Profiling Bortezomib Resistance Identifies Secondary Therapies in a Mouse Myeloma Model

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Abstract

Multiple myeloma is a hematologic malignancy characterized by the proliferation of neoplastic plasma cells in the bone marrow. Although the first-to-market proteasome inhibitor bortezomib (Velcade) has been successfully used to treat patients with myeloma, drug resistance remains an emerging problem. In this study, we identify signatures of bortezomib sensitivity and resistance by gene expression profiling (GEP) using pairs of bortezomib-sensitive (BzS) and bortezomib-resistant (BzR) cell lines created from the Bcl-x1/Myc double-transgenic mouse model of multiple myeloma. Notably, these BzR cell lines show cross-resistance to the next-generation proteasome inhibitors, MLN2238 and carfilzomib (Kyprolis) but not to other antimyeloma drugs. We further characterized the response to bortezomib using the Connectivity Map database, revealing a differential response between these cell lines to histone deacetylase (HDAC) inhibitors. Furthermore, in vivo experiments using the HDAC inhibitor panobinostat confirmed that the predicted responder showed increased sensitivity to HDAC inhibitors in the BzR line. These findings show that GEP may be used to document bortezomib resistance in myeloma cells and predict individual sensitivity to other drug classes. Finally, these data reveal complex heterogeneity within multiple myeloma and suggest that resistance to one drug class reprograms resistant clones for increased sensitivity to a distinct class of drugs. This study represents an important next step in translating pharmacogenomic profiling and may be useful for understanding personalized pharmacotherapy for patients with multiple myeloma.

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Introduction

Multiple myeloma is a hematopoietic neoplasm characterized by the proliferation of neoplastic plasma cells in the bone marrow (1). Each year about 22,000 new cases arise in the United States, accounting for approximately 2% of all cancer deaths (2). Standard treatments for patients with multiple myeloma use combination chemotherapies (i.e., alkylating agents and corticosteroids) along with autologous stem cell transplants. However, in the past decade, a number of novel classes of agents have been developed for the treatment of multiple myeloma, including the proteasome inhibitor, bortezomib (Velcade; Millennium Pharmaceuticals, Inc.), which is approved for the treatment of multiple myeloma and relapsed mantle cell lymphoma (3). Despite the initial success of bortezomib therapy, multiple myeloma remains incurable due in part to the emergence of bortezomib-resistant (BzR) cells in the majority of patients (4, 5).

The primary target of bortezomib, the proteasome, is part of the highly regulated ubiquitin–proteasome system (UPS) necessary for intracellular proteolysis. The UPS plays a critical role in cellular homeostasis, cell-cycle progression, and DNA repair (6, 7). The constitutive proteasome, a primary UPS player, is composed of the catalytic 20S core barrel and 19S regulatory caps (together called the 26S proteasome). Bortezomib is a boronic acid dipeptide that is highly selective for inhibition of the chymotryptic activity of the 26S proteasome via reversible binding of its target, PSMB5, a subunit of the 20S catalytic core (8, 9). Bortezomib treatment has been shown to inhibit the transcriptional activity of NF-κB as well as trigger the unfolded protein response (UPR), leading to cell stress and apoptosis (10–12). With the advent of next-generation proteasome inhibitors, it has become
imperative that the bortezomib response and signatures that are associated with bortezomib-resistance can be further defined to identify those patients who (i) will benefit most from proteasome inhibitor treatment, (ii) will show signs of emerging resistance, and (iii) will benefit from selective secondary therapies.

Double-transgenic Bcl-XL/Myc mice develop plasma cell tumors (mean onset of 135 days) with full (100%) penetrance that possess many of the karyotypic, phenotypic, and gene expression features of human multiple myeloma (13, 14). Furthermore, malignant plasma cells can be isolated from these animals, expanded, modified in vitro, and subsequently transferred back into syngeneic mice for drug treatment in the presence of an active immune system, which includes a complete bone marrow microenvironment (13, 14). We have chosen to use this system to model bortezomib response to better understand acquired bortezomib-resistance within malignant plasma cells for potential application to the human multiple myeloma patient population. Although therapeutic response in human multiple myeloma is likely dependent on a combination of tumor genetic variation and inherited population variation, the mouse model provides a common strain background, allowing us to focus on tumor variation and tumor evolution that lead to drug-resistance.

