Article

Novel combinatorial therapeutic strategy to overcome gain-of-function p53 mutations

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Abstract: Mutations in the "guardian of the genome" *TP53* predominate in solid tumors. In addition to loss of tumor suppressor activity, a specific subset of missense mutations confers additional oncogenic properties. These "gain-of-function" (GOF) mutations portend poor prognosis across cancer types regardless of treatment. Our objective in this study was to identify novel therapeutic opportunities to overcome the deleterious effects of GOF *TP53* mutants. Using gynecologic cancer cell lines with known *TP53* mutational status, we established that treatment with a proteasome inhibitor induced cell death in cells with two recurrent GOF *TP53* mutations (R175H and R248Q), and addition of a histone deacetylase inhibitor (HDACi) enhanced this effect. By contrast, p53-null cancer cells were relatively resistant to the combination. Towards understanding the mechanism, we found that proteasome inhibition promotes apoptosis of cells with *TP53* GOF mutations, potentially through induction of the unfolded protein response. In line with the reported hyperstabilization of GOF p53 protein, cells treated with HDACi exhibited reduced levels of p53 protein. Together, these data form the basis for future clinical studies examining therapeutic efficacy in a preselected patient population with GOF *TP53* mutations.

Keywords: p53; proteasome inhibitor; endometrial cancer; ovarian cancer; gain-of-function mutation

1. Introduction

The Cancer Genome Atlas (TCGA) project has substantiated the long-held notion that the "guardian of the genome" *TP53* is the most mutated gene in tumors [1]. Certain tumor types have an exceptionally high preponderance of mutations in *TP53*: for example, mutations in *TP53* occur in 96% of all serous ovarian tumors [2], and nearly all serous and ~25% of high grade endometrioid endometrial cancers have mutations in *TP53*[3]. The prevalence of *TP53* mutations is also particularly high in head and neck cancer and breast cancer [1,4].

While it is appreciated that *TP53* mutations occur in a substantial number of tumors, it is critically important to note that varying types of p53 mutant proteins exist with different implications for chemosensitivity. Some mutations are relatively inconsequential from the perspective of p53 function, and proteins of this type retain wild type activity. Other mutations are loss of function (LOF) or p53-null in which single amino acid changes completely inactivate or destabilize the protein. Finally, an interesting category is the gain of function (GOF) or "oncogenic" *TP53* mutations that convert p53 from a tumor suppressor to an oncogene. The majority of LOF and GOF *TP53* mutations result in loss of DNA binding to canonical p53 targets. However, GOF mutants also have new protein:protein interactions and/or transcriptional targets that confer an additional "oncogenic" function [5-8]. To date, eight missense mutations in human *TP53* have been established as GOF mutations and result in the following amino acid changes: P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L and R282W.

Substantial clinical and preclinical data from a wide range of cancers demonstrate that GOF *TP53* mutations predict for poor response to treatment. In recently published work, we evaluated the relationship of the eight GOF *TP53* mutations with progression-free survival (PFS), risk of recurrence, and response to standard platinum and taxane chemotherapy in serous ovarian cancer [9]. We found that 21.2% of serous ovarian cancer patients in the TCGA cohort have a GOF *TP53* mutation, whereas 18.9% are LOF [9]. Ovarian cancer patients with GOF *TP53* mutations have worse clinical outcomes compared to patients with unclassified *TP53* mutations (i.e., variants of unknown significance), including a shorter PFS and a 60% greater risk of recurrence [9]. These findings have important potential implications for all cancers characterized by mutations in *TP53.*

Analysis of *TP53* mutational status is now included in many next-generation sequencing tests. An obvious question, therefore, is how to convert this deadly oncogenic mutation into an actionable mutation. Herein we identify the combination of a proteasome inhibitor with an epigenetic modulator (histone deacetylase inhibitor (HDACi)) as a potent therapeutic strategy to overcome the deleterious effects of *TP53* GOF mutations. These preclinical data serve as the proof-of-concept for future phase I trials evaluating specific combinatorial therapies in patients whose tumors contain *TP53* GOF mutations.

