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Research article

The inhibitory effect of indisulam-coumarin combined therapy on glioblastoma multiforme

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Abstract

Cancer is a disease that occurs due to irregular growth and proliferation of body cells and can be caused by many factors. One of these factors is carbonic anhydrase 9 (CAIX). While its expression is high in malignant cells, it is a molecule whose presence is difficult to detect in healthy tissues. Glioblastoma multiforme (GBM) is one of the fast-spreading brain cancers, and unlike healthy tissues, overexpressed CAIX in its cell receptors. Indisulam, one of the new-generation drug candidates for the treatment of solid tumours, is a type of CAIX inhibitor that affects cell division progression in human tumour cells. Similarly, it is known that coumarin, as one of the new-generation drugs in cancer treatment, is used together with chemotherapy. In this study, combined treatment of indisulam and coumarin was investigated on glioblastoma multiforme cells to evaluate their cytotoxicity, cell migration and antiproliferative effects. The effects of combined treatment on cell migration and proliferation were investigated with the IC_{50} values determined after the cytotoxicity test. As a result of the wound healing assay, it was determined that the control cells were closed by 69.6%, while the combined treatment closed the wound by 32% and seriously prevented cell migration. The percentage of proliferative cell nuclear antigen (PCNA) positive cells decreased significantly after combined treatment, cell proliferation was 93% in the control group and 77% in the combined treatment group.

Keywords: *Cancer; carbonic anhydrase IX; coumarin; indisulam; glioblastoma*

1. Introduction

Glioblastoma multiforme (GBM) is a primary brain tumour with a genetically and phenotypically heterogeneous group of tumours (Lam et al., 2000; Karcher et al., 2006). GBM has aggressive characteristics such as rapid proliferation, invasion into brain tissue, necrosis, angiogenesis, microvascular proliferation, and migration. The development of GBM is associated with the deregulation of the G1/S checkpoint in cell division and the emergence of numerous genetic disorders in glioma cells (Robert and Wastie, 2008; Hanif et al., 2017). The histological characteristics of glioma models, such as foci of tumour necrosis, nuclear polymorphism, and a high mitotic index, are like those of human GBM and Wistar rat GBM. When C6 cells are implanted into the brains of newborn rats, they mimic human GBM (Giakoumettis et al., 2018). The C6 glioma models investigate several biological processes, including the development, dissemination, migration, angiogenesis, control of growth factor production, and disruption of the blood-brain barrier in tumours (Giakoumettis et al., 2018; Li et al., 2020).

CAIX is a transmembrane glycoprotein that consists of an ECD, a single-pass transmembrane region, and an intracellular tail. It is one of three exofacial CA isoforms, along with CAIX, CAIV, and CAXII, that is closely linked to cancer. It has a unique position in the enzyme family due to its expression pattern associated with hypoxia. Required for energy metabolism of most tumour cells during hypoxia, glycolysis results in accumulation of lactic acid, lowering of pH, and acidification of the environment. The acidic microenvironment, which is characteristic of solid tumours with hypoxic regions, is

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associated with tumour invasiveness and adversely affects anticancer therapy (Stubbs et al., 2000; Gatenby and Gillies, 2004; Kroemer and Pouyssegur, 2008).

The sulfonamides are bonded in a tetrahedral geometry. In its deprotonated forms, it interacts directly with catalytic zinc and inhibits CA activity by displacing zinc-bound water hydroxide ions. Due to its chemical structure, indisulam is one of the best examples of carbonic anhydrase inhibitors (Cuffaro et al., 2020). The inhibitory effect of indisulam, a new anti-cancer drug currently in phase II clinical development for the treatment of solid tumours, is associated with a cell division arrest in the G1 phase. Specific inhibitors such as indisulam establish a definitive pathway for CAIX in tumorigenesis, making the mechanism reversible. Therefore, selective CAIX inhibitors illuminate the role of CAIX in controlling pH imbalance in tumour cells in hypoxic cancers and developing diagnostic therapeutic applications for tumour management (Supuran, 2003).

