

Original Article

Smart drug combinations for cervical cancer: dual targeting of Bcl-2 family of proteins and aurora kinases

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Abstract: Human papillomavirus (HPV) is the main causative agent in cervical cancers. Recurrent cervical cancer is refractory to currently available treatments. Clearly there is an urgent unmet need to investigate new therapeutic strategies for both the newly diagnosed and recurrent patient populations. We have previously shown that the presence of HPV oncogenes sensitizes cells to inhibition of aurora kinases (AURKs), which induces mitotic delay eventually leading to apoptotic cell death. In this study, we explored whether a dual approach of combining an AURK inhibitor, MLN8237 (Alisertib), with a range of Bcl-2 family anti-apoptotic protein inhibitors would accelerate cancer cell killing. Enhanced and rapid cervical cancer cell killing was observed when Alisertib was combined with inhibitors of either Bcl-2 (Venetoclax), Bcl-XL (A1331852) or Mcl-1 (A1210477) proteins, likely by accelerating apoptosis during mitotic delay due to the loss of functional Bcl-2, Mcl-1, or Bcl-XL. This study presents a promising approach to treating aggressive cervical cancers and may apply to other HPV-related cancers.

Keywords: HPV, aurora kinase, cervical cancer, alisertib

Introduction

Human papillomavirus (HPV) is the fourth most prominent cause of cancer in women, the primary cause of cervical cancer. Despite the overwhelming success of HPV vaccines and pap smears, they do not guarantee a lifetime protection against HPV-related cancers and previous exposure prior to vaccination can still lead to the development of cervical carcinomas. Current therapy for cervical cancer involves a combination of surgery, radiotherapy, and chemotherapy that often results in permanent, life-altering adverse effects. We have previously shown that HPV oncogene, E7, sensitizes cells to the inhibition of the aurora kinases (AURKs) and treatment is highly effective at eliminating early tumours and reducing large, late tumours [1, 2]. AURKs have key roles in the transition into, through and out of mitosis. AURKA is required for progression into mitosis and establishing a proper spindle pole, and AURKB is required for exit from mitosis and correct cell division. Functionally, AURKA and B inhibition using MLN8237 (Alisertib) cause HPV

positive (+) cells to take longer to traverse mitosis and key anti-apoptotic proteins degrade and apoptosis is induced (1). As we now understand how AURK inhibition affects HPV+ cancer cells, we wish to explore if we can exploit secondary vulnerabilities in cervical cancer cells, using a second molecular inhibitor, to push cells more quickly and effectively towards death. Our previous work showed that Alisertib treatment induces cell death of HPV+ cancer cells via an Mcl-1 sensitive apoptotic mechanism [1, 3]. We also observed that Alisertib treatment had some effect on the expression of other Bcl-2 family members, Bcl-2 and Bcl-XL [1]. Overall, sensitivity to Alisertib was defined by E7 expression and also potentially by the level of Bcl-2 related anti-apoptotic proteins. Therefore, in this study we wish to enhance the effect of Alisertib by adding inhibitors of anti-apoptotic Bcl-2 family of proteins, Bcl-2, Bcl-XL and Mcl-1.

Results and discussion

To date, no work has been done to assess the effect of combining other drugs with AURK

inhibitors (AURKi) in HPV+ cancers. We firstly queried the Cancer Target Discovery and Development (CTD2) (<https://ocg.cancer.gov/programs/ctd2>) and Genomics of Drug Sensitivity in Cancer (GDSC) (<https://www.cancerrxgene.org/>) databases to assess any correlation between the level of expression of anti-apoptotic proteins with sensitivity to Alisertib. The cell lines represented in the databases were initially defined as either sensitive or insensitive based on natural inflection points in the drug sensitivity data of all cell lines. The expression levels of the components of the apoptotic machinery, Bcl-2, Bcl-XL, Bcl-W, A1, Mcl-1, Bid, Bim, Bad, Bax, PUMA, NOXA and XIAP genes were assessed. Mcl-1 expression was relatively constant across all cell lines, but Mcl-1 levels are controlled by the E3 ligase, FBXW7 [4], and this was added. It showed that lower levels of the anti-apoptotic Bcl-XL and Bcl-W were significantly associated with increased sensitivity to Alisertib in both datasets (**Figure 1A** and **1B**). Increased FBXW7, suggesting lower Mcl-1 levels, was also associated with sensitivity. Increased pro-apoptotic NOXA, a selective inhibitor of Mcl-1 was associated with sensitivity. Interestingly, a modest decrease in Bad expression, a selective inhibitor of Bcl-2, Bcl-XL and Bcl-W, is also associated with sensitivity. Another mitotically targeted drug, PLK1 inhibitor, BI-2536, showed a similar profile of sensitivity (**Figure 1C**). Surprisingly, increased Bcl-2 expression was associated with increased sensitivity to both drugs. Unsurprisingly, high Bcl-2 expression is associated with sensitivity to the Bcl-2 inhibitor, Venetoclax (**Figure 1D**). We have previously reported that Alisertib treatment only affected Mcl-1 (decreased) and Bim (increased) levels [1]. These findings suggested that inhibiting specifically Bcl-2 and Mcl-1 might increase the sensitivity to Alisertib.

