Synthetic Lethal Approaches Exploiting DNA Damage in Aggressive Myeloma

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ABSTRACT

Ongoing DNA damage is a common feature of epithelial cancers. Here, we show that tumor cells derived from multiple myeloma, a disease of clonal plasma cells, demonstrate DNA-replicative stress, leading to DNA damage. We identified a poor-prognosis subset of multiple myeloma with extensive chromosomal instability and replicative stress, which rely on ATR to compensate for DNA-replicative stress; conversely, silencing of ATR or treatment with a specific ATR inhibitor triggers multiple myeloma cell apoptosis. We show that oncogenes, such as MYC, induce DNA damage in multiple myeloma cells not only by increased replicative stress, but also via increased oxidative stress, and that reactive oxygen species-inducer piperlongumine triggers further DNA damage and apoptosis. Importantly, ATR inhibition combined with piperlongumine triggers synergistic multiple myeloma cytotoxicity. This synthetic lethal approach, enhancing oxidative stress while concomitantly blocking replicative stress response, provides a novel combination targeted therapy to address an unmet medical need in this subset of multiple myeloma.

SIGNIFICANCE: Multiple myeloma remains an incurable disease. We have identified a subset of multiple myeloma patients with poor prognosis, whose tumors present chromosomal instability, replicative and oxidative stress, and DNA damage. We define a synthetic lethal approach enhancing oxidative stress while targeting replicative stress response, inducing tumor cell apoptosis in this patient subset. Cancer Discov; 5(9):972–87. © 2015 AACR.

INTRODUCTION

Multiple myeloma is a clonal proliferation of malignant plasma cells. The genome of multiple myeloma cells is remarkably complex with profoundly altered karyotypes, including aneuploidies, chromosomal translocations, and frequent DNA copy-number variations (1–3). The causes underlying these extensive genomic rearrangements in multiple myeloma remain largely unknown. We and others have recently shown that hematologic cancers, including multiple myeloma, have constitutive ongoing DNA damage, evidenced by high number of phospho-H2AFX (γH2A.X) foci in their nuclei (4, 5). As a result, the DNA damage response is activated, with phosphorylation of ATM and CHK2, as well as ATR and CHK1. These findings suggest that the complex karyotypes in multiple myeloma cells may result from ongoing intrinsic DNA damage in multiple myeloma cells.

The mechanisms responsible for DNA damage in hematologic cancers remain unclear. In epithelial cancers, activated oncogenes elicit double-strand breaks (DSB) and ultimately genomic instability (6–10). Classic studies on instability arising from overexpression of mutated HRAS (11) and MYC (12) support this notion. A recent model in epithelial cancers proposed that activated oncogenes trigger inordinate DNA replication, thereby leading to replicative stress (6, 8, 9, 13), which results in DNA DSBs. For example, MYC interacts with the prereplicative complex and localizes in proximity of replication origins early in S phase; when overexpressed, it increases replication due to unscheduled origin activation (14). Through CDC45, MYC overexpression reduces interorigin distances, independent of its transcriptional activity (15). In addition, oncogenes can upregulate components of the prereplicative complex (pre-RC) and the replication machinery, including CDC6 and members of the origin recognition complex (ORC) and mini chromosome maintenance complex (MCM) families. These proteins are frequently overexpressed and amplified in various cancers, associated with a poor prognosis, and behave as oncogenes when overexpressed both in vitro and in vivo (7, 16).

Oncogene-induced replicative stress also triggers a DNA damage response and senescence (7), and cancer cells overcome these suppressive responses via several compensatory mechanisms. For example, tumors frequently inactivate the PI3K-related protein kinase (PIKK) ATM and its downstream target TP53 (p53), thereby abrogating apoptosis after DSBs. However, the relevance of the inactivation of the p53 pathway in hematologic cancers is unclear, because we have recently shown that hematologic cancers preferentially activate an alternative pathway in response to DNA damage. Specifically, after ATM activation, the serine-threonine kinase ABL1 relocalizes to the nucleus, where it interacts with the Hippo cofactor YAP1 and the tumor suppressor TP73 to induce apoptosis. Importantly, a subset of hematologic malignancies...
genetically or functionally disables YAP1, thereby preventing apoptosis (4).

Surprisingly, ATR, the other major PIKK, has an opposite effect from ATM, because it is required for survival of cancer cells under conditions of increased DNA damage (17). Unlike ATM, ATR is activated during S phase and regulates firing of replication origins and repair of damaged replication forks. Indeed, during replicative stress, single-stranded DNA (ssDNA) increases, which is coated by the ssDNA-binding replication protein A (RPA2, also known as RPA32), thereby activating ATR and its main downstream target, CHK1. As a result, replication forks are stabilized and stalled forks resolved, in order to assure completion of replication. If the resolution of the stalled forks does not succeed, ssDNAs evolve into DSBs, followed by ATM and H2AX phosphorylation and apoptosis.

An intact ATR–CHK1 pathway is crucial for the survival of tumor cells in vivo (18), especially in the presence of activated oncogenes. For instance, Myc transgenic mice develop B-cell lymphomas with ATM activation (19) and intense replicative stress, as well as ATR and CHK1 phosphorylation (20). Remarkably, crossing the Myc transgenic mice with a hypomorphic ATR mouse strain (Atr-Seckel; Atr–/–; ref. 21) prevents this development of lymphoma (18). ATR also suppresses MYC-induced replicative stress and apoptosis. Accordingly, treatment with CHK1 inhibitors (UCN-01 or SB-218078) was highly effective in human cancer cell lines and tumors from mice overexpressing MYC, especially in a TAp53-null background. Similar results can be observed in tumors with replicative stress due to dysregulation of other oncogenes, including members of the RS family and MLL-ENL (22), although the effect of mutated RAS might be context-dependent (18). Importantly, ongoing replicative stress may allow specific targeting of cancer cells in a synthetic lethal approach, because reduction of ATR levels induces apoptosis only in tumor cells, with no apparent effects on normal cells. These studies have provided the rationale for the development of ATR inhibitors for the treatment of cancers with enhanced replicative stress (22-24).

