

AWARD NUMBER: W81XWH-15-1-0513

TITLE: Itraconazol, an antifungal and a hedgehog pathway inhibitor for treatment of prostate cancer

PRINCIPAL INVESTIGATOR: Hasan Mukhtar

CONTRACTING ORGANIZATION: The Board of Regents of the University of Wisconsin System (UW-Madison) MADISON, WI 53705

REPORT DATE: DECEMBER 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE DECEMBER 2019		2. REPORT TYPE Final		3. DATES COVERED 30SEP2015 - 29SEP2019	
4. TITLE AND SUBTITLE Itraconazol, an antifungal and a hedgehog pathway inhibitor for treatment of prostate cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-15-1-0513	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Hasan Mukhtar, PhD E-Mail:hmukhtar@dermatology.wisc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Board of Regents of the University of Wisconsin System 21 N. Park Street, Suite 6401 Madison, WI 537151218				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT A commonly used antifungal, Itraconazole targets a pathway that is also upregulated in human prostate cancer. It was observed that Itraconazole synergizes with cyclopamine to induce superior therapeutic effects. Cyclopamine is toxic, however, when combined with itraconazole the dose requirement for each drug was considerably reduced. This combination therefore has the potential to be more effective and at the same time less toxic. The most important aspect of this finding is that these are existing drugs whose safety and toxicological profiles are known. This project sought to investigate the efficacy of Itraconazol in combination with cyclopamine against prostate cancer with an intention to accelerate their rapid translation into human clinical trials. Treatment of prostate cancer cells with the combination of itraconazole and cyclopamine led to increased cell growth inhibition that was greater than the sum of the individual agents alone suggesting synergism. We also analyzed the effect of the combination on the growth of tumors in vivo conditions using athymic nude mice. In control animals, targeted tumor volume of 1200 mm ³ was reached at 6 weeks post cell inoculation and significantly later time in animals treated with itraconazole alone (9 weeks) and cyclopamine alone (8 weeks). Notably, tumor volumes stayed under 165 mm ³ in mice that received both itraconazole and cyclopamine. In the prostate specific Pten knockout mice combination of itraconazole and cyclopamine produced 57% inhibition of tumor growth compared to 30% in cyclopamine alone and 45% in itraconazole alone treated mice. These data show that a combination of itraconazole and cyclopamine is considerably more efficient in inhibiting tumor growth.					
15. SUBJECT TERMS: NONE LISTED					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			USAMRMC
			Unclassified	11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	2
2. Keywords.....	2
3. Accomplishments.....	2-10
4. Impact.....	10
5. Changes/Problems.....	10
6. Products.....	11
7. Participants & Other Collaborating Organizations.....	11
8. Special Reporting Requirements.....	N/A
9. Appendices.....	N/A

1. INTRODUCTION:

Hedgehog (Hh) signaling plays an important role in prostate development and is also a characteristic feature of prostate cancer (PCa). The ability of Hh pathway activation to promote cell invasiveness, epithelial to mesenchymal transition and metastasis suggest that targeting Hh pathway may provide an important clinical avenue for the treatment of advanced PCa. Loss of PTEN function is considered a characteristic feature of advanced PCa. To gain insight into the status of Hh signaling in PCa in relation to PTEN status we selected three cell lines based on their PTEN expression. Gli1 mRNA was upregulated 2000-fold in PC3 (PTEN^{-/-} null) cells, 800-fold in DU145 (PTEN^{+/-}) cells when compared with normal prostate epithelial cells suggesting that PTEN loss is associated with increased Hh signaling. We also observed significant increase in the mRNA levels of Hh ligand (Shh), Hh receptors (PTCH2 and SMO) and effectors of Hh signaling (Gli1 and Gli2) in the prostate tissues of PTEN knockout mice. A 400-fold increase in the mRNA levels of Gli1 and 15-fold increase in the levels of Gli2 mRNA levels was also observed. We also observed significant decrease in the mRNA levels of the negative regulators of Hh signaling, HHIP and SuFu in the prostate tissues of PTEN knockout mice with similar inhibition in the transcript levels of GAS1. We next investigated the effect on Hh signaling in PCa cells treated with itraconazole and cyclopamine alone and in combination. PCa 22Rv1 and PC-3 cells transfected with Gli-dependent luciferase reporter construct were treated with itraconazole (1 and 2 μ M), cyclopamine (5 and 10 nM) and combination of itraconazole (1 μ M) and cyclopamine (5 nM) for 40 hours. Gli-dependent luciferase reporter activity was inhibited by itraconazole and cyclopamine treatments; however, the inhibition was significantly greater in combination. Treatment of PCa cells with the combination of itraconazole and cyclopamine led to increased cell growth inhibition that was greater than the sum of the individual agents alone suggesting synergism. We next analyzed the effect of the combination on the growth of CWR22Rv1 tumors under in vivo conditions using athymic nude mice. In control animals, targeted tumor volume of 1200 mm³ was reached at 6 weeks post cell inoculation and significantly later time in animals treated with itraconazole alone (9 weeks) and cyclopamine alone (8 weeks). Notably, tumor volumes stayed under 165 mm³ in mice that received both itraconazole and cyclopamine. In the prostate specific Pten knockout mice combination of itraconazole and cyclopamine produced 57% inhibition of tumor growth compared to 30% in cyclopamine alone and 45% in itraconazole alone treated mice. These data show that a combination of itraconazole and cyclopamine is considerably more efficient in inhibiting tumor growth highlighting the synergistic efficacy of this combination for the treatment of PCa.

2. KEYWORDS:

Prostate cancer, itraconazole, cyclopamine, combination, hedgehog, signaling, antifungal, PTEN, metastasis, cell growth, viability

3. ACCOMPLISHMENTS:

What were the major goals of the project?

- a. Evaluate the effect of itraconazole and cyclopamine, alone and in combination on the growth and metastasis of human PCa cells *in vitro*.
- b. Investigate the effect of itraconazole and cyclopamine, alone and in combination, on the growth and metastasis of human PCa cells *in vivo* implanted in mice.
- c. Investigate the effect of itraconazole and cyclopamine alone and their combination on the progression of PCa in the PTEN knockout mouse model of PCa.

What was accomplished under these goals?

