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TITLE: Development of Novel Drugs That Target Coactivation Sites of the Androgen Receptor for Treatment of Antiandrogen-Resistant Prostate Cancer

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14. ABSTRACT
Interest in developing androgen receptor (AR) inhibitors with novel mechanism of action is slowly increasing since commercial anti-androgens (Bicalutamide, Flutamide, Nilutamide and Enzalutamide) face therapeutic limitations. Current therapies fail over a period of time because they all target hormone binding pocket on AR to which the receptor has already developed effective resistance mechanisms. One of the promising strategies to combat drug resistance is to develop the inhibitors that target an alternative binding pocket of the AR, called Binding Function 3 (BF3). In the current study, we report indole chemical series, identified through systematic *in silico* screen, as leading AR BF3 inhibitors. The most potent inhibitor (compound VPC-13566**)** demonstrated excellent anti-androgen potency, anti-PSA activity and abrogates androgen-induced proliferation of LNCaP and Enzalutamide-resistant prostate cancer cell lines. Moreover, it demonstrated clear reduction of tumour growth in tumor xenograft models in mice. Based on these results new derivatives have been developed to improve stability and better efficacy in *in-vivo* models. These findings provide evidence that targeting AR BF3 pocket using small molecule inhibitors is a viable therapeutic approach for patients with advanced prostate cancer.

15. SUBJECT TERMS
P**rostate cancer, small molecule drugs, androgen receptor, chemical genomics, drug resistance, hormone resistance,**computer-aided drug design

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1. **INTRODUCTION:** Androgen receptor (AR), a member of the nuclear hormone receptor (NHRs) is a ligand-dependent transcription factor (1) with significant therapeutic relevance in prostate cancer (PCa) (2). Conventional AR-based therapeutics have mainly focused on targeting the traditional hormone binding pocket of the receptor (3;4). However, over a period of time, therapeutic efficacy of these drugs suffered from the problem of drug resistance (5) due to 1) mutations at the ligand binding pocket causing structural and functional changes of the receptor and 2) conversion of antagonist into an agonist in a physiological context. Antiandrogen-based therapy including second generation androgen receptor (AR) inhibitors, Enzalutamide represents a typical case, where drug resistance is inevitable and its onset is associated with poor prognosis and high mortality (6). This highlights the urgency to develop entirely new types of anti-AR therapeutics with novel mode of action *i.e.* rather than blocking ligand interaction with AR the novel class of drugs should ideally bind to alternative sites on the receptor thereby effectively disrupting the association of coactivators that bind to these sites. Recently, a co- regulatory surface site called the Binding Function 3 (BF3) has been identified by Fletterick *et al* (7). The specific function of this pocket has not yet been fully characterized, although recent reports provide some evidence of its possible involvement in the AR association with FKBP52 - an important positive regulator of this receptor (8) and possible cross-talk with the AF2 site (9). In addition, BF3 is conserved among other members of NHR family, thereby offering a better opportunity to understand the molecular mechanism of co-factor recruitment and subsequently its inhibition. Since AF2 and BF3 surface pockets play pivotal role in mediating AR function, therapeutic targeting of these sites offers a rich vein for the discovery of novel drugs for PCa with alternative mechanism of action and thereby circumvents treatment resistance seen with conventional anti-androgens. These drugs would provide an additional line of therapy for patients combating castration-resistant PCa thereby considerably expanding their life span.

2. **KEYWORDS:** Prostate cancer, small molecule drugs, androgen receptor, chemical genomics, drug resistance, hormone resistance, computer-aided drug design

3. **OVERALL PROJECT SUMMARY:**

Specific Aim 1: to use the generated structure-activity data and the resolved crystal structure to design and synthesize AR AF2 and AR BF3 binders with enhanced target-affinity and 'drug-like' properties.

Task 1.1. Molecular modeling of derivatives of lead BF3 binders VPC-0098 and a closely related VPC-4035.

During the first year of the project we identified 2 derivatives of VPC-0098 with excellent activity in the low nanomolar range (VPC-13562 and 13566). In the second year of the project, we made multiple attempts to enhance the activity profile of these new BF3 leads via structure based lead optimization as well as to improve their stability and their formulation so that ultimately these lead derivatives will be compatible for oral dosing which would be more appropriate for a potential treatment option. Each subsequent chemical series this year was developped based on the scaffold of active compounds VPC-13566 from the previous series.

Task 1.2. Synthesis of derivatives of our lead AF2binders VPC-0061 and lead BF3 binders VPC-0098 and a closely related VPC-4035.

Based on these findings, 170 small molecule compounds were synthesized either at Enamine **(**http://www.enamine.net/**)** or at our collaborator Robert Young's laboratory and send to the VPC for further testing (see Specific Aim 2).