Coumarins are polyphenolic chemicals that are members of the group of colorless heterocyclic compounds discovered in the plant *Dipteryx odorata* Wild. Coumarin-based carbonic anhydrase inhibitors are studied in association with the tumour-associated isoforms CAIX and CAXII. Apart from being a CAIX inhibitor, coumarin has a wide range of pharmacological effects such as anti-tumour, anti-coagulant, anti-inflammatory, antioxidant, anti-HIV and anti-bacterial. Recent studies highlight the potential for use of gambling CAIs in combination with standard chemotherapies in cancer therapy (Touisni et al., 2011; Sumorek-Wiadro et al., 2020).

CAIX inhibition is attributed to coumarin among the selected active ingredients (Ismail, 2023). A series of sulfonamide designs containing coumarin moieties have been discussed for combining these two active ingredients. Also, indisulam is an orally active agent that has anti-cancer properties and induces p53 and p21 through the downregulation of cognate cell division checkpoint molecules. It is a sulfonamide anti-tumour agent (Moncao et al., 2022). The combined use of indisulam and coumarin active ingredients has been evaluated by Chahal et al. (2022) with a database that predicts the most likely macromolecular targets of a presumed bioactive small molecule by using Swiss Target Prediction tool of Swiss Institute of Bioinformatics.

This study investigates whether the two compounds chosen are active on the CAIX enzyme. The dynamic table in the database provides the predicted target molecules for the queried molecule. The result obtained indicates the probability of targeting indisulam on CAIX and coumarin on CAIX (Teixeira et al., 2021; Pontecorvi et al., 2022). When the required scanning was performed for coumarin, the probability ratio against the CAIX enzyme was 0.86. As a result of scanning for indisulam, the probability ratio against the CAIX enzyme is 0.83. A probability value of 1 indicates that targeting is quite strong (Chahal and Kakkar, 2023).

In this study, indisulam+coumarin combined therapy was investigated on GBM cells in terms of cytotoxic effect, wound healing and antiproliferative properties. The main reason for the preference for the indisulam+coumarin combination was their anticancer activities. Carbonic anhydrase IX inhibition is attributed to coumarin among the selected active ingredients (Ismail, 2023). A series of sulfonamide designs containing coumarin moieties have been discussed for combining these two active ingredients. Also, indisulam is an orally active agent that has anti-cancer properties and induces p53 and p21 through the downregulation of cognate cell division checkpoint molecules. It is a sulfonamide anti-tumour agent (Moncao et al., 2022). When comparing the results of the combined treatment with indisulam and coumarin, the cytotoxic effect exhibited similarity to indisulam, surpassing the effectiveness of coumarin. Combined therapy decreased wound healing in cancer cells compared to the control group and significantly reduced proliferation. Indisulam+coumarin combined therapy was overall more effective than single-drug therapy for C6 GBM cells.

2. Materials and methods

Indisulam, coumarin, thiazolyl blue tetrazolium bromide %98 (MTT), trypsin EDTA and DMSO were obtained from Sigma Aldrich (St. Louis, USA). Gibco provided the Dulbecco's Modified Eagle Medium (DMEM) / F-12 Nutrient Mixture (Ham), L-glutamine, Penicillin-streptomycin (PEST), and Fetal Bovine Serum (FBS). The C6 glioblastoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Thermo Scientific™ supplied the proliferating cell nuclear antibody (MS-106-P). All the chemicals and solvents used were of analytical quality and were not purified further.

2.1. Cell culture studies

C6 glioma cells (ATCC-CCL-107) were cultured in a Dulbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 medium (1:1) with 5% fetal bovine serum (FBS), 100 µg/mL Penicillin-Streptomycin and 0.2 mM L-Glutamine. Cells were kept at 37°C in a humidified incubator with 5% CO_2. Fresh growth media was used to replenish the cells' growth medium 2 to 3 times each week. During the incubation period, the cells were passaged every two to three days.

2.2. Cytotoxic effect of indisulam, coumarin and indisulam+coumarin combine therapy

The MTT 3-(4,5-dimethyl-thiazolyl 2,5-diphenyltetrazolium bromide) is a sensitive reliable indicator of cellular metabolic activity and is used to determine and measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity (Welder et al., 1991). To determine cell viability levels and cytotoxic activity of indisulam, coumarin and indisulam+coumarin combined therapy on C6 cells, MTT assay was used.

