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TITLE: Super-Penetrant Androgen Receptor: Overcoming Enzalutamide Sensitivity in Castration-Resistant Prostate Cancer

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14. ABSTRACT Prostate cancer cells rely critically on the androgen receptor (AR) for initiation, growth and progression to castration resistant prostate cancer (CRPC) - providing a compelling molecular basis for it as a therapeutic target. Enzalutamide or MDV3100, an AR antagonist, is now routinely used for the treatment of CRPC patients. Enzalutamide impedes both the nuclear translocation and chromatin recruitment of AR. However, recent studies reveal that while Enzalutamide provides palliative benefits, even the most responding patients relapsed within ~2 years. AR, being a versatile transcriptional co-activator, interacts with critical proteins to adapt rapidly to androgen deprivation. In recent years, distinct AR modifications, have garnered considerable attention, primarily due to their direct correlation with pathogenic AR activation in CRPCs. Notably, some of these mechanisms confer both androgen-independence and anti-androgen resistance to PC cells -an underlying basis for their clinical association with hormone refractory and metastatic prostate cancers. The hypothesis is that CRPCs could endow 'super-penetrance' to AR, via camouflaging it with post-translational marks. Modified AR can translocate to the nucleus in the presence of Enzalutamide due to its tight association with the modifying enzymes. Subsequently, <i>super-penetrant phosphoAR</i> , is recruited to the chromatin in an androgen independent manner to regulate distinct transcription program, making CRPC cells resistant to Enzalutamide treatment. Importantly, this proposal will use an innovative chemical proteomics- mass spectrometry based method to uncover novel targets and inhibitors to overcome Enzalutamide resistance of CRPCs. The objectives are; First, examine whether modifications in AR promote differential association with Enzalutamide. Second, determine whether Enzalutamide-bound phosphoAR translocates to the nucleus and is recruited to the AR- target gene promoters. Finally, evaluate whether tyrosine kinase inhibitors synergize with Enzalutamide to inhibit CRPC xenograft growth.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
4. Impact.....	5
5. Changes/Problems.....	6
6. Products.....	6
7. Participants & Other Collaborating Organizations.....	7
8. Special Reporting Requirements.....	8
9. Appendices.....	8

INTRODUCTION

Prostate cancers (PC) rely critically on the androgen receptor (AR) for initiation, growth and progression to a highly metastatic stage called as castration resistant prostate cancer (CRPC)-providing a molecular basis as an amenable therapeutic target. Not surprisingly, AR blockers, the anti-androgens such as Bicalutamide (Casodex) or Flutamide (Eulexin), have emerged to be a preferred therapeutic option in advanced PCs and have provided patients with a desperately needed relief. However, their short-term affectivity soon became evident; PC developed resistance, often within 24 months, progressing to the metastatic and highly lethal CRPC stage. In this bleak scenario, a second generation anti-androgen, Enzalutamide or MDV3100 (Xtandi), has been introduced for CRPC patients (1). Enzalutamide binds the ligand binding domain of the AR and obstructs binding of AR to the chromatin. Significantly, unlike earlier anti-androgens, Enzalutamide impedes the nuclear translocation of AR (1). Although highly effective, as seen by significant decrease in serum PSA levels, Enzalutamide provided palliative benefits only in a subset of CRPC patients (12 out of 30 patients) (1) and often relapsed within ~2 years (2). How prostate cancer cells circumvent AR inhibition has now become one of the most vexing problem in prostate cancer therapy. AR, a versatile transcriptional co-activator, seems to adapt to androgen-deprived environment by distinct modifications. This proposal will use an innovative chemical proteomics-mass spectrometry method to uncover novel targets that modify AR to mediate enzalutamide resistance.

Rationale/Hypothesis: The hypothesis is that **CRPCs could endow 'super-penetrance' to AR, via camouflaging it with post-translational marks such as tyrosine-phosphorylation.** Modified AR, now Enzalutamide-bound, 'piggybacks' activated NRTKs to the nucleus and allows PCs to overcome enzalutamide mediated inhibition of AR nuclear translocation. Subsequently, *super-penetrant AR* is recruited to the chromatin in an androgen independent manner to regulate distinct transcription program, making CRPC cells resistant to Enzalutamide treatment.

