Moving synergistically acting drug combinations to the clinic by comparing sequential versus simultaneous drug administrations

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Running Title: Effective AKT and WEE1 targeting in melanoma.

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Nonstandard Abbreviations: FDA (Food and Drug Administration), IND (Investigational New Drug), CI (Combination Index), DMSO (Dimethyl Sulfoxide), DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum), QOD (Once-a-day), BID (Twice-a-day).

ABSTRACT

Drug combinations acting synergistically to kill cancer cells have become increasingly important in melanoma as an approach to manage the recurrent resistant disease. AKT is a major target in this disease but its inhibitors are not effective clinically, which is a major concern. Targeting AKT in combination with WEE1 seems to have potential to make AKT based therapeutics effective clinically. Since agents targeting AKT and WEE1 have been tested individually in the clinic, the quickest way to move the drug combination to patients would be to combine them sequentially, enabling the use of existing phase-I clinical trial toxicity data. Therefore, a rapid preclinical approach is needed to evaluate whether simultaneous or sequential drug treatment has maximal therapeutic efficacy, which is based on a mechanistic rationale. To develop this approach, melanoma cell lines were treated with AKT inhibitor AZD5363 and WEE1 inhibitor AZD1775 using simultaneous and sequential dosing schedules. Simultaneous treatment synergistically reduced melanoma cell survival and tumor growth. In contrast, sequential treatment was antagonistic and had a minimal tumor inhibitory effect compared to individual agents. Mechanistically, simultaneous targeting of AKT and WEE1 enhanced deregulation of the cell cycle and DNA damage repair pathways by modulating the transcription factors p53 and FOXM1, which was not observed with sequential treatment. Thus, this study identifies a rapid approach to assess the drug combinations with a mechanistic basis for selection, which suggests combining AKT and WEE1 inhibitors is needed for maximal efficacy.

INTRODUCTION

Despite recent advances in targeted therapies and immune system modulators, development of resistance continue to be a major concern for melanoma treatment, requiring the identification of drug combinations to retard this process (Paraiso et al., 2011; Villanueva et al., 2010). Drug combinations effective for treating melanoma often involve use of agents that have undergone FDA required toxicity evaluation as single agents in human trials (Al-Lazikani et al., 2012). Movement of drug combinations rapidly to the clinic is not generally a fast process; however, if the agents can be combined sequentially, this process can move more quickly and efficiently. Furthermore, a solid mechanistic basis for the selection would be needed. If the sequential approach were feasible, it could potentially simplify the process, as the toxicity of the individual agents would be known (US-FDA, 2013). In contrast, combining agents requires additional toxicity assessments and the filing of a new IND with the FDA, which adds cost and increases time to clinical evaluation (US-FDA, 2013).

AKT signaling is activated in up to 70% of sporadic melanomas and targeting this protein in preclinical models effectively inhibits tumor development (Cheung et al., 2008; Davies et al., 2008; Shao and Aplin, 2010; Stahl et al., 2004). Unfortunately, these studies did not translate clinically and AKT inhibitors tested on melanoma patients had little efficacy (Dinavahi et al., 2015; Pal et al., 2010). Therefore a screen was undertaken to identify targets and agents that could be combined with AKT inhibition to more effectively target this pathway (Pal et al., 2010). Genetic and pharmacologically targeting of AKT and WEE1 was found to be an effective approach,

leading to dramatic and highly synergistic killing of melanoma cells in culture and in mice tumors (Kuzu et al., 2017a; Kuzu et al., 2017b).

WEE1 kinase lies downstream of V600E-BRAF in the MAPK signaling cascade (Sharma et al., 2013). Interestingly, combined targeting of AKT and V600E-BRAF was additive while targeting AKT and downstream WEE1 was highly synergistic making this a potentially more important actionable target (Kuzu et al., 2017a; Kuzu et al., 2017b). WEE1 responds to cellular DNA damage by regulating the cell cycle through phosphorylation-mediated inactivation of cyclin-dependent kinase-1 (CDK1), which halts the cell cycle in the G2/M phase until the damage is repaired (Madhunapantula et al., 2013; Watanabe et al., 1995).