2. Results

2.1. Sensitivity of cancer cells with known p53 status to proteasome inhibitors

We first examined the sensitivity of two well-characterized endometrial cancer cell lines with known p53 mutational status to the proteasome inhibitor, bortezomib (Velcade®). We made the unexpected discovery that KLE cells with the R175H GOF mutation were highly sensitive to the proteasome inhibitor bortezomib, whereas Hec50 cells with LOF p53 mutation were relatively resistant to bortezomib (**Figure 1A**).

2.2. Addition of HDACi enhances sensitivity to proteasome inhibitor treatment in cells with endogenous TP53 GOF mutation

Next, we examined the impact of addition of a histone deacetylase inhibitor. The combination of bortezomib with the HDACi LBH589 (panobinostat) further increased cell killing in KLE cells (R175H GOF) as compared to bortezomib alone (**Figure 1B**).
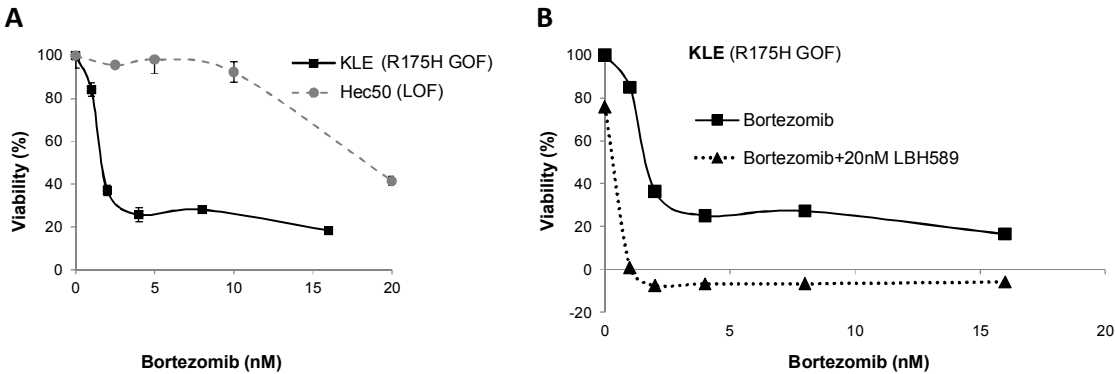
Figure 1. Endometrial cancer cells with GOF *TP53* mutation are highly sensitive to the proteasome inhibitor bortezomib alone or in combination with the histone deacetylase inhibitor LBH589/panobinostat. (**a**) Bortezomib induces massive cell killing in endometrial cancer cells with *TP53* GOF mutation R175H (KLE cells) but not LOF mutation (Hec50 cells). (**b**) Addition of histone deacetylase inhibitor LBH589 further sensitizes KLE cells with *TP53* GOF to bortezomib.

Studies were also performed in ovarian cancer cell lines with LOF or GOF *TP53* mutations. Consistent with our findings in endometrial cancer cells, OVCAR3 cells with a GOF *TP53* mutation were highly sensitive to bortezomib alone or in combination with HDACi (**Figure 2A**), was much less responsive to bortezomib (**Figure 2B**). OVCAR3 cells contain a different *TP53* GOF mutation, R248Q, than KLE cells, suggesting that this effect is not restricted to the R175H mutation.
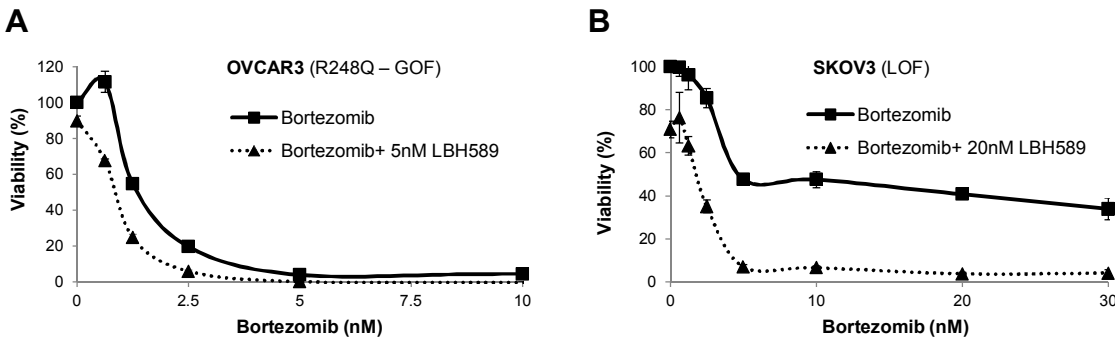

Figure 2. Ovarian cancer cells with GOF *TP53* are highly sensitive to bortezomib alone or in combination with LBH589/panobinostat. Sensitivity (as measured by percent viability) of OVCAR3 cells with R248Q GOF mutant (**a**) or SKOV3 cells with LOF *TP53* mutant (**b**) to bortezomib alone or in combination with LBH589/panobinostat.

