

## Interactions of the Hdm2/p53 and Proteasome Pathways May Enhance the Antitumor Activity of Bortezomib

Melissa G. Ooi,<sup>1</sup> Patrick J. Hayden,<sup>1</sup> Vassiliki Kotoula,<sup>4</sup> Douglas W. McMillin,<sup>1</sup> Elpida Charalambous,<sup>4</sup> Emily Daskalaki,<sup>4</sup> Noopur S. Raje,<sup>2</sup> Nikhil C. Munshi,<sup>1,3</sup> Dharminder Chauhan,<sup>1</sup> Teru Hideshima,<sup>1</sup> Leutz Buon,<sup>1</sup> Martin Clynes,<sup>5</sup> Peter O'Gorman,<sup>6</sup> Paul G. Richardson,<sup>1</sup> Constantine S. Mitsiades,<sup>1</sup> Kenneth C. Anderson,<sup>1</sup> and Nicholas Mitsiades<sup>1</sup>

**Abstract Purpose:** p53 is inactivated in many human malignancies through missense mutations or overexpression of the human homologue of Mdm2 (Hdm2), an E3 ubiquitin ligase that ubiquitinates p53, thereby promoting its proteasomal degradation. The *cis*-imidazoline nutlin-3 can disrupt the p53-Hdm2 interaction and activate p53, inducing apoptosis *in vitro* in many malignancies, including multiple myeloma (MM).

**Experimental Design:** We hypothesized that suppression of Hdm2-mediated p53 ubiquitination may augment sequelae of p53 accumulation caused by proteasomal inhibition. We compared the response of MM cells versus several epithelial cancer models to the proteasome inhibitor bortezomib in combination with nutlin-3.

**Results:** The combination of sublethal concentrations of bortezomib plus nutlin-3 induced additive cytotoxicity against bortezomib-sensitive MM cell lines. Importantly, however, in breast, prostate, colon, and thyroid (papillary, follicular, anaplastic, and medullary) carcinoma cell lines, this combination triggered synergistic cytotoxicity, and increased expression of p53, p21, Hdm2, Bax, Noxa, PUMA, and cleavage of caspase-3 and poly ADP ribose polymerase. Coculture with bone marrow stromal cells attenuated MM cell sensitivity to nutlin-3 monotherapy and was associated with evidence of suppression of p53 activity in MM cells, whereas combined bortezomib-nutlin-3 treatment maintained cytotoxicity even in the presence of bone marrow stromal cells.

**Conclusions:** This differential response of MM versus epithelial carcinomas to combination of nutlin-3 with bortezomib sheds new light on the role of p53 in bortezomib-induced apoptosis. Concurrent Hdm2 inhibition with bortezomib may extend the spectrum of bortezomib applications to malignancies with currently limited sensitivity to single-agent bortezomib or, in the future, to MM patients with decreased clinical responsiveness to bortezomib-based therapy. (Clin Cancer Res 2009;15(23):7153-60)

The “guardian of the genome” p53 is a transcription factor with multiple regulatory roles in DNA repair, cell cycle, and apoptosis. It is inactivated in most human cancers, frequently through missense mutations in its DNA binding core domain or through overexpression of the human homologue of Mdm2 (Hdm2), an E3 ubiquitin ligase that binds and ubiquitinates p53, thereby leading to its degradation through the

ubiquitin/proteasome pathway (1). Direct inhibition of Hdm2 function can stabilize p53, increase its protein levels, and activate the p53 apoptotic pathway in a nongenotoxic manner. Nutlin-3 is a *cis*-imidazoline small molecule with affinity for the p53-binding pocket of Hdm2. It is capable of disrupting the p53-Hdm2 interaction, thus protecting p53 from proteasomal degradation and activating the p53 pathway,

**Authors' Affiliations:** <sup>1</sup>Medical Oncology, Jerome Lipper Multiple Myeloma Center, Dana Farber Cancer Institute; <sup>2</sup>Massachusetts General Hospital, and <sup>3</sup>VA Boston Healthcare System, Boston, Massachusetts; <sup>4</sup>Department of Pathology, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece; and <sup>5</sup>National Institute for Cellular Biotechnology, Dublin City University and <sup>6</sup>Department of Haematology, Mater Misericordiae University Hospital, Dublin, Ireland  
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M.G. Ooi and P.J. Hayden contributed equally to this work.

**Requests for reprints:** Constantine S. Mitsiades, Department of Medical Oncology, Dana-Farber Cancer Institute, Mayer Building, Room M555, 44 Binney Street, Boston MA 02115. Phone: 617-792-7240; Fax: 617-812-7701; E-mail: Constantine\_Mitsiades@dfci.harvard.edu.

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### Translational Relevance

The proteasome inhibitor bortezomib has proven clinical activity against multiple myeloma (MM). Our research program has investigated several bortezomib-based combinations with the goal of enhancing the anti-MM activity of bortezomib or overcome resistance to it, while maintaining a manageable profile of adverse events. We have previously reported the *in vitro* synergistic activity of combinations between bortezomib and DNA-damaging, p53-activating, chemotherapeutic agents, such as doxorubicin and melphalan. The anti-MM activity of these combinations has now been validated in the clinical setting and represents an established approach for MM treatment. We now hypothesized that the nongenotoxic stabilization of p53, caused by the E3 ubiquitin ligase Hdm2 inhibitor nutlin3 through its suppressive effect on Hdm2-mediated p53 ubiquitination, may synergize with p53 accumulation caused by bortezomib. Our results suggest that concurrent Hdm2 inhibition with bortezomib may extend the spectrum of malignancies sensitive to bortezomib treatment and overcome bortezomib resistance.

leading to apoptosis in various malignancies, including multiple myeloma (MM; refs. 2–5).