For this study, existing drugs targeting AKT and WEE1 that have undergone phase-I evaluation were tested (Pal et al., 2010). Simultaneous treatment of WEE1 inhibitor AZD1775 and with AKT inhibitors AZD5363 or GDC0068, synergistically killed cultured melanoma cells and inhibited melanoma tumor growth by greater than 90% irrespective of BRAF mutational status (Kuzu et al., 2017a). Mechanistically, simultaneous drug treatment enhanced the effects modulated by each of the individual drugs leading to significant deregulation of the cell cycle and DNA damage repair pathways mediated through the transcription factors p53 and FOXM1 (Kuzu et al., 2017a; Kuzu et al., 2017b).

To design a strategy for clinical evaluation, a rapid approach was needed to assess whether sequential drug treatment would have the same effect as simultaneous drug treatment and mechanistically validate the approach. Simultaneous treatment with the drug combination in

cultured melanoma cells was highly synergistic in contrast to sequential treatment, which was antagonistic. Similarly, simultaneous treatment led to >90% reduction of xenograft tumor development without toxicity. In contrast, sequential treatment was less effective and showed signs suggestive of toxicity. Simultaneous treatment increased p53 pathway activity while decreasing FOXM1 signaling, which did not occur with sequential treatment, providing a solid mechanistic basis for selecting the simultaneous rather than sequential approach for clinical evaluation of the drug combination.

MATERIALS AND METHODS

Cell lines and culture conditions. UACC 903, 1205 Lu, SK-MEL-28 and WM164 melanoma cell lines were grown in DMEM with 1% GlutaMAX and 10% FBS in a 37°C humidified 5% CO_2 incubator. Cell lines were periodically monitored for genotypic characteristics, phenotypic behavior and tumorigenic potential to confirm identity (Gowda et al., 2017).

Drug compounds. AstraZeneca generously gifted AZD5363 and AZD1775. The compounds were dissolved in DMSO at a concentration of 20 mM for cell cultured-based synergy analysis. For tumor-based synergy studies, drugs were prepared in 0.5% methylcellulose, 1.0% Tween 80, 5% DMSO and 200 µL administered orally (Kuzu et al., 2017a).

Cell culture based synergy analysis. Cell viability was assessed by MTS assay as described previously (Kuzu et al., 2017a). Cells were treated with AKT inhibitor, AZD5363; WEE1 inhibitor AZD1775; and a combination of both at the same time for simultaneous treatment for 72 hours. For sequential treatment, 1 µM of AZD1775 was treated for 24 hours followed by treatment of AZD5363 for 48 hours. The Combination Index (CI) values were calculated using CalcuSyn software (Kuzu et al., 2017a; Kuzu et al., 2017b). Briefly, data were expressed as fraction of cells affected by the dose in drug-treated cells compared with DMSO treated cells. CalcuSyn program is based on the Chou-Talalay method according to the following equation: CI = (D)1/(Dx)1 + (D)1(D)2/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have the same x- effect when used alone. The CI values of <0.9 were considered synergistic, >1.1 considered antagonistic, and values 0.9–1.1 considered as additive (Chou, 2010).

Tumor based synergy analysis. Xenografted tumor assessment studies were undertaken in athymic-Foxn1^{nu} nude mice as described previously (Kuzu et al., 2017a). Eight days following cell injection when a fully vascularized tumor had formed, daily oral treatments of AZD1775 (50 mg/kg) and AZD5363 (150 mg/kg) were initiated either alone or in combination for simultaneous treatment. For sequential treatment, the oral regimen was adapted from clinical usage and included BID treatment of AZD5363 (130 mg/kg) for 4-days and BID treatment of AZD1775 (30 mg/kg) for 3-days. Tumor volumes and animal weights were measured on alternate days (N=8). At the end of the drug treatment, blood samples were collected; serum was separated, and major organ biomarkers were assessed.

Mechanistic basis for synergy efficacy. Western blotting was used to determine the mechanistic basis for selection of the dosage regimen as described previously (Kuzu et al., 2017a). For simultaneous treatment, melanoma cells were treated with AZD1775 and AZD5363 for 48 hours. For sequential treatment, cells were treated with AZD1775 for 24 hours and AZD5363 for another 24 hours. p53, H2AX and secondary antibodies are from Santa Cruz Biotechnology and FOXM1 from Cell Signaling.

