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Short Communication

Effects of metformin and silodosin as supplementary treatments to abiraterone on human telomerase reverse transcriptase (hTERT) level in metastatic castration-resistant prostate cancer (mCRPC) cells: An in vitro study

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Abstract

The antiproliferative properties of metformin and silodosin have been observed in prostate cancer. Furthermore, it is hypothesized that the molecular pathways related to these drugs may impact the levels of human telomerase reverse transcriptase (hTERT) in prostate cancer cells. The aim of this study was to assess the effect of metformin and silodosin on the levels of hTERT in metastatic castration-resistant prostate cancer (mCRPC) cells. The present study employed an experimental design with a post-test-only control group. This study utilized the PC3 cell line as a model for mCRPC. A viability experiment was conducted using the CCK-8 method to determine the inhibitory concentration (IC_{50}) values of metformin, silodosin, and abiraterone acetate (AA) after a 72-hour incubation period of PC3 cells. In order to investigate the levels of hTERT, PC3 cells were divided into two control groups: a negative control and a standard therapy with AA. Additionally, three experimental combination groups were added: metformin with AA; silodosin with AA; and metformin, silodosin and AA. The level of hTERT was measured using sandwich ELISA technique. The difference in hTERT levels was assessed using ANOVA followed by a post hoc test. The IC_{50} values for metformin, silodosin, and AA were 17.7 mM, 44.162 mM, and 66.9 µM, respectively. Our data indicated that the combination of metformin with AA and the combination of metformin, silodosin and AA decreased the hTERT levels when compared to control, AA, and silodosin with AA. The administration of metformin resulted in a reduction of hTERT levels in the PC3 cell line, but the impact of silodosin on hTERT levels was not statistically significant compared to AA group.

Keywords: Prostate cancer, hTERT, metformin, silodosin, abiraterone acetate

Introduction

Prostate cancer is the second most common form of cancer in men worldwide, with over 1.4 million new cases each year and approximately 350,000 deaths [1]. Metastasis, which has few curative options, is responsible for the preponderance of prostate cancer-related deaths. Although the causes of metastasis of any malignancy are extremely complex, a number of

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aberrant cellular pathways have been linked to metastatic formation [2]. Several of these aberrations, including mutations in serine/threonine kinase or Ataxia-telangiectasia mutated (*ATM)* gene and amplifications of *MYC* gene leading to double-strand break (DSB) repair and cell proliferation, respectively [3]. Intriguingly, both of these mutations have been linked to the maintenance and shortening of telomeres in certain cancer cells, including prostate cancer [4-6].

Telomeres, which comprise the outermost regions of chromosomes, consist of a repeating TTAGGG sequence and shelterin-bound proteins [7]. Through acting as chromosomal end-protectors, telomeres defend against genomic instability by shielding chromosome ends from constant exposure to the DNA damage response (DDR) machinery [7,8]. Activation or upregulation of the normally dormant human telomerase reverse transcriptase (*hTERT*) gene results in the synthesis of telomerase, a protein with reverse transcriptase activity and a functional RNA (encoded by *hTR*, also called *hTERC*) to form a ribonucleoprotein enzyme complex, allowing cancer cells to become proliferatively immortal [9,10]. Despite the fact that telomerase reverse transcriptase (*TERT)* is normally silenced in virtually all somatic cells, it is substantially expressed in 85–95% of human malignancies including prostate cancer cell [11]. Telomerase, and specifically its catalytic component hTERT, have become an appealing target for anticancer treatment due to their near universality, high selectivity for cancer cells, and ability to provide replicative immortality.

Metformin is one of the most widely prescribed anti-diabetic medications in the world [12]. Epidemiological investigations have revealed that metformin exerts a protective effect on individuals with cancer as well as diabetes [13,14]. Recent evidence demonstrates that metformin inhibits tumor growth, invasion, and metastasis for a variety of malignancies in both in vitro and murine tumor models, including prostate cancer cells [13,15,16]. Metformin's anticancer activity is mediated by the activation of the adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway [13]. Metformin also induces apoptosis and decreases hTERT levels and this effect may be a result of mTOR inhibition [17].

