Supplementary Data:
Combined TRIP13 and Aurora kinase inhibition induces apoptosis in human papillomavirus–driven cancers

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Methodology for Supplementary data
Supplementary Figure S1: Quality control metrics for the HTDS demonstrating reproducibility. **A.** The 2 biological replicates for every drug–cell line combination in the HTDS were plotted and analyzed with concordance analysis to calculate the correlations using the concordance correlation (0.809), Pearson (0.81), and Spearman (0.70) coefficients. These data showed a strong, significant correlation between the biological replicates (Pearson \( r = 0.81; P < 10^{-16} \)), indicating that the assay was biologically reproducible. **B.** Heatmap showing an unsupervised clustering analysis of the residual standard error (RSE) values for all cell lines and drug combinations across the HTDS to ascertain the goodness of fit and to measure its technical reproducibility. CCC, concordance correlation coefficient.
Supplementary Figure. 2. Landscape of drugs that cause cell death in squamous carcinoma.

A, Normalized growth rate model adapted from Hafner et al. (1) demonstrating the area-over-the-curve lethal dose (AOC_LD; orange area). The AOC_LD value is 0 when a drug does not kill cancer cells at any concentration and greater than 0 when cancer cell death occurs.

B, Eight hundred and sixty-four unique drugs were divided into 51 classes based on the drug target and classified based on sensitivity (AOC_LD) in 33 cancer cell lines. The histogram on the left shows the frequency of each class in the high throughput drug screen. Orange indicates enrichment. Blue indicates depletion.

C-D, Unsupervised clustering of effective compounds on the basis of their AOC_LD values in each of the 33 cell lines defined 6 clusters. We combined drugs targeting AKT, DNAPK, GSK3β, mTOR, PDK1, PI3K, and PIKFYVE into 1 category (PI3K/AKT/mTOR); all CDK inhibitors into 1 category (CDK2/3/5/7/9); and drugs targeting MAPK/ERK and P38 MAPK into one category (MAPK), as these drugs target overlapping signaling pathways. Drugs with similar targets, particularly those targeting EGFR/HER2, CHK/WEE, and microtubules, were clustered together. We excluded classifiers with 5 or fewer drugs per category and drugs classified as “other” as it was composed of drugs with diverse targets. C, Dendrogram showing the drug clusters. D, Heatmap demonstrating the number of drugs in each of the 6 clusters (columns) for each drug classification (rows). Drugs in the same classification were mostly clustered together as evidenced by the chi-square test (P < 0.001) comparing drug classifications and cluster memberships. The color of each cell corresponds to the standardized Pearson residual measuring the strength of enrichment for the drugs allocated to each cluster.
Supplementary Figure S3: Landscape of chemotherapy and PI3K/AKT/mTOR inhibitors. A, B. Chemotherapy drugs were divided into 7 categories based on their mechanisms of action. A. Unsupervised clustering of the effective chemotherapy drugs by efficacy based on the AOC_LD values in each of the 33 cell lines defined 3 clusters. Data are represented as a heatmap with a dendrogram (A) or a table (B) demonstrating the number of drugs in each of the 3 clusters (columns) for each drug classification (rows). Drugs of the same classification were mostly clustered together, as evidenced by the chi-square test (P < 0.001) comparing drug classification and cluster membership. C, D. Drugs targeting the PI3K/AKT/mTOR pathway were divided into 7 categories based on their primary targets. Data are represented as (C) a table with the number of drugs in each of the 3 clusters (columns) for each drug classification (rows) or (D) a heatmap with a dendrogram. Unsupervised clustering of the effective drugs by efficacy based on the AOC_LD values in each of the 33 cell lines defined 3 clusters as demonstrated in the heat map of Pearson residual values. Orange indicates enrichment. Blue indicates depletion.
AMG900_AOCLD by HPV group
- t-test p = 0.0425
- Wilcoxon p = 0.0178

