Investigating the Role of Novel Fusion Proteins of Interferon in Melanoma

A dissertation presented to

the faculty of

the College of Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy

Elroy C. Fernandes

August 2010

This dissertation titled

Investigating the Role of Novel Fusion Proteins of Interferon in Melanoma

by

ELROY C. FERNANDES

has been approved for

the Department of Chemistry and Biochemistry

and the College of Arts and Sciences by

Susan C. Evans

Associate Professor of Chemistry and Biochemistry

Benjamin M. Ogles

Dean, College of Arts and Sciences

ABSTRACT

FERNANDES, ELROY C., Ph.D., August 2010, Chemistry and Biochemistry

Investigating the Role of Novel Fusion Proteins of Interferon in Melanoma (117 pp.)

Director of Dissertation: Susan C. Evans

Melanoma is an aggressive form of skin cancer with high occurrence in the United States. Interferon α2b (IFNα2b/IFNα2) has been used in high doses to treat melanoma. However, problems associated with small therapeutic proteins, such as with interferon treatment, include degradation by serum proteases and rapid kidney clearance because of small molecular size. Pegylation increases the size of the molecule but creates a host of other issues, such as decrease receptor binding, non-specific chemical derivatization, low overall yields and additional purification steps. In this study we used an alternative approach of IFN produced as an arabinogalactan fusion protein in plant cells. These IFN analogues bind to IFN receptors and follow the IFN induced JAK-STAT signaling pathway in melanoma cells. Experiments also demonstrate that these fusion proteins of higher molecular weight cause similar growth inhibition and affect cell cycle distribution. Further, the fusion proteins increased translation of 2'5' OAS1 and PKR, known IFN induced proteins, showing similar downstream signaling as native recombinant IFNα2. The tumor suppressor p53 gets activated in response to DNA damage and has interferon stimulated response elements (ISREs) in its promoter region and hence can be induced by IFN. Additionally, it has a significant role in mediating apoptosis by activating several intracellular pathways as well as up regulating proteins involved in cell cycle arrest. In this study we show that the fusion analogue IFNα2-

$(SO)_{20}$,as well as recombinant IFNα2b, were able to stabilize p53 protein levels and its pro-apoptotic target Bax. Also, there was a decrease in HDM2 levels, the negative regulator of p53. These results suggest that p53 is a downstream signaling target of IFNs and has a possible role in IFN mediated effects in these melanoma cells.

Approved: _____

Susan C. Evans

Associate Professor of Chemistry and Biochemistry

ACKNOWLEDGMENTS

There are several people that I would like to thank who have helped me during the last five years of graduate school both with my research endeavors as well my personal growth and success.

Firstly, I would like to thank my research advisor Dr. Susan Evans, for giving me the opportunity to work on this project. She has been a good mentor and friend and has always helped me with insightful discussions, experimental techniques and reviewed my work. Also, she has given me the freedom to plan my research, pursue ideas and pace my project to completion.

I would also like to extend my sincere gratitude to Dr. M. Kieliszewski, who in addition to being a collaborator and committee member has helped me tremendously during my research work with valuable suggestions and manuscript review.

In addition, I also thank my committee members Drs. Jackson and Chen for their insight and suggestions on my research. I am grateful to Dr. Kohn and Dr. A. Weeraratna for providing the melanoma cell line. Also, Dr. Kohn, Dr. K. Mccall and Dr. Kopchick for allowing me to use instruments within their laboratories for my experiments. My lab mates Eroica Soans, Dr. Yan Liu and Shuhua Du deserve special mention for all the experimental help and meaningful discussions. Former lab mates Dr. Martin Schmerr, Dr. Chrisanne Dias and Dr. Bernard Ayanga helped me get started when I first joined the lab in 2005. I am grateful to Seth Kettle, Cynthia Cippolla, Dr J. Xu and Dr. Li Tan for their help in some of the experiments.

My sincere gratitude goes out to the Department of Chemistry and Biochemistry as well as the staff of Edison Biotechnology Institute for all the help over the past five years and making it an enjoyable experience.

I am thankful to my family for all their support through the years. A special thanks goes out to my wife, lab mate, friend and partner Eroica Soans, without whom none of this would have been possible. She has given me the strength, advise and courage to face the many challenges of everyday life as well as helped me with discussions, planning and implementation of my research work.

Finally, I would like to thank Ohio University, all its members and facilities for making my graduate experience pleasant and memorable.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

CHAPTER 1: SIGNIFICANCE AND HYPOTHESIS

Melanoma is a deadly form of skin cancer with a high incidence rate and relatively high mortality rates (see table 1). The occurrence of this disease is primarily due to ultraviolet (UV) radiation, but also by inherited mutations (ACS, 2009; Thompson, Scolyer, & Kefford, 2005; Tucker & Goldstein, 2003). The continued exposure to the sun's UV radiation causes damage to DNA in the melanocytes within the epidermal layer of the skin which eventually leads to several mutations in the DNA that accumulate over time (Gilchrest, Eller, Geller, & Yaar, 1999). Eventually, the melanocytes begin to proliferate and transform into a tumor growth seen on the skin surface. Malignant melanoma occurs when, in addition to the primary melanoma site, the lymph nodes and the lymphatic system also becomes involved and cancer cells circulate throughout the body into distant sites (Thompson, et al., 2005).

After surgery and radiation, a potent systemic adjuvant therapy for melanoma, which has shown success, is the use of a high dose regimen of IFNα2b. However, there is considerable toxicity associated with a high dose intravenous regimen of IFNα2b including chronic fatigue, fever, nausea and liver toxicity among others. In any case, it still remains the only drug approved by the FDA for treatment of high-risk malignant melanoma (Kirkwood, et al., 2002; Thompson, et al., 2005).

However, like most small protein therapeutics, therapeutic regimens are limited and high doses required because of short serum half-lives, degradation by the serum proteases and rapid clearance by the kidneys (Harris & Chess, 2003; Xu, Tan, Goodrum,

& Kieliszewski, 2007). For this reason, several attempts have been made to improve the stability of IFNs by increasing its molecular weight while still keeping its biological activity intact, including pegylation. The addition of a chemical polyethylene glycol group (PEG) to interferon increases its molecular weight and stability but lowers receptor binding affinity and biological activity. Moreover, it is inefficient and expensive (Tsubery, Mironchik, Fridkin, & Shechter, 2004; Xu, et al., 2007).

The production of native recombinant IFNα2b as an arabinogalactan (AGP) fusion protein offers a solution to tackle the inherent problem of the use of small protein therapeutics. This synthetic gene approach employs the use of previously predicted O-glycosylation codes that can cause hydroxyproline O-glycosylation in plants. The hydroxylation of proline to hydroxyproline (Hyp) is a posttranslational modification unique to plants and green algae. This method produces high molecular weight IFN as arabinogalactan fusion proteins, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$, where the $(SO)_{10}$ and $(SO)_{20}$ refer to Ser-Pro in 10 and 20 tandem repeats, respectively. These fusion proteins are produced in cultured tobacco cells and have higher molecular weights as well as greater stability as evidenced by as much as 13-fold greater serum half-life as compared with the native protein. They are also not susceptible to serum proteases and the AGP domain itself shows very little immunogenicity in vivo. Importantly, it maintains it's biological activity in the in-vitro antiviral assays performed (Xu, et al., 2007). As mentioned above, IFN has already been approved in high doses for the treatment of malignant melanoma (Thompson, et al., 2005). Therefore, to test these modified fusion proteins, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$, along with recombinant IFNα2b, we

investigated their effect on growth inhibition of human melanoma cell lines M92-047 and SKMEL-28.

Interferons transmit their signals in cells through the already well studied JAK-STAT signaling pathway to influence transcription of effector genes containing interferon stimulated response elements (ISREs) in their promoter regions (Heim, 1999; Schindler & Darnell, 1995). Thus, we tested whether IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ was able to bind to the IFNα receptor and trigger the JAK-STAT signaling pathway similar to IFNα2b. Further, we studied the effect on downstream signaling of the JAK-STAT signaling pathway. For this, we determined the expression of two IFN inducible proteins,2'5' OAS1 and PKR, which play an important role in IFN signaling in mediating its antiviral properties. The significance of this part of the project was mainly to determine the biological activity of the fusion proteins as compared with IFNα2b and to investigate whether these glycosylated fusions were still able to bind to the IFNα receptor and trigger downstream signaling similar to the native recombinant protein.

Interestingly, the tumor suppressor p53 was found to have 2 distinct ISREs in its promoter region suggesting that transcription of the p53 gene could be IFN mediated (Takaoka, et al., 2003). Although IFN has been used to treat a variety of cancers, the actual nature of its antiproliferative mechanisms remain poorly understood (Ezekowitz, Mulliken, & Folkman, 1992; Selleri, et al., 1997; Tagliaferri, et al., 2005).

In hepatic cancer cells, p53 responses have been induced by IFNs; both transcription and translation of p53 increased with IFN treatment (Takaoka, et al., 2003). Moreover, the tumor suppressor p53, which plays a crucial role in apoptosis and cell

cycle regulation, is rarely mutated in melanoma (Thompson, et al., 2005) and therefore could serve as an important target to mediate growth inhibition in melanoma cells. Thus, our next aim was to investigate the role of p53 in melanoma cells M92-047 treated with IFNα2b as well as the fusion protein IFNα2-$(SO)_{20.}$ We determined the activity of the p53 promoter as well as p53 transcript and protein levels at different time points after IFNα2b/IFNα2-$(SO)_{20}$ treatments. Finally, to determine p53 transcriptional activity we assessed the transcription and translation of Bax, which is a direct target or p53 and is involved in the p53 mediated mitochondrial apoptosis pathway.

In summary, this research project consists of two aims to investigate the role of fusion proteins of IFN in melanoma. Aim 1 is to demonstrate the biological effects of these fusion proteins as well as their ability to adhere to IFN signaling as compared with IFNα2b. Aim 2 is to determine the role of p53 in these melanoma cells after treating them with both IFN as well as the fusion proteins.

This novel approach of producing higher molecular weight IFNs with longer serum half-lives will be extremely beneficial to overall management of malignant melanoma as well as other IFN treated cancers. The next step would be to develop a tumor mouse model to test the efficacy of these fusion proteins in reducing tumor burden. If found to be efficacious, this approach could offer several advantages in dosing regimens, reducing side effects and lowering treatment costs of IFN therapy.

CHAPTER 2: INTRODUCTION

2.1. History and Background of Melanoma:

Melanoma is a form of skin cancer in which the normal skin cells, melanocytes, acquire the properties of cancerous cells, which include uncontrolled growth and metastatic invasion, giving rise to a malignant tumor. This epidemic cancer has very high incidence rates and is increasing annually, with almost a million new cases being diagnosed every year in the United States. The relatively high mortality rates as a result of this disease make this form of skin cancer extremely dangerous and the need for therapeutics very necessary (H.Irene Hall, 1999). The relative incidence of melanoma among young women is greater than in young men, but at age 45 or greater, a reversal of this trend is seen with men becoming more susceptible to this dreadful disease (Tucker & Goldstein, 2003). Table 1 shows the estimated new cases and deaths in the US in 2009 by American Cancer Society (ACS) for different cancer types. For melanoma specifically, the estimated number of new cases of melanoma are 68,720 across both sexes and the estimated number of deaths are 8650 (ACS, 2009).

Table 1

Estimated New Cases & Deaths for Different Cancer Types, US, 2009

	New Cases (estimated; 2009)			Deaths (estimated; 2009)		
	Male	Female	Both Sexes	Male	Female	Both Sexes
Cancer Type						
Breast	1,910	192,370	194,280	440	40,170	40,610
Prostate	192,280	-	192,280	27,360	-	27,360
Melanoma	39,080	29,640	68,720	5,550	3,100	8,650
Lung	116,090	103,350	219,440	88,900	70,490	159,390
Brain	12,010	10,060	22,070	7,330	5,590	12,920

(as adapted from Cancer Facts & Figures, ACS, 2009)

The diverse distributions of melanoma occurrences can be characterized by different factors which include age, sex and place of residence along with site of primary tumor, stage and depth of the tumors (H.Irene Hall, 1999).

In addition to these factors, melanoma susceptibility has been linked to 2 genes, cyclin dependent kinase inhibitor 2A (CDKN2A) and cyclin dependent kinase 4 (CDK4) that are related to cyclins, which play an important role in the division of a cell. The protein products of the CDKN2A gene are p16 and p14ARF; the former plays a vital role in the retinoblastoma pathway while the latter has been implicated in the p53 pathway (Tucker & Goldstein, 2003). However, even though germ-line mutations in the CDKN2A gene do occur in melanoma, which can be inherited, generally this predisposition is not as

much of a risk in melanoma as compared to exposure to UV radiation (sunlight), which is the major contributing factor to the incidence of this disease. This indicates that the origin of melanoma is closely related to genetic susceptibility and environmental UV exposure (Thompson, et al., 2005).

In humans, melanoma pathogenesis generally begins with continued exposure to UV radiation, causing mutations in the skin cells. These mutations accumulate over time resulting in the production of growth factors, such as fibroblast growth factor (FGF), stem cell factor and transforming growth factor-α (TGF-α),leading to the proliferation of the melanocytes. This leads to an increase in reactive oxygen species (ROS) of melanin within these proliferating melanocytes and they acquire properties of host immune defense evasion. Eventually this leads to a malignant condition characterized by improper functioning of cellular pathways related to cell cycle arrest or apoptosis, which are necessary for normal cellular functions.

2.1.1 Treatment of Melanoma:

There is growing need for effective therapy for melanoma. Several treatment options exist, which are described below:

Surgery: surgical excision is one of the first line of treatments for patients with melanoma that is diagnosed early and is more optimal if the tumor is localized to its primary site. Metastasized tumors involving the sentinel lymph node require the dissection of regional lymph nodes to prevent further metastases of the disease. However

after surgery, post-operative care is most often managed by radiation therapy (Thompson, et al., 2005).

Radiotherapy: involves the use of ionizing radiation to kill malignant cells. It is the favored treatment when the brain and bone are the sites of metastasis (Fife, et al., 2004; Thompson, et al., 2005).

Chemotherapy and biological therapy: consists of the use of chemotherapeutic drugs for management of the spread of the disease. Most often this treatment is used in combination with vaccines and biological drugs (Flaherty, et al., 2001; Thompson, et al., 2005).

Immunotherapy: is one of most investigated therapies in melanoma, used for advanced melanoma treatment as well as adjuvant therapy. This treatment involves the use of the body's own immune system to boost an immune response towards tumor cells. Interferons and other cytokines are naturally produced proteins in the body in response to inflammation; they help improve the immune response to foreign tumor specific antigens. Interleukin-2, which is a kind of cytokine, has been used in very high doses of intravenous injections for the treatment of melanoma. However, the response rates are not very high and the toxicity related to these high doses makes it a poor option to treat melanoma. Another problem that arises while using immunotherapy for melanoma treatment is that in some cases melanoma cells become resistant to apoptosis and tolerant to immune responses, hence, higher doses and the use of other biological molecules are needed for effective treatment. A potent systemic adjuvant therapy for melanoma is the

use of a high dose regimen of interferon alpha 2b (IFNα2b/IFNα2) (Kirkwood, et al., 2002; Rosenberg, 2001; Thompson, et al., 2005).

After surgical resection of the primary tumor site, only high dose treatment with IFNα2b administered as adjuvant has shown relapse free survival. The disease free survival rates, as well as the overall survival (OS) rates, are much higher in patients treated with the high dose interferon treatment regimen, although the high dose therapy is associated with related toxicities as well. (Jonasch, et al., 2000; Kirkwood, et al., 2002; Moschos, et al., 2006; Muggiano, et al., 2004). Also, besides a high dose treatment regimen of IFNα2b alone, various clinical trials have used or are using combinatorial therapy, involving the use of chemotherapy and IFN to achieve better OS rates. Different types of treatment regimens using different chemotherapeutics have been used in several clinical trials for the treatment of metastatic melanoma (Agarwala & Kirkwood, 2003; Atkins, et al., 2002; Kaufmann, et al., 2005; Shah & Chapman, 2007).

2.2 Interferons:

Interferons were the first discovered and studied cytokines and are archetypes for cytokine research. Evidence of interferons antiviral effects were uncovered as early as 1804 by Jenner while studying viral interference. He observed that in individuals with herpetic infections, vaccination was not possible (Morris & Zvetkova, 1997). Isaacs and Lindenmann, in 1957, observed that the supernatant of cells treated with heat-killed virus had the ability to prevent infection in other cells infected with the live strain. Furthermore, experiments showed that this particular factor in the supernatant was not an

antibody but was a protein of non-viral origin. It was also found that this protein factor acted on target cells and needed some time to render the cells resistant or induce an antiviral state(Lindenmann, 1988). Around the mid-1970s it was known that Interferon (IFN) could be produced by a variety of cells in response to viral infections as well as non-viral factors (mostly double stranded polynucleotides).Interferons are of more than one type as they are produced by a variety of cells. Therefore, depending on the factors that induce their production, they are classified as type 1 and type II IFNs (see Table 2).

Table 2

Classification of Different Types and Subtypes of Interferon Based on Cells that Produce Them in Response to Different Stimuli

Interferon	**Cells producing**	**Induced by**
Type 1		
Interferon alpha (IFNα)	Leucocytes	Virus
Interferon beta (IFNβ)	Fibroblasts	Virus
Type II		
Interferon gamma (IFNγ)	T-lymphocytes	Mitogenic stimulation of T-cells

A characteristic property of cytokines is that they have multiple effects, which is seen in IFNs as well. The first non-viral activity of IFN observed was inhibition of cell growth or proliferation, indicating its use in cancer. It also has effects on the

histocompatibility antigen and subsequent natural killer (NK) cell activity, which provide a link between IFN and the immune system. The production of IFNs was done with the advent of genetic engineering. The fact that human genes could be cloned and then expressed in a bacterial system like Escherichia Coli (E.coli) suggested that IFN could be produced using this approach as well. Hence, by the 1980s the cDNA's for IFNα and IFNβ were cloned and IFNγ followed later (Morris & Zvetkova, 1997).

2.2.1 Biological Roles of Interferons:

Interferons are cytokines with antiviral, antiproliferative, and immunomodulatory properties (Schindler & Darnell, 1995; Wong, et al., 1997). The biological role of type I IFNs, namely IFNα/IFNβ, differ from type II IFNs, IFNγ, wherein the latter induces expression of Class II MHC molecules along with other antigen presenting cells and activating macrophages. Hence, IFNγ carries out antiviral effects by boosting the immune response. In general, type I IFNs have more potent antiviral activity than type II IFNs (Morris & Zvetkova, 1997). The biological role in terms of antitumor activities of type I IFNs and their effects on tumor cells is shown in Fig. 1 (Pestka, 2003).

2.2.2 Interferon Signaling:

IFNs transmit their extracellular signals into transcriptional responses by the JAK-STAT signaling pathway (Fig.2). IFNα/β type 1 IFNs bind to a common IFNAR (interferon alpha receptor), which exists as at least 2 receptor chains having 2 tyrosine kinases, JAK-1 (Janus Kinase1) and Tyk-2 (tyrosine kinase2), as intracellular domains.

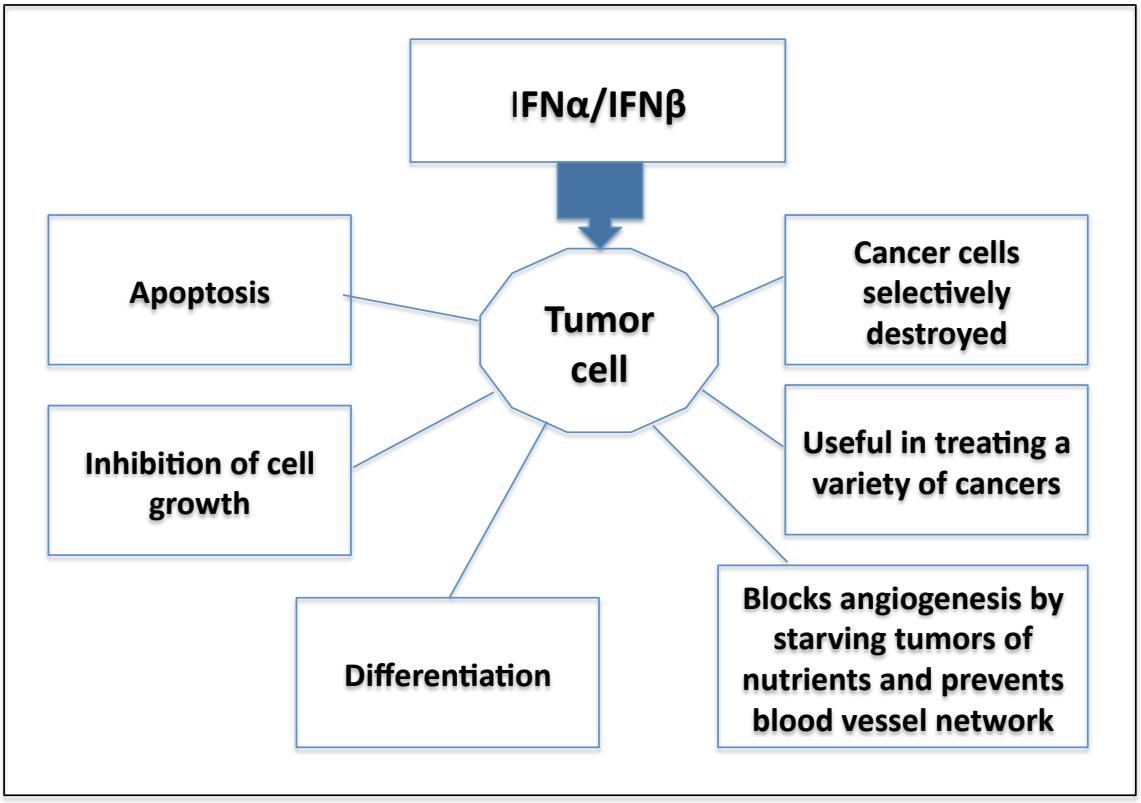


Fig 1: Schematic representation of the different anti-tumor effects of type I IFNs. IFNs can cause tumor cell and tumor stroma destruction in many different ways, which are summarized in the figure, (as adapted from S. Pestka, 2003).

Ligand binding induces receptor multimerization and activation of the receptor tyrosine kinases, which in turn phosphorylate tyrosine residues within the receptors. This then acts as a docking site for STATs (signal transducers and activators of transcription) to bind and activate by phosphorylation on a tyrosine residue in the SH2 (src homology) domain. The STATs then form homodimers (Type 2 signaling) or STAT1-STAT2 heterodimers (Type 1 signaling) (Heim, 1999; Rawlings, Rosler, & Harrison, 2004).

