SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): ___Relazione Finale_____

TITOLO DELLA RELAZIONE: Effects of AZD5363 in xenograft model and in Advanced or Metastatic Breast Cancer, Stratified by *PIK3CA* Mutation Status (BEECH clinical trial).

RELAZIONE:

AZD5363 is a potent, selective inhibitor of the kinase activity of the serine/threonine AKT/PKB (protein kinase B) that is being developed as a potential treatment for solid and haematological malignancies.

AKT is part of the AGC family of kinases. Mammalian cells express three closely related AKT isoforms: AKT1, AKT2 and AKT3, all encoded by different genes. AKT is a node of multiple signalling pathways promoting tumorigenesis, inhibiting apoptosis, impacting on cell cycle and promoting invasion and migration. The Phosphoinositide 3-kinase (PI3K)/AKT/Phosphatase and Tension Homolog (PTEN) pathway is frequently deregulated in cancer and drives tumour growth and cell survival (Lindsley 2010). All 3 AKT isoforms are activated in different tumour types including breast, prostate, ovarian, pancreatic and gastric cancers, and this activation is often associated with resistance to established cancer therapies as well as advanced disease and/or poor prognosis (Altomere and Testa 2005). AKT activation in tumours is largely due to input from other signalling pathways upstream of AKT (eg, mutation of oncogenes such as Ras, Bcr-abl, mutation of receptor tyrosine kinases such as EGFR, amplification of HER2, loss of PTEN function, mutations of PI3K).

Inhibitors of AKT are anticipated to have efficacy when dosed when combined with cytotoxic chemotherapies or when combined with targeted or antihormonal agents. AZD5363 inhibits all three AKT isoforms (AKT1, AKT2 and AKT3) and therefore has the potential to provide clinical benefit over a range of therapeutic indications.

A significant relationship has been found in pre-clinical studies between the presence of a phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*) mutation and sensitivity to monotherapy AZD5363 in a panel of 182 cancer cell lines ($p = 0.0059$; T test). Mutations in *PIK3CA* are common in human breast cancer; 27% of breast cancers harbour *PIK3CA* mutations. They are most common in Estrogen receptor–positive (ER+) breast tumours (35% have *PIK3CA* mutations). However, the relationship between *PIK3CA* mutation and sensitivity to AZD5363 is not absolute; some *PIK3CA* mutation-negative cell lines are also sensitive to AZD5363.

Non-clinical information and correlative studies

AZD5363 is a potent inhibitor of AKT 1, 2 and 3 in enzyme assays and inhibits the phosphorylation of AKT substrates in cells. AZD5363 inhibits the proliferation of a range of cell lines derived from solid and haematological tumours. Breast cancer cell lines appear to be the tumour types that show the greatest sensitivity to AZD5363. AZD5363 shows dose dependent pharmacodynamic and antitumour activity in xenografts at well-tolerated doses, and can enhance the efficacy of existing treatment (trastuzumab and docetaxel) in appropriate xenograft models.

Studies *in vitro* show AZD5363 to be a potent inhibitor of AKT 1, 2 and 3 (concentration giving 50% of the drug-induced inhibitory effect [IC50] <10 nM), and to inhibit protein kinase A (PKA) with a similar potency. AZD5363 inhibits Rho associated protein kinase (ROCK) 1 and ROCK2 with moderate potency (IC50: 126 nM and 56 nM, respectively). In a counter screen against 75 other kinases, AZD5363 showed activity against 11 other kinases, with >75% inhibition at 1 nM.

In cell lines, AZD5363 inhibits the phosphorylation of substrates of glycogen synthase kinase 3 (GSK3) and proline rich AKT substrate of 40 kilodaltons (PRAS40) with a potency of <1 nM. AZD5363 also inhibits the kinase activity of PKA with a potency of approximately 1 nM in a tumour cell line, but displays low activity for phosphorylation of cofilin, which lies downstream of ROCK.

AZD5363 inhibits the proliferation of 22 tumour cell lines with a concentration causing 50% inhibition of cell growth (GI50) of <1 nM, including the 2 tumour cell lines in which we have demonstrated inhibition of AKT substrates. Breast cancer cell lines appear to be the tumour type that show the greatest sensitivity to AZD5363 as monotherapy *in vitro*.