In light of this, we explored the effectiveness of combining Alisertib with commercially available inhibitors of Bcl-2 (Venetoclax), Bcl-XL (A1331852) and Mcl-1 (A1210477) on Alisertib-sensitive HPV+ cervical cancer cell lines [1, 2], HeLa and CaSki. It is important to note that these cells express varying levels of Bcl-2 proteins (**Figure 1E**). There was higher Bcl-2 and Mcl-1 and lower Bcl-xL expression in HeLa cells, compared to CaSki cells, consistent with our previous observations [1]. Indeed, combining Alisertib with a range of Bcl-2 family anti-apoptotic protein inhibitors were more effective than inhibitors alone (**Figure 1F**). Importantly, the

effect of combining Alisertib with any of the tested anti-apoptotic protein inhibitors was synergistic (**Figure 1G**). Dual inhibition of AURKs and Bcl-2 family anti-apoptotic proteins have been explored in other cancer types [5-11], but not HPV+ cancers. Given the premise that AURK inhibition drives cells to undergo apoptosis through the loss of Bcl-2 family expression in other cancer types [12, 13], these cells are vulnerable to further inhibition of these Bcl-2 family members.

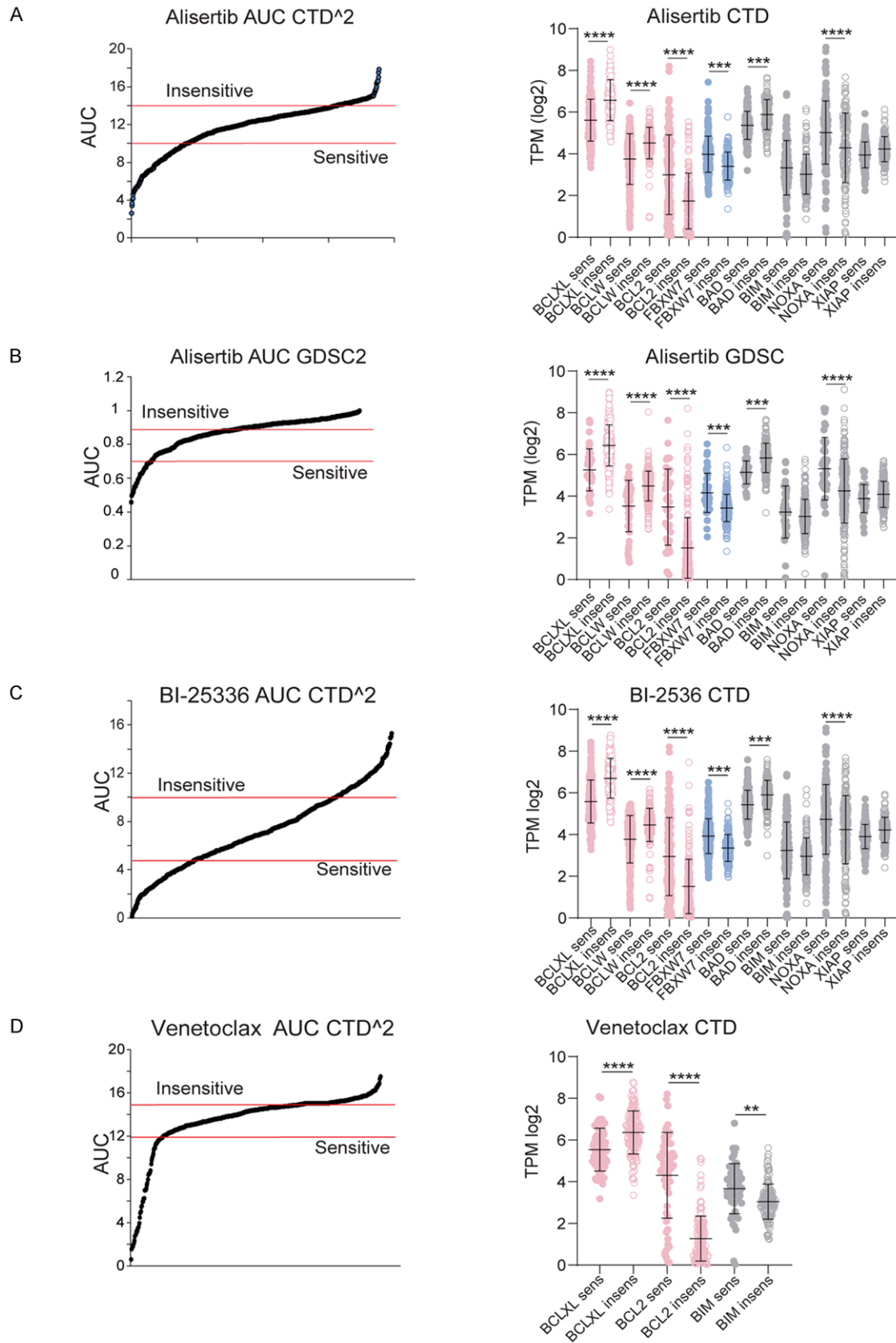
We then focused on elucidating the onset of cell killing by these dual combinations on HeLa cells. Compared to drugs alone, dual Alisertib and Bcl-2 family anti-apoptotic protein inhibitor combinations shorten the time that cell death events first appeared (**Figure 2A**), concurring with the occurrence of cells undergoing a smaller number of cell divisions (**Figure 2B**). Collectively, this suggests that dual combinations induce the rapid onset of cell death. Notably, combination of Alisertib with A1331852 significantly induced cell death as early as 6 hr post-treatment when compared to A1331852 treatment alone (**Figure 2C** and **Supplementary Table 1**). Indeed, Alisertib combined with A1331852 produced the most prominent apoptotic killing effect, which is confirmed by higher levels of PARP protein cleavage seen with this combination compared to other combinations (**Figure 2D**). Co-targeting AURKs and Bcl-2 proteins together has been done in a number of cancer models [14-16]. This is the first study testing this combination for cervical cancers. Enhanced and rapid cervical cancer cell killing observed with this combination likely occurs by accelerating apoptosis during mitotic delay due to the loss of functional Bcl-2 family of proteins. Taken together, our study showed that co-targeting AURK and Bcl-2 family of proteins could represent a novel alternative treatment strategy for cervical cancer. Importantly, this combination could be applied to other HPV-driven cancers. Combination treatment between Alisertib and inhibitors of Bcl-2 family members should be subjected to future clinical trials to overcome specific molecular vulnerabilities in cervical cancer.

