), mRNA.	0,69	0,41

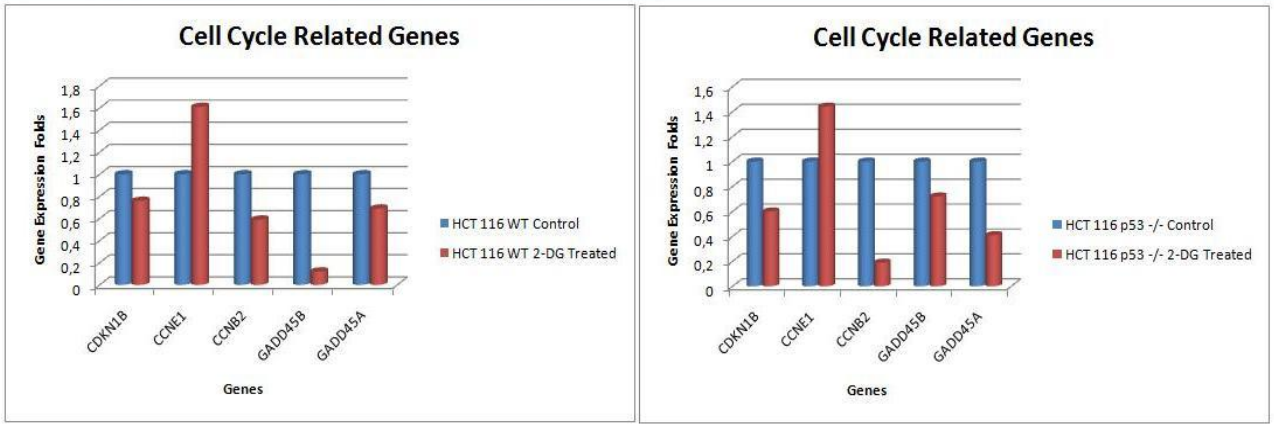


Figure 5. 16 Microarray Results: Expresion folds of the genes related to cell cycle.

5.1.11.4 Genes related to cell growth, proliferation and tumorigenesis

TPD52 gene produces tumor protein D52. It has been shown to be overespressed in ovarian and prostate cancer cells(127, 128).

EGR1 codes for early growth response 1 nuclear protein functioning as a transcription factor. It serves for transducing the proliferative signal upon its rapid induction by growth factors, and it has various roles in cell growth, programmed cell death and tumor progression(129). The decrease in the expression levels might mean that 2-DG may cause some kind of a growth arrest.

BRMS1 has the coding information of breast cancer metastasis-suppressor 1 protein. Identified in and named after breast cancer cells, this metastasis suppressor gene is involved in reducing the metastatic potential, blocking tumor cells' ability to spread and inhibits gene expression in cancer cells(130, 131, 132). The increased expression in this study for both WT and p53 -/- HCT 116 cell lines provide an interesting and promising result.

ING1 gene has the codes for inhibitor of growth protein 1. It is referred to as a candidate tumor suppressor(133). Downregulation of ING1 is associated with the sensitization of p53-deficient glioblastoma cells to cisplatin induced cell death(134).

CELL GROWTH, PROLIFERATION AND TUMORIGENESIS			
		HCT 116 WT	HCT 116 p53 -/-
Gene	Definition	Fold(2-DG/C)	Fold(2-DG/C)
TPD52	Homo sapiens tumor protein D52 (TPD52), transcript variant 3, mRNA.	0,66	0,52
EGR1	Homo sapiens early growth response 1 (EGR1), mRNA.	0,48	0,45
BRMS1	Homo sapiens breast cancer metastasis suppressor 1 (BRMS1), transcript variant 3, mRNA.	1,65	2,01
ING1	Homo sapiens inhibitor of growth family, member 1 (ING1), transcript variant 1, mRNA.	0,5	0,62
FRAP1	Homo sapiens FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), mRNA.	1,61	1,95
CAPRIN1	Homo sapiens cell cycle associated protein 1 (CAPRIN1), transcript variant 2, mRNA.	0,32	0,5
HIF1AN	Homo sapiens hypoxia inducible factor 1, alpha subunit inhibitor (HIF1AN), mRNA.	2,21	1,58

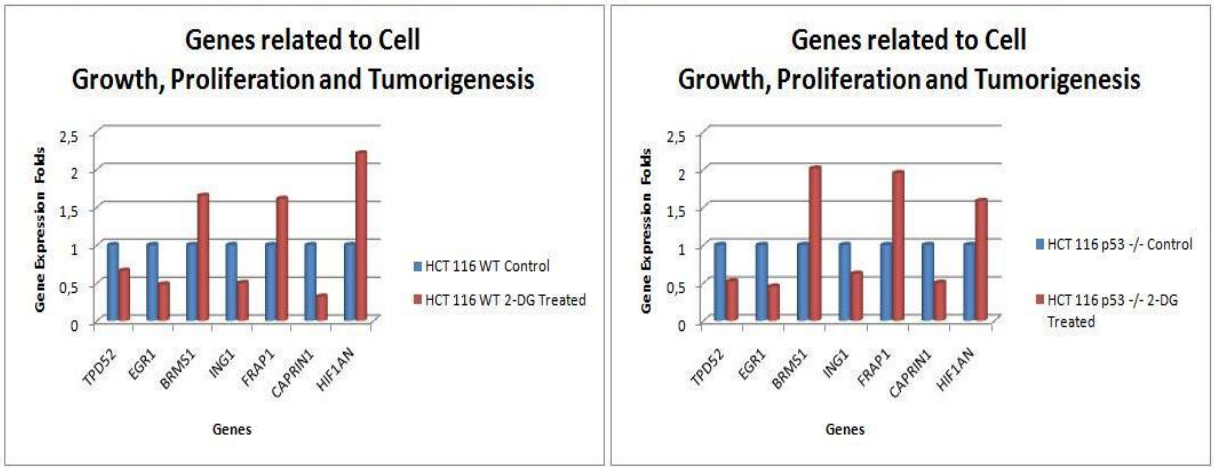


Figure 5. 17 Microarray Results: Expresion folds of the genes related to cell growth, proliferation and tumor formation.

FRAP1 codes for mammalian target of rapamycin, mTOR. Our western blot experiments gave out the decrease in activation of mTOR's downstream target p70 S6K and this is associated with inhibition of mTOR kinase activity(135). However, this is also interesting to see that it gets upregulated in the gene level after 2-DG treatment.

Cytoplasmic activation/proliferation-associated protein-1 is encoded by CAPRIN1 gene. This protein is associated with cellular proliferation, its suppression is shown to be responsible for slowing of the proliferation rate and prolongation of the G1 phase of the cell cycle(136). CAPRIN1 expression levels decreased in this study, which might explain the accumulation at G1 phase in cell cycle analysis.

It was pleasing to see another familiar name here as we detected HIF1AN upregulation. The gene codes for Hypoxia-inducible factor 1-alpha inhibitor (aka FIH-1). We talked about HIF-1 transcription factor and its effects over glycolysis, cell growth etc. The product of this certain gene HIF1AN works as an inhibitor of HIF-1 alpha therefore inhibiting the activities of a highly studied cancer metabolism related gene(137).

6. DISCUSSION AND CONCLUSION

One of the biggest problems of cancer treatment, and probably the most crucial one is that the treatments based on killing cancer cells also damage healthy cells in the organism. This drawback is eliminated to an extent with radiation therapy where a degree of specificity is achieved by localizing the radiation to the tumor(56). However, it is still needed to distinguish between cancer cells and normal cells for a treatment to provide the ultimate cure. This idea, and the urge for finding better ways to cancer treatment introduced some promising targets that specifically selects and affects cancer cells. One great target is, as the study of this project, glycolysis which is hailed as one of the hallmarks of cancer(53).

