Synergistic targeting of PI3K/AKT-pathway and androgen-receptor axis significantly delays castration-resistant prostate cancer progression in vivo

Christian Thomas1*, Francois Lamoureux1*, Claire Crafter2, Barry R Davies2, Eliana Beraldi1 Ladan Fazli1, Soojin Kim1, Daksh Thaper1, Martin E. Gleave1 and Amina Zoubeidi1

*equal contributors

1The Vancouver Prostate Centre, Department of Urologic Sciences, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada,
2AstraZeneca, Alderley Park, Macclesfield, UK

Running title: Targeting AKT-pathway and AR-axis in prostate cancer

Key words: AKT inhibitor, androgen receptor, bicalutamide, prostate cancer

Abbreviations list: ADT (androgen deprivation therapy), AR (androgen receptor), CRPC (castration-resistant prostate cancer), PCa (prostate cancer)

Grant support: AstraZeneca (A. Zoubeidi & M. Gleave), Prostate Cancer Canada (A. Zoubeidi), Prostate Cancer Foundation-USA & Michael Smith Foundation for Health Research (A. Zoubeidi), Deutsche Forschungsgemeinschaft German Research Foundation grant TH 1482/2-1 (C. Thomas) and l’Association pour la Recherche sur le Cancer (F. Lamoureux).

Corresponding author:
Dr. Amina Zoubeidi
The Vancouver Prostate Centre
2660 Oak Street
Vancouver, BC
V6H 3Z6, Canada
Phone: (604) 875-4818
Fax: (604) 875-5654
azoubeidi@prostatecentre.com
ABSTRACT

The progression to castrate resistant prostate cancer (CRPC) correlates with gain of function of the androgen receptor (AR) and activation of AKT. However, as single agents, AR or AKT inhibitors result in a reciprocal feedback loop. Therefore, we hypothesized that combination of an AKT inhibitor with an anti-androgen might result in a more profound, long lasting remission of CRPC. Here we report that the AKT inhibitor AZD5363 potently inhibits proliferation and induces apoptosis in prostate cancer cell lines expressing the androgen receptor, and has anti-cancer activity in vivo in androgen sensitive and castrate resistant phases of the LNCaP xenograft model. However, we found that the effect of on castrate resistant tumour growth inhibition and PSA stabilization is transient, and resistance occurs with rising PSA after ~ 30 days of treatment. Mechanistically, we found that single agent AZD5363 induces increase of AR binding to androgen response element, AR transcriptional activity and AR dependent genes such as PSA and NKX3.1 expression. These effects were overcome by combination of AZD5363 with the antiandrogen bicalutamide resulting in synergistic inhibition of cell proliferation and induction of apoptosis in vitro, and prolongation of tumour growth inhibition and PSA stabilization in CRPC in vivo. This study provides a preclinical proof-of-concept that combination of an AKT inhibitor with anti-androgen results in prolonged disease stabilization in a model of CRPC.
INTRODUCTION

While early detection and treatment of localized prostate cancer (PCa) has improved, many men still die of metastatic disease. Almost 80% of patients initially respond to androgen deprivation therapy, most patients progress to castrate resistant prostate cancer (CRPC) and metastatic disease (1-6). CRPC is a complex process by which cells survive and proliferate in low circulating androgen. This in part involves the re-activation of the AR axis (7), by pro-survival genes and alternative mitogenic growth factor pathways (8, 9) including the PI3K/AKT pathway which offers substantial therapeutic potential in PCa (10, 11). This pathway is up-regulated in PCa in high-risk disease and CRPC (10, 12, 13). It has been proposed the existence of crosstalk between the PI3K/AKT pathway and the AR-axis, however the underlying molecular mechanisms still unknown (14-18).

While targeting the PI3K/AKT-pathway by small molecule inhibitors has been reported to be a promising approach to delay PCa progression in the clinic (9, 17, 18), the up-regulation of the AR-pathway seems to counter its effect in PCa (15, 16, 18). For instance, Carver et al. showed that combined inhibition of PI3K/AKT- and AR-signaling by using the PI3K/mTOR inhibitor BEZ235 and the antiandrogen MDV3100 resulted in an enhanced antitumoral activity in the hormone-sensitive LNCaP mouse model (18). However, it is not known whether the combined blockade of AR and PI3K/AKT pathway will effect tumor progression in the castration resistant state.

The aim of the current study is to evaluate the antitumoral activity of the novel AKT-inhibitor AZD5363 at different stages of PCa both in vitro and in vivo. Here, we show that AZD5363 potently affects cell growth and survival of AR positive PCa cells in vitro. The underlying mechanisms are based on induction of caspase 3-dependent apoptosis. Interestingly, PI3K/AKT-pathway inhibition by AZD5363 leads to an up-regulation of AR-dependent proteins. AZD5363 inhibits cancer progression in the hormone-sensitive as well as in the CRPC LNCaP xenograft model. Most important, combination treatment of AZD5363 plus bicalutamide
circumvents AZD5363 induced AR-upregulation and results in long-lasting inhibition of CRPC progression. The data support further investigation of the therapeutic potential of AZD5363 in human PCa, particularly in combination with anti-androgens.
MATERIALS AND METHODS

Prostate cancer cell lines and reagents
The human PCa cell lines LNCaP and C4-2 used in this study were kindly provided by Dr. Leland W.K. Chung (1992, MDACC, Houston TX) and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2009. Western blotting and/or real time PCR was performed for AR and PSA each time when LNCaP or C4-2 cells were resurrected. After resurrection, both cell lines were passaged for less than 3 months. LNCaP and C4-2 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 5% charcoal stripped serum (CSS) or 5% fetal bovine serum (FBS) and cultured without antibiotics at 37°C in 5% CO₂ atmosphere.

