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NEÜ YAYINEVİ

Investigation of the Anticancer Activity of DCZ0415, a Small Molecular Inhibitor of TRIP13, in U87 Human Glioblastoma Multiforme Cells

TRIP13'ün Küçük Bir Moleküler İnhibitörü Olan DCZ0415'in, U87 İnsan Glioblastoma Multiforme Hücrelerindeki Antikanser Etkinliğinin İncelenmesi

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Öz

Amaç: Tiroid Hormon Reseptörü Etkileşimli Protein 13 (TRIP13); mayotik rekombinasyonda rol oynayan, iğ-toplanma kontrol noktasında görevli bir proteindir. Son yıllarda yapılan çalışmalar TRIP13'ün glioblastoma multiforme (GBM) de dahil olmak üzere çok sayıda kanserde potansiyel bir tümör indükleyicisi olabileceğini ortaya koymuştur. Bu çalışmada TRIP13'ün küçük bir moleküler inhibitörü olan DCZ0415'in U87 insan GBM hücrelerindeki antikanser etkinliğinin araştırılması amaçlandı.
Gereçler ve Yöntem: DCZ0415'in U87 hücrelerindeki olası antikanser etkisi sitotoksisite analizi, koloni formasyon analizi ve apoptoz analizi ile belirlendi. Ayrıca qRT-PZR analizi ile DCZ0415'in apoptoz, invazyon ve Transforme Edici Büyüme Faktörü-Beta (TGF-β) sinyal yolağı ile ilişkili genlerin mRNA seviyeleri üzerine etkisi araştırıldı.
Bulgular: DCZ0415, U87 hücre proliferasyonunu doz ve zaman bağımlı şekilde inhibe etti. U87 hücrelerinde DCZ0415'in 48 saat için IC_{50} dozu 19,77 µM olarak belirlendi. Bu dozda DCZ0415 uygulaması U87 hücrelerinde apoptozu indükledi ve hücrelerin koloni oluşturma yeteneklerini baskıladı. Ayrıca DCZ0415 apoptoz, invazyon ve TGF-β sinyal yolağı ile ilişkili genlerin mRNA seviyelerini antikanser etkiye yol açabilecek şekilde değiştirdi.
Sonuç: Kanserde yeni bir onkogenik faktör olarak değerlendirilen TRIP13'ün bir inhibitörü olan DCZ0415, GBM hücrelerinde antikanser etkiye sahiptir. Bu açıdan, TRIP13'ün GBM için önemli bir terapötik hedef olabileceği ve DCZ0415'in GBM hücrelerinde antikanser etkiye yol açan etkili bir inhibitör olarak değerlendirilebileceği düşünülmektedir.

Anahtar Kelimeler: TRIP13, DCZ0415, Glioblastoma multiforme, TGF-β sinyal yolağı

Abstract

Aim: Thyroid Hormone Receptor Interacting Protein 13 (TRIP13) is a protein involved in spindle-aggregation checkpoint, which plays a role in meiotic recombination. Recent studies have revealed that TRIP13 may be a potential tumor inducer in many cancers, including glioblastoma multiforme (GBM). We aimed to investigate the anticancer activity of DCZ0415, a small molecule inhibitor of TRIP13, in U87 human GBM cells in this study.
Materials and Methods: The possible anticancer effect of DCZ0415 on U87 cells was determined by cytotoxicity, colony formation, and apoptosis assays. In addition, the effects of DCZ0415 on mRNA levels of genes which were involved in apoptosis, invasion and Transforming Growth Factor-Beta (TGF-β) signaling pathway were investigated by qRT-PCR analysis.
Results: DCZ0415 inhibited U87 cell proliferation in a dose and time dependent manner. The IC_{50} dose of DCZ0415 for 48 hours was determined as 19.77 µM in U87 cells. DCZ0415 treatment at this dose induced apoptosis and suppressed colony forming abilities of U87 cells. In addition, DCZ0415 altered mRNA levels of genes associated with apoptosis, invasion and TGF-β signaling pathway, which could lead to anticancer effects.
Conclusion: DCZ0415, an inhibitor of TRIP13 which has been evaluated as a new oncogenic factor in cancer, has an anticancer effect on GBM cells. In this respect, it is thought that TRIP13 may be an important therapeutic target for GBM and DCZ0415 may be considered as an effective inhibitor that causes anticancer effects in GBM cells.

