



The multifaceted antitumor effects of the ALK5 inhibitor BI 4659 in colon cancer: An *in vitro* study of cytotoxicity, clonogenicity, migration, and 3D spheroid formation

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Abstract

Purpose: Inhibition of ALK5, the main receptor of the TGF- β pathway, is one of the new strategies in colon cancer treatment. In this study, the anticancer effects of the small molecule BI 4659 were investigated in the colon cancer cell line Caco-2.

Method: Caco-2 cells were treated with increasing doses of BI 4659 for 72 hours. WST-1 was used for the viability assay, colony formation was used to observe the long-term effect, wound healing was used to determine migration ability and hanging drop assays were performed for 3D tumors.

Findings: BI 4659 exhibited high cytotoxicity in a dose-dependent manner in the Caco-2 cell line (IC₅₀ = 29.33 \pm 2.35 nM). Treatment resulted in significantly reduced colony formation, migration ability, and 3D spheroid size (p < 0.05).

Conclusion: Treatment with BI 4659 produced a multifaceted antitumor effect in the Caco-2 cell line. Inhibition of ALK5 reduced proliferation, migration, and 3D organization. These results suggest that BI 4659 may be a potential candidate for colon cancer treatment. Further experiments are recommended to elucidate the underlying molecular mechanisms and demonstrate *in vivo* effects.

Keywords: colon cancer, ALK5 inhibitor, BI 4659, TGF- β , cell migration, tumor spheroids

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Kolon kanserinde ALK5 inhibitörü BI 4659'un çok yönlü antitümör etkileri: sitotoksosite, klonojeniklik, göç ve 3D sferoid oluşumu üzerine bir *in vitro* çalışma

Özet

Amaç: TGF- β yolunun ana reseptörü olan ALK5'in inhibe edilmesi, kolon kanseri tedavisinde yeni stratejilerden birisidir. Bu çalışmada kolon kanser hücre hattı Caco-2'de küçük molekülü BI 4659'un antikanser etkileri incelendi.

Metod: Caco-2 hücreleri artan dozlarda BI 4659 ile 72 saat süre ile tedavi edildi. Canlılık deneyi için WST-1, uzun süreli etkiyi görmek için koloni formasyon, migrasyon yeteneğini belirlemek için Wound healing, 3B tümör için ise hanging drop deneyleri yapıldı.

Bulgular: BI 4659, Caco-2 hücre hattında doza bağlı olarak yüksek sitotoksosite gösterdi (IC₅₀ = 29.33 \pm 2.35 nM). Tedavi ile koloni oluşumu, migrasyon yeteneği ve 3B sferoit büyüklüğü anlamlı düzeyde düşük çıktı (p < 0.05).

Sonuç: BI 4659 tedavisiyle Caco-2 hücre hattında çok yönlü antitümör etki ortaya çıkmıştır. ALK5'in inhibisyonu proliferasyon, göç ve 3B organizasyonunda baskılayıcı etki göstermiştir. Bu sonuçlar kolon kanseri tedavisinde BI 4659'un potansiyel bir aday olabileceğini göstermektedir. Altta yatan moleküler mekanizmaların ve *in vivo* etkilerin gösterilmesi için ileri deneylerin yapılması tavsiye edilmektedir.

Anahtar kelimeler: kolon kanseri, ALK5 inhibitörü, BI 4659, TGF- β , hücre göçü, tümör sferoidleri

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1. Introduction

Cancer is a group of diseases that are difficult to treat, have devastating effects, and have significant consequences for humanity. The number of colon cancer cases and the mortality rates they cause are increasing worldwide for various reasons. Colorectal cancer, the third most common cancer type in both men and women, is expected to continue to increase. The five-year survival rate has been reported to be around 67% [1]. The number of cases is expected to double by 2040 [2]. Because it often does not present symptoms in the early stages, most cases are diagnosed at a later stage. In conventional treatment, surgery and chemotherapy are the primary options. [3] As with many types of cancer, despite numerous treatment methods, sufficient success has not been achieved in this type of cancer. Therefore, new treatment approaches are needed [4]. In addition to the fact that current treatment methods have failed to demonstrate sufficient efficacy, the need for alternative treatment methods is growing due to the serious side effects they cause. Inhibitors, which offer high selectivity and tolerability compared to traditional treatments, have recently attracted significant attention. To date, nearly 100 small-molecule inhibitors have been approved for use in cancer treatment. In this regard, small-molecule inhibitors hold great promise [5,6].

