Aqila Ahmed ORCID iD: 0000-0003-2337-5465

Targeted Notch1 inhibition with a Notch1 antibody, OMP-A2G1, decreases tumor growth in two murine models of prostate cancer in association with differing patterns of DNA damage response gene expression.

Aqila A. Ahmed[1], Tyler Robinson[1], Monica Palande[1], June Escara-Wilke[1], Jinlu Dai[1], Evan T. Keller[1,2*]

[1]Department of Urology and University of Michigan, Ann Arbor, Michigan.

[2]Biointerfaces Institute, University of Michigan, Ann Arbor, Michigan.

[*]Correspondence Evan T. Keller Department of Urology and University of Michigan, Ann Arbor, Michigan. Email etkeller@umich.edu

Abstract

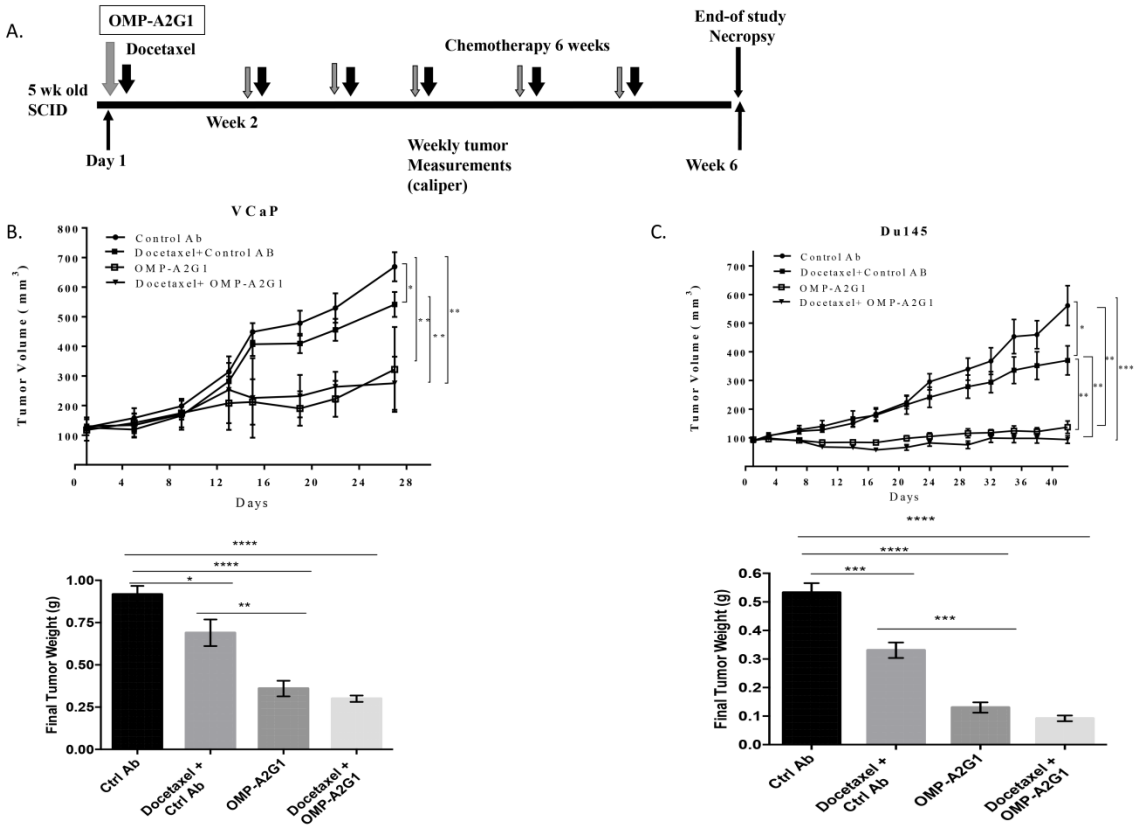
Notch plays a pro-tumorigenic role in many cancers including prostate cancer (PCa). Global notch inhibition of multiple Notch family members using γ-secretase inhibitors (GSI) has shown efficacy in suppressing PCa growth in murine models. However, global Notch inhibition is associated with marked toxicity due to the widespread function of many different Notch family members in normal cell physiology. Accordingly, in the current study we explored if specific inhibition of Notch-1 would effectively inhibit PCa

This is the author manuscript accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jcb.28954.

growth in a murine model. The androgen-dependent VCaP and androgen-independent DU145 cell lines were injected subcutaneously into mice. The mice were treated with either control antibody 1B7.11, anti-Notch1 antibody (OMP-A2G1), docetaxel or the combination of OMP-A2G1 and docetaxel. Tumor growth was measured using calipers. At the end of the study, tumors were assessed for proliferative response, apoptotic response, Notch target gene expression and DNA damage response (DDR) expression. OMP-A2G1 alone inhibited tumor growth of both PCa cell lines to a greater extent than docetaxel alone. There was no additive or synergistic effect of OMP-A2G1 and docetaxel. The primary toxicity was weight loss that was controlled with dietary supplementation. Proliferation and apoptosis were affected differentially in the two cell lines. OMP-A2G1 increased expression of the DDR gene GADD45α in VCaP cells, but downregulated GADD45α in Du145 cells. Taken together, these data show that Notch-1 inhibition decreases PCa xenograft growth but does so through different mechanisms in the androgen dependent VCaP cell line versus the androgen independent DU145 cell line. These results provide a rationale for further exploration of targeted Notch inhibition for therapy of PCa.

Graphical Abstract

We examined whether specific inhibition of Notch1, as opposed to global inhibition of multiple Notch molecules, would effectively inhibit PCa growth in a murine model. We showed that anti-Notch1 (OMP-A2G1) treatment in androgen-dependent VCaP and androgen-independent DU145 xenograft were effective in reducing tumor growth through different mechanisms.



