Enhanced Apoptosis in Melanoma Cells Following Treatment with Bortezomib (Velcade ®, PS-341) and Interferon Alpha (IFN-α)

A Senior Honors Thesis

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By

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Abstract

The 26S proteasome is a protein complex that plays a critical role in the degradation of cellular proteins including transcription factors, cyclins, and other proteins required for cell cycle progression in normal and malignant cells. Bortezomib (PS-341, Velcade) is a specific and selective inhibitor of the 26S proteasome that has cytotoxic activity against tumor cells. Treatment with this compound has been shown to induce apoptosis in malignant cells. Interferon-alpha (IFN-α) is an immunostimulatory cytokine that is known to inhibit proliferation and to promote apoptosis in tumor cells. Apoptosis of human melanoma cells was evaluated following treatment with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or both agents combined by Annexin V/ propidium iodide (PI) staining and demonstrated that cell death was synergistic in nature. The pro-apoptotic activity of this treatment combination was evident in multiple malignant melanoma cell lines (1259 MEL, 18105 MEL, A375) indicating that this effect was not cell line specific. Cell death induced by bortezomib and IFN-α was associated with cleavage of caspase proteins (caspase-3, -7) and poly ADP-ribose polymerase (PARP). Interestingly, levels of the cyclin dependent kinase inhibitor, p21, were increased slightly following treatment with bortezomib plus IFN-α. Levels of the pro-survival mitochondrial membrane protein Bcl-2 were decreased in a synergistic manner following treatment of human melanoma cells with bortezomib and IFN-α combined. Real-Time PCR analysis demonstrated that pre-treatment of melanoma cells with bortezomib enhanced IFN-α induced gene expression. Together these data suggest that bortezomib and IFN-α exert complimentary pro-apoptotic effects on the tumor cell and that pre-treatment with bortezomib can sensitize melanoma cells to the direct effects of IFN-α. The safety of this treatment combination is being evaluated in a Phase I clinical trial in patients with metastatic melanoma at The Ohio State University (OSU 04105; PI: Carson).

Introduction

Interferon-alpha (IFN-α) has been utilized in the treatment of malignant melanoma and renal cell carcinoma for the past three decades. Although its mechanism of action has not been fully elucidated, IFN-α possesses potent immunostimulatory properties and direct anti-proliferative, pro-apoptotic, and anti-angiogenic effects on the tumor cell (1-2). Specifically, IFN-α has been shown to sensitize malignant cells to pro-apoptotic stimuli by increasing the expression of death receptors (TRAIL, Fas) and by upregulating the expression of cell cycle regulatory proteins (e.g. the CDK inhibitor p21) (3-6). These observations suggest that IFN-α may be an effective means to enhance the pro-apoptotic effects of other targeted therapeutics.

Treatment of melanoma patients with IFN-α as a single agent yields clinical responses in approximately 10-20% of patients (7, 8, 9). While this response is modest, IFN-α is one of the limited options for patients with metastatic melanoma and is the only FDA-approved agent for the adjuvant therapy of patients who have undergone tumor excision. However, chemotherapy and high dose cytokine therapy is often poorly tolerated by patients with advanced disease.

Bortezomib (Velcade®, PS-341) is a novel compound that specifically inhibits the 26S proteasome, which plays a critical role in many intracellular events including cell cycle progression, transcription factor activation, and apoptosis (10). The 26S proteasome is responsible for ordered, temporal degradation of cyclins and CDK inhibitors (p21 and p27) required for cell cycle progression (10, 11). In the presence of bortezomib, these proteins are stabilized, thus arresting the cell cycle and promoting apoptosis.

Bortezomib possesses in vitro activity against a variety of tumor cell types including multiple myeloma, prostate cancer, pancreatic cancer, renal cell carcinoma, and squamous cell carcinoma (12-17). Clinical trials involving treatment with bortezomib demonstrate that it

possesses activity in patients with advanced multiple myeloma and has a tolerable toxicity profile (15). It has also demonstrated some clinical activity in other solid tumors, including advanced renal cell carcinoma where it produced a partial response in 11% of patients and stable disease in 38% of patients (17). These results suggest that the effects of bortezomib on solid tumor cells may be enhanced when used in combination with other pro-apoptotic agents.

We hypothesized that IFN-α would enhance the anti-tumor effects of bortezomib by activating common pro-apoptotic pathways. We have demonstrated that treatment of malignant melanoma cells with the combination of bortezomib and IFN-α synergistically induced apoptotic cell death. Apoptosis induced by this combination was associated with increased cleavage of caspase-3 and PARP, slightly increased levels of p21, and decreased levels of Bcl-2. These data demonstrate that the pro-apoptotic effects of bortezomib against malignant melanoma tumors are enhanced in the presence of IFN-α and have identified potential molecular targets of this treatment combination.

