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Original Article

The Anti-proliferative Effect of a Novel Glutaminase Inhibitor IN-3 on Prostate Cancer Cells

Yeni Bir Glutaminaz Baskılayıcı Olan IN-3'ün Prostat Kanseri Hücrelerinde Büyüme Karşıtı Etkisi

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ABSTRACT

Objective: This study aimed to evaluate anti-cancer potential of a novel glutaminase (GLS) inhibitor IN-3 in prostate cancer cells.

Methods: The cell viability upon IN-3 treatment was examined using crystal violet staining and IC_{50} values were calculated for cancer cell lines PC-3 and LNCaP and normal fibroblasts CCD1072sk. The expression levels of GLS isoforms were determined by real-time polymerase chain reaction after IN-3 treatment. The metastatic prostate cancer dataset was downloaded from C-Bioportal and the expressions of GLS isoforms were analyzed.

Results: The IC_{50} values of IN-3 for LNCaP, PC-3 and CCD1072sk were 2.13, 6.14 and 15.39 μM respectively. The dose dependent effect of IN-3 was evident even in low concentration with 1 μM in LNCaP and 2 μM in PC-3 and these anti-proliferative effects of IN-3 were highly significant with p-values lower than 0.0001. The treatment of PC-3 cells with 10 μM IN-3 elevated the expression of kidney type GLS isoform of GLS1 but not GLS2. Comparison of metastatic and localized prostate cancer tissues showed that GLS1 was highly expressed not only in primary but also in metastatic prostate cancer tissues. GLS1 expression was significantly higher than GLS2 expression with p-values lower than 0.001.

Conclusions: The GLS inhibitor IN-3 may be a potent anti-cancer agent in prostate cancer by demonstrating its differential effect between cancer and normal cells. Further studies are warranted to elucidate its drug potential in prostate cancer.

Keywords: Prostate cancer, GLS1, IN-3

ÖZ

Amaç: Bu çalışma yeni bir glutaminaz (GLS) baskılayıcı olan IN-3'ün prostat kanseri hücrelerinde kanser karşıtı etkisini değerlendirmeyi amaçlamaktadır.

Yöntemler: IN-3'ün hücre canlılığına etkisi kristal viyole boyaması ile incelenerek PC-3 ve LNCaP prostat kanseri hücrelerinde ve normal fibroblastik CCD1072sk hücrelerinde IC_{50} değerleri hesaplanmıştır. GLS izoformlarının ekspresyon düzeyleri gerçek zamanlı polimeraz zincir reaksiyonu ile belirlenmiştir. C-Bioportal veri bankasından metastatik prostat kanseri verileri indirilerek GLS izoformlarının ekspresyonları analiz edilmiştir.

Bulgular: LNCaP, PC-3 ve CCD1072sk için IC_{50} değerleri sırasıyla 2,13, 6,14 ve 15,39 μM'dır. LNCaP hücrelerinde 1 ve PC3 hücrelerinde 2 μM gibi düşük dozlarda bile IN-3'ün doz bağımlı etkisi belirgindir ve IN-3'ün büyüme karşıtı etkisi 0,0001'in altında p-değeri ile yüksek derecede anlamlıdır. PC-3 hücrelerinin 10 μM IN-3 ile muamelesi sonrası GLS1'in böbrek tipi GLS izoformunun ekspresyonu artarken GLS2 ekspresyonu değişmemiştir. Metastatik ve lokalize prostat kanseri örneklerinin karşılaştırılması göstermiştir ki; GLS1 sadece primer prostat kanseri örneklerinde değil metastatik prostat kanseri örneklerinde de yüksek düzeyde eksprese edilmektedir. GLS1 ekspresyonu 0,001'in altında p-değeri ile GLS2 ekspresyonundan anlamlı şekilde yüksektir.

Sonuçlar: GLS baskılayıcı IN-3 normal ve kanser hücrelerinde farklı etki göstermesi sebebiyle önemli bir kanser karşıtı ajan olabilir. Prostat kanserinde ilaç olma potansiyelinin ortaya çıkarılması için daha ileri çalışmalar yapılması gerekmektedir.