Materials and methods

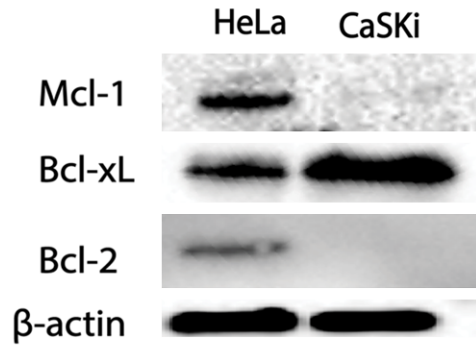
Cell culture

HeLa and CaSki cells were obtained from the ATCC. Cells were maintained in complete me-

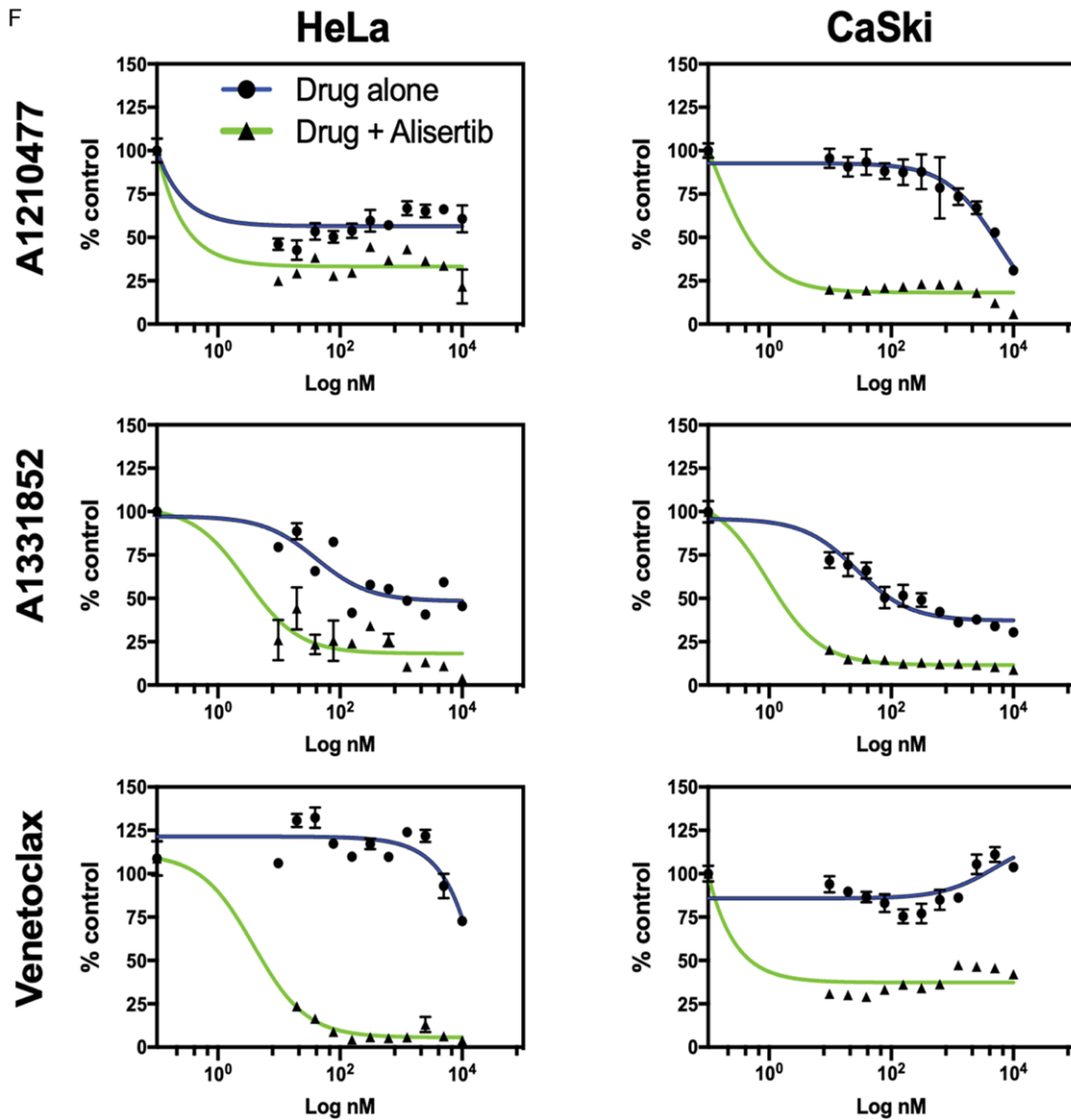