Starting with the studies of Warburg in the early 20th century and the progress coming with his contemporaries, cancer is linked to altered metabolism i.e. cancer cells switching to the glycolytic phenotype as they choose it over aerobic respiration for their metabolic requirements(1, 2, 3, 4). This altered metabolism is referred to as the Warburg Effect. Studying this phenomenon and targeting the Warburg effect in terms of an anticancer treatment gained attention and renewed interest in recent years. Glycolysis inhibition is infact a promising therapeutic strategy considering the increased dependence of cancer cells on glycolysis for their metabolic needs. It also serves for the idea of preferential killing of cancer cells without damaging the healthy ones in cancer chemotherapy(59).

There are various inhibitors and compounds targeting specific enzymes of the glycolytic pathway. 2-Deoxyglucose, a synthetic, non-metabolizable analogue of glucose is one of these compounds(69). It inhibits phosphorylation of glucose by hexokinase. 2-DG itself gets phosphorylated and this causes an accumulation of non-metabolizable 2-DG-P(59). It is currently at clinical trials.

In this present study, we chose 2-DG as our glycolysis inhibitor to see the effect of glycolysis inhibition as an anticancer strategy. We wanted to work on colon cancer as it is one of the most commonly diagnosed cancers. Therefore, colon cancer cell lines

HCT 116 WT, p53 -/- and Bax -/- were selected. That left us with the selection of anticancer agents. By diving the cell lines into two groups being WT | p53 -/- and WT | Bax -/-, we wanted to work on two different anticancer agents. For the first set of cells, we selected cisplatin which is a widely used chemotherapy drug exerting its activity by damaging DNA(138). For the other set, we went for TNF-related apoptosis-inducing ligand (TRAIL) which is a promising candidate for cancer treatments due to its therapeutic selectivity by inducing apoptosis through death receptors in malignant cells(139).

After the determination of dose and time for treatments, we started to check out 2-DG effect on the cells. Prior to cisplatin and Killer TRAIL treatments, the cells underwent a pretreatment session of 10mM 2-DG for 24 hours. Following this, they were given decided concentrations of anticancer agents for 48 hours. The expectation was to observe increased cell death due to sensitization of the cells and potentiation of the agents. The results were satisfying as cell death responses were significantly increased(FIG. 5.3 and FIG. 5.4). However, potentiation by 2-DG showed its first requirement: the apoptotic signaling must be over a threshold that is the used anticancer agent must start an apoptotic response causing a considerable amount of cell death. That is why 2-DG did not potentiate lower concentrations(lower than 12μM) of cisplatin. It also did not potentiate Killer TRAIL for HCT 116 Bax -/- cells because of no proper apoptosis induction due to that cell line's resistance.

The sensitization effect was further confirmed with the proliferation assay for the first set. The DNA content of HCT 116 WT and p53 -/- cells were checked after the treatments. 2-DG enhanced the antiproliferative properties of cisplatin, causing a significant decrease in DNA content after combined use(FIG. 5.5). We did not perform the proliferation assay for the other set because even though 2-DG sensitized HCT 116 WT cells to Killer TRAIL-induced cell death, we couldn't sensitize HCT 116 Bax -/- cells. Therefore, that set has entered a different path. We wanted to sensitize Bax -/- cells and to do that we tried two completely different compounds for different purposes. One of them was 3-methyladenine, a compound used for autophagy inhibition. This was selected to prevent autophagic cell survival if there ever was. The other compound was the BH3 mimetic ABT-737 working as an inhibitor of Bcl-2 and Bcl-XL. The idea behind selecting this one was to inhibit Bcl-2 and Bcl-XL, the antiapoptotic members of the Bcl-2 protein family, so that their negative regulation of apoptosis induction could be stopped if they were responsible. However, in both cases

HCT 116 Bax -/- cells showed that same resistance to Killer TRAIL(FIG. 5.6). Treatment of HCT 116 WT cell lines with the same compounds did not provide anything either(FIG. 5.7).

To check the apoptosis markers on protein level, we performed western blotting experiments. We decided to look for caspase 3 activation for the first set and caspase 8 activation for the second because cisplatin and Killer TRAIL induce apoptosis by different pathways. Cisplatin induces apoptotic signaling via intrinsic (mitochondrial) pathway whereas Killer TRAIL cause apoptosis from extrinsic pathway through death receptors. Each experiment showed caspase cleavage therefore confirming apoptosis induction(FIG. 5.8). BID, a proapoptotic member of Bcl-2 protein family, was also checked for the second set as caspase 8 activation cause the truncation of BID. HCT 116 Bax -/- cells did not show apoptosis induction as BID remained intact in all the samples, unlike HCT 116 WT.

BID opened the way for the idea of taking a look at some of the Bcl-2 protein family members. At this part of the study, we left the anticancer agents behind us and put the emphasis on 2-DG alone to understand the mechanisms as to how it sensitizes the cells to the anticancer agents. With the very first try we obtained an important result with Mcl-1, one of the antiapoptotic members of the family. 2-DG treatment caused a decrease in Mcl-1 protein levels for all three cell lines, HCT 116 WT, p53 -/- and Bax -/-(FIG. 5.9). This was the second successful step after the flowcytometric analysis of the sensitization effect of 2-DG. At this point we thought of Mcl-1 as the highly possible key player of the sensitization effect, because of its unique properties unlike other antiapoptotic members. A detailed literature research gave us the reason why Mcl-1 could be so important: first it has a very short life which helps for its being highly regulated and second in the name of its role in apoptosis regulation Mcl-1 is beyond other antiapoptotic members because its expression can be stimulated by growth factors and it has connections to cell cycle control(103). These attributes make it a pivotal survival protein having roles in normal animal development(140). We also leaned over some other Bcl-2 family members that have interaction to Mcl-1. However, protein levels of antiapoptotic Bcl-2 and proapoptotic Bim and Bax were all unaffected(FIG. 5.9).

Assessment of ATP levels was always in our minds just to show the effect of 2-DG over the energy metabolism. We performed ATP assay with all three cell lines after 24 hours of 2-DG treatment. But here, the ATP concentrations showed very little or no

decrease. Therefore, we wanted to see an early response and checked the changes after 5 hours of 2-DG treatment(FIG. 5.10). For this condition, ATP concentrations were decreased 20% in each cell line. Actually we expected a decrease for 24-hour treatment as well in the first place. However, the medium used for the cells (McCoy's 5A modified medium) had glucose and L-glutamine as ingredients. 2-DG effectiveness is affected to an extent by the presence glucose as both compete for phosphorylation, and there was also something we underestimated: the alternative energy sources such as fatty acids and amino acids(especially L-Glutamine)(59). Therefore, the cells probably overcame the ATP depletion by alternative energy pathways, however, the ATP depletion in the early hours did actually turn into signals. With western blotting experiments, we wanted to confirm the ATP decrease by checking the phosphorylation level of AMPK, the energy sensor protein. Phospho-AMPK levels increased after 2-DG treatment (FIG. 5.10) for all three cell lines which was what we wanted, because when ATP levels decrease AMPK gets phosphorylated(activated) and signals for this stress condition(31). Being an ancient metabolic regulator found in all eukaryotes, AMPK couples energy status to growth signals(32, 1). It provides a metabolic checkpoint and regulates the cellular response to energy availability(1). One other feature is that it inhibits mTOR resulting in inhibition of cell proliferation under energetic stress conditions. This makes AMPK signaling another candidate mechanism for 2-DG-induced sensitization. Apart from confirming the ATP decrease, AMPK activation also led us to check the mTOR pathway as it was shown to be responsible for mTOR inhibition in a similar study(36).