Materials and Methods

Cell Lines. The HT144 and A375 (human) melanoma cell lines were purchased from the American Type Cell Culture collection (ATCC, Manassas, VA). The 1259 MEL and 18105 MEL human melanoma cell lines were obtained from Dr. Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY).

Reagents. Murine (mu) IFN-α (specific activity = 3.3×10^6 U/mg) was purchased from Access Biochemical (San Diego, CA). Recombinant human (hu) IFN-α (specific activity = 2×10^8 IU/mg) was purchased from Schering-Plough, Inc. (Nutley, NJ). Bortezomib (Velcade®, PS-341) was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA) and reconstituted with normal saline (0.9%), to a stock concentration of 1 mg/ml prior to use in all assays.

Analysis of Apoptosis with Annexin V/Propidium Iodide (PI) Staining. The exposure of phosphatidyl serine on the cell membrane, a marker of apoptosis, was assessed in tumor cells following treatment with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or both agents combined by flow cytometry using APC-Annexin V and propidium iodide (PI; BD Pharmingen, San Diego, CA) (18). Quantitative data were obtained using a Becton Dickinson FACScalibur cytometer (BD Immunocytometry systems, San Jose, CA) and analyzed using WinMDI v. 2.8 software (created by Joseph Trotter; available at: http://pingu.salk.edu/software.html). Early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and non-apoptotic (Annexin V^-/PI^-) events were quantified utilizing at least 10,000 events.

Immunoblot Analysis. Cytoplasmic lysates (to measure caspase proteins) were prepared with 1X CHAPS lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). Whole cell lysates (to measure Bcl-2, p21, PARP) were prepared by harvesting cells into lysis buffer (10% SDS, 1M Tris-HCl pH 6.8, 0.5M EDTA) containing the following protease inhibitors (1M Sodium Flouride, 100mM Sodium Orthovanadate, 100mM Sodium Pyrophosphate). Loading dye (2X SDS-2% 2-beta-mercaptoethanol) was added to samples in a 1:1 ratio and samples were boiled for 5 minutes. Protein in cell extracts was resolved on SDS-polyacrylamide gels and transferred via electrophoresis to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk or 5% BSA in TBS-T (100mM Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20). Immunoblots were probed overnight with antibodies (Ab) specific for p21 (Pharmingen, San Diego, CA), human Bcl-2, caspase-3, and poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology). Membranes were next washed twice (5 minutes per wash) with TBS-T to rid the nitrocellulose membrane of excess Ab and incubated with appropriate horseradish-peroxidase-conjugated secondary Ab. Membranes were washed again with TBS-T and immune complexes were detected using the ECL Plus detection kit (Amersham Biosciences, Ayesbury, UK). β-actin, a housekeeping gene, was utilized as a control to confirm equal loading for all immunoblots (Sigma).

Real Time PCR. Total RNA was harvested from cells using TRIzol, a reagent that maintains the integrity of RNA while disrupting cell membranes and solubilizing cell components (Invitrogen). Chloroform was then added to samples prior to centrifugation to separate aqueous and organic phases. Following centrifugation, total cellular RNA remained in the aqueous phase and was then precipitated using isopropanol. The resulting RNA pellet was washed with ethanol

and resuspended in sterile water. Reverse transcription was performed using 2 μg of total RNA and random hexamers (PerkinElmer, Norwalk, CT) as primers for first-strand synthesis of cDNA and the following conditions: 70°C for 2 minutes, 42°C for 60 minutes, and 94°C for 5 minutes. We used 2 μL of the resulting cDNA as template to measure the levels of mRNA for IFIT2 by real-time RT-PCR with pre-designed primer/probe sets (Assays on Demand; Applied Biosystems, Foster City, CA) and 2X TaqMan Universal PCR Master Mix (Applied Biosystems). Pre-designed primer/probe sets for human β-actin (Applied Biosystems), a housekeeping gene, were used as an internal control in each reaction well. Real-time RT-PCR reactions were performed in triplicate in capped 96-well optical plates. The following amplification scheme was used: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Real-time RT-PCR data were analyzed using Sequence Detector software, version 1.6 (PE Applied Biosystems, Foster City, CA).

Results

Combined treatment of melanoma cell lines with bortezomib and IFN-α leads to enhanced apoptotic cell death.