Specific Aim 2: to experimentally evaluate the developed synthetic derivatives.

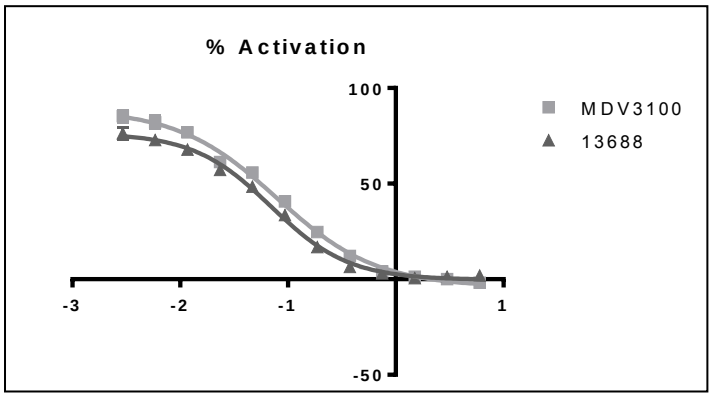
Task 2.1. eGFP Cellular AR Transcription Assay.

All the 170 compounds were screened for their ability to inhibit AR transcriptional activity using a nondestructive, cell-based enhanced green fluorescent protein (eGFP) AR transcriptional assay (10). In this assay, the expression of eGFP is under the direct control of an androgen responsive probasin-derived promoter and enables quantification of AR transcriptional activity. All compounds that exhibited >75% inhibition of AR transcription at a screening concentration of 3μM were then subjected to concentration-dependent titration to establish their corresponding IC_{50} values (Figure 1). To ensure these values are true positive hits in the AR transcriptional eGFP assay, we validated their activity by a second transcription assay, based on light detection instead of fluorescence, by quantifying their effect on the production of the prostate specific antigen (PSA) in prostate cancer cell lines (11). PSA is AR-regulated serine protease and is widely used as a biomarker for PCa. As expected, hit compounds induced a equivalent decrease in PSA levels in LNCaP (12) cells as the IC_{50} values found with the eGFP assay. Table 1 below summarize compounds that were found to have an IC50 below 100 nM. For comparison purposes, in this assay, gold standards Enzalutamide and Bicalutamide shows IC_{50} of 100 nM and 600 nM respectively. Importantly, nine compounds exhibit exceptional activity under 50 nM. Figure 1 shows a typical experiment for determination of IC_{50} (in this case compound VPC-13688) using Enzalutamide (MDV3100) as comtrol. In figure 1, typical curves obtained with these assays are described using compound VPC-13688 as an example.

Table 1: Compounds that exhibited an IC_{50} under 0.1 μM in transcription assays (eGFP and PSA)

Internal Number	IC50_eGFP	PSA IC50 (uM)
13566	0.01	0.011
13591	0.012	0.027
13621	0.02	0.008
13697	0.029	0.029
13642	0.032	0.035
13610	0.033	0.013
13713	0.037	0.044
13698	0.041	0.041
13696	0.042	0.042
13582	0.051	0.058
13676	0.053	0.034
13585	0.057	0.028
13716	0.067	0.058
13688	0.069	0.068
13695	0.069	0.087
13694	0.070	0.067
13699	0.073	0.073
13674	0.075	0.051
13703	0.087	0.097
13584	0.088	0.050
13579	0.1	0.04270
13622	0.1	0.075
13692	0.1	0.082

A)