STAT1 exists as STAT1α (91 kDa) and STAT1β (84 kDa); STAT2 is homologous to STAT1 but is larger, about 113 kDa. STAT1-STAT2 heterodimers associate with a third non-STAT protein called p48 (ISGF-3γ- interferon stimulated gene factor-3 gamma; DNA binding protein) to form a trimeric protein complex, ISGF-3, which in turn moves into the nucleus and binds to ISREs present in promoters of IFN inducible genes (Pansky, et al., 2000; Schindler & Darnell, 1995). Additionally, STAT1 expression is required for an IFN response and STAT2 mediates the formation of the functional ISGF-3 complex, by associating both with STAT1 and p48 (Martinez-Moczygemba, Gutch, French, & Reich, 1997).

IFN also induces dsRNA-activated enzymes, PKR (Protein Kinase R) and 2'5' OAS1 (Oligo adenylate synthetase 1), which have important biological functions in cellular events leading to differentiation and apoptosis as well the antiviral properties of IFN (Hovanessian, 2007; Hovanessian & Justesen, 2007). Both of these proteins are known IFN induced proteins and their activation signifies downstream signaling (Fernandes, Soans, Xu, Kieliszewski, & Evans, 2010).

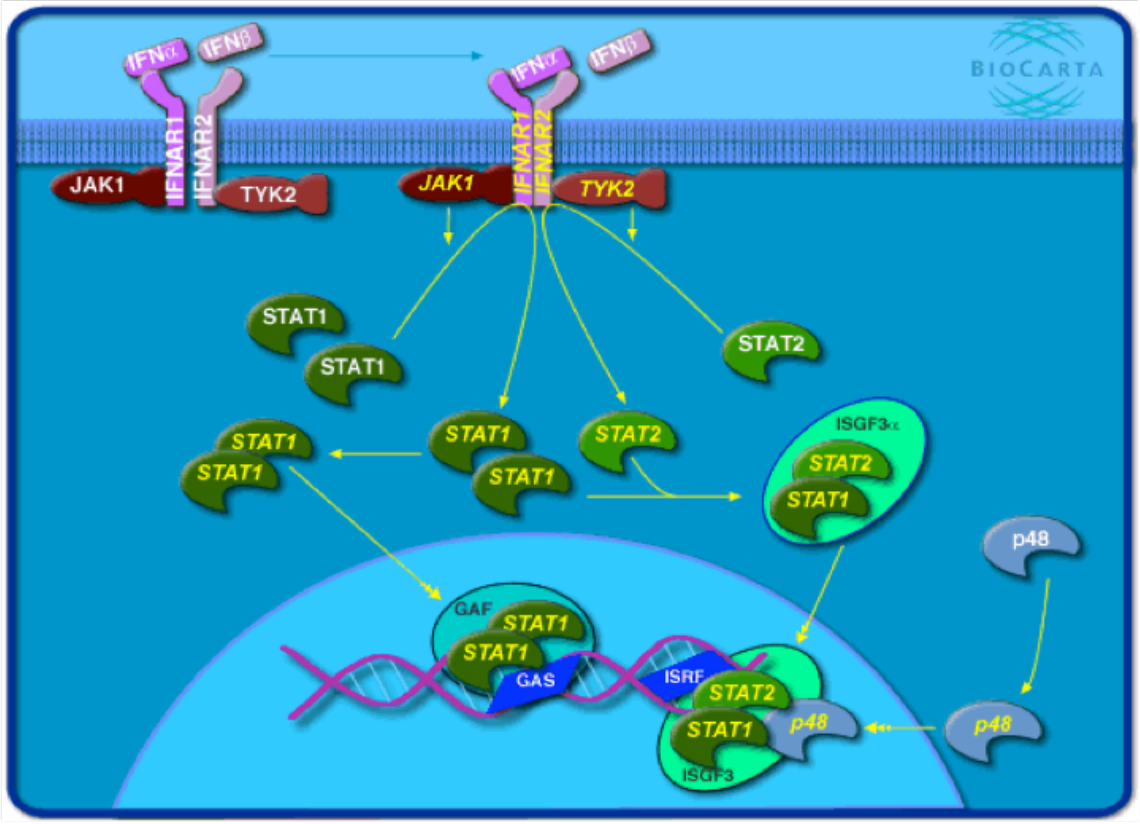


Fig. 2: Interferon, JAK-STAT signaling pathway. IFNs transmit their signals intracellularly by binding to cell surface receptors IFNAR1 (Interferon alpha-receptor 1) and IFNAR2 (Interferon alpha-receptor 2) which are associated with receptor tyrosine kinases belonging to the JAK family of proteins. Another family of transcription activators STATs (signal transducers and activators of transcription) are involved in this signaling pathway. As adapted from Biocarta website(ODell, 2000).

2.2.3 Interferons Have Been Used to Treat a Variety of Other Cancers:

IFNs were among the very first proteins produced from human cells and found to be effective in treatment of cancer. Among the different types of IFNαs only IFNα2 has been assessed clinically in a broad spectrum of cancers, specifically IFNα2b (Borden,

Lindner, Dreicer, Hussein, & Peereboom, 2000). There are studies that showIFNα2b as a potential therapeutic in chronic myelogenous leukemia (CML) with both hematologic and cytogenetic responses in the range of 46-80% and 13-32%, respectively (Alimena, et al., 1988; Kantarjian, O'Brien, Anderlini, & Talpaz, 1996; Mahon, et al., 1998).In addition, therapeutic activity is seen in lymphomas as well (both B and T-cell origin) (Borden, 1994). IFNα2a is effective in 45% of patients with cutaneous T-cell lymphoma, while in poorly defined nodular B-cell lymphoma the response rate is about 45% when it is used as standalone treatment (Bunn, et al., 1984; Foon, et al., 1984; O'Connell, et al., 1986). Studies show partial response of IFNβ in T-cell leukemias (Tamura, Makino, Araki, Imamura, & Seita, 1987). Even in multiple myeloma (highly prevalent in the United States and Europe) IFNα shows antiproliferative effects as well as mediating oncogene expression (Borden & Ball, 1981; Mandelli, et al., 1990). In addition to these types of cancers, IFNs have been investigated in melanoma (as described earlier), urologic malignancies, primary brain metastases and ovarian cancers with promising effects. Besides IFNs being used as single agent treatments, it has been used in combination therapy not only for melanoma but for other cancer types as well (Borden, et al., 2000). In CML, IFNα2 is used in combination with cytosine arabinoside (ara-C), an inhibitor of c-abl kinase. In randomized trials of IFNα2 in one arm versusIFNα2 and ara-C on the other arm, the combination shows better hematologic and cytogenetic responses and results in complete disease remission (Arthur & Ma, 1993; Guilhot, et al., 1997; Kantarjian, et al., 1992; Kantarjian, et al., 1999; Thaler, et al., 1997). In non-Hodgkin's lymphomas, it is used in combination with doxorubicin and shows better disease free

survival and overall survival (Borden, 1994; Smalley, et al., 1992; Solal-Celigny, et al., 1993). In multiple myeloma, combinations of chemotherapy and IFNα2 improves overall survival rate (Borden, et al., 2000).

2.2.4 Structure and Molecular Weight of Interferons:

Both human IFNα and IFNß are structurally very similar proteins and are encoded by genes located on chromosome 9. IFNα has two recombinant forms, IFNα2a and IFNα2b, having molecular masses of 19241 and 19269 daltons, respectively. Each of these proteins contains about 165 amino acids with an additional 20 amino acids as part of the secretory peptide at the amino-terminal end.

IFNβ also has two recombinant forms, IFNβ1a and IFNβ1b, in which the former is produced in Chinese hamster ovary cells (CHO) and the latter in E.coli. Moreover, IFNβ1a is a single glycosylated polypeptide chain of 165 amino acids having a molecular mass of 22500 daltons while IFNβ1b is a single non-glycosylated polypeptide chain of 166 amino acids where serine is substituted for cysteine at position 17. It has a molecular mass of 18510 daltons.

IFNγ is a single non-glycosylated polypeptide containing 144 amino acids and has a molecular weight of 16879 daltons, which is the smallest of the IFNs (Borden, et al., 2000).

2.2.5 Problems Associated with the Use of Low Molecular Weight IFN as a Therapeutic:

IFNs relatively small molecular weight (approximately 19.2 kDa), allows its rapid clearance by the kidneys and it is susceptible to degradation by serum proteases. This leads to a short serum half-life when administered as a therapeutic (Harris & Chess, 2003; Xu, et al., 2007). Chemical modifications such as 'pegylation' (addition of polyethylene glycol groups)overcome this problem by increasing molecular mass and stabilizing small proteins. Pegylated interferon was produced by the addition of a 12000 daltons monomethoxypolyethylene glycol (PEG-12000) to IFNα2b to form a semi synthetic protein polymer complex, which increased the serum half-life of the protein considerably (Grace, et al., 2001). However, pegylation results in low receptor binding affinity thereby reducing in vitro biological activity(Harris & Chess, 2003; Ramon, Saez, Baez, Aldana, & Hardy, 2005; X. Wu, Li, Zeng, Zheng, & Wu, 2006). Additionally, PEG groups attach non-specifically to the amino group of lysines, the N-terminus, the α-amino group of cysteines, imidazole nitrogens of histidines and the hydroxyl groups of serines, threonines, and tyrosines (Ramon, et al., 2005; Y. Yamamoto, et al., 2003). Although, several alternate pegylation strategies exist, it seems that irrespective of the approach, the derivatized protein polymer requires a number of purification steps and gives low yields, thus making the process expensive and cost ineffective (DeFrees, et al., 2006; Fernandes, et al., 2010; Tsubery, et al., 2004; Xu, et al., 2007).

2.3 Novel Approach to Synthesize High Molecular Weight IFN in Plants:

An advancement in this field was made by Xu et al, by expressing human interferon α2b (IFNα2b/IFNα2) as an arabinogalactan protein fusion (AGP) in tobacco cells (Xu, et al., 2007). In hydroxyproline rich glycoproteins (HRGPs) of plants, there exist a unique posttranslational modification known as O-glycosylation in which the hydroxyproline (Hyp) residues in these HRGPs undergo hydroxyproline-O-glycosylation (Hyp-O-glycosylation) (Kieliszewski & Shpak, 2001; Xu, et al., 2007). The codes that cause this modification depend on the proximity of Hyp in the glycomodule. Therefore, based on this, there are two possible types of Hyp-glycomodules, one in which to these contiguous Hyp residues there is the addition of arabinooligosaccharides, which are short and unbranched, and usually characterized by pentapeptide motif Ser-Hyp_4 as seen in extensins (Shpak, Barbar, Leykam, & Kieliszewski, 2001; Xu, et al., 2007). On the other hand, the addition of branched arabinogalactan polysaccharides to Hyp residues, which are not in close proximity to each other, results in larger glycomodules characterized by the sequence of Ser/Ala-Hyp-Ser/Ala-Hyp, where the presence of Ser/Ala states the type of arabinogalactan proteins (AGPs) (Shpak, et al., 2001; Shpak, Leykam, & Kieliszewski, 1999; Tan, Leykam, & Kieliszewski, 2003; Tan, Qiu, Lamport, & Kieliszewski, 2004; Xu, et al., 2007). Further, the demonstration of adding the AGP module to enhanced green fluorescent protein (EGFP) (non plant protein) at either N or C termini resulted in a fusion protein with an unaltered function and culture medium containing reasonable yields of this fusion protein (Held, et al., 2004; Shpak, et al., 2001; Shpak, et al., 1999; Tan, et al., 2003; Xu, Shpak, Gu, Moo-Young, & Kieliszewski, 2005; Xu, et al., 2007). Hence, using a synthetic gene approach, involving the use of the cDNA of IFNα2 and subsequent cloning and subcloning steps, 2 genes were designed which encoded 10 and

20 tandem Ser-Pro repeats. These were then subcloned into transformed tobacco cells in culture and the hydroxylation of each Pro residue in the repeats could be predicted based on the Hyp-O-glycosylation codes described above. As a result, AGP fusion proteins IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ were expressed in tobacco cells, where $(SO)_{10}$ and $(SO)_{20}$ refer to the number of arabinogalactosylated serine (S) hydroxyproline (O) repeats at the C-terminus of interferonα2b (Xu, et al., 2007). Also, this post-translational modification is only seen in plants and green algae (Kieliszewski & Shpak, 2001). The resultant AGP fusions have greatly increased molecular weights (55 kDa for IFNα2-$(SO)_{10}$ and 80 kDa for IFNα2-$(SO)_{20}$) as compared to 19.2 kDa for native recombinant IFNα2b (Fig. 3), and are resistant to proteolytic degradation.

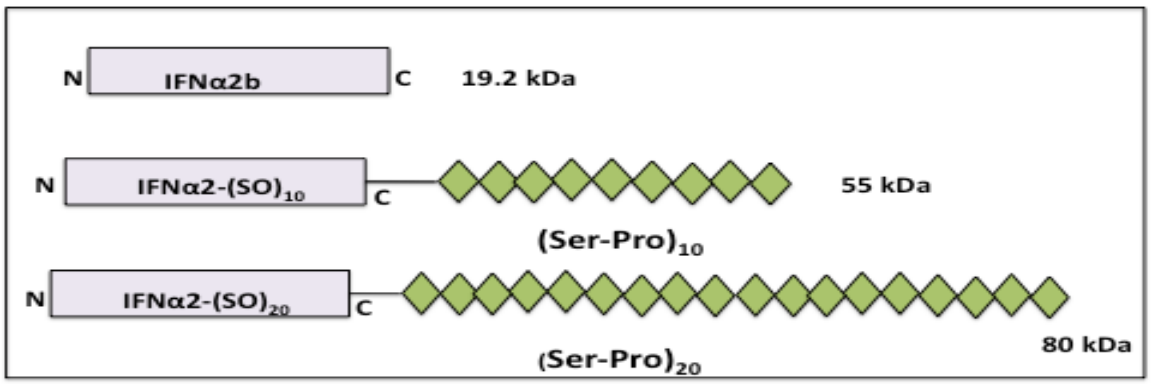


Fig. 3: Schematic representation of the higher molecular weight AGP fusion IFNs. Recombinant IFNα2b has a molecular weight of 19.2 kDa, however, the addition of 10 $(Ser\text{-}Pro)_{10}$ and 20 $(Ser\text{-}Pro)_{20}$ tandem repeats of Ser-Pro to the C-terminal end of the recombinant IFN protein yields higher molecular weight fusion derivatives IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ of 55 kDa and 80 kDa, respectively.

In addition, the higher molecular weights are accompanied by longer serum half-lives (greater than 13 fold in vivo as compared with native recombinant interferon) and the AGP component shows almost no immunogenicity when injected in mice (Xu, et al., 2007). This represents a novel approach to solve the inherent problem of small protein therapeutics and to produce higher molecular weight IFNα2b in plants on a relatively cheap and large scale.

2.4 Interrelationship between IFNα2b Signaling and Tumor Suppressor p53:

Both IFNα2b and p53 are known to be involved in tumor suppression. However, Takaoka et al first described the interrelationship between them in 2003, when they showed that type 1 IFN signaling was linked to p53 responses. Specifically, they showed that there exist 2 distinct ISREs present in the promoter region of the p53 gene. Moreover, their results indicate that in hepatic cancer cells, IFN signaling was able to increase the transcription of the p53 gene accompanied by a concomitant increase it its protein levels (Takaoka, et al., 2003).

2.4.1 Role of p53 in Melanoma:

As described above, the role of p53 in melanoma is related to one of the products of the CDKN2A gene, p14 ARF that is sometimes mutated in melanoma (Gilchrest, et al., 1999; Sharpless & Chin, 2003; Thompson, et al., 2005). Several studies have shown that ARF may play a role in stabilizing p53, by regulating its degradation by human double minute 2 (HDM2) by itself binding to HDM2. However, mutations in ARF will lead to

HDM2 mediated degradation of p53 (Kamijo, et al., 1998; Pomerantz, et al., 1998; Sharpless & Chin, 2003; Stott, et al., 1998). Also, p53 is rarely mutated early in melanoma and therefore could be an important target in this type of cancer (Thompson, et al., 2005; M. Yamamoto, Takahashi, Saitoh, Horikoshi, & Takahashi, 1995).

2.5 Tumor Suppressor p53:

The p53geneis located on human chromosome 17 and is about 20 kb in size, comprised of 11 exons and 10 introns (Isobe, Emanuel, Givol, Oren, & Croce, 1986; Lamb & Crawford, 1986). It is a highly conserved gene family including at least 2 more genes, p63 and p73. The products of these genes yield 3 proteins, namely p53, p63 and p73, of which p63 and p73 are thought to be involved in normal development (Irwin & Kaelin, 2001; Kaghad, et al., 1997; Schmale & Bamberger, 1997). However, the 53 kDa protein, p53, is the most extensively studied protein of this family because of its role in various cellular events. The p53 protein is comprised of 393 amino acids and is associated with several structural and functional domains. At the N-terminal end, it has the transactivation domain (mdm2 binding domain) and the proline rich region. The core region contains the DNA binding domain, which is a hot spot for several mutations that occur in many different kinds of cancers. The C-terminal end contains the tetramerization domain, the nuclear localization sequence (NLS), 3 nuclear export sequences (NES) as well as the regulatory domain. The regulatory domain is where posttranslational modifications such as phosphorylation and acetylation occur (Fig. 4) (Zhu, 2006).

The p53 protein is a nuclear phosphoprotein with a short half-life of 6-20 min and is negatively regulated by mdm2 (mouse double minute2), known as Hdm2 in humans (Fields & Jang, 1990; Lin, Chen, Elenbaas, & Levine, 1994; Oren, 2003; Soussi, 2005). When phosphorylated, p53 remains in the nucleus where it performs transcriptional activation of several genes involved in apoptosis and cell cycle arrest. However, p53 is sequestered into the cytosol for degradation. As mentioned above, several mutational hot spots are present in the DNA binding domain of p53 and are mutated in almost 50 % of carcinomas, leading to a loss of its transcriptional activity. It has various other biological roles in development, gene amplification, cellular senescence, chromosomal segregation, genetic recombination and cell differentiation (Oren & Rotter, 1999; Zhu, 2006).

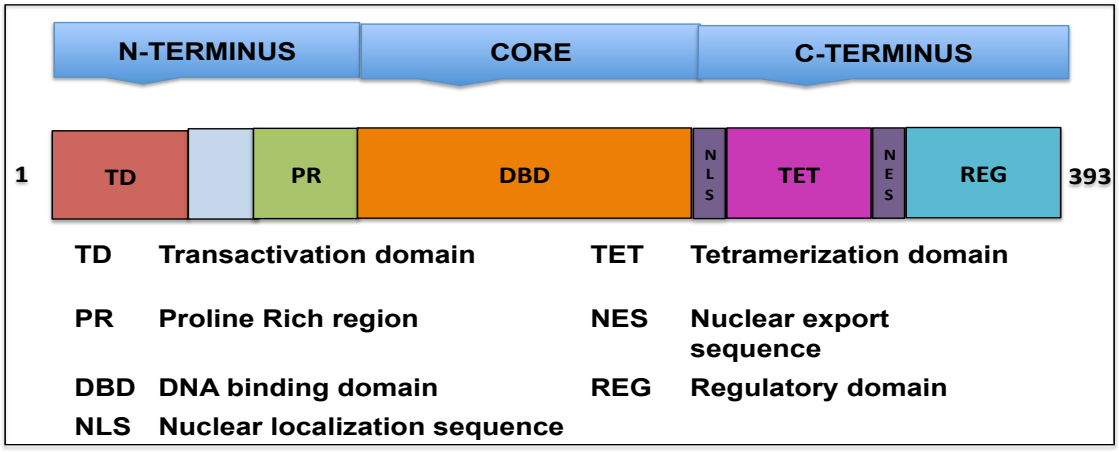


Fig 4: Schematic representation of the p53 protein showing the structural and functional domains. The p53 protein contains different functional domains within the N and C terminal end of the protein. The DNA binding domain is contained within the central core while the transactivation domain, which also contains the HDM2 binding site, is present at the N terminus **(**as adapted from L. Bai and W. Zhu, 2006).

2.5.1 Cellular role of p53 in response to stress:

P53 is extremely important in maintaining the integrity of the genome and it gets activated in response to cellular stresses such as UV radiation, gamma ionizing radiation and cytotoxic agents, all of which cause DNA damage thereby activating p53. In addition to the stresses mentioned earlier causing DNA damage, p53 can also be activated by other factors such as conditions of low oxygen (hypoxia), heat shock and over expression of oncogenes (Lane, 1992; Levine, 1997; Vogelstein, Lane, & Levine, 2000; Vousden & Lu, 2002). The activation of p53 results in an increase in amount of p53 protein which undergoes posttranslational modifications, increasing its stability and keeping it in the nucleus where it can perform its transcriptional activity by binding to its target genes.

The multifunctioning protein p53 has various targets, which are involved in varied cellular responses like apoptosis (activation of pro-apoptotic Bax, Bak, Apaf-1, PUMA, NOXA; repression of anti-apoptotic Bcl-2, Bcl-X_l, MAP4), cell cycle arrest ($p21^{Waf1/Cip1}$, GADD45), DNA repair mechanisms (GADD45, Pol β) and senescence ($p21^{Waf1/Cip1}$, $p66^{SHC}$) (Fig. 5) (Fritsche, Haessler, & Brandner, 1993; Zhu, 2006).