In this study, we have used gene expression profiling (GEP) in the mouse model system to identify bortezomib-responsive genes in vitro. To identify signatures of bortezomib-resistance, we created BzR mouse cell lines and examined their response to bortezomib compared with sensitive controls by GEP, revealing signatures of bortezomib-resistance over the course of bortezomib treatment. Finally, we used the Connectivity Map (CMAP) database to further identify individual differences in bortezomib resistance in our representative bortezomib-sensitive (BzS) and BzR lines, which identified a unique response to histone deacetylase (HDAC) inhibitors, a class of drugs currently in early clinical trials for the treatment of multiple myeloma (15–21).

Materials and Methods

Mouse and human plasma cell lines

The mouse cell lines 595, 589, and 638 were isolated from 3 individual Bcl-XL/Myc double transgenic mice and cultured in Cell Signaling Technologies (CST) media as previously described (14) and cultured for greater than 40 passages before any experimentation, after which no changes in rate of growth, clonal drift, and response to drug or gene expression were observed. The human myeloma cell lines MM1.S and U266 (obtained from American Type Culture Collection) were maintained in human myeloma cell line (HMCL) media: RPMI-1640 (Lonza) supplemented with 15% FBS (Cellgro), 50 U/mL of penicillin and streptomycin (HyClone), and 2 mmol/L L-glutamine (Life Technologies). Cell lines were not authenticated.

Drugs and treatment conditions

Bortezomib (Millennium Pharmaceuticals, Inc.) was dissolved in serum-free RPMI-1640 (Lonza) and stored at −80°C. MLN2238 (Millennium Pharmaceuticals, Inc.), MG-132 (American Peptide Company), trichostatin A (Cell Signaling Technology), and vorinostat (SAHA; LC Laboratories) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), melphalan (Sigma-Aldrich) was dissolved in EtOH, and panobinostat (LC Laboratories) was dissolved in double-distilled water (ddH2O); all drugs were stored at −20°C.

BzR cells were generated by dose escalation of bortezomib over 6 months by once weekly treatment with bortezomib starting at 16 nmol/L; the bortezomib concentration was doubled every 3 weeks until a final growth concentration of 64 nmol/L was reached. Cultures were removed from bortezomib for 14 days or 6 months before analysis and cultured in a manner consistent with the parental lines.

Preparation of RNA for GEP

The mouse cell lines 595 BzS, 595 BzR, 638 BzS, and 638 BzR, removed from bortezomib selection for 14 days, were plated at a density of 4 × 10⁵ cells per mL in CST media with mIL-6. In addition, 595 BzS (595.2), 595 BzR (BzR 595.2), 589 BzS, and 589 BzR, removed from bortezomib selection for 6 months, were plated at a density of 4 × 10⁵ cells per mL in CST media with mIL-6. After 24-hour incubation, 66 nmol/L bortezomib was added to each well, and the cells were collected at 0, 2, 8, 16, and 24 hours after treatment. Similarly, MM1.S and U266 were plated 24 hours before 33 nmol/L bortezomib treatment and cells were collected at 0, 16, and 24 hours. Cells were lysed and total RNA was extracted using QIAshredder and RNeasy RNA purification columns (Qiagen). RNA concentration and integrity were analyzed using the Nanodrop-8000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies). cDNA was prepared and labeled with the Illumina TotalPrep-96 RNA Amplification Kit (Life Technologies). Samples were hybridized to the Illumina MouseWG-6 v2.0 Expression BeadChip according to manufacturer’s protocols and read on the Illumina iScan (Illumina). Microarray data have been made available at the Gene Expression Omnibus (GEO) website (accession no. GSE41930).

Cell viability assay

Cells were seeded at a concentration of 4 × 10⁵ cells per mL. After 24 hours, the cells were treated with the indicated concentrations of drug for 48 hours. Cell viability was measured by CellTiter-Glo Luminescent cell viability assay according to manufacturer’s instructions (Promega) using the Synergy 2 Microplate Reader (Biotek). Values were normalized to untreated controls and IC₅₀ values were estimated by calculating the nonlinear regression using the sigmoidal dose–response equation (variable slope) in GraphPad (Prism). In some experiments, cell
growth was calculated by Trypan blue (Life Technologies) exclusion with a hemocytometer.