CCT137690_AOCLD by HPV group
- t-test p = 0.0885
- Wilcoxon p = 0.0804

CCT137690_AOCLD by HPV group
- t-test p = 0.0885
- Wilcoxon p = 0.0804

CYC116_AOCLD by HPV group
- t-test p = 0.0277
- Wilcoxon p = 0.0088

DANUSERTIB_AOCLD by HPV group
- t-test p = 0.1078
- Wilcoxon p = 0.1643

TAK901_AOCLD by HPV group
- t-test p = 0.4269
- Wilcoxon p = 0.5278

ZM447439_AOCLD by HPV group
- t-test p = 0.1188
- Wilcoxon p = 0.0234

TOZASERTIB_AOCLD by HPV group
- t-test p = 0.0348
- Wilcoxon p = 0.0068

CCT129202_AOCLD by HPV group
- t-test p = 0.3308
- Wilcoxon p = 0.1508

MK5108_AOCLD by HPV group
- t-test p = 0.1742
- Wilcoxon p = 0.1124
Supplementary Figure S4: Aurora kinase inhibitors were more effective in HPV positive cells using AOC_LD as the measure of efficacy. A. Enlargement of the 30 drugs from Fig 2B that reached a statistically significant differential effect ($P \leq 0.05$). B. Heatmap of AOC_LD values for 16 Aurora kinase inhibitors and 3 additional drugs that inhibit Aurora kinase (rows) in all 33 cell lines (columns). C. AOC_LD values of 14 Aurora kinase inhibitors as well as hesperidin, AT9283, and JNJ7706621. AOC-LD values were used as continuous variables, and cell lines were grouped by HPV status. Box plots show the median AOC_LD levels and 95% confidence intervals across all cell lines in the category. Each data point represents 1 cell line. D. IC$_{50}$ values ($\mu$M) for all 16 Aurora kinase inhibitors in HPV-positive cell lines represented as a heat-map. E. HPV-positive and HPV-negative cell lines were incubated with alisertib at drug concentrations ranging from 0.01 $\mu$M to 3.16 $\mu$M for 72 hours. Cells were counted immediately before incubation with alisertib and after 72 hours. Data are graphed as growth relative to baseline cell number (i.e., zero growth means that the cell number is the same as the cell number before drug treatment and negative values indicate less cells after drug treatment than prior to drug treatment)
Supplementary Figure S5: Aurora kinase inhibitors are more effective in HPV-positive cells with low Rb levels. **A.** Protein levels measured by RPPA were compared with drug sensitivities to 16 Aurora kinase inhibitors. Differentially expressed proteins (columns) that were statistically significant for at least 4 drugs (rows) are included on the plot. The size of the data point corresponds to the P value from a 2-sample t-test, and the color indicates the direction of the correlation. A negative correlation indicates that low levels of that protein were correlated with drug sensitivity. **B.** Representative immunoblots of the basal expression levels of pRb S807/S811, Rb, and p16 in HPV-positive and -negative cell lines. **C.** Levels of protein expression from immunoblots in all cell lines were quantitated and normalized for β-actin and then the positive control. Protein levels were compared to drug sensitivities for all Aurora kinase inhibitors. Representative box plots of pRb (S807/S811), Rb, and p16 expression are shown according to alisertib’s AOC_LD values.
Supplementary Figure S6: Expression of Rb protein predicts response to Aurora kinase inhibitors. A. Proteins levels of pRb (S807/S811) measured by RPPA were compared with drug sensitivities to 15 Aurora kinase inhibitors. Each data point represents 1 cell line. Box plots demonstrate the median pRb (S807/S811) level and the 95% confidence intervals across all cell lines. B. Proteins levels of Rb measured by immunoblotting were compared with drug sensitivities for 16 Aurora kinase inhibitors. C. Proteins levels of pRb (S807/S811) measured by RPPA were compared with drug sensitivities to 15 Aurora kinase inhibitors in sensitive and resistant HPV-positive cell lines as defined by AOC_LD values. Each data point represents one cell line. D. Representative immunoblots of the basal expression levels of 11 proteins that correlated with sensitivity to Aurora kinase inhibitors from Fig S5A in HPV-positive and -negative cell lines. E. Levels of protein expression from immunoblots in all cell lines were quantitated and normalized for β-actin and then the positive control. Protein levels were compared to drug sensitivity for all Aurora kinase inhibitors. Representative box plots of 9 proteins’ expression levels compared to AOC_LD values for alisertib are shown.
Supplementary Figure S7: *TP53* mutations correlated with drug resistance in 7 Aurora kinase inhibitors. **A.** Drug sensitivities of 16 Aurora kinase inhibitors were compared with the gene mutation status of the 50 most common HNSCC mutations in TCGA. Thirty-five of those 50 genes (rows) were mutated in 2 or more cell lines in the HTDS. The size of the data point corresponds to the *P* value from a 2-sample *t*-test. The color indicates the odds ratio. **B.** Aurora B mRNA, Aurora B protein, and Aurora A mRNA expression levels did not correlate with Aurora kinase inhibitor efficacy. Representative dot blots showed correlations between alisertib efficacy (AOC_LD) and Aurora B mRNA (*AURKB*, RNA-seq), Aurora B protein (RPPA), and Aurora A mRNA (*AURKA*, RNA-seq) expression.
Control ShRNA
Rb
Rb Sh1
Rb Sh2
Rb Sh4
Rb Sh6
Actin
HN31
UMSCC4

Fold change relative to Actin

Stable cells
Empty vector
E7-Ox (1)
E7-Ox (2)
E7
Rb
GAPDH
HN31
MDA886LN

mRNA fold change relative to Actin

Apoptotic + cells %

Cl. Caspase3
GAPDH
MDA886LN

Control
Aisertib
E7-Ox Clone(1)
E7-Ox (2)
E7-Ox (1) + Aisertib
E7-Ox (2) + Aisertib