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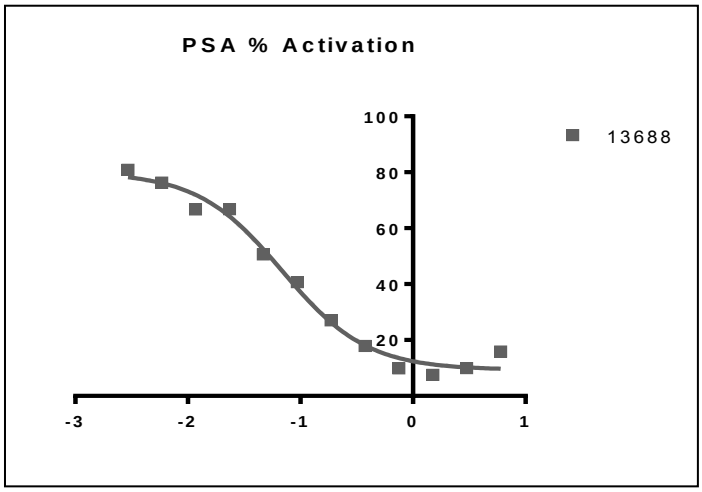
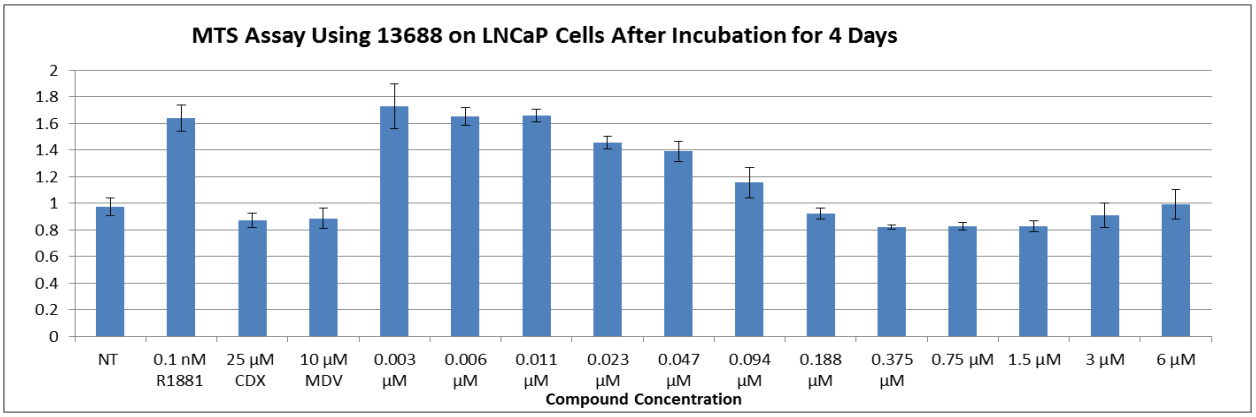


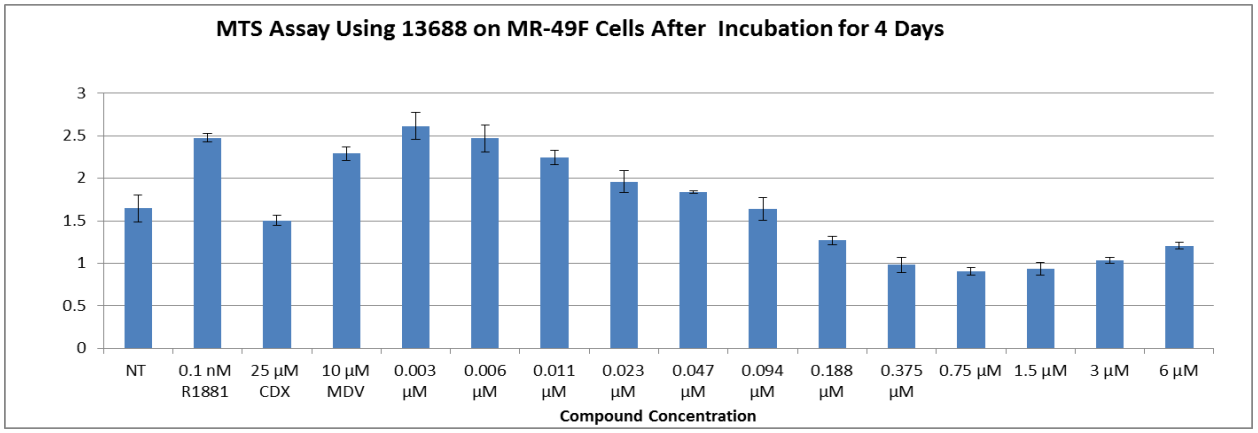
Figure 1: Sigmoidal curves of compound VPC 13688 to determine IC50 in A) eGFP and B) PSA. The Y-axis represents the percentage of activity remaining after treatment with the compound.

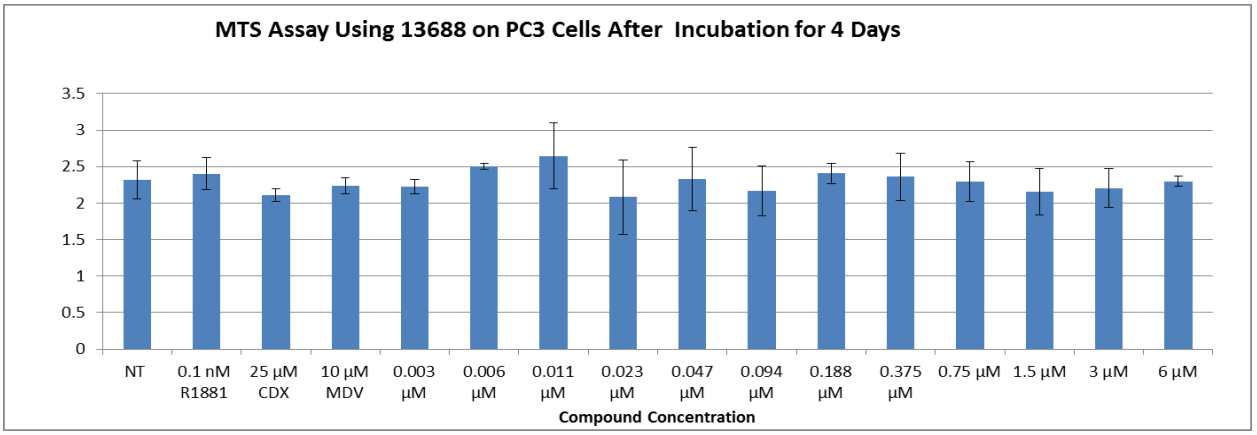
Task 2.2. *MTS assay*.