**CMAP drug prediction**
Genes were assigned a binary distinction of "up" or "down," respectively, by selecting genes with at least 2-fold higher or lower expression in the BzS cell lines relative to the derived BzR lines at the 24-hour time point (post-bortezomib treatment). These "up" and "down" sets were queried against the CMAP database (22, 23). The given connectivity score estimates the similarity between the input and database expression signatures. A positive score indicates that the input signature is similar to, and a negative score indicates that the input signature is opposite to what would be expected by the predicted compound. Predicted compounds were ranked in ascending order of P value and viable compounds were chosen on the basis of significance of correlation with the input signature (P < 0.05).

**Animal care, tumor injection, and drug treatment**
FVBN/B6 recipient mice were generated as previously described (13, 14). Mice were maintained in a controlled environment receiving food and water *ad libitum*. Fresh, ficolloid (Ficoll-Paque PLUS, GE Healthcare) 595 BzS and BzR cells (1 × 10⁶) suspended in 100 μL of serum-free RPMI-1640 media were injected into 6 mice per group (3 groups/cell line) via the lateral tail vein of age-matched recipient mice. Mice were weighed and treated by intraperitoneal injection twice per week starting 5 days after cell transfer with vehicle (5% dextrose in water), bortezomib (1.2 mg/kg dissolved in 0.9% saline), or panobinostat (10 mg/kg suspended in vehicle) until moribund. Kaplan–Meier curves were generated using GraphPad Prism. Statistical significance was determined using a Student t test. Tumor cell homing was monitored by positron emission tomography (PET) imaging (see Supplementary Methods). All mouse veterinary care, colony maintenance, and PET imaging experiments were carried out in accordance with University of Iowa Institutional Animal Care and Use Committee guidelines and approvals.

**Results**

**Bcl-xL/Myc transgenic mouse plasma cell tumor lines show similar significant shifts in gene expression upon bortezomib treatment as human myeloma**
In this study, 3 representative clonal cell lines isolated from the Bcl-xL/Myc double transgenic mouse model of plasma cell malignancy were used (13, 14) to identify transcriptional responses to bortezomib in BzS and BzR cells *in vitro*. The 595, 589, and 638 plasma cell lines derived from individual mice showed IC₅₀ values within a 22 to 32 nmol/L range to bortezomib by cell viability assay following 48 hours of drug treatment (Supplementary Fig. S1A). Consistent with previous reports (24), bortezomib treatment of these cell lines resulted in programmed cell death as evidenced by caspase-3 cleavage and annexin V-positive/propidium iodide-negative staining by flow cytometry (data not shown). This cytotoxic profile was similar to 2 representative, well-described BzS HMCLs, MM1.S and U266 (Supplementary Fig. S1B; refs. 25–27). Taken together, these data suggest that, such as in human multiple myeloma, the treatment of these mouse cell lines *in vitro* with bortezomib induces a cytotoxic response.

We next asked whether the transcriptional profile induced over time by exposure to bortezomib was similar across both species. The 3 BzS mouse lines (595 shown in duplicate as 595.2) were treated with a sublethal 66 nmol/L dose of bortezomib over a time course of 24 hours and analyzed using GEP. This dose was chosen because it resulted in less than 20% death at 24 hours (Supplementary Fig. S1C) but greater than 50% death at 48 hours (data not shown), suggesting that it was an optimal concentration for collecting kinetic data within a 24-hour time frame. In addition, the HMCLs, U266 and MM1.5, were also treated with a sublethal 33 nmol/L dose (Supplementary Fig. S1D) of bortezomib (equitoxic to the dose used on the mouse cell lines) and analyzed by time course GEP. The variant transcripts identified from these human (genes = 1,421; variance > 0.1) and mouse (genes = 1,021; variance > 0.1) time course data shared 132 genes in common, 58% of which also shared a common, kinetic pattern of response to bortezomib across all cell lines analyzed across both species (Fig. 1B and Supplementary Table S1).