The proteasome inhibitor bortezomib, the prototypic member of this class of agents, is approved by the U.S. Food and Drug Administration for the treatment of MM (6). Our research program has investigated several bortezomib-based drug combinations with the goal of identifying combinations that will achieve enhanced anti-MM activity or overcome bortezomib resistance, with minimal toxicity. We have previously reported the *in vitro* synergistic activity of the combination between bortezomib and DNA-damaging, p53-activating, chemotherapeutic agents, such as doxorubicin and melphalan (7). This concept has been validated in the clinical setting by trials demonstrating the superiority of combined pegylated liposomal doxorubicin plus bortezomib compared with bortezomib monotherapy for the treatment of patients with relapsed or refractory MM (8), as well as the superiority of combined bortezomib plus melphalan and prednisone compared with only melphalan plus prednisone to treat newly diagnosed MM patients (9). These findings support the role of combining p53-activating chemotherapeutics with proteasome inhibitors as a promising novel therapeutic approach in MM.

The ubiquitin/proteasome pathway is responsible for the degradation of many tumor-suppressing and proapoptotic proteins that are important for survival and proliferation of cancer cells, including p53. We hypothesized that nongenotoxic stabilization of p53, caused by nutlin-3 through suppression of Hdm2-mediated p53 ubiquitination, may synergize with accumulation of p53 caused by bortezomib through proteasome inhibition, thereby leading to increased antitumor activity. In this study, we have shown the activity of combined nutlin-3 with bortezomib against bortezomib-sensitive MM cell lines and, for comparison, epithelial cell carcinoma lines, which have lower sensitivity to bortezomib than MM cells. We also assessed

the impact of the microenvironment (stromal cells) on the activity of nutlin-3 on MM cells versus other neoplasias.

### Materials and Methods

**Reagents.** Racemic nutlin-3 was purchased from Cayman Chemical Company. Bortezomib [pyrazylCONH(CHPh)<sub>2</sub>CONH(CHisobutyl)B(OH)<sub>2</sub>] was obtained from Millennium Pharmaceuticals, and ZVAD-FMK was purchased from Calbiochem.

**Cells lines.** The bortezomib-sensitive MM cell lines MM1.S, MM1.R, KMS-11, OPM-2, and NCI-H929 have been described previously (10). The breast carcinoma cell line MDA-MB-231met-LN and the prostate carcinoma cell line DU145 were generous gifts of Dr. Andrew L. Kung (Dana-Farber Cancer Institute, Boston, MA). The SW579 (papillary thyroid) and TT (medullary thyroid) cell lines were obtained from American Type Culture Collection. The WRO (follicular thyroid) and FRO (anaplastic thyroid) lines were generous gifts of Dr. James A. Fagin (Memorial Sloan Kettering Cancer Center, New York, NY). The ARO cells were provided by Dr James A. Fagin as well, and were recently found to be a derivative of the HT-29 colon carcinoma cell line (11). The HRO85-1 and DRO81-1 medullary thyroid cell lines were a generous gift of Dr. Guy J.F. Juillard (University of California at Los Angeles School of Medicine, Los Angeles, CA).

Myeloma cell lines were grown in RPMI 1640 (Mediatech) with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. All carcinoma cell lines were grown in DMEM (Mediatech) with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (BioWhittaker).

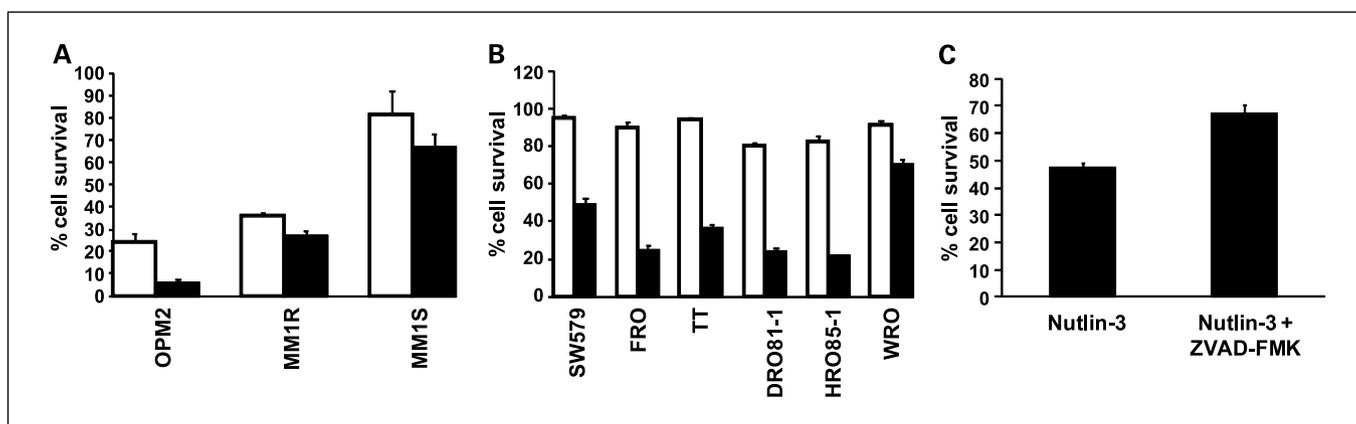
**Cell viability assessment.** Cell viability was measured by MTT (Chemicon International) colorimetric survival assay, as described previously (10). Cell viability was expressed as a percentage of the value of untreated controls. Each experimental condition was repeated at least in triplicate wells in each experiment. Data reported are average values ± SD of representative experiments.

**Immunoblotting.** Immunoblotting and enhanced chemiluminescence (Amersham) were performed as previously described (10). The primary antibodies (Ab) used for immunoblotting were anti-p53 monoclonal Ab (mAb) from Upstate Biotechnologies, anti-Hdm2 and glyceraldehyde-3-phosphate dehydrogenase mAb from Santa Cruz Biotechnology, Rabbit polyclonal Ab to PUMA from Axxora LLC, anti-Noxa mAb from Imgenex, and Polyclonal Abs to caspase-3 and poly ADP ribose polymerase from Cell Signaling. Peroxidase-labeled secondary Abs were purchased from Jackson ImmunoResearch.

