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14. ABSTRACT
During the past year I have setup a transplant androgen sensitive and castrate resistant prostate tumor mouse model for pre-clinical evaluation of novel targeted therapies. Further, this tumor is derived from a transgenic mouse of prostate cancer and all studies are performed in immune-competent animals. We have currently submitted 2 manuscripts, the first describing the creation of the transplant tumor model, and the second describing the increased therapeutic efficacy of HDAC/mTOR inhibitor combination for the treatment of advanced and castrate resistant prostate cancer. We also describe that increased inhibition of androgen receptor and HIF-1 alpha signaling as a major reason for greater therapeutic efficacy by combination therapy. Lastly, this manuscript demonstrates that microRNA may provide novel biomarkers for indication of therapy response. This work is inline with a current clinical trial under recruitment to treat patients with HDAC/mTOR inhibitor combination who have castrate resistant prostate cancer.

15. SUBJECT TERMS
Mouse models of prostate cancer, HDAC inhibitors, mTOR inhibitors, targeted therapies

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Introduction:

Specific inhibitors towards Histone Deacetylases (HDACs) and Mammalian Target of Rapamycin Complex 1 (mTORC1) have been developed and demonstrate potential as treatments for patients with advanced and/or metastatic and castrate resistant prostate cancer (PCa). Further, deregulation of HDAC expression and mTORC1 activity are documented in PCa and provide rational targets to create new therapeutic strategies to treat PCa. Here we report the use of the c-MYC adenocarcinoma cell line from the *c-myc* transgenic mouse with prostate cancer to evaluate the *in vitro* anti-tumor and *in vivo* therapeutic efficacy of the combination of the HDAC inhibitor panobinostat with the mTORC1 inhibitor everolimus. Panobinostat/everolimus combination treatment resulted in both greater antitumor activity and therapeutic efficacy in mice bearing androgen sensitive Myc-CaP and castrate resistant Myc-CaP tumors compared to single treatments. We identified that panobinostat/everolimus combination resulted in enhanced anti-tumor activity mediated by decreased tumor growth concurrent with augmentation of p21 expression and the attenuation of angiogenesis and tumor proliferation via androgen receptor, c-MYC and HIF-1α signaling. Also, we observed altered expression of microRNAs associated with these three transcription factors. Overall, our results demonstrate that low dose concurrent panobinostat/everolimus combination therapy is well tolerated and results in greater anti-tumor activity and therapeutic efficacy compared to single treatments in tumor bearing immuno-competent mice. Finally, our results suggest that response of specific miRs could be utilized to monitor panobinostat/everolimus *in vivo* activity.

Body

Specific Aim 1: Analyze the expression of HDACs in prostatic tissue from transgenic mouse models of prostate cancer. (Months 1-12)

Task 1: Generate Hi-myc Tg mice and litter mate controls (Months 1-3)

The Hi-myc transgenic mouse was purchased from NCI-Fredrick is now being successfully bred at RPCI, generating litters which consist of transgenic and wild type male and female mice.

Task 2: Generate Myc-CaP/PTEN shRNA cell lines (Months 1-2)

Knockdown of Pten was abandoned because shRNA a concurrent loss of androgen receptor expression was also observed was stable Myc-CaP cells selected by antibiotic resistance. To produce a castrate resistant model with an over expression of Myc for further PCR and/or therapeutic studies, we first generated subcutaneous Myc-CaP cell line derived androgen sensitive tumors in intact male FVB mice. Once tumors were established and measured ≥500mm^3, tumor bearing animals were surgically castrated. Post-castration 50% of mice (*n=2*) developed castration refractory Myc-CaP tumors. To produce a *bone fide* castrate resistant Myc-CaP tumor, we serially passaged these tumors subcutaneous in pre-castrated mice for at least 5 passages before being used in experiments (Ellis et al, Fig 1). Interestingly, we observed that these tumors did initially lose androgen receptor (AR) nuclear localization as well as AR transcriptional activity as determined by loss of its downstream transgene target, *c-MYC*, and concurrent with loss of proliferation. At round four of passaging we noted that these tumors now phenocopied the androgen sensitive Myc-CaP tumor, by regaining AR nuclear localization and transcriptional activity as seen by re-expression of c-MYC and gain in tumor proliferation (Ellis et al, Fig 2). Castration resistance was further confirmed by treatment of castrated mice bearing Myc-CaP/CR tumors with bicalutamide. Treated with either 25mg/kg or 50mg/kg daily with bicalutamide resulted in no anti-tumor activity (Ellis et al, Fig 3).

Task 3: Conduct aging studies in Hi-myc Tg mice and litter mate controls (Months 3-12)

Hi-myc transgenic male mice and their male wild type litter mates have been successfully aged to 1, 3, 5, 7 and 9 months of age (Fig 1 and Fig 2) and consequent total RNA was collected as well as samples for H&E histology staining. Currently, we are designing custom quantative real-time PCR plates to carry out Task 3 of specific aim 1.

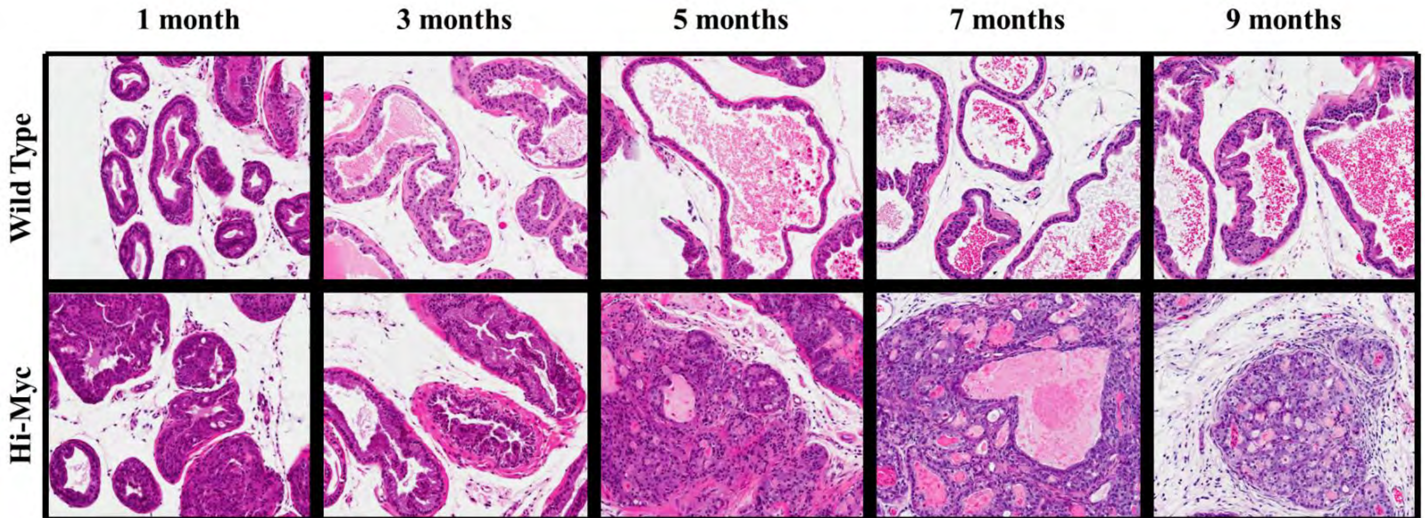

Fig 1: Transgenic and wild type FVB male mice where aged to indicated ages, at which they were sacrificed and prostatectomies performed. H&E staining of lateral prostate gland tissue indicates that aging wild type mice maintain normal luminal morphology. Conversely, aging transgenic Hi-Myc male mice at 1 and 3 months display mPIN and 5 months begin to develop adenocarcinoma.

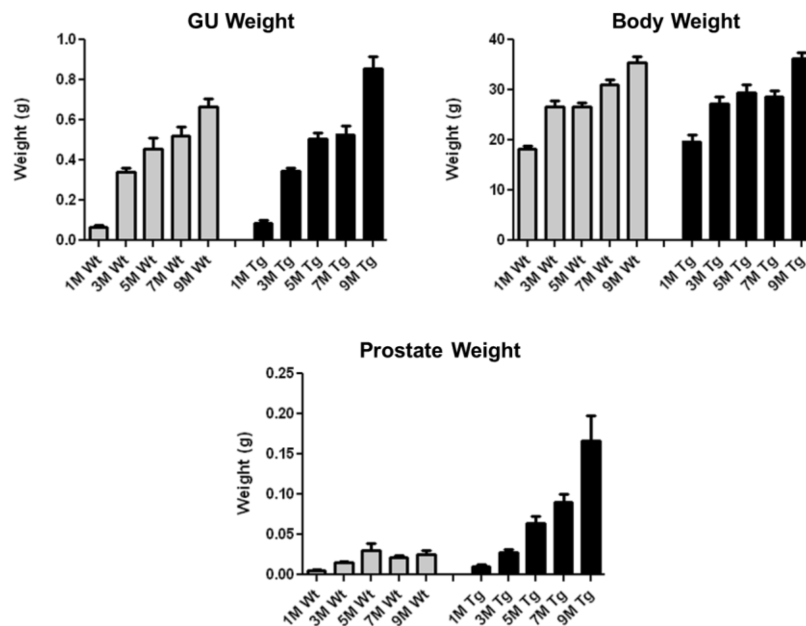

Fig 2: Transgenic and wild type FVB male mice where aged to indicated ages, at which they were sacrificed and prostatectomies performed. No difference was observed in overall GU and body weight of aging mice, though as expected increased prostate weight was seen as adenocarcinoma developed in transgenic mice.

Specific Aim 2: Evaluate the therapeutic efficacy of the HDACi LBH589 and the mTOR inhibitor RAD001 as both monotherapy and in combination therapy in mouse models of prostate cancer. (Months 1-12)

Task 1: In vivo therapy experiments in a Myc-CaP transplant mouse model (Months 1-6)
Task 2: In vivo therapy experiments in a Myc-CaP/PTEN-shRNA transplant mouse model (Months 6-12)

Because the generation of Myc-CaP cell lines with stable knock down of pten was not pursued we conducted in vivo therapy experiments in intact and castrated FVB male mice bearing Myc-CaP androgen sensitive and Myc-CaP castrate resistant subcutaneous tumors respectively. Before conducting in vivo experiments we first investigated the sensitivity of Myc-CaP cell lines to panobinostat and everolimus individual treatments. Fig 3A shows that panobinostat induces loss of cell membrane permeability in a time and dose dependent manner, where as Myc-CaP cell lines were resistant to the cytotoxic effects of everolimus. Treatment with low dose (non-cytotoxic) concentrations of panobinostat also inhibited cell growth. Similar concentrations of everolimus also inhibited cell growth (Fig 3B). Combination of panobinostat and everolimus with concentrations that only inhibited cell growth also enhanced the inhibition of the clonogenic capabilities of Myc-CaP cells when compared to single agent treatment. This effect was dose and time dependent (Fig 3C-F). Further in vitro analysis revealed that combination treatment induced a greater response of p21 though this did not result in an increase in accumulation of Myc-CaP in the G1 phase of the cell cycle (Fig 4A-C). Either single treatment or combination only modestly increased levels of apoptosis (Fig 4D-E).

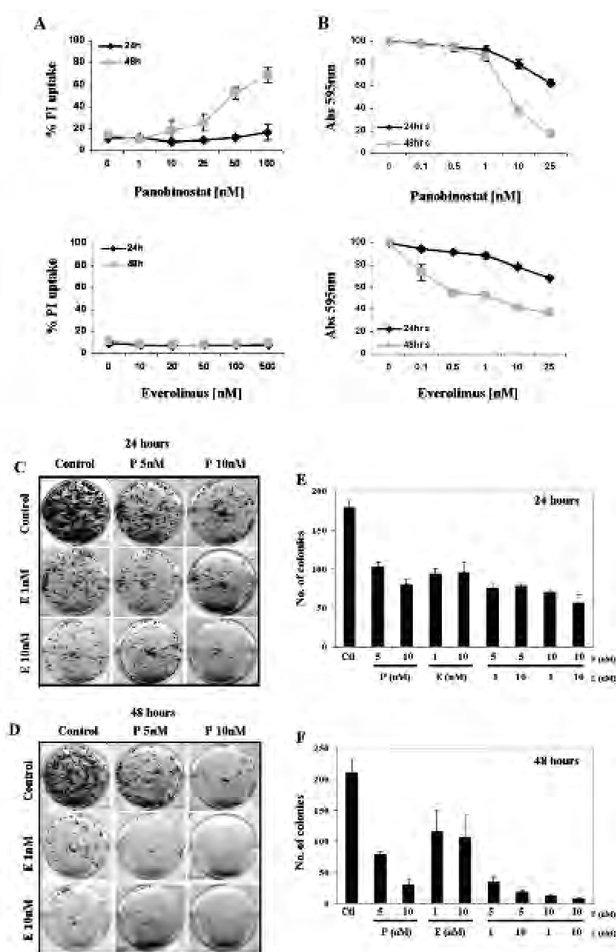

Fig 3: Myc-CaP cells treated with indicated concentration of panobinostat, everolimus or combination and assessed for **(A)** loss of cell membrane permeability, **(B)** inhibition of cell growth and **(C)** clonogenic potential.

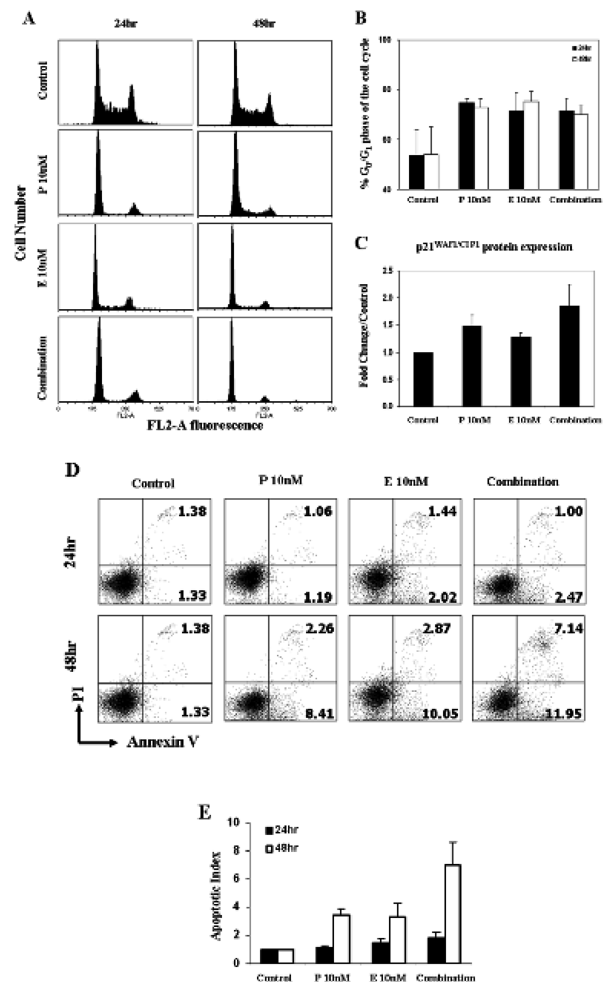

Fig 4: Myc-CaP cells treated with indicated concentration of panobinostat, everolimus or combination and assessed for **(A-B)** cell cycle profile, **(C)** protein levels of p21 and **(D-E)** levels of apoptosis.

In vivo analysis of the anti-tumor and therapeutic benefit mediated by the combination of panobinostat with everolimus was assessed as mentioned above. Excitingly we demonstrated that panobinostat/everolimus combination resulted in greater anti-tumor activity and therapeutic efficacy compared to each single treatment in both our androgen sensitive and castrate resistant Myc-CaP tumor models. Also both compounds were well tolerated and exhibited low toxicities as measured by mouse body weight (not shown) and platelet and white blood cell counts (Fig 5).

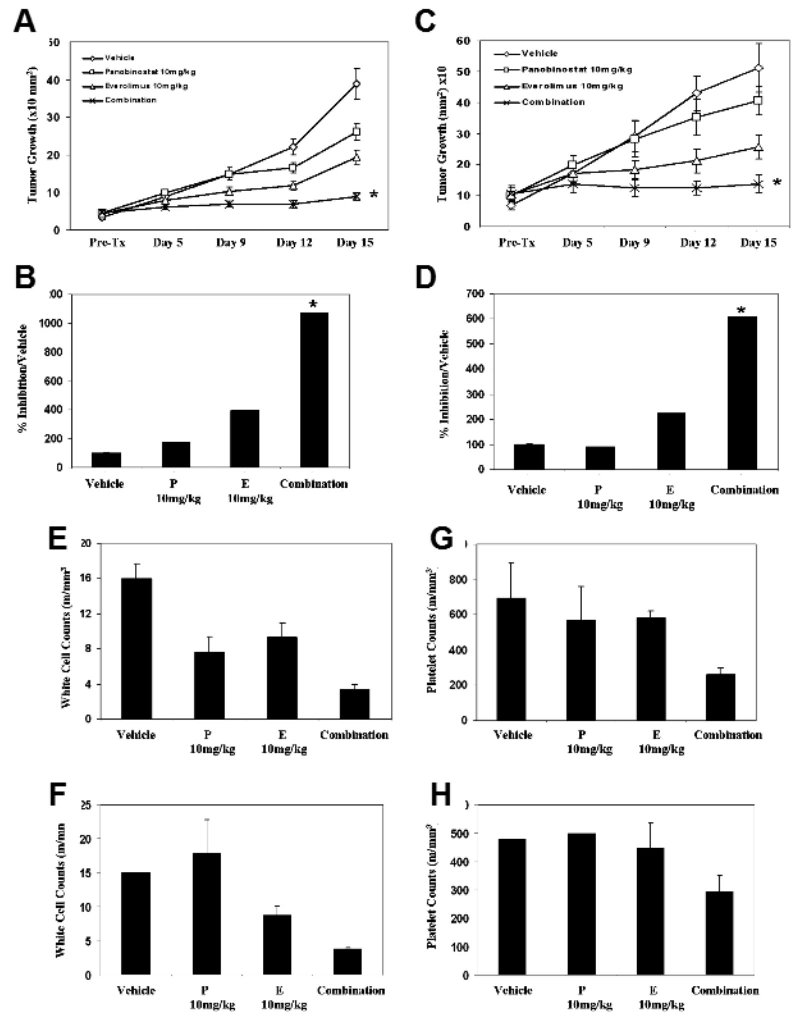

Fig 5: Male FVB intact or castrated mice were subcutaneously grafted with Myc-CaP androgen sensitive or Myc-CaP castrate resistant tumors. Mice were treated daily with panobinostat 10mg/kg, everolimus 10mg/kg or combination. **(A-D)** Serial caliper measurements and endpoint tumor weights indicate that combination therapy significantly enhanced anti-tumor activity compared to single treatments. **(E-H)** Blood analysis revealed that single and combination treatments did not induce dose limiting toxicities as assessed by platelet and white blood cell counts.