Ixazomib (MLN2238) is a next-generation proteasome inhibitor that has replaced bortezomib in the clinic for multiple myeloma due to its improved activity and other characteristics, such as oral bioavailability [10,11]. Therefore, we repeated the above experiments using ixazomib, either alone or in combination with the HDACi LBH589/panobinostat in KLE and OVCAR3 cells that express different *TP53* GOF mutations. Similar to the bortezomib studies, both KLE and OVCAR3 cells responded well to MLN2238/ixazomib (**Figure 3A, B**). Moreover, MLN2238/ixazomib was highly synergistic with the HDACi. In contrast, the p53-null SKOV3 cells were completely resistant to MLN2238/ixazomib at these low doses, with or without addition of the HDACi (**Figure 3C**).
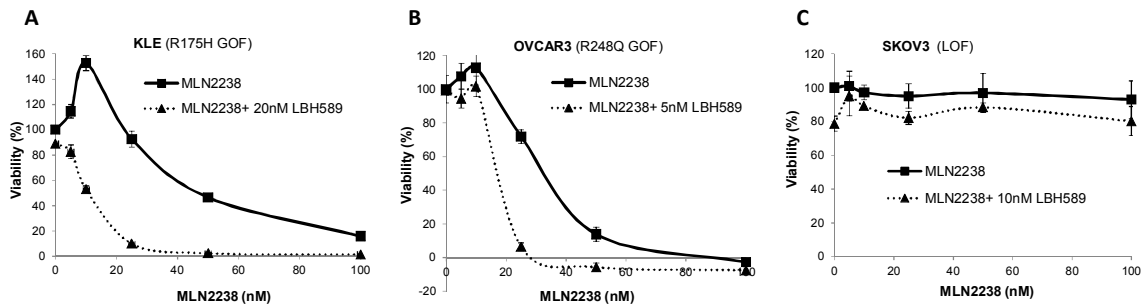
118   **Figure 3. Cells with *TP53* GOF mutants are sensitive to next-generation proteasome inhibitor**
119   **ixazomib/MLN2238.** Sensitivity of KLE cells with R175H GOF mutant (**a**), OVCAR3 cells with R248Q
120   GOF mutant (**b**) or SKOV3 cells with LOF *TP53* mutant (**c**) to MLN2238 alone or in combination with
121   LBH589/panobinostat.

122   *2.3. Exogenous expression of GOF TP53 in p53-null cells sensitizes cells to proteasome inhibitor + HDACi*
123   *therapy*

124   To further address the specific role of *TP53* GOF mutations in response to proteasome
125   inhibitor+HDACi treatment, we introduced the p53 GOF mutant, R175H, in p53-null cells by
126   exogenous expression [12]. As compared to parental cells, expression of p53R175H partially restored
127   sensitivity to MLN2238/ixazomib (**Figure 4A**), and the addition of the HDACi LBH589 to the
128   proteasome inhibitor backbone treatment substantially increased cell death (**Figure 4B**).
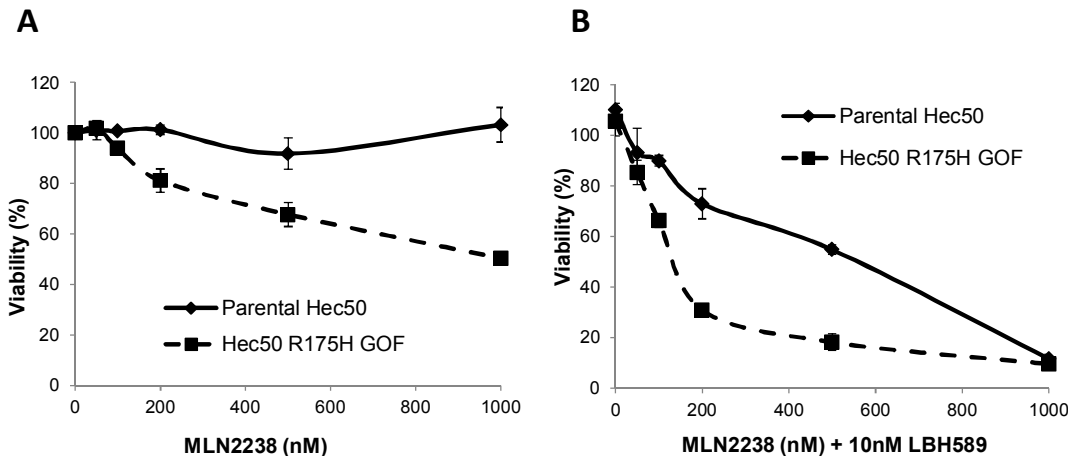

