

***In vitro* antitumor effects of FGFR and PI3K inhibitors on human papillomavirus positive and negative tonsillar and base of tongue cancer cell lines**

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Abstract. Human papillomavirus positive (HPV⁺) tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC) have better outcomes than corresponding HPV⁻ negative (HPV⁻) cancer cases. Our previous study demonstrated that fibroblast growth factor receptor 3 (FGFR3) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit a (PIK3CA) are often mutated in HPV⁺ cancer. To investigate whether targeted therapy is an option for TSCC/BOTSCC, two HPV⁺ and one HPV⁻ TSCC/BOTSCC cell lines were tested for their sensitivity towards FGFR and PI3K inhibitors. The HPV⁺ cell lines UM-SCC-47 and UPCI-SCC-154, and the HPV⁻ cell line UT-SSC-60A were tested by competitive allele-specific TaqMan-PCR for presence/absence of frequently occurring FGFR3 and PIK3CA mutations. All cells were then treated with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120 alone, or with AZD4547 and BEZ235 in combination. Viability was analyzed using a WST-1 assay, cytotoxicity tested by a CellTox Green cytotoxicity assay, apoptosis analyzed by a Caspase Glo 3/7 assay and proliferation examined with the xCELLigence system. HPV⁺ UM-SCC-47 and UPCI-SCC-154 cells, and HPV⁻ UT-SSC-60A cells, did not exhibit any common FGFR3 or PIK3CA mutations, but were all sensitive to FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120. Notably, HPV⁺ UPCI-SCC-154 cells were more sensitive than the other two cell lines. Furthermore, when AZD4547 and BEZ235 treatment was combined in HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SSC-60A cells, potentiated

combination effects were observed. HPV⁺ UM-SCC-47 and UPCI-SCC-154 cells, and HPV⁻ UT-SSC-60A cells had no common FGFR3 or PIK3CA mutations, but were sensitive to FGFR inhibitor AZD4547, and PI3K inhibitors BEZ235 and BKM120. Furthermore, the latter two cell lines were particularly sensitive to combinations of AZD4547 and BEZ235.

Introduction

Human papillomavirus positive (HPV⁺) tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC) generally have a better outcome than the corresponding HPV⁻ negative (HPV⁻) cancers and most other head neck squamous cell carcinoma (HNSCC), and their incidences have increased considerably (1-15). Most HPV⁺ TSCC/BOTSCC patients do therefore not need the intensified treatment, more recently given to HNSCC patients in general, and which has not improved survival for patients with HPV⁺ TSCC/BOTSCC (5,16-18). To better individualize treatment, experimental approaches have been made to find predictive biomarkers (19-27). Such markers were e.g. high CD8⁺ tumor infiltrating lymphocyte counts, HPV16 E2 mRNA expression, absent/low HLA class I, HLA-A*02, CD44, LMP10 expression, high LRIG1 or CD98 expression, and combined in mathematical models, they identified ~50% of patients with high survival probability (16-27).

To identify even more markers for prognostication, we performed next-generation sequencing (NGS) of hot spot mutations in 50 cancer related genes and found, similar to others, that PIK3CA mutations are common in HPV⁺ TSCC/BOTSCC (28,29). In our study, however presence of FGFR3 mutations were also relatively common and correlated to worse prognosis, and later similar to others we found that FGFR3 was overexpressed in TSCC/BOTSCC and oropharyngeal cancer (29-31). In view of the fact, that targeted therapy against FGFR3 and PIK3CA has recently been initiated in other types of cancers, it could also be useful to consider for TSCC/BOTSCC, and for reviews see e.g. (32,33). This would especially be important for HPV⁺ TSCC/BOTSCC patients with high risk of a poor outcome, where today's chemotherapy and radiotherapy is suboptimal (18,32,33).

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Here, the HPV⁺ cell lines UM-SCC-47 and UPCI-SCC-154, and the HPV⁻ cell line UT-SCC-60A were tested for possible presence of the most common FGFR3 and PIK3CA mutations by competitive allele-specific TaqMan-PCR (CAST-PCR). They were then treated with FGFR inhibitor AZD4547, or PI3K inhibitors BEZ235 and BKM120, alone or the former two combined, to investigate the sensitivity of the cell lines to any of the drugs alone or combined, and to whether the combination of drugs was beneficial or antagonistic.

Materials and methods

Cell line characteristics and cell culture. All cell lines were derived from primary tumors prior to treatment. HPV⁺ UM-SCC-47, a squamous cell carcinoma isolated from the primary tumor of the lateral tongue and UPCI-SCC-154, a squamous cell carcinoma of the tongue were provided by Susan Gollin, University of Pittsburgh USA (34,35). HPV⁻ UT-SCC-60A, a tonsillar squamous cell carcinoma, was kindly obtained from Reidar Grénman, University of Turku, Finland (36). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco), with 10% fetal bovine serum (FBS; Gibco), 1% L-glutamin, 100 U/ml of penicillin, and 100 µg/ml streptomycin. All cells were maintained at 37°C in a humidified incubator with 5% CO₂. Possible presence of FGFR3 or PIK3CA mutations were analyzed as described below.

Competitive allele-specific TaqMan PCR (CAST-PCR). Detection of FGFR3 and PIK3CA mutations was performed by Competitive Allele-Specific TaqMan[®] PCR technology (Thermo Fischer Scientific) as described in detail earlier (30). Briefly the analysis was performed in 384-well plates, in 10 µl comprising 5 µl 2X TaqMan Genotyping Mastermix (Thermo Fischer Scientific), 0.2 µl 50X Exogenous Internal Passive Control (IPC) template DNA, 1 µl 10X Exogenous IPC mix, µl Mutation Detection Assay, 1.8 µl deionized water and 20 ng DNA (in 1 µl). Runs were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using a following set of reaction conditions described in detail before (30). The PCR result was analyzed with the SDS 2.3 software program and Mutation Detector Software 2.0 (Thermo Fischer Scientific). Ct was determined for exogenous IPC reagents added to every reaction to evaluate PCR failure or inhibition in a reaction. The Mutation Detection Assays detecting the reference FGFR3 gene and its variants p.R248C, p.S249C and p.K650Q have been described before (30). The same system was used to detect the reference gene PIK3CA and its variants p.E542K, p.E545K and p.H1047R. Mutation Detection Assays were Hs00000822_mu, Hs00000824_mu, Hs00000831_mu, which detects variants p.E542K, p.E545K and p.H1047R in the PIK3CA gene respectively, and reference assay Hs00001025_rf was used for detection of wild-type PIK3CA.

Treatment with FGFR and PI3K inhibitors after cell seeding and experimental set up

Cell seeding. For most assays, 5,000 cells were seeded out into 80-100 µl medium (without penicillin and streptomycin) per well of 96 well plates, where the outer wells were not used, but filled with medium to avoid any edge effects.

