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PRINCIPAL INVESTIGATOR: Hasan Mukhtar

CONTRACTING ORGANIZATION: University of Wisconsin, Madison
Madison, WI 53706-1509

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Hasan Mukhtar, PhD

E-Mail:hmukhtar@dermatology.wisc.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

A commonly used antifungal, Itraconazol 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 seeks to investigate the efficacy of Itraconazol against prostate cancer with an intention to accelerate its rapid translation into human clinical trials. We are in the process of identifying a combination of drugs for the treatment of advanced prostate cancer. Although these are preclinical studies, however, if the efficacy of the drug can be established against prostate cancer in this proof-of-principle study then clinical trials could be immediately started based on the fact that these are existing drugs with all the information about toxicity and dose. The drugs do not have to undergo the mandatory initial trials to establish dose and toxicity. This report outlines our progress to date.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:**

Hedgehog (Hh) signaling is activated in advanced prostate cancer (PCa) and is required for proliferation, viability, and invasive behavior. The levels of Hh activity also correlate with the severity of the tumor and are both necessary and sufficient for metastatic behavior. Blockade of Hh signaling leads to tumor shrinkage and remission in preclinical tumor models. We hypothesize that targeting Hh pathway activation in advanced PCa will result in decreased Hh signaling and subsequent inhibition of prostate tumorigenesis. Current Hh inhibitors such as cyclopamine present with severe side effects in non-tumor tissues. Itraconazole, a commonly used antifungal agent with a well-known safety profile was observed to inhibit the hedgehog (Hh) pathway by acting on the essential Hh component Smoothened (SMO) through a mechanism distinct from cyclopamine and other SMO antagonists. We will evaluate the effect of itraconazole alone and in combination with cyclopamine, on the growth and metastasis of human PCa *in vitro* and *in vivo* with an intention to accelerate their rapid translation into human clinical trials. We propose to study the effect of itraconazole and cyclopamine alone and in combination against PCa both under *in vitro* and *in vivo* conditions. We will evaluate the effect of the combination on the growth and metastasis of human PCa cells representative of the advanced disease; the effect of the combination, on the growth and metastasis of human PCa cells implanted orthotopically and subcutaneously in male athymic nude mice. PC-3 cells labeled with luciferase and green fluorescent protein will be used to monitor the effect of drugs and the metastatic dissemination pattern to various tissues. We will investigate the effect of the combination on the progression of PCa in the PTEN knockout mouse model that recapitulates features of advanced human PCa. The data obtained will be extremely valuable in providing information on the usefulness of novel Hh signaling inhibitors against PCa for their rapid translation into human clinical trials.

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?**

  1. Evaluate the effect of itraconazole and cyclopamine, alone and in combination on the growth and metastasis of human PCa cells *in vitro*.
  2. 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.
  3. Investigate the effect of itraconazole and cyclopamine alone and their combination on the progression of PCa in the PTEN knockout mouse model of PCa.

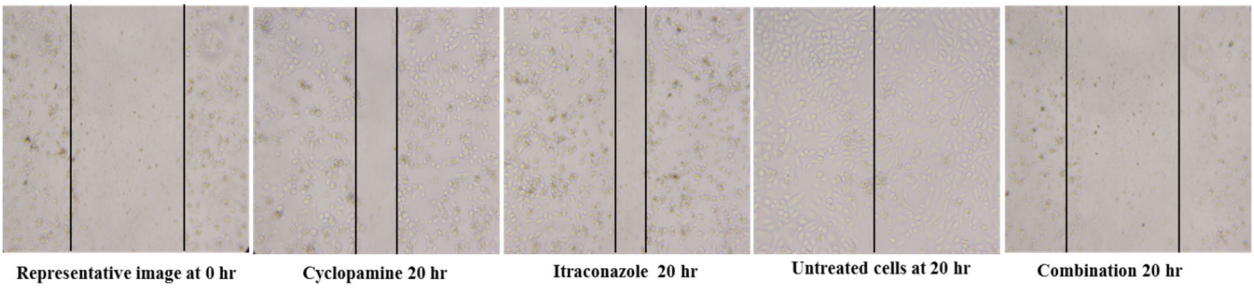
- **What was accomplished under these goals?**

  - 1) Major activities: We repeated many of these experiments 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.

- 2) Specific objectives: We ascertained the effect of itraconazole and cyclopamine and their combination on the migration and invasion and conducted in vivo experiments in the xenograft mouse model implanted with human prostate cancer cells.
- 3) 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 cells 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.

