

Supplementary Table Legends

Supplementary Table 1. The 5 most enriched pathways in MM cell lines with increased DNA damage (high expression levels in cell lines with increased DNA damage).

Supplementary Table 2. The 5 most enriched pathways in the K2 MM patient subgroup in the KEGG MSigDB collection (high expression levels in K2).

Supplementary Table 3. The 5 most enriched pathways in the K2 MM patient subgroup in the BIOCARTA MSigDB collection (high expression levels in K2).

Supplementary Table 4. Patient survival data and classification, based on (1), and the K1/K2 clustering.

Supplementary Table 5. Multivariate analysis of survival groups.

Supplementary Table 6. Pathways implicated in ROS and NOS metabolism collected from MSigDB.

Supplementary Figure Legends

Supplementary Fig. S1. 53BP1 and RPA32 staining in MM cells. **A,** immunofluorescence staining for 53BP1 in H929, MM.1S, and RPMI/8226 cells. **B,** immunofluorescence staining for RPA32 in H929 and RPMI/8226 cells.

Supplementary Fig. S2. Replicative stress markers in MM cells. **A,** immunofluorescence staining for RAD9A in H929, MM.1S, and RPMI/8226 cells. **B,** quantification of replicative stress markers in a panel of MM cells.

Supplementary Fig. S3. Replicative stress markers in peripheral blood mononuclear cells (PBMCs). Peripheral blood mononuclear cells were isolated from healthy donors, and immunofluorescence staining was performed immediately with antibodies against 53BP1, RAD51, RPA32, and RAD9A.

Supplementary Fig. S4. Phospho-histone H3 and phospho-H2A.X staining in MM cells. Immunofluorescence staining for phospho-histone H3 and γ -H2A.X in H929, OPM-2, and MM.1S MM cell lines.

Supplementary Fig. S5. 53BP1 and RPA32 in primary MM cells. Immunofluorescence staining for 53BP1 and RPA32 in MM primary cells.

Supplementary Fig. S6. ATR in MM patient and cell lines. A, heat map in the K1 and K2 groups showing the expression levels corresponding to probe sets included in the ATR/BRCA1 BIOCARTA pathway, as annotated in MSigDB. **B,** GSEA ES enrichment profile for 559 patients divided in K1 and K2 groups corresponding to the ATR/BRCA1 BIOCARTA pathway, as annotated in MSigDB. **C,** U266 cells were transfected with EGFP-MYC and EGFP-LACZ and treated with 1-5 μ M VE-821 for 48 h, starting from Day 0 of transfection. MTT absorbance assay was used to measure the fraction of affected cells.

Supplementary Fig. S7. Gene expression levels of probe sets related to oxidative stress in MM patients. Heat map in the K1 and K2 groups showing the expression levels corresponding to probe sets included in the pathway MOOTHA_MITOCHONDRIA, KEGG_OXIDATIVE_PHOSPHORYLATION and MOOTHA_VOXPPOS respectively, as annotated in MSigDB.

Supplementary Fig. S8. Oxidative stress markers in MM cells upon MYC modulation and N-Acetyl Cysteine, VE-821, or Ku55933 treatment A, immunofluorescence staining for RNS in U266 cells upon transfection with EGFP-MYC or EGFP-LACZ. As positive control, U266 were treated with 1mM L-arginine for 30 minutes. DAPI stains the nuclei. **B,** U266 cells were transfected for EGFP-MYC and EGFP-LACZ and incubated with DMSO or 5mM N-Acetyl Cysteine (NAC) for 48 hours. Immunofluorescence for γ -H2A.X foci is shown. **C,** Western

blot analysis for γ -H2A.X and GAPDH in H929 cells upon 5 mM NAC treatment for 24 hours. **D**, H929 and OPM-2 cells were treated with 2.5 μ M VE-821 and 5mM NAC, alone or in combination. Apoptosis with Annexin V-FITC/PI staining was measured at 48 hours. **E**, Evaluation of ROS levels by flow cytometry staining in a panel of MM cell lines, after incubation for 24 hours with DMSO, 1 μ M VE-821, or 10 μ M Ku55933, an ATM inhibitor. **F**, Western blot analysis for γ -H2A.X and GAPDH in U266 cells upon treatment for 24 hours with 1 mM L-arginine; H929 were incubated with 1 mM L-NAME for 24 hours and western blot was performed using antibodies against γ -H2A.X and GAPDH.