To check the mTOR inhibition we had several alternative ways. mTOR signaling regulates cell growth, proliferation, metabolism and survival by controlling protein synthesis(mRNA translation, ribosomal biogenesis)(43). mTOR directly phosphorylates ribosomal protein S6 kinase(p70 S6K), eukaryotic initiation factor 4E binding protein 1(4E-BP1) and eukaryotic elongation factor 2 kinase(EEF2K), all of which have crucial roles in protein synthesis and help mTOR in the regulation of cell growth(43). Coming from this point, a possible mTOR inhibition should decrease the phosphorylation levels of these three regulators. We were thinking of performing a western blot, and p70 S6 kinase was selected because of its importance as a translation regulator(37). Figure 5.11 has the results of the western blot experiment, phospho-p70 S6K antibody was used to detect activated p70 S6 kinase. It was decreased for all cell

lines' 2-DG samples, confirming the inhibition of mTOR as the phosphorylated p70 S6 kinase protein levels were reduced.

Considering the potential connection of Mcl-1 to cell cycle control, we performed cell cycle analysis in order to see the distribution and how 2-DG treatment did affect the overall process. All cell lines showed further accumulation at G1 phase in comparison to control samples, suggesting a cell cycle arrest at this phase(FIG. 5.12). p53 has well known functions in controlling cell cycle arrest, so we checked its protein levels. It was not present in HCT 116 p53 -/- cells, but it showed a decrease in other cell lines. Absence in one and reduction in the other two kept it in discussions that p53 might play an important role. We did a literature search about p53 roles in cell cycle control and metabolism to obtain details. What we got out of this is that p53 gets in contact with AMPK and mTOR. It works together with AMPK for the induction of a cell cycle check point, causing cell cycle arrest under energy related stress conditions(46). Also, p53 interacts with mTOR in the regulation of cell growth. Down-regulation and/or absence of p53, on the other hand might be understood as a contribution to cancer cells in the name of reducing (or eliminating) the guardian of the cell, the tumor suppressor. And inactivation of p53 is like a common feature in many cancers, however its absence under a metabolic stress condition may sensitize the tumor cells(54). In the end it all comes down to the double nature of p53, again.

To make sure about the contribution of Mcl-1 to 2-DG-induced sensitization effect, we performed overexpression experiments. We did transient transfection of HCT 116 WT and p53 -/- cells with an Mcl-1 overexpression vector and then we followed the same treatment procedures with 2-DG and cisplatin. The results were highly successful as Mcl-1 overexpression resulted in a significant decrease in cell death numbers(FIG. 5.13). With this result, Mcl-1 guaranteed its place in this study as the leading actor of the sensitization effect.

RNA Microarray analysis was done to provide us information on the changes of gene expression levels after 2-DG treatment. Control and 2-DG treated RNA samples from HCT 116 WT and p53 -/- were analyzed. The selected genes were divided in four groups in terms of their activity: apoptosis related, cell cycle related, metabolism related and growth-proliferation-tumorigenesis related. The list was created with the following criteria: a p-value for differential gene expression of 0.05 or better and changes that happened in both WT and p53 -/- samples. Apart from the list, we did not observe the names of Mcl-1 and p53 among the genes, this gave us the idea that the changes for

these most probably happened at the protein level. AMPK activation is shown to cause Mcl-1 block in translation(36). And also there is the fact that mTOR inhibition should cause problems at protein synthesis. A 2009 dated paper stated that AMPK-mTOR pathway controls Mcl-1 levels by controlling its translation(36). We also showed AMPK activation and mTOR inhibition correlating with their findings.

Going back to the actual list under the group of apoptosis related genes(FIG. 5.14), we observed that 2-DG induced the expression of caspases 2 and 3 along with programmed cell death proteins 2, 5 and 6. It also induced the expression of two proapoptotic Bcl-2 family members Bcl-AF1 and BID. These can be explained as a preparation for apoptotic signaling.

There were a couple of highly important metabolic enzymes in the list of genes related to cellular metabolism(FIG. 5.15). Hexokinase 2 downregulation was spot on as the first enzyme of glycolysis got downregulated after 2-DG treatment. GAPDH and LDHA were the other glycolytic enzymes showing downregulation. ACADM upregulation was the confirmation of the cells' search for alternative energy sources as it is important for fatty acid oxidation. TIGAR, negative regulator of glycolysis was upregulated. Pyruvate dehydrogenase kinase (PDK3) was downregulated, it is an important enzyme contributing to glycolysis by phosphorylating and inactivating pyruvate dehydrogenase complex which converts pyruvate into Acetyl-CoA. COASY and SCO2 upregulation might mean some kind of a revival in the mitochondrial energy pathway, forcing the cells to go for oxidative phosphorylation. This also made us think of ROS production as a cause of sensitization to apoptosis because of the sudden abnormal activation of the mitochondrial energy pathway. p53 has roles in the regulation of oxidative phosphorylation and ROS production as well as activating an antioxidant response against oxidative stress(54). A reduction in (or absence of) p53 protein levels may cause inability to prevent ROS production. These all suggest that 2-DG causes serious changes in the expression of some important metabolic enzymes and it basically targets cellular metabolism.

Cell cycle related genes showed downregulation(FIG. 5.16): CDKN1B, CCNE1, CCNB2, GADD45A, GADD45B. Growth arrest and DNA damage(GADD45) genes are shown as p53 targets and they are responsible for cell cycle arrest control(45). The effect on cell cycle should be studied in details as dysregulation of the cell cycle might contribute to sensitization. In this study, we had shown cell cycle arrest at phase G1 after 2-DG treatment.

HIF1 alpha came into prominence out of the list of genes related to cell growth-proliferation-tumorigenesis(FIG. 5.17). We did not observe its name among the genes actually, so there was not any indication of it being upregulated or downregulated. However, we observed the upregulation of HIF1AN (aka FIH-1) which inhibits the activity of HIF1(13). HIF1, hypoxia-inducible factor 1, works as a transcription factor regulating glucose metabolism by stimulating glytcolytic energy production by transactivating genes such as LDH and PDK(which can be spotted in our list under genes related to cell metabolism), but it also regulates glycolysis by downregulating oxidative phosphorylation within the mitochondria(13). Therefore, considering the upregulation of HIF1AN, we can say that 2-DG treatment causes HIF-1 inhibition via the induction of its inhibitor. This in turn results in the downregulation of glycolytic enzymes and upregulation of some mitochondrial metabolic enzymes. HIF-1 inhibition might also relate to ROS production as when it is active it is responsible for the decreased mitochondrial function meaning decreased ROS production(13). In this case, HIF-1 inactivation may cause an increase in the formation of reactive oxygen species.

HIF-1 interacts with both mTOR and p53. mTOR is actually an upstream activator of HIF-1, seeing both getting inhibited after 2-DG treatment is so appropriate. HIF-1's interaction with p53 has been shown as low level of p53 attenuates HIF-1 transactivation(141).

The other significant gene expression changes were the suppression of proliferation-tumorigenesis related genes such as CAPRIN1, TPD52, EGR1, ING1 and the potential causes of these changes are slowing of the proliferation rate and growth arrest. mTOR showed an upregulation in the gene level which was interesting and might be some kind of a recovery mechanism to take over following its inhibition at protein level. Another interesting finding was to observe BRMS1, a tumor suppressor, getting upregulated after 2-DG treatment. This metastasis suppressor gene may contribute to the sensitization effect.

