Overcoming Drug-resistance in Multiple Myeloma by CRM1 Inhibitor Combination Therapy

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Abstract

Introduction
Significant progress has been made over the past several years in the treatment of multiple myeloma (MM). However patients eventually develop drug resistance and die from progressive disease. The incurable nature of MM clearly demonstrates the need for novel agents and treatments.
The overall objective of this study was to investigate the use of CRM1 inhibitors (KPT330 and KOS2464) to sensitize de novo and acquired drug-resistant MM cells to the proteosome inhibitors bortezomib (BTZ)and carfilzomib (CFZ) and to the topoisomerase II (topo II) inhibitor doxorubicin (DOX).
Methods
Drug resistant U266 and 8226 MM cell lines were developed at VCU (Steven Grant) and the Moffitt Cancer Center (Ken Shain) respectively by the incremental exposure to BTZ. Cells were treated in vitro with CRM1 inhibitors (KPT330 or KOS2464) +/- either BTZ, CFZ or DOX. Sensitivity was measured by both cell viability (CellTiter-Blue) and apoptosis (activated caspase 3). U266 resistant cells were also used to challenge NOD/SCID-gamma mice which were then treated with combinations of KPT330 and BTZ or DOX. Mononuclear cells were isolated from patients with newly diagnosed and refractory (drug-resistant) MM. Patient bone marrow aspirates were treated with drug combinations (KPT330 or KOS2464 +/- either BTZ, CFZ or DOX) and CD138/light chain positive MM cells assayed for apoptosis.
Results
BTZ selected U266 and 8226 MM cells were assayed and found to be resistant to BTZ, CFZ, and DOX as compared to parental cell lines. Both U266 and 8226 resistant MM cell lines were found to be >10-fold resistant to BTZ. Cell viability was decreased synergistically when the CRM1 inhibitor KPT330 was used in combination with BTZ or DOX. Combinatorial index values were < 1.0 (synergistic) in both parental and drug resistant U266 and 8226 MM cells. Resistant U266 and 8226 MM cell lines were sensitized by the CRM1 inhibitors KPT330 and KOS2464 to both BTZ and CFZ as shown by apoptosis assay. CD138/light chain double-positive MM cells derived from both newly diagnosed and refractory (drug-resistant) MM patients were sensitized by CRM1 inhibitors to BTZ, CFZ and DOX as shown by apoptosis. NOD/SCID-gamma mice treated with CRM1 inhibitors and either BTZ or DOX had a significantly better response to combined treatment than single agents.

Background: Drug Resistance

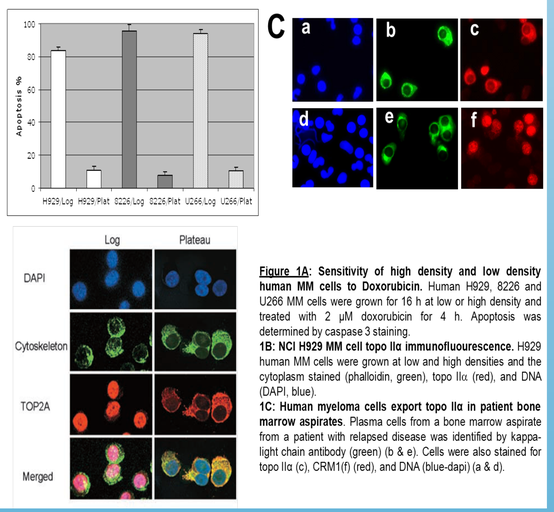

Figure 1A: Sensitivity of high density and low density human MM cells to Doxorubicin. Human H929, 8226 and U266 MM cells were grown for 16 h at low or high density and treated with 2 μM doxorubicin for 4 h. Apoptosis was determined by caspase 3 staining.
1B: NCI H929 MM cell topo IIα immunofluourescence. H929 human MM cells were grown at low and high densities and the cytoplasm stained (phalloidin, green), topo IIα (red), and DNA (DAPI, blue).
1C: Human myeloma cells export topo IIα in patient bone marrow aspirates. Plasma cells from a bone marrow aspirate from a patient with relapsed disease was identified by kappa-light chain antibody (green) (b & e). Cells were also stained for topo IIα (c), CRM1(f) (red), and DNA (blue-dapi) (a & d).