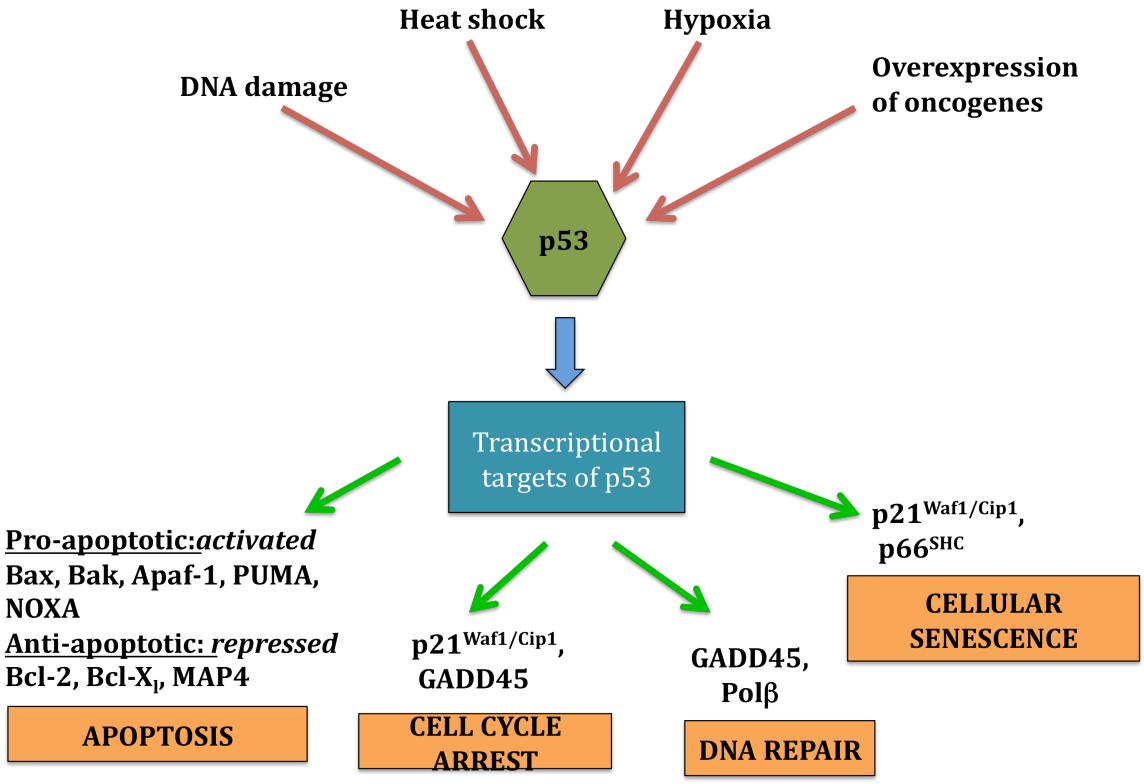


Fig 5: Schematic representation of the different cellular stresses which can activate p53 and different responses induced by its transcriptional targets. p53 can be activated by different cellular stresses, thus in turn activates several transcriptional targets, including pro-apoptotic proteins, cell cycle inhibitory proteins, DNA repair proteins, and cellular senescence proteins (as adapted from L. Bai & W. Zhu, 2006).

2.5.2 Regulation of p53:

P53 regulation occurs by increasing the stability of the protein as well as influencing the activity of the protein. The former is done by various factors depending on the cell's conditions and external environment, but more notably by its negative

regulator MDM2 (HDM2). The latter involves various post-translational modifications such as phosphorylation, acetylation, dephosphorylation, glycosylation and covalent/noncovalent modifications at the C-terminal end, modulating its sequence specific DNA binding, thereby influencing its transcriptional activity (Shaw, Freeman, Bovey, & Iggo, 1996). DNA damage can induce phosphorylation of p53 at the N-terminus by various activated kinases (ATM, ATR, CHK2) this in turn can cause acetylation of C-terminal lysine residues, thereby stabilizing p53. MDM2 regulates p53 negatively by binding to an MDM2 binding site within p53's transactivation domain lowering p53 activity (Balint & Vousden, 2001; Gu & Roeder, 1997; Sakaguchi, et al., 1998). In addition, a degradation effect is seen by MDM2's ubiquitin protein ligase (E3) activity (described below, 2.6), in which MDM2 ubiquitinates p53 and targets it for degradation by the proteosome (Y. Haupt, Maya, Kazaz, & Oren, 1997; Kubbutat, Jones, & Vousden, 1997). MDM2 can also change the subcellular localization of p53 from the nucleus to the cytoplasm where it can be degraded. This is probably a result of ubiquitination, which can expose the NES in the C-terminus of p53 since it exists in a monomeric form when ubiquitinated (Stommel, et al., 1999). However, not only does MDM2 degrade p53 but p53 also transactivates MDM2; hence there exist an autoregulatory feedback loop between the two proteins (Balint & Vousden, 2001; Barak, Juven, Haffner, & Oren, 1993). As mentioned earlier p14 ARF (mouse p19 ARF) regulates p53 by binding to MDM2 and preventing it from binding to p53 and degrading it. Additionally, MDMX, an MDM2 like protein, regulates p53 activity by binding to its

transactivation domain like MDM2 but does not degrade p53 probably because it lacks the ubiquitin ligase activity (Balint & Vousden, 2001; Shvarts, et al., 1996).

2.6 MDM2 (HDM2), the Negative Regulator of p53:

HDM2 is an oncogene that is overexpressed in tumors, mainly soft tissue sarcomas and gliomas (Brown, Thomas, & Deb, 1998). The *Hdm2* gene encodes a protein containing 5 polypeptides with 491 amino acids and several functional domains (Fig. 6) (Marechal, et al., 1997; Olson, et al., 1993). At the N-terminal end there is a p53 binding site as well as NLS and NES sequences. Binding of p53 transcriptionally activates the *Hdm2*gene and thereby increases HDM2 protein levels that can in turn degrade p53 as described above (section 2.5.2). Moreover, the NLS and NES signals play important roles in HDM2's ability to sequester p53 to the cytoplasm for degradation (Boyd, Vlatkovic, & Haines, 2000; Geyer, Yu, & Maki, 2000; Roth, Dobbelstein, Freedman, Shenk, & Levine, 1998). The central core of the protein contains the acidic domain, which can bind both ribosomal RNA 5S and protein L5 (Marechal, Elenbaas, Piette, Nicolas, & Levine, 1994). The highly conserved Zinc (Zn) binding domain facilitates binding to DNA and protein (Honda, Tanaka, & Yasuda, 1997; Honda & Yasuda, 1999). The C-terminal end contains the RING finger domain that has ubiquitin protein ligase (E3) activity and is able to ubiquitinate itself as well as p53 (Joazeiro & Weissman, 2000). The ligase activity allows the ubiquitination of p53 at several lysine residues, which is followed by polyubiquitination of p53 by a transcriptional coactivator

p300, which possesses E4 ligase activity (Fig. 6)(Grossman, et al., 2003; Grossman, et al., 1998).

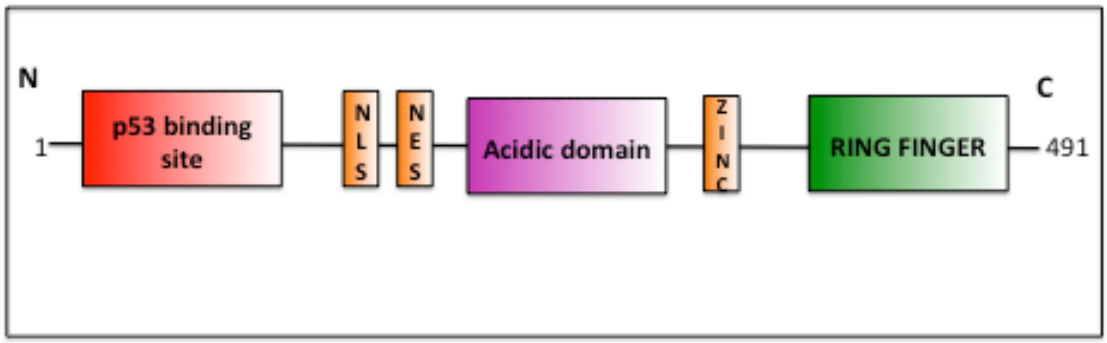


Fig. 6: Schematic representation of the MDM2 (HDM2) protein. The MDM2 (human homologue HDM2) contains several functional domains. The N-terminus contains the p53-binding site; the central portion contains the acidic domain, which has amino acid residues that can be post-translationally phosphorylated. The nuclear localization sequences (NLS) and nuclear export sequences (NES) are also contained in the N-terminal end. In addition there is Zinc binding domain and the ring finger domain at the C-terminal end, which has the ubiquitin (E3) ligase activity (as adapted from E. Balint & K. Vousden, 2001).

2.6.1 Regulation of HDM2:

As mentioned earlier, regulation of HDM2 is primarily through transactivation by p53 and degradation by an autoregulatory loop. Further, the importance of this kind of regulation is heightened by the fact that MDM2 deficient mice, with unrestrained p53 activity (wild type p53) are embryonic lethal, while those with a p53 null status can be

rescued (Jones, Roe, Donehower, & Bradley, 1995; Montes de Oca Luna, Wagner, & Lozano, 1995). This suggests that HDM2 mediated degradation of p53 is needed for normal growth and development. However, overexpression of HDM2is associated with tumor development presumably by inhibiting wild type p53 activity (Oliner, Kinzler, Meltzer, George, & Vogelstein, 1992). HDM2 is also inhibited by ARF binding, which specifically inhibits its ubiquitin ligase activity (Honda & Yasuda, 1999). Also, HDM2 can be regulated independent of p53. The HDM2 protein is made up of several serine and threonine amino acid residues (~20 %), which serve as putative phosphorylation sites within the N-terminus as well as in the acidic domain of the central region(Zhang & Prives, 2001). Various kinases get activated within the cell in certain stress conditions, which can phosphorylate HDM2. This leads to an accumulation of HDM2 in the nucleus and prevents its binding to ARF, thereby increasing p53 activity (Mayo & Donner, 2001, 2002). Phosphorylation of HDM2 at Ser 17 causes disruption in the p53-HDM2 binding, thereby stabilizing p53 (Mayo, Turchi, & Berberich, 1997). Interestingly, there are also alternatively spliced products of HDM2, produced after certain stresses, such as UV and ionizing radiations (IR), that inhibit full-length HDM2 mRNA as well as protein levels further stabilizing p53 (Chandler, Singh, Caldwell, Bitler, & Lozano, 2006; Dias, Liu, Yau, Westrick, & Evans, 2006). Finally, posttranslational modifications to HDM2 are involved in its regulation, such as phosphorylation by p38, which alters the E3 ligase activity so that MDM2 is ubiquitinated, and rapidly degraded (Buschmann, Fuchs, Lee, Pan, & Ronai, 2000).

2.7 Bax (Bcl-2-associated X protein), a Pro-apoptotic p53 Transcriptional Target:

Bax, an apoptotic gene product induced by p53, is involved in the intrinsic apoptosis pathway, also called the mitochondrial pathway (described in section 2.7.1) (Cory & Adams, 2002; Korsmeyer, 1999). It belongs to a family of Bcl-2 related proteins, which are comprised of 3 main classes based on the type of Bcl-2 homology domains (BH) they contain. The first class of proteins are pro-apoptotic, such Bax, Bak, Bcl-Xl, and contain homology domains BH1, BH2, BH3 also known multi-BH domain proteins. The second class of proteins is anti-apoptotic, namely Bcl-2 and Bcl-X_l, and contains three or four BH domains. These protect the integrity of the outer mitochondrial membrane. The third class of apoptotic proteins is the 'BH3 domain only' proteins such as, Bid, Bad, PUMA and NOXA, which transmit apoptotic signals to the mitochondria (George, Targy, Evans, Zhang, & Luo, 2010; S. Haupt, Berger, Goldberg, & Haupt, 2003; Kuwana, et al., 2005). Bax was the first identified primary response gene of p53 and is a direct transcriptional target of p53 containing p53 response elements. Bax plays an important role in p53 mediated apoptosis (Thornborrow, Patel, Mastropietro, Schwartzfarb, & Manfredi, 2002).

2.7.1 Role of Bax in Apoptosis:

In p53 dependent apoptosis, activated p53 can transcriptionally activate Bax thereby leading to elevated Bax levels. Bax in its inactive form resides in the cytosol; upon activation Bax is recruited to the mitochondria wherein the outer mitochondrial membrane potential changes leading to mitochondrial outer membrane permeabilization (MOMP). This causes the release of cytochrome c leading to oligomerization of Apaf-1 (apoptotic protease activating factor-1), and activation of caspases, the proteases involved

in the biochemical sequence of events described as apoptosis (George, et al., 2010; Green, 2005; Kuwana, et al., 2005).

CHAPTER 3: MATERIALS AND METHODS

3.1. Cell Culture:

Human melanoma cell line M92-O47 was obtained from Dr Ashani T Weeraratna of the National Institute of Health (National Institute on Aging, Baltimore, MD) through Dr. Leonard Kohn of Ohio University. Melanoma cell line SKMEL-28 (ATCC, Manassas, VA) was also used. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, USA) supplemented with 10% bovine growth serum (Hyclone, USA) and 1% penicillin streptomycin (GIBCO, USA) and kept in an incubator (NAPCO, USA) in an environment containing 95% air, 5% carbon dioxide at 37˚C (Fernandes, et al., 2010).

3.2. Recombinant Interferonα2b, Interferonα2-$(SO)_{10}$ and Interferonα2-$(SO)_{20}$:

Recombinant Interferon α2b (IFNα2) was purchased from Pro Spec-Tany Technogene Ltd, Israel and had a specific activity of 2.8 x 10^8 IU/mg. Interferon α2-$(SO)_{10}$ and Interferon α2-$(SO)_{20}$, was prepared in Dr. Kieliszewski's laboratory at Ohio University, as described earlier (Xu, et al., 2007) and had specific activities of 2.0 x 10^8 IU/mg and 1.9 x 10^8 IU/mg, respectively (Fernandes, et al., 2010).

3.3. Cell Viability Analysis:

5 x 10^4 cells were seeded in 24 well plates and allowed to attach for 4 -6 hrs followed by treatment with interferon α2b (IFNα2b/IFNα2) (Pro Spec-Tany TechnoGene Ltd, Rehovot, Israel) and fusion protein of interferon:IFNα2-$(SO)_{20}$ (Dr. Kieliszewski's

laboratory, Ohio University, Athens, USA) for 24 h, 48 h and 72 h. The analysis was carried out using the Cell Titer-Glo®luminescent cell viability assay (Promega, Madison, WI). At each of the time points the media was removed and cells were washed twice with 1X PBS and further processed as per the manufacturer's protocol. Experiments were done in triplicate and significance was calculated (Fernandes, et al., 2010).

3.4. Morphological Change Analysis:

M92-047 and SKMEL-28 were seeded at 1 x 10^6 cells in 60mm plates and allowed to attach for 4-6 hrs followed by either treatment with IFNα2/IFNα2-$(SO)_{20}$ for 24 h,48 h and 72 h or left untreated. At each time point, morphological changes in the cells were visualized under an inverted light microscope, Nikon Eclipse TS 100 (DC Imaging,W. Chester, OH) and cell pictures were taken at 200x magnification using a digital camera (Nikon cool pix, Nikon, USA).

3.5. Cell Cycle Analysis:

1 x 10^6 cells were seeded in 60mm plates and allowed to attach for 4-6 hrs followed by either treatment with IFNα2/IFNα2-$(SO)_{20}$ for 48 h or left untreated. The cells were collected and fixed with ice cold 70% ethanol and stored at -20 ˚C overnight. The next day, the ethanol in the supernatant was carefully removed from the cell pellet after centrifuging the tubes at 1200 rpm at 4 ˚C. The propidium iodide (PI) mix (PI 40 µl, RNase 10µl and PBS 950µl) was added to the cell pellet and mixed thoroughly. The tubes were incubated at 37 ˚C for 30 min prior to being analyzed by flow cytometry

(FACS analyzer, Becton Dickinson, San Jose, CA). 10,000 cells were counted and analyzed by the instrument in triplicate. The resultant data analysis was done using the ModFit LT^{TM} software (Verity software sales, Topsham, ME). Experiments were done in triplicate and significance calculated.

3.6. Western Analysis:

Total protein from whole cell lysates were extracted with lysis buffer (50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P40, IX Protease inhibitor) on ice for 30-40 min. Lysed cells were subjected to centrifugation at 13,000 rpm to separate out the cell debris. The resultant protein extract was quantified and separated on an SDS gel. The separated proteins were transferred onto an Immobilon-P membrane (Millipore, USA) followed by blocking the membrane in 5% non-fat dry milk prepared in PBS-T (0.1% tween-20 added) for 1 hr at RT. After appropriate primary and secondary antibodies were added and washing steps were done, the signal was detected using Amersham ECL plus (GE healthcare, USA). Primary antibodies used were anti-tyk-2, anti-JAK-1, anti-Stat1, anti-Stat2, anti-ISGF3γ-p48, anti-OAS1, anti-PKR, anti-p53 (Bp53-12), anti-Mdm2 (N-20) and anti-Bax (B-9). Primary and secondary antibodies used were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-β-actin (Sigma, St. Louis, MO) and anti-GAPDH (Santa Cruz Biotechnology, Inc) was used as loading controls for the analysis.

3.7. Immunoprecipitation (IP):

Total protein aliquots (200 mg) were immunoprecipitated using anti-Stat-2 antibody (Santacruz, CA, USA) and Protein A sepharose beads (Sigma, St. Louis, MO). The beads were then washed with 1X SNNTE buffer (5% sucrose, 500 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% NP40) and boiled before separation on an SDS-gel. Western blotting for Stat1 and ISGF3γ-p48 was performed as described above (Fernandes, et al., 2010).

3.8. RNA Isolation, Quantification and Reverse-transcription Polymerase Chain Reaction (RT-PCR):

M92-047 cells were grown as described above and treated with IFNα2b/IFNα2-$(SO)_{20}$ for 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, 72 h and left untreated (0 h). After each time point, the total RNA was isolated from the cells using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA) as per the manufacturers protocol. Treating with RNase free DNase for 15 min during the isolation eliminated DNA contamination. The RNA isolated was quantified spectrophotometrically (260 nm) and the concentration was calculated. The purity and integrity of the RNA isolated was examined by separation on a 1-2% agarose gel and subjected to gel electrophoresis. The RNA was then used for RT-PCR using the RT^2 First strand kit C03 (SABiosciences, USA) as per the manufacturers protocol. The RT-PCR conditions were incubation at 42 °C for exactly 15 min and immediately stopping the reaction by heating at 95 °C for 5 min. The resultant cDNA was used for p53 and Bax transcript quantification using the RT^2 SYBR® Green qPCR

master mix kit (SABiosciences, USA) and PCR reactions were carried out in the Biorad iCycler (Biorad). Hercules, CA). The RT^2PCR primer sets used were for human Bax and human p53 (purchased from SABiosciences, Frederick, MD) and human ß actin primers (Biosynthesis, Lewisville). TX)

5'–TGTGATGGTGGGAATGGGTCAG-3'(sense) and5'–TTTGATGTCACGCACGATTTCC-3' (antisense). The ß actin mRNA was used as a control to account for differences in RNA quantity between the samples. The amounts of p53 and Bax transcripts at different time points of IFNα2b/IFNα2-$(SO)_{20}$ treatments were calculated using the ΔCt method. 2 independent experiments were done in triplicate and significance was calculated.

3.9. Dual Reporter Luciferase Assays:

M92-047 cells were seeded in a 24 well plate and allowed to attach and grow for 24 h. The cells were then transfected with p53 luciferase reporter plasmid pp53-Luc (Panomics, Fremont, CA) which is a firefly luciferase reporter plasmid designed by using cis-acting enhancer element sequence with a p53 binding site or bax luciferase reporter plasmid. Transfection was done using lipofectamine 2000 (Invirogen, Carlsbad, CA) as per the manufacturers protocol. The transfection efficiency was evaluated by co-transfection of an internal control reporter plasmid pRL-TK (renilla luciferase, HSV-thymidine kinase) vector (Promega, Madison, WI). After 6 h of transfection the media was removed and replaced with regular growth media for about 8 h followed by treatment with IFNα2b/IFNα2-$(SO)_{20}$or left untreated. Cells were treated with IFNα2b/IFNα2-

$(SO)_{20}$ for 12 h and 18 h for p53 expression and 18 h for Bax expression. The luciferase expression was measured using the dual luciferase reporter assay kit (Promega, Madison, WI) as per the manufacturers protocol. The cells were lysed using lysis buffer and 20 µl of the supernatant was used to measure the amount of luminescence using a luminometer (Lumat LB 9507, Berthold, Oak Ridge, TN). The respective promoter activities, p53 and Bax, were normalized using Renilla luciferase activity as the transfection control. The experiments were performed in triplicate and significance was calculated.

3.10. Statistical Analysis:

The data were analyzed using the student-t test to determine significance. A p value of ≤ 0.05 was considered statistically significant. Also, standard deviation was determined as applicable.

CHAPTER 4: RESULTS

4.1. IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ Treatment of SKMEL-28 Cells Sensitive to IFNα2b, Showed Increase in STAT1 levels:

The sensitivity of SKMEL-28 cells to IFNα2b was confirmed using the amount of STAT1 protein level as a marker of sensitivity/resistance. The cells were treated with IFN α2 /IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ or left untreated for 24 h. Cells treated with IFNα2b showed an increase in STAT1 proteins levels as compared with untreated control cells (Figure 7A). Also, cells treated with IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ showed increase in STAT1 levels as compared with untreated control cells (Figure 7B), thus confirming sensitivity to interferon.

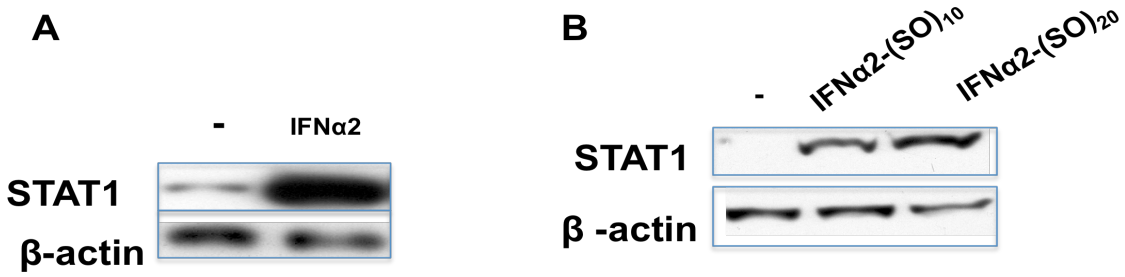
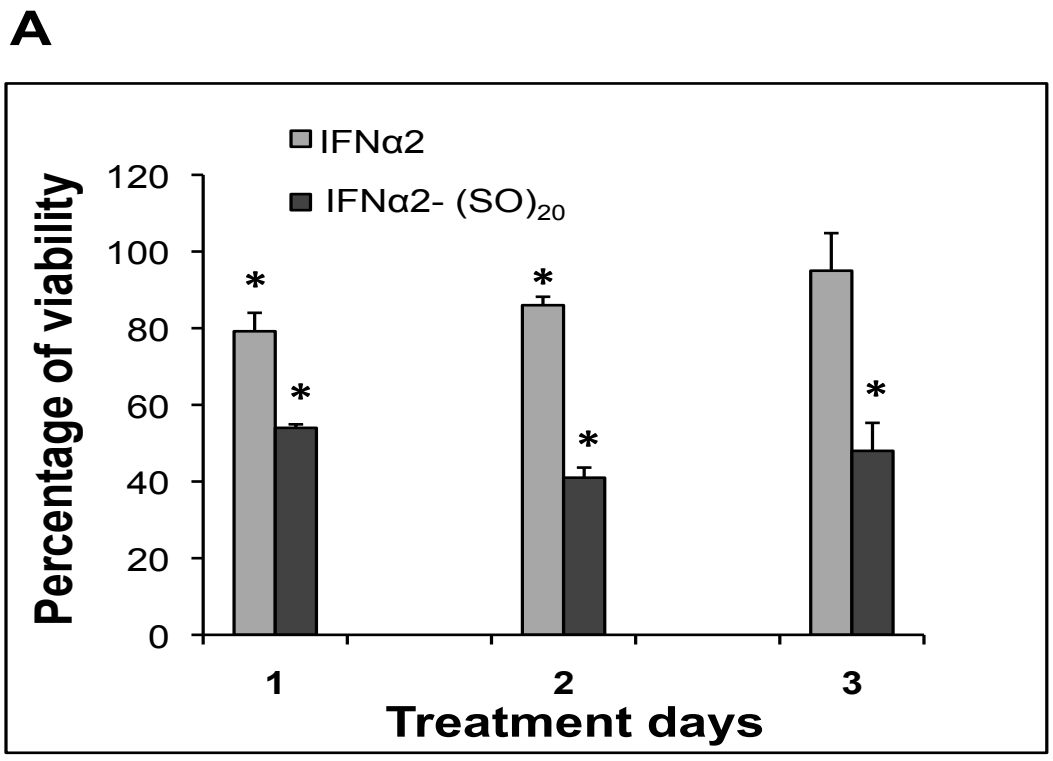


Fig 7: SKMEL-28 cells showing the level of STAT1, as a marker of IFNα2 resistance. SKMEL-28 cells were treated with A) IFNα2 and B) IFNα2-$(SO)_{10}$ & IFNα2-$(SO)_{20}$ or left untreated (-) and STAT1 protein levels were detected by western blot analysis. ß-actin was used as a loading control.

