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# Pegylated IFN-α sensitizes melanoma cells to chemotherapy and causes premature senescence in endothelial cells by IRF-1 mediated signaling

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## Abstract

Pegylated Interferon-α2b (pIFN-α) is an integral part of the drug regimen currently employed against melanoma. Interferon Regulatory Factor-1 (IRF-1) plays an important role in the transcriptional regulation of the IFN response, cell cycle and apoptosis. We have studied pIFN-α induced responses when combined with the chemotherapy agent, vinblastine in tumor and endothelial cell lines and the connection to IRF-1 signaling. Levels of IRF-1/IRF-2 protein expression were found to be decreased in tumor versus normal tissues. pIFN-α induced IRF-1 signaling in human melanoma (M14) and endothelial (EA.hy926) cells and enhanced cell death when combined with vinblastine. Upon combined IFN-α and vinblastine treatment, p21 expression, PARP cleavage and activated Bak levels were increased in M14 cells. An increase in p21 and cyclin D1 expression occurred in EA.hy926 cells after 6 h of treatment with pIFN-α which dissipated by 24 h. This biphasic response, characteristic of cellular senescence, was more pronounced upon combined treatment. Exposure of the EA.hy926 cells to pIFN-α was associated with an enlarged, multinucleated, β-galactosidase-positive senescent phenotype. The overall therapeutic mechanism of IFN-α combined with chemotherapy may be due to both direct tumor cell death via IRF-1 signaling and by premature senescence of endothelial cells and subsequent effects on angiogenesis in the tumor microenvironment.

## Introduction

The abysmal median survival for patients with metastatic melanoma ranges from 6 to 9 months, causing it to be one of the most aggressive human cancers. The p53 protein acts as a checkpoint in the cell cycle, either preventing or initiating programmed cell death. A p53 mutation remains the most common genetic change identified in human neoplasia [1] with melanoma exhibiting one of the highest rates of this oncogenic event [2].

Interferon Regulatory Factor-1 and -2 are two structurally related members of the IRF family of transcription factors. While IRF-1 expression has been associated with tumor suppressor activity, IRF-2 expression has an oncogenic effect [3–5]. IRF-1 acts as a tumor

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suppressor very similar in function to p53 and regulates expression of most of the p53 target genes [6]. It is critical to cell cycle and induction of apoptosis in response to stress signals [7-8]. The expression of several genes involved in cell cycle regulation and apoptosis such as $p21^{WAF/cip}$ [8], $p27^{Kip1}$ [9], lysyloxidase [10], caspase-1 [11], cyclin D1 and CDK4 [12], survivin [13] and Noxa [14] are transcriptionally regulated by IRF-1. By modulating the expression of VEGF[15] and MMP-9 [16], IRF-1 has also been shown to inhibit angiogenesis. Thus, the induction of apoptosis or modification of angiogenic controls by normal IRF-1 driven processes provide alternative pathways influential to tumor growth control. However, similar to mutant p53, this growth control may be evaded by mutant tumor cells and/or the tumor microenvironment to allow tumor progression.

The combination of cytotoxic chemotherapy with active biological agents like IL-2 and IFN-α, termed biochemotherapy, has generated a great deal of interest over the last several years [17-18]. Adjuvant IFN-α treatment to prevent the recurrence of melanoma has been encouraging, yet is likely far from optimized. An additional important mechanism of the IFN-α mediated antitumor activity appears to rely on the interference with tumor-mediated angiogenesis [19-20]. There are a number of clinical studies that focus on the use of IFN-α as an anti-angiogenic agent. However, the true effects of IFN-α in combination with various standard chemotherapy regimens on the endothelium and/or the viability of tumor cells are not clearly understood. Interestingly, interferon-α (a standard of care in melanoma) is one of the agents that induces IRF-1, activating its tumor suppressor function.

In the present study, using human melanoma and endothelial cell lines, we have observed that treatment with a clinically used form of pegylated IFN-α treatment in combination with the chemotherapeutic agent vinblastine induces cell death via IRF-1 mediated signaling in melanoma cells and concurrently induces premature senescence in endothelial cells. The induction of senescence may be a novel explanation for the anti-angiogenic effects that have been indicated with the clinical use of IFN-α.

## Results

### Decrease in the IRF-1/IRF-2 ratio in tumor tissues

The expression of IRF-1 and IRF-2 can be altered by a host of factors/agents and thus they may serve as possible targets for the treatment of cancer. They have been most well studied in their role as transcription factors active in (IFN-α) and type II (IFN-γ) signal transduction pathways[21]. There are several reports indicating that a decrease in the ratio of IRF-1/IRF-2 expression is prevalent in most tumor tissue types. In an analysis to study the protein expression profiles of IRF-1 and IRF-2 we were able to show that while most normal and tumor tissues expressed IRF-1 and IRF-2 the ratio of IRF-1 to IRF-2 dropped dramatically in the corresponding tumor tissue samples (Figure. 1). This information builds a case for the relevance of IRF-1 and 2 in cancer biology and treatment.

### Enhanced sensitivity of M14 melanoma cells to combined treatment with IFN-α and Vinblastine

To determine the cytotoxicity of vinblastine and pIFN-α, the cells were incubated with varying concentrations of the drugs at 37 °C for 72 h. Tetrazolium (MTT) cell proliferation assay was used to estimate the concentration required to inhibit cell growth by 50% ($IC^{50}$) for each drug. The $IC_{50}$ values of vinblastine and pIFN-α were approximately 1 nM and 0.5 μg/ml respectively for M14 cells (Figure 2a & 2b). Cells were treated with constant concentration of pIFN-α (0.5 μg/ml) and the indicated concentration of vinblastine (0 - $10^4$ pM) for 72 h. A greater than additive cell growth inhibition by MTT assay (70%) was

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observed when the cells were treated with a combination of 1nM ($10^3$ pM) vinblastine and 0.5 μg/ml of pegylated IFN-α (Figure 2c).

