ANTI-CANCER ACTIVITY OF A NOVEL SELECTIVE CYP17A1 INHIBITOR IN PRE-CLINICAL MODELS OF CASTRATE RESISTANT PROSTATE CANCER

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Running Title: Pre-clinical evaluation of VT-464 in prostate cancer models

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Abstract

VT-464 is a novel, non-steroidal, small molecule CYP17A1 inhibitor with 17,20-lyase selectivity. This study evaluates the anti-cancer activity of VT-464 compared to abiraterone (ABI) in castrate resistant prostate cancer cell lines and xenograft models that are ENZ-responsive (C4-2) or ENZ-resistant (MR49C, MR49F). In vitro, androgen receptor (AR) transactivation was assessed by probasin luciferase reporter, while AR and AR-regulated genes and steroidogenic pathway enzymes were assessed by western blot and/or qRT-PCR. The MR49F xenograft model was used to compare effects of oral VT-464 treatment to vehicle and abiraterone acetate (AA). Steroid concentrations were measured using LC-MS chromatography. VT-464 demonstrated a greater decrease in AR transactivation compared to ABI in C4-2 and both ENZ-resistant cell lines. At the gene and protein level, VT-464 suppressed the AR axis to a greater extent compared to ABI. Gene transcripts StAR, CYP17A1, HSD17B3 and SRD5A1 increased following treatment with ABI and to a greater extent with VT-464. In vivo, intratumoral androgen levels were significantly lower after VT-464 or AA treatment compared to vehicle, with the greatest decrease seen with VT-464. Similarly, tumor growth inhibition and PSA decrease trends were greater with VT-464 than with AA. Finally, an AR-antagonist effect of VT-464 independent of CYP17A1 inhibition was observed using luciferase reporter assays and a direct interaction was confirmed using an AR ligand binding domain bio-layer interferometry. These pre-clinical results suggest greater suppression of the AR axis with VT-464 than ABI that is likely due to both superior selective suppression of androgen synthesis and AR antagonism.
Introduction

Recent clinical trials have clearly established that castrate resistant prostate cancer (CRPC) continues to remain sensitive to agents that inhibit the AR pathway (1, 2). Targeting androgen biosynthesis in CRPC dates back to the use of ketoconazole almost 25 years ago(3). More recently, abiraterone acetate (AA) in combination with prednisone has demonstrated improved overall survival (OS) in both pre- and post-chemotherapy patients with CRPC in phase III trials (2, 4). AA specifically and irreversibly inhibits both CYP17 hydroxylase and lyase (5). Inhibition of 17α-hydroxylase by AA prevents the synthesis of glucocorticoids and produces side-effects due to mineralocorticoid excess, which are partially suppressed by co-administration of the cortisol replacement, prednisone(6). The development of selective CYP17A1 lyase inhibitors has potential to obviate the need for concomitant prednisone administration in patients which is associated with its own toxicities.

CYP17A1 is the rate-limiting enzyme in the biosynthesis of androgens. CYP17A1 is a dual function enzyme with both 17-hydroxylase and 17, 20-lyase functions sharing the same active site, which makes selective therapeutic inhibition of the lyase function more challenging. Inhibition of the hydroxylase function in patients results in an ACTH feedback-mediated mineralocorticoid excess which can be suppressed through prednisone administration (Supplementary Figure 1). In addition to avoidance of side effects of prednisone, avoiding prednisone may have potential oncologic benefits in patients (7, 8).

VT-464 is a novel small molecule CYP17A1 inhibitor with selectivity for the 17, 20-lyase activity of this dual enzyme (9-11)(Figure 1A). The objective of this study was to evaluate the anti-cancer activity of VT-464 compared to abiraterone in pre-clinical models of CRPC, particularly in the enzalutamide-resistant cell lines since preliminary clinical reports indicate that abiraterone is less effective in patients with a prior enzalutamide history(12, 13). We used both a CRPC cell line model (C4-2) and two previously reported(14) enzalutamide (ENZ)-resistant cell lines (MR49C, MR49F) to model advanced disease responses to this novel inhibitor and to abiraterone in vitro and in vivo.