To determine the translational potential of the most potent BF3 inhibitors listed in Table 1, we evaluated their ability to reduce growth of PCa models stimulated by the androgen R1881 *i.e.* LNCaP, and androgen-independent PC3 cell line. The cell viability was assessed after 4 days of incubation with the test compound in a concentration dependent manner. Figure 2 shows a typical experiment with one of our lead compound VPC-13688 where the compound is very effective in inhibiting the growth of LNCaP cells (Figure 2A), establishing an IC_{50} values of 100 nM. This compound was also very effective in inhibiting the growth of MR49F cells resistant to Enzalutamide (Figure 2B) at an IC50 similar to what was observed in LNCaP cells. Moreover, compound 13688 did not show any effect on AR independent PC3 cell lines (figure 2C), confirming its AR-specific activity. MTS experiments are performed on all our compounds that are under 1 µM in eGFP activity.

Figure 2: The effect of compounds VPC-13566 on cell viability in LNCaP and PC3 cells. In all our MTS experiments Enzalutamide (MDV3100) is used as control.







LNCaP Cells (A) and Enzalutamide resistant cells(B) stimulated by androgen R1881 (0.1 nM) are inhibited by a dose dependent concentration of compound VPC-13688, to a level comparable to the controls Bicalutamide (CDX) and Enzalutamide (MDV). PC3 cells (C) lacking the AR were not inhibited.

Table 2: Effect on proliferation of LNCaP cells (MTS assays) with effective BF3 compounds

Compound Number	Approximate IC50 (nM)
13688	0.094
13692	0.094
13703	0.14
13654	0.15
13628	0.188
13676	0.188
13601	0.188
13593	0.188
13646	0.188
13702	0.188
13699	0.188
13655	0.188
13630	0.28
13674	0.375
13691	0.375
13658	0.56
13602	0.56
13660	0.75

Task 2.3.*Biolayer interferometry*.

Biolayer interferometry (BLI) studies demonstrated a direct reversible interaction between these compounds and a purified AR ligand binding domain in a dose dependent manner. When a compound binds to the AR, there is a shift in wavelength that is detectable and measurable using the instrument. Figure 3 shows the BLI data for the example compound VPC-13688. There is a clear binding pattern for this compound to the AR. All BF3 compounds exhibited this behavior with more or less efficiency depending of the potency of the compound. As a further task in 2.3 we also tested the ability of the compound to displace a peptide that bind to an adjacent site (AF2) but none worked, suggesting that binding of our BF3 compounds does not occur to alternate sites such as the AF2 (not shown)

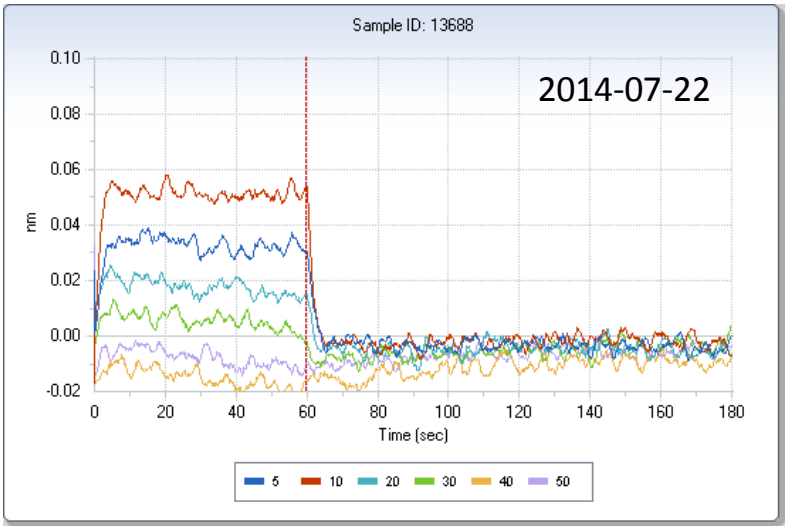


Figure 3: Binding of the VPC-13688 compound to AR determined by Biolayer Interferometry analysis

Task 2.4. *Protein expression, purification, crystallization and data collection.*

This part of the work is still in progress. On the third year of this project we will try to obtain a crystal structure of one of our most potent inhibitor VPC-13688.