The effect of vinblastine and pegylated IFN-α on cell survival was also studied by clonogenic assay. Combination of vinblastine (1 nM) and pIFN-α (0.5 μg/ml) was 1.75-fold more toxic to the M14 cells than treatment with vinblastine (1 nM) alone. Treatment with half the concentration of vinblastine (0.5 nM) and pIFN-α (0.25 μg/ml) was as toxic as treatment with 1 nM of vinblastine alone (Figure 2d). These studies suggest that combination of pIFN-α and vinblastine may enhance the induction of cell death compared to either treatment alone.

## Induction of IRF-1 is specific to IFN-α exposure and enhances cell death via IRF-1 mediated signaling in M14 melanoma cells

IRF-1 is a critical transcriptional regulator in the IFN signaling pathway[5]. Therefore, we investigated the expression of IRF-1 in response to pIFN-α and vinblastine in M14 melanoma cells. A dose of 30 nM for vinblastine was selected for treatment to ensure complete cell death. Cells were treated at the indicated time points with vinblastine (30 nM) or pIFN-α (0.5 μg/ml) alone and in combination at the same (30 nM Vinblastine + 0.5 μg/ml pIFN-α) or half the concentration of each agent (15 nM Vinblastine + 0.25 μg/ml pIFN-α). IRF-1 was induced by 3-6 h and then decreased by 24 h in response to pIFN-α treatment. Interestingly, treatment with 0.25 μg/ml or 0.5 μg/ml pIFN-α induced the same level of IRF-1. Vinblastine treatment alone did not cause any noticeable induction of IRF-1. In addition, IRF-2 did not show any significant change in M14 cells after treatment with either vinblastine or pIFN-α (Figure 3a).

Subsequently, we looked at the Induction of IRF-1 downstream targets and cell death in M14 melanoma cells, as assessed by PARP-cleavage, in response to pIFN-α and vinblastine individually or combined against M14 melanoma cells. Transcriptional induction of p21 is dependent on both p53 and IRF-1[8]. M14 cells are p53 defective and possibly evade cell death by downregulation of p21. We have observed induction of p21 in M14 melanoma cells by 6 h and subsequent down regulation by 24 h in response to pIFN-α but not vinblastine (Figure 3b). Except for a delay in the induction of p21 by 3 h, the pattern of p21 protein expression in response to pIFN-α was similar to the pattern of IRF-1 induction suggesti ve of its transcriptional regulation by IRF-1.

Bak is a proapoptotic member of the Bcl-2 family of proteins and induces cell death by undergoing activation and homo-oligomerization22-[23]. We observed upregulation of total Bak in M14 melanoma cells upon treatment with pIFN-α but not vinblastine. Further, immunoblotting for PARP revealed that PARP cleavage, an indicator of cell death, occurs only upon treatment with vinblastine. However, combined treatment of vinblastine and pIFN-α at half the normal concentration of each agent (VBL, 15 nM and pIFN-α, 0.25 μg/ml) caused the same level of PARP cleavage as treatment with 30 nM vinblastine alone or the combined treatment (VBL, 30 nM and pIFN-α, 0.5 μg/ml). Treatment with pIFN-α alone did not cause PARP cleavage even by 48 h (Figure 3b).

To determine whether combined treatment with pIFN-α and vinblastine results in elevated activation of Bak, the conformationally active form was immunoprecipitated under native conditions from M14 cells treated for 36 h with vinblastine (30 nM) or pIFN-α (0.5 μg/ml) alone and in combination (30 nM Vinblastine + 0.5 pIFN-α μg/ml). For immunoprecipitation we used rabbit anti-Bak (NT) antibody (Millipore), which recognizes the conformationally active form of Bak. Cells treated with pegylated IFN-α had an increased level of inactive Bak (Supplemental Fig. 1). Treatment with vinblastine caused inactive Bak to undergo a conformational change and increased levels of activated protein

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were detected. Combined treatment with pIFN-α and vinblastine induced a further accumulation of active Bak leading to an increase in the total amount of cell death (Figure 3c).

Using the technique of 'BH3 profiling' developed by Letai and colleagues [24-25], we observed that a buildup of inactive Bak in the mitochondria as a consequence of pIFN-α treatment made the M14 melanoma cells more sensitive to vinblastine-induced cell death (i.e. pIFN-α conditions or primes the cells to death signaling induced by other agents). The essence of this technique is to use isolated mitochondria which contain inactive Bak, and induce cytochrome c release through the addition of tBid or Bid (BH3 peptide) which promotes Bak activation. Mitochondria isolated from M14 melanoma cells treated with 0.5 μg/ml pIFN-α and subsequently incubated with an activator (BH3 peptide) of Bak, induced cytochrome c release in a dose dependent manner (Figure 3d). In contrast, only low background levels of cytochrome c are released from mitochondria of untreated cells under similar conditions (Figure 3d).