Materials and Methods

Materials: Stock solutions of testosterone (T) (Sigma) 4-androstene-3,17-dione (Sigma), 4-pregnen-17-ol-3,20-dione (Steraloids, Inc.), 5α-androstan-17β-ol-3-one (Sigma), dihydrotestosterone (DHT) (Sigma), 5β-pregn-3a-27-diol-20-one (Steraloids), 5β-pregn-3,20-dione (Steraloids), androsterone (Aldrich), cortisol (Sigma), pregnenolone (Sigma), progesterone (Sigma), R1881 (DuPont), and were prepared in 100% methanol as MS standards. VT-464, abiraterone, enzalutamide, and TAK-700 were provided by Viamet Pharmaceuticals, Inc.; stock solutions were prepared in DMSO. Abiraterone acetate (MedChem Express) was used for in vivo evaluations. VT-464 design and synthesis has been described elsewhere(9).

In vitro models: C4-2 cells were provided as a gift from Dr. Leland Chung and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2013. MR49C and MR49F cells were derived from LNCaP cells through serial xenograft passage in ENZ-treated mice as described previously(14). All cell lines were treated in RPMI 1640 in 10% charcoal-stripped serum (CSS; Hyclone), with 10µM ENZ present in the media of MR49C and MR49F cells. For all 6-day experiments, media with corresponding treatments were changed at 72 hours.

In vivo models: All animal experimentation was conducted in accordance with institutional standards and the Canadian Council of Animal Care (CCAC). Male athymic mice (Harlan
Sprague-Dawley, Inc.) were castrated 1-2 weeks prior to tumor inoculation with enzalutamide 10mg/kg being started the day prior to inoculation and continued until the total tumor volume was >200mm³. Two million MR49F cells with 0.5mL Matrigel were inoculated in both flanks of mice for the pharmacodynamic study and in the left flank for the full study, similar to previous experiments(14). ENZ 10mg/kg was given by oral gavage on a 5 day on, 2 day off cycle starting the day of tumour inoculation and stopped at the time of treatment initiation. PSA levels were measured by tail vein sera samples weekly using the Cobas automated enzyme immunoassay (Montreal, PQ). Tumor measurements were made twice weekly using calipers (volume = length x width x height x 0.5326). Treatments began once the total tumor size exceeded 200mm³. For the pharmacodynamic study, tumors were collected after 3-10 days (for each treatment: 2 mice after 3 days, 1 after 7 days and 2 after 10 days) and fragments were either immediately frozen in liquid nitrogen, fixed in formalin or preserved in RNAlater (Ambion). Treatments were cycled for 5 days on, 2 off. All xenograft tumors were harvested approximately 3 hours after their last treatment dose. Mice were sacrificed when tumor volume exceeded 1500mm³ or loss of >20% body weight.

**Steroid analysis by LC-MS.** Cell pellets and tissue homogenates were extracted 1:4 (v:v) with 60/40 hexane/ethyl acetate (hex/EtOAc) and media with EtOAc. Cell lines studies were performed in technical triplicates with biologic duplicate experiments and each tumour sample was measured in technical triplicates. Extracted steroids were dried (CentriVap) and reconstituted in 50-100 µL of 50 mM hydroxylamine, incubated 1hr at 65°C and the resulting oximes analyzed using a Waters Acuity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer. Separations were carried out with a 2.1x100mm BEH 1.7µM C18 columns, mobile phase water (A) and 0.1% formic acid in acetonitrile (B) (gradient: 0.2min, 20%B; 8min, 80%B; 9-10min, 100%B; 10.2min, 20%B; 12min run length). All data was collected in ES+ by multireaction monitoring (mrm) with instrument parameters optimized for the m/z’s and corresponding fragments of the oxime-steroids. Data processing was done with Quanlynx (Waters) and exported to Excel for addition normalization to weights and volumes as required. Deuterated T and DHT were used as internal standards (IS) and a curve of 6 calibration standards (0.01-10ng/ml) used for quantification (R2> 0.98). Recoveries were greater than 80% including extraction, conversion and matrix effects. The extraction protocol was found to also be effective for VT-464 and ABI and they withstood the derivatization procedure essentially intact, however some conversion of ABI to DHEA was observed (<0.01%) rendering samples very high in ABI unusable for that endpoint. Deuterated T was used as internal standard and a curve of 6 calibration standards (0.005-10µM, VT-464; 0.0015-3µg/ml, ABI) used for quantification (R2> 0.98). Samples were diluted as needed with IS blank to be within calibration range.