This transcriptional response was further validated using GEP data from a recently published human MMTT3 drug trial by Shaughnessy and colleagues where RNA was collected from newly diagnosed patients with multiple myeloma before and following a single, 48-hour test dose of bortezomib (28). Gene set enrichment analysis (GSEA) of the mouse *in vitro* transcriptional response to bortezomib (24 vs. 0 hours) showed remarkable enrichment [nominal P value (NOM), P < 0.05; false discovery rate (FDR) < 25%] for the Shaughnessy and colleagues 80-gene model. This 80-gene model included those genes that were (i) most changed among all patients by the bortezomib treatment at 48 hours and (ii) were associated with progression-free survival (PFS), of which 46 genes were unique and shared a gene symbol with mouse (Supplementary Fig. S2A; ref. 28). Twenty-nine genes were shown to significantly contribute to the enrichment between the mouse *in vitro* and the human *in vivo* data (Supplementary Fig. S2B) many of which are known components of the proteasome ubiquitination pathway (28), which was found to be enriched in this dataset by Ingenuity Pathway Analysis (IPA). In addition, the kinetic GEP response in both the human and mouse cell lines was enriched for downstream targets of the transcription factor NFE2L2 (NRF2; P < 1 × 10⁻¹⁰; z score > 4; IPA) a transcription factor known to be involved in the oxidative stress response to proteasome inhibition (29). Taken together, these data show a robust transcriptional...
response to bortezomib that is conserved not only between mouse and human plasma cell malignancies, but also within isolated tumor cells in vitro and tumor cells in their native microenvironment in human patients.

**Generation of clonally related bortezomib-sensitive and -resistant cell line pairs**

Because the mouse model provides a drug-naive system with a common strain background and the ability to transfer cells back into syngeneic animals with a competent immune system, we chose to further profile the transcriptional response associated with bortezomib-resistance within the tumor cell using this model system. The 3 BzS mouse cell lines characterized earlier, were dose-escalated with bortezomib over 6 months to obtain resistant (BzR), clonally related (data not shown), daughter cell lines with a 2- to 5-fold increase in IC50 in response to bortezomib (Fig. 2A and Table 1). Doubling rates for the pairs of cell lines were similar (data not shown), suggesting that the increased IC50 in BzR lines is not due to an increase in cell growth. To determine whether these BzR mouse cell lines were capable of maintaining their bortezomib-resistance over time, the cells were removed from drug selection, and bortezomib cell viability assays were conducted after 1 year. The BzR lines maintained their resistant phenotype showing virtually identical IC50 values (data not shown).

Others have reported that bortezomib-resistance is often associated with increases in chymotrypsin-like proteasome activity (30–32). Indeed, we find that 2 representative BzR cell lines have significantly increased chymotrypsin-like activity, whereas trypsin- and caspase-like activity is decreased (Supplementary Fig. S3A and S3B) compared with their BzS counterparts. This observation directly correlated with a similar increase in PSMB5 protein expression by Western blotting in the BzR cells (data not shown) in the absence of Psmb5 mutations by sequencing (data not shown), suggesting that higher baseline proteasome activity (via increased PSMB5 protein expression), not Psmb5 inactivating mutations, likely contribute to the resistance observed in these BzR lines. In addition, these BzR lines showed cross-resistance to the boronic acid next-generation proteasome inhibitor, MLN2238, as well as the epoxyketone next-generation proteasome inhibitor, carfilzomib (Kyprolis). Although the 595 BzR line also showed cross-resistance to the classical aldehyde proteasome inhibitor, MG-132, this line showed increased sensitivity to the multiple myeloma drug, melphalan, whereas the 589 BzR line maintained sensitivity to these compounds (MG-132, melphalan; Table 1; Fig. 1A). Neither the BzS nor the BzR lines responded to drugs known to be ineffective as single agents against multiple myeloma: vincristine, hydroxyurea, and fludarabine (data not shown). These data suggest that not only is the resistance...
observed in the BzR lines specific to bortezomib and its next-generation derivative (MLN2238) and sustained over time but that the resistant phenotype may be overcome with other drugs.

**Bortezomib resistance is associated with changes in transcription that are predictive of patient outcomes**

We were particularly interested in determining the transcriptional differences in gene expression between...
these BzS and BzR cells lines and, thus, examined time courses of response by GEP following the same, sublethal bortezomib treatment in BzR lines (Supplementary Fig. S1E) as used on their BzS counterparts (Fig. 1). To globally visualize these expression patterns, we analyzed the BzR transcriptional response to bortezomib as we had the BzS cell lines described earlier.

First, a pairwise comparison of the BzS and BzR baseline gene expression in the absence of bortezomib treatment was conducted revealing a 51-gene expression signature that statistically distinguished (fold change $> 2$; $P < 0.05$; Student t test) sensitive and resistant lines (Supplementary Fig. S3C). Of these 51 mouse genes, 23 had human homologs. To test the predictive power of this 23-gene model, we queried the gene expression signatures of 210 patients from the MMTT3 human drug trial (28). Unsupervised clustering of these patients based on the 23-gene model identified 2 groups whose PFS and overall survival (OS) were significantly different (log-rank test; Fig. 2B). These results suggest that our in vitro mouse model of bortezomib-resistance has predictive value in human multiple myeloma drug trials, which include bortezomib.

