CHAPTER - 3

TO EXPLORE THE ROLE OF METFORMIN ON DIFFERENT PROSTATE CANCER CELL LINES

Objective: To explore the role of metformin on different prostate cancer cell lines

Introduction

Cancer is a disease where cells start to grow uncontrollably, due to disruption of regulatory mechanisms. In 2018, GLOBACON reported approximately 15 types of cancer. Amongst them, prostate cancer (1.27 million cases) ranked as the second most common cancer in males, which was responsible for approximately 0.37 million deaths. (2020) Three different type of prostate cells basal, luminal and neuroendocrine show molecular and morphological heterogeneity in the expression of AR, MyrAKT and N-Myc or loss of PTEN, smad4, TR53 and Epithelial-mesenchymal transition, which play an important role in cancer progression leading to metastasis.

Androgen deprivation therapy (ADT) stands as a gold standard treatment for advanced prostate cancer (PCa). but castration-resistant prostate cancer (CRPC) following ADT has become a major obstacle. The androgen receptor plays a pivotal role in the occurrence and progression of prostate cancer. The AR is activated by androgens, causing the A-AR complex to enter the nucleus, and activate the expression of genes responsible for cell survival and proliferation. ADT treatment causes a reduction in androgen production or blocks the interaction of hormones with the AR-LBD, eventually, leading to a castration-resistant form (CRPC). LBD mutations render anti-androgen ineffective or constitutively active AR splice variants, lacking the LBD, [(Dalal et al., 2014)] Docetaxel, a non-hormonal drug is first-line therapy for CRPC but shows cytotoxic side effects [(Tannock et al., 2004)]. Hence there is a need to explore better molecules for effective therapy.

Metformin belongs to the biguanide class of drugs, which has the property of lowering blood glucose and increasing insulin sensitivity. Libby and colleagues stated patients with Type 2 Diabetes Mellitus(T2DM), who are on Metformin medication demonstrated a lower chance of Cancer compared to nonusers (7.3% vs 11.6%) ([(Libby et al., 2009)]. Further high dose of metformin is associated with the greatest

reduction in cancer risk. Thus, metformin drew the attention of scientists for the scope of its anti-cancer effect. The primary site of action of metformin is complex -1 of the electron transport chain of mitochondria and plays a role in ATP reduction, and increased AMP levels which drives the activation of AMPK, an energy sensor and regulator of metabolic and growth pathways. [(Pernicova & Korbonits, 2014)]. AMPK activation leads to inhibition of mTOR via activation of TSC. Thus, Metformin plays an important role in Cell proliferation and cellular degradation by autophagy, and affects apoptosis via inhibition of NF-κβ. Apart from this metformin has been shown to decrease AR and ARV7 expression and degradation of ER-alpha and ER-Beta [(Kim et al., 2016)] In light of this metformin seems to be good candidate, hence in the present paper we explored anticancer efficacy of metformin in AR dependent, AR independent prostate cancer using appropriate cell lines.

Materials and Methods:

Cell lines:

selected three different models to check the metformin effect and its cause of inhibition relative to AR presence. We opted to evaluate the metformin effect in the PC-3(CRL-1435, ATCC) cell line which is AR- Negative cell line, Lncap cell(CRL-1740, ATCC) line which is an AR -Positive cell line with 877A mutation in the LBD and 22RV-1(CRL-2505, ATCC) cell line with 874A mutation in the LBD along with a splice variant of AR. Splice variant lacking a ligand binding domain called ARV7 which can activate AR signaling pathway by binding to Androgen Responsive Element (ARE) of DNA Binding Domain (DBD).

Media:

PC-3 was cultured in DMEM F12 + 10%FBS + 1% Pen strep media. Lncap and 22RV-1 cells were cultured in RPMI-1640 + 10%FBS+1% Pen strep media.

Chemicals:

MTT Reagent (TC191, Himedia), Presto blue (A13261, Thermo Fischer) Paraformaldehyde (TC703, Himdeia), crystal violet (TC510, Himedia), Bradford Reagent (B6916, Sigma), Ponceau S treatment (P7170-1L, Sigma), Blotting grade

blocker (1706404, Biorad), Tween20 (P1379, Sigma), Clarity Max Western ECl (1705062, BioRad), Trizol (9109, Takara), C-DNA synthesis kit (TAKARA -9109), Metformin (317240-5GM, sigma), Fetal bovine serum (10270106, Gibco), Charcoal stripped fetal bovine serum (12-676-029, Gibco), RPMI 1640 (AT171, Himedia), DMEM Ham's F12 (AL127A, Himedia), Trypsin EDTA (TCL179, Himedia), Pensterp(15140122, Gibco)

Growth inhibition assay:

