**SYNERGY OF INTERFERONS AND BORTEZOMIB: ADVANTAGES OF COMBINATION TREATMENTS IN FACILITATING APOPTOSIS IN MULTIPLE MYELOMA CELLS.**

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**ABSTRACT**

Intracellular (IFN-γ) and the proliferation inhibitor bortezomib have both been studied as a means to enhance the treatment of radiation-refractory multiple myeloma. Current radiation treatment modalities are unable to control the expansion of thousands of genomes in multiple myeloma. Bortezomib is a reversible 26S proteasome inhibitor that allows multiple targets to be selected independently in tumor cells and has demonstrated low toxicity in patients. Combination therapy in myeloma is a widely researched treatment methodology that has led to improved patient outcomes. The cell cycle analysis. At the indicated time points cells were washed with PBS and Annexin V binding buffer (BD Biosciences, San Jose, CA). Flow cytometry revealed increased hypodiploid population in the combination of the individual therapies thus demonstrating increased efficacy compared to levels at day 6. Combination of each IFN and bortezomib further decreased the IC50 values in Figure 3) for two days.

**MATERIAL AND METHODS**

**Human multiple myeloma U266 and cell line.** The cell line was obtained from the American Type Culture Collection (Manassas, VA). U266 cells were treated as described for the cell viability assay. U266 cells were seeded in 96-well plates at 1 x 10^5 cells/well in triplicate wells in 200 µl of RPMI media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and incubated under a fully humidified atmosphere of 95% air-5% CO2. Human multiple myeloma U266 cell line was obtained from the American Type Culture Collection.

**Three-color flow cytometry.** Three-color flow cytometry was performed using the CellQuest (BD Biosciences) and a FACSCalibur flow cytometer (BD Biosciences). The flow cytometric analysis was performed using FlowJo software (TreeStar, Ashland, OR). The effectiveness of each treatment varied in a concentration and time-dependent manner.

**Caspase 3/7 activity assay.** Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay kit (Promega, Madison, WI) as described in the manufacturer’s instructions. Absorbance at 450 nm was recorded and results were expressed as a percentage of untreated cells.

**Cell viability assay.** Cell viability was assessed by the ability to exclude trypan-blue (0.5% serum (FBS) and incubated under a fully humidified atmosphere of 95% air-5% CO2). Human multiple myeloma U266 cell line was obtained from the American Type Culture Collection. Growth inhibition effects of IFN and bortezomib alone or in combination suggested synergistic activity via the effectors caspase 3/7 activation leading to cell death [1]. IFN-γ (10 ng/ml) and bortezomib (2.5 nM) alone or in combination for two days.

**Results.** We have determined the individual antiproliferative (AP) activities of Interferon-Alpha (IFN-α) and Interferon-Gamma (IFN-γ) for the following time points: 24, 48, and 72 hours. IFNs and bortezomib were found to have a synergistic effect in combination.

**Conclusion.** Our results demonstrate differential efficiency of selected IFN species to increase antitumor activity of bortezomib in multiple myeloma cells. This program is based upon the Chou-Talalay method [1,2]. A synergy is defined when CI is less than 1.0, additive effect when CI equals 1.0, and antagonistic effect when CI is greater than 1.0. A three-color flow cytometric analysis was performed using the CellQuest program (BD Biosciences). The effectiveness of each treatment varied in a concentration and time-dependent manner.

**Figure 4.** Medium-effect analysis reveals concentration-dependent growth inhibition of IFNs and bortezomib antiproliferative treatment. Cells were treated with indicated interferon concentrations (ng/ml) of Type I and II IFN in a single agent and in combination with 2.5 nM bortezomib for four days. Combination index (CI) values below 1.5 indicate strong synergy.

**Figure 5.** Enhanced apoptosis of U266 cells treated with IFN and bortezomib combination is mediated by activation of caspase 3/7 in a concentration-dependent manner. Cells were treated with indicated interferon concentrations (ng/ml) alone or in combination with 2.5 nM bortezomib for four days. Combination index (CI) values below 1.5 indicate strong synergy.

**Figure 6.** Time and concentration-dependent survival of U266 in bortezomib as single agent. IC50 values are depicted in (A) bortezomib at the corresponding day of treatment.

**Figure 7.** Concentration between caspase 3/7 and cytotoxicity level activity in U266 cells treated with IFN (10 ng/ml) and bortezomib (2.5 nM) alone or in combination for two days.

**Figure 8.** Apoptotic quantification by Annexin V-FITC binding analysis of U266 cells treated with IFN (10 ng/ml) and bortezomib (2.5 nM) for four days. Treatment of the cell line with different drug agents are present in figures of all conditions (100,000 cells); see color code and table below.

**Figure 9.** Caspase 3/7 activity analysis of cells exposed to single agents and combination treatment. We have determined the individual antiproliferative (AP) activities of selected IFN species to increase antitumor activity of bortezomib in multiple myeloma cells. This program is based upon the Chou-Talalay method [1,2]. A synergy is defined when CI is less than 1.0, additive effect when CI equals 1.0, and antagonistic effect when CI is greater than 1.0. A three-color flow cytometric analysis was performed using the CellQuest program (BD Biosciences). The effectiveness of each treatment varied in a concentration and time-dependent manner.

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