## Evaluation of the response of EA.hy926 endothelial cells to vinblastine and/or pIFN-α

Using the MTT assay, EA.hy926 human endothelial cells were found to be relatively more sensitive to vinblastine than the M14 melanoma cells with an $IC_{50}$ between 500 pM and 1 nM (Fig. 4A). The $IC_{50}$ for pIFN-α in these cells was approximately 0.5 μg/ml (Fig. 4B). To test the combined effect of the drugs, cells were treated with a constant concentration of pIFN-α (0.5 μg/ml) and indicated concentration of vinblastine (0 - 500 pM) or indicated concentrations of vinblastine alone for 72 h. Cell growth inhibition measured by MTT assay was 40% greater with the combined treatment of 500 pM vinblastine and 0.5 μg/ml of pIFN-α in comparison to treatment with 500 pM vinblastine alone (Figure 4c). In subsequent clonogenic studies, cells were treated with the indicated concentration of pIFN-α and Vinblastine alone and in combination and medium replaced with fresh medium without the drugs after 24 h. In contrast to the results from the MTT assay, the clonogenicity of EA.hy926 cells was found to be markedly reduced after pIFN-α treatment alone. Combination of vinblastine (500 pM) and pIFN-α (0.5 μg/ml) had a similar effect to that of pIFN-α alone (Figure. 4d).

## Induction of IRF-1 in response to pIFN-α and synergistic response of IRF-1 downstream targets to vinblastine and pIFN-α in EA.hy926 endothelial cells

Induction of IRF-1 in EA.hy926 cells that have wild type p53 was found to occur by 6 h in the presence of pIFN-α but not vinblastine (Figure 5a, top panel). Cyclin D1, like p21 is a downstream target of p53 [26] and IRF-1[12]. We observed induction of p21 and Cyclin D1 by both vinblastine and IFN-α. Upregulation of p21 at 6 h post treatment was much greater when EA.hy926 endothelial cells were treated with vinblastine and pIFN-α. Half the normal concentration of the drugs in the combined treatment was sufficient to cause maximum induction of p21 (Figure 5a, upper middle panel). Upregulation of Cyclin D1 at 6 h was followed by its downregulation by 24 h. However, the combined treatment caused a greater reduction in Cyclin D1 at 24 h (Figure 5a, lower middle panel). While the level of Bak expression tended to vary in the presence of pIFN-α alone or in combination with vinblastine it was completely absent at 48 h post-treatment with vinblastine alone (Figure 5b). This suggests that IFN-α treatment increases and maintains the level of Bak expression to some extent, a function likely mediated through IRF-1 activity and also indicated by our results from western blotting described in Supplemental Fig. 1 and the BH3 profiling results shown in Figure 3.

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### Exposure to Interferon-α induces a senescent phenotype in EA.hy926 endothelial cells

We did not see substantial cell death even at 1 μM IFN-α treatment in the 72 h MTT assay (Figure 4b) but observed a significant reduction in cell survival in the clonogenic studies (Figure 4d). These perplexing results led us to inspect the doubling time of the cells after treatment with pIFN-α. Interestingly, while the control cells doubled in 0.9167 days (about 24 h) the pIFN-α treated cells failed to increase in total number (Figure 6a). This loss of the proliferative capacity of EA.hy926 cells was subsequently observed to be at least partially caused by pIFN-α induced senescence. We observed that upon exposure to pIFN-α cells became enlarged, flattened, irregularly shaped and significantly more cells expressed SA β-gal+ suggesting a senescent phenotype (Figure 6b, middle and lower panel) as compared to untreated cell cultures (Figure 6b, upper panel).

To observe the effect of pIFN-α on the morphology of EA.hy926 endothelial cells over time, $1\times10^5$ cells were treated for 1, 2 and 6 days with 0.25 and 0.5 μg/ml of pIFN-α and observed at 200x magnification. Cells treated with IFN-α for 1 and 2 days were rinsed and grown in fresh medium until the $6^{th}$ day. There were significantly more cells exhibiting a senescent phenotype in cultures treated continuously with 0.5 μg/ml of pIFN-α compared to cells treated with 0.25 μg/ml of pIFN-α (Supplemental Figure 2a). The cells were subsequently harvested and replated with fresh medium into new culture vessels for two weeks and again observed by phase contrast microscopy. In order to prevent the cells from being over confluent, only one-fifth of the total cells in the untreated plate were replated. The senescent phenotype continued to persist in these freshly plated cells previously exposed to pIFN-α independent of total length of the initial exposure to pIFN-α (Supplemental Figure 2b). Treatment with the microtubule inhibitor, vinblastine causes the cells to arrest at G2/M. Cells tend to get rounded, start floating and eventually undergo cell death. In EA.hy926 cells a similar morphological change was observed by 48 h (Supplemental Figure 3a) upon treatment with 30 nM vinblastine or with a combination of 15 nM vinblastine and 0.25 μg/ml pIFN-α. A quantification of adherent versus non-adherent cells after treatment with pIFN-α and vinblastine is shown in Supplemental Figure 3b and represents data from three replicates per condition. Since unsynchronized cells were used for plating we did observe a percentage (11%) of non-adherent cells upon IFN-α treatment as compared to almost 50% of non-adherent cells after vinblastine or combined treatment with IFN-α and vinblastine.

## Discussion

The use of pegylated IFN-α2b in monotherapy [27] and in combination with chemotherapy has been evaluated in several different types of cancers [28-29]. Pegylated IFN-α2b with its improved pharmacokinetic profile has been successfully introduced into melanoma therapy [30]. However, the most common adverse effects of neuro-psychiatric, hematologic and hepatic toxicity as a consequence of IFN therapy have not been able to be negated and therefore hold the full potential of this agent in check. Development of optimal protocols for the use of pegylated IFN-a2b to maximize benefit and minimize toxicity will allow for wider use with fewer adverse effects. A detailed understanding of the mechanism of action of IFN-α alone and in combination with chemotherapy is required to achieve this goal.