In addition, oxidative stress, an imbalance between the production and elimination of free radicals and reactive metabolites [so-called reactive oxygen species (ROS)] of mitochondrial origin, can lead to DNA damage and promote neoplastic transformation. ROS can also activate cellular proliferation via AKT activation or modulate the expression and enzymatic activity of the DNA mismatch repair genes (25). Interestingly, mutated RAS (26), MYC (27), and cyclin E have been reported to increase ROS (28) without eliciting replicative stress (29, 30), and therefore represent an independent manner to induce DNA damage in cancer.

Several oncogenes have been associated with multiple myeloma pathogenesis. MYC in particular is overexpressed in multiple myeloma, and it has been proposed that low-level overexpression of MYC drives the transition from precursor stages to active multiple myeloma. In addition, genomic rearrangements directly targeting the MYC locus occur during disease progression, resulting in further upregulation of MYC expression levels (31). In this study, we delineated mechanisms underlying constitutive ongoing DNA damage in multiple myeloma and identified a novel synthetic lethal approach to target these cells and induce their apoptosis.

RESULTS

Intense Replicative Stress in Multiple Myeloma Cell Lines with Constitutive DNA Damage

To obtain insights into the mechanisms underlying widespread DNA damage in multiple myeloma cells, we first compared gene expression profiles of multiple myeloma cell lines with ongoing DNA damage with those without this phenotype (4) using Gene Set Enrichment Analysis (GSEA; ref. 32). Remarkably, examination of the Kyoto Encyclopedia on Genes and Genomes (KEGG) repository revealed that the DNA replication pathway was the most significantly enriched in multiple myeloma cells with enhanced DNA damage (normalized enrichment score (NES) = 1.89, 
P < 0.0001, FDR < 0.014; Fig. 1A; Supplementary Table S1). Indeed, oncogene-induced replicative stress is often associated with enhanced expression of components of the replication machinery (7). Cell-cycle regulatory genes were also significantly altered in these cells (NES = 1.92, 
P < 0.0001, FDR = 0.047), suggesting an association between enhanced proliferation and replicative stress in multiple myeloma cells.

To explore whether ongoing DNA damage in multiple myeloma cells led to increased replicative stress, we next assessed a large panel of replicative stress markers, including TP53BP1 (S3BP1), RPA32, RAD9A, and RAD51. Multiple myeloma cell lines (H929, MM.1S, and RPMI/8226) all demonstrated markers of replicative stress (Fig. 1B and C; Supplementary Figs. S1A and S1B; S2A and S2B) that were absent in peripheral blood mononuclear cells (PBMC; Supplementary Fig. S3) or only minimally expressed in multiple myeloma cell lines without DNA damage.

Moreover, we found a significant colocalization of γ-H2A.X with phospho-histone H3 (Fig. 1D and Supplementary Fig. S4), a marker of proliferation phosphorylated only during mitosis (33), further indicating that cells with DNA damage have activated replicative capacity. We then explored whether replicative stress was also evident in patient multiple myeloma cells. As shown in Fig. 1E and Supplementary Fig. S5, patient multiple myeloma cells showed signs of replicative stress, evidenced by increased expression of MYC and RAD51 positivity, as well as by colocalization of γ-H2A.X and phospho-histone H3. Taken together, these results indicate that multiple myeloma cells with DNA damage also have increased replicative stress.

A Signature of Chromosomal Instability Defines a Subset of Multiple Myeloma Patients with Increased Expression of Replicative Genes and Poor Prognosis

We next sought to determine whether patient multiple myeloma cells also show DNA damage associated with replicative stress. To this end, we examined a widely used gene expression signature (34) to identify patients characterized by increased chromosomal instability and DNA damage, and then applied this signature to our published multiple myeloma gene expression profiling (GEP) data, which include healthy as well as patient-derived plasma cells (1). Remarkably, a subset of approximately 20% multiple myeloma patients demonstrated overexpression of the probesets belonging to the chromosomal instability signature (Fig. 2A). In contrast, plasma cells derived from healthy individuals consistently showed the lowest level of expression of these probesets.
probesets. We next extended our analysis to a GEP set that includes 559 multiple myeloma patients (35). K means clustering sharply divided these patients into two groups, including 96 patients (17%) with overexpression of the instability signature (K2; Fig. 2B). We then compared and contrasted this subset with the remaining K1 patients using GSEA. As in multiple myeloma cell lines with DNA damage, GSEA in the KEGG repository revealed that DNA replication was the most significantly altered pathway in the K2 subgroup, compared with the remaining patients (NES = 2.49, $P < 0.0001$, FDR $< 0.0001$; Fig. 2C and D and Supplementary Table S2); the cell-cycle pathway also significantly differed in these patient subgroups (NES = 2.20, $P < 0.0001$, FDR $= 0.003$; Fig. 2C).

We similarly explored the BioCarta dataset and identified the pathway centered on ATM to be among the most significantly dysregulated in the K2 patient subgroup (NES = 1.84, $P < 0.006$, FDR $= 0.05$), again with significant differences in the cell-cycle pathway as well (Fig. 2C; Supplementary...
Figure 2. Definition of a multiple myeloma patient subset with increased genomic instability, enhanced expression of DNA replication and cell-cycle genes, and poor prognosis. A, expression levels in plasma cells derived from healthy individuals and patients with multiple myeloma for the probesets corresponding to the chromosomal instability signature described in ref. 34. B, heatmap showing the K-means clustering (clusters = 2) of 559 multiple myeloma patient GEP data using the probesets corresponding to the chromosomal instability signature described in ref. 34. C, heatmap in the K1 and K2 groups showing the expression levels corresponding to probesets included in the DNA replication (top) and cell-cycle (bottom) KEGG pathways as annotated in MSigDB. D, GSEA enrichment score profiles for 559 patients divided in K1 and K2 groups of KEGG DNA replication (left) and BioCarta ATM (right) pathways as annotated in MSigDB. E, Kaplan–Meier survival curves of K1 and K2 patients (414 patients analyzed).