**PCR and Western blot analysis.** Steroidogenic enzyme RNA quantification in tumor samples was assayed by quantitative reverse transcription-PCR (qRT-PCR) with primers for STAR, HSD3B2, CYP17A1, HSD17B3, SRD5A1 and AKR1C3 (sequences in Supplementary Table 1) Reactions were conducted with 0.4 µL of RT-PCR cDNA, 0.8 µL each of forward and reverse primers (3µM), 2 µL nuclease-free water (Ambion), and 4 µL Roche SYBR Green qPCR MasterMix. Triplicates of samples were run on the default settings of the ABIiia7 real-time PCR machine. For western blot analysis, cell pellets or homogenized tumor tissue was processed in RIPA buffer (50 mM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mM NaCl and 1× Roche complete protease inhibitor cocktail). 30-50ug of protein were loaded and run on gel electrophoresis. After washing thrice with washing buffer, membranes were incubated with Alexa Fluor 680 or 800 secondary antibodies (Invitrogen) for 1h. Detection of specific bands with their densitometric quantification was done using the ODYSSEY IR imaging system (Li-COR.
Biosciences). AR (sc-816 / sc-7305), PSA (sc-7638 / sc-69664), and CYP17A1 (sc-46084) antibodies were purchased from Santa Cruz Biotechnology and Vinculin (V 9131) with an antibody from Sigma-Aldrich (St. Louis, MO). For immunoprecipitation, total proteins were precleared with protein-G sepharose (Invitrogen Life Technologies), immunoprecipitated with 2 μg anti-CYP17A1 prior to western blotting as previously described(15). Western blots and qPCR analyses were performed in at least duplicate.

**AR transactivation assays.** C4-2, MR49C or MR49F cells were plated on 6 well plates and transfected with 1.0 µg per well of ARR3-Luciferase-Plasmid using lipofectin (4.5uL per well; Invitrogen Life Technologies, Inc.) overnight in serum-free media. Treatments in 10% CSS RPMI 1640 with VT-464, ABI or DMSO control +/- 0.1nM R1881 (PerkinElmer) were performed at indicated times. Luciferase activity (relative light units) was measured using a microplate luminometer (Tecan) in duplicate and normalized to the cell lysate protein concentration. All experiments were carried out in triplicate.

The AR agonist / antagonist activity of VT-464, abiraterone, and TAK-700 was assessed at Indigo Biosciences (State College, PA) using CHO cells transfected with an expression vector that encoded a hybrid receptor comprised of full length wild-type AR and an AR response element functionally linked to firefly luciferase. Reporter cell AR response was validated using α-fluoro-testosterone as an agonist (EC50 = 60 pM). For antagonist assays (n = 3), cells were co-mixed with 2X the EC80 of α-fluoro-testosterone and the CYP17 inhibitor. Per cent antagonist inhibition was calculated as: 100*(1-[Average relative light units (RLU) test compound / Average RLU EC80 agonist). Dose-response non-linear curve-fits were generated using GraphPad Prism software and IC50s were calculated.

**Bio-Layer Interferometry (BLI) assay** The direct reversible interaction between small molecules and the AR was quantified by BLI using OctetRED (ForteBio) as previously described(16). The LBD of the biotinylated androgen receptor (bAR) was produced in situ with AviTag technology. The AviTag sequence (GLNDIFEAQKIEWHE) followed by a six residue glycine serine linker (GSGSGS) was incorporated at the N-terminus of the AR LBD (669-919). Escherichia coli BL21 containing both biotin ligase and AR LBD vectors were induced with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of dihydrotestosterone (DHT) and biotin at 16°C overnight. The bacteria were then lysed by sonication, and the resulting lysate was purified by immobilized metal ion affinity chromatography (IMAC) with nickel_nitrilotriacetic acid (Ni_NTA) resin and cation-exchange chromatography (HiTrap SP). Purified bAR LBD (50µg/mL) was bound to the super-streptavidin sensors over 50 min at room temperature. The sensor was kept in assay buffer [20 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES), 150mM NaCl, 500µM tris(2-carboxyethyl)phosphine (TCEP), 500mM DHT, and 1% dimethylsulfoxide (DMSO)]. In all experiments, a known AR ligand was used as a control to confirm functionality of the bAR LBD(17).