Supplementary Fig. S9. Piperlongumine effects on MM cells. **A**, Cellular growth by cell count with trypan blue exclusion in U266 cells transfected with EGFP-MYC or EGFP-LACZ upon treatment with DMSO or 1mM L-NAME. Experiment was performed in duplicate and results at 48 hours are shown. **B**, Evaluation of ROS and superoxide (SOD) levels by flow cytometry staining in a panel of MM cell lines. Cells were treated for 24 hours with 1 μ M PL and stained with CellROX™ Deep Red Reagent or MitoSOX reagent. **C**, Combination studies by MTT viability assay using 1-1.5 μ M PL and 5 mM NAC in H929, U266, and RPMI/8226 MM cells after 48 hour treatment. **D**, Combination studies by MTT viability assay using 1-2.5 μ M PL and 1 mM L-NAME in H929, MM.1S, and OPM-2 MM cells after 48 hour treatment.

Supplementary Fig. S10. Effects of arsenic trioxide treatment of MM cells. **A**, Evaluation of ROS and superoxide (SOD) levels by flow cytometry staining in a panel of MM cell lines. Cells were treated for 24 hours with 1 μ M arsenic trioxide (ATO) and stained with CellROX™ Deep Red Reagent or MitoSOX reagent. **B**, Western blot analysis for γ -H2A.X and GAPDH in H929 treated with DMSO and 1-2.5 μ M ATO for 24 hours. **C**, Apoptosis is evaluated with Annexin V FITC-PI

staining in a panel of cell lines to compare the effects of PL and ATO. Both compounds are administrated to the cells at 1 μ M concentration for 48 hours.

Supplementary Fig. S11. Piperlongumine and glutathione in MM cells. A, Glutathione reductase (GR) activity in MM.1S and H929 MM cells upon treatment for 24 hours with DMSO or 1 μ M PL. **B,** Gene expression profiling data comparing plasma cells from healthy individuals and MM cells from GSE4452 dataset. Probe set used: 200824_at for GSTP1; 201470_at for GSTO1; 200681_at for GLO1. Statistical comparisons (student t-test) between the two groups are shown.

Supplementary Fig. S12. Piperlongumine on peripheral blood mononuclear cells (PBMCs). Evaluation of PBMC cells viability by MTT absorbance assay upon PL incubation at increasing doses for 72 hours.

Supplementary Fig. S13. Cytotoxicity effects of piperlongumine in combination with VE-821. A, Combination studies by MTT viability assay using 0.5-1.5 μ M VE-821 and 1-1.5 μ M PL were performed in MM.1S and RPMI/8226 cells after 48 hour treatment. Combination Index (CI) plot with CI values are shown as insets. **B,** Combination studies by MTT viability assay in U266 cells as above.

Supplementary Fig. S14. Cytotoxicity effects of arsenic trioxide in combination with VE-821. Combination studies by MTT viability assay using 0.5-1.0 μ M arsenic trioxide (ATO) and 1.0-1.5 μ M VE-821 in MM.1S, H929, RPMI/8226, and KMS-20 MM cells. Combination Index (CI) plot with CI values are shown. Data were collected after 48 hour treatment

Supplementary Fig. S15. Cytotoxicity effects of piperlongumine in combination with proteasome inhibitors. A-B, Combination studies by MTT viability assay using 50-300 nM PL, 1.25-2.5 nM bortezomib (BTZ), and 2.5-5 nM carfilzomib (CFZ) in MM.1S, OPM-2, and U266 MM cells after 48 hour treatment. Combination Index

(CI) plot with CI values are shown as insets in **A**. In **B**, a full table showing CI and fraction of affected cells (Fa) is shown on the left panel. **C**. Western blot analysis for γ -H2A.X and GAPDH in H929 cells upon treatment for 24 hours with DMSO, 1 μ M PL, 1.25 nM BTZ, and combination of PL with BTZ.

