AZD3514: A Small Molecule That Modulates Androgen Receptor Signaling and Function In Vitro and In Vivo

Sarah A. Loddi1, Sarah J. Ross1, Andrew G. Thomason1, David M. Robinson1, Graeme E. Walker1, Tom P.J. Dunkley2, Sandra R. Brave1, Nicola Broadbent1, Natalie C. Stratton1, Dawn Trueman1, Elizabeth Mouchet1, Fadhel S. Shaheen2, Vivien N. Jacobs1, Marie Cumberbatch1, Joanne Wilson1, Tom P.J. Dunkley1, Sandra R. Brave1, Nicola Broadbent1, Natalie C. Stratton1, Dawn Trueman1, Elizabeth Mouchet1, Fadhel S. Shaheen2, Vivien N. Jacobs1, Marie Cumberbatch1, Joanne Wilson1, Rhys D.O. Jones1, Robert H. Bradbury1, Alfred Rabow1, Luke Gaughan2, Chris Womack1, Simon T. Barry1, Craig N. Robson2, Susan E. Critchlow1, Stephen R. Wedge1, and A. Nigel Brooks1

Abstract

Continued androgen receptor (AR) expression and signaling is a key driver in castration-resistant prostate cancer (CRPC) after classical androgen ablation therapies have failed, and therefore remains a target for the treatment of progressive disease. Here, we describe the biological characterization of AZD3514, an orally bioavailable drug that inhibits androgen-dependent and -independent AR signaling. AZD3514 modulates AR signaling through two distinct mechanisms, an inhibition of ligand-driven nuclear translocation of AR and a downregulation of receptor levels, both of which were observed in vitro and in vivo. AZD3514 inhibited testosterone-driven seminal vesicle development in juvenile male rats and the growth of androgen-dependent Dunning R3327H prostate tumors in adult rats. Furthermore, this class of compound showed antitumor activity in the HID28 mouse model of CRPC in vivo. AZD3514 is currently in phase I clinical evaluation.

Mol Cancer Ther; 12(9); 1715–27. ©2013 AACR.

Introduction

Prostate cancer is the most common form of malignancy in men, and remains the second leading cause of male cancer-related death (1). This disease is dependent upon the hormone testosterone (2), which activates androgen receptor (AR) signaling, with surgical (orchiectomy) and chemical antiandrogen approaches being established as first-line therapy. Although treatments are initially highly effective, resistance ultimately develops in the majority of patients. Despite castrate levels of androgen, in many cases, this resistance is still dependent upon AR signaling (3, 4).

The AR is a member of the steroid hormone receptor family and functions as a ligand-dependent transcription factor. In the absence of androgens, AR is predominantly inactive and present in the cytoplasm bound to HSPs. Binding of androgens to AR induces conformational changes, dimerization, and translocation into the nucleus. In the nucleus, AR binds to androgen response elements (ARE) within regulatory elements of target genes [such as prostate-specific antigen (PSA) and TMPRSS2] and regulates gene expression through the recruitment of cofactors (5–7).

There are a number of potential mechanisms by which AR signaling can occur in the presence of antiandrogen therapies. These include: an increase in the expression of AR protein which can sensitize cells to low levels of androgen (8–11), AR mutations that can alter transactivation or sensitize AR to alternative ligands (3, 5), and those that cause classical AR antagonists such as flutamide and bicalutamide to behave as agonists (12, 13). In addition, prostate tumors may also synthesize their own androgens, thereby increasing the local intratumoral testosterone levels available to activate the AR (4, 5, 14–16). Prostate cancer acquiring resistance to conventional treatment was initially termed androgen-independent or hormone-refractory, but is now widely described as castration-resistant prostate cancer (CRPC). The change in terminology reflects emerging data that show prostate cancer in this resistant state to have a continued dependency upon AR signaling. This is exemplified by the recent overall survival benefit shown in patients with CRPC following treatment with either abiraterone acetate or enzalutamide (17–22). Abiraterone acetate inhibits 17-α-hydroxylase/17,20-lyase (CYP17) activity resulting in a reduction in residual androgens synthesized by the...
Enzalutamide inhibits the key events that must occur to enable AR signaling such that it prevents testosterone from binding to AR, translocating to the nucleus, and binding to DNA (24). However, although the activity of these agents in CRPC is very encouraging, neither works in all patients and both are associated with the development of additional resistance. Although the mechanisms by which tumors become refractory to abiraterone acetate and enzalutamide treatment have not yet been clearly elucidated, current clinical (4, 25) and preclinical (26–28) data suggest that re-activation of the AR will remain a primary mechanism. Thus, there is a continued need to identify alternative therapies for the treatment of CRPC, and in particular those that have a mechanistically distinct inhibitory effect on AR signaling.