Confluent C6 cells were removed from the plate by trypsinization, and they were seeded at $1x10^4$ cells/mL in 96-well culture plates. The microplate was incubated at 37°C in a 5% CO_2 incubator for 24 hours. At the end of the incubation time, the medium was replaced with a fresh medium containing different concentrations (10, 25, 50, 100, 250, 500, µM) of indisulam, coumarin and indisulam+coumarin combinations, and no application was made to the control group. Microplates were incubated for 24 and 48 hours, and at the end of the incubation times, the medium was discarded from each well and 50 µL of MTT reagent was added. Cells were incubated for 3 hours at 37°C with 5% CO_2. After 3 hours, the MTT solution was removed from the cells and the formazan crystals were dissolved by adding 100 µL of DMSO. Absorbance values (OD) were measured with a microplate reader at 570 nm and the cell

viability value was calculated with the following equation after 3 repetitions.

Cell Viability (%) = OD570e / (OD 570b) * 100

OD570e: It is the absorbance value of the samples at 570 nm.

OD570b: It is the absorbance value given by the control group at 570 nm.

The concentration values were calculated that caused 50% inhibition (IC_{50}) of cells with the % cell viability results obtained.

2.3. Wound healing assay

A wound healing test is a common *in vitro* method for studying collective cell migration. In this test, a cell-free region is formed in a confluent monolayer either by physical, mechanical, thermal, or chemical damage (Jonkman et al., 2014). C6 glioblastoma cells were seeded in a 24-well plate as a $1x10^5$ cells/well and incubated for 24 hours until cells attached to the plate to form a confluent monolayer. A sterile 200 µL pipette tip was used to make a physical scratch in the center of each monolayer cell well. To eliminate non-adherent cells and debris, cells were gently washed with new media. The IC_{50} values for indisulam, coumarin, and indisulam+coumarin combinations were applied to the wells. Images were obtained immediately after injury (0 h), and 6 hours, 12 hours, and 24 hours afterward. GraphPad Prism was used to quantify the closure distance of the scratch after randomly selecting photos from each well using phase-contrast inverted microscopy (X10 magnification).

2.4. Determination of cell proliferation with PCNA method

The proliferating cell nuclear antigen is an evolutionarily well-conserved protein found in all eukaryotic species and Archaea (Strzalka and Ziemienowicz, 2011). PCNA was first shown to function as a transactivation factor of DNA polymerase δ, which is required for DNA synthesis during replication (Prelich et al., 1987). PCNA gene expression is involved with cell proliferation and consequently DNA synthesis during genome replication during the S phase of cell division in all species. PCNA forms a homotrimeric ring around the double helix of DNA and acts as a movable sliding clamp to attract other proteins involved in cell division control as well as DNA synthesis and repair (Yu et al., 2013). PCNA can stop damaged cell DNA replication and repair the damage. In cells that proliferate, that is, increase because of cell growth and cell division, PCNA levels rise in the middle of the G1 phase and remain high throughout the S phase. For PCNA, the DNA replication protein PCNA, which is synthesized in the G1 and S phases of the cell division, shows proliferation with the presence of anti-PCNA monoclonal antibodies. PCNA is a protein used in the analysis to show the proliferation rate. Cells in all phases of the cycle are marked with PCNA immunocytochemistry (Matsumoto et al., 1987). Cells in all phases of the cycle are marked with PCNA immunocytochemistry.

