Synthetic lethal targeting of *PSMC2*-dependent p97-proteasome complexation overcomes chemoresistance in multiple myeloma

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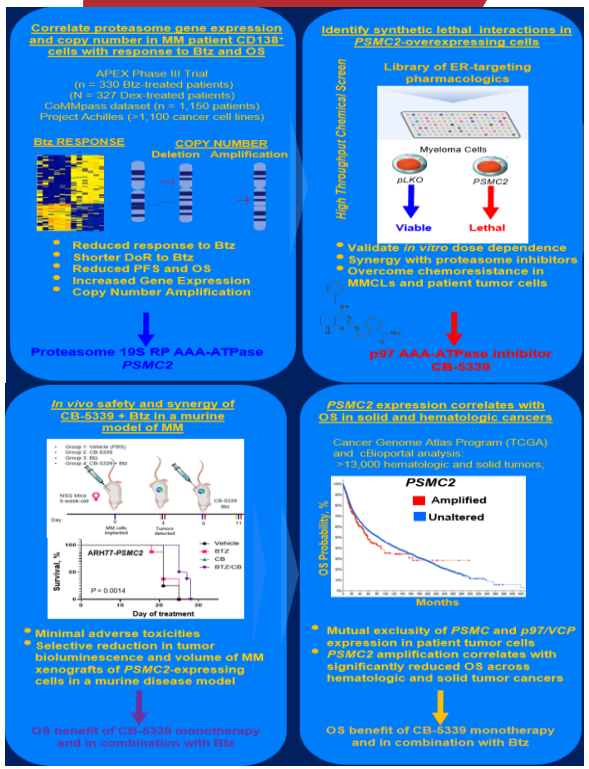
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INTRODUCTION

- Multiple myeloma (MM) remains an incurable plasma cell malignancy that produces large amounts of protein, making it exquisitely sensitive agents that disrupt proteostasis.
- The 26S proteasome is a 2.5 MegaDalton multisubunit proteolytic machine that is composed of a 20S catalytic core particle capped at either or both ends by 19S regulatory particles.
- Proteasome inhibitors (PI), e.g., bortezomib (BTZ), primarily bind the PSMB5 subunit that has chymotrypsin-like peptidase activity. Bortezomib yields clinical responses alone and in multidrug regimens, but drug resistance and relapse eventually occur.
- We hypothesized that adaptations within the ubiquitin proteasome system may decrease PI sensitivity of MM cells but also reveal actionable targets that sensitize BTZ-resistant cells to novel therapies.

METHODS



RESULTS

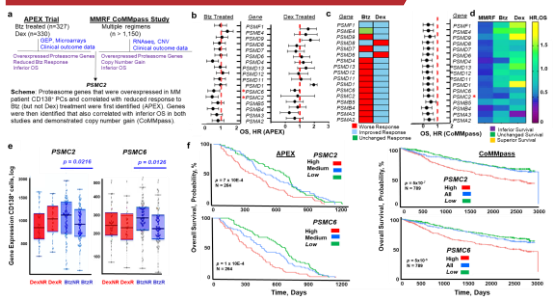


Fig 1. Proteasome gene expression correlates with patient response to Btz and OS. a. Schema for bioinformatic analysis of the APEX and CoMMpass study datasets to identify proteasomal genes that correlated with BTZ-resistance and inferior OS. **b.** Univariate Cox proportional hazard analysis of the APEX dataset was performed to identify proteasome genes that correlated with lack of response to Btz or Dex and also correlated with clinical outcome. OS of patients in the upper tertile of expression was compared to the OS of patients in the lower tertile of expression in patients treated with either Btz or Dex monotherapy. **c.** Univariate Cox proportional hazard analysis of the CoMMpass dataset to correlate proteasome gene expression with outcome. **d.** Heat map of proteasome gene e **e.** Box and whisker plot for *PSMC2* and *PSMC6* RNA expression levels stratified by treatment with either Btz or Dex and response status (NR = non-responder, R = responder). Btz R (n = 55) on average exhibited lower levels of *PSMC2* and *PSM6* compared to Btz NR (n= 61). Data was analyzed utilizing a two-tailed student's t-test with *p*-values of 0.0216 and 0.0126, respectively. **f.** KM analysis of APEX patients stratified by low, medium, and high tertiles of *PSMC2* and *PSMC6* expression. Analysis of the CoMMpass IA22 patient database revealed worse OS in patients in the high tertile of *PSMC2* and *PSMC6* expression than those in the low tertile *p*-value = $5x10^{-7}$ and $5x10^{-6}$ respectively.