- a. Major activities: We evaluated the effect of itraconazole and cyclopamine in vitro and in vivo and repeated many of these experiments to make sure we made consistently similar observations. We observed and found a comparable trend and similar results. We ascertained the effects of the compounds on the metastasis and invasion and observed that the combination was very effective compared to the individual compounds.
- b. Specific objectives: We ascertained the effect of itraconazole and cyclopamine and their combination on the migration and invasion and conducted in vitro and in vivo experiments in cell culture and in the xenograft mouse model implanted with human prostate cancer cells and in the Pten mouse model of prostate cancer.
- c. Significant results: Existing studies including our own observations suggest that Hh signaling is active during the development and progression of prostate cancer in humans. Specific Hh inhibitors inhibit the growth of prostate cancer cell lines including PC3, DU145 and 22Rv1 cells. However, clinical application of Hh inhibitors has been slow due to the fact that available inhibitors are associated with severe side effects. The discovery of itraconazole provides an opportunity to target the Hh signaling in prostate cancer and help its rapid translation into the clinic. Itraconazole synergizes with the known Hh inhibitor cyclopamine and the combination results in several fold lower dose requirements. In the prostate specific Pten knockout mice combination of itraconazole and cyclopamine produced 57% inhibition of tumor growth compared to 30% in cyclopamine alone and 45% in itraconazole alone treated mice. These data show that a combination of itraconazole and cyclopamine is considerably more efficient in inhibiting tumor growth highlighting the synergistic efficacy of this combination for the treatment of PCa.

Methods:

Cell lines and reagents: The human prostate cancer cells were obtained from ATCC and cultured in RPMI supplemented with 10% fetal bovine serum and 1% antibiotic penicillin and streptomycin. The cells were maintained under standard cell culture conditions at 37° C and 5% CO₂ in a humid environment. PCa 22Rv1 and PC-3 cells were transfected with Gli-dependent luciferase reporter construct. Eighteen hours post transfection cells were treated with itraconazole (1 and 2 μ M), cyclopamine (5 and 10 nM) and combination of itraconazole (1 μ M) and cyclopamine (5 nM) for 40 hours. Luciferase and β -galactosidase activities were measured using commercially available kits (Promega, Pierce Biotechnology). Combination index, a quantitative measure of the degree of drug interaction was evaluated by the median-effect equation using the software program Calcsyn (Biosoft, Cambridge, UK).

Protein extraction and Western blotting: Cells/tissues were incubated in ice-cold lysis buffer, 40 μ g of protein was resolved over 12% polyacrylamide gels, transferred onto a nitrocellulose membrane, probed with appropriate monoclonal primary antibody, incubated with appropriate secondary antibody horseradish peroxidase conjugate, and detected by chemiluminescence and autoradiography.

Tumor xenograft study: Male athymic^{nu/nu} were obtained from Harlan Laboratories (Madison, WI). Mice were subcutaneously implanted with 22Rv1 cells in 1:1 (media:matrigel) and randomized into four groups of eight mice each. Group I served as the control; Group II received itraconazole (10 mg/Kg by oral gavage daily); Group III received Cyclopamine (4 mg/Kg by oral gavage daily); Group IV received combination of itraconazole (10 mg/Kg by oral gavage daily) and Cyclopamine (4 mg/Kg by oral gavage daily). Once tumors started growing, their sizes were measured weekly and tumor volumes were calculated using the formula for calculating the volume of a hemi-ellipsoid. Tumor measurements were taken till tumors reached a targeted volume of 1200 mm³.

Pten mice study: Mice were generated by crossing C57/BL6J Pten floxed (loxp/loxp) with Probasin-Cre (PB-Cre4). Starting at 24 weeks of age mice were divided into four groups of 6 mice each. Group I served as the

control; Group II received itraconazole (10 mg/Kg by oral gavage daily); Group III received Cyclopamine (4 mg/Kg by oral gavage daily); Group IV 4 received combination of itraconazole (10 mg/Kg by oral gavage daily) and Cyclopamine (4 mg/Kg by oral gavage daily).

Results:

Itraconazole and cyclopamine synergize to inhibit Gli2 luciferase activity in PCa cells: To determine the effect of itraconazole and cyclopamine alone and in combination we conducted studies to investigate the effect on Hh signaling in PCa cells. PCa 22Rv1 and PC-3 cells were transfected with Gli-dependent luciferase reporter construct. Eighteen hours post transfection cells were treated with itraconazole (1 and 2 μ M), cyclopamine (5 and 10 nM) and combination of itaconazole (1 μ M) and cyclopamine (5 nM) for 40 hours. Luciferase and β -