Collectively, these results gave an idea of how 2-DG sensitizes HCT 116 colon cancer cell lines and potentiates apoptosis inducer anticancer agents, cisplatin and Killer TRAIL. AMPK activation, mTOR inhibition, HIF-1 inhibition, cell cycle arrest, Mcl-1 downregulation all contribute to the sensitization effect from various perspectives(FIG. 6.1).

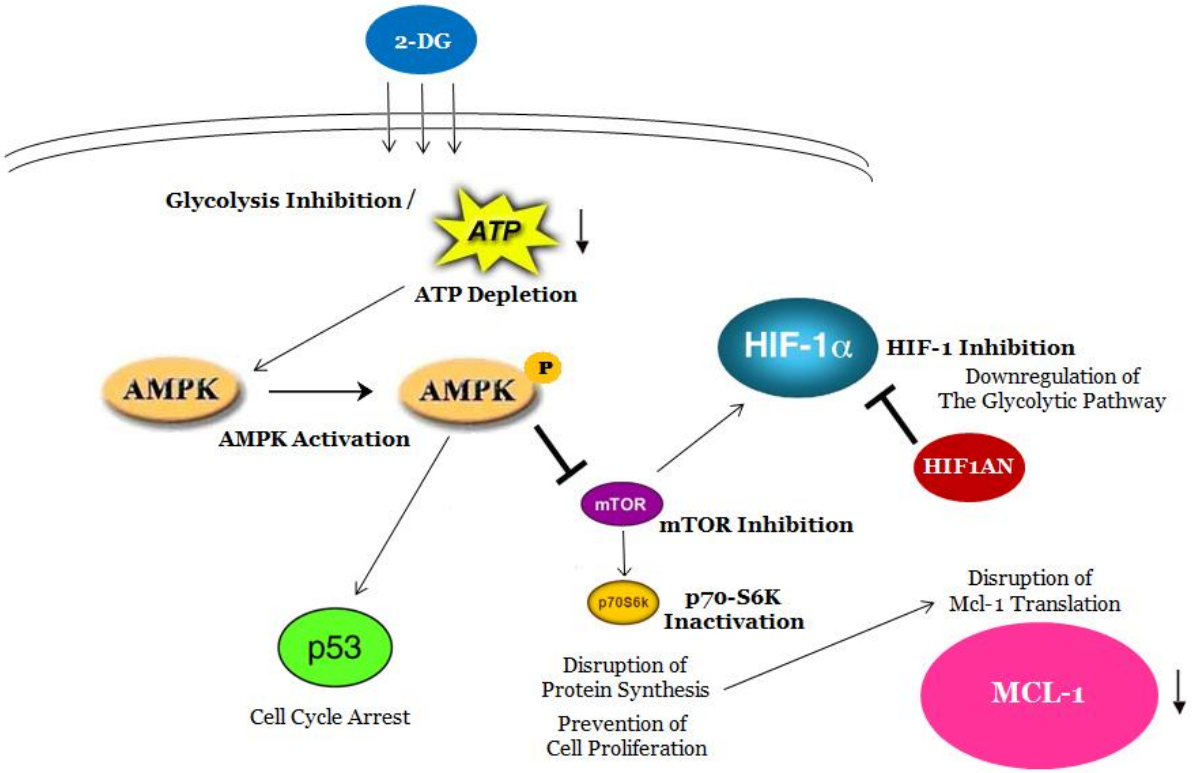


Figure 6. 1 A Schematic Presentation as to how 2-Deoxyglucose Treatment Effect Certain Signaling Pathways Leading to Sensitization of Cancer Cells.

Coming all the way from Warburg's first observations, targeting cancer metabolism has gained renewed interest in cancer therapy. Glycolytic inhibitors is now seen as potential anticancer agents. Several of them are already at various stages of pre-clinical and clinical development(10). With each new study over the Warburg effect, cancer cell metabolism is explored deeper and so making it even more exciting for designing new therapeutic methods that might ultimately give what scienctist want: win the war against cancer.

7. FUTURE STUDIES

Clinical significance of cancer's glycolytic phenotype has proved itself with the development of F-fluorodeoxyglucose positron emission tomography (F-FDG-PET). It gave an enormous contribution to cancer treatment as the system bases itself upon cancer cells' increased glucose uptake and metabolism. Relying on the Warburg effect, F-FDG-PET is used successfully in the diagnosis, staging and prognosis of cancer(53). After the injection of radioactive pharmaceutical fluorodeoxyglucose (FDG), the sister molecule of 2-DG, PET scanner forms images of the distribution of FDG around the body. FDG, just like 2-DG, is an anti metabolic glucose analog. Once it gets inside the cells and gets phosphorylated, it can not be further metabolized. Therefore it causes an accumulation of phosphorylated FDG molecules inside the cells with higher glucose uptake.

The use of F-FDG-PET scan in clinical oncology consolidates glycolytic metabolism as one of cancer's most vulnerable phenotype. Therefore, targeting cancer cell metabolism is as promising as ever. Glycolysis inhibition proves itself as a promising therapeutic anticancer strategy. More clinical studies should be done for the further development of this strategy.

For the future of this particular study, we are thinking of expanding it to several other cancer cell lines such as breast cancer cells and leukemia cells. We are considering to work on other glycolysis inhibitors as well for comparative analysis. If we go into some specific details, the role of hypoxia inducible factor 1 (HIF-1) should be analyzed thoroughly. The proteins responsible for cell cycle control should be studied in order to understand their contribution to cellular metabolism. And of course we will select and go for the genes that came out of our microarray analysis, especially the ones that are related to cell growth and tumorigenesis. There can be some important aspects hiding in the background but responsible for cancer cells' metabolic switch.

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APPENDIX A

Name of Antibody	Supplier Company	Catalog Number
2-Mercaptoethanol	Fluka, Switzerland	63700
Acrylamide/Bis-Acrylamide	Sigma, Germany	A3699
Agarose	AppliChem, Germany	-
Ammonium persulfate	Sigma, Germany	A3768-25G
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Glycine	Molekula, UK	M10795955
HCl	Merck, Germany	100314
Hepes	Molekula, UK	M55704197
Isopropanol	Merck, Germany	1009952500
KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haén, Germany	4243
Liquid nitrogen	Karbogaz, Turkey	-
Methanol	Sigma-Aldrich	24229
Na_2HPO_4	Merck, Germany	1065791000
NaCl	Duchefa Biochemie	S05205000
Phosphatase Inhibitor coctail tablet	Roche, Germany	4906837001
PMSF	Amresco®,USA	0754-25G
Protease Inhibitor coctail tablet	Roche, Germany	4693124001
TEMED	Sigma, Germany	T7024-100ml
Tris	Molekula, UK	M11946779
Trypsin/EDTA	PAN, Germany	P10-0231SP
Tween 20	Molekula, UK	18945167