Drug Resistant Myeloma Cell Lines

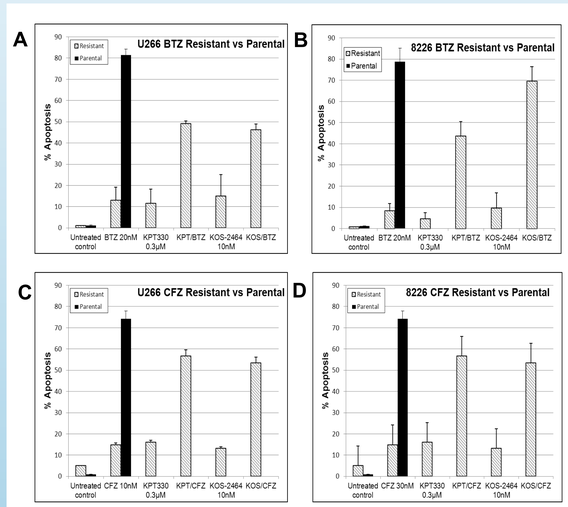

Figure 2: CRM1 inhibition sensitizes drug-resistant human myeloma cell lines to bortezomib. Human U266 (A,C) and 8226 (B,D) drug resistant and parental myeloma cell lines were treated concurrently for 20 hours with KPT-330 (300nM) or KOS-2464 (10nM) with and without bortezomib (A,B) or carfilzomib (C,D) and assayed for apoptosis by flow cytometry (activated caspase 3). Resistant cell lines, U266R and 8226R were up to 10-fold resistant to bortezomib as compared to parental cells. The addition of the CRM1 inhibitors, KPT-330 or KOS-2464 sensitized drug resistant cells to bortezomib and carfilzomib. All cells were grown at log-phase growth conditions (5x10^5 cells/ml).

Viability Assay				
Cell line	**CombiGrp**	**CI(mean)**	**CI(SEM)**	**CI(n)**
RPMI 8226-parental	**KOS-2464 & Bortezomib**	0.992	0.028	3
RPMI 8226-parental	**KPT 330 & Bortezomib**	0.715	0.015	4
RPMI 8226-parental	**KOS-2464 & Carfilzomib**	1.059	0.105	3
RPMI 8226-parental	**KPT 330 & Carfilzomib**	0.633	0.007	3
RPMI 8226-parental	**KOS-2464 & Doxorubicin**	0.914	0.004	3
RPMI 8226-parental	**KPT 330 & Doxorubicin**	0.547	0.014	3
RPMI 8226-BTZ	**KOS-2464 & Bortezomib**	0.351	0.095	3
RPMI 8226-BTZ	**KPT 330 & Bortezomib**	0.417	0.095	4
RPMI 8226-BTZ	**KOS-2464 & Carfilzomib**	0.590	0.043	3
RPMI 8226-BTZ	**KPT 330 & Carfilzomib**	0.680	0.108	3
RPMI 8226-BTZ	**KOS-2464 & Doxorubicin**	0.740	0.048	2
RPMI 8226-BTZ	**KPT 330 & Doxorubicin**	0.521	0.074	2

Figure 3: Cell Viability Assay: KOS-2464 & KPT 330 were found to synergize with BTZ, CFZ and doxorubicin in 8226 parental & BTZ resistant human myeloma cell cultures. Combination index values of log phase cells co-treated 48 hours with KOS or KPT-330 and BTZ, CFZ or DOX. Drug concentration ranges were as follows: KOS-2464 (0.9 nM- 50nM), KPT 330 (0.4 μM -5 μM), doxorubicin (90 nM -5 μM), bortezomib (0.9 nM -100 nM), carfilzomib (0.9 nM -100 nM). CI, combination index. <1 = synergy

In Vivo Mouse Studies

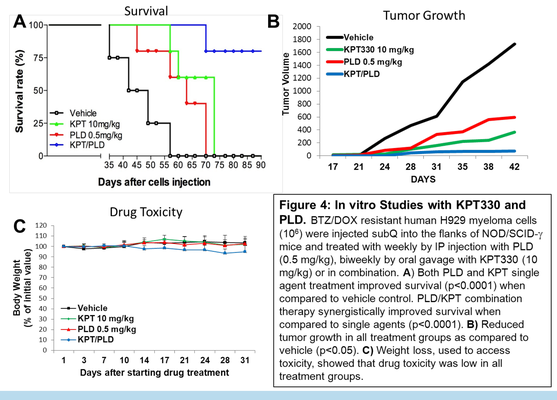

Figure 4: In vitro Studies with KPT330 and PLD. BTZ/DOX resistant human H929 myeloma cells (10^6) were injected subQ into the flanks of NOD/SCID-γ mice and treated with weekly by IP injection with PLD (0.5 mg/kg), biweekly by oral gavage with KPT330 ((10 mg/kg) or in combination. **A**) Both PLD and KPT single agent treatment improved survival (p<0.0001) when compared to vehicle control. PLD/KPT combination therapy synergistically improved survival when compared to single agents (p<0.0001). **B)** Reduced tumor growth in all treatment groups as compared to vehicle (p<0.05). **C)** Weight loss, used to access toxicity, showed that drug toxicity was low in all treatment groups.

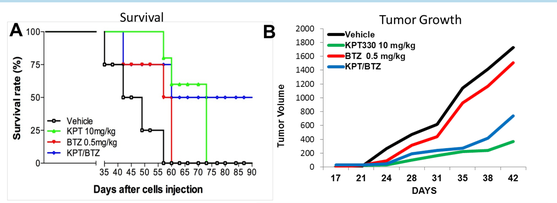

Figure 5: In vitro Studies with KPT330 and BTZ. BTZ/DOX resistant human H929 myeloma cells (10^6) were injected subQ into the flanks of NOD/SCID-γ mice and treated biweekly by IP injection with BTZ (0.5 mg/kg), or oral gavage with KPT330 (10 mg/kg) or in combination. **A**) Both BTZ and KPT single agent treatment improved survival (p<0.0001) when compared to vehicle control. BTZ/KPT combination therapy synergistically improved survival when compared to single agents (p<0.0001). Fifty percent of the BTZ/KPT mice were completely tumor-free. **B)** Tumor growth was reduced in all treatment groups (p<0.05). **C)** Weight loss, used to access toxicity, showed that drug toxicity was low in all treatment groups.