Here, we describe the characterization of AZD3514, an orally bioavailable drug that binds to AR, inhibits its nuclear translocation, and ligand-dependent and -independent transcriptional activity. In addition, AZD3514 is differentiated from existing pharmacologic approaches in that it is also able to induce AR downregulation in vitro and in vivo.

Materials and Methods

**AZD3514**

6-(4-{4-[2-(4-Acetylpiperazin-1-yl)ethoxy]phenyl)piperidin-1-yl)-3-(trifluoromethyl)-7,8-dihydro[1,2,4]triazolo[4,3-b]pyridazine (AZD3514; AstraZeneca; Fig. 1) was synthesized according to the published procedures (29). For in vitro studies, AZD3514 was formulated in a solution of 20% Captisol (β-cyclodextrin sulfobutyl ethers sodium salts) at pH 4.

**ARD1**

6-(4-{4-Cyanobenzyl)piperazin-1-yl]-3-(trifluoromethyl) [1,2,4]triazolo[4,3-b]pyridazine (ARD1; AstraZeneca; Fig. 6C) was obtained by the published procedure (30). For in vitro studies, ARD1 was prepared in a suspension of 1% polysorbate 80.

**Abiraterone acetate**

Abiraterone acetate was obtained according to the published procedures (31) and was prepared in a suspension of 1% polysorbate 80. Animals were given between 4 and 40 mg/kg, administered once daily by oral gavage.

**Enzalutamide**

Enzalutamide was obtained according to the published procedures (32).

**Testosterone propionate**

Testosterone propionate (TP) was prepared in stripped corn oil at 0.2 mg/ml and administered subcutaneously at 0.4 mg/kg once a day.

**Cell culture**

LNCaP, DU145, and HCT116 cells were purchased from American Type Culture Collection, LAPC4 were obtained from Dr. Beth Pflug (Indiana University School of Medicine, Indianapolis, IN), and AR U2OS were purchased from Thermo Fisher Scientific. All cell lines were maintained under standard cell culture conditions and were tested and authenticated at AstraZeneca cell banking using DNA fingerprinting short-tandem repeat (STR) assays. All revived cells were used within 15 passages, and less than 6 months.

**Assessment of protein and mRNA levels**

Cell lysates were prepared with Lysate Buffer (25 mmol/L Tris pH 6.8, 3 mmol/L EDTA, 3 mmol/L EGTA, 50 mmol/L NaF, 2 mmol/L Na3VO4, 0.27 mol/L sucrose, 10 mmol/L β-glycerolphosphate, 5 mmol/L pyrophosphate, and 0.5% Triton X-100) containing EDTA-free protease inhibitor cocktail (Cat# 11873580001; Roche) for assessment of total protein by Western blotting following 24-hour treatment of appropriate test compound. Western blotting was conducted using standard SDS-PAGE procedures with antibodies to AR (AR441; cat#M3562; Dako), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clone6C5; BioScientific), PSA (Cat#AF1344; R&D Systems), or PARP (cat#51-6639GR; BD Pharmingen). Protein levels were determined using the ChemiGenius imaging system and GeneTools analysis software (Syngene). For mRNA analysis, cell lysates were prepared using FastLane Cell RT-PCR kit (Qiagen) and TaqMan real-time PCR (RT-PCR) conducted to measure mRNA levels of PSA and TMPRSS2. Expression was normalized to 18s ribosomal protein and quantified using the 2−ΔΔCT method. Data were analyzed relative to a dimethyl sulfoxide (DMSO)-treated control in the absence of dihydrotestosterone (DHT). Primer probe assays used were: 18S Hs00455840_s1, PSA Hs00237175_s1, and TMPRSS2 Hs00237175_s1 from Applied Biosystems (Life Technologies).

