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Investigation of the Effects of PFKFB3 Small Molecule Inhibitor KAN0438757 on Cell Migration and Expression Level of N-cadherin Protein in Glioblastoma Cell Lines

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Abstract: Reprogramming of energy metabolism in cancerous cells plays a crucial role in promoting the epithelial-mesenchymal transition (EMT) program that is linked to malignancy. PFKFB3 (6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase 3), which is responsible for energy metabolism, is a significant glycolytic activator involved in the progression of various types of tumours, including glioblastoma. PFKFB3 has the capacity to alter the expression of EMT-related proteins, thereby influencing the EMT program amongst tumour cells. When undergoing EMT, glioblastoma cells acquire a mesenchymal phenotype associated with augmented cellular motility, invasiveness, and resistance to therapy. Inhibition of PFKFB3 in glioblastoma cells has promising potential as a therapeutic strategy to target EMT and halt cancer progression. PFKFB3 inhibitors are compounds that can block PFKFB3 activity, inhibiting glycolysis in cancer cells. KAN0438757 is a novel, selective inhibitor of PFKFB3 that exhibits anti-tumour effects in various cancer models both *in vitro* and *in vivo*. The study evaluated the impact of a novel inhibitor on the viability, migration, and death of glioblastoma cancer cell lines U373 and U251 using WST-1 cell viability, AO/EtBr staining western blotting, and wound healing assays. The results showed that cell viability decreased and dose-dependent apoptotic morphological changes were observed in glioblastoma cells after KAN0438757 treatment. Moreover, the protein level of EMT-associated N-cadherin decreased, leading to reduced cell migration. In conclusion, it is possible that KAN0438757 could elicit anti-tumour effects in glioblastoma by reversing the EMT programme and inducing apoptotic morphological changes in cancer cells.

PFKFB3 Küçük Molekül İnhibitörü KAN0438757'nin Glioblastoma Hücre Hatlarında Hücre Migrasyonu ve N-kadherin Proteininin Ekspresyon Düzeyi Üzerine Etkilerinin Araştırılması

Anahtar Kelimeler
KAN0438757, PFKFB3, Glioblastoma, Migration

Öz: Tümör hücrelerinde enerji metabolizmasının yeniden programlanmasının, malign özelliklerle ilişkili epitelyal-mezenkimal geçiş (EMT) programının desteklenmesinde önemli bir rol oynadığı bilinmektedir. Enerji metabolizmasında görev alan PFKFB3 (6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase 3), glioblastoma dahil olmak üzere çoklu tümör tipi ilerlemesinde rol oynayan önemli bir glikolitik aktivatördür. PFKFB3, EMT ile ilişkili proteinlerin ekspresyonunu modüle ederek tümör hücrelerinde EMT programını etkileyebilmektedir. EMT sırasında glioblastoma hücreleri, artan hücre hareketliliği, istilacılık ve tedaviye direnç ile ilişkili bir mezenkimal fenotip kazanmaktadır. Glioblastoma hücrelerinde PFKFB3'ün inhibisyonu, EMT'yi hedeflemek ve kanser ilerlemesini engellemek için potansiyel bir terapötik strateji olarak görülmektedir. PFKFB3 inhibitörleri, PFKFB3'ün aktivitesini bloke edebilen ve dolayısıyla kanser hücrelerinde glikoliz sürecini inhibe edebilen bileşiklerdir. KAN0438757, PFKFB3'ün yeni ve seçici bir inhibitörüdür. KAN0438757'nin hem *in vitro* hem de *in vivo* olarak çeşitli kanser modellerinde anti-tümör etkilerine sahip olduğu gösterilmiştir. Yeni inhibitörün glioblastoma kanseri hücre hatları U373 ve U251'de hücrelerin canlılığı, hücre

göçü ve hücre ölümü üzerindeki etkisi, WST-1 hücre canlılığı, AO/EtBr, boyama western blotlama ve yara iyileştirme testleri ile araştırıldı. Elde ettiğimiz sonuçlarda, glioblastoma hücrelerinde, KAN0438757 tedavisinden sonra hücre canlılığının azaldığı ve doza bağlı apoptotik morfolojik değişiklikler görüldü. Ayrıca EMT ilişkili N-cadherin proteininin düzeyinin azaldığı ve hücre göçünün de baskılandığını gözlemlendi. Sonuç olarak, KAN0438757'nin glioblastomada, EMT programını tersine çevirerek ve kanser hücrelerinin apoptotik morfolojik değişikliklere yol açarak anti-tümör aktiviteye sahip olabileceğini düşündürmektedir.