4.2. IFNα2b/IFNα2 and IFNα2-$(SO)_{20}$ Decreased Melanoma Cell Viability:

The antiproliferative/growth inhibitory effects of IFNα2 and IFNα2-$(SO)_{20}$ were assessed by treating human melanoma cells, M92-047 and SKMEL-28, over a period of 72 h and measuring the cell viability. Although there was decrease in percent cell viability of M92-047 treated with IFNα2 and IFNα2-$(SO)_{20}$, the decrease was greater in IFNα2-$(SO)_{20}$ cell treatment, with the greatest effect observed at 48 h (Figure 8A). SKMEL-28 cells, known to be sensitive to interferon, showed an even greater decrease in cell viability, with better effects by IFNα2-$(SO)_{20}$ at 48 h and 72 h treatment compared with the IFNα2 treatment (Figure 8B) (Fernandes, et al., 2010).



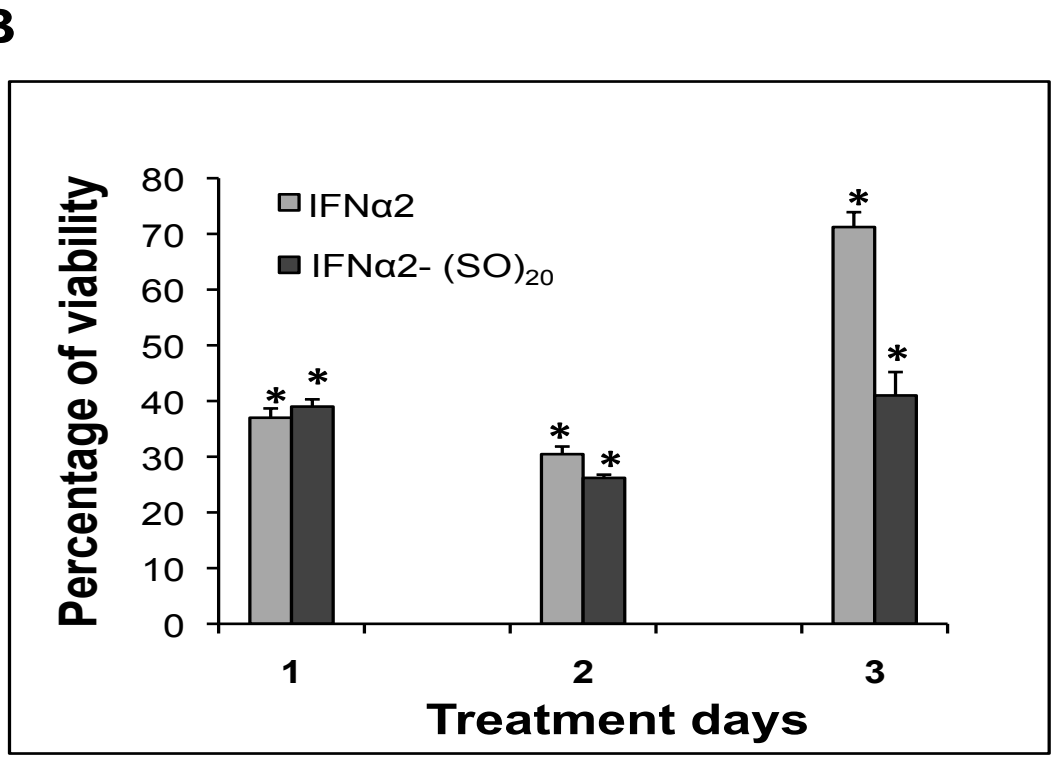
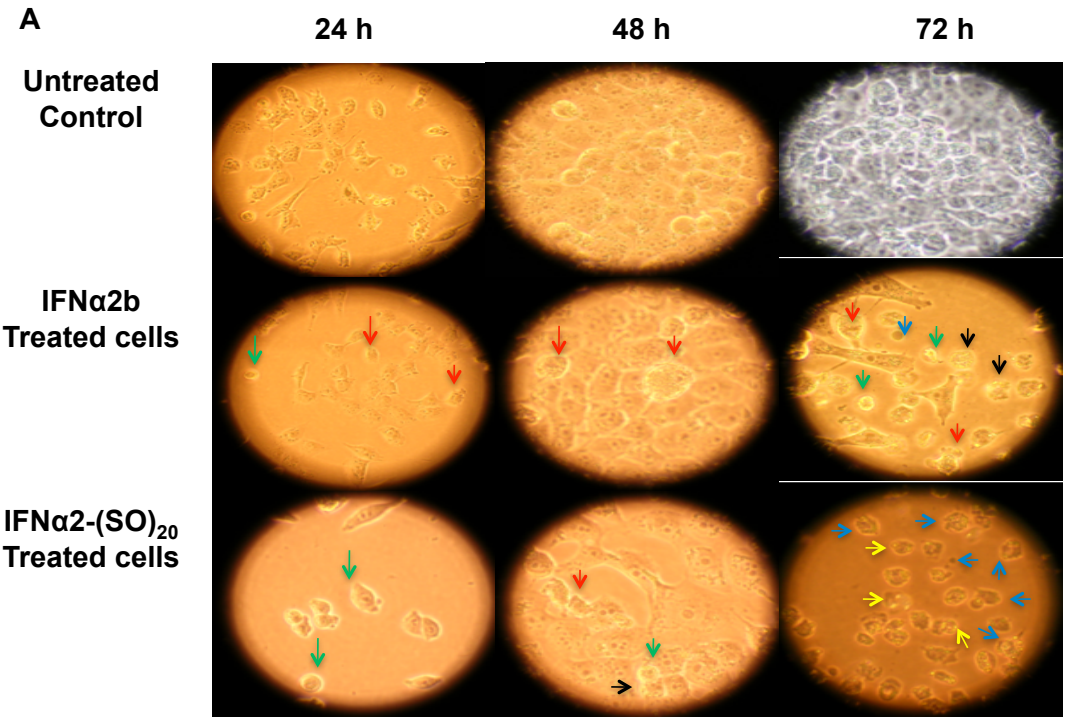


Fig 8: Cell viability analysis for melanoma cell lines. Cells were treated with IFNα2 and IFNα2-$(SO)_{20}$, for 24 h, 48 h and 72 h, respectively. The percentage of viable cells after different treatment periods is shown after being normalized with untreated controls. A) M92-047 cells and B) SKMEL-28 cells. The data are a representation of three independent experiments performed in triplicate. Standard deviation was determined and significance compared to untreated controls on each day was calculated (* $p \leq 0.05$).

4.3. IFNα2b/IFNα2 and IFNα2-$(SO)_{20}$ Treatment Affects Melanoma Cell Morphology:

Morphological changes visualized at 24 h, 48 h and 72 h after treatment show both M92-047 (Fig. 9A) and SKMEL-28 (Fig. 9B) undergoing the different characteristic

features of apoptosis. The sequence of events include initial rounding of cells, followed by nuclear blebbing, surface blebbing and the formation of the cell blister. These events eventually lead to nuclear condensation and subsequent cell lysis.



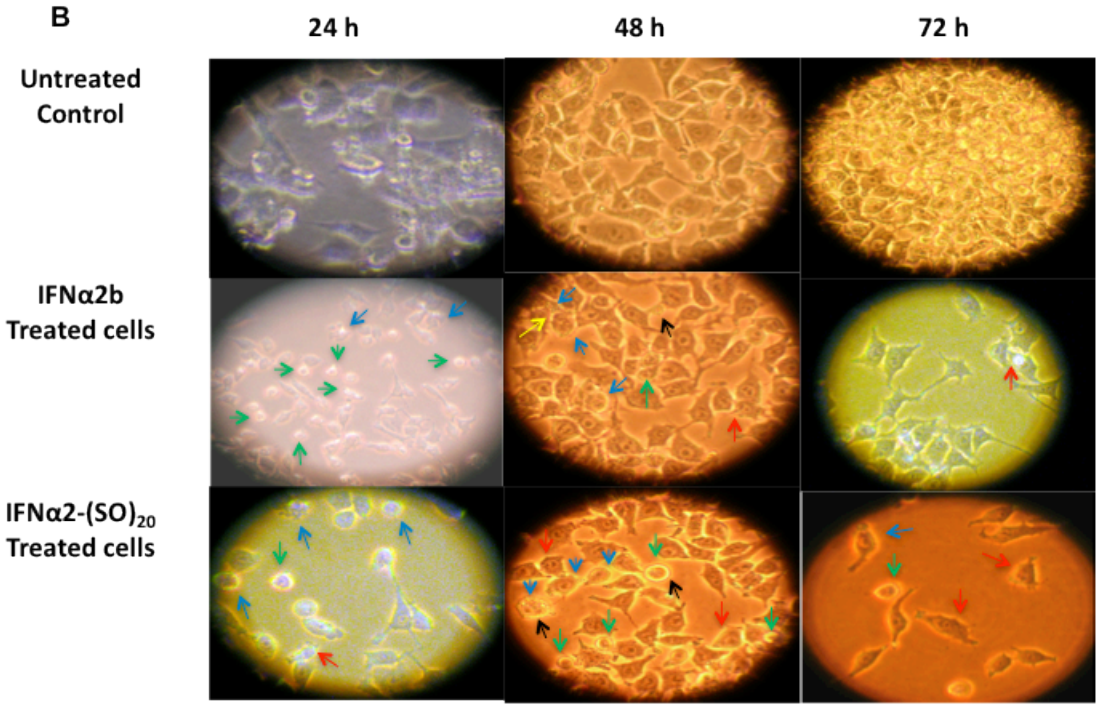


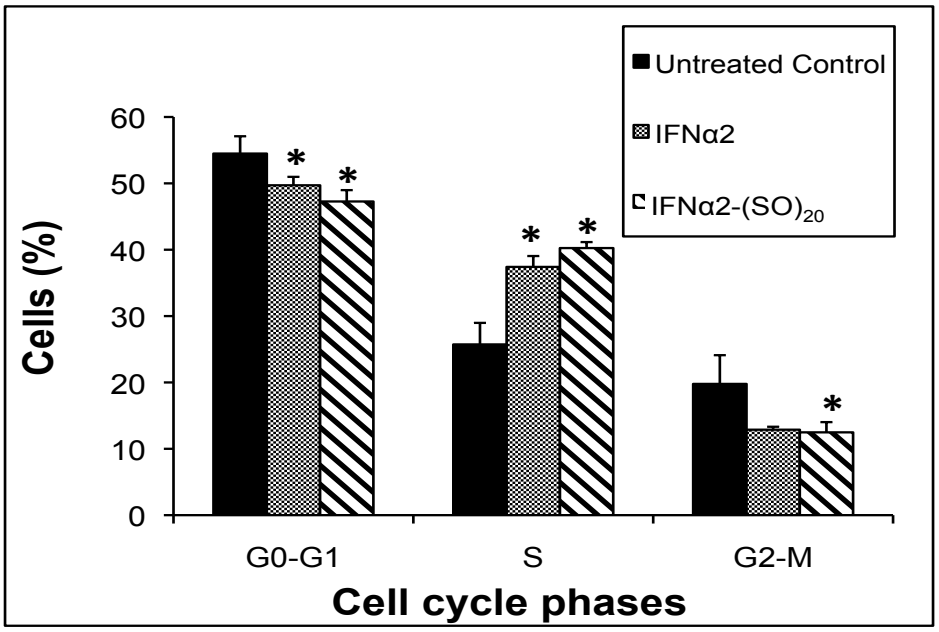
Fig 9: Cell morphological changes observed for melanoma cells. Cells were treated with IFNα2 and IFNα2-$(SO)_{20}$, for 24 h, 48 h and 72 h or left untreated. The pictures shown were taken under inverted light microscope (Nikon Eclipse 5100) at 200x magnification using a digital camera (Nikon cool pix), after respective treatment times. A) M92-047 cells and B) SKMEL-28 cells. The arrows indicate typical features of apoptosis; red arrows show cells undergoing early stages of apoptosis, blue arrows indicate cell surface blebbing and blister formation, yellow arrows indicate surface blister, green arrows show nuclear condensation and black arrows show nuclear blebbing.

4.4. IFNα2b/IFNα2 and IFNα2-$(SO)_{20}$ Treatment Affects Cell Cycle Progression:

An increase in the number of cells arrested at the 'S' phase of the cell cycle, compared with untreated control, was observed in M92-047 cells. This 'S' phase arrest

was higher when M92-047 cells were treated with IFNα2-$(SO)_{20}$ as compared with IFNα2 (Figure 10A). When SKMEL-28 cells were similarly treated and analyzed, the percentage of cells in the 'G1' phase of the cell cycle was higher than the untreated control, showing a G1 arrest in this cell type. Moreover, this arrest was highest in cells treated with IFNα2-$(SO)_{20}$ as compared with native IFNα2 (Figure 10B) (Fernandes, et al., 2010).

A



B

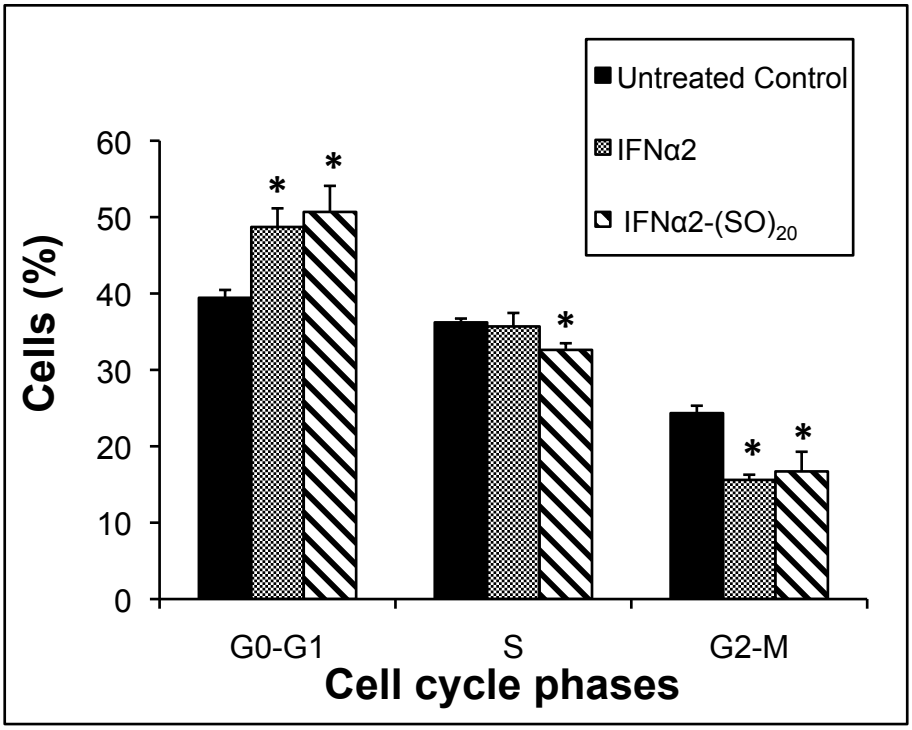


Fig 10: Cell cycle analysis for melanoma cells. Cells were left untreated or treated with IFNα2/IFNα2-$(SO)_{20}$ for 48 h and stained using Propidium iodide (PI, 40 μg/ml). Histograms representing the percentage of cells in the different phases of cell cycle are shown. A) M92-047 cells and B) SKMEL-28 cells. Analysis was done by a flow cytometer and the Modfit software was used to analyze the data obtained. The data shown is a representation of three independent experiments performed, standard deviation was determined and significance compared to untreated control was calculated (* $p \leq 0.05$).

4.5. The Fusion Derivatives IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ Bind Cell Surface Receptors and Trigger the JAK-STAT Signaling Pathway as Effectively as Native IFNα2:

To determine if IFNα2-$(SO)_{10\&20}$ bind to cell surface receptors and trigger the JAK-STAT signaling pathway as effectively as native IFNα2, we analyzed the protein levels of the receptor tyrosine kinases, Tyk-2 and JAK-1, which function upstream in the interferon signaling pathway. M92-047 cells stimulated with both IFNα2 and IFNα2-$(SO)_{20}$ showed elevated levels of total Tyk-2 (Figure 11A) and JAK-1 (Figure 11B) as compared with untreated control cells (Fernandes, et al., 2010).

4.6. JAK-STAT Signaling Components Have Increased Proteins Levels as Compared with Untreated Control Cells:

Both STAT1 and STAT2 protein levels increased in cells stimulated by IFNα2, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ over non-stimulated control cells (Figure 12A). In addition, p48 (ISGF-3γ) the third protein required to form the functional ISGF-3 complex in the signal transduction pathway increased. The increase in p48 was seen in cells treated with IFNα2, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$(Figure 12A). We then assayed STAT1/STAT2 heterodimerization, which is necessary for formation of the ISGF-3 complex. Our results indicated that although the heterodimer is present in the control cells (untreated cells, lane 1), the amount of heterodimer increased after treatment with IFNα2, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$. Immunoprecipitation of STAT2 followed by western blot analysis using an anti-p48 antibody (Figure 12B) indicated an association between STAT2 and p48 (Fernandes, et al., 2010).

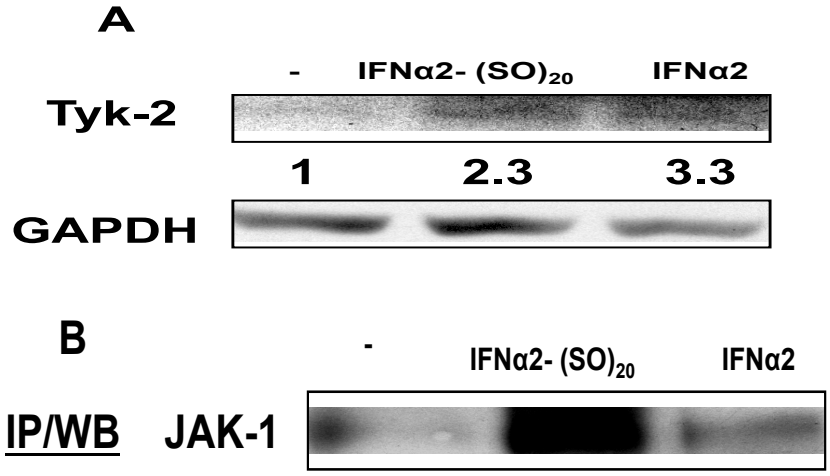
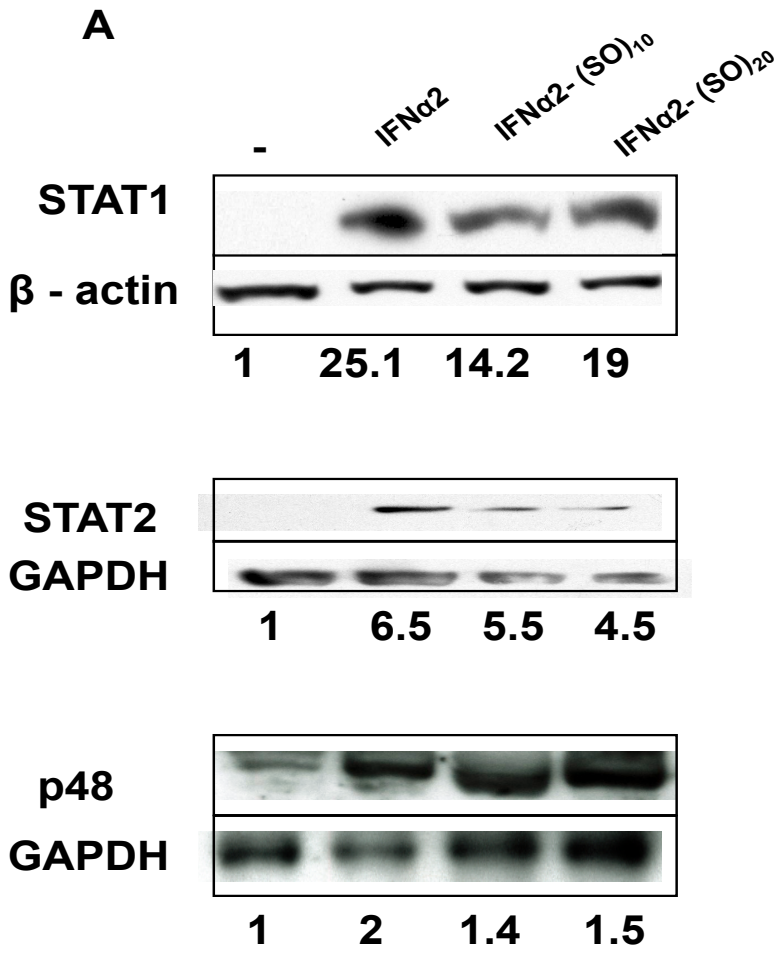


Fig 11: Representative western blots of M92-047 cells showing the receptors Tyk-2 and Jak-1 involved in IFN signaling. A) Tyk-2 detected by western analysis using anti-tyk-2 antibody after cells were stimulated with IFNα2, IFNα2-$(SO)_{20}$ or left untreated (-). GAPDH was used as a loading control. The numbers are representative of a fold change in pixel density values of Tyk-2 compared to the untreated control (-) and normalized to respective GAPDH pixel density values. B) Shows JAK-1 detected by immunoprecipitation followed by western analysis using anti-JAK-1 antibody.