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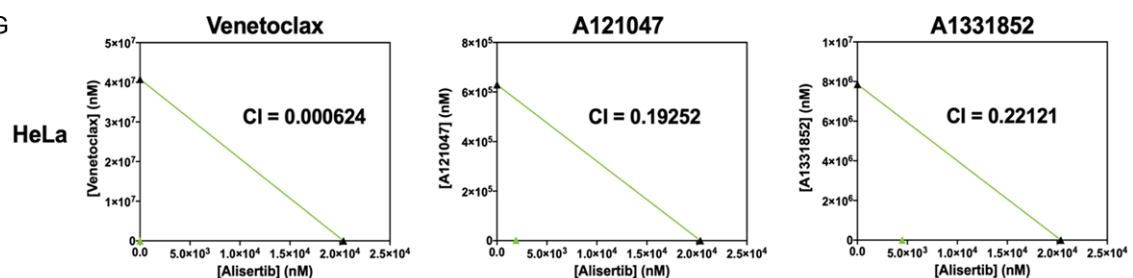
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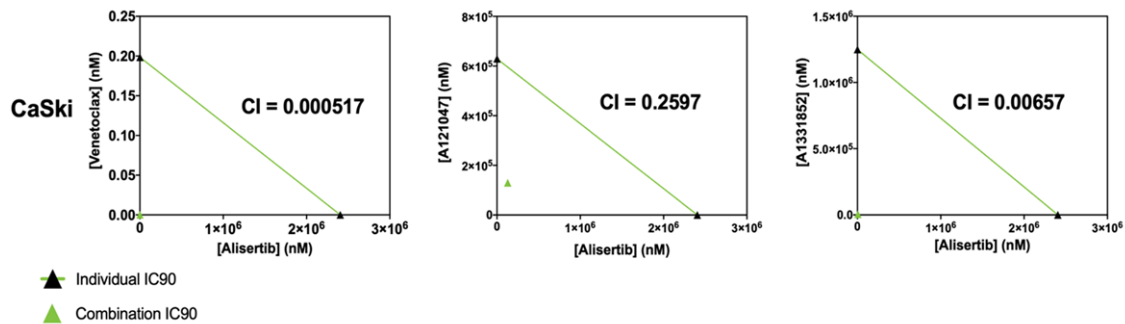
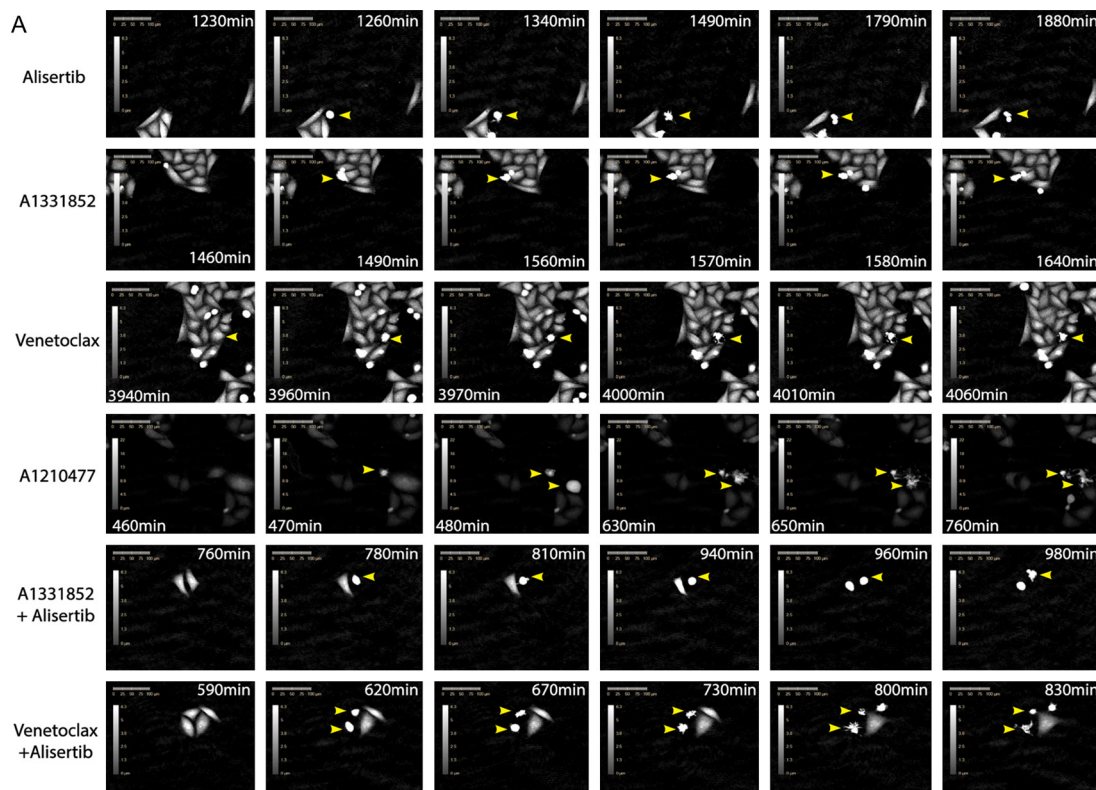