1. INTRODUCTION

Gliomas constitute approximately 80% of malignant tumours and 30% of brain tumours. Gliomas are classified by the World Health Organization (WHO) as types 1, 2 (low-grade gliomas), 3 (anaplastic glioma) and 4 (Glioblastoma (GBM)) according to their general characteristics [1]. GBM is a highly aggressive form of this type of this classification. Although many treatments have been applied for glioblastoma, according to studies, no further progress has been made in the last decade [2]. To this end, blocking the growth-promoting kinase targets of glioblastoma seems to be a reasonable treatment strategy that requires further study [3]. As with most cancers, glioblastomas tend to favour aerobic glycolysis over oxidative phosphorylation, a phenomenon known as the Warburg effect [4,5]. Glioblastoma cells exhibit elevated levels of fructose-2,6-bisphosphate (F2,6BP), essential controller of 6-phosphofructo-1-kinase, significant enzyme responsible for regulating the rate.Glycolytic flux is controlled through several enzyme steps in glycolysis, including those catalysed by the enzymes phosphofructokinase-1 (PFK1) and 6-phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase (PFKFB) [6]. The bifunctional enzyme family known as PFKFB is expressed by four different genes and is important for cancer metabolism. PFKFB enzymes are crucial for regulating glycolysis. They can be classified into four groups: PFKFB1, PFKFB2, PFKFB3 and PFKFB4[7]. The activity of the PFKFB3 enzyme plays an important role in glycolysis metabolism because the kinase/phosphatase ratio of PFKFB3 is very high, thus enabling the formation of F-2,6-BP and increased glycolysis [8]. As a result of various studies, PFKFB3 was found to be overexpressed in different human cancers, including malignant glioma [9]. PFKFB3 is overexpressed in GBM and has been shown to promote tumour growth and invasive ligation. PFKFB3 promotes GBM growth and invasiveness through several mechanisms; Increasing the production of F2,6BP, a master regulator of glycolysis, can promote Warburg effect, a metabolic change characteristic of cancer cells, and suppress programmed cell death. PFKFB3 can also promote epithelial-mesenchymal transition (EMT) in GBM cells by activating a number of signalling pathways, including the PI3K/AKT/mTOR and the TGF-β1 pathways [10]. EMT is a complex biological process in which epithelial cells mislay their epithelial properties and take on mesenchymal ones. That procedure crucial for normal embryonic development but may also play a role in cancer progression. Glioblastoma cells often receive EMT to obtain increased invasiveness and disseminate to other parts of the brain [11]. EMT is an a hallmark of cancer and has been demonstrated to enhance tumor invasion, metastasis, and treatment resistance [12]. The downregulation of epithelial markers (E-cadherin) and the overexpression of mesenchymal markers (N-cadherin) characterize EMT [13]. EMT is a significant factor in the progression of GBM. Promisingly, PFKFB3 inhibitors can halt EMT and offer a new therapeutic strategy for GBM [14]. Furthermore, targeted PFKFB3 inhibition may be an effective treatment for CNS patients. PFKFB3 inhibitor treatment have shown its potential to improve the sensitivity of tumors that are resistant to treatment, both to chemotherapy and radiation [15, 16]. Anti-PFKFB3 and anti-VEGF combination treatment significantly increased the survival rate of preclinical models of glioblastoma and eliminated resistance to anti-angiogenic therapy, as reported by Zhang et al. [17]. In 2018, a specific small molecule inhibitor called KAN0438757 was discovered. It was found to have a high level of selectivity for the PFKFB3 kinase domain. KAN0438757 induced an increase in cell permeability and a decrease in cell viability in various types of cell lines via suppression of PFKB3-118-20]. Conversely, KAN0438757 treatment produced radiosensitivity and cytotoxicity in cancer cells at concentrations that normal cells tolerated. The use of KAN0438757 after ionising radiation has inhibited the PFKFB3 molecule. This inhibition also prevented PFKFB3, BRCA1 and RAD51 from being nuclear localised. All of these molecules have been deemed essential for proper homologous recombination repair. Furthermore, KAN0438757 efficiently inhibited dNTPs during double-stranded DNA repair by inhibiting homologous recombination repair activity. As a result, H2AX levels increased and ionizing radiation caused cell cycle arrest [19]. In the case of human colorectal cancer cells, cell migration has been inhibited by KAN0438757, along with a decrease in associated PAXLLIN, VINCULIN and CORTACIN genes. *İn vivo* administration of KAN0438757 has been found to be non-toxic [20]. However, the effect of KAN0438757 specifically on cell migration in glioblostoma is still mysterious.