KEYWORDS:

AR (Androgen Receptor); CRPC (Castration resistant prostate cancer; Enzalutamide, Src, resistance, Xtandi, ACK1/TNK2, Androgen deprivation therapy.

OBJECTIVES/GOALS:

- (1) Examine whether modifications in AR promote differential association with Enzalutamide.
- (2) Determine whether Enzalutamide-bound AR translocates to the nucleus and is recruited to the AR- target gene promoters.
- (3) Evaluate whether tyrosine kinase inhibitors synergize with Enzalutamide to inhibit CRPC xenograft growth.

ACCOMPLISHMENTS: As described below the major accomplishments were (a) the development of enzalutamide resistant C42B metastatic prostate cancer cell line and (b) the identification of a novel AR acetylation site (K609) in the DNA binding domain of the AR in Enzalutamide resistant metastatic CRPC cells. Future studies may reveal whether this novel acetylation site can retain functionality of the androgen receptor even in the presence of antiandrogens and the clinical significance of this modification.

Experimental Results

Aim 1. Examine whether modifications in AR promote differential association with Enzalutamide

An unbiased chemical proteomic analysis was performed to determine whether modified AR promotes differential association with Enzalutamide.

Methodology Briefly, drug affinity chromatography technology enabled by downstream mass spectrometry (LC-MS/MS) was utilized to evaluate whether AR that

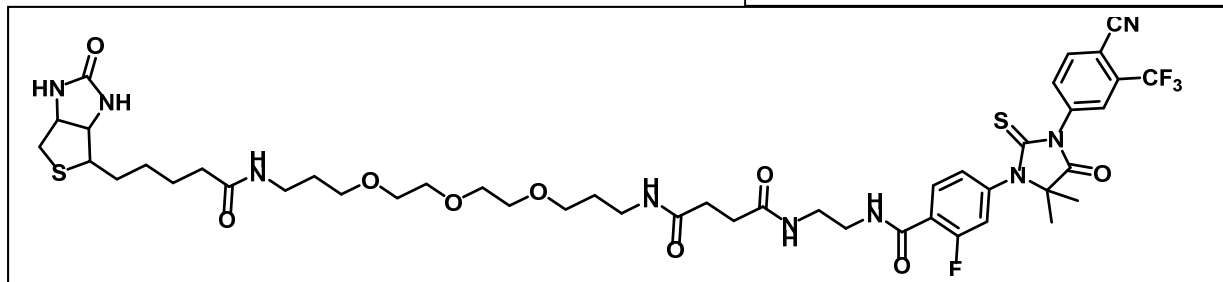
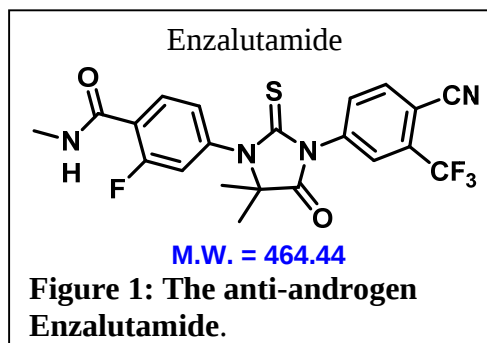


Figure 2. The methyl group of the Enzalutamide (**Figure 1**) was replaced by a PEG linker with a terminal amine attached to biotin so that the bound proteins can be captured on the streptavidin coated sepharose beads.

is bound to Enzalutamide is post translationally modified. As a first step we attached a tether, suitable for securing Enzalutamide. The methyl group of the compound Enzalutamide (**Figure 1**) was replaced by a PEG linker with a terminal amine attached to biotin so that the bound proteins can be captured on the streptavidin coated sepharose beads (**Figure 2**), at the final drug concentration of 25 nmol/50 μ l beads. The Enzalutamide-biotin conjugate bound streptavidin sepharose beads (beads linked to another inhibitor DZ1-067-PEG linker, as negative control) were incubated with the nuclear lysates prepared from prostate cancer cells, LNCaP, LAPC4 and VCaP. Beads were washed and bound proteins were eluted run on SDS PAGE gels, the bands were cut out followed by mass spectrometry.