Keywords: Prostate Cancer, Notch signaling, Notch1, DNA damage response, Hormone sensitivity

Introduction

Notch is an evolutionarily conserved pathway with roles in various cellular processes including proliferation, apoptosis, migration, growth and differentiation. Notch signaling is mediated by four receptors (Notch 1–4) and five ligands (Delta-like 1, 3, and 4 & Jagged-1 and 2)(Su & Xin, 2016). The transmembrane surface receptors (Notch1-4) on one cell interact with membrane-bound ligands expressed on the surface of an adjacent cell. Through a series of proteolytic events mediated by γ-secretase, the notch intracellular domain (NICD) is released from the interior cell membrane into the cytoplasm and translocates into the nucleus, where it participates in a core transcriptional complex that turns on target genes. Notch signaling is deregulated during initiation and progression of prostate cancer (PCa) (Bertrand, McCubrey, Angus, Nutter, & Sigounas, 2014).

(PCa) is the most common cancer of men. Proliferation, maintenance and function of prostate cells is regulated by a number of signaling pathways, including Notch, DNA damage response (DDR) and the androgen receptor (AR). In both clinical samples and in cell culture, multiplicity in AR, Notch and DDR activity has been shown to impact PCa biology and therapy response. It has been demonstrated that the androgen signaling pathway and Notch pathway can regulate each other in PCa. Yu and colleagues showed that in PCa patients, Jagged1 expression correlates with cancer progression in a process mediated through AR overexpression (Y. Yu et al., 2014). Another study reported that Hey1, a transcription factor activated by Notch1, repressed AR-dependent transcriptional activity (Belandia et al., 2005). However, this repression is absent in PCa tumors, due to exclusion of Hey1 from the nucleus in tumor cells, suggesting that progression to

aggressive AR-independent PCa could in part be due to nuclear exclusion of repressors (Belandia et al., 2005). Therefore, further understanding the interplay between AR and Notch signaling in PCa may uncover additional mechanisms through which Notch contributes to PCa progression.

Previously, we explored the impact of global Notch inhibition using a γ-secretase inhibitor (GSI) on two PCa hormone insensitive cell lines (PC3 and Du145). The GSI induced a combination of decreased proliferation, induced apoptosis, and blocked tumor angiogenesis (D. Cui et al., 2015). There are several drawbacks to use of global Notch inhibition including marked toxicity and targeting multiple Notch receptors as opposed to those specifically shown to be oncogenic. This is of particular therapeutic relevance as Notch can function either as tumor suppressive or oncogenic mediator in PCa (Carvalho, Simons, Eberhart, & Berman, 2014). Hence, targeting specific Notch pathway molecules that have pro-tumor promoting activity, while sparing Notch pathway molecules that have anti-tumor activity may provide a therapeutic benefit. Therefore, in this study, we investigated the therapeutic efficacy of anti-Notch1 monoclonal antibody on both hormone-independent and dependent PCa xenografts.

Methods

Antibodies:

OMP-A2G1(Anti-Notch1) and Control antibody 1B7.11 (against dinitrophenol) monoclonal antibody were provided by OncoMed Pharmaceuticals.

Cell lines and Cell culture condition:

Human prostate cancer cell lines Du145 was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI 1640 (Invitrogen Co., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Inc.)(Sampson, Neuwirt, Puhr, Klocker, & Eder, 2013; S.-Q. Yu et al., 2009). VCaP cells (kindly provided by Dr. Kenneth Pienta, University of Michigan, Ann Arbor, MI) were maintained in DMEM (Life Technologies, Inc.)(Korenchuk et al., 2001). with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Inc.). All cultures were maintained at 37°C, 5% CO2, and 100% humidity. Cell identification is completed semi-annually using PCR for short tandem repeats.

Cell Growth Assay:

Du145 and VCaP cells were grown in 96-well plates. Cells were then treated with either GSI(R04), OMP-A2G1 (0.1μg/ml, 1μg/ml, 10μg/ml, 100 μg/ml) or vehicle for 72hrs. Cell proliferation reagent WST-1 was added and incubated at 37°C and 5% CO2 for 4hr. Absorbance was then measured at 440 nm with a SpectraMax M5 plate reader. Data are presented as mean ± SD from triplicate determinations.

Animal Studies:

6-7 week-old male CB17/Icr-Prkdcscid/IcrIcoCrl (SCID) mice (Charles River, Wilmington, MA) were housed under pathogen-free conditions. All experimental protocols were approved by the University of Michigan Animal Care and Use

Committee. For subcutaneous injection, 1×10^6 of Du145 cells and 3×10^6 of VCaP cells were injected subcutaneously into the flank. When cohorts of tumors reached 100-200 mm^3, mice were randomly assigned to control and three treatment groups (13-15 mice per group): (1) Control antibody 1B7.11 (against dinitrophenol; (2) OMP-A2G1 (5mg/kg weekly); (3) Control antibody plus docetaxel (5mg/kg weekly); (4) OMP-A2G1 plus docetaxel (same dosage as above). Tumor size and body weight were measured twice a week. Tumor volume was calculated following the formula: tumor volume V = a $\times b^2\times0.52$, where a is the length of the tumor and b is the width of the tumor (D. Cui et al., 2015).

Western blot analysis:

Du145 and VCaP subcutaneous xenografts were harvested after treatment and stored at −80°C. Tumor tissues were homogenized in ice-cold RIPA lysis buffer (Millipore, Billerica, MA) containing protease inhibitors and phosphatase inhibitors. The protein concentration of tumor extracts was determined using the BCA Protein Assay Kit (Pierce). Target protein expression was analyzed using Western blot analysis, with β-tubulin/GAPDH used as loading controls. Notch1, NICD, GADD45α, and p53 were obtained from Cell Signaling Technology Company (Beverly, MA); β-tubulin and GAPDH were obtained from Sigma Aldrich (St Louis, MO). The antibodies were diluted as recommended by the manufacturers.

Quantitative real-time PCR:

Total RNA was extracted from Du145 and VCaP subcutaneous xenografts using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Using

Superscript III first-strand synthesis system (Invitrogen, Inc.), mRNA was reverse-transcribed into cDNA. SYBR green (Qiagen) was used to amplify cDNA using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Relative quantification was done using the ΔΔCt method, normalizing to GAPDH mRNA.