All cell lines were seeded at a density of 5000 cells/well in 96-well plates and starved for 24 hours for the determination of IC_{50} of Metformin. The experiments were conducted in the presence or absence of 1nM R1881, with a 24-hour incubation before drug treatment. In the Second experiment, cells underwent 5 days of starvation to assess the efficiency of Metformin in androgen-deprived conditions. In these conditions, 10% charcoal-stripped serum was used instead of 10% fetal bovine serum. After 72 hours of Metformin treatment, cell viability was measured in both cases using an MTT reagent or Presto blue by Multimode Reader (Synergy H1, BioTek)

Clonogenic Assay:

Around 3000 Cells/well were seeded in 6 well plates(90mm/Well) followed by 24 hrs serum starvation using 10% charcoal-stripped media respective to its culturing media. Metformin IC_{50} concentration was calculated by Growth inhibition assay which was administered along with 1nM R1881 to all wells except media control in all cell lines. After 6 Days of treatment, clonogenicity was measured by fixation with 4% PFA and 0.2% crystal violet treatment after one PBS wash

Migration Assay:

Cells were plated at a density of 0.5×10^6 Cells/Well in the six-well plate and kept for culturing for 24Hrs-48Hrs based on Confluency which is supposed to be good enough to cover whole well surface area. the cell monolayer was scraped in a straight line to with a sterile 200µL pipet tip and media was subsequently replaced with Metformin and R1881 containing media (Final concentration equivalent to IC_{50}) every alternate day. Mark the reference point by razor blade at outer bottom of the plate. Used this reference point every time to acquire an image and analyzed quantitatively using

ImageJ software

Western Blot Assay:

Cells were plated at a density of 0.5× 10^6 Cells/Well in the six-well plate. After 24 h cells were starved for 24hrs using 10% charcoal stripped media. After 24 hrs starvation, Metformin treatment for 24hrs was given along with R1881. Cells were harvested in Laemmli Sample Buffer (after an ice-cold PBS wash. Protein The protein concentration of samples was determined by Bradford assay. The equal protein was loaded and resolved on 10% SDS-PAGE. The electro-blotting of protein onto an activated PVDF membrane (methanol charged) was conducted at a voltage of 100 V for 90 minutes, with the incorporation of an ice pack within the chamber. Transfer of protein was checked by Ponceau S treatment for 3-5min. After PBS wash the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS), 0.1% Tween-20 in TBS-0.02 M Tris-Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with a specific primary antibody (1:1000). After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and the signal was visualized by using Clarity Max Western ECl and the signal was visualized by the UVTEC gel documentation system.

PCR Method:

1X 10^6Cell/well were seeded in 6 well plates and incubated in 5%C02, 37^0C incubator for 24hrs. Cell The cell was starved for 24hrs followed by R1881 and Metformin treatment for 24hrs. The next day Cells were harvested by Trizol (9109, Takara, Canada) treatment after PBS wash. RNA was isolated according to the protocol provided in the manufacturer's leaflet. Purity was checked by using a nanodrop instrument and then the RNA sample was used for C-DNA synthesis which was carried out by using a C-DNA synthesis kit (TAKARA -9109, Canada). PAGE was run and treated with EtBr to evaluate the amplicon by the UVTEC gel documentation system.

Results:

We performed a cell growth inhibition assay in all three cell lines in the presence and absence of R1881. (a synthetic AR ligand). It shows a difference in $IC_{50.}$ of Metformin (Figure 1 A) PC3 cell lines showed 30.98 mM without R1881 whereas 40.67 IC_{50} was obtained with 1nM R1881 treatment. A similar experiment was performed with two other cell lines. A significant alteration was observed in Lncap an AR-positive cell line 21.29mM IC_{50} without R1881 and 26.29mM IC_{50} with 1nM R1881. In the case of 22RV-1 cells (21.88 Vs 56.43mM) which is a splice variant of AR-positive cell line.