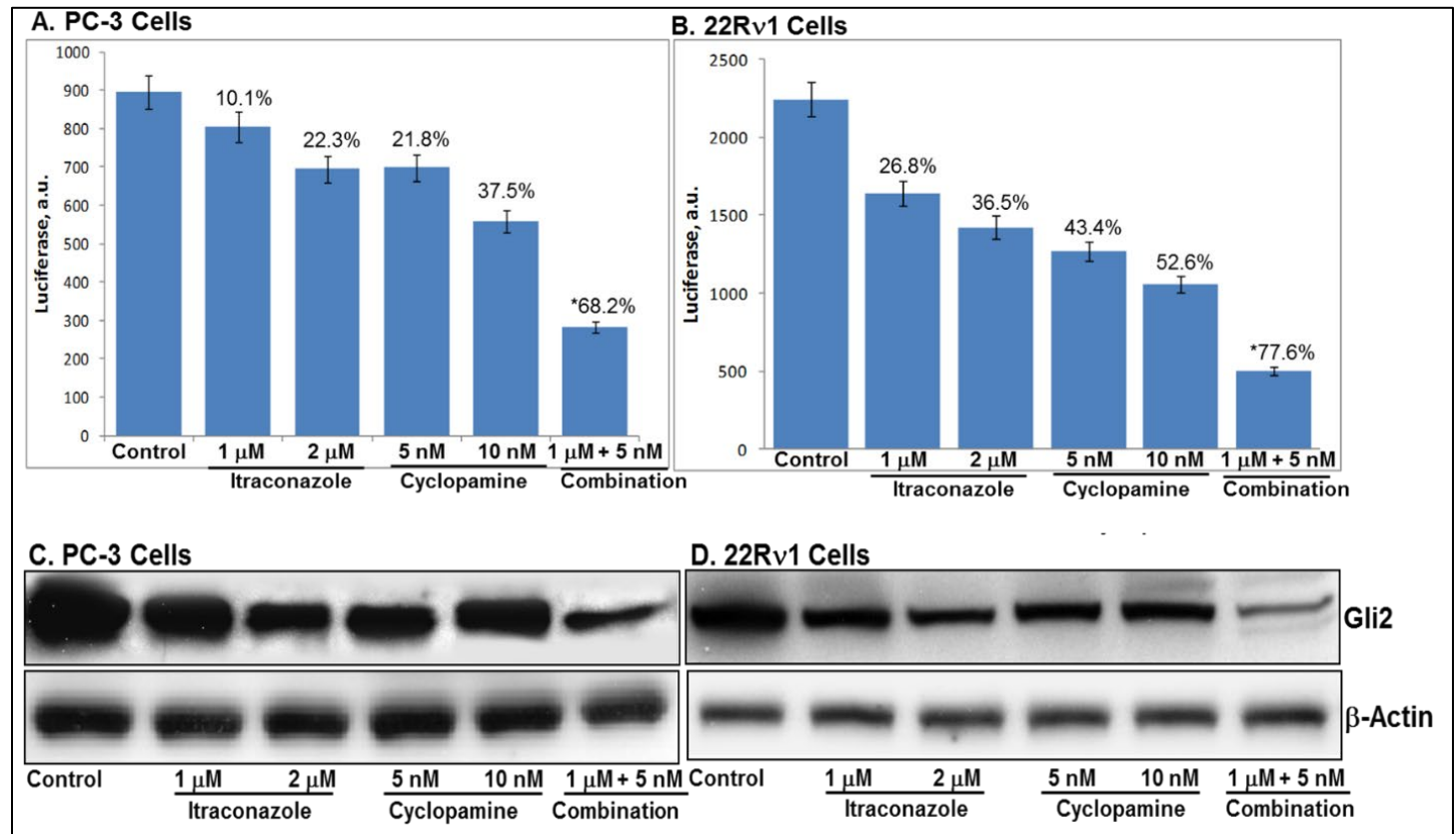
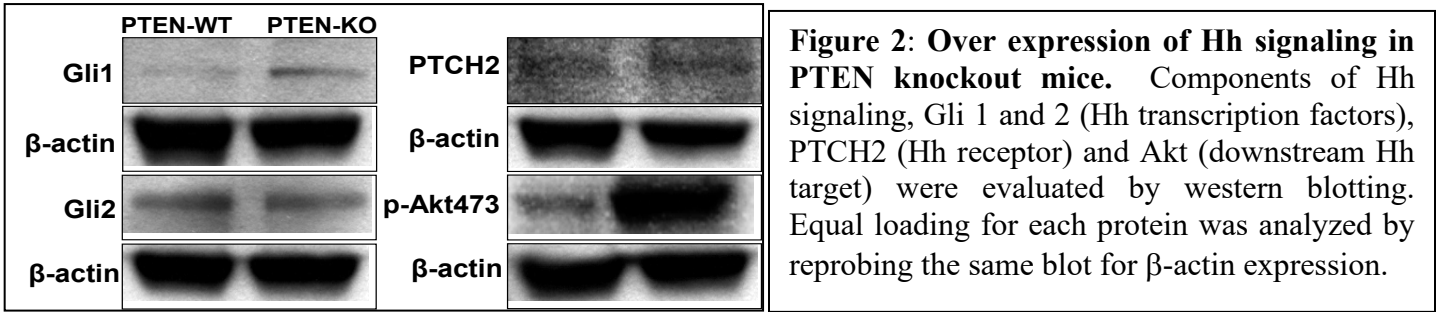


Figure 1: Itraconazole and Cyclopamine Synergize to Inhibit Gli2 Luciferase Activity and Protein Expression: Cells were transfected with Gli-dependent luciferase reporter construct. Eighteen hours post transfection cells were treated with itraconazole and cyclopamine and their combination for 24 hours. Luciferase and β -galactosidase activities were measured using commercially available kits. Cell lysates were evaluated by western blotting for Gli2 protein expression as a readout for Hh activity.

galactosidase activities were measured using commercially available kits (Promega, Pierce Biotechnology). Combination index (CI), a quantitative measure of the degree of drug interaction was evaluated by the median-effect equation using the software program Calcsyn (Biosoft, Cambridge, UK). Gli-dependent luciferase reporter activity was inhibited by itraconazole and cyclopamine treatments; however, the inhibition was significantly greater when itraconazole and cyclopamine were used in combination (Figure 1 A & B). Cells were treated with itraconazole (1 and 2 μ M), cyclopamine (5 and 10 nM) and a combination of itaconazole (1 μ M) and cyclopamine (5 nM) for 40 hours. Cell lysates were prepared and the lysates were subjected to western blot analysis for Gli2 protein. Treatment with either itraconazole or cyclopamine inhibited protein expression of Gli2, however the decrease in expression was significantly greater when cells were treated with a combination of itraconazole and cyclopamine. (Figure 1 C & D).

Hedgehog signaling is activated in mice with targeted deletion of PTEN in the prostate: Fifteen weeks old mice with targeted prostate specific deletion of PTEN were evaluated for the protein expression of various



components of Hh signaling and compared with appropriate wild type mice. We observed increased protein expression of Gli1 (Hh transcription factor), PTCH2 (Hh receptor) and Akt (activated following loss of PTEN) in mice with PTEN deletion compared to mice with wild type PTEN (Figure 2).