This study aimed to is to focus and investigate the anticancer status, cell viability and migration of KAN0438757 in glioblostoma and the EMT-amplified protein N-cadherin.

2. MATERIAL AND METHOD

2.1 Cell Culture

In this study, human glioblastoma U373 and U251 cell lines purchased from ATCC (American Type Culture

Collection) were used. The cells were grown DMEM medium supplemented with 10% fetal bovine serum and 64 µg/ml penicillin + 0.1 mg/ml streptomycin in in 5% CO_2/95% air at 37 °C unless otherwise stated.

2.2 WST-1 Viability Test

Water-soluble tetrazolium salts, known as WSTs, are commonly used for testing cell viability. These tests determine cell viability using spectrophotometry. The viability of untreated cells was set as 100%, and the viability of treated cells is expressed as a percentage (%) in comparison. Once the cells had reached a specific density, they were transferred to a 96-well plate with 3000 cells per well and incubated overnight in a CO_2 environment. The plate was then kept in the CO_2 incubator for several hours. Following this, glioma cell lines were treated with varying doses of KAN0438757 for 48 hours. WST-1 dye was added to each well at a volume of 10 µl and left to mix for 2-3 minutes. Afterwards, the plate was measured at 450 nm using the ELISA reader device [21].

2.3 Wound-Healing Migration Assay

The cells were plated onto a 6-well plate with a density of 8,000 cells per well and grown to reach 85-90% confluence. Subsequently, a 100 µL tip were used to produce uniform strips in the cell wells by making a scratch, followed by washing the plates with PBS. After this step, the cells were treated with serum-free DMEM/medium that contained different concentrations of KAN0438757 (5, 10, and 25 µM).The gap regions were photographed with a phase contrast microscope (Olympus, CKX41, Tokyo, Japan) at certain time intervals (0, 24, 48, 72 hours). Wound closure percentage was calculated using ImageJ software based on the cell-free area, as has been previously described [22, 23]. (Wound Closure % = {(At=0 – At=Δh)/At=0} *100. At=0 = area of the wound measured immediately after scratching. At=Δh = area of the wound measured h hours after the scratch is performed.)

2.4 AO/EBr Staining

In a 6-well plate, U373 and U251 cells were sown ($15x10^4$ cells per well) and incubated for overnight. The cells were treated with different concentrations of KAN0438757 (5, 10, and 25 µM). After the treatment period was over, acridine orange ethidium bromide dye was prepared and added to each well from the mixture. Imaging was then carried out under a microscope. Live cells were green under the microscope, but dead cells appeared red [24].