Supplementary Fig. S16. Cytotoxicity effects of VE-821 in combination with proteasome inhibitors. Combination studies by MTT viability assay using 300-1000 nM VE-821 and 1-5 nM bortezomib (BTZ) in a panel of MM cells at 24 hours. Combination Index (CI) plot with CI values are shown.

Supplementary Methods.

Western blotting. MM cells were harvested and lysed using lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM NaF, 2 mM Na_3VO_4 , 1mM PMSF, 5 $\mu\text{g mL}^{-1}$ leupeptine, and 5 $\mu\text{g mL}^{-1}$ aprotinin). Nuclear extracts were prepared using Nuclear Extraction Kit (Affymetrix, Santa Clara, CA, United States). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with different antibodies: phospho-ATM (Ser1981), ATR, phospho-ATR (Ser428), p21, total PARP, p-CHK2, APE1, NRF2, BACH1, and GAPDH (from Cell Signaling, Beverly, MA, United States); p-RPA32 (S4/S8) (from Bethyl Laboratories, Montgomery, TX), RPA32 (from Pierce-Thermo Scientific, Rockford, IL, United States), RAD51 and γ -H2A.X (Ser139) (from Millipore/Merck, Darmstadt, Germany); as well as actin, c-MYC, and thioredoxin (from Santa Cruz Biotechnology, Dallas, TX, United States). All antibodies were diluted 1:1000, except for GAPDH antibody (1:2000 dilution).

RNA extraction and reverse transcription polymerase chain reaction.

RNA was extracted using Trizol (Life technologies, Carlsbad, CA, United States) and

quantified by a Nanodrop spectrophotometer (Labtech). Specifically, cells were pelleted, washed with cold PBS, and resuspended in 1 mL Trizol. They were then incubated with 1-Bromo-3-chloropropane (Sigma–Aldrich (St. Louis, MO, United States), washed first with Isopropyl alcohol and then with 75% Ethanol, and resuspended in Nuclease Free-water (Life technologies, Carlsbad, CA, United States). After quantification, 2000 ng of RNA was used to synthesize cDNA via the Superscript II First strand synthesis Kit (Life technologies, Carlsbad, CA, United States), according to the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using SYBR GREEN PCR Master Mix (Applied Biosystem, Life technologies, Carlsbad, CA, United States), after optimization of the primer conditions. cDNAs were diluted 1:100 or 1:1000 and amplified in a 20µL reaction. Primers were used at 200 nmol or 400 nmol concentration. Thermal cycling conditions were: 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, followed by 1 minute at 60°C. Real-time quantitative PCR was performed on ABI Prism 7300 Sequence Detection System (Applied Biosystems, Life technologies, Carlsbad, CA, United States). Data were analyzed using the delta delta Ct method. GAPDH was used as a loading control.

| Primer name | Sequence |
|--------------------|-----------------------|
| MYC-F | TTTTTCGGGTAGTGGAAA |
| MYC-R | GCAGTAGAAATACGGCTGCAC |
| GCLC-F | GGCGATGAG GTGGAATACAT |
| GCLC-R | GTCCTTCCCCCTTCTCTTG |
| GCLM-F | GCGAGGAGCTTCATGATTGT |
| GCLM-R | CTGGAAACTCCCTGACCAA |
| GSTP1-F | TTGGGCTCTATGGGAAGGAC |

| | |
|---------|------------------------|
| GSTP1-R | GGGAGATGTATTTGCAGCGGA |
| GSTO1-F | GAACGGCTGGAAGCAATGAAG |
| GSTO1-R | TGCCATCCACAGTTTCAGTTT |
| GLO1-F | AGCAGACCATGCTACGAGTGA |
| GLO1-R | GAGAGCGCCCAGGCTATTT |
| GAPDH-F | GAAGGTGAAGGTCGGAGTCA |
| GAPDH-R | GGGGTCATTGATGGCAACAATA |