Figure 1. Inhibitors of anti-apoptotic BCL-2 family proteins enhanced Alisertib-mediated toxicity in cervical cancer cells. A-D. Anti-apoptotic family gene expression correlates with sensitivity to Alisertib. GDSC2 [18] and CTD² [19] small molecule viability datasets in DepMap were analysed using the DepMap portal Data Explorer (<http://www.depmap.org>). Alisertib (MLN8237) was present in both datasets (725 cell lines in CTD²; 404 cell lines in GDSC2). The area under the inhibition curves (AUC) was used for each dataset, with lower AUC indicating sensitivity to the drug. Sensitive and insensitive cell lines were defined by natural inflection points in the drugs sensitivity data for all cell lines and gene expression for the sensitive and insensitive cells was reported from the Cancer Cell Line Encyclopedia. The mean and SD of the gene expression (TPM) were calculated for each sample set and the significance determined using one-way ANOVA with Tukey's multiple comparison test. E. HeLa and CaSki cells express varying levels of BCL-2 anti-apoptotic protein family members. Protein lysates from cells were immunoblotted for Mcl-1, Bcl-xL and Bcl-2 proteins. β -actin was used as a loading control. Individual blots shown are representative of three independent experiments. F. Combination of Alisertib with anti-apoptotic protein inhibitors enhances cervical cancer cell killing. Cells were treated with Venetoclax, A1331852 and A1210477 at increasing doses either in the presence or absence of Alisertib (500 nM) before measuring cell viability after 3 days by the MTT assay. Data is representative of one out of three independent experiments. Data points represent the mean $\pm$ SEM. G. The effect of combining anti-apoptotic protein inhibitors and Alisertib in cervical cancer cells is synergistic. Standard isobologram analysis of cell killing by different drug combinations. IC90 values of each drug are plotted on the axes; the solid line represents the additive effect, while the points represent concentrations of each drug resulting in 90% inhibition of growth. Points falling below the line indicate synergism between drug combinations whereas those above the line indicate antagonism. Combination index (CI) at fractional response 0.9 (90% killing) are also shown. All CI values are <1 , indicative of a synergistic effect. Data is representative of three independent experiments.