Statistical Analysis. Descriptive statistics including means and standard errors for the serum toxicity parameters are prepared in **Table 1**. The measurements were analyzed with a one-way analysis of variance to identify statistically significant difference among the four treatment groups (control, AZD5363, AZD1775, and combination). As 8 different parameters are being considered, a Bonferonni adjustment has been applied (by multiplying the p-value by 8) to adjust for multiple testing. The sequential and simultaneous experiments are analyzed independently. For

comparison between more than two groups, one-way ANOVA was used followed by Dunnett's as post hoc analysis. For analysis of xenograft data, two-way ANOVA was used followed by Dunnett's as post hoc analysis. The software package used was GraphPad Prism version 5.0. Results with a *P* value less than 0.05 (95% confidence interval) were considered significant. The experimentwise error threshold is 5%.

RESULTS

Assessing whether simultaneous versus sequential targeting of AKT and WEE1 more effectively kills cultured melanoma cells. Targeting AKT and WEE1 following simultaneous or sequential treatment was tested on cultured UACC 903 or 1205 Lu cell lines (**Fig. 1**). A single treatment of 1 μM of AZD1775 or 0.31-20 μM of AZD5363 reduced cell viability by 20-50% in 72 hours for UACC 903 cells (**Fig. 1A; left panel**). In contrast, combination of the two agents led to 80% decrease in cell viability (**Fig. 1A; left panel**). The calculated CI values ranging from 0.1 to 0.3 suggested strong synergism **(Fig. 1B, left panel)**. For sequential treatment, UACC 903 cells were treated with AZD5363 for 24 hours and subsequently, cells were then treated with AZD1775. However, sequential treatment led to an antagonistic relationship between the two agents as evident from the calculated CI values above 2.0 (**Fig. 1B, right panel**). An alternate approach, with cells treated first with AZD1775 and then with AZD5363, was also employed with similar antagonistic response between the drugs (data not shown). Comparable synergistic results were obtained with the 1205 Lu cell line, suggesting that synergy between AKT and WEE1 inhibitors is not a cell line specific phenomenon **(Figs. 1C & 1D)**.

Evaluating simultaneous versus sequential targeting of AKT and WEE1 synergistically inhibited xenografted melanoma tumor growth. To assess whether simultaneous or sequential regimens of AZD5363 and AZD1775 would synergistically inhibit tumor growth, efficacy of the drug combinations were evaluated in UACC 903 and 1205 Lu xenografts **(Fig. 2)**. For simultaneous treatment, oral administrations of AZD5363 (150 mg/kg), AZD1775 (50 mg/kg) or the combination, were initiated 8-days post-melanoma cell implantation when the tumors were

vascularized and continued daily. For sequential treatment, oral administrations of AZD5363 (130 mg/kg) BID were initiated for 4-days followed by AZD1775 (30 mg/kg) BID for the next 3-days. The dosage for simultaneous treatment was established in-house and utilized the doses calculated based on the studies with individual agents (Kuzu et al., 2017a). The dosage for sequential treatment was established in consultation with AstraZeneca to best mimic the regimen used for the phase-II trials of AZD5363 (NCT02338622) and AZD1775 (NCT01357161). Individually, AZD1775 and AZD5363 led to 30% and 50% reduction in tumor sizes in UACC 903 cells respectively while simultaneous treatment led to 90% decrease (**Fig. 2A**). In sequential treatment, individual AZD5363 and AZD1775 led to 50% and 15% reduction of tumor sizes, respectively. The combination led to only 50% reduction in tumor volume with no difference between the AZD5363 treated group and combination (**Fig. 2B**). For the simultaneous treatment regimen, no significant changes were observed in animal body weights (**inset in Fig. 2A)** or blood biomarkers indicative of vital organ function (**Table 1**)**.** Although there were no changes in animal body weights for the sequential treatment regimen, significant differences were observed in blood glucose and blood urea nitrogen levels in serum, suggesting possible toxicity (**Table 1**). Similar results were observed in xenograft studies with 1205 Lu cell line **(Figs. 2B & 2D)**; where simultaneous treatment led to 90% tumor reduction compared to a 50% reduction for the sequential drug approach. Morbidity and subsequent death occurred in one of the sequential combination group in the 1205 Lu xenograft study (**Fig 2D inset**; death of the animal indicated by ϕ) further suggesting possible toxicity of the sequential dosage regimen.