Treatment with FGFR and PI3K inhibitors and experimental set up. The cells were treated with different inhibitors 24 h (h) after seeding. For treatment, the PI3K inhibitors BEZ235, which is a PI3K and mTOR inhibitor and BKM120, which is a pan-class I PI3K inhibitor and the selective FGFR inhibitor AZD4547 a potent selective ATP-competitive receptor tyrosine kinase inhibitor of FGFR1-3 (all from Selleck Chemicals) were used. The drugs were diluted in DMSO in order to generate the stock concentrations and stored at -20°C. These stocks were further diluted with PBS and added directly to the cells in media to achieve the end concentrations. For BEZ235 and BKM120 concentrations of 0.25-5 µM were used, while for AZD4547, concentrations of 5-50 µM were used, and in combination experiments the highest concentrations were generally avoided. All experiments were repeated at least three times. Overall, after treatment the cells were incubated for 24-72 h, or up to 120 h, and assays were applied to investigate cell viability, cytotoxicity and apoptosis for all cell lines when using one drug at a time. Thereafter, proliferation tests were performed on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A using all three drugs one by one. After these initial experiments, combinations of AZD4547 and BEZ235 were tested on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A, and viability, cytotoxicity, apoptosis and proliferation was followed for 24-72 h or up to 120 h. All experiments were repeated at least three times unless indicated otherwise. For most figures, a summary of at least three experiments are presented, however, for proliferation tests, representative experiments are shown. The ratio-to-control parameter was calculated by the following formula: Ratio/Control=(mean experimental values-mean blank values)/(mean PBS control values-mean blank values), for both the WST-1 and the cytotoxicity assay. For the apoptosis assay the ratio-to-control parameter was calculated in comparison to the PBS control.

WST-1 viability assay. Three 96-well plates were seeded and treated the same way to examine three time-points (for details of seeding see above). Duplicates were used for each single drug concentration, and triplicates for drug combinations, PBS was used as a control and two wells with medium alone, were used to measure the blank background. After 24, 48 and 72 h, 10 µl of the WST-1 reagent (Roche, Mannheim, Germany) was added. The plates were incubated for 1 h at 37°C in the incubator and the absorbance was measured at 450 nm with a Versa Max microplate reader (Molecular Devices). In the analysis the average blank was subtracted from every value. The average of the duplicates/triplicates was calculated. The average values were normalized to the average control treated with PBS. In addition, the IC₅₀ (inhibitory concentration 50%) of the drugs on different cell lines were determined from log concentrations-effect curves in GraphPad Prism (GraphPad Software Version 8) using non-linear regression analysis.

CellTox Green Cytotoxicity assay. The CellTox Cytotoxicity assay (Promega) was performed after 48, 72, 96 and 120 h and 5,000 cells were seeded out in 96-well plates Corning Costar black (Merk) with clear flat bottom and an end-volume of 80 µl. For each drug concentration triplicates were prepared, PBS was added to the negative and positive controls and

medium was filled into two blank wells. Before measurement 4 μ l cell lysis solution (Promega) was added to the positive controls and the plates were incubated for 30 min (min) at 37°C in the incubator. Then 20 μ l CellTox green dye solution, 1:100 dye to buffer assay (both from Promega Corporation), was added to treated, blank, and control wells. The plates were wrapped in tin foil to avoid light irradiation and incubated on the shaker at RT for 15 min. The read out was performed on the SPARK 10M multimode microplate plate reader (Tecan Group Ltd.) with 485 nm excitation and 520 nm emission.

Caspase-Glo 3/7 apoptosis assay. The Caspase-Glo 3/7 Assay was done in duplicates immediately afterwards in the plates used for the CellTox Green Cytotoxicity Assay. 100 μ l reagent (Promega Corporation) was added to every treated well except the positive control of the Cell Tox Green Cytotoxicity Assay. The plates wrapped in foil were incubated at RT for one hour on the shaker. Luminescence was measured with the Centro LB 960 Microplate Luminometer (Berthold Technologies).

Proliferation assay. Cell proliferation was measured within the xCELLigence RTCA DP instrument, and by using E-plate VIEW 16 plates (ACEA Biosciences Inc.). In order to measure the background, 50 μ l medium was added into each well and set in the cradle and starting step one to measure the background. The reads should be below a cell index of 0.6, where the cell index is defined as changes in response to cell number and morphology. Afterwards, 5,000 cells in 50 μ l medium of the UT-SCC-60A and UPCI-SCC-154 cell lines were seeded out in the E-Plate VIEW 16 to a total end volume of 100 μ l. The plates were left under the hood for 30 min at RT to settle and then inserted back into the reader. Step two was continued with sweeps of the plates initiated every 2 h during the 72 h experimental run. Step two was paused after 24 h. The drugs and PBS as a control were added; afterwards step three was continued for at least 84 h, also with sweeps every 2 h.

Statistical analysis. To evaluate the effects of the single and the combination treatments a multiple t-test followed by a correction for multiple comparison of the means according to the Holm-Sidak method was performed. The combination effects were evaluated according to the effect-based approach 'Highest Single Agent' (37), which reports if the resulting effect of a drug combination (E_{AB}) is greater than the effects produced by any of combined individual drugs (E_A and E_B). A combination index (CI) was calculated with the following formula: $CI = \text{Max}(E_{A \text{ or } B})/E_{AB}$. A $CI < 1$ was defined as a positive combination effect and $CI > 1$ as a negative combination effect. Two-tailed unpaired Student's t-test was used to examine the difference in means between two treatments. A $P < 0.05$ was considered as statistically significant.

Results

Analysis of FGFR3 and PIK3CA mutations in HPV⁺ UPCI-SCC-154 and UM-SCC-47 cells, and HPV⁻ UT-SCC-60A cells by CAST-PCR. DNA was extracted successfully from all three cell lines, but none of the FGFR3 and PIK3CA mutations, assayed for by the CAST-PCR, were detected (data not shown).

Treatment of HPV⁺ UPCI-SCC-154 and UM-SCC-47 and HPV⁻ UT-SCC-60A with FGFR and PI3K inhibitors independently

WST-1 viability analysis after treatment with FGFR and PI3K inhibitors independently on HPV⁺ UPCI-SCC-154 and UM-SCC-47 and HPV⁻ UT-SCC-60A. To examine effects of FGFR and PI3K inhibitors on viability of TSCC/BOTSCC cell lines, WST-1 viability assays were performed 24, 48 and 72 h after treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120 on HPV⁺ UM-SCC-47 and UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Data for three combined experiments using different concentrations of the drugs are shown in Fig. 1.

In general, all cell lines were sensitive for treatment with the FGFR and PI3K inhibitors. HPV⁺ UPCI-SCC-154 was slightly more sensitive than the other two cell lines to AZD4547, and showed $\geq 50\%$ decreased absorbance representing cell viability after treatment with 5 μ M AZD4547 after 72 h, and with 10, 25 and 50 μ M AZD4547 after 24, 48 and 72 h (at least $P < 0.05$ for all) (Fig. 1B). Similar effects with $\geq 50\%$ decreased absorption were obtained for HPV⁺ UM-SCC-47 and HPV⁻ UT-SCC-60A after treatment with 25 and 50 μ M AZD4547 at 24, 48 and 72 h (at least $P < 0.05$ for all) (Fig. 1A and C, respectively).