Results from Mass spectrometry analysis

We searched the mass spectrometry data for phosphorylation, ubiquitination and acetylation of AR protein and the only possible post-translational modification (PTM) we identified from the sample is K609 acetylation of the androgen receptor in the LAPC4 cells sample. The intact peptide measurement mass was very accurate (m/z ratio of 694.8545 and 0.72 ppm) (**Figure 3**). This acetylation site mapped to the DNA binding domain of AR.

Aim 2. Determine whether Enzalutamide-bound AR translocates to the nucleus and is recruited to the AR-target gene promoters One mechanism by which Enzalutamide antagonizes AR transcriptional activity is attributed to impaired nuclear translocation and significant decrease in AR target gene expression due to the inhibition of chromatin binding.

Methodology To evaluate AR cellular distribution, we developed an enzalutamide resistant cell line of the metastatic castration resistant prostate cancer (CRPC) cell line C42B by long term propagation in the presence of enzalutamide. The C42B initially had an IC_{50} of 1.14 μ M for Enzalutamide and were the most sensitive of all prostate cancer cell lines tested (LNCaP, LAPC4, 22RV1 and RWPE) by Cell titre Glo analysis (Promega). However, by culturing them in the presence of enzalutamide these cells are able to thrive in presence of up to 25 μ M of enzalutamide.

Results: To determine the effect of enzalutamide on AR translocation on C42B and C42B ENZ resistant cells,

we treated cells with either DMSO control or 10 μ M of Enzalutamide for 24 hours and fractionated the cells into nuclear and cytosolic fractions. The protein extracts were immunoblotted with AR and Actin antibodies. Interestingly, the translocation of AR into the nucleus was not impeded in naïve C42B cells exposed to Enzalutamide. In contrast, AR translocation was significantly suppressed in C42B enzalutamide resistant cells cultured long term in the presence of enzalutamide. These results suggest that castration resistant prostate cancer cells required much higher concentrations of enzalutamide and long term exposure to the AR antagonist to be effective in restraining AR in the nucleus.

Next, we performed quantitative RT-PCR to analyze expression of AR its target gene PSA in C42B and C42B-ENZ resistant cells as a more sensitive readout for the effect of enzalutamide on AR expression and functionality in metastatic castration resistant prostate cancer cell lines. We found that AR mRNA expression was increased two-fold in the presence

