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ARAŞTIRMA YAZISI / RESEARCH ARTICLE

PROSTAT KANSERİ HÜCRE HATTINDA (PC-3) DL-FENİLALANİN VE DL-ALANİN'İN HEDGEHOG YOLU İLE İLİŞKİSİNİN ARAŞTIRILMASI

INVESTIGATION OF THE RELATIONSHIP OF DL-PHENYLALANINE AND DL-ALANINE WİTH THE HEDGEHOG PATHWAY IN THE PROSTATE CANCER CELL LINE (PC-3)

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ÖZET

AMAÇ: Bu çalışmada, DL-Fenilalanin ve DL-Alanin'nin PC-3 prostat kanser hücre hattında Hedgehog sinyal yolağı üzerinden etkisinin araştırılması amaçlanmıştır.

GEREÇ VE YÖNTEM: DL-Fenilalanin ve DL-Alaninin PC-3 kanser hücrelerinde hücre canlılığına etkisi MTT yöntemi ile belirlemiştir. DL-Fenilalanin için belirlenen IC50 değeri PC-3 ve HEK-293 hücrelerine uygulandı. Total RNA izolasyonu ve cDNA sentezi yapıldı. Hedgehog yolağı ile ilişki anahtar genlerin (*SHH, PTCH, SMO, GLI-1*) gen ifadesi qPCR (Kantitatif PCR) ile belirlendi.

BULGULAR: PC-3 hücreleri üzerinde DL-Alanin sitotoksik etki göstermedi. DL-Fenilalanin hücre canlılığını zamana ve doza bağlı olarak azalttı. DL-Fenilalanin için IC50 değeri 48.saatte 500 µg/mL olarak belirlendi. PC-3 hücrelerinde DL-Fenilalanin uygulamasından sonra tüm genlerin ekspresyonunda aşağı regülasyon, HEK-293 hücrelerinde ise yukarı regülasyon tespit edildi ($p<0.05$).

SONUÇ: DL-Fenilalanin prostat kanseri hücrelerinde Hedgehog yolağını inhibe etmiştir. Bu inhibisyon kontrol grubu HEK-293 hücrelerinde gözlenmemiştir. DL-Fenilalanin prostat kanseri tedavisi için terapötik bir aday olabilir ancak, DL-Alanin'nin böyle bir etkisi bulunmamıştır.

ANAHTAR KELİMELER: PC-3, DL-Fenilalanin, DL-Alanin, Hedgehog.

ABSTRACT

OBJECTIVE: In this study, it was aimed to investigate the effect of DL-Phenylalanine and DL-Alanine on the Hedgehog signaling pathway on the PC-3 prostate cancer cell line.

MATERIAL AND METHODS: The effects of DL-Phenylalanine and DL-Alanine on cell viability in PC-3 cancer cells were determined by the MTT method. The IC50 value determined for DL-Phenylalanine was applied to PC-3 and HEK-293 cells. Total RNA isolation and cDNA synthesis were performed. Gene expression of key genes related to the Hedgehog pathway (*SHH, PTCH, SMO, GLI-1*) was determined by qPCR (Quantitative PCR).

RESULTS: DL-Alanine showed no cytotoxic effect on PC-3 cells. DL-Phenylalanine decreased cell viability in a time- and dose-dependent manner. The IC50 value for DL-Phenylalanine was determined to be 500 µg/mL at 48 hours. After DL-Phenylalanine application in PC-3 cells, down-regulation in the expression of all genes was detected, while up-regulation was detected in HEK-293 cells ($p<0.05$).

CONCLUSIONS: DL-Phenylalanine inhibited the Hedgehog pathway in prostate cancer cells. This inhibition was not observed in the control group HEK-293 cells. While DL-Phenylalanine may be a therapeutic candidate for the treatment of prostate cancer, DL-Alanine was not found to have such an effect.

KEYWORDS: PC-3, DL-Phenylalanine, DL-Alanine, Hedgehog.

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INTRODUCTION

According to World Health Organization (WHO) GLOBOCAN data for 2022, prostate cancer is the 2nd most common type of cancer globally in terms of incidence and mortality rate (1). Well-known risk factors for prostate cancer include: age, ethnicity, family history and mutations, obesity, tobacco consumption, metabolic syndromes (2). Heterogeneous prostate cancers can remain silent for a long time or behave very aggressively. For this reason, it is important to determine tumor behavior as well as making the diagnosis (3). The best known prognostic factors regarding the tumor are preoperative PSA score, Gleason score (histological grade), tumor volume, surgical margin status and pathological stage (4).

Besides DNA copy number changes, some genetic changes occur in prostate cancer, including inactivation of tumor suppressor genes, loss of specific genomic sequences, and gain of other genes associated with the activation of oncogenes (5, 6). Although the heterogeneity and changes in analytical and post-analytical data observed in prostate cancer create obstacles to the development of appropriate models for treatment; the clinical use of biological, genetic, and molecular pathological data that benefit cancer prevention, and survival continues to become increasingly important day by day (7).