HPV⁺ UPCI-SCC-154 was in general also slightly more sensitive to treatment with BEZ235 and BKM120 compared to the other two cell lines. HPV⁺ UM-SCC-47 and UPCI-SCC-154 both showed $\geq 50\%$ decreased absorbance after treatment with 1 and 5 μ M BEZ235 after 24 and 48 h, and UPCI-SCC-154 also at 72 h (at least $P < 0.01$) (Fig. 1D and E, respectively). Moreover, UPCI-SCC-154 also showed $\geq 50\%$ decreased absorbance with 0.25 and 0.5 μ M BEZ235 at 48 and 72 h (at least $P < 0.01$ for all) (Fig. 1E). For HPV⁻ UT-SCC-60A a $\geq 50\%$ decrease was obtained only with the highest concentration i.e. 5 μ M BEZ235 after 48 and 72 h (at least $P < 0.05$, for all) (Fig. 1F).

For BKM120, all cell lines reached a significant decrease for 5 μ M BKM120 at 24, 48 and 72 h (at least $P < 0.001$ for all) (Fig. 1G-I). A significant decrease was obtained for UPCI-SCC-154 also at 1 μ M of BKM120 at 48 and 72 h, while for UM-SCC-47 and UT-SCC-60A respectively, significance was obtained only sporadically i.e. after 48 and 24 h respectively, (at least $P < 0.05$, for all) (Fig. 1G-I).

Finally, the IC_{50} for each cell line for each drug were determined (Table I). Taken together these data confirm that the UPCI-SCC-154 was in general more sensitive than the other two cell lines to all drugs.

CellTox Green Cytotoxicity Assay with FGFR and PI3K inhibitors independently on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. CellTox Green Cytotoxicity Assays on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A were performed in four different experiments 48, 72, 96 and 120 h after treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 to assess cytotoxic effects in correlation to PBS and lysis buffer respectively (Fig. 2).

After treatment with 5, 10 and 25 μ M AZD4547, UPCI-SCC-154 showed a trend of increased cytotoxicity as compared to the PBS control, but this was significant only for 5 μ M at 72 h ($P < 0.05$) (Fig. 2A). For UT-SCC-60A, the cytotoxic effect seemed lower, however at 48 and 120 h after treatment with 25 μ M AZD4547 there was a significant

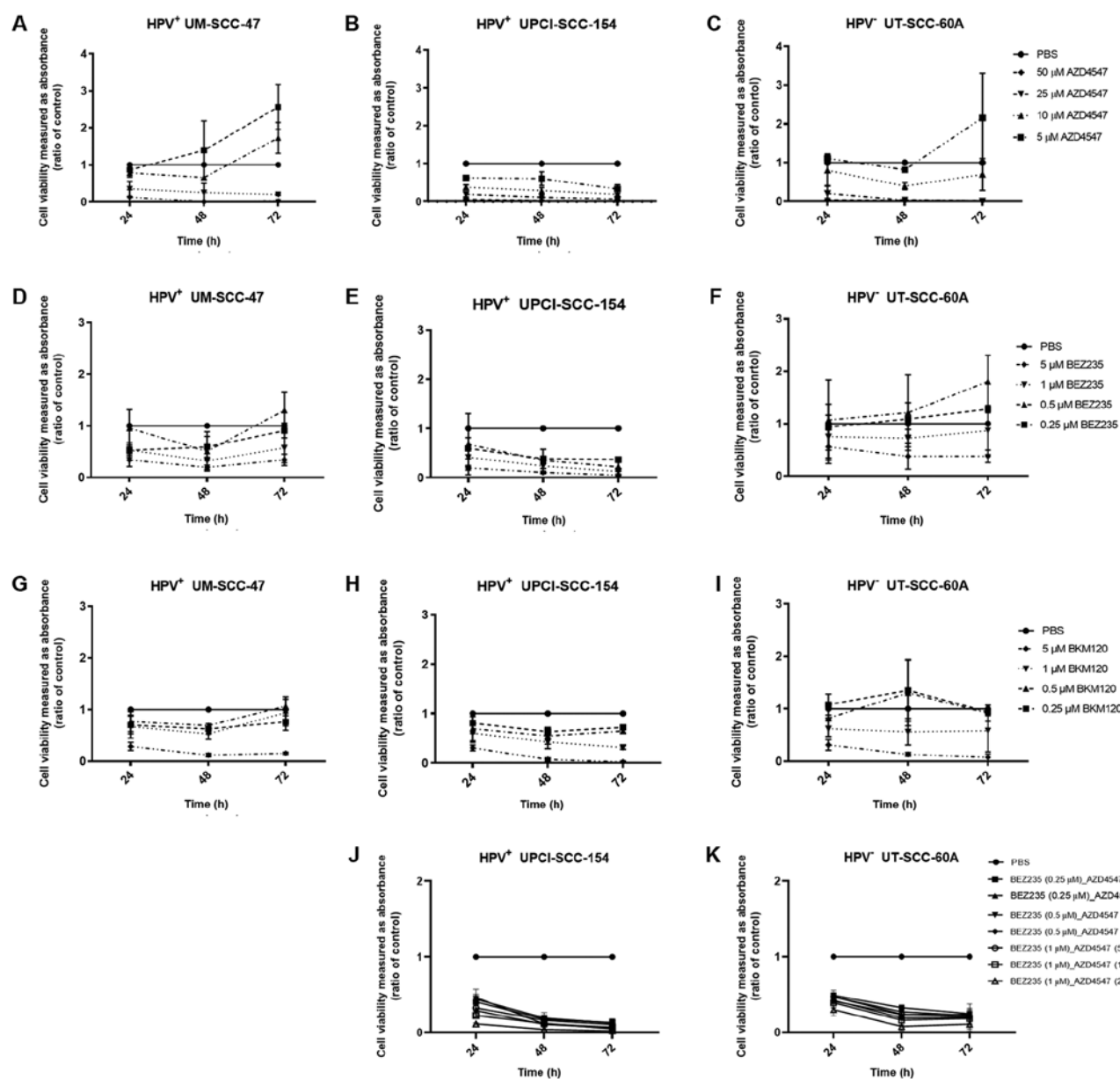


Figure 1. WST-1 viability assays on cell lines HPV⁺ UPCI-SCC-154, UM-SCC-47 and HPV⁻ UT-SCC-60A. WST-1 viability analysis measured as absorbance, after treatment for 24, 48 and 72 h with FGFR inhibitor AZD4547 for (A) HPV⁺ UM-SCC-47, (B) HPV⁺ UPCI-SCC-154 and (C) HPV⁻ UT-SCC-60A cells. Effect of treatment with PI3K inhibitors BEZ235 on (D) HPV⁺ UM-SCC-47, (E) HPV⁺ UPCI-SCC-154 and (F) HPV⁻ UT-SCC-60A cells, and BKM120 on (G) HPV⁺ UM-SCC-47, (H) HPV⁺ UPCI-SCC-154 and (I) HPV⁻ UT-SCC-60A cells. Effect of combined treatment of FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on (J) HPV⁺ UPCI-SCC-154 and (K) HPV⁻ UT-SCC-60A cells. Data are presented as the mean \pm SD of three experimental runs per cell line. FGFR, fibroblast growth factor receptor; HPV, human papillomavirus.

difference in cytotoxicity when compared to the PBS control (at least $P < 0.05$) (Fig. 2B). For treatment with BEZ235, there were no significant increases in cytotoxicity compared to PBS (Fig. 2C and D).