Immunohistochemistry (IHC):

Subcutaneous tumors were fixed in 10% formalin. Five-micron (5μM) sections were used for H&E and IHC. Ki67 (1:500, Santa Cruz Biotechnology) and cleaved-caspase 3 (1:300, Cell Signaling Biotechnology) were stained as per the manufacturer's protocols. Sections were examined for positive staining and quantified as previously described (D. Cui et al., 2015). Representative fields were photographed under 40× magnification.

Statistical analysis:

Numerical data are expressed as mean ± SD. Statistical analysis was performed by One-way ANOVA for multiple comparisons and Student's t-test for independent analysis. Differences with $P < 0.05$ were determined as statistically significant.

Results:

Anti-notch1 antibody decreases proliferation of hormone-sensitive prostate cancer cells

We first explored baseline gene expression levels of the Notch ligands, receptors and Notch pathway activation in several prostate cancer cells. Initially, RT-PCR analysis was

performed revealing a varied expression of among the PCa cell lines. Downstream target genes of Notch pathway were higher in VCaP compared to the other two cell lines (Fig. 1A). In terms of protein expression, Notch1 was highest in DU145 and lowest in VCaP; whereas, it was expressed at various levels among the other prostate cancer cell lines (Fig. 1B). These results confirm previous work that VCaP cells express Notch1 protein (Litvinov et al., 2006) as did DU145 cells (Shou et al., 2001; Zayzafoon et al., 2004; Patrawala et al., 2006). We next explored if Notch-1 inhibition impacted growth of the PCa cells lines. First we treated cells with the GSI R04 for 72 hours to determine the impact of global Notch inhibition on these cells. R04 inhibited overall cell growth of both cell lines in a dose-responsive fashion (Fig. 1C). We next determined the impact of specific inhibition of Notch1 on prostate cancer cell growth. Accordingly, we cultured the prostate cancer cells with increasing doses of anti-Notch-1 monoclonal antibody OMP-A2G1 for 72 hours. OMP-A2G1 reduced cell growth of both cell lines in a dose responsive fashion with more of an inhibitory impact VCaP compared to DU145 (Fig. 1D). The response in VCaP with low Notch1 expression compared to Du145 supports the efficacy of OMP-A2G1. These results indicate that Notch1 can promote growth of both androgen-sensitive and insensitive PCa cells.

Notch1 inhibits the growth of both hormone-dependent and independent xenografts

To investigate the potential therapeutic efficacy of OMP-A2G1 in vivo we subcutaneously injected VCaP and Du145 into mice and allowed tumors to become established to a volume range of 100-200mm^3. At this point, mice were randomized into four treatment groups consisting of control antibody 1B7.11 (against dinitrophenol),

control antibody plus docetaxel, OMP-A2G1 alone or OMP-A2G1 plus docetaxel and treated as indicated in Figure 2A over 6-week period. Docetaxel inhibited growth of VCaP xenografts compared to the control group (Fig. 2B). OMP-A2G1 inhibited growth of VCaP xenografts to a greater extent than docetaxel alone did. The combination of docetaxel and OMP-A2G1 had an inhibitory effect similar to that of OMP-A2G1 alone indicating that there was no additive or synergistic effect for these two compounds. These growth effects are reflected in the final tumor weights of all four groups of VCaP xenografts (Fig. 2B). OMP-A2G1 had a similar impact on the Du145 xenografts with OMP-A2G1 inhibiting tumor growth more than docetaxel (Fig. 2C). Taken together, these results indicate that OMP-A2G1 has a strong anti-tumor effect; however, there was no additive or synergistic activity between docetaxel and OMP-A2G1. OMP-A2G1 primary toxicity was weight loss, which was controlled with dietary supplementation.

Anti-Notch1 exerts mixed effects

To determine if Notch1 inhibition mediates its anti-tumor effect through modulating proliferation and/or apoptosis in vivo we assessed expression of Ki67 (proliferation marker) and cleaved-caspase 3 (apoptosis marker) in the VCaP and DU145 xenografts. None of the solitary or combination treatments had an impact on the number of cells expressing Ki67 in the VCaP xenografts (Fig. 3A). In contrast, OMP-A2G1 and docetaxel alone increased the number of cleaved-caspase 3 positive cells; however, the combination of OMP-A2G1 and docetaxel had no impact on the number of cleaved-caspase 3 positive cells. In VCaP xenograft, OMP-A2G1 and docetaxel alone had no impact on the number of cells expressing Ki67; although, the combination of both

decreased the proportion of Ki67 positive cells in the Du145 xenografts (Fig. 3B). Furthermore, none of the individual or combination treatments had an impact on the number of cells expressing Caspase 3.

The response of Notch target genes to Notch-1 varies in xenografts

To determine the effect of Notch1 inhibition on Notch pathway-related genes in the tumor tissues, we subjected the VCaP and Du145 xenografts to western blot analysis. In response to OMP-A2G1 antibody alone, VCaP xenografts did not show a decrease of NICD protein expression (Fig. 4A); whereas, the Notch target, Hes1 mRNA expression was significantly decreased (Fig. 4C). In contrast in DU145, the OMP-A2G1 antibody alone decreased NICD protein expression, but not Hes1 mRNA expression (Fig. 4B and 4D). The combination of docetaxel and OMP-A2G1 did significantly decrease Hes1 mRNA expression in Du145 xenografts (Fig. 4D). We looked at additional Notch1 targets such as Hes6, Hey1, and Notch3. In VCaP xenografts, OMP-A2G1 treatment led to a downregulation trend in these transcripts; whereas, there was no impact on them in DU145 cells (data not shown). Taken together, these results indicate that OMP-A2G1 antibody impacts Notch1-mediated signaling in these PCa cells and this might be by different mechanisms.