Owing to the ability of IFNs to enhance immunogenicity, Interferon signaling and IFN therapy have been extensively studied with regard to levels of tumor immunity attained. Other non-immune based molecular mechanisms have been studied at the cellular level in response to IFNs but have not readily been connected with tumor/ tumor microenvironment response. Recent evidence has broadened the scope of IRF-1 tumor suppressor functions in a variety of cancers including ovarian cancer [31], melanoma [4], breast cancer [32] and cervical cancer [33]. Here, we have shown that there is a substantial fall in the IRF-1/IRF-2 ratio in tissues from 9 different types of human tumors when compared to the corresponding normal

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tissue (Fig. 1). This may not just serve as a useful indicator for diagnosis and prognosis of most tumors (Fig. 1) but enables their use as possible targets in cancer treatment.

A series of viability and survival studies indicated that pIFN-α enhances the sensitivity of the M14 melanoma cells to cell death by the cytotoxic agent, vinblastine (Figure 2). At the molecular level, while IRF-1 gets specifically induced in response to pIFN-α, the level of IRF-2 remains more or less constant ( Figure 3a) and it is known that IRF-1 is involved in cell cycle regulation and induction of apoptosis. For instance, transcriptional induction of p21 by γ-irradiation was dependent on both IRF-1 and p53 [8]. p21 is a cyclin dependent kinase inhibitor (CDKI) and regulates cell cycle progression at S phase [34]. The M14 melanoma cell line is p53 defective and thus one possible alternate route to induction of p21 is through upregulation of IRF-1. (Figure 3b).

We also observed distinct PARP cleavage, suggestive of cell death induction, upon vinblastine treatment after 24 h. pIFN-α treatment alone did not cause any PARP cleavage even by 48 h (Figure 3b). We investigated the possible role of Bak induction and activation in the greater than additive cell death caused by vinblastine combined with pIFN-α. Bak, a distal mediator of apoptosis, undergoes conformational changes and homo-oligomerization in response to diverse apoptotic signals, leading to pore formation in the mitochondria and release of apoptosis-promoting factors [22-23]. The expression of Bak has been shown to be upregulated by IRF-1 [35] (Figure 3b). Activation of Bak is a critical step towards apoptosis involving the exposure of its N-terminal epitope. Previously, we showed that vinblastine induces activation of Bak in KB-3 cells [23]. Importantly, while there is a significant increase in Bak expression in response to pIFN-α (Figure 5b) we show in the present report, using a conformationally-dependent Bak antibody, that only vinblastine induces Bak activation in M14 melanoma cells (Figure 3c). It appears that the combined treatment of pIFN-α and vinblastine allows for an accumulation of total Bak and a subsequent increase in overall activation of Bak in M14 melanoma cells which leads to enhanced cell death by the combined treatment.

Using an alternate strategy we have also demonstrated that the build-up of inactive Bak in response to pIFN-α treatment sensitizes the M14 melanoma cell mitochondria to BH3-peptide mediated cytochrome c release (Figure 3d). This relationship thus suggests that the pIFN-α treatment primes M14 melanoma cells to more rapidly respond to cell death stimuli such as that exerted by vinblastine and possibly other chemotherapeutics. Induction of p21 and Bak via upregulation of IRF-1 in response to pIFN-α might partially explain how the IFN signaling pathway crosstalks with the other apoptotic and cell cycle pathways to enhance cell death in M14 melanoma cells and may have implications for other tumor cell types.

The progressive growth and metastasis of most tumors are angiogenesis dependent and therefore controlled by not only tumor cell proliferation but also proliferation and activity of cells that form the microvasculature[19-20]. We selected the human EA.hy.926 endothelial cell line to better understand the possible effects of pIFN-α exposure on tumor microvasculature mediated by endothelial cell responses (Figure 4). EA.hy926, is an immortalized endothelial cell line harboring wild type p53. It has been shown that the transcriptional induction of the p21 gene is dependent on both p53 and IRF-1 [8]. Cyclin D1,which works in concert with p21 to promote progression through G1 [36], has also been shown to be transcriptionally regulated by p53 [26] and IRF-1 [12]. These two tumor suppressor transcription factors therefore converge functionally to regulate cell cycle and possibly senescence through the activation of common target genes. It appears that sensitivity to cell death is enhanced in cells where both p53 and IRF-1 mediated pathways can be activated such as in normal cells of the stromal compartment that supports the tumor microenvironment (Figure 5)

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Our initial results from the treatment of EA.hy926 cells with pIFN-α were perplexing. While on one hand MTT cell viability assay did not show complete or substantial cell death even at 1 μM IFN-α treatment (Figure 4b) long term survival studies showed a significant reduction in number of colonies when treated with 0.5 μg/ml IFN-α (Figure 4d). This increase in sensitivity to IFN-α was not due to the extent of apoptotic cell death (data not shown) but rather associated with enlarged, multinucleated, β-galactosidase-positive senescent EA.hy926 endothelial cells (Figure 6a). While the normal cells doubled in less than one day the number of cells in tissue culture plates after 5 days of IFN-α treatment remained constant. This suggested an alternate mechanism to apoptotic or clonogenic cell death may be at play. Indeed, we observed a premature senescence phenotype induced in IFN-α treated cells that persisted long-term over subsequent passages of the cells. Induction of a senescent phenotype has previously been observed after treatment with several DNA-binding drugs [37]. The EA.hy926 endothelial cells studied here exhibited a similar phenotype and an induction followed by a subsequent decrease in the levels of p21 and cyclin D1 upon treatment with pIFN-α and vinblastine (Figure 5). This biphasic response of p21 and cyclin D1 is a further characteristic of cellular senescence [38-39]. Therefore, our results suggest that the normally proliferative tumor endothelium in a growing malignant tissue environment may be sensitive to pIFN-α-induced senescence which would be expected to modify the angiogenic activity in the tumor. We have also demonstrated (Supplemental Figure 3) that vinblastine treatment which causes G2/M arrest followed by apoptosis results in non-adherent cells that do not undergo senescence.