Caspase-Glo 3/7 apoptosis-assay with FGFR and PI3K inhibitors independently on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. To evaluate the effects on viability Caspase-Glo 3/7 apoptosis assays were done 48, 72, 96 and 120 h after treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 at various concentrations normalized against PBS on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Again, HPV⁺ UPCI-SCC-154 had a tendency to be more sensitive than HPV⁻ UT-SCC-60A as shown in Fig. 3.

After 48 h HPV⁺ UPCI-SCC-154 showed an increase in apoptosis with 10 and 25 μ M AZD4547 (at least $P < 0.05$) (Fig. 3A). For UT-SCC-60A a similar weak trend was observed for the 25 μ M concentration, however statistical significance was not reached (Fig. 3B).

Neither HPV⁺ UPCI-SCC-154 (Fig. 3C) nor HPV⁻ UT-SCC-60A (Fig. 3D) showed increased luminescence representing apoptosis, after treatment with any of the concentrations of BEZ235.

Proliferation assay using the xCELLigence System after treatment with FGFR and PI3K inhibitors independently of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Lastly, to evaluate effects on cell growth, proliferation assays using the xCELLigence

Table I. WST-1 viability analysis following treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120 for 24, 48 and 72 h.

Cell line	Target	Drug	IC ₅₀ (μM)		
			24 h	48 h	72 h
UPCI-SCC-154 (HPV ⁺)	FGFR	AZD4547	7.24	6.29	2.86
	PI3K	BKM120	1.67	0.57	0.62
		BEZ235	0.74	0.12	0.14
UM-SCC-47 (HPV ⁺)	FGFR	AZD4547	18.24	16.32	NA
	PI3K	BKM120	1.85	0.89	2.78
		BEZ235	1.90	0.44	2.71
UT-SCC-60A (HPV ⁻)	FGFR	AZD4547	16.33	8.55	10.41
	PI3K	BKM120	2.12	1.01	1.21
		BEZ235	5.99	3.47	4.29

The IC₅₀ for each cell line for each drug was determined from log concentrations-effect curves in GraphPad Prism using non-linear regression analysis. FGFR, fibroblast growth factor receptor; HPV, human papillomavirus; NA, not available.

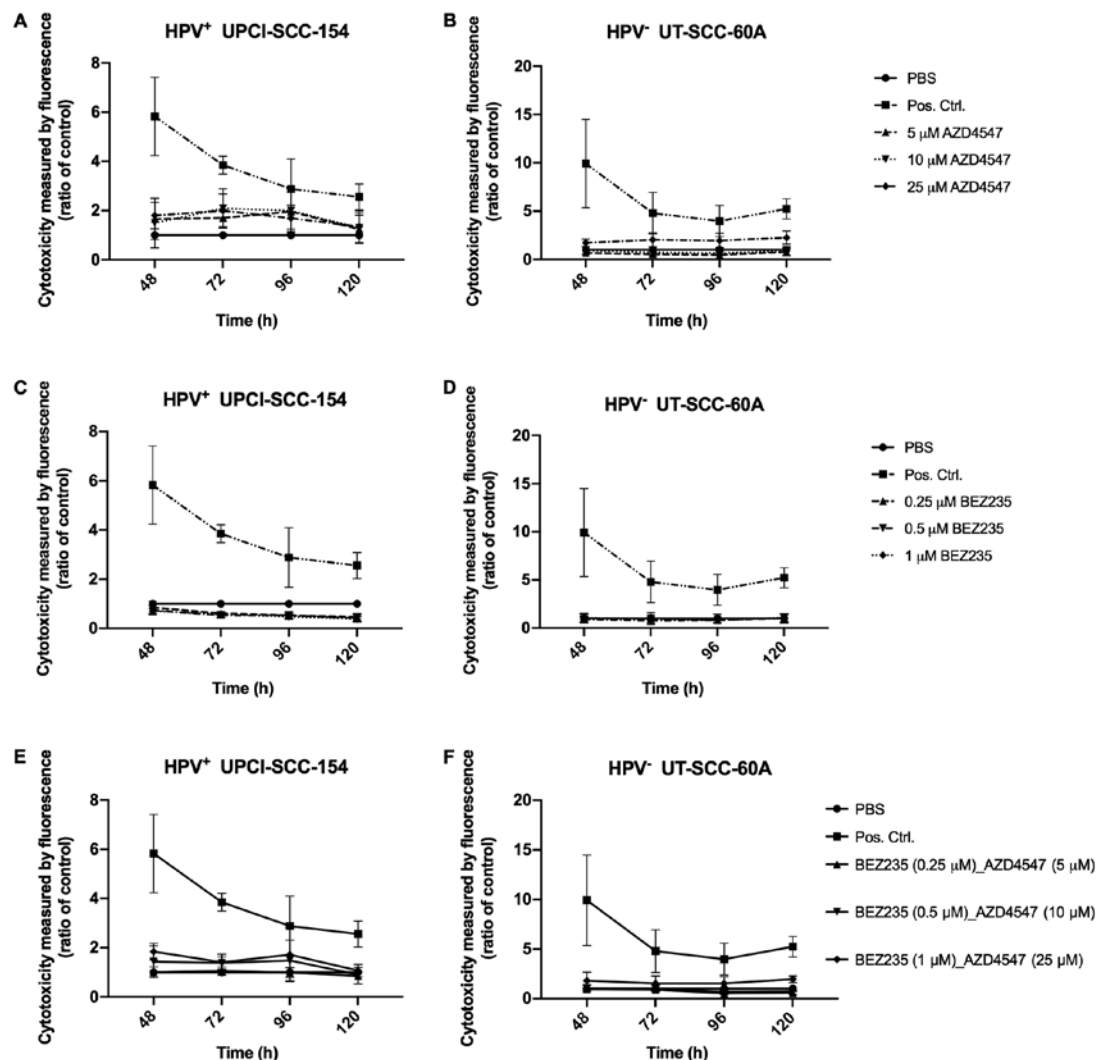


Figure 2. CellTox Green Cytotoxicity assays of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A cells. CellTox Green cytotoxicity measured by fluorescence after treatment for 48, 72, 96 and 120 h with FGFR inhibitor AZD4547 for (A) HPV⁺ UPCI-SCC-154 and (B) HPV⁻ UT-SCC-60A cells. Effect of treatment with PI3K inhibitor BEZ235 on (C) HPV⁺ UPCI-SCC-154 and (D) HPV⁻ UT-SCC-60A cells. Effect of combined treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on (E) HPV⁺ UPCI-SCC-154 and (F) HPV⁻ UT-SCC-60A cells. The data are presented as the mean \pm SD of four experimental runs per cell line. FGFR, fibroblast growth factor receptor; HPV, human papillomavirus; Pos. Ctrl., positive control.