Specific Aim 3: to select several lead compounds for pharmacological development.
Task 3.1. *Solubility, Stability and formulation for in vivo studies.*

Analytical methods have been developed for each compound upon chromatographic isolation in liquid (LC) phases and mass spectrometry. Stability testing has been carried out (temperature and time dependent) in order to provide assurance that the compounds tested for *in vitro* activity are chemically intact and not derivatives produced as a result of chemical instability. Thus, all compounds that were shown to be effective in *in vitro* assays were evaluated for their stability. All compounds tested were soluble up to 50 μM in media (not shown).

Metabolic stability refers to the susceptibility of compounds to biotransformation in the context of selecting and/or designing drugs with favourable pharmacokinetic properties. Metabolic stability results are usually reported as measures of intrinsic clearance, from which secondary pharmacokinetic parameters such as bioavailability and half-life can be calculated when other data on volume of distribution and fraction absorbed are available. These parameters are very important in defining the pharmacological and toxicological profile of drugs as well as patient compliance. Preliminary data using microsomes studies have shown that many of our compounds did not show a great half-life in metabolic stability (Figure 4). However, based on this information we will be capable of identifying weak points in the structure of the compounds and improve this aspect in year 3.

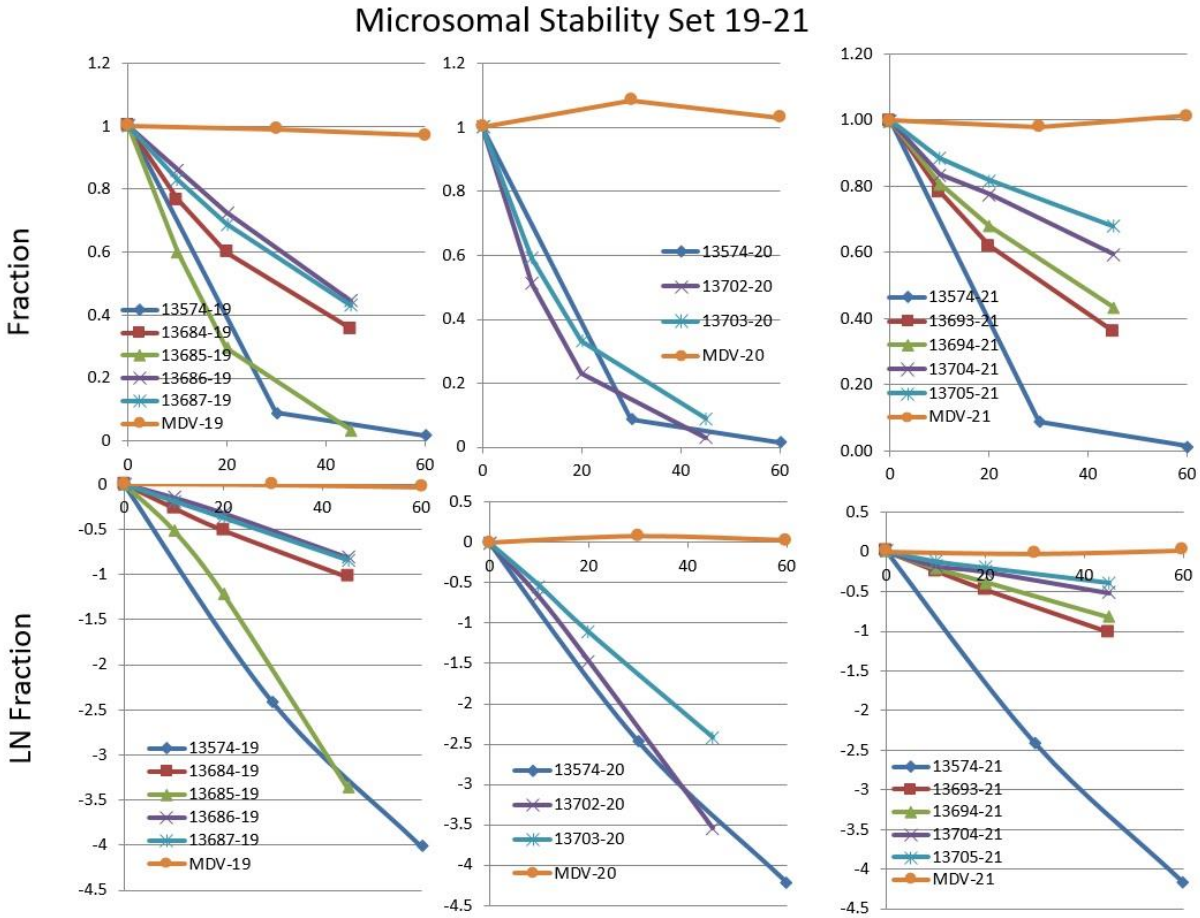


Figure 4: Microsomes study of VPC compounds.