In summary our results suggest that the overall therapeutic mechanism of IFN-α combined with chemotherapeutic agents may be due to direct tumor cell death via IRF-1 based signaling and by inducing premature senescence in endothelial cells which could reduce the angiogenic activity in the tumor. These results further uncover alternate mechanisms by which IFN-α functions in cancer therapy. An increased understanding of how the IFN-α pathway crosstalks with other pathways via IRF-1 based signaling to control apoptosis in the tumor microenvironment appears to be a valid approach to better identify drug regimens suited to block angiogenesis and cell proliferation in melanoma and other solid tumors. Efforts are underway to study these interconnections in co-cultures and in *in vitro* angiogenic co-culture constructs of tumor and endothelial cells.

## Materials and Methods

### Cell lines and culture

M14 is a human malignant melanoma cell strain, originally denoted as UCLA-SO-M14, later called M14, which expresses most melanoma associated markers 40-41. EA.hy926 is an immortalized human endothelial-like cell line, derived from the fusion of primary human endothelial vein endothelial cells (HUVEC) with the lung carcinoma cell line A549 [42]. The cell lines were maintained in monolayer culture at 37 °C and 5% $CO_2$. The M14 human melanoma cell line was maintained in RPMI (Cellgro), supplemented with 10% fetal bovine serum (Atlas), 50 units/ml penicillin, and 50 μg/ml streptomycin (Hyclone). The EA.hy926 endothelial cell line was cultured in DMEM/F-12 (Cellgro) with 10% Bovine Calf Serum (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 1:50 HAT (Cellgro).

### Chemicals and reagents

Pegylated Interferon-α2b (PEGINTRON®) referred as pIFN-α was purchased from Schering-Plough. Vinblastine and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma-Aldrich. IRF-1, IRF-2 and Cyclin D1 (DCS-6) antibodies were from Santa Cruz Biotechnology. Bak (NT) antibody for immunoprecipitation was from Millipore

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and Bak (Ab-1) antibody for immunoblotting was from Oncogene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Cell Signaling Technology. Cytochrome-c and p21 antibodies were from BD Pharmingen. Crystal Violet (Fisher Scientific) and Protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology.

### MTT cell viability assay

Cells (2000/well) were seeded in 96-well plates and the following day vinblastine and/or pIFN-α was added. After 72 h, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) (50 mg/well) was added for 4 h followed by dimethylsulfoxide solubilization of the cells, and an absorbance reading was done at 540 nm.

### Clonogenic assay

Cells were seeded at 200 cells/well in 6 well tissue culture plates and the next day treated with the indicated concentration of drugs for 24 h and then colony formation was assessed. Cells were allowed to grow for 2 weeks after rinsing and replenishing the wells with fresh media without the drugs at 24 h. Colonies were stained with crystal violet and counted to calculate the surviving fraction.

### Expression of IRF-1 and IRF-2 in normal human and tumor tissues

Customized western blots (ProSci, CA) of 15 normal human and tumor tissues each were probed for IRF-1(1:1000). The blots were stripped and reprobed for IRF-2(1:1000). The level of IRF-1 and IRF-2 protein expression was estimated by analyzing the relative band intensities using the Image J software.

### Preparation of cell extracts and immunoblotting

Whole cell extracts were prepared by suspending cells in 0.25 ml of lysis buffer (25 mM HEPES, pH 7.5, 0.5% sodium deoxycholate, 5 mM EDTA, 5 mM dithiothreitol, 20 mM -glycerophosphate, 1 mM Na3VO4, 50 mM NaF, 1% Triton X-100, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μM pepstatin, 1 μM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride). Cells were incubated at 4°C on a nutator for 1 h, insoluble material was removed by centrifugation (15 min at 12,000 x g), and protein concentration determined using the Bio-Rad protein assay.

### BH3 peptide mediated mitochondrial cytochrome c release

Mitochondria (25 μg/reaction) isolated from M14 cells without and after treatment with 0.5 μg/ml pIFN-α for 48 h were incubated with the indicated concentrations of BH3 peptide for 30 minutes at room temperature. Supernatant was collected and Cytochrome c (Cyt.c) release observed by immunoblotting. DMSO was used as the vehicle (2% final concentration in all samples) [24].

### Immunoprecipitation

To evaluate Bak activation status, the cells were lysed in 0.5 ml of 10 mM HEPES (pH 7.4), 150 mM NaCl, 1% CHAPS, plus protease inhibitors (as above) and incubated at 4°C for 45 min on nutator. The cell extract was centrifuged at 12,000 x g for 15 min, and 500 μg of cell lysate was precleared using protein A/G-PLUS agarose beads for 30 min. followed by incubation with 4 μg of Bak (NT) polyclonal antibody (Millipore) in a total of 500 μl of lysis buffer for 1 h. The lysates were then incubated with 25 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The beads were pelleted by centrifugation and washed three times with 0.2 ml of lysis buffer. The beads were resuspended in 50 μl of 2x SDS loading buffer and electrophoresed on 12.5% SDS-PAGE precast acrylamide gels

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(Biorad). Following electrophoresis, immunoblotting was performed using mouse anti-Bak (Ab-1) monoclonal antibody (Oncogene) at 1:1000 dilution [23].