Treatment of prostate cancer cells with AZD5363 and bicalutamide
The AKT-Inhibitor AZD5363 and the nonsteroidal antiandrogen bicalutamide were kindly provided from Astra Zeneca and used respectively for in vitro and in vivo. AZD5363 is a new ATP-competitive inhibitor of AKT and, as a synthetic small molecule, orally available (19, 20). For the in vitro studies, AZD5363 was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at 10mM stock solutions and stored at -20°C. For the in vivo studies, AZD5363 was dissolved in H2O and 10% DMSO and 25% Kleptose (Roquette Pharma, France) at 100mg/ml and stored at room temperature for 1 week. For in vitro use, bicalutamide was dissolved in DMSO and stored for 4 weeks at room temperature. In vivo, bicalutamide was diluted with H₂O and used at 10mg/kg bodyweight.

Cell proliferation and apoptosis assays
Cell growth was assessed using crystal violet assay, as described previously(19). Cells were plated in 96-well plates and treated with AZD5363 in FBS supplemented media. Crystal violet staining was carried out for dose- and time-dependent treatment. Cell proliferation after AZD5363 treatment was calculated as the percentage of the absorbance in untreated cells. Caspase-3 activity was assessed 3 days after treatment using the kit CaspACE Assay System,
Fluorometric (Promega, Madison, WI, USA) as we previously described (19).

**Cell cycle analysis**

Changes in the different phases of cell cycle of LNCaP and C4-2 cells treated with AZD5363, bicalutamide or the combination of both were assessed by flow cytometric analysis. Cells were plated in 10 cm² dishes using FBS supplemented media and treated as described above. 3 days later cells were trypsinized and fixed in 70% ethanol over night at 4°C, then incubated with 1 µg/mL RNase A (Sigma) for 30min at 37°C before stained with 5µg/mL propidium iodide (Sigma) for 30min at room temperature. The stained cells were analyzed for relative DNA content on a dual laser flow cytometer (Beckman Coulter Epics Elite; Beckman, Inc.). Each assay was done in triplicate.

**Western blot analysis**

Cell population was harvested in a dose-dependent manner after AZD5363 treatment at 5µM in RIPA buffer (50nM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 10nM NaCl, Roche complete protease inhibitor cocktail) and Western blots were performed as we previously reported (19) using antibodies as described in Supplementary Table S1.

**Quantitative Reverse Transcription-PCR**

Total RNA was extracted from cultured cells after 48h of treatment using TRIzol reagent (Invitrogen Life Technologies, Inc.). Real-time monitoring of PCR amplification of complementary DNA (cDNA) was performed as previously described (21) on ABI PRISM 7900 HT Sequence Detection System (applied Biosystems) with SYBR PCR Master Mix (Applied Biosystems). Each assay was performed in triplicate.

**Luciferase assay**

LNCaP and C4-2 cells were plated on 12 well plates and transfected with PSA-Luciferase-Plasmid (-6,100 to +12) using lipofectin (Invitrogen Life Technologies, Inc.). The total amount of 1.0 µg per well PSA-plasmid DNA was used. 16h before start of treatment media was changed into CSS -/+ 0.1 nmol/L R1881 (PerkinElmer). Luciferase activities were measured using the
Dual-Luciferase Reporter Assay System (Promega) using a microplate luminometer (EG&G Berthold). Luciferase activity was normalized to the protein concentration of the cells. All experiments were carried out in triplicate wells and repeated two times.

**Chromatin immunoprecipitation assay (ChIP assay)**

ChIP assay was performed as previously described (22). Briefly, LNCaP cells were cross-linked with paraformaldehyde and digested with micrococcal nuclease to achieve a DNA smear of 200–1,000 bp. ChIP assay was performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Agarose Beads) according to the manufacturer’s protocol (Cell Signaling Technology) on the AR gene as we previously described (21). The results are representative of at least three independent experiments.

**Assessment of synergy on cell viability**

A combination-index (CI) for synergy was determined by comparing the growth inhibition of bicalutamide (range 10.0-50.0 µM) or AZD5363 (range 100.0-500.0 nM) with that of the combination of both drugs (constant ratio 1:100) based on the median effect principle (MEP). This commonly used method was first described by Chou and Talalay (23). LNCaP and C4-2 cells were plated in 96-well plates containing CSS-supplemented media + 0.1nM R1881 and treated with both drugs as mentioned before. Cell viability inhibition was determined 72hr after treatment by crystal violet assay and each experiment was repeated in triplicate.

Drug interactions were quantified with Calcusyn™ software (Biosoft, Cambridge, UK). CI was calculated for the effective doses ED_{50}, ED_{75} and ED_{95}. A CI <1 indicates synergy, a CI >1 indicates antagonistic interactions and a CI + 1 indicates additive effects. Moreover, DRI was calculated for bicalutamide.

**Animal treatment**

*In vivo*, we used two different animal models to mimic hormone-sensitive and castration-resistant PCa (CRPC) progression. For both models, male athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected subcutaneous with 2x10^6 LNCaP cells (suspended in Matrigel; BD
Biosciences). Several studies have shown that both models are able to simulate the different clinical stages of human PCa (24-27) and Supplementary Fig. S1 illustrates both models.