2.5 Western Blotting

Under denaturing conditions, the cells were separated using SDS-PAGE (12%) gel [25]. Proteins were subsequently transferred onto a PVDF membrane following their separation on the gel. Following transfer, the membrane was incubated for 1 hours at room temperature with 5% BSA (Bovine Serum Albumin) dissolved in 1X TBS-T (TrisBuffered Saline and Tween 20) solution. Membrane were shaken overnight at +4 °C with the appropriate primary antibody (anti-N-cadherin (Santa Cruz, sc-7939, 1:1000) and anti-GAPDH (Santa Cruz, sc-365062, 1:1000). Membrane was rinsed with 1X TBS-T for 5 minutes/5 times the next day before being incubated with secondary antibody (anti-mouse or anti-rabbit) for 60 minutes at room temperature. Images were acquired using the chemiluminescence method after the membrane was washed with 1X TBS-T for 5 minutes/5 times. Image-j was used to determine band intensities densitometrically (National Institute of Health, Bethesda, MD; Image J). Each band's intensity was normalized to the intensity of the matching GAPDH band.

2.6. Statistical Analyses

All results were assessed using the Graph Pad Prism 5.01 software. Statistical analysis was performed through one-way ANOVA followed by post-hoc Tukey test and was repeated three times for reproducibility.

3. RESULTS

3.1 Effect of KAN0438757 on Cell Viability

To investigate the effects of KAN0438757 cell viability, U373 and U251 cell line were treated with different doses of KAN0438757 (5, 10, 25, 50 and 100 µM). According to the data obtained as a result of the measurement, KAN0438757 was shown to impair cell viability in the U373 cell line in a dose-dependent manner, with a sharper drop noted at 25 µM as shown in Figure 1A. Similarly, KAN0438757 reduced cell viability in the U251 cell line Figure 1B. Cell viability was shown to be significantly reduced in both cell lines, particularly after the 10 µM dosage.

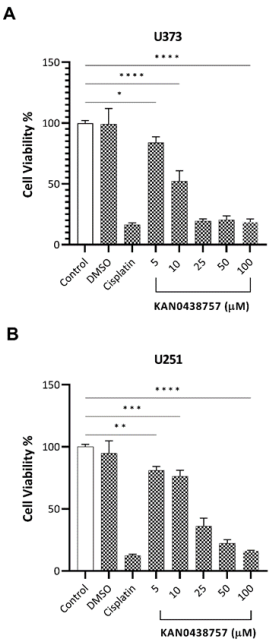

Figure 1. Effect of KAN0438757 on cell viability of human glioblastoma (U373 and U251) cell lines. (A, B) U373, U251 cells were exposed to for 48 hours with KAN0438757 and cell viability was evaluated using WST-1 assays.

3.2 Effect of KAN0438757 on Cell Migration

We further investigated KAN0438757 effects on migration of U373 and U251 cells. The cells received treatment with KAN0438757 (5, 10, and 25 M), as indicated in Figs. 2A and 2B. For 72 hours, wound closure percentages in U373 cells were 20,78% at 10 M and 8,13% at 25 M. Similar to U373 cells, the percentages of wound closure were 38,18 % at 10 µM and 13,19 % at 25 µM, respectively in U251 cells. U373 cells treated with low doses of KAN0438757 demonstrated that the intercellular gap was closed after 48 hours. On the other hand, it was showed that the intercellular distance in the cells treated with 25 µM of KAN0438757 had a very low mobility after 48 hour compared to the control group. At 5 µM of KAN0438757, there was no statistically significant difference in inhibition effect in both cells lines.

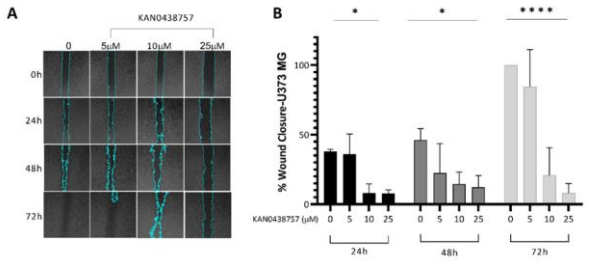

Figure 2. Effect of KAN0438757 on Cell Migration in U373 cell line. KAN0438757 (5-25 M) was applied to U373 cells for 72 hours. Subsequently, the wound-healing assay was conducted to determine cell migration. (A) Microscopic images (4x-500µm) of U373 cells following various treatments with KAN0438757. (B) The wound was measured for closure. All of the data given are from three distinct tests.

