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Modulation of *TGF-B* Signaling to Enhance Irinotecan Cytotoxicity in Colorectal Cancer

Kolorektal Kanserde İrinotekan Sitotoksisitesini Arttırmak için *TGF-B* Sinyalizasyonunun Modülasyonu

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Abstract

Background: Impaired Transforming Growth Factor Beta (*TGF-β*) signaling contributes to colorectal cancer (CRC) progression. Targeting *TGF-β* receptors (*TGFβR*) in combination with established chemotherapies may enhance treatment efficacy. This study investigates the effects of a *TGFβR-I/II* inhibitor (LY2109761) on the cytotoxic activity of irinotecan in the human colorectal cancer cell line HCT-116.

Materials and Methods: LY2109761 was used to pharmacologically inhibit *TGFβR-I/II* in HCT-116 cells. Cell viability following treatment with irinotecan, LY2109761, or their combination was assessed using the MTT assay. Real-time quantitative polymerase chain reaction (RT-qPCR) quantified mRNA levels of genes associated with cell death, cell cycle, and *TGF-β* signaling.

Results: Combination therapy of LY2109761 and irinotecan significantly reduced cell viability in HCT-116 cells in a dose- and time-dependent manner. Enhanced expression of cell cycle inhibitor *P21* and increased markers of apoptosis and autophagy were observed.

Conclusions: The combination of LY2109761 and irinotecan exhibited enhanced cytotoxicity compared to irinotecan alone, suggesting this approach as a promising therapeutic strategy for CRC.

Keywords: Colorectal cancer, *TGFβR-I/II*, Irinotecan, LY2109761, Cytotoxicity

Öz

Amaç: Bozulmuş Transforme edici büyüme faktörü beta (Transforming Growth Factor Beta, *TGF-β*) sinyalizasyonu kolorektal kanser (CRC) ilerlemesine katkıda bulunur. TGF-β reseptörlerini (*TGFβR*) bilinen kemoterapilerle birlikte hedeflemek tedavi etkinliğini artırabilir. Amacımız insan kolorektal kanser hücre hattı HCT-116'da *TGFβR-I/II* inhibitörü (LY2109761) ile irinotekanın sitotoksik aktivitesine olan etkilerinin belirlenmesidir.

Materyal ve metod: HCT-116 hücrelerinde *TGFβR-I/II*'yi farmakolojik olarak inhibe etmek için LY2109761 inhibitörü kullanıldı. MTT analizleri, irinotekan, LY2109761 ve bunların kombinasyonu ile tedaviden sonraki hücre canlılığı değerlendirildi. Kantitatif Real Time PCR (RT-qPCR) ile, hücre ölümü, hücre döngüsü ve *TGF-β* sinyalizasyonu ile ilişkili genlerin mRNA düzeyleri değerlendirildi.

Bulgular: LY2109761 ve irinotekanın kombinasyon tedavisi, HCT-116 hücrelerinde hücre canlılığını doza ve zamana bağlı bir şekilde önemli ölçüde azalttı. Hücre döngüsü inhibitörü *P21*'in artan ekspresyonu ve apoptoz ve otofaji belirteçlerinin arttığı gözlemlendi.

Sonuç: LY2109761 ve irinotekanın kombinasyonu, irinotekanın tek başına kullanımına kıyasla artan sitotoksisite sonucu CRC için umut verici bir tedavi stratejisi olduğunu göstermektedir.

Anahtar Kelimeler: Kolorektal kanser, *TGFβR-I/II,* İrinotekan, LY2109761, Sitotoksisite

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Introduction

Colorectal cancer (CRC) is a malignant tumor of the colon or rectal mucosa and a leading cause of cancer-related mortality worldwide (1).