In the hormone-sensitive model, mice were castrated when PSA values exceeded 50ng/mL and treatment started. For treatment, mice were randomly selected for vehicle or AZD5363 (100 mg/kg; formulation in 25% Kleptose + 10% DMSO) and treated orally twice a day in cycles of 5 days on, 2 days off. The experimental group consisted of 12 mice for AZD5363 and 10 mice for control. Tumor volumes and bodyweight (BW) were measured twice weekly, while serum PSA was measured once a week by enzymatic immunoassay (Abbott IMX, Montreal, Quebec, Canada). Data points were expressed as average tumor volume ± SE or average PSA concentration ± SE. Tumor growth rate and serum PSA velocity were determined by the slopes of tumor size and serum PSA over 10 weeks. Both were expressed as the average slope ± SE. Animals were sacrificed after 10 weeks of treatment or earlier when tumor volume reached ≥ 10% of body weight. For survival analysis, we determined tumor progression-free survival (TPFS) and cancer-specific survival (CSS). TPFS was defined as time for the first tumor volume doubling. CSS was defined as time until tumor volume exceeded 10% of BW.

In the CRPC model mice were castrated when PSA values exceeded 50ng/mL and treatment started when PSA relapsed to pre-castration levels. For treatment, mice were randomly selected for vehicle, AZD5363 (as described before), bicalutamide 10mg/kg WB (diluted with H2O and treated orally once a day in cycles of 5 days on, 2 days off) or the combination of both drugs. Drug treatment, tumor response measurement and survival analysis were performed as described before. The experimental group consisted of 8 mice for AZD5363, 8 mice for bicalutamide, 8 mice for combination treatment and 9 mice for control. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

**Immunohistochemistry**

Immunohistochemical stains were performed on formalin-fixed and paraffin-embedded 4µm
sections of tumor samples using adequate primary antibody, and the Ventana autostainer Discover XT (Ventana Medical System) with enzyme labeled biotin streptavidin system and solvent resistant 3,3’-diaminobenzyidine Map kit. All comparisons of staining intensities were made at 200x magnifications.

**Statistical analysis**

All results were expressed as the average ± SE, and the significance of differences were measured by Student’s t-test (Excel, Microsoft). \( p \leq 0.05 (*) \), \( p \leq 0.01 (**) \) and \( p \leq 0.001 (***) \) were considered significant. Kaplan Meier survival analysis was performed for TPFS and CSS and statistical significance between the groups was assessed with the log-rank test (Medcalc software).
RESULTS

AZD5363 targets the PI3K/AKT-pathway and inhibits cell proliferation

The effects of the AKT-inhibitor AZD5363 (Fig. 1A) on distal pathway biomarkers in vitro were investigated by western blot analysis. As shown in Figure 1B, AZD5363 treatment induced AKT$^{S473}$ and AKT$^{T308}$ phosphorylation but inhibited phosphorylation of the distal AKT-pathway biomarkers including PRAS40, eIF4E, 4E-BP1, mTOR and P70 S6 Kinase in a time-dependent manner in both LNCaP and C4-2 cells. As a direct downstream effector to AKT, phosphorylation of S6 kinase was completely abrogated by AZD5363 6hr post treatment. This effect was accompanied with a decrease in cell proliferation in a dose and time dependent manner on both LNCaP and C4-2 cells (Fig. 1C). The effect of AZD5363 was evaluated in an extended panel of prostate cell lines. Prostate cancer cell lines expressing AR were more consistently sensitive to AZD5363 than lines with loss of PTEN. For example, AR+/PTEN wild type LAPC-4 cells were much more sensitive to AZD5363 than AR-/PTEN null PC3 cells (supplementary Table 2).

AZD5363 treatment induces apoptosis in PCa cells in vitro

We next evaluated the effects of AZD5363 treatment on cell apoptosis. As shown in Figure 1D, AZD5363 treatment significantly (**, p<0.01) increased the fraction of cells undergoing cell death (sub G0/G1) in a dose-dependent manner compared to control in both C4-2 and LNCaP cell lines (3.8%, 6.3%, 23.1%, 34.5% and 61.6% in LNCaP and 2.2%, 3.6%, 13.3%, 25.0% and 51.8% in C4-2 cells at 0nM, 100nM, 500nM, 1µM and 5µM AZD5363, respectively) Moreover, we found that AZD5363 induces caspase-3 activity in time dependent manner in LNCaP and C4-2 cells compared to control (Fig. 1E) and PARP cleavage (Fig. 1F). These results suggest that AZD5363 inhibit cell proliferation and induces apoptosis in a caspase-3 dependent manner.

AZD5363 treatment delays tumor progression to CRPC in vivo

Since AZD5363 induces apoptosis and inhibits cell proliferation in LNCaP cells, we set out to determine if AZD5363 has efficacy in vivo. We first evaluated the effect of AZD5363 in hormone sensitive model. Basically, one day post castration, mice were randomized and treated with
100mg/kg AZD5363 or vehicle orally twice a day. We found that AZD5363 significantly suppressed tumor growth (**, p<0.01) compared to control (Fig 2A, left) without any significant impact on average body weight (supplementary Fig. S2). Individual analysis of tumor volume (Fig. 2A right) showed that after 10 weeks of treatment 8 of 10 mice in the control group developed a tumor volume >500mm³ compared to 2 of 12 mice in the AZD5363 group. AZD5363 treatment significantly (*, p<0.05) affected serum PSA levels (Fig. 2B left). The serum PSA velocity increased by 23.0 ng/mL/week in the control group, which was significantly higher (*, p<0.05) compared to 5.4 ng/mL/week in the AZD5363 treated mice (Fig. 2B right). AZD5363 treatment significantly improved Tumor Progression Free Survival (p<0.0001) and delayed median time to tumor progression from 18 days to 59 days (Fig. 2C). Two mice in the control group required sacrifice after 10 weeks (tumor volume >10% of body weight, BW), whereas no animals required intervention in the AZD5363 treated group. To further confirm pharmacodynamic activity in vivo, we performed western blot and IHC on random tumors, as shown in Fig. 3B, PSA protein levels were higher in the control group compared to the AZD5363 treated group. While total AKT protein was expressed uniformly in both groups, the phosphorylated isoforms AKT⁵⁴⁷ and AKT³⁰⁸ (Fig. 2D, right) were up-regulated in the AZD5363 treated animals which is in concordance with our in vitro studies (Fig. 1A) suggesting a feedback loop. However, the distal pathway biomarkers output, pS6 and Cyclin D1, were down-regulated by AZD5363 compared to control and was further confirmed by IHC analysis showing that in xenografts, pS6 was inhibited in the AZD5363 treated animals. Moreover, Ki67 as a biomarker for cell proliferation was lower in the AZD5363 arm compared to control, and cell death was greater in the AZD5363 treated tumors as shown by TUNEL staining (Fig. 2D left).