Both bortezomib and IFN-α have been shown to induce tumor cell apoptosis when administered as single agents. It was hypothesized that the pro-apoptotic effects of these two agents would be superior to either agent alone when used against melanoma cell lines. Human and murine melanoma cells (B16, MEL 39, HT144) were treated with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or both agents combined for 48 hours. Phosphatidyl-serine exposure on the cell membrane was then measured by flow cytometry at various time points using fluorescently labeled Annexin V in combination with propidium iodide (PI) staining as previously described (18). The combination of bortezomib and IFN-α induced a greater level of apoptosis compared to either agent alone in three separate melanoma cell lines (Fig. 1). For example, treatment of the B16 murine melanoma cell line with IFN-α resulted in 6.57% apoptotic cells and bortezomib yielded 29.57% apoptotic cells. However, treatment with both agents combined induced apoptosis in 46.81% of cells.

Combined treatment of melanoma cell lines with bortezomib and IFN-α induces cleavage of caspase-3.

Caspase-3 is a major downstream effector caspase and the cleaved form of this protein is partially responsible for the induction of apoptosis (2). Human melanoma cells were treated with PBS, bortezomib (10nM, 20nM), IFN-α (10^4 U/mL), or both agents combined for 48 hours. Immunoblot analysis indicated that treatment of melanoma cells with bortezomib (20nM) or

bortezomib (20nM) and IFN-α induced cleavage of caspase-3 at the 48 hour time point (Fig. 2). This pattern of caspase activation was reproducible and routinely observed in multiple human melanoma cell lines (A375, MEL 1259, MEL 18105; data not shown).

Combined treatment of melanoma cell lines with bortezomib and IFN-α induces cleavage of PARP.

The cleavage of poly ADP-ribose polymerase (PARP), an enzyme involved in the surveillance of genome integrity and DNA repair, is a valid marker of apoptosis (19). Human melanoma cells were treated with PBS, bortezomib (10nM, 20nM), IFN-α (10^4 U/mL), or both agents combined for 48 hours. Immunoblot analysis demonstrated that there was an increased level of cleaved PARP in melanoma cells that had been treated with the combination as compared to either agent alone indicating that a greater level of apoptosis was reached when treated with the combination (Fig. 3; arrow). This pattern of PARP cleavage was reproducible and routinely observed in multiple human melanoma cell lines (MEL 1259, MEL 18105).

Combined treatment of melanoma cell lines with bortezomib and IFN-α leads to decreased expression of Bcl-2.

The Bcl-2 family of mitochondrial proteins are key regulators of apoptosis that promote cell survival by stabilizing the mitochondrial membrane (20, 21). Modulation of these proteins has been shown to contribute to the pro-apoptotic effects of bortezomib in Jurkat cells (22). Human melanoma cells were treated for 48 hours with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or

both agents combined. Immunoblot analysis demonstrated a significant decrease in the level of Bcl-2 protein following treatment of human melanoma cell lines with the combination of bortezomib and IFN-α (Fig. 4). This pattern of decreased Bcl-2 protein was reproducible in other human melanoma cell lines treated with bortezomib and IFN-α (A375, HT144).

Combined treatment of melanoma cell lines with bortezomib and IFN-α leads to increased levels of p21.

Both bortezomib and IFN-α have been shown to induce the accumulation of the cyclin dependent kinase inhibitor p21 as single agents in human multiple myeloma and lymphoma cells (3, 23). This molecule is known to promote cell cycle arrest and can serve to sensitize the cell to the induction of apoptosis. To determine whether p21 accumulates prior to the onset of apoptosis human melanoma cells were treated for 24 hours with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or both agents combined. Enhanced expression of p21 protein was observed following treatment with bortezomib alone, while treatment with both agents combined led to only a modest enhancement of p21 protein (Fig. 5).

Combined treatment of melanoma cell lines with bortezomib and IFN-α induces increased expression of the IFN-α responsive gene IFIT2.

Since bortezomib and IFN-α induce synergistic apoptosis, we hypothesized that the combined treatment of melanoma cells with bortezomib and IFN-α may affect the transcription of IFN-α stimulated genes. Human melanoma cells were treated for 16 hours with bortezomib (10nM,

20nM) or PBS and then stimulated with IFN-α (10^4 U/mL) for 4 hours. Total cellular RNA was isolated and the transcription of the IFN-α-responsive gene IFIT2 was measured by Real Time PCR. Proteasome inhibition resulted in a dose-dependent increase in the expression of IFIT2. This effect occurred prior to the onset of cell death (36 – 48 hours). Similar results were observed following an 18-hour co-treatment with bortezomib and IFN-α (Fig.6).

Discussion

We have shown that bortezomib and IFN-α can induce synergistic apoptosis in melanoma cell lines. This apoptosis was associated with cleavage of the downstream effector caspase (caspase-3) and PARP. In addition, we demonstrate that this treatment combination resulted in decreased levels of the pro-survival Bcl-2 protein and modest increases in the level of the cyclin dependent kinase inhibitor p21. Finally we have shown that pre-treatment or co-treatment with bortezomib leads to enhanced transcription of IFN-α induced genes. These data demonstrate that this treatment combination may be an effective means of inducing apoptosis in human melanoma cells or sensitizing cells to the direct actions of IFN-α.