In this context, the discovery of new inhibitors is of interest. Activin receptor-like kinase-5 (ALK5) is a member of the tumor growth factor family. It plays a role in processes such as cell growth, proliferation, and differentiation. Elevated levels of the ALK5 protein are known to be associated with various diseases, including cancer. This makes the ALK5 protein a potential target. Selective ALK5 inhibitors affect cancer formation, cancer prognosis, and the metastatic ability of cells [7]. Selective ALK5 inhibitors are known to play a regulatory role in tumor formation, prognosis, and metastatic effects. The significant effects of the TGF- β signaling pathway in cancer development play a key role in the interest in ALK5 inhibitors as a therapeutic target. In colon cancer, TGF- β /ALK5 activation is known to be associated with poor prognosis and chemotherapy resistance. Therefore, pharmacological inhibition of ALK5 has the potential to inhibit cancer cell proliferation as well as prevent cancer progression and spread (metastasis). ALK5 inhibitors, which are part of targeted treatment strategies, are being evaluated as innovative treatment approaches [8]. It is emphasized that ALK5 levels in cancer cells are closely associated with treatment resistance [9]. Various studies indicate that ALK5 inhibitors exhibit cytotoxic, anticlonogenic, and anti-migratory effects in cancer cells [10,11].

BI 4659 is a highly selective, small-molecule ALK5 inhibitor developed by Boehringer Ingelheim and is currently in the preclinical research phase [12]. BI 4659, which is used in the treatment of various diseases, has not been sufficiently studied in the treatment of colon cancer. [13].

This study aims to evaluate the cytotoxic and apoptotic effects of BI 4659, a selective ALK5 inhibitor, in the human colon cancer cell line Caco-2. To this end, experiments were conducted on the proliferation, invasion, and tissue-like formation capabilities of cells, going beyond the cytotoxic effect at the single-cell level.

2. Materials and methods

2.1. Chemicals, Reagents

ALK5 inhibitor (BI 4659) was kindly provided by Boehringer Ingelheim via its open innovation platform *openMe* (available at <https://openme.com>) Phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, trypsin-EDTA, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Trypan blue, dimethyl sulfoxide (DMSO), methanol, acetic acid, and ethanol were obtained from Sigma Aldrich (St. Louis, MO, USA). 25 and 75 cm² cell culture flasks (Sarstedt, Numbrecht, Germany); microcentrifuge tubes, 15 and 50 mL Eppendorf tubes (Isolab, Eschau, Germany); 6- and 96-well microplates (Corning, NY, USA); 5-, 10-, and 25-mL sterile pipettes and plastic pipette tips, 3- and 6-cm Petri dishes (Costar, Washington, DC, USA).

2.2. Cell culture

The Caco-2 colon cancer cells used in the study were obtained from the Cancer Research Laboratory at Bingöl University. All cell culture studies were performed in accordance with GCCP (Good Cell Culture Practice) standards. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 64 μ g/mL penicillin, and 100 μ g/mL streptomycin in a humidified cell culture incubator at 37°C containing 5–6% CO₂.

2.3. Cytotoxicity analysis

A WST-1 viability assay was performed to determine the changes observed in the Caco-2 cell line following the applied treatment. This assay involved the following steps: 8,000 cells were seeded into each well of a 96-well plate. After confirming that the cells had adhered, increasing doses of (0, 1, 2, 4, 8, 16, 32, 64, and 128 nM) BI4659 inhibitor and (0, 1, 2, 4, 8, 16, 32, 64, and 128 μ M) cisplatin for 72 hours. At the end of 72 hours, the DMEM was removed from

the wells, and 90 μL of DMEM and 10 μL of WST-1 solution were added to each well, followed by 3 hours of incubation. At the end of the incubation period, the plates were read at 480 nm using an ELISA microplate reader. The formula used to calculate the percentage of viability was: [Optical density (OD) of treated cells / Optical density (OD) of control cells] \times 100 [14].