Table S3). We next sought to identify any potential association with prognosis and found that patients in the K2 group demonstrated a poor prognosis when compared with the other patients (P < 0.0001; Fig. 2E; and Supplementary Table S4). Importantly, in a multivariate analysis, this instability signature was independent from other poor prognostic criteria, including the proliferation signature and the presence of MMSET/FGFR3 or MAF translocations (ref. 35; Supplementary Table S5). These results therefore indicate that there is a subset of multiple myeloma with genomic instability associated with replicative stress, ATM activation, and active proliferation, and that patients in this subgroup have a poor prognosis.

MYC Triggers Replicative Stress and DNA Damage in Multiple Myeloma Cells

One of the mechanisms by which mutated and dysregulated oncogenes induce DNA damage is via induction of replicative stress (6, 8, 9, 13). Because the oncogene MYC can induce replicative stress (14, 15) and plays a prominent role in multiple myeloma pathogenesis (31), we next explored its expression in the K2 patient subgroup overexpressing genes included in the chromosomal instability signature. MYC was significantly upregulated in the K2 subgroup (Fig. 3A), suggesting its potential role in replication stress and DNA damage in this multiple myeloma subset. We therefore next...
Figure 3. MYC is involved in DNA damage and replicative stress in multiple myeloma. A, MYC expression levels in K1 (blue) and K2 (red) patient groups. B, U266 multiple myeloma cells were transiently transfected with MYC-EGFP or LACZ-EGFP. Lysates were obtained at 48 hours after transfection and assayed for Western blot analysis using antibodies against MYC, γ-H2A.X, pATM, and GAPDH. DAPI is used as loading control. C, immunofluorescence staining for γ-H2A.X at 48 hours from transfection. Right, number of γ-H2A.X foci in U266 MYC-EGFP cells in comparison with LACZ-EGFP cells at 48 hours. D, replicative stress markers by Western blot analysis and immunofluorescence evaluated at 48 hours. Left, Western blot analysis for pRPA32, RAD51, pATM, MYC, and GAPDH in U266 MYC-EGFP and U266 LACZ-EGFP transfected cells. Right, RPA32 and RAD51 immunofluorescence staining in U266 MYC-EGFP cells versus U266-LACZ-EGFP cells. E, number of RPA32, RAD51, and RAD9A foci in U266 MYC-EGFP cells in comparison with LACZ-EGFP cells. F, MM.1S cells were silenced for MYC using shRNAs #3 and #5 and a vector including a scrambled sequence. Western blot analysis was performed on lysates obtained at 48 hours after transfection using antibodies against MYC, γ-H2A.X, pATM, pCHK2, and GAPDH. G, immunofluorescence to detect γ-H2A.X foci was performed in MM.1S and H929 48 hours after transfection with scrambled or shRNAs against MYC. Number of γ-H2A.X foci is shown on the right from 2 representative experiments and 10 different cells. H, replicative stress markers in MM.1S and H929 silenced for MYC by Western blot analysis (left) in H929 cells and by immunofluorescence with foci count (right). **, P < 0.001; *** P < 0.0001 (Student's t test).
determined whether MYC overexpression triggers replicative stress in multiple myeloma cells. As a gain-of-function model, we chose the U266 multiple myeloma cell line, which expresses MYCL (L-MYC) and does not have either genomic rearrangements affecting the MYC locus nor MYC overexpression (36). Despite a reported role for L-MYC in inducing DNA damage (14), we were not able to detect ongoing DNA damage in U266 cells, evidenced by the lack of γ-H2A.X foci or of markers of DNA damage response activation, including phosphorylated (p) ATM and pATR, and their downstream effectors pCHK2 and pCHK1, respectively (ref. 4; Fig. 3 and data not shown). The overexpression of MYC in U266 cells triggered an increase of γ-H2A.X, as assessed by Western blotting (Fig. 3B). γ-H2A.X foci also appeared after MYC overexpression, detected by immunofluorescence (from 1.75 ± 1.7 to 19.0 ± 5.2, on average, \( P = 0.0052 \); Fig. 3C). These changes were associated with activation of the DNA damage response, because both pATM and pCHK2 were increased after MYC overexpression (Fig. 3B).

We next explored whether the increased DNA damage triggered by MYC overexpression was associated with enhanced replicative stress. We first assayed for ssDNA, typically associated with stalled DNA replication forks, both by immunofluorescence using antibodies specific for RPA32 and RAD51 and by Western blotting (Fig. 3D and E). RPA32, RAD51, and RAD9 all increased after MYC overexpression. Phosphorylation of ATR also increased, further confirming the notion that overexpression of MYC in multiple myeloma cells induces DNA-replicative stress that in turn leads to enhanced DNA damage.