Immunofluorescence staining. 15,000 cells from MM cell lines, PBMCs or cells obtained from subjects affected by MM were cytopun for 5 min at 350–500 rpm, fixed in 4% paraformaldehyde (PFA) for 15 min, washed three times with PBS, and incubated with 0.1 M glycine for 10 min to quench PFA autofluorescence. After washing again, cells were permeabilized and stained for 90 min with a solution of 0.1% Triton X–100 and PBS + BSA 1%, containing primary antibodies at a ratio of 1:100. Cells were washed and incubated for 45 min with appropriate secondary–fluorescent antibodies. Alexa Fluor 488 anti–rabbit and Alexa Fluor 488 and Alexa Fluor 568 anti–mouse antibodies were purchased from Invitrogen (Life technologies, Carlsbad, CA, United States). After washes, the nuclear content was stained with DAPI reagent (Invitrogen, Life technologies, Carlsbad, CA, United States) for 5 min and washed. The entire procedure was performed at room temperature. The slides were then mounted with ProLong Gold Antifade Reagent (Invitrogen, Life technologies, Carlsbad, CA, United States), and images were taken using a Zeiss microscope (Carl Zeiss, Jena, Germany) equipped with Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with ImageJ

software. Anti- γ -H2A.X and anti-RAD51 were obtained from Millipore/Merck, (Darmstadt, Germany), Anti-phospho H3 and anti-53BP1 from Cell signaling (Beverly, MA, United States) and RPA32 and RAD9A from Pierce-Thermo Scientific, Rockford, IL, United States.

Transient transfection of MM cell lines. MSCV-MYC-EGFP plasmid, shRNAs against ATR and scrambled were purchased from Addgene (Cambridge, MA, United States); MSCV-LACZ and MSCV-MYC were subcloned from the original vector. shRNAs against MYC were kindly provided by Dr. William Hahn (Dana-Farber Cancer Institute). MM.1S, H929, OPM-2 and U266 cells were transiently transfected using 'Cell Line Nucleofector Kit V or C (Amaxa Biosystems, Köln, Germany), according to the manufacturer's instructions. Specifically, 3,000,000 cells were resuspended in 100 μ L V solution and 2500 ng of plasmid. Following transfection, MM cells were subjected to mRNA analysis, Western blotting, as well as apoptosis, cell counting, and MTT assays.

| Primer name | Sequence |
|----------------|---|
| ShRNA c-MYC #1 | CCATAATGTAAACTGCCTCAA |
| ShRNA c-MYC #3 | CAGTTGAAACACAAACTTGAA |
| ShRNA c-MYC #5 | CCTGAGACAGATCAGCAACAA |
| shRNA-ATR | GATCCCCGGCGTCGTCTCAGCTCGTCTTCAAGAGAGACGAGCTG AGACGACGCCTTTTTG GAAA |

Foci number quantification. MM cell lines and MM cells were stained and images acquired as described above. For each sample, at least three different images were taken, representative of different fields of the slide. Each image contained a field of at least 30 cells. Number of RPA32, RAD51 and γ -H2A.X foci was determined in

different conditions. Mean and standard deviation were calculated among the triplicates.