Pharmacokinetic exposure increased dose proportionally in the dog but more than dose proportionally in the rat. Minimal accumulation was seen after multiple daily dosing in both the rat and dog. AZD5363 free fraction in human serum albumin and human "1-acid glycoprotein were 29.5% and 66.5-75.8%, respectively. *In vitro* the major human metabolite was a direct glucuronide conjugate (via UGT1A9 and UGT2B7). CYP3A4 was mainly responsible for the formation of monooxygenated metabolites, with contributions from CYP2C9 and CYP3A5. AZD5363 produced reversible inhibition of CYP2D6, CYP3A4/5, CYP2C9, CYP2B6 and CYP2C19. Time-dependent inhibition of CYP3A4/5 was observed.

In a rat quantitative whole-body autoradiography (QWBA) study there was persistence of radioactivity up to at least 168 and 504 hours after dosing in pigmented skin and the uveal tract of the eye respectively.

AZD5363 has been found in an *in vitro* assay to inhibit Organic Cation Transporter 2 (OCT2), found in the human kidney. In a clinical setting this has the potential to increase a patient's serum creatinine level, and also to increase the plasma of drugs known to be excreted by this transporter, including metformin. Metformin is currently recommended for the management of hyperglycaemia occurring in patients participating in studies of AZD5363.

AZD5363 was well tolerated in rats at daily doses of up to 100 mg/kg/day, for up to 28 days. Decreases in body weight and food consumption, increases in plasma levels of glucose and insulin and polyuria, glycosuria and proteinuria were noted. Histopathological changes in the liver (hypertrophy and glycogen accumulation), pancreas (hypertrophy/hyperplasia), male reproductive tract (reduced weight and degeneration), ovaries and uterus (decreased weight), bone marrow and thymus (hypocellularity) and hypertrophy in the pituitary, thyroid and adrenals were seen.

Rationale for conducting this study

Breast Cancer

Breast cancer is one of the most common malignancies in women; it is also one of the most common causes of cancer deaths in women. Although earlier detection and improving treatments have led to a decrease in the mortality rate in some countries, the overall number of deaths from breast cancer has continued to rise in Europe (130,000 in 2004 vs 132,000 in 2006) (Ferlay et al 2007). In the US, breast cancer is the most common cancer in women with an incidence rate of 123.6 per 100,000 from 2001 to 2005 (Jemal et al 2009). The treatment of breast cancer is determined by the extent of the disease and a variety of other prognostic factors, including hormone receptor status. The most important factor determining response to hormonal manipulation is the

presence of the ER in the target tissue (Fisher et al 2001). The choice of treatment sequence is complex and dependent on a number of factors, including prior endocrine treatments received. Irrespective of the treatment sequence received a number of patients will experience disease progression, and therefore there remains a need to identify further treatment options for those patients who progress on or shortly after
endocrine therapy.

Rationale for evaluation of *PIK3CA* mutation sub-population.

Molecular aberrations, including *PIK3CA* activating mutations, in the PI3K/AKT pathway have been implicated in the development and progression of breast cancer and also as a mechanism of resistance to breast cancer therapy. (Gonzalez-Angulo et al 2010) A significant relationship was found between the presence of a *PIK3CA* mutation and sensitivity to monotherapy AZD5363 (see section 1.1.1).

Activation of the PI3K-AKT–mTOR network is common in human breast cancer (27% of breast cancers have a *PIK3CA* mutation), and most common in ER+ve breast cancer (35% *PIK3CA*, 3% PTEN and 3% AKT1 mutation frequency). (Kalinsky et al 2009).

The frequency of *PIK3CA* mutations in samples has been studied performing a mutational analysis of exons 9 (HD) and 20 (KD) of the *PIK3CA* gene using tumour deoxyribonucleic acid (DNA) obtained from patients with recurrent disease. Mutations in *PIK3CA* were identified in 24.5% of patients (11.3% in HD and 13.2% in KD). *PIK3CA* mutation was significantly correlated with lower tumour grade (47% in grade 1/2 vs 8% in grade 3, p=0.004), positive ER (35% in ER+ vs 5% in ER-, p=0.017), and PR (37% in PR+ vs 5% in PR-, p=0.011). Overall survival (OS) was 139.5 and 53.7 months for mutation and nonmutation carriers respectively (p=0.014). (Ma et al 2009).