To further define the differences in the transcriptional response to bortezomib in BzS and BzR cell lines, we conducted a combined analysis of the BzS and BzR bortezomib transcriptional profiles identifying 219 genes that changed significantly in both the sensitive or the resistant group in response to drug ($P < 0.001$; [fold change] > 2; pairwise 2 group t test; Supplementary Table S2). Examination of heatmap clusters showed that the majority of the response to bortezomib in the sensitive cell lines was conserved in the resistant cell lines (Fig. 2C and Supplementary Table S2), illustrating a common programmed transcriptional response of cell lines to bortezomib regardless of their overall sensitivity (measured by IC$_{50}$) to the compound. However, 29 genes were differentially responsive to bortezomib in vitro between BzS and BzR lines ($P<0.001$; [fold change] > 2; Fig. 2D). Among these were the upregulation of a cluster of NRF-2–mediated oxidative stress response genes: Hspa1a, Hspa1b, Hspa1l, Hspa1l2, and Ddit3 [also known as CCAAT/enhancer-binding protein homologous protein (CHOP); ref. 29], which has been observed in other BzS human cell lines (12, 29), suggesting that these may serve as a signature of bortezomib-mediated cell death, which is unique to BzS compared with derived BzR cells.

Using CMAP to identify drugs with high correlation to bortezomib-associated death by GEP

Biomarkers that are associated with emerging resistance to bortezomib and yet may define sensitivity to alternative therapies, remain ill defined (33). Although we do see a common gene signature of response to bortezomib and have identified similar biomarkers across BzR cell lines compared with their sensitive counterparts, we have also observed differences in response to secondary therapies across BzR cell lines (Table 1). Indeed, not all patients with bortezomib-refractory multiple myeloma respond similarly to secondary therapies. Given these differences, we aimed to use the individual cell line GEP data described earlier to identify non-proteasome inhibitor drugs that could target BzR cell populations using CMAP (Broad Institute, Boston, MA; ref. 22). The CMAP database contains treatment-induced transcriptional signatures from 1,309 bioactive compounds in 4 human cancer cell lines. An input signature can be used to query the database for correlated drug signatures. To create these input signatures, GSEA was used to identify in individual paired mouse GEPs those genes that were most different in BzS versus BzR lines following drug treatment (24 vs. 0 hour data comparison; [fold change] > 2). These genes were then used to query CMAP to identify drugs that induced expression signatures that were similar (positive correlation) or dissimilar (negative correlation) to the differential expression response of each BzS lines relative to its BzR line when treated with bortezomib. We hypothesized that such drugs, particularly those with positive correlations to the differential response, may have the potential to kill the BzR lines.

The CMAP query produced a number of compounds with signatures that were predicted as significantly correlated (positive) or anticorrelated (negative) with the BzS/BzR differential response (Table 2 and Supplementary Table S3). Broadly, drugs promoting inhibition of the proteasome–ubiquitin pathway, NF-$k$B, HSP90, protein synthesis, and microtubules showed positive correlation,
Interestingly, several HDAC inhibitors (HDACi) were not surprising given the known modes of action for these drugs (Table 2 and Supplementary Table S3). These predictions correlated with the differential responses in all pairs of BzR mouse lines (595 and 589) for bortezomib (34). Interestingly, several HDAC inhibitors were chosen to test 2 representative pairs of BzS and BzR lines would respond differently to HDACi treatment than their BzR counterparts (Fig. 3A–C, bottom). This panobinostat-sensitive phenotype has been observed in an additional BzR mouse cell line (bortezomib IC50: 34 nmol/L (BzS) and 63 nmol/L (BzR); panobinostat IC50: 37 nmol/L (BzS) and 22 nmol/L (BzR)) and indicates that CMAP may have the ability to identify differences in secondary drug response in these cell line models.

**A subset of bortezomib-resistant mouse cell lines has greater in vivo sensitivity to HDAC inhibitors**

It is well documented that the myeloma response to some chemotherapeutic agents is dependent not only on the stromal microenvironment of the bone marrow (37), but also on a complete immune system (38) highlighting the use of our immunocompetent mouse model system. To determine whether the differential in vitro drug responses were maintained in vivo, the 595 BzS and BzR cell lines were adoptively transferred back into syngeneic recipients. The untreated BzS mouse phenotype seemed to be significantly less severe compared with BzR mice, which reached moribundity quickly at a median time of 16 days (P = 0.033).