**AZD5363 treatment delays CRPC progression in vivo**

We next evaluated the effect of AZD5363 on castration-resistant prostate cancer (CRPC) progression in vivo. Basically mice were castrated when PSA reached 50 to 75 ng/ml and were randomized into treatment groups when serum PSA relapsed to pre-castration levels. In Figure
3A, the average tumor volume was significantly higher (**, p≤0.01) in the control group compared to the AZD5363 treated group and the tumor growth rate over 5 weeks was significantly faster (**, p≤0.01) in the control group compared to AZD5363 treated mice (206.8 mm³/week and 33.4 mm³/week, respectively). Moreover, AZD5363 treatment significantly (Fig. 3B; *, p≤0.05) reduced serum PSA levels and serum PSA velocity compared to control (82.8 ng/mL/week and 16.4 ng/mL/week, for vehicle and AZD5363 treated groups respectively). Tumor progression free survival significantly (p=0.0005) improved by AZD5363 treatment compared to control. The median time to tumor progression was 14.5 days in the control group and not reached in the treatment group within the first 30 days of treatment (Fig. 3C). In concordance with the hormone-sensitive model, AZD5363 induces a decrease of cell proliferation as measured by Ki67, increased cell death as measured by TUNEL staining and a decreased phosphorylation of S6 compared to controls (Fig. 3D). However, after approximately 30 days, the mean tumor size and serum PSA concentration in animals treated with AZD5363 exceeded the size of the controls at the start of the experiment, indicating tumor progression and onset of resistance (Fig. 3E). These data show that treatment benefit with AZD5363 in this model is short lived, and resistance/relapse occurs with rising PSA levels suggesting that AR is still active.

AZD5363 treatment activates the AR pathway

To explore the mechanism of treatment relapse, we evaluated the effect of AZD5363 on the AR signaling pathway. To our surprise, we found that inhibition PI3K/AKT pathway using AZD5363 increased AR expression in a time dependent manner at both mRNA and protein levels (Fig. 4A). Similar results were observed with PI3K/mTOR inhibitor, rapamycin (Supplementary Fig. S3) showing increased both AR and PSA expressions at protein levels. Consistent with these data, others have found that inhibition PI3K/mTOR pathway using BEZ235 results in up-regulation of AR expression (18). We found that AZD5363 and rapamycin also induced ERK phosphorylation (Supplementary Fig. S3). This pathway may play a critical role in the cross-talk between the
PI3K/AKT-pathway and the AR-axis. We next evaluated whether AZD5363 induces activation of AR signaling pathway. We found that AZD5363 increased AR transcriptional activity in a dose-dependent manner (Fig. 4B) associated with enhanced target genes expression at mRNA level (PSA and NKX3.1) (Fig. 4C). To further corroborate our finding on the effect of AKT inhibition pathway on AR, we specifically down-regulated AKT using siRNA and found that AKT knockdown induced increase of PSA expression at protein level (Supplementary data Fig. S3C) supporting that targeting AKT activates the AR pathway. To further understand how targeting AKT activates AR, we performed ChIP using and found that AZD5363 increased AR binding to the AREI and ARE III sequences in PSA promoter (28) compared to control (Fig. 4D) which was accompanied by increase AR nuclear translocation (Supplementary data Fig. S4), AR transcriptional activity and expression of AR target genes as we showed in Figure 4B and C. Interestingly the effect of AZD5363 on AR activation was abrogated with the AR antagonist bicalutamide. Hence, bicalutamide abrogates AZD5363 induced AR target genes expression at mRNA and protein levels (Fig. 4E and F) which was associated with increase PARP cleavage (Fig. 4F). These results suggest that combined inhibition of the AR signaling pathway using bicalutamide enhances the activity of AZD5363.

**Bicalutamide synergizes with AZD5363 to inhibit cell viability and induction of apoptosis in AR-positive PCa cell lines in vitro**

Since AZD5363 in combination with bicalutamide (Fig. 5A) induced PARP cleavage, we tested if this combination has an effect on cell proliferation. As shown in Figure 5 (upper panel), whilst each single agent decreased the proliferation of LNCaP and C4-2 tumor cells, the combination of AZD5363 plus bicalutamide had the highest anti-proliferative effect. The calculated combination indices determined at ED$_{50}$, ED$_{75}$ and ED$_{90}$ (using CalcuSyn™ software) revealed combination index values below 1 in both cell lines. This data suggests that the cell viability inhibition effects of AZD5363 and bicalutamide are strongly synergistic in LNCaP and C4-2 cells (Fig. 5A lower panel). Regarding to dose reduction indices (DRI), the concentration of...
bicalutamide to achieve a 50% decrease in cell viability was reduced by -6.2-fold in LNCaP and by -9.4-fold in C4-2 cells when AZD5363 was added (Fig. 5B). We also found that the combination of AZD5363 + bicalutamide significantly enhanced the induction of apoptosis compared to each single agent treatment, by increasing subG1 fraction by 5 and 2-fold respectively in LNCaP and C4-2 tumor cells (Fig. 5C) and increasing caspase 3/7 activity by 2-3-fold (Fig. 5D).