129   **Figure 4. Sensitivity to MLN2283/ixazomib and LBH589/panobinostat combination treatment is**
130   **dependent on expression of GOF *TP53*.** (**a, b**) Sensitivity of Hec50 cells with exogenous R175H GOF
131   mutant to MLN2238 alone (**a**) or in combination with LBH589/panobinostat (**b**).

132   We also attempted to confirm these findings by silencing endogenous p53 using shRNAs in
133   KLE and OVCAR3 cells that express R175H and R248Q GOF mutants, respectively. However,
134   silencing p53 was highly toxic for both cell lines (**Figure S1**). This is likely because these cells are
135   addicted to the GOF mutant for survival.

136   *2.4. Potential mechanisms for increased drug sensitivity in cells with TP53 GOF mutation*

137   Our next objective was to interrogate possible mechanisms by which cells with *TP53* GOF
138   mutations become sensitized to proteasome and HDAC inhibitors.   We found that treatment with
139   MLN2238/ixazomib promoted cleavage of caspase 3, a marker for apoptosis, in KLE and OVCAR3
140   cells, which harbor two different *TP53* GOF mutants (**Figure 5**). Others have shown that proteasome
141   inhibitors induce apoptosis by activating the unfolded protein response (UPR) pathway, a
142   homeostatic mechanism that is normally triggered by accumulation of misfolded proteins in the

143   endoplasmic reticulum [13]. A hallmark of the UPR pathway is increased expression of Bip/GRP78, a
144   chaperone protein that induces proper folding of misfolded proteins such as GOF p53.
145   Immunoblotting our samples for Bip revealed that treatment with MLN2238/ixazomib increased
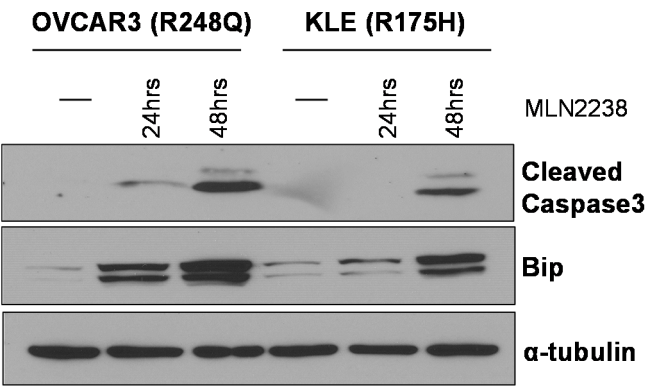146   expression of Bip (**Figure 5**).

147

148   **Figure 5. Treatment with MLN2238/ixazomib promotes apoptosis, potentially through the UPR**
149   **pathway.** Cells were treated for 0, 24 or 48 hrs with MLN2238/ixazomib and cell lysates analyzed by
150   western blotting with the indicated antibodies (*α*-tubulin served as loading control).