Specific Aim 3: Characterize the *in vivo* anti-tumor effect observed from the combination of LBH589 and RAD001. (Months 12-24)

<u>Task 1: Conduct in vivo analysis of anti-tumor effects of LBH589 and RAD001: Drug pharmacodynamics (PD) (Months 12-15).</u> To assess the effectiveness of LBH589 and RAD001 to 'hit its target' antibodies towards acetylated histone H3, p-mTOR and p-pS6K will be used to stain collected samples described in specific aim 2.

Western blot analysis revealed that in vivo treatment with panobinostat and everolimus were active and targeting their 'primary' targets (Fig 6).

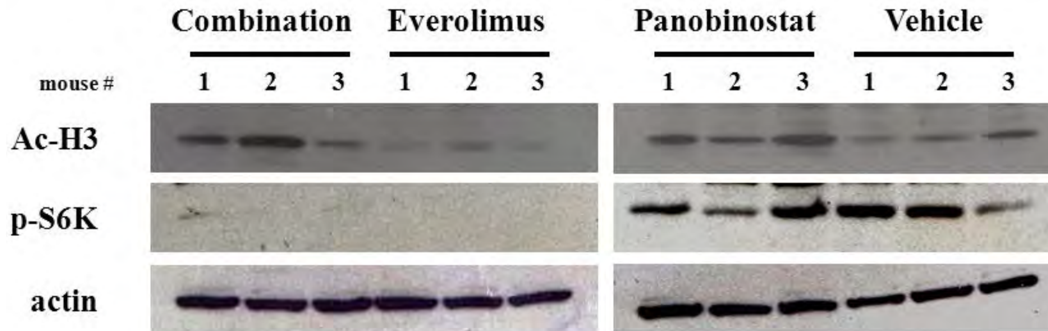

Figure 6: Western blot analysis whole cell lysates from Myc-CaP androgen sensitive tumors revealed that panobinostat and everolimus was active in vivo and no negative drug interactions were encountered in combination. **(top row)** reveals that single and combination treatment involving panobinostat induces histone H3 acetylation. **(middle row)** reveals that single and combination treatment involving everolimus inhibits phosphor-S6K, a down stream target of mTORC1. **(bottom row)** β-actin served as a protein loading control.

<u>Task 2: Assess the anti-angiogenic in vivo properties of LBH589 and RAD001 (Months 15-18).</u> To assess the anti-angiogenic properties of LBH589 and RAD001, antibodies towards CD31, HIF-1α and AR will be used to stain tissue samples collected from therapy experiments.

In vitro and in vivo analysis revealed that combination treatment significantly attenuated both HIF-1α and androgen receptor activity as well as protein expression, which was associated with significant anti-tumor effect (Fig 7 and Fig 8).

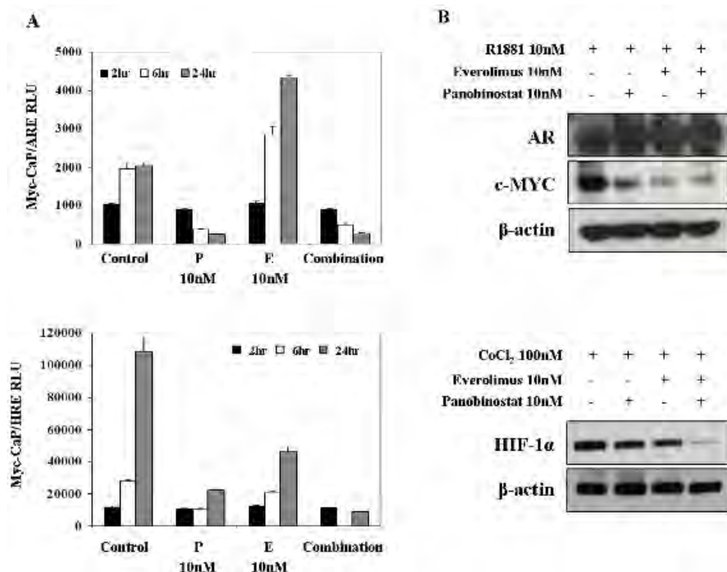

Fig 7: Myc-CaP cells treated with indicated concentration of panobinostat, everolimus or combination and assessed for **(A)** AR and HIF-1α transcriptional activity by reporter assay method, **(B)** AR and HIF-1α protein expression by western blot technique.

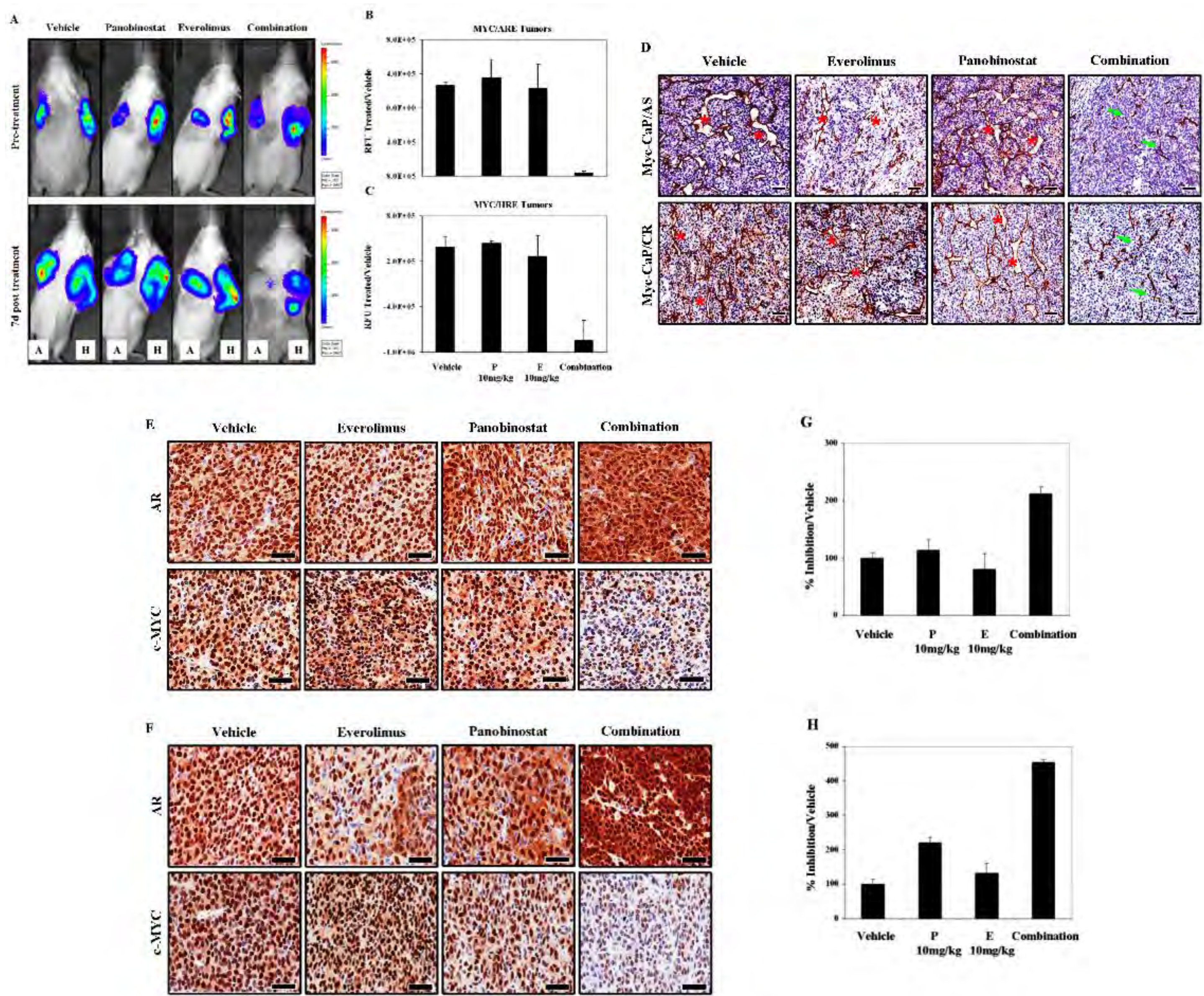

Figure 8: (A) Intact FVB male mice were subcutaneously grafted with Myc-CaP cells (1x10^6) stably expressing reporter plasmids for AR and HIF-1α transcriptional activity. Mice were treated daily for 7 days with vehicle, panobinostat 10mg/kg, everolimus 10mg/kg or combination. **(B-C)** Quantitation of relative luciferase units from (A) indicates that combination treatment significantly attenuated both AR and HIF-1α in vivo transcriptional activity. **(D)** CD31 staining of Myc-CaP androgen sensitive and castration resistant tumors shows that a loss tumor vascular size is associated with loss of HIF-1α transcriptional activity. **(E-H)** IHC staining for AR and c-Myc indicate that combination induces AR cytoplasmic localization which is associated with concurrent loss of AR transcriptional activity and c-Myc protein expression.

Task 3: Assess the anti-proliferative properties and induction of apoptosis induced in vivo by LBH589 and RAD001 (Months 18-21). Tissue sections from conducted therapy experiments will be stained with the proliferation marker Ki-67 to assess the anti-proliferative properties of LBH589 and RAD001. Furthermore TUNEL and/or caspase-3 staining will be utilized to evaluate the apoptotic properties of LBH589 and RAD001.

Previous in vitro data revealed that (Fig 3 and Fig 4) panobinostat/everolimus combination resulted in growth inhibition associated with arrest in G1 phase of the cell cycle, with significant increases in apoptosis. Similar to in vitro results, IHC staining of treated in vivo tumor tissue showed that anti-tumor activity was mediated through loss of tumor proliferation as indicated by the loss of Ki67 staining (Fig 9). Tumor apoptosis was assessed by activated caspase 3 staining and no increase throughout treatment was observed (data not shown).

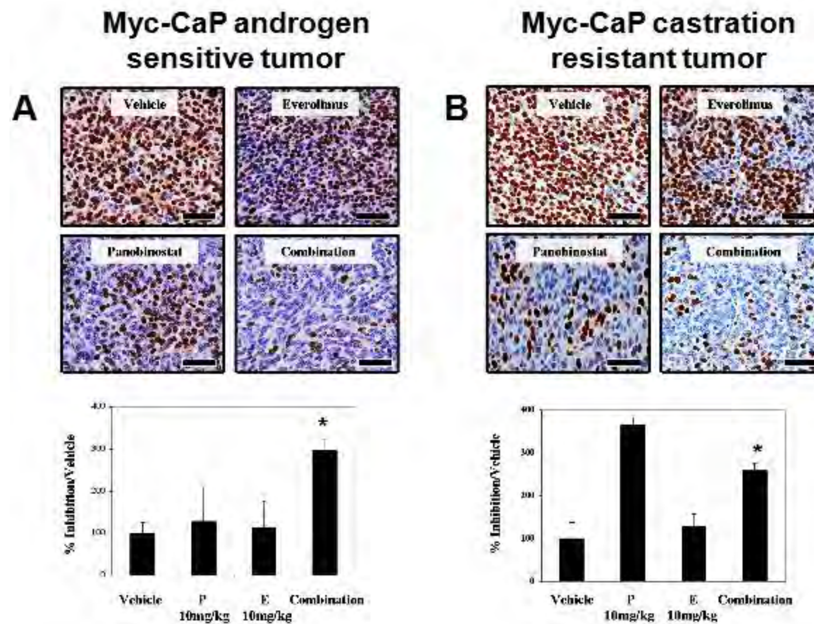

Figure 9: Intact and castrated FVB male mice were subcutaneously grafted with androgen sensitive or castrate resistant Myc-CaP tumors. Mice were treated with vehicle, panobinostat 10mg/kg, everolimus 10mg/kg or combination daily. Panobinostat/everolimus combinations significantly inhibits androgen sensitive tumor proliferation compared to single treatments **(A)**, whereas combination significantly inhibits castrate resistant tumor proliferation compared to everolimus single treatment but not panobinostat treatment.

Task 4: Assess the induction in vivo of autophagy induced by LBH589 and RAD001 (Months 21-24). Protein lysates produced from snap frozen tissue sections will be separated by western blot technique and probed using antibodies towards beclin-1 and LC3 proteins to assess whether induction of autophagy plays a role in the anti-tumor effects of LBH589 and RAD001.

Because mTORC1 inhibits autophagy we assessed to in vivo induction of autophagy within tumors by western blot analysis for the autophagy marker LC3 (Fig 10). There was no significant increased conversion of LC3-I to LC3-II conversion as seen by western blot. Therefor we did not pursue this any further as a possible anti-tumor mechanism.

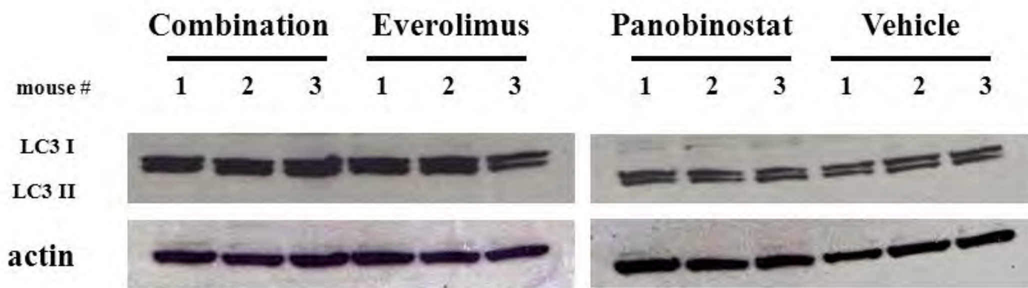

Figure 10: Western blot analysis whole cell lysates from Myc-CaP androgen sensitive tumors revealed that panobinostat and everolimus did not induce autophagy as shown by levels of LC3-I/II protein levels **(top row).** β-actin served as a protein loading control **(bottom row).**

Additional Data: Regulation of oncogenic microRNA in vivo by Panobinostat and Everolimus.

We went onto to also show that recently published microRNA relevant to prostate cancer also show distinctive responses to drug treatment (Fig 11) which suggests that miRs could strengthen monitoring a patient's response to therapy, possibly in line or independent of PSA. From this and previous pre-clinical data we have also started recruitment of patients for a phase I clinical trial investigating the potential of this combination to treat castrate resistant, chemotherapy naive prostate cancer patients.

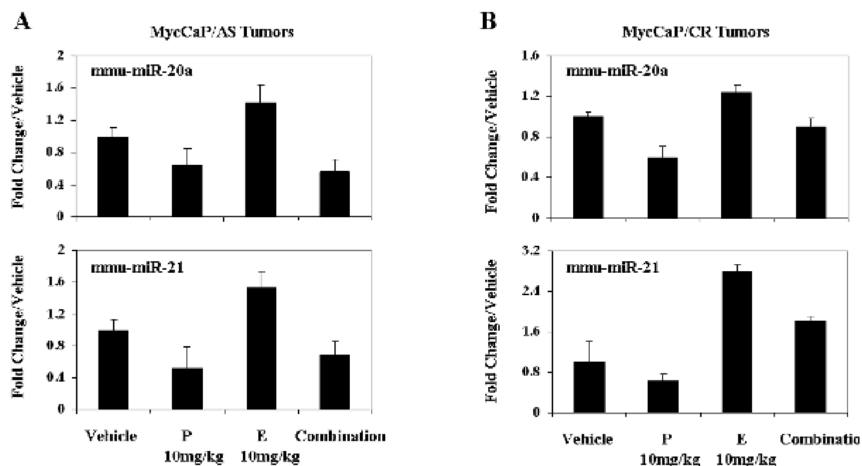

Figure 11: RNA was extracted from (A) Intact mice bearing Myc-CaP/AS tumors and (B) castrated mice bearing Myc-CaP/CR tumors that were treated daily with 10mg/kg everolimus (E) orally, 10mg/kg panobinostat (P) IP or combination for a total of 7 days. QRT-PCR analysis was used to investigate microRNA expression. $n=2$. Mean ± SE of Ct values from 2 independent tumors.

Key Research Accomplishments:

- Myc-CaP androgen sensitive tumors have the ability to progress to castrate resistant Myc-CaP tumors. This progression shares features with the clinic. (Published in *The Prostate*, paper attached).
- Combination of panobinostat/everolimus results in significant increase in anti-tumor activity compared to each single treatment.
- Panobinostat/everolimus combination mediates its antitumor activity through significant attenuation of HIF-1α and AR transcriptional activity, resulting in reduced tumor vascular and loss of tumor proliferation.
- We identified that 2 key oncogenic miRs are also regulated by everolimus and panobinstat treatment. This data further strengthens that continued efforts are needed to establish miRs as biomarkers to monitor response to therapy as well as indicators of disease progression.

Reportable Outcomes:

- **Two manuscripts submitted.**
- **Development of a castrate resistant transplant model from a transgenic murine model of prostate cancer. *The Prostate* May 2011.**
  Leigh Ellis, Kristin Lehet, Swathi Ramakrishnan, Remi Adelaiye and Roberto Pili
- **Concurrent HDAC and mTORC1 inhibition attenuate androgen receptor and hypoxia signaling associated with alterations in microRNA expression. Accepted with revision to *PLoS ONE* June 2011.**
  Leigh Ellis, Kristin Lehet, Swathi Ramakrishnan, Kiersten M Miles, Dan Wang, Song Liu, Peter Atadja, Michael A Carducci and Roberto Pili.