Intrinsic PCa cell line properties influences response to Notch-1 inhibition

We next explored why the two different PCa cell lines demonstrated differential responses to OMP-A2G1 therapy. Notch signaling is involved in a wide range of cellular processes and any one of these can be altered depending on the state of cell. We focused on the underlying differences between our models. VCaP is AR and ERG positive with a

functional mutated p53 protein (Carroll, Voeller, Sugars, & Gelmann, 1993; Chappell et al., 2012; Mohamed et al., 2017) while DU145 is both AR and ERG negative with a nonfunctional p53 possible (Carroll et al., 1993). Mohamed et al. reported synergy between Notch and AR inhibition in VCaP but not DU145. VCaP cells had decreased cell growth, cell survival, and enhanced apoptosis in response to Notch and the AR inhibitor bicalutamide while no change was observed in AR negative DU145 cells (Mohamed et al., 2017). DU145, showed no change in protein expression after treatment with Notch and AR inhibitors. Another inherent difference is the p53 status of VCaP and DU145. DU145 harbors mutant Leu-223 and Phe-274 that renders p53 nonfunctional, while VCaP (mutant p53, Trp-248) has been shown to have a functional p53 protein (Carroll et al., 1993; Chappell et al., 2012). Carroll et al. showed that the DNA damage response (DDR) in PCa cell lines was dependent on p53 status and that alteration of this pathway is vital in the survival of advanced PCa cells during exposure to anticancer therapies, specifically agents that induce DNA damage. Since DDR is dependent on p53 functional status, which is different between VCaP and DU145 cells, we evaluated the effect of OMP-A2G1 on DDR. To perform this, we utilized an array consisting of genes involved in Ataxia telangiectasia mutated and RAD3-related (ATM/ATR) signaling, DDR, apoptosis and cell cycle. Equal amounts of RNA from each xenograft was characterized for same 84 genes involved in DNA damage signaling pathways. VCaP tumors treated with OMP-A2G1 had dysregulation of genes involved in cell cycle and apoptosis (Fig. 5A). In contrast, DU145 did not show any dysregulated genes in common with VCaP but had dysregulation in several DDR genes (Fig. 5B). Although the dysregulated genes were different among VCaP and DU145 cells, they have a p53 pathway in common. Notch

signaling has been shown to regulate p53 expression via both Hey1 and Hes1 (Huang et al., 2004) as well as DDR (Vermezovic et al., 2015). Accordingly, we explored for an impact of Notch-1 inhibition on p53 and Gadd45α, which is a downstream target of p53 in DDR. In VCaP xenografts, OMP-A2G1 downregulated p53 expression (Fig. 5B). This was associated with an increase in downstream target Gadd45α. In contrast, in DU145 cells, which lack a functional p53, OMP-A2G1 had no impact on p53 expression, but did decrease Gadd45α (Fig. 5C). These results suggest that p53 function could contribute to a differential impact of Notch-1 inhibition in PCa cells.

Discussion

Notch signaling is multifaceted and is known to associate with multiple processes including p53 and androgen signaling pathways. In our study, we evaluated the response to OMP-A2G1 in androgen-dependent and independent cells. OMP-A2G1 reduced growth of both DU145 and VCaP xenografts. The overall impact of OMP-A2G1 on cellular mechanisms (i.e., decreased proliferation or increased apoptosis) accounting for the decreased tumor growth appeared different between the DU145 and VCaP xenografts. Specifically, while VCaP showed a significant upregulation in cleaved-caspase 3, DU145 xenografts showed no difference between treatment groups. Both cell lines have inherent differences not only in AR but ERG and p53 expression, which may account for the differential response. Canonical and non-canonical Notch are known to crosstalk with AR and p53 (Chappell et al., 2012; Haffner et al., 2010; Leong & Gao, 2008; Thompson & Li, 2017; Vermezovic et al., 2015). Considering all of these, we took a closer look at p53 and DDR and showed differences in response between the xenografts exposed to OMP-

A2G1. Gadd45α, a pro-apoptotic and p53-dependent DDR protein was upregulated in VCaP xenografts in response to OMP-A2G1. Recent work has attributed GADD45α upregulation to VCaP expression of fusion protein TMPRSS2-ERG (Chatterjee et al., 2015). DU145 cells express mutant nonfunctional p53 that causes a defective G_1 checkpoint (Chappell et al., 2012) which can account for the lack of Gadd45α upregulation upon OMP-A2G1 treatment. These results are supportive of the possibility that DNA damage–induced p53 contributes to the increased apoptosis seen in VCaP when Notch1 is inhibited. Loss of p53 function can relieve apoptosis and lead to growth advantage (Lin et al., 2018). It's been reported in multiple cancers that p53 loss is synergistic with Notch1 expression (Carvalho et al., 2015; Chanrion et al., 2014; Deng et al., 2016; Qiu et al., 2018; Zhang et al., 2017)

Given that Notch1 has a spectrum of activities, our data suggest that Notch1 inhibition can impede PCa growth through various mechanisms such as DDR, AR and p53 signaling. Recent work on Notch signaling and DDR shows that Notch1 can displace key DDR proteins and alter the response to damage (Vermezovic et al., 2015). The crosstalk between AR and Notch1 is also crucial in the response to Notch inhibition. Downstream targets of Notch signaling Hey1, Hey2 and HeyL, were shown to repress AR signaling (Belandia et al., 2005; Lavery et al., 2011). Our data shows an upregulation of AR in anti-Notch1 treated VCaP tumors (data not shown). Mohamed et al., showed that Notch1 and Notch2 have ERG binding sites upstream of promoters in the TMPRSS2-ERG positive VCaP but not DU145. They also show that dual AR and Notch inhibition repressed proliferation and increased apoptosis in VCaP but not DU145. Kron et al. recently showed that TMPRSS2-ERG fusion activates Notch signaling in primary

PCa. Furthermore, Notch inhibition was shown to overcome resistance to androgen deprivation in PCa cells, strengthening the link between Notch and AR signaling (J. Cui et al., 2018).