Apoptosis or programmed cell death consists of the ordered disassembly of the cell from within as opposed to necrosis or accidental cell death. Apoptosis may be induced through the extrinsic pathway (activation of cell surface death receptors) or the intrinsic pathway (alterations in the integrity of the mitochondrial membrane that induce the release of cytochrome c) (24). These pathways converge at the level of the effector caspases (caspase-3, -6, and –7) (24). Once activated, these effector caspases cleave cytoskeletal and nuclear proteins such as poly ADP-ribose polymerase (PARP) thereby initiating cellular disassembly (24). We have demonstrated that both caspase-3 and PARP are cleaved in response to treatment of melanoma cells with bortezomib and IFN-α combined, supporting the premise that cell death occurs in a manner consistent with apoptosis.

The Bcl-2 family of proteins function as key regulators of apoptosis that promote cell survival by controlling mitochondrial membrane permeability and the release of cytochrome c into the cytoplasm (20-21). The Bcl-2 protein is an anti-apoptotic member of this family that appears to antagonize the actions of the pro-apoptotic counterparts in this family. Although the

exact mechanism remains unknown, the Bcl-2 protein has been shown to inhibit the pro-apoptotic Bcl-2 family protein, Bak, and to regulate cytochrome c release from the mitochondria (22, 25). Interestingly, the Bcl-2 family of proteins has been shown to play a role in the resistance of Jurkat cells to apoptosis (26). In the present study, decreased levels of Bcl-2 were observed following the treatment of human melanoma cell lines with bortezomib, and this effect was markedly enhanced by the combination of bortezomib and IFN-α. Future studies will define the role of Bcl-2 in promoting the pro-apoptotic effects of this treatment combination.

The cyclin dependent kinase inhibitor, p21, is involved in regulating the cell cycle (3, 23). IFN-α has been shown to increase levels of p21 which in turn leads to cell cycle arrest (23). The ordered degradation of CDK inhibitors is required for cell cycle progression in normal and malignant cells. Following treatment with bortezomib, the proteasome is inhibited thereby disrupting the necessary degradation of p21 and leading to cell cycle arrest. There was a modest increase observed in the level of p21 protein when melanoma cells were treated with the combination of bortezomib and IFN-α as compared to either agent alone.

In addition to its immune-enhancing effects, IFN-α is also known to act on the tumor cell to decrease cell proliferation and promote apoptosis (2). These processes are mediated in part through the transcription and translation of IFN-α stimulated genes. In the present study, we have shown that both pre-treatment with bortezomib and co-treatment with bortezomib and IFN-α increases the expression of IFN-α-stimulated genes. These data suggest that inhibition of the proteasome via treatment with bortezomib may enhance the sensitivity of melanoma cells to the direct effects of IFN-α. IFN-α is one of the few treatment options available to those afflicted with malignant melanoma and these data show that there may be ways to enhance the activity of this compound via proteasome inhibition with bortezomib.

We have demonstrated that bortezomib and IFN-α can effectively induce apoptosis in human melanoma cell lines. This treatment combination is currently being tested in a Phase I Clinical Trial at The Ohio State University Medical Center (OSU 04105; PI: W.E. Carson). Recently published research indicates that this drug combination may also be used to combat bladder cancer as well (27). Further research is necessary to fully elucidate the mechanism by which bortezomib and IFN-α work together to induce apoptosis in tumor cells. Establishing this mechanism is of particular importance since it may provide novel targets for cancer prevention and therapy.

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Figure Legends

Figure 1: *Combined treatment of melanoma cell lines with bortezomib and IFN-α induces apoptotic cell death.* (A-C) Apoptosis was measured by flow cytometry following a 48 hour treatment with PBS, bortezomib (10nM), IFN-α (10^4 U/mL), or both agents combined. Data in each dot plot were derived from a minimum of 10,000 events. Annexin-V staining is depicted on the x-axis while PI staining is shown on the y-axis. The percentage of Annexin-V positive cells is included on each dot plot for comparison.

Figure 2: *Combined treatment of melanoma cell lines with bortezomib and IFN-α induces cleavage of caspase-3.* Human melanoma cells (1259 MEL) were treated for 48 hours with PBS, bortezomib (10-20nM), IFN-α (10^4 U/mL), or both agents combined. Cell lysates were analyzed by immunoblot analysis for caspase-3 protein or its cleaved forms (arrow). Membranes were also probed with an anti-β-actin Ab to control for equal loading. Similar results were observed in the A375, 1259 MEL and 18105 MEL human melanoma cell lines. B = Bortezomib.