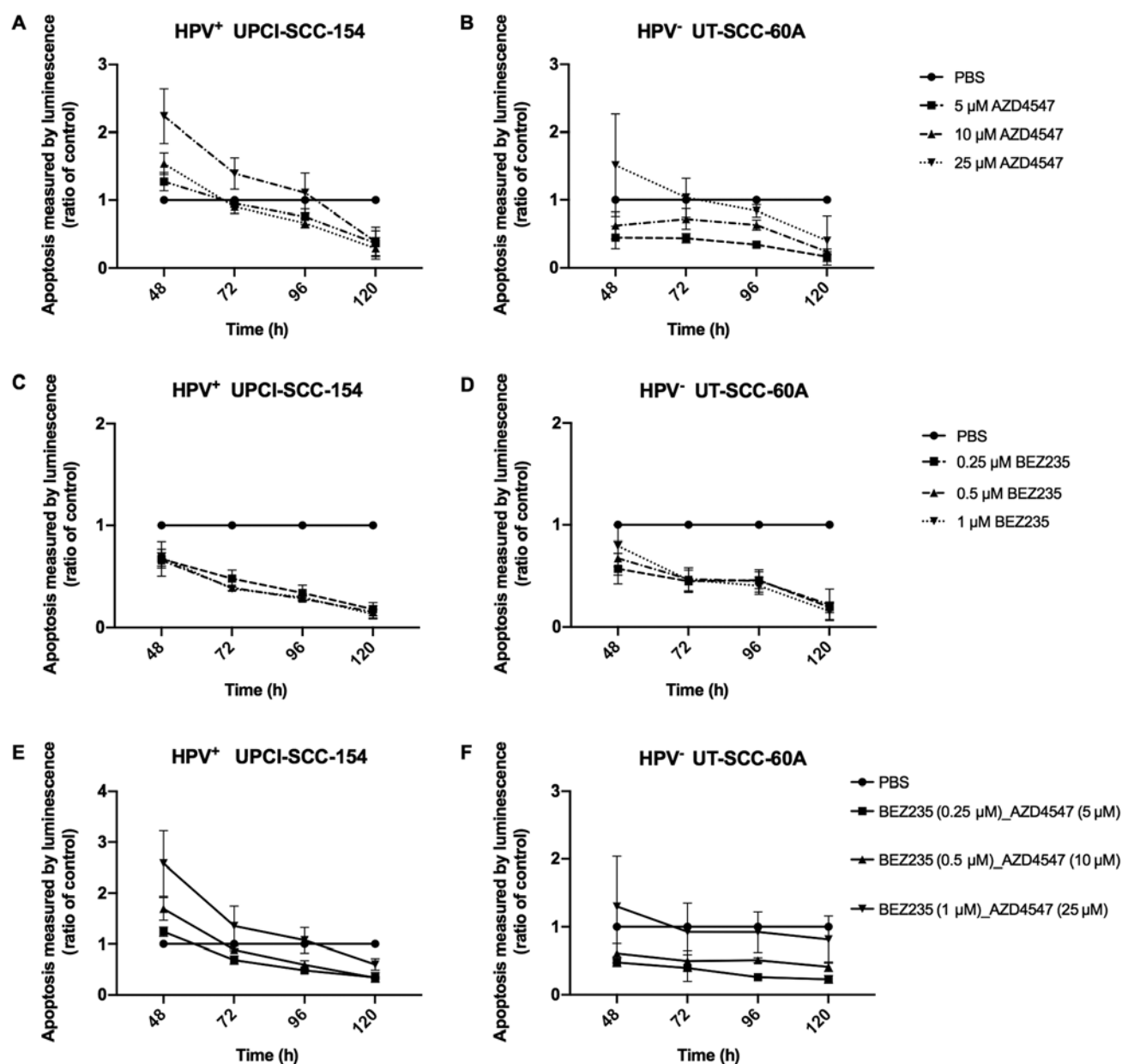


Figure 3. Caspase-Glo 3/7 apoptosis-assays of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A cells. Caspase-Glo 3/7 apoptosis-assay measured by luminescence after treatment for 48, 72, 96 and 120 h with FGFR inhibitor AZD4547 for (A) HPV⁺ UPCI-SCC-154 and (B) HPV⁻ UT-SCC-60A cells. Effect of treatment with PI3K inhibitor BEZ235 on (C) HPV⁺ UPCI-SCC-154 and (D) HPV⁻ UT-SCC-60A cells. Effect of combined treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on (E) HPV⁺ UPCI-SCC-154 and (F) HPV⁻ UT-SCC-60A. The data are presented as the mean \pm SD of four experimental runs per cell line. FGFR, fibroblast growth factor receptor; HPV, human papillomavirus.

System after treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120 on the cell lines HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A were performed (Fig. 4).

HPV⁺ UPCI-SCC-154 showed a complete inhibition of proliferation after treatment with 10, 25 and 50 μ M AZD4547 as compared to treatment with PBS during the whole observation period of 96 h (Fig. 4A). For HPV⁻ UT-SCC-60A treatment with 50 μ M AZD4547 also inhibited proliferation almost throughout the whole observation of 96 h as compared to treatment with PBS, and in addition, treatment with 25 μ M, but not with 10 μ M AZD4547 eventually inhibited proliferation between 60 and 96 h (Fig. 4B).

Treatment with 1 and 5 μ M of BEZ235, and 5 μ M BKM120 almost completely inhibited HPV⁺ UPCI-SCC-154

proliferation through the 96 h observation period as compared to PBS, and when treating with lower doses, both BEZ235 and BKM120 induced decreased proliferation when compared to PBS (Fig. 4C and E, respectively). For HPV⁻ UT-SCC-60A treatment with 1 and 5 μ M BEZ235 and 5 μ M BKM120 respectively inhibited proliferation almost completely as compared to PBS, during most of the 96 h observation period, while lower concentrations were not as efficient, and especially not for BKM120 (Fig. 4D and F).

Treatment of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A with FGFR and PI3K inhibitors AZD4547 and BEZ235 combined. Clearly there were inhibitory effects on growth using FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235