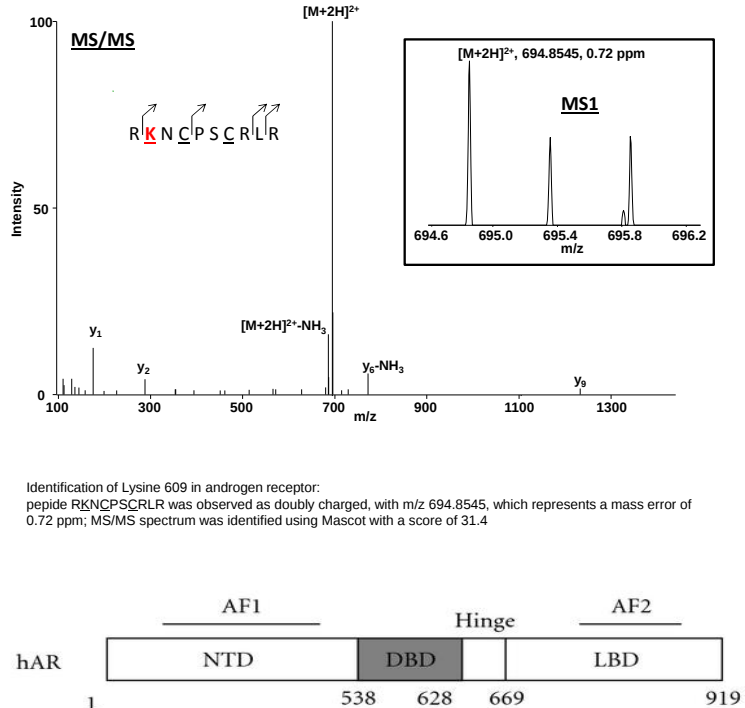


Figure 3: Identification of a novel acetylated Lysine 609 residue in the DNA binding domain (DBD) of the androgen receptor in the presence of Enzalutamide AR peptide RKNCPSCRLR was observed as doubly charged, with m/z 694.8545, which represents a mass error of 0.72 ppm; MS/MS spectrum was identified using Mascot with a score of 31.4.

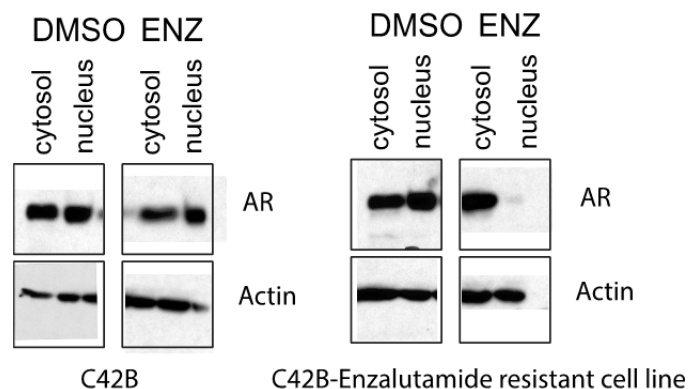


Figure 4: AR translocation to the nucleus is impeded in cells that are resistant to Enzalutamide C42B or C42B enzalutamide resistant cells were treated with enzalutamide and nuclear cytosolic fractionation was performed. Immunoblot was performed with AR and Actin antibodies as control.

of enzalutamide while PSA expression was downregulated in C42B ENZ resistant cells but not completely abolished, suggesting that AR is continued to be recruited to chromatin despite Enzalutamide exposure (**Figure 5**).

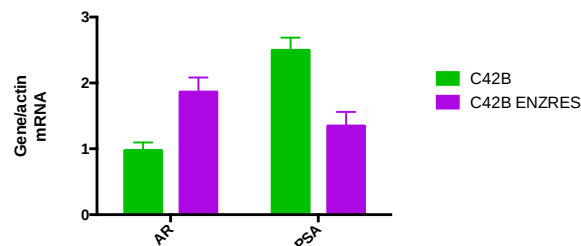


Figure 5: AR mRNA expression is increased in Enzalutamide Resistant Cells AR and PSA levels were quantitated in C42B and C42B Enz resistant cells. Actin was used as a normalization control.

Aim 3. Evaluate whether tyrosine kinase inhibitors synergize with Enzalutamide to inhibit CRPC xenograft growth. We did not detect any tyrosine phosphorylation in the androgen receptor by mass spectrometry. It is well known that tyrosine phosphorylations are often transient, represent less than 1% of total protein and are often difficult to determine by mass spectrometry.

Methodology To ascertain conclusively whether tyrosine kinase inhibitors synergize with Enzalutamide to inhibit CRPC xenograft growth, mice were injected with Enzalutamide & Sarcatinib (both 25 mg/Kg) or Enzalutamide & (R)9b- -Ack1 inhibitor (both 25 mg/Kg) at suboptimal concentrations. Comparison with Enzalutamide alone was done to determine whether non-receptor tyrosine kinase inhibitors synergize with Enzalutamide to eliminate CRPC xenograft growth. The results from these experiments revealed opposing effects of two kinase inhibitors. While the Src tyrosine kinase selective inhibitor, Saracatinib (IC₅₀ 2.7nm) potentiated castration resistant growth of VCaP cells and did not synergize with Enzalutamide, the Ack1 selective inhibitor, (R)9b was effective in restraining CRPC tumor growth alone and synergized with Enzalutamide to completely inhibit the growth of the cancer cells (**Figure 6 and 7**). Knockdown of ACK1 was not very effective in inhibiting CRPC growth suggesting that it could be attributed to the transient nature of repression (**Figure 8**).