VCaP AR expression may explain the lack of difference in proliferation of OMP-A2G1 treated tumors. Another possibility is the ERG expression present in VCaP but not DU145. Mohamed et al. showed that AR and Notch inhibition delivered significant increase in apoptotic cells and decrease in cell growth and cell survival in VCaP, while DU145 showed no difference (Mohamed et al., 2017).

In summary, our study demonstrates that specific Notch1 inhibition reduces PCa xenograft growth. The data indicates the anti-tumor effects may be associated with DDR and AR status. These results suggest that further exploration of targeted Notch inhibition in combination with DNA damaging agents and AR inhibitors may lead to promising PCa therapies.

Acknowledgements:

We thank Tim Hoey and Oncomed for provision of OMP-A2G1 and 1B7.11 antibodies. This work was supported in part by NIH CA093900. No authors have conflict of interest.

References:

Belandia, B., Powell, S. M., García-Pedrero, J. M., Walker, M. M., Bevan, C. L., & Parker, M. G. (2005). Hey1, a Mediator of Notch Signaling, Is an Androgen Receptor Corepressor. *Mol Cell Biol, 25*(4), 1425-1436. doi:10.1128/MCB.25.4.1425-1436.2005

Bertrand, F. E., McCubrey, J. A., Angus, C. W., Nutter, J. M., & Sigounas, G. (2014). NOTCH and PTEN in prostate cancer. *Adv Biol Regul, 56*, 51-65. doi:10.1016/j.jbior.2014.05.002

Carroll, A. G., Voeller, H. J., Sugars, L., & Gelmann, E. P. (1993). p53 oncogene mutations in three human prostate cancer cell lines. *Prostate, 23*(2), 123-134.

Carvalho, F. L., Marchionni, L., Gupta, A., Kummangal, B. A., Schaeffer, E. M., Ross, A. E., & Berman, D. M. (2015). HES6 promotes prostate cancer aggressiveness independently of Notch signalling. *J Cell Mol Med, 19*(7), 1624-1636. doi:10.1111/jcmm.12537

Carvalho, F. L., Simons, B. W., Eberhart, C. G., & Berman, D. M. (2014). Notch signaling in prostate cancer: a moving target. *Prostate, 74*(9), 933-945. doi:10.1002/pros.22811

Chanrion, M., Kuperstein, I., Barrière, C., El Marjou, F., Cohen, D., Vignjevic, D., . . . Robine, S. (2014). Concomitant Notch activation and p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. *Nature Communications, 5*, 5005. doi:10.1038/ncomms6005 https://www.nature.com/articles/ncomms6005#supplementary-information

Chappell, W. H., Lehmann, B. D., Terrian, D. M., Abrams, S. L., Steelman, L. S., & McCubrey, J. A. (2012). p53 expression controls prostate cancer sensitivity to chemotherapy and the MDM2 inhibitor Nutlin-3. *Cell Cycle, 11*(24), 4579-4588. doi:10.4161/cc.22852

Chatterjee, P., Choudhary, G. S., Alswillah, T., Xiong, X., Heston, W. D., Magi-Galluzzi, C., . . . Almasan, A. (2015). The TMPRSS2-ERG Gene Fusion Blocks XRCC4-Mediated Nonhomologous End-Joining Repair and Radiosensitizes Prostate Cancer Cells to PARP Inhibition. *Molecular cancer therapeutics, 14*(8), 1896-1906. doi:10.1158/1535-7163.MCT-14-0865

Cui, D., Dai, J., Keller, J. M., Mizokami, A., Xia, S., & Keller, E. T. (2015). Notch Pathway Inhibition Using PF-03084014, a gamma-Secretase Inhibitor (GSI), Enhances the Antitumor Effect of Docetaxel in Prostate Cancer. *Clin Cancer Res, 21*(20), 4619-4629. doi:10.1158/1078-0432.CCR-15-0242

Cui, J., Wang, Y., Dong, B., Qin, L., Wang, C., Zhou, P., . . . Gao, W. Q. (2018). Pharmacological inhibition of the Notch pathway enhances the efficacy of

androgen deprivation therapy for prostate cancer. *Int J Cancer, 143*(3), 645-656. doi:10.1002/ijc.31346

Deng, G., Ma, L., Meng, Q., Ju, X., Jiang, K., Jiang, P., & Yu, Z. (2016). Notch signaling in the prostate: critical roles during development and in the hallmarks of prostate cancer biology. *J Cancer Res Clin Oncol, 142*(3), 531-547. doi:10.1007/s00432-015-1946-x

Haffner, M. C., Aryee, M. J., Toubaji, A., Esopi, D. M., Albadine, R., Gurel, B., . . . Yegnasubramanian, S. (2010). Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements. *Nature genetics, 42*(8), 668-675. doi:10.1038/ng.613

Huang, Q., Raya, A., DeJesus, P., Chao, S.-H., Quon, K. C., Caldwell, J. S., . . . Schultz, P. G. (2004). Identification of p53 regulators by genome-wide functional analysis. *Proceedings of the National Academy of Sciences of the United States of America, 101*(10), 3456.

Korenchuk, S., Lehr, J. E., L, M. C., Lee, Y. G., Whitney, S., Vessella, R., . . . Pienta, K. J. (2001). VCaP, a cell-based model system of human prostate cancer. *In Vivo, 15*(2), 163-168.