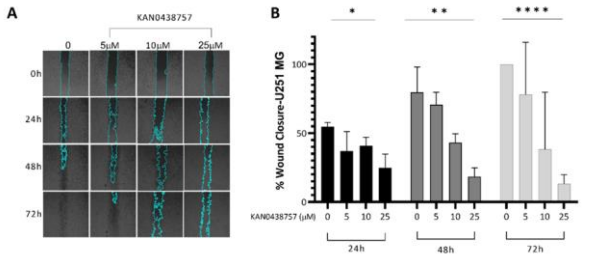

Figure 3. Effect of KAN0438757 on Cell Migration in U251 cell line. The U251 cells were treated with KAN0438757 (5-25 µM) for 72 hours. Subsequently, the wound-healing assay was conducted to determine cell migration. (A) Images (4x-500µm) taken using a microscope of U25 cells following various treatments with KAN0438757. (B) The wound was measured for closure. All of the data given are from three distinct tests.

3.3. Effect of KAN0438757 on Cell Death in U251 and U373 Cells

In order to determine whether the growth inhibition by KAN0438757 was associated with cell death, gliobalstoma cells treated with KAN0438757 were analyzed for dual staining (AO/EB staining). Figure. 4-A and B shows that when treated with KAN0438757, the ratio of apoptotic cells significantly increased in cells at high dose of KAN0438757. Given the administered dosage of 5 and 10 µM, the number of viable cells is relatively considerable. The cells treated with 25 µM of KAN0438757 were observed to change from green to yellow and orange which suggests that when exposed to KAN0438757, U251 and U373 cells underwent the typical changes of apoptosis.

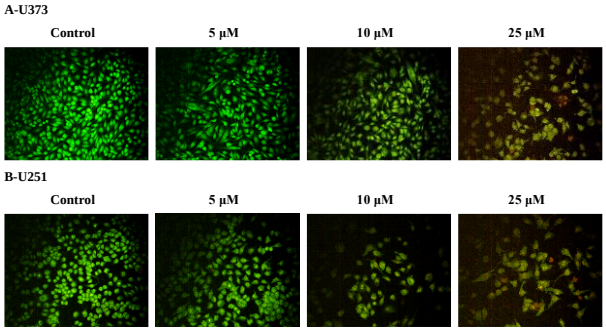

Figure 4. Effect of KAN0438757 on the apoptotic cell death in human glioma cells was evaluated by fluorescence microscopy using acridine orange/propidium iodide double staining. Detection of apoptotic morphology was performed using fluorescent staining of U251 and U373 cell lines with acridine orange-ethidium bromide (AO/EB) after treatment with KAN0438757 (5-25 µM) for 72 hours. Apoptotic cells emit red fluorescence while as viable cells emit green fluorescence. An increase in the number of apoptotic cells was observed as the KAN0438757 dose increased. This figure presents the outcomes of at least three separate trials (Original magnification 20x).

3.4. Western Blotting

To further investigate the molecular basis effect of KAN0438757 on N-cadherin in gliobasltoma cancer cells, we examined the expression N-cadherin after treatment with different concentrations of KAN0438757 by western blot analysis. At 5 µM of KAN0438757, there was no statistically significant difference in N-cadherin expression in both cells lines (Figure 5B and 5C). N-cadherin, expression levels were decreased after exposure to 25 µM KAN0438757 compared with the levels in control cells.