Anahtar kelimeler: Prostat kanseri, GLS1, IN-3

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INTRODUCTION

The metabolic vulnerabilities of cancer cells have been gaining much more attention recently because of the promising new treatment options. The dependence of cancer cells on glutamine has long been known. Although glutamine is a non-essential amino-acid meaning that there is cell-intrinsic biosynthesis of glutamine, the cancer cells highly depend on external glutamine supply. Indeed, from the very early stages of *in vitro* cancer research, glutamine-supplemented growth media have been shown to promote better growth of cancer cells. Glutamine is utilized by the cancer cells for several purposes such as energy production, nucleotide and amino acid biosynthesis. Glutaminase (GLS) converts glutamine to glutamate to further fuel Krebs cycle with α-ketoglutarate[1]. GLS has two isoforms as GLS1 and GLS2. GLS1 has been commonly studied in several cancer types with an oncogenic role. However, GLS2 is known for tumor suppressor function and its expression is low in cancer cells. GLS1 is represented with two isoforms produced from the same gene with an alternative splicing event. Kidney type glutaminase (KGA) isoform has lower catalytic activity than glutaminase C (GAC) isoform[2].

First inhibitor reported in the literature to target GLS was bis-2-(5-phenylacetamido-1, 3, 4-thiadiazol-2-yl) ethyl sulphide (BPTES). Because of its promising anti-cancer effect both in *in vitro* and *in vivo*, more potent derivatives of BPTES have been developed. Telaglenastat (CB839) is a BPTES derivative already progressing to the clinical trials[3]. There are several cancers, including breast, kidney, colon and leukemia, where promising results of combination therapies with CB839 have been shown[4,5].

Several therapy options are available when prostate cancer (PCa) is in early, localized stage. However, in advanced stage, castration resistant prostate cancer (CRPC) and metastatic CRPC (mCRPC) emerge and the therapy options for them are limited. Therefore, many current studies have been prospecting single or combination therapy alternatives in mCRPC.

In our study, anti-proliferative effect of glutaminase inhibitor 3 (IN-3) was explored in cells for the first time in the literature. To understand the differential effect of IN-3 inhibition on expression of GLS isoforms, real-time polymerase chain reaction (PCR) analysis was performed. The expression levels of GLS isoforms were also investigated in PCa tissue samples using publicly available datasets.

MATERIALS and METHODS

Chemicals

GLS-IN-3 was supplied by Medchem. The primary stock solution was prepared by diluting the compound in dimethyl sulfoxide (DMSO) to a concentration of 20 mM and stored at -20 °C. The final concentrations of 1 µM, 2 µM, 5 µM, and 10 µM were used to treat the cells.

Cell Culture

PC-3 cell line is a bone metastatic and LNCaP cell line is a lymph node metastatic PCa cell lines. PC-3 and LNCaP cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Normal human fibroblast cells CCD-1072Sk was cultured in Dulbecco's modified Eagle's medium with 10% FBS and 1% penicillin-streptomycin. The cells were incubated at 37 °C and 5% CO_2 conditions.

Cell Viability

The cell viability was determined using the crystal violet staining. Firstly, 6×10^3 cells for PC-3 and 12×10^3 cell for LNCaP and CCD-1072sk were seeded per well in 96-well plate and incubated at 37 °C with 5% CO_2 for 24 hours. Afterwards, GLS-IN-3 was applied to the cells at three different concentrations with three technical replicates. 2 µM, 5 µM, and 10 µM for PC-3 cells and 1 µM, 5 µM, and 10 µM for LNCaP and CCD-1072Sk cells were used as final concentrations. An equal volume of DMSO was added as a control, and the cells were incubated for 48 hours. Then, the medium was removed, and the cells were washed with 200 µL of phosphate buffer saline (PBS). 50 µL of 0.05% crystal violet solution was added to the wells and incubated for 20 minutes. The dye was discarded, and the cells were washed first with 200 µL of PBS and then with 400 µL of distilled water. Finally, 200 µL methanol was added to the wells and incubated for another 20 minutes to release the dye, followed by measuring the absorbance at 590 nm in a plate-based spectrophotometer.

RNA Extraction, cDNA Synthesis and qRT-PCR

For RNA isolation, 2×10^5 PC-3 cells were seeded per well in a 6-well plate and incubated at 37 ˚C with 5% CO_2 for 24 hours. After 24 hours, 10 µM of GLS-IN-3 was applied to the cells and an equal amount of DMSO was used as a control. After 24-hour incubation, the cell pellets were collected and stored at -80 ˚C. After three biological replicates, RNA was isolated by Innu PREP RNA Mini Kit 2.0 (Analytik Jena, Cat #845-KS-2040050) and stored at -80 ˚C. The cDNA was synthesized according

to the Gscript First-Strand Synthesis Kit (Gene DireX, Cat #MB305-0050) and stored at -20 ˚C. PCR reaction was set up for each sample with two technical replicates, following the instructions provided in the GoTaq® qPCR Master Mix manual (Promega, Cat #A6001). The cDNA was diluted at a 1:5 ratio. The sequences for the primers GAC, KGA, and GLS2 were selected from the literature (Table 1), and were synthesized by Bioligo. As a housekeeping gene, B-Actin was used. The expression levels of GAC, KGA, and GLS2 were calculated with the ΔΔCt method.