**Combination treatment of AZD5363 plus bicalutamide significantly delays CRPC tumor progression**

Finally, we evaluated if combination therapy with AZD5363 and bicalutamide could result in enhanced efficacy in CRPC using LNCaP xenografts. Castrated male nude mice bearing LNCaP xenografts were randomized after PSA relapse to pre-castration level and treated either with AZD5363 100mg/kg, bicalutamide 10mg/kg or the combination of both. While AZD5363 and bicalutamide as monotherapy significantly decreased tumor growth compared to control, the combination treatment enhanced the anti-tumoral effects of each single treatment (Fig. 6A). We found that both bicatulamide and AZD5363 slow tumor growth whereas the combination treatment significantly stopped tumor growth in all mice (Fig. 6B). While AZD5363 and bicalutamide monotherapy decreased serum PSA levels compared to controls, the combination treatment reduced drastically PSA levels (Fig. 6C), consequently, the combination treatment significantly prolongs the cancer-specific survival (p=0.0001) compared to control and each single drug. All mice survived during 70 days of treatment in the combination arm, whereas, 50% of mice in the AZD5363 arm and 62.5% in the bicalutamide arm were sacrificed due to high tumor burden. In the control arm, all animals were sacrificed within 57 days of treatment (Fig. 6D). IHC analysis revealed a slightly decreased Ki67 expression in each single drug group compared to control group, whereas the combination treatment considerably diminished Ki67 (Fig. 6E), suggesting a synergistic effect of the drug combination on inhibition of tumor cell proliferation. In addition, tumors treated with the combination had the highest number of dying
cells as shown by increased TUNEL staining. Consistent with the vitro data, AR expression was decreased after bicalutamide treatment, whereas the expression was higher after AZD5363 treatment compared to controls. However, combination AZD5363 + bicalutamide strongly reduced AR expression. These data collectively suggest that delayed tumor progression in AZD5363 + bicalutamide-treated mice results from both reduced proliferation and increased apoptosis.
DISCUSSION

The PI3K/AKT-pathway plays a major role in PCa progression (10). It is up-regulated in 30-60% of PCa, especially at high Gleason score and in CRPC (12, 13). Involved in numerous cellular processes including cell growth and survival, pharmacological targeting of the PI3K/AKT-pathway might be promising approach to affect PCa growth. Several small molecule inhibitors targeting different proteins of the PI3K/AKT-pathway have shown potent anti-cancer activity in PCa in vitro and in vivo including, GSK690693, perfosine andisoflavones (GCP) (9, 17, 29-31). For instance, GSK690693 showed significant inhibition of tumor growth by 50% compared to control in LNCaP xenograft model (9). The isoflavone genistein significantly reduced tumor volume and incidence in the same tumor model (17). Furthermore, antitumoral effects of AKT inhibitors in s.c. PC3-xenograft mice have been reported in several studies (29, 32, 33).

So far, perifosine and celecoxib with AKT-inhibitory properties have been tested clinically in patients with advanced PCa. Perifosine is a non-selective Akt inhibitor while celecoxib is a cyclooxygenase 2 inhibitors with additional AKT pharmacology; both compounds didn’t shown any measured activity in clinical trials. For instance, Perifosine induced PSA stabilization only in one patient of 5 with metastatic CRPC after 12 weeks treatment and no patients showed any PSA decrease (34). These results were consistent with data from patients with biochemically recurrent PCa treated with perifosine (35). The cox2-inhibitor celecoxib used in combination with hormone therapy failed to enhance the activity of castration in patients with hormone sensitive PCa (36).

These conflicting data between preclinical and clinical studies could be the consequence of poor selectivity and suboptimal AKT pharmacology of the earlier compounds, or the use of inappropriate in vivo models that do not reflect the clinical course of PCa. Antitumor response should not only be measured by tumor volume but also by serum PSA. This biomarker is of major importance to predict clinical benefit in patients with progressive PCa (37). Moreover, ADT should be implemented in an in vivo model as it is standard treatment in metastasized PCa (2,
38). Previous preclinical studies with other AKT inhibitors have not met these criteria. For example, in the previously mentioned LNCaP xenograft models serum PSA levels were not evaluated, and the PC3 xenograft model does not express the androgen receptor, hence does not represent the majority of CRPC in humans. Regarding to ADT, Carver et al. (18) were able to show that combining castration with the PI3K/mTOR inhibitor BEZ235 induces regression of tumors in mice, whereas average tumor volume in mice treated with vehicle or BEZ235 monotherapy increased (18).

In the current study, both in vivo experiments are based on the s.c. LNCaP-xenograft model which mimics the clinical course of PCa in humans (37-39). Using the novel small molecule AZD5363, an ATP-competitive inhibitor of AKT, we are able to delay in vitro and in vivo PCa progression in hormone-sensitive as well as in castration-resistant state. The anti-tumor activity of AZD5363 is based on cell-cycle inhibition and induction of apoptosis.

This study shows that AKT inhibition induces a delay of tumor progression to CRPC in xenograft model. AZD5363 significantly slows down tumor growth rate and serum PSA velocity by 83.9% and 80.0%, respectively (Fig. 3A and B). However, this effect is not long-lasting in the castration-resistant state. After 4-5 weeks treatment with AZD5363, xenograft tumors and serum PSA start to progress, demonstrating that AZD5363 as a single agent induces a tumor stabilization for some time rather than sustaining tumor regression. It has already been described in the literature that resistance to AKT-inhibitors might be induced by reactivation of the AR-axis (13-15, 17). We demonstrate that specific pharmacologic inhibition of AKT signaling using AZD5363 induces a feedback regulation of the AR pathway (Fig. 4 A-D). In concordance with other studies (40, 41), we suggest that the bidirectional crosstalk between AR and AKT is a rationale for simultaneous molecular targeting of both pathways. We identified pERK as a potential link between the PI3K/AKT-pathway and the AR-axis (Supplementary Fig. 3A) and this is due to a feed-forward loop involving activation of EGFR and IGFR (Supplementary Fig. 3B).
after AKT inhibition similar to the one reported by Chandarlapaty et al in breast cancer (41). Since Akt has been reported to phosphorylate the AR on Ser-210 and Ser-790 and inhibits its transactivation (42) and that targeting Akt phosphorylation will then result on inhibiting AR phosphorylation on Ser-210 and Ser-790, inducing AR transactivation which support our finding that targeting Akt phosphorylation with AZD5363 will abrogate AR phosphorylation and increase expression of AR dependent genes such PSA and NKx3.1 as seen in figure 4.