Simultaneous but not sequential targeting of AKT and WEE1 enhanced the effects of the signaling pathways. Simultaneously targeting AKT and WEE1 demonstrated synergism due to

enhanced modulation of pathways regulated by the transcription factors p53 and FOXM1 (Kuzu et al., 2017a). To validate the mechanism for the efficacy of simultaneous rather than sequential targeting, Western blotting analysis of effects of the drugs on p53 and FOXM1 protein levels were examined. For simultaneous treatment, melanoma cells were treated with AZD5363 alone or in combination with AZD1775 for 48 hours. Similarly, for sequential treatment, cells were treated with AZD5363 for 24 hours followed by a washout with PBS and then treated with AZD1775 for another 24 hours. Simultaneous treatment led to a 17.5-fold and 7.5-fold increase in pH2AX and p53 respectively **(Fig. 3)**. In stark contrast, sequential treatment led to only 1.3-fold and 2.7-fold increase in pH2AX and p53, respectively. Similar results were observed for 1205 Lu cells. To substantiate the significance of p53 activity in the mechanism for synergy, cell viability experiments were performed in TP53 mutated (dominant negative) melanoma cell lines SK-MEL-28 and WM164 (**Supplementary Fig. 1**). No synergy was observed in the simultaneous treatment of AZD1775 and AZD5363 indicating that p53 activity is essential for synergy between AKT and WEE1 inhibitors. Collectively, data suggested that the synergy between AZD5363 and AZD1775 required an increase in pH2AX and p53 levels in cells, which is not enhanced in sequential treatment compared to simultaneous treatment (**Fig. 3B**).

DISCUSSION

The underlying rationale for drug combination treatment in cancer has been to co-administer drugs that act by different mechanisms, thereby increasing tumor cell killing while reducing the likelihood of drug resistance (Al-Lazikani et al., 2012). To rapidly move drug combinations to the clinic, studies need to compare the effects of simultaneous versus sequential administration of targeted therapy and select the best approach based on a solid mechanistic justification (Dos Santos et al., 2015; Fung et al., 2013; Song et al., 2015; Wang et al., 2012; Wang et al., 2013; Wild et al., 2013). If sequential is as effective as simultaneous administration, existing clinical trial toxicity data can be used to expedite clinical testing.

While many AKT inhibitors are effective in preclinical studies, they fail in clinical trials due to lack of efficacy (Davies et al., 2012). AZD5363 is a promising ATP-competitive type I inhibitor of AKT signaling with a potency of <10 nmol/L for all three AKT isoforms (Davies et al., 2012). Likewise, WEE1 is expressed at high levels in many cancers including melanoma, where this protein regulates cell cycle progression by controlling the G2/M checkpoint; through catalyzing the inhibitory phosphorylation of CDK1 (Madhunapantula et al., 2013; Visconti et al., 2016). As a single agent, AZD1775 reduced tumor development in several preclinical cancer models (Do et al., 2015; Guertin et al., 2013). Furthermore, a recent phase I trial led to a partial response in two of 25 enrolled patients (Do et al., 2015; Guertin et al., 2013). Currently, it is being tested in 20 clinical trials, in various cancer types including solid tumors, where it is combined with DNA-damaging agents such as carboplatin, cisplatin, paclitaxel, temozolomide or UV irradiation (Visconti et al., 2016).

Current clinical testing of AZD5363 uses 240 to 480 mg BID for a 4-day on and 3-day off cycle (NCT02338622; NCT02121639, NCT0220837 & NCT01226316). Similarly, clinical testing for AZD1775 includes 225 mg BID for a 3-day on and 4-day off cycle (NCT01357161 & NCT01164995). The dosage regimen for sequential treatment used for this study was established to mimic the current individual trials of both AZD5363 and AZD1775. Hence, a 4-day on and 3-day off AZD5363 with intermittent usage of AZD1775 was employed for sequential treatment. Dosage regimen for simultaneous treatment was determined based on the previous empirical preclinical results with individual agents; synergy analysis inhibiting cultured melanoma cell lines and the 1:3 ratio of AZD1775 to AZD5363 for inhibiting melanoma xenografts (Kuzu et al., 2017a).

Simultaneous targeting of AZD5363 and AZD1775 inhibited melanoma tumor growth more synergistically than sequential treatment and did not lead to obvious toxicity. In contrast, sequential treatment elevated blood glucose and urea nitrogen levels. A possible explanation for reduced toxicity when using the simultaneous approach could be due to the lower amount of each drug used per day (150 mg/kg QOD and 50 mg/kg QOD) compared to sequential treatment (130 mg/kg BID and 30 mg/kg BID for AZD5363 and AZD1775 respectively).