### Senescence Associated-ß-Gal assay

The SA-ß-Gal assay was performed as previously described in the manufacturer's instructions (Senescence β-galactosidase staining kit, Cell Signaling Technology). Briefly, cells were washed in phosphate-buffered saline and fixed in 2% formaldehyde-0.2% glutaraldehyde. Then the cells were washed and incubated at 37°C overnight with fresh senescence-associated ß-Gal stain solution (1 mg of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside [X-Gal per ml, 40 mM citric acid-sodium phosphate [pH 6.0, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). For all visualization and photography a Nikon Eclipse Te2000-U microscope was used.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **pIFN-α** | pegylated Interferon-α2b |
| **IRF-1** | Interferon regulatory factor-1 |
| **IRF-1** | Interferon regulatory factor-1 |
| **PARP** | Poly (ADP-ribose) polymerase (PARP) |

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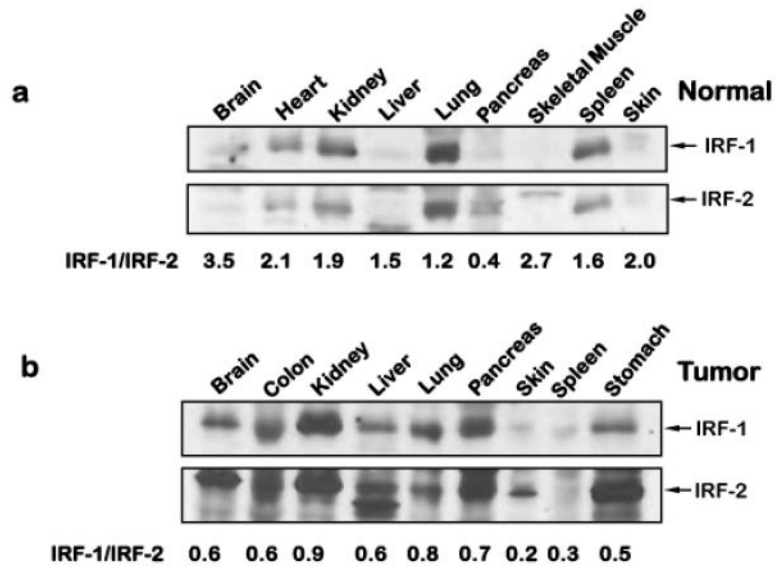


**Figure 1. Western analysis of IRF-1 and IRF-2 expression in normal human and tumor tissues**

Expression profile of IRF-1 and IRF-2 in normal human tissues (**a**) or in human tumor samples (**b**) were analyzed by western immunoblotting (ProSci) and the IRF-1/IRF-2 ratio was quantified by densitometry using the Image J software.

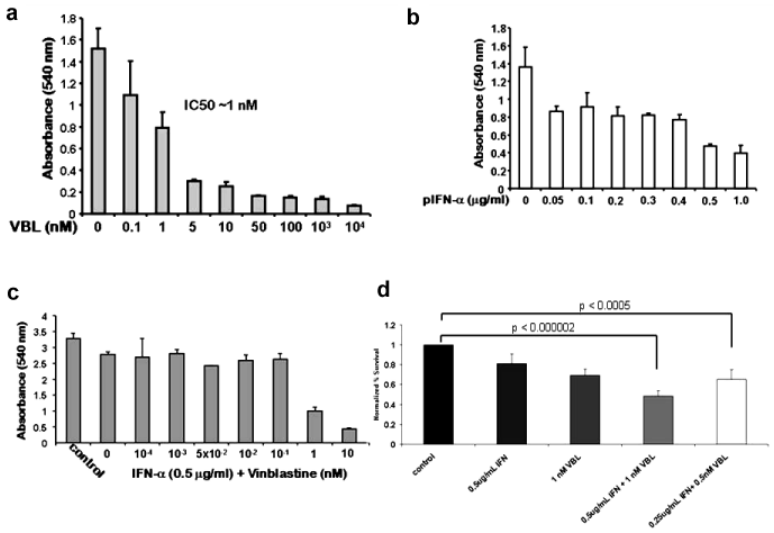


**Figure 2. Enhancement of M14 cell sensitivity to vinblastine cytotoxicity by pegylated IFN-α**

3000 Cells /well in a 96-well plate were treated with indicated concentrations of vinblastine (**a**) or pegylated IFN-a2b (**b**) for 72 h and subjected to MTT cell viability assay. (**c**) Cells were treated with the pegylated IFN-a2b (0.5 mg/ml) in combination with the indicated concentrations of vinblastine (0 -10 nM) for 72 h. Cell proliferation was estimated by MTT assay, (**d**) Cells (200 cells/well) were treated with the indicated concentration of drugs for 24 h. The cells were rinsed, fresh medium was added and after 2 weeks, colonies were stained with crystal violet, counted and surviving fraction calculated. Results in each of the above experiments are the means ± standard deviation (n = 6). The p values are shown.