**Western translocation assays**

LNCaP cells were plated in media containing steroid-free serum and treated with AZD3514, enzalutamide, or DMSO for 2 hours before treating with 1 nmol/L of DHT or DMSO for 30 minutes. Nuclear and cytoplasmic cell lysates were prepared using the NE-PER Extraction Kit (Pierce; Thermo Fisher Scientific) according to the
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Relative peptide quantification by SRM

Relative quantification of two AR peptides was conducted by SRM using an Ultimate 3000 nano liquid chromatography (LC) system ( Dionex) coupled to either a TSQ Vantage (Thermo Fisher Scientific; experiment 3) or a 4000 QTRAP (Applied Biosystems; experiments 1 and 2) triple quadrupole mass spectrometer. Further details of instrument set up are included in Supplementary Methods for reference. Both the TSQ Vantage and 4000 QTRAP were operated in SRM mode to selectively measure the following AR peptides: GAFQNLFQSVR (21–31) and LLDSVQPIAR (862–871). The unlabeled, “heavy” labeled ([13C6, 15N4] R, +10 Da), and “medium” labeled ([13C6, +6 Da) forms of both peptides were measured in parallel. Instrument settings are shown in Supplementary Tables S1 and S2.

AR degradation and synthesis by radiolabeled pulse chase

LNCaPs were seeded in RPMI containing 5% dialyzed charcoal-stripped FBS. Radiolabeling of AR protein used Labeling Media [RPMI without amino acids methionine and cysteine supplemented with 100 μCi [35S] methionine/cysteine labeling mix (PerkinElmer)] with cells incubated for 1 hour. Lysates were prepared from the cells by the addition of Lysate Buffer supplemented with 0.1% SDS and AR protein immunoprecipitated by incubating 500 μg protein with 50 μL of Protein G–coated paramagnetic beads (Invitrogen) bound with 4 μg PG21 anti-AR antibody. The beads were washed three times in Lysate Buffer without SDS supplemented with 500 mmol/L NaCl, and AR eluted in LDS Loading Buffer (Invitrogen) at 95°C, 5 minutes. Samples were run on polyacrylamide gels, transferred to nitrocellulose, and levels of radiolabeled AR present determined by autoradiography.

In vivo physiologic studies

Male 33-day-old Hans Wistar/RCC Hans Wistar rats were obtained from Harlan and randomized into cages of five at AstraZeneca. All experiments were carried out in full accordance with the UK Home Office Animal (Scientific Procedures) Act 1986.

For intact studies, 49-day-old rats (Hans Wistar) were assigned to vehicle or treatment groups and dosed for 6 days, animal body weight and condition were recorded daily. On day 7, 24 hours after the last dose, animals were terminated and seminal vesicle weight recorded. For castrated rat studies, 42-day-old rats (RCC Hans Wistar) were surgically castrated under isoflurane anesthesia and left for a period of 7 days. Animals were assigned to vehicle or treatment groups and dosed for 6 days, animal body weight and condition were recorded daily. On day 7, 24 hours after the last dose, animals were terminated and seminal vesicle weight recorded.