Future treatment strategies may be aligned to the presence or absence of *PIK3CA* mutations. The presence of a *PIK3CA* mutation is correlated with sensitivity to monotherapy AKT inhibition (Courtney et al 2010) – and it is therefore considered appropriate to explore AZD5363 in this setting for patients in this population.

Rationale for study design.

Pre-clinical data has shown that AZD5363 when given in combination with docetaxel is efficacious. This study will test the hypothesis that this pre-clinical finding is applicable to paclitaxel in the clinical setting, utilising the following design:

Part A. Safety run-in: to identify an appropriate and tolerable dose of AZD5363 and dosing schedule, when in combination with weekly paclitaxel, to take forward to Part B.

Part B. Randomised Expansion: to make an assessment of efficacy of the combination (paclitaxel plus AZD5363) against an active control (paclitaxel alone) in a specific target patient population (ER+ve breast cancer) and to explore whether additional efficacy is likely to be present in a *PIK3CA* mutation-positive sub-group for whom it is hypothesised that there will be greater sensitivity to AZD5363.

Rationale for Paclitaxel as combination therapy.

Chemotherapy remains the mainstay of treatment in metastatic breast cancer (MBC) and the goals in this setting are to prolong survival, alleviate or prevent tumour-related symptoms and improve quality of Life.

The taxanes, specifically paclitaxel and docetaxel, are amongst the most active agents in MBC (Ghersi et al 2005) and have demonstrated significant activity in MBC in terms of response rate (RR) and PFS.

In current clinical practice, paclitaxel is used in doses ranging from 135 to 225 mg/m2 administered every 3 weeks; however, its weekly administration usually at a dose of 80 mg/m2 provides superior RR, PFS and overall survival (OS) (Seidman et al 2008).

Part B. Randomised expansion (objectives applicable to all randomised patients and to the *PIK3CA* tumour mutation-positive sub-population alone): To assess the relative efficacy of AZD5363 when combined with weekly paclitaxel compared with weekly paclitaxel plus placebo by assessment of best objective response and duration of response. To assess the relative anti-tumour activity of AZD5363 when combined with weekly paclitaxel compared with weekly paclitaxel plus placebo. To assess the safety and tolerability of AZD5363 when combined with weekly paclitaxel compared with weekly paclitaxel plus placebo. To investigate the effect on patients' quality of life of AZD5363 when combined with weekly paclitaxel, compared with weekly paclitaxel plus placebo. To compare overall survival in patients treated with AZD5363 in combination with weekly paclitaxel compared with weekly paclitaxel plus placebo.

A randomised double-blind assessment of the safety, tolerability, pharmacokinetics, pharmacodynamics and anti-tumour activity of the dose and schedule of AZD5363 selected from

Part A when combined with weekly paclitaxel - versus weekly paclitaxel plus placebo matched to AZD5363.

Patients will be assessed at screening for presence/absence of *PIK3CA* tumour mutations and will be stratified in to *PIK3CA* mutation-positive and *PIK3CA* mutation-not detected arms. Within each stratum, patients will be randomised to receive either paclitaxel plus AZD5363 or paclitaxel plus placebo.

Patient assessments will continue up to death or withdrawal of consent. A 28-day safety follow-up assessment should be conducted following cessation of all study therapy (AZD5363/placebo and paclitaxel). Patients who discontinue treatment for reasons other than progression, death or withdrawal of consent will continue to be followed for objective disease progression status as defined by RECIST 1.1.