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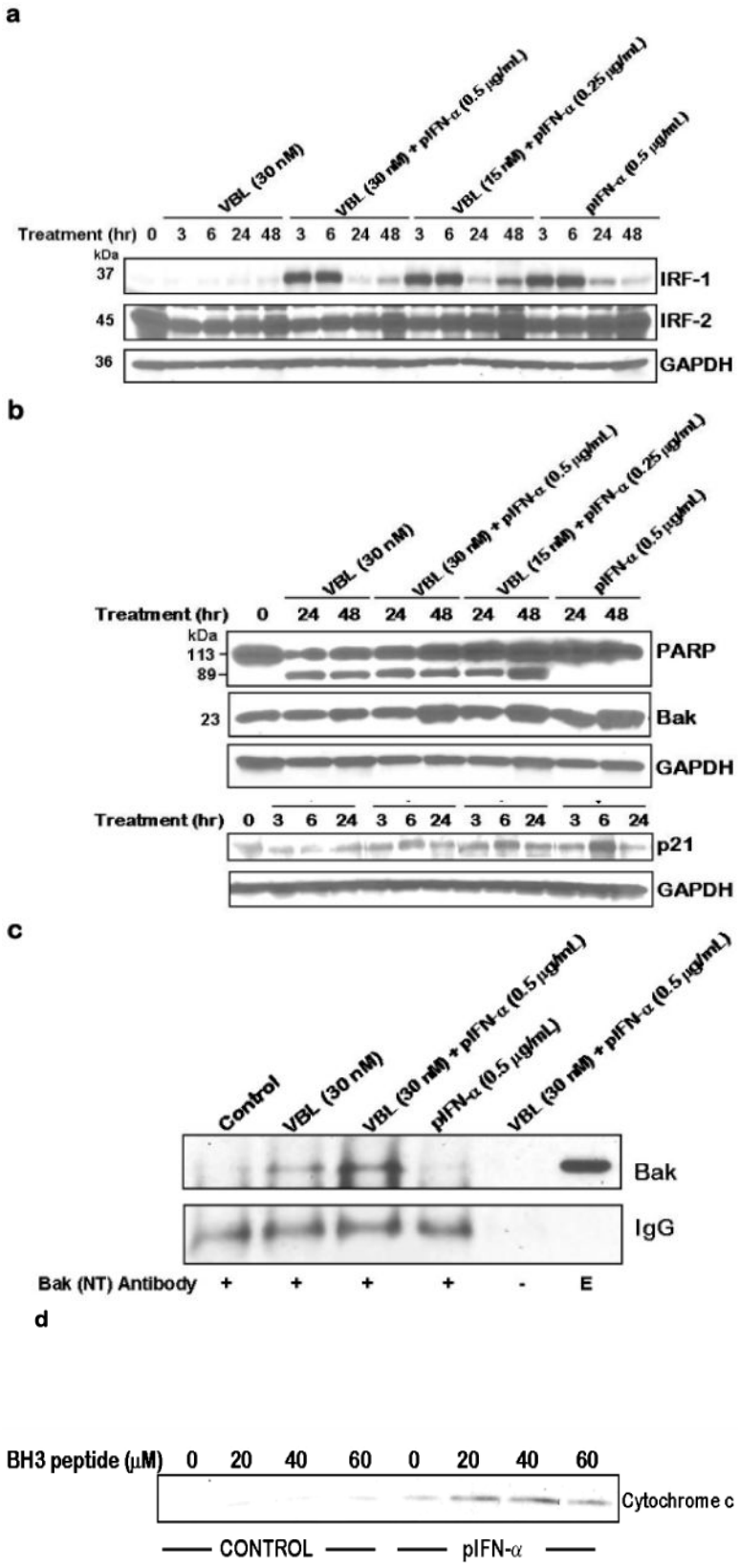

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**Figure 3. Molecular response of M14 melanoma cells to vinblastine and IFN-α**

(**a**) IRF-1 and not IRF-2 is induced specifically in response to pegylated IFN-α in M14 melanoma cells. M14 cells were treated for the indicated time with either vinblastine (VBL), pegylated IFN-α2b (pIFN-α), or a combination of both at the indicated concentrations. Cell extracts (50 mg/lane) were immunoblotted for IRF-1 or IRF-2. Immunoblotting for GAPDH served as the loading control. (**b**) Induction of IRF-1 downstream targets in response to IFN-α and combined effect of IFN-α and vinblastine on PARP induced apoptosis in M14 cells. Cells were treated with the indicated drug concentrations of vinblastine or pegylated IFN-α (pIFN-α) and harvest at the given time points. Cell extracts were subjected to immunoblotting for PARP, Bak and p21. Immunoblotting for GAPDH served as the loading control. (**c**) Treatment of M14 cells with IFN-α and vinblastine causes induction and activation of Bak. M14 melanoma cells were untreated or treated with Vinblastine (30 nM) or pIFN-α (0.5 mg/mL) alone or in combination (30 nM Vinblastine + 0.5 pIFN-a mg/mL) for 36 h. Lysates were immuno-precipitated with anti-Bak-NT antibody for active form of Bak followed by immunoblotting for Bak. Precipitates prepared in the absence of the antibody (−) and whole cell extract (E) were also examined as controls. (**d**) BH3 peptide mediated cytochrome c release from mitochondria of M14 melanoma cells. Mitochondria (25 μg/reaction) isolated from M14 cells without and after treatment with 0.5 mg/ml IFN-α for 48 h were incubated with the indicated concentrations of BH3 peptide for 30 minutes at room temperature. Supernatant was collected and cytochrome c (cyt.c) release observed by immunoblotting. DMSO vehicle was 2% final in all samples.

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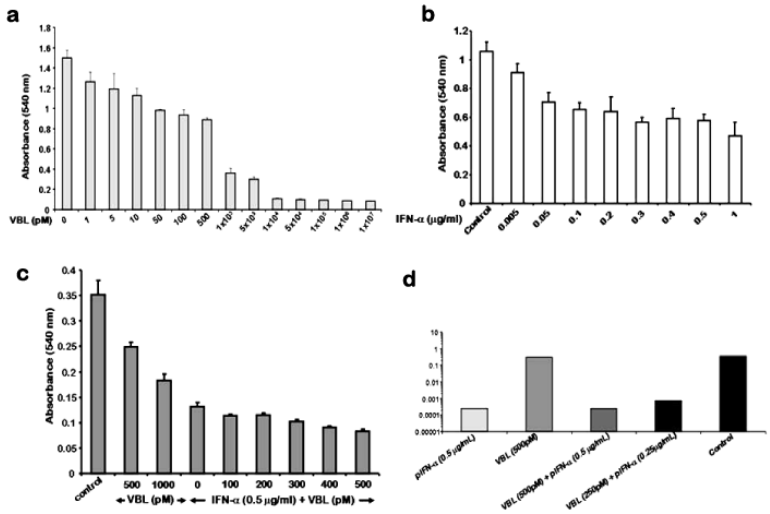