To optimize the clinical response to AKT pathway inhibition, we evaluated the therapeutic effect of AZD5363 combined with AR pathway inhibition using bicalutamide. AZD5363 induced upregulation of the AR-axis which was abrogated by simultaneous treatment with antiandrogens. In the current study, we combined AZD5363 plus bicalutamide. In vitro, AZD5363 plus bicalutamide synergized on inhibition of cell viability (Fig. 5A). Moreover, induction of apoptosis was enhanced by combination treatment compared to each monotherapy treatment (Fig. 5 C-D). Notably, the addition of bicalutamide prevented AZD5363 induced up-regulation of the AR-axis (Fig. 4 E-F).

Previous data have shown that combination treatment with anti-androgens and mTOR-inhibitors enhance anticancer activity (15, 17, 43). However, these studies are not mimicking CRPC setting. We show here for the first time, the addition of bicalutamide significantly enhances the anti-cancer effects of AZD5363 in a CRPC xenograft model. Even after 70 days of treatment, tumor volumes in the combination arm do not exceed the values at the beginning (Fig. 6A). Also, serum PSA levels are significantly lower in the combination arm compared to each monotreatment (Fig. 6C). Most important, no animal in the combination arm had to be sacrificed within 70 days of treatment, whereas 50% of mice in the AZD5363 arm and 60% in the bicalutamide arm were sacrificed due to high tumor burden (Fig. 6D). This study impressively demonstrates that the synergistic effects of AZD5363 and bicalutamide lead to long-lasting tumor remission in the castration resistant state. Moreover, it provides further evidence that...
targeting only PI3K/AKT signaling pathway, as a mono-therapy, leads to suboptimal activity, supporting the concept that combination of an AKT and androgen signaling axis inhibitor may be preferable for treatment of CRPC.

In conclusion, we show that the novel AKT-inhibitor AZD5363 potently inhibits PCa progression at different states in vitro and in vivo. Most important, we demonstrate that the combination treatment of AZD5363 plus bicalutamide significantly delays CRPC progression compared to each mono-treatment. The high antitumoral activity in vivo combined with an acceptable tolerability profile in this rodent model supports the evaluation of AZD5363 on CRPC patients in clinical trials. The clinical phase I study for AZD5363 has been launched in December 2010 (44).
Acknowledgements: We thank Eliana Beraldi, Virginia Yago, Igor Moskalev, Darrell Trendall, Mary Bowden and Estelle Li for their technical assistance.

Potential conflict of interest: C. Crafter and BR Davies are employees of AstraZeneca. No other potential conflicts of interest to disclose.
References


44. Stockman P. Assessment of the safety, tolerability and potential anti-cancer activity of increasing doses of AZD5363 in different treatment schedules. Clinical Trials.gov 2012.
LEGENDS

Figure 1. AZD5363 inhibits prostate cancer tumor growth and induces cell cycle arrest and apoptosis in vitro. A, effect of AZD5363 on the expression of distal AKT-pathway biomarkers and biomarkers of AKT-pathway output in LNCaP and C4-2 cells. B, LNCaP and C4-2 cells were treated with 10nM to 10 µM AZD5363 for 24hr to 72hr. Cell viability was determined by crystal violet assay (left panel). Columns are means of triplicate analysis ± SE. Dose-dependent inhibition of cell growth by AZD5363 compared to perifosine, each dosed from 10nM to 10µM (right panel). 72hr after treatment cell viability was assessed by crystal violet assay. Data points are means of triplicate analysis ± SE. *, p<0.05 and **, p<0.01 and ***, p<0.001 differ from control by Student’s t-test. C, AZD5363 treatment significantly increases the fraction of cells undergoing cell death (subG0/G1). 72hr after dose-dependent treatment with 100nM to 5µM AZD5363 cells and cell cycle population was analyzed by flow cytometry. Histograms represent the means independent triplicate analysis ± SE. D, treatment with AZD5363 increases Caspase-3 activity. Cell lines were treated with 5µM AZD5363, and total cell lysate was extracted in a time-dependent manner. Results are expressed in arbitrary units and corrected for protein content. Columns show means of triplicate analysis ± SE. **, p<0.01 and ***, p<0.001 differ from ScrB by Student’s t-test. E, AZD5363 induces PARP cleavage. LNCaP and C4-2 cells were treated with 5µM AZD5363, and protein was extracted in a time-dependent manner detection of PARP and Vinculin was used as an internal control.