A
-
IFNα2
IFNα2- (SO)10
IFNα2- (SO)20
STAT1
β - actin
1
25.1
14.2
19
STAT2
GAPDH
1
6.5
5.5
4.5
p48
GAPDH
1
2
1.4
1.5

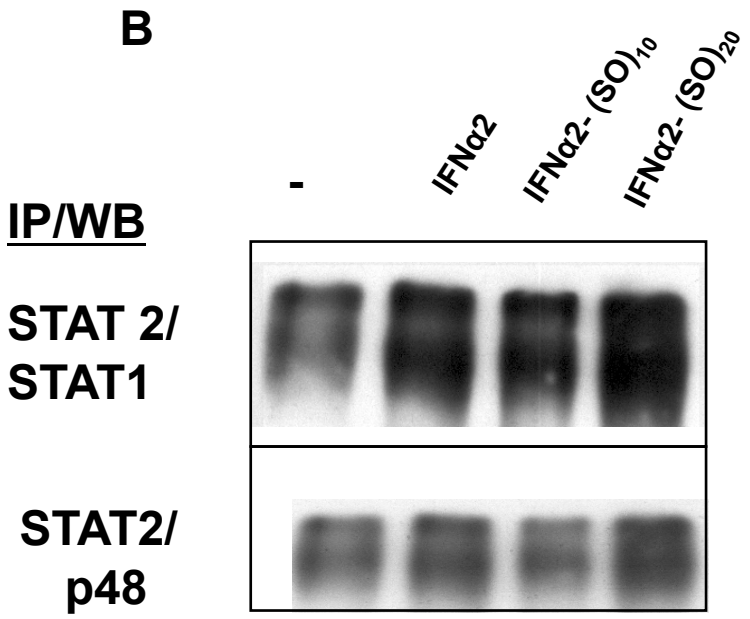


Fig 12: Representative western blots showing the key signaling components of the Interferon signaling pathway. M92-047 cells stimulated with IFNα2, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ for 24 h in comparison with untreated control. A) Western analysis of proteins Stat1, Stat2 and p48. ß-actin or GAPDH were used as loading controls. The numbers are representative of a fold change in pixel density values of STAT1, STAT2 and p48 compared to their respective untreated controls (-) and normalized with their respective GAPDH/ß-actin pixel density values. B) Immunoprecipitation/western analyses of Stat2/Stat1 and Stat2/p48.

4.7. The Fusion Derivative IFNα2-$(SO)_{20}$ and IFNα2 Up Regulate Downstream Signaling Proteins OAS1 and PKR:

Next, we showed that IFNα2 and IFNα2-$(SO)_{20}$ treatment lead to an increase in expression levels as assayed by western blot analysis of both 2'5'OAS1 (Figure 13A) and

PKR both of which are downstream proteins of the IFN signaling pathway (Figure 13B) (Fernandes, et al., 2010).

4.8. IFNα2 and IFNα2-$(SO)_{20}$ Increased p53 Promoter Activity in M92-047 Cells after Treatment for 12 h and 18 h:

We demonstrated IFN signaling via the JAK-STAT pathway in human melanoma cells M92-047 and formation of an active ISGF3 complex, evidenced by activation of downstream proteins 2'5' OAS1 and PKR. Therefore, our next step was to test if the ISGF3 complex was able to bind to the ISREs in the p53 promoter and transcriptionally activate p53 after treatment. For this, we treated M92047 cells with IFNα2 and IFNα2-$(SO)_{20}$ or left them untreated for 12 h (Fig. 14A) and 18 h (Fig. 14B) and we determined the p53 promoter transcriptional activity after these treatments using the dual luciferase reporter assay. Our results showed that p53 promoter transcriptional activity increases in both treatments at the indicated time points as compared with untreated control cells. Moreover, the fusion protein IFNα2-$(SO)_{20}$ showed the higher p53 transcriptional activity at these time points.

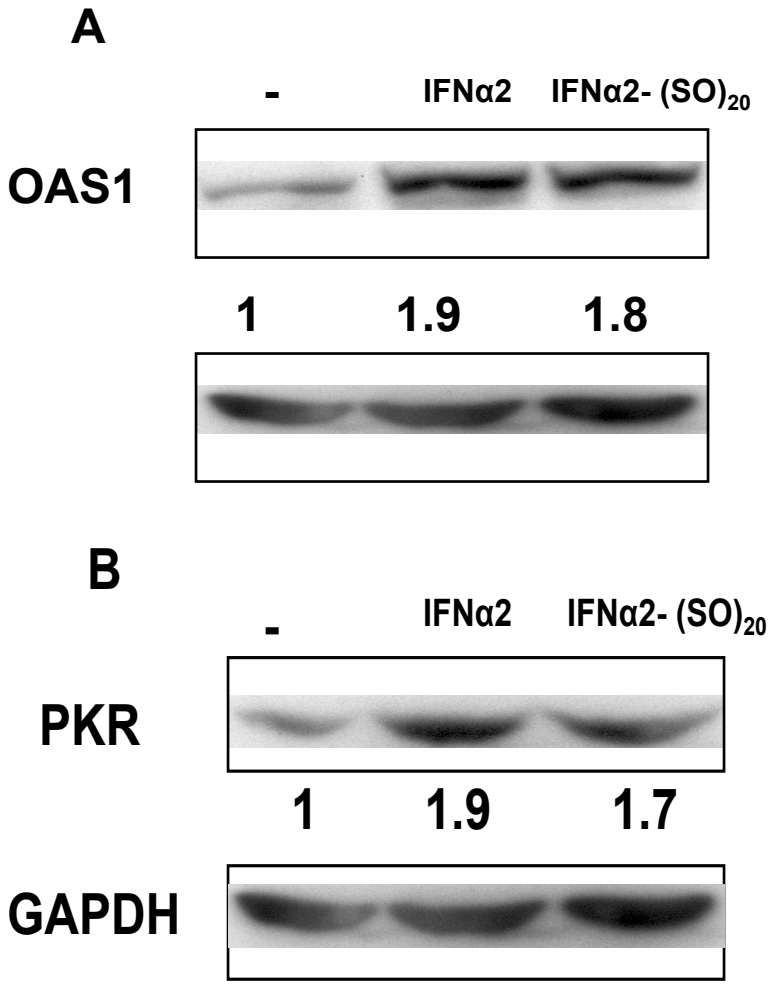
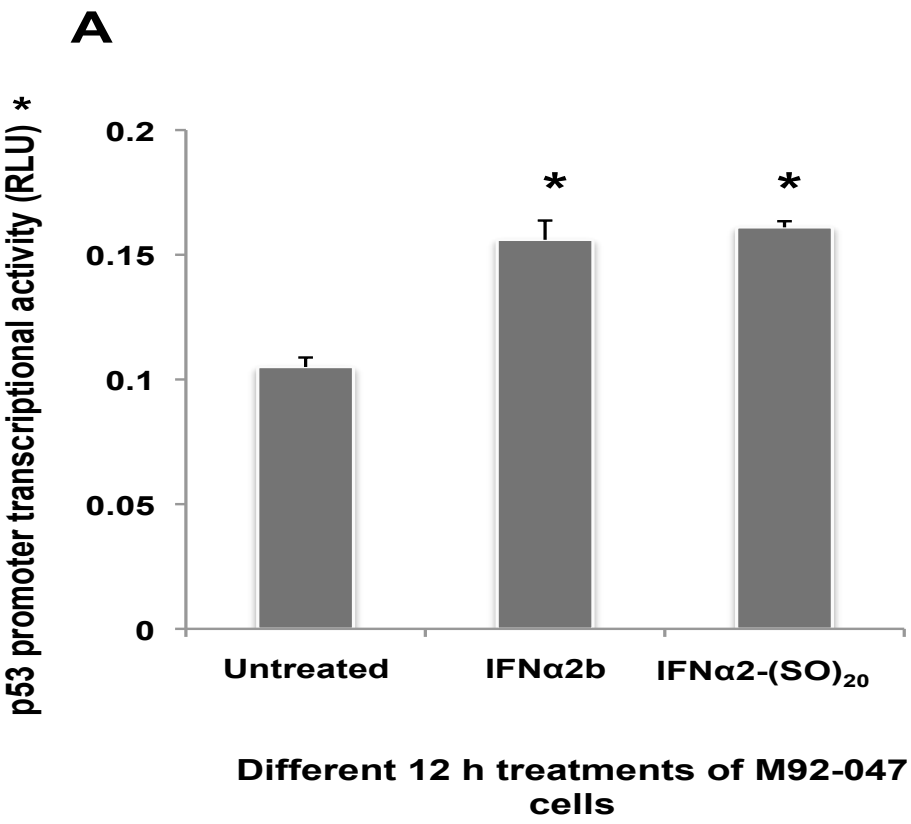


Fig 13: Representative western blots showing the protein levels of downstream targets of Interferon. M92-047 cells were treated with IFNα2/IFNα2-$(SO)_{20}$ or left untreated. Western blot analysis of A) 2'5' OAS1 protein B) PKR protein. GAPDH was used as a loading control. The numbers are representative of a fold change in pixel density values of OAS1 and PKR compared to their respective untreated controls (-) and normalized with their respective GAPDH pixel density values.


A
p53 promoter transcriptional activity (RLU) *
0.2
0.15
0.1
0.05
0
*
*
Untreated
IFNα2b
IFNα2-(SO)20
Different 12 h treatments of M92-047 cells

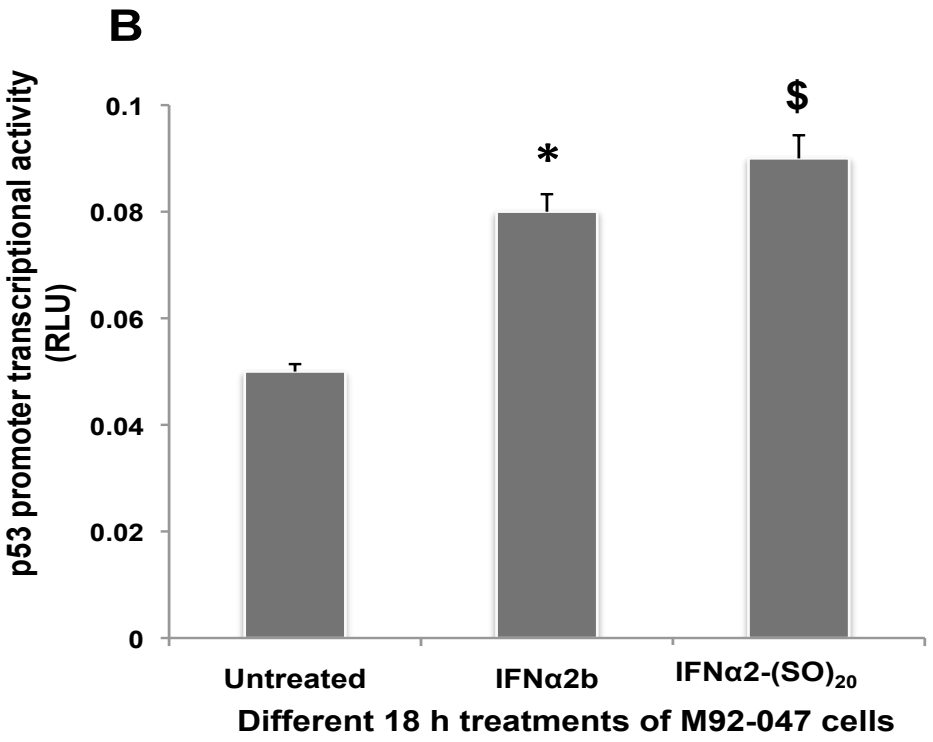


Fig 14: p53 promoter activity analysis performed using the dual luciferase reporter assay. M92-047 cells were transfected with p53 reporter plasmid and control reporter plasmid. The cells were then treated with IFNα2/IFNα2-$(SO)_{20}$ or left untreated for A) 12 h and B) 18 h. The data are a representation of two independent experiments done in triplicate. Standard deviation was determined and significance as compared to untreated control calculated (* $p \leq 0.05$, \$ $p \leq 0.06$).

4.9. IFNα2 and IFNα2-$(SO)_{20}$ Increased p53 transcript Levels in Melanoma Cells M92047:

After determining an increase in the p53 promoter activity in cells treated with IFNα2b and the fusion derivative, we next investigated the effect of the same treatments on p53 transcript levels when cells were treated for different time points, 6 h, 12 h, 18 h,

24 h, 36 h, 48 h and 72 h or left untreated (0 h), by real time RT-PCR. The results showed that both IFNα2 & IFNα2-$(SO)_{20}$ increased p53 transcript levels over the untreated (0 h) control. Also, the fusion protein IFNα2-$(SO)_{20}$ increased p53 transcript levels as early as 6 h after treatment as compared with IFNα2 which increased at 12 h after treatment. Moreover, p53 transcript level increased in a time dependent manner in cells treated with IFNα2-$(SO)_{20}$, with the highest fold change seen at 48 h after treatment. In cells treated with IFNα2, there is a decrease in transcript level at 18 h, 24 h and 36 h as compared with 12 h treatment and another increase at 48 h, with the highest fold change seen at 72 h after treatment (Fig. 15).

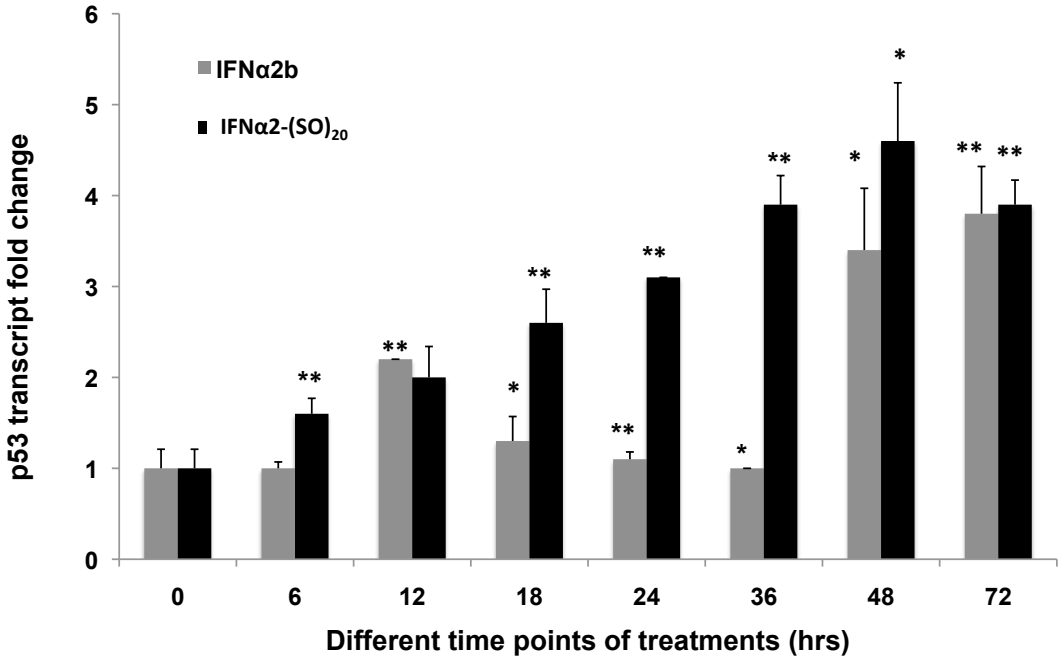


Fig 15: p53 transcript level was determined by real time RT-PCR at different time points after treatment. M92-047 cells were treated with IFNα2/IFNα2-$(SO)_{20}$ or left untreated (0 h) for 6 h, 12 h,18 h, 24 h, 36 h, 48 h and 72 h. At each time point the RNA was isolated and used to quantify p53 transcript levels expressed as a fold change. The data are a representation of two independent experiments done in triplicate and the significance compared to the 0 h control was calculated (* $p \leq 0.05$, ** $p \leq 0.01$, n = 2).

4.10. IFNα2 and IFNα2-$(SO)_{20}$ Treatment of M92047 Cells Increased p53 protein Levels in a Time Dependent Manner:

Next, we assessed the p53 protein levels in these cells after treatment at different time points 6 h, 12 h, 24 h, 48 h and 72 h with both IFNα2 & IFNα2-$(SO)_{20}$ or left untreated (0 h), by Western blot analysis. In the IFNα2 treated cells, the highest increase

is at 24 h/48 h after treatment, while in IFNα2-$(SO)_{20}$ treated cells, the highest increase in p53 protein levels is seen at 12 h after treatment. Also, in IFNα2 treated cells there is a decrease in p53 protein level at 72 h as compared with 24 h/48 h treatments. However, in cells treated with the fusion protein, the p53 protein level remains stable through 72 h of treatment (Fig.16).

4.11. IFNα2 and IFNα2-$(SO)_{20}$ treatment of M92047 Cells Lowered HDM2 levels Over Time:

After examining p53 protein levels, the next step was to measure the levels of its negative regulator HDM2 after M92-047 cells were treated with IFNα2 & IFNα2-$(SO)_{20}$ for 6 h, 12 h, 24 h, 48 h, 72 h or left untreated (0 h). For this, we used western analysis and the results indicate that HDM2 protein levels decreased for both treatments. However, in IFNα2 treated cells, there was a transient increase in HDM2 levels at 12 h and 24 h over the untreated (0 h) control, but these levels were again significantly lower at 48 h and 72 h after treatment compared with untreated cells. Where as in cells treated with IFNα2-$(SO)_{20}$, HDM2 protein levels decreased by 6 h and remained low as time increased (Fig. 17).

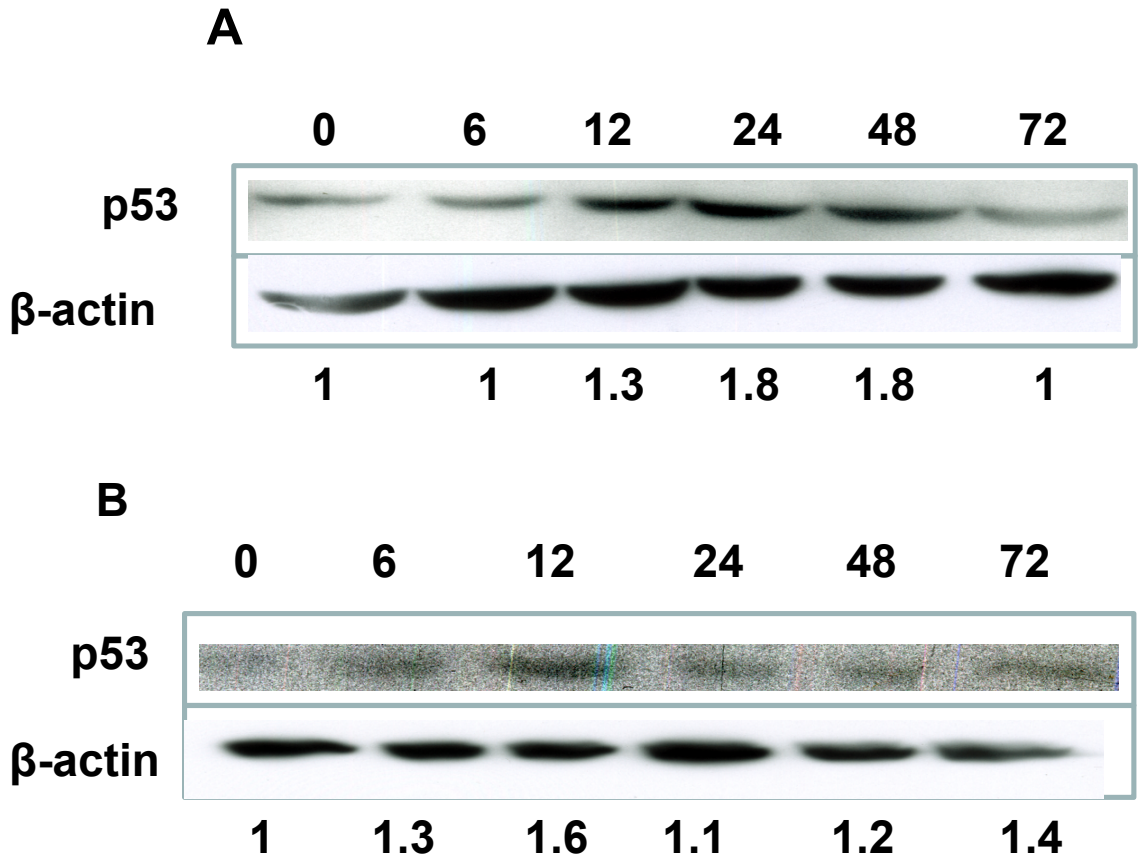


Fig 16: Representative western blots of M92-047 cells showing p53 protein levels at different time points after treatment. M92-047 cells were treated with A) IFNα2 and B) IFNα2-$(SO)_{20}$ or left untreated (0 h) for 6 h, 12 h, 24 h, 48 h and 72 h. p53 protein levels were detected by western analysis using anti-p53 antibody (Bp53-12). The amount of p53 protein was normalized by ß-actin. The numbers are representative of a fold change in pixel density values of p53 compared with the untreated control (0 h) and normalized with ß-actin.