- **Two abstracts submitted and presented as posters.**

- **4th Annual Prostate Cancer Program Retreat (SPORE), 2011, Poster Presentation.**
  Title: Concurrent HDAC and mTOR inhibition attenuate Androgen Receptor and Hypoxia Signaling associated with alterations in MicroRNA expression.
  L Ellis, K Lehet, S Ramakrishnan, MA Carducci and R Pili

- **Innovative Minds in Prostate Cancer Today (IMPaCT) Conference, 2011, Poster Presention.**
  Title: Concurrent HDAC and mTOR inhibition attenuate Androgen Receptor and Hypoxia Signaling associated with alterations in MicroRNA expression.
  L Ellis, K Lehet, S Ramakrishnan, MA Carducci and R Pili

Abstract for both conferences

Background and Objective: Limited therapies are available to patients with advanced prostate cancer (PCa) and castrate resistant PCa. Molecular mechanisms involved in PCa have indentified histone deacetylases (HDACs) and the mammalian target of rapamycin complex 1 (mTORC1) as potential therapeutic targets. Moreover, specific inhibitors towards HDACs and mTORC1 have been clinically developed and demonstrate great potential as novel treatments for patients with PCa. The specific objective of this study was to investigate whether tumor bearing mice would receive greater therapeutic benefit from low-dose concurrent combination treatment with Panobinostat (LBH589) and Everolimus (RAD001) over mice treated with each agent as a monotherapy. Also, it was our goal to identify potential molecular mechanisms underlying any observed antitumor effect mediated by these compounds. **Methods**: We have utilized the murine MYC-CaP epithelial cell line. This is a unique epithelial cell line generated from the Hi-MYC murine model of PCa, and represents androgen-dependent undifferentiated adenocarcinoma overexpressing the human oncogene c-MYC. We have also transplanted this cell line to intact and castrated wild-type male mice to generate both androgen-sensitive and castrate-resistant tumor banks for *in vivo* preclinical studies. **Results**: We demonstrate that combinational treatment with the HDACI Panobinostat and the mTORC1-I Everolimus results in greater antitumor activity and therapeutic efficacy in an androgen-sensitive and castrate-resistant immunocompetent murine MYC model of PCa. Further, we identified that combinational treatment resulted in augmentation of p21 expression concurrent with attenuation of androgen receptor, c-MYC and HIF-1α signaling. Inhibition of these signaling pathways was also associated with altered expression of microRNAs involved as effectors or regulators of these transcription factors. Overall, our results confirm that low dose concurrent combination of Panobinostat and Everolimus is well tolerated and results in greater antitumor activity and therapeutic efficacy in tumor bearing immunocompetent mice. This combinational strategy warrants further clinical development for the treatment of patients with advanced and castrate-resistant PCa.

Conclusion

Upon conclusion of specific aim 1 we are certain that by obtaining an understanding of critical epigenetic events occurring under the influence of a defined genetic background will allow us to stratify more insightful links to disease development and progression. This knowledge will greatly benefit the clinic and potential directions for prevention medicine. From specific aims 2 and 3 we have demonstrated that we can successfully treat both advanced and castrate resistant forms of prostate cancer. Because we did not induce tumor apoptosis we propose to try alternative methods of panobinostat administration (pulsing higher doses IV to achieve greater anti-tumor concentrations), or try panobinostat with other novel chemotherapy combinations. In saying that, we have achieved a milestone with this work, which is successful bench to bedside medicine where our pre-clinical studies as resulted in the initiation of a phase I clinical trial for this combination strategy in the treatment of prostate cancer.

Concurrent HDAC and mTOR inhibition attenuate Androgen Receptor and Hypoxia Signaling associated with alterations in MicroRNA expression.

Leigh Ellis[1], Kristin Lehet[1], Swathi Ramakrishnan[1], Michael A Carducci[2#] and Roberto Pili[1#]
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Background and Objective: Limited therapies are available to patients with advanced prostate cancer (PCa) and castrate resistant PCa. Molecular mechanisms involved in PCa have indentified histone deacetylases (HDACs) and the mammalian target of rapamycin complex 1 (mTORC1) as potential therapeutic targets. Moreover, specific inhibitors towards HDACs and mTORC1 have been clinically developed and demonstrate great potential as novel treatments for patients with PCa. The specific objective of this study was to investigate whether tumor bearing mice would receive greater therapeutic benefit from low-dose concurrent combination treatment with Panobinostat (LBH589) and Everolimus (RAD001) over mice treated with each agent as a monotherapy. Also, it was our goal to identify potential molecular mechanisms underlying any observed antitumor effect mediated by these compounds. **Methods:** We have utilized the murine MYC-CaP epithelial cell line. This is a unique epithelial cell line generated from the Hi-MYC murine model of PCa, and represents androgen-dependent undifferentiated adenocarcinoma overexpressing the human oncogene c-MYC. We have also transplanted this cell line to intact and castrated wild-type male mice to generate both androgen-sensitive and castrate-resistant tumor banks for *in vivo* preclinical studies. **Results:** We demonstrate that combinational treatment with the HDACI Panobinostat and the mTORC1-I Everolimus results in greater antitumor activity and therapeutic efficacy in an androgen-sensitive and castrate-resistant immunocompetent murine MYC model of PCa. Further, we identified that combinational treatment resulted in augmentation of p21 expression concurrent with attenuation of androgen receptor, c-MYC and HIF-1α signaling. Inhibition of these signaling pathways was also associated with altered expression of microRNAs involved as effectors or regulators of these transcription factors. Overall, our results confirm that low dose concurrent combination of Panobinostat and Everolimus is well tolerated and results in greater antitumor activity and therapeutic efficacy in tumor bearing immunocompetent mice. This combinational strategy warrants further clinical development for the treatment of patients with advanced and castrate-resistant PCa.

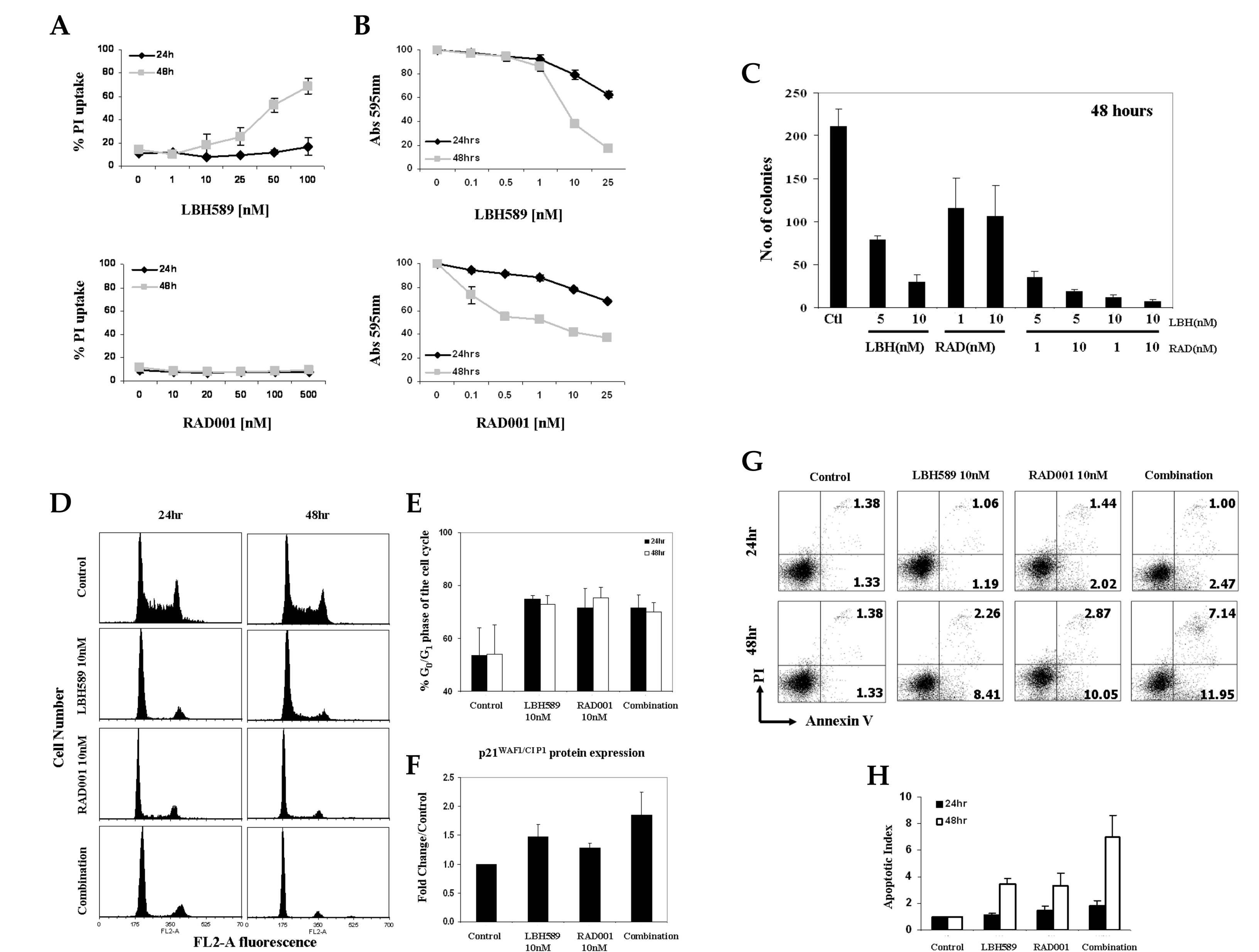


Figure 1: MYC-CaP sensitivity to HDAC and mTOR inhibition. (A) LBH589 induces loss of cell membrane permeability in MYC-CaP cell lines in a dose and time dependent manner (upper panel), whereas MYC-CaP cell lines remain resistant to RAD001 induced cytotoxicity (lower panel). **(B)** Non-cytotoxic concentrations of LBH589 (upper panel) and RAD001 (lower panel) inhibit cell growth of MYC-CaP cells in a time and dose dependent manner. Treatment of MYC-CaP cells with non-cytotoxic concentrations of LBH589 and RAD001 in combination for **(C)** at 48hrs reduce the clonogenicity of MYC-CaP cells greater than single agent treatments. **(D-E)** Flow cytometry analysis detecting propidium iodide (PI) staining of MYC-CaP cells reveals that LBH589 and RAD001 treatments induce an accumulation of 2N DNA (G_1 cell cycle arrest). **(F)** Flow cytometry quantitation of p21 indicates a greater induction by combination treatment. **(G)** Flow cytometry scatter plots displaying MYC-CaP cells stained with PI and annexin-V. **(H)** Quantitation of (G) indicates that combination treatment results in the increase of apoptotic cells over treatment with single agents.

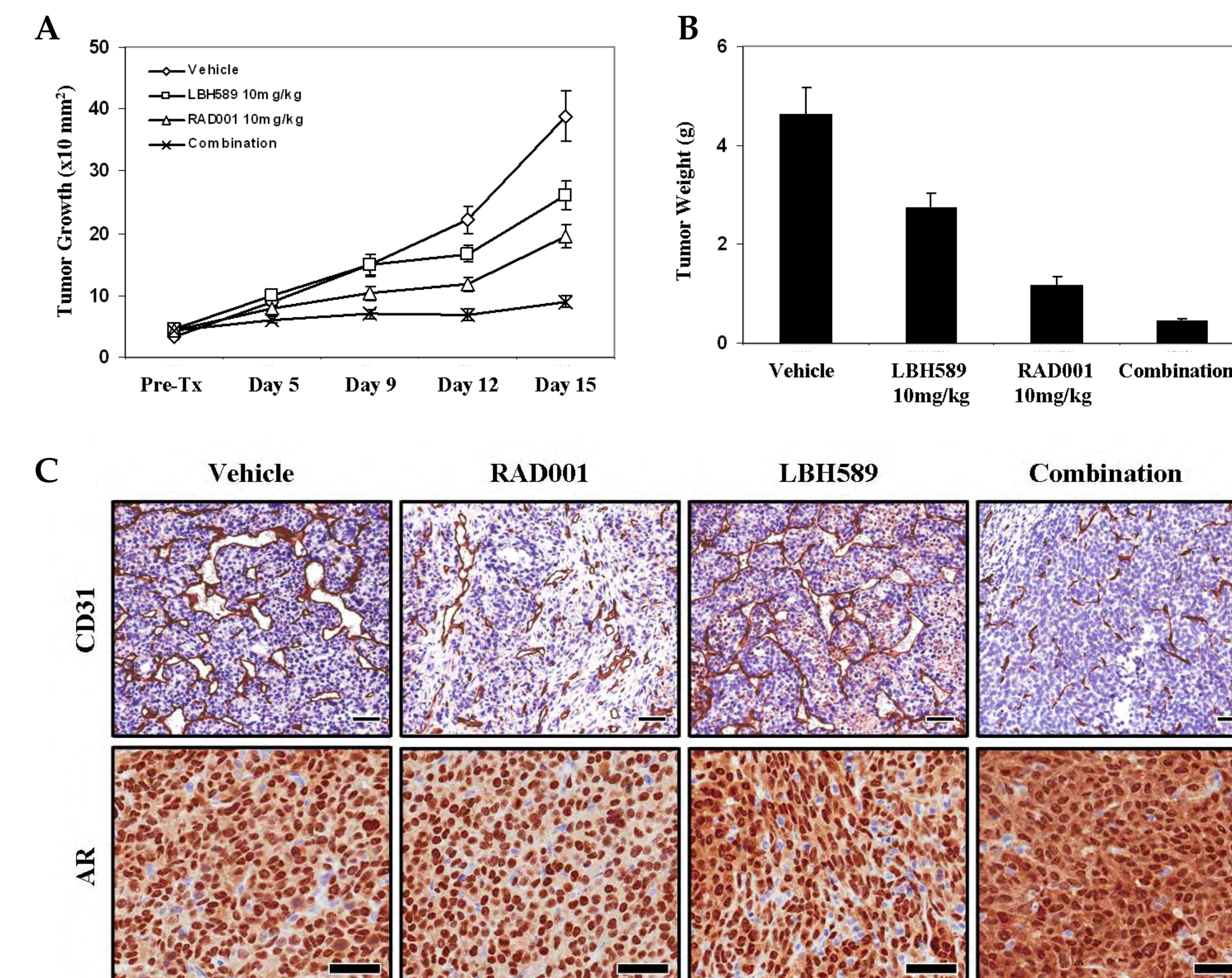


Figure 2: Mice bearing MYC-CaP tumors receive greatest therapeutic efficacy from LBH589 and RAD001 in combination. (A) Serial caliper measurement of subcutaneous tumors implanted to wild type intact FVB male mice. **(B)** Tumor weights at the conclusion of therapy (day 16). **(C)** IHC staining for CD31 x20 (endothelial marker; top panel) and androgen receptor x40 (bottom panel). Scale bars = 50μM

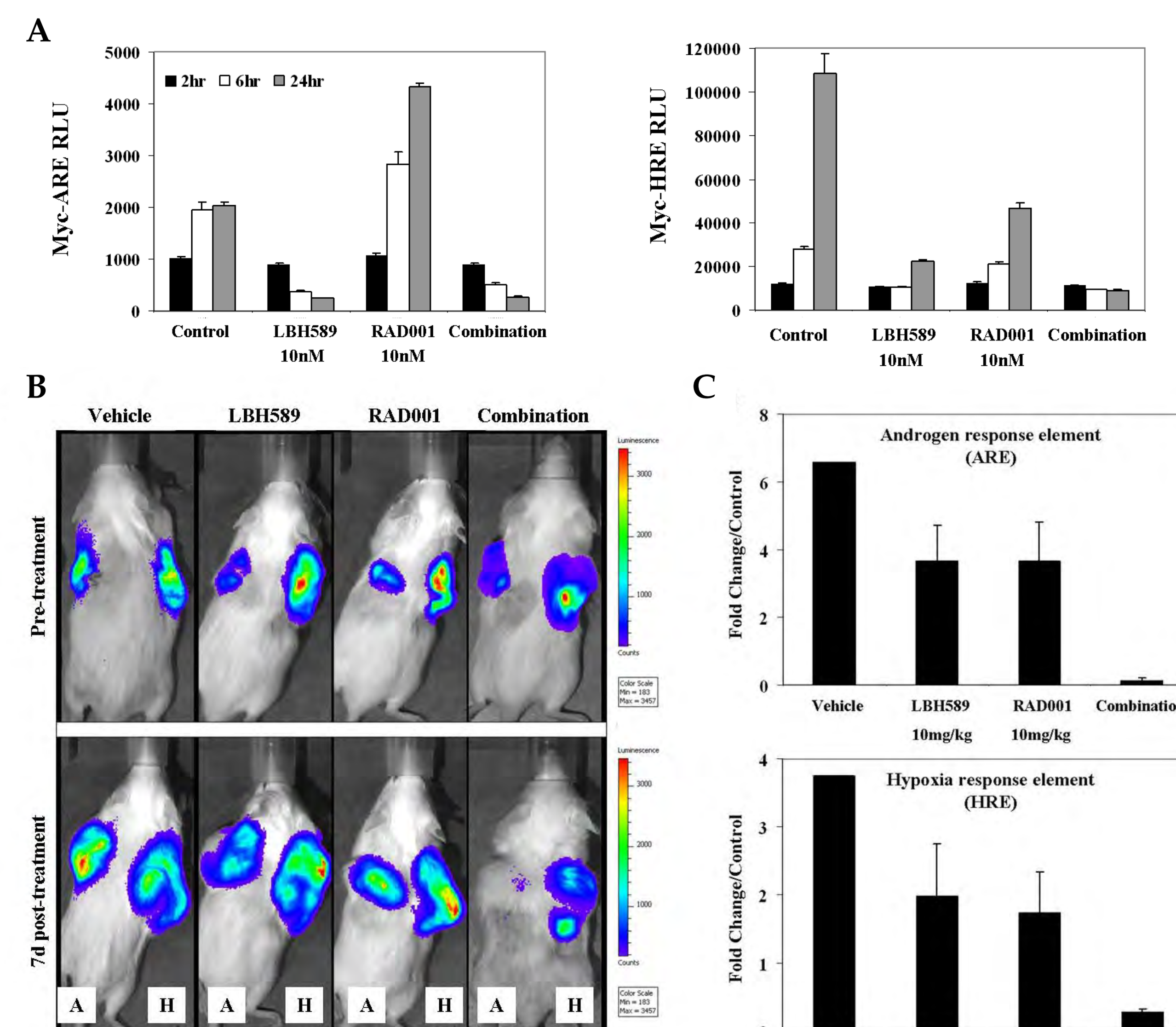


Figure 3: Combination therapy of LBH589 with RAD001 result in greater attenuation of Androgen Receptor and HIF-1α transcriptional activity *in vitro* and *in vivo*. (A) *In vitro* luminescence quantitation of MYC-CaP cell lines treated with indicated concentrations of LBH589 and/or RAD001. Cell lines used stably express reporter plasmids expressing luciferase driven expression by either an androgen response element (ARE) or a HIF-1α response element (HRE). **(B)** Seven (7) day treatment of wild type intact FVB male mice bearing bi-lateral subcutaneous MYC-CaP/ARE [A] and MYC-CaP/HRE [H] tumors. **(C)** Quantitation of tumor luminescence in (B) by a Xenogen® IVIS 50 imaging sytem.