The Hedgehog signaling pathway, which is important in embryonic development, and tissue regeneration, is linked to many biological processes. It also regulates the activity of stem cells in vertebrate, and invertebrate organisms, as well as maintaining homeostasis in various tissues, and organs (8). Three Hedgehog proteins, Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh), are found in mammals (9). The main target genes of the Hh signaling pathway are PTCH1, PTCH2, and GLI1 (10). Abnormal postnatal Hedgehog signaling has been linked to many malignancies, including basal cell (11, 12, 13). In other tumors such as stomach, and prostate, activation of Hh signaling is associated with tissue invasion, and increased metastatic potential. Hh inhibition reduces tumor cell proliferation in prostate, and stomach cancer (14).

In recent years, L-Alanine amino acid polymorphism has attracted great attention due to its use in drug production (15). DL-Alanine, a non-essential amino acid consisting of amino ,and carboxyl groups, is a racemic mixture of D- , and L- form, ,and is an important source of energy for muscles ,and the central nervous system (16). Phenylalanine is an important component of body proteins, and is an important precursor of many aromatic compounds that make up more than half of the Aspartame molecule, and are necessary for normal body function (17). Phenylalanine, an essential amino acid, is converted into tyrosine by the phenylalanine hydroxylase enzyme (PAH) in the liver with the help of the tetrahydrobiopterin cofactor in the first step of the catabolic pathway. Decreased or absent activity of the PAH enzyme causes blood phenylalanine levels to increase, and phenylalanine to have a toxic effect on the brain (18). Additionally, cytotoxic, and anticancer effects of phenylalanine have been reported in various cancer cells (19, 20). In this study, it was aimed to investigate the cytotoxic effects of DL-Alanine, and phenylalanine on prostate cancer cells, and their relationship with the Hedgehog pathway.

MATERIALS AND METHODS

Cell Culture

PC-3 (CRL-1435™) grade IV prostatic adenocarcinoma cells, and HEK293 control cell line were obtained from ATCC. Cells were incubated with 10% filtered fetal bovine serum (FBS), 1% Penicillin/streptomycin solution, and DMEM (Dulbecco's Modified Eagle's Medium) fresh medium by keeping them in a humidified environment at 37°C with 95% free air, and 5% CO_2. According to the manufacturer's instructions, a sufficient number of passages were frozen with medium containing 10% DMSO (dimethyl sulfoxide), and stored in a sterile cryotube.

MTT Cytotoxicity Test

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) test was applied as mentioned previously (21). The dose range was prepared as 1,5,50,100,125,250,350,500,1000 µg/mL for DL-Alanine and DL-Phenylalanine (24h, 48h, 72h). MTT dye was treated with cells cultured approximately overnight in a 96-well cell culture plate. Crystal structures were dissolved with DMSO, and spectrophotometric absorbance measurement was taken at 570 nm.

RNA Isolation

After the IC_{50} (Half maximal inhibitory concentration) value determined in the MTT test was applied to the cells, the cells were removed with Trypsin-EDTA. Experimental groups were created for quantitative PCR (qPCR) by applying the DL-Phenylalanine IC_{50} value to both PC-3 cells and HEK293 cells. The cells, precipitated by centrifugation at 1500 g for 5 minutes, were lysed by applying TRIzol. After applying chloroform, and isoamyl alcohol respectively, RNA was precipitated with 75% ethanol, and cleaned of residues. After the alcohol was evaporated at room temperature, the RNA pellet was gently dissolved in different volumes of 75-100 µl.

cDNA Synthesis

The reverse transcriptase enzyme catalyzed the cDNA synthesis reaction of total RNA samples. In this reaction, the Revertaid First Strand cDNA synthesis kit (Thermo Fisher Scientific) was used. Appropriate primers, dNTP, reverse transcriptase, buffer, and RNase inhibitor were mixed with RNA (1000 ng). Reaction steps were followed according to the kit's instructions.

Real Time PCR

All primers used in this step were designed using the IDT Primer Quest Tool. These designed primers **(Table 1)** were mixed with iTaq universal SYBRGreen supermix, and nuclease-free water. The mixture was added to the fast optical 96-well plate, and covered with a cohesive seal. Amplification was performed using the Quantstudio 3 qPCR (Quantitative polymerase chain reaction) system (Thermofisher Scientific). The qPCR reaction (SYBRGreen Master Mix, cDNA, nuclease-free water) was set up (Five minute 95 °C denaturation step followed by 40 cycles of 5 s at 95 °C and 30 s at 57-58°C).