Proliferation was determined using the PCNA antibody and the Invitrogen Histostain-Plus Kit (Cat:85-9043). C6 cells were grown as $1x10^5$ cells/well on sterile coverslips in a 24-well plate and incubated for 24 hours until cells joined to the plate to create a confluent monolayer. The cells were then treated with 24h IC_{50} values of indisulam, coumarin, and indisulam+coumarin and incubated at 37°C for 24 hours. The media was taken from the wells at the conclusion of the incubation time, and the cells were rinsed with phosphate-buffered saline (PBS). For 5 minutes, the cells were fixed in ice-cold methanol. After the samples fixed with methanol, they were washed with PBS for 5 minutes, a blocking solution was added to each sample and incubated at room temperature for 20 minutes. After blocking, PCNA primary antibody prepared in PBS, and it was added to the samples at a ratio of 1/150. Antibody-added samples were incubated at room temperature for 2 hours and they were washed 3 times for 5 minutes with 1xPBS. Following washing with PBS, a biotinylated secondary antibody was added to the samples and incubated for 20 minutes at room temperature and washed 3 times for 5 minutes with 1x PBS. After the washing process, Streptavidin-peroxidase (HRP) was applied to the samples and incubated for 20 minutes at room temperature and washed again with 1x PBS for 3x5 minutes. After the last washing process, AEC chromogen (Invitrogen, USA) was added to the samples and incubated for 10-20 minutes at room temperature. Cells labelled with PCNA were stained and hematoxylin-eosin was used as counterstain. Coverslips, containing the PCNA positive and negative cells, was covered on the slides with a mounting medium and analysed under a light microscope. Proliferation levels were determined by identifying marked and unlabeled cells in 10 randomly selected areas at 100x magnification with an immersion oil objective. The percentage of immune-reactive cells was calculated with the formula.

Cell Proliferation (%) = (Number of PCNA positive cells)/(Number of total cells) *100

2.5. Statistical analysis

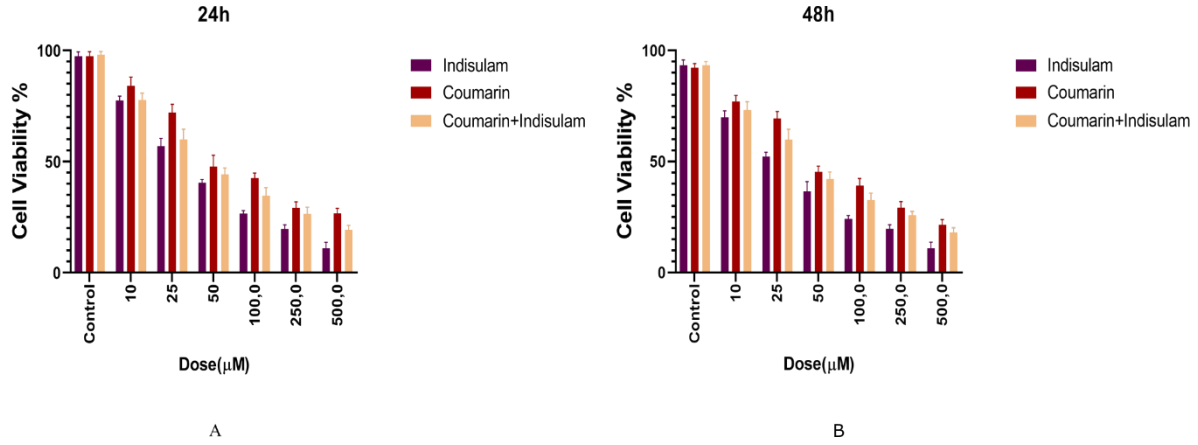
GraphPad Prism software was used for all statistical analyses (GraphPad Software, La Jolla, CA). Data were presented as mean standard deviation (SD), and ANOVA was used to compare groups, with $p<0.05$ deemed statistically significant. ns indicates that the p-value is greater than 0.05 and is statistically insignificant; *, **, ***, and **** represent increasingly significant statistical data, respectively.

3. Results and discussion

3.1. Cytotoxic effect of indisulam, coumarin and indisulam+coumarin combine therapy

In a study using indisulam as the active ingredient, the application of indisulam impaired a cell division progression of P388 murine leukemia cells in the G1 phase. Complete regression of advanced LX-1 tumours was observed in 80% of mice treated with E7070 (Ozawa et al., 2001).

