Replication stress leading to apoptosis within the S-phase contributes to synergism between vorinostat and AZD1775 in HNSCC harboring high risk *TP53* mutation

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Supplementary Materials and Methods

Analysis of combined drug effects

Drug synergy was determined by the combination-index and isobologram analyses, which were generated based on the median-effect method of Chou and Talalay using the CalcuSyn software (Biosoft, Ferguson, MO).The combination-index (CI) is a quantitative representation of the degree of drug interaction. On the basis of the dose-response curves using clonogenic assay for HNSCC cells treated with AZD1775 and vorinostat either alone or in combination as previously indicated, the CI values were generated over a range of fraction affected (Fa) levels from growth inhibition percentages. The isobologram is formed by plotting the individual drug concentrations required to achieve 50% inhibitory effect on their respective x- and y- axes. For conservative estimate of synergism, the conservative isobologram plots of the combination of vorinostat and AZD1775 used at fixed constant molar ratios (Vorinostat: AZD1775; 2:1or 10:1 respectively) were generated. The molar ratios were determined using the IC50 of each agent in all HNSCC cells examined. A straight line connecting the two points is made and the

concentration (combination data point) of the two drugs used in combination to achieve the 50% inhibitory effect is plotted on the isobologram. Combination data points that fall on the line represent an additive drug effect (CI = 1), whereas data points that fall below or above the line represent synergism (CI < 1) and antagonism (CI > 1), respectively.

Live cell imaging and EdU labeling

For live cell imaging studies, PCI13-G245D cells were transfected with a Histone H2B-RFP lentiviral vector, selected with 2 μg/mL blasticidin, and sorted by flow cytometry to enrich for highly expressing cells. Depending on the particular experiment, cells were plated at a density of 1-2×10^5 cells/well in a 6-well plate, or 2×10^4 cells/well in a 12-well plate, or 2 to 4×10^4 cells/well in 4-well chamber slides, and grown overnight prior to drug treatment. Cell were then treated with 0.01% DMSO alone, 0.25 μmol/L AZD1775 alone, 4 μmol/L vorinostat alone, or in combination. The dishes were then placed in an automated Olympus IX81 inverted microscope system equipped with a Solent 37°C Incubator (Solent Scientific Limited, Portsmouth, Great Britain) to maintain a temperature of 37° C and 5% CO_2, a Photometrics Coolsnap cooled digital monochrome microscope camera (Roper Scientific, Munich, Germany), and driven by MetaMorph Multidimensional Imaging software for Olympus (Universal Imaging and Olympus America Inc., Center Valley, PA). Live cell imaging was carried out by separately capturing bright field Images (either under Phase or Hoffman optics) and red fluorescence images at 10× magnification at 10 minute intervals at multiple stage positions for each time point. Live cell imaging movies were created by overlaying the bright field and fluorescence images at each time point and the movies were analyzed for cell cycle status (interphase or metaphase) and

cellular morphology (e.g., mitotic fidelity) using MetaMorph software. Event charts of the live cell imaging results were created using R Studio and the Flexible Event Chart for Time-to-Event Data developed by Drs. J. Jack Lee and Kenneth R. Hess (Department of Biostatistics, the University of Texas MD Anderson Cancer Center) and Joel A. Dubin (Department of Statistics, the University of Waterloo) and modified by Dr. Hsiang-Chun Chen, Department of Biostatistics, the University of Texas MD Anderson Cancer Center. In some short term experiments, cells were labeled with EdU (20 μmol/L) for 1 hour prior to fixation with 4% paraformaldehyde followed by 70% ethyl alcohol/PBS treatment. The slides were then processed for EdU click-iT chemistry (Invitrogen/Molecular Probes, Eugene, OR) using an Alexa-Fluor 488 probe and counterstained with TOPRO-3 at 5 μmol/L for DNA content analysis. The slides were then scanned for EdU uptake and DNA content using a Compucyte laser scanning cytometer. Note that the acquisition parameters were identical within different chambers of the same slide.

Immunofluorescence

Cells were plated on glass coverslips and treated with drugs the following day as previously described. Cells were then fixed in 2% paraformaldehyde for 1 hour, washed, permeabilized in 0.2% Triton X-100 in PBS for 20 minutes, washed, and blocked for 1 hour at room temperature in PBS buffer containing 2% normal goat serum, and 0.3% Triton X-100. Then, glass coverslips were incubated with primary Rad51 (MA5-14419; Thermo Scientific) antibody overnight at 4°C. After washing with PBS, primary antibody was visualized with secondary Alexa Fluor-conjugated antibody as appropriate. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images were acquired on a Leica confocal microscope. For Rad51 focus formation, cells containing 20 or more Rad51 foci were counted in 4 different high-power fields (hpf) each

containing at least 100 cells. Data were presented as percentage of cells with Rad51 foci. All experiments were performed three times independently in duplicate. Cells exposed to cisplatin (10 μmol/L) for 4 hours were used as positive controls for detection of Rad51 focus formation.