**Coculture of MM and breast carcinoma cells with stromal cells.** For coculture experiments, MM.1S and MDA-MB-231met-LN cells stably expressing a luciferase (luc) vector were cultured in the presence or absence of the bone marrow stromal cell (BMSC) line HS-5 in 96-well optical bottom tissue culture plates (Nunc), as previously described (12).<sup>7</sup> The luc-expressing cell line MM.1S-GFP/luc cells were generated by retroviral transduction with the pGC-gfp/luc vector (kind gift of C.G. Fathman, Stanford University, Palo Alto, CA). The MDA-MB-231met-LN luc-expressing cells were kindly provided by Andrew L. Kung (Dana-Farber Cancer Institute, Boston, MA). Briefly, HS-5 stromal cells were plated in 96-well plates and allowed to attach overnight. Tumor cells were then plated and treated with nutlin-3, with or without bortezomib, for 48 h at the indicated concentrations. At the end of the incubation period, luciferin substrate (Xenogen Corp.) was then added to the culture, and the resulting bioluminescence signal was measured using a Luminoskan luminometer (Labsystems).

**Relative quantification of selected transcripts in MM cells cocultured with BMSCs.** MM.1S-GFP/luc cells were cultured for 24 h in the presence or absence of the BMSC line HS-5 and then sorted by fluorescence-activated cell sorting (FACS) cell sorter (BD FACSAria cell sorter, Becton Dickinson). RNA was extracted with TRIZOL-LS (Invitrogen), further

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**Fig. 1.** *In vitro* activity of nutlin-3 against MM cells and other malignant cells. **A**, MM cell lines are sensitive to nutlin-3. Viability of the MM1.S, MM1.R, and OPM-2 cell lines after exposure to nutlin-3 (white columns, 5  $\mu$ mol/L; black columns, 10  $\mu$ mol/L) for 48 h was assessed by MTT. Cell survival was expressed as a percentage (mean  $\pm$  SD) compared with vehicle-treated controls. The treatment was repeated thrice with similar results. Columns, mean values from representative experiments; bars, SD. **B**, response of a panel of 6 carcinoma cell lines (SW579, papillary thyroid; FRO, anaplastic thyroid; TT, DRO81-1, and HRO85-1, medullary thyroid; WRO, follicular thyroid) to treatment with nutlin-3 (white columns, 10  $\mu$ mol/L; black columns, 30  $\mu$ mol/L) for 48 h, as assessed by MTT. Cell survival was expressed as a percentage (mean  $\pm$  SD) compared with vehicle-treated controls. The treatment was repeated thrice with similar results. Columns, mean values of representative experiments; bars, SD. **C**, anaplastic thyroid carcinoma FRO cells were treated with nutlin-3 (20  $\mu$ mol/L) for 48 h in the presence or absence of the pan-caspase inhibitor ZVAD-FMK (20  $\mu$ mol/L). Cell survival was quantified by MTT and expressed as a percentage (columns, mean; bars, SD) compared with vehicle-treated controls.

treated with DNase I, and purified with the RNeasy MinElute Cleanup kit (Qiagen). Reverse transcription was accomplished with random hexamers and Superscript II, followed by incubation with RNase H (Invitrogen). Amplification reactions (25  $\mu$ L, 100 ng cDNA/reaction) with Taqman FAM/MGB probes were done in a 7500 Real-time PCR System (Applied Biosystems) for select p53-regulated transcripts. Relative quantification for each target versus a reference gene transcript [glucuronidase  $\beta$  (GUSB)] was assessed with the SDS v1.3 software (Applied Biosystems). For all assessments, the evaluation threshold was set at 0.3.

**Statistical analysis.** To evaluate the differences across various experimental conditions, one-way ANOVA was done, and *post hoc* tests (Duncan and Dunnett's T3 tests) served to evaluate differences between individual pairs of experimental conditions. The additive or synergistic nature of the interaction between nutlin-3 and bortezomib was evaluated by two-way ANOVA (GraphPad Prism 4.03). In all analyses,  $P < 0.05$  was considered statistically significant.

**Transcriptional signature of stroma-responsive genes and relationship with clinical outcome in bortezomib-treated MM patients.** A transcriptional index of tumor cell response to nutlin-3 was identified, based on previously published studies of transcripts selectively downregulated by nutlin-3 treatment in responsive, but not in unresponsive, tumor lines (5). The probes of that signature for U133plus2.0 oligonucleotide microarray chips were used to filter the log<sub>2</sub>-transformed and median-centered gene expression data set of tumor cells from bortezomib-treated MM patients enrolled in phase II SUMMIT and phase III APEX clinical trials of this agent (13–15). After the filtering, the transcriptional index of nutlin-3-suppressed genes was calculated as the average of the log<sub>2</sub>-transformed and median-centered values for each probe of the signature. Patients were then classified as having low (bottom tertile of expression) versus high (top two tertiles) expression of nutlin-3-suppressed genes, and Kaplan-Meier survival analyses for progression-free and overall survival of bortezomib-treated patients were done using SPSS 17.0.