The FDA authorized silodosin in 2008 for the treatment of the symptoms of benign prostatic hyperplasia, and it is a selective α1A-adrenergic receptor (α1-AR) antagonist. Silodosin demonstrates the ability to impede the growth of bladder cancer cells, including cells expressing androgen receptor (AR) [18]. Latest studies have shown that silodosin reduced the expression and activity of ELK-1 pathways, hence reducing the migration of both AR-positive and AR-negative cancer cells [18,19]. The use of AR targeted agent (ARTA), such as abiraterone acetate (AA), is indicated for the treatment of metastatic prostate cancer (mPCa). The usage of ARTA is recommended in combination with androgen deprivation therapy (ADT) or in triplet combination with ADT and chemotherapy in mPCa. Although the triplet combination has shown superior efficacy compared to the doublet combination in high-volume prostate cancer, it is important to note that the incidence of adverse events is also higher [20]. The potential relationship between the molecular pathways of metformin and silodosin in the treatment of prostate cancer suggests that both drugs should be considered as viable adjunctive therapies in the management of mPCa. Both metformin and silodosin have been found to exhibit an antiproliferative effect on prostate cancer [19,21,22]. Additionally, their molecular pathways have been shown to potentially influence the expression of hTERT in prostate cancer [13,23]. The aim of this study was to investigate the impact of metformin and silodosin as supplementary treatments to AA on the level of hTERT in metastatic castrate resistant prostate cancer (mCRPC) cell.

Methods

Study design and setting

An in vitro experimental study was conducted at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. PC3 cells, a human mCRPC cell line, were divided into five groups and treated with AA alone; combination of metformin+AA; combination of silodosin+abiraterone acetate; and combination of AA+metformin+silodosin. After 72 hours exposure, the level of the hTERT was measured using ELISA method.

Materials and cell culture

The PC3 cells, F-12K medium, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), penicillin/streptomycin solution, and phosphate buffered saline (PBS) were acquired from American Type Culture Collection (ATCC, Manassas, VA, US). Metformin, silodosin, and AA were purchased from the Tokyo Chemical Industry (Tokyo, Japan). The Human TERT ELISA Kit was purchased from Elabsciences (Houston, TX, US). All tissue culture plasticware and additional substances were acquired from ThermoFisher Scientific.

PC3 cells were maintained in F-12K supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 until they reached 80% confluency. All these cell lines were then cultured in complete medium culture at least 24 hours before experimental treatment.

Cell viability assay and inhibitory concentration (IC_{50}) measurement

For viability assays, PC3 cells were plated in 96 well plates at a density of 3,000–4,000 cells per well and allowed to attach overnight. The cells were then treated for 72 hours with complete medium, metformin (0.1–100 mM), silodosin (0.1–100 mM), and AA (0.1–100 µM). The cells were next incubated at 37°C for 1–2 hours with the CCK-8 viability assay (50µl CCK-8/ml culture media). The amount of relative absorbance of the cell culture was then measured at an excitation wavelength of 485 nm.

Quantitative measurement of hTERT level

The determination of serum hTERT was conducted using a polyclonal sandwich ELISA kit in accordance with the guidelines provided by the manufacturer (Elabsciences, Houston, Texas). The PC3 cells were seeded in a 96-well plate at a density of 3000–400 cells per well and incubated overnight to facilitate attachment. The cells were subjected to a 72-hour treatment with drug combinations: (a) AA; (b) metformin+AA; (c) silodosin+abiraterone acetate; and (d) AA+metformin+silodosin, at their respective IC_{50} concentrations. Then, the cells were treated with trypsin-EDTA and subsequently centrifuged for 5 min (1000×*g*). The cells underwent a process of suspension and subsequent washing, which was repeated three times using pre-cooled PBS. The freeze-thaw process was iterated three times. Cells were centrifuged for 10 min at 1500x*g* at 2–8°C. The samples were maintained at a temperature of 37°C for 90 min. The detection of hTERT antibody, which was biotinylated, was introduced into each well and allowed to incubate for 60 min. The working solution containing HRP conjugate was introduced into each well and incubated at a temperature of 37°C for 30 min. Following this, 90 µl of substrate reagent was added and 50µl of stop solution was added to each well. The relative absorbance of the cell culture was subsequently quantified at an excitation wavelength of 450 nm.

Statistical analysis

The optical density (OD) values of hTERT obtained from ELISA were analyzed. A normality test was conducted in order to ascertain the distribution of the data. The one-way analysis of variance (ANOVA) test was employed. Following this, a subsequent multiple comparison test or post hoc test were performed. The statistical analysis was conducted using SPSS version 25 (IBM, New York, US).

Results

IC_{50} of PC3 cells for abiraterone acetate, silodosin, and metformin

In order to determine the IC_{50} of each drug, a viability CCK8 assay was conducted. Three iterations were performed for each drug. The results of absorbance were collected three days afterwards. The viability of PC3 cells was substantially declined upon administration of metformin, as demonstrated by an IC_{50} value of 17.7 mM (**Figure 1A**). The identical result was observed when silodosin was administered; viability decreased significantly with an IC_{50} of 44.162 mM (**Figure 1B**). The IC_{50} value for AA as standard therapy was 66.9 mM (**Figure 1C**).