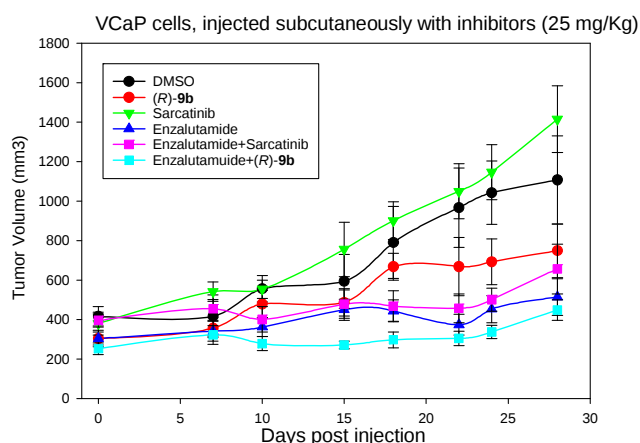


Figure 6: ACK1 inhibitor synergizes with Enzalutamide to inhibit CRPC tumor growth. VCaP cells were either treated individually or in combination with the inhibitors (inset) control.

Conclusions

(a) At this time, it is not known how the K609 acetylation of the androgen receptor affects its transcriptional coactivator function or its DNA binding property or whether it influences AR translocation to the nucleus in the presence of Enzalutamide. However, future studies will be aimed at addressing this aspect and establishing the clinical relevance of this modification in conferring enzalutamide resistance in patient samples, once we have validated and characterized the newly generated ARK609 acetylation antibodies.

(b) The results from this in vivo experiments suggest that on successful completion of this proposal, it would not only divulge why certain subset of CRPC patients may be insensitive to Enzalutamide but also opens the possibility wherein ACK1 inhibitors could be combined with Enzalutamide for better therapeutic efficacy.

What opportunities for training and professional development has the project provided?

As a result of the DOD exploratory hypothesis award and long term research productivity, Dr. Kiran Mahajan had advanced career development as a tenure track Assistant Professor. It also provided opportunity as a speaker at the Florida Prostate Cancer Symposium.

How were the results disseminated to communities of interest?

"Nothing to Report at this time"- Eventually will submit a manuscript and give presentations.

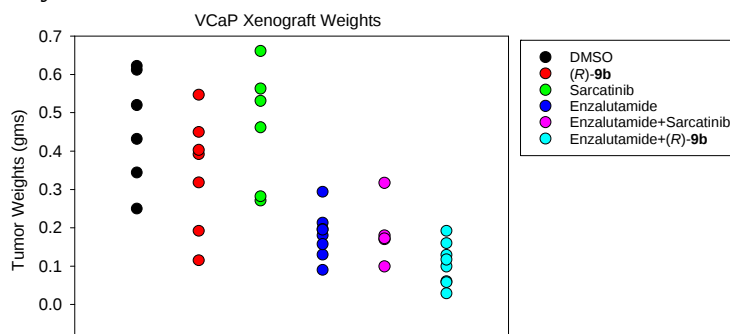


Figure 7: ACK1 inhibitor synergizes with Enzalutamide to inhibit CRPC tumor growth. VCaP cells were either treated individually or in combination with the inhibitors (inset) control and tumor weights were taken at the end of the experiment. The Enzalutamide + (R)9b combination was most effective in inhibiting tumor growth.

What do you plan to do during the next reporting period to accomplish the goals?

