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

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ABSTRACT

Interferon-Alpha (IFN-α) and the proteasome inhibitor bortezomib are both clinically indicated for the treatment of specific hematological cancers. Interferons function by controlling the expression of hundreds of genes in multiple pathways. Bortezomib is a reversible 26S proteasome inhibitor that affects multiple signaling pathways selectively in tumor cells and has demonstrated low toxicity in patients. Combination therapy in oncology is a widely practiced treatment methodology that can lead to improved clinical outcomes. We have determined the individual antiproliferative (AP) activities of bortezomib, Type I IFNs (α, β, ω) and Type II IFN (IFN-γ) in the U266 human multiple myeloma cell line by colorimetric and luminescent cell viability assays. The individual effectiveness of each treatment varied in a concentration and time-dependent manner. The IC_{50} for bortezomib at day 1 (6.8 nM) dropped to 2.3 nM at day 6. The IC_{50} for Type I IFNs were at ng/ml level at day 2 and decreased to pg/ml levels at day 6. Combination of each IFN and bortezomib further decreased the IC_{50} of the individual therapies thus demonstrating increased efficacy compared to individual treatment AP effects. Cell cycle analysis by propidium iodide (PI) staining flow cytometry revealed increased hypodiploid population in the combination treatment. Functional studies revealed a synergy of growth inhibition due to increased apoptosis (Annexin V/PI staining) and caspase 3/7 activation. Overall, Type I IFNs (α, β, ω) displayed greater synergy indices (CI 0.3-0.5) than IFN-γ (CI 0.6-0.7) in both growth inhibitory and apoptotic assays.

MATERIALS AND METHODS

Cell culture. Human multiple myeloma U266 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD), maintained in RPMI-1640 medium (ATCC) supplemented with 15% fetal bovine serum (FBS) and incubated under a fully humidified atmosphere of 95% air- 5% CO_2 at 37ºC. Exponentially growing cells were used in all experiments. Cell viability was assessed by the ability to exclude trypan-blue (0.5% w/v, Invitrogen) and counted in Countess Cell Counter (Invitrogen). Cells were seeded at 5×10^5 cells/ml for regular maintenance and treatment in specified plate formats.

Interferons. Type I (IFN-α, IFN-β, IFN-ω) and II (IFN-γ) human recombinant IFN proteins were expressed in bacteria and purified at PBL InterferonSource. All IFNs were diluted in tissue culture media immediately before use.
Proteasome inhibitor **bortezomib** (Velcade, PS-341) was purchased from LC laboratories (B-1408), solubilized in DMSO to final 13 mM stock concentration and stored at -20ºC. Working concentrations were prepared by diluting in complete cell growth media supplemented with 0.2% DMSO before cell treatment.

Cell viability assays. Cells were counted and seeded at 5×10^4 cells per well (100µl) in triplicate wells in 96-well microtiter plates, pre-incubated for 4-5 hours and treated with IFN and bortezomib at various concentrations as single agents or in combination. Viable cells were determined by colorimetric tetrasolium reduction (CellTiter 96® Aqueous Non-radioactive Cell Proliferation Assay, Promega, Madison, WI) or luminescent assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega) according to the manufacturer's instructions. Absorbance at 490 nm (colorimetric) or luminescence was recorded in Molecular Devices UVMax or Victor 1420 multi-label plate reader (PerkinElmer), respectively. Viability is presented as percent of treated over the control cells and the IC_{50} concentrations were calculated using GraphPad Prizm software (Version 4.02, 2004).

Median-effect analysis. The interaction between IFN and bortezomib was analyzed using CalcuSyn software program (Biosoft, Cambridge, UK). Data from cell viability assays were expressed as fraction of cells with growth affected (*fa*) in drug-treated versus untreated cells (*fu*). This program is based upon the Chou-Talalay method to determine synergy of two or more drugs by calculating the combination index (CI) value [Chou T, 2008; Chou T, 2010]. A synergy is defined when CI is less then 1.0, additive effect when CI equals 1.0, and antagonism when CI is more than 1.0.

Caspase 3/7 activity assay. 96-well white-wall plates were seeded and treated as described for the cell viability assays. At the indicated time post-treatment 33 µl of cell suspension was added to equal volume caspase 3/7-Glo reagent (Caspase 3/7-Glo Assay, Promega, Madison, WI), plates were incubated in dark for 30 min and luminescence was recorded using Victor 1420 multi-label counter (PerkinElmer, Waltham, MA).