151   Mutant p53 has also been shown to interact with histone deacetylases (e.g., HDAC2/6), which
152   contributes to its stabilization and aberrant functions [14,15]. Published evidence suggests that
153   HDACi like LBH589/panobinostat may decrease the stability of mutant p53 [14,15]. Indeed, we
154   found that treatment with LBH589/panobinostat caused a marked decrease in the total protein levels
155   of p53 in KLE cells with the R175H GOF mutant (**Figure 6**). As a control, we also examined p21
156   levels, which are known to be increased following treatment with HDACi regardless of p53
157   expression. Consistent with this notion, LBH589/panobinostat increased p21 in all cell lines
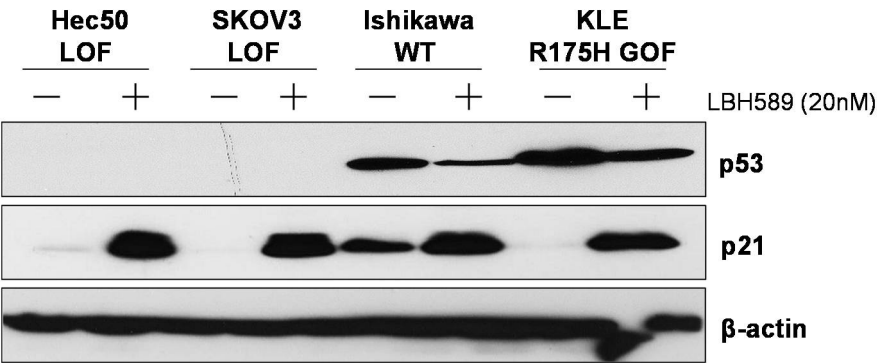158   examined.

159   **Figure 6. Treatment with LBH589/panobinostat reduces p53 protein levels.** Cells lacking p53 (LOF)
160   or expressing the indicated forms of p53 (WT or R175H GOF mutant) were treated with 20 nM
161   panobinostat and levels of the indicated proteins measured by western blotting. B-actin served as a
162   loading control.

3. Discussion

164   Despite clear data in multiple cancer types that *TP53* GOF mutations predict for poor outcomes,
165   including resistance to therapy, to date no clinical trials have tested treatment strategies designed to
166   specifically overcome the effects of *TP53* GOF mutations. In fact, TP53 mutational status is widely
167   ignored when making treatment decisions. Herein we present a novel combinatorial strategy that
168   effectively induces cell death specifically in cancer cells bearing GOF mutations. Of note is that the
169   combinatorial strategy of proteasome inhibitor plus HDACi was highly effective in cells with

170 different recurrent *TP53* GOF mutations. We demonstrate this effect with two different proteasome
171 inhibitors, bortezomib and ixazomib, indicating the potential generality of the approach. The
172 mechanism of cell death upon treatment with proteasome inhibition is induction of apoptosis,
173 whereas HDAC inhibition with panobinostat destabilizes mutant p53 protein levels. These data set
174 the stage for future clinical studies in patients with GOF *TP53* mutations.

175   The cornerstone of personalized medicine is designing treatment strategies that overcome
176 driver mutations in patients. However, mutations in *TP53* are not considered actionable in the
177 traditional sense. One strategy to make *TP53* mutations druggable is based upon the principles of
178 synthetic lethality, the term for an historical genetic observation that in the presence of certain single
179 gene mutations, blocking or mutating a second gene leads to cell death, though neither mutation
180 alone has a phenotype.[16] With respect to cancer therapy, synthetic lethality means capitalizing on
181 the presence of a driver mutation to design novel treatments which block the compensatory survival
182 pathways activated as a result of the mutation. To create therapeutic synthetic lethality, one must
183 first know the driver mutation, understand the compensatory survival pathway that has been
184 activated as a result of the mutation, and have an agent which can block this critical pathway. In a
185 series of published studies, our group has established that treatment with a tyrosine kinase inhibitor
186 (e.g, gefitinib, nintedanib, cediranib) sensitizes p53-null cancer cells to paclitaxel-containing
187 chemotherapy [12,17,18]. The mechanism is through abrogation of the G2/M cell cycle checkpoint.
188 Enforcing the G2/M cell cycle checkpoint allows tumor cells to repair DNA before entering M,
189 leading to chemoresistance [19-25]. Wild-type p53 normally maintains both the G1/S and G2/M
190 checkpoint. However, emerging data suggest that p38MAPK can also maintain the G2/M checkpoint
191 [26-28]. In cells with LOF p53, p38 is activated as an alternative means to maintain the G2/M
192 checkpoint [24]. Therefore treatment with an upstream agent that blocks p38 activation (e.g.,
193 tyrosine kinase inhibitors) sensitizes p53-null cells to paclitaxel, resulting in accumulation of cells in
194 mitosis and massive cell death via mitotic catastrophe [12,17].