To further demonstrate the link between MYC overexpression and replicative stress with DNA damage, loss-of-function studies were next undertaken. In this case, we chose two multiple myeloma cell lines, MM1.S and H929, with robust MYC expression and γH2A.X staining. To reduce MYC levels, we chose two shRNA constructs targeting MYC, based on their ability to decrease MYC expression (Fig. 3F). Upon MYC knockdown, a reduction of γ-H2A.X staining, assessed by Western blot and immunofluorescence, was evident, indicating a concurrent reduction in DNA damage upon MYC downregulation (Fig. 3F and G). We further explored the effects of MYC downregulation on markers of DNA damage activation, and found that pATM and pCHK2 levels were greatly reduced. To assess the effect of MYC downregulation on markers of replicative stress, we carried out both immunofluorescence and Western blotting: a marked reduction in replicative stress markers, including RPA32, RAD51, and pATR, was observed in both MM1.S and H929 multiple

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**Figure 4.** ATR modulation by shRNAs or using a specific inhibitor (VE-821) reduces multiple myeloma cell growth. A, H929 multiple myeloma cells (left plots, blue) and OPM-2 multiple myeloma cells (right plots, red) were transiently transfected with an shRNA against ATR or with a scrambled shRNA. Data derived from two independent experiments in triplicate are reported. For each cell line, cellular growth is shown on the left and apoptosis on the right. Cellular growth is measured by cell count with trypan blue exclusion at days 0 to 3, whereas apoptosis is evaluated by Annexin V–PE/7-Aminoactinomycin D (7-AAD) on GFP-gated cells at 48 hours. B, left, immunofluorescence staining for γ-H2A.X foci was performed in H929 and OPM-2 cells silenced with scrambled and ATR shRNAs at 48 hours. Right, number of γ-H2A.X foci with average ± SD is reported. C, lysates were obtained at 48 hours after transfection. Western blot analysis using antibodies against ATR, γH2A.X, and GAPDH (as loading control) was used. (continued on following page)
Targeting DNA Damage in Aggressive Myeloma

**ATR Is Required for Survival of MYC-Overexpressing Cells**

To mend stalled forks and prevent the apoptotic response triggered by replicative stress, epithelial cancer cells require ATR and its downstream target CHK1. Recent evidence suggests that ATR may play a similar role in hematologic cancers. Indeed, ATR inactivation prevents MYC-induced lymphomas (18). Notably, our K2 multiple myeloma patient subset demonstrated significant upregulation of genes belonging to the ATR pathway, suggesting that these tumors rely on activated ATR for survival (Supplementary Fig. S6A and S6B; Supplementary Table S3). We therefore next assessed whether ATR is required for survival of multiple myeloma cells. To this end, we downregulated ATR in H929 and OPM-2 multiple myeloma cells using a specific shRNA. ATR knockdown significantly reduced viable cell count and increased apoptosis in both cell lines, assessed with Annexin V-propidium iodide (PI) staining (Fig. 4A). ATR knockdown was also associated with enhanced DNA damage, as evidenced by increased γH2A.X levels both by immunoﬂuorescence (Fig. 4B) and Western blotting (Fig. 4C).

A potent and selective small-molecule ATR inhibitor, VE-821, has been recently described (37), which we next used to determine whether pharmacologic inhibition of ATR could trigger apoptosis in multiple myeloma cells. As anticipated, U266 cells were only modestly sensitive to the drug (Fig. 4D); however, other multiple myeloma cells with ongoing DNA damage responded to VE-821 treatment, as evidenced by reduced viability and increased apoptosis (Fig. 4D). Consistent with previous results showing that cells without p53 are more sensitive to ATR inhibitors (18, 24), TP53-mutant multiple myeloma cell lines (OPM-1, OPM-2, and RPMI/8266) showed greater response to VE-821 than p53 wild-type multiple myeloma cell lines (MM.1S and H929). Moreover, treatment with VE-821 induced PARP cleavage and increased γH2A.X (Fig. 4E). To determine the potential role of MYC-induced replicative stress in this phenotype, we re-expressed MYC in U266 cells. Remarkably, U266 cells became more sensitive to VE-821 after MYC re-expression (Fig. 4F and Supplementary Fig. S6C). In contrast, silencing of MYC in MM.1S and H929 multiple myeloma cells blocks the effect of VE-821 treatment (Fig. 4G). These results suggest that inhibition of ATR can selectively target the subset of multiple myeloma with increased replicative stress and ongoing DNA damage.

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Figure 4. (Continued) D, left, MTT viability assay was used to evaluate growth-inhibitory effects upon incubation for 72 hours with VE-821, with doses ranging from 0 to 10 μmol/L. Right, apoptosis was measured with Annexin V–FITC/PI staining, upon incubation with DMSO, 1 μmol/L, and 2.5 μmol/L for 48 hours. Data are obtained from two independent experiments, performed in triplicate. E, Western blot analysis for total PARP, γH2A.X, and GAPDH after treatment with DMSO, 1 μmol/L, and 2.5 μmol/L for 48 hours. F, U266 cells were transfected with MYC-EGFP and LACZ-EGFP and then treated with 5 μmol/L VE-821 for 48 hours, starting from day 0 of transfection. Annexin V–PE/7-AAD staining was used to measure the percentage of dead cells. G, H929 and MM.1S myeloma cells were silenced with shRNAs targeting MYC and scrambled and then treated with 2.5 μmol/L VE-821 for 48 hours. Apoptosis was evaluated as in F. ***P < 0.001; ****P < 0.0001 (Student t test).
Genes Associated with ROS Are Dysregulated in the K2 Multiple Myeloma Patient Subgroup

In a few cellular settings, oncogenes, such as MYC, have been shown to enhance DNA damage through enhanced production of ROS (27, 30). Although oncogene-induced DNA damage in epithelial cancer cells has been primarily attributed to increased replicative stress (29), a recent report in glioblastoma multiforme showed that MYC and RAS can elicit DNA damage through both enhanced replicative stress and increased ROS production, depending on the cellular context (38). High levels of ROS in the setting of oxidative stress reflect an imbalance between production and termination of reactive species.