To evaluate the status of hedgehog signaling in the PTEN mouse model at the transcriptional level we used a

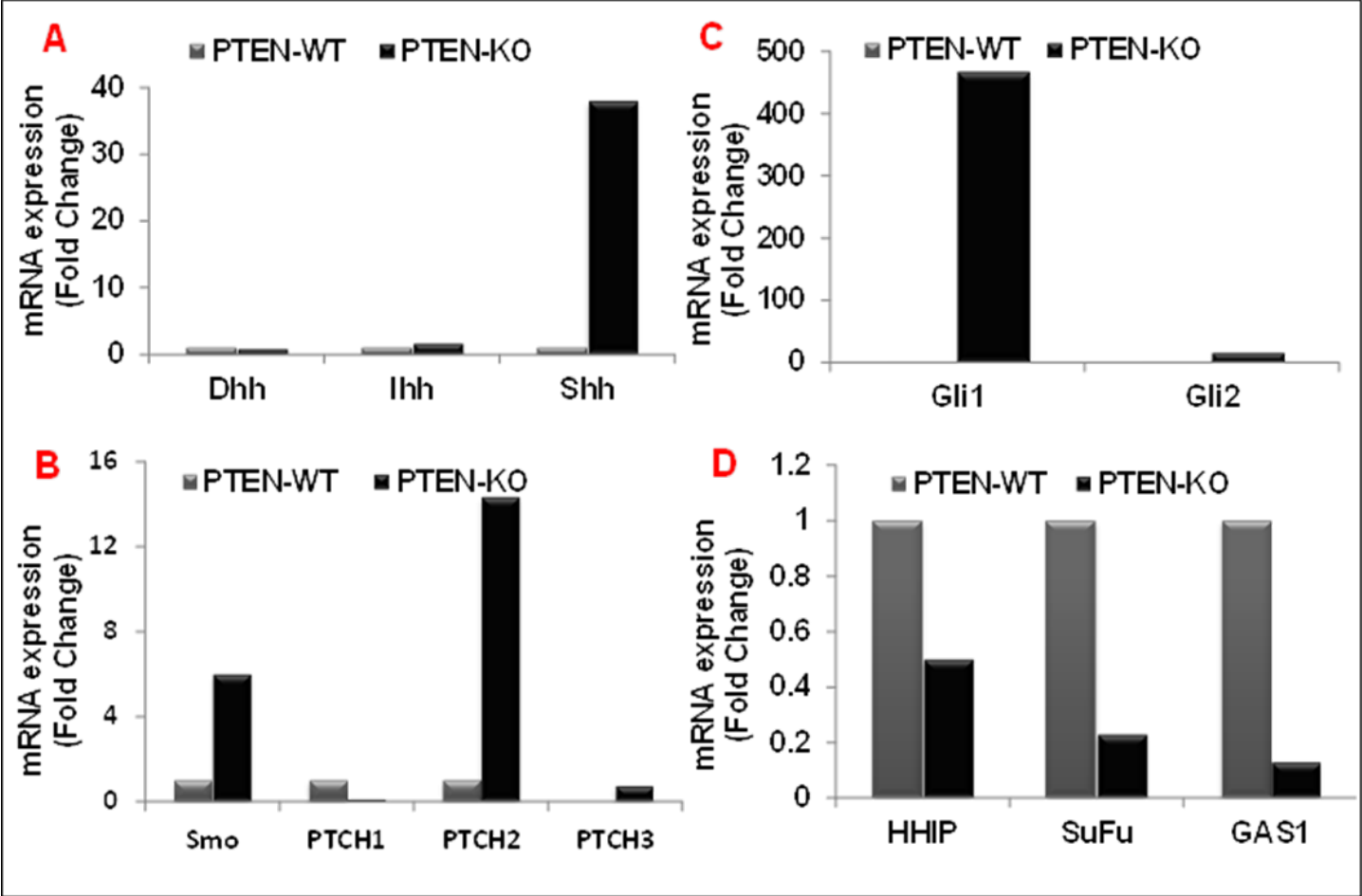
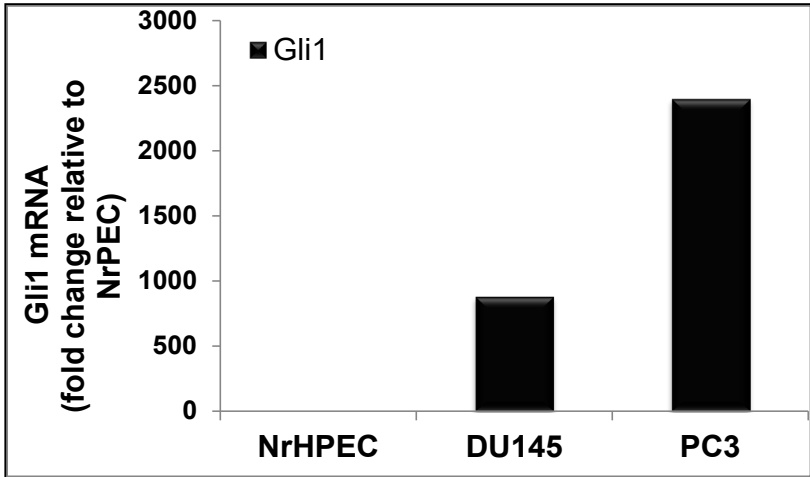


Figure 3: Over expression of Hh signaling in PTEN knockout mice at the mRNA level: Components of Hh signaling evaluated by Hh-pathway focused primer panel designed to detect pathway-specific genes. A. Hh ligands (Shh, Dhh and Ihh). B. Hh receptors (Smo, PTCH1-3). C. Hh specific transcription factors (Gli1-2). D. Negative regulators of Hh signaling (HHIP and SuFu). All the data were normalized to β-actin and compared with age matched PTEN wild-type mice.

Hh-pathway focused qPCR primer panel (Origene, Rockville, MD) designed to detect pathway-specific genes. Prostate tissue and tumor samples from fifteen weeks old PTEN knockout mice were used to assess mRNA

transcript levels. Data were normalized to β -actin and compared with age matched PTEN wild-type mice. We found significant increase in several components of the Hh signaling in the prostate tissue samples of PTEN knockout mice. Among the three known Hh ligands, we observed significant increase in the mRNA levels of Shh ligand only and no changes were observed in the levels of Desert Hh (Dhh) and Indian Hh (Ihh) (Figure 3A). Among the Hh receptors we observed significant increase in the mRNA levels of PTCH2 and SMO (Figure 3B). The Gli proteins are the effectors of Hh signaling and have been shown to be involved in embryo development and tumorigenesis (1). The Gli transcription factors activate/inhibit transcription by binding to Gli responsive genes and by interacting with the transcription complex. We observed a significant (467 fold) increase in the mRNA levels of Gli1 and 15 fold increase in the levels of Gli2 mRNA levels in the prostate tissues of PTEN knockout mice (Figure 3C). The Hh signaling pathway is negatively regulated by three different proteins namely Hh interacting protein (HHIP), suppressor of fused protein (SuFu) and Growth arrest-specific protein 1 (GAS1). Among them HHIP and SuFu are the most established negative regulators of Hh signaling. Interestingly, we observed significant decrease in the levels of both HHIP and SuFu mRNA transcripts in the prostate tissues of PTEN knockout mice (Figure 3D). Similar inhibition was also observed in the transcript levels of GAS1 (Figure 3D).