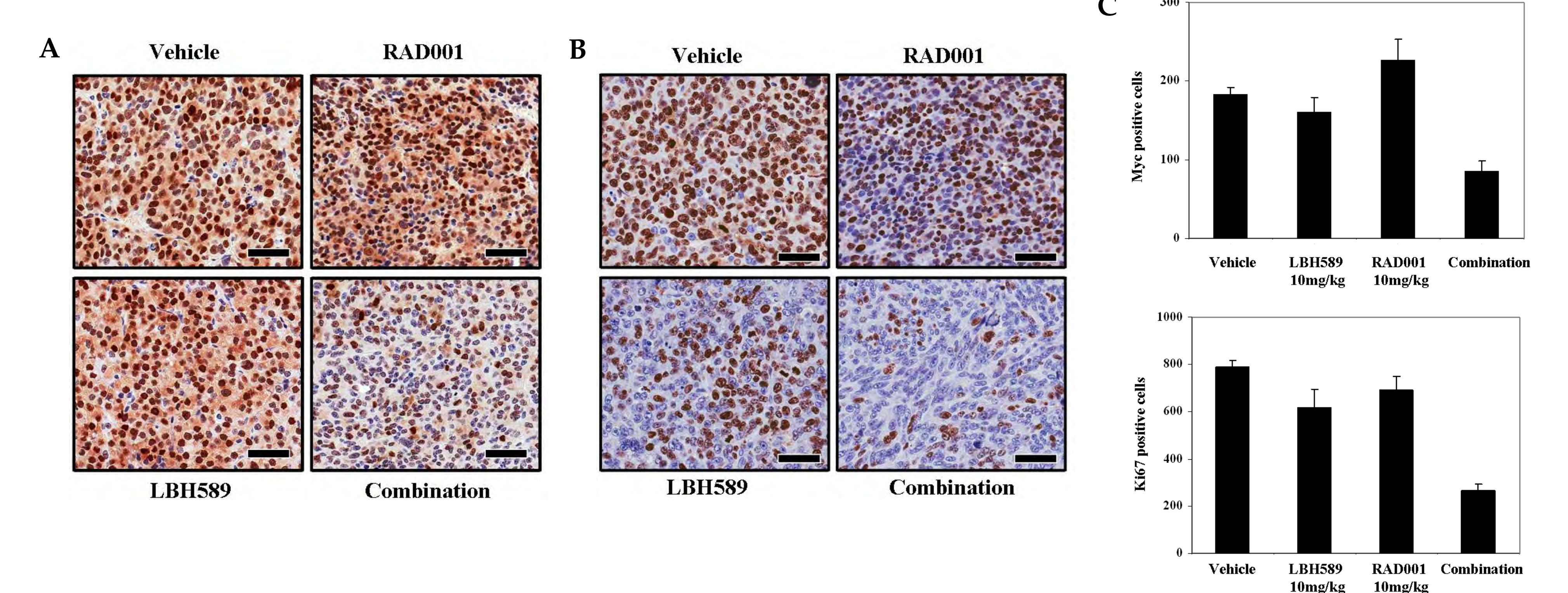
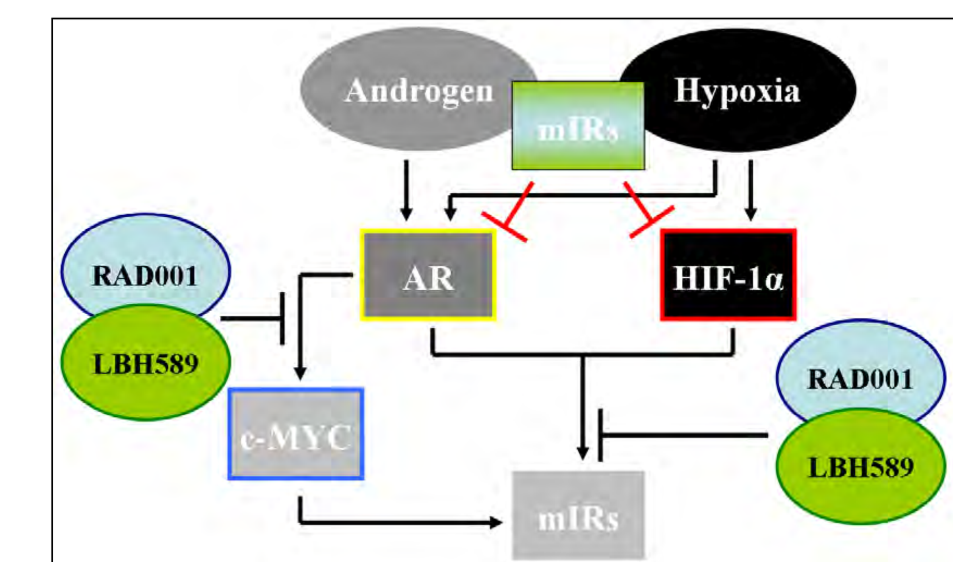


Figure 4: Combination therapy of LBH589 with RAD001 result in greater loss of c-MYC expression and increased inhibition of tumor proliferation. IHC staining of tumor tissue samples from figure 2 for **(A)** c-MYC and **(B)** Ki-67. **(C)** Quantitation of IHC staining for cells staining positive for c-MYC (top panel) or Ki-67 (bottom panel).

Table 1: Example miRs regulated in Myc-CaP cell lines treated *in vitro* with 10nM LBH589

Relative Expression post LBH589 treatment (10nM) Treated/Untreated Control					
miR	Pathway	2h	4h	24h	Target mRNA
miR-20a	MYC	1.08	0.98	0.84	p21
miR-92	MYC	1.04	0.95	0.77	p63 and CDH1
miR-18a	MYC	1.14	0.94	0.75	ER alpha
miR-331-3p	AR	1.00	2.07	1.76	Erb-B2 and E2F1
miR-125b-3p	AR/Hyp	1.06	1.03	0.67	Bak-1
miR-125b-5p	AR/Hyp	1.09	0.94	0.88	



Summary

- Concurrent HDAC and mTORC1 inhibition results in greater antitumor and therapeutic efficacy in an androgen-sensitive and castrate-resistant MYC murine model of PCa.
    - Inhibition of clonogenic potential
    - Inhibition of cell cycle progression (p21 induction)
    - Attenuate AR and HIF-1α transcriptional activity
    - Decrease tumor proliferation (Ki-67) and tumor vasculature (CD31)
    - Decrease c-MYC expression/activity (AR transcription dependent)
    - Alter expression of onco/tumor suppressor miRs
    - This pre-clinical study has also been conducted in castrated male FVB mice bearing castrate-resistant MYC-CaP tumors with the same results, indicating this combinational treatment is well warranted for further investigation to treat patients with advanced and castrate-resistant PCa.

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Abstract: Specific inhibitors towards Histone Deacetylases (HDACs) and Mammalian Target of Rapamycin Complex 1 (mTORC1) have been developed and demonstrate potential as treatments for patients with advanced and/or metastatic and castrate resistant prostate cancer (PCa). Further, deregulation of HDAC expression and mTORC1 activity are documented in PCa and provide rational targets to create new therapeutic strategies to treat PCa. Here we report the use of the c-MYC adenocarcinoma cell line from the c-myc transgenic mouse with prostate cancer to evaluate the in vitro anti-tumor and in vivo therapeutic efficacy of the combination of the HDAC inhibitor panobinostat with the mTORC1 inhibitor everolimus. Panobinostat/everolimus combination treatment resulted in both greater antitumor activity and therapeutic efficacy in mice bearing androgen sensitive Myc-CaP and castrate resistant Myc-CaP tumors compared to single treatments. We identified that panobinostat/everolimus combination resulted in enhanced anti-tumor activity mediated by decreased tumor growth concurrent with augmentation of p21 expression and the attenuation of angiogenesis and tumor proliferation via androgen receptor, c-MYC and HIF-1α signaling. Also, we observed altered expression of microRNAs associated with these three transcription factors. Overall, our results demonstrate that low dose concurrent panobinostat/everolimus combination therapy is well tolerated and results in greater anti-tumor activity and therapeutic efficacy compared to single treatments in tumor bearing immuno-competent mice. Finally, our results suggest that response of specific miRs could be utilized to monitor panobinostat/everolimus in vivo activity.

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To Whom It May Concern:

Attached you will find our manuscript entitled '**Concurrent Histone Deacetylase and Mammalian Target of Rapamycin inhibition attenuate androgen receptor and hypoxia signaling associated with alterations in microRNA expression**' which we would like to submit as a research article for publication in PLoS ONE.

Limited therapies to patients with prostate cancer (PCa) are a major concern. Within we have utilized the murine Myc-CaP prostate cancer cell line to generate both androgen sensitive and castrate resistant 'Myc-CaP tumors', allowing us to evaluate the potential of concurrent HDAC and mTOR inhibition as a novel therapeutic intervention in an immuno-competent mouse model of PCa. We demonstrate that combination treatment increases anti-tumor effect and enhances therapeutic efficacy by attenuating HIF-1α, androgen receptor (AR) and c-MYC expression and transcriptional activity, associated with changes in microRNA expression that are known to be down stream effectors of hypoxia, AR and c-MYC signaling, ultimately resulting in an overall loss of tumor cell proliferation. These data also depict that this combination therapy approach is well tolerated with minimal toxicities, and warrants further pre-clinical and clinical evaluation for the treatment of patients with PCa.

Thank you very much for your consideration of our manuscript, which we hope is suitable for publication in PLoS ONE. We look forward to hearing from you.

Kind Regards,

Roberto Pili, M.D.
Professor of Oncology

RP/km

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Concurrent HDAC and mTORC1 inhibition attenuate androgen receptor and hypoxia signaling associated with alterations in microRNA expression

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Running Title: Inhibition of HDACs and mTOR to treat prostate cancer

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Keywords: histone deacetylase inhibitors, mTOR inhibitors, therapy, prostate cancer

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Abstract:

Specific inhibitors towards Histone Deacetylases (HDACs) and Mammalian Target of Rapamycin Complex 1 (mTORC1) have been developed and demonstrate potential as treatments for patients with advanced and/or metastatic and castrate resistant prostate cancer (PCa). Further, deregulation of HDAC expression and mTORC1 activity are documented in PCa and provide rational targets to create new therapeutic strategies to treat PCa. Here we report the use of the c-MYC adenocarcinoma cell line from the *c-myc* transgenic mouse with prostate cancer to evaluate the *in vitro* anti-tumor and *in vivo* therapeutic efficacy of the combination of the HDAC inhibitor panobinostat with the mTORC1 inhibitor everolimus. Panobinostat/everolimus combination treatment resulted in both greater antitumor activity and therapeutic efficacy in mice bearing androgen sensitive Myc-CaP and castrate resistant Myc-CaP tumors compared to single treatments. We identified that panobinostat/everolimus combination resulted in enhanced anti-tumor activity mediated by decreased tumor growth concurrent with augmentation of p21 expression and the attenuation of angiogenesis and tumor proliferation via androgen receptor, c-MYC and HIF-1α signaling. Also, we observed altered expression of microRNAs associated with these three transcription factors. Overall, our results demonstrate that low dose concurrent panobinostat/everolimus combination therapy is well tolerated and results in greater anti-tumor activity and therapeutic efficacy compared to single treatments in tumor bearing immuno-competent mice. Finally, our results suggest that response of specific miRs could be utilized to monitor panobinostat/everolimus *in vivo* activity.

Introduction

Treatment for advanced prostate cancer currently involves hormone therapies that lowers serum testosterone and antagonizes the transcriptional capabilities of the androgen receptor (AR) by targeting its ligand binding domain. Initially effective, these therapies are eventually 'adapted' to, enabling the cancer to survive in a low androgen environment. This results in the development of a lethal PCa phenotype, castrate-resistant prostate cancer (CRPC). Currently, therapies including the microtubule inhibitors docetaxel and cabazitaxel, and the recently approved abiraterone and the autologous immunotherapy sipuleucel T are available therapies to patients with CRPC. Although these therapies are life prolonging, additional treatment options are still required.

Targeted therapies have emerged as promising agents for novel therapeutic interventions in PCa. Thereby understanding specific genetic and/or epigenetic alterations we can better strategize how to utilize targeted therapies to their fullest potential. PCa can be characterized by four predominant genetic and cellular modifications which include the presence of the *TMPRSS2-ERG* gene fusion [1]; loss of phosphatase and tensin homolog *(PTEN)* tumor suppressor function ultimately resulting in constitutive PI3K-pathway activation [2]; amplification of the oncogene *MYC* [3]; and the amplification, over-expression or mutation of the AR [4,5]. More recently epigenetic changes including deregulation of small non-coding RNAs called microRNA as well as histone deactylases (HDACs) have been documented in PCa pre-clinical and clinical studies [6,7].

A primary target of the PI3K-pathway is AKT and its downstream effector mammalian target of rapamycin (mTOR) [8]. mTOR promotes cellular protein synthesis and is highly involved in cell cycle progression, proliferation, apoptosis, autophagy and

angiogenesis [9]. mTOR signaling is organized into two main mutiprotein complexes; mTORC1 (mTOR complex 1) and mTOR2 (mTOR complex 2). mTORC1 is the molecular target of the FDA approved mTOR inhibitor rapamycin and its analogs everolimus and temsirolimus which act to antagonize mTORC1 activity via allosteric inhibition [10,11].

HDACs are documented to play a major role in the progression of PCa [6,12]. While HDACs are an important component of transcriptional co-repressor complexes mediating gene transcription via deacetylation of histones, they also regulate the activity of non-histone proteins including two critical transcription factors in PCa, HIF-1α [13] and AR [14] via deacetylation. The HDAC inhibitors romidepsin and vorinostat, have been approved to treat cutaneous T cell lymphomas. While mTORC1 [15] and HDAC [12] inhibitors show great promise as monotherapies, it maybe in combination strategies where these agents reach their fullest clinical potential. For that reason, multiple clinical trials are currently pursuing optimum combination strategies to best utilize these targeted therapies in multiple cancer types, including PCa.

Within, we utilize the mouse prostate cancer cell line Myc-CaP generated from the Hi-Myc murine model of PCa [16,17] to demonstrate that low dose combination of the HDAC inhibitor panobinostat and the mTORC1 inhibitor everolimus *in vitro* and *in vivo* result in greater anti-tumor activity and therapeutic efficacy than single agent treatment in a murine model of PCa. Overall panobinostat/everolimus combination resulted in a significant reduction in angiogenesis and tumor cell proliferation when compared to single agent treatments. These combination effects were associated by induction of the cyclin dependent kinase inhibitor p21$^{WAF1/CIP1}$, loss of oncogenic

microRNA expression secondary to a significant loss of transcriptional activity driven by HIF-1α, c-MYC and AR. Further, we demonstrate a distinct regulation of two oncogenic miRs associated with PCa and HIF-1α, c-MYC and AR signaling. These miRs could be utilized to monitor response to therapy. The cooperative effect from combination therapy on these signaling pathways likely explains the greater therapeutic effect *in vivo*.

Results

Myc-CaP cell line *in vitro* sensitivity to panobinostat and everolimus. Myc-CaP cell lines cultured *ex vivo* were exposed to increasing concentrations of panobinostat and everolimus for 24 and 48 hours and cell membrane permeability was assessed by uptake of propidium iodide (PI). As shown in Figure 1A (top and bottom panel), Myc-CaP cells were sensitive to the cytotoxic effects of panobinostat in a dose and time dependent manner. Conversely, increasing concentrations of everolimus did not display any cytotoxic effects towards Myc-CaP cells. Because Myc-CaP cell lines remained resistant to the cytotoxic effects of everolimus it was hypothesized that Myc-CaP cells would be sensitive to everolimus growth inhibitory effects. Myc-CaP cells treated with non-cytotoxic concentrations of panobinostat and everolimus for 24 and 48 hours were assessed for cell growth by colorimetric absorbance of Myc-CaP cells fixed and stained with 10% MeOH in crystal violet. Figure 1B (top and bottom panel) shows that Myc-CaP cells were sensitive to growth inhibitory effects induced by panobinostat and everolimus in a time and dose dependent manner. From figure 1A and B we chose to explore clonogenic survival assays with non-cytotoxic concentrations of panobinostat and everolimus to evaluate the long term effects of panobinostat and everolimus as single or combination treatments. Non-cytotoxic concentrations were based on concentrations of either compound that did not induce loss of cell viability but induced decrease in cell growth. Figure 1C and D demonstrates quantitation of colony growth. These results indicate that low non-cytotoxic concentrations of panobinostat (10nM) and everolimus (10nM) in combination have significant inhibition of clonogenic survival over single

treatments at 24 hours. For further *in vitro* analysis concentrations were panobinostat 10nM and everolimus 10nM for single and combination treatments were chosen.

Non-cytotoxic concentrations of panobinostat/everolimus combination induce cell cycle arrest and not apoptosis. Because low dose concentrations of panobinostat and everolimus in combination resulted in greater loss of clonogenic survival it was our objective to determine if this was due to inhibition of cell cycle progression or induction of apoptosis. Treatment of Myc-CaP cells with 10nM panobinostat and 10nM everolimus individually or in combination for 24 and 48 hours indicates that both single and combination treatments did not induce cell death as no accumulation of cells in $SubG_1$ (Fig 2A) or increase in the positive apoptotic staining of cells with annexin V and propidium iodide were observed (data not shown). Inhibition of cell cycle progression was induced however, evident by a loss of S phase and a concomitant increase in the G_0/G_1 phase (Fig 2A) and induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ (Fig 2B).

Panobinostat/everolimus combination results in reduced tumor burden in mice bearing androgen sensitive or castrate resistant Myc-CaP tumors. To further investigate the therapeutic potential of panobinostat/everolimus combination for the treatment of prostate cancer, pre-clinical therapy studies were conducted. Myc-CaP/AS (androgen sensitive) or Myc-CaP/CR (castrate resistant) tumor pieces were transplanted unilateral to intact or castrated male FVB mice respectively. Tumor bearing animals were then treated with 10mg/kg panobinostat, 10mg/kg everolimus, or the combination for 15 days on a QD x7 schedule. Treatment with panobinostat alone resulted in a modest

decrease in mean tumor proliferation and volume in androgen sensitive (Fig. 3A-D) and castrate resistant Myc-CaP tumors (Fig. 3E and F). Interestingly, panobinostat single treatment mediated a strong reduction in tumor proliferation as indicated by IHC staining for Ki67 (Fig. 3G and H) compared to vehicle treated controls. Everolimus also induced a modest decrease in tumor growth, size and proliferation of androgen sensitive (Fig. 3A-D) and castrate resistant Myc-CaP tumors (Fig. 3E-H), while panobinostat/everolimus combination therapy significantly reduced tumor proliferation and volume in both Myc-CaP/AS (Fig. 3A-D) and Myc-CaP/CR (Fig. 3E-H) tumor models. Further, all therapies were well tolerated without overt signs of toxicities and significant weight loss. Importantly, white cell and platelet counts, though reduced, stayed within normal ranges for all treatment groups (data not shown).