Mechanistic basis for synergism was validated with only simultaneous treatment and did not occur with sequential treatment. In melanoma cells, p53 activity is tightly regulated by both AKT and WEE1 pathways (Kuzu et al., 2017a). WEE1 inhibitor induces DNA damage as indicated by the upregulation of phospho H2AX and p53 signaling (Kuzu et al., 2017a). AKT interferes with the DNA damage response by inducing ubiquitin-mediated degradation of p53 and stimulating

the catalytic activity of PLK1, which inhibits the pro-apoptotic functions of p53 (Kuzu et al., 2017a). Simultaneous treatment enhances p53 activity due to the increase in both the pathways whereas sequential treatment causes an increase in p53 only due to one dominant pathway thereby limiting this activity (Kuzu et al., 2017a).

This study stratifies a rapid approach to evaluate whether simultaneous versus sequential drug combinations is effective and could be advance rapidly to the clinic. It provides novel insights regarding the dosage regimen for AKT and WEE1 drug combinations and a solid mechanistic rationale for selecting a simultaneous rather than sequential treatment strategy. Furthermore, this approach demonstrates the importance of increasing the p53 and H2AX levels for enhanced efficacy of AKT and WEE1 inhibitors suggesting the potential biomarkers for evaluating the drug combination in clinical trials.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dinavahi, Noory, Gowda, Drabick, Berg, Neves, Robertson

Conducted experiments: Dinavahi, Noory, Gowda, Neves, Robertson

Contributed new reagents or analytic tools: Drabick, Robertson

Performed data analysis: Dinavahi, Noory, Gowda, Drabick, Berg, Neves, Robertson

Wrote or contributed to the writing of the manuscript: Dinavahi, Noory, Gowda, Drabick, Berg, Neves, Robertson

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FOOT NOTES

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LEGENDS FOR FIGURES

Table 1. Measurement of serum biomarkers following simultaneous and sequential treatment with AKT and WEE1 inhibitors. Levels of blood biomarkers indicating major organ-related toxicity were measured at the endpoint following daily drug treatment. * represents significantly difference ($p < 0.05$) compared to the individual agents.

Figure 1. Comparing simultaneous versus sequential treatment with AKT and WEE1 inhibitors to inhibit cultured melanoma cell survival. Dose-response curves of melanoma cell lines UACC 903 **(1A)** or 1205 Lu **(1C)** simultaneously or sequentially treated with AZD5363 and AZD1775. For synergy analysis, the concentrations of each drug were varied and combination index (CI) values were calculated (**1B & 1D**).

Figure 2. Evaluation of simultaneous and sequential treatment with AKT and WEE1 targeting drugs to inhibit melanoma tumor growth. Inhibition of UACC 903 **(2A & 2B)** and 1205 Lu **(2C & 2D)** xenografts following simultaneous (**2A & 2C**) or sequential (**2B & 2D**) treatments with AZD5363 and/or AZD1775 (N=8). Insets: Animal weights to indicate gross animal toxicity of the dosage regimen. * was used to represent a significant difference of $p < 0.05$; *** to represent a significant difference of $p<0.001$. ϕ represent animal mortality within the experiment.

Figure 3. Effects of simultaneous or sequential treatment with AKT or WEE1 inhibitors on p53 and FOXM1 pathway signaling. Simultaneous but not sequential targeting of AKT and

WEE1 enhanced the inhibitory effects occurring when targeting each pathway individually **(3A)**. Alpha-enolase served as a control for protein loading. Numbers in **Fig. 3A** represent quantification using ImageJ of protein normalized to loading control. Combination of WEE1 and AKT inhibitors caused increased p53 and decreased FOXM1 **(**signaling cascade represented in **3B)**.