To gain insight into the status of Hh signaling in PCa cells in relation to PTEN status we selected three cell lines based on their PTEN expression. Gli1 mRNA was upregulated

Figure 4: Over expression of Hh signaling in cells with loss of PTEN function: Gli1 mRNA as a read out for Hh signaling was evaluated in DU145 and PC3 cells and compared with NrPEC by qPCR. All the data were normalized to β -actin and compared NrPEC.

>2000 fold in PC3 (PTEN^{-/-} null) cells, >800 fold in DU145 (PTEN^{+/-}) cells when compared with normal prostate epithelial cells (NrPEC, PTEN wild type) cells suggesting that PTEN loss is associated with increased Hh signaling and underscoring the use of cells with loss of PTEN for evaluating the effect of Hh inhibitors (Figure 4). Loss of PTEN function is considered a characteristic feature of advanced PCa.

Itraconazole and cyclopamine synergize to inhibit metastatic potential of PCa cells: We undertook studies to ascertain the effect of itraconazole and cyclopamine on metastasis and epithelial to mesenchymal transition.

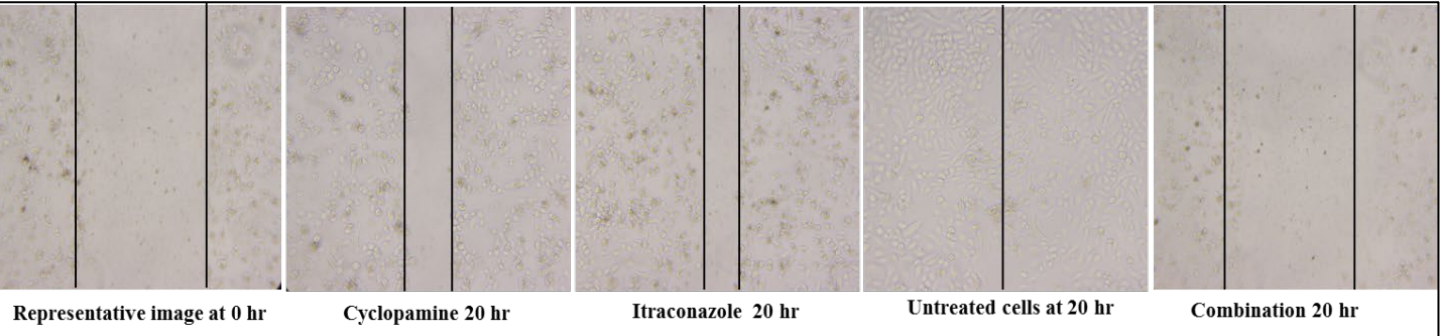


Figure 5: Effect of itraconazole, cyclopamine and their combination on the migration potential of PC-3 cells. Representative images showing migration of cells across a wound over a 20 h period. PC3 cells were cultured and treated with itraconazole, cyclopamine and their combination for 20 hours. A scratch was pinched in the middle of the plate from one end to other. Plated cells were incubated for 20 h and pictures were taken at both time points for untreated and treated cells. Migration of cells across the scratch was analyzed slides were photographed.

The primary purpose was to see if the hedgehog pathway inhibitors affect cell migration, proliferation and cell cycle. Both primary (RWPE1) as well as metastatic (PC3, C4-2B) PCa cells were treated with itraconazole (0-10 μ M), cyclopamine (2-50 nM) and their combination and cell motility as a measure of metastatic potential was determined by the wound closure assay. We observed that both drugs affected the invasion of cells when given alone; however the combination of the two drugs resulted in greater inhibition of cell invasion supporting our hypothesis for the use of hedgehog inhibitors in combination.

Itraconazole, cyclopamine and their combination inhibit tumor growth in mice implanted with 22Rv1 cells. We analyzed the effect of the combination on the growth of 22Rv1 tumors under in vivo conditions using athymic nude mice. To establish tumor xenografts, 6-8-week-old athymic nu/nu male mice were injected subcutaneously with 1×10^6 22Rv1 cells mixed with 50 μ l RPMI and 50 μ l matrigel on the back of each mouse. The mice were then randomly divided into four groups: Group I (4 mice) served as the control; Group II (4 mice) received itraconazole (25 mg/Kg by oral gavage daily); Group III (4 mice) received Cyclopamine (5 mg/Kg by oral gavage daily); Group IV (4 mice) received combination of itraconazole (25 mg/Kg by oral gavage daily) and Cyclopamine (5 mg/Kg by oral gavage daily). Once tumors started growing, their sizes were measured weekly and tumor volumes were calculated using the formula for calculating the volume of a hemi-ellipsoid. Tumor measurements were taken till tumors reached a targeted volume of 1200 mm³. In control animals, targeted tumor volume of 1200 mm³ was reached at 6 weeks post cell inoculation and significantly later time in animals treated with itraconazole alone (9 weeks) and cyclopamine alone (8 weeks). Notably, tumor volumes stayed under 165 mm³ in mice that received both itraconazole and cyclopamine. These data show that a combination of itraconazole and cyclopamine is considerably