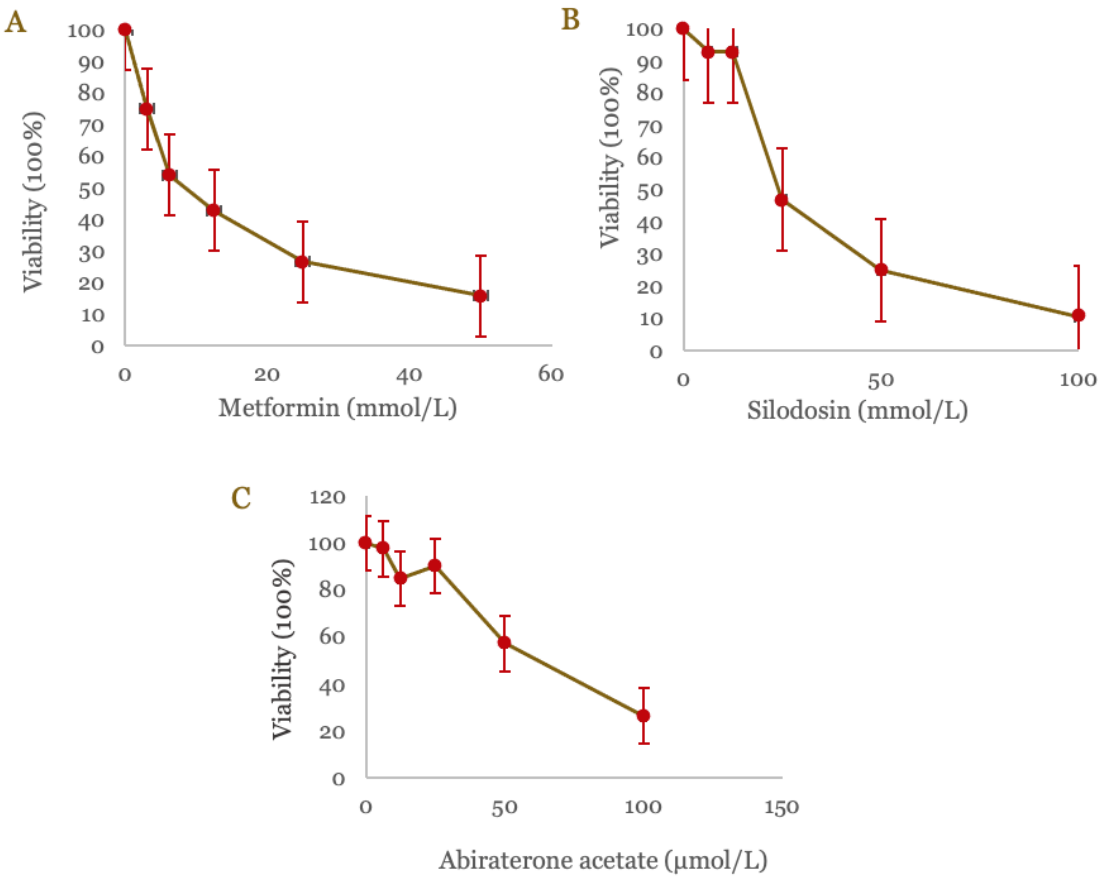

Figure 1. Assessment of the PC3 cell line viability during a 72-hour period exposed to three drugs: (A) metformin, (B) silodosin, and (c) abiraterone acetate (AA).

Impact of silodosin, metformin, abiraterone acetate, and drug combinations on hTERT levels

The PC-3 cells were exposed to the intended drugs and combinations for 72 hours with the dosage administered based on the IC_{50} value obtained. Subsequently, hTERT levels were measured using sandwich ELISA. Our data indicated that the levels of hTERT were statically significant among groups with a $p<0.001$ (**Table 1**). A post hoc test was conducted to further compare the hTERT level between the two groups. The results showed that the group receiving a combination of metformin and AA had significantly lower hTERT levels than the control group, AA group, and combination of AA and silodosin group ($p<0.05$). The level of hTERT in the combination of metformin, AA, and silodosin group was significantly lower compared to the control group, AA group, and AA+silodosin group ($p<0.05$) (**Table 1**). The concurrent administration of silodosin and AA led to a significant reduction in hTERT levels in comparison to the control group only (**Figure 2**).