Key words: TRIP13, DCZ0415, Glioblastoma multiforme, TGF-β signaling pathway

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INTRODUCTION

Glioblastoma multiforme (GBM), also called Grade IV glioma, is the most common malignant primary brain tumor (1). Treatment approaches for GBM include surgery, temozolomide-based chemotherapy, radiotherapy, and their combinations. However, GBM is a highly lethal type of cancer with a high recurrence rate. Accordingly, median survival of the disease is 15 months (2, 3). The identification and targeting of new molecules associated with GBM is crucial for the development of new therapeutic approaches.

TRIP13 (Thyroid Hormone Receptor Interaction Protein 13), first identified as a protein that interacts with human papillomavirus protein E1, is found in AAA+ (ATPases associated with various cellular activities) family. TRIP13, a protein consisting of 432 amino acids, is encoded by a gene on chromosome 5p15.33. This protein plays a role in meiotic recombination and is involved in the spindle-assembly checkpoint that maintains genomic stability and prevents aneuploidy (4, 5). Recent studies show that TRIP13 plays an oncogene role in cancer pathogenesis. It has been found that TRIP13 expression is increased in thyroid cancer (4), ovarian cancer (6), and prostate cancer (7) tissues compared to adjacent tissues.

In hepatocellular carcinoma (8), bladder cancer (9), esophageal cancer (10) and cervical cancer (11), increased TRIP13 expression was associated with lower survival and advanced tumor stage. It was stated that TRIP13 mRNA level is high in advanced gliomas and a negative correlation is found between TRIP13 and the overall survival rate. Therefore, TRIP13 is also considered as an oncogenic factor for GBM (12). And, targeting TRIP13 in tumor tissues with high TRIP13 expression may be a therapeutic approach as well as important for identifying TRIP13-related signaling pathways. DCZ0415 is a small molecule inhibitor that has been shown to bind to TRIP13 by nuclear magnetic resonance (NMR) spectroscopy and pull-down, and is used to target TRIP13 and has shown anticancer activity in several cancer cells (13-15).

Transforming growth factor (TGF)-β is another molecule associated with malignancy in gliomas, and the signaling pathway involving TGF-β is associated with processes such as angiogenesis, invasion and stem cell maintenance in gliomas (16). In this respect, TGF-β signaling pathway is also considered an important therapeutic target (17). In this study, we aimed to investigate possible anticancer activity of DCZ0415, an inhibitor of TRIP13, which is also evaluated as an oncogene for GBM, in U87 cells and to evaluate this effect in terms of genes associated with apoptosis, invasion, and TGF-β signaling pathway.

MATERIALS AND METHODS

Cell Culture and DCZ0415 Treatment

Human U87 GBM cell line was obtained from American Type Culture Collection. U87 cells were cultured in DMEM containing 2 mM L-glutamine in the presence of 1 % penicillin/streptomycin and 10% FBS and the cells were grown at 37°C, 5% CO_2 and 95% humidity. DCZ0415 was purchased commercially (Biosynth, SPD47043) and stock solution was prepared with 0.1% DMSO in medium.

Cytotoxicity Assay

U87 cells were seeded in 96-well plates and after 24 hours, cells were incubated with 5, 10, 15, 20, 25, 50, 75, 100, 150 and 200 μM DCZ0415 for 24, 48, and 72 hours. And, XTT assay was performed as described in our study (18). The half-maximal inhibitory concentration (IC_{50}) of DCZ0415 was determined by GraphPad Prism software using % cell inhibition values.

Colony Formation Assay

In order to evaluate the effect of DCZ0415 treatment at IC_{50} dose on the colony forming abilities, U87 cells were seeded in 6-well plates. Then, the cells were cultured by changing the medium every other day for approximately 10 days. Afterwards, cells were treated with cold methanol and stained with crystal violet dye and colonies were counted.