Statistical Analysis

The statistical analyses were conducted using GraphPad Prism. Student's t-test was performed to assess the differences between groups. The statistical significance was considered at p-values less than 0.05 (*: p<0.05, **: p<0.01, ***: p<0.001, and ****: p<0.0001).

Analysis of GLS Expression

The mRNA data of GLS were downloaded from C-Bioportal database[6]. Fred Hutchinson 2016 data were analyzed to compare the expressions of GLS1 and GLS2 in primary and metastatic samples using SPSS. Wilcoxon signed rank test was applied to determine statistical significance between expression of GLS1 and GLS2.

RESULTS

Cytotoxic Effect of GLS-IN-3

The cytotoxic effect of GLS-IN-3 on PCa cells (PC-3 and LNCaP) and normal cell (CCD 1072sk) has been examined. IC_{50} graphs and dose-dependent cell viability graphs were presented in Figure 1. All applied doses diminished the cell viability of PC-3 and LNCaP cancer cells in a highly significant manner with p-values lower than 0.001 according to Student t-test. The IC_{50} values for PC-3, LNCaP, and CCD1072sk cells were determined as 6.14 μM, 2.13 μM, and 15.39 μM, respectively (Table 2). IN-3 decreased the viability of highly aggressive bone metastatic PC-3 cells and lymph node metastatic LNCaP cells in a lower concentration than the normal CCD1072sk cells.

Effect of GLS-IN-3 on the Expression of GLS Isoforms

After the application of 10 μM GLS-IN-3 to PC-3 cells, the expression levels of *KGA*, *GAC*, and *GLS-2* genes were examined (Figure 2). In PC-3 cells, GLS2 isoform was expressed lower than both KGA and GAC isoforms. GAC isoform is the highly expressed form in PC-3 cells. Upon IN-3 treatment, while GLS2 expression stayed stable, the fold changes in the expression of KGA and GAC were 2.64 and 1.5 respectively.

mRNA Expression Data of GLS1 and GLS2 in mCRPC Tissue

mRNA expression levels of GLS1 and GLS2 in primary and metastatic PCa tissue samples were analyzed (Figure 3). Primary PCa tissue and matched metastatic samples from 16 patients were included. GLS1 expression was significantly higher than GLS2 expression both in primary and metastatic PCa tissue samples according to Wilcoxon signed rank test with p-value lower than 0.001. GLS1 expression kept its high level also in metastatic samples.

DISCUSSION

In this study, the anti-cancer activity of GLS inhibitor IN-3 on PCa cells was evaluated. The PCa cell lines PC-3 and LNCaP presented the differential sensitivity to IN-3 compared to normal fibroblasts CCD1072Sk cells. Inhibition of GLS1 isoform of GLS by IN-3 in PC-3 cells did not cause increase in the expression of GLS2 isoform.

Table 1. Sequences of GAC, KGA, and GLS2 in qPCR.

	Forward sequence (5'to 3')	Reverse sequence (5'to 3')
GAC	GAAGGTGGTGATCAAAGGCATTC	CCTCATTTGACTCAGGTGACACT
KGA	TGGAGATGTGTCTGCACTTCG	AACTTCAACATGACCCTCTGC
GLS2	ACACCCTCAGCCTCATGCAT	ATGGCTCCTGATACAGCTGACTT

KGA: Kidney (K-type) glutaminase, GAC: Glutaminase C, GLS2: Glutaminase 2

Table 2. IC_{50} values of glutaminase-IN-3.