2.4. Clonogenic assay

A colony formation assay was performed to determine the altered colony-forming ability of Caco-2 cells following the administered treatments. For this purpose, 2×10^3 Caco-2 cells were seeded into each well of 6 cm Petri dishes. After ensuring that the cells had adhered, treatment was performed for 72 hours with BI 4659 at doses of 4 and 8 nM and cisplatin at a dose of 4 μM . At the end of the period, the drug-containing DMEM was removed and fresh DMEM was added. After 9-12 days of incubation, the necessary washes were performed, and the cells were exposed to the fixation solution for 5 minutes. Following fixation, after 15 minutes of staining with 0.5% crystal violet, more than 50 cell clusters were considered colonies. The colony changes resulting from the treatment effect were analyzed by comparison with the control group [15].

2.5. Wound healing assay

A wound healing assay was performed to observe the altered migration ability of Caco-2 cells following appropriate treatment [16]. For this experiment, 4×10^5 Caco-2 cells were seeded into each well of a 6-well plate. When the cell density reached 80-90%, a linear wound area was created in the wells using a 100 μL sterile pipette tip. The surface was washed twice with PBS to remove the shielding cells. Photographs were taken under a microscope at 0 and 24 hours to detect changes in migration ability depending on the treatments. The obtained photographs were analyzed using the Image J program.

2.6. Hanging drop

The hanging drop method is a fundamental technique with various applications in cancer research and cell biology [17]. Ramsey and colleagues' method was used as a reference for applying this technique [18]. In summary, cells were seeded in drug-containing DMEM onto the plate cover so that each drop contained 25,000 cells. At the end of the period (5 days), the droplets were left intact, and the spheroids were directly imaged through the cover using an inverted microscope. The images were recorded for morphological evaluation.

2.7. Statistical analysis

Unless otherwise stated, all experiments were repeated three times. Graphical and statistical analyses were performed using GraphPad Prism (GraphPad Software, USA). The obtained data were analyzed using Tukey's multiple comparison and One-Way ANOVA tests. Those with a p-value < 0.05 were considered statistically significant.

3. Results

3.1. Cell viability

The graph and microscope image of the WST-1 viability assay performed to measure the cytotoxic effect of the inhibitor and cisplatin used in Caco-2 cells after 72 hours are shown in Figure 1. According to the results obtained, the ALK 5 IC₅₀ value was found to be 29.33 ± 2.35 nm and cisplatin 30.67 ± 1.99 μm .