This is a one year project

IMPACT

The major impact is the identification of a previously unknown AR acetylation site (K609) in the DNA binding domain of the Androgen Receptor in Enzalutamide resistant metastatic castration resistant prostate cancer cells. Future studies may reveal whether this novel acetylation site is important to maintain the transcriptional coactivator activity of the androgen receptor even in the presence of antiandrogens and the clinical significance of this modification and whether prostate cancers acquire this modification during the course of development of resistance.

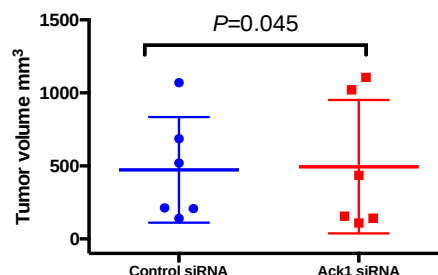


Figure 8: ACK1 RNA interference has only partial effect on inhibiting CRPC growth .

In addition, the novel monoclonal antibodies developed against the AR acetylation site may be used to screen primary patient samples acquired before and after androgen deprivation therapies, to determine if this is a significant mechanism of acquisition of enzalutamide resistance. Future studies may also reveal whether K609 may serve as biomarker of enzalutamide resistance.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Not significant, but a change was made to use SCID mice instead of nude mice and ACURO approval was taken for the amendment.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS

Publications, conference papers, and presentation:

Journal publications

Mahajan K, Malla P, Lawrence H, Chen Z, Sinha CK, Malik R, Shukla S, Kim J, Coppola D, Lawrence N and Mahajan NP. Epigenetic Reprogramming of Androgen Receptor by Histone H4 Tyr88-phosphorylation Promotes Castration Resistance. *Cancer Cell*, 2016. (Under Revision).

Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications.

Nothing to report

Presentations.

1. Session Chair and Speaker-Dr. Nupam Mahajan (Co-investigator), at the Florida Prostate Cancer Research Symposium- *Prostate Cancer Epigenetic Reprogramming of the Androgen Receptor in Castration Resistant Prostate Cancer*, May19-20, 2016.
2. Speaker- Kiran Mahajan (Principal Investigator), Moffitt Cancer Center, Tumor Biology Department Retreat, Tampa, Florida, *Recalcitrant cancer, cancer stem cells and targeted therapies*, Dec 4, 2015.
3. Speaker-Kiran Mahajan Moffitt Cancer Center, Tumor Biology Research In Progress, Tampa, Florida *Exiting the Comfort Zone: Tackling the Complexity of Castration Resistant Prostate Cancer*, Nov 3, 2015

4. Speaker-Kiran Mahajan, Moffitt Cancer Center, Drug Discovery Group Seminar Series, Tampa, Florida, *Epigenetic Underpinnings of the DNA damage response pathways*, June 23, 2015.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

1. Enzalutamide resistant C42B metastatic prostate cancer cell line.
2. Generated antibodies for the novel Androgen Receptor Acetylation site.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	<i>Kiran Mahajan</i>
Project Role:	<i>Assistant Professor</i>
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	<i>Dr. Mahajan has performed animal experiments and cell culture studies</i>
Funding Support:	NA.
Name:	<i>Nupam Mahajan</i>
Project Role:	<i>Assistant Professor</i>
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3
Contribution to Project:	<i>Dr. Nupam Mahajan has performed the pull down studies with the Enzalutamide linked sepharose beads and control beads.</i>
Funding Support:	NA
Name:	<i>Niveditha Nerlakanti</i>

Project Role:	<i>Research Associate</i>
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	7
Contribution to Project:	<i>Niveditha performed western blotting and quantitative RT-PCR experiments</i>
Funding Support:	NA

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

APPENDICES

Nothing to Report.

References

1. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* (New York, NY. 2009;324(5928):787-90. PubMed PMID: 19359544.
2. Bennett LL, Ingason A. Enzalutamide (Xtandi) for patients with metastatic, resistant prostate cancer. *The Annals of pharmacotherapy*. 2014;48(4):530-7. Epub 2014/01/25. doi: 10.1177/1060028013518899. PubMed PMID: 24458946.