Cell line	IC50 value (μM)
PC-3	6.14 μM ±4.35
LNCaP	2.13 μM ±0.62
CCD1072sk	15.39 μM ±2.97

IC_{50}: Half inhibitory concentration

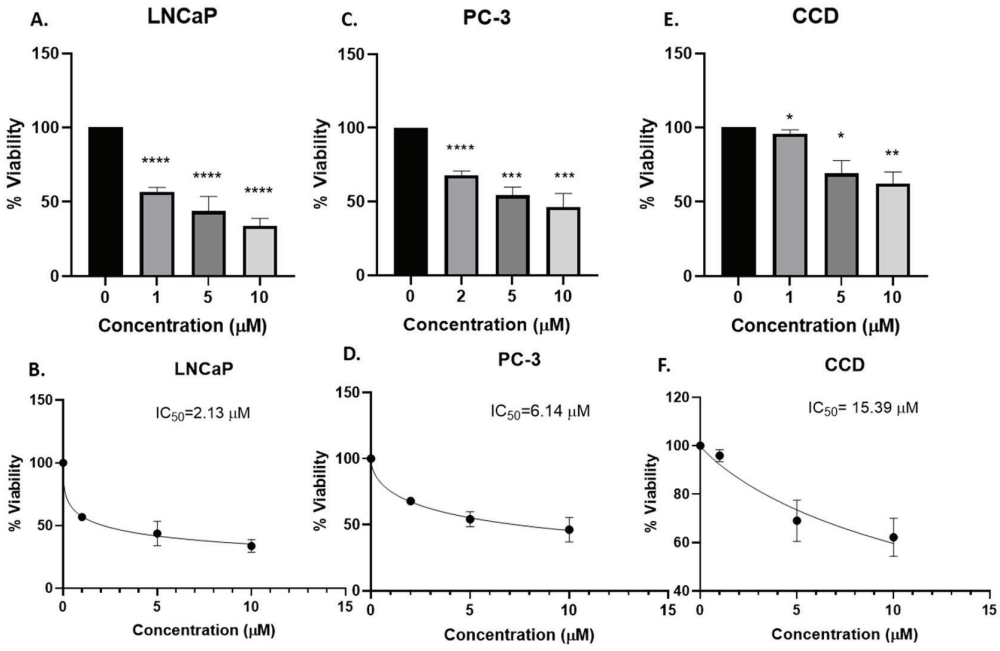

Figure 1. IC_{50} graphs of glutaminase-IN-3 on prostate cancer cells LNCaP **A)**, PC-3 **C)**, and normal cell CCD1072sk **E)** and the dose dependent effect of glutaminase-IN-3 on prostate cancer cells LNCaP **B)**, PC-3 **D)**, and normal cell CCD1072sk **F)** (Student t-test was applied to determine statistical significance. *: p<0.05, **: p<0.01, ***: p<0.001, and ****: p<0.0001).

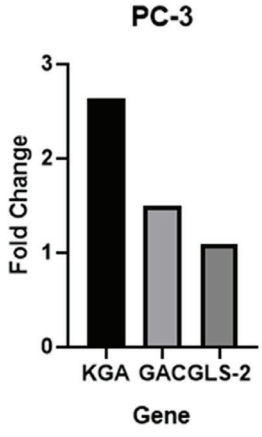

Figure 2. Changes in the expression levels of KGA, GAC, and GLS2 in response to glutaminase IN-3 in PC-3 cells.

KGA: Kidney (K-type) glutaminase, GAC: Glutaminase C, GLS2: Glutaminase 2

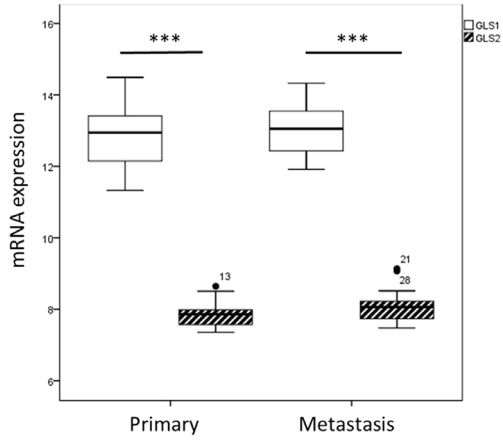

Figure 3. mRNA expression of GLS1 and GLS2 in primary and metastatic prostate cancer tissue. (Wilcoxon signed-rank test was applied to determine statistical significance. *: p<0.05, **: p<0.01, ***: p<0.001).

Our analysis in publicly available dataset in metastatic PCa tissue confirmed that GLS1 was the highly expressed isoform in PCa patients and this high expression level was maintained in the metastatic PCa tissue samples.