Figure 3: *Combined treatment of melanoma cell lines with bortezomib and IFN-α induces cleavage of PARP.* Human melanoma cell lines (1259 MEL and 18105 MEL) were treated for 48 hours with PBS, bortezomib (10-20nM), IFN-α (10^4 U/mL), or both agents combined. Cell lysates were analyzed by immunoblot analysis for PARP or its cleaved form (arrows). Membranes were also probed with an anti-β-actin Ab to control for equal loading. Similar results were observed in A375 human melanoma cell line. B = Bortezomib.

Figure 4: *Combined treatment of melanoma cell lines with bortezomib and IFN-α leads to decreased levels of Bcl-2.* Immunoblot analysis of Bcl-2, expression in human A375 melanoma cells following treatment with PBS, IFN-α (10^4 U/mL), bortezomib (10nM) or both agents combined for 48 hours. Membranes were also probed with an anti-β-actin Ab to control for equal loading. Similar results were also observed in HT144 melanoma cells (data not shown).

Figure 5: *Combined treatment of melanoma cell lines with bortezomib alone or bortezomib and IFN-α leads to increased p21.* Immunoblot analysis of p21 expression in human 1259 MEL melanoma cells following treatment with PBS, IFN-α, bortezomib (10nM; B) or both agents combined for 48 hours. Membranes were also probed with an anti-β-actin Ab to control for equal loading. Similar results were also observed in HT144 and A375 human melanoma cells (data not shown).

Figure 6: *Combined treatment of melanoma cell lines with bortezomib and IFN-α induces increased expression of the IFN-α responsive gene IFIT2.* A) Human melanoma cells were treated with PBS, IFN-α (10^4 U/mL), bortezomib (10-20nM), or both agents combined. RNA was isolated, converted to cDNA via RT-PCR, and then analyzed using Real-Time PCR. All gene expression levels were normalized to β-actin expression, and expressed as fold-increase versus PBS treated cells. Similar results were observed following A) an 18 hour pre-treatment with bortezomib followed by a 4 hour stimulation with IFN-α in the A375 cell line, B) an 18 hour co-treatment with bortezomib and IFN-α in the A375 cell line, and C) a 24 hour co-treatment with bortezomib and IFN-α in the 18105 MEL cell line.