Apoptosis Analysis

The effect of DCZ0415 treatment at IC_{50} dose on apoptosis was determined with FITC Annexin V method (BioLegend, 640.922). For this, cell suspensions were prepared using Annexin V Binding Buffer from control and DCZ0415-treated cells. Cell suspensions (100 μl) were transferred to test tubes. Then, FITC Annexin V and 7-ADD dye were added. After 15 minutes, samples were placed on a flow cytometer and percentages of early apoptosis, late apoptosis, and necrosis were determined.

qRT-PCR Analysis

The changes observed in mRNA level of genes associated with apoptosis, invasion, and TGF-β signaling pathway after DCZ0415 treatment at IC_{50} dose were evaluated by qRT-PCR analysis. For this, firstly, total RNA was isolated (GeneAll, 301-001) and then cDNA was synthesized (Bio-Rad, 170-8891). Preparation of the reaction components and setting up the reaction were performed as previously described

Table 1. Primer sequences of target and reference genes

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
CASP3	CTCTGGAATATCCCTGGACAAC	ACATCTGTACCAGACCGAGA
CASP7	GTCACCATGCGATCCATCAA	CGCCCATACCTGTCACTTTATC
CASP8	GATTCAGAGGAGCAACCCTATT	AGCAGAAAGTCAGCCTCATC
CASP9	CGACCTGACTGCCAAGAAA	GACAGCCGTGAGAGAGAATG
BAX	GTCACTGAAGCGACTGATGT	ACTCCCGCCACAAAGATG
BCL2	GGAGGATTGTGGCCTTCTTT	GTTCAGGTACTCAGTCATCCAC
CYCS	GGAGAGGATACACTGATGGAGTA	GTCTGCCCTTTCTTCCTTCTT
FAS	CTTTTCGTGAGCTCGTCTCTGA	CTCCCCAGAAGCGTCTTTGA
FADD	GACAGCATCGAGGACAGATAC	CTGTTGCGTTCTCCTTCTCT
TNFA	CCAGGGACCTCTCTCTAATCA	TCAGCTTGAGGGTTTGCTAC
TNFR1	CTCCTTCACCGCTTCAGAAA	GTCCACTGTGCAAGAAGAGAT
MMP2	GGCACCCATTTACACCTACA	CCAAGGTCAATGTCAGGAGAG
MMP9	GGGCTTAGATCATTCCTCAGTG	GCCATTCACGTCGTCCTTAT
TIMP1	GTCAACCAGACCACCTTATACC	TATCCGCAGACACTCTCCA
TIMP2	AAGGAAGTGGACTCTGGAAAC	CAGGCCCTTTGAACATCTTTATC
TGFβ1	CGTGGAGCTGTACCAGAAATAC	CTAAGGCGAAAGCCCTCAAT
TGFβR1	GTTCCGTGAGGCAGAGATTTAT	ACCAGAGCTGAGTCCAAGTA
TGFβR2	GTCGCTTTGCTGAGGTCTATAA	CTCTGTCTTCCAAGAGGCATAC
SMAD2	GGGACTGAGTACACCAAATACG	TACCTGGAGACGACCATCAA
SMAD3	CCTGAGTGAAGATGGAGAAACC	GGCTGCAGGTCCAAGTTATTA
SMAD4	TCCAGCATCCACCAAGTAATC	GCAGTGCTGGTAGCATTAGA
SMAD7	AGGCTGTGTTGCTGTGAA	TCCATCGGGTATCTGGAGTAA
ZEB1	GGCTCCTATAGCTCACACATAAG	TGCTGAAAGAGACGGTGAAG
ZEB2	CCCATTCTGGTTCCTACAGTTC	GGGAAGAACCCGTCTTGATATT
ACTB	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT

(18). Primers designed for target and reference genes are presented in Table 1.

Statistical Analysis

Data from experiments performed in triplicate are presented as mean ± SD. qRT-PCR results were analyzed using $2^{-\Delta\Delta CT}$ method by normalizing with ACTB reference gene. Control and dose groups were compared using t-tests in GraphPad Prism software. And, $P < 0.05$ was considered significant.

RESULTS

DCZ0415 Suppresses Proliferation and Colony Forming Capacity of U87 Cells

The effect of DCZ0415 on proliferation of U87 cells was investigated by XTT method. Accordingly, DCZ0415 suppressed the proliferation of U87 cells (Fig 1). IC_{50} doses of DCZ0415 for 24, 48 and 72 hours in U87 cells were found as 82.56 µM, 19.77 µM, and 13.16 µM, respectively.

Since it would be more convenient to treat U87 cells with DCZ0415 at a low dose and in a short time, in subsequent experiments, U87 cells were treated with 19.77 µM DCZ0415 for 48 hours. According to colony analysis results, DCZ0415 significantly inhibited the colony forming capacity of U87 cells ($P = 0.0011$) Colony numbers in control and DCZ0415-treated cells were found as 125.33±10.05 and 66.33±4.03, respectively (Fig 2).