According to GLOBOCAN 2022 data, the number of new CRC cases worldwide has been determined to be approximately 1,926,000. The age-based incidence rate is 27.3/100,000 in men and 18.7/100,000 in women (2). These rates indicate that the incidence of the disease increases significantly with age and is higher in men than in women (3,4). According to GLOBOCAN 2022 data for Türkiye, the annual number of new CRC cases is approximately 21,718, and the death rate is 11,698. It is reported to be the third most common cancer type in Türkiye (5). CRC treatment depends on disease stage and includes surgery, radiotherapy, and chemotherapy, yet high recurrence and metastasis rates underscore the need for new combination strategies (6-8).

Irinotecan is a DNA topoisomerase I inhibitor used in the FOLFIRI regimen as a first-line treatment for colorectal cancer, particularly in metastatic disease (9). However, the clinical use of Irinotecan is limited by low tumor selectivity, variable response, and immunosuppressive side effects; therefore, combination with targeted therapies may enhance its antitumor efficacy (10,11).

The progression of CRC is a multistep molecular process characterized by mutations or expression disorders of several critical genes, particularly APC, KRAS, BRAF, P53, SMAD4, TGFBR2, and PIK3CA (12-14). Transforming growth factor-β (TGF-β) regulates cell differentiation, proliferation, and apoptosis via TGF-β receptors I, II and III (15). Ligand-induced activation of TGF-β receptors triggers SMAD2/3–SMAD4 signaling and nuclear gene regulation; while tumor suppressive early, TGF-β promotes progression at advanced stages (16,17). In colorectal cancer, mutations in the TGF-β pathway, particularly TGF-βRII and SMAD4, promote tumor progression by enhancing proliferation and inhibiting apoptosis (17,18). Disruptions in this signaling pathway are similarly observed in other malignancies such as gastric, prostate, and ovarian cancer (19-21).

Accordingly, our study aimed to investigate the efficacy of suppressing the TGF-β pathway on irinotecan activity using LY2109761, a TGF-βR-I/II inhibitor, in HCT-116 CRC cells.

Material and Methods

Chemicals and Anticancer Drugs

TGFßR-I/II inhibitor LY2109761 (Molecular Formula: $C_{26}H_{27}N_5O_2$, Cayman Chemical, CAS No: 700874-71-1) was obtained from Cayman. Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, CAS No. 67-68-5) and diluted with culture medium to a final DMSO concentration of 0.1% (v/v). Working dilutions of the drugs were prepared from stock solutions by diluting them in the appropriate culture medium. For the drug, 3 different concentrations were used and defined as test drug concentrations. Drug concentrations used for in vitro experiments were 1µM, 100 µM, 200 µM (22,23).

Generation of HCT-116 Cell Line

The CRC cell line HCT-116, which was used in the experiments, was cultured in the medium formed by adding 10% Fetal Bovine Serum (FBS) (Capricon, cat no: FBS-11A) into RPMI-1640 (Roswell Park Memorial Institute-1640) (Capricorn, cat-no: RPMI-HA-P10) medium. The cells were propagated in the medium using 100 U/ml penicillin and 100 mg/ml streptomycin (Capricon, cat no:PS-B) at 37°C, 5% CO_2.

MTT Cytotoxicity Assay

The cell viability was assessed with MTT, a tetrazolium salt. This salt is specific to the succinate dehydrogenase enzyme found in the mitochondria of living cells and is based on the principle that this enzyme breaks down the tetrazolium ring of the MTT dye and forms water-insoluble formazan salts. This formation is only seen in living cells with active mitochondria and the value obtained by dissolving water-insoluble formazan crystals with DMSO and measuring them in a spectrophotometer indicates the number of living cells. Since the MTT reagent is light-sensitive, the experiments were carried out without direct exposure to light. After the cells were removed from the surface of the flask they were in, the cell suspension formed was centrifuged, and the cell pellet was collected. The pellet was resuspended in the medium, seeded in a 96-well plate at $1x10^5$ cells/well and incubated overnight at 37°C.