Table 3: Half life of the most stable compounds in microsomes studies

Internal Numbering	Half Life (min.)
13673	1330
13666	642
13648	503
13665	186
13627	154
13677	144
13663	126
13593	105
13646	103
13592	94
13606	89
13630	77
13651	76
13602	75
13601	66
13669	66
13645	61
13597	59
13652	58
13611	53
13670	51
13586	50
13599	50

Task 3.4. Efficacy studies in human xenograft tumour bearing mice; (months 13-36).

In year 2 we proceeded to test compound VPC-13566 identified in Year 1 for its ability to reduce the growth of tumor cell lines in xenograft models (Figure 5-7). Interestingly, this compound have shown great efficacy to reduce growth of all these prostate tumor cell lines.

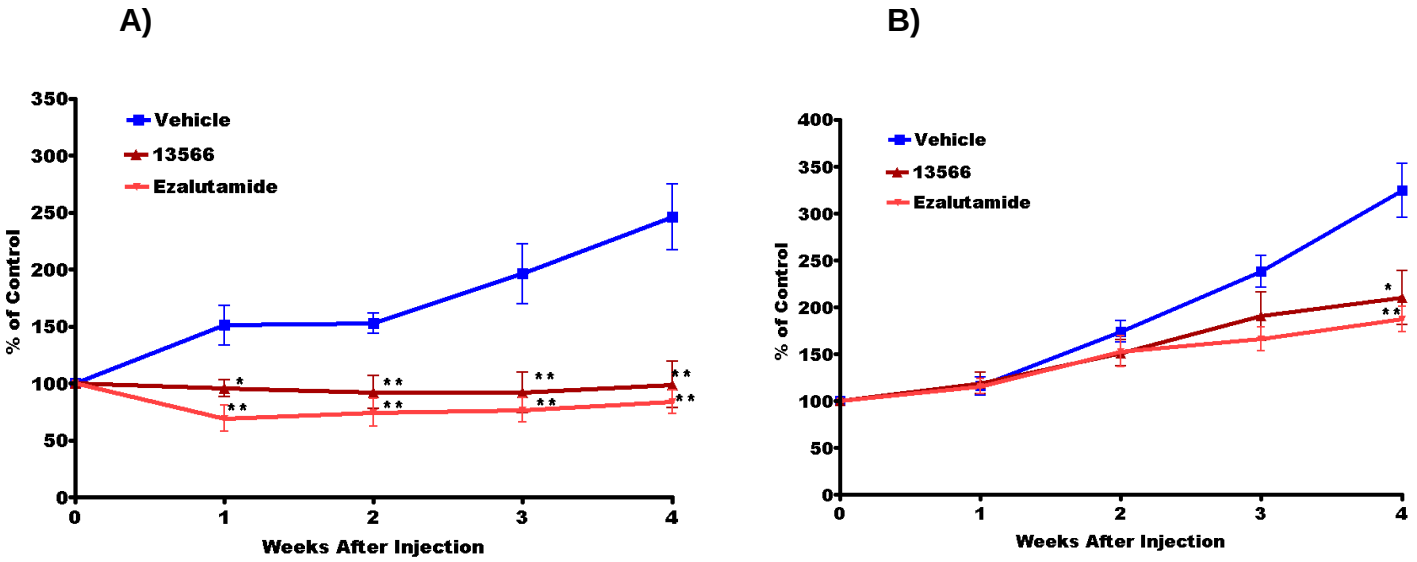


Figure 5: Reduction of A) tumour growth and B) PSA in LNCaP xenograft model compared to vehicule control and gold standard Enzalutamide