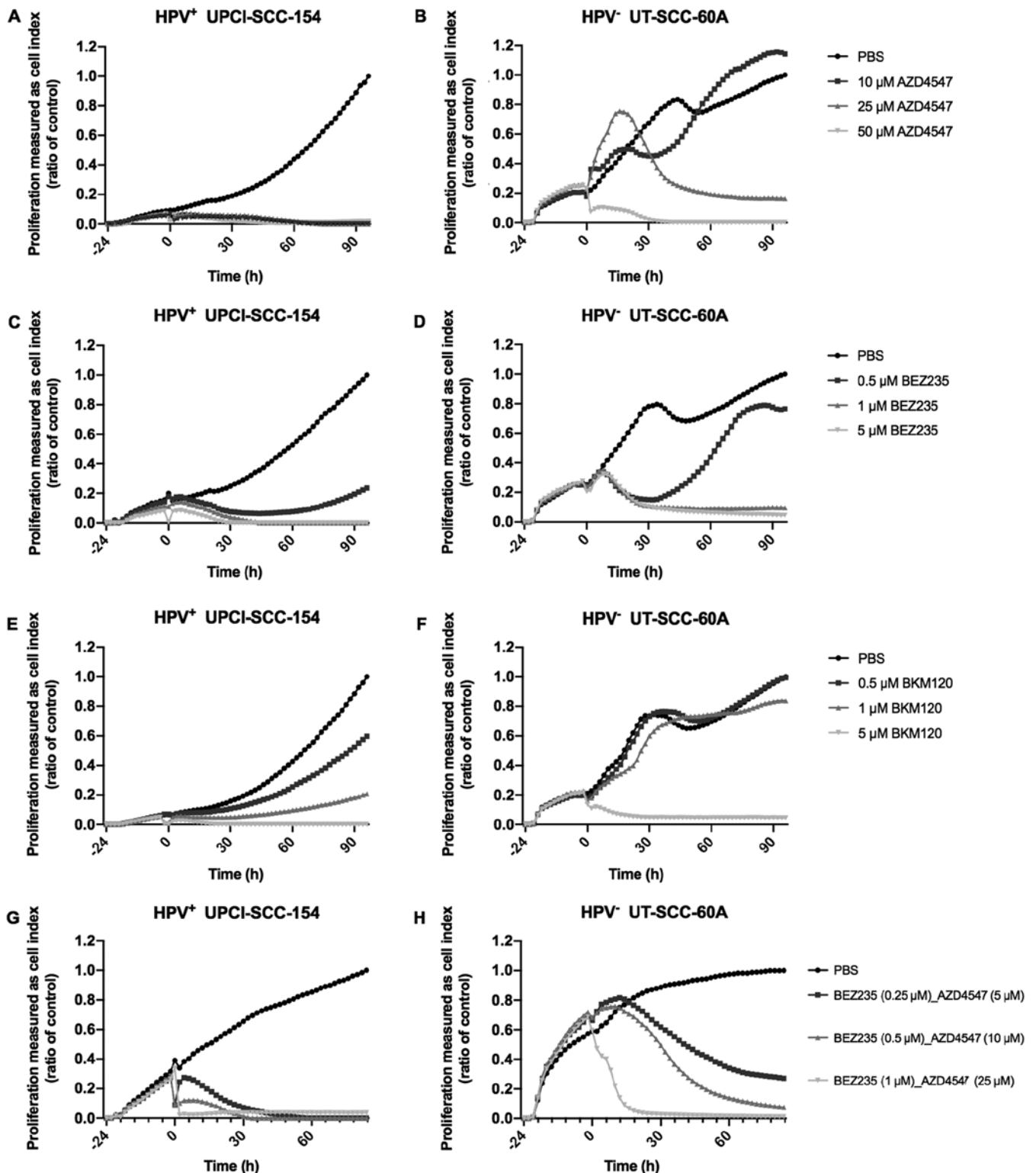


Figure 4. Proliferation assays using HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A cells. Results of proliferation assays presented as cell index after treatment with FGFR inhibitor AZD4547 for (A) HPV⁺ UPCI-SCC-154 and (B) HPV⁻ UT-SCC-60A cells. Effect of treatment with PI3K inhibitors BEZ235 on (C) HPV⁺ UPCI-SCC-154 and (D) HPV⁻ UT-SCC-60A cells, and BKM120 on (E) HPV⁺ UPCI-SCC-154 and (F) HPV⁻ UT-SCC-60A. Effect of combined treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on (G) HPV⁺ UPCI-SCC-154 and (H) HPV⁻ UT-SCC-60A cells. The graphs represent one typical experiment, with the mean and SD of two wells shown. FGFR, fibroblast growth factor receptor; HPV, human papillomavirus.

and BKM120 on all the cell lines as shown above. To further investigate their therapeutic potential we now wanted to examine if lower drug concentrations could be used when combining two different types of drugs or whether they had an antagonistic effect. For this purpose, cell viability,

cytotoxicity, apoptosis and proliferation (using the WST-1 assay, Cell-Tox Green Cytotoxicity Assay, Caspase-Glo 3/7 assay, and the xCELLigence System respectively) of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A were measured after combined treatment with the FGFR AZD4547 and the PI3K

BEZ235 inhibitors. In this respect, as indicated above HPV⁺ UPCI-SCC-154 was an example of a generally more sensitive cell line, while HPV⁻ UT-SCC-60A was an example of a more resistant cell line.

WST-1 viability analysis after treatment with FGFR and PI3K inhibitors AZD4547 and BEZ235 combined on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. WST-1 viability assays were performed 24, 48 and 72 h after treatment with FGFR inhibitor AZD4547 at concentrations 5, 10 and 25 μ M combined with PI3K inhibitor BEZ235 at concentrations 0.25, 0.5 and 1 μ M on cell lines HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A (for details see Fig. 1J and K, respectively). All combinations decreased viability $\geq 50\%$ at 24, 48 and 72 h of both cell lines (at least $P < 0.001$ for all) (Fig. 1J and K). Furthermore, for HPV⁺ UPCI-SCC-154 viability decreased to $\sim 90\%$ for all concentrations at 72 h (Fig. 1J).

Viability was also illustrated as staple diagrams for single doses of AZD4547 and BEZ235 as well as combined for HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A at 48 h in Fig. 5 (with the same concentrations of drug combinations shown in Figs. 1-3) and Fig. S1 (with additional drug combinations, not tested in the other assays). We also calculated the combination index (CI) for each combination in order to investigate whether we had a positive or negative combination effect, these CIs are displayed in Figs. 5 and S1 as well as summarized for all time-points in Fig. S2.

At 48 h, a decrease in viability was significant for HPV⁺ UPCI-SCC-154 in four out of seven tested combinations (Fig. 5A-C and S1A-D together) and for HPV⁻ UT-SCC-60A in six of seven (Figs. 5D-F and S1E-H together). Especially for HPV⁻ UT-SCC-60A in the lower dose combinations (AZD4547 5 μ M and BEZ235 0.25 or 0.5 μ M) a clear synergistic effect was observed, where the single drugs had minor (AZD4547) or no effect (BEZ235), while the combination induced a strong effect on cell viability (Figs. 5D and S1E).

In most cases, with four statistically non-significant exceptions, the highest concentrations of AZD4547 and BEZ235 in HPV⁻ UT-SCC-60A at all time-points, and the 10 μ M of AZD4547 and 0.25 μ M BEZ235 combination for HPV⁺ UPCI-SCC-154 at 24 h) the combined treatment tended to induce greater viability effect than single treatments, shown by CIs < 1 (Fig. S2).

CellTox Green Cytotoxicity Assay with FGFR and PI3K inhibitors AZD4547 and BEZ235 combined on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. CellTox Green Cytotoxicity assays were done 48, 72, 96 and 120 h after treatment with FGFR inhibitor AZD4547 at concentrations of 5, 10 and 25 μ M combined with PI3K inhibitor BEZ235 at concentrations of 0.25, 0.5 and 1 μ M, respectively on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Except for UPCI-SCC-154 at the highest combination at 48 h, and for UT-SCC-60A at the highest combination at 120 h (at least $P < 0.05$ for both), there were no other significant increases in induced cytotoxicity with any of the drug combinations at any of the time-points as compared to PBS (Fig. 2E and F).

Caspase-Glo 3/7 apoptosis-assay with FGFR and PI3K inhibitors AZD4547 and BEZ235 combined on HPV⁺

UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Caspase-Glo 3/7 apoptosis-assays were performed 48, 72, 96 and 120 h after treatment with FGFR inhibitor AZD4547 at concentrations 5, 10 and 25 μ M combined with PI3K inhibitor BEZ235 at concentrations 0.25, 0.5 and 1 μ M on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. An increase in apoptosis being proportional to measured luminescence $> 50\%$ was observed for UPCI-SCC-154 with all drug combinations at 48 h (at least $P < 0.05$ for all) (Fig. 3E). For UT-SCC-60A, possibly a tendency of an increase in luminescence was observed for the highest drug combination at 48 h only ($P = ns$) (Fig. 3F).