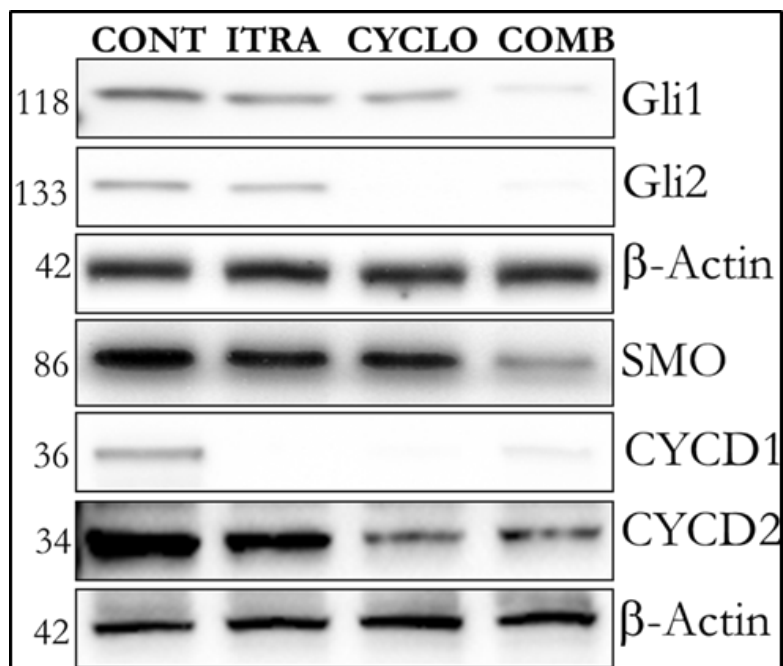


Figure 6: Effect of treatment with itraconazole, cyclopamine and their combination on Hh pathway and related cell cycle regulatory molecules. Mice were treated with itraconazole and cyclopamine and their combinations as described in the text. At the end of the treatment mice were killed and the tissues harvested. Harvested tissues were processed for protein expression by western blotting. Equal loading for each protein was analyzed by reprobing the same blot for β -actin expression.

more efficient in inhibiting Hh signaling pathway in PCa cells and in the process significantly reducing the effective concentration of cyclopamine. We further examined hedgehog pathway and related cell cycle regulatory molecules and observed significant inhibition in the group that received a combination of itraconazole and cyclopamine (Figure 6). These observations provide evidence that both itraconazole and cyclopamine inhibit tumor growth in mice.

Itraconazole, cyclopamine and their combination inhibit tumor growth in PTEN knockout/mutant mice.

We generated the prostate specific knockout mice in our in house animal core facility at the UW Madison Biotron Center. The original PTEN^{loxP/loxP} mice were obtained Jackson laboratories (Bar Harbor, ME) and crossed with ARR2 probasin-cre transgenic line PB-cre4 obtained from the NIH mouse repository. The subsequent breeding of the F1 generation produce F2 offsprings with a homozygous deletion of Pten in the prostates. Pten deletion in the mice was confirmed using tail DNA. PTEN knockout mice (8 weeks old) were divided into four groups. Group 1 received vehicle only and served as the control. Group 2 mice received itraconazole (10 mg/Kg by oral gavage, five days a week). Group 3 received cyclopamine (4 mg/Kg by oral

gavage, five days a week). Group 4 mice received itraconazole (10 mg/Kg daily by oral gavage five days a week) and cyclopamine (4 mg/Kg daily by oral gavage five days a week). The mice were monitored every four weeks for prostate tumor progression. We observed that at the end of the study, in the prostate specific Pten knockout mice combination of itraconazole and cyclopamine produced 57% inhibition of tumor growth compared to 30% in cyclopamine alone and 45% in itraconazole alone treated mice (Figure 7). These data show that a combination of itraconazole and cyclopamine is considerably more efficient in inhibiting tumor growth highlighting the synergistic efficacy of this combination for the treatment of PCa.

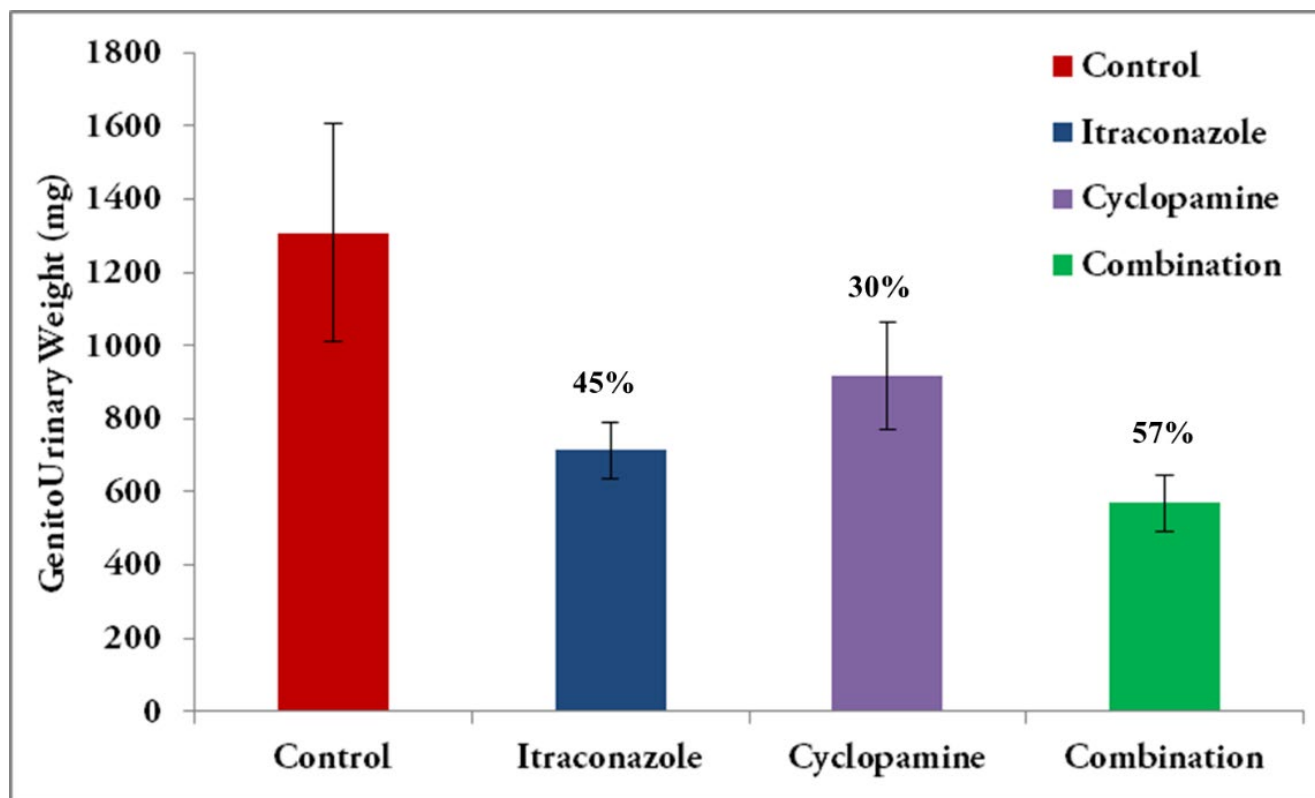


Figure 7: Effect of treatment with itraconazole, cyclopamine and their combination on tumor growth and in PTEN knockout. Mice were treated with itraconazole and cyclopamine and their combinations as described in the text. Tumor progression was monitored over time and the mice were killed at the end of the study and the genitourinary (GU) apparatus was removed *en bloc*, including the seminal vesicles and weighed. Histograms represent mean GU weight $\pm$ SEM.