APPENDIX B

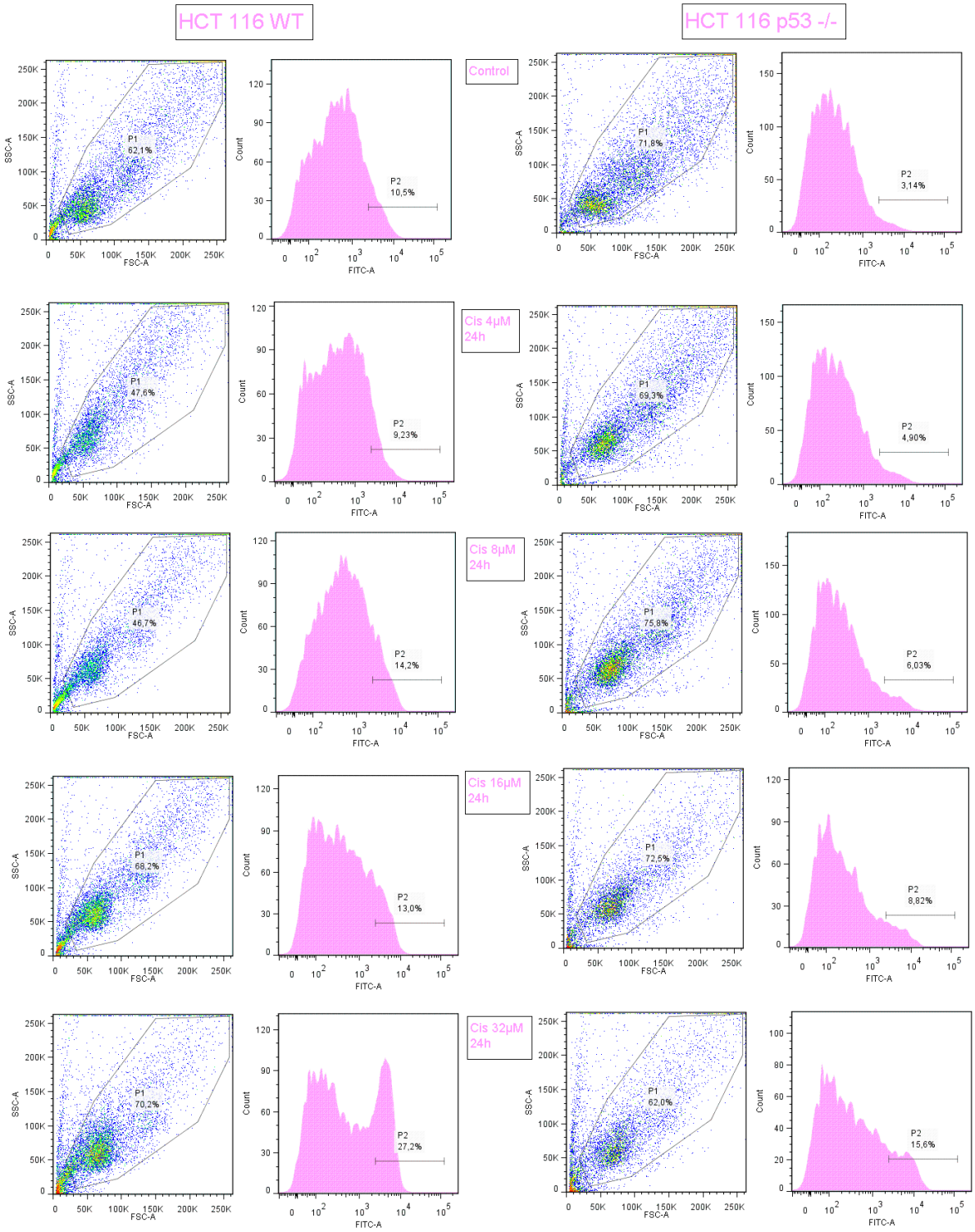
Name of Antibody	Supplier Company	Catalog Number
Anti Mcl-1	Cell Signal Tech., USA	4572
Anti Bcl-2	Cell Signal Tech., USA	2870
Anti Bim	Cell Signal Tech., USA	2933
Anti BID	Cell Signal Tech., USA	2002
Anti β-Actin	Cell Signal Tech., USA	4970
Anti p53	Cell Signal Tech., USA	9282
Anti Bax	Cell Signal Tech., USA	2772
Anti cleaved caspase 3	Cell Signal Tech., USA	9661
Anti caspase 8	Cell Signal Tech., USA	9746
Anti Phospho-AMPKα (Thr172)	Cell Signal Tech., USA	2535
Anti Phospho-p70 S6 Kinase (Thr389)	Cell Signal Tech., USA	9234