Fig. 1

Figure 2

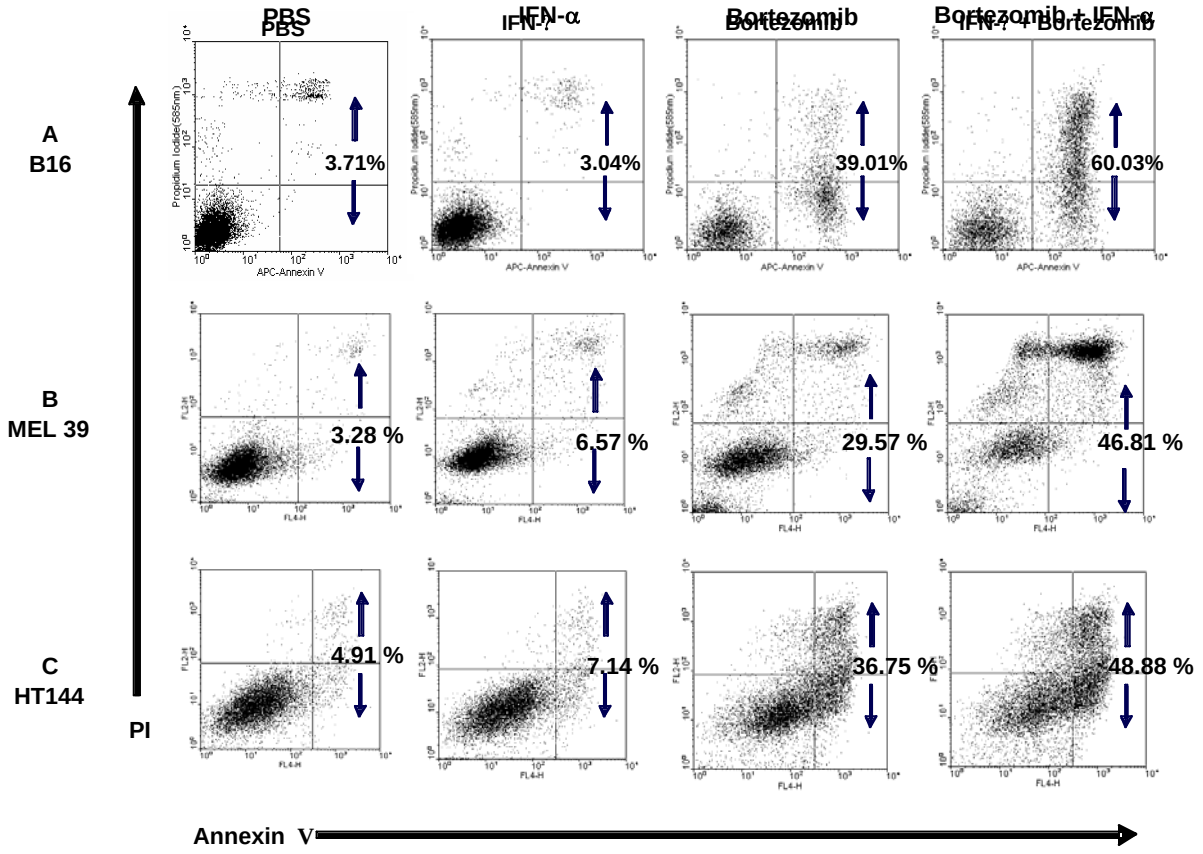
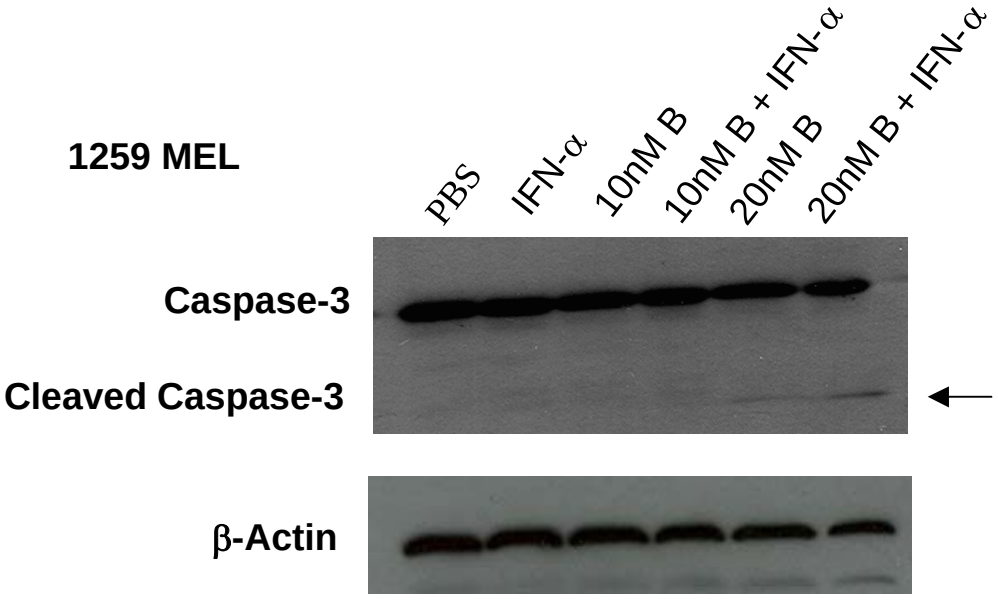


Fig. 2


1259 MEL
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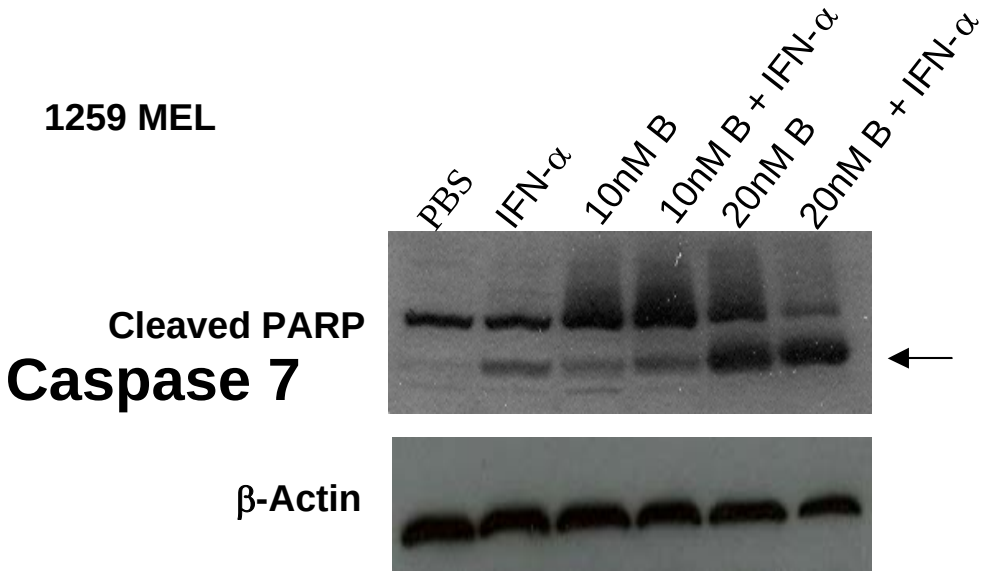
10nM B
10nM B + IFN-α
20nM B
20nM B + IFN-α
Caspase-3
Cleaved Caspase-3
β-Actin