## Results

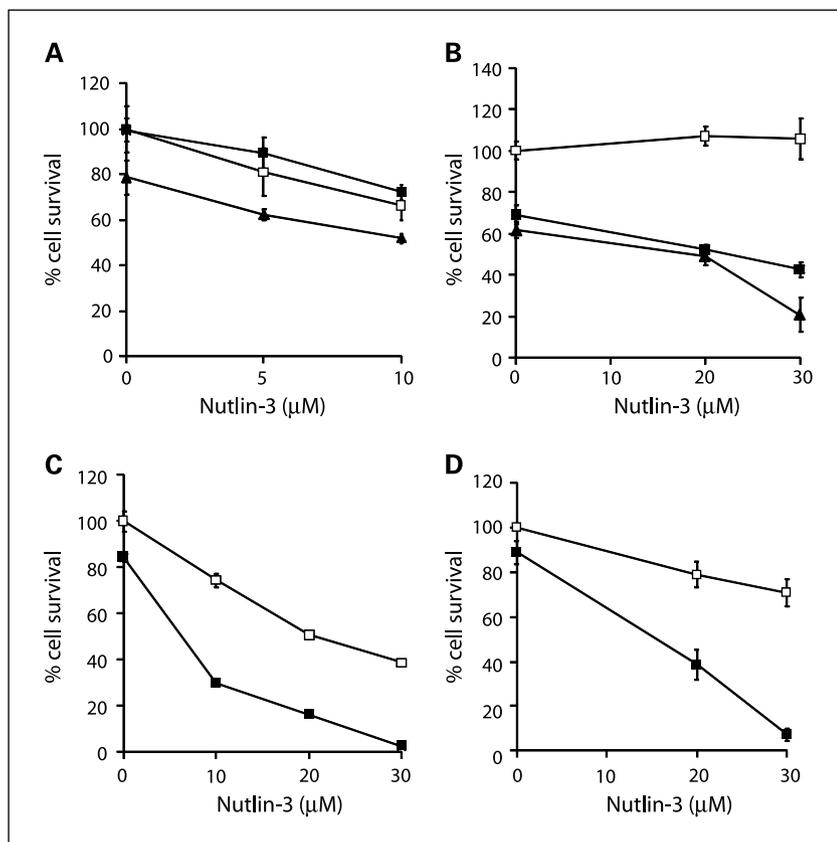
**Racemic nutlin-3 activates the p53 apoptotic pathway and induces apoptosis.** A panel of MM and carcinoma cell lines were treated with racemic nutlin-3. In agreement with a previous report (4), MM cell lines were sensitive to nutlin-3, with decreased cell viability upon treatment; representative results for MM1.S, MM1.R, and OPM-2 are shown in Fig. 1A. Epithelial

tumor cell lines were also sensitive to nutlin-3, although typically to a lesser extent than MM cells (Fig. 1B). Nutlin-3 upregulated expression of several p53-dependent targets including Bax, PUMA, Noxa, p21, and Hdm2 itself (Table 1), and induced caspase-3 cleavage (Supplementary Fig. 1). The proapoptotic effect of nutlin-3 was partially attenuated by the pan-caspase inhibitor ZVAD-FMK (Fig. 1C).

**Differential effect of nutlin-3 on activity of sublethal doses of bortezomib against MM versus epithelial tumor cells.** We have previously reported that bortezomib increases p53 protein levels, at least in part due to inhibition of its proteasomal degradation, as well as upregulates p53 mRNA expression and

**Table 1.** Anaplastic thyroid carcinoma FRO cells were treated with nutlin-3 (30  $\mu$ M) for 0, 24 and 48 h and mRNAs levels for a panel of p53-responsive transcripts were then analyzed by qRT-PCR and expressed as fold change ( $2^{-\Delta\Delta CT}$ ) relative to control cells (i.e., controls are assigned a value of 1 by definition)

$2^{-\Delta\Delta CT}$	24 h	48 h
BAK	4.201	2.220
BAX	5.458	2.658
PUMA (BBC3)	7.816	2.564
BTG1	4.814	4.030
CDKN1A (p21/CIP1/WAF1)	10.984	5.544
CDKN1B (p27/KIP1)	10.834	3.258
CDKN1C (p57/KIP2)	2.426	1.560
CDKN2B (p15/INK4B)	2.552	11.519
GADD45B	4.342	1.880
GADD45G	19.671	12.158
HSP90AA1	5.481	3.704
HSP90B1 (GRP94)	6.944	5.737
HSPCB (HSP90AB1)	3.901	2.905
HDM2/MDM2	7.515	2.610
PMaip1 (NOXA)	8.829	2.972
VEGFA	1.781	4.657
CD44, isoforms 1-4	0.573	0.568

