# SYNOPSIS

**SYNOPSIS OF THE THESIS ON**

# EXPLORING THE POTENTIAL OF METFORMIN AND ITS TARGET(S) TO CELL DEATH PATHWAY IN PROSTATE CANCER

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**BHATT ANKIT, M.SC.**

**UNDER SUPERVISION OF**

**PROF. SARITA GUPTA**

**DEPARTMENT OF BIOCHEMISTRY**

**FACULTY OF SCIENCE**

**THE M. S. UNIVERSITY OF BARODA**

**VADODARA- 390 002**

## Introduction

Cancer is one of the most dreadful diseases, and is leading cause of death. According to WHO nearly 10 million deaths occurred in 2020 due to cancer. Sedentary lifestyle, habits, pollution, and genetic mutations and viral infections such as hepatitits and HPV (human papillomavirus) emerge as major contributing factors for increasing incidences of cancer with 244 million reported in 2016. Among males, prostate cancer (1.27 million cases) is the most common cancer after lung cancer (1.36 million cases). Estimated data indicates that 0.37 million deaths occurred in 2020 due to prostate cancer. India reported 34,540 new cases and 16,783 deaths, representing over 60% of the prostate cancer burden in South-Central Asia. The average annual percent changes from 2015 to 2019 showed a significant increase of 0.49% in the incidence rate and 0.48% in the mortality rate [ (Sung, 2021)].

Prostate cancer is further divided in to three categories based on the types of cells of involved in the tumor formation like luminal, basal type and neuroendocrine and termed as squamous/adenocarcinoma, adenocarcinoma and neuroendocrine carcinoma respectively. Several large-scale genomic studies in both primary prostate tumors and metastatic castration-resistant prostate cancer (mCRPC) have identified recurrent DNA copy number changes, mutations, rearrangements, and gene fusions. [ (Taylor, 2010) , (Abeshouse, 2015). Signature genetic alterations target the pathways of AR, PI3K–PTEN, Wnt, and DNA repair and components of the cell cycle in nearly all metastatic prostate cancers and a high fraction of primary prostate cancers (Taylor et al. 2010; ; Robinson et al. 2015).,. Increasing ratio of relapse due to drug resistance and metastastis in various oragan with primary tumor leads more than 90 % of death in cancer [ (Harbeck, 2016)Thus, it is a challenge to find a novel therapy for drug acquired resistance cancer. However, the different physiological conditions, origins, metastasis and re-occurance of prostate cancer still not understood well.

Due to high rate of occurrence and mortality in prostate cancer need further understanding of pathogenesis of prostate cancer. To identify molecular mechanisms of prostate cancer pathogenesis and novel therapeutic targets is important to be addressed.

Androgens are needed to ensure that the prostate grows and functions normally. Androgens can lead to prostate cancer, because they bind to and activate (turn on) the 'androgen receptor (AR)', The AR gene is encoded on Xq11–12 and consists of eight exons which correspond to different parts of the modular protein. Once these androgen receptors have been bound to androgen, they then stimulate certain genes that cause prostate cells to grow. At early stages of prostate cancer, prostate cancers "feed off" androgens, depending on them to grow. These types of prostate cancers are known as "androgen-dependent" or "androgen-sensitive". Androgen Deprivation Therapy (ADT) works to 'deprive' these prostate cells of androgen, by blocking the growth promoting effects. In many cases cancer relapsed after androgen deprivation therapy and cancerous cells starts to grow independent to androgens called "androgen independent". and have potential to produce a cell proliferation signaling. There were various splice variants of androgen receptor who have lost their ligand binding sites called ARVs and specifically ARV7 is main prognostic factor in case of Androgen independent prostate cancer [ (Beltran, 2013)]. Lowering a androgen levels by castration (Prostatectomy) or inhibitor of androgen synthesis (abiraterone acetate shows effective treatment But AR signaling not completely inhibited. Despite the significant advances in the targeting of AR which have been translated to survival improvement for patients with mCRPC, this state of disease remains incurable and is associated with significant morbidity and mortality [ (Toren, 2013)].

Other important pathway is mTOR/PI3K/Akt pathway which appears to be involved in crosstalk with the AR pathway. Other kinase signaling pathways such as IRS-1, c-met, MAPK and SEMA3C may also be relevant targets. Transcription factors such as Stat3 and cytokines such as NF-κB and IL-6 are also implicated in CRPC progression and are used as novel targets.

Induction of apoptosis and autophagy are two major signaling pathways responsible for cell death and drugs targeting these pathways are important anticancerous agents. In Apoptosis extrinsic pathways are initiated by death ligands and intrinsic pathways are induced by cellular and DNAdamage. On one hand, clustering of death receptors in death inducing signaling complexes leads to the activation of initiator caspase-8 and -10; on the other hand, depolarization of the mitochondrial membrane release of cytochrome c, may induce activation of initiator caspase-9,activate the executioner caspases (-3, -6 and -7), which irreversibly trigger apoptosis by cleavage of a multitude of death substrates (e.g. PARP1). Mitochondrial apoptosis pathways are critically controlled by Bcl-2 proteins, enclosing antiapoptotic (e.g. Bcl-2 and Mcl-1), proapoptotic multidomain (e.g. Bax and Bak) and proapoptotic BH3-only proteins (e.g. Puma, Noxa and Bid).5 The extrinsic pathway may also be enhanced via the mitochondria, ascaspase-8 can process and activate the proapoptotic BH3-only protein Bid leading to truncated Bid (tBid) [ (Quast, 2013)].