Proliferation assay using the xCELLigence System after combined treatment with FGFR and PI3K inhibitors AZD4547 and BEZ235 of HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A. Proliferation assays were performed up to 84 h after combined treatment with FGFR inhibitor AZD4547 at concentrations 5, 10 and 25 μ M and PI3K inhibitor BEZ235 at concentrations 0.25, 0.5 and 1 μ M, respectively, on HPV⁺ UPCI-SCC-154 and HPV⁻ UT-SCC-60A (Fig. 4G and H). Compared to PBS alone, treatment with all combination treatments inhibited proliferation of HPV⁺ UPCI-SCC-154 during most of the observation period of 84 h (Fig. 4G).

Likewise, compared to PBS alone, treatment with all combinations inhibited the proliferation of HPV⁻ UT-SCC-60A, however the lower doses of 5 and 10 μ M AZD4547 and 0.25 and 0.5 μ M of BEZ235, were not as efficient as the highest dose (Fig. 4H).

Discussion

This study has investigated the sensitivity of HPV⁺ and HPV⁻ TSCC/BOTSCC cell lines UPCI-SCC-154, UM-SCC-47 and UT-SCC-60A to FGFR inhibitor AZD4547, and PI3K inhibitors BEZ235 and BKM120 and disclosed that they were more or less sensitive to all inhibitors. Furthermore, taken together the data indicated that HPV⁺ UPCI-SCC-154 was generally more sensitive than the HPV⁺ UM-SCC-47 and HPV⁻ UT-SCC-60A cell lines to these afore mentioned drugs. In addition, combination treatments with the FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235, allowed for the possibility to decrease the concentrations of the respective FGFR and PI3K inhibitors, especially for the less sensitive HPV⁻ UT-SCC-60A cell line.

The above was illustrated e.g. by the fact that viability for HPV⁺ UPCI-SCC-154 was decreased for all doses of each drug at all three time-points 24, 48 and 72 h. In contrast, for cell lines HPV⁺ UM-SCC-47 and HPV⁻ UT-SCC-60A, viability went down early on at 24 h and then increased considerably for the two lower doses of AZD4547 and BEZ235 at later time-points 48 and 72 h, although at higher AZD4547 and BKM120 concentrations both latter cell lines did also respond. We also analyzed the combinations according to the Highest Single Agent approach. It is a simple, but yet clinically relevant method which provides evidence of the superiority of the drug combination compared to its single agents. However, it offers limited evidence of synergy except when at least one drug is inactive as a single treatment, which was the case for several combinations in the resistant cell line HPV⁻ UT-SCC-60A. Likewise, in the WST-1 viability assay, HPV⁺ UPCI-SCC-154 was sensitive at all doses at all time-points 24-72 h in contrast