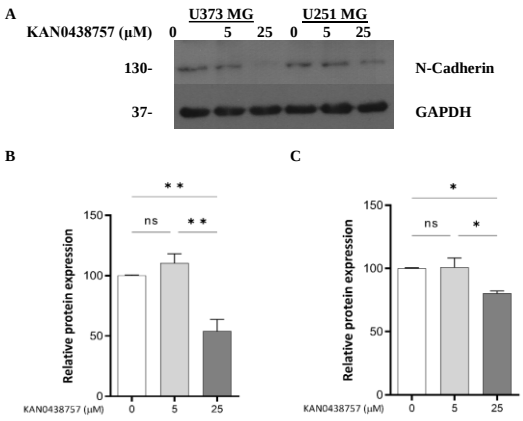

Figure 5. Effect of KAN0438757 on N-cadherin. A) Westsern blot bands. (N-cadherin and GAPDH Western blotting was used to evaluate protein expression levels.), B-C) N-cadherin protein expression levels levels. GAPDH was used as control. Control group and compared, *P < 0.05, **P < 0.01

4. DISCUSSION AND CONCLUSION

Metastasis, known as the process in which the cancerous cell leaves the primary tumor tissue, passes through the basement membrane to the circulatory system, reaches different tissues and forms a secondary tumor, is the main cause of death from cancer [26]. Despite this important effect on cancer deaths, the molecular

mechanisms of invasion and migration, which form the basis of metastasis, remain unknown. The escape of cancerous cells from the primary tumor tissue is associated with increased migration potential with the reduction of intercellular junction proteins and acquisition of mesenchymal phenotype, called EMT, which has basic functions such as embryogenesis and wound healing in the normal physiological process [27]. The EMT process is mediated by various transcription factors, mainly the Snail, Twist and Zeb family [28]. The roles of transcription factors in EMT are mainly by the regulation of various proteins as an example E and N-cadherin, β-catenin, vimentin and fibronectin and suppression of intercellular tight junctions [29]. Various growth and differentiation factors such as fibroblast growth factor (Fibroblast Growth Factor; FGF), wnt and notch proteins are involved in the initiation of the EMT process. Among these factors, transforming growth factor β (Transforming Growth Factor β; TGFβ), which is an important inducer of EMT, is largely involved in cancer studies [30].

PFKFB3 is an enzyme that is essential in glycolysis, the process by which cells convert glucose into energy [8]. PFKFB3 has been shown to be activated by hypoxia and other stress signals and promotes EMT [31]. EMT is a process in which epithelial cells that are normally tightly packed and adhere to each other lose their cell-cell adhesion and become more mesenchymal-like. Mesenchymal cells are more mobile and can migrate to other parts of the body [32]. EMT is crucial for a diversity of processes, including development, wound healing, and cancer metastasis. In cancer, EMT is thought to promote metastasis by allowing cancer cells to escape from the primary tumor and travel to other parts of the body. PFKFB3 has been shown to be involved in EMT in several ways. First, PFKFB3 can activate transcription factors that promote EMT, such as Snail and Twist [33]. Second, PFKFB3 can increase the production of extracellular matrix proteins that may aid in the migration of cancer cells [34]. Third, PFKFB3 can enhance the generation of reactive oxygen species, which can cause cell-cell adhesions to break down and induce EMT[35]. Here are some additional research findings regarding the relationship between PFKFB3 and EMT; The study in human breast cancer cells showed that inhibition of PFKFB3 reduced EMT and increased the sensitivity of cells to chemotherapy [36]. According to another research in colorectal cancer cells, inhibition of PFKFB3 reduced the cells' ability to metastasize [37]. Overall, the research suggests that PFKFB3 plays a role in EMT and that inhibiting PFKFB3 could be a potential therapeutic strategy for cancer. More study is needed, however, to fully understand the role of PFKFB3 in EMT and to create safe and effective PFKFB3 inhibitorsPFKFB3 is a key enzyme in glycolytic tumor metabolic reprogramming, and protein levels of PFKFB3 are substantially higher in high-grade gliomas (HGGs) than in low-grade gliomas. The significance of PFKFB3 splice variants in glioblastoma development and prognosis is still poorly known. Therefore, it is desired to reveal a new strategy to treat cancer using PFKFB3 inhibitors in the treatment of glioblastoma. Inhibitors that provide inhibition of PFKFB3 appear as four different molecules: 3PO, PFK15, PFK158 and KAN0438757 [19,40]. Among these inhibitors, KAN0438757 is a new inhibitor that has been studied in recent years, and it has been determined that it selectively inhibits the proliferation of cancer cells. It was also concluded that inhibition of PFKFB3 with KAN0438757 impairs DNA repair, resulting in death of cancer cells. However, there is no study yet to reveal the effects of KAN0438757 inhibitor on glioblastoma cells. As a result, we explored the consequences of the KAN0438757 on cell migration and N-cadherin protein expression levels in glioblastoma cell lines in this study.