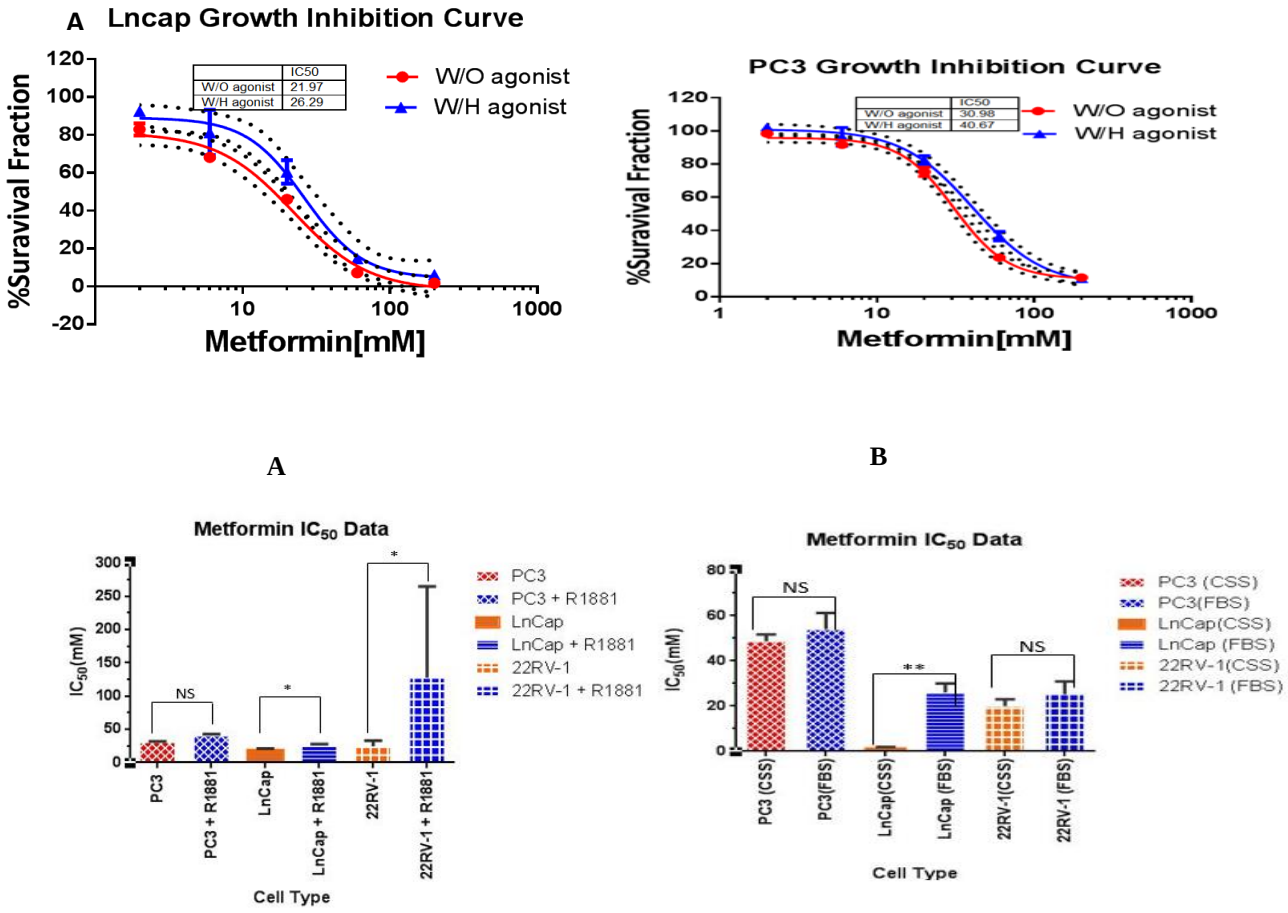


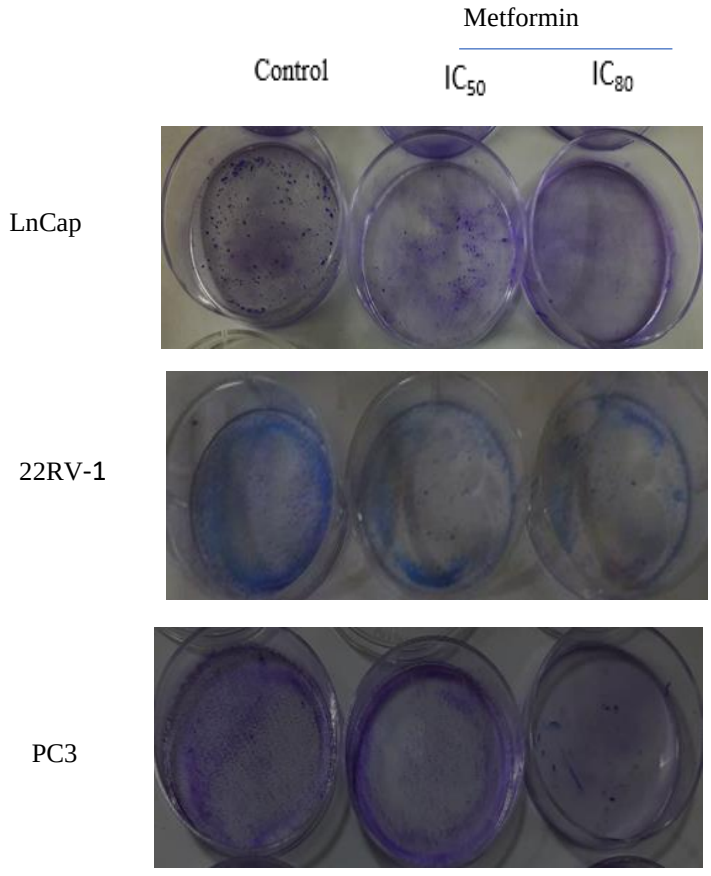
Figure 1 (A) Cell viability accessed after 72 hours following 1nM R1881 and Metformin treatment (B) Cell viability accessed after 72 hours following 5 Day starvation and Metformin treatment The data are expressed as mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e Nonsignificant

The findings indicated a significant contrast in the effectiveness of metformin, especially in AR-positive cell lines. Lower efficacy was observed in media containing androgen (fetal bovine serum), whereas higher efficacy was noted in media with androgen-deprived (charcoal-stripped serum) (Figure 1B). clonogenicity and cell migration have been assessed in Lncap and PC3 cell lines as per Figure 2. showing a reduction in % survival and % migration in treated wells for both AR negative and AR/variant positive cell lines. Inhibition is shown in a representative image.