Panobinostat/Everolimus combination attenuates HIF-1α transcriptional activity and angiogenesis. Angiogenesis is an important signaling pathway in PCa and is primarily driven by the transcriptional activity of HIF-1α. Our laboratory had previously demonstrated that the combination of rapamycin and panobinostat resulted in HIF-1α protein degradation associated with a reduction in tumor angiogenesis of prostate cancer and renal cell carcinoma xenograft models [19]. Because of this previous work and increased vasculature of Myc-CaP tumors, we assessed the effects of everolimus and panobinostat on angiogenesis by measuring HIF-1α protein levels, transcriptional activity and intratumoral vasculature (using CD31 as an endothelial cell marker). The transcriptional activity of HIF-1α was assessed by the use of reporter plasmids expressing hypoxia response element specific for the recognition by HIF-1α and not HIF-2α. Myc-

CaP/HRE cells treated *in vitro* with cobalt chloride to mimic hypoxia show a HIF-1α time dependent response that is inhibited by panobinostat and everolimus single treatments, but combination of these two drugs produced an enhanced reduction of HIF-1α transcriptional activity (Fig. 4C). Further, panobinostat/everolimus combination greatly reduced HIF-1α protein levels compared to single treatments (Fig. 4D). The combination of panobinostat/everolimus *in vivo* also resulted in a significant loss of HIF-1α transcriptional activity (Fig. 5A and Fig. 5C). Combination therapy also induced dramatic reduction of luminal structures throughout the tumor vasculature (*) as well as a dramatic reduction in the size of these vessels (indicated by arrows) compared to single treatment of Myc-CaP/AS and Myc-CaP/CR tumors (Fig. 5D). These results suggest that inhibition of angiogenesis may also play a significant role towards the therapeutic efficacy of the panobinostat/everolimus combination.

Panobinostat/everolimus combination attenuates Androgen Receptor transcriptional activity. HDACI have previously been shown to disrupt AR protein stability and transcription [14] and there has been recent work investigating mTOR and AR cross talk [20]. We therefore investigated the effects of panobinostat and everolimus on AR protein stability and transcriptional activity in the Myc-CaP model. Myc-CaP/ARE cells cultured in low androgen conditions supplemented with 10nM R1881 demonstrate that ligand dependent AR transcriptional activity is inhibited by panobinostat, whereas everolimus treatment resulted in an increased AR transcriptional response. Combination treatment interestingly showed that panobinostat was able to inhibit the activation of AR transcriptional response mediated by everolimus (Fig. 4A).

Investigation of whole cell Myc-CaP lysates by immunoblot indicate that either single or combination treatment does not result in AR protein degradation, though as indicated by decreased proteins levels of c-MYC (specific transcriptional target of AR in this model), AR transcriptional activity is suppressed (Fig. 4B). We believe that the opposing effects on AR transcriptional activity in Fig. 4A and Fig. 4B by treatment with everolimus maybe explained through the inability of mRNA translation due to the inhibition of mTORC1 by everolimus. Immunohistochemical analysis was performed to determine if the effects seen *in vitro* were also observed *in vivo*. Transcriptional AR activity *in vivo* was dramatically reduced by panobinostat/everolimus combination over each single treatment (Fig. 5A and Fig. 5B). Paraffin embedded Myc-CaP/AS (Fig. 5E) and Myc-CaP/CR (Fig. 5F) tumor sections were stained to investigate AR protein levels and cellular location or anti-c-MYC to monitor AR transcriptional activity *in vivo*. Single treatment of both tumors did not result in major changes of AR or c-MYC expression. Interestingly, combination treatment strongly induced cytoplasmic localization of AR with an associated loss of c-MYC expression (Fig. 5G and Fig. 5H). These results indicate that combination therapeutic efficacy may be in part mediated by the inhibition of HIF-1α and AR transcriptional response and loss of proliferation.

Panobinostat/everolimus combination reduces known onco-microRNA expression *in vivo*. Hypoxia, AR and c-MYC signaling have been documented to target downstream microRNA's via their transcriptional activity. Because our previous results demonstrate decreased oncogene signaling via attenuation of HIF-1α and AR (also c-MYC) transcriptional activity we investigated known associated onco-miRs downstream

of these transcription factors that may indicate potential mechanisms of panobinostat/everolimus combination anti-tumor activity. Using QRT-PCR, we determined the expression levels of a documented miR associated with AR/hypoxia signaling, miR-21 [21] and the c-Myc/hypoxia associated miR-20a [22]. Single and combination treatments of intact and castrated mice bearing Myc-CaP/AS and Myc-CaP/CR tumors respectively resulted in miR expression patterns highly similar to AR signaling from our *in vitro* data (Fig.5A). Regulation of miR expression patterns in both Myc-CaP/AS and Myc-CaP/CR by panobinostat single treatment resulted in down-regulation of miR-20a and miR-21 compared to vehicle treated mice. Response to everolimus single treatment however resulted in both miRs being up-regulated respective to control treated mice. The up-regulation of these two onco-miRs was interestingly attenuated in the panobinostat/everolimus combination treated mice (Fig. 6A and Fig. 6B). Taken together these data demonstrate that inhibition of HDACs and mTORC1 can affect androgen and hypoxia signaling at multiple levels (Fig. 7). Further, HDAC and mTORC1 regulate miR expression inversely and may offer a potential explanation towards the current failure of mTOR inhibitors in the clinic. By combining everolimus with panobinostat we elude possible tumor escape mechanisms in response to mTOR inhibition, resulting in, at least with this combination, cytostatic anti-tumor activity.

Discussion

HDAC inhibitors exhibit pleiotropic molecular and biologic effects [12,23] and have shown clinical activity in the treatment of cutaneous T cell lymphoma [24]. Because of HDAC inhibitors ability to affect multiple pathways and genes involved in apoptosis [18], cell cycle arrest [25] and angiogenesis [24,26,27], their greatest potential as targeted therapies maybe to utilize them in novel combinational therapeutic strategies in PCa with already existing chemotherapies such as docetaxel [28], or with other novel targeted chemotherapies including mTOR inhibitors [19].

Within, we have utilized the mouse cell line Myc-CaP [16] generated from the Hi-Myc transgenic mouse model of prostate cancer [17], which drives the expression of c-Myc by the androgen receptor dependent rat probasin promoter, to assess the *in vitro* and *in vivo* anti-tumor activity and therapeutic efficacy of combination treatment with low dose HDACI panobinostat and the mTOR inhibitor everolimus. It has been documented that PCa involves deregulated expression of HDACs [6] and activation of mTORC1 [29,30] signaling and therefore provide rationale to target these in combinational therapeutic strategies.

Initial *in vitro* data demonstrates that low dose panobinostat/everolimus combination did not result in tumor cell apoptosis, but rather reduced the tumor growth and clonogenic capacity of Myc-CaP cell lines through induction of cell cycle arrest associated with enhanced p21 protein expression. This is consistent with recent data published that demonstrated HDAC/mTOR inhibitor combination treatment of PCa cell lines resulted in increased inhibition of cell growth and cell cycle progression concurrent with increased levels of p27 and p21 proteins [31]. Moreover, p21 repression is also

mediated by c-Myc and induced acetylation of the p21 promoter through HDAC inhibition and loss of c-Myc expression is correlated with induced p21 expression [32]. Likewise, our treatment of Myc-CaP cell lines with panobinstat did induce histone H3 acetylation (data not shown) and combination with everolimus resulted in greatest inhibition of c-Myc protein expression. *In vitro* growth inhibition by panobinostat/everolimus combination was also correlated *in vivo* in our immunocompetent mouse transplant model, where Myc-CaP/AS and Myc-CaP/CR tumor growth was inhibited without induction of tumor apoptosis as determined by caspase 3 staining (data not shown). Reduction in tumor burden has been previously demonstrated in a PCa xenograft model treated with concurrent HDAC/mTOR inhibition [19] and more recently in renal cell carcinoma (RCC) xenograft models [33]. Interestingly, HDAC/mTOR inhibition in this RCC model also resulted in tumor apoptosis via greater inhibition of the anti-apoptotic, pro-angiogenic protein survivin [33,34]. These differences maybe explained by model specific responses to HDAC/mTOR inhibition. Further, the RCC model was treated with vorinostat at a dose 10 fold greater than panobinostat used in this study, possibly allowing for greater alteration of the pro/anti-apoptotic balance. Another possibility is that although HDAC inhibitors of the same class, such as vorinostat and panobinostat elicit overlapping gene transcription patterns, they can also mediate specific genetic signatures, possibly because of different HDAC inhibition capabilities [12,35]. These data highlight the possible importance of understanding HDAC expression underlying specific tumor types which may aide in HDAC inhibitor type and dose used to treat PCa patients.

HDAC and mTOR inhibitors also demonstrate greater anti-angiogenic activity in combination [19,33]. Recent data published from our laboratory, displays combination of rapamycin and panobinostat significantly reduced HIF-1α protein and vessel density in xenograft models with constitutive mTOR activity, either through loss of *PTEN* (PC3 cells) or *VHL* (C2 cells) [19]. Myc-CaP/AS and Myc-CaP/CR tumors express wild type PTEN and low levels of activated mTOR (data not shown). Even so, we observed substantial activity in HIF-1α transcriptional activity associated with densely vascularized tumors, and panobinostat/everolimus combination resulted in abundant inhibition of tumor angiogenesis in androgen sensitive and castrate resistant tumors. We believe that the highly vasculature phenotype in Myc-CaP tumors is driven by c-Myc expression itself, as c-Myc has been shown to be essential for vasculogenesis and angiogenesis during tumor development and progression [36]. Further, increased proliferation of c-MYC driven tumors creates a greater environment of tumor hypoxia which in turn activates HIF-1α activity. Also, enhanced metabolic stress within the tumor cell could allow for mTORC1 inhibition to elicit a therapeutic response in combination with HDAC inhibitors.

Critical to androgen sensitive and castrate resistant prostate cancer growth and survival is the transcriptional activity of the AR. Myc-CaP cell lines express an amplification of their wild type AR though this phenomenon was independent of androgen withdrawal [16]. HDAC inhibitors have previously demonstrated the ability to attenuate AR transcriptional activity by either loss of protein expression or by disabling the ability of AR to bind DNA [14,37]. Conversely, inhibition of mTORC1 signaling activates AR signaling [20,38]. Consistent with these reports, our data indicates elevated

AR transcriptional activity resulting from mTORC1 inhibition which was significantly inhibited by panobinostat in combination treatment, indicating increased AR function can maintain survival in the presence of mTORC1 inhibition, and to perturb AR function with HDAC inhibitors offers a novel therapeutic strategy to over come this.

Recently, microRNAs importance as effectors of hypoxia, c-Myc and AR signaling has been recently highlighted [39,40,41,42]. Of specific interest to us was the response of two documented microRNAs to exhibit oncogenic activity in PCa and whose expression is mediated by these signaling pathways, miR-20a [43] and miR-21 [44]. Recently, miR-21 was observed to be elevated in patient serum levels with metastatic hormone-refractory PCa [45]. Further, when serum miR-21 and miR-141 levels was integrated with PSA serum levels, positive prediction of PCa was increased from 40% to 87.5% success [46]. Also, data from clinical samples defined patients with a Gleason score ≥7 had significant increase of miR-20a expression compared to patients with Gleason score ≤6 [43]. Treatment of Myc-CaP/AS and Myc-CaP/CR tumors resulted in similar responses where again both onco-miRs were up-regulated in response to everolimus treatment, though excitingly panobinostat treatment attenuated this increase in onco-miR expression. The down regulation of these two associated PCa microRNAs raises the opportunity to evaluate patient response to therapy and to predict the efficacy of these targeted therapies on important signaling pathways involved in PCa. Whereas PSA allows for surveillance of AR transcriptional activity, microRNAs including miR-20a and miR-21 would allow monitoring multiple pathways within PCa patients being treated with novel targeted therapies.

Androgen receptor, c-Myc and HIF-1α activity are associated with poor prognosis in many cancers, including PCa [47,48]. Previous work from this laboratory has demonstrated panobinostat to be potent inhibitor of tumor angiogenesis as a single agent [26] and also in combination with the mTORC1 inhibitor, rapamycin [19]. These studies were conducted in the PC3 PCa tumor model which has constitutive activation of the PI3K-Akt-mTOR pathway through loss of Pten. Those data focus on the mediation of antitumor activity by panobinostat's ability to induce HIF-1α protein degradation in endothelial cells, thus inhibiting tumor angiogenesis. Our current investigation utilizes an immunocompetent mouse model of PCa which is Pten expressing and does not involve constitutively activated PI3K-Akt-mTOR signaling. Collectively, our data presented within demonstrate that only panobinostat/everolimus combination therapy result in the degradation of HIF-1α protein and inhibits HIF-1α and AR transcriptional activity *in vivo*. The low dose biological effects of this combination are of particular interest in view of a previous report showing poor tolerability and limited activity of full dose of vorinostat in patients with advanced CRPC [49]. Also, to date single agent clinical activity of either HDAC or mTOR inhibition in PCa has been limited [49,50]. This study provides strong rationale for the continued clinical investigation and design of clinical trials with rational combinations of targeted therapies including HDAC and mTOR blockade for the treatment of patients with advanced and castrate resistant PCa.

Methods and Materials

Ethics Statement

The Institute Animal Care and Use Committee (IACUC) at Roswell Park Cancer Institute (RPCI) approved all mouse protocols used in this study. Our approval/protocol ID is 1137M.

Cell culture and reagents

The Myc-CaP cell line [16] was a kind gift from Dr Charles Sawyers and were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C, 5% CO_2. *In vitro*, panobinostat and everolimus powder (Novartis) were dissolved in DMSO as 10mM stocks and diluted in cell culture medium prior to experiments. *In vivo*, panobinostat powder was dissolved in D5W (5% dextrose in distilled water) at a concentration of 1mg/mL. everolimus was supplied as an aqueous solution at 20mg/mL and diluted in distilled water to a final concentration of 1mg/mL. A placebo (vehicle) was also supplied as an aqueous solution and diluted in distilled water the same as everolimus. Cobalt chloride was purchased from Sigma-Aldrich. Lentiviral particles containing reporter element constructs for androgen receptor (AR) and hypoxia inducible factor-1 alpha (HIF-1α) response elements, which drive firefly luciferase expression, where purchased from SABiosciences. Bright-Glo™ Luciferase Assay System (Promega) was used to detect luciferase luminescence for *in vitro* assays.

In vitro cell death and cell growth assays

Myc-CaP cells (4x10^5/mL) were left to adhere overnight in 24-well plates (BD Biosciences) then incubated in the presence of indicated treatments for 24-48 hours in 1 mL normal cell culture medium. Viability (cell death) was measured by propidium iodide (Sigma) (PI) uptake. Apoptosis was measured by annexin V (BD Bioscience) and PI double staining. Cell growth was measured by fixation and staining of cells with 10% Methanol/Crystal Violet solution. Stained cells were made soluble in absolute methanol and absorbance was detected at an emission length of 595nm.

Clonogenic survival assays

Myc-CaP cells (5x10^2/mL) were left to adhere overnight in 6-well plates. Cells were then treated as indicated for 24-48 hours. Post drug treatment cells were washed in fresh media and grown in the absence of drug for 12 days. Developed cell colonies were fixed and stained in 10% Methanol in Crystal violet solution. Colony counts were performed using Image J software.

Western blot analysis

Myc-CaP were washed in PBS and lysed in RIPA buffer (Sigma-Aldrich) containing 1x protease and phosphatase inhibitors (Sigma-Aldrich). Equal amounts of protein were separated by electrophoresis using 4-15% SDS-PAGE gradient gels (Bio-rad) as previously described [18]. Protein was transferred to nitrocellulose membranes. Immunoblotting was performed with anti-AR (Santa Cruz Biotechnology), anti-c-MYC (Epitomics), anti-HIF-1α (Cayman Chemicals) and anti-β-actin (Sigma-Aldrich). Anti-

rabbit and mouse horseradish peroxidase-conjugated secondary antibodies were from Dako (Carpinteria, CA). Immunoblots were visualized using enhanced chemiluminescence (PerkinElmer).

Cell Cycle Analysis

Myc-CaP cells ($4x10^5$/mL) were left to adhere overnight in 6-well plates. Cells were then treated with indicated compounds for 24 and 48 hours. Adherent and non-adherent cells were collected and washed in PBS. Cells were fixed over night in 50% ethanol and stained with PI solution containing RNase A (Sigma) for 15 minutes at 37°C. DNA content was analyzed using a FACs Caliber cytometer.

Measurement of $p21^{WAF1/CIP1}$

Myc-CaP cell lines were cultured in normal culture conditions and treated with panobinostat and/or everolimus as indicated. One million cells was fixed/permealized according the protocol of Cell Signaling. Cells were incubated with anti-p21 (Santa Cruz) followed with anti-rabbit-FITC. Fluorescence was detected using a FACs Caliber cytometer.

***In vitro* analysis of AR and HIF-1α transcriptional activity**

To generate Myc-CaP cell lines stably expressing ARE/luciferase (Androgen Response Elements; Myc/ARE) or HRE/luciferase (HIF-1α Response Elements; Myc/HRE), Myc-CaP cells were grown to 70% confluency in a 96 well plates (BD Biosciences) and transduced with lentiviral particles containing ARE/luciferase or

HRE/luciferase expression plasmids according to manufactures instructions (SABiosciences). Stably expressing cells were selected by resistance to puromycin (2μg/mL) over 14 days. Luminescence quantitation was measured from Myc-CaP cell lines by Bright-Glo™ Luciferase Assay System.