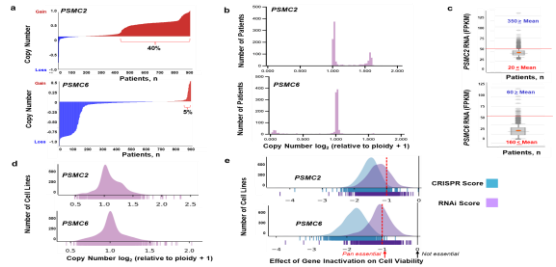


Fig 2. *PSMC2* copy number alteration and RNA upregulation in MM patients. a. Waterfall plot to represent the magnitude of *PSMC2* and *PSMC6* copy number alteration in CD138+ cells from patients enrolled on the CoMMpass study (n=1077). Increased *PSMC* copy nuumber (red) and patients with decreased *PSMC* copy number are indicated in blue (X-axis). **b.** Number of patients (Y-axis) vs. copy number $\log_2$ (relative to ploidy + 1) for *PSMC2* or *PSMC6* using the CoMMpass dataset. **c.** Box and whisker plots of RNA expression levels of *PSMC2* and *PSMC6* from CoMMpass. ~350 patients exhibited RNA levels > the mean for *PSMC2* (n = 420) while 60 patients exhibit *PSMC6* levels > the mean (n = 220). **d.** *PSMC2* and *PSMC6* DNA CN in cell lines interrogated using the DEPMAP Portal. DEPMAP portal's relative copy number pipeline varies by cell line using Sanger whole exome sequencing (WES) data, and 700 cell lines. Data is graphed as $\log_2$ (n+1) ploidy. **e. S**ensitivity of cell lines to *PSMC2* perturbation. 1150 cell lines underwent *CRISPR*-based *PSMC2* KO and 708 cell lines underwent RNAi-based *PSMC2* knockdown, respectively. A score of 0 is the median for non-essential genes, while a score of -1 is the median for essential genes.

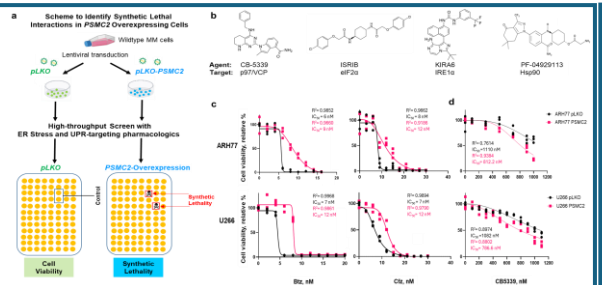


Fig 3. *PSMC2* overexpression sensitizes MMCLs to ER stress inhibition. a. Scheme for the high throughput pharmacologic screen to detect synthetic lethal interactions in *PSMC2*-overexpressing cells compared to cells expressing the empty vector control *pLKO*. MMCLs were transduced with either *pLKO* or *PSMC2-FLAG* inserted within *pLKO* and challenged with a library of 200 small molecules that target effectors of the ER stress response. After three days of treatment, relative cell viability was assayed using the XTT assay. Hits were identified as compounds that increased cell killing in *PSMC2*-overexpressing cells two-fold greater than that measured in *pLKO*-expressing cells. **b.** Chemical structures of the top four HTS hits identified: CB-5339 (p97 inhibitor), ISRIB (EIF2α inhibitor), KIRA6 (IRE1α inhibitor) and PF04929113 (HSP90 inhibitor). **c.** *pLKO* or *PSMC2*-transduced MM cells were treated with increasing concentrations of Btz or Cfz, and viability was measured using the XTT assay. Results are expressed as relative absorbance compared to untreated cells with n=3 in all cases. Data was analyzed using nonlinear regression analysis in Graphpad. R^2 and LD_{50} values are reported. **d.** *pLKO* or *PSMC2*-transduced MM cells were treated with CB-5339 for three days before viability was measured using the XTT assay. Results are expressed as relative absorbance compared to untreated cells with n=3 in all cases. Data was analyzed using nonlinear regression analysis in Graphpad. R^2 and LD_{50} values are reported.