Table 1: The nucleotide sequences of the primer pairs

Gene	Oligonucleotide sequence (5′-3′)	Amplicon size
SHH	F-CCGAGCGATTTAAGGAACTCA R-ATCACCGAGATGGCCAAAG	133
PTCH	F-GGGTGGCACAGTCAAGAACAG R-TACCCCTTGAAGTGCTCGTACA	108
SMO	F-ATCGCTACCCTGCTGTTATTC R-GGGCACCATCCATGAACT	90
GLI-1	F-GAGCTGGACATGCTGGTT R-ATTCAGGCTCACGCTTCTC	148
GAPDH	F-GGAGCGAGATCCCTCCAAAAT R-GGCTGTTGTCATACTTCTCAT	197

Ethical Committee

The study protocol was approved by the ethics board of Karatay University Drug and Non-Medical Device Research Ethics Committee (2023/025).

Statistical Analysis

Gene expression levels were evaluated through Ct (threshold cycle) values. Differences in gene expression level between control and dose groups were calculated according to Livak's $2^{-\Delta\Delta CT}$ (2(-Delta Delta C(T)) method (22) and normalized using the GAPDH housekeeping gene. Observed differences in gene expression between groups were calculated as fold increases and evaluated with student's t-test. Statistically $p<0.05$ significance level was accepted.

RESULTS

MTT Assay Results

The cytotoxic effect on PC-3 cells was investigated in the dose range of 1-1000 µg/mL for DL-Alanine, and DL-Phenylalanine. While no cytotoxic effect was observed for DL-Alanine in the specified dose range, the IC_{50} value for DL-Phenylalanine was determined as 500 µg/mL at the 48th hour. It was determined that phenylalanine suppressed cell viability depending on concentration, and time **(Figure 1)**.

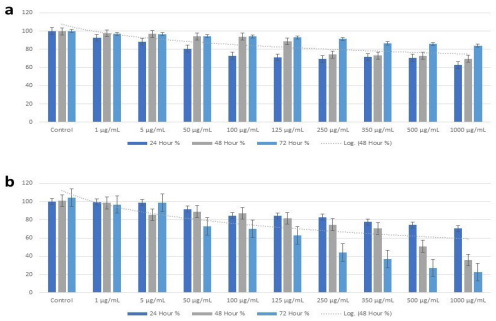

Figure1: MTT cell viability graph at different doses. a. DL-Phenylalanine treatment results, b. DL-alanine treatment results

After applying the determined IC_{50} value to PC-3 cells (control, phenylalanine, and alanine applied), they were examined under an inverted microscope. A decrease in cell number was detected in the DL-Phenylalanine applied group compared to control PC-3 cells. Additionally, no significant change was detected in the morphology of the cells **(Figure 2)**.

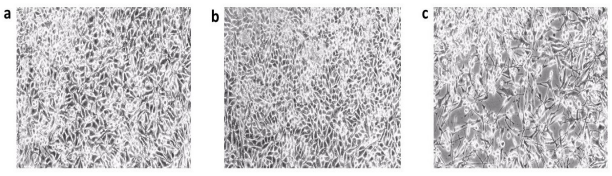

Figure 2: Invert microscope cell morphology images. a. PC-3 control cells, b. PC-3 cells after application of 1000 µg/mL 72 h DL-alanine c. PC-3 cells after application of 500 µg/mL 48 h (IC50) DL-phenylalanine

Real Time PCR Results

After applying the IC_{50} value of DL-Phenylalanine to PC-3 cells, down-regulation was detected in all Hedgehog-related genes (Table 1). Among these gene expression changes, the greatest change was observed in the *PTCH* gene. The order of gene expression changes from largest to smallest is as follows; *PTCH* (fold=2.24, p=0.002), *SMO* (fold=2.19, p=0.013), *SHH* (fold=1.98, p=0.003), *GLI-1* (fold=0.28, p=0.001), respectively **(Figure 3)**.

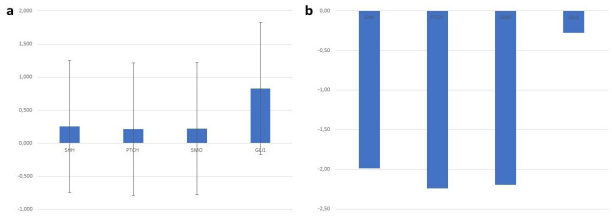

Figure 3: . PC-3 cells qPCR results. a.Relative gene expression fold change $2^{-\Delta\Delta Ct}$ graph b. Fold change log 2 graph.

On the contrary, after applying the IC_{50} value of DL-Phenylalanine to HEK-293 cells, up-regulation of the relevant genes was detected. Among these expression changes, the most changes were detected in the *GLI-1* gene. The order of gene expression changes from largest to smallest is as follows; *GLI-1* (fold=1.52, p=0.002), *PTCH* (fold=1.25, p=0.001), SMO (fold=0.86, p=0.013), *SHH* (fold=0.32, p=0.017), respectively **(Figure4)**.