In vivo tumor studies

All animals were housed in specific pathogen-free facilities and all experiments were carried out in accordance
with approved ethical standards appropriate to the establishment. R3327H tumor studies were carried out by Oncodesign. R3327H tumors were established by implanting male Copenhagen rats (8- to 12-week-old; obtained from Harlan) with fresh tumor fragments obtained from donor rats. When tumors reached the determined size, animals were randomized into control and treated groups. Tumor volume (measured by caliper), animal body weight, and tumor condition were monitored twice weekly for the duration of the study.

HID28 tumor studies were carried out by Xentech. HID28 tumors were established by implanting male athymic nude mice (Hsd:Athymic Nude-Fox1nu; Harlan; 6- to 9-week-old) with HID28 tumor fragments established previously in either intact (for intact study) or castrated (for castrated study) mice. When tumors reached the determined size, animals were randomized into control and treated groups.

Tumor volume (measured by caliper), animal body weight, and tumor condition were monitored twice weekly. Tumor volumes were assessed by bilateral Vernier caliper measurement at least twice weekly and calculated using the formula $(length \times width)^{2} \times \frac{\sqrt{3}}{4}$, where length was taken to be the longest diameter across the tumor and width the corresponding perpendicular.

**Immunohistochemical methods**


AR protein was observed within the first 6 hours (Fig. 3C). However, downregulation was observed either with or without DHT at 24 hours. In contrast, enzalutamide had minimal effects on the growth of DU145 (Supplementary Fig. S2A), an AR-negative prostate cancer cell line, and in HCT116 (Supplementary Fig. S2B), a colon cell line negative for AR and other nuclear hormone receptors, estrogen receptor (ER), progesterone receptor (PR), and glucocorticoid receptor (GR).

We explored the ability of AZD3514 to inhibit AR functional activity in vitro using physiologic assays that measure the activity against endogenous (intact rat) or exogenous (castrated rat) testosterone-induced growth of seminal vesicles (35). In the intact rat assay, AZD3514 reduced seminal vesicle weight, similar to the CYP17 inhibitor of testosterone production, abiraterone acetate (Fig. 2H). In the castrated rat assay, AZD3514 inhibited the ability of exogenous testosterone propionate to cause an increase in seminal vesicle weight, consistent with a direct inhibition of AR signaling. Abiraterone acetate was inactive in this assay consistent with its mechanism of action (Fig. 2I).

**Mechanism of action of AZD3514**

The mechanism by which AZD3514 inhibits AR function was explored in vitro. With 24-hour treatment, AZD3514 reduced AR protein expression in LNCaP cells in a dose-dependent manner (Fig. 3A and B) although complete reduction of AR protein was not observed at any dose. A time-course of treatment with 10 μmol/L of AZD3514 in LNCaP cells showed that no downregulation of AR protein was observed within the first 6 hours (Fig. 3C). However, downregulation was observed either with or without DHT at 24 hours. In contrast, enzalutamide had no significant effect on AR expression at any time point.
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Figure 2. A to C, LNCaP and LAPC4 growth in steroid-free media supplemented with 1 nmol/L of DHT and AZD3514 or enzalutamide. Cell number determined after 7 days. Data shown are growth relative to vehicle (Veh) control and are the mean of three (AZD3514) or two (enzalutamide) independent experiments. Error bars, SEM. D to G, PSA and TMPRSS2 gene expression normalized to levels of 18s after AZD3514 treatment of cells ± DHT. Data shown are gene expression relative to the vehicle control in the absence of ligand and are a mean of two independent experiments. Error bars, SEM. H, AZD3514 or abiraterone acetate was administered by oral gavage once daily for 6 days to intact 42-day-old male rats at the doses indicated. On day 7, animals were terminated and seminal vesicles weighed. Data shown are the mean values of 5 animals. Error bars, SEM. I, testosterone propionate (TP; 0.4 mg/kg) was administered by subcutaneous injection along with either vehicle; AZD3514 or abiraterone acetate were administered by oral gavage once daily for 6 days to castrated 42-day-old male rats at the doses indicated. On day 7, animals were terminated and seminal vesicles weighed. Data shown are the mean values of 5 animals. Error bars, SEM.
Downregulation of AR protein was also observed in LAPC4 cells treated with 10 \( \mu \text{mol/L} \) of AZD3514 for 24 hours (Supplementary Fig. S3).