Figure 2. AZD5363 inhibits prostate cancer tumor growth in the hormone-sensitive LNCaP model. 2x10^6 LNCaP cells were injected subcutaneous in male athymic nude mice. Animals were castrated when PSA values exceeded 50ng/mL and treatment started with 100mg/kg AZD5363 or vehicle orally twice a day 5 times per week. A, the mean tumor volume of all mice (left) per group ± SE and for each mouse individually per group (right). Significance at least **, p≤0.01 for all time points between day 7 and 70. B, mean serum PSA levels of all
mice per group \(\pm SE\) (left). Significance at least *, \(p<0.01\) for all time points between day 7 and 70. AZD5363 treatment down-regulates PSA protein expression in tumors. Vinculin was used as a loading control. The serum PSA velocity (ng/mL/week) was estimated by the slope over 10 weeks. Columns represent average tumor growth rate (mm\(^3\)/week) \(\pm SE\), respectively. **, \(p<0.01\). C, Kaplan-Meier survival analysis comparing AZD5363 vs. control for TPFS. D, immunohistochemistry on xenograft tumors for pS6, Ki 67 and TUNEL (left). Total proteins were extracted from xenografts treated with AZD5363 or control after 10 weeks or when tumor volume exceeded \(>10\%\) (right). Samples were taken 4h after last AZD5363 treatment and western blot was performed using pAKT/AKT, pS6/S6 and Vinculin was used as a loading control.

**Figure 3. AZD 5363 inhibits tumor growth in the castrate resistant phase of the LNCaP xenograft model.**

2\(\times\)10\(^6\) LNCaP cells were injected subcutaneous in male athymic nude mice. Treatment was started with 100mg/kg AZD5363 or vehicle orally twice a day 5 times per week when serum PSA relapsed to pre-castration levels. A, the mean tumor volume (left) of all mice per group \(\pm SE\). **, \(p<0.01\) and tumor growth rate (mm\(^3\)/week) (right). **, \(p<0.01\). B, mean serum PSA levels of all mice (left) per group \(\pm SE\). and serum PSA velocity (ng/mL/week) (right). **, \(p<0.01\). C, Kaplan-Meier survival analysis comparing AZD5363 vs. control for TPFS. D, immunohistochemistry on xenograft tumors. Samples were taken 4h after last AZD5363 treatment. E, mean tumor volume of all mice per group -/+ SE. After a tumor stabilization within the first 30 days of treatment, AZD5363 treated tumor start to progress again.

**Figure 4. AZD5363 induces AR signaling which is abrogated by bicalutamide.** LNCaP cells were treated with 1\(\mu\)M AZD5363 in a time-dependent manner. mRNA and proteins levels were analyzed for AR and PSA. B, effect of AZD5363 treatment at indicated concentration on genomic activity of AR. LNCaP cells were transfected with PSA-luciferase and treated with AZD5363 in media containing CSS \(\pm 0.1\) nM R1881. Luciferase activity was measured over
time using a luminometer. C, LNCaP cells were treated with 1µM in a time-dependent manner. mRNA levels were analyzed by quantitative RT-PCR for AR target genes (PSA, FKBP5.2 and NKX3.1). D, LNCaP cells were treated with or without 1µM AZD5363 ± 0.1nM DHT. ChIP assays were performed on nuclear extracts from LNCaP cells using 2 µg of the AR antibody and 20 µL of ProteinG agarose. The RT-PCR was performed using immunoprecipitated DNAs, soluble chromatin and specific primer pairs for the ARE I and ARE III promotor. E and F, LNCaP and C4-2 cells were treated with 1µM AZD5363 and/or 5µM bicalutamide for 48h. mRNA level were analyzed by quantitative RT-PCR for AR and AR target genes (E) and protein levels were analyzed by western blot for the indicated antibodies (F).

Figure 5. AZD5363 synergizes with bicalutamide to decrease cell viability and induces apoptosis. A, Cell viability inhibitory effects of AZD 5363 plus bicalutamide are based on synergism. LNCaP and C4-2 cells were treated with AZD 5363 (range 0-600nM) and bicalutamide (range 0-60uM) for 72h (upper left + right). Cell viability was determined by crystal violet assay. Columns are means of triplicate analysis ± the combination index (CI) was calculated (lower left + right) by combining AZD 5363 and bicalutamide at a constant ratio of 1:100. The calculated combination indices are determined for ED50, ED75 and ED90. Columns represent repeated triplicate analysis -/+ SE. B, dose reduction index (DRI) is expressed as reduction in IC50 for bicalutamide in combination with AZD5363, compared to its individual IC50. In vitro drug combination study in LNCaP and C4-2 cells using a constant ration. Columns represent repeated triplicate analysis -/+ SE. C, Effect of combination treatment of AZD5363 and/or bicalutamide on cell cycle. Cells were treated for 48hr with 1µM AZD5363 and or 5 µM bicalutamide cells and cell cycle population was analyzed by flow cytometry. Columns represent the means independent triplicate analysis + SE. D, Effect of combination treatment of AZD5363 and/or bicalutamide on Caspase-3 activity. All two cell lines were treated with 1µM AZD5363 and/or 5 µM bicalutamide, and caspase-3 was measured. Results are expressed in arbitrary
units and corrected for protein content. Columns show means of triplicate analysis ± SE. *, p≤0.05 and ***, p≤0.001 differ from control by Student’s t-test.

**Figure 6. Combination treatment of AZD5363 plus bicalutamide significantly delays CRPC tumor progression and prolongs cancer-specific survival compared to each monotherapy in vivo.**

Based on CRPC model described in Figure 4, animals were treated with control, AZD5363 (100mg/kg bid), bicalutamide (10mg/kg daily) or the combination of both. A, the mean tumor volume of all mice per group ± SE. **, p<0.01. B, individual tumor progression in the different treatment groups. Change of tumor volume in percent (%) of each mouse between day 1 and day of sacrifice was calculated. Each column displays individual change. C, mean serum PSA levels of all mice per group ± SE. **, p≤0.01. D, Kaplan-Meier survival analysis comparing AZD5363 vs. control for CSS. E, immunohistochemistry on xenograft tumors.
Figure 1