The idea of glutamine metabolism as a drug target is based on the information about high levels of GLS1 enzyme in cancer tissues. Several studies analyzed the expression level of GLS enzymes in primary PCa. Pan et al.[7] compared PCa with bening prostatic hyperplasia

(BPH) in terms of GLS1 protein expression and reported that GLS1 protein levels were elevated in PCa tissue compared to BPH. In addition, they showed a correlation with the increased GLS1 expression and the advanced Gleason scores and TNM stage. In another study, high GLS1 expression was correlated with shorter overall survival in 87 PCa patients[8]. Zhang et al.[9] compared mRNA levels of GLS1 in PCa tissue in respect to adjacent normal tissue. They reported that GLS1 mRNA expressions were high in mCRPC compared to normal tissue and there was a correlation between GLS1 mRNA expression and Gleason score and TNM stage. In contrast to these studies, Myint et al.[10] unable to show any correlation between GLS1 expression and the survival in both publicly available mRNA data from TCGA and protein data from their tissue microarray. However, this study still confirmed high GLS1 expressions in PCa compared to benign tissues. They concluded that their patient population have less advanced disease compared to the other studies[10]. In our study we compared GLS1 and GLS2 mRNA levels in primary and metastatic PCa tissue samples using publicly available data. GLS1 mRNA level was higher than GLS2 both in primary and metastatic PCa. The maintenance of high GLS1 level in metastatic samples ensures that glutamine metabolism is still active and viable target of therapy both in primary and metastatic PCa. Our *in vitro* results also confirmed this finding by demonstrating the high anti-proliferative potential of IN-3 in metastatic PCa cell lines.

The anti-proliferative activity of GLS inhibitor IN-3 has not been tested in any other study before. It is a patent derived compound. In our study, we found out that the IC_{50} values of IN-3 against PC-3 and LNCaP cell lines were 6.14 and 2 μM respectively. Xu et al.[11] reported that androgen receptor (AR) negative PC-3 cells displayed much higher sensitivity to CB839 compared to AR positive LNCaP cells. They explained this discrepancy with isoform switch of GLS1 from KGA isoform to GAC isoform in AR negative or castrate resistant conditions and claimed that the sensitivity to CB839 was correlated with GAC expression level. Because of the higher sensitivity of AR positive LNCaP cells to IN-3 compared to AR negative PC-3 cells in our study, it was suggested that sensitivity to IN-3 was not correlated with AR status. The prominent anti-proliferative activity of IN-3 both in AR positive and negative cells is an indicator of superior potential of IN-3 over CB839 as a GLS1 inhibitor.

Another concern about CB839 activity in PCa was upregulation of GLS2 level upon CB839 treatment. GLS2 compensates inactivity of GLS1 and maintain GLS activity in PCa cells[12]. In our study, we showed that inhibition of GLS1 with IN-3 had no effect on the expression of GLS2. However, the increase in the expression of KGA isoform of GLS1 was a main concern. It is a common phenomenon that when the activity of an enzyme is inhibited, the mRNA expression for this protein is increased to compensate the inhibitory effect. In parallel to that, Timofeeva et al.[13] also showed that upon CB839 treatment in lymphocytes from chronic lymphoblastic leukemia patients, GAC expression was increased. However, after a prolonged inhibition of the activity, mRNA levels would be decreased also. In our study, we evaluated the expression levels of GLS isoforms in 24 hours. Further experiments for time-dependent changes in the expression levels of GLS isoforms were warranted.

Although our study presented preliminary results about potential of IN-3 as an anti-cancer therapy, only two mCRPC cell lines were included in IC_{50} calculations. It is foremost important to expand the study to confirm this finding in other cell lines in PCa or in any other cancer type. Several studies about mRNA or protein levels of GLS isoforms in PCa tissue samples have been highlighted in the literature. However, our study analyzed the mRNA expression of GLS isoforms in metastatic PCa for the first time. The downsides for that part is the limited number of metastatic samples and unavailability of KGA and GAC isoforms data separately. It is crucial to explore KGA and GAC isoform levels in a larger metastatic PCa cohort.

CONCLUSION

This was the first study in the literature showing anti-cancer potential of GLS inhibitor IN-3. The differential effect of IN-3 in cancer cells compared to normal cells was noteworthy. This warrants further study about IN-3 as a potential drug in cancer. This study analyzed the expression level of GLS1 in metastatic PCa tissues for the first time. The high expression levels of GLS1 in metastatic PCa tissue samples also further prove GLS1 as an important drug target in PCa.

Ethics

Ethics Committee Approval: The authors declare that this study did not include any human or animal subjects.

Informed Consent: The authors declare that this study did not include any human or animal subjects.

Author Contributions

Concept: U.D., A.B.C., Design: U.D., A.B.C., Data Collection and/or Processing: U.D., A.B.C., Analysis and/or Interpretation: U.D., A.B.C., Literature Search: U.D., A.B.C., Writing: U.D., A.B.C.

Conflict of Interest: The authors have no conflict of interest to declare.

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