To better compare the disease burden in these animals, both 2-[18F]fluoro-2-deoxy-glucose (FDG)- and 18-fluoro-2-deoxy-glucose (FDG)- imaging of representative animals from each group showed that the BzS cells home to the bone marrow, whereas the BzR cells are dispersed in moribund animals with fewer characteristic “hot spots” in the long bones (Fig. 4A). Representative histopathologic analyses of bone marrows and soft tissues from these animals showed that the malignant plasma cells were present in the marrows of both animals but were notably absent or minimal in the soft tissues of BzS mice (Supplementary Fig. S4B). Those BzS and BzR cells that home to the bone marrow had similar metabolic activity (FDG-PET); however, BzS cells had significantly higher rates of proliferation (FLT-PET) in vivo even though the BzS and BzR growth rates were similar in vitro (Fig. 4B). Treatment of BzS and BzR mice with bortezomib in vivo (n = 6 mice/group) showed that BzS mice received a significantly greater survival advantage with bortezomib treatment than their BzR counterparts (Fig. 4C) whose survival did not differ from BzR vehicle-treated mice (data not shown) in agreement with our in vitro data (Fig. 2A). This was directly correlated with significantly decreased tumor burden (FDG-PET) in BzS mice treated with bortezomib compared with vehicle (Fig. 4D). These results indicate that myeloma cell homing may play a role in bortezomib sensitivity and that the immunophenotype associated with extramedullary homing may be associated with bortezomib resistance in vivo.

To specifically determine whether 595 BzR mice receive a survival advantage from panobinostat treatment as predicted in vitro, mice were injected with BzR cells and treated with either vehicle (n = 5) or panobinostat (n = 6), and the time to death for each group was compared.

### Table 2. Families of drugs with expression patterns significantly correlated to the bortezomib response in mouse lines using CMAP

<table>
<thead>
<tr>
<th>Drug family</th>
<th>Prediction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>595sp&lt;sup&gt;b&lt;/sup&gt;</th>
<th>638bm</th>
<th>589bm</th>
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<td>Proteasome inhibitors and UPS modulators</td>
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<tr>
<td>CDK/TopoI/TopoII/cell-cycle inhibitors</td>
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<td>+/np</td>
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<td>HDAC inhibitors</td>
<td>+/np</td>
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<sup>a</sup>Significant predictions (P < 0.05) were made for each of the mouse line pairs individually. A positive prediction (+) indicates that the drug family induces a similar expression signature to the BzS signature. A negative prediction (−) indicates that the drug family induces an opposite expression signature to the BzS signature. An “np” indicates that no drug prediction was made.

<sup>b</sup>595 is a combination of the 595 and 595.2 datasets.

...as well as panobinostat (Fig. 3C, top). This was a cytotoxic response as evidenced by CHOP induction (Supplementary Fig. S4A; refs. 12, 34, 36). In contrast, the 589 BzR line (not predicted for enhanced sensitivity to HDACIs by CMAP) showed cross-resistance to all 3 HDACi compounds compared with the BzS line (Fig. 3A–C, bottom). This panobinostat-sensitive phenotype has been observed in an additional BzR mouse cell line (bortezomib IC50: 34 nmol/L (BzS) and 63 nmol/L (BzR); panobinostat IC50: 37 nmol/L (BzS) and 22 nmol/L (BzR)) and indicates that CMAP may have the ability to identify differences in secondary drug response in these cell line models.

whereas drugs that inhibit the cell cycle showed negative correlation with the differential responses in all pairs (Table 2 and Supplementary Table S3). These predictions were not surprising given the known modes of action for bortezomib (34). Interestingly, several HDAC inhibitors (HDACi) were significantly positively correlated with the differential bortezomib response in 1 of 3 pairs of cell lines, 595 (P < 2 × 10⁻⁵; Table 2; Supplementary Table S3), but were not predicted in the other 2 highlighting some heterogeneity among these cell line pairs. In fact, this was the only drug family with consistent drug predictions for each drug across the 3 cell lines queried (Supplementary Table S3). Because HDACIs have been reported as having synergistic effects when combined with other multiple myeloma treatments (34) and are currently in clinical trials for refractory multiple myeloma (34) and are currently in clinical trials for refractory multiple myeloma (34) and are currently in clinical trials for refractory multiple myeloma (34) and are currently in clinical trials for refractory multiple myeloma (34) and are currently in clinical trials for refractory multiple myeloma (34) and are currently in clinical trials for refractory multiple myeloma (34), we chose to evaluate this result further. We hypothesized that the 595 pair of lines would respond differently to HDACi treatment than the other pairs of lines based on the CMAP results.