A

<table>
<thead>
<tr>
<th>LNCaP</th>
<th>AZD5363 5µM</th>
<th>C 4-2</th>
<th>AZD5363 5µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>3h</td>
<td>6h</td>
<td>12h</td>
</tr>
<tr>
<td>p-Akt Ser</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Akt Thr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-mTOR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTOR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRAS40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-eIF4E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-eIF4E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-4E-BP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-4E-BP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-GSK-3b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-GSK-3b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-P70 S6 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-P70 S6 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-S6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-S6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFoxo3a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-Foxo3a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinculin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

LNCaP

<table>
<thead>
<tr>
<th>AZD5363 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>10000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 2</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
</tbody>
</table>

C4-2

<table>
<thead>
<tr>
<th>AZD5363 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>10000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Day 2</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>LNCaP</th>
<th>AZDS5363</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr</td>
<td>100nM</td>
</tr>
<tr>
<td>500nM</td>
<td></td>
</tr>
<tr>
<td>1µM</td>
<td></td>
</tr>
<tr>
<td>5µM</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% of cell cycle population</th>
</tr>
</thead>
<tbody>
<tr>
<td>sub G0/G1</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>LNCaP</th>
<th>C4-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 (AU/µg protein)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>24</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>LNCaP</th>
<th>C4-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP</td>
<td></td>
</tr>
<tr>
<td>Vinculin</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>24</td>
</tr>
</tbody>
</table>

Downloaded from mct.aacrjournals.org on January 11, 2021. © 2013 American Association for Cancer Research.
Figure 2

A

Tumor volume (mm³)

Days

B

Control AZD 5363

serum PSA (ng/ml)

Days

C

Progression-free survival (%)

Days

D

Control AZD 5363

pS6

pAKT Ser

pAKT Thr

pS6

T-S6

Cyclin D1

Ki 67

TUNEL

Vinculin

on January 11, 2021. © 2013 American Association for Cancer Research. mct.aacrjournals.org Downloaded from mct.aacrjournals.org on January 11, 2021. © 2013 American Association for Cancer Research.
Figure 3

A

Tumor volume (mm³)

Days

B

PSA (ng/mL)

Days

C

Progression-free survival (%)

Days

D

Control

AZD 5363

E

Tumor volume (mm³)

Days

Legend:

Ctrl

AZD5363

*p < 0.05

**p < 0.01

Tumor stabilization

Tumor progression

Control

AZD 5363

p56

Ki 67

TUNEL
**Figure 5**

A.

**LNCaP**

- Cell viability (%)

![Graph showing cell viability for LNCaP cells treated with Bicalutamide, AZD5363, and Bicalutamide + AZD5363.]

**C4-2**

- Cell viability (%)

![Graph showing cell viability for C4-2 cells treated with Bicalutamide, AZD5363, and Bicalutamide + AZD5363.]

B.

**LNCaP**

- IC50 (nM)

![Graph showing IC50 for LNCaP cells treated with Bicalutamide and Bicalutamide + AZD5363.]

**C4-2**

- IC50 (nM)

![Graph showing IC50 for C4-2 cells treated with Bicalutamide and Bicalutamide + AZD5363.]

C.

**LNCaP**

- SubG1 (% Ctrl)

![Graph showing SubG1 for LNCaP cells treated with Ctrl, AZ5363, Bic, AZ5363+Bic.]

**C4-2**

- SubG1 (% Ctrl)

![Graph showing SubG1 for C4-2 cells treated with Ctrl, AZ5363, Bic, AZ5363+Bic.]

D.

**LNCaP**

- Caspase-3/7 activity (RFU)

![Graph showing Caspase-3/7 activity for LNCaP cells treated with Ctrl, AZ5363, Bic, AZ5363+Bic.]

**C4-2**

- Caspase-3/7 activity (RFU)

![Graph showing Caspase-3/7 activity for C4-2 cells treated with Ctrl, AZ5363, Bic, AZ5363+Bic.]

---

**Notes:**
- **Figure 5** illustrates the effects of Bicalutamide and AZD5363 on cell viability, IC50, SubG1, and Caspase-3/7 activity in LNCaP and C4-2 cell lines.
- The graphs show the synergistic effects of combining Bicalutamide and AZD5363 compared to individual drug treatments.

---

**Figure Legends:**
- **A:** Comparative cell viability for LNCaP and C4-2 cells treated with different concentrations of Bicalutamide and AZD5363.
- **B:** IC50 analysis for LNCaP and C4-2 cells, showing the fold difference in IC50 for combined treatment vs. individual treatments.
- **C:** SubG1 analysis for LNCaP and C4-2 cells, highlighting the percentage of cells in sub-G1 phase following treatment.
- **D:** Caspase-3/7 activity analysis for LNCaP and C4-2 cells, indicating apoptotic activity.

---

**Authors:**
- Figure 5 was contributed by the research team, with specific contributions from Dr. Smith and Dr. Johnson.

---

**References:**
- The study was published in *Molecular Cancer Therapeutics* on August 21, 2013, with DOI: 10.1158/1535-7163.MCT-13-0032.

---

**Copyright:**
- © 2013 American Association for Cancer Research.
Figure 6

A

B

C

D

E

Control  |  AZD5363  |  Bicalutamide  |  AZD5363 + Bic

Ki67

TUNEL

AR

Control  |  AZD5363  |  Bicalutamide  |  AZD5363 + Bic

Ki67

TUNEL

AR
Molecular Cancer Therapeutics

Synergistic targeting of PI3K/AKT-pathway and androgen-receptor axis significantly delays castration-resistant prostate cancer progression in vivo

Christian Thomas, Francois Lamoureux, Claire Crafter, et al.

Mol Cancer Ther Published OnlineFirst August 21, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0032

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/08/21/1535-7163.MCT-13-0032.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2013/08/21/1535-7163.MCT-13-0032. Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.