Orthotopic mouse model of oral tongue cancer and tumor growth delay

All animal experimentation was approved by the Institutional Animal Care and use Committee (IACUC) of the University of Texas MD Anderson Cancer Center. The high-risk *TP53* mutant HNSCC (PCI13-G245D) cells were harvested from subconfluent culture by trypsinization and washed with PBS. Groups of 10 athymic nude mice were injected into the lateral tongues with $5x10^4$ cells suspended in 30 μL of PBS. Eight to 10 days after injection, four groups of mice with similar average tumor volumes were created. Treatment schedule is shown in Supplementary Figure S10. Treatment was initiated when tumors were less than 3 mm^3 in size. Mice were treated with vorinostat dissolved in DMSO and PBS at 1:9 ratio and administrated at a dose of 65 mg/kg by oral gavage (p.o.) bid daily five days a week, followed by AZD1775 in 0.5% methylcellulose at a dose of 45 mg/kg given by oral gavage (p.o.) bid on days 2 and 4. Control mice were given PBS five days a week. Tumor size and weight loss of the mice were assessed and recorded twice a week for 4 weeks. Tongue tumors were measured with microcalipers, and tumor volume was calculated as $(A)(B^2)\pi/6$, where A is the longest dimension of the tumor and B is the dimension of the tumor perpendicular to A. Mice were euthanized by CO_2 asphyxiation when they lost more than 20% of their preinjection body weight or 30 days after the initiation of treatment. For immunohistochemical analysis, mice were sacrificed and tongue tumors were collected 72 hours after the final dose of vorinostat and AZD1775. Tissues were then fixed in

4% paraformaldehyde, paraffin-embedded, sectioned and subjected to the immunohistochemical analysis.

Immunohistochemistry

Sections were prepared from formalin-fixed paraffin embedded mice tumor tissues. The sections were incubated with 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity. The tissue slides were heated in citrate buffer (pH 6.0) for 15 minutes using a microwave oven and blocked with 0.2% Tween20, 2.5% normal goat serum in TBS for 60 minutes. The tissue slides were incubated at 4^0C overnight with following antibodies; p21 Waf1/Cip1 (CST: 12D1; #2947), CD31 (abcam: ab28364), p-CDK1 (Y15) (abcam: ab133463), RRM2 (Novus Biologicals: NBP1-31661) diluted at 1:50, survivin (CST: 71G4B7; #2808) diluted at 1:100, and PCNA (Millipore: MAB424R) diluted at 1: 200 in 0.2% Tween 20, 2.5% normal goat serum made in TBS. The slides were then incubated with goat anti-rabbit/mouse IgG-HRP antibody (Santa Cruz, 1:100 dilution) for 60 minutes and 3, 3'-diaminobenzidine (DAB) chromogen for 10 minutes at room temperature. The sections were counterstained with hematoxylin for 1 minute, dehydrate and mounted with Permount mounting media (Fisher Scientific). Images of the slides were captured with a Leica DMLA microscope (Leica Microsystems) using a cooled charge-coupled Hamamatsu C5810 camera (Hamamatsu Corp.) and Image Pro MC 6.1 software (Media Cybernetics). Three slides were selected from each group. CD31 and p-CDK1 positive and negative cells were quantified in several fields containing more than 700 cells for each control and treatment group using ImageJ software (NIH) and analyzed with GraphPad Prism 6 (GraphPad Software, San Diego, CA).

In vivo TUNEL assay

Apoptosis was assessed in mice tissue sections with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay with DeadEnd^{TM} Fluorometric TUNEL System (Promega) according to the manufacturer's protocol with some modifications. Briefly, mice tissues were fixed with 4% paraformaldehyde, incubated with equilibration buffer. Then, the tissues were incubated with reaction buffer (44 µL equilibration buffer, 5 µL nucleotide mix, and 1 µL TDT) at 37°C for 1 hour in the dark. The reaction was terminated by immersing the samples in 2× standard saline citrate (SSC) for 15 minutes. The tissue samples were counterstained with DAPI and mounted using Vectashield Mounting Medium (Vector Laboratories Inc.). Immunofluorescence microscopy was performed using Olympus IX71 microscope equipped with a 100-W high pressure mercury burner and filter sets (Olympus) to reveal green and blue fluorescent images individually. Images were captured using Hamamatsu ORCA-AG camera system and cellSens Dimension software (Olympus).