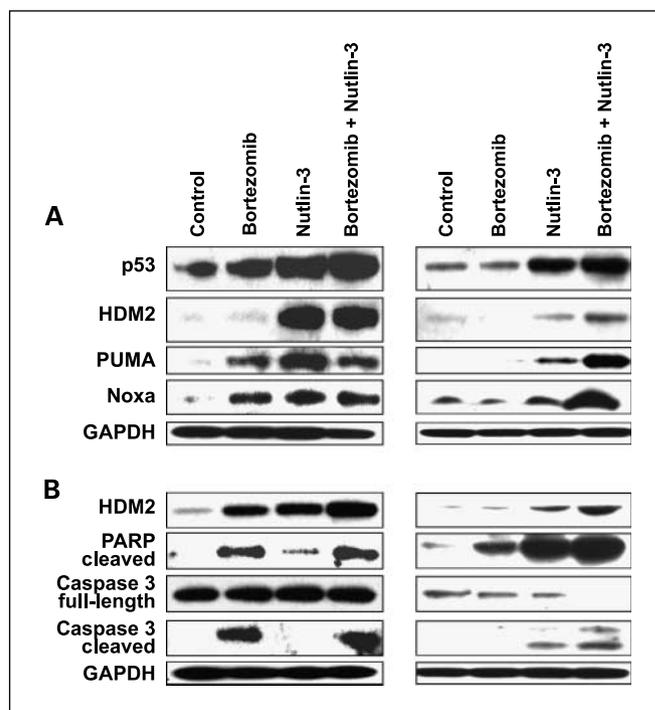


**Fig. 2.** Effect of combined Hdm2 and proteasome inhibition on malignant cells. Treatment of the MM cell lines MM1.S, MM1.R, KMS-11, OPM-2, and NCI-H929 with combinations of sublethal concentrations of nutlin-3 and bortezomib exhibited an additive cytotoxicity. Nutlin-3 also potentiates the activity of sublethal concentrations of bortezomib against several types of epithelial tumors, including thyroid, breast, prostate, and colon carcinomas. Data from representative cell lines are shown: *A*, MM1.S cells were treated for 48 h with nutlin-3 (0-10  $\mu\text{M}$ /L) in the absence ( $\square$ ) or presence of bortezomib ( $\blacksquare$ , 2 nmol/L;  $\blacktriangle$ , 4 nmol/L). *B*, follicular thyroid carcinoma WRO cells were treated for 48 h with nutlin-3 (0-30  $\mu\text{M}$ /L) in the absence ( $\square$ ) or presence of bortezomib ( $\blacksquare$ , 20 nmol/L;  $\blacktriangle$ , 40 nmol/L). *C*, anaplastic thyroid carcinoma FRO cells were treated for 48 h with nutlin-3 (0-30  $\mu\text{M}$ /L) in the absence ( $\square$ ) or presence ( $\blacksquare$ ) of bortezomib (4 nmol/L). *D*, breast carcinoma MDA-MB-231met-LN cells were treated for 48 h with nutlin-3 (0-30  $\mu\text{M}$ /L) in the absence ( $\square$ ) or presence ( $\blacksquare$ ) of bortezomib (5 nmol/L). Cell survival was quantified by MTT and expressed as a percentage (points, mean; bars, SD) relative to vehicle-treated controls.

induces p53 phosphorylation (Ser15; refs. 16, 17). We hypothesized that combined treatment with the Hdm2 ubiquitin ligase inhibitor nutlin-3 would enhance the p53-stabilizing activity and hence the proapoptotic effect of bortezomib. MM cell lines are highly sensitive to *in vitro* single-agent bortezomib, including MM1.S (Fig. 2A), MM1.R, KMS-11, OPM-2, and NCI-H929 (data not shown), and combinations of sublethal concentrations of nutlin-3 and bortezomib triggered additive cytotoxicity. In contrast, nutlin-3 exhibited synergistic cytotoxicity with bortezomib against epithelial tumor cell lines including thyroid, breast, and prostate carcinomas (Supplementary Fig. S2; Fig. 2B-D), as well as colon carcinoma.

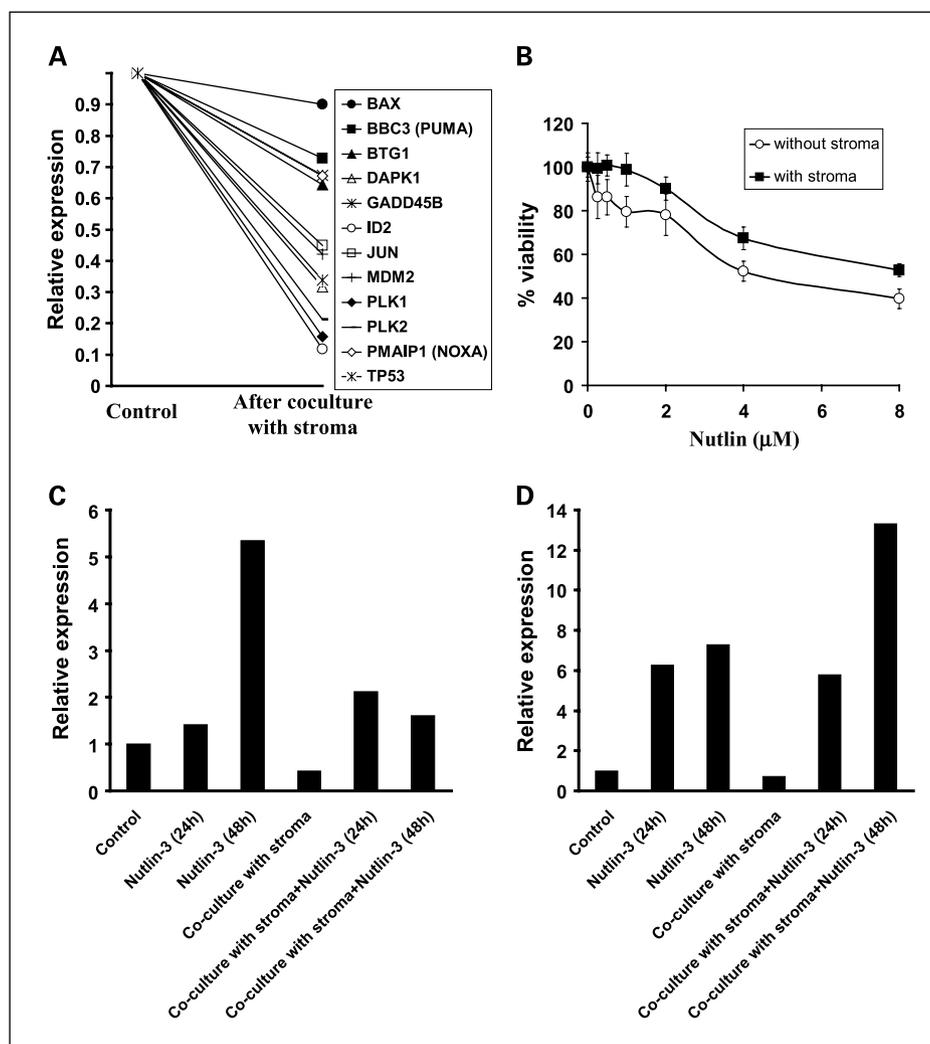
**Nutlin-3 enhances the activation of the p53 apoptotic pathway by sublethal concentrations of bortezomib in epithelial carcinoma cell lines, but not in bortezomib-sensitive MM cells.** We next investigated the mechanism for the differential sensitivity of MM versus epithelial cell lines to the combination of bortezomib with nutlin-3. Immunoblotting analysis of bortezomib-sensitive MM1.S cells treated with this combination for 8 or 24 hours showed no evidence of synergistic activation of p53 pathway and apoptosis mediators (Fig. 3), consistent with the lack of enhanced killing effect. In contrast, FRO cells treated with sublethal concentrations of nutlin-3 and bortezomib for 8 or 24 hours revealed synergistic activation of p53 pathway and apoptosis, associated with upregulation in p53, Hdm2, Noxa, and PUMA expression, as well as enhanced cleavage of poly ADP ribose polymerase and caspase-3 (Fig. 3), consistent with the synergistic activity of this drug combination.