195   Unfortunately, this same combinatorial strategy is not effective in cells with GOF p53.
196 Specifically, our published data from cell models with endogenous and exogenous expression of
197 GOF p53 mutants demonstrate that, in contrast to LOF p53, GOF forms of p53 constitutively enhance
198 the G2/M checkpoint and are resistant to paclitaxel + tyrosine kinase inhibitors [12,17]. Others have
199 reported that p53GOF mutants R175H, R273H and R280K aberrantly induce p38 activation via
200 transcriptional activation of MKK3 (an upstream kinase of p38), thereby maintaining the G2/M
201 checkpoint [29]. Other established cancer therapeutics, such as temozolomide and tamoxifen,
202 likewise are ineffective against tumor cells expressing *TP53* GOF mutants due to specific effects of
203 mutant p53 on 06-methylguanine DNA-methyl-transferase (MGMT) and estrogen receptor
204 expression, respectively [30]. Therefore, alternative strategies are necessary to overcome the effects
205 of GOF p53. One approach is to use small molecule drugs to restore the wild-type p53 conformation
206 and thereby restore normal p53 anti-cancer function [30]. Our approach instead takes advantage of
207 the unique properties of GOF p53 mutants, namely aberrant folding and increased stability.

208   Here, we discovered that cells with GOF but not LOF *TP53* mutations are hyper-sensitive to
209 proteasome inhibition, and addition of an HDACi (here, panobinostat) further enhanced cell killing.
210 Both histone deacetylase inhibitors (vorinostat, panobinostat) and proteasome inhibitors
211 (bortezomib, ixazomib) have been extensively studies in preclinical and clinical models of multiple
212 cancer types [31]. Our intent was not to establish novel mechanisms of cell death in p53-GOF cell
213 models, but rather to extend prior findings with unique drug combinations in our cell models of
214 ovarian and endometrial cancer. Studies in multiple myeloma have provided significant mechanistic
215 insight into why proteasome inhibitors are highly toxic to the cancer cells. For example, proteasome
216 inhibition has been shown to promote apoptosis via terminal UPR [13]. Consistent with these data,
217 we found that ixazomib treatment induced caspase 3, a canonical marker of apoptosis, as well as
218 expression of Bip/GPR78, a marker for ER stress. Since p53 GOF mutant protein is a misfolded
219 protein, proteasome inhibition may induce cell death through excessive accumulation of misfolded
220 proteins. Several studies have reported hyperstabilization of GOF p53 protein in cancer [32], which

221   has been postulated to occur through more than one mechanism. First, p53 GOF proteins are unable
222   to bind the E3 ligase Mdm2, which negates the typical pathway of p53 ubiquitination and
223   degradation via the proteasomal pathway [33]. Instead, p53 GOF protein is thought to be degraded
224   by the lysosome in a process termed "chaperone-mediated autophagy" (CMA) [34]. Intriguingly,
225   inhibition of the proteasome results in a compensatory induction of the activity of the CMA pathway
226   [34]. Published evidence suggests that HDACi may decrease the stability of mutant p53 by
227   disrupting its association with heat shock proteins [14,15]. In addition, mutant p53 has been shown
228   to interact with histone deacetylases, which contributes to its stabilization and aberrant functions
229   [14,15]. Therefore, one possibility is that HDACi potentiates the effects of the proteasome inhibitor
230   by removing components of the chaperone complex, improving uptake in the lysosome and leading
231   to CMA-mediated p53 GOF degradation. Supporting this notion, we observed decreased total p53
232   protein levels via treatment with panobinostat. In addition, histone deacetylase inhibitors have been
233   shown to induce cell cycle arrest at the G1/S transition via upregulation of p21, which we also
234   demonstrate in cells with both WT (Ishikawa cells) and p53 GOF mutant (KLE, R175H p53). Whereas
235   early studies (PMID: 12531799) with vorinostat suggested that G1/S cell cycle arrest was also
236   accomplished through upregulation of p53, others have established that HDACi treatment
237   destabilizes mutant p53, resulting in a marked decreased in p53 levels (PMID: 8710879). Our data are
238   in line with the latter findings whereby treatment with LBH589/panobinostat resulted in a 50% or
239   greater decrease in both GOF p53 (KLE cells) and WT p53 (Ishikawa cells).