On the basis of the HDACi prediction, 3 HDAC inhibitors were chosen to test 2 representative pairs of BzS and BzR mouse lines (595 and 589) for in vitro sensitivity. The 595 BzR line showed enhanced sensitivity to the HDACi trichostatin A and vorinostat (SAHA; Fig. 3A and B, top), which were chosen from the CMAP-predicted drug list (Supplementary Table S3), as well as panobinostat (Fig. 3C, top). This was a cytotoxic response as evidenced by CHOP induction (Supplementary Fig. S4A; refs. 12, 34, 36). In contrast, the 589 BzR line (not predicted for enhanced sensitivity to HDACIs by CMAP) showed cross-resistance to all 3 HDACi compounds compared with the BzS line (Fig. 3A–C, bottom). This panobinostat-sensitive phenotype has been observed in an additional BzR mouse cell line (bortezomib IC50: 34 nmol/L (BzS) and 63 nmol/L (BzR); panobinostat IC50: 37 nmol/L (BzS) and 22 nmol/L (BzR)) and indicates that CMAP may have the ability to identify differences in secondary drug response in these cell line models.
Consistent with our in vitro findings (Figs. 2A and 3C), panobinostat treatment significantly increased the OS of BzR mice (Fig. 4E) and decreased the tumor burden (Fig. 4F) in these animals. These in vivo results not only confirm our in vitro findings but further suggest that there may be a greater benefit from HDAC inhibitor therapy as a salvage therapy in some refractory myelomas.

Discussion

In this study, we defined the malignant plasma cell response to bortezomib and characterized signatures of bortezomib-sensitivity and -resistance by GEP using cell lines derived from the Bcl-XL/Myc double transgenic mouse model. Although a number of bortezomib treatment studies using HMCLs, primary patient samples, and nonmyeloma primary patient samples have been reported (25, 28, 39–41), they did not provide a kinetic analysis of BzS and BzR cells exposed to bortezomib over time nor did they explore individual differences in bortezomib-resistance that may contribute to secondary drug efficacy. In addition, the availability of published bortezomib data has allowed us to validate the Bcl-XL/Myc cell lines’ response to drugs in vitro further illustrating their use as a preclinical tool for asking pharmacogenomic questions.

Malignant plasma cells isolated in cell culture from these animals were generally sensitive (nmol/L concentrations) to bortezomib. In addition, we found evidence of a robust, conserved, and likely complex response to bortezomib in our mouse cell lines consistent with recent reports (28, 29, 39). This response was highly conserved in well-characterized HMCLs and in human patient clinical trial samples. Perhaps, the most striking trend was the coordinated upregulation of the majority of the constitutive proteasomal subunits in response to bortezomib treatment in both BzS and BzR cell lines, which has been previously described (28, 29, 39) further validating our model system.

The transcriptional response to bortezomib that we observed in all cells regardless of their sensitivity to the drug included the induction of the gene \textit{Psmd4}, which is part of the 19S proteasomal cap complex that selectively targets and binds ubiquitinated substrates for degradation by the 20S proteasome and whose amplification has been associated with high-risk myeloma (28). We did not
identify higher baseline Psmd4 expression in our BzR cell lines, nor did we identify increases in copy number at the mouse Psmd4 locus by array comparative genomic hybridization (mouse 3qF2.1 syntenic to human 1q21; data not shown). Therefore, although 1q21 amplification has been associated with a high-risk multiple myeloma phenotype in patients, our data would suggest variations in bortezomib response may be more complex than Psmd4 expression levels alone. Interestingly, we do observe differences in baseline expression of Eno1 and Cxcr4 in BzR compared with BzS cell lines, both of which have been associated with a poor prognostic outcome in patients with multiple myeloma (42, 43). We believe that these data further highlight the use of this system and underscore the need for companion analyses to validate these potential biomarkers using clinical samples, which we are currently developing.