RNA-seq Profiling

HNSCC cell lines, PCI-13 that stably expresses high-risk *TP53* mutation (G245D) were treated for 48 hours with either vorinostat (4 µmol/L), AZD1775 (0.25 µmol/L) alone or in combination. Total RNA was isolated 36 hours after treatment using the RNeasy mini kit reagents (QIAGEN) according to the manufacturer's instructions. Cells treated with 0.1% DMSO served as untreated controls. For RNA-seq, total RNA was submitted to the Sequencing and Microarray Facility at MD Anderson Cancer Center for next-generation sequencing. For each of the samples, three replicates were performed using two sequencing lanes. Quality control (QC) was performed

using FastQC and FastQ Screen. Sequencing reads were aligned to GRCh37, using tophat2. Read counts for each gene region were calculated using SAMtools and normalized utilizing trimmed mean of M (TMM) method. Weakly expressed and noninformative (non-aligned) genes were filtered out before normalization. Data were then transformed to log2 counts-per-million (CPM). Differentially expressed genes (DEGs) were identified using linear model likelihood ratio and ANOVA-like tests implemented in the edgeR package with Benjamini-Hochberg multiple testing correction. Significance was set based on the FDR cutoff of 0.05 and in the meanwhile the genes are significant based on the ANOVA-like test results. Filtering criteria include selection of DEGs with an adjusted *P*-value of < 0.01, keeping genes that have at least 50 average reads and 4-fold change in the higher of the two comparison groups (vorinostat versus untreated controls), and exclusion of noncoding RNAs. Significant DEGs among the treatment groups were subjected to Gene Ontology (GO) and Ingenuity Pathway (IP) analysis to identify cell function. mRNA expression data from the Cancer Genome Atlas HPV-negative HNSCC cohort (*n* = 449) was also analyzed.

Reverse phase protein array (RPPA)

Samples were prepared as described previously (28). Briefly, PCI13-G245D cells were treated for 48 hours with vorinostat, AZD1775 alone or in combination, washed in ice-cold PBS containing protease and phosphatase inhibitors, vortexed and centrifuged. The resulting supernatants were collected and total protein content for each sample was quantitated using a BCA kit (Pierce Biotechnology Inc., Rockford, IL). RPPAs were printed from each lysate, with each sample printed in triplicate for quality control (QC). RPPA was performed by the RPPA core facility at the University of Texas MD Anderson Cancer Center. Protein expression data were generated by RPPA for PCI13-G245D using 190 antibodies. RPPA slides were quantified

using ArrayPro (Media Cybernetics) to generate signal intensities that were further processed by SuperCurve to estimate relative protein levels (in log2 scale). RPPA samples quality was monitored by a QC classifier and only the slides whose QC scores were above 0.8 (on a 0–1 scale) were used for further analysis. Distinct protein expression patterns present across the cell lines were assessed by unsupervised two-way hierarchical clustering using Pearson correlation distance between proteins (rows), Euclidean distance between cell lines (columns), and the Ward's linkage rule and by first principal component analysis (PCA). An analysis of variance (ANOVA) model was applied on a protein-by-protein basis to identify differentially expressed proteins. FDR and Benjamini-Hochberg corrected *P*-values were used to identify statistically significant and nonsignificant differences in protein expression patterns. In the analysis, we used a FDR of 1% (corresponding *P*-value = 0.0388) plus and additional requirement that the fold difference between treated and untreated groups be > 1.5 (to try to minimize inclusion of proteins where difference in expression might be statistically significant, but were less likely to be biologically significant) (28). All data analysis was conducted using R statistical software (version 3.1.). Significant differentially expressed proteins and phosphoproteins among the treatment groups were subjected to GO and Ingenuity Pathway (IP) analysis to identify cell function.

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Supplementary Figure Legends

Supplementary Figure S1. Synergistic effects of vorinostat and AZD1775 *in vitro* in established isogenic pair HNSCC cell lines with known *TP53* mutational status. A and B, clonogenic survival curves and images for HNSCC wildtype p53 (HN30) and p53 mutant (HN31) cells treated with a range of vorinostat (0.01-20 μmol/L) concentrations for 48 hours to determine the drug IC50. C and D, assessment of the degree of synergy between vorinostat and AZD1775 in HN30 and HN31 cells using the Chou and Talalay method (Median Dose-Effect analysis). AZD1775 and vorinostat were used at constant ratios (2:1 and 1:1 for HN30 and HN31 respectively). E-F, Fa-CI plots generated to determine the CI values (< 1.0 indicate synergism). The CI values for combination of effective drug doses (ED) that result in clonogenic survival inhibition of 50% (ED_{50}; fa = 0.5), 75% (ED_{75}; fa = 0.75), and 90% (ED_{90}; fa = 0.90) were generated from the conservative isobolograms (G-H). The ED_{50} (red X), ED_{75} (green crosses)

and ED_{90} (blue circles) graphed against fractional concentrations of vorinostat and AZD1775 on the y and x axis, respectively are indicated. All treatments were performed in triplicate and each experiment was repeated at least three times.