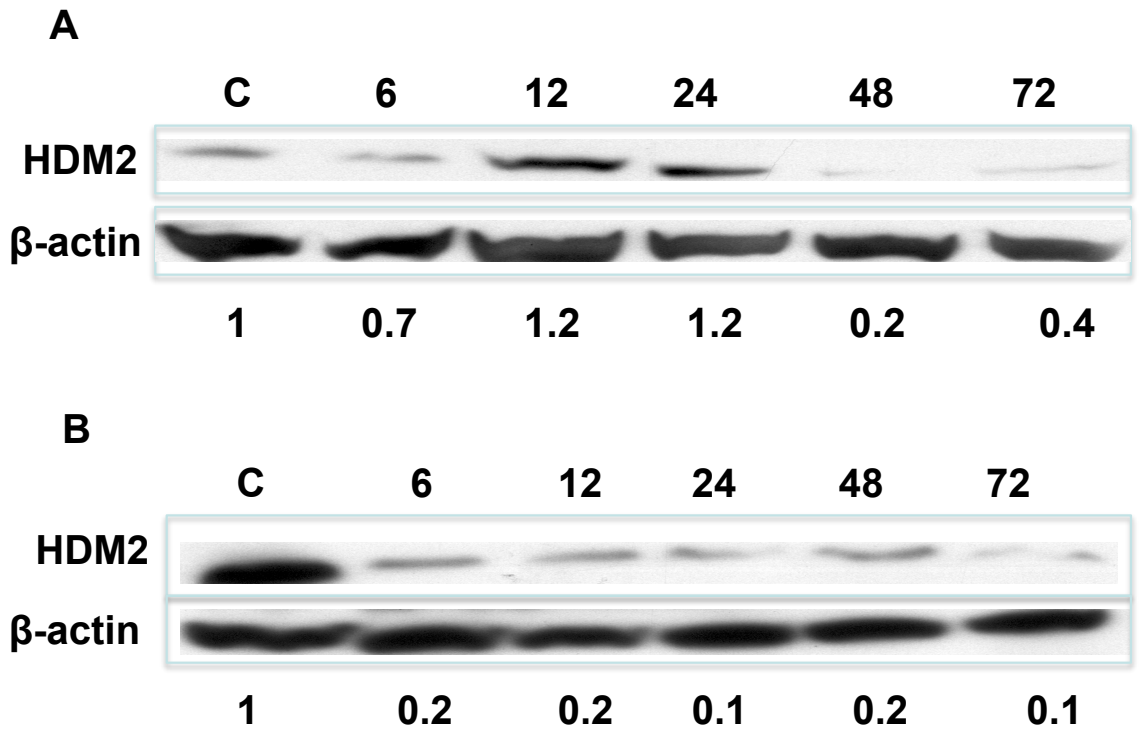


Fig 17: Representative western blots of M92-047 cells showing HDM2 protein levels at different time points after treatment. M92-047 cells were treated with A) IFNα2 and B) IFNα2-$(SO)_{20}$ or left untreated (0 h) for different time points of 6 h, 12 h, 24 h, 48 h and 72 h. HDM2 proteins levels were detected using anti-MDM2 antibody (N-20). The numbers are representative of a fold change in pixel density values of HDM2 compared with the untreated control (0 h) and normalized with ß-actin.

4.12. IFNα2 and IFNα2-$(SO)_{20}$ Increased Bax Promoter Transcriptional Activity after M92-047 Cells Were Treated for 18h:

Bax is a transcriptional target downstream of p53 and plays a crucial role in the apoptotic effects mediated by p53. Therefore, our next step was to evaluate the effect on

Bax transcriptional activity in these cells after treatment. The Bax promoter transcriptional activity was assayed using dual luciferase reporter assay and it was found that as compared with the untreated control, the Bax promoter activity increased albeit slightly when cells were treated with both IFNα2 and IFNα2-$(SO)_{20}$, with a significant increase seen with the fusion protein treatment (Fig. 18).

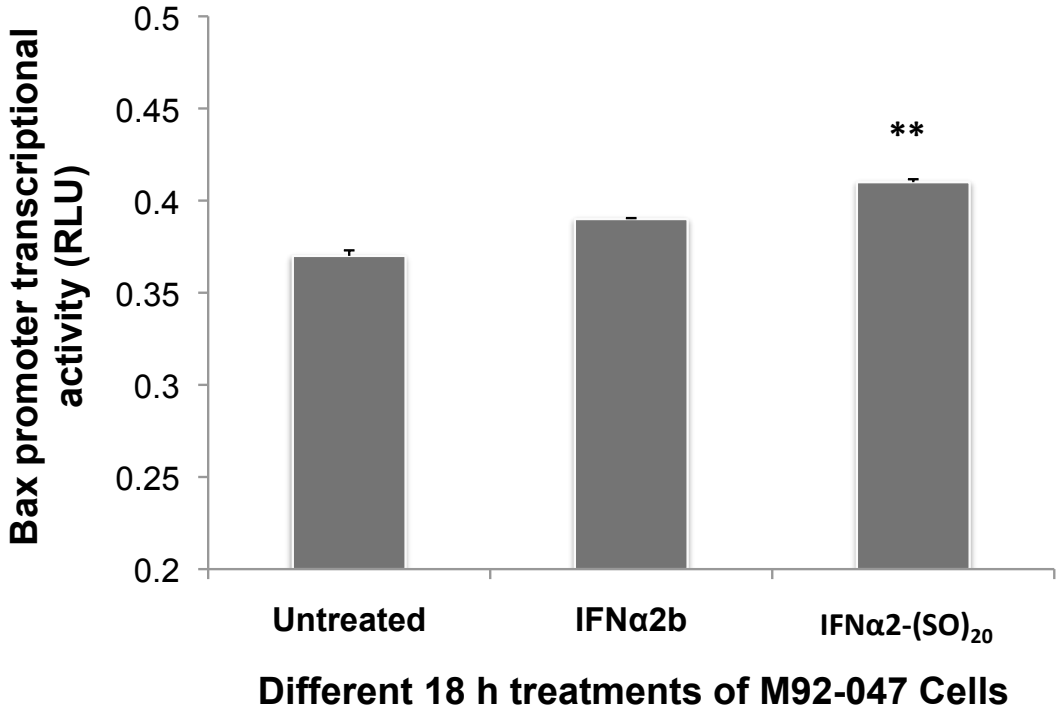


Fig 18: Bax promoter activity analysis performed using the dual luciferase reporter assay. M92-047 cells were transfected with Bax reporter plasmid and control reporter plasmid. The cells were then treated with IFNα2/IFNα2-$(SO)_{20}$ or left untreated for 18 h. The data are a representation of two independent experiments done in triplicate. Standard deviation was determined and significance as compared with untreated control was calculated (** $p \leq 0.01$).

4.13. IFNα2 and IFNα2-$(SO)_{20}$ increased Bax Transcript Levels Quantitated by Real Time RT-PCR after Cells Were Treated for Different Time Points:

We next examined the Bax transcript levels at different time points, as was done previously for p53 (described in section 4.9). Our results indicate an increase in the Bax

transcript levels (represented as a fold change) with time for both treatments as compared with the untreated (0 h) control. Also, as seen with p53 transcript levels, in cells treated with IFNα2 the increase was seen at 12 h as compared with 6 h for IFNα2-$(SO)_{20}$. Moreover, with IFNα2-$(SO)_{20}$ treatment, Bax transcript levels continuously increased with time, with the highest fold change seen at 48 h and 72 h after treatment. However, with IFNα2 treatments, Bax transcript levels did not increase to the same degree as the fusion protein but the highest fold change was also observed at 72 h after treatment (Fig. 19).

4.14. IFNα2 and IFNα2-$(SO)_{20}$ Increased Total Bax Protein Levels after Cells Were Treated for Different Time Points:

After quantitating Bax transcript levels, we determined the total protein levels of Bax at various time points after the cells were left untreated (0 h) or treated with IFNα2b and IFNα2-$(SO)_{20}$. The western blot analysis revealed a steady increase in Bax protein levels for both treatments, with the highest increase seen at 72 h after IFNα2 treatment; in IFNα2-$(SO)_{20}$ treated cells the highest increase was seen at 24 h and 48 h after treatment (Fig. 20).

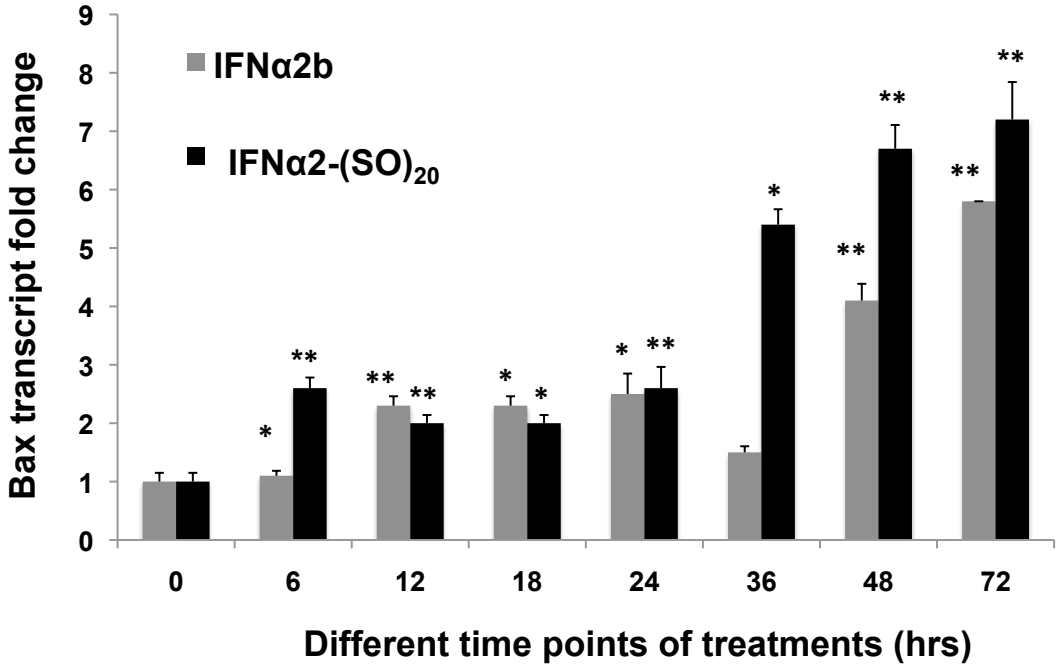


Fig 19: Bax transcript level was determined by real time RT-PCR at different time points after treatment. M92-047 cells were treated with IFNα2/IFNα2-$(SO)_{20}$ or left untreated (0 h) for 6 h, 12 h, 18 h, 24 h, 36 h, 48 h and 72 h. At each time point the RNA was isolated and used to quantify Bax transcript levels expressed as a fold change. The data are a representation of two independent experiments done in triplicate and the significance compared with the 0 h control was calculated (* $p \leq 0.05$, ** $p \leq 0.01$, n = 2).

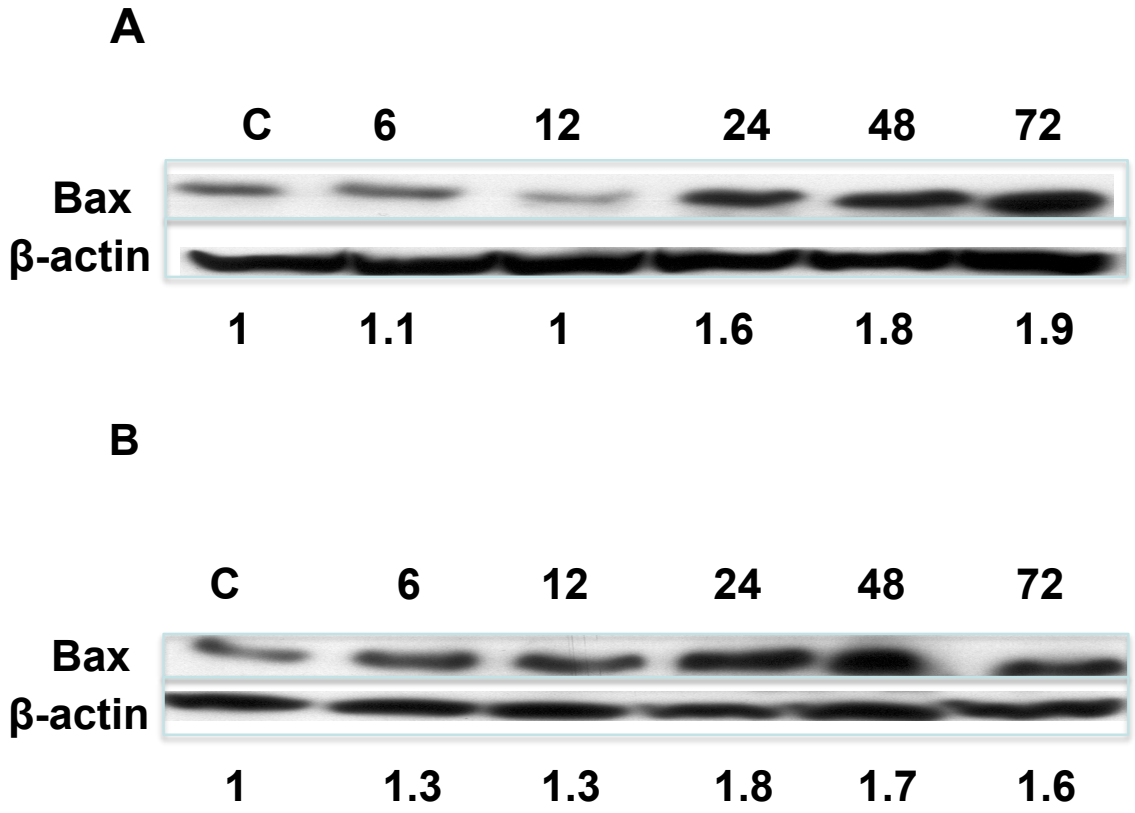


Fig 20: Representative western blots of M92-047 cells showing Bax protein levels at different time points after treatment. M92-047 cells were treated with A) IFNα2 and B) IFNα2-$(SO)_{20}$ or left untreated (0 h) for different time points of 6 h, 12 h, 24 h, 48 h and 72 h. The amount of Bax protein loaded was normalized by the ß-actin. The numbers are representative of a fold change in pixel density values of Bax compared with the untreated control (0 h) and normalized with ß-actin.

CHAPTER 5: DISCUSSION

Small protein therapeutics possess a variety of problems arising from their small size and hence short serum half-life. Possible solutions involve the use of pegylation, which increases the molecular weight of the protein, thereby increasing the half-life in the body system(Roberts, Bentley, & Harris, 2002). Pegylation also prevents degradation caused by serum proteases, thereby increasing the overall stability of the protein. Disadvantages of pegylation include low reaction yields, many added purification steps, low biological activity and hence higher production costs (Wang, et al., 2002; Xu, et al., 2007). A novel method was developed to produce IFNα2 as an AGP fusion in tobacco cells(Xu, et al., 2007). These proteins can be readily isolated in bulk using relatively few steps (1 or 2) resulting in high molecular weight IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ fusion glycoproteins having increased serum half life compared to non-glycosylated native recombinant IFNα2, yet retain virtually all biological activity (Fernandes, et al., 2010; Xu, et al., 2007).

Here, our first step was to confirm the sensitivity of SKMEL-28 cells toIFNα2 and determine if IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ would have a similar effect.STAT1 levels detected in the cells after IFNα2 treatment correlates with sensitivity to IFNα2 treatment(Wong, et al., 1997). In this study, treating SKMEL-28 cells with IFNα2, IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ increased STAT1 protein levels compared with the untreated control cells, confirming the sensitivity of these cells to IFN treatment. Furthermore, the fusion proteins had a similar effect as IFNα2.

Next, we expanded our approach to determine if IFNα2-$(SO)_{20}$ caused growth inhibition in human melanoma cell lines, M92-047 and SKMEL-28. Decreased cell viability was observed in both cell lines over a period of 72 h treatment with IFNα2 and IFNα2-$(SO)_{20}$,with a greater percent decrease seen in IFNα2-$(SO)_{20}$ treated cells compared with IFNα2 treated cells (Fernandes, et al., 2010).In addition, our observations of the morphological changes occurring in the cells throughout the treatment time course show typical features of cells undergoing apoptosis, characterized by rounding of cells, followed by surface blebbing, blister formation and cell lysis (Collins, Schandi, Young, Vesely, & Willingham, 1997; Saraste, 1999). These morphological changes, although qualitative, were seen in both cell types suggesting apoptosis as a possible mechanism of cell death leading to lower cell viability induced by IFNα2 and IFNα2-$(SO)_{20}$ treatment in these cells.

The effect of interferon on cell cycle regulation has been previously investigated in many different cell lines, with arrest seen most often at G1 phase of the cell cycle; however, there have also been reports of prolongation of S phase of the cell cycle with IFNα2 (Lundblad & Lundgren, 1981; Murphy, Detjen, Welzel, Wiedenmann, & Rosewicz, 2001; Panniers & Clemens, 1981; Sangfelt, Erickson, & Grander, 2000). We show that M92-047 cells remain for a prolonged period of time in the S phase of the cell cycle after 48 h treatment of IFNα2 and IFNα2-$(SO)_{20}$, whereas SKMEL-28 cells arrest at the G1 phase of the cell cycle under similar treatment conditions, presumably due to the difference between the two cell lines. Our data indicated that interferon produced as an AGP fusion protein has the ability to inhibit cell growth and alter the cell cycle in

melanoma cells. However, an important objective of our study was to show that these fusion proteins were comparable to native recombinant IFNα2 in terms of intracellular signaling events.

Modification of IFNα2 to produce the fusion proteins IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$ results in an increased molar mass compared with native recombinant protein because of the glycosylation by arabinogalactan (AG), which accounts for the larger size and greater serum stability (Xu, et al., 2007). Thus, we hypothesized that this larger molecular weight IFNα2 produced in plant cells would exert the same or better effects as native IFNα2. IFN exerts its effects intracellularly through the previously described JAK-STAT signaling pathway (Heim, 1999; Larner & Reich, 1996; Magrassi, et al., 1999; Martinez-Moczygemba, et al., 1997; Pansky, et al., 2000; Pestka, Krause, & Walter, 2004; Rawlings, et al., 2004; Schindler & Darnell, 1995; Wong, et al., 1997). In particular, IFNα2 binds to cell surface Tyk-2 and JAK-1 tyrosine kinase receptors. The signaling proteins STAT1, STAT2 and p48 (ISGF-3) are then up regulated in cells after IFNα2 treatment. We showed that both Tyk-2 and JAK-1 total receptor increased after the cells were treated with IFNα2 and IFNα2-$(SO)_{20}$ compared with untreated control cells. Thus, IFNα2-$(SO)_{20}$ initiates the same signaling pathway as IFNα2. Increased levels of STAT1in melanoma cells, which is extremely important in IFN signaling, is a marker indicating their sensitivity to IFN (Bromberg, Horvath, Wen, Schreiber, & Darnell, 1996; Carson, 1998; Wong, et al., 1997). Experiments showed the expression level of STAT1 protein increased when cells were treated with both fusion proteins IFNα2-$(SO)_{10}$ and IFNα2-$(SO)_{20}$, similar to IFNα2 treated cells.

Often in melanoma cells, resistance to IFNα2 is due to a deficiency in the signaling components of the ISGF3 complex, namely STAT1, STAT2 and p48 (Wong, et al., 1997). For the formation of the functional ISGF3 complex, STAT2 is thought to be extremely important, as it binds to both STAT1 to form a heterodimer and with the DNA binding protein p48. Evidence also suggests that two distinct complexes, STAT1-STAT2 and STAT2-p48, enter the nucleus as ISGF3 and bind to ISREs (Berger, et al., 1995; Lupas, 1996; Martinez-Moczygemba, et al., 1997). Our western blotting results showed that IFNα2-$(SO)_{10}$ and IFNα2$(SO)_{20}$,when added to M92-047 cells, increased expression of signaling components STAT1, STAT2 and p48 similar to cells treated with IFNα2. In addition, immunoprecipitation results showed the formation of the ISGF3 via 2 distinct complexes, STAT1-STAT2 heterodimer formation and the STAT2-p48 binding complex. These data suggest that the higher molecular weight IFNα2 proteins are able to communicate using the JAK-STAT signaling pathway used by cytokines, which was in agreement with our primary objective of the study.

After the ISGF3 complex enters the nucleus, it binds to ISREs present in promoters of IFN stimulated genes (ISGs). There are many ISGs that help in the mediation of IFN stimulated effects, but the two most commonly discussed ISGs for viral infections are the double stranded RNA activated enzymes, 2'5' OAS1 and PKR. These proteins are known to be induced in cells treated with IFNα2 and are hence called IFN induced proteins (Borden, et al., 2007; Chelbi-Alix & Wietzerbin, 2007; Gale & Katze, 1998; Hovanessian, 2007; Zilberstein, Kimchi, Schmidt, & Revel, 1978). As an extension to our primary objective of examining initial signaling events, we further hypothesized

that the fusion protein IFNα2-$(SO)_{20}$ would adhere to the downstream events in the IFN signaling pathway. For this, the effect on downstream proteins when cells were treated with IFNα2-$(SO)_{20}$ and IFNα2 was examined. Both 2'5' OAS1 and PKR protein expression increased as compared to untreated control cells showing that the fusion protein IFNα2-$(SO)_{20}$ was able to induce both 2'5' OAS1 and PKR in the same manner as native IFNα2. Together these results show that the higher molecular weight fusion protein,IFNα2-$(SO)_{20}$,binds to the interferon α/β receptor, activates the JAK-STAT signaling pathway components and, finally, up regulates expression of downstream proteins in a similar manner as compared with native recombinant IFNα2 (Fernandes, et al., 2010).

IFNs influence the transcription of their target genes by the binding of the trimeric protein complex of ISGF3 to ISREs present in the promoters of IFN inducible genes (Bluyssen, Durbin, & Levy, 1996; Darnell, Kerr, & Stark, 1994; Haque & Williams, 1994). The tumor suppressor p53 is involved in several signaling pathways in response to DNA damage including apoptosis and cell cycle arrest, (Lane, 1992; Levine, 1997; Vogelstein, et al., 2000; Vousden & Lu, 2002) which we observed upon IFNα2 treatment in our study. Moreover, the promoter of the p53 gene contains 2 distinct ISREs (Takaoka, et al., 2003) and therefore could be a potential transcriptional target of IFN mediating its effects in cancer. Therefore, we investigated the role of p53 in human melanoma cells M92-047 in response to stimulation by both IFNα2 and IFNα2-$(SO)_{20}$.

An increase in the gene expression of p53 could be related in part to its increase in promoter activity due to the presence of the ISREs in the promoter of the p53 gene

(Hamid & Kakar, 2004). p53 promoter activity increased at both time points after treatment and the increase was more in the cells treated with the fusion protein IFNα2-$(SO)_{20}$ than cells treated by IFNα2. An enhanced p53 promoter activity may be a result of an indirect activation of IFN through signaling of the active ISGF3 complex or another mechanism or the result of direct transactivation of the p53 promoter. However, irrespective of whether or not an increase in p53 gene expression is an indirect/direct effect of IFN signaling in these cells, the more stable fusion protein contributed to higher gene expression possibly due to more sustained signaling.

As an extension to the promoter activity analysis, we determined p53 transcript and protein levels after treating cells for different time points. Increases in p53 protein levels do not always coincide with similar accumulation of p53 mRNA transcript levels; the steady state p53 mRNA levels may either remain constant or even decrease (McVean, Xiao, Isobe, & Pelling, 2000). For example mouse fibroblasts, when growth arrested show higher levels of p53 protein with a simultaneous decline in p53 mRNA levels as cells re-enter the cell cycle. Furthermore, elevated levels of the p53 protein were observed in the presence of transcriptional inhibitors (Mosner, et al., 1995). In our study there appeared to be a steady increase in p53 transcript level in a time dependent manner when M92-047 cells were treated with the fusion protein IFNα2-$(SO)_{20}$. Also, there is a steady increase in p53 protein levels over time with IFNα2-$(SO)_{20}$ compared with the untreated control levels, which begins at 6 h and peaks at 72 h post treatment. However, when cells were treated with IFNα2b, the p53 transcript level increased at 12 h, decreased between 18 and 36 hrs, then once again increased at 48 h and 72 h post treatment, all the

while remaining above control levels. In this case, the increase in p53 protein levels seems to be independent of p53 transcript level. Interestingly, similar to our observations of p53 transcript levels, p53 protein levels increase earlier (6 h) when treated with the fusion derivative and later (12 h) with IFN treatment. A possible reason for steady time dependent increases in p53 at both transcriptional and translational levels in IFNα2-$(SO)_{20}$ treated cells could be related to the greater stability and more continuous IFN signaling by the fusion protein compared with the recombinant protein.