The long-term survival of a patient with metastatic melanoma treated with indisulam is elaborated in the study conducted by Baur et al. (2007) supports this study and the patient with metastatic malignant melanoma was treated at varying indisulam doses. While the tumour burden was quite high at the start of treatment, after 2.5 years of treatment with Indisulam, the tumour appeared to shrink significantly. Finn et al. (2002) conducted a comparative trial of paclitaxel, doxorubicin and coumarin anti-cancer agents in HEp-2, HCT-8 and Caco-2 cell lines. Coumarin alone has been shown to be less effective in growth inhibition in all three tumour cell lines. However, it has been revealed that coumarin, which creates different mechanisms in each cell line, has lower cytotoxicity



A B

Fig. 1. The IC_{50} values of indisulam, coumarin and indisulam+coumarin treatment on C6 glioma cells at the end of the 24th (A) and 48th (B) hour with the cytotoxicity. $IC_{50\ Coumarin}$: 45.98 µM, $IC_{50\ Indisulam}$: 32.64 µM, $IC_{50\ Combined}$: 39.11 µM for 24th hour; $IC_{50\ Coumarin}$: 39.80 µM, $IC_{50\ Indisulam}$: 28.34 µM, $IC_{50\ Combined}$: 31.41 µM for 48th hour.

compared to other anti-cancer drugs applied.

In this study, the IC_{50} values of indisulam in C6 Glioma cells were determined as 32.64 µM and 28.34 µM for 24 and 48 hours, respectively (Fig. 1A-B). In support of the experimental data obtained, it is seen that similar IC_{50} values were obtained because of MTT in the HCT116-C9 human colon cancer cell line (Oda et al., 2003).

The IC_{50} values of coumarin in C6 Glioma cells were determined as 45.9 µM and 39.80 µM for 24 and 48 hours, respectively (Fig. 1A-B). Experimentally obtained IC_{50} values are consistent with the study realized by Gkionis et al. (2021) comparing the cytotoxicity of coumarin analogues on breast cancer.

The combined application has been preferred for the purpose of providing both cytotoxicity balance and therapeutic effect. While indisulam alone has limited anti-tumour effects, studies evaluating its effects in combination with other chemotherapeutic agents have provided significant results. Because of this coumarin is included in this study as the before mentioned bioactive and chemotherapeutic agent (Touisni et al., 2011). The IC_{50} values of indisulam+coumarin combined treatment on C6 glioma cells were determined as 39.11 µM and 31.41 µM for 24 and 48 hours, respectively (Fig. 1A-B). In the literature, there is no study yet on the C6 GBM cells with combined treatment of indisulam+coumarin.

3.2. Cell proliferation assay with PCNA method

PCNA immunohistochemical analysis was performed to determine the effect of indisulam, coumarin and indisulam+coumarin combined treatment on the proliferation on C6 Glioma cells. In the application with 24-hour IC_{50} doses, it was observed that indisulam, coumarin and indisulam+coumarin treatment decreased the proliferation rate of the cells compared to the control group.

A study involving mice demonstrated that coumarin inhibits cell proliferation in skin papillom (Bhattacharyya et al., 2010). Another study reported that coumarin ameliorates benign prostatic hyperplasia by inhibiting the progression of G1/S phase cell turnover (Imani et al., 2021). It was also clarified that indisulam disrupts cell proliferation by targeting RBM39, a crucial mRNA splicing factor (Bussiere et al., 2020).

In this study, the percentage of proliferation was 93% in the control group compared to the indisulam and coumarin,