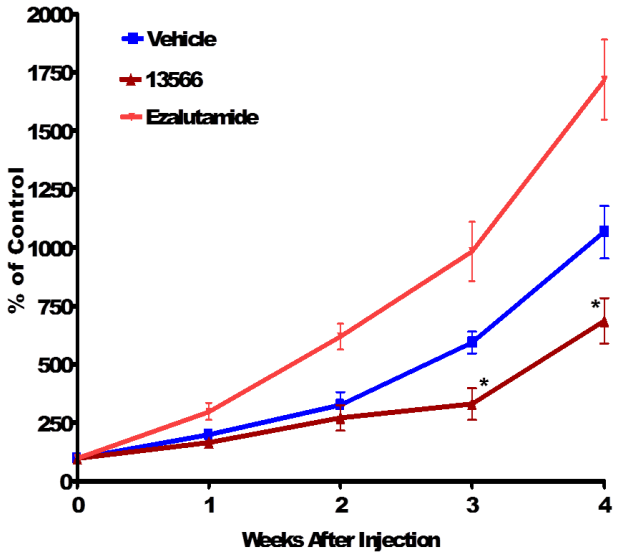


Figure 6: Reduction of tumor growth in MR49F xenograft model using VPC-13566

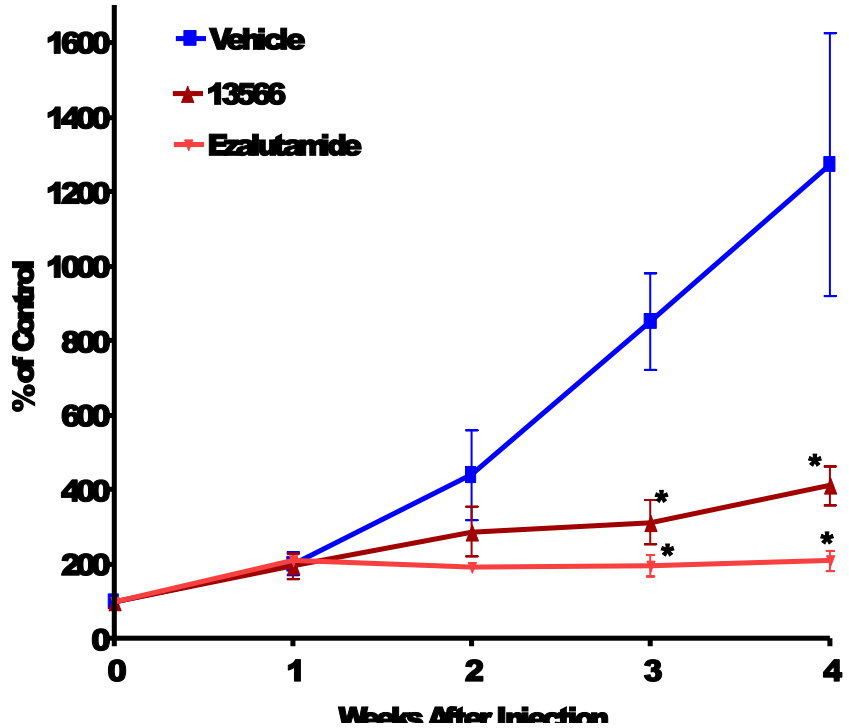


Figure 7: Reduction of tumor growth in C4-2 xenograft model using compound VPC-13566

4. KEY RESEARCH ACCOMPLISHMENTS:

- Uding our in-silco model we identified and characterized 23 small molecule compounds inhibitor of the AR with an IC50 under 100 nM in transcription assay
- Nine of these compounds exhibit exceptional activity under 50 nM which is twice more potent than gold standard Enzalutamide
- These compounds show a direct interaction with the AR by BLI and are effective to reduce the growth of Enzalutamide-resistant cell lines
- Half-life of these compounds in metabolic conditions remain to be improved, as few of the very active compounds have a half-life over 60 minutes in microsomal studies.
- However, parental compound VPC-13566 identified in year 1 of the project is very effective in reducing tumor growth of LNCaP and Enzalutamide-resistant cell lines in mice.
- Based on these outcomes, it can be concluded that BF3 specific inhibitors can act as complementary therapeutics to treat castrate-resistant prostate cancer

5. CONCLUSION:

Prostate cancer is the most commonly diagnosed non-skin cancer in Canadian men and one of the leading causes of cancer-related death. If diagnosed early, when still confined to the prostate, it is frequently curable by surgery or radiotherapy. Treatment for locally advanced, recurrent or metastatic prostate cancer is primarily some form of androgen withdrawal therapy, which is generally designed to block either the production of androgens or their binding to the androgen receptor. Unfortunately, the effectiveness of this type of treatment is usually temporary due to progression of surviving tumor cells to a castration-resistant state. With no curative treatment options for castration-resistant prostate cancer, the median life expectancy is approximately 18 months. Part of the problem is that all anti-AR agents currently used to treat patients act by direct binding to the AR hormone binding site and hence are vulnerable to mutations which frequently arise in this region of the molecule. The proposed research aims to address this problem by using computer modeling, biological screening, and structural biology to develop an entirely new class of anti-AR drugs which will target an distinct region of the AR called binding function-3 (BF3) to inhibit its activity. We anticipate that these new anti-AR drugs will replace or supplement existing anti-AR therapeutics and will provide new options for treating patients with metastatic, castration-resistant prostate cancer.