**Statistics.** Tumor growth velocity was calculated using linear regression of the log tumor volume over time. Student t-test was used to compare means. Means were plotted +/- SEM. Significant differences (p≤0.05 (*), p≤0.01 (**) and p≤0.001) were assessed using a Student’s t-test. Kaplan-Meier curves compared overall (OS) and cancer-specific survival (CSS). CSS was defined by the time from treatment until animal sacrifice for tumor size exceeding endpoint.
Results

**VT-464 suppresses the AR signaling pathway**

The presence of CYP17A1 protein in our LNCaP-based cell lines was confirmed using immunoprecipitation with CYP17A1 antibody (Figure 1B). To evaluate the activity VT-464 on AR signaling pathway, we first analyzed its effect on AR transactivation using an ARR3 luciferase reporter assay following treatment with 1, 5, and 10μM of ABI, VT-464 or control for six days in androgen-depleted media. In C4-2 cells, both ABI and VT-464 significantly decreased AR-transactivation (Figure 2A). At the 1μM and 5μM doses, this was significantly greater with VT-464 compared to ABI (P<0.01). Notably, in the ENZ-resistant MR49C and MR49F cell lines, only VT-464 demonstrated decreases compared to control, while ABI was similar to controls or increased transactivation. With VT-464 treatment, a dose-dependent trend in decreasing transactivation was also observed in both MR49C and MR49F cells. Similar results were observed for all cell lines and doses after 72h of treatment (Supplementary Figure 2A).

Since VT-464 induced a decrease in AR transactivation, we next assessed changes in AR-dependent genes were evaluated following six days in androgen-deprived conditions. The treatment-containing media was changed after 72h. In C4-2 cells, we found that both ABI and VT-464 stimulated an increase in AR mRNA transcripts, but a decrease in AR-dependent mRNA transcripts (e.g. PSA, NKX3.1) with greater decreases seen with VT-464 (Figure 2B). In the ENZ-resistant MR49C cell line, ABI did not produce any decrease in AR-dependent transcript levels relative to control; VT-464 at equivalent doses lowered transcript levels compared to ABI. Similar to C4-2 cells, there was a VT-464 dependent decrease in AR-dependent genes compared to AR transcript levels suggesting a feedback-induced increase in AR levels due to greater androgen suppression with VT-464. In MR49F cells, 10 μM VT-464 induced a sharp decrease in AR and PSA mRNA (Figure 2B) and PSA protein (Figure 2C) possibly due to tumor cell apoptosis as evidenced by increased PARP cleavage (Supplementary Figure 2B).

There were no differences in AR protein levels in C4-2 and MR49C cells following ABI or VT-464 treatment at the dose range tested, though AR levels did seem to increase more in response to VR-464 in MR49F cells as noted above (Figure 2C). However, PSA protein levels were significantly lower after VT-464 treatment, with a dose-dependent decrease in the ENZ-resistant cell lines, compared to ABI treated cells.

**Changes in androgen synthesis pathway following CYP17A1 inhibition in vitro**

Since VT-464 and ABI inhibit the AR signaling pathway by inhibiting CYP17(9, 11), we evaluated mRNA transcript levels for selected steroidogenesis enzymes in all three cell lines following 6 days of treatment in androgen-depleted media. Both ABI and VT-464 treatments increased STAR, HSD3B2, HSD17B3, AKR1C3, and SRD5A1 enzyme transcript levels in a dose-dependent fashion (Figure 3A); VT-464 was much more potent than ABI, presumably due to greater suppression of androgens. Similar trends were seen with upstream steroid and fatty acid pathway transcription factor SREBP-1 and HMGCR gene transcripts (Supplementary Figure 3A-D). With the exception of outliers, the levels of CYP17A1 expression were higher in VT-464- than ABI-treated cells with no evident dose response observed (Figure 3A).