On the second day, the cells in the 96-well plates were exposed to the test substances at five different concentrations in three repetitions and incubated for 24 hours. After the 24-hour incubation period, 5 mg/ml of powdered MTT (Sigma-Aldrich M5655, cas no:298-93-1) prepared in PBS was added to each well of the 96-well plates and after incubation for 4 hours, 100 µl of DMSO was added to dissolve the formazan crystals and measured at 570 nm with a spectrophotometer. The data repeated at least 3 times for each substance were compared and the concentration-dependent graph was drawn, and the relative % cell viability was determined. At the end of the measurement, the percentage proliferation value was calculated using the formula; Viability % = [100 × (Mean absorbance of cells treated with compound - blank mean) / (Mean absorbance of control cells - blank mean)] (24).

Real Time Quantitative PCR (RT-qPCR)

Total RNA was isolated from control and treatment cells. Cells were dissolved in TRI Reagent. The Direct-zol RNA MiniPrep Plus Kits (Zymo Research, cat no:R2072USA) kit was used according to the manufacturer's guidelines. RNA concentration and purity was measured using NanoDrop system (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was generated with SensiFAST cDNA Synthesis Kit (Meridian Bioscience, cat no: BIO-98005 Germany). The cDNAs were kept at -20 °C. mRNA expression levels of TGF-β1, TGFβRII, SMAD2, SMAD4, BCL2, BAX, BECLIN 1, ATG7, P21, and P53 genes were determined quantitatively. The sense and antisense primers, as well as the PCR product lengths for the expression analysis of ACTB, BCL2, BAX, TP53, P21, TGF-β1, SMAD2, SMAD4, TGFβRII, BECLIN 1, ATG7, and ATG5 genes, are presented in Table 1. Primer pairs for the genes were designed using bioinformatics tools such as Ensemble, NCBI, Primer3, Primer Blast, and UCSC. Forward and reverse primers were designed to span exon-exon junctions and include at least two introns to prevent amplification from genomic DNA. The primer design was tested using the BLAST program, ensuring that the primer pairs did not form self/heterodimers. Lyophilized primers were diluted according to the manufacturer's protocol.

Table 1. The primer sequences and product lengths of all genes are shown

Gene Name	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product length (bp)
ACTB	TTCCTGGGCATGGAGTCCT	AGGAGGAGCAATGATCTTGATC	204
BCL2	TCATGTGTGTGGAGAGCGTC	TCACTTGTGGCCCAGATAGG	254
BAX	CTGACGGCAACTTCAACTGG	CAACCACCCTGGTCTTGGATC	172
TP53	TTTAAGGTTTTTACTGTGAGGGATG	GCATTTCACAGATATGGGCCTTG	175
P21	CCAGCATGACAGATTTCTACCAC	GATGTAGAGCGGGCCTTTGA	122
TGF-β1	GTGGAAACCCACAACGAAA	CACGTGCTGCTCCACTTTTA	165
SMAD2	GCTGCTCTTCTGGCTCAGTC	GTCTGCCTTCGGTATTCTGC	119
SMAD4	GCACTGCCAACTTTCCCAAC	AGGTGGTAGTGCTGTTATGATGG	174
TGFβRII	ATGACATCTCGCTGTAATGC	GGATGCCCTGGTGGTTGA	163
BECLIN1	ACAGTGGACAGTTTGGCACA	CGGCAGCTCCTTAGATTTGT	218
ATG7	TCGAAAGCCATGATGTCGTCTT	CCAAAGCAGCATTGATGACCA	108
ATG5	GGCCATCAATCGGAAACTCAT	AGCCACAGGACGAAACAGCTT	124

SensiFAST SYBR Kit (Meridian Bioscience, Germany) was used for qPCR experiments. CFX96 Real-Time PCR Detection System (Bio-Rad, USA) was used for amplification. Melting curve analysis was performed. All genes were normalized to ACTB. Each sample was studied in triplicate. 2^–ΔΔCt is a common analysis method used to quantify relative gene expression levels. This method calculates the change in the expression level of a target gene by normalizing it to a reference gene (housekeeping gene) and comparing it to a control group. The ΔCt (ΔCt=CT target gene-CT reference gene) value represents the difference between the cycle threshold (Ct) of the target gene and the Ct of the reference (housekeeping) gene. The 2-ΔΔCT method was used to compare gene expressions of control and treatment cells (25).