To determine whether pathways involved in ROS metabolism are dysregulated in multiple myeloma patients, especially in the subset with enhanced DNA damage, we first determined whether the K2 patient group demonstrated dysregulated expression of genes implicated in oxidative stress. To this end, we compiled a list of pathways implicated in ROS and nitrous oxide (NOS) metabolism collected from the Molecular Signature Database (MSigDB; ref. 32; Supplementary Table S6). Remarkably, in the K2 group, there was a highly significant enrichment for pathways related to ROS metabolism, including Mootha_Mitochondria, Kegg_Oxidative Phosphorylation, and Mootha_Voophos (NES = 2.23, 2.04, and 1.90; P < 0.0001, P = 0.002 and 0.006; and FDR < 0.0001, FDR = 0.002 and 0.014, respectively; Fig. 5A and data not shown). In fact, most genes within these pathways were upregulated in the K2 compared with the K1 multiple myeloma subgroups (Supplementary Fig. S7A–S7C). Notably, at the gene expression level, NOS signaling was not enriched in this patient subset, and inducible NOS was barely detectable in the tested multiple myeloma cell lines (data not shown). Taken together, these results indicate that the K2 multiple myeloma subgroup demonstrates replicative stress, as well as activation of pathways implicated in generation and metabolism of ROS.

MYC Induces Oxidative Stress in Multiple Myeloma Cells

We next sought to test whether the oncogene MYC can elicit ROS in multiple myeloma cells. To this end, we first tested the levels of both free-radical ROS (superoxide) and nonradical ROS (hydrogen peroxide). Overexpression of MYC in U266 cells increased both superoxide and hydrogen peroxide levels (Fig. 5B), suggesting that MYC regulates ROS levels in multiple myeloma cells. Conversely, downregulation of MYC in MM.1S and H929 multiple myeloma cell lines reduced these ROS levels (Fig. 5B and data not shown). Notably, MYC overexpression did not increase levels of nitrogen oxide (NO) species, unlike the NO inducer L-arginine, which bly, MYC overexpression did not increase levels of nitrogen oxide (NOS inhibitor, L-NG-Nitroarginine Methyl Ester (L-NAME), which scavenges ROS by replenishing glutathione stores. NAC reduced DNA DSBs, assessed by γ-H2A.X staining (Fig. 5E and Supplementary Fig. S8B). Interestingly, NAC did not completely block the formation of DNA DSBs in the presence of MYC, and a significant increase in γ-H2A.X foci remained after MYC overexpression even in the presence of NAC. We next explored the effects of NAC treatment on multiple myeloma cell lines with replicative stress. NAC markedly reduced γ-H2A.X in multiple myeloma cells (Supplementary Fig. S8C), and concomitant downregulation of MYC further decreased its levels (Fig. 5F). Notably, NAC treatment did not affect replicative stress, assessed by the number of RPA32, RAD51, and 53BP1 foci (Fig. 5G), and minimally reduced the efficacy of VE-821 treatment (Supplementary Fig. S8D).

In addition, neither VE-821 nor an ATM inhibitor, Ku55933, increased ROS (Supplementary Fig. S8E). These data indicate that DNA damage in multiple myeloma cells induced by MYC through ROS is not mediated by and is independent of replicative stress (Fig. 5G). Because reactive nitrogen species (RNS) can also be important in inducing DNA DSBs, we treated U266 and H929 cells with an RNS inducer, L-arginine, and an NOS inhibitor, L-NG-Nitroarginine Methyl Ester (L-NAME), respectively. Although we observed modulation of γ-H2A.X levels (Supplementary Fig. S8F), the effects of RNS in inducing DNA damage are likely independent of MYC, because MYC overexpression was not modulating RNS levels (Supplementary Fig. S8A). Altogether, these results demonstrate that MYC elicits DNA damage in multiple myeloma cells both by inducing DNA-replicative stress and by increasing ROS levels.

MYC Modulates Antioxidant Pathways

Elevated oxidative stress is often present in cancer, and strong evidence links ROS to carcinogenesis. Conversely, other data indicate that ROS increase in cancer cells as a byproduct of the tumorigenic process and that excessive ROS may be detrimental to cancer growth (39). As such, cancer cells tightly regulate ROS levels via various antioxidant pathways (40). Glutathione and thioredoxin are among the main mechanisms involved in ROS detoxification. We therefore next tested whether MYC overexpression was able to modulate the levels of the enzymes involved in glutathione production, including glutamate-cysteine ligase, catalytic subunit (GCLC), and glutamate-cysteine ligase complex modifier subunit (GCLM). MYC overexpression increased mRNA levels of both enzymes; conversely, MYC downregulation reduced their mRNA levels (Fig. 6A). MYC similarly modulated the levels of thioredoxin,
MYC induces oxidative stress–mediating DNA damage in multiple myeloma cells. **A**, GSEA enrichment score profiles for 559 patients divided in K1 and K2 groups of MOOTHA_MITOCHONDRIA (left), MOOTHA_OXIDATIVE PHOSPHORYLATION (middle), and MOOTHA_VOXPHOS (right) pathways as annotated in MSigDB. **B**, U266 cells were transfected with MYC-EGFP and LACZ-EGFP; superoxide levels were detected by immunofluorescence (left), whereas ROS levels were evaluated by flow cytometry at 48 hours. **C**, tetramethylrhodamine, ethyl ester (TMRE) staining to detect active mitochondria upon MYC silencing in MM.1S and H929 or overexpression of MYC in U266 cells. Cells were gated to exclude Annexin V–FITC-positive cells. Western blot analysis using antibodies against RPA32, RAD51, and RAD9A foci is shown. **NS**, P < 0.001; ***, P < 0.0001 (Student t test).
APE1 (important in hydrogen peroxide–induced oxidative stress in the mitochondria), and BACH1, which blocks oxidative stress–induced senescence either directly or through nuclear factor (erythroid-derived 2)–like 2 (NRF2) (Fig. 6B). We therefore next tested glutathione and thioredoxin activity after MYC modulation. MYC overexpression increased, whereas MYC downregulation decreased, glutathione and thioredoxin activities, indicating that MYC regulates ROS levels through the modulation of these proteins (Fig. 6C).