RNA extraction and Quantitative real-time PCR

RNA was extracted from treated and untreated Myc-CaP/AS and Myc-CaP/CR tumors by TRI Reagent® (TRIzol) method. For first strand cDNA synthesis 40ng of total RNA was reverse transcribed into a final volume of 20ul using the miRCURY LNA™ Universal RT cDNA synthesis kit (Exiqon) as per manufacturer's instructions. One microliter of synthetic spike-in was added to 40ng of FirstChoice Human Placental Total RNA (Ambion) and reverse transcribed. This sample was run as an inter-plate calibrator on every plate using control primers supplied with the Exiqon SYBR green master mix. This control allows for the detection of run-to-run variation between plates. Upon completion of the RT process, the template is used for real-time PCR amplification. For QRT-PCR amplification, the cDNA template is diluted 80-fold in nuclease-free water prior to use. Ten Microliter reactions were carried out, in triplicate, according to manufacturer's specifications (Exiqon) using SYBR Green master mix (Exiqon) and pre-designed LNA PCR primer sets (listed below). 40 cycles of PCR amplification, (initial 95˚C denaturation step for 10 minutes followed by 40 cycles of 95C for10 seconds then 60˚C for one minute) were performed using relative quantitation on a 7900HT Sequence Detection System (ABI) with optical reading of the SYBR green signal during the 60˚C annealing/extension step. Data analysis was performed using the ABI 7900HT SDS

software v2.4 and RQ manager 1.2.1. The microRNA target sequences are miR21* (CAACACCAGUCGAUGGGCUGU), miR21 (UAGCUUAUCAGACUGAUGUUGA) and miR20a (UAAAGUGCUUAUAGUGCAGGUAG). U6 was used has a control reference gene. For proprietary reasons the Exiqon oligonucleotide sequences are unable to be published.

***In vivo* mouse experiments**

For *in vivo* therapy experiments the generation of Myc-CaP tumor banks was first established. These tumor banks consisted of Myc-CaP androgen sensitive tumors (Myc-CaP/AS), Myc-CaP castrate resistant tumors (Myc-CaP/CR) (manuscript submitted), Myc-CaP/ARE and Myc-CaP/HRE. All mice were purchased from NCI Frederick (Maryland, USA).

Development of Myc-CaP/ARE and Myc-CaP/HRE tumor banks: Myc-CaP/ARE and Myc-CaP/HRE ($1x10^6$ cells/mouse) cells were injected subcutaneous into wild-type FVB male mice. Established tumors were confirmed by bioluminescence imaging using the Xenogen® IVIS 50 system. Tumors positive for ARE or HRE driven luciferase expression were stored at -80°C until use.

In vivo therapy experiments with mice bearing Myc-CaP/AS and CR tumors: Intact or castrated male FVB mice received small pieces of Myc-CaP/AS or Myc-CaP/CR tumor tissue (~$25mm^2$) respectively by subcutaneous implantation. Tumor growth was monitored by caliper measurement. Upon indication of tumor growth mice received treatment with everolimus by oral gavage (10mg/kg) daily, panobinostat (10mg/kg) by intraperitoneal (IP) injections daily, or both therapies in combination daily.

Mice in the control group received a corresponding amount of placebo administered by oral gavage. Therapeutic efficacy was determined by serial caliper measurements and all tumor tissue collected post-mortem was weighed and used in immunohistochemical studies. Blood was collected by retro-orbital methods at the experiments conclusion to investigate peripheral white cell and platelet counts.

In vivo therapy experiments with mice bearing Myc-CaP/ARE and Myc-CaP/HRE tumors: Intact male FVB mice received small pieces of Myc-CaP/ARE and Myc-CaP/HRE tumor tissue (~25mm^2) bilateral by subcutaneous implantation. Mice were treated as described above for a total of 7 days. *In vivo* imaging to determine tumor androgen receptor and HIF-1α transcriptional activity,Myc-CaP/ARE and Myc-CaP/HRE tumor bearing mice were anesthetized using isoflorane and bioluminescence imaging was conducted using a Xenogen® IVIS 50 system.

Immunohistochemistry

Formalin fixed, paraffin-embedded tissue (4μm) were stained with primary antibodies to detect androgen receptor (Santa Cruz Biotechnology), Ki67 (Thermo Scientific), c-MYC (Epitomics). Zinc fixed paraffin-embedded tissue (4μm) were stained with primary antibodies to detect CD31 (BD Pharmingen). All sections were incubated overnight with primary antibodies at 4˚C and then incubated with ImmPRESS™ reagent kit HRP anti-rabbit IgG antibodies (Vector Laboratories). Staining was developed by incubation with 3,3'-diaminobenzide (Dako), and counterstained with hematoxylin. Images were captured using a Scanscope XT system (Aperio Imaging) and analyzed using Imagescope software (Aperio).

Statistics analysis

Statistical significance between treatment groups was determined using a Student's *t* test.

Differences at $P < 0.05$ were considered significant.

Acknowledgements

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Figure Legends

Figure 1: (A) Myc-CaP cell lines were incubated with indicated concentrations of panobinostat or everolimus for 24 and 48 hours. Cell viability was determined by the uptake of PI and FACs analysis. (B) Myc-CaP cells were incubated with indicated concentrations of panobinostat or everolimus for 24 and 48 hours. Cell were fixed and stained with 10% MeOH in crystal violet. Final cell growth was determined by quantitating the absorbance at an optical density of 570nm. (C-D) The clonogenic potential of Myc-CaP cell lines treated for 24 and 48 hours with panobinostat or everolimus alone or in combination and quantitated by image J analysis. Results shown represent the mean ± SE of 3 separate experiments. * $p<0.05$.

Figure 2: (A) Myc-CaP cells treated with indicated concentrations of panobinostat (P) and everolimus (E) alone or in combination for 24 and 48 hours. Cell cycle analysis was performed by FACs after staining fixed/permeabilized cells with PI. Cell cycle distribution of cells in G_0/G_1, S, G_2/M and SubG_1 (< 2N DNA content) are indicated. (B) Myc-CaP cell lines treated with indicated concentrations of panobinostat or everolimus alone or in combination. Quantitation of $p21^{WAF1/CIP1}$ was performed by FACs after staining fixed/permeabilized cells with anti-p21 followed by anti-rabbit FITC secondary antibody. Results shown in B represent the mean ± SE of 3 separate experiments.

Figure 3: (A) Myc-CaP/AS tumors ($\sim 25mm^2$) were transplanted into the flank of wild type FVB intact male mice. Approximately fourteen (14) days post transplantation mice were treated daily with 10mg/kg everolimus (E) orally, 10mg/kg panobinostat (P) IP or both agents concurrently for a total of 15 days. Tumors were measured twice weekly. Mean ± SE, $n \geq 10$. (B) Excised tumors were weighed to assess the therapeutic efficacy of each treatment schedule. Mean ± SE, $n \geq 7$. (C-D) Tumor samples from A ($n = 3$) were collected and fixed in formalin for IHC analysis. Four micron (4μM) sections were stained for the cell proliferation marker Ki67. Magnification (x40); Scale bar = 50μM. (E) Myc-CaP/CR tumors ($\sim 25mm^2$) were transplanted into the flank of wild type castrated FVB male mice. Mice were treated daily with 10mg/kg everolimus orally, 10mg/kg panobinostat IP or both agents concurrently for a total of 15 days. Tumors were measured twice weekly. Mean ± SE, $n \geq 10$. (F) Excised tumors were weighed to assess the therapeutic efficacy of each treatment schedule. Mean ± SE, $n \geq 7$. (G-H) Tumor samples from A ($n = 3$) were collected and fixed in formalin for IHC analysis. Four micron (4μM) sections were stained for the cell proliferation marker Ki67. Magnification (x40); Scale bar = 50μM. * indicates that combination treatment is significant compared to either single agent treatment. $P < 0.05$. # Note in figure 3H, combination was significant when compared to everolimus treatment ($p<0.05$) but not to panobinostat treatment.

Figure 4: (A) Myc-CaP cell lines with stable expression of androgen response element [Myc-ARE] plasmid treated with 10nM R1881 ± 10nM panobinostat (P), 10nM everolimus (E) or combination. At indicated times cells were lysed and luminescence

intensity was measured and quantitated. Mean ± SE from 3 independent experiments. (B) AR and human c-MYC protein was investigated by immunoblot using whole cell Myc-CaP lysates. β-actin was used as loading control. (C) Myc-CaP cell lines with stable expression of hypoxia response element [Myc-HRE] plasmid treated with 100μM $CoCl_2$ ± 10nM panobinostat (P), 10nM everolimus (E) or combination. At indicated times cells were lysed and luminescence intensity was measured and quantitated. Mean ± SE from 3 independent experiments. (D) HIF-1α protein was investigated by immunoblot using whole cell Myc-CaP lysates treated with 10nM Panobinostat, 10nM everolimus or combination. β-actin was used as loading control.

Figure 5: (A) Myc-CaP tumors expressing either ARE [A] or HRE [H] plasmids were transplanted bi-laterally to wild type FVB intact male mice. Mice were treated daily with 10mg/kg everolimus orally, 10mg/kg panobinostat IP or combination for a total of 7 days. (B-C) Luminescence was measured and quantitated pre- and post therapy. Mean ± SE, *n* = 3. (D) Tumor samples from figure 3A and 3E were collected and placed in zinc fixative. Four micron (4μM) sections were stained for the endothelial marker CD31. Magnification (x20); Scale bars = 50μM. (E-F) Tumor samples from figure 3A and 3E were collected and fixed in 10% neutral buffered formalin. Four micron (4μM) sections were stained for AR and human c-MYC. Magnification (x40); Scale bars = 50μM. (G-H) Quantitation of human c-MYC inhibition from Fig.4E and F. Mean ± SE, *n* = 3 mice.

Figure 6: RNA was extracted from (A) Intact mice bearing Myc-CaP/AS tumors and (B) castrated mice bearing Myc-CaP/CR tumors that were treated daily with 10mg/kg

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4 everolimus (E) orally, 10mg/kg panobinostat (P) IP or combination for a total of 7 days.
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7 QRT-PCR analysis was used to investigate microRNA expression. *n=2*. Mean ± SE of Ct
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9 values from 2 independent tumors.
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14 **Figure 7:** Schematic diagram illustrating potential points where combination therapy
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17 attenuates hypoxia and androgen signaling in murine Myc-CaP PCa tumors. (i) Upstream
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19 of ligand mediated AR activity, (ii) Upstream of hypoxia mediated HIF-1α activity, (iii)
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21 Downstream of AR transcriptional activity and (iv) Downstream of HIF-1α
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Figure 1

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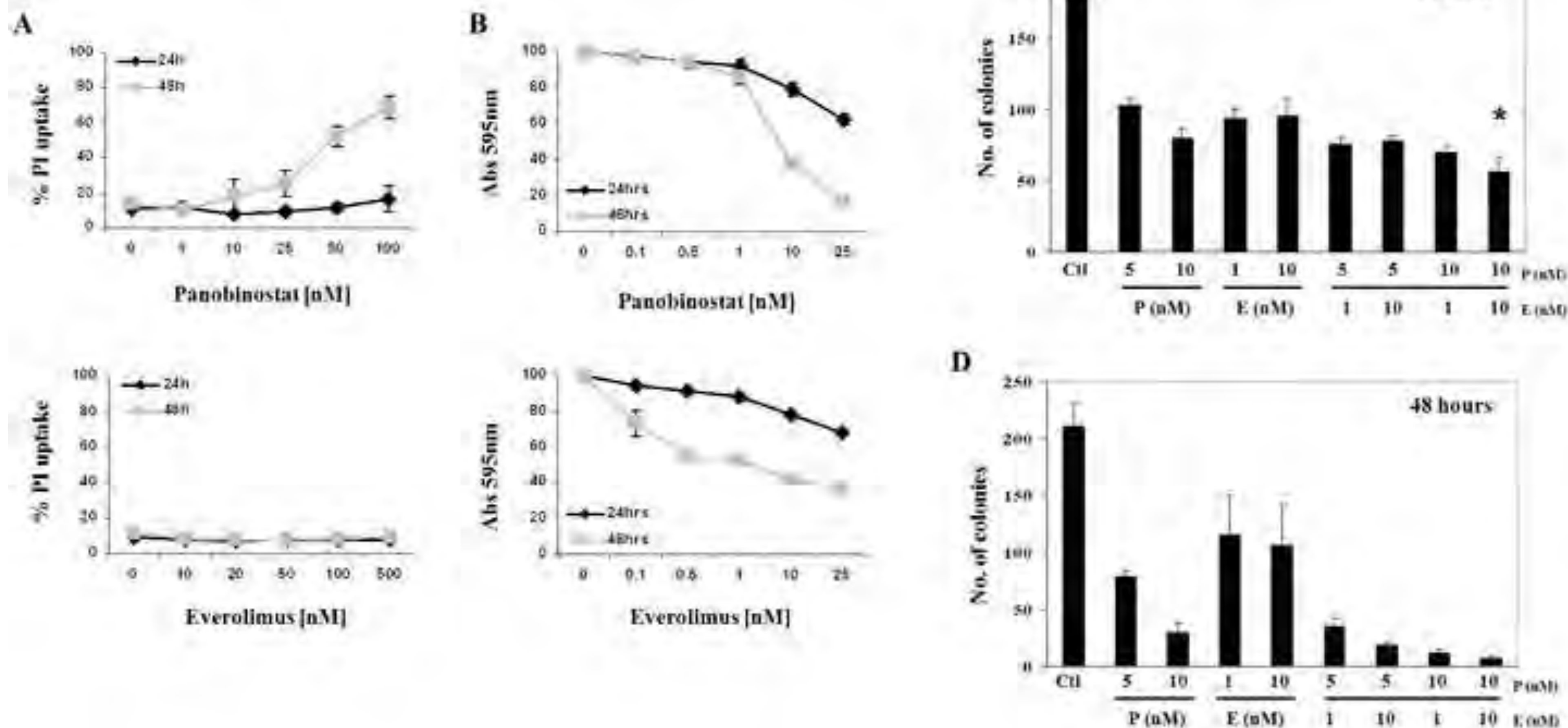

Figure 2

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A

24hr

48hr

Cell Number

Control

G1 51%
S 24%
G2M 25%

G1 55%
S 25%
G2M 20%

P 10nM

G1 70%
S 10%
G2M 20%

G1 73%
S 10%
G2M 17%

E 10nM

G1 61%
S 16%
G2M 23%

G1 75%
S 9%
G2M 16%

Combination

G1 74%
S 8%
G2M 18%

G1 78%
S 6%
G2M 16%

FL2-A

FL2-A

FL2-A fluorescence

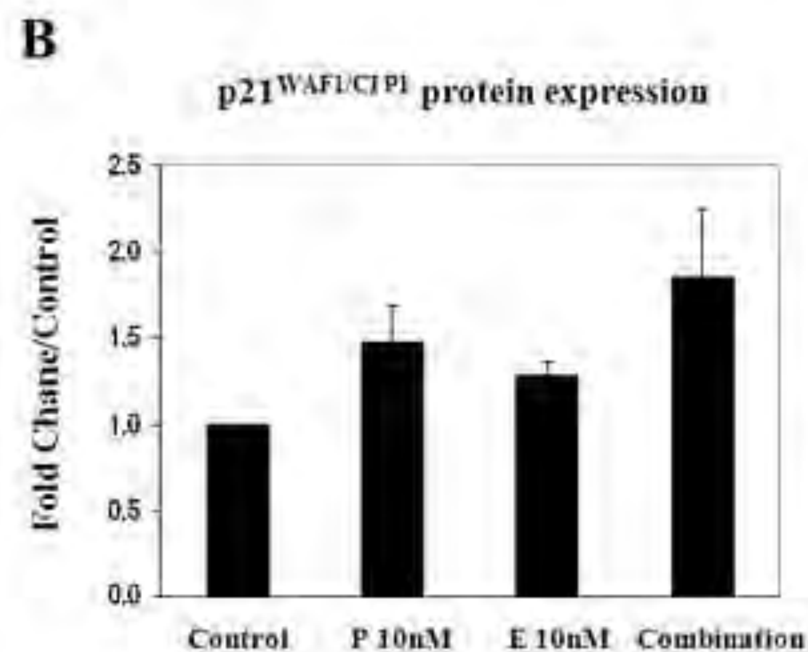

Figure 3

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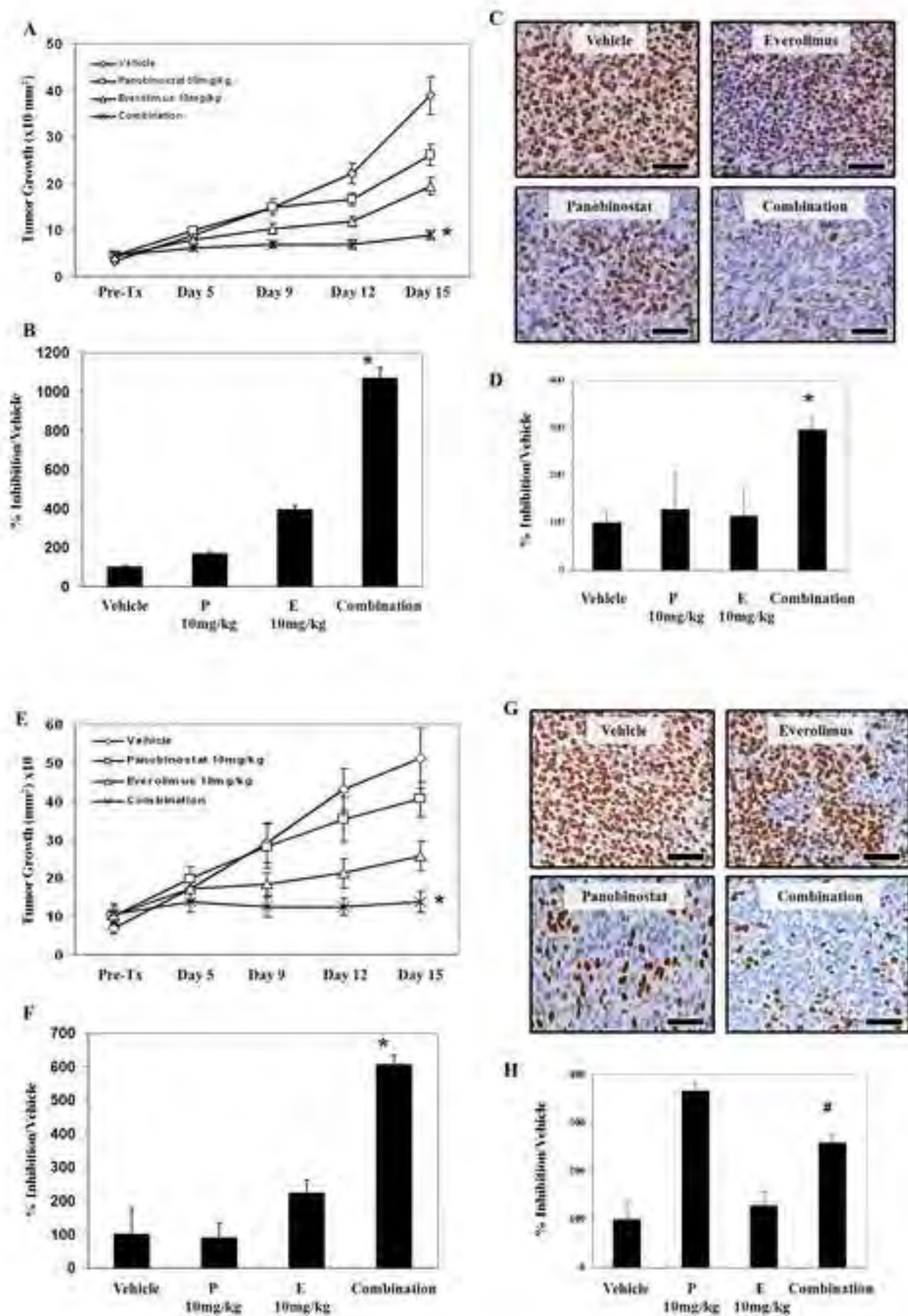