Part B Randomised Expansion

The primary outcome variable of progression-free survival and the secondary efficacy variables will be analysed formally in Part B, the randomised expansion. Each variable will be analysed for all patients and then separately in those patients with *PIK3CA* mutation positive tumour(s). All safety data collected during Part B will be summarised descriptively. Progression free survival will be analysed in the Part B randomised expansion using a Cox proportional hazards model allowing for the effect of treatment and with a term for *PIK3CA* mutation status. Progression-free survival in patients with *PIK3CA* mutation-positive tumours will be estimated from a Cox model with a term for treatment only. The hazard ratios for the treatment effect in the overall population (AZD5363 + paclitaxel: placebo + paclitaxel), and in the subgroup of patients with *PIK3CA* mutation-positive tumours will be estimated together with their 80% confidence intervals and one-sided p-values (a hazard ratio less than 1 would favour AZD5363 + paclitaxel). Kaplan-Meier plots of progression-free survival and estimates of median progression-free survival will be presented by treatment group in the overall population and within the subgroup of patients with *PIK3CA* mutation-positive tumours.

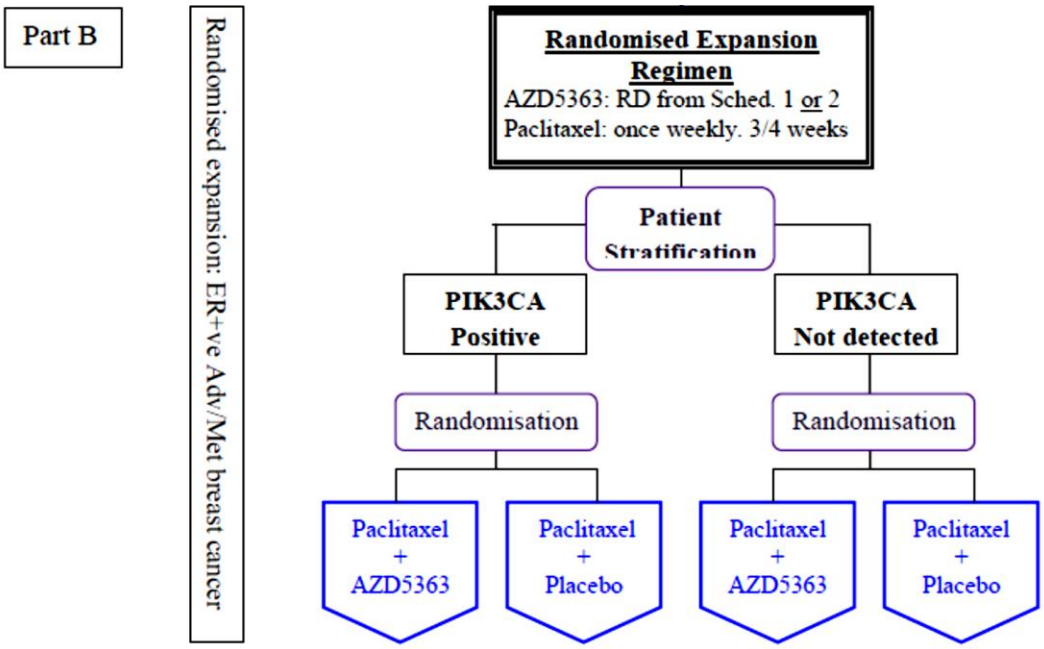
A sample size of 50 patients in each of the Paclitaxel + placebo and AZD5363 + Paclitaxel groups (100 in total) is required to detect a hazard ratio of 0.61, corresponding to median improvements in PFS from 5 months to 8.2 months. A total of at least 76 PFS events will be required to ensure power is at least 80%; the type 1 error assumed is a 1 sided 10% level. The critical value of the HR is 0.74 (i.e. If the observed HR is >0.74, the upper 90% CI for the HR is likely to extend beyond 1, resulting in non significance at the 10% level).

A sample size of 50 patients who are *PIK3CA* tumour mutation positive, (25 in each of the Paclitaxel + Placebo and AZD5363 + Paclitaxel groups) is required to detect a hazard ratio of 0.50, corresponding to median improvements in PFS from 5 months to 10 months. A total of at least 38 PFS events will be required to ensure power is at least 80%; the type 1 error assumed is a 1 sided 10% level. The critical value of the HR is 0.66 (i.e. If the observed HR is >0.66, the upper 90% CI for the HR is likely to extend beyond 1, resulting in non significance at the 10% level).

All efficacy data in Part B of the study will be analysed on an intention-to-treat basis including all randomised patients and comparing treatment groups on the basis of randomised treatment, regardless of the treatment they actually received.