To test the hypothesis that multiple myeloma cells tightly control ROS levels and that unrestrained ROS may be detrimental to multiple myeloma cells, we overexpressed MYC in U266 cells, in the presence of NAC. Remarkably, the addition of NAC increased MYC-induced proliferation (Fig. 6D), which was not rescued by L-NAME (Supplementary Fig. S9A). These results suggest that MYC both increases and at the same time stringently controls ROS levels, to prevent ROS-mediated apoptosis in multiple myeloma cells.
Targeting DNA Damage in Aggressive Myeloma

Enhanced Oxidative Stress Promotes Multiple Myeloma Cell Toxicity

Prompted by these data, we reasoned that increasing ROS levels in multiple myeloma cells could further promote DNA damage and induce apoptosis. Indeed, novel compounds have recently been described that exploit induction of ROS for treatment of cancer (41). Specifically, a recent cell-based small-molecule screening identified piperlongumine (PL) as a compound that increases ROS levels, thereby selectively inducing apoptosis in cancer cells while sparing normal cells (42). We therefore next tested whether further increasing ROS levels in multiple myeloma cells could enhance DNA damage and induce cell death. Indeed, treatment of U266 cells for a short period (30 minutes) with PL triggered a surge in ROS levels (Fig. 6E), which was enhanced by MYC overexpression and abrogated by treatment with NAC. PL also dramatically increased apoptosis and decreased proliferation, both of which were surprisingly enhanced by MYC overexpression (Fig. 6F). Indeed, PL induced DNA DBSs, which were further increased by MYC overexpression (Fig. 6G). As expected, PL induced a robust increase of both ROS and superoxide levels in multiple myeloma cell lines (Supplementary Fig. S9B). Most multiple myeloma cell lines were sensitive to PL; OPM2 cells were least sensitive, whereas U266 were the most sensitive to PL (Fig. 6H). To confirm that the activity of PL was mediated by an increase in ROS, H929 and OPM2 cells were treated with PL, with or without NAC. Indeed, concomitant treatment of multiple myeloma cells with PL and NAC significantly reduced the apoptotic effects of PL (Fig. 6I and Supplementary Fig. S9C), whereas L-NAME did not affect the activity of PL (Supplementary Fig. S9D). Finally, we assessed the overall effects of PL on apoptosis and DNA damage. PL significantly induced PARP cleavage, as well as increased γH2A.X and p21 levels, in both H929 and OPM-2 multiple myeloma cells (Fig. 6J). We then tested the effect of arsenic trioxide (ATO), a well-known inducer of ROS. Indeed, ATO increased ROS/superoxide levels and γH2A.X (Supplementary Fig. S10A and S10B). Importantly, in our study, PL was more potent in terms of growth inhibition than ATO, despite inducing lower levels of ROS and superoxide dismutase (SOD; Supplementary Fig. S10C). PL acts predominantly by reducing glutathione levels, without affecting glutathione reductase activity (Supplementary Fig. S11A; ref. 42). On the other hand, PL directly interacts with several proteins, including glutathione S-transferase pi 1 (GSTP1), glutathione S-transferase omega 1 (GSTO1), and glyoxalase I (GLO1), which are important in catalyzing the conjugation of reduced glutathione to electrophilic substances (43). Interestingly, we found that these proteins are significantly upregulated in multiple myeloma in comparison with plasma cells from healthy individuals (Supplementary Fig. S11B). We also evaluated whether their expression was also modulated by MYC and found that MYC overexpression increased, whereas MYC silencing decreased, the levels of GSTP1, GSTO1, and GLO1 (Fig. 6K). Of note, PL did not exhibit any cytotoxic effect on PBMCs (Supplementary Fig. S12), suggesting a favorable therapeutic index. Altogether, these data suggest that increasing ROS levels in multiple myeloma induces apoptosis and may represent a novel therapeutic option.

ATR Inhibition Combined with PL Treatment Induces Synergistic Multiple Myeloma Cell Death

To assess whether combining therapies that elicit oxidative stress, like PL, with ATR inhibition may have higher cytotoxic effects on multiple myeloma cells, we next examined the effect of combined genetic knockdown of ATR with PL treatment. In H929 and OPM-2 cells transfected with ATR shRNAs, we observed decreased proliferation (Fig. 7A) and enhanced apoptosis (Fig. 7B) when we combined ATR inactivation and PL treatment. Similar results were obtained with VE-821 (1–1.5 μmol/L) in combination with PL (0.5–1.5 μmol/L). The combination had synergistic effects, with a combination index (CI) below 1 for both H929 and OPM-2 cells (Fig. 7C). Similar synergistic effects were observed in the multiple myeloma cell lines MM.1S and RPMI/8226, but no synergism was detectable in U266 cells (Supplementary Fig. S13A and S13B). Multiple myeloma cells isolated from patients also showed a similar pattern, with synergistic/additive effects of combination treatment (Fig. 7D). Interestingly, the combination treatment of ATO with VE-821 was less effective than the combination of PL with VE-821 (Supplementary Fig. S14). Indeed, a recent study reported that ROS are not essential for the induction of multiple myeloma cell death by ATO (44). Interestingly, the proteasome cascade was one of the pathways most prominently associated with the K2 subgroup, and bortezomib can induce a “BRCAness” phenotype by abrogating ubiquitination of H2A.X, thereby reducing recruitment of repair proteins, such as RAD51 and BRCA1, and acting as a stress sensitizer (45). We therefore next assessed whether the combination of PL and VE-821 demonstrated synergistic effects with proteasome inhibitors. To this end, we have tested both multiple myeloma cell lines and patient multiple myeloma cells with bortezomib and carfilzomib. Indeed, we detected a robust synergy between proteasome inhibitors and PL (Fig. 7E and Supplementary Fig. S15A and S15B). On Western blot analysis, we observed that combination treatment with bortezomib and PL induced higher levels of γH2A.X than either single agent alone (Supplementary Fig. 15C). Similar results were obtained combining proteasome and ATR inhibitors (Supplementary Fig. S16). Taken together, these results indicate that combined inhibition of ATR with pharmacologic induction of ROS may trigger synergistic cytotoxicity and apoptosis in multiple myeloma cells with ongoing DNA damage (Fig. 7F).