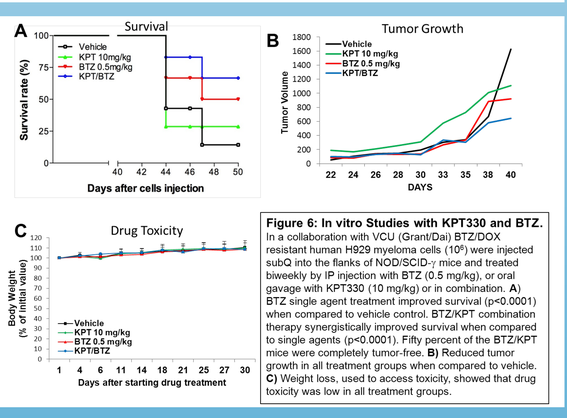

Figure 6: In vitro Studies with KPT330 and BTZ. In a collaboration with VCU (Grant/Dai) BTZ/DOX resistant human H929 myeloma cells (10^6) were injected subQ into the flanks of NOD/SCID-γ mice and treated biweekly by IP injection with BTZ (0.5 mg/kg), or oral gavage with KPT330 (10 mg/kg) or in combination. **A**) BTZ single agent treatment improved survival (p<0.0001) when compared to vehicle control. BTZ/KPT combination therapy synergistically improved survival when compared to single agents (p<0.0001). Fifty percent of the BTZ/KPT mice were completely tumor-free. **B)** Reduced tumor growth in all treatment groups when compared to vehicle. **C)** Weight loss, used to access toxicity, showed that drug toxicity was low in all treatment groups.

Ex vivo Apoptosis Assay

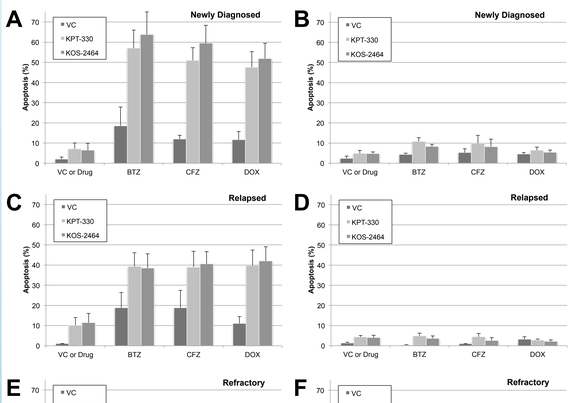

Figure 7. KOS-2464 and KPT330 sensitize newly diagnosed, relapsed and refractory patient myeloma cells to doxorubicin, bortezomib and carfilzomib. Bone marrow mononuclear cells were isolated and treated with KPT-330 or KOS-2464 (300 nM) +/-DOX, CFZ, BTZ or DMSO (VC) for 20 hours. Treated cells were fluorescently labeled with antibodies against activated caspase 3, CD138, and light chain (kappa or lambda). Results are shown in newly diagnosed (A,B) (n=8), relapsed (C,D) (n=5) and refractory (E,F) (n=10) MM patient samples. KPT330 and KOS-2464 sensitized patient myeloma cells when compared to the vehicle control. Non-myeloma CD138/ light-chain double-negative patient cells (B,D,F) were not sensitized by CRM1 inhibitors.

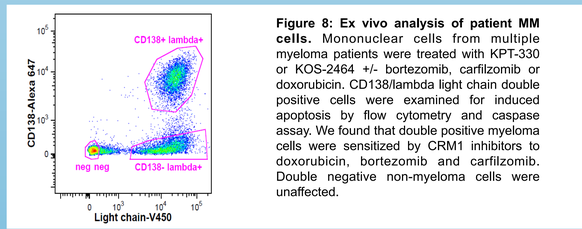

Figure 8: Ex vivo analysis of patient MM cells. Mononuclear cells from multiple myeloma patients were treated with KPT-330 or KOS-2464 +/- bortezomib, carfilzomib or doxorubicin. CD138/lambda light chain double positive cells were examined for induced apoptosis by flow cytometry and caspase assay. We found that double positive myeloma cells were sensitized by CRM1 inhibitors to doxorubicin, bortezomib and carfilzomib. Double negative non-myeloma cells were unaffected.

Conclusions

- CRM1 inhibitors KPT-330 and KOS-2464 sensitized drug-resistant multiple myeloma to the proteasome inhibitors (BTZ and CFZ) and the topo II inhibitor DOX.
- Studies were performed in BTZ resistant MM cell cultures, BTZ resistant MM animal models and *ex vivo* CD138+light chain+ patient MM cells including newly diagnosed, relapsed and refractory patients.
- These combination therapies may be effective for the treatment of refractory multiple myeloma.