Steroid level measurements were attempted for all cell lines but were only feasible in C4-2 cells due to detection limitations in MR49C and MR49F cells. Surprisingly, testosterone and dihydrotestosterone (DHT) levels were increased in ABI-treated cells after 6 days of treatment. This was consistent with the increase in PSA protein levels and AR and PSA mRNA transcripts observed in ABI-treated, but not VT-464-treated cells (Figure 2B, C). Decreasing concentrations of progesterone and increasing concentrations of pregnenolone occurred with higher doses of
ABI, consistent with prior reports of HSD3B2 inhibition with higher doses of ABI (18) (Figure 3B). In C4-2 cells, the decreased AR transactivation with ABI compared to DSMO control, despite increased concentrations of the androgens DHT, T, and androsterone, as well as pregnenolone, suggesting that non-steroid factors must be involved, such as drug AR antagonism. DHEA levels were very low in VT-464 treated cells, but could not be measured accurately in abiraterone-treated cells due to the low level conversion of abiraterone to DHEA noted in the methods. Overall, these results indicated that in C4-2 cells, suppression of the AR pathway by ABI was not due to androgen concentration decreases. Further, for VT-464, the increase in steroid enzyme transcripts did not appear to be related to resistance as observed increases did not result in increased androgen biosynthesis or AR transactivation. In contrast, the increased androsterone and DHT that occurred in response to ABI in C4-2 cells does suggest that increased expression of AKR1C3, and SRD5A1 may be relevant to resistance through activation of alternate androgen synthetic pathways as previously reported (19, 20).

**AR antagonism effect of VT-464**

To investigate a possible AR antagonistic effect, we repeated transactivation assays with ABI and VT-464 in the presence of 0.1nM R1881. ENZ as well as the CYP17 inhibitor TAK-700, were also tested as controls. Both abiraterone and VT-464 suppressed R1881-stimulation of AR transactivation (Fig. 4A). In C4-2 cells, the decrease in R1881-stimulated transactivation due to VT-464 was similar to that observed with ENZ, while no effect was seen with TAK-700. As expected, ENZ and TAK-700 again showed minimal changes in reporter activity in the ENZ-resistance cell lines. Both abiraterone and VT-464 decreased R1881-induced AR-transactivation in MR49F cells. Corroborating prior results, VT-464 demonstrated greater suppression of transactivation in the presence of R1881 than abiraterone in C4-2 and MR49C cells.

To further evaluate a direct antagonistic effect on the AR, VT-464 and ABI were tested in a wild-type AR luciferase assay transfected into CHO cells. AR-antagonism was observed with both ABI and VT-464, but not with TAK-700 (Fig. 4B). Assessment of AR protein levels following VT-464 treatment at different time points and different doses indicated that AR degradation is not a major contributor to the AR-antagonism observed (Fig. 4C, Supplementary Fig. 4). A competitive binding assay using purified AR ligand binding domain (LBD) and bio-layer interferometry demonstrated a clear dose-dependent effect, evidence of direct binding between VT-464 and the AR LBD (Figure 4D).

**VT-464 and abiraterone acetate decrease tumor growth and serum PSA levels in an in vivo model of enzalutamide resistance**