All patients who receive at least one dose of randomised treatment in Part B will be included in the assessment of the safety data, and patients will be assessed according to treatment actually received. Any patient who receives one or more doses of paclitaxel and does not commence dosing with investigational product (i.e. AZD5363 or placebo) will not be included in the assessment of safety data.

In Part B, the effect of AZD5363 + paclitaxel on change in tumour size will be estimated from an analysis of covariance model including covariates for baseline tumour size (log transformed), *PIK3CA* mutation status and treatment. Change in tumour size in patients that have *PIK3CA* mutation-positive tumours will be estimated from an analysis of covarince model with baseline tumour size and treatment as covariates. Estimates of the treatment effects (ratio of glsmeans, AZD5363 + paclitaxel: placebo + paclitaxel) will be calculated together with their 2-sided 80% confidence intervals. Waterfall plots showing the percentage change in tumour size for individual patients will also be presented.



Aims

The aim of this study is to set up a reliable and reproducible method to isolate low molecular weight cfDNA from serum in order to improve the sensitivity of detection of somatic mutations in metastatic Breast Cancer (MBC) Patients.

A secondary objective of this study was to compare automated versus manual cfDNA extraction using two different systems, the circulating nucleic acid (cna) kit from Qiagen and the MagMax cfDNA extraction kit on an automated system from Thermo.

Furthermore a xenograft analysis has been carried out in order to evaluate the time schedule of AZD5363 administration. As concerns the clinical data, this is an ongoing study and the results can't be shown.

Material and Methods

HCC Xenograft Model

The HCC cell line MCF-7 was acquired from the American Type Culture Collection. Cells were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum and maintained under a 5% CO2-humidified atmosphere at 37°C. Cells growing exponentially in vitro were trypsinized and harvested for tumor implantation. All animal manipulations were performed under sterile conditions. To create orthotopic HCC models, mice were anesthetized with intraperitoneal injection of a ketamine/diazepam solution (50 mg/kg ketamine and 5 mg/kg diazepam) and operated on a pre-warmed operation table. A subcutaneous dose of baytril 5 mg/kg injection was given before operative procedure. Briefly, an upper midline laprotomy was performed. Aided by the use of an operating microscope (Leica, Switzerland), the portal vein was exposed by displacing the duodenum through a midline incision of the abdomen and a suspension of 1 × 106 HepG2 cells in MEM medium was injected via the portal vein using a 30-gauge needle within a period of a minute. The abdominal incision was sutured close with 5/0 maxon (monofilament polyglyconate synthetic absorbable suture) and skin was closed with 5/0 prolene (polypropylene suture). Postoperatively, the mice were given a subcutaneous injection of 5 mg/kg caprofen for analgesia. The animals were kept warm and returned to their cages when fully awake. PET scanning was performed on the mice periodically after day 4.

Study Cohort

13 paired plasma and serum samples from MBC patients from the Royal Marsden Hospital sponsored trial "*Plasma DNA as a surrogate for breast cancer phenotype study*".

Processing of plasma and serum

Blood collected in EDTA K2 tubes was processed within two hours of sample collection and centrifuged at 1600 rpm for 20 min, with plasma stored at -80°C until DNA extraction. Serum samples were allowed to clot at room temperature for approximately 45 minutes. Subsequently and within 4 hours of collection, they were centrifuged for 15 minutes at 1600 g to separate the serum from the clot. Serum was stored at -80°C until DNA extraction.

Manual extraction of circulating DNA from plasma and serum

DNA was extracted from 2 ml of plasma or serum using the QIAamp circulating nucleic acid (cna) kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted into buffer AVE in two independent 50µl aliquots (elution 1 and elution 2) and stored at -20°C.