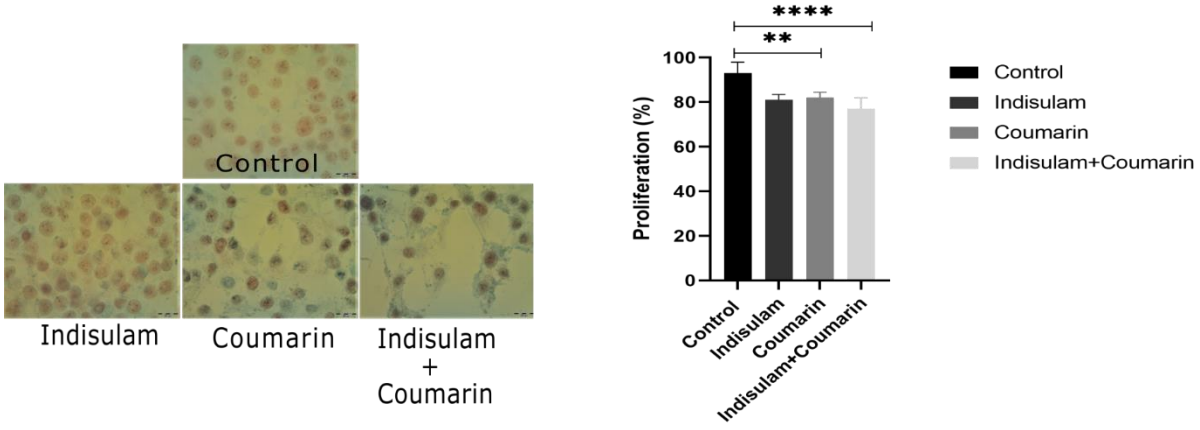


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Fig. 2. Indisulam, coumarin, and indisulam+coumarin therapy inhibited the proliferation of C6 Glioma cells using proliferating cell nuclear antigen (PCNA) labeling. Quantification of PCNA-positive immunostained cells demonstrated that the indisulam+coumarin combination therapy significantly reduced cell proliferation for 24 hours compared to the control group (****$p \leq 0.0001$) (B). PCNA-positive tagged cells (red) are visible in the brightfield microscope pictures (A). The data were reported as means standard deviations (n=3) for each group.

which were 81% and 82%, respectively. While these two compounds alone did not make a significant difference, when applied together, the percentage of proliferation decreased by 23% and was determined as 77% (Fig. 2B). This shows that the combined therapy suppresses the growth of cancer cells compared to single-dose therapy. According to the results obtained, the combined therapy of coumarin and indisulam on C6 glioma cells significantly reduces the proliferation rates of cells entering the progression of the cell division and causes the cells to be more susceptible to apoptosis after these active substances applications.

3.3. Wound healing

Wound healing assay has been preferred because it is low cost and the easiest test for cell migration *in vitro*. This method allows to simulate cell migration of an artificial wound created with a pipette tip and to examine cell-cell, active substance-cell interactions (Rodriguez et al., 2005). The most common information to come from the wound healing assay is the gap closure rate as a measure of the collective movement rate of cells. Wound healing was determined as 69.6% in the control group. Sayed et al. (2021) was stated that sulfonamides, which are the group including indisulam, can be used to reduce wound infection in addition to providing wound healing on rats. Lee et al. (2006) determined that certain coumarin molecules synthesized by different methods showed growth inhibition activity against HUVEC cells.

In this study, there was 57.38% wound closure with coumarin therapy, and 50.94% wound closure with indisulam therapy. The closure value of the combined application group was determined as 32% (Fig. 3). The reason for the almost complete closure of the wound created with a pipette tip in the control group in the C6 glioma cell line after 24 hours is the lack of an anti-tumour agent. The reason cells treated with indisulam and coumarin showed less closure at the end of 24 hours is that it causes inhibition of cell migration in a concentration-dependent manner. Microtubule binding agents are exemplary as anti-migration agents. In the study in which the effectiveness of migration inhibitors was measured, it was stated that active substances with anti-cancer activity such as doxorubicin and paclitaxel prevented wound healing compared to the control group (Wang et al., 2019). The combination of indisulam and coumarin can be classified as an anti-migration agent in tumour cells.

4. Conclusion

Indisulam inhibits cyclin-dependent kinases (CDKs), which control cell division progression and are frequently overexpressed in cancer cells. Inhibiting CDK causes cell division G1/S phase arrest, which can lead to induction apoptosis and tumour cell proliferation inhibition. It has been determined that coumarin induces apoptosis by causing morphological changes in human cervical cancer HeLa cell line studies (Chuang et al., 2007). The most important reason the coumarin active ingredient is preferred is that it is a natural inhibitor. Anti-tumour mechanisms of coumarin-derived compounds are remarkably diverse; inhibition of carbonic anhydrase (CA), targeting of PI3K/Akt/mTOR signaling pathway, inhibition of multi-drug resistance (MDR), and induction of apoptosis are some of them (Feitelson et al., 2015). A common effect of coumarin and indisulam is inhibition of CAIX. Inhibition of CAIX is another target because it is highly expressed in tumour cells (Fukuoka et al., 2001). The main goal is to identify less toxic and more effective anti-cancer agents. In these contexts, indisulam and coumarin were preferred in this study among the CAIX enzyme inhibitors, whose expression increased in the tumour microenvironment and in the tumour region (Abel and Baird, 2018).