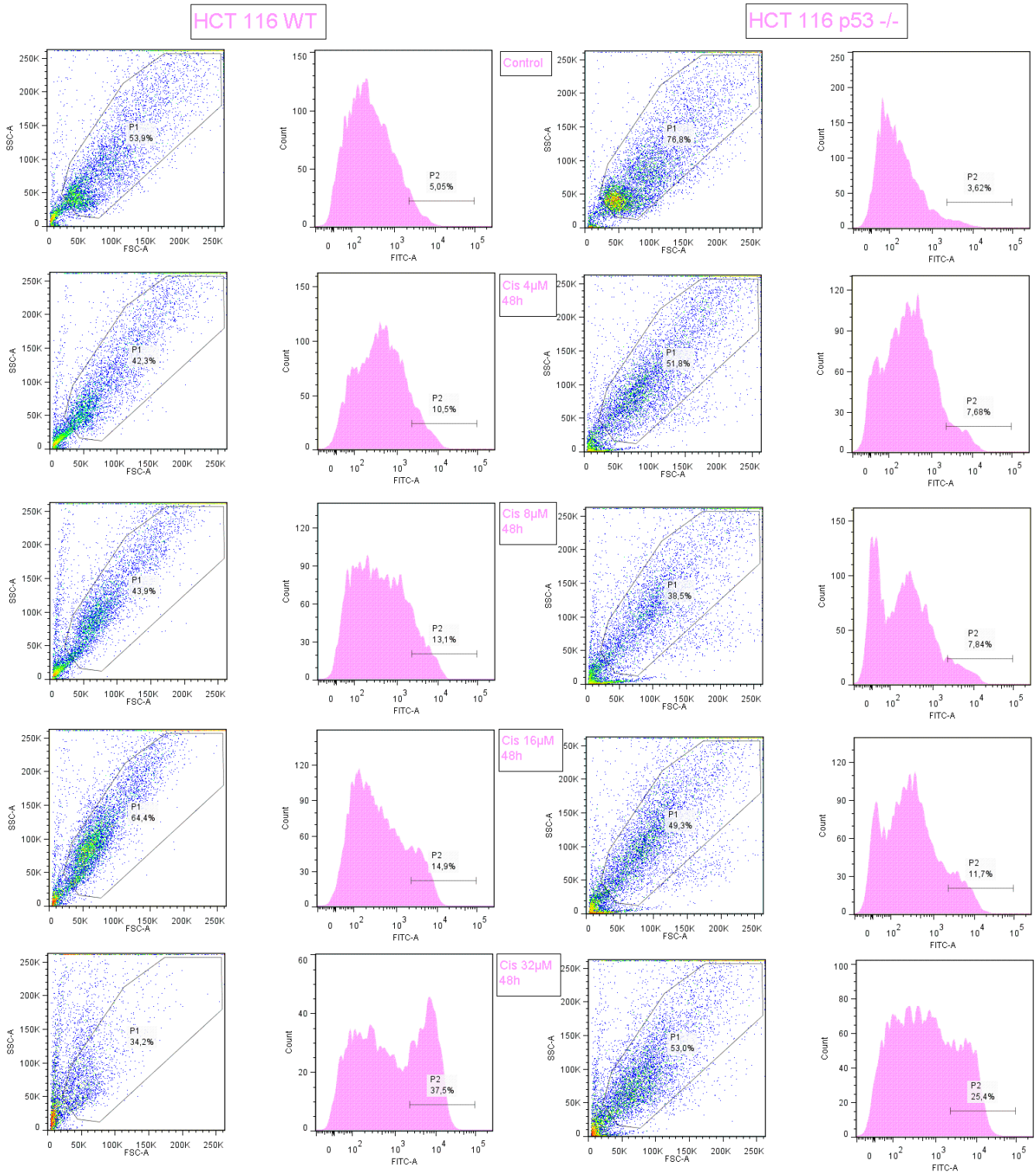
Appendix C

Name of Material	Supplier Company	Catalog Number
ECL Advance Chemiluminescence	Amersham Biosciences, UK	RPN2135
Blocking agent	ECL Advanced TM blocking agent	CPK1075
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
PVDF membrane	Roche, Germany	3010
Developer Solution	Agfa, USA	-
Bradford Solution	Biorad Inc., USA	500-0006
Whatman Papers	-	-
Page Ruler Prestained Ladder	Fermentas, Germany	#SM1811
Plasmid pCMV-Flag-hMcl-1	Addgene	25392
McCoy's 5A Medium (Modified)	Pan Biotech, Germany	-
Fetal Bovine Serum	PAN, Germany	-
Penicilin/Streptomycin	PAN, Germany	P06-07100
Genopure Plasmid Midi Kit	Roche	03143414001
X-tremeGENE 9 Reagent	Roche	-
CyQUANT Cell Prolif. Assay	Invitrogen	-
ATP Bioluminescence Assay Kit HS II	Roche	11699709001

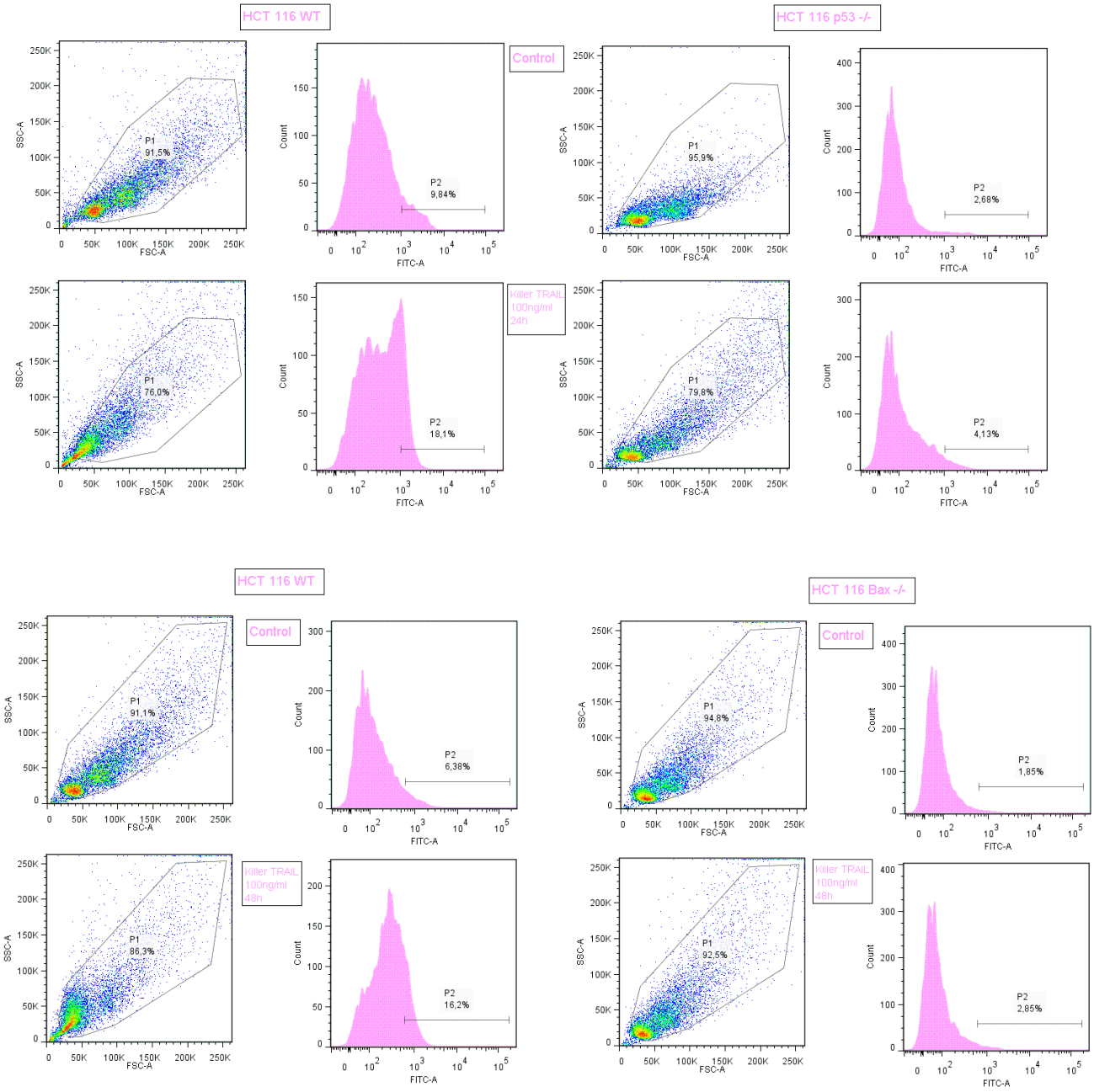
Appendix D

Part of Figure 5.1: Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.