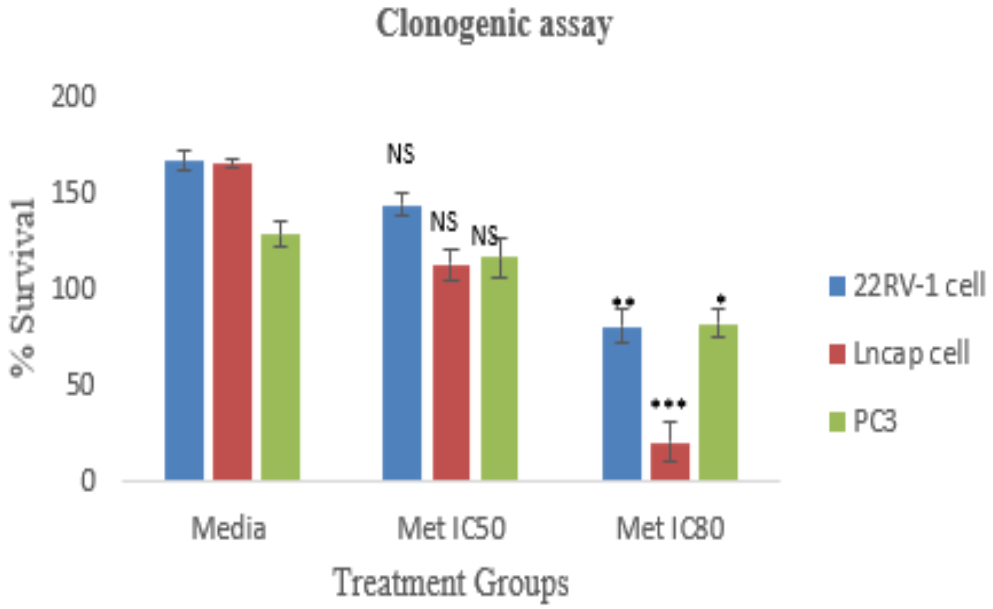
After treatment with R1881 AR signaling expression was modulated with time and concentration (Figure 3A& 3B) and AR was inhibited while treated with Metformin 21mM (IC_{50}) in both AR positive cell line LnCap and 22RV-1(Figure 3C & 3D) In case of ARV-7 expression in 22RV-1 was also induced by R1881 (Figure 4A) and increased level till 24Hrs which inhibited by Metformin (Figure 4A)