According to the obtained data, indisulam and coumarin have anti-proliferative and cytotoxic activity in the C6 glioma cell line. But also, indisulam+coumarin combined therapy results showed us that combined therapy is more effective on C6 glioma cells. IC_{50} values were determined as 39.11 μM for 24th hour and 31.41 μM for 48th hour, respectively for combined therapy. As a result of the wound healing and proliferation studies with IC_{50} doses, it was determined in wound healing that the control cells closed at the rate of 69.6%, while the combined treatment closed the wound by 32% and seriously prevented cell migration. On the other hand, the percentage of proliferative cell nuclear antigen (PCNA) positive cells decreased significantly

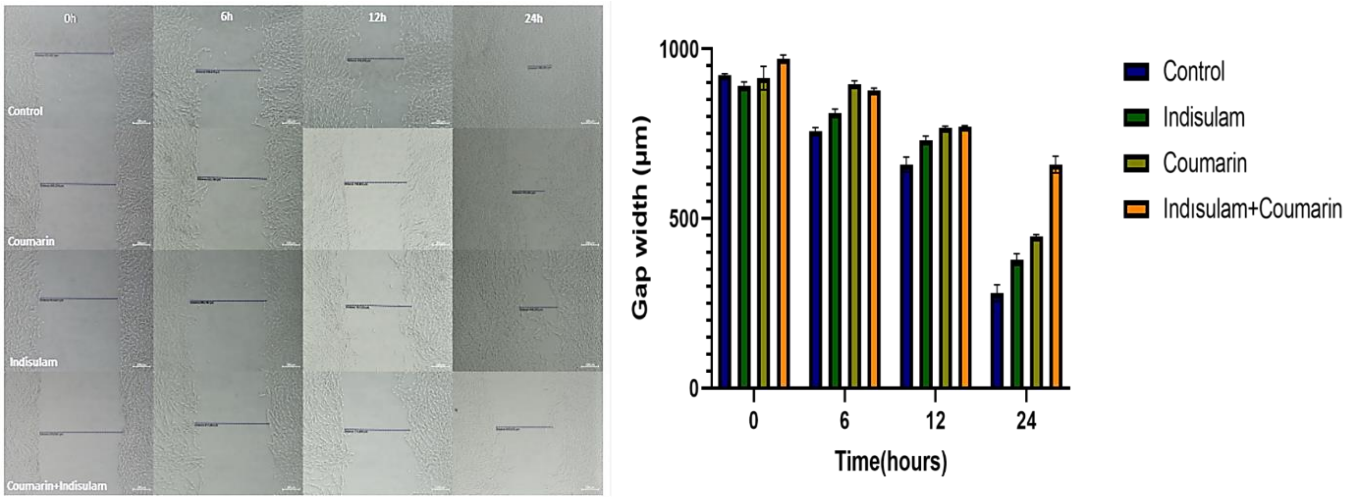

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Fig. 3. Effect of indisulam, coumarin and indisulam+coumarin combine treatment on C6 glioblastoma cell migration was determined by wound healing assay (A). Quantification of migration after 6, 12 and 24h treatment with indisulam, coumarin and indisulam+coumarin (n = 3) compared to control (B).

after the combined treatment, with cell proliferation regressed from 93% in the control group to 77% in the combined treatment group.

The results of the study show that the combined therapy of indisulam and coumarin can be used as an alternative chemotherapeutic therapy in the treatment of brain tumours. Inhibition of the CAIX enzyme, which participates in the common mechanism of action of indisulam and coumarin, in the tumour microenvironment may lead to original studies that will shed light on alternative treatments. The results of this study should be enriched with *in vivo* and clinical trials.

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