**Figure 4. Sensitivity of EA.hy926 endothelial cells to vinblastine and pegylated IFN-α**

3000 cells/well in a 96-well plate were treated with indicated concentrations of vinblastine (**a**) or pegylated IFN-a2b (**b**) for 72 h and subjected to MTT colorimetric assay. (**c**) Cells were treated with the pegylated IFN-a2b (0.5 mg/ml) in combination with the indicated concentrations of vinblastine (0 - 500 pM) for 72 h. Cell viability was estimated by MTT assay. The above three figures were representative of three independent experiments the results of each are the means ± standard deviation (n = 6), (**d**) 2000 cells/well in a 6-well plate were treated with the indicated concentration of drugs for 24 h. The cells were rinsed, fresh medium was added and after 2 weeks, colonies were stained with crystal violet, counted and surviving fraction calculated.

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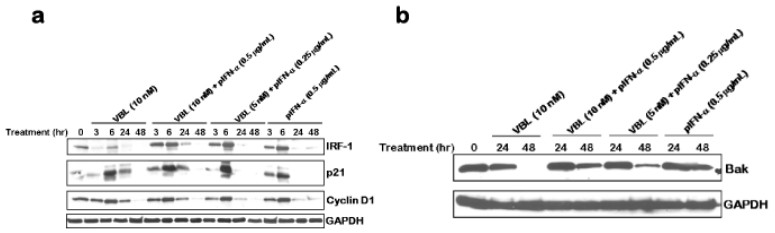


**Figure 5. Induction of IRF-1 and its downstream targets in response to pIFN-α and vinblastine in EA.hy926 endothelial cells**

Cells were treated with the indicated concentrations of vinblastine or pegylated IFN-α (pIFN-α) and harvested at the given time points. Cell extracts were subjected to immunoblotting for (**a**) IRF-1, p21 and cyclin D1 and (**b**) Bak. Immunoblotting for GAPDH served as the loading control.

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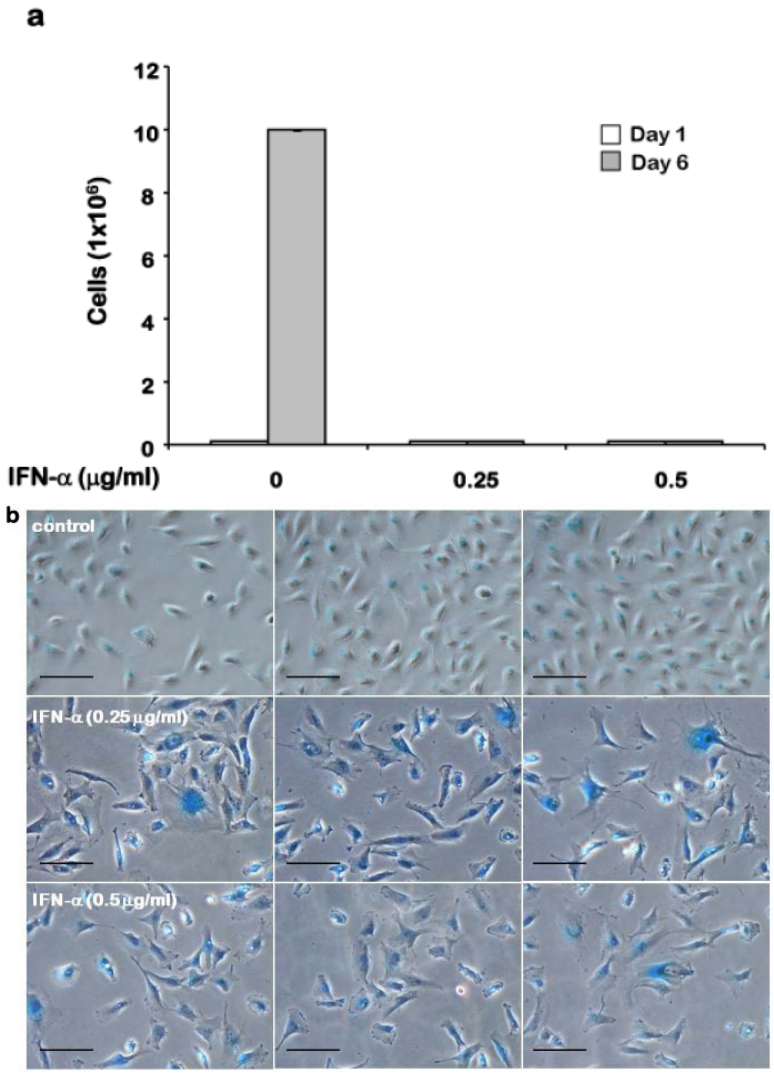


**Figure 6. Induction of senescence like growth arrest in EA.hy926 endothelial cells in response to pIFN-α**

(**a**) 5 ×104 EA.hy926 cells were plated onto 60 mm petriplates and treated the next day with 0.25 mg/ml or 0.5 mg/ml of IFN-α and counted on the 6th day post treatment. (**b**) The cells treated in the same manner were stained for senescence associated β-galactosidase 48 h post treatment and phase contrast images of cells were taken at 200X magnification. Scale Bar = 100 μm