Lavery, D. N., Villaronga, M. A., Walker, M. M., Patel, A., Belandia, B., & Bevan, C. L. (2011). Repression of androgen receptor activity by HEYL, a third member of the Hairy/Enhancer-of-split-related family of Notch effectors. *J Biol Chem, 286*(20), 17796-17808. doi:10.1074/jbc.M110.198655

Leong, K. G., & Gao, W. Q. (2008). The Notch pathway in prostate development and cancer. *Differentiation, 76*(6), 699-716. doi:10.1111/j.1432-0436.2008.00288.x

Lin, R. W., Ho, C. J., Chen, H. W., Pao, Y. H., Chen, L. E., Yang, M. C., . . . Wang, C. (2018). P53 enhances apoptosis induced by doxorubicin only under conditions of severe DNA damage. *Cell Cycle, 17*(17), 2175-2186. doi:10.1080/15384101.2018.1520565

Mohamed, A. A., Tan, S. H., Xavier, C. P., Katta, S., Huang, W., Ravindranath, L., . . . Srivastava, S. (2017). Synergistic Activity with NOTCH Inhibition and Androgen Ablation in ERG-Positive Prostate Cancer Cells. *Mol Cancer Res, 15*(10), 1308-1317. doi:10.1158/1541-7786.MCR-17-0058

Qiu, S., Deng, L., Bao, Y., Jin, K., Tu, X., Li, J., . . . Wei, Q. (2018). Reversal of docetaxel resistance in prostate cancer by Notch signaling inhibition. *Anticancer Drugs, 29*(9), 871-879. doi:10.1097/CAD.0000000000000659

Sampson, N., Neuwirt, H., Puhr, M., Klocker, H., & Eder, I. E. (2013). In vitro model systems to study androgen receptor signaling in prostate cancer. *Endocr Relat Cancer, 20*(2), R49-64. doi:10.1530/erc-12-0401

Su, Q., & Xin, L. (2016). Notch signaling in prostate cancer: refining a therapeutic opportunity. *Histol Histopathol, 31*(2), 149-157. doi:10.14670/HH-11-685

Thompson, T. C., & Li, L. (2017). Connecting androgen receptor signaling and the DNA damage response: Development of new therapies for advanced prostate cancer. *Molecular & cellular oncology, 4*(4), e1321167-e1321167. doi:10.1080/23723556.2017.1321167

Vermezovic, J., Adamowicz, M., Santarpia, L., Rustighi, A., Forcato, M., Lucano, C., . . . d'Adda di Fagagna, F. (2015). Notch is a direct negative regulator of the DNA-damage response. *Nat Struct Mol Biol, 22*(5), 417-424. doi:10.1038/nsmb.3013

Yu, S.-Q., Lai, K.-P., Xia, S.-J., Chang, H.-C., Chang, C., & Yeh, S. (2009). The diverse and contrasting effects of using human prostate cancer cell lines to study androgen receptor roles in prostate cancer. *Asian journal of andrology, 11*(1), 39-48. doi:10.1038/aja.2008.44

Yu, Y., Zhang, Y., Guan, W., Huang, T., Kang, J., Sheng, X., & Qi, J. (2014). Androgen receptor promotes the oncogenic function of overexpressed Jagged1 in prostate cancer by enhancing cyclin B1 expression via Akt phosphorylation. *Mol Cancer Res, 12*(6), 830-842. doi:10.1158/1541-7786.mcr-13-0545

Zhang, L., Sha, J., Yang, G., Huang, X., Bo, J., & Huang, Y. (2017). Activation of Notch pathway is linked with epithelial-mesenchymal transition in prostate cancer cells. *Cell Cycle, 16*(10), 999-1007. doi:10.1080/15384101.2017.1312237

Figure 1: Notch signaling evaluation in hormone dependent and hormone independent prostate cancer cell lines. A. To measure mRNA, total RNA was extracted from 3 different prostate cancer cell lines (LNCaP, DU145 and VCaP) and 1 μg of total RNA was reverse transcribed and subjected to qRT–PCR for the indicated target gene and normalized to GAPDH and then reported relative to VCaP. **B**. To measure protein, indicated cell lines were subjected to immunoblot for anti-Notch1 antibody. β-Tubulin was used as loading control. **C**. 3×10^3 DU145 and VCaP cells were treated with GSI R04 for 72 hours. Cell viability was measured using resazurin cell viability assays. **D**. 3×103 DU145 and VCaP cells were treated with OMP-A2G1 (0-80 ug/ml) 72 hours. Cell viability was measured using resazurin cell viability assays. *p<0.05, **p<0.01.