Figure 4
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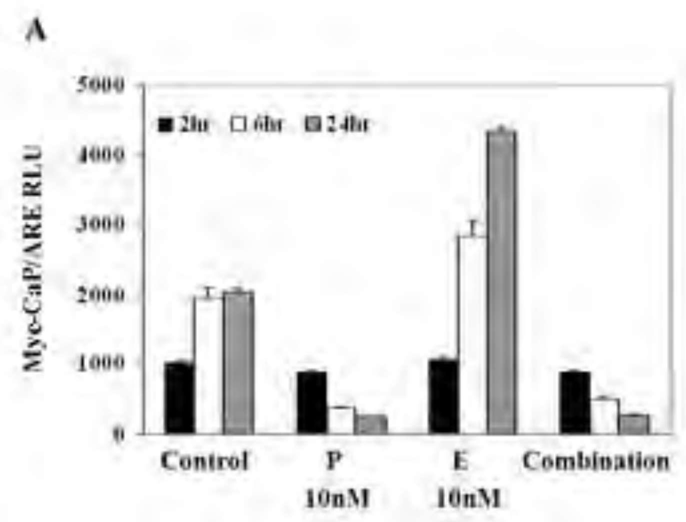

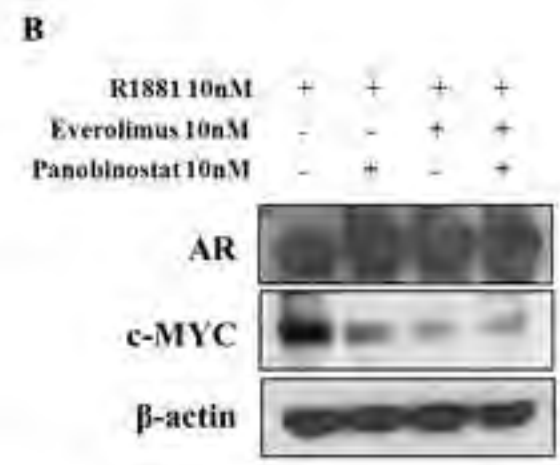

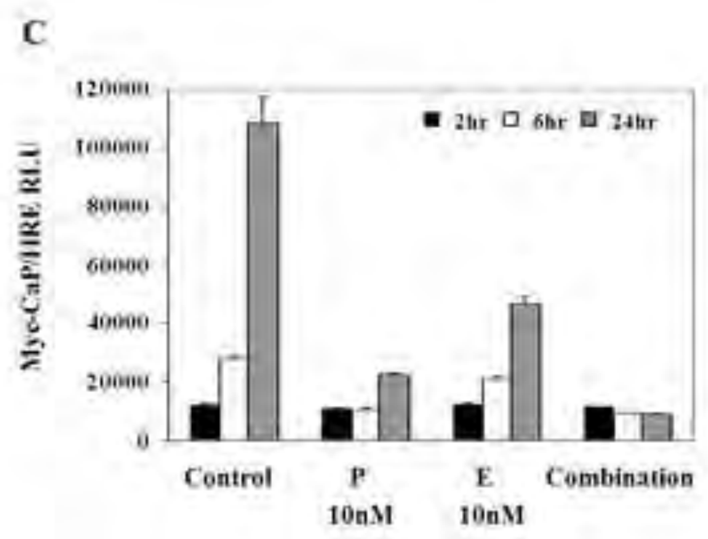

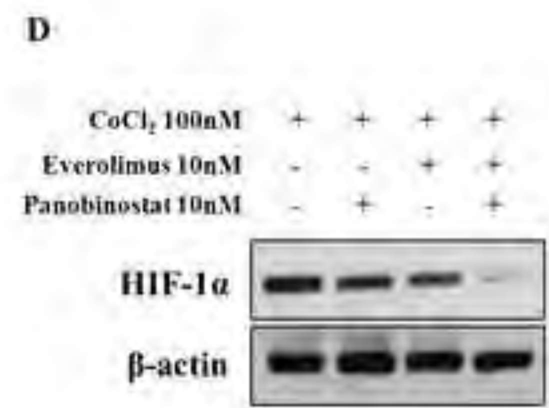

Figure 5

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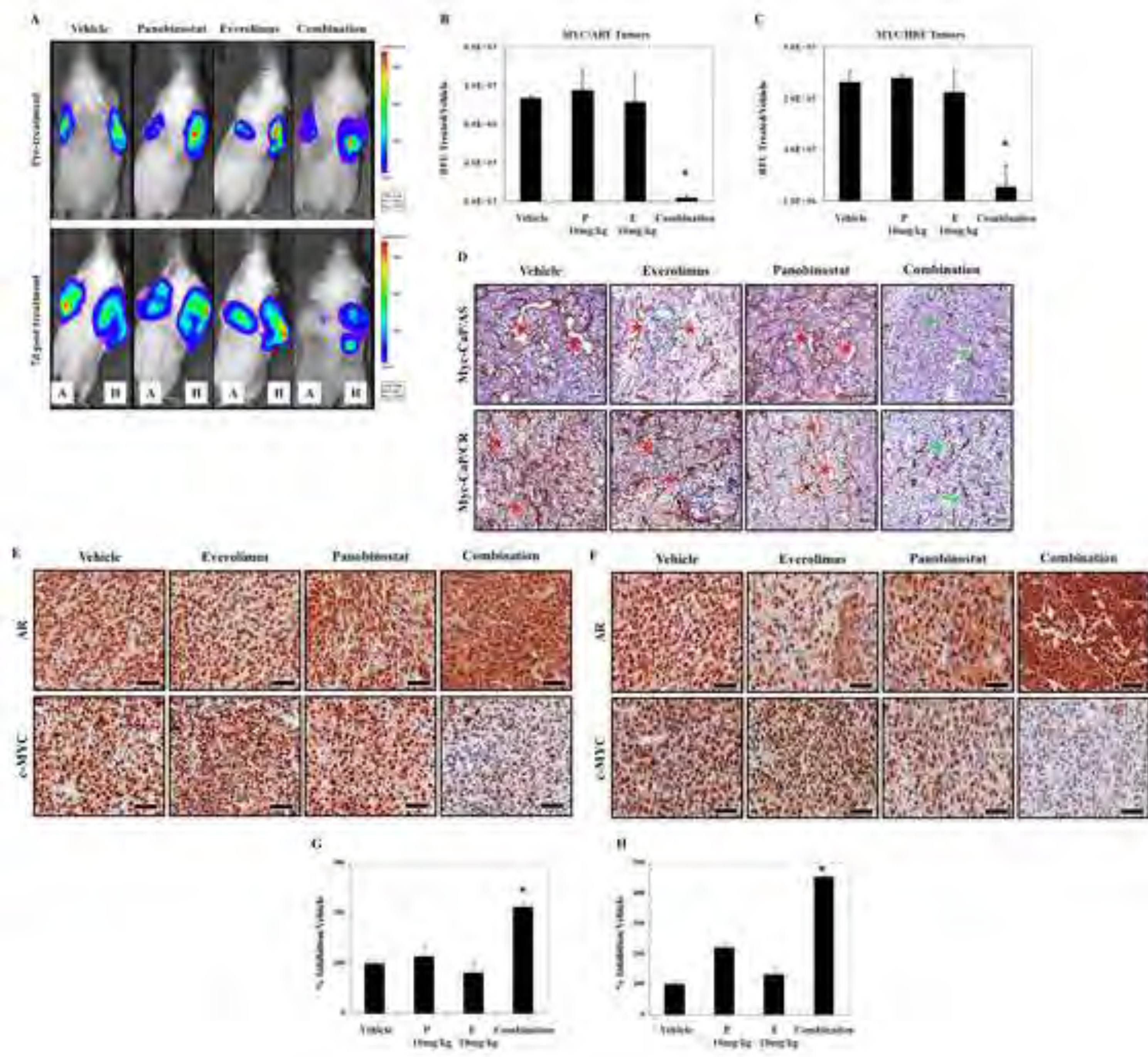

Figure 6

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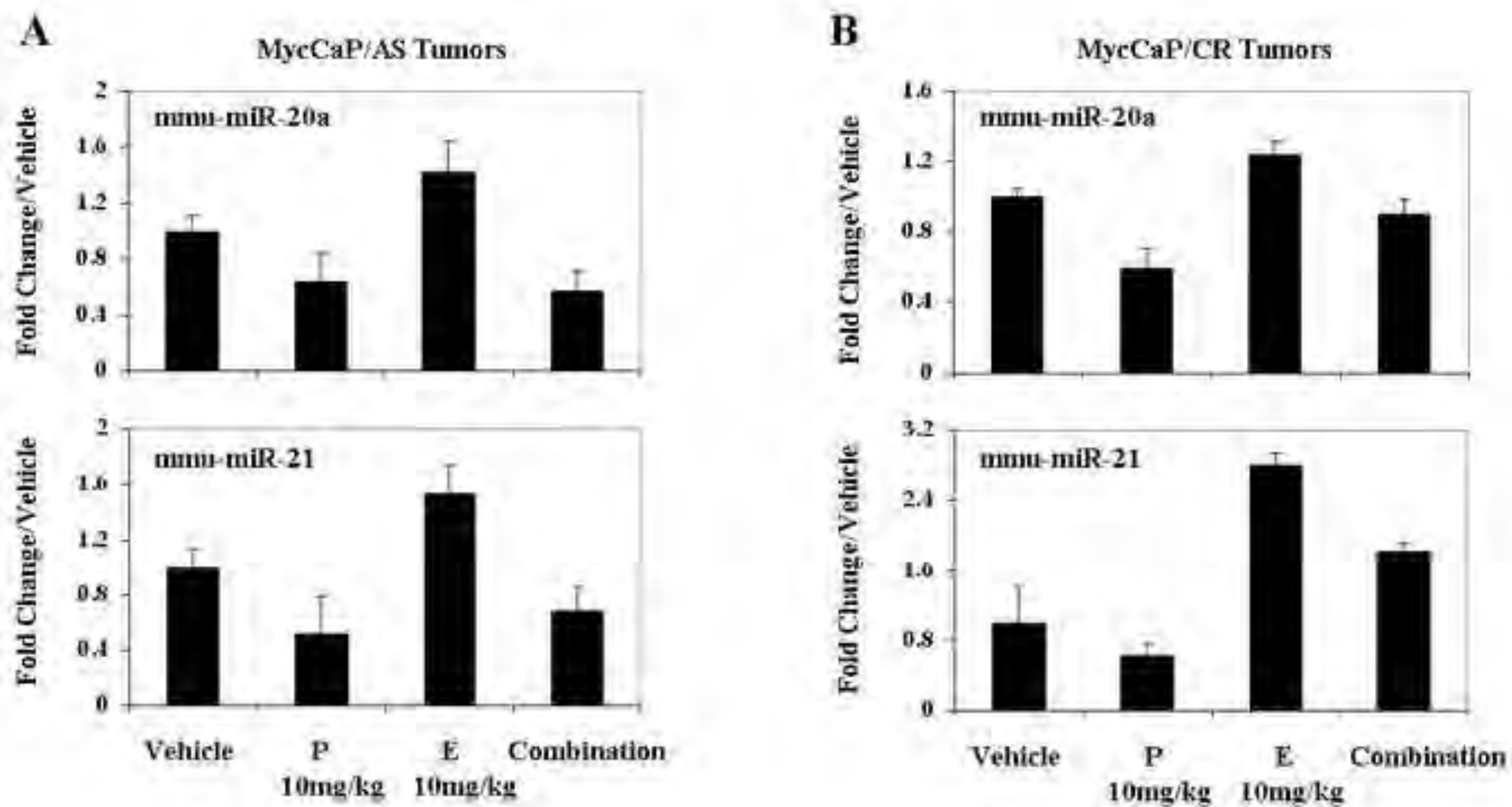

Figure 7

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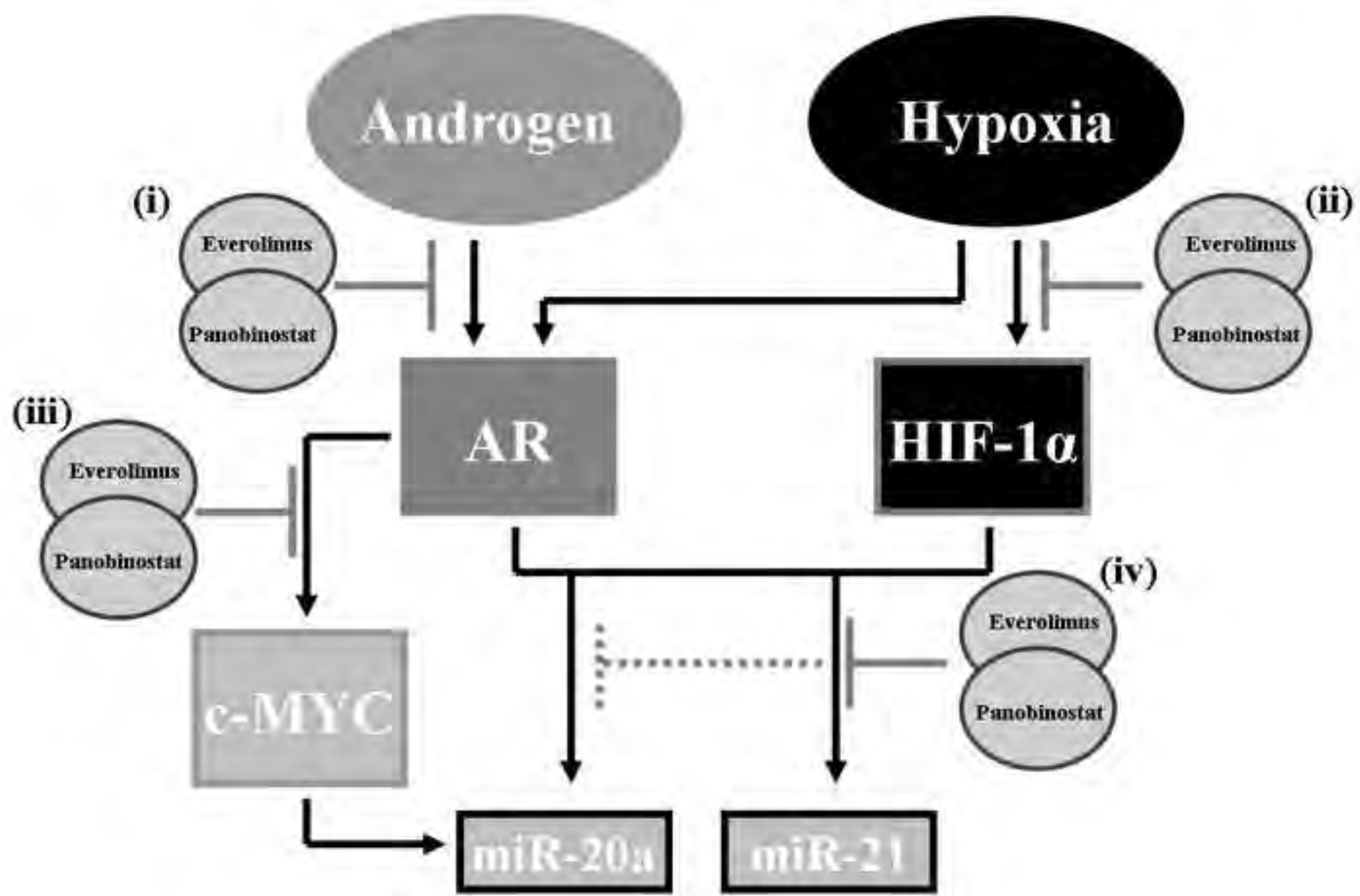

Development of a Castrate Resistant Transplant Tumor Model of Prostate Cancer

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BACKGROUND. Currently, limited mouse models that mimic the clinical course of castrate resistant prostate development currently exist. Such mouse models are urgently required to conduct pre-clinical studies to assist in the understanding of disease progression and the development of rational therapeutic strategies to treat castrate resistant prostate cancer.
METHODS. Wild type intact FVB male mice were injected by subcutaneous injection with Myc-CaP cells to establish androgen sensitive Myc-CaP tumors. Tumor bearing mice were castrated and resulting tumors serially passaged in pre-castrated FVB male mice to produce a bone fide Myc-CaP castrate resistant tumor.
RESULTS. Immunohistochemical analysis revealed that initial androgen sensitive Myc-CaP tumors had strong nuclear transcriptional active androgen receptor expression, as indicated by marked c-MYC staining and were highly proliferative. Castration of tumor bearing animals resulted in cytoplasmic relocation of androgen receptor concurrent with loss of transcriptional activity and tumor proliferation. Serial passaging of castrate refractory Myc-CaP in pre-castrated male FVB mice resulted in the development of a bona fide castrate resistant Myc-CaP tumor which pheno-copied the original androgen sensitive parental Myc-CaP tumor.
CONCLUSIONS. Developing a murine castrate transplant resistant tumor model that mimics the clinical course of human castrate resistant prostate cancer will create better opportunities to understand the development of castrate resistant prostate cancer and also allow for more rapid pre-clinical studies to stratify rational novel therapies for this lethal form of prostate cancer. *Prostate* © 2011 Wiley-Liss, Inc.