HN31
Supplementary Figure S8: Effect of Rb expression on Aurora kinase A inhibition–induced apoptosis. A. Two HPV-negative cell lines were transfected with 10 nmol/L siRNA oligonucleotides pooled specific to RB1 or with control oligonucleotides. Twenty-four hours later, the cells were treated with 300 nmol/L alisertib for an additional 24 hours and then harvested and immunoblotted for the indicated proteins. B-C. HN31, UMSCC4, and MDA886LN cells were infected with lentivirus-based shRNAs, to stably knock down the expression of Rb. Four different shRNAs were introduced into each cell line. Cells were harvested, lysed, and immunoblotted for Rb expression, which was reported relative to that of cells infected with the scrambled control shRNA. ** P ≤ 0.001; *** P ≤ 0.0001. D. HN31 and MDA886LN cells were stably transfected with an E7-overexpressing (Ox) plasmid. Cells were harvested and immunoblotted for the indicated proteins. E. Quantitative polymerase chain reaction was performed in the indicated cells as in (D) to determine the E7 and Rb1 mRNA levels. Signals were normalized to actin transcripts. *** P ≤ 0.0001 (unpaired, 2-tailed Student t test). F. MDA886LN cells were stably transfected with E7-overexpressing plasmid and treated with 300 nmol/L alisertib for 24 hours. Cells were harvested and immunoblotted for the indicated proteins. G. Samples from (F) were used for annexin-PE staining to measure apoptosis and analysis with flow cytometry after treatment with 300 nmol/L alisertib for 24 hours. The bar graph represents the ratio of apoptosis (late plus early) among different groups. Values are the mean ± the standard deviation for 3 independent experiments. * P ≤ 0.02; ** P ≤ 0.002 (unpaired, 2-tailed Student t test). Cl, cleaved; Fl, full length; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. H. HN31 cells expressing RB1 or control shRNA were treated with 300 nmol/L alisertib for 24 hours before lysis and immunoblotting with the indicated antibodies. I. Two HPV-negative cell lines were seeded on coverslips (5 × 10^5). Twenty-four hours later, the cells were treated with 300 nmol/L alisertib for an additional 24 hours. Thereafter, cells were fixed and incubated with Rb-FITC conjugated or cleaved caspase 3-TRITC-conjugated antibody. Bars, 50 μm.
Supplementary Figure S9: Depletion of TRIP13 with aurora kinase inhibition demonstrated enhanced cell death in Rb deficient cell lines in vitro. A-C. mRNA levels of (A)TRIP13, (B) BUB1B and (C) MAD2L1 were compared with drug sensitivities to 9 Aurora kinase inhibitors. Each data point represents one cell line. Box plots show the median TRIP13 or BUB1B levels and 95% confidence intervals across all cell lines in the category. D-E. CASKI cells were transfected transiently with two different siRNAs targeting TRIP13 for 48h before lysis or mRNA extraction. Lysates were then immunoblocked (D) with the indicated antibodies and densitometric analysis of the intensities of the bands was calculated relative to the control siRNA (E). F. Quantitative polymerase chain reaction was performed to determine the TRIP13 mRNA levels. Signals were normalized to actin transcripts. G. TRIP13 was depleted by siRNA (2) in CASKI) cells. Cells were then treated with 100 nmol/L alisertib for 36 hours before being subjected to lysis and immunoblotting with the indicated antibodies. ** P ≤ 0.001; *** P ≤ 0.0001 (unpaired, 2-tailed Student t test). H. TRIP13 was depleted by siRNA in NSCLC cell lines followed by treatment with 100 nmol/L alisertib for 36h before Annexin-PE staining to measure apoptosis and analysis with flow cytometry. The bar graph represents the ratio of apoptosis (late plus early) among different groups. I-J. Levels of protein expression from immunoblots in seven NSCLC cell lines (J) were quantitated and normalized for β-actin and then the positive control. Protein levels were compared to drug sensitivity to combination (I).
Methodology for Supplementary data:

HTDS

We performed an HTDS using 864 unique drugs (Table S2) in 33 HNSCC and cervical squamous carcinoma cell lines (Table S3) as described previously (2). Briefly, 24 hours after plating, drugs were added to cells at concentrations ranging from 0.01 to 3.16 µmol/L and were then incubated for 72 hours at 37 °C. Cells were then fixed, permeabilized, stained with diamidino-2-phenylindole, and counted. Two biological replicates were performed at least 1 week apart for all cell lines. Ganetespib and paclitaxel were included on every assay plate as positive controls.

We performed quality control metrics in accordance with the National Center for Advancing Translational Sciences Assay Guidance Manual (3-10). This process includes calculating the Z-prime (Z′) from on-plate positive and negative controls to evaluate assay robustness, plotting the concordance of biological replicates to determine inter-batch effects, using the RSE to determine goodness of fit for dose-response curves, and calculating the minimum significant ratio for ganetespib and paclitaxel (Table S7) to track intra-batch reproducibility (4).

Calculation of AOC_LD curves

We used the growth normalization method proposed by Hafner et al (1) to quantify the extent of growth inhibition or cytotoxicity caused by treatment with individual drugs. In total, 6 doses were tested in duplicate from 10 nM to 3.16 uM in half log steps, which are then fitted using a cascade of logistic regression models. The normalized AOC_LD was then calculated by integrating the area above the negative (lethal) region of the fitted growth response curve. This resulted in an interpretable metric in which 0 represented drugs that did not kill cells and values approaching 1 represented drugs that had increasing toxicity.
References