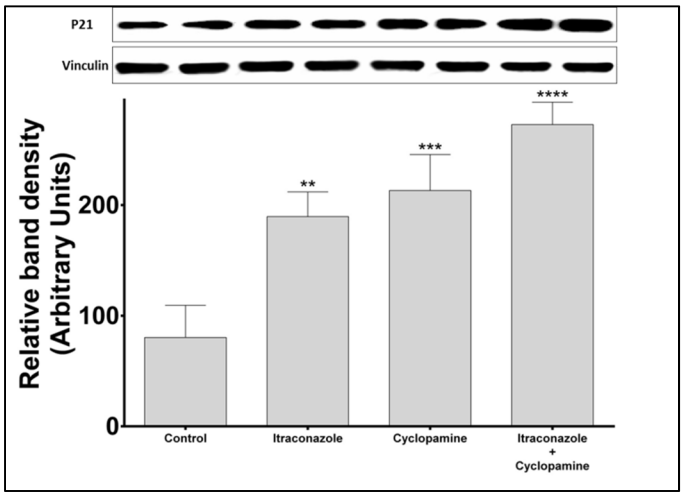
  We undertook studies to ascertain the effect of itraconazole and cyclopamine on metastasis and epithelial to mesenchymal transition. The primary purpose was to see if the hedgehog pathway inhibitors affect cell migration, proliferation and cell cycle..

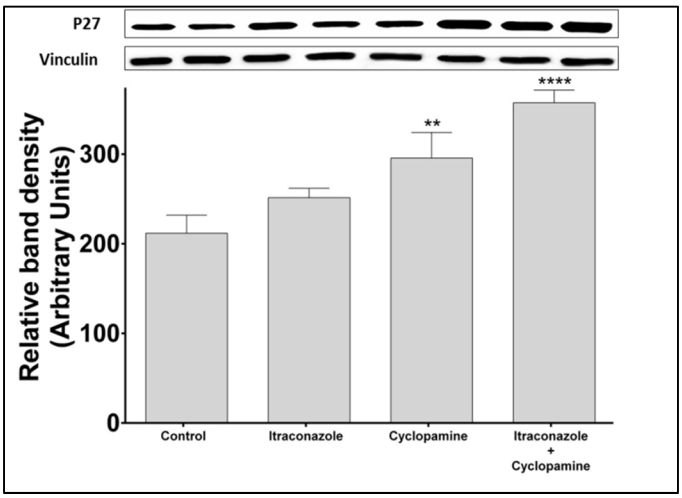
  Both primary (RWPE1) as well as metastatic (PC3, C4-2B) PCa cells were treated with itraconazole (0-10 μM), cyclopamine (2-50 nM) and their combination and cell motility as a measure of metastatic potential was determined by the wound closure assay. We observed that both drugs affected the invasion of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