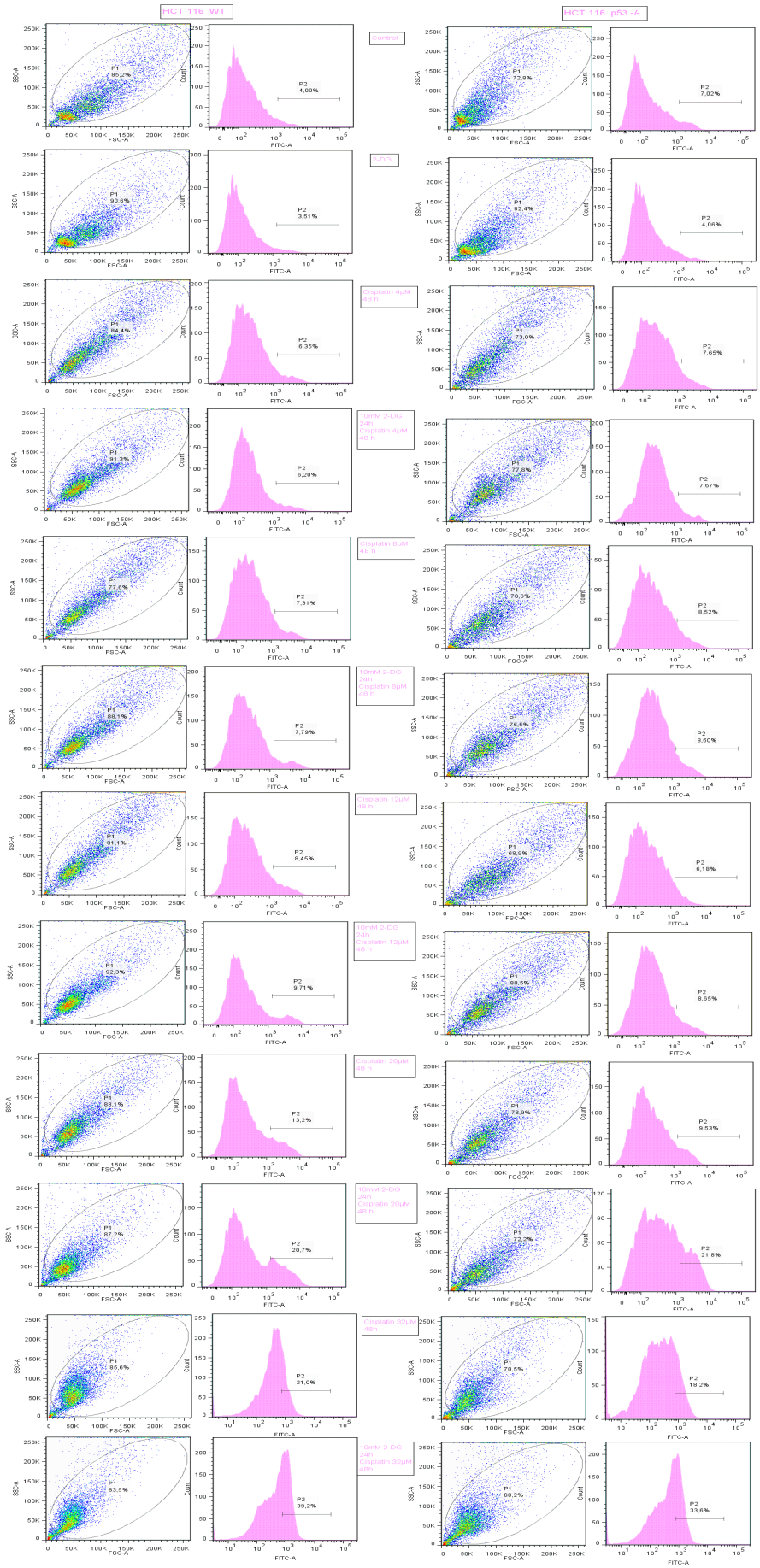



HCT 116 WT
HCT 116 p53 -/-
Control
P1
53,9%
P2
5,05%
P1
76,8%
P2
3,62%
Cis 4µM
48h
P1
42,3%
P2
10,5%
P1
51,8%
P2
7,68%
Cis 8µM
48h
P1
43,9%
P2
13,1%
P1
38,5%
P2
7,84%
Cis 16µM
48h
P1
64,4%
P2
14,9%
P1
49,3%
P2
11,7%
Cis 32µM
48h
P1
34,2%
P2
37,5%
P1
53,0%
P2
25,4%
SSC-A
FSC-A
Count
FITC-A

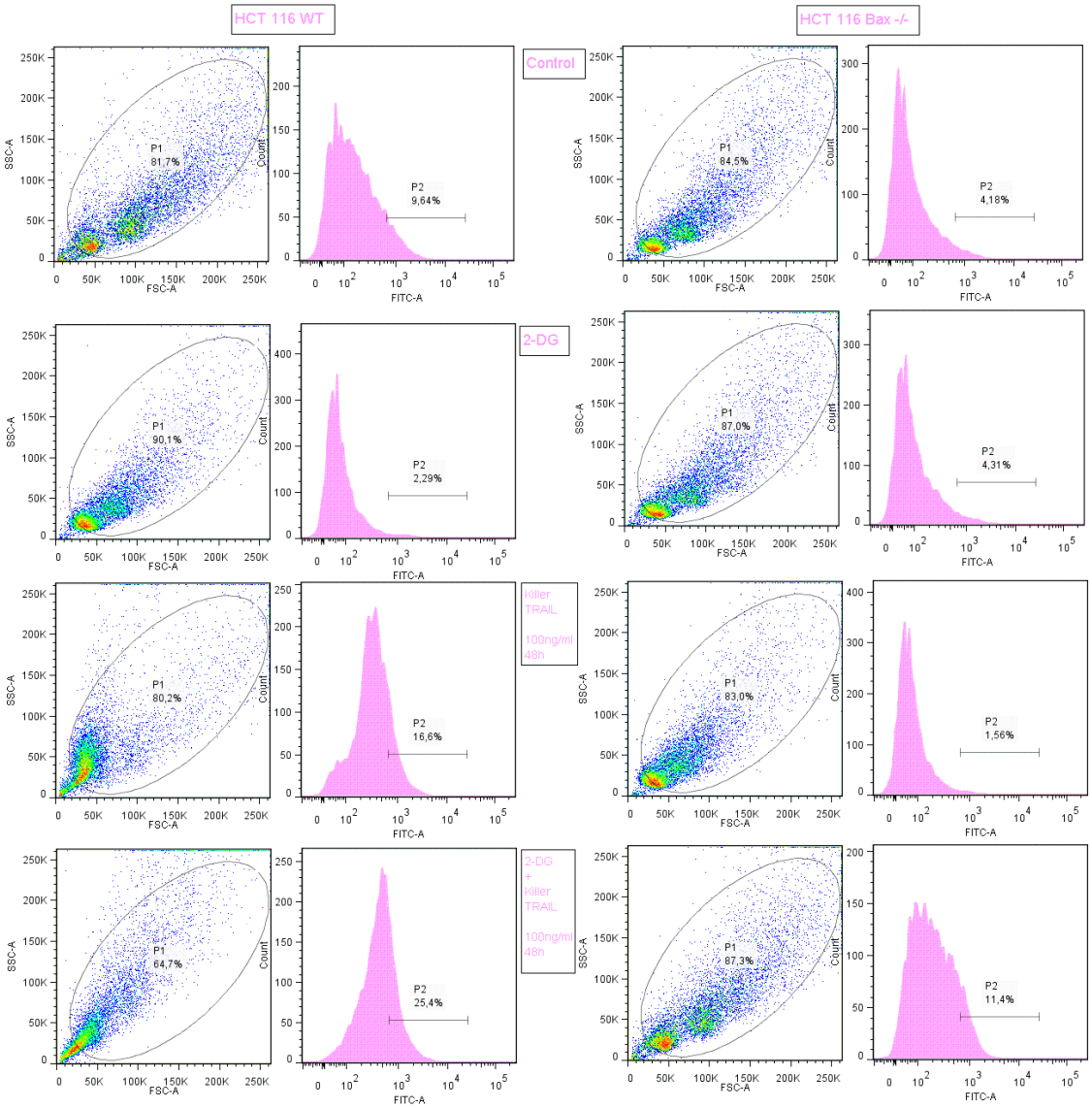
Part of Figure 5.2:
Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.