Autophagy is a highly conserved intracellular degradation system that is activated in response to physiological or pathological stressors, such as starvation, hypoxia, oxidative stimulation, infection, and oncogene activation Autophagy-related (ATG) genes and proteins play an essential role in the regulation of the process and function of autophagy in human health and diseases. Autophagy can protect healthy cells from environmental stress to promote survival [ (Kroemer, 2010).]. However, the excessive or impaired autophagic activity can trigger cell injury and death.,. Conversely, autophagy suppression is also often associated with certain diseases, including a subset of cancers,  Initiation of autophagy requires the ULK1 kinase complex, which is tightly regulated by AMPK and mTOR, which act as an activator and inhibitor, respectively. AMPK activates ULK1 through phosphorylation. ULK1 complex comprise of ATG proteins (ATG1,101 and FIP200) which stimulate class III phosphatidylinositol 3-kinase complex(comprises BECN1).this stimulation leads to the cleavage of LC3 into LC3 I and conjugation with phosphatidylethanolamine (PE) .. LC3-II binds to LIR-containing autophagy receptors (AR; such as p62) bound to cargo targeted for degradation.

Differences in oxidative stress and antioxidant capacities in different populations may cause different risk level of prostate cancer. Indeed, clinical evidence of faster PCa disease progression in obese individuals further corroborates the hypothesis that oxidative stress and inflammation, , are key players in the initiation, progression and therapeutic resistance of PCa[ (Mondal, 2021)]. Furthermore, ADT enhances the vulnerability of PC cells to toxic oxidative stress induced by radiation. Ionizing radiation  which generates multiple highly reactive oxygen species (ROS) from cellular water, which oxidize DNA, proteins and lipids and may cause, disruption of plasma, mitochondrial, nuclear, endoplasmic reticulum membranes and as well as alteration of cellular protein functions and their degradation[ (Lu, 2010)] A dysfunctional or overwhelmed ETC can result in the leakage of electrons, which are captured by molecular oxygen [O2] to ultimately generate free oxygen radicals such as the superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH·)[133]. Chronic exposure to high levels of these ROS can cause significant damage to DNA, proteins and lipids, and ultimately result in decreased antioxidant defense mechanisms.The ataxia-telangiectasia-mutated (ATM) protein is a key regulator of the DNA damage response following oxidative stress. Interestingly, exposure to AR antagonists induces both telomere DNA damage and damage response inhibition in the CRPC cell line 22Rv1(Splice variant ARV7 +Ve cell line)[ (Reddy, 2019).]. Changes in the behavior of the PCa cells undergoing ADT is likely due to an adaptive response to a combination of stresses from the hypoxic tumor environment, which results in the activation of multiple alternate second messenger signaling that increase both AR gene expression and ligand-independent AR activation. Interleukins (ILs) like IL-6 and its receptor IL-6Ra signalling multiple downstream signaling and many of these utilize ROS secondary messengers. NF-κB, STAT and C/EBP, which are activated during IL-6 signaling, can directly interact with AR and regulate its function

Metformin, marketed under the trade name Glucophage among others, is the first-line medication for the treatment of type 2 diabetes. Its lowering glucose effect require activation of AMP-activated protein kinase (AMPK) to inhibit hepatic gluconeogenesis [ (Zhou, 2001).], increase peripheral uptake of glucose, and delay gastrointestinal glucose absorption. Metformin received considerable attention as potential anti-cancer agent as its exhibits a strong and consistent ant proliferative action on several cancer cell lines, including breast, colon, ovarian, pancreatic, lung, and prostate cancer cells. Metformin is a hydrophilic molecule and therefore cannot pass through the cell's membrane by passive diffusion. The organic cation transporter family (OCT), and specifically the organic cation transporter 1 (OCT1), is responsible for the uptake of metformin into hepatocytes and tumor cells[Ikhlas S and Ahmad M et.al].AMPK activation by metformin may happen by indirect upstream activation of LKB1 kinases which is known as tumor suppressor gene[Carling D, Mayer FV et al.]. Metformin is generally well-tolerated and has a low risk of adverse effects. Combining it with specialized anticancer drug may not only enhance its anticancer effects but also reduce the side effects associated with more aggressive cancer treatments.