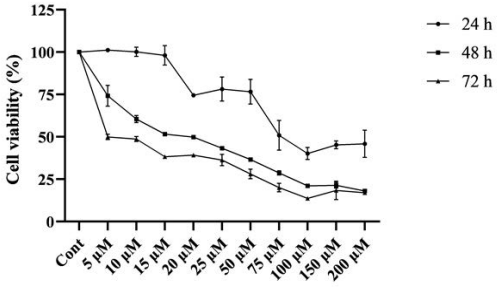

Figure 1. Effect of DCZ0415 treatment at different doses and times on U87 cell proliferation.

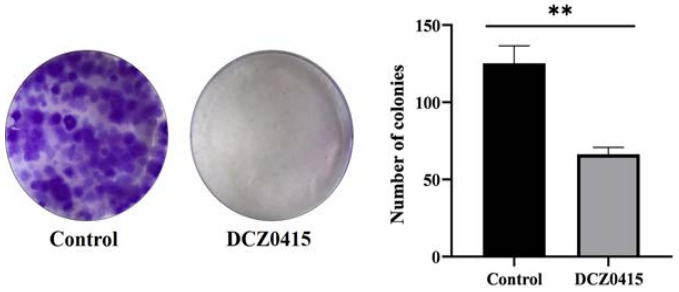

Figure 2. Effect of DCZ0415 treatment on colony forming capacity of U87 cells. **P < 0.01.

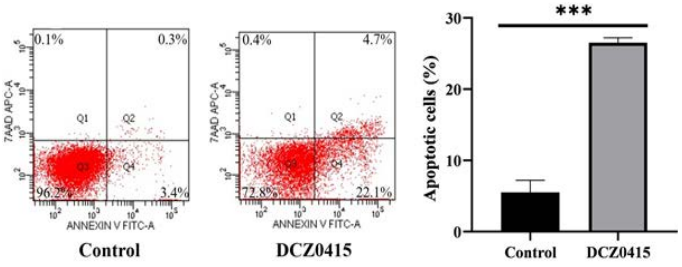

Figure 3. Effect of DCZ0415 treatment on apoptosis in U87 cells. ***P < 0.001.

DCZ0415 Induces Apoptosis in U87 Cells

Apoptotic effect of DCZ0415 was evaluated with flow cytometer-based FITC Annexin V method and the percentages of early apoptosis, late apoptosis, and necrosis were determined in the control and the DCZ0415-treated cells. Accordingly, % total apoptosis was determined as 5.5±1.52 in control group and 26.5±0.63 in DCZ0415-treated group. Accordingly, DCZ0415 treatment significantly increased the apoptosis rate in U87 cells (Fig 3; P < 0.001).

DCZ0415 Affects mRNA Level of Important Apoptosis Genes

The effect of DCZ0415 on mRNA levels of CASP3, CASP7, CASP8, CASP9, BAX, BCL2, CYCS, FAS, FADD, TNFA, and TNFR1 in U87 cells was evaluated by qRT-PCR analysis. Accordingly, a significant increase was observed in mRNA levels of CASP3, CASP9, BAX, and CYCS as 4.41 (P= 0.004672), 9.07 (P= 0.000238), 1.79 (P= 0.009565) and 9.7 (P= 0.000898) fold, respectively, in DCZ0415-treated cells compared to the control group (Fig 4).

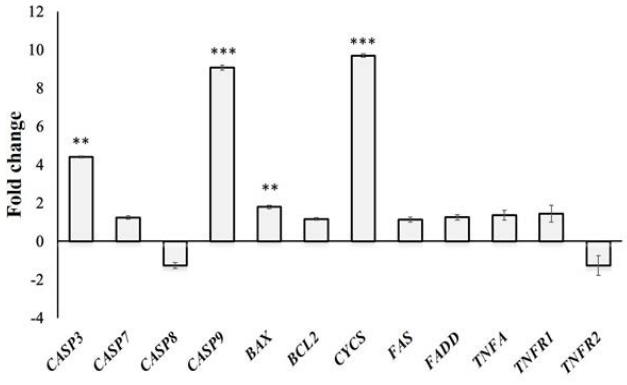

Figure 4. Effect of DCZ0415 treatment on apoptosis-related genes in U87 cells. **P < 0.01; ***P < 0.001.