KEY WORDS: **mouse models; prostate cancer; MYC; castrate resistant prostate cancer**

INTRODUCTION

A major cause of mortality and morbidity in men world wide is prostate cancer, or more specifically, the lethal form castrate resistant prostate cancer. The research field, in this case prostate cancer, relies on the use of pre-clinical animal models to understand disease development and progression, and to also strategize novel rational therapeutic interventions. Over the years, multiple xenograft and genetically engineered mouse models of prostate cancer have been generated to attempt to bridge our success of bench to bedside medicine. The Prostate Cancer Foundation, in 2007, held a Prostate Cancer Models Working Group to discuss relevant issues in prostate cancer animal models [1].

Within the last decade two transgenic mouse models were reported which had specific genetic manipulation highly relevant to the development and progression of human prostate cancer, the PtenL/L;C+ model [2], and myc model [3] of prostate cancer. These two mouse models give rise to mPIN at approximately 6 weeks and 2 weeks of age, respectively and progress to invasive adenocarcinoma at

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approximately >6 months of age. Array data collected from both models revealed a molecular signature that closely mimics human prostate cancer [2,3]. A further advantage was the generation of cell lines from these mouse models, Pten-CaP [4] and Myc-CaP [5], allowing for the generation of transplant tumor models in an immunocompetent host. This approach has been greatly utilized in the Eμ-myc model of B cell lymphoma [6,7] and allows for rapid pre-clinical studies. The PtenL/L;C+ model was reported to develop castrate resistant disease post surgical castration [2] and consequent cell lines had been developed and reported [8]. Currently to our knowledge, no further development of an in vivo castrate resistant tumor model from the myc mouse model has been reported. The original report from Ellwood-Yen et al. [3] demonstrated that castration of myc mice with developed PIN reverted progression to cancer, but castration of mice with prostate cancer resulted in residual quiescent tumor burden that was non-proliferating and lacked nuclear androgen receptor staining and myc transgene expression, though no evidence of progression to castrate resistant prostate cancer was documented [3]. Further work with the Myc-CaP cell line found that expressing an androgen-independent myc was sufficient to rescue in vitro, but not in vivo androgen independent growth [5], though knockdown of Pten could rescue androgen independent Myc-CaP tumor growth in vivo [4]. Interestingly, it was observed that Myc-CaP tumor growth >500 mm^3 did posses the potential to regain proliferation post castration. Further, when tumors that increased in size were harvested and placed in short term culture without androgen they were found to express c-Myc mRNA levels similar to the parental androgen dependent Myc-CaP cells [5].

MATERIALS AND METHODS

Cell Culture and Reagents

The Myc-CaP cell line [5] was a generous gift from Dr. Charles Sawyers and were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C, 5% CO_2.

Bicalutamide (casodex) for in vivo experiments was purchased from Toronto Research Chemicals (Ontario, Canada). Stock solution was made in distilled water (2.5 mg/ml) and stored at 4°C until used in experiments.

Development of Myc-CaP Transplant Models of Prostate Cancer

The Institute Animal Care and Use Committee (IACUC) at Roswell Park Cancer Institute (RPCI) approved all mouse protocols used in this study. All mice were purchased from NCI Frederick (MD, USA).

Development of Myc-CaP/AS (Androgen Sensitive) and Myc-CaP/CR (Castrate Resistant) Tumor Banks

Wild-type FVB mice were injected subcutaneous with Myc-CaP cell lines (1×10^6 cells/mouse). Tumor bearing mice were sacrificed and Myc-CaP/AS tumors from non-castrated mice were collected, or mice were surgically castrated to produce castrate resistant tumors. Myc-CaP/CR tumors were passaged through four rounds of surgically castrated wild-type FVB male mice before they were believed to be bona fide castrate resistant tumors. Tumor proliferation was monitored by serial caliper measurement.

In vivo therapy experiments. Six-week old male FVB mice were anesthetized using isoflourane and surgically castrated. Ten-days post castration, Myc-CaP castrate resistant tumor pieces (~20–30 mm^2) were subcutaneously grafted to these mice and left for an additional 10 days before being treated with bicalutamide at a dose of 25 mg/kg/day or 50 mg/kg/day by oral gavage. Control mice were treated with a corresponding volume of distilled water.

Immunohistochemistry

Formalin fixed, paraffin-embedded tissue (4 μm) were stained with primary antibodies to detect androgen receptor (Santa Cruz Biotechnology), Ki67 (Thermo Scientific), c-MYC (Epitomics). All sections were incubated overnight with primary antibodies at 4°C and then incubated with ImmPRESS™ reagent kit HRP anti-rabbit IgG antibodies (Vector Laboratories). Staining was developed by incubation with 3,3′-diaminobenzide (Dako), and counterstained with hematoxylin. Images were captured using a Scanscope XT system (Aperio Imaging) and analyzed using Imagescope software (Aperio).

Staining Quantification and Statistical Analysis

Ki-67 immuno-staining quantitation was performed using Image J software. Myc-Cap tumor doubling times were calculated by non-linear regression analysis using GraphPad Prism software. Statistical significance between treatment groups was determined using a Student's *t* test. Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

In this present study, we wanted to identify if indeed androgen sensitive Myc-CaP tumors developed in intact mice did progress to a bona fide

castrate resistant phenotype, and if so does the development/progression to castrate resistance reflect the clinical course of castrate resistant prostate cancer development. We initially examined the ability of Myc-CaP cells to develop androgen sensitive tumors within the major graft sites utilized with in vivo mouse models. Figure 1 demonstrates the schema used to develop a bona fide castrate resistant Myc-CaP tumor in vivo. Mice bearing $\geq$500 mm^2 established subcutaneous androgen sensitive Myc-CaP tumors were castrated and monitored over 1 month at which time proliferating tumors where excised and viable tissue was transplanted to pre-castrated male mice for a serious of four passages.

Figure 2A demonstrates that Myc-CaP tumors are capable of proliferating in castrated mice post engraftment (Fig. 2A, CR-P1) and further, gain the ability to be successfully grafted and proliferate in pre-castrated mice (Fig. 2A, CR-P2-P4). As previously shown [5], we established Myc-CaP androgen sensitive tumors by subcutaneous injection of 1×10^6 Myc-CaP cells to intact male mice (Fig. 2B, top panel). Myc-CaP androgen sensitive tumors displayed abundant nuclear expression of androgen receptor that was transcriptionally active as indicated by the increased expression of the androgen dependent transgene c-MYC. Myc-CaP tumors were also highly proliferative as indicated by IHC staining for the proliferation marker Ki67 (Fig. 2B and C). Figure 2B further demonstrates that castration of tumor bearing mice and subsequent passaging of tumor to pre-castrated mice as indicated by IHC staining, highly mimics the clinical progression of androgen sensitive prostate to castrate resistant prostate cancer. That is, as shown in panels CR-P1 and CR-P2 following castration, AR is located to the cytoplasm (red arrows). Further, because of AR translocation to the cytoplasm, lesser transcriptional activity occurs as indicated by loss of c-MYC staining concurrent with a decrease in proliferation as indicated by less Ki67 staining and over all tumor size (Fig. 2C). At passage 3 (Fig. 2B panel CR-P3) a heterogeneous phenotype is indicated by nuclear recapture of AR (green arrow) while other tumor cells still have greater AR cytoplasmic staining (red arrow). Tumor cells started to display nuclear staining for c-MYC (green arrows) indicating AR transcriptional activity though tumor proliferation was still decreased as shown by Ki67 staining and tumor size (Fig. 2B panel CR-P3, Fig. 2C). Excitingly, by passage 4 of these tumors to castrated mice it was evident that these Myc-CaP tumors indeed phenocopy the IHC staining of Myc-CaP tumors in their intact mice counterparts. Figure 2B (panel CR-P4) clearly demonstrates that these tumors have progressed to a castrate resistant phenotype as indicated by recapturing AR nuclear localization (green arrows) as well as its down stream transcriptional target, c-MYC (green arrows) and increases in tumor proliferation (Ki67 IHC) and tumor size (Fig. 2B CR-P4, Fig. 2C). Non-linear regression analysis revealed that tumor doubling time was similar between each passage (Fig. 2D) and raises and interesting question of signaling pathway compensation in the absence of AR transactivation.

We next investigated the sensitivity of Myc-CaP/CR tumors to the AR antagonist, bicalutamide (casodex). We treated castrated FVB male mice bearing subcutaneous Myc-CaP/CR tumors by oral gavage with a daily dosing schedule of 25 mg/kg or 50 mg/kg. Surprisingly, tumor growth was not inhibited by bicalutamide treatment (Fig. 3A). Conversely, bicalutamide treatment with 25 mg/kg/day significantly increased endpoint tumor weights ($P = 0.027$), whereas bicalutamide treatment with 50 mg/kg/day increased tumor weight, though was not significant ($P = 0.215$) compared to control treated mice (Fig. 3B). This resistance to bicalutamide is attributed to the amplification of AR in the Myc-CaP cell

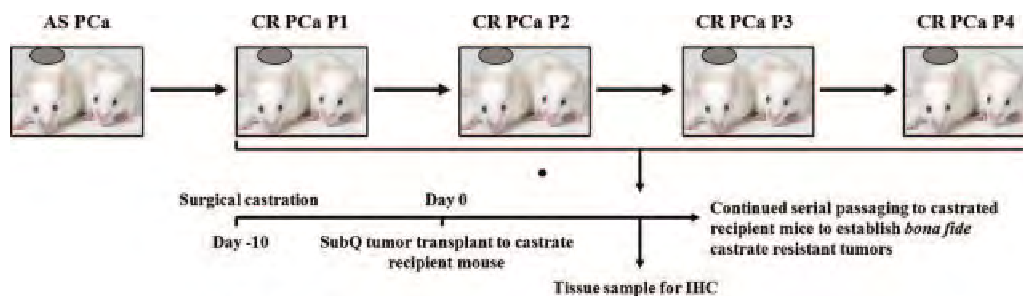

Fig. 1. Schema for the generation of an in vivo Myc-CaP castrate resistant tumor model of prostate model. Intact FVB male mice were grafted with 1×10^6 Myc-CaP cells subcutaneous and grown to $\geq$500 mm^2 determined by serial caliper measurement. Once tumors were $\geq$500 mm^2, mice were surgically castrated and monitored for 1-month post castration. Tumors that demonstrated proliferation as determined by caliper measurements were surgically excised and transplanted to castrated FVB male mice for a total of four serial passages. At the time of transplant, tumor tissue was also fixed in 10% normal buffered formalin for immunhistochemical analysis.

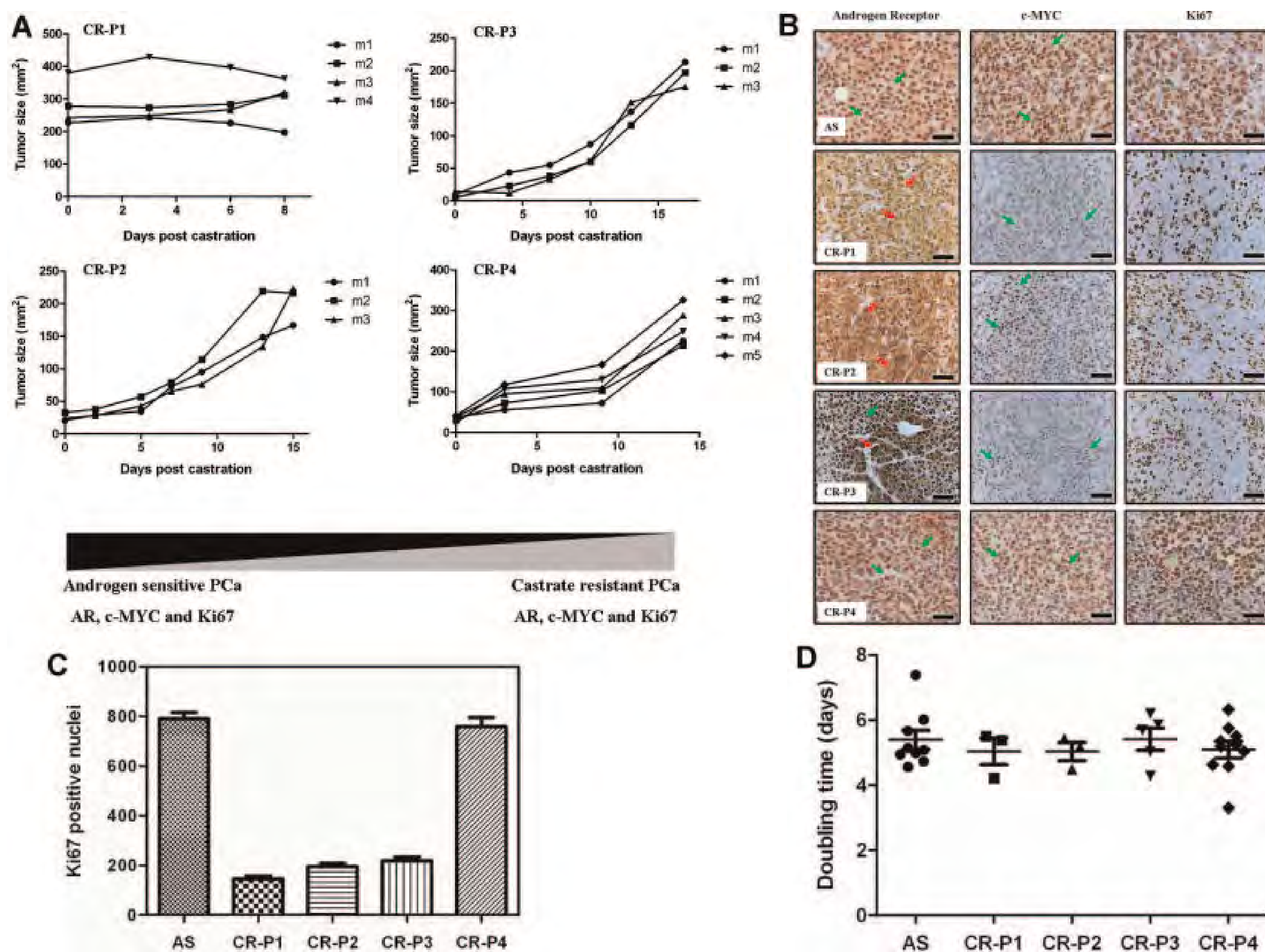

Fig. 2. **A**: Myc-CaP tumor growth curves. Each line represents an individual tumor bearing mouse. **B**: Immunohistochemical staining of 4 μM paraffin embedded Myc-CaP tumor tissue samples pre- and post-castration. Antigen retrieval was performed in 10 mM sodium citrate buffer under microwave-heated conditions. Primary antibodies used were androgen receptor (Santa Cruz Biotechnology), Ki67 (Thermo Scientific), c-MYC (Epitomics). All sections were initially blocked with horse serum (Vector Laboratories) before incubation overnight with primary antibodies at 4°C and then incubated with ImmPRESS^{TM} reagent kit HRP secondary IgG antibodies (Vector Laboratories). Staining was developed by incubation with 3,3′-diaminobenzide (Dako), and counterstained with hematoxylin. Images were captured using a Scanscope XT system (Aperio Imaging) and analyzed using Imagescope software (Aperio). AS, androgen sensitive; CR-P, castrate resistant passage number. Magnification (40×); Scale bar = 50 μM. **C**: Quantitation of Ki-67 IHC staining from Figure 2A using image J software. **D**: Non-linear regression analysis of Myc-CaP tumor doubling time for each individual passage from Figure 2A using GraphPad Prism software. Sample number for Figure 2C and D were; AS n = 9, CR-P1 n = 3, CR-P2 n = 3, CR-P3 n = 5, CR-P4 n = 10.

line, which was previously documented [5]. Increased expression of AR primarily through gene amplification [9] is common in castrate resistant prostate cancer and is sufficient to mediate resistance to bicalutamide therapy in mouse xenograft models [10,11].

Overall, we demonstrate the development of a novel murine transplant model of castrate resistant prostate cancer that initially undergoes a latency period (possibly quiescent low proliferating tumors as documented in the transgenic myc model [3]) before progressing to a hormone refractory state, as indicated by these tumors ability to proliferate in low androgen levels, and finally progressing to a castrate resistant phenotype. This transition closely follows the course of progression to castrate resistant prostate cancer in the clinic.

Development of castrate resistant tumor models in immunocompentent mice will allow for a closer understanding of not only the development and progression of prostate cancer to its most lethal castrate resistant form, but also impact design

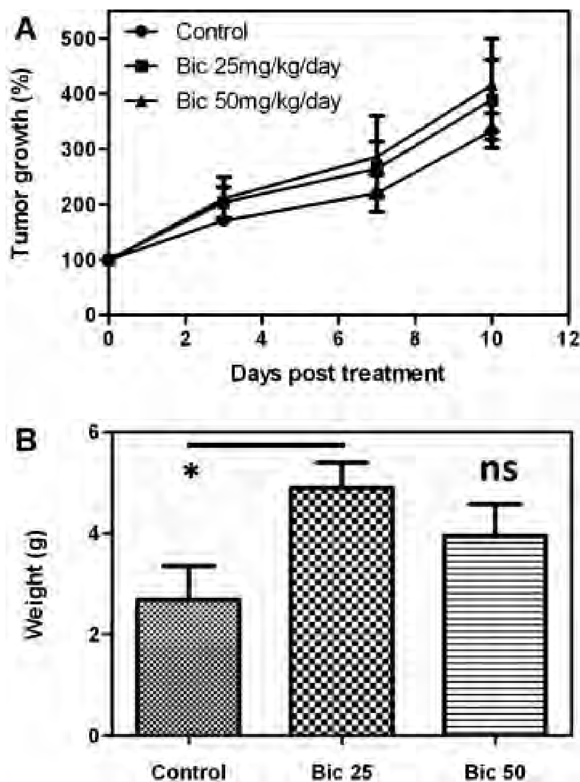

Fig. 3. Surgically castrated male FVB mice were grafted subcutaneously with Myc-CaP/CR tumor pieces (~20–30 mm^2). Tumor bearing mice were treated daily with control (distilled water) (n = 4), 25 mg/kg bicalutamide (n = 6) or 50 mg/kg bicalutamide (n = 5) by oral gavage. **A**: Tumor growth was monitored by serial caliper measurements. **B**: At the experiments conclusion all tumors were excised and weighed to determine final tumor weights. $^{*}P = 0.027$, ns = not significant.

strategies for novel therapeutic interventions that will have the greatest implications in clinical trials.

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