240   Multiple myeloma is typified by accumulation of high levels of immunoglobulin, and thus the
241   cells are extremely dependent upon proteasomal pathways for survival. We speculate that GOF p53
242   mutants create a similar "prion-like" scenario that also necessitates a functional proteasome to
243   maintain cell survival. Indeed, it has been suggested in the literature that excessive accumulation of
244   mutant p53 may be more sensitive to proteasome and/or histone deacetylase inhibition [35].
245   Inhibiting the proteasome pathway would create a reliance on the lysosomal pathway for degrading
246   the mutant p53, which is recognized as a misfolded protein. In line with this concept, we observed
247   increased expression of Bip, a marker of the misfolded protein response pathway.

248   It is probably that distinct GOF mutations may differentially affect sensitivity to the single or
249   combinatorial treatment regimen presented in this manuscript. In contrast to these findings, other
250   studies have provided evidence that bortezomib sensitivity is dependent on wild-type p53
251   expression, whereby apoptosis is induced through p53-mediated downregulation of the
252   pro-survival factor, survivin [36,37]. Cells that express a mutant p53 or p53-null cells were found to
253   be resistant to bortezomib-induced apoptosis through sustained expression of survivin [37].
254   However, the specific GOF p53 mutants included in a previous study were R280K (MDA-MD-231
255   breast cancer cells) and E285K in RPMI-8226 multiple myeloma cells. While our data show similar
256   results using cells expressing the R175H (Hec50 endometrial cancer cells) and R248Q (OVCAR3
257   ovarian cancer cells) mutants, a comprehensive analysis of cells expressing other recurrent p53 GOF
258   mutants is warranted.

259   One potential interpretation of the differential sensitivity of cells with loss-of-function (LOF) vs.
260   gain-of-function (GOF) p53 is that the cells proliferate at different rates. Thus, cells that are less
261   sensitive to proteasome inhibitor alone (i.e., Hec50 cells with LOF p53) would be anticipated to have
262   a slower doubling time as compared to sensitive cells (i.e., KLE cells with GOF p53). However, we
263   have found that Hec50 cells have a much shorter doubling time and proliferate three times faster
264   than KLE cells. Moreover, expression of the R175H GOF p53 mutant in Hec50 cells did not markedly
265   affect the proliferation rate yet sensitized cells to drug treatment.

266   In summary, we present a novel therapeutic strategy for tumors with GOF *TP53* mutations
267   using drugs that are already being advanced in clinical trials. These data suggest that p53 mutational
268   status can be used as the foundation for defining personalized treatments.

269

4. Materials and Methods

Reagents. All antibodies were purchased from Cell Signaling. Bortezomib, LBH589 (panobinostat), and MLN2238 (ixazomib) were purchased from Selleck Chemicals and suspended in DMSO.

Cell lines and culture conditions. All cell lines used in this study were purchased from ATCC, except for Hec50 endometrial cancer cells that were kindly provided by Dr. Erlio Gurpide (New York University) as previously described [12]. Hec50 cells expressing R175H *TP53* GOF have been previously described [12]. All cell lines have been authenticated using STR analysis by BioSynthesis.

Cell viability assays. Beginning 24 h after plating equal numbers of cells, cells were treated for 72 h followed by assessment of cell viability using the Wst-1 assay per manufacturer's instructions (Clontech). Data were quantitated relative to values obtained for control (untreated) cells, which was set at 100% viability.

Western blot analysis. As previously described [12], cells were plated in 100 mm dishes and were allowed to grow for 24 h prior to treatment. After treatment, cells were harvested, lysed with extraction buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 20 μg/ml aprotinin, 1 mM PMSF, and 2 mM Na_3VO_4), and subjected to three freeze/thaw cycles. Equal amounts of protein (determined by the method of Bradford, BioRad) were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes (BioScience). Membranes were probed with primary antibodies against cleaved caspase 3, Bip, α-tubulin, p53, p21 or β-actin followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibody. The signal was visualized by chemiluminescence using ECL Western blotting detection reagents (Pierce).

Author Contributions: X.M., S.Y., K.W.T., and K.K.L. conceived and designed the experiments; X.M., S.Y., Y.L., and Y.L. performed the experiments; X.M., E.J.D., D.E.Q., K.W.T., and K.K.L analyzed the data; X.M., K.W.T., D.E.Q., and K.K.L. wrote the paper.

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