**Impact of BMSCs on p53 activity and sensitivity to nutlin-3 in MM cells.** BMSCs confer a protective effect on MM cells against several conventional proapoptotic agents, such as dexamethasone



**Fig. 3.** Immunoblotting analysis of MM1.S (left) and FRO (right) cells treated with sublethal concentrations of nutlin-3 and bortezomib for 8 h (A) or 24 h (B). Total cell lysates were assayed for p53, Hdm2, Noxa, PUMA, and cleaved poly ADP ribose polymerase (PARP) and caspase-3. There was no synergistic activation of p53 pathway and apoptosis in MM1.S cells (nutlin-3, 2  $\mu\text{M}$ /L; bortezomib, 2 nmol/L). In contrast, the combination of nutlin-3 (10  $\mu\text{M}$ /L) with bortezomib (4 nmol/L) resulted in synergistic activation of p53 pathway and apoptosis in the anaplastic thyroid FRO cells.

**Fig. 4.** Impact of coculture with BMSCs on the p53 pathway in MM cells. **A**, MM.1S-GFP/luc cells were cultured for 24 h in the presence or absence of BMSCs line HS-5, and then sorted by FACS. Levels of mRNAs for a panel of p53-responsive transcripts were quantified by qRT-PCR and expressed relative to the respective levels in control cells. Coculture with BMSCs suppressed levels of these p53-responsive transcripts. **B**, MM.1S-GFP/luc cells were treated with nutlin-3 for 48 h, in the presence or absence of BMSCs. MM cell viability was assessed using the CS-BLI approach and expressed as a percentage (points, mean; bars, SD) relative to vehicle-treated controls. Coculture with HS-5 BMSCs partially attenuated the proapoptotic effect of nutlin-3 on MM1S. **C**, effect of nutlin-3, in the presence or absence of BMSC, on the expression of Hdm2 in MM cells. MM.1S-GFP/luc cells were treated with nutlin-3 (8  $\mu$ mol/L) for 0, 24, or 48 h in the presence or absence of the BMSC line HS-5, and then sorted by FACS. Levels of Hdm2 mRNA were quantified by qRT-PCR and expressed as a ratio relative to respective levels in control cells. **D**, effect of nutlin-3, in the presence or absence of BMSC, on the expression of PUMA in MM cells. MM.1S-GFP/luc cells were treated with nutlin-3 (8  $\mu$ mol/L) for 0, 24, or 48 h in the presence or absence of the BMSC line HS-5, and then sorted by FACS. Levels of PUMA mRNA were quantified by qRT-PCR and expressed as a ratio relative to respective levels in control cells.



or cytotoxic chemotherapy (18, 19). We therefore next investigated the effect of coculture with BMSCs to the activation of the p53 pathway in MM cells and to sensitivity to nutlin-3. Human MM1S-GFP/luc cells were cocultured with HS-5 BMSCs for 24 hours and were separated using a FACS cell sorter. Quantitative reverse transcription-PCR in MM1S cells cocultured with BMSCs showed decreased expression of p53 and gene transcripts known to be induced by p53 including Hdm2 (20), Bax (21), Noxa (22), PUMA (23), BTG1, DAPK1 (24), GADD45B, ID2, Jun, Plk1, and Plk2 (Fig. 4A; ref. 25). These results suggest that coculture with BMSCs triggers downregulation of the p53 pathway in MM cells, which can contribute to resistance to apoptosis.

We next assessed the impact of coculture with BMSCs on MM cell sensitivity to nutlin-3. We found that MM1S-GFP/luc cells were less sensitive to the proapoptotic effect of nutlin-3 in the presence of HS-5 BMSCs (Fig. 4B). Importantly, however, treatment with nutlin-3 was able to induce the expression of Hdm2 (Fig. 4C), PUMA (Fig. 4D), Noxa, Bax, and GADD45B (data not shown), even in the presence of BMSCs.

**Impact of BMSCs on p53 activity and sensitivity to nutlin-3 in epithelial carcinoma cells.** As noted above, the combination of nutlin-3 with bortezomib mediates additive cytotoxicity against bortezomib-sensitive MM cells, but triggers synergistic cytotoxicity against epithelial carcinoma cell lines. We investigated

whether this latter effect persists in the presence of BMSC. We specifically evaluated the impact of BSMCs on the effect of the combination of nutlin-3 with bortezomib on MDA-MB-231metLN cells stably transfected with a luc vector. Coculture with BMSCs did not protect against the synergistic proapoptotic effect of the combination of nutlin-3 with bortezomib in this epithelial carcinoma model (Fig. 5). These data suggest that the enhanced activity of nutlin-3 combined with bortezomib against epithelial models of cancer is unlikely to be inhibited by microenvironmental interactions.