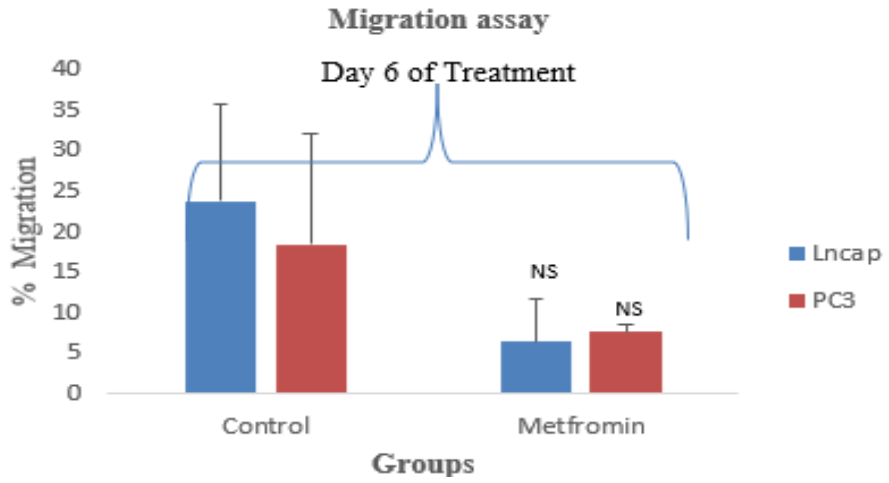
A

Clonogenic Assay

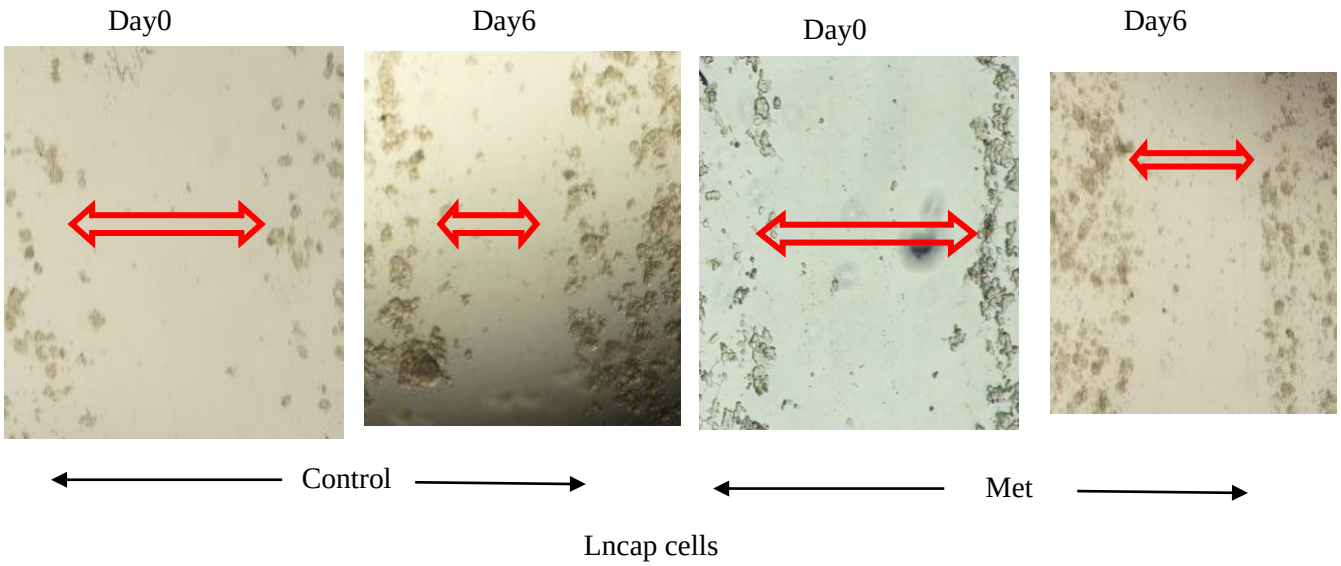


A


Clonogenic assay
200
150
100
50
0
% Survival
NS
NS
NS
***
*
Media
Met IC50
Met IC80
Treatment Groups
22RV-1 cell
Lncap cell
PC3


Migration assay
Day 6 of Treatment
40
35
30
25
20
15
10
5
0
% Migration
NS
NS
Control
Metfromin
Groups
Lncap
PC3