HDM2 is a transcriptional target of p53 and p53 is negatively regulated by HDM2 creating an auto regulatory feedback loop (Balint & Vousden, 2001; Zhu, 2006). Therefore, in order to further study the effect of HDM2 on p53 levels in these cells after IFN treatment, we investigated HDM2 expression levels after similar time points and treatments. It is important to note that although the up regulation of HDM2 is most often p53 dependent, in many cases it has been observed that the down regulation of HDM2 is p53 independent (Alarcon, Koumenis, Geyer, Maki, & Giaccia, 1999; Arriola, Lopez, & Chresta, 1999; L. Wu & Levine, 1997). In our study, HDM2 protein in IFNα2b treated cells showed an initial decrease at 6 h, transciently increased from 12 h to 24 h, then decreased again. Therefore, it appears that up to 24 h after IFNα2b treatment, the induction of HDM2 is p53 dependent; however, it is possible that as the cellular stress of the treatment increases, other p53 independent pathways down regulate HDM2. For the cells treated with the fusion protein, the down regulation of HDM2 appears to be p53 independent because over time as p53 levels are increased and stabilized HDM2 levels are decreased.

P53 mediated apoptosis involves a variety of different signaling pathways (Kinzler & Vogelstein, 1997; Liu, Yue, Khuri, & Sun, 2004; O'Connor, Harris, & Strasser, 2000; Sheikh, et al., 1998; Takimoto & El-Deiry, 2000; Zhu, 2006). However, one of most important pathways involves the mitochondrial membrane protein Bax, which is a direct target of p53 and a key player involved in p53's apoptotic effects (Erster, Mihara, Kim, Petrenko, & Moll, 2004; Mihara, et al., 2003). Our next aim was to determine if increased p53 protein in treated cells had an effect on Bax activity. Since the Bax promoter contains p53 responsive elements to which p53 can bind and increase expression of the Bax gene (Miyashita & Reed, 1995; Thornborrow, et al., 2002), we would expect that if p53 is being stabilized in these cells then the promoter activity of Bax should increase, thereby increasing gene expression. Moreover, being a direct target of p53, an increase in Bax would indicate that treatment of these melanoma cells with IFN and the fusion protein of IFN, not only increases p53 protein levels, but this increased p53 protein is also trancriptionally active in up regulating its downstream targets. Our results, showed an increase in Bax promoter activity for both treatments. Interestingly however, the increase is significantly higher in the cells treated with the fusion protein. In addition, we observed an increase in Bax transcript levels over time which correlates with increased p53 levels and observed that, IFNα2-$(SO)_{20}$ treatment increased Bax levels earlier (6 h) than IFNα2b (12 h) and with higher levels, probably due to its ability to sustain stabilized levels of p53.

The role of Bax in p53 mediated apoptosis has been well studied (Chipuk, et al., 2004; Mihara, et al., 2003; Zhu, 2006). Its ability to form homodimers and disrupt the

mitochondrial outer membrane potential (MOMP), which releases cytochrome c, details its role as an accelerator of apoptosis (Chipuk, Bouchier-Hayes, & Green, 2006; Chipuk, et al., 2004; Green, 2005; Kuwana, et al., 2005). Hence, our next step was to investigate if the increased Bax transcript levels were accompanied by a subsequent increase in its protein levels so as to further emphasize the role of p53 in lowering cell viability after treating the cells with both IFN types. Western blot analysis revealed that both IFN treatments increased Bax protein levels in a time dependent manner. Interestingly, after treatment with the fusion derivative of IFN, an increase in Bax protein levels was observed as early as 6 h, which further increased and remained high up to 72 h post treatment. However, in recombinant IFN treated cells there appears to be a later increase in Bax protein levels (24 h), which stays elevated through 72 h after treatment. This finding is suggestive of the ability of IFNα2-$(SO)_{20}$ to stabilize p53 levels to a greater extent than IFNα2 and this stabilized p53 directly activates its downstream target Bax, which is a crucial apoptotic protein involved in the intrinsic apoptotic pathway, leading to cell death and lower cell viability in tumor cells.

In summary, we have shown that when IFNα2 is produced as a higher molecular weight AGP fusion in tobacco cells, it is able to cause growth inhibition in melanoma cells M92-047 and SKMEL-28. In addition, there is a direct affect on the cell cycle distribution, whereby M92-047 cells stay for a prolonged period of time in the S phase, while SKMEL-28 cells get arrested at the G1 phase, both of which are indirectly controlled by p53. Next we demonstrated that these fusion proteins bind to the IFN receptors and trigger signaling via the JAK-STAT signaling pathway. For this we showed

increased expression of receptor tyrosine kinases Tyk-2 and JAK-1 in cells treated with IFNα2 and IFNα2-$(SO)_{20}$ and increased expression of proteins involved in the initial events of the signaling pathway (experiments done with both fusion proteins IFNα2-$(SO)_{20}$ & IFNα2-$(SO)_{10}$). Downstream signaling was demonstrated by the ability of fusion protein IFNα2-$(SO)_{20}$ to increase expression of known ISGs by examining the protein expression of 2'5'OAS1 and PKR, both of which are induced by interferons (Fernandes, et al., 2010).

Our next set of experiments showed that the tumor suppressor p53 had increased gene expression, mRNA transcript levels and protein levels in M92-047 cells treated with both recombinant IFN as well as the fusion protein at different time points. Moreover, declining levels of HDM2 protein was observed over time. Finally, we found increased gene expression, transcript levels and protein levels for pro-apoptotic protein Bax at the time points investigated. Treatment of cells with the fusion protein IFNα2-$(SO)_{20}$ often showed higher responses in all these experiments as compared with IFNα2.

IFNα2 fusion proteins produced in plants represent a novel approach to overcome problems associated with small protein therapeutics. Our results show that modification of the protein does not alter the native IFN signaling pathway, as evidenced by both IFN types behaving in the same manner, decreasing cell viability and affecting cell cycle distribution in these cells (Fernandes, et al., 2010). Also, the antitumor activity of IFN and the fusion protein appear to be related to stabilized p53 levels, a decline in HDM2 levels and an increase in Bax levels. Furthermore, morphological changes characteristic of apoptosis were observed when cells were treated over a period of 3 days. Together

these results indicate a relationship between IFN (as well as the fusion derivative) and p53 in mediating cell viability in these cells. Additional characterization of this relationship is needed to determine if the effect is direct or indirect. Plant produced IFN fusion proteins with increased serum half-life represent a paradigm shift in IFN treatment and our results exhibit that these fusion proteins display the same signal transduction properties as IFNα2 (Fernandes, et al., 2010). Importantly, the fusion protein lowered cell viability in these cells probably due to greater accumulation of p53 along with increase Bax levels. However, in vivo efficacy studies need to be done in order to examine the implications of this type of therapeutic in IFN treated cancers (Fernandes, et al., 2010).

CHAPTER 6: FUTURE WORK

The synthesis of IFNα2b as an arabinogalactan fusion protein represents a novel approach to mitigate the problems associated with therapeutic proteins of low molecular weights. Our work described thus far reinforces the knowledge about the stability of these IFNs and describes their ability to retain their biological activity, transmit their signals internally, as well affect the downstream target genes (Fernandes, et al., 2010). However, to further strengthen the evidence of growth inhibition of human melanoma cells and the stability of these fusion proteins to exert therapeutic effects at equivalent or lower doses than currently used for recombinant IFN, in-vivo efficacy studies using a SCID (severe combined immunodeficient) mouse model need to be done. These studies will aim to investigate if these fusion proteins are able to reduce tumor burden in mice after the mice have been injected with human melanoma cells M92-047. Further, in addition to tumor burden, the ability of these fusions to prevent metastasis of the tumor can also be studied. The former was studied in our initial work; however, there were several problems we encountered while performing the study. The study was designed to compare recombinant IFNα2b, pegylated IFNα2b and the fusion proteins in their ability to reduce tumor volume in mice. One problem encountered was difficulty in getting the tumors to grow; therefore, different baseline tumor volume across all groups was seen. However, several modifications can be made to our protocol in order to better perform the study. These include subcutaneous injection (s.c.) instead of intraperitoneal (i.p.) of the human melanoma cells M92-047 resuspended in 200 μl of PBS (phosphate buffered saline) into the lower left flank. Also, our protocol involved waiting for tumors to grow prior to injecting the different types of IFNs. However, it has been described that same day

injections into the opposite flank have been used successfully to reduce tumor volume. Weekly assessments of tumor size using a caliper would be done and at the end of 4 weeks the mice would be euthanized and tumor weight measured (Krepler, et al., 2004). This would be done for the different dose escalations and efficacy evaluated.

In addition to demonstrating the ability of the fusion proteins to bind to IFN receptors and transmit their signals, our work also showed the involvement of p53 in the growth inhibition of melanoma cells by IFNα2-$(SO)_{20}$ and IFNα2b. We showed that treatment of cells with IFNα2-$(SO)_{20}$ stabilized p53 levels to a greater extent than IFNα2b treatment. Moreover, the stabilized p53 induced its apoptotic target Bax and HDM2 levels decreased. However, the exact nature of this relationship needs to be further evaluated. For this, the dependence of IFN mediated antitumor effects on p53 can be assessed by using short interference RNA (si RNA) to down regulate p53 expression and quantitate cell death after this down regulation (Shen, Buck, Liu, Winkler, & Reske, 2003). In addition to using si RNA, p53 null cell lines can be used and the effect of the fusion proteins on cell growth can be studied in the absence of p53 (Dumble, Croager, Yeoh, & Quail, 2002). Also, more experiments can be done to characterize the nature of p53 stabilization, whether it is phosphorylation, acetylation(Sakaguchi, et al., 1998)or other mechanisms that may be involved in this process.

In summary, the work proposed here would be extremely important in shaping the benefits and usefulness of the IFN fusion proteins as recombinant IFN substitutes to treat melanoma. Also, the involvement of p53 represents a new target, which can be pursued in melanoma therapy.

REFERENCES

ACS (2009). Cancer Facts & Figures 2009. Atlanta.

Agarwala, S. S., & Kirkwood, J. M. (2003). Temozolomide in combination with interferon alpha-2b in patients with metastatic melanoma: a phase I dose-escalation study. *Cancer, 97*(1), 121-127.

Alarcon, R., Koumenis, C., Geyer, R. K., Maki, C. G., & Giaccia, A. J. (1999). Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. *Cancer Res, 59*(24), 6046-6051.

Alimena, G., Morra, E., Lazzarino, M., Liberati, A. M., Montefusco, E., Inverardi, D., et al. (1988). Interferon alpha-2b as therapy for Ph'-positive chronic myelogenous leukemia: a study of 82 patients treated with intermittent or daily administration. *Blood, 72*(2), 642-647.

Arriola, E. L., Lopez, A. R., & Chresta, C. M. (1999). Differential regulation of p21waf-1/cip-1 and Mdm2 by etoposide: etoposide inhibits the p53-Mdm2 autoregulatory feedback loop. *Oncogene, 18*(4), 1081-1091.

Arthur, C. K., & Ma, D. D. (1993). Combined interferon alfa-2a and cytosine arabinoside as first-line treatment for chronic myeloid leukemia. *Acta Haematol, 89 Suppl 1*, 15-21.

Atkins, M. B., Gollob, J. A., Sosman, J. A., McDermott, D. F., Tutin, L., Sorokin, P., et al. (2002). A phase II pilot trial of concurrent biochemotherapy with cisplatin, vinblastine, temozolomide, interleukin 2, and IFN-alpha 2B in patients with metastatic melanoma. *Clin Cancer Res, 8*(10), 3075-3081.

Balint, E. E., & Vousden, K. H. (2001). Activation and activities of the p53 tumour suppressor protein. *Br J Cancer, 85*(12), 1813-1823.

Barak, Y., Juven, T., Haffner, R., & Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. *EMBO J, 12*(2), 461-468.

Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., & Kim, P. S. (1995). Predicting coiled coils by use of pairwise residue correlations. *Proc Natl Acad Sci U S A, 92*(18), 8259-8263.

Bluyssen, A. R., Durbin, J. E., & Levy, D. E. (1996). ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. *Cytokine Growth Factor Rev, 7*(1), 11-17.

Borden, E. C. (1994). Innovative treatment strategies for non-Hodgkin's lymphoma and multiple myeloma. *Semin Oncol, 21*(6 Suppl 14), 14-22.

Borden, E. C., & Ball, L. A. (1981). Interferons: biochemical, cell growth inhibitory, and immunological effects. *Prog Hematol, 12*, 299-339.

Borden, E. C., Lindner, D., Dreicer, R., Hussein, M., & Peereboom, D. (2000). Second-generation interferons for cancer: clinical targets. *Semin Cancer Biol, 10*(2), 125-144.

Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R., et al. (2007). Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov, 6*(12), 975-990.

Boyd, M. T., Vlatkovic, N., & Haines, D. S. (2000). A novel cellular protein (MTBP) binds to MDM2 and induces a G1 arrest that is suppressed by MDM2. *J Biol Chem, 275*(41), 31883-31890.

Bromberg, J. F., Horvath, C. M., Wen, Z., Schreiber, R. D., & Darnell, J. E., Jr. (1996). Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci U S A, 93*(15), 7673-7678.

Brown, D. R., Thomas, C. A., & Deb, S. P. (1998). The human oncoprotein MDM2 arrests the cell cycle: elimination of its cell-cycle-inhibitory function induces tumorigenesis. *EMBO J, 17*(9), 2513-2525.

Bunn, P. A., Jr., Foon, K. A., Ihde, D. C., Longo, D. L., Eddy, J., Winkler, C. F., et al. (1984). Recombinant leukocyte A interferon: an active agent in advanced cutaneous T-cell lymphomas. *Ann Intern Med, 101*(4), 484-487.

Buschmann, T., Fuchs, S. Y., Lee, C. G., Pan, Z. Q., & Ronai, Z. (2000). SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell, 101*(7), 753-762.

Carson, W. E. (1998). Interferon-alpha-induced activation of signal transducer and activator of transcription proteins in malignant melanoma. *Clin Cancer Res, 4*(9), 2219-2228.

Chandler, D. S., Singh, R. K., Caldwell, L. C., Bitler, J. L., & Lozano, G. (2006). Genotoxic stress induces coordinately regulated alternative splicing of the p53 modulators MDM2 and MDM4. *Cancer Res, 66*(19), 9502-9508.

Chelbi-Alix, M. K., & Wietzerbin, J. (2007). Interferon, a growing cytokine family: 50 years of interferon research. *Biochimie, 89*(6-7), 713-718.

Chipuk, J. E., Bouchier-Hayes, L., & Green, D. R. (2006). Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ, 13*(8), 1396-1402.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., et al. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science, 303*(5660), 1010-1014.

Collins, J. A., Schandi, C. A., Young, K. K., Vesely, J., & Willingham, M. C. (1997). Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem, 45*(7), 923-934.

Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer, 2*(9), 647-656.

Darnell, J. E., Jr., Kerr, I. M., & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science, 264*(5164), 1415-1421.

DeFrees, S., Wang, Z. G., Xing, R., Scott, A. E., Wang, J., Zopf, D., et al. (2006). GlycoPEGylation of recombinant therapeutic proteins produced in Escherichia coli. *Glycobiology, 16*(9), 833-843.

Dias, C. S., Liu, Y., Yau, A., Westrick, L., & Evans, S. C. (2006). Regulation of hdm2 by stress-induced hdm2alt1 in tumor and nontumorigenic cell lines correlating with p53 stability. *Cancer Res, 66*(19), 9467-9473.

Dumble, M. L., Croager, E. J., Yeoh, G. C., & Quail, E. A. (2002). Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma. *Carcinogenesis, 23*(3), 435-445.

Erster, S., Mihara, M., Kim, R. H., Petrenko, O., & Moll, U. M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol, 24*(15), 6728-6741.

Ezekowitz, R. A., Mulliken, J. B., & Folkman, J. (1992). Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med, 326*(22), 1456-1463.

Fernandes, E., Soans, E., Xu, J., Kieliszewski, M., & Evans, S. C. (2010). Novel fusion proteins of interferon alpha2b cause growth inhibition and induce JAK-STAT signaling in melanoma. *J Immunother, 33*(5), 461-466.

Fields, S., & Jang, S. K. (1990). Presence of a potent transcription activating sequence in the p53 protein. *Science, 249*(4972), 1046-1049.

Fife, K. M., Colman, M. H., Stevens, G. N., Firth, I. C., Moon, D., Shannon, K. F., et al. (2004). Determinants of outcome in melanoma patients with cerebral metastases. *J Clin Oncol, 22*(7), 1293-1300.

Flaherty, L. E., Atkins, M., Sosman, J., Weiss, G., Clark, J. I., Margolin, K., et al. (2001). Outpatient biochemotherapy with interleukin-2 and interferon alfa-2b in patients with metastatic malignant melanoma: results of two phase II cytokine working group trials. *J Clin Oncol, 19*(13), 3194-3202.

Foon, K. A., Sherwin, S. A., Abrams, P. G., Longo, D. L., Fer, M. F., Stevenson, H. C., et al. (1984). Treatment of advanced non-Hodgkin's lymphoma with recombinant leukocyte A interferon. *N Engl J Med, 311*(18), 1148-1152.

Fritsche, M., Haessler, C., & Brandner, G. (1993). Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene, 8*(2), 307-318.

Gale, M., Jr., & Katze, M. G. (1998). Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. *Pharmacol Ther, 78*(1), 29-46.

George, N. M., Targy, N., Evans, J. J., Zhang, L., & Luo, X. (2010). Bax contains two functional mitochondrial targeting sequences and translocates to mitochondria in a conformational change- and homo-oligomerization-driven process. *J Biol Chem, 285*(2), 1384-1392.

Geyer, R. K., Yu, Z. K., & Maki, C. G. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nat Cell Biol, 2*(9), 569-573.

Gilchrest, B. A., Eller, M. S., Geller, A. C., & Yaar, M. (1999). The pathogenesis of melanoma induced by ultraviolet radiation. *N Engl J Med, 340*(17), 1341-1348.

Grace, M., Youngster, S., Gitlin, G., Sydor, W., Xie, L., Westreich, L., et al. (2001). Structural and biologic characterization of pegylated recombinant IFN-alpha2b. *J Interferon Cytokine Res, 21*(12), 1103-1115.

Green, D. R. (2005). Apoptotic pathways: ten minutes to dead. *Cell, 121*(5), 671-674.

Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., et al. (2003). Polyubiquitination of p53 by a ubiquitin ligase activity of p300. *Science, 300*(5617), 342-344.

Grossman, S. R., Perez, M., Kung, A. L., Joseph, M., Mansur, C., Xiao, Z. X., et al. (1998). p300/MDM2 complexes participate in MDM2-mediated p53 degradation. *Mol Cell, 2*(4), 405-415.

Gu, W., & Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell, 90*(4), 595-606.

Guilhot, F., Chastang, C., Michallet, M., Guerci, A., Harousseau, J. L., Maloisel, F., et al. (1997). Interferon alfa-2b combined with cytarabine versus interferon alone in chronic myelogenous leukemia. French Chronic Myeloid Leukemia Study Group. *N Engl J Med, 337*(4), 223-229.

H.Irene Hall, D. R. M., Joseph D. Rogers and Barbara Bewerse (1999). Update on the incidence and mortality from melanoma in the United States. *Journal of the American Academy of Dermatology, 40*(1), 35-42.

Hamid, T., & Kakar, S. S. (2004). PTTG/securin activates expression of p53 and modulates its function. *Mol Cancer, 3*, 18.

Haque, S. J., & Williams, B. R. (1994). Identification and characterization of an interferon (IFN)-stimulated response element-IFN-stimulated gene factor 3-independent signaling pathway for IFN-alpha. *J Biol Chem, 269*(30), 19523-19529.

Harris, J. M., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov, 2*(3), 214-221.

Haupt, S., Berger, M., Goldberg, Z., & Haupt, Y. (2003). Apoptosis - the p53 network. *J Cell Sci, 116*(Pt 20), 4077-4085.

Haupt, Y., Maya, R., Kazaz, A., & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature, 387*(6630), 296-299.

Heim, M. H. (1999). The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J Recept Signal Transduct Res, 19*(1-4), 75-120.

Held, M. A., Tan, L., Kamyab, A., Hare, M., Shpak, E., & Kieliszewski, M. J. (2004). Di-isodityrosine is the intermolecular cross-link of isodityrosine-rich extensin analogs cross-linked in vitro. *J Biol Chem, 279*(53), 55474-55482.

Honda, R., Tanaka, H., & Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett, 420*(1), 25-27.

Honda, R., & Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J, 18*(1), 22-27.

Hovanessian, A. G. (2007). On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2'-5'oligoadenylate synthetases and the protein kinase PKR. *Cytokine Growth Factor Rev, 18*(5-6), 351-361.

Hovanessian, A. G., & Justesen, J. (2007). The human 2'-5'oligoadenylate synthetase family: unique interferon-inducible enzymes catalyzing 2'-5' instead of 3'-5' phosphodiester bond formation. *Biochimie, 89*(6-7), 779-788.

Irwin, M. S., & Kaelin, W. G. (2001). p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ, 12*(7), 337-349.

Isobe, M., Emanuel, B. S., Givol, D., Oren, M., & Croce, C. M. (1986). Localization of gene for human p53 tumour antigen to band 17p13. *Nature, 320*(6057), 84-85.

Joazeiro, C. A., & Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell, 102*(5), 549-552.

Jonasch, E., Kumar, U. N., Linette, G. P., Hodi, F. S., Soiffer, R. J., Ryan, B. F., et al. (2000). Adjuvant high-dose interferon alfa-2b in patients with high-risk melanoma. *Cancer J, 6*(3), 139-145.

Jones, S. N., Roe, A. E., Donehower, L. A., & Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature, 378*(6553), 206-208.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., et al. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell, 90*(4), 809-819.

Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., & Sherr, C. J. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc Natl Acad Sci U S A, 95*(14), 8292-8297.

Kantarjian, H. M., Keating, M. J., Estey, E. H., O'Brien, S., Pierce, S., Beran, M., et al. (1992). Treatment of advanced stages of Philadelphia chromosome-positive chronic myelogenous leukemia with interferon-alpha and low-dose cytarabine. *J Clin Oncol, 10*(5), 772-778.