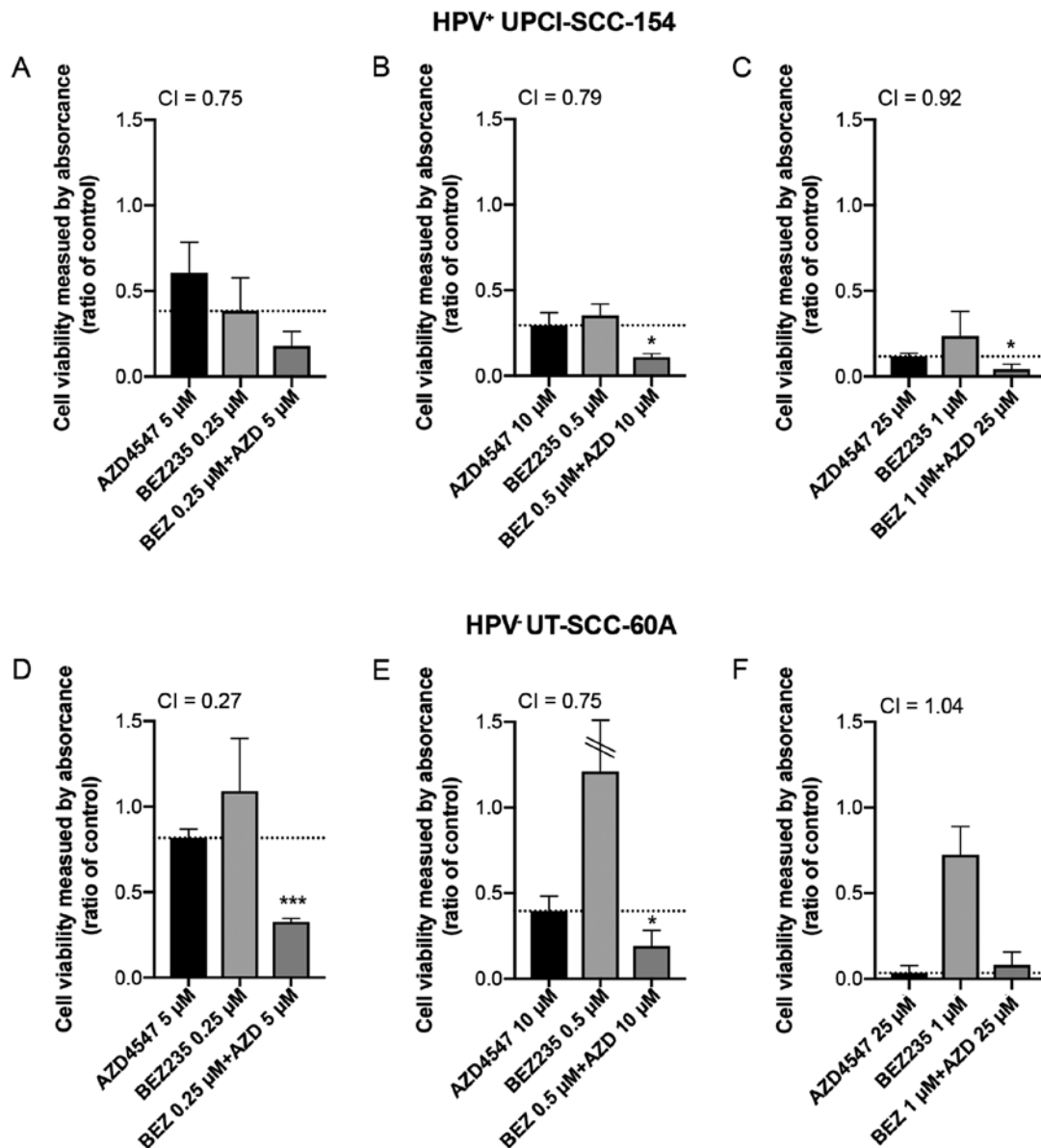


Figure 5. Combinational effects of AZD4547 and BEZ235 in HPV+ UPCI-SCC-154 and HPV- UT-SCC-60A cells. Cell viability after single and combination treatments with AZD4547 and BEZ235 after 48 h treatment, as determined with WST-1 analysis for HPV+ UPCI-SCC-154 and HPV- UT-SCC-60A cells. CI was calculated according to the Highest Single Agent approach. A CI <1 indicates a positive combination effect and a CI >1 a negative effect. The dotted line represents the effect of the best single drug, thus a combinational effect below that line shows a positive combination effect (equivalent with CI <1). Significance was tested for HPV+ UPCI-SCC-154 cells between (A) the combination of AZD4547 (5 μ M) and BEZ235 (0.25 μ M), and the best single drug effect of AZD4547 (5 μ M) or BEZ235 (0.25 μ M); (B) the combination of AZD4547 (10 μ M) and BEZ235 (0.5 μ M) and the best single drug effect of AZD4547 (10 μ M) or BEZ235 (0.5 μ M); and (C) the combination of AZD4547 (25 μ M) and BEZ235 (1 μ M), and the best single drug effect of AZD4547 (25 μ M) or BEZ235 (1 μ M). Significance was tested for HPV- UT-SCC-60A cells between (D) the combination of AZD4547 (5 μ M) and BEZ235 (0.25 μ M), and the best single drug effect of AZD4547 (5 μ M) or BEZ235 (0.25 μ M); (E) the combination of AZD4547 (10 μ M) and BEZ235 (0.5 μ M) and the best single drug effect of AZD4547 (10 μ M) or BEZ235 (0.5 μ M); and (F) the combination of AZD4547 (25 μ M) and BEZ235 (1 μ M), and the best single drug effect of AZD4547 (25 μ M) or BEZ235 (1 μ M). Significance was determined using Student's t-test between the combination and the best single drug effect. *P<0.05 and ***P<0.001 vs. best single drug effect. The mean \pm SD of three individual experiments is presented. CI, combination index; HPV, human papillomavirus.

to the two other cell lines, where sensitivity was only observed at 24 and 72 h only with the highest doses.

Cytotoxicity measured by CellTox Green Cytotoxicity assay was not really obvious for any of the cell lines, and apoptosis was observed at 48 h only for the most efficient concentrations of the AZD4547. For BEZ235 none of the concentrations showed efficient increases in luminescence for HPV+ UPCI-SCC-154 nor HPV- UT-SCC-60A, suggesting that apoptosis was not observed during the 48-120 h observation period. The proliferation assays however, showed a similar

profile to the viability assays, with a very strong inhibition of HPV+ UPCI-SCC-154 with the majority of the used concentrations of AZD4547 and BEZ235, while the proliferation curves were more diverse for HPV- UT-SCC-60A.

Together the data indicated a sensitivity of these cell lines to the above-mentioned drugs. Furthermore, when combining FGFR and PI3K inhibitors, we found that the positive effects were mainly seen on the more resistant cell line, since the sensitive one was more or less sensitive to all doses. Moreover, the different assays complemented each other quite well,

since e.g. viability (WST-1 assay), apoptosis (Caspase-Glo 3/7 apoptosis-assay) and proliferation (xCELLigence System) for each cell line treated with a specific drug and dose seemed to correlate relatively well. Nonetheless, the cytotoxicity assay (CellTox Green Cytotoxicity Assay) did not indicate that much, which we interpret as that the obtained effect, was an inhibition of proliferation, rather than a cytotoxic crisis.

The use of these types of drugs have frequently been tested on urothelial cancer cell lines *in vitro* and synergistic effects have also been reported, as has the documentation that using one drug alone often can result in the gain of resistance to that drug (38-40). The same type of drugs have also been used on head and neck cancer cell lines *in vitro*, and in some cases on HPV⁺ and HPV⁻ head and neck squamous cell carcinoma lines and similar drug concentrations were used (41-43).

To our knowledge this is the first time, that drugs against FGFR and PI3K were used in combination for treatment of HPV⁺ and HPV⁻ TSCC/BOTSCC cell lines, although the combination of AZD4547 and an MTOR inhibitor has been used on a nasopharyngeal carcinoma cell line (44). Furthermore, to our knowledge, these drugs have not been used under physiological conditions as single treatments on patients with HPV⁺ TSCC/BOTSCC or combined treatments for patients with HPV⁺ or HPV⁻ TSCC/BOTSCC. Nevertheless, when comparing the doses to those used on urinary bladder cell lines, where data are more frequently available, similar doses are used, but our cell lines are possibly slightly more resistant than the urinary bladder cancer cell lines (38). Then on the other hand, in contrast to the urinary bladder cancer cell lines, our cell lines do to our knowledge not contain any modifications/fusion products of the most commonly mutated FGFR or PI3K gene variants. Such cell lines remain to be tested. However, it has also been reported in a phase I study, that the presence of PI3K mutations or not, does not always influence the sensitivity to a clinical response (45). Nevertheless, in other situations PI3K mutations have been reported to influence the responses of FGFR inhibitors used to combat cervical tumors with FGFR3-TACC3-fusion genes (46). In that paper, the authors therefore suggest the use of combination treatments.

There are limitations in this study, since only three cell lines were tested. On the other hand, the cell lines that have been tested are representative of the types of cell lines used in these circumstances (34-36). Furthermore, several types of assays were used and they all indicated that the UPCI-SCC-154 cell line was most likely more sensitive than the UM-SCC-47 or the UT-SCC-60A. For future studies, other cell lines with FGFR or PI3K mutations could be of use and such cell lines could very well be more sensitive to FGFR and PI3K targeted therapy. On the other hand, several publications have indicated that this is the case in some, but not all circumstances (47). Definitely, more knowledge on the subject will be required.

There are also other limitations, in that resistance can arise to either FGFR and PI3K inhibitors when used as single treatments and that these treatments can also result in serious side effects (38,47-49). However, if lower doses can inhibit both the development of resistance and induce fewer side effects this would definitely be of benefit for the patients.

Finally, one last limitation in this study was that detailed mechanistic studies were not performed. It is known that BEZ235 is both a PI3K and MTOR inhibitor, this allows for

multiple effects. In this study, we only were aware of that our cells did not exhibit PIK3CA mutations, and we do not know if MTOR was deregulated. Therefore, it is possible that BEZ235 exhibited multiple effects on our cell lines. Likewise, AZD4547 can target more than one tyrosine kinase, and we have on our cell lines focused on FGFR3 mutations, therefore also here AZD4547 could also have more than one effect. To scrutiny such mechanisms in more detail, additional studies with more selective drugs, and more detailed information on the nature of targets of the drugs utilized in the studied cell lines could be of interest.

To conclude, FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BKM120 were tested alone on HPV⁺ UM-SCC-47, and UPCI-SCC-154 and HPV⁻ UT-SCC-60A, and AZD4547 and BEZ235 combined on the latter two cell lines. All cell lines were found more or less sensitive to treatment with these drugs, and especially for the latter two cell lines when used in combination. Nonetheless, more studies also testing TSCC/BOTSCC cell lines with FGFR3 and PIK3CA mutations should be useful in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SH and ONK performed the majority of the experiments, interpreted the data, calculated the statistics and contributed to the writing of the manuscript. AO initiated the experiments and the interpretation of the initial experiments and contributed to the writing of the material and methods section. CB supervised AO, and planned the initial experiments and performed the CAST-PCR. BKAL and TA collaborated with SH and ONK and performed several experiments. BKAL contributed to the writing of the manuscript together with SH and ONK. TR assisted AO when necessary and contributed to the calculation of the statistics. MW assisted TA with the planning of some experiments, and performed final interpretation and presentation of the data and the statistics of the manuscript. TD made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data and has been involved in drafting the manuscript and revising it critically for important intellectual content. TD has also given final approval of the version to be published and has agreed

to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved. All authors critically read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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