Table 1

Dosage regimen			Simultaneous treatment					Sequential treatment				
Test	**Units**	**Reference**	**Control**	**AZD5363**	**AZD1775**	**Combination**	***p*-value**	**Control**	**AZD5363**	**AZD1775**	**Combination**	***p*-value**
GLU *	**mg/dL**	**90-192**	192 ± 11	214 ± 30	237 ± 31	238 ± 47	0.6677	160 ± 12	185 ± 35	193 ± 22	350 ± 38	0.0011
BUN *	**mg/dL**	**18-29**	20 ± 2.7	25.8 ± 2.2	25.3 ± 1.9	20.7 ± 1.5	0.1941	18.2 ± 1.4	16.7 ± 2.7	24.0 ± 2.7	36.0 ± 1.5	0.0002
TP	**g/dL**	**3.6-6.6**	5.3 ± 0.2	5.1 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	0.7090	5.9 ± 0.9	5.2 ± 1.0	7.0 ± 1.0	6.9 ± 0.9	0.1616
ALB	**g/dL**	**2.5-4.8**	2.5 ± 0.1	2.5 ± 0	2.6 ± 0.1	2.6 ± 0	0.5626	1.1 ± 0.3	1.2 ± 0.4	2.1 ± 0.6	1.6 ± 0.1	0.4379
GLOB	**g/dL**	**-**	2.8 ± 0.1	2.6 ± 0.1	2.6 ± 0	2.5 ± 0.1	0.0805	4.7 ± 0.5	3.9 ± 0.6	4.9 ± 0.7	5.4 ± 0.5	0.6287
ALT	**U/L**	**28-132**	42.5 ± 2.2	41.3 ± 7.4	55 ± 10.1	46 ± 4.2	0.5047	124.5 ± 14.0	105.5 ± 24.1	107.5 ± 15.0	122.6 ± 19.2	0.8424
CHOL	**mg/dL**	**36-96**	116 ± 5.3	113.5 ± 7.6	142.5 ± 4.6	132.7 ± 7.8	0.0203	63.2 ± 5.7	50.75 ± 32.0	98.2 ± 8.4	71.3 ± 20.2	0.3691
AMYL	**U/L**	**1691-3615**	1830 ± 67	1891 ± 130	2251 ± 50	1966 ± 135	0.0410	1201 ± 89	1114 ± 165	1710 ± 219	2085 ± 324	0.0268