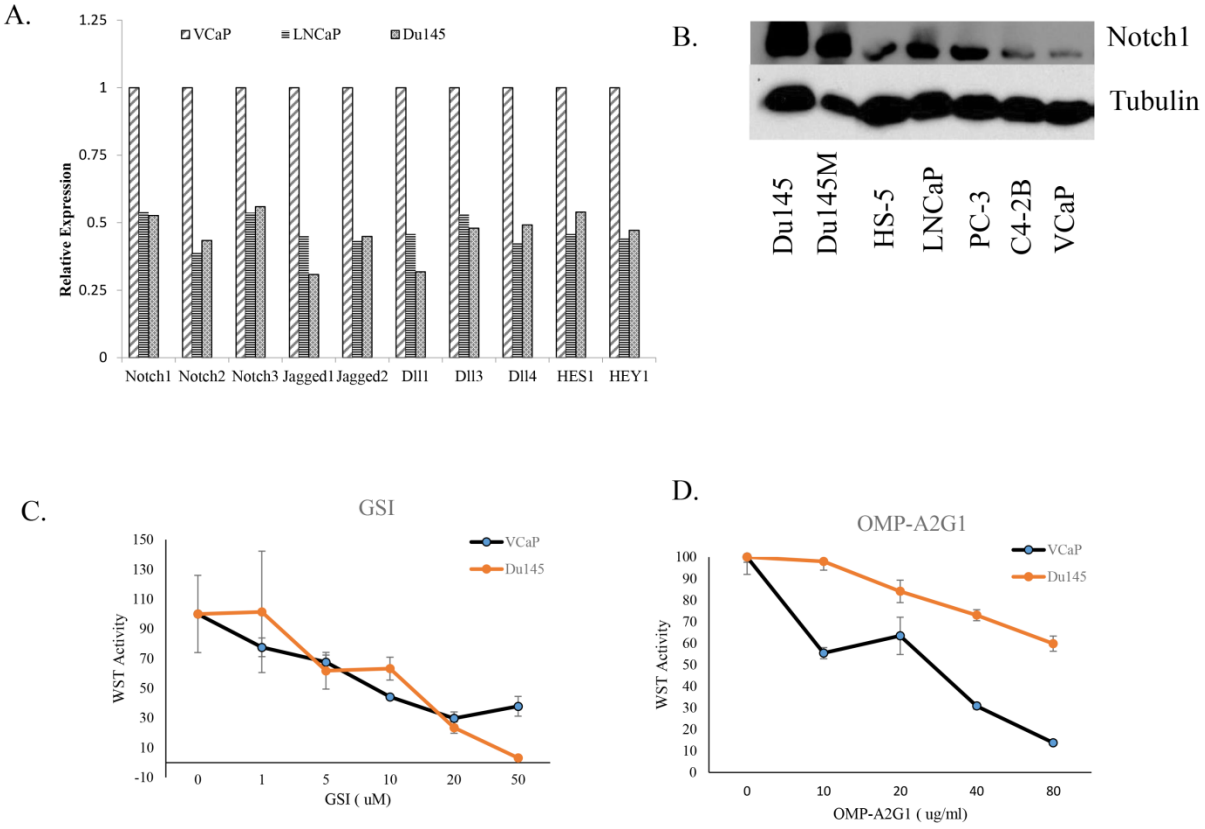


Figure 2: OMP-A2G1 had a significant antitumor effect in VCaP and Du145 xenografts. A General scheme for OMP-A2G1 and docetaxel treatment. **B-C.** Du145 (1×10^6) and VCaP (3×10^6) were injected subcutaneously into mice. When cohorts of tumors reached 100-200 mm^2 mice were divided into four treatment groups consisting of control antibody 1B7.11 (against dinitrophenol), control antibody plus docetaxel, OMP-A2G1 alone or OMP-A2G1 plus docetaxel and treated with as described in Materials and Methods (N=12-14 mice per group). **D**. At end of study, tumors were harvested and weighted, mean weights were then graphed (*p<0.05, **p<0.001, ***p<0.0001; NS=no significant difference).

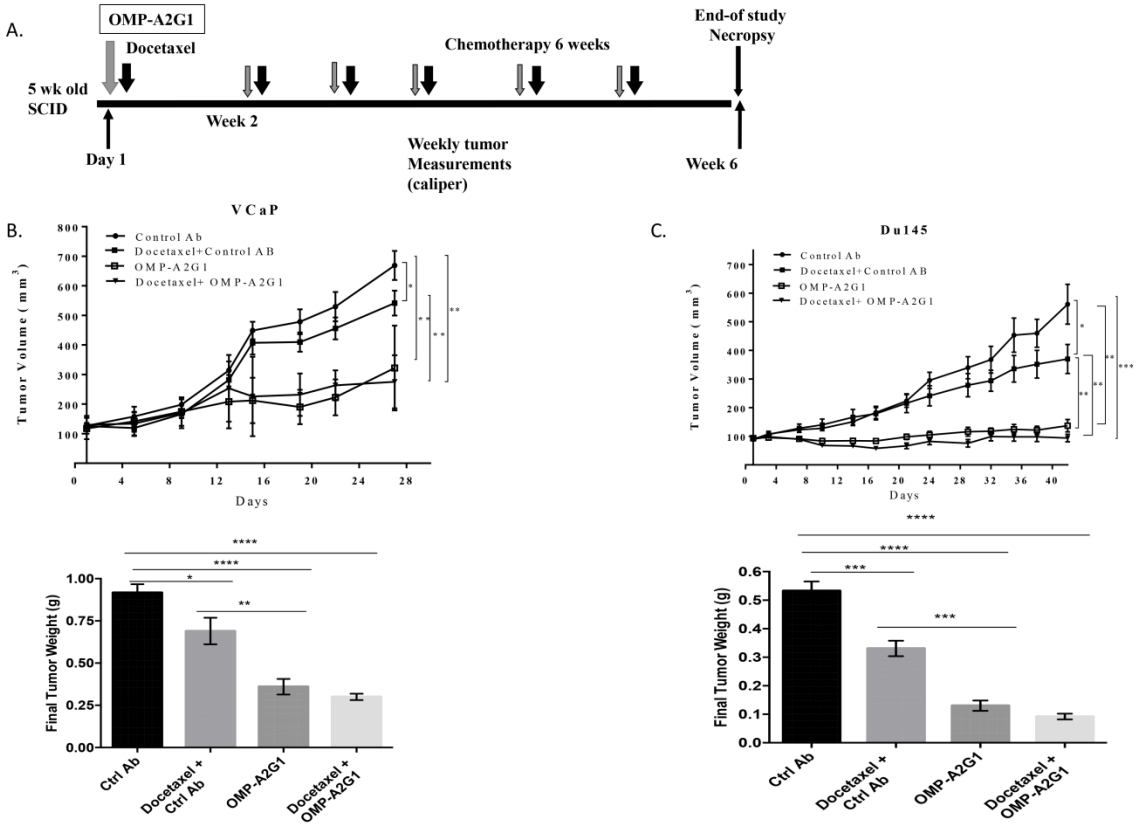


Figure 3: OMP-A2G1 induces apoptosis alone and in combination with docetaxel in DU145 and suppresses tumor cell proliferation in VCaP. A -B. At end of study, tumors were harvested from the mice as described in methods. A portion of the DU145 and VCaP tumor xenografts (n=5) were subjected to immunohistochemistry for proliferation (anti-Ki67) and apoptosis (anti-cleaved caspase 3). Left: Representative photomicrographs of Ki67 and cleaved-caspase 3 stained tumor sections (×40). Right: Quantitation of Ki-67 and cleaved-caspase 3 positive percentage (* $p<0.05$; ** $p<0.01$).