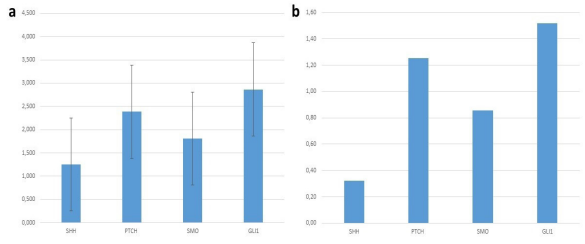

Figure 4: HEK-293 cells qPCR results. a.Relative gene expression fold change $2^{-\Delta\Delta Ct}$ graph b. Fold change log 2 graph

When all these results are evaluated together, gene expression changes on HEK-293 cells are minimal, but significant gene expression changes were detected on prostate cancer cells.

DISCUSSION

Prostate cancer is one of the common types of cancer that is very difficult to diagnose, and treat. Due to the difficulty of treatment in prostate cancer tumors associated with poor prognosis, various alternative treatment methods are needed (3). Especially in functional medicine applications, supplementation of essential, and organic molecules has recently been preferred in cancer treatment (23). Because while many chemotherapeutic drugs inhibit cancer cells, they can also negatively affect many healthy cells (24). Innovative approaches in medicine support cancer therapy with smart drug technology, and functional medicine.

Alanine, and phenylalanine are essential amino acids encoded in the cell genome, and low phenylalanine can cause genetic/metabolic syndromes such as phenylketonuria (25). Thanks to different derivatives, and modifications of L-Alanine, its use in cancer treatment is predicted (26, 27). In addition, the effect of β-alanine, one of the different alanine forms, on cervix, and renal tumors in particular, has been demonstrated (28). However, in our study, Alanine did not show a cytotoxic effect on prostate cancer cells within the given dose range. This may be related to the overaggressive, and poor prognosis of the grade IV PC-3 prostate cancer cell line used in our study.

The role of extracellular alanine in cell metabolism is not yet fully understood. Recent have reported that pancreatic cancer cells use extracellular alanine as a carbon source to regulate the TCA (tricarboxylic acid) cycle in a GPT2 (glutamate-pyruvate transaminase 2)-dependent manner (29). It was also discovered that extracellular alanine activates T cells (30). No change in alanine levels was detected in extracellular alanine catabolism before, and after the threatment of CB839 in resistant non-small cell lung cancers (31). Another study predicted that alanine was secreted at higher levels in prostate cancer cells compared to normal prostate cells, and could be a potential biomarker (32). This suggests that prostate cancer cells secrete alanine extracellularly, and do not contain it intracellularly. In the MTT test, externally administered DL-Alanine was not used within the framework of the cell's needs, and could not show any effect. Perhaps, in overdoses, alanine could have a toxic effect. In contrast, DL-Phenylalanine showed a cytotoxic effect on prostate cancer cells. These results are consistent with the fact that phenylalanine is a biologically active molecule, and phenyl rings can also have anticancer effects

(33, 34). In addition, studies conducted in recent years have revealed that the use of phenylalanine in metal complexes such as boron, poly-phenylalanine structures or dipeptides is important in the fight against cancer (20, 35).

Matijin-Su (MTS) is a phenylalanine dipeptide monomer compound with various biological activities (36, 37), and MTS derivatives exhibit various pharmaceutical activities (38). These pharmaceutical activities include antitumor ability, and anti-inflammatory activities (36, 39, 40). Additionally, MTS derivatives significantly inhibited proliferation, and prevented metastasis in HepG2 hepatocellular carcinoma cells. Additionally, MTS derivatives exhibited low toxicity in normal hepatocyte cells (38). In our study, it is not surprising that DL-Phenylalanine did not have a toxic effect on HEK-293 normal embryonic kidney cells, and did not inhibit the Hedgehog pathway, one of the self-renewal pathways, but it is compatible with the literature. DL-Phenylalanine has an inhibitory effect on PC-3 prostate cancer through the Hedgehog pathway. This effect can be evidenced by the down-regulation of *SMO*, and *GLI-1* following down-regulation of the *SHH* ligand. Additionally, in our study, it was observed that PTCH receptor gene expression was parallel to the decrease in *SHH* gene expression level.

Investigating whether many biocompatible molecules such as phenylalanine are effective in cancer therapies is very important for anticancer therapies. In the future, *in vitro*, and *in vivo* studies on the modification of different biological molecules, and their use in the fight against cancer should be expanded. In particular, understanding the relationship of cancer-related proliferative, apoptotic, and self-renewal signaling pathways with biological molecules will play a key role in targeted therapies.

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