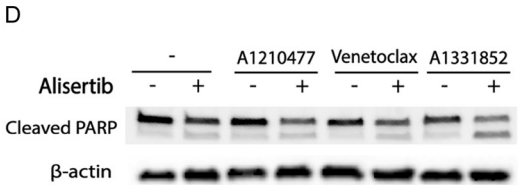
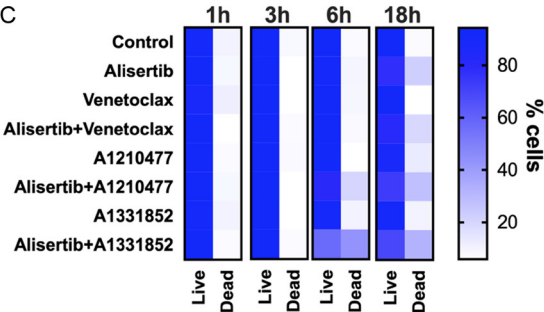
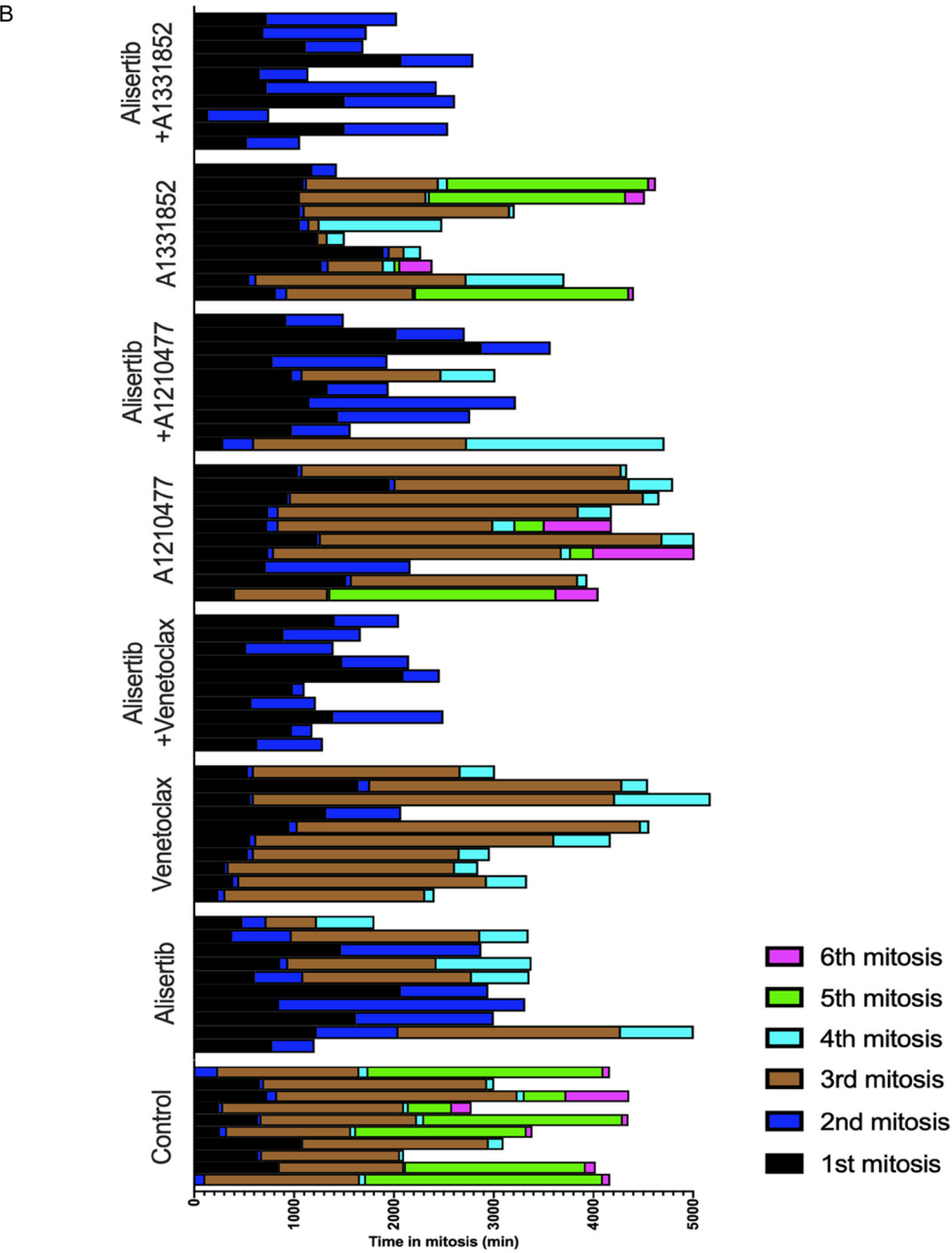
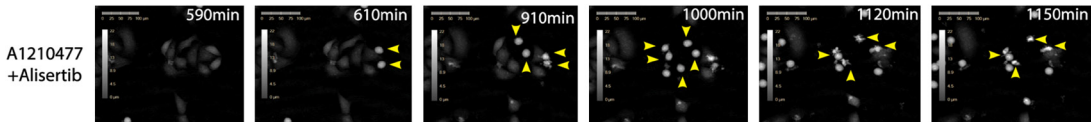