Table 1. Quantitative analysis of hTERT of expression in each treatment group

Comparison of hTERT levels between groups		Mean difference of hTERT levels	Confidence interval 95% Lower bound	Upper bound	*p*-value
Control	AA	0.7220	0.120	0.14	0.023
	AA+Met	0.2462	0.180	0.311	<0.001
	AA+Sil	0.1120	0.468	0.177	0.002
	AA+Sil+Met	0.2314	0.166	0.296	<0.001
Abiraterone acetate (AA)	AA+Met	0.1690	0.103	0.234	<0.001
	AA+Sil	0.3480	-0.030	0.100	0.279
	AA+Met+Sil	0.1540	0.089	0.219	<0.001
AA+metformin (Met)	AA+Sil	-0.1340	-0.1994	-0.690	<0.001
	AA+Met+Sil	-0.1480	-0.800	0.050	0.641
AA+Silodosin (Sil)	AA+Met+Sil	0.1194	0.542	0.184	0.001

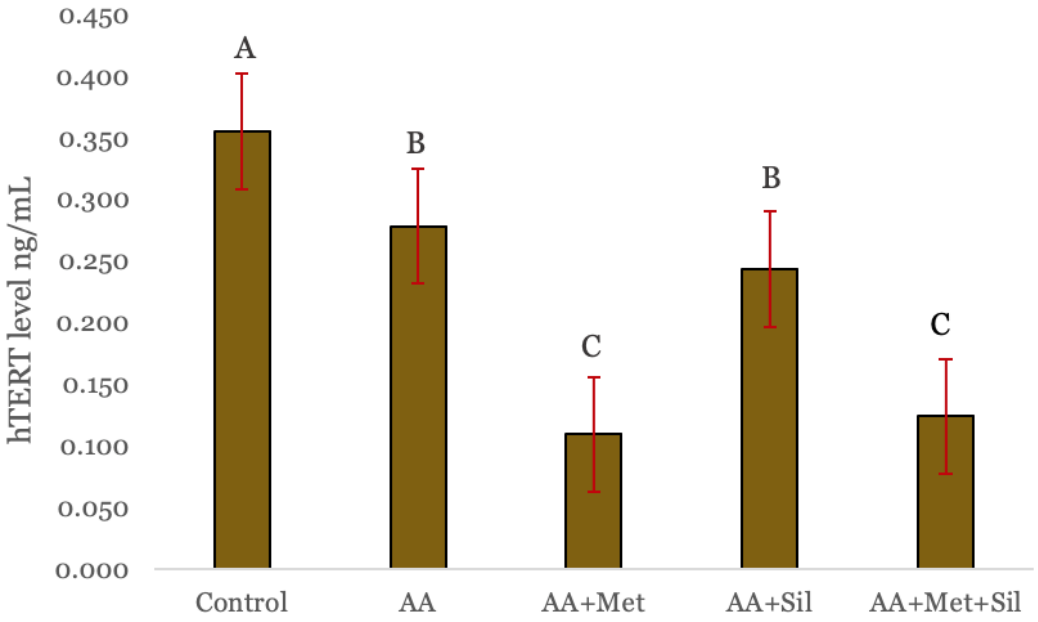

Figure 2. The impact of abiraterone acetate (AA), metformin (Met), and silodosin (Sil) on hTERT levels. In contrast to the silodosin combination, the hTERT level was significantly reduced by the metformin combination. The bars in the graph represent standard errors; different letters indicate statistically significant with a significance level of $p<0.05$.

Discussion

The present study examined the impact of silodosin and metformin as additional therapy on the levels of telomerase in mCRPC model using PC3 cells. The first stage involved acquiring a drug IC_{50} for each drug. The IC_{50} value is the threshold at which a drug elicits a biological effect of 50% on a specific population of cells [24]. There is a noticeable discrepancy in the IC_{50} dosage when comparing the results of this study with those of previous investigations [25-27]. The IC_{50} dosage of metformin has been reported in prior experiments to range from 1 to 20 mM [25–27]. The findings of this investigation indicated that the IC_{50} of metformin was 17.7 mM. The IC_{50} obtained for silodosin also revealed differences in the doses of silodosin. Differences in IC_{50} doses observed between the present study and previous studies could potentially be attributable to variations in the sources of metformin, silodosin, or AA, as these were obtained from different pharmaceutical companies. Previous studies also employed different types of prostate cancer cells. Some studies utilize hormone-sensitive prostate cancers (HSPCs), whereas other investigations employ castration-resistant prostate cancer cells (CRPC) [25-27]. Furthermore, there is significant variation in the duration of treatment observed across different studies. Multiple studies utilized different incubation periods, with certain studies utilizing a duration of 48 hours while others opted for a duration of 72 hours [25-27]. The determination of the IC_{50} value was conducted in this study using the CCK-8 test method. Differences in IC_{50} calculations can arise due to the utilization of alternative techniques, such as the MTT test or trypan blue.