Because the effects of AZD3514 on AR protein expression did not seem to fully account for its ability to inhibit AR signaling, we explored whether AZD3514 impacted on AR nuclear translocation, a key process in AR transactivation in response to androgens. By Western blot analysis in the absence of DHT, AR is predominantly cytoplasmic in LNCaPs, with a small fraction detected in the nucleus (Fig 3D). In response to 30-minute treatment with 1 nmol/L of DHT, an increase in the levels of nuclear AR was detected (Fig. 3D). AZD3514 caused a dose-dependent reduction in this ligand-driven AR nuclear localization, with 10 \( \mu \text{mol/L} \) of AZD3514 almost completely inhibiting the increase in nuclear AR (Fig. 3D). As has been reported previously, enzalutamide was also able to inhibit ligand-driven translocation of nuclear AR in this assay (Fig 3D; ref. 24). No effects of AZD3514 on total levels of AR were observed following 2 hours of treatment (Fig. 3C). AZD3514 also inhibited ligand-induced AR nuclear translocation in engineered U2OS cells expressing GFP-tagged AR (Fig 3E and F and Supplementary Fig. S7).

**Mechanism of AR downregulation by AZD3514**

We next investigated whether the mechanism of AR downregulation by AZD3514 was via an increase in the rate of AR degradation or a decrease in AR synthesis. A mass spectrometry approach was used which measured AR degradation by tracking the decrease of two \(^{13}\text{C}_6^{15}\text{N}_4\) arginine-labeled (heavy) AR peptides. A composite measure of AR degradation and synthesis was obtained by tracking the increase of the unlabeled (light) AR peptides. Treatment of LNCaPs with 10 \( \mu \text{mol/L} \) of AZD3514 was unable to increase the rate of AR degradation as compared with vehicle control (Fig. 4A and Supplementary Fig. S4A). However, geldanamycin, an Hsp90 inhibitor, was able to increase the rate of AR degradation as previously reported.
reported (Fig. 4A; Supplementary Fig. S4A; ref. 39). The half-life of AR in LNCaPs quantified using this approach for both vehicle- and geldanamycin-treated cells is consistent with that previously reported (Supplementary Table S3; refs. 11, 39). Levels of light AR peptide were significantly reduced following AZD3514 treatment (Fig. 4B and Supplementary Fig. S4B). No increase in the rate of AR degradation was observed (Fig. 4A and Supplementary Fig. S4A), therefore these data suggest that AZD3514 is reducing the rate of AR synthesis.

These observations were confirmed measuring AR turnover by pulse chase labeling of LNCaPs with 35S-methionine and cysteine. AZD3514 had no effect on AR degradation rate although DHT stabilized AR as expected (Fig 4C and D; refs. 40, 41). In contrast, a 24-hour treatment with 10 μmol/L of AZD3514 was shown to decrease AR synthesis in the absence or presence of ligand (Fig. 4E and F).

The effects of AZD3514 on AR mRNA levels were variable between experiments with 0% to 50% reduction in steady state AR mRNA observed as measured by RT-PCR (data not shown), suggesting that an effect on AR transcription is unlikely to be the only mechanism by which AZD3514 regulates AR protein levels.