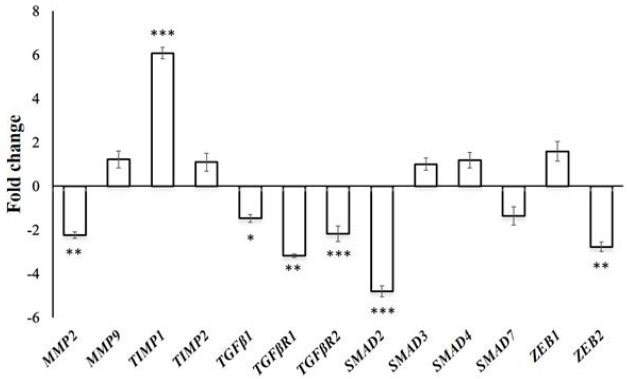

Figure 5. Effect of DCZ0415 treatment on invasion and TGF-β signaling-related genes in U87 cells. *P < 0.05; **P < 0.01; ***P < 0.001.

DCZ0415 Affects expression level of Invasion and TGF-β Signaling-Related Genes

The effect of DCZ0415 treatment at IC50 dose on mRNA levels of MMP2, MMP9, TIMP1, and TIMP2 genes which are associated with invasion, and, TGFβ1, TGFβR1, TGFβR2, SMAD2, SMAD3, SMAD4, SMAD7, ZEB1 and ZEB2 genes associated with TGF-β signaling were evaluated. Accordingly, after DCZ0415 treatment, MMP2 (2.23 fold; P=0.006202), TGFβ1 (1.47 fold; P= 0.018663), TGFβR1 (3.17 fold; P= 0.001715), TGFβR2 (2.17 fold; P= 0.000749), SMAD2 (4.81 fold; P= 0.000087) and ZEB2 (2.77 fold; P= 0.006447) gene expressions were significantly decreased while TIMP1 (6.08 fold; P= 0.000001) gene expression was significantly increased when compared to the control group (Fig 5).

DISCUSSION

Identification of new molecules that are effective in cancer development and determination of the relationship between these molecules and known signaling pathways are very important for improving the new treatment approaches. Recent studies have revealed that expression of TRIP13 gene is increased in many cancers, including GBM, and in this respect, TRIP13 is considered as a new oncogenic factor (12). In support of this, silencing of TRIP13 has been shown to have anticancer effects in various cancer types. It has been shown that TRIP13 expression is high in bladder cancer tissues, and increased TRIP13 expression is related to advanced tumor stage, metastasis and low survival. Silencing TRIP13

in bladder cancer cells resulted in suppression of proliferation, increase in apoptosis, and cell cycle arrest (9). Similarly, it has been shown that TRIP13 expression is high in ovarian cancer tissues and cells, and silencing of TRIP13 induces apoptosis and inhibits cell proliferation, invasion, and migration (6). Zhang et al. (5) also stated that TRIP13 plays an important role in tumorigenesis of GBM. And, silencing of TRIP13 in GBM cells has been shown to inhibit proliferation, migration, and invasion.

DCZ0415, whose anticancer activity was investigated in GBM cells in this study, is a small molecule inhibitor used to target TRIP13. In a study with multiple myeloma cells, DCZ0415 has been shown to inhibit cell growth and induce apoptosis. It has also been noted that the combination of DCZ0415 with melphalan or panbinostat has a synergistic effect (13). Similarly, it has been reported that DCZ0415 treatment reduces cell proliferation, arrests the cell cycle, and promotes apoptosis in colon cancer cells (14). Xu et al. (15) also stated that DCZ0415 suppresses proliferation, migration, and invasion of hepatocellular carcinoma cells. And, researchers have demonstrated that DCZ0415 has a synergistic effect with olaparib, a PARP1 inhibitor, in hepatocellular carcinoma cells.

In this study, we first performed cytotoxicity analysis to investigate the possible anticancer activity of DCZ0415 in U87 human GBM cells and treated the cells with different concentrations of DCZ0415 for 24, 48, and 72 hours. Accordingly, DCZ0415 suppressed U87 cell proliferation, and, IC_{50} dose of DCZ0415 for 48 hours was determined as 19.77 μM. We performed colony analysis after cytotoxicity analysis, and accordingly, treatment of DCZ0415 at IC_{50} dose for 48 hours also inhibited the colony forming capacity of U87 cells. These results demonstrate the suppressive effect of DCZ0415 on U87 cell proliferation.