Castrated mice inoculated with MR49F cells were randomized to oral gavage treatment with vehicle (0.5% methylcellulose) twice-daily (BID), abiraterone acetate (AA) 196mg/kg BID, VT-464 75mg/kg BID and VT-464 150mg/kg once-daily (QD). The AA oral gavage dose of 1 mmol/kg daily is higher than previously published doses of 0.5mmol/kg/d(20), but was tolerated in prior unpublished experiments; the VT-464 dose of 0.375 mmol/kg daily (75 mg/kg BID or 150 mg/kg OD) was used due to mouse weight loss at higher BID doses . Efficacy of the VT-464 150mg/kg OD arm was assessed preliminarily in 5 mice. AA and both VT-464 regimens demonstrated tumor growth inhibition (Fig. 5A). Mean tumor growth velocity was significantly lower in the VT-464 BID arm compared to vehicle (P=0.03); tumour growth velocity in AA arm did not different statistically compared to vehicle (P=0.06). Differences between AA and VT-464 were not significant (p=0.64).
Serum PSA results was suppressed in all three treatment arms (Fig. 5B). After 3 weeks of treatment the median PSA was lowest in the VT-464 BID arm (Fig. 5B). The mean PSA velocity was significantly lower for AA compared to vehicle (P=0.03), but not for VT-464 BID (P=0.38). The waterfall plots after three weeks (Fig. 5B) show that there was a small subgroup of AA-treated mice with very low PSA that skewed the mean versus median values. Similarly, there were high PSA outliers in the VT-464 BID treatment arm.

Results in the VT-464 150mg/kg QD arm were generally similar to 75 mg/kg BID regimen. Weight loss was similar between all treatment arms (Supplementary Fig. 5A). Two mice in the AA arm and one mouse in the VT-464 75mg BID arm were euthanized for weight loss >20% after 19, 33 and 39 days of treatment, respectively. One mouse in the VT-464 150mg OD arm was found dead one day after starting treatment. Significant improvements in OS and CSS were found only in the VT-464 75mg/kg BID arm compared to vehicle (p=0.03 and p=0.009, respectively) (Fig. 5C).

End-of-study intratumoral AR-dependent mRNA transcripts in VT-464 75mg/kg BID treated-mice were lower compared to AA (Supplementary Fig. 5B). End-of-study tumor samples also had steroidogenesis enzyme mRNA transcripts (Supplementary Fig. 5C) which were lower in both AA and VT-464 treatment arms compared to vehicle, with the lowest levels in the VT-464 arm.

End-of-study intratumoral AR-dependent mRNA transcripts levels (Supplementary Fig. 5B) were lower in VT-464 75mg/kg BID treated-mice compared to AA. Abiraterone-treated mice had AR mRNA transcript levels slightly increased compared to vehicle-treated mice. Changes in AR-regulated genes PSA and NKX3.1 were also lower in the VT-464 compared to the AA arm, which were increased relative to vehicle. TMPRSS2 showed a different trend, with higher values in the VT-464-treated group compared to AA and vehicle. In contrast to the in vitro results above, select steroid synthetic enzyme mRNA transcripts (Supplementary Fig. 5C) were significantly lower intratumorally in the treatment arms (AA and VT-464) compared to vehicle, with the lowest levels in the VT-464 arm.

Pharmacodynamic study

To investigate pharmacodynamic effects, castrated mice implanted with MR49F xenografts in both flanks were treated orally with VT-464 at 100mg/kg BID or AA at 196mg/kg BID once tumors reached 200mm³ in size as detailed in methods. AR-dependent gene transcripts and AR and PSA protein level data collected from the tumors demonstrated significant heterogeneity of response (Fig. 6A,B), with a similar suppression of the AR-pathway transcripts with both abiraterone and VT-464. Analysis of intratumoral drug levels indicated similar heterogeneity in intratumoral drug levels (Fig. 6C) though the intratumoral VT-464 concentrations were greater on average and somewhat less variable.

Analysis of intratumoral steroid levels demonstrated that testosterone and DHT levels were significantly lower following VT-464 or AA treatment compared to vehicle, with the greatest decreases seen with VT-464 (Fig. 6D). Upstream pregnenolone levels were significantly higher in tumors of AA-treated animals (P=0.03).

Steroidogenic enzyme mRNA transcripts were assessed in the collected tumor samples. Here, in contrast to the prior in vivo findings, but similar to the in vitro findings, we found a trend toward increased steroid enzyme synthesis in AA- and VT-464 treated mice (Supplementary Fig.
6), with the greater up-regulation seen in the VT-464 treated mice, though androgen levels were lower in this group (Fig. 6D).