DISCUSSION

Ongoing DNA damage is a hallmark of epithelial cancers that leads to genomic instability and ultimately to more aggressive tumors, often resistant to current therapies. Dysregulated oncogene expression triggers replicative stress in these cancers, leading to DSBs. We have recently demonstrated constitutive ongoing DNA damage in hematologic malignancies (4). In this study, we show that replicative stress causes DNA damage in blood cancers, specifically in multiple myeloma. Indeed, we identified a subset of multiple myeloma characterized by chromosomal instability, replicative stress, and poor prognosis. These patients had increased expression of the oncogene MYC, suggesting a prominent role for MYC in conferring this phenotype. We show that MYC is able to trigger replicative stress in multiple myeloma cells. Besides replicative stress, oncogenes,
**Figure 7.** ROS induction and ATR inhibition synergize in inducing multiple myeloma (MM) cell death. 

A, H929 multiple myeloma cells (left) and OPM-2 multiple myeloma cells (right) were transiently transfected with ATR shRNAs or scrambled shRNAs and incubated with DMSO or 1 μmol/L PL starting from day 0 of transfection. Cell growth by cell counting with trypan blue exclusion was performed in triplicate. 

B, apoptosis by Annexin V–PI staining was evaluated in the same conditions as A at 48 hours from transfection. Summary of two independent experiments is shown. 

C, combination studies by MTT viability assay using 0.5 to 1.5 μmol/L VE-821 and 1 to 1.5 μmol/L PL were performed in H929 and OPM-2 cells after 48-hour treatment. CI plot with CI values are shown as insets. 

D, combination studies by MTT viability assay in multiple myeloma patient cells as in C. 

E, combination studies by MTT viability assay using 50 to 300 nmol/L PL, 1.25 to 2.5 nmol/L bortezomib (BTZ), and 2.5 to 5 nmol/L carfilzomib (CFZ) in H929 and 1 representative patient. CI plot with CI values are shown as insets. 

F, schematic model. Top, oncogene activation (as for example MYC) increases replicative stress and ROS, thus triggering DNA damage in multiple myeloma cells that is maintained below a critical threshold by ATR-mediated resolution of DNA-replicative stress and increase of enzymes reducing ROS levels. Bottom, combined inhibition of ATR (stress sensitization) and increase in ROS (stress overload) elicits an increase in unresolved DNA damage that leads to apoptosis. **, P < 0.001 (Student t test).
including MYC, can induce DNA damage by increasing production of ROS (27, 46). We here provide evidence that MYC also triggers DNA damage by increasing ROS levels in multiple myeloma cells. Furthermore, a recently described ROS inducer, PL, enhances DNA damage via increased ROS. These data indicate that both replicative stress and ROS induction trigger DNA damage in multiple myeloma cells, providing the basis for novel combination treatment strategies.

A model has been recently proposed in which a step-wise increase in MYC levels is associated with disease progression. For example, a relatively modest, yet critical, increase in MYC levels is implicated in driving the evolution from monoclonal gamopathy of undetermined significance (MGUS) toward multiple myeloma (47). As the disease progresses, genomic rearrangements affecting the MYC locus occur, resulting in much higher levels of MYC expression (31). Indeed, mouse models support the notion that distinct expression thresholds may direct the role of MYC in oncogenesis (48, 49). Although our data demonstrate that MYC contributes to the replicative stress and enhanced proliferation in the K2 multiple myeloma subgroup, we would argue that oncogenes other than MYC might also play a role. Further studies are needed to comprehensively evaluate the genetic or epigenetic anatomy of this patient subset.

Oncogenesis causes cellular stresses in cancer cells that are not evident in normal cells (50). As a consequence, tumor cells become dependent on stress response pathways for their survival. These stresses include DNA damage/replication, proteotoxic, mitotic, metabolic, and oxidative stresses. This so-called nononcogene addiction represents a vulnerability of cancer cells that can be targeted therapeutically. For example, PARP inhibitors are under clinical evaluation in patients with breast and ovarian cancers with hereditary mutations of BRCA1 or BRCA2 (51) to exploit one of these liabilities. This approach offers several advantages over the current model of targeting proteins arising from mutated genes. First, the development of compounds against members of a pathway, rather than against single mutated proteins, is more feasible from a medicinal chemistry perspective. Indeed, recent large sequencing efforts have revealed a panoply of mutations in various unrelated genes. As a consequence, the development of compounds targeting a large series of mutated proteins remains a formidable challenge. Second, drug resistance develops more promptly against compounds targeting single mutated amino acids, as is often the case in conventional targeted therapies, than against compounds targeting an entire pathway. Third, recent reports in multiple myeloma (52, 53) have demonstrated exceedingly high levels of genetic and clonal heterogeneity. Therefore, targeting one clone endowed with a specific set of mutations may not be effective, because additional clones may quickly overcome the targeted one. Hence, it may be more effective to target a pathway to which a cancer in its entirety has become addicted, irrespective of the mutations present in each single clone. In this regard, multiple myeloma represents a particularly apt example. Multiple myeloma plasma cells are characterized by increased protein synthesis, well in excess of the levels present in healthy cells, irrespective of their genetic makeup. Multiple myeloma cells cope with this stress by increasing the activity of the proteasome to facilitate the degradation of unfolded proteins. Importantly, the introduction of proteasome inhibitors, such as bortezomib, for the treatment of multiple myeloma is a landmark achievement, targeting a vulnerability that is based on non-oncogene addiction. Additional such approaches, such as the one described here, may similarly provide an important advance in the treatment of this disease.