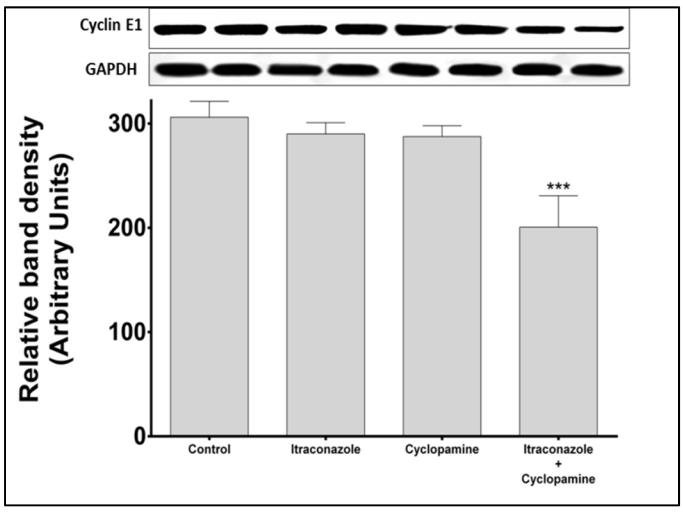


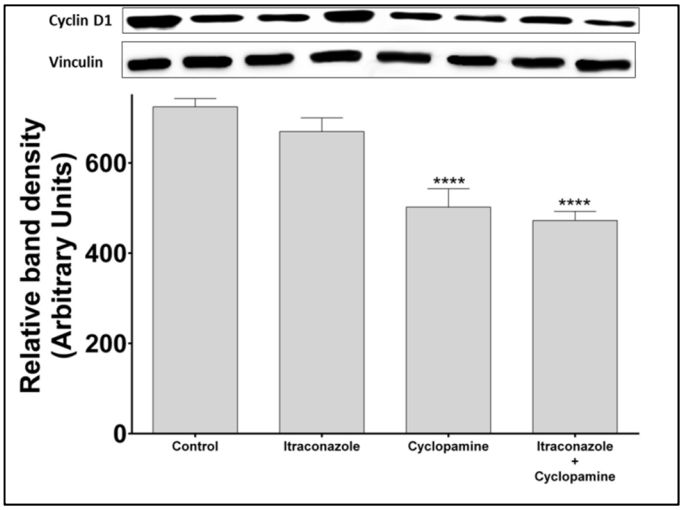
Effect of itraconazole, cyclopamine and their combination on the migration potential of PC-3 cells. Representative images showing migration of cells across a wound over a 20 h period. PC3 cells were cultured and treated with itraconazole, cyclopamine and their combination for 20 hours. A scratch was 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.

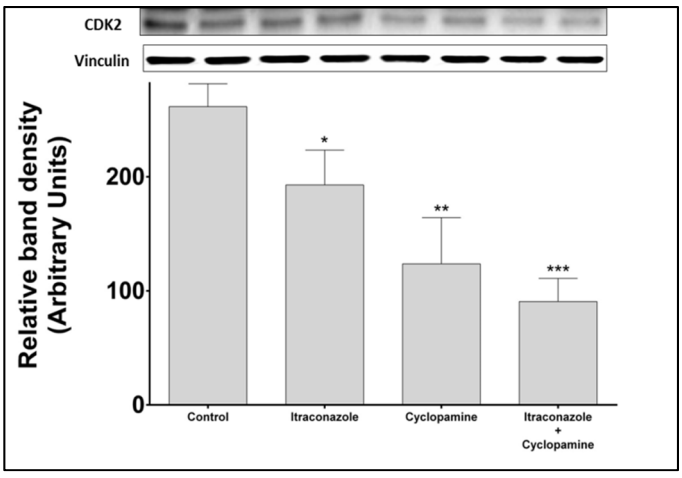
To investigate the effect of itraconazole and cyclopamine and their combination on proliferation and cell cycle, we examined the expression of molecules related to proliferation in particular and also others related to cell cycle. We observed that both drugs affected the expression of molecules related to proliferation alone, but the combination of the two drugs resulted is 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.