Figure 2. Anti-apoptotic BCL-2 inhibitors accelerate Alisertib-mediated killing. HeLa cells were treated either alone, with Alisertib (500 nM) or in combination with either venetoclax (15 nM), A1331852 (40 nM) or A1210477 (1 nM). A. Anti-apoptotic Bcl-2 inhibitors induce early killing when combined with Alisertib. Images were captured by time-lapse microscopy over 72 h. Still images shown were captured at times indicated. Yellow arrows denote cells that are undergoing cell death. Data presented are representative of one out of three independent experiments. B. Combined drug treatments underwent fewer rounds of mitosis. 10 individual cells were tracked from time-lapse microscopy videos using the Hstudio 2.7.5™ live imaging software and the time in mitosis and number of cell divisions were plotted on the graph over a 72 h period. C. Alisertib combined with anti-apoptotic inhibitors induces early cell death onset. Dead (not double negative for annexin V and propidium iodide) and live (double negative for annexin V and propidium iodide) cell populations were determined by flow cytometry via Annexin V/Propidium Iodide staining at the indicated time points. Percentage of cells are represented on a heat map plot. Data is representative of one out of at least three independent experiments. One-way Anova analysis was done for the 6 h time point to highlight significantly induced cell death seen with the combination of Alisertib with A1331852 (Supplementary Table 1). D. Enhanced killing seen with combined Alisertib and A133185 treatment correlated with elevated PARP cleavage. Proteins from cells collected at 24 h were immunoblotted for cleaved PARP. β -actin was used as a loading control. Individual blots shown are representative of three independent experiments.

dia; DMEM (Gibco-Invitrogen, Waltham, MA) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Gibco-Invitrogen, Waltham, MA) and 1% of antibiotic/glutamine preparation (100 U/ml penicillin G, 100 U/ml streptomycin sulphate, and 2.9 mg/ml of L-glutamine) (Gibco-Invitrogen, Waltham, MA).

Chemicals

MLN8237 (Alisertib), Venetoclax, A1331852, and A1210477 were all purchased from Selleck Chemicals (Houston, TX) and dissolved in sterile-grade DMSO (Sigma-Aldrich, St Louis, MI).

Cell viability assessment

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent was added at a final concentration of 0.5 mg/ml for an additional 1 h. MTT crystals were dissolved in 100% DMSO before reading the colorimetric absorbance at 544 nm on a FLUOstar OPTIMA microplate reader (BMG LabTech, Germany).

Time-lapse microscopy

Treated cells were followed by time-lapse microscopy using Holometer®, a cell stain-free phase holographic imager (PHI AB, Lund, Sweden) at 37°C and 5% CO₂ and data analysed in Hstudio 2.7.5™ (PHI AB, Lund, Sweden) on 24-well plate (STARSTED, Nümbrecht, Germany). Images were captured at 10 min intervals.

Drug synergism determination

Drug interactions between Alisertib and other drugs were assessed using CompuSyn soft-

ware version 1.0 (ComboSyn, Inc., Paramus, NJ), and determined by isobologram analysis at fraction response 0.9 (90% killing). Combination index (CI) analysis was based on the median-effect principle and computed using the following formula: $CI = D1/(Dx)1 + D2/(Dx)2$. D1 and (Dx)1 are concentrations of Alisertib and the added drug, respectively, that inhibit cell growth by 90% of control when used alone. D2 and (Dx)2 are concentrations of Alisertib and the added drug, respectively, that inhibit cell growth by 90% of control when used in combination. The combined effects of various concentrations at a ratio of 1:1 of Alisertib and other drugs were assessed and the CI was calculated according to the Chou-Talalay method [17]. In brief, a CI value which was <1 = synergistic effect, 1 = additive effect and >1 = antagonistic effect.