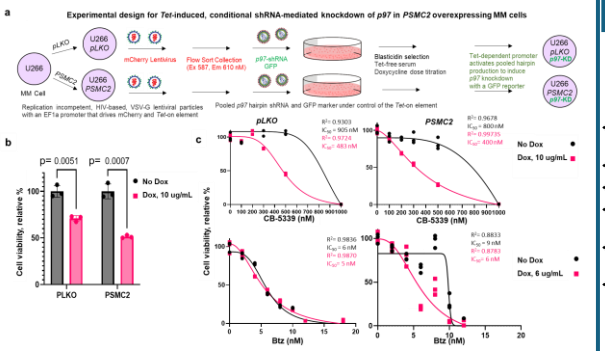


Fig 4. Conditional KD of *p97* sensitizes *PSMC2*-overexpressing MM cells to Btz and CB-5339. a. Schema of conditional *p97* knockdown system in MM cells. *pLKO* or *PSMC2-FLAG* expressing U266 cells were sequentially transduced to stably express a Dox-responsive transcription cofactor and selected by mCherry flow sorting. mCherry positive cells were then transduced with three shRNAs to knockdown *p97* and expressed a GFP marker under control of a third generation, blasticidin-selectable, *Tet-On* promoter. Representative flow cytometry and fluorescent microscopy data are provided (Fig. S6). **b.** U266-pLKO-Cherry and U266-PSMC2-Cherry were pretreated with 10μg/ml Dox and analyzed by XTT assay for relative viability. Viability of *pLKO* cells decreased by ~30%, while *PSMC2* viability decreased ~50% after Dox treatment. Differences between Dox and no Dox treatment mean values were compared by Sidak's multiple comparison testing (n=3 with exact *p*-values given). **c**. U266-pLKO-Cherry and U266-PSMC2-Cherry cells were pretreated with 10μg/ml Dox for three days, at which point an equal number of cells were removed and treated with increasing doses of CB-5339 for three days in triplicate prior to measuring cell viability using the XTT assay. A nonlinear curve was extrapolated with R^2 and LD_{50} values reported.

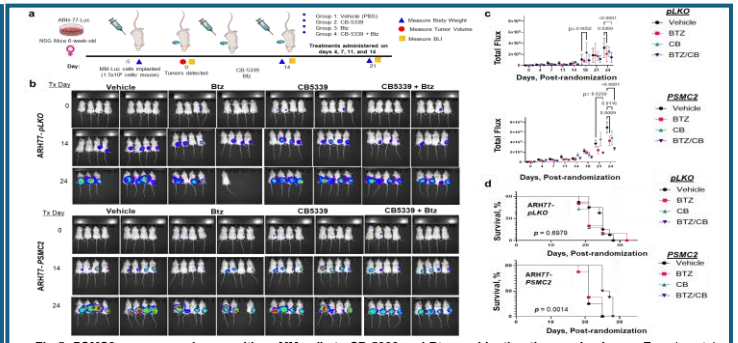


Fig 5. *PSMC2* overexpression sensitizes MM cells to CB-5339 and Btz combination therapy *in vivo*. a. Experimental scheme: 32 female NSG mice ≥ 6 weeks of age were injected with $1.5x10^6$ ARH77-*pLKO-LUC* or ARH77-*PSMC2-LUC* cells subcutaneously (sc). Five days after injection, mice were injected with luciferin SC. Ten minutes later, total photon flux was quantified using the Spectrum IVIS with a whole mouse gate using LivingImage software. Mice were randomized into four groups, with eight mice/group. Two mice in the pLKO Btz group and one in the CB-5339 group did not develop tumors and were excluded from analysis, thus n=6 and n=7 for these groups, respectively. Groups were treated with either 0.5 mg/kg Btz sc, 45 mg/kg CB-5339 by oral gavage or combination therapy 2X weekly for 2 weeks. Mice were monitored by 2X weekly for tumor size by BLI and for morbidity. **b.** Normalized total photon flux was analyzed at the start of therapy (t=0), and at 14 and 24 days. **c.** Average photon flux/treatment group plotted over time. Dunnett's multi-comparison analysis compared total flux between treatment groups with exact p-values given in the figure. n=8 for each group except n=6 for pLKO Btz and n=7 for pLKO-CB-5339. **d**. Kaplan-Meier OS analysis of ARH77-*pLKO-LUC* and ARH77-*PSMC2-LUC* injected mice treated with vehicle, 0.5 mg/kg Btz 2X weekly for 2 weeks, 45 mg/kg CB-5339 2X weekly for 2 weeks or combination treatment. Median OS improved in ARH77-*PSMC2-LUC* mice from 21 days in vehicle to 24 days in the combination treatment. OS was analyzed by log-rank test, revealing a statistically significant difference between ARH77-*PSMC2-LUC* treatments (p = 0.0014).

CONCLUSIONS

- Transcriptomic analysis of MM patient tumor cells reveals that *PSMC2* expression correlates with OS in both the APEX and CoMMpass datasets.
- *PSMC2* overexpression increases resistance to BTZ and mitigates ER stress in MM cells.
- HTS reveal that *PSMC2*-overexpressing cells exhibit synthetic lethality to the p97 ATP competitive inhibitor CB-5339.
- BTZ and CB-5339 overcome BTZ resistance and synergize *ex vivo* to kill CD138+ cells isolated from MM patients and *in vivo* to prolong survival in a murine MM model.
- Finally, affinity purification and analytic size exclusion chromatography have revealed that PSMC2 is required for the binding of p97 to proteasomes forming structures distinct from canonical 26S proteasomes that confer BTZ-resistance.
- Taken together,PSMC2 overexpression is both prognostic and predictive, potentially identifying patients uniquely sensitivity to regimens targeting the proteasome and p97.

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