Discussion

This study demonstrated more potent inhibition of androgen synthesis by VT-464 in CRPC models compared to abiraterone particularly in the enzalutamide resistant cells, MR49C and MR49F. Further, direct AR antagonism was demonstrated as a novel mechanism of action of VT-464. The use of LNCaP-based models mirrors the clinical situation wherein the T887A AR mutation renders tumors sensitive to activation by non-canonical androgens such as progesterone, pregnenolone, and prednisone (21, 22).

AR antagonism has been demonstrated in pre-clinical studies for the steroid-based CYP17 inhibitors AA and galeterone (VN/124, TOK001) (23-26) but this mechanism has not previously been reported for any non-steroidal CYP17A1 inhibitor. The clinical results with TAK-700(21) and enzalutamide (1) confirm that selective androgen synthesis inhibition or AR antagonism alone have activity in CRPC. Combination AR antagonism with CYP17A1 inhibition has previously been reported to contribute to the pre-clinical efficacy of galeterone and abiraterone (23, 24). Our results suggest that AR antagonism is best combined with selective inhibition of CYP17A1 lyase, compared to the complete inhibition of both lyase and hydroxylase functions.

Prior in vitro studies evaluating the selectivity for the lyase versus hydroxylase activity of CYP17A1 suggest VT-464 has approximately 50X more selectivity compared to abiraterone (27). In castrate rhesus monkeys, similar to our results, decreases in testosterone with VT-464 were not accompanied by accumulation of progesterone or pregnenolone seen with abiraterone(28). This selectivity allows for dosing of VT-464 without prednisone in ongoing Phase II trials in populations of patients who have previously failed enzalutamide (NCT02012920) or prior regimens of enzalutamide, abiraterone, or chemotherapy (NCT02117531); the oncologic significance of avoiding prednisone continues to be explored.

Recent pre-clinical studies suggest glucocorticoids may activate tumors through -AR-redundant transactivation pathways following ENZ-resistance (7). Therefore the combination of lyase-selective CYP17 inhibition and AR antagonism without prednisone supplementation is a promising strategy for patients who progress on ENZ. MR49C and MR49F cells have two notable characteristics which model clinical ENZ-resistant disease (29, 30). We previously demonstrated higher levels of nuclear AR in these cells(14). Further, in addition to the T887A AR mutation, the F876L mutation is present in ~50% of MR49C and MR49F cells (unpublished data). The mutation alters the AR ligand binding domain, resulting in ENZ acting as an agonist (29, 31). In our study, we found VT-464 had anti-AR activity against both MR49C and MR49F cells.

The lack of relative up-regulation of steroidogenic enzymes in VT-464-treated tumours collected at animal endpoints differs from our in vitro and pharmacodynamic study results. This may be related to up-regulated metabolism of VT-464 observed in regulatory safety studies in rodents, where the exposure to the drug after 3 weeks is substantially diminished. A preliminary study in our lab in the LNCaP CRPC xenograft model showed that co-administration of a CYP3A4 inhibitor in mice along with VT-464 improved the PSA response (data not shown). This is consistent with the diminished PSA response observed in the VT-464 once daily arm after 3 weeks. Our in vivo results are also limited by some heterogeneity. Nonetheless, this
heterogeneity mirrors early clinical results of CYP17A1 inhibition in ENZ-resistant patients (12, 13)

In summary, the novel CYP17 inhibitor VT-464 demonstrated anti-cancer activity in preclinical models of CRPC and ENZ-resistance, decreasing androgen levels significantly in castrate mice. The *in vitro* and *in vivo* results suggest greater suppression of the AR-axis with VT-464 compared to abiraterone due to the selective suppression of androgen synthesis through CYP17 lyase inhibition, as well as AR antagonist effects.
References


Figure Captions

**Figure 1.** A. Molecular Structure of abiraterone acetate (AA) and VT-464, a highly-selective 4-(1,2,3-Triazole)-Based P450c17a 17,20-Lyase Inhibitor B. Immunoprecipitation demonstrating CYP17A1 protein in indicated LNCaP based cell lines with densitometry quantification below.