Supplementary Figure S2. Synergistic effects of vorinostat and AZD1775 *in vitro* in various established HNSCC cell lines with known *TP53* mutational status. A and B, clonogenic survival curves for HNSCC cells p53 null (OSC-19) and mutant p53 (Detroit562 and HN5) treated for 48 hours with a range of vorinostat concentration (0.01-10 μmol/L) and AZD1775 concentration (0.01-1.0) to determine the IC_{50}. C, E and G, representative images of the results of clonogenic survival assays. Assessment of the degree of synergy between vorinostat and AZD1775 in these cell lines was determined using the Chou and Talalay method (median dose-effect analysis) as previously indicated. Vorinostat and AZD1775 were used at constant ratios (10:1 respectively). The CI values for combination of effective drug doses (ED) that result in clonogenic survival inhibition of 50% (ED_{50}; fa = 0.5), 75% (ED_{75}; fa = 0.75), and 90% (ED_{90}; fa = 0.90) were generated from the conservative isobolograms. The ED_{50} (red X), ED_{75} (green crosses) and ED_{90} (blue circles) graphed against fractional concentrations of vorinostat and AZD1775 on the y and x axis, respectively are indicated. D, F and H, conservative isobologram plots demonstrate that vorinostat and AZD1775 acts synergistically to inhibit *in vitro* clonogenic survival of HNSCC cells. All treatments were performed in triplicate and each experiment was repeated at least three times.

Supplementary Figure S3. Laser scanning confocal microscopy showing stress-induced membrane blebbing prior to apoptosis in selected populations of PCI13-G245D cells. A and B, examples of 20x and 40x magnification images from control and combination treated samples pulsed with EdU prior to fixation as indicated previously and imaged using laser scanning confocal microscopy. The arrows indicate cells showing the stressed morphology (DIC images, left side) along with the associated EdU and nuclear images (right side), suggestive of cells about to undergo apoptosis during an impeded S phase.

Supplementary Figure S4. Combination of vorinostat and AZD1775 is associated with subsequent accumulation of cells in G2/M phase in HNSCC mutp53 cells. Unsynchronized PCI13-G245D cells were treated with either vorinostat (4 μmol/L), AZD1775 (0.25 μmol/L) alone or in combination for 48 hours. A and B, percentage of cell cycle distribution determined by flow cytometry is presented as cell cycle chromatograms and bar graphs respectively. C, mutp53 (G245D) HNSCC cells were subjected to FITC-APO-BrdU tunnel assay and cells stained positive for APO-BrdU indicative of apoptosis were monitored by flow cytometric analysis. An increase in sub G1 fraction indicative of cumulative apoptosis was also observed. Data shown are representative of two independent experiments.

Supplementary Figure S5. Vorinostat interacts synergistically with AZD1775 in HNSCC to induce markers of DNA damge, replication stress and apoptosis. HNSCC (OSC-19, Detroit562 and HN5) cells were treated with either vorinostat, AZD1775 alone or in combination for 16 and/or 48 hours and subjected to immunoblot analysis using antibodies as indicated. A-C, protein expression levels of p53 and p21 respectively. D-F, levels of phosphorylation of

double-strand break indicator H2AX (S139), Chk1 (S345), total Chk1, hyperphosphorylation of RPA32, Rad51, and RRM2. The cyclin B1 total protein, CDK1 (CDC2) tyr15 phosphorylation, and total protein levels were also analyzed. The cells were also examined for the presence of PARP-1 cleavage as marker of apoptosis. Lysates from staurosporine-treated (1 μmol/L) cells were used as positive controls for apoptosis. The β-actin served as loading control.