Swertiamarin is one of the bioactive in Qing Ye Dan & Enicostemma littorale plant which is a seco-iridoid glycoside. It possesses multifunctional properties, including analgesic, anti-inflammatory, antiarthritis, hepatoprotective, antidiabetic, antioxidant, antimicrobial, anticancer, neuroprotective and gastroprotective properties. Swertiamarin has been extensively studied in our lab for its antidiabetic,hepatoportoective and antiadipogenic activity It modulates a wide range of molecular targets, including IR ,PI3Kinase inflammatory cytokines, transcription factors, , growth factors, apoptosis-related proteins, receptors, in various pathological conditions. [ Nur Sakinah Muhamad Fadzil[1]Siew Hua Gan[2] et al, Patel T Rawal KSoni S et al, (Tushar P. Patel, 2013),.] In light of this combinatorial efficacy of metformin along with SM is evaluated for effective prostate cancer therapy.

**Specific objectives:**

Major Objectives of the present study are.

1. To explore the role of metformin on different prostate cancer cell lines
2. To Study mechanism of action of metformin on cell death.
3. To modulate the efficacy of Metformin with bio actives.

**Objective-1: To explore the role of metformin on different prostate cancer cell lines.**

To investigate the role of Metformin's anticancer effects on prostate cancer, we selected three cell lines: Lncap and 22RV-1, which express AR (Androgen Receptor) and ARV7 (Androgen Receptor Variant 7), and PC-3, which lacks AR/ARV7 expression. The reason for choosing these three cell lines was to evaluate the impact of AR and ARV7 on Metformin's anticancer effects. We conducted cell growth inhibition assays in the presence and absence of the AR synthetic compound R1881 and observed varying efficacies of Metformin across the cell lines.

Metformin demonstrated higher efficacy in Lncap (AR-positive) compared to PC-3 (AR-negative) and 22RV-1 (ARV7-positive). Interestingly, Metformin showed lower efficacy in 22RV-1 than PC-3 despite being ARV7 positive. We also conducted clonogenic assays for Lncap and 22RV-1 cells, which revealed significant inhibition at the $IC_{80}$ of Metformin. Once again, Lncap exhibited higher inhibition compared to 22RV-1.

We evaluated the migration efficacy of Metformin in Lncap cells, which showed a significant difference in cell migration on day 12. Given the differing AR expression among the cell lines, when we assessed AR/ARV-7 inhibition in the presence and absence of R1881 with Metformin. It showed significant inhibition in Lncap cells in the absence of AR ligand treatment, while there was no significant inhibition in the presence of AR ligand treatment. In the case of 22RV-1, no significant AR inhibition was observed in either scenario—whether AR was present or absent. Notably, AR treatment had a substantial impact on ARV7 inhibition, demonstrating nonsignificant degradation when cells were treated with Metformin and 1nM R1881 (AR ligand).

**Objective-2: 2. To Study the mechanism of action of metformin on cell death**

We observed cell death through both apoptosis and autophagy pathways. To evaluate the apoptotic effect of Metformin, we examined caspase 3 and PARP-1 inhibition in the presence and absence of AR ligand treatment.

In Lncap cells, the expression of cleaved Caspase 3 increased non-significantly due to Metformin treatment with AR ligand and amino acid starvation, which induces autophagy. However, the expression of PARP-1 showed considerable variation with or without Metformin treatment.

In the PC-3 cell line, C-Caspase 3 expression decreased with Metformin treatment, and total PARP-1 expression also decreased. In the 22RV-1 cell line, cleaved PARP-1 expression decreased with Metformin treatment. The decrease in Caspase-3 expression in PC-3 cells indicates a lesser apoptotic effect of Metformin, suggesting that cell death in this case may be attributed to alternative pathways.

In autophagy, Metformin exhibits an increase in fluorescence when we introduce an LC-3/p62 GFP-mcherry construct under androgen-starvation conditions, similar to the observed increment in LC3 II protein levels in Lncap and 22RV-1 cells under androgen starvation and autophagic induction.

The ROS levels in Lncap cells also increase when we treat the cells with Metformin. This increase may be the reason for the heightened autophagy in these cells. Consequently, AR-positive cells are consistently sensitive to Metformin treatment, leading to cell death through autophagy, apoptosis, and due to ROS. In the case of AR-negative cells, the response to Metformin needs further investigation.

**Objective3: To modulate the efficacy of Metformin with bio actives**

Metformin has been used in combination therapy for various cancers due to its autophagic, anti-inflammatory, and glucose-lowering activities, which may enhance the overall survival (OS) or progression-free survival (PFS) of cancer patients. Since Metformin has shown efficacy in milimolar(mM), we aimed to improve its effectiveness through combination therapy or replace it with a similar antidiabetic drug to achieve better clinical outcomes in prostate cancer. Our overarching goal is to overcome the limitations associated with Metformin.

To achieve this, we have selected a compound called Swertiamarin, which exhibits a similar antidiabetic activity and has a stronger binding capacity. Swertiamarin is a molecule derived from Enicostemma littorale. To pursue this research, we conducted growth inhibition assay other parameters like Clonogenic assay Migration study, is underway further its efficacy in combination with metformin will be assed for apoptosis, autophagy and oxidative stress parameters.

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**Achievements:**

1. Presented ideation for SiRNA therapy of Hedgehog singling pathway in Prostate cancer in Entrepreneurship Capacity Building Program at STB I(Savali Technology and Business Incubator) powered by GSBTM (Gujarat State Biotechnology Mission)