B


Day0
Day6
Day0
Day6
Control
Met
Lncap cells

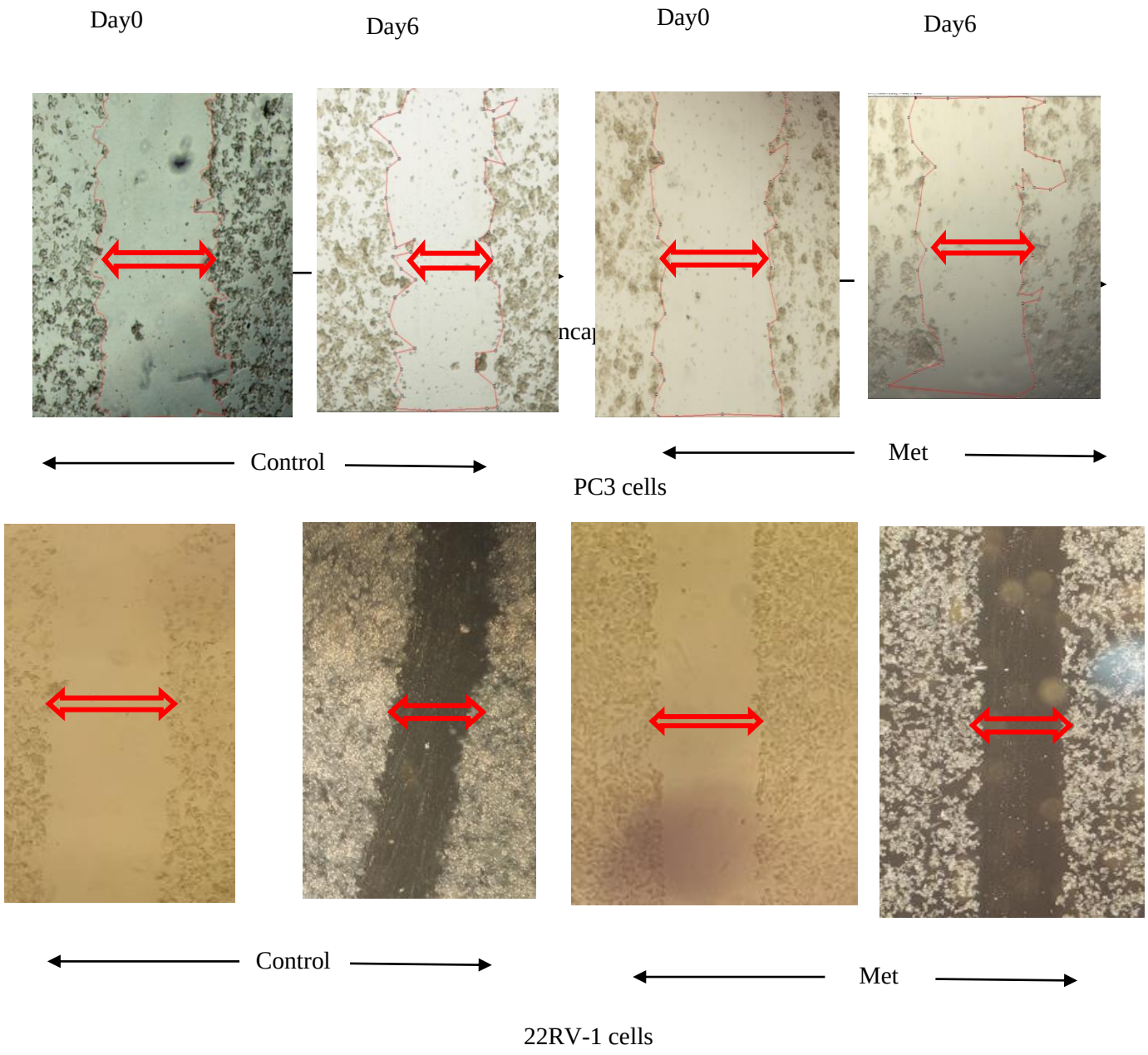


Figure 2 (A) Colony formation was assessed after 6 days of Metformin treatment, utilizing concentrations based on IC_{50} and IC_{80} values. (B) The assessment of cell migration in Lncap and PC3 cells was conducted after six days of Metformin treatment. Note:1nM R1881 was added in all wells. ***Representative images are expressed as mean ± SEM (n = 3) ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i, e Nonsignificant***

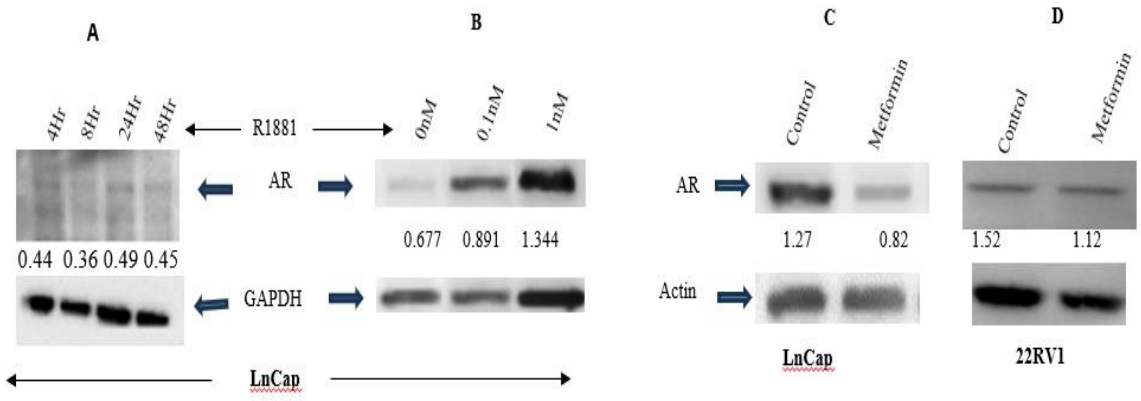


Figure 3 (A) and (B) present a time course and dose-dependent analysis of R1881 following treatment. (C) and (D) demonstrate AR expression in Lncap and 22RV-1 cells after treatment with 21mM Metformin

mRNA level of AR and PSA downstream gene of AR both were affected by the treatment of Metformin even in the presence of R1881 (figure 5A & 5B) means Metformin can inhibit androgen-dependent and independent signaling pathway.

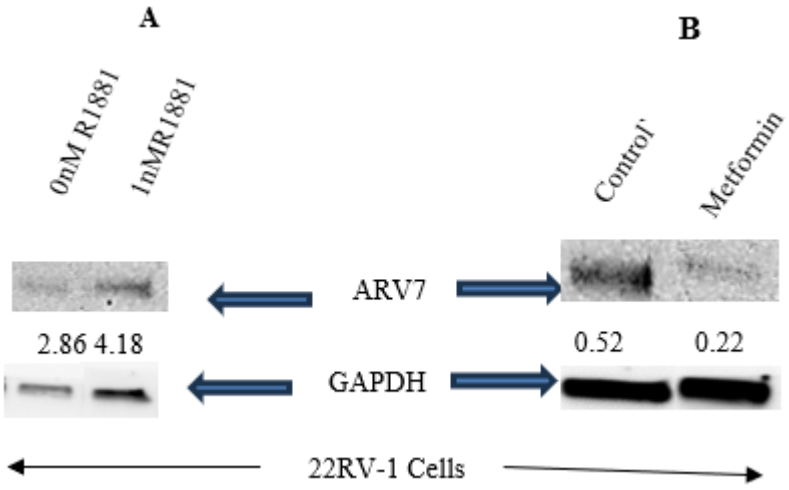


Figure 4 (a) and (b) level of ARV7 was assessed after 24 hours with/without 20mM of Metformin treatment in the presence of 1nM R1881