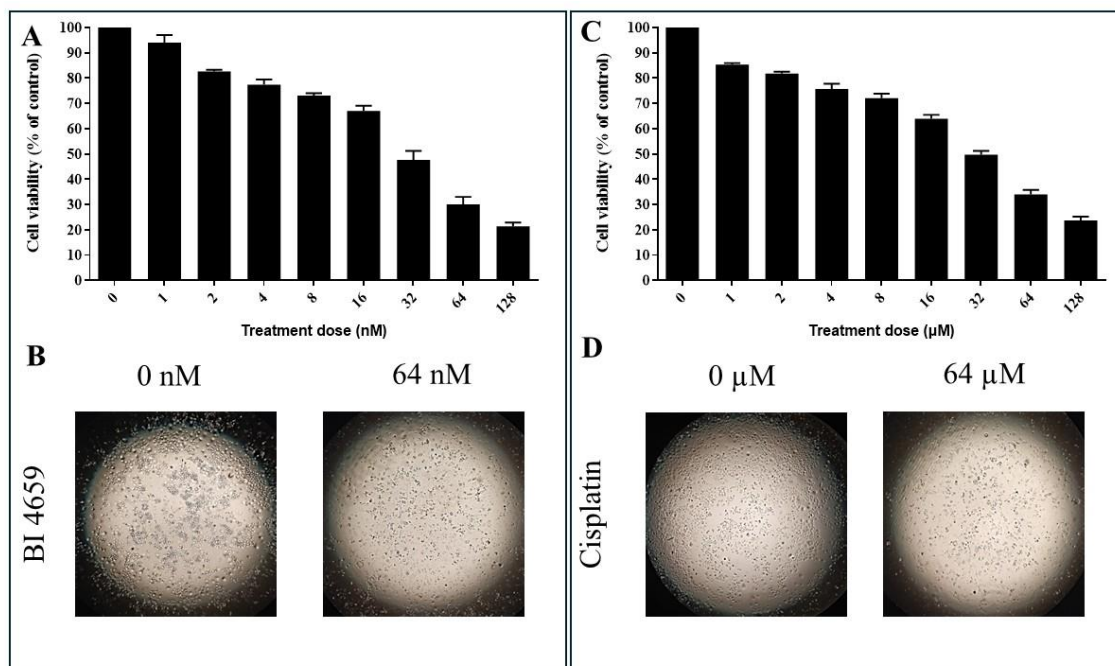


Figure 1. Effects of BI 4659 inhibitor application on cell viability and morphology in Caco-2 cells. (A) Dose-response curves showing the cytotoxic effects of increasing doses of BI 4659 (0–128 nM) and (C) cisplatin (0–128 μM) after 72 hours. (B), (D) Representative microscopic images showing changes in cell density and morphology after application of the control group and (64 nM) BI 4659 and 64 μM cisplatin. Data are presented as mean ± standard deviation (SD)

3.2. Clonogenic assay

Graphs and visuals from colony formation assays conducted to determine the long-term effect of BI 4659 inhibitor on the Caco-2 colon cancer cell line are presented in Figure 2. Considering the IC50 value obtained in viability assays, a significant decrease was observed at the end of the 72-hour treatment compared to the control ($p < 0.05$).

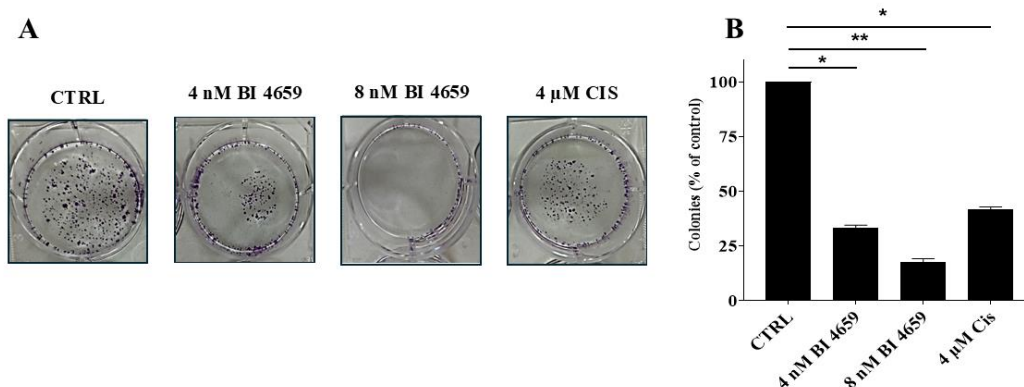


Figure 2. Changes in colony formation ability in Caco-2 cells following BI 4659 inhibitor application (A), Control (CTRL), 40 nM and 80 nM BI 4659, and 4 μM Cis after 72 hours of treatment, as shown by Petri dish images. (B) Bar graph analysis of colony counts normalized relative to the control group. Treatment groups statistically inhibited colony formation more strongly compared to the control. Data are presented as mean ± SD (* $p < 0.05$, ** $p < 0.01$)

3.3. Wound-healing assay

The wound healing experiment graph and microscope images conducted to determine the change in Caco-2 cell migration ability with BI 4659 treatment are shown in Figure 3. It was observed that the migration ability of cells with BI 4659 treatment decreased significantly compared to the control group at the end of 24 hours ($p < 0.05$).