Figure 1

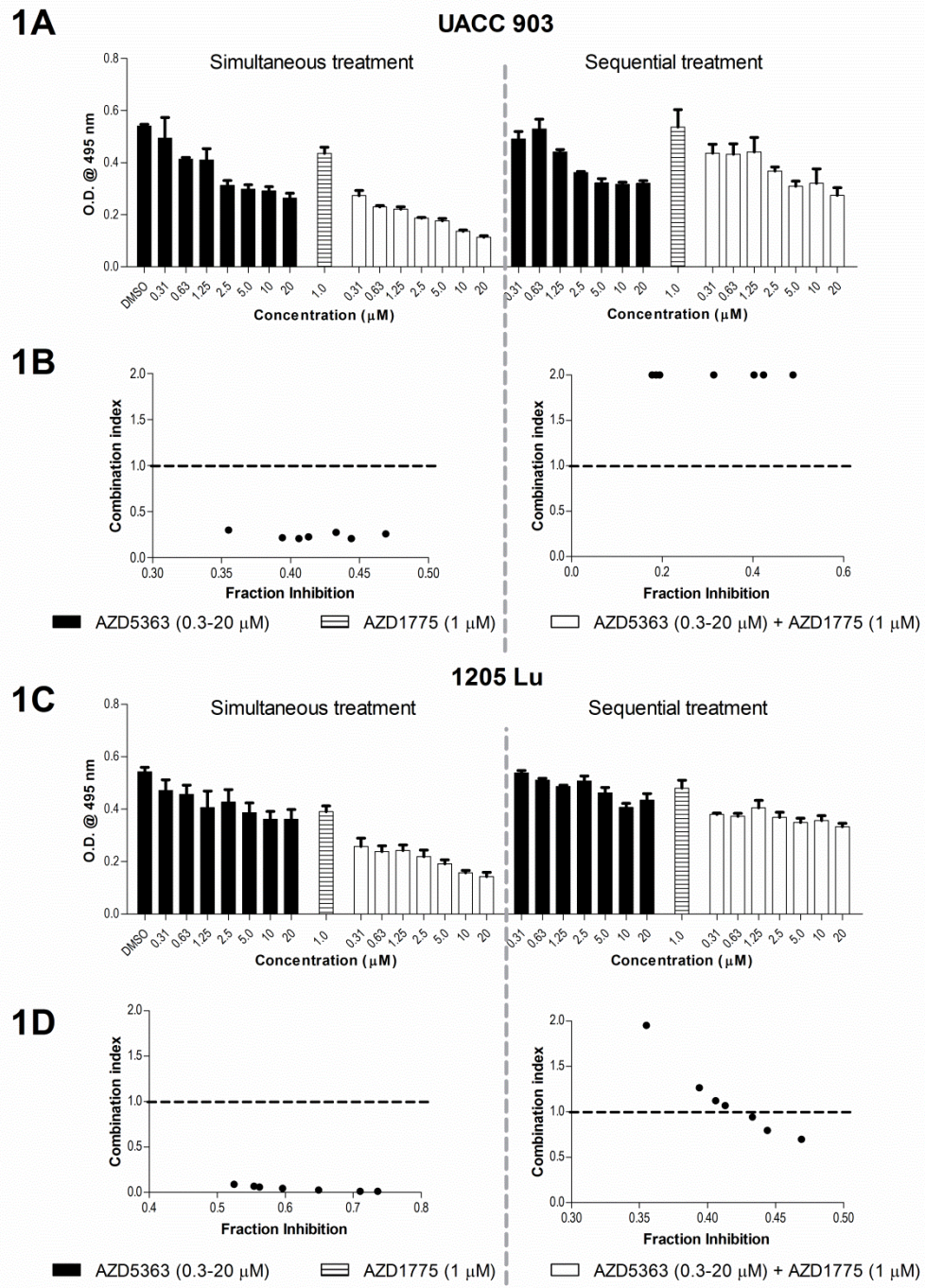

1A
UACC 903
Simultaneous treatment
Sequential treatment
O.D. @ 495 nm
Concentration (μM)
DMSO
0.31
0.63
1.25
2.5
5.0
10
20
1.0
1B
Combination index
Fraction Inhibition
AZD5363 (0.3-20 μM)
AZD1775 (1 μM)
AZD5363 (0.3-20 μM) + AZD1775 (1 μM)
1205 Lu
1C
1D