One of the important indicators of anticancer activity is the induction of apoptosis. In other words, anticancer drugs are expected to induce apoptosis in cancer cells (19). In our study, the anticancer activity of DCZ0415 was evaluated in terms of apoptosis with the FITC Annexin V method, and accordingly, apoptosis was induced in GBM cells by DCZ0415 treatment at IC_{50} dose for 48 hours. In mammalian cells, apoptosis occurs in two ways as intrinsic and extrinsic pathways in which many proteins are involved, and these two pathways can be induced in anticancer activity (20). The intrinsic pathway, also called the mitochondrial pathway, is characterized by Cytochrome-c release from mitochondria to the cytoplasm, depending on the activity of Bcl-2 protein members. The release of Cytochrome-c leads to the formation of apoptosome, resulting in the activation of Caspase-9. And then, effector Caspases (Caspase-3, -6 and -7) are activated. Apoptotic cell death is triggered by the cleavage of substrates by effector Caspases (21). The extrinsic pathway is triggered by binding of extracellular ligands to tumor necrosis factor (TNF) receptor superfamily members, which are located on the cell surface and are called death receptors. Intracellular domains of death receptors, called the death domain, are effective in the transmission of the apoptotic signal (22, 23). In this study, the effect of DCZ0415 on apoptosis was also evaluated at the molecular level, and the expression levels of genes encoding important proteins that function in intrinsic and extrinsic pathways of apoptosis were evaluated by qRT-PCR analysis. Accordingly, DCZ0415 significantly increased mRNA levels of CASP3, CASP9, BAX, and CYCS genes, which encode Caspase-3, -9, Bax and Cytochrome-c proteins, are involved in intrinsic pathway of apoptosis, in GBM cells. In this respect, DCZ0415 is thought to be effective on the intrinsic pathway of apoptosis.

Distant organ metastases are rarely encountered in gliomas. However, the cells have property of infiltrative growth, which provides invasiveness to tumor. Tumor invasiveness is one of the most important causes of recurrence and GBM cells can invade as individually or collectively by remodeling the extracellular matrix (24, 25). Matrix metalloproteinases (MMPs) are enzymes that play a role in tumor invasion by degrading extracellular matrix components. MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (26). To evaluate the effect of DCZ0415 on invasion, we analyzed mRNA levels of MMP2, MMP9, TIMP1, and TIMP2 genes. And, DCZ0415 decreased MMP2 gene expression while increased TIMP1 gene expression. This result suggests that DCZ0415 may exert an antiinvasive effect in U87 cells by regulating mRNA levels of invasion-related genes.

In this study, anticancer activity of DCZ0415 in GBM cells was also evaluated in terms of the TGF-β signaling. TGF-β family members bind to two receptors in the cell. As a result of this binding, Smad2 and -3 are activated by phosphorylation and form a complex with Smad4. This complex imports to the nucleus and regulates expression of TGF-β target genes such as ZEB1 and ZEB2. One of the TGF-β target genes is Smad7 which acts as a negative regulator in this

pathway (27, 28). It has been shown that activation of the pathway is increased in aggressive and highly proliferating gliomas and this increase is associated with poor prognosis (29). In this study, the effect of DCZ0415 on mRNA levels of TGF-β signaling-related genes was evaluated and it was determined that TGF-β pathway was suppressed at mRNA level. This result shows that the anticancer effect of DCZ0415 in GBM cells may be related to suppression of TGF-β pathway. In addition, considering that DCZ0415 is a TRIP13 inhibitor, it is thought that there may be a possible relationship between TRIP13 and TGF-β signaling pathway.

In this study, the anticancer effect of DCZ0415, a small molecule inhibitor of TRIP13, which has been shown to have an oncogenic role in many cancers in recent years, was investigated in GBM cells. Our findings revealed that DCZ0415 suppresses proliferation and induces apoptosis in GBM cells, and DCZ0415 regulates the expression of genes associated with apoptosis, invasion, and TGF-β signaling. And, it is thought that TRIP13 can be evaluated as a new target for GBM and DCZ0415 can be an effective inhibitor in this respect. However, this study, which presents the first data on the activity of DCZ0415 in GBM cells, has several limitations, such as the fact that it was performed with a single cell line representing GBM, and changes in expression level of genes were investigated only at mRNA level. In order to fully elucidate the molecular mechanism of DCZ0415 in GBM cells, further studies are needed. In this respect, it is also believed that this study will contribute to further studies that will investigate the efficacy of DCZ0415.

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