**AZD3514 modulates nuclear AR protein and inhibits growth of the androgen-dependent Dunning R3327H rat prostatic adenocarcinoma model *in vivo***

To assess AZD3514 antitumor activity *in vivo* against an androgen-dependent model, male Copenhagen rats bearing Dunning R3327H prostate tumors (42) were randomized and treated with AZD3514. Once daily oral administration of AZD3514 at 50 mg/kg caused a statistically significant inhibition of tumor growth (64%; \( P < 0.001 \)) compared with vehicle-treated controls (Fig. 5A).
Figure 4. A and B, LNCaP cells grown in steroid-free conditions in SILAC media containing 5% dialyzed fetal calf serum, $^{13}$C$_6$$^{15}$N$_4$ arginine, and $^{13}$C$_6$ lysine (to label proteins as “heavy”) were treated with 10 μmol/L of AZD3514 or vehicle for 24 hours, then switched to grow in SILAC media containing unlabeled arginine (to label newly synthesized protein as “light”). Cells previously dosed with AZD3514 were treated for a further 24 hours with 10 μmol/L of AZD3514. Cells previously dosed with vehicle were treated for a further 24 hours with either 3 μmol/L of geldanamycin or vehicle. Samples were collected at indicated time points following the media switch and protein lysates prepared. AR protein levels were measured in the samples by immunoprecipitation with an AR-specific antibody followed by LC–mass spectrometry quantifying levels of N-terminal peptide of AR. A, AR shown is percentage of heavy-labeled AR peptide at each time point relative to the level of AR present at the time of the media switch. B, AR shown is the increase in light-labeled AR peptide at each time point following the media switch. Both graphs are representative data of three separate experiments ±SD. Half-life values obtained in three independent experiments are shown in Supplementary Table S3. C and D, radiolabeled pulse chase analysis of AR degradation in LNCaP cells. Cells were incubated with radiolabel, washed, and label-free media added containing 10 μmol/L of AZD3514 or 1 nmol/L of DHT. Lysates were prepared, levels of radiolabeled AR measured and expressed relative to AR levels at time 0. Data are represented as mean levels from two independent experiments. Error bars, SD. E and F, radiolabeled pulse chase analysis of AR synthesis in LNCaP cells. Cells were treated with 10 μmol/L of AZD3514 or vehicle control for 24 hours. Labeling Media containing treatments was added, and the cells were incubated for 1 hour. Lysates were prepared and levels of radiolabeled AR measured and expressed relative to the DMSO control -DHT. The graph shows the mean AR levels from two independent experiments. Error bars, SD.
AZD3514 also reduced nuclear AR protein expression in these tumors (data not shown). However, as tumor size and morphology was modulated by chronic treatment, we investigated the impact of AZD3514 on AR protein expression in R3327H tumors in vivo following acute dosing. Rats were treated with 50 or 100 mg/kg AZD3514 for 3 days and tumors were harvested 24 hours after the last dose for assessment of AR protein expression by immunohistochemistry (IHC; Fig. 5B). No conclusive results were obtained from the analysis of cytoplasmic AR

Figure 5. A to D, AZD3514 was administered by oral gavage once daily to Copenhagen rats bearing established R3327H tumors at the doses indicated. A, tumor volumes are plotted against time. B, i, representative images showing AR IHC from animals administered with vehicle or AZD3514 at 50 or 100 mg/kg for 3 days; ii, the associated Genie annotation grading intensity of nuclear AR. Graphical representation of the percentage of tumor nuclei with 0+, 1+, 2+, or 3+ intensity of AR protein as quantified by Genie (C) and scored by a pathologist (D). qd, every day.
expression, which was highly variable and challenging to assess (data not shown). However, 3-day dosing of AZD3514 caused a significant reduction in nuclear AR protein at both the 50 and 100 mg/kg dose, with 100 mg/kg having the greatest effect (Fig. 5B). Nuclear AR expression was quantified by both conventional pathology scoring and the use of Aperio Genie software to determine the proportion of tumor cells expressing different intensities of AR, and both analyses confirmed a reduction in nuclear AR by AZD3514 (Fig. 5C and D).