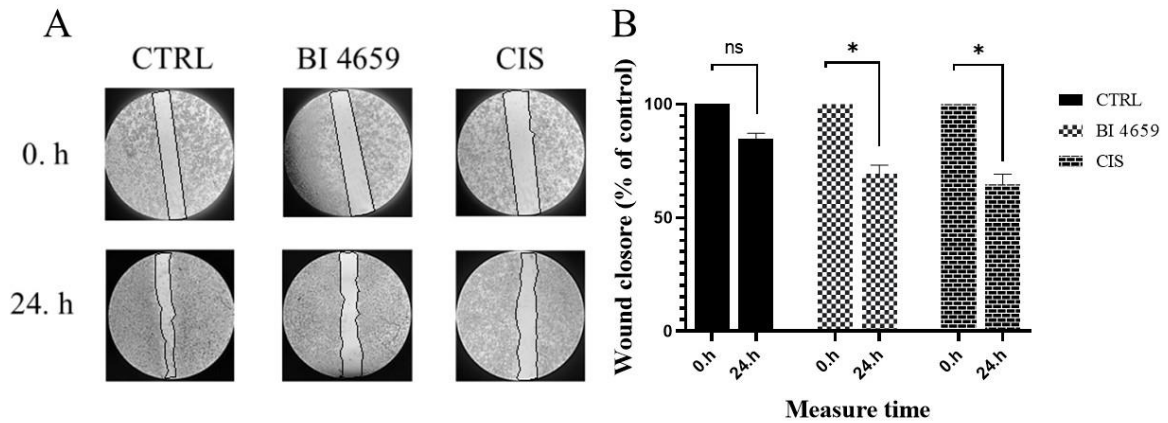


Figure 3. Inhibitory effects of BI 4659 inhibitor on lateral migration (cell migration) in the Caco-2 cell line. (A) Phase-contrast microscope images showing wound healing (closure) at 0 and 24 hours. Black lines indicate wound margins (B). Statistical comparison of wound closure rates between groups. Treatment groups significantly delayed wound closure compared to the control group. Data were analyzed using Image J software. (* $p < 0.05$, ns: non-significant difference)

3.4 Hanging drop assay

The hanging drop assay graph and microscope images used to determine the tumor spheroid formation ability of Caco-2 cells under the effect of BI 4659 are shown in Figure 4. It was observed that the spheroid formation ability of the cells decreased significantly under the effect of the inhibitor ($p < 0.05$).