Automated extraction of circulating DNA from plasma

DNA was extracted from 2ml of plasma using the automated KingFisher Flex purification system (Thermo) using the MagMax cfDNA extraction kit (Thermo) as per manufacturer instructions. DNA was eluted either on 50µl or 100µl elution buffer and stored at -20°C.

cfDNA quantification from serum and plasma

DNA isolated from plasma or serum was quantified on a Bio-Rad QX-200 droplet ddPCR using RNase P as the reference gene. One µl of eluate was added to a digital PCR reaction containing 10 µl ddPCR Supermix for probes (Bio-Rad) and 1 µl of TaqMan Copy Number Reference Assay, human, RNase P (Life Technologies) on a total volume of 20 µl. The reaction was partitioned into ~14,000 droplets per sample in a QX-200 droplet generator according to manufacturer's instructions. Emulsified PCR reactions were run on a 96 well plate on a G-Storm GS4 thermal cycler incubating the plates at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec, followed by 10 min incubation at 98°C. The temperature ramp increment was 2.5°C/sec for all steps. Plates were read on a Bio-Rad QX-200 droplet reader using QuantaSoft v1.7.4 software from Bio-Rad. At least two negative control wells with no DNA were included in every run. The amount of amplifiable RNase P DNA was calculated using the Poisson distribution in QuantaSoft.

Size analysis of cfDNA extracted from plasma and serum

1 µl of each eluate of plasma or serum extracted cfDNA was analysed using Agilent High Sensitivity DNA Kit as per manufacturer instructions on a 2100 Bioanalyzer.

Results

PIK3CA mutational status

We analysed 0.25, 0.5 or 1 ml of plasma or serum equivalent by mdPCR to test for the most commonly occurring *PIK3CA* hotspot mutations in 12 paired plasma and serum samples from our cohort.

We only found 1 sample, 7018, harbouring a *PIK3CA* mutation on exon 9, *PIK3CA c.1633 G>A; pE545K*. We were able to detect this mutation in as low as 0.25 ml plasma equivalent at roughly the same Allele Frequency (AF) or mutant copies per ml in all the plasma volumes analysed.

However, in order to detect the same mutation in serum extracted ctDNA we had to use a higher volume of serum equivalent. We were able to detect the same mutation, albeit at a lower AF and mutant copies per ml in 1 ml serum equivalent. This result would point to the hypothesis that serum ctDNA might be more difficult to detect due to gDNA contamination from WBC.

When we analysed the dPCR plots we found that plasma-derived ctDNA containing the *PIK3CA* mutation was clearly observed, while in serum-derived ctDNA the mutation was not detected.

As concerns the clinical data and the correlation between progression free survival vs *PIK3CA* mutation status and Overall survival vs *PIK3CA* mutation status can't be shown since this is an ongoing study funded by Astra Zeneca.

Xenograft Analysis

AZD5363 has been shown to greatly increase sensitivity to the taxane docetaxel (Taxotere) in several breast cancer xenograft models. For example, both continuous and intermittent schedules of AZD5363 enhanced the efficacy of docetaxel in the HCC-1187 xenograft (Figure 1). The sequence of administration appears to be very important; in the HCC-1187 model, administering docetaxel after an intermittent dosing schedule of AZD5363 (4 days on, 3 days off) was found to be antagonistic, whilst administering docetaxel before the intermittent schedule resulted in enhanced efficacy (Figure 2).

Efficacy equivalence has been shown for AZD5363 combined with Taxotere under continuous, 4 days-on, 3 days-off and 2 days-on 5 days-off schedules at adjusted dose levels (Figure 3).

When tumours are re-challenged after a period of recovery, both schedules (continuous and intermittent) cause sustained tumour regressions, whilst docetaxel monotherapy results in progressive tumour growth.

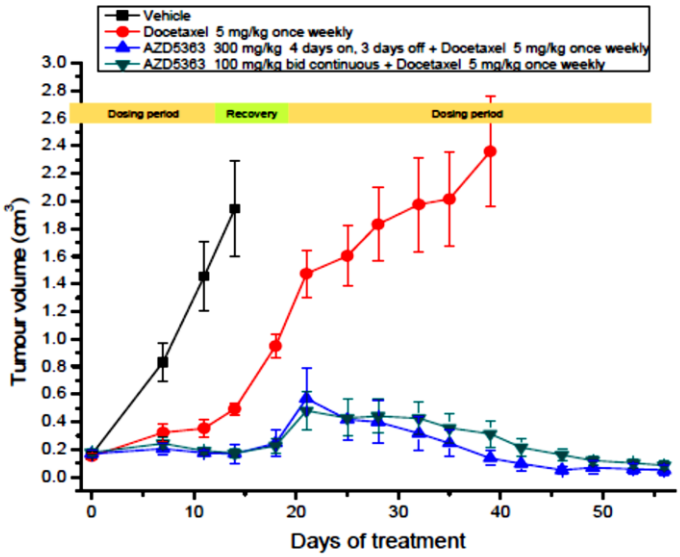


Figure 1 AZD5363 sensitises HCC-1187 xengrafts to docetaxel (Taxotere)