ARD1, a compound with similar biologic properties to AZD3514 inhibits androgen-independent HID28 prostate tumor growth in vivo

We subsequently wanted to assess whether the AZD3514 mechanism of action could deliver in vivo efficacy in a castrate-resistant setting using the murine HID28 model, an androgen-independent variant of PAC120 prostate tumors (43). Oral dosing with AZD3514 at 100 mg/kg in the mouse had poor pharmacokinetic (PK) profile (Supplementary Fig. S5C and Supplementary Table S4). This was such that we would not have been...
able to increase the dose sufficiently to a dose that would have been required for adequate exposure. Thus, the hypothesis was tested with ARD1, a compound from the same chemical series (Fig. 6 D-F) which had improved mouse PK versus AZD3514 (Supplementary Fig. S5C).

Intact male nude mice bearing the HID28 tumors were randomized and treated with ARD1 or vehicle. Twice daily oral administration of ARD1 at 100 mg/kg caused a statistically significant inhibition of tumor growth (54%; \( P < 0.01 \)) compared with vehicle-treated controls (Fig. 6A). AR expression in these tumors detected by IHC was highly variable and not quantified, however, some modulation of nuclear AR levels was observed with ARD1 (Supplementary Fig. S6A). In this HID28 study, the AR antagonist bicalutamide was inactive, however, the gonadotropin-releasing hormone antagonist degarelix (44) also caused a significant inhibition of tumor growth (40%; \( P < 0.05 \); Supplementary Fig. S6B), suggesting that androgens were contributing to HID28 tumor growth in intact animals. However, ARD1 was also able to inhibit HID28 growth (95%; \( P < 0.05 \)) in castrated mice (Fig. 6B), suggesting that ARD1 is able to inhibit tumor growth under castrate-resistant levels of androgen.

Discussion

A body of data now indicate that AR continues to play a key role in driving progression of CRPC after classical antiandrogen therapies have failed. Multiple mechanisms have been implicated in the re-activation of AR signaling within castrate levels of androgen, including overexpression, mutation, or alternative splicing of the receptor, or the intratumoral synthesis of testosterone. Given the diversity of available mechanisms, re-activated AR signaling may also have the potential to mediate intrinsic and acquired resistance to alternative inhibitors of AR signaling such as those described recently to have activity in CRPC (19, 20). Thus, a strategy involving the removal of AR protein should have use within different stages of current prostate cancer treatment practice, such an approach having the ability to inhibit both androgen-dependent and -independent AR signaling. To obtain such an inhibitor, we established a cellular assay that was designed to identify compounds that modulated the level of AR in the nucleus of LNCaP prostate cancer cells in androgen-free conditions (30). This work yielded AZD3514, a small-molecule inhibitor of AR signaling that is also able to downregulate AR.

Consistent with inhibitors of AR, AZD3514 was shown to inhibit ligand-driven proliferation and expression of AR-dependent genes (PSA and TMPRSS2) in LNCaP and LAPC-4s. Although the potency of AZD3514 was influenced by the concentration of DHT, consistent with an antagonist mechanism of action, inhibition of AR signaling was still observed at the highest level of DHT tested (10 nmol/L). AZD3514 was also able to inhibit the basal expression of PSA and TMPRSS2 in LNCaPs consistent with a mode of action that is dependent upon AR but independent of androgens. In vivo AZD3514 treatment inhibited the growth of Dunning R3327H tumors, an androgen-dependent prostate cancer model. The compound also inhibited the increase in seminal vesicle weight in juvenile male rats that was driven by either physiologic testosterone, or exogenous testosterone in castrated rats. This is in contrast to the androgen synthesis inhibitor abiraterone acetate, which was active against physiologic testosterone only and is consistent with AZD3514 having a direct effect on the AR.