To therapeutically target these hallmarks of stress, one could envision two approaches, stress overload or stress sensitization (50). We have explored both paths, with the goal of maximizing the effects on tumor cells (Fig. 7F). Blocking ATR (stress sensitization) prevents the repair of stalled forks during proliferation induced by unconstrained oncogenic stimuli, thereby leading to DNA damage and cell death. In parallel, PL treatment increases ROS levels and exacerbates the oncogene-induced DNA damage (stress overload).

Although drugs targeting MYC or MYC interactors have recently been proposed (54–56), in our approach interfering with MYC activity would be detrimental, because it would reduce the stress overload on multiple myeloma cells. Based on gene expression profiling, patients with multiple myeloma have been subdivided into 7 subgroups, each endowed with specific features, plus an additional subgroup characterized by a “myeloid” signature (35). Among these 8 subtypes, one subgroup of patients, the proliferation (PR) subgroup, specifically overexpresses cell-cycle- and proliferation-related genes, and has a significantly higher gene expression–defined proliferation index than the other groups. Importantly, patients in the PR group have the worst prognosis. Remarkably, however, a multivariate analysis demonstrated that the chromosomal instability classification that we propose herein was nevertheless able to identify patients with a poor prognosis who are not captured by other genomic features, including the proliferation group, as outlined in Supplementary Table S5. Specifically, 33 patients belonged to both the K2 and high-proliferation groups. However, 34 patients in the low-proliferation group were also included within the K2 group, and these patients did show a poor prognosis, despite being included in the low-proliferation group (P < 0.013). Importantly, a likelihood ratio test comparing the multivariate model with and without the K1/K2 clusters was significant (P = 0.023). Therefore, the K1/K2 classification provides additional information and is independent of other established prognostic factors derived from gene expression profiling, such as proliferation and the presence of MMSET/FGFR3 translocations.

We demonstrate here the rationale for a novel therapeutic approach specifically targeting this patient group with aggressive disease, a poor prognosis, and lack of effective therapeutic options. In the context of their endogenous rapid tumor cell proliferation and associated DNA damage, ATR inhibition coupled with the induction of further oxidative stress could both inhibit proliferation and trigger apoptosis. Our study provides the framework for derived combination clinical trials to address an unmet medical need and improve outcome in this patient subgroup.

**METHODS**

**Reagents**

The ATR inhibitor VE-821, bortezomib, and carfilzomib were purchased from Selleck Chemicals LLC; PL and NAC from Sigma-Aldrich; and L-NAME from Santa Cruz Biotechnology.
Cell Lines and Culture

The multiple myeloma cell lines MM.1S, U266, RPMI/8226, and H929 were purchased from the American Type Culture Collection (ATCC); plasma cell leukemia cells OPM-1 and OPM-2 were provided by Dr. Edward Thompson (University of Texas Medical Branch, Galveston, TX). Cell lines have been tested and authenticated by STR DNA fingerprinting analysis (Molecular Diagnostic Laboratory, DFCI), and used within 3 months after thawing. All multiple myeloma cell lines were cultured in RPMI/1640 media containing 10% FBS (GIBCO; Life Technologies), 2 μmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO; Life Technologies).

Primary Cells

Blood samples collected from healthy volunteers were processed by Ficoll-Paque (GE Healthcare) gradient to obtain PBMCs. Multiple myeloma cells from individuals affected by multiple myeloma were obtained from bone marrow samples after informed consent. Gene expression analysis was performed in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute. Mononuclear cells were separated using Ficoll-Paque density sedimentation, and plasma cells were purified (>95% CD138+) by positive selection with anti-CD138 magnetic activated cell separation micro beads (Miltenyi Biotec).

Western Blotting, RNA Extraction and Reverse Transcription PCR, Immunofluorescence Staining, Transient Transfection of Multiple Myeloma Cell Lines, Foci Number Quantification, Viability and Cellular Growth Assays, Apoptotic Assays, and ROS Assays

Detailed protocols are included in Supplementary Methods.

Gene Expression Analysis

The Affymetrix H133A gene expression dataset for multiple myeloma cell lines (57) was downloaded from the Multiple Myeloma Research Consortium Genomics Portal at http://www.broad.mit.edu/mmpg. The cell lines used for the analysis were KMS-34, U266, and Karpas-620 (DNA damage-negative; ref. 4), and INCA-1, NCI-H929, OCI-MY5, RPMI/8226, and UTMC-2 (DNA damage–positive cell lines; ref. 4). The Affymetrix U133 Plus 2.0 GEPS were derived from ref. 4. The Affymetrix U133 Plus 2.0 GEPS were derived from ref. 4 (GEO accession number GSE4452) and from ref. 35 (GEO GSE2658). GSEA was performed as previously described (refs. 1, 32; GSEA v2.0 at http://www.broad.mit.edu/gsea) using gene set as permutation type and 1,000 permutations and signal to noise as metric for ranking genes. Both absolute and real data preprocessing and K-means clustering were performed with GenePattern (http://genepattern.broadinstitute.org/gp/pages/login.jsf).

Statistical Analysis

Statistical significance was determined by the Student t test on average values ± SD. Survival analysis was assessed with the “survival” package in R. For the survival analysis, a subset of 414 patients was analyzed (patients with the myeloid signature were excluded), in line with ref. 35.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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