Propidium iodide (PI) staining for cell cycle analysis. PI is a fluorescent nucleic acid binding dye that binds preferentially to double-stranded nucleic acids, allowing fluorescence intensity to be used as an indicator of the cellular DNA content. U266 cells were seeded at 5×10^5 cells/ml in 6-well tissue culture plates (5 ml per well) and incubated for 12-18 hr at 37ºC. After treatment with IFN (10 ng/ml) and/or bortezomib (2.5 nM) for the indicated time periods, cells were fixed in 70% ethanol for 30 min on ice and kept at 4ºC. Cells were washed with PBS, treated with 100 µg/ml RNase A (Sigma, St. Louis, MI), stained with 10 µg/ml PI (Sigma) and flow cytometry was performed using a Beckman Coulter FC500 analyzer.

Flow cytometry analysis for apoptosis quantification. U266 cells were seeded and treated as described for the cell cycle analysis. At the indicated time points cells were washed with PBS and Annexin V binding buffer (eBioscience, San Diego, CA). Cell samples of 100µl were stained with 5µl Annexin V-APC and 10 µg/ml PI, incubated for 15 min in the dark and analyzed with a Beckman Coulter MoFlo XDP Cell Sorter. The degree of Annexin V and PI staining was evaluated and presented as per cent of gated cells (10 000 events).

Figure 1. Time and concentration-dependent sensitivity of U266 to bortezomib as single agent. IC_{50} values are displayed in [nM] bortezomib at the corresponding day of treatment.

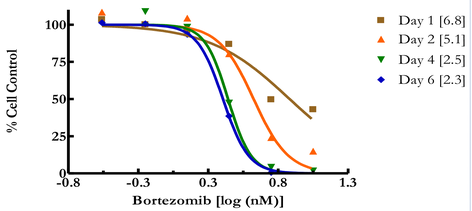


Figure 2. U266 is sensitive to IFN-α2 treatment in a time and concentration-dependent manner. IC_{50} values are shown as [ng/ml] after the corresponding time of treatment.

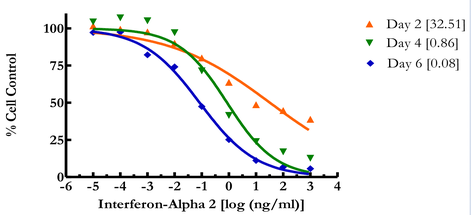
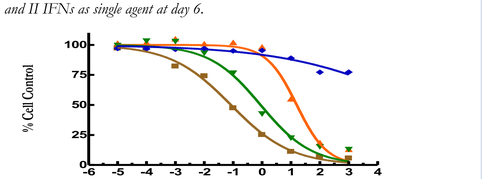


Figure 3. Comparison of U266 sensitivity to increasing concentrations individual Type I and II IFNs as single agent at day 6.



IFN	IC_{50} (ng/ml)
IFN-α2	0.08
IFN-β	15.92
IFN-ω	0.89
IFN-γ	>100

Figure 4. Median-effect analysis reveals concentration-dependent growth inhibition synergy of IFN and bortezomib combination treatment. U266 cells were treated with indicated concentrations (ng/ml) of Type I and II IFN as single agents and in combination with 2.5 nM bortezomib for five days. Combination index (CI) values below 1.0 indicates strong synergy.

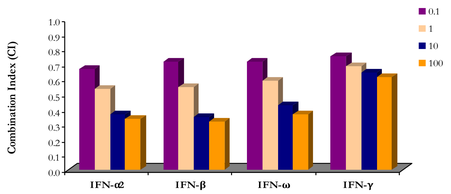


Figure 5. Increased apoptosis of U266 cells treated with IFN and bortezomib combination is mediated by activation of caspase 3/7 in a concentration-dependent manner. Cells were treated with indicated bortezomib concentrations (nM) alone or with individual IFNs (IC_{50} values in Figure 3) for two days.

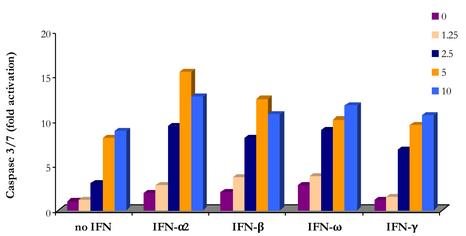


Figure 6. Dose-response of caspase 3/7 activation in U266 cells treated with indicated concentrations (ng/ml) of IFN alone or with 2.5 nM bortezomib for two days.

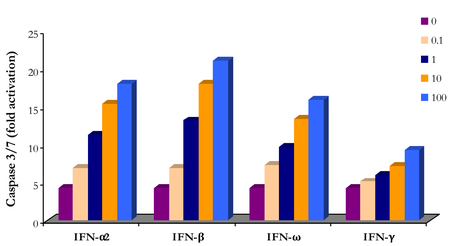


Figure 7. Correlation between caspase 3/7 and cytotoxicity level activation in U266 cells treated with indicated concentrations of IFN-α2 (ng/ml) alone or with 2.5 nM bortezomib for two days.