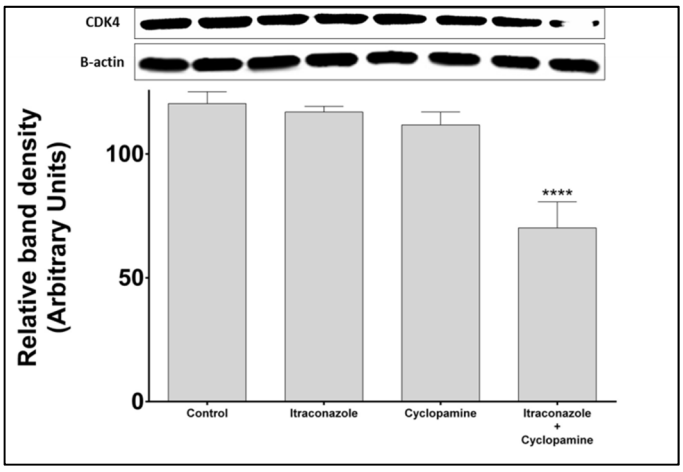






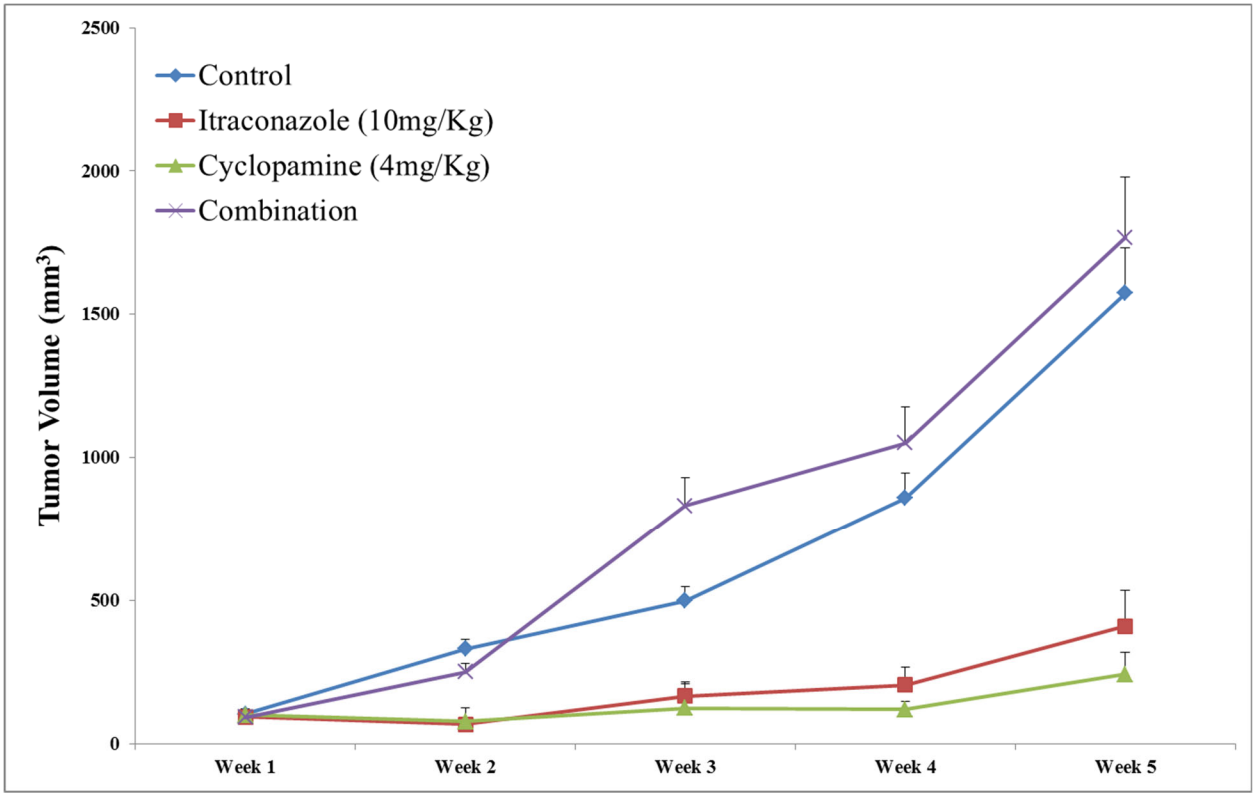






Effect of itraconazole, cyclopamine and their combination on the expression of markers related to the proliferation and cell cycle. Prostate cancer cells 22Rv1 were treated with itraconazole, cyclopamine and their combination for 24 hours; cell lysates were prepared and subjected to protein expression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

- *4) Other achievements.* The primary purpose of our initial experiments was to study the effect of each individual drug on growth and cell viability with the objective to identify ideal doses for use in combination. We are also conducting studies to investigate the therapeutic efficacy in vivo using animal models. An unexpected observation was the lack of growth inhibition when itraconazole and



Effect of treatment with itraconazole, cyclopamine and their combination on tumor growth and in mice implanted with 22Rv1 cells. Line graph showing tumor volume growth determined by weekly measurements. Each value in the graph is the mean ± SE from six mice.

cyclopamine were administered together orally at the same time in animals. This lack of growth inhibition may be due to drug interaction and possible interference with drug absorption when administered together. We are repeating these experiments to understand whether these are real observations or affected by other factors. Our studies with the Pten mouse model are currently underway and we have completed the tumor analysis studies. We are analyzing the data and validating with western blot and immunochemistry.

Hedgehog signaling has an important role in prostate development and it appears to be a characteristic feature of prostate cancer. This application proposes to test the hypothesis that itraconazole will inhibit growth and metastasis of human prostate cancer and will synergize with cyclopamine through inhibition of hedgehog signaling. The data obtained will be extremely valuable in providing information on the usefulness of novel hedgehog signaling inhibitors against prostate cancer for their rapid translation into human clinical trials.

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

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

- **What opportunities for training and professional development has the project provided?**
  - "Nothing to Report."
- **How were the results disseminated to communities of interest?**
  - "Nothing to Report."
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Review all in vitro experiments and repeat any experiments in necessary
  - Investigate the effect of itraconazole on the growth and metastasis of human prostate cancer cells implanted in the Pten mouse model.
  - Tumor growth studies in the Pten mouse model
  - Metastasis monitoring
  - Immunohistochemistry
  - Review nude mice data and repeat experiments if necessary

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
Imtiaz Siddiqui	Scientist	None	9.0	Contributor, PCa cell growth kinetics, Animal Studies	Depart-mental

- **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:** ***N/A***