Flow cytometry

The percentage of dead cells (cells that are not double negative for annexin V and propidium iodide) post-treatment was determined by flow cytometry using the Annexin V-FITC Apoptosis Kit (#K101) (BioVision, Milpitas, CA) as per manufacturer's protocol. Samples were analysed on a BD LSR FORTRESSA cell analyser (BD bioscience, San Jose, CA).

Immunoblotting and antibodies

Protein from cells were extracted in boiling sodium dodecyl sulfate (SDS) cell lysis buffer (50 mM Tris-HCL pH 7.48 + 2% SDS). Immunoblots were probed with antibodies against PARP (PARP) (Cell Signaling Technologies, Danvers, MA, #9524) and β -actin (Cell Signaling Technologies, Danvers, MA, #4967). Rabbit

(Cell Signaling Technologies, Danvers, MA #7074) and mouse (Cell Signaling Technologies, Danvers, MA, #7076) secondary antibodies and ECL were used to detect the signals on a Chemidoc XRS Visualiser (BioRad, Hercules, CA).

Statistical analysis

The mean and standard deviation (SD) of the gene expression (TPM) were calculated for each sample set and the significance determined using one-way ANOVA with Tukey's multiple comparison test. Comparison of percentage of dead cells between different drug combinations at 6 h post-treatment was determined using one-way ANOVA with Holm-Sidak's multiple comparisons test. All statistical analyses were performed using the statistical software package GraphPad Prism 8.4.2.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Comparison of percentage of dead cells between different drug combinations at 6 h post-treatment using Ordinary One-Way Anova analysis

	Mean difference	Standard Error of difference	n1	n2	Significant?	Summary	Adjusted P Value
Control vs. Alisertib	0	3.975	3	3	No	ns	>0.9999
Control vs. Venetoclax	-0.9933	3.975	3	3	No	ns	>0.9999
Control vs. Alisertib+Venetoclax	-1.063	3.975	3	3	No	ns	>0.9999
Control vs. A1210477	-2.603	3.719	3	4	No	ns	0.9989
Control vs. Alisertib+A1210477	-8.83	3.719	3	4	No	ns	0.4523
Control vs. A1331852	-5.65	3.975	3	3	No	ns	0.9507
Control vs. Alisertib+A1331852	-22.97	3.719	3	4	Yes	***	0.0002
Alisertib vs. Venetoclax	-0.9933	3.975	3	3	No	ns	>0.9999
Alisertib vs. Alisertib+Venetoclax	-1.063	3.975	3	3	No	ns	>0.9999
Alisertib vs. A1210477	-2.603	3.719	3	4	No	ns	0.9989
Alisertib vs. Alisertib+A1210477	-8.83	3.719	3	4	No	ns	0.4523
Alisertib vs. A1331852	-5.65	3.975	3	3	No	ns	0.9507
Alisertib vs. Alisertib+A1331852	-22.97	3.719	3	4	Yes	***	0.0002
Venetoclax vs. Alisertib+Venetoclax	-0.07	3.975	3	3	No	ns	>0.9999
Venetoclax vs. A1210477	-1.609	3.719	3	4	No	ns	0.9999
Venetoclax vs. Alisertib+A1210477	-7.837	3.719	3	4	No	ns	0.6119
Venetoclax vs. A1331852	-4.657	3.975	3	3	No	ns	0.9841
Venetoclax vs. Alisertib+A1331852	-21.97	3.719	3	4	Yes	***	0.0003
Alisertib+Venetoclax vs. A1210477	-1.539	3.719	3	4	No	ns	0.9999
Alisertib+Venetoclax vs. Alisertib+A1210477	-7.767	3.719	3	4	No	ns	0.6119
Alisertib+Venetoclax vs. A1331852	-4.587	3.975	3	3	No	ns	0.9841
Alisertib+Venetoclax vs. Alisertib+A1331852	-21.9	3.719	3	4	Yes	***	0.0003
A1210477 vs. Alisertib+A1210477	-6.228	3.443	4	4	No	ns	0.7845
A1210477 vs. A1331852	-3.048	3.719	4	3	No	ns	0.998
A1210477 vs. Alisertib+A1331852	-20.36	3.443	4	4	Yes	***	0.0003
Alisertib+A1210477 vs. A1331852	3.18	3.719	4	3	No	ns	0.998
Alisertib+A1210477 vs. Alisertib+A1331852	-14.14	3.443	4	4	Yes	*	0.0132
A1331852 vs. Alisertib+A1331852	-17.32	3.719	3	4	Yes	**	0.0039