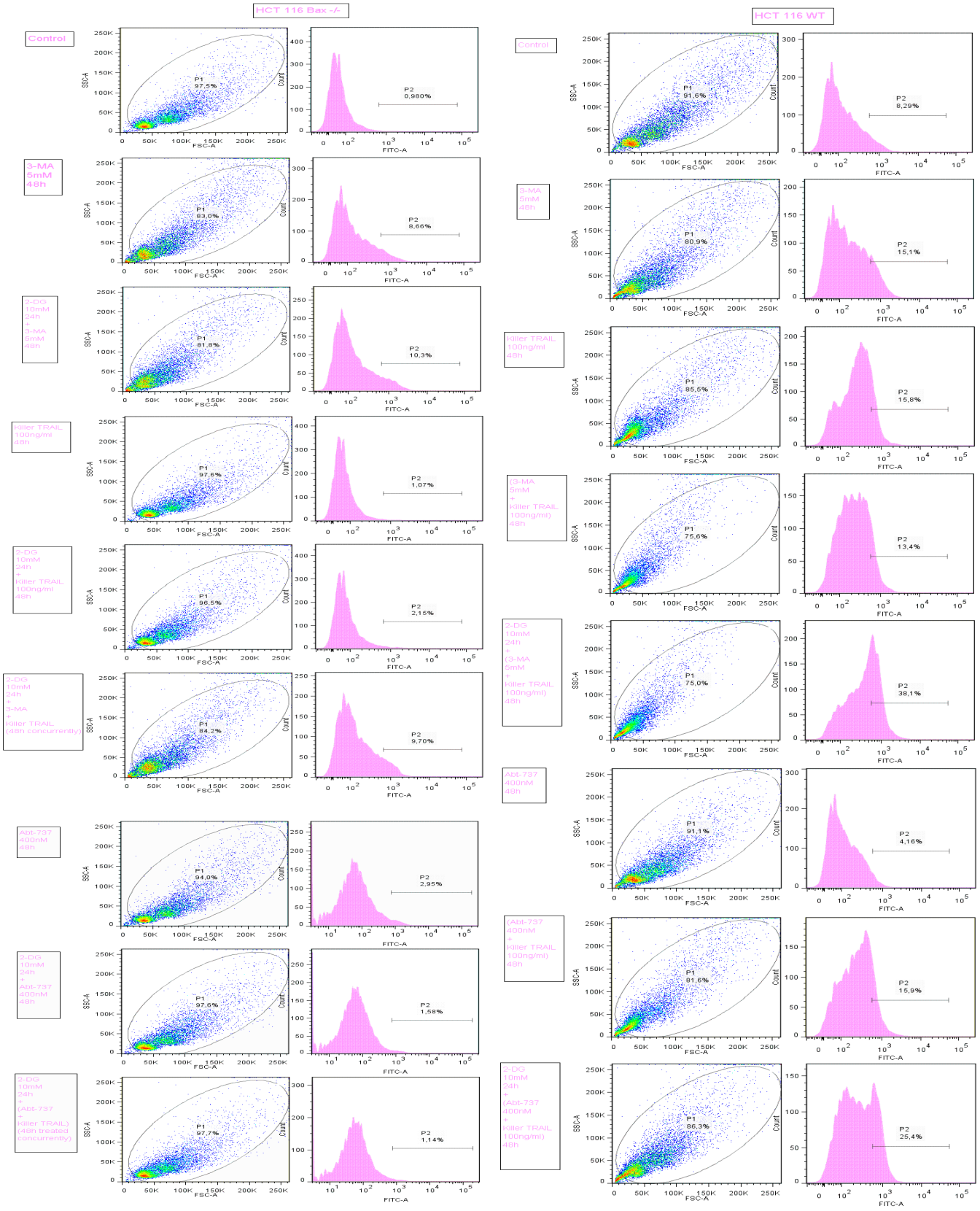
Part of Figure 5.3: Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.



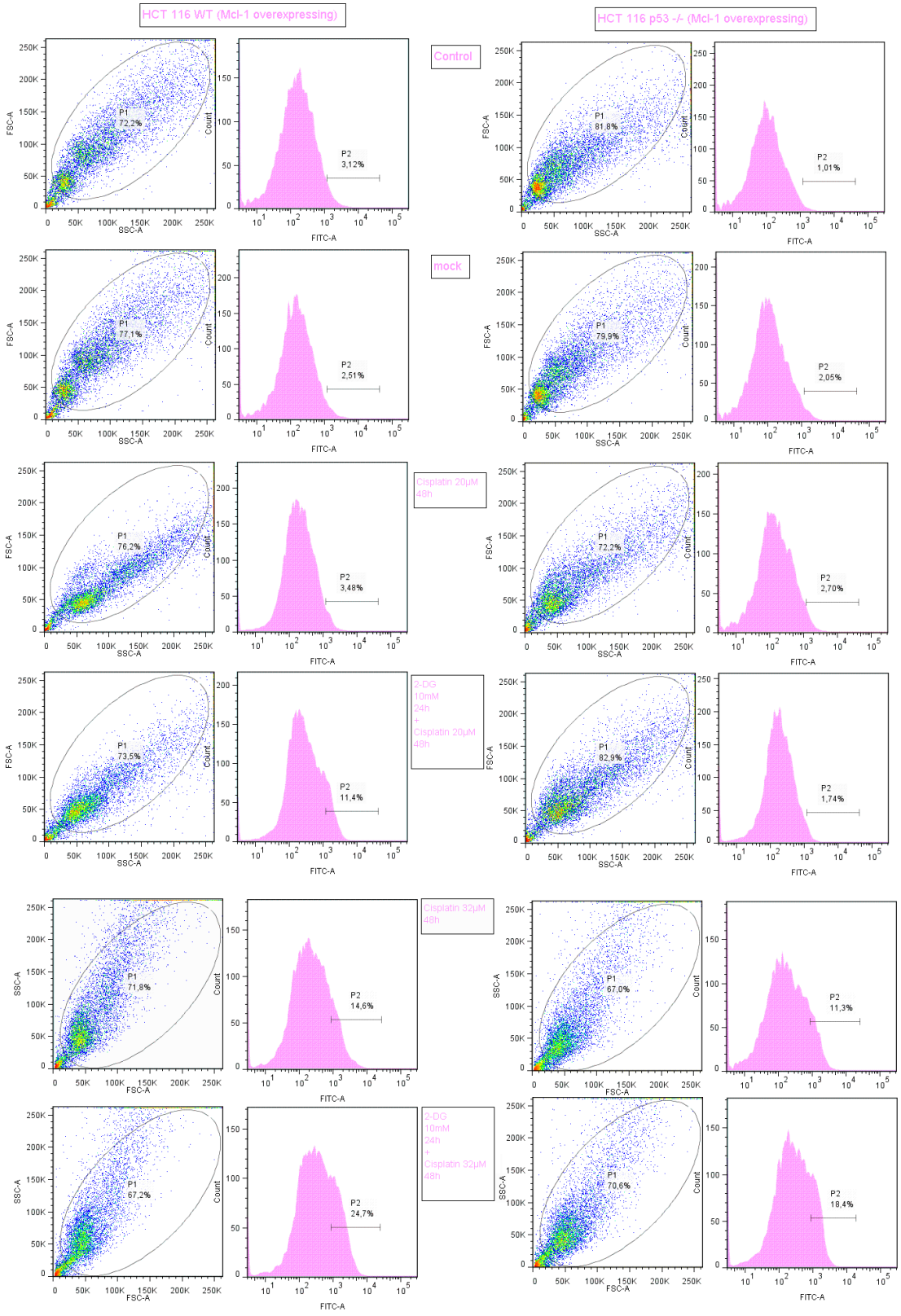
Part of Figure 5.4:
Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.



Part of Figure 5.6 – 5.7:
Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.



Part of Figure 5.13:
Detailed flow-jo graphs of populations selected for analysis and the histograms showing the shift in the intensity of fluorescence dye between control and cisplatin treated cells.



APPENDIX E

Autoclave:	Hirayama, Hi-Clave HV-110, JAPAN
Balance:	Sartorius, BP211D, GERMANY
Centrifuge:	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod. Heraues Multifuge 3L, GERMANY
Deepfreeze:	-70^{0}C, Kendro Lab. Prod. Hearus Hfu486 Basic, GERMANY
	-20^{0}C, Bosch, TURKEY
Distilled Water:	Milipore, MiliQ Academic, FRANCE
Electrophoresis:	Biorad Inc., USA
Flow Cytometry:	BD FACS Canto™, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
Laminar Flow:	Kendro Lab. Prod. Heraues, HeraSafe, HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
Microliter Pipette:	Eppendorf, Research
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4o C, Bosch, TURKEY
Shaker:	Stuart® Gyratory rocker, SSL3, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Water Bath:	Huber, Polystat cc1, GERMANY