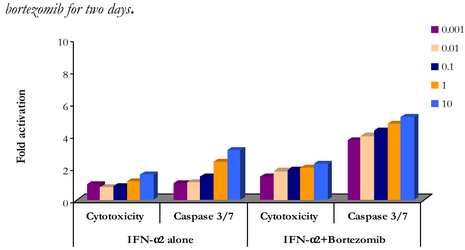
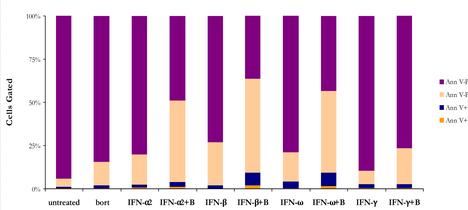


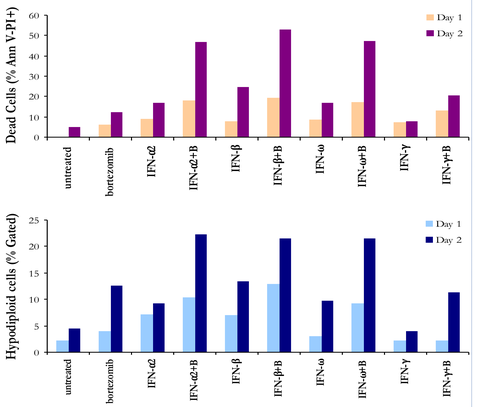
Figure 8. Apoptotic quantification by Annexin V-APC binding analysis of U266 cells treated with IFNs (10 ng/ml) and bortezomib (2.5 nM) for two days. Fractions of the cells in different apoptotic status are presented as percent of all gated cells (10 000 events). See also color code and table below.



Ann V-PI+	Ann V+PI+
dead	late apoptotic
Ann V-PI-	Ann V+PI-
viable	early apoptotic

Untreated	4.80	0.84	Bortezomib	12.50	1.46
	91.72	0.27		78.51	0.40
IFN-α2	17.01	1.74	IFN-α2+B	46.67	2.83
	78.74	0.75		48.35	1.03
IFN-β	24.51	1.78	IFN-β+B	52.83	7.21
	71.11	0.19		35.40	1.87
IFN-ω	16.72	3.78	IFN-ω+b	47.33	7.64
	79.06	0.45		43.46	1.57
IFN-γ	7.86	2.35	IFN-γ+B	20.74	2.28
	89.51	0.29		76.59	0.41

Figure 9. Correlation between the increase of dead cells (Annexin V-PI+ populations) and hypodiploid fractions (PI staining) of U266 treated with IFN (10 ng/ml) and bortezomib (2.5 nM) alone or in combination for one or two days.



CONCLUSIONS

We have analyzed the individual growth inhibition properties of bortezomib, Type I IFNs (α, β, ω) and Type II IFN (γ) in the U266 human multiple myeloma cell line. The effectiveness of each treatment varied in a concentration and time-dependent manner. The IC_{50} for bortezomib at day 1 (6.8 nM) dropped to 2.3 nM at day 6 (**Figure 1**). The IC_{50} for Type I IFNs were at ng/ml level at day 2 which decreased to pg/ml levels at day 6 (**Figure 2, 3**). IFN-γ didn't have AP activity on U266 cells for the concentrations and time points tested (**Figure 3**). Colorimetric and luminescent cell viability assays demonstrated similar result (data not shown).

Combination of each IFN and bortezomib further decreased the IC_{50} of the individual treatments (data not shown) thus demonstrating increased efficacies compared to the single agent treatment growth inhibition effects.

Growth inhibition effects of IFN and bortezomib alone or in combination triggered increased apoptosis via the effectors caspase 3/7 activation leading to cell death [caspase 3/7 activity assessment (**Figure 5, 6, 7**), Annexin V/PI staining (Figure 8, 9) in fresh cells and cell cycle analysis by PI staining (Figure 9) of ethanol fixed cells). In addition, evaluation of IFN and bortezomib cytotoxicity (**Figure 7**, ApoTox-Glo Triplex assay, Promega) revealed correlation with the increase of caspase 3/7 activity and the decreased level of viable cells much better pronounced in the combination treatment again in a concentration and time-dependent manner.

Median-effect analysis (**Figure 4**) demonstrated that Type I IFN displayed greater synergy indices (CI 0.3-0.5) than IFN-γ (CI 0.6-0.7) in growth inhibition assays.

Our results demonstrate differential efficiency of selected IFN species to increase the anti-tumor activity of bortezomib in multiple myeloma cells.