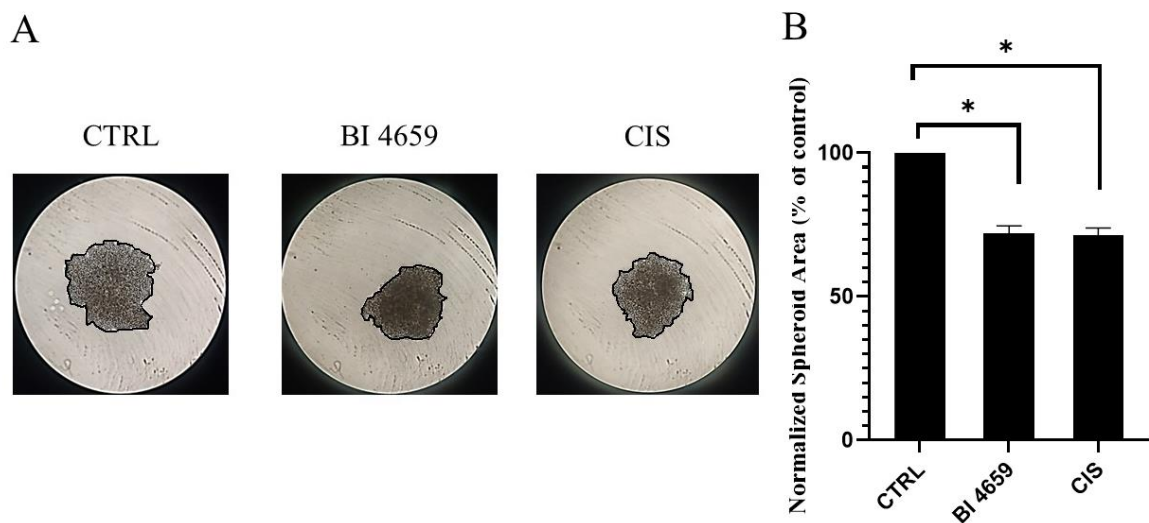


Figure 4. Effects of BI 4659 inhibitor on spheroid formation in Caco-2 cells. (A) Microscopic images showing 3D spheroid development in different treatment groups. (B) Graphical analysis showing the difference in spheroid areas ($p < 0.05$)

4. Conclusions and discussion

Colorectal cancer remains a significant problem worldwide and is the third most common cancer in both men and women. Due to late diagnosis, treatment failure rates are increasing. The outlook for colorectal cancer statistics is not encouraging. The number of cases is expected to double by 2040. [17]. Insufficient success in cancer treatment and the development of resistance over time necessitate new treatment strategies. Inhibitors have the potential for success in this regard [18]. ALK5 is an important component of the TGF- β signaling pathway. It has critical effects on tumor formation, inflammation, and fibrosis. Due to their anticancer effects, selective ALK5 inhibitors are emerging as a strategy in cancer treatment [19]. Research in this field is attracting attention because ALK5 inhibitors exhibit cytotoxic effects in many types of cancer [7,20].

The compound BI 4659 investigated in this study is a small-molecule ALK5 inhibitor and is being evaluated as part of targeted therapy. It shows promise as a new treatment option for patients resistant to standard agents used in chemotherapy [10].

Various studies have shown that ALK5 inhibitors have cytotoxic effects [7,21]. Consistent with the literature, our study detected a potent cytotoxic effect at the nanomolar level in Caco-2 cells. The nanomolar IC50 detected has the potential to be an advantage in colon cancer treatment. The high sensitivity of the cytotoxic effect suggests that BI 4659 may prove effective in targeted therapeutic approaches. Given the high incidence of side effects in colorectal cancer treatment, this finding is significant and holds potential for clinical application.

It has been reported that ALK5 inhibitors inhibit colony formation in various cancer cell lines [22,23]. In our study, the dramatic decrease in colony-forming ability observed with treatment is an important finding demonstrating the long-term effect of BI 4659. Given that colony formation is considered significant in terms of indicating cancer cells' ability to proliferate and their potential to develop resistance to treatment, this finding suggests that BI 4659 may have not only short-term but also long-term antitumor effects.

Studies with ALK5 inhibitors have reported a decrease in the migration ability of cancer cells [8,22,24]. In our study, consistent with the literature, a significant decrease in the migration ability of cells was detected with BI 4659 treatment applied to the Caco-2 cell line. Considering that the migration ability of cells has an important effect on the course of cancer, the decreased migration ability has the potential to yield important results. This finding indicates that BI 4659 not only has a cytotoxic effect on colon cancer cells but also has the potential to alter cell behavior.

Studies using ALK5 inhibitors have reported a decrease in the ability of cells to form spheroids [25]. Determining the response of cancer cells to treatment using 3D spheroid models can provide more important information than two-dimensional cell culture models [18]. In our study, the significant decrease in spheroid formation ability in Caco-2 cells treated with BI 4659 can be considered promising information before *in vivo* studies. This finding suggests that the drug may be effective not only in monolayer cultures but also in models that more closely resemble the clinical environment.

In conclusion, as a contribution to new treatment strategies in colon cancer therapy, which is a major problem to be overcome due to the mortality and treatment costs it causes, the ALK5 inhibitor BI 4659 was studied in detail *in vitro* for the first time in the Caco-2 cell line. The limitations of our study are that it was conducted on a single colon cancer cell line and that animal experiments were not performed. Further experiments are required to elucidate the molecular mechanism. Testing the data obtained in different cell lines and *in vivo* conditions will yield important.

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