Kantarjian, H. M., O'Brien, S., Anderlini, P., & Talpaz, M. (1996). Treatment of myelogenous leukemia: current status and investigational options. *Blood, 87*(8), 3069-3081.

Kantarjian, H. M., O'Brien, S., Smith, T. L., Rios, M. B., Cortes, J., Beran, M., et al. (1999). Treatment of Philadelphia chromosome-positive early chronic phase chronic myelogenous leukemia with daily doses of interferon alpha and low-dose cytarabine. *J Clin Oncol, 17*(1), 284-292.

Kaufmann, R., Spieth, K., Leiter, U., Mauch, C., von den Driesch, P., Vogt, T., et al. (2005). Temozolomide in combination with interferon-alfa versus temozolomide alone in patients with advanced metastatic melanoma: a randomized, phase III, multicenter study from the Dermatologic Cooperative Oncology Group. *J Clin Oncol, 23*(35), 9001-9007.

Kieliszewski, M. J., & Shpak, E. (2001). Synthetic genes for the elucidation of glycosylation codes for arabinogalactan-proteins and other hydroxyproline-rich glycoproteins. *Cellular and Molecular Life Sciences, 58*(10), 1386-1398.

Kinzler, K. W., & Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature, 386*(6627), 761, 763.

Kirkwood, J. M., Bender, C., Agarwala, S., Tarhini, A., Shipe-Spotloe, J., Smelko, B., et al. (2002). Mechanisms and management of toxicities associated with high-dose interferon alfa-2b therapy. *J Clin Oncol, 20*(17), 3703-3718.

Korsmeyer, S. J. (1999). BCL-2 gene family and the regulation of programmed cell death. *Cancer Res, 59*(7 Suppl), 1693s-1700s.

Krepler, C., Certa, U., Wacheck, V., Jansen, B., Wolff, K., & Pehamberger, H. (2004). Pegylated and conventional interferon-alpha induce comparable transcriptional responses and inhibition of tumor growth in a human melanoma SCID mouse xenotransplantation model. *J Invest Dermatol, 123*(4), 664-669.

Kubbutat, M. H., Jones, S. N., & Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. *Nature, 387*(6630), 299-303.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., et al. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell, 17*(4), 525-535.

Lamb, P., & Crawford, L. (1986). Characterization of the human p53 gene. *Mol Cell Biol, 6*(5), 1379-1385.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. *Nature, 358*(6381), 15-16.

Larner, A., & Reich, N. C. (1996). Interferon signal transduction. *Biotherapy, 8*(3-4), 175-181.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell, 88*(3), 323-331.

Lin, J., Chen, J., Elenbaas, B., & Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev, 8*(10), 1235-1246.

Lindenmann, A. I. a. J. (1988). Virus interference: I. The interferon. *CA Cancer J Clin, 38*(5), 280-290.

Liu, X., Yue, P., Khuri, F. R., & Sun, S. Y. (2004). p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res, 64*(15), 5078-5083.

Lundblad, D., & Lundgren, E. (1981). Block of glioma cell line in S by interferon. *Int J Cancer, 27*(6), 749-754.

Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends Biochem Sci, 21*(10), 375-382.

Magrassi, L., De-Fraja, C., Conti, L., Butti, G., Infuso, L., Govoni, S., et al. (1999). Expression of the JAK and STAT superfamilies in human meningiomas. *J Neurosurg, 91*(3), 440-446.

Mahon, F. X., Faberes, C., Pueyo, S., Cony-Makhoul, P., Salmi, R., Boiron, J. M., et al. (1998). Response at three months is a good predictive factor for newly diagnosed chronic myeloid leukemia patients treated by recombinant interferon-alpha. *Blood, 92*(11), 4059-4065.

Mandelli, F., Avvisati, G., Amadori, S., Boccadoro, M., Gernone, A., Lauta, V. M., et al. (1990). Maintenance treatment with recombinant interferon alfa-2b in patients with multiple myeloma responding to conventional induction chemotherapy. *N Engl J Med, 322*(20), 1430-1434.

Marechal, V., Elenbaas, B., Piette, J., Nicolas, J. C., & Levine, A. J. (1994). The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol Cell Biol, 14*(11), 7414-7420.

Marechal, V., Elenbaas, B., Taneyhill, L., Piette, J., Mechali, M., Nicolas, J. C., et al. (1997). Conservation of structural domains and biochemical activities of the MDM2 protein from Xenopus laevis. *Oncogene, 14*(12), 1427-1433.

Martinez-Moczygemba, M., Gutch, M. J., French, D. L., & Reich, N. C. (1997). Distinct STAT structure promotes interaction of STAT2 with the p48 subunit of the interferon-alpha-stimulated transcription factor ISGF3. *J Biol Chem, 272*(32), 20070-20076.

Mayo, L. D., & Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A, 98*(20), 11598-11603.

Mayo, L. D., & Donner, D. B. (2002). The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci, 27*(9), 462-467.

Mayo, L. D., Turchi, J. J., & Berberich, S. J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res, 57*(22), 5013-5016.

McVean, M., Xiao, H., Isobe, K., & Pelling, J. C. (2000). Increase in wild-type p53 stability and transactivational activity by the chemopreventive agent apigenin in keratinocytes. *Carcinogenesis, 21*(4), 633-639.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., et al. (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol Cell, 11*(3), 577-590.

Miyashita, T., & Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell, 80*(2), 293-299.

Montes de Oca Luna, R., Wagner, D. S., & Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature, 378*(6553), 203-206.

Morris, A., & Zvetkova, I. (1997). Cytokine research: the interferon paradigm. *J Clin Pathol, 50*(8), 635-639.

Moschos, S. J., Edington, H. D., Land, S. R., Rao, U. N., Jukic, D., Shipe-Spotloe, J., et al. (2006). Neoadjuvant treatment of regional stage IIIB melanoma with high-dose interferon alfa-2b induces objective tumor regression in association with modulation of tumor infiltrating host cellular immune responses. *J Clin Oncol, 24*(19), 3164-3171.

Mosner, J., Mummenbrauer, T., Bauer, C., Sczakiel, G., Grosse, F., & Deppert, W. (1995). Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J, 14*(18), 4442-4449.

Muggiano, A., Mulas, C., Fiori, B., Liciardi, G., Pintus, M., Tanca, L., et al. (2004). Feasibility of high-dose interferon-alpha2b adjuvant therapy for high-risk resected cutaneous melanoma. *Melanoma Res, 14*(2), S1-7.

Murphy, D., Detjen, K. M., Welzel, M., Wiedenmann, B., & Rosewicz, S. (2001). Interferon-alpha delays S-phase progression in human hepatocellular carcinoma cells via inhibition of specific cyclin-dependent kinases. *Hepatology, 33*(2), 346-356.

O'Connell, M. J., Colgan, J. P., Oken, M. M., Ritts, R. E., Jr., Kay, N. E., & Itri, L. M. (1986). Clinical trial of recombinant leukocyte A interferon as initial therapy for

favorable histology non-Hodgkin's lymphomas and chronic lymphocytic leukemia. An Eastern Cooperative Oncology Group pilot study. *J Clin Oncol, 4*(2), 128-136.

O'Connor, L., Harris, A. W., & Strasser, A. (2000). CD95 (Fas/APO-1) and p53 signal apoptosis independently in diverse cell types. *Cancer Res, 60*(5), 1217-1220.

ODell, W. (2000). IFN alpha Signaling pathway Retrieved June 29, 2010, from http://www.biocarta.com/pathfiles/h_ifnaPathway.asp

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., & Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature, 358*(6381), 80-83.

Olson, D. C., Marechal, V., Momand, J., Chen, J., Romocki, C., & Levine, A. J. (1993). Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. *Oncogene, 8*(9), 2353-2360.

Oren, M. (2003). Decision making by p53: life, death and cancer. *Cell Death Differ, 10*(4), 431-442.

Oren, M., & Rotter, V. (1999). Introduction: p53--the first twenty years. *Cell Mol Life Sci, 55*(1), 9-11.

Panniers, L. R., & Clemens, M. J. (1981). Inhibition of cell division by interferon: changes in cell cycle characteristics and in morphology of Ehrlich ascites tumour cells in culture. *J Cell Sci, 48*, 259-279.

Pansky, A., Hildebrand, P., Fasler-Kan, E., Baselgia, L., Ketterer, S., Beglinger, C., et al. (2000). Defective Jak-STAT signal transduction pathway in melanoma cells resistant to growth inhibition by interferon-alpha. *Int J Cancer, 85*(5), 720-725.

Pestka, S. (2003). A dance between interferon-alpha/beta and p53 demonstrates collaborations in tumor suppression and antiviral activities. *Cancer Cell, 4*(2), 85-87.

Pestka, S., Krause, C. D., & Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunol Rev, 202*, 8-32.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., et al. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell, 92*(6), 713-723.

Ramon, J., Saez, V., Baez, R., Aldana, R., & Hardy, E. (2005). PEGylated interferon-alpha2b: a branched 40K polyethylene glycol derivative. *Pharm Res, 22*(8), 1374-1386.

Rawlings, J. S., Rosler, K. M., & Harrison, D. A. (2004). The JAK/STAT signaling pathway. *J Cell Sci, 117*(Pt 8), 1281-1283.

Roberts, M. J., Bentley, M. D., & Harris, J. M. (2002). Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev, 54*(4), 459-476.

Rosenberg, S. A. (2001). Progress in human tumour immunology and immunotherapy. *Nature, 411*(6835), 380-384.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., & Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53

protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J, 17*(2), 554-564.

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., et al. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev, 12*(18), 2831-2841.

Sangfelt, O., Erickson, S., & Grander, D. (2000). Mechanisms of interferon-induced cell cycle arrest. *Front Biosci, 5*, D479-487.

Saraste, A. (1999). Morphologic criteria and detection of apoptosis. *Herz, 24*(3), 189-195.

Schindler, C., & Darnell, J. E., Jr. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem, 64*, 621-651.

Schmale, H., & Bamberger, C. (1997). A novel protein with strong homology to the tumor suppressor p53. *Oncogene, 15*(11), 1363-1367.

Selleri, C., Sato, T., Del Vecchio, L., Luciano, L., Barrett, A. J., Rotoli, B., et al. (1997). Involvement of Fas-mediated apoptosis in the inhibitory effects of interferon-alpha in chronic myelogenous leukemia. *Blood, 89*(3), 957-964.

Shah, G. D., & Chapman, P. B. (2007). Adjuvant therapy of melanoma. *Cancer J, 13*(3), 217-222.

Sharpless, E., & Chin, L. (2003). The INK4a/ARF locus and melanoma. *Oncogene, 22*(20), 3092-3098.

Shaw, P., Freeman, J., Bovey, R., & Iggo, R. (1996). Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. *Oncogene, 12*(4), 921-930.

Sheikh, M. S., Burns, T. F., Huang, Y., Wu, G. S., Amundson, S., Brooks, K. S., et al. (1998). p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res, 58*(8), 1593-1598.

Shen, C., Buck, A. K., Liu, X., Winkler, M., & Reske, S. N. (2003). Gene silencing by adenovirus-delivered siRNA. *FEBS Lett, 539*(1-3), 111-114.

Shpak, E., Barbar, E., Leykam, J. F., & Kieliszewski, M. J. (2001). Contiguous hydroxyproline residues direct hydroxyproline arabinosylation in Nicotiana tabacum. *J Biol Chem, 276*(14), 11272-11278.

Shpak, E., Leykam, J. F., & Kieliszewski, M. J. (1999). Synthetic genes for glycoprotein design and the elucidation of hydroxyproline-O-glycosylation codes. *Proc Natl Acad Sci U S A, 96*(26), 14736-14741.

Shvarts, A., Steegenga, W. T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., et al. (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J, 15*(19), 5349-5357.

Smalley, R. V., Andersen, J. W., Hawkins, M. J., Bhide, V., O'Connell, M. J., Oken, M. M., et al. (1992). Interferon alfa combined with cytotoxic chemotherapy for patients with non-Hodgkin's lymphoma. *N Engl J Med, 327*(19), 1336-1341.

Solal-Celigny, P., Lepage, E., Brousse, N., Reyes, F., Haioun, C., Leporrier, M., et al. (1993). Recombinant interferon alfa-2b combined with a regimen containing doxorubicin in patients with advanced follicular lymphoma. Groupe d'Etude des Lymphomes de l'Adulte. *N Engl J Med, 329*(22), 1608-1614.

Soussi, T. (2005). The p53 pathway and human cancer. *Br J Surg, 92*(11), 1331-1332.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., & Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J, 18*(6), 1660-1672.

Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., et al. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J, 17*(17), 5001-5014.

Tagliaferri, P., Caraglia, M., Budillon, A., Marra, M., Vitale, G., Viscomi, C., et al. (2005). New pharmacokinetic and pharmacodynamic tools for interferon-alpha (IFN-alpha) treatment of human cancer. *Cancer Immunol Immunother, 54*(1), 1-10.

Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., et al. (2003). Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature, 424*(6948), 516-523.

Takimoto, R., & El-Deiry, W. S. (2000). Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene, 19*(14), 1735-1743.

Tamura, K., Makino, S., Araki, Y., Imamura, T., & Seita, M. (1987). Recombinant interferon beta and gamma in the treatment of adult T-cell leukemia. *Cancer, 59*(6), 1059-1062.

Tan, L., Leykam, J. F., & Kieliszewski, M. J. (2003). Glycosylation motifs that direct arabinogalactan addition to arabinogalactan-proteins. *Plant Physiol, 132*(3), 1362-1369.

Tan, L., Qiu, F., Lamport, D. T., & Kieliszewski, M. J. (2004). Structure of a hydroxyproline (Hyp)-arabinogalactan polysaccharide from repetitive Ala-Hyp expressed in transgenic Nicotiana tabacum. *J Biol Chem, 279*(13), 13156-13165.

Thaler, J., Hilbe, W., Apfelbeck, U., Linkesch, W., Sill, H., Seewann, H., et al. (1997). Interferon-alpha-2C and LD ara-C for the treatment of patients with CML: results of the Austrian multi-center phase II study. *Leuk Res, 21*(1), 75-80.

Thompson, J. F., Scolyer, R. A., & Kefford, R. F. (2005). Cutaneous melanoma. *Lancet, 365*(9460), 687-701.

Thornborrow, E. C., Patel, S., Mastropietro, A. E., Schwartzfarb, E. M., & Manfredi, J. J. (2002). A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine bax genes. *Oncogene, 21*(7), 990-999.

Tsubery, H., Mironchik, M., Fridkin, M., & Shechter, Y. (2004). Prolonging the action of protein and peptide drugs by a novel approach of reversible polyethylene glycol modification. *J Biol Chem, 279*(37), 38118-38124.

Tucker, M. A., & Goldstein, A. M. (2003). Melanoma etiology: where are we? *Oncogene, 22*(20), 3042-3052.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature, 408*(6810), 307-310.

Vousden, K. H., & Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer, 2*(8), 594-604.

Wang, Y. S., Youngster, S., Grace, M., Bausch, J., Bordens, R., & Wyss, D. F. (2002). Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. *Adv Drug Deliv Rev, 54*(4), 547-570.

Wong, L. H., Krauer, K. G., Hatzinisiriou, I., Estcourt, M. J., Hersey, P., Tam, N. D., et al. (1997). Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J Biol Chem, 272*(45), 28779-28785.

Wu, L., & Levine, A. J. (1997). Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Mol Med, 3*(7), 441-451.

Wu, X., Li, X., Zeng, Y., Zheng, Q., & Wu, S. (2006). Site-directed PEGylation of human basic fibroblast growth factor. *Protein Expr Purif, 48*(1), 24-27.

Xu, J., Shpak, E., Gu, T., Moo-Young, M., & Kieliszewski, M. (2005). Production of recombinant plant gum with tobacco cell culture in bioreactor and gum characterization. *Biotechnol Bioeng, 90*(5), 578-588.

Xu, J., Tan, L., Goodrum, K. J., & Kieliszewski, M. J. (2007). High-yields and extended serum half-life of human interferon alpha2b expressed in tobacco cells as arabinogalactan-protein fusions. *Biotechnol Bioeng, 97*(5), 997-1008.

Yamamoto, M., Takahashi, H., Saitoh, K., Horikoshi, T., & Takahashi, M. (1995). Expression of the p53 protein in malignant melanomas as a prognostic indicator. *Arch Dermatol Res, 287*(2), 146-151.

Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., et al. (2003). Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat Biotechnol, 21*(5), 546-552.

Zhang, T., & Prives, C. (2001). Cyclin a-CDK phosphorylation regulates MDM2 protein interactions. *J Biol Chem, 276*(32), 29702-29710.

Zhu, L. B. a. W.-G. (2006). p53: Structure, Function and Therapeutic Applications. [Review]. *J. Cancer Mol, 2*(4), 141-153.

Zilberstein, A., Kimchi, A., Schmidt, A., & Revel, M. (1978). Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. *Proc Natl Acad Sci U S A, 75*(10), 4734-4738.

APPENDIX A: LIST OFABBREVIATIONS

2'5' OAS1: 2'5' oligoadenylate synthetase 1

ACS: American cancer society

AG: arabinogalactan

AGP: arabinogalactan

AGPs: arabinogalactan proteins

Apaf-1: apoptotic protease activating factor -1

ara-C: cysosine arabinoside

ARF: alternate reading frame

ATCC: American type culture collection

ATM: ataxia telangiectasia mutated

ATR: ataxia telangiectasia related

Bad: Bcl-2 associated agonist of cell death

Bak: Bcl-2 antogonist/killer

Bax: Bcl-2-associate X protein

Bcl-2: B cell leukemia-2 proto-oncogene

Bcl-Xl: B cell leukemia Xl

Bcl-X_L: B cell lymphoma extra large

BH: Bcl-2 homology

Bid: BH3 interacting domain death agonist

Caspases: cysteine-aspartic-acid-proteases

CDK4: cyclin dependent kinase 4

CDKN2A: cyclin dependent kinase 2A

cDNA: complementary DNA

CHK2: checkpoint kinase 2

CHO: Chinese hamster ovary

CML: chronic myelogenous leukemia

DBD: DNA binding domain

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

EGFP: enhanced green fluorescent protein

FACS: Fluorescent activated cell sorter

FGF: fibroblast growth factor

GADD45: growth arrest and DNA damage induced-45

***Hdm2*:** human double minute 2 gene/RNA

HDM2: human double minute 2 protein

HRGPs: hydroxyproline rich glycoproteins

Hyp-O-glycosylation: hydroxyproline O glycosylation

Hyp: hydroxyproline

IFN: interferon

IFNAR: interferon alpha-receptor

IFNAR1: interferon alpha-receptor 1

IFNAR2: interferon alpha-receptor 2

IFNs: interferons

IFNß: interferon beta

IFNα2-$(SO)_{10}$: interferon alpha 2b with 10 repeats of Serine and Hydroxyproline

IFNα2-$(SO)_{20}$: interferon alpha 2b with 20 repeats of Serine and Hydroxyproline

IFNα2b/IFNα2: interferon alpha 2b/interferon alpha 2

IFNγ: interferon gamma

IR: ionizing radiation

ISGF-3: interferon stimulated gene factor-3

ISGF-3γ: interferon stimulated gene factor -3 gamma

ISREs: interferon stimulated response elements

IU: International units

JAK- STAT:janus kinase 1- signal transducers and activators of transcription

JAK-1:janus kinase 1

MAP4: Microtubule associated factor 4

***Mdm2*:** murine double minute 2 gene/RNA

MDM2: murine double minute 2 protein

MOMP: Mitochondrial outer membrane potential

NES: nuclear export sequence

NK: natural killer

NLS: nuclear localization sequence

NOXA: Phorbol-12-myristate-13-acetate-induced protein 1 (latin word for damage)

O: hydroxyproline

OS: overall survival

PBS: Phosphate Buffered Saline

PEG-12000: monomethoxypolyethylene glycol

PEG: polyethylene glycol

PI: Propidium Iodide

PKR: protein kinase R

Polβ: DNA polymerase beta

PR: Proline rich region

PUMA: p53-upregulated modulator of apoptosis

REG: regulatory domain

RLU:relative luminescence units

RNA: ribonucleic acid

ROS: reactive oxygen species

RT-PCR: Reverse transcription polymerase chain reaction

RT: room temperature

S: serine

SCID: Severe Combined immunodeficient

Ser-Hyp$_4$: Ser-Hyp-Hyp-Hyp-Hyp

SH2: src homology 2

TD: transactivation domain

TET: tetramerization domain

TGFα: transforming growth factor α

Tyk-2: tyrosine kinase 2

UV: ultraviolet

APPENDIX B: PERMISSION OBTAINED FROM LWW PUBLISHERS TO USE THE CONTENT OF MANUSCRIPT CJ1, 2010 IN THIS DISSERTATION

Lippincott Williams & Wilkins
351 W. Camden Street
Baltimore, MD 21201

410 528 4000 tel
www.LWW.com

DATE: 5/5/10

Elroy Fernandes
Ohio University, Konneker Research Laboratories
The Ridges
Athens, OH 45701

Fee: $0.00

Re: ***Journal of Immunotherapy***
Spec Mat: CJI, 2010; In press
Author Request / to use in a PhD dissertation

CONDITIONS

Permission is granted for your requested use. Retain this copy for your records. This permission is subject to the following conditions:

A credit line will be prominently placed and included: for books – the author(s), title of book, editor, copyright holder, year of publication; for journals – the author(s), title of article, title of journal, volume number, issue number and inclusive pages.

1) The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or author(s) of the material or to Wolters Kluwer Health.

2) Permission is granted for one time use only as specified in your correspondence. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works.

3) Permission granted is non-exclusive, and is valid throughout the world in the English language only.

4) Wolters Kluwer Health cannot supply the requestor with the original artwork or a "clean copy."

5) The requestor agrees to secure written permission from the author (for book material only).

6) Permission is valid if the borrowed material is original to a Wolters Kluwer Health imprint (Lippincott, Williams & Wilkins, Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown and Company, Harper & Row Medical American Journal of Nursing Co, and Urban & Schwarzenberg – English Language).

APPENDIX C: PERMISSION TO USE IFN ALPHA SIGNALING PATHWAY' FROM BIOCARTA IN THE DISSERTATION

Dear Elroy,
BioCarta hereby grants you a permission to use the figure of its 'IFN alpha signaling pathway' in your PhD dissertation. The dissertation will be published online only by Ohio University after it has been accepted and graduation completed.

You may cite however you want. Please refer to our "Disclaimer" as well as "Terms and Conditions" in our web site to ensure proper use of pathway information.

Best Regards,
Terry Huang
President
BioCarta, LLC
3525 Del Mar Heights Rd., #244
San Diego, CA 92130
(858) 200-2037 x808