To investigate the effect of itraconazole and cyclopamine and their combination on proliferation and cell cycle, we examined the expression of molecules related to proliferation in particular and also others related to cell cycle in prostate cancer 22Rv1 cells. We observed that both drugs affected the expression of molecules related to proliferation alone, but the combination of the two drugs resulted in more effective modulation of these markers. We observed significant increase in the expression of cyclin dependent kinase inhibitors p21 and p27 in cells treated with a combination of itraconazole and cyclopamine. Other cell cycle regulatory proteins cyclins D1 and E1 were also observed to be inhibited by the treatment with the combination of itraconazole and cyclopamine (Figure 8).

Summary:

Components of the Hedgehog pathway are constitutively activated in prostate cancer. Loss of PTEN function, considered a characteristic feature of advanced prostate cancer, is also associated with increased activation of the Hh pathway. High levels of Shh and GLI1 mRNA expression strongly correlate with Hh pathway activation in Pten-knock out mouse model of prostate cancer. High levels of Gli1 mRNA expression is associated with loss of Pten expression in prostate cancer cells. Itraconazole and cyclopamine synergize to inhibit Gli2

luciferase activity and protein expression. Treatment with either itraconazole or cyclopamine inhibited tumor growth in athymic mice, however the decrease is significantly greater with a combination of itraconazole and cyclopamine. Itraconazole and cyclopamine synergize to inhibit Hh signaling in tumors from athymic nude mice. Itraconazole and cyclopamine inhibit tumor growth in Pten-KO mice. Itraconazole and cyclopamine synergize to inhibit Hh signaling in Pten-KO mice.

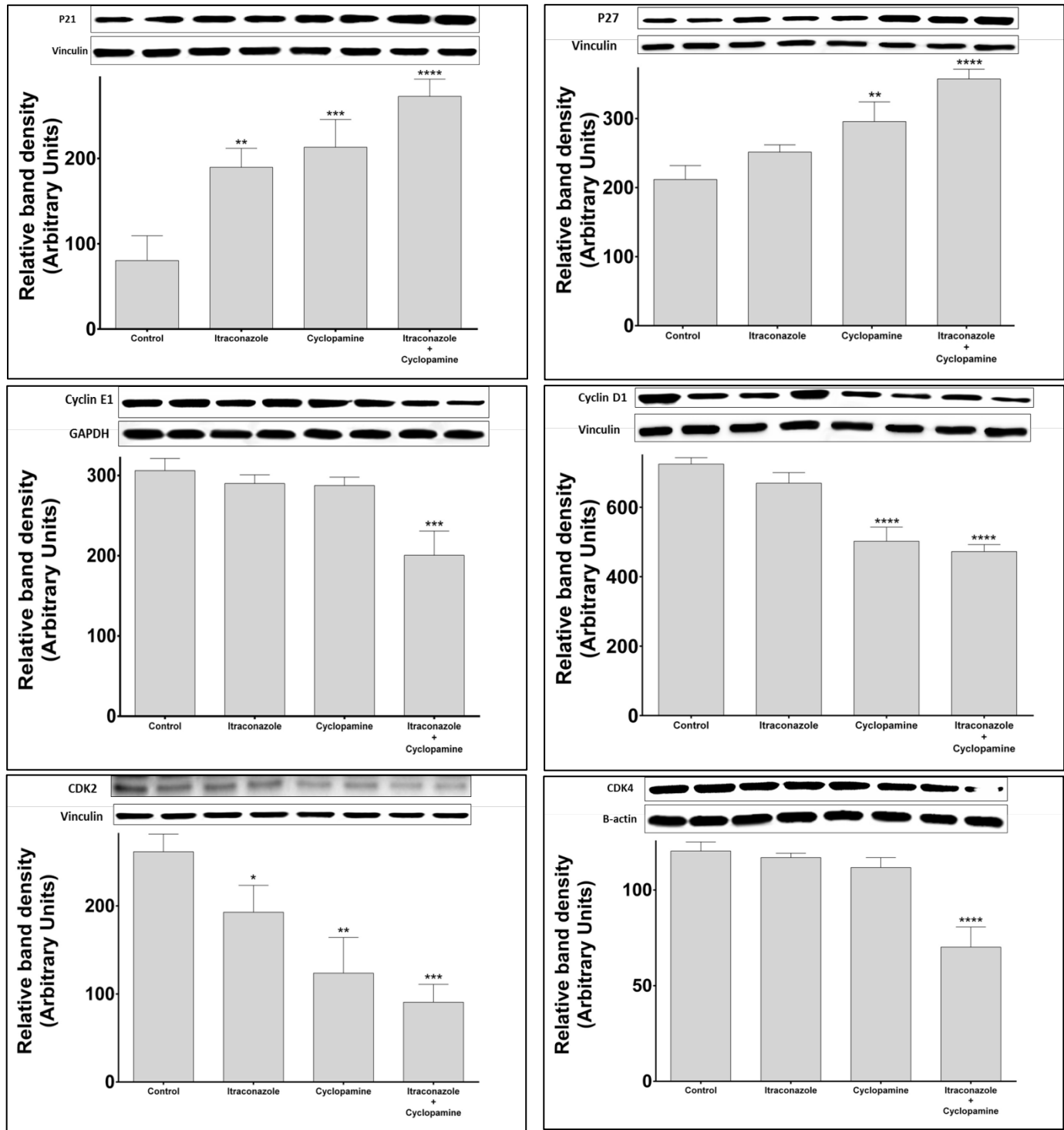


Figure 8: Effect of itraconazole, cyclopamine and their combination on the expression of markers related to the proliferation and cell cycle. Prostate cancer cells 22Rv1 were treated with itraconazole, cyclopamine and their combination for 24 hours; cell lysates were prepared and subjected to protein expression analysis by immunoblotting. Blots were scanned and quantitated. Bars are mean protein expression $\pm$ SEM. Similar results were obtained with PC3 and DU145 cells.