Fig. 3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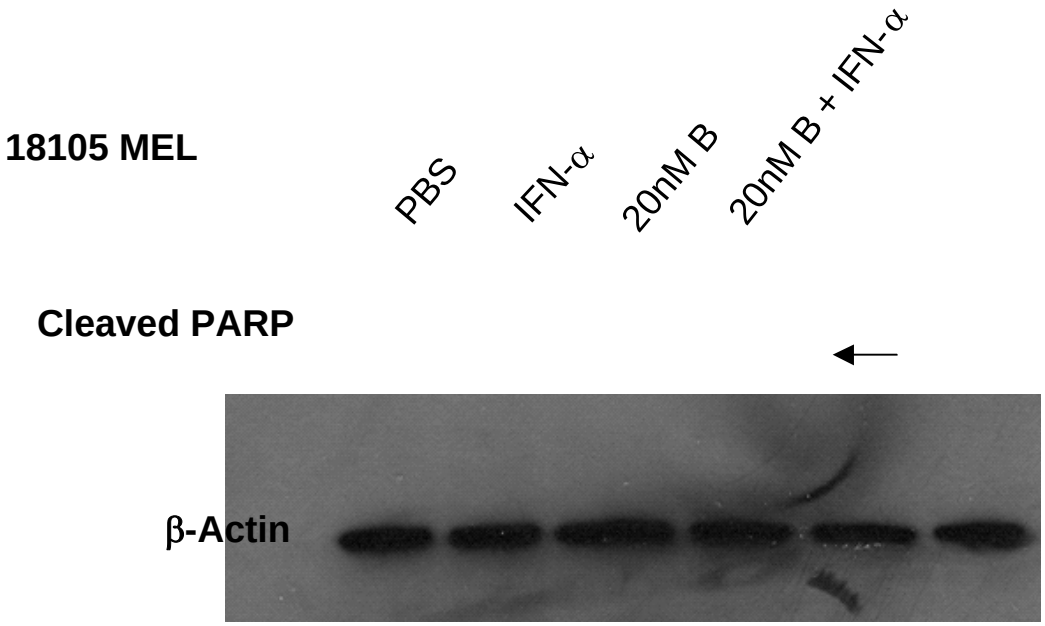
Cleaved PARP
Caspase 7
β-Actin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20nM B
20nM B + IFN-α
Cleaved PARP
β-Actin