**Figure 2.** Effect of VT-464 and abiraterone on the AR-pathway. AR-transactivation evaluated using a ARR3 luciferase plasmid(A). Transfected C4-2, MR49C and MR49F cell lines were treated in androgen-depleted media for 6 days with 1, 5 or 10μM of treatment with ABI or VT-464(VT). Results are normalized to total protein concentration. Representative results of at least three independent experiments +/- SEM are presented. Significant differences are indicated (p<0.05 (*), p<0.01 (**) and p<0.001) (B) Under identical conditions, AR and AR-dependent transcript levels were assessed using quantitative real-time PCR. (AR and PSA protein levels were also similarly assessed in all three cell lines(C). Densitometry was relative to vinculin and control treatment. The media and drug treatments were changed at 72hrs for all experiments. For PCR and protein results, representative results of at least duplicate experiments are presented.

**Figure 3.** The effect of VT-464 and abiraterone on the steroid synthesis pathway *in vitro*. C4-2, MR49F and MR49C cells were cultured in 1, 5 or 10μM of abiraterone, VT-464 for 6 days. The media and drug treatments were changed at 72hrs and MR49C and MR49F cells were maintained in 10μM ENZ. Transcript levels of STAR, HSD3B2, CYP17A1, HSD17B3, AKR1C3 and SRD5A1 enzyme mRNA transcripts were measured using SYBR green primers (see methods) for all cell lines (A). Values relative to DMSO control are plotted +/-SEM. Using LC-MS, steroid levels were measured in C4-2 cells at the same time period (B).

**Figure 4.** AR antagonism with VT-464 and abiraterone. AR transactivation results using transient transfection of an ARR3 luciferase reporter in androgen-depleted conditions in C4-2, MR49C and MR49F cell lines. In the presence of 0.1nM R1881, both abiraterone 5μM and VT-464 5μM decreased AR-transactivation after 24h of treatment (A) in all cell lines. No decrease was seen with the negative control TAK-700 in all cell lines; ENZ potency was also consistent as a positive and negative control in C4-2 and ENZ-resistant cells, respectively. Representative results of at least 3 replicates +/- SEM are displayed. AR antagonism with abiraterone and VT-464, but not TAK-700 was further seen using a wild-type AR luciferase reporter assay in CHO cells(B) No AR degradation was noted in time course of AR protein levels over time under treatment with 10 μM ABI(left) or 10 μM VT-464(right) in MR49C and MR49F cell lines(C). Representative results of duplicate time courses are presented. D. Bio-Layer interferometry assay demonstrating a dose-dependent effect with VT-464 binding to a purified androgen receptor ligand binding domain. Doses listed are in μM.

**Figure 5.** *In vivo* evaluation of VT-464 and abiraterone. Castrated MR49F xenografts were randomized to treatment once tumor size exceeded 200mm³. Treatments were vehicle (0.5% MC) BID, abiraterone 196mg/kg BID, VT-464 75mg/kg BID and VT-464 150mg/kg QD. Mean tumor size is plotted +/- SEM(A, left). The T waterfall plot details % change from baseline tumor size after 3 weeks of treatment for all mice in study(A, right). Waterfall plot of serum PSA after 3 weeks of treatment for all mice in study (B, left). Mean M PSA values +/-SEM areare plotted (B, right). Kaplan-Meier survival for all treatments: cancer specific survival(C, left) and overall survival (C, right).
Figure 6. Pharmacodynamic evaluation of VT-464 and abiraterone in MR49F xenograft model. Fifteen xenograft–bearing mice were treated for 3-10 days with abiraterone 196mg/kg BID, VT-464 100mg/kg BID or vehicle and tumors were harvested. All mice were sacrificed approximately 3 hours after last dose (for details, see methods). mRNA transcript levels of AR related transcripts in tumor samples were evaluated with qRT-qPCR relative to GAPDH(A). Western blot analysis for AR, PSA and CYP17A1 levels demonstrates significant tumor heterogeneity between tumours from individual mice (B). Intratumoral drug levels for abiraterone and VT-464 as measured using LC-MS chromatography in triplicate (C). Steroid analysis results for androgens and upstream pre-cursors(D).
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ANTI-CANCER ACTIVITY OF A NOVEL SELECTIVE CYP17A1 INHIBITOR IN PRE-CLINICAL MODELS OF CASTRATE RESISTANT PROSTATE CANCER

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