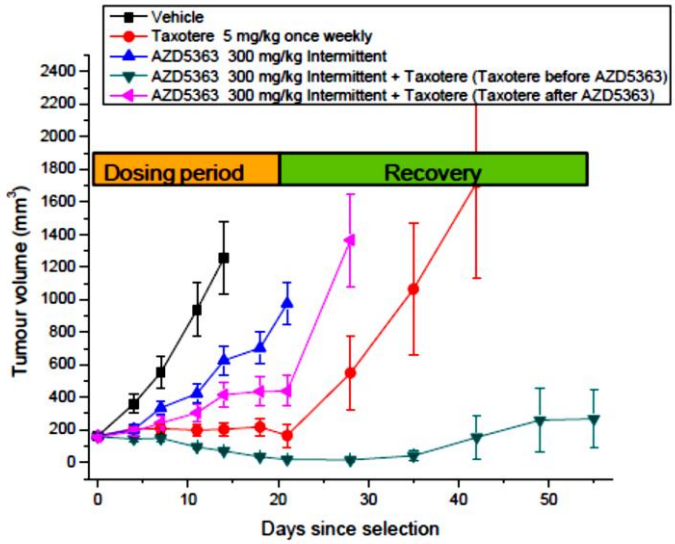


Figure 2 AZD5363 sensitises HCC-1187 xengrafts to docetaxel (Taxotere)

Using an intermittent dosing schedule (300 mg/kg qd, 4 days on, 3 days off), AZD5363 sensitises HCC-1187 xenografts to docetaxel provided the weekly docetaxel is administered 1- 24 h before the first dose of AZD5363; the combination is antagonistic if the docetaxel is given 24 h after the AZD5363 dosing sequence.

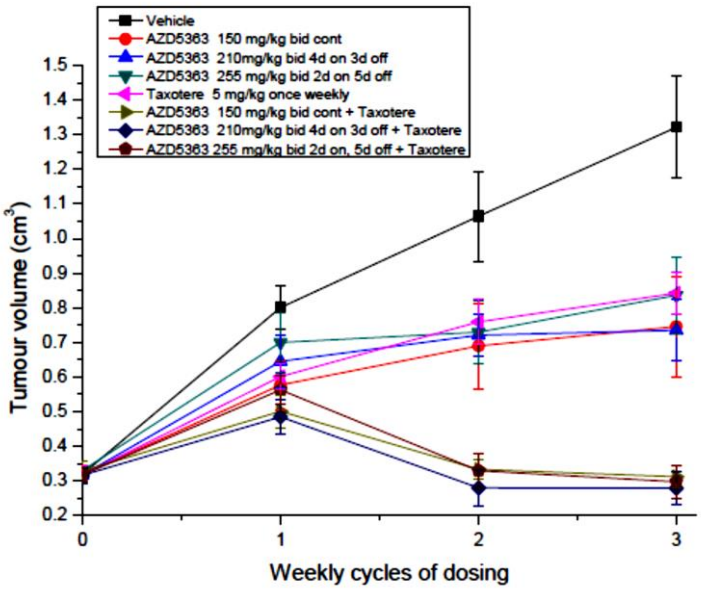


Figure 3 Different dosing schedules of AZD5363 deliver equivalent efficacy, as monotherapy or in combination with taxotere, in the BT474c xenograft model.

In this model, a dose of 255 mg/kg bd of AZD5363 combined with Taxotere given in a 2 days-on 5 days-off intermittent schedule delivers equivalent efficacy to that obtained from a combination of Taxotere with either 150mg/kg bd of AZD5363 given in a continuous schedule and 210mg /kg bd of AZD5363 given in a 4 days on, 3 days off schedule.

As concerns the clinical data, this is an ongoing study and the results can't be shown.

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