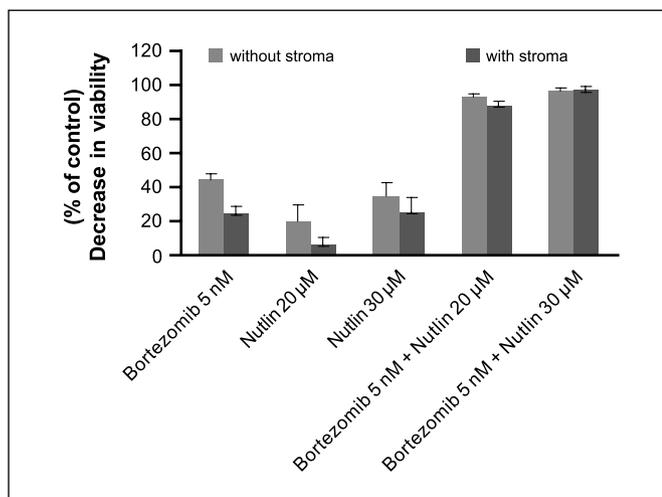
**A transcriptional signature of nutlin-3-suppressed genes correlates with clinical outcome in bortezomib-treated MM patients.** To probe the clinical relevance of molecular sequelae triggered by nutlin-3, we next evaluated whether the transcriptional signature of tumor cell response to nutlin-3 treatment is enriched in genes that may correlate with clinical outcome. To test this hypothesis, a previously reported signature of genes suppressed in tumor cells by nutlin-3 treatment was used to classify bortezomib-treated MM patients enrolled in phase II SUMMIT and phase III APEX trials into cohorts with high versus low expression of nutlin-3-suppressed genes. Bortezomib-treated patients who had at baseline high expression of nutlin-3-suppressed genes had significantly shorter progression-free (A,  $P = 0.001$ , log-rank test) and overall

survival ( $B$ ,  $P = 0.002$ , log-rank test) compared with those with low expression levels (Fig. 6).

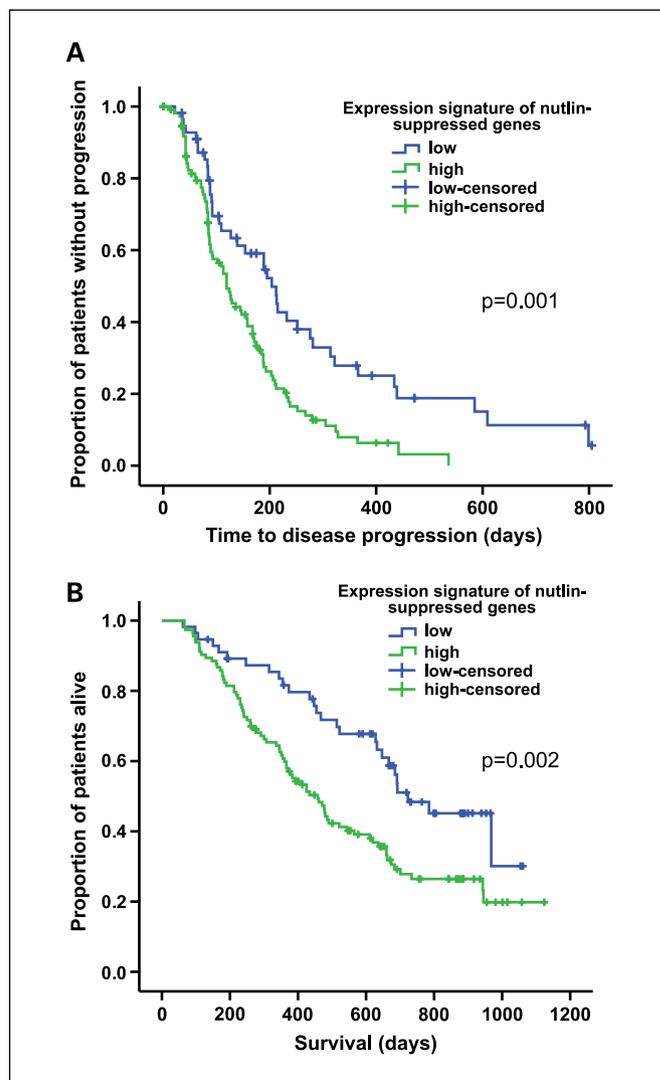
## Discussion

The proteasome inhibitor bortezomib represents a significant advance in the therapeutic armamentarium against MM. However, resistance to bortezomib, *de novo* or acquired, is an important obstacle to achieving better clinical results. Furthermore, bortezomib has not been shown to be active against most solid tumors. We are therefore trying to identify clinically applicable approaches that would allow us to extend bortezomib activity against a broader spectrum of tumors. In that context, we compared responses to bortezomib in MM versus solid tumor models to delineate mechanisms of differential bortezomib responsiveness. Specifically, proteasome inhibition leads to increased p53 mRNA and protein levels (16), but the role of p53 signaling to MM cell death from bortezomib is unclear. We hypothesized that inhibition of Hdm2, the E3 ubiquitin ligase that ubiquitinates p53 and promotes its proteasomal degradation, could enhance p53 stabilization in bortezomib-treated MM cells, with a resultant synergistic effect on apoptosis induction. We found that the combination of the Hdm2 inhibitor nutlin-3 with bortezomib mediated additive cytotoxicity against bortezomib-sensitive MM cell lines and synergistic activity against epithelial cancer cell lines. Coculture of MM cells with stroma cells suppressed p53 signaling and attenuated sensitivity to single-agent nutlin-3.

Direct inhibition of the E3 ubiquitin ligase Hdm2 stabilizes p53 and activates the p53 apoptotic pathway in a nongenotoxic manner and, therefore, represents a promising approach for the treatment of cancer (26). Treatment of MM cells with nutlin-3, which interrupts the p53-Hdm2 interaction, increases protein levels of p53, thereby inducing p53 targets and apoptotic cell



**Fig. 5.** Impact of coculture with BMSCs on effect of combined Hdm2 and proteasome inhibition. The breast carcinoma MDA-MB-231met-LN cells stably expressing luc were treated with nutlin-3 (0, 20, or 30 μmol/L) and bortezomib (5 nmol/L) for 48 h, in the presence or absence of HS-5 stromal cells. MDA-MB-231met-LN viability was assessed using the CS-BLI approach. Decrease in viability was expressed as % of control cells. Synergistic cytotoxicity of the combination of nutlin-3 and bortezomib persisted despite coculture with BMSCs.