Supplementary Figure S6. Correlation between overall survival and expression levels of genes regulated by vorinostat alone or in combination with AZD1775 in HNSCC TCGA dataset. A, heatmap describes levels of 130 differentially expressed genes determined by RNA Seq in 449 patients with HPV-negative HNSCC in TCGA cohort. These genes were downregulated by vorinostat alone or in combination with AZD1775 in PCI13-G245D as shown in Supplementary Table S3. The hierarchical clustering analysis shows separation of these patients into two distinct clusters. Group 1 (red) shows lower expression and group 2 (blue) shows higher expression of the 130 genes. B and C, Kaplan-Meier plots represent long-term and 5-year overall survival curves for groups 1 and 2 respectively. Univariate analysis was carried out using Log-rank test. Multivariate analysis was done using the cox proportional hazards model adjusting sex, age, tumor grade, smoking, alcohol use, and tumor site to correlate patient clusters to overall survival. Tumors with lower expression of the genes downregulated by vorinostat alone or in combination with AZD1775 (group 1) correlated with better overall survival with almost statistical significance.

Supplementary Figure S7. Proteomic profiling of HNSCC mutant p53 cells treated with vorinostat and AZD1775 identifies proteins involved in cell cycle regulation. A, reverse-phase protein array analysis of PCI13-G245D cells treated with either vorinostat, AZD1775 alone or in combination for 48 hours, illustrated as hierarchical clustering and associated with the target GO terms. Shown are 118 proteins and phosphoproteins which are differentially expressed between treatment groups. Red and green colors indicate increased or decreased proteins or phosphoproteins levels, respectively. *P*-values are shown and fractional numbers (in parenthesis) on top indicate the proteins from the list of 118 proteins that linked to a cell function (bottom and pink boxes) divided by the total number of proteins listed for the function by GO enrichment. Protein level changes of > 1.5 fold, with an FDR ≥ 1% and *P*-value of < 0.05 revealed downregulation of a number of proteins and phosphoproteins involved in cell cycle regulation, including CDK1, cyclin B1, PLK1, EMA (mucin), FoxM1 and Rb_pS807_S811. B, western blot analysis confirmed downregulation of these proteins.

Supplementary Figure S8. **Tumor volume growth curves for individual mice in each treatment group.** PCI13-G245D cells expressing-high-risk mutp53 were injected into the oral tongues of mice and treatment with vorinostat and/or AZD1775 was initiated when tumors were less than 3 mm^3 in size as previously described. Mice were examined twice a week for 4 weeks where tumor size was measured with microcalipers, and tumor volume calculated as described previously. Individual tumor growth curves in each group are shown.

Supplementary Figure S9. Immunostaining of p21, PCNA, RRM2 and survivin in tumor of HNSCC oral xenografts mice. Tissue sections from tumors of mice bearing oral xenografts with PCI13-G245D cells treated with drugs as indicated were obtained, and subjected to immunohistochemistry with indicated antibodies as described in Supplementary Materials and Methods. A and B, immunostaining and quantification of immunohistochemical images of p21. C, D and E, F, are immunostaining and quantification of immunohistochemical images of PCNA and survivin respectively. G, immunostaining levels of RRM2. Quantification of immunohistochemical images was assessed and presented as percent positive and negative tumor cells for each protein examined in the tumor sections. Chi-square is used to compare immunostaining differences between vorinostat, vorinostat + AZD1775 vs AZD1775 and untreated control groups. Results demonstrate statistical significance between vorinostat, vorinostat + AZD1775 vs. AZD1775 and untreated controls ($P < 0.0001$). Images (A, C, E), (G) were taken at X100 and X200 magnification respectively.

Supplementary Figure S10. Combination of vorinostat and AZD1775 is well tolerated in an orthotopic mouse model of oral cancer. Athymic nude mice were injected into the lateral tongues with $5x10^4$ PCI13-G245D cells. Treatment was initiated when tumors were less than 3 mm^3 in size. Mice were treated with vorinostat (65 mg/kg i.p., daily), AZD1775 (45 mg/kg p.o., bid) alone or in combination. Control mice received equal volumes of vehicle. A, treatment schedule. B, average body weight during the course of treatment.

Supplementary Video S1: Live cell imaging of histone-H2B-RFP expressing PCI13-G245D cells in the presence of AZD1775. The yellow arrow indicates a cell undergoing an extended mitosis. . The green arrow indicates a cell with an extended mitosis followed by interphase entry and subsequent apoptosis.

Supplementary Video S2: Live cell imaging of histone-H2B-RFP expressing PCI13-G245D cells in the presence of vorinostat. The yellow arrow indicates a cell undergoing an extended mitosis, and subsequent binucleation and apoptosis. The green arrow indicates a cell with an extended mitosis and apoptosis.

Supplementary Video S3: Live cell imaging of histone-H2B-RFP expressing PCI13-G245D cells treated with vorinostat and AZD1775 combination. The yellow and green arrows indicate cells undergoing an extended mitosis with subsequent massive apoptosis in the next cell cycle interphase.