Other achievements. Part of the data was presented at the 2018 annual meeting of the American Association for Cancer Research.

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

- "Nothing to Report."

○ **How were the results disseminated to communities of interest?**

- The findings from this proposal were presented at the Annual meeting of the American Association for Cancer Research, 2018.

Vaqar M. Adhami, Imtiaz A. Siddiqui, Mohammad Imran Khan, Islam Rady, Leanna Sako, Hasan Mukhtar. Hedgehog pathway inhibitors itraconazole and cyclopamine produce synergistic suppression of Pten deficient prostate cancer [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl): Abstract nr 5808.

Experimental and Molecular Therapeutics

Abstract 5808: Hedgehog pathway inhibitors itraconazole and cyclopamine produce synergistic suppression of Pten deficient prostate cancer

Vaqar M. Adhami, Imtiaz A. Siddiqui, Mohammad Imran Khan, Islam Rady, Leanna Sako, and Hasan Mukhtar
Cancer Res July 1 2018 78 (13 Supplement) 5808-5808; DOI:10.1158/1538-7445.AM2018-5808

See Appendix for abstract

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

Not applicable.

4. IMPACT:

○ **What was the impact on the development of the principal discipline(s) of the project?**

- "Nothing to Report."

○ **What was the impact on other disciplines?**

- "Nothing to Report."

○ **What was the impact on technology transfer?**

- "Nothing to Report."

○ **What was the impact on society beyond science and technology?**

- "Nothing to Report."

5. CHANGES/PROBLEMS:

○ **Changes in approach and reasons for change**

- "Nothing to Report"

○ **Actual or anticipated problems or delays and actions or plans to resolve them**

- "Nothing to Report"

○ **Changes that had a significant impact on expenditures**

- "Nothing to Report"

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

- "Nothing to Report"

- Significant changes in use or care of human subjects

- "Nothing to Report"

- Significant changes in use or care of vertebrate animals.

- "Nothing to Report"

- Significant changes in use of biohazards and/or select agents

6. **PRODUCTS:** "Nothing to Report."

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS—**

Name:	Project Role:	Researcher Identifier	Nearest person month worked:	Contribution to Project:	Funding Support:
Hasan Mukhtar	PI	None	1.8	Overall project administration	This grant
Vaqar Adhami	Co-Investigator	None	3.6	Contributor, PCa cell sensitivity, growth kinetics Animal Studies	This grant

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

- "Nothing to Report."

- What other organizations were involved as partners?

- "Nothing to Report"

8. **SPECIAL REPORTING REQUIREMENTS—**Nothing to report

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

Abstract 5808: Hedgehog pathway inhibitors itraconazole and cyclopamine produce synergistic suppression of Pten deficient prostate cancer

Vaqar M. Adhami, Imtiaz A. Siddiqui, Mohammad Imran Khan, Islam Rady, Leanna Sako and Hasan Mukhtar

Experimental and Molecular Therapeutics

DOI: 10.1158/1538-7445.AM2018-5808 Published July 2018



Proceedings: AACR Annual Meeting 2018; April 14-18, 2018; Chicago, IL

Abstract

Hedgehog (Hh) signaling plays an important role in prostate development and is also a characteristic feature of prostate cancer (PCa). The ability of Hh pathway activation to promote cell invasiveness, epithelial to mesenchymal transition and metastasis suggest that targeting Hh pathway may provide an important clinical avenue for the treatment of advanced PCa. Loss of PTEN function is considered a characteristic feature of advanced PCa. To gain insight into the status of Hh signaling in PCa in relation to PTEN status we selected three cell lines based on their PTEN expression. Gli1 mRNA was upregulated 2000 fold in PC3 (PTEN^{-/-} null) cells, 800 fold in DU145 (PTEN^{+/-}) cells when compared with normal prostate epithelial cells suggesting that PTEN loss is associated with increased Hh signaling. We also observed significant increase in the mRNA levels of Hh ligand (Shh), Hh receptors (PTCH2 and SMO) and effectors of Hh signaling (Gli1 and Gli2) in the prostate tissues of PTEN knockout mice. A 400 fold increase in the mRNA levels of Gli1 and 15 fold increase in the levels of Gli2 mRNA levels was also observed. We also observed significant decrease in the mRNA levels of the negative regulators of Hh signaling, HHIP and SuFu in the prostate tissues of PTEN knockout mice with similar inhibition in the transcript levels of GAS1. We next investigated the effect on Hh signaling in PCa cells treated with itraconazole and cyclopamine alone and in combination. PCa 22Rv1 and PC-3 cells transfected with Gli-dependent luciferase reporter construct were treated with itraconazole (1 and 2 μ M), cyclopamine (5 and 10 nM) and combination of itraconazole (1 μ M) and cyclopamine (5 nM) for 40 hours. Gli-dependent luciferase reporter activity was inhibited by itraconazole and cyclopamine treatments; however

the inhibition was significantly greater in combination. Treatment of PCa cells with the combination of itraconazole and cyclopamine led to increased cell growth inhibition that was greater than the sum of the individual agents alone suggesting synergism. We next analyzed the effect of the combination on the growth of CWR22Rv1 tumors under in vivo conditions using athymic nude mice. In control animals, targeted tumor volume of 1200 mm³ was reached at 6 weeks post cell inoculation and significantly later time in animals treated with itraconazole alone (9 weeks) and cyclopamine alone (8 weeks). Notably, tumor volumes stayed under 165 mm³ in mice that received both itraconazole and cyclopamine. In the prostate specific Pten knockout mice combination of itraconazole and cyclopamine produced 57% inhibition of tumor growth compared to 30% in cyclopamine alone and 45% in itraconazole alone treated mice. These data show that a combination of itraconazole and cyclopamine is considerably more efficient in inhibiting tumor growth highlighting the synergistic efficacy of this combination for the treatment of PCa.

Citation Format: Vaqar M. Adhami, Imtiaz A. Siddiqui, Mohammad Imran Khan, Islam Rady, Leanna Sako, Hasan Mukhtar. Hedgehog pathway inhibitors itraconazole and cyclopamine produce synergistic suppression of Pten deficient prostate cancer [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl):Abstract nr 5808.

- ©2018 American Association for Cancer Research.