**Fig. 6.** A transcriptional signature of nutlin-responsive genes correlates with clinical outcome in bortezomib-treated MM patients. Gene expression data were analyzed from tumor cells of bortezomib-treated MM patients (relapsed and/or refractory) enrolled in the phase II SUMMIT and phase III APEX trials. A previously reported signature of genes suppressed in tumor cells by nutlin-3 treatment was used to classify bortezomib-treated patients having high versus low expression of nutlin-3-suppressed genes. Bortezomib-treated patients who had baseline high expression of nutlin-3-suppressed genes had significantly shorter progression-free (A,  $P = 0.001$ , log-rank test) and overall survival (B,  $P = 0.002$ , log-rank test) compared with those with low expression levels.

death (4). The differential response of MM versus epithelial malignancies to the combination of bortezomib with nutlin-3 sheds light on the role of p53 in bortezomib-induced apoptosis and suggests a complex, tissue-dependent, interaction between the HDM2/p53 and proteasome pathways. It is possible that p53 is more important for bortezomib-induced apoptosis in epithelial malignancies than in MM. Mutations in p53 are very common in epithelial malignancies, whereas in MM, they have been historically considered to be present in only late stage disease (e.g., plasma cell leukemia/extramedullary MM). However, their prevalence may increase in the future, because patients with advanced MM survive longer due to novel agents including thalidomide, bortezomib, and lenalidomide. It is also possible

that epithelial cancers that are bortezomib refractory may have more potential for sensitization upon Hdm2 inhibition than MM cells, which are more responsive to single-agent bortezomib and nutlin-3. Importantly however, in advanced MM with p53 pathway lesions and bortezomib-resistance/refractoriness, concurrent Hdm2 inhibition may restore bortezomib sensitivity. This hypothesis is further supported by our observation that those MM patients with high baseline levels of expression of genes suppressed *in vitro* by nutlin-3 have short progression-free and overall survival after treatment with bortezomib. This observation suggests that treatment with nutlin-3 may counteract molecular pathways associated with decreased responsiveness to bortezomib and thereby overcome clinical bortezomib resistance. This hypothesis warrants testing in future studies.

Ubiquitinated p53, in particular Hdm2-ubiquitinated p53, is in general transcriptionally inactive. Ubiquitination targets p53 not only for proteasomal degradation, but also for nuclear export and cytosolic localization, thus eliminating the opportunity for binding to DNA (27). Also, many of the same sites that are ubiquitinated by Hdm2 can also be acetylated by p300/CBP to promote p53 activation. Acetylation of p53 is essential for the binding of p53 to p53-binding elements promoting transcription of various apoptosis or cell cycle arrest genes, including p21, PUMA, BAX, and Noxa (27). However, when Hdm2 ubiquitinates p53, the latter cannot be acetylated by p300/CBP and becomes transcriptionally inactive, even in the absence of proteasome-mediated degradation (27). This could explain why p53 does not seem to play a major role as a mediator of the effects of bortezomib monotherapy, because, although its total levels are increased in the presence of bortezomib (16), it is still ubiquitinated and functionally inactivated by Hdm2. Also, it provides another mechanism to explain the synergistic activity of bortezomib together with nutlin-3, which we describe in this study: the addition of Hdm2 inhibition to proteasome inhibition not only further stabilizes p53 and increases its protein levels, but also blocks its ubiquitination and transcriptional inactivation, thus making it more functional.

Interactions of MM cells with normal cells of the bone marrow milieu can attenuate the antitumor activity of conventional therapies, such as glucocorticoids, standard cytotoxic agents, such as alkylators, and novel targeted therapies, such as bortezomib (18, 28). In this study, we found that coculture with BMSCs partially attenuates the response of MM cells to nutlin-3. This is associated with evidence that interaction with BMSCs suppresses p53 activity in highly bortezomib-responsive MM cells. However, the synergistic effect of nutlin-3 with bortezomib in solid carcinoma cell lines persists unaffected despite coculture with BMSCs. This further supports the promise of anticancer activity of Hdm2 inhibitors in combination with proteasome inhibitors.

Clinically relevant preclinical models can help identify drug combinations with enhanced anticancer activity. We have previously reported that DNA-damaging chemotherapeutics including doxorubicin and melphalan induce synergistic MM cytotoxicity when combined with bortezomib (7). This approach has been validated clinically (8, 9), and the combination of bortezomib with pegylated liposomal doxorubicin or with melphalan plus prednisone are now Food and Drug Administration–approved anti-MM combination regimens. We now report that nongenotoxic activation of the p53 pathway using nutlin-3 can sensitize epithelial carcinoma cells to bortezomib in a manner that is not suppressed by microenvironmental interactions. These observations suggest that concurrent Hdm2 inhibition with nutlin-3 may extend the spectrum of bortezomib activity to solid tumors, and also overcome bortezomib resistance in advanced MM. Our studies further provide the framework for derived combination clinical trials to improve outcome in patients with solid tumor and hematologic malignancies.

### Disclosure of Potential Conflicts of Interest

K. Anderson, commercial research grants, Celgene, Millennium; honoraria, consultancy, Celgene, Novartis, Millennium. N. Munshi, honoraria, Celgene, Novartis, Millennium. P.G. Richardson, honoraria, consultancy, Celgene, Millennium. C. Mitsiades, consultancy, Novartis, Millennium, Pharmion.

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## Interactions of the Hdm2/p53 and Proteasome Pathways May Enhance the Antitumor Activity of Bortezomib

Melissa G. Ooi, Patrick J. Hayden, Vassiliki Kotoula, et al.

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