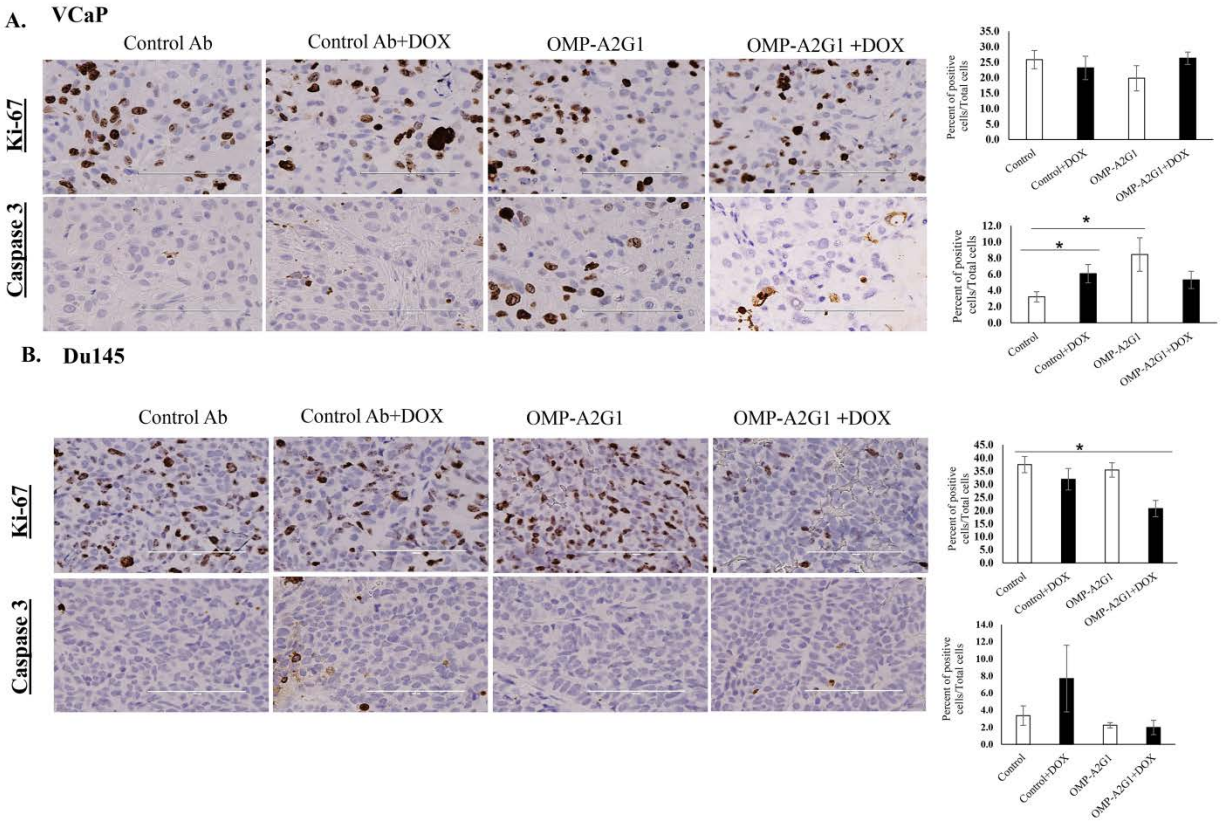


Figure 4: Differential notch signaling response to OMP-A2G1 treatment. A-B. Total protein was isolated from OMP-A2G1 and control treated DU145 and VCaP and subject to immunoblot for NICD1 as described in figure 1. GAPDH was used as loading control. Bands were measured using densitometry and values first normalized to respective GAPDH bands. **C-D**. To measure mRNA, total RNA was extracted from OMP-A2G1 and control treated DU145 and VCaP. Total RNA (1 μg) was subjected to qRT-PCR using primers for Hes1 and GAPDH (as loading control). (* $p<0.05$; ** $p<0.01$).

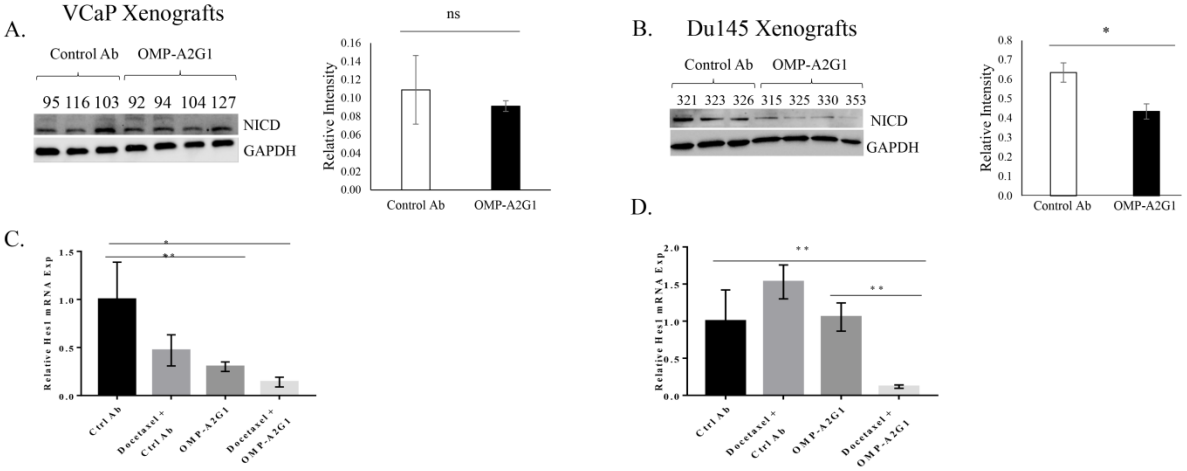


Figure 5: OMP-A2G1 treated VCaP and Du145 xenografts have differential DNA damage response. A. 400 ng of total RNA was extracted from DU145 and VCaP treated tumors and their respective control antibody treated tumors and was reverse transcribed and subjected to qRT–PCR using the Human DNA Damage Signaling Pathway PCR array. CT values were analyzed using the GeneGlobe Data Analysis Center, relative gene expression between of top genes in each xenograft were then plotted. **B-C.** Total protein was isolated from OMP-A2G1 and control treated DU145 and VCaP and subject to immunoblot for p53 and GADD45α as described in Figure 1. GAPDH and β-tubulin were used as loading control.