We found it particularly interesting that HDACis were predicted by CMAP in this study because bortezomib is known to downregulate the expression of class I HDACs (44), and HDACis have been shown to decrease the 20S chymotryptic activity of the proteasome (45), both mediating cell death through the induction of CHOP and NOXA (46). In addition, HDAC6 has been shown to be a key regulator of aggresome activity, an alternative pathway for protein degradation in the absence of proteasome activity (47). Bortezomib response is correlated to the HDAC inhibitor response in only 1 of 3 pairs of mouse cell lines, suggesting that (i) we might further identify a small, very specific gene signature that is associated with an HDAC inhibitor response in vitro in these mouse cell lines, and (ii) not all drug-resistant myelomas will respond similarly to HDACis highlighting some heterogeneity within our in vitro system that is also likely present in the bortezomib-refractory patient population. Although there is a conserved transcriptional response to bortezomib between these 2 pairs of BzS and BzR cell lines, they respond very differently to HDACi compounds, suggesting that a more-favorable response might be achieved with HDACis such as panobinostat or vorinostat as a secondary therapy in some, but not all relapsing myelomas. Indeed, we have identified those genes that uniquely predicted the HDACi response in the 595 cell lines (Supplementary Figure 4).

Figure 4. BzR cells respond favorably to panobinostat (Pan) in vivo. A, representative FDG-PET imaging of untreated 595 BzS and BzR cell-injected syngeneic mice (1 mouse shown from each group). Red arrows indicate tumor “hotspots.” B, quantification of FDG- (blue) and FLT-PET (red) imaging of untreated BzS and BzR mice [n = 2 per group (4 tissue sites scored from each)]; *, P < 0.05. BM, bone marrow; SUV, standardized uptake value. C, Kaplan–Meier curve of BzS (black) and BzR (red) cell-injected mice treated with bortezomib (Bz). Statistical significance was determined using a one-tailed Student t test. D, quantification of FDG-PET–imaged BzS mice treated with either vehicle (left; white bar) or bortezomib (right; gray bar; n = 2 per group (4 tissue sites scored from each)); ***, P < 0.0005. E, Kaplan–Meier curve of BzR cell-injected mice treated with vehicle (n = 5; black) or panobinostat (n = 6; blue) until death. Statistical significance of both Kaplan–Meier curves was determined using a one-tailed Student t test. F, quantification of FDG-PET–imaged BzR mice treated with either vehicle (left; white bar) or panobinostat (right; gray bar; n = 2 per group (4 tissue sites scored from each)); *, P < 0.05. Statistical significance of all PET imaging was determined using a two-tailed, nonparametric Mann–Whitney test [95% confidence interval (CI)].
Table S4). Interestingly, these genes were enriched for biosynthetic and metabolic pathways (IPA), which are currently investigating in BzR disease. We did not include a dual treatment (bortezomib and panobinostat) arm in our in vivo study; however, dual treatment of the cell lines in vitro indicates that there is synergy between these 2 compounds (data not shown), which has been shown in another mouse model of multiple myeloma (48). The mechanism of this synergy may be further informed by these studies.

Although CMAP produced additional predictions to other classes of drugs, the true predictive power of this approach remains unclear. For example, alkylating agents were predicted by CMAP but did not include melphalan for which we have observed differential response between our BzR cell lines. In fact, HDACis were the only drug family that had a consistent prediction pattern across all 3 cell line pairs for all drugs predicted. This suggests that perhaps the most viable secondary drug candidates will be those with consistent predictions. It remains to be seen if there are similar predictions of response in subsets of patients receiving bortezomib/panobinostat combination therapy; however, our data suggest that refinement of this model may be effective for predicting these cases in advance.

With the initial success of combination bortezomib and HDACi treatments for refractory patients in the clinic (49) and our indication of differential response to HDACis in vitro and in vivo, our model system provides additional opportunities to characterize sensitivity and resistance to HDACis in vitro and in vivo. It is apparent that the approach taken in this study identifies a novel use for GEP in the repurposing of drugs as secondary therapies in myeloma. The prediction of HDACis as a secondary therapy here suggests that a panobinostat response profile of emerging resistance might be further elucidated that could provide preclinical support for personalized medicine approaches in multiple myeloma.

Disclosure of Potential Conflicts of Interest
H.A.F. Stessman has a commercial research grant from Millennium Pharmaceuticals, Inc: The Takeda Oncology Company. No potential conflicts of interest were disclosed by the other authors.

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