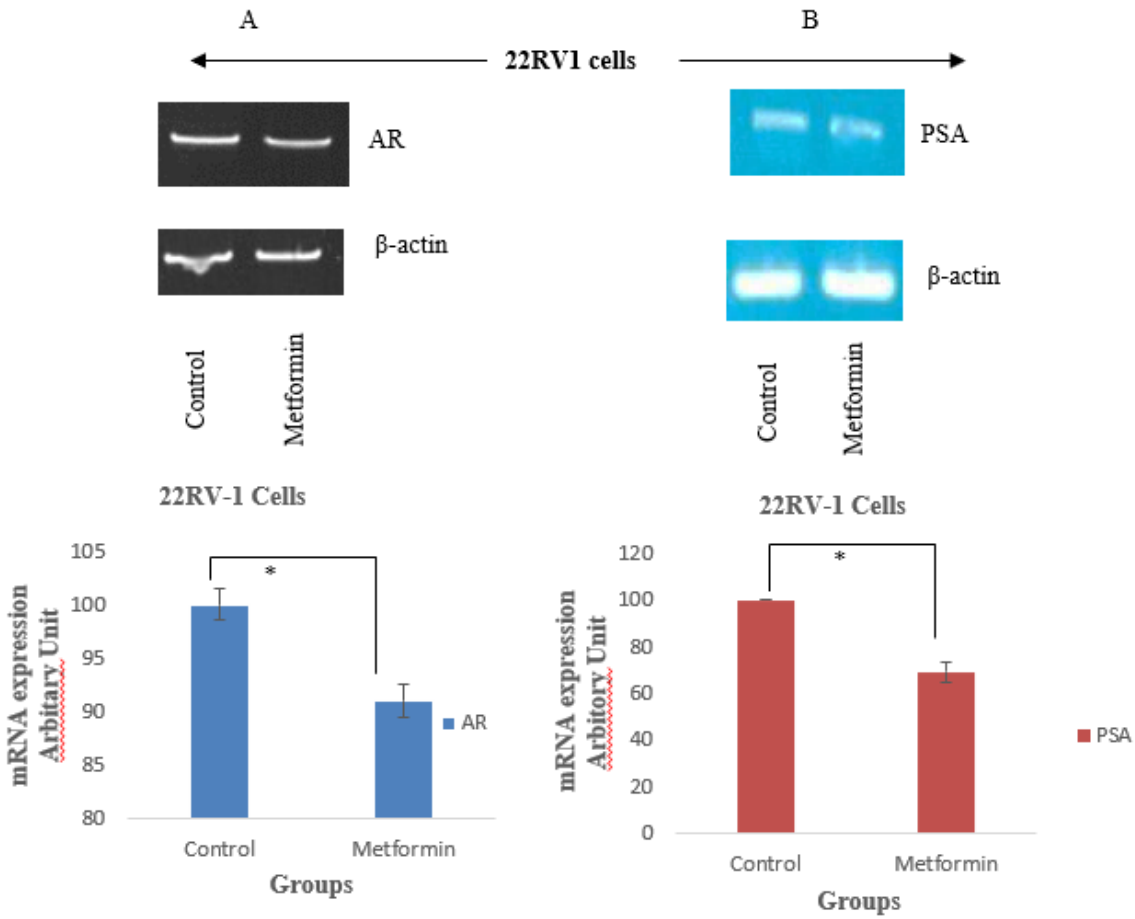


Figure 5 (A) The mRNA levels of AR and PSA from 22RV-1 cells subjected to treatment with 1nM R1881 and 20mM of Metformin presentation of it. Representative image is expressed as mean ± SEM (n = 3) ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i, e Nonsignificant

Discussion:

Cancer is a multi-faceted disease that commences with cellular transformation and uncontrollable growth arising from disruption in a variety of regulatory mechanisms. Prostate cancer has been one of the most prevalent cancers in males (GLOBACON, 2018). Androgen deprivation therapy (ADT) remains the most promising avenue of its treatment. However, studies have demonstrated that the LBD (Ligand binding domain) of AR leads to the activation of AR splice variants and making the anti-androgen therapy ineffective [(Dalal et al., 2014)]. Hence, various non-hormonal molecules are being explored to targets the splice variants. metformin is one such molecule. In the current study, we have demonstrated the inhibition of cellular growth in prostate cancer cellular models using metformin treatment which was supported by the study by Zao et.al. where it was studied that metformin treatment exhibits growth inhibition in cancer by promoting the cell cycle arrest and thus increasing the apoptotic and autophagic activity of the cells [(Zhao et al., 2019)]. Since the prostate cancer demonstrate molecular heterogenicity, it is crucial to assess the sensitivity of the drug across various cellular variants for which the in-vitro clonogenic assay was performed in presence of androgen agonist to simulate tumour microenvironment. It was observed that even though metformin exhibited inhibitory effect on various cells (C. Chen et al., 2021), AR mutant 22RV1 cells was less sensitive to metformin compared to the AR negative PC3 and AR positive Lncap cells which was supported by the microscopic observations of metformin treatment on AR negative cell lines by Zao et. Al. and Chunyang chang et. al. Moreover, to explore the effect of metformin on cell invasive properties and to obtain the additional information on its growth inhibition and apoptotic effects on the cancer cells, we performed the scratch assay in AR-positive and AR-negative cell lines and found a reduction in cell invasion in both cases which shows that cell invasion was not affected by AR presence and Absence. However, scratch assay provides primary information in relation to metastatic transformation. The Metformin treatment after Androgen starvation in all three in vitro cells have depicted a different half-maximal growth inhibition (IC_{50}) relative to AR and its variant presence. LnCap cells has been found to be more sensitive to Metformin in hormonal starvation compared to 22RV-1 and PC3 because of WtP53 expression (promoted by metformin). While PC3 cells lacks AR and p53 expression, making it the lowest sensitive cell towards metformin, 22RV-1 that possesses AR and

its variant but not the functional p53 dealt with metformin treatment very strongly in the presence of AR ligand R1881 which manifests wide difference in IC_{50} in the absence and presence of ligand. Thus, we showcased growth inhibition in all three cell lines, both in the presence and absence of androgen receptor (AR) because the varying sensitivity observed is attributed to the heterogeneity of AR indicated by clonogenic assay.

To further prove the efficacy of metformin in AR positive and its splice variant ARV7, we analysed translational expression of AR and ARV7 in both Androgen sensitive Lncap and 22RV-1 cell lines which was reduced in both cases. Earlier, Yi Xie et.al have shown the usage of LNCaP and VCaP cells in which AR/ARV7 expression increased due to Androgen receptor targeted agent (ARAT) like abiraterone and enzalutamide which was inhibited in present study when androgen agonist and metformin treated together, emphasizing the anticancer role of metformin and its better efficacy for CRPC. An increase in androgen receptor mRNA is observed consistently in prostate cancer resistance to antiandrogen therapy [(M. Chen et al., 2009)]. Hence, we also assessed transcriptional expression of AR, where it was observed that metformin inhibits the mRNA levels of AR in the AR and variant-positive 22RV-1 cell line which indicates the role of metformin in the transcriptional regulation of AR and its variants. Similar results were observed by Wang et.al. and in Prostatic hyperplasia by Mosli et.al[(Mosli et al., 2015)]. Also, PSA gene was found to be reduced by metformin which is representing a downstream signal of both AR and ARV7 and an important biomarker to assess prostate growth in clinical situation.

In other cancers like breast cancer, ER (Estrogen receptor) plays a crucial oncogenic role. However, the relative heterogenicity among the nuclear hormone in TNBC, non-TNBC and ER negative cell lines for metformin was not observed [(Lee et al., 2014)] This variation in the efficacy of metformin as observed in our study has been observed by Sarah et al in clinical cases [(Skuli et al., 2022)] We did a detailed analysis of all three in vitro models and we assumed that apart from AR heterogeneity, P53 and PTEN status also varies significantly and can play a vital role in the alteration of efficacy Since the WtP53 is promoted by metformin [(Zhao et al., 2019)] Lncap shows the highest sensitivity while PC3 does not have AR and p53 makes it the lowest sensitive cell towards metformin, and 22RV-1 that possesses AR

and its variant but not the functional p53, has dealt with metformin treatment very strongly in the presence of AR ligand R1881 and this manifests wide difference in IC_{50} in the absence and presence of ligand. This can be addressed and studied constructively in the future.

Conclusion:

As per this data, metformin can be a good combinatorial drug with AR blocker in mutated Androgen receptor/ variant positive cells and thus a better candidate for CRPC.

TABLE – 3.1

Prostate Cancer Cell Line Details

Cell line	AR	ARV7	PTEN	P53
PC3	Absent	Absent	Loss	Deficient
LnCap	Mutated (877A)	Absent	Loss	Wild type
22RV-1	Mutated (874A)	Present	Present (+/+)	type/1 copy Mutant (Q31 copy Wild11R)

Source:(Sampson et al., 2013)**,** (Ullén et al., 2005), (Fraser et al., 2012), (Rossini et al., 2020)