In this study, cell viability, cell proliferation, cell death, cell migration, and protein levels such as N-cadherin were analyzed in glioblastoma cell lines U373 and U251 cells. In our study, firstly, the effect of KAN0438757 inhibitor on cell viability were assessed in U373 and U251 cell lines. The WST-1 viability test revealed that cell viability reduced dose-dependently in both cell lines. In the study of Oliveira et al., it was observed that after treatment with KAN0438757 in colorectal cancer cell proliferation was significantly reduced, especially at 50 and 75 μM doses. In a separate investigation Yan et al. reported that inhibition of PFKFB3 by oxaplatin in colon cancer not only reduces cell viability but also promotes apoptotic cell death [38]. Cell migration was determined by our next experiment, Wound Healing. Glioblastoma cell lines U373 and U251 were treated with KAN0438757 for 0-72 hours. In the U373 cells, it was observed that it inhibited the migration ability of the cells depending on the dose and time. It was concluded that the U251 cell line significantly affected cell migration compared to the U373 cell line and reduced the migration abilities of the cells. According to the information we obtained as a result of these findings, it was determined that the KAN0438757 inhibitor inhibited the migration ability of cells in glioblastoma cells in a time and dose dependent manner. Yan et al. [38] has shown that it significantly reduced cell migration and invasion activity in colorectal cancer cell lines. In another study, Veseli et al. observed that treatment with 3PO, significantly inhibited cell migration in endothelial cells. These results, like the results we obtained in our study, show that PFKFB3 inhibitors have a significant decrease in the migration ability of cancer cells and this has an important role in leading cancer cells to death [41].

Preceding studys has shown that the overexpression of PFKFB3 elevates the migratory and invasive capabilities in tumour cell, while reducing the expression of E-cadherin and upregulating N-cadherin. Specifically, the transcription factors Snail and Twist are notably induced, which have been found to contribute significantly to EMT [14]. Western blot results of the N-cadherin showed that the KAN0438757 caused a decrease in U251 cell lines, especially in the high-dose group compared to the control. In addition, it was determined that KAN0458757 treatment caused a very dramatic decrease in N-cadherin protein expression in U373 cell lines. Yalçın et al. in their study in 2023 showed that silencing of PFKFB3 caused changes in the expressions

of E-cadherin, Vimentin and EMT genes in different cancer cell lines [33]. We have shown that PFKFB3 inhibitor can cause a phenomenon that supports the changes in EMT proteins that we have obtained consistent with this result.

Apoptosis is the removal of infected, damaged, or undesirable cells in order to preserve cell homeostasis. Another experiment, AO/EtBr staining experiments, examined cell death in glioblastoma cell lines after treatment of the KAN0438757 inhibitor. Firstly, it was observed that the cells tended towards death with a reduction in cell count in the U251 cell line, especially in the 25 μM dose group. On the other hand, in the U373 cell line, it was also detected that in the 25 μM dose group, significant cells are close to death and some of these even death. In the work of Wang et al. [39] apoptosis was looked at to determine cell death. It was determined that PFK15, an inhibitor of PFKFB3 in rhabdomyosarcoma cells, had a stimulating effect on cell viability loss after induction with 3MA. It has also been observed that PFK15 has multiple cell death-inducing effects other than caspase-dependent apoptosis.

The study shows the significance of PFKFB3-mediated metabolism in the development of EMT in glioblastoma cells and indicates that KAN0438757 could be a fresh approach to treating glioblastoma.

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