Fig. 4

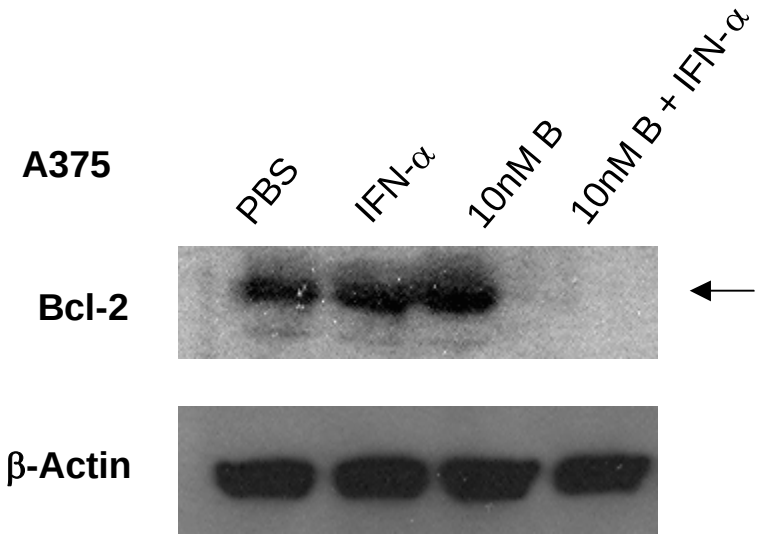


Fig. 5

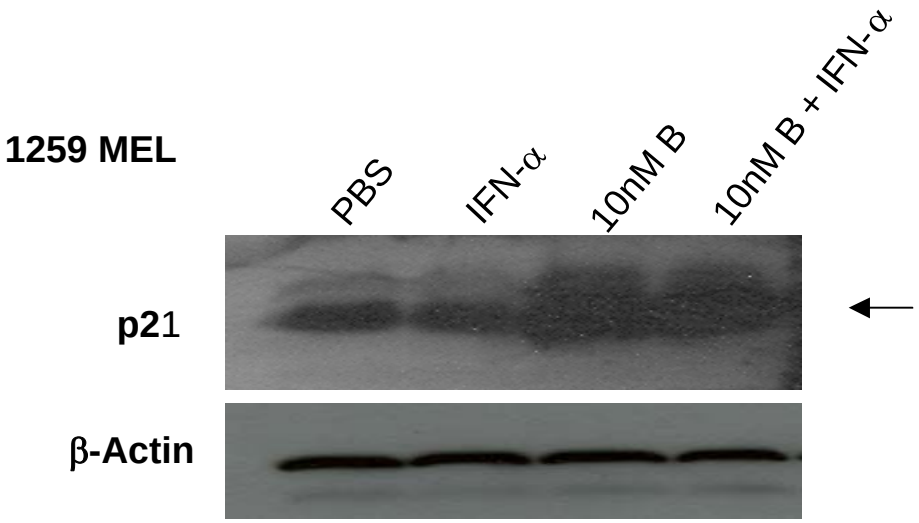
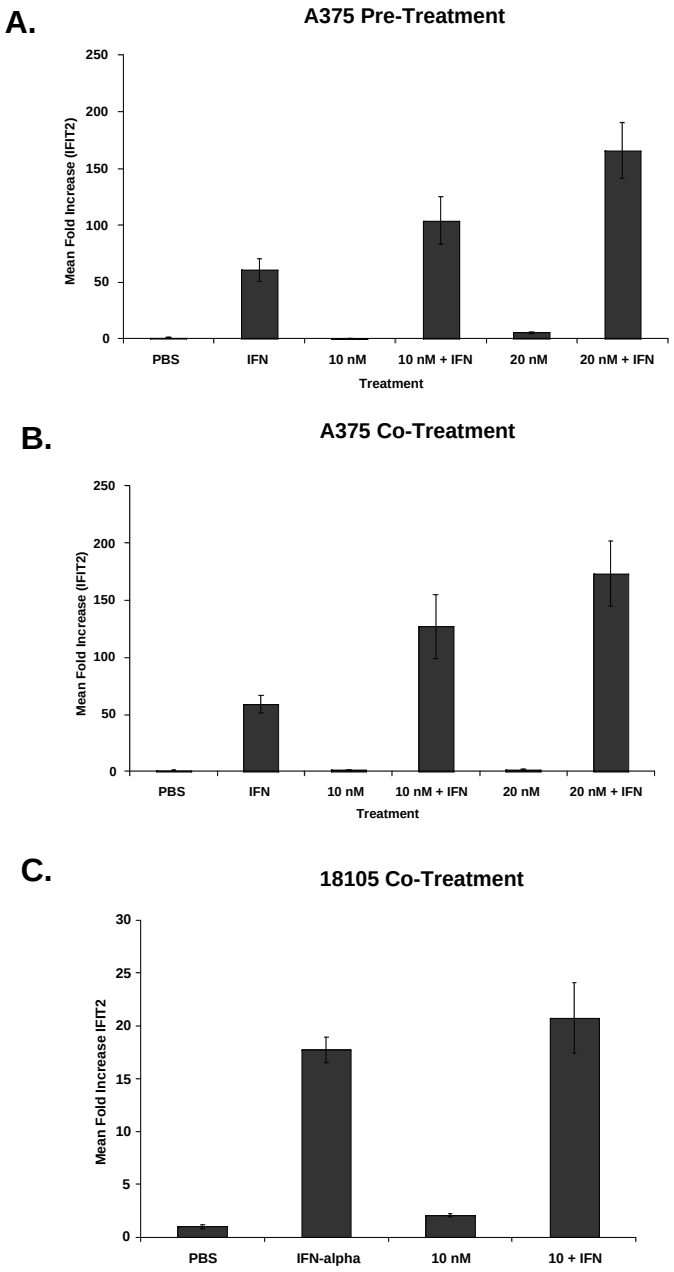


Fig. 6


A.
A375 Pre-Treatment
Mean Fold Increase (IFIT2)
250
200
150
100
50
0
PBS
IFN
10 nM
10 nM + IFN
20 nM
20 nM + IFN
Treatment
B.
A375 Co-Treatment
Mean Fold Increase (IFIT2)
250
200
150
100
50
0
PBS
IFN
10 nM
10 nM + IFN
20 nM
20 nM + IFN
Treatment
C.
18105 Co-Treatment
Mean Fold Increase IFIT2
30
25
20
15
10
5
0
PBS
IFN-alpha
10 nM
10 + IFN