Figure 2

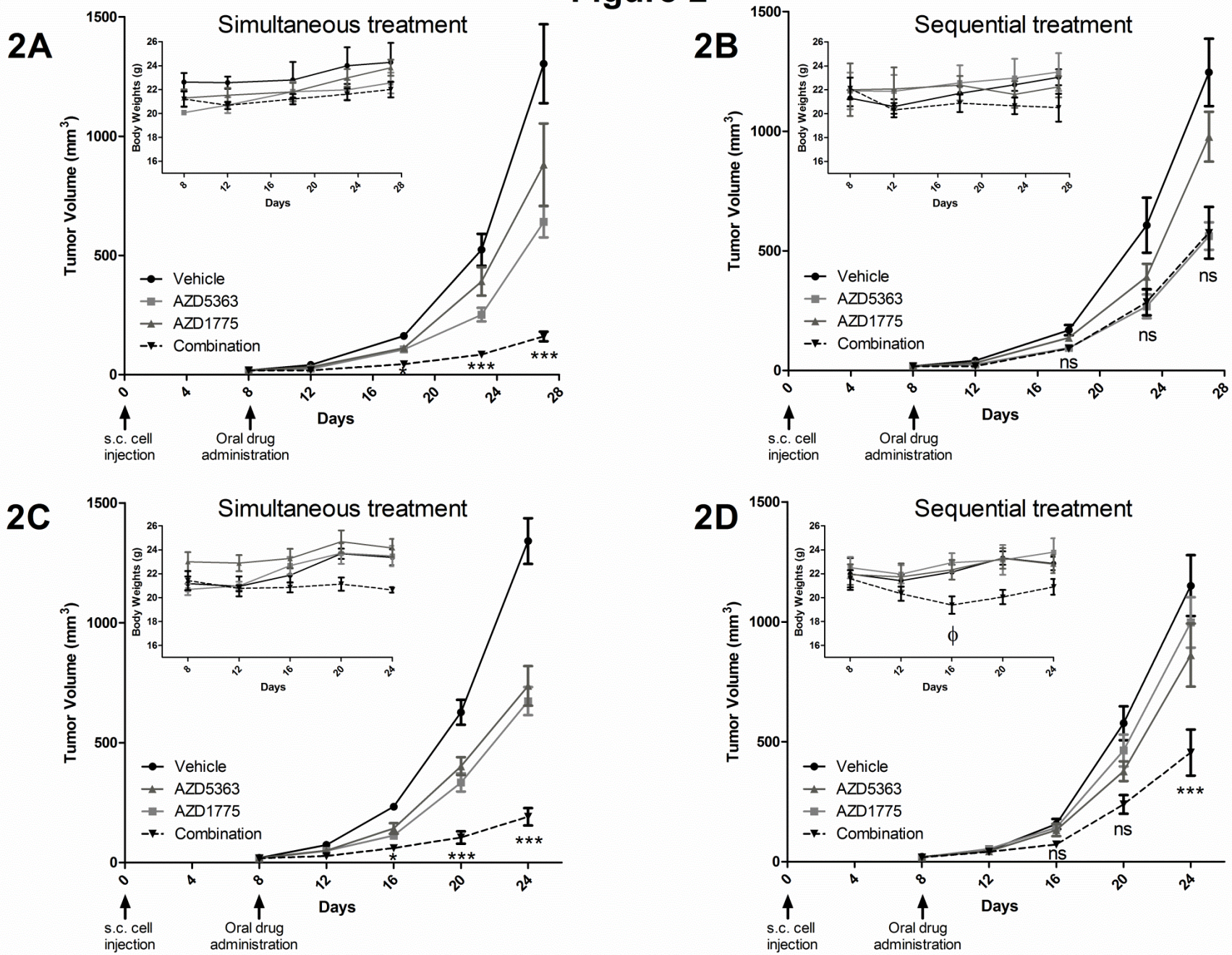
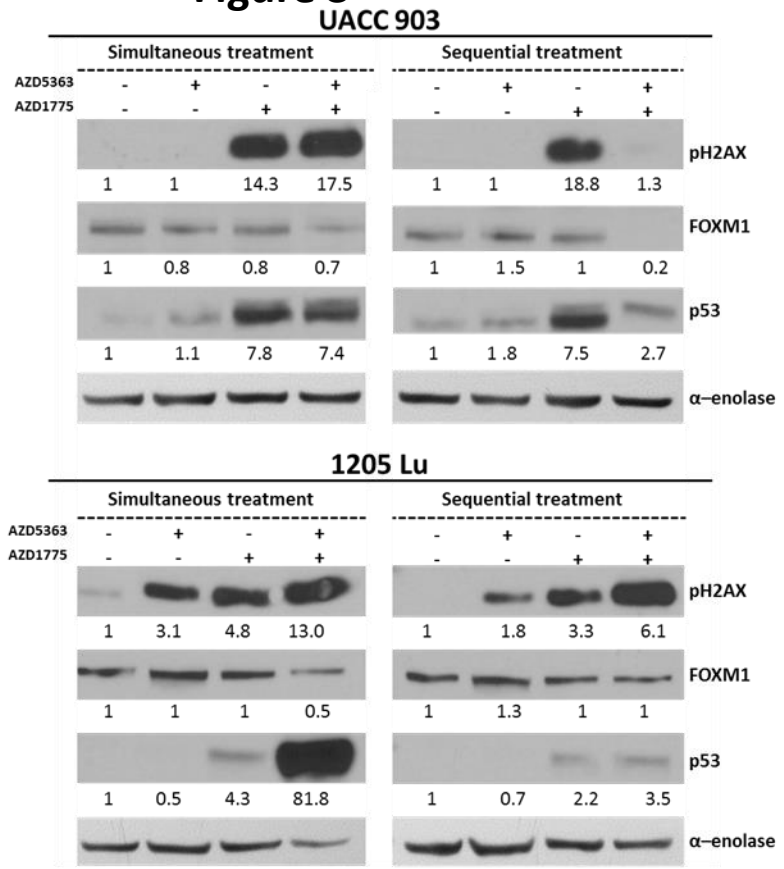

2A
Simultaneous treatment
Tumor Volume (mm3)
Body Weights (g)
Days
Vehicle
AZD5363
AZD1775
Combination
*
***
***
s.c. cell injection
Oral drug administration
2B
Sequential treatment
ns
ns
ns
2C
Simultaneous treatment
*
***
***
2D
Sequential treatment
ϕ
ns
ns
***

Figure 3

3A



3B