The findings of this study indicated a reduction of hTERT levels in cells that were treated with metformin and AA combination in comparison to untreated group. The activation of the AMPK pathway is a prominent energy-generating cascade in cancer cells. Metformin exerts its influence on cellular proliferation by modulating the aforementioned route via the activation of the LKB1 protein, the primary upstream kinase of AMPK pathway. The activation of the LKB1 protein leads to a reduction in the mTOR protein, which serves as a crucial regulatory protein within the AMPK pathway [28]. The P13K/AKT/mTOR pathway has been empirically demonstrated to induce a reduction in hTERT mRNA expression within malignant cells [28]. This finding is substantiated by previous studies that elucidated the impact of rapamycin, an inhibitor of the mTOR, on cancer stem cells, leading to a concomitant reduction in the production of hTERT [23,29]. Our findings are consistent with earlier studies showed that metformin could

lower hTERT levels in a variety of cancer cell types and decrease cancer cell proliferation in a dose-dependent way [23,29].

Metformin therapy is commonly utilized as a supplementary treatment for cancer cells [30]. The effectiveness of metformin and chrysin in inducing apoptosis in T47D breast cancer cells provided evidence for their potential therapeutic use [30]. Additionally, a negative correlation was identified between the dosage of metformin, chrysin, or a combination of these medications and the expression of hTERT [30].

As indicated by the IC_{50} of PC3 cells, exposure to silodosin decreased PC3 cell proliferation, according to the present study findings. This is different from the previous study by Kawahara *et al.* that demonstrated silodosin inhibited proliferation only in androgen-sensitive prostate cancer cells [19]. Silodosin exhibited inhibitory effects on the migration of DU145, LNCAP, PC-3, and C4-2 prostate cancer cells, but it only induced reduction of proliferation in prostate cancer cells that are androgens sensitive [19]. ELK-1 is a protein that exerts a modulatory effect on the growth of prostate cancer subsequent to the administration of silodosin [18,19]. Additional proteins that might have an impact due to the inactivation of ELK-1 include c-fos and c-junc, both of which serve as regulators in the activation of telomerase [31]. A reduction of c-fos expression leads to a decrease in the expression of hTERT in laryngeal cancer [31]. Nevertheless, conflicting findings from another study indicated that the downregulation of AP-1 proteins (c-fos, c-junc, juncB, and junc D) did not elicit a noticeable effect on hTERT [32]. The study also indicated that the suppression of AP-1 in mice lead to a decrease in mouse telomerase reverse transcriptase (mTERT) expression. This finding suggests the existence of divergent mechanisms behind the regulation of the telomerase gene across different species [32]. The findings of our study offer a novel viewpoint on the correlation between silodosin exposure and hTERT levels. Specifically, the study elucidates that the administration of silodosin does not enhance the effectiveness of AA in lowering hTERT levels in PC3 cells.

The findings of this study together elucidate that the levels of hTERT are proteomically associated with exposure to metformin, but not with exposure to silodosin. Several limitations need to be considered in this investigation. Firstly, the measurement of hTERT levels was conducted using proteomic techniques, which means that observations of the *hTERT* gene following exposure to treatment or mRNA products were not performed. Disparities in expression patterns may exist between proteomics and mRNA. Furthermore, there can be variations in telomerase activity in relation to the quantities of telomerase, whether in terms of RNA or proteome expression. It is imperative to comprehend the unique nature of DNA, RNA, and protein expression and regulation and their connection. Differences in hTERT activity and *hTERT* expression can arise as a consequence of DNA methylation and post-translational modifications.

Additional investigation stemming from this work may delve more comprehensively into the cellular mechanisms underlying the interactions of silodosin, metformin, and abiraterone acetate, with a particular focus on exploring the potential synergistic impact on cellular proliferation and hTERT expression. Moreover, performing a comprehensive study on the mechanism by which metformin reduces hTERT levels could enhance the scientific validity of this study and reinforce its findings.

Conclusion

The findings of this study demonstrate that the viability of PC3 prostate cancer cells is decreased by the administration of metformin, silodosin, and AA, as evidenced by the IC_{50} dose. The combination of metformin as adjunctive therapy with AA decreased the levels of hTERT while adding silodosin with AA did not result in any significant changes in the levels of hTERT in PC3 cells.

Ethics approval

Not required.

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Competing interests

Authors have no conflict of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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