Over the last year, there has been considerable progress in our project to develop small molecules inhibitors that target the BF3 site of the AR for the treatment of prostate cancer. Based on the chemical scaffold VPC-13566 previously reported in year 1 of the project, we conducted a systematic *in silico* screen and identified approximately 170 indole based compounds for biological testing. These compounds were evaluated successfully using a series of *in vitro* assays confirming their potency via AR guided mechanism of action in various prostate cancer cell lines. Several compounds demonstrated inhibition of AR in low nanomolar range. It should be noted that these derivatives show two to five fold increase in their potency when compared to gold standard Enzalutamide. Additionally, the Biolayer Interferometry (BLI) assay detected direct reversible interactions between the AR and these derivatives. This class of inhibitors is promising and is currently under further investigation. Importantly, VPC-13566 exhibited a strong anti-proliferative effect on both LNCaP and Enzalutamide-resistant cell lines (MR49F) in xenograft mouse models, confirming that thes compounds are truly effective anti-tumor agents. Based on our outcomes using original scaffold and an alternative binding pocket BF3, it can be concluded that BF3 specific inhibitors can act as complementary therapeutics to treat castrate-resistant prostate cancer.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

1) Discovery of 1H-indole-2-carboxamides as novel inhibitors of the androgen receptor binding function 3 (BF3). Ban F, Leblanc E, Li H, Munuganti RS, Frewin K, Rennie PS, Cherkasov A. J Med Chem. 2014 Aug 14;57(15):6867-72. doi: 10.1021/jm500684r. Epub 2014 Jul 25. PMID:25025737

2) Identification of a Potent Antiandrogen that Targets the BF3 Site of the Androgen Receptor and Inhibits Enzalutamide-Resistant Prostate Cancer Ravi S.N. Munuganti, Mohamed D.H. Hassona, Eric Leblanc, Kate Frewin, Kriti Singh, Dennis Ma, Fuqiang Ban,Michael Hsing, Hans Adomat, Nada Lallous, Christophe Andre, Jon Paul Selvam Jonadass, Amina Zoubeidi, Robert N. Young, Emma Tomlinson Guns, Paul S. Rennie, and Artem Cherkasov, Chemistry and Biology*, In press*

3) The development of anti-androgens with a new mechanism of action for treatment of castration resistant prostate cancer.
Ravi SN Munuganti, Mohamed H Hassona, Eric Leblanc, Fuqiang Ban, Emma T. Guns, Paul S. Rennie, Artem Cherkasov. Poster presented at the AACR meeting , April 05-09, 2014 at San Diego, USA:

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report.

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

Nothing to report.

10. REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in Science, Military Medicine, etc.)

1. Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, et al. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: Glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacological Reviews. 2006;58:782-97.
2. Denmeade SR, Isaacs JT. A history of prostate cancer treatment. Nature Reviews Cancer. 2002;2:389-96.
3. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, et al. Development of a Second-Generation Antiandrogen for Treatment of Advanced Prostate Cancer. Science. 2009;324:787-90.
4. Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A, et al. ARN-509: A Novel Antiandrogen for Prostate Cancer Treatment. Cancer Research. 2012;72:1494-503.
5. Lassi K, Dawson NA. Emerging therapies in castrate-resistant prostate cancer. Current Opinion in Oncology. 2009;21:260-5.
6. Adamo V, Noto L, Franchina T, Chiofalo G, Picciotto M, Toscano G, et al. Emerging targeted therapies for castration-resistant prostate cancer. Frontiers in endocrinology. 2012;3:73-.
7. Estebanez-Perpina E, Arnold AA, Nguyen P, Rodrigues ED, Mar E, Bateman R, et al. A surface on the androgen receptor that allosterically regulates coactivator binding. Proceedings of the National Academy of Sciences of the United States of America. 2007;104:16074-9.
8. De Leon JT, Iwai A, Feau C, Garcia Y, Balsiger HA, Storer CL, et al. Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2011;108:11878-83.
9. Grosdidier S, Carbo LR, Buzon V, Brooke G, Nguyen P, Baxter JD, et al. Allosteric Conversation in the Androgen Receptor Ligand-Binding Domain Surfaces. Molecular Endocrinology. 2012;26:1078-90.
10. Tavassoli P, Snoek R, Ray M, Rao LG, Rennie PS. Rapid, non-destructive, cell-based screening assays for agents that modulate growth, death, and androgen receptor activation in prostate cancer cells. Prostate. 2007;67:416-26.
11. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. Journal of Clinical Oncology. 2003;21:383-91.
12. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, et al. The LNCaP cell line--a new model for studies on human prostatic carcinoma. Progress in clinical and biological research. 1980;37:115-32.