A number of approaches were used to gain insight into the mechanisms through which AZD3514 drives modulation of AR function. AZD3514 inhibited ligand-driven translocation of AR to the nucleus in LNCaPs and in engineered U2OS cells expressing AR-GFP. This occurred within 2 hours of AZD3514 treatment and is consistent with the early effects observed on AR-driven gene expression. The mechanism by which AR nuclear translocation is inhibited has not been explored fully, but is also reported to occur following treatment with the AR antagonist enzalutamide (24). Ligand displacement assays using either rat AR ligand-binding domain or full-length AR derived from LNCaP lysates has shown that AZD3514 binds to AR (data not shown). In addition, as AZD3514 also competes with DHT-induced responses, these acute effects are anticipated to be a consequence of a direct interaction with AR. Longer term treatment with AZD3514 in vitro (24 hours in LNCaPs) caused a reduction in total AR protein levels in both the absence and presence of androgen, providing an additional mechanism by which AZD3514 could reduce AR-signaling independent of androgens. These effects on AR were also evident in vivo. In the Dunning R3327H tumor model, AR staining in the nucleus of tumor cells was significantly reduced following 3 days of AZD3514 treatment.

AR downregulation could be achieved by enhancing the rate of AR degradation and/or reducing the rate of synthesis. Although the effect of AZD3514 in the cellular assay was attenuated by proteasome inhibition (data not shown), no effects on the rate of AR degradation were observed in our assays. Indeed our data suggest that AZD3514 treatment reduces the rate of AR synthesis, which is perhaps consistent with the time frame that the AR downregulation is observed. These observations were made using two approaches, a mass spectrometry pulse chase method and a radiolabeled pulse chase method, and consistent results were obtained. Importantly, in these studies the AR half-life measured, and the impact of DHT and geldanamycin on AR stability was consistent with published data. Whether the reduced rate of protein synthesis by AZD3514 is due to inhibition of mRNA transcription or translation is as yet unclear and inconsistent effects on steady state levels of AR mRNA were observed. microRNAs have been suggested to regulate AR levels in prostate cancer cells (45) and EBP1 has been reported to inhibit the translation of AR mRNA (46, 47). These and other potential mechanisms cannot be ruled out and will be the focus of future investigation.
Using ARD1, which is structurally related to AZD3514 and is similarly able to inhibit AR nuclear translocation and reduce AR levels, we were able to examine the effectiveness of this class of compound in the HID28 tumor model; an androgen-independent variant of the PAC120 patient-derived prostate tumor model grown in nude mice (40). ARD1 was active in this model when grown in both intact and castrated mice, suggesting that this class of compound is active under low/castrate levels of androgen. Although the circulating testosterone levels in rat are not affected by the administration of pharmacologically active doses of ARD1 (data not shown), we cannot conclusively exclude the possibility of a direct effect on androgen synthesis in the tumor. In addition, although AR protein expression is variable in the HID28 tumors, we were able to detect a reduction in nuclear AR protein following ARD1 treatment, consistent with a direct modulation of AR signaling. This effect on AR protein, independent of effects on androgen synthesis differentiates the mechanism of AZD3514 from other known agents that target AR signaling such as abiraterone.

Collectively, these preclinical data indicate that AZD3514 has a novel mechanism and is able to inhibit AR signaling in androgen-dependent and -independent conditions. The compound prevents nuclear translocation of AR and inhibits AR synthesis, with sustained exposure leading to a detectable downregulation of the receptor. Because intrinsic or acquired resistance to current antiandrogen therapies is frequently mediated by AR, an inhibitor that also reduces AR levels may conceivably be beneficial in circumventing, or helping to delay the emergence of resistance. Such an approach may deliver benefit in CRPC either alone or in combination with other therapies.

Disclosure of Potential Conflicts of Interest
S.R. Wedge holds stock in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.A. Loddick, A.G. Thomason, G.E. Walker, S.R. Brave, N. Broadbent, N.C. Stratton, D. Trueman
Study supervision: S.A. Loddick, G.E. Walker, L. Gaughan, C.N. Robson, A.N. Brooks

Grant Support
This study was supported by MRC Industrial Collaboration Award, G2800889/1 awarded to C.N. Robson, L. Gaughan, S.A. Loddick, and N.A. Brooks.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 18, 2012; revised May 28, 2013; accepted June 19, 2013; published OnlineFirst July 16, 2013.

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