Viability and cellular growth assays. Viability of MM cells was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-MTT (Sigma-Aldrich, St. Louis, MO, United States) colorimetric survival assay. MM cells (20,000–50,000) were plated in 100 μ L medium. At the various time points (24–96 h), 10 μ L 5mg mL⁻¹ MTT were added to cells. After 4 h incubation at 37 °C, medium was discarded and 100 μ L MTT stop solution (Isopropanol with 1 N HCl) was used to dissolve MTT metabolic products. Absorbance was read at 570 nm and background was subtracted at 630 nm, using the spectrophotometer SPECTRAMAX M2 and Softmax Pro v5 software. Cellular growth was estimated by cell counting in triplicates, excluding dead cells stained by trypan blue.

Apoptosis assays. Dead cells were detected by propidium iodide (PI) staining. Apoptosis was quantified using Annexin V-FITC/PI staining or Annexin V-PE/7-AAD staining on GFP-positive cells (BD Biosciences, San Diego, CA United States). Specifically, cells were washed twice with room-temperature PBS, resuspended in 100 μ L of Annexin binding buffer, and stained with specific antibodies for 20 min. After adding additional 400 μ L of Annexin binding buffer, samples were acquired using FACS Canto II machine from Becton Dickinson, BD (Franklin Lakes, NJ, United States) and analyzed with FCS EXPRESS 4 Flow Research Edition software. The percentage of apoptotic cells was defined as the sum of early apoptotic (Annexin V⁺, PI⁻) and late apoptotic (Annexin V⁺, PI⁺) cells.

ROS quantification. Cells were washed twice with PBS, resuspended in PBS + 5mM CellROX™ Deep Red Reagent and incubated for 30 minutes at 37 °C, washed again and evaluated by flow cytometry.

Tetramethylrhodamine, ethyl ester (TRME) staining or mitochondrial membrane potential assay. Cells were incubated with 50 nM TRME (Abcam, Cambridge, MA, United States) for 15 minutes at 37 °C, and evaluated by flow cytometry in FL2 channel.

MitoSOX™ Red mitochondrial superoxide staining. 5mM MitoSOX™ reagent from Molecular Probes, Life technologies (Carlsbad, CA, United States) was resuspended in DMSO and diluted in HBSS/Ca/Mg buffer (GIBCO, GIBCO, Life technologies, Carlsbad, CA, United States) to obtain a final concentration of 5µM as working solution. Cells were incubated in 2mL of solution for 10 minutes at 37°C, washed and resuspended in plain HBSS/Ca/Mg buffer, and evaluated by flow cytometry.

Superoxide and RNS detection. Cells were resuspended in pre-warmed tissue culture media containing superoxide detection reagent and RNS detection reagent (superoxide/RNS detection mix -Abcam, Cambridge, MA, United States) and incubated at 37°C for 2 hours. Cells were then centrifuged at 400g for 5 minutes, washed in 5mL washing buffer, and cytospun in 100 µL. DAPI staining was added for 5 minutes and the slides were then mounted and observed as above. NO inducer (L-arginine) was added to control cells at 1mM for 30 minutes as recommended.

Glutathione reductase activity. Glutathione reductase (GR) activity was tested using a specific kit purchased from Cayman chemical (Ann Arbor, MI, United States). Briefly, cell lysates were quantified and incubated with GR assay buffer, GSSG and

NADPH in triplicate. Positive controls and background wells were set up as well in triplicate. Absorbance at 340 nM was read at different time points and changed in absorbance were calculated following the protocol.

Thioredoxin reductase activity. Thioredoxin reductase (TXN) activity was tested using a specific kit purchased from Cayman chemical (Ann Arbor, MI, United States). Briefly, sample cell lysates and control samples (rat liver TXN reductase) in triplicate will be incubated with NADPH and DTNB in the presence or absence of a specific thioredoxin inhibitor to test the specific contribution of thioredoxin in the reduction of DTNB products. Absorbance once every minute at 405-414 nM was obtained for five time points and changed in absorbance were calculated following the protocol.

References:

1. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. *Blood*. 2006;108(6):2020-8.