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 9.3.0 (GraphPad Software, Demo Version). All results were given as mean ± SD. Differences between means were analyzed using two-way ANOVA with significance $p<0.05$, $p<0.01$, and $p<0.001$.

Results

Boosting of Irinotecan Cytotoxic Activity in HCT-116 Cell Line

An irinotecan dose–response analysis was performed on

cell lines with target gene regulation. Irinotecan dose trials were conducted at the 1µM, 100 µM and 200 µM and were identified as 200 µM for IC50 dose. Studies were followed with the doses chosen according to the 24-hour MTT assay result. The appropriate dose of irinotecan was dissolved in the culture medium, and its cell cytotoxicity was investigated. MTT viability assay for irinotecan was performed in 96-well cell culture plates, each dose tested 3 different times as at least 2 separate independent experiments (Figure 1). In this study, 10 µM dose concentration of LY2109761 was studied on HCT-116 cell lines. MTT cell viability assay results are given in Figure 1. The most appropriate combination therapy for cytotoxic activity was determined to be 200 µM irinotecan with 10 µM LY2109761 in HCT-116 cells. According to MTT assay results, LY2109761 enhanced cytotoxicity of irinotecan in HCT-116 (Figure 1).

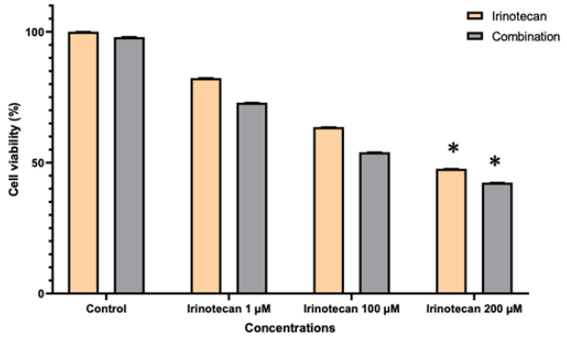

Figure 1. Relative cell viability of HCT-116 cells exposed to various concentrations of irinotecan and 10 µM LY2109761 for 24 h, calculated as percentage of control cells as measured by the MTT assay. Data is shown as mean ± SE derived from a minimum of three independent experiments. *p<0.05

Determination of TGF-β signaling pathway, cell death and cell cycle genes expression changes

The mRNA expression levels of TGF-β signaling pathway genes (TGF-β1, TGFβRII, SMAD2, SMAD4), apoptosis-related genes (BCL2, BAX), cell cycle-related genes (P21, TP53), and autophagy-related genes (BECLIN1, ATG5, ATG7) were quantified. Following treatment of HCT-116 cells, the mRNA expression levels of TGF-β signaling pathway genes, including TGF-β1, TGFβRII, SMAD2, and SMAD4, were determined (Figure 2).

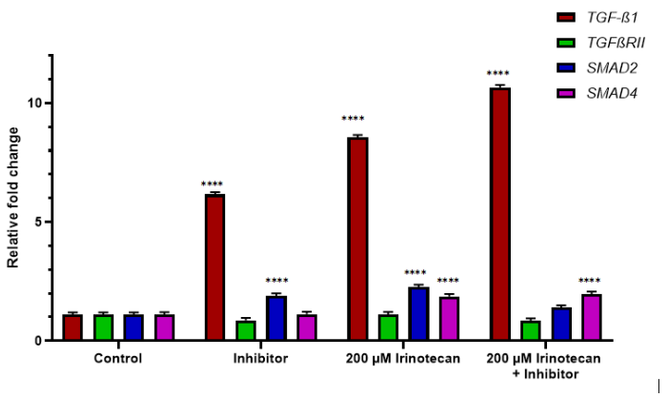

Figure 2. mRNA levels of TGF-β signaling pathway genes (TGF-β1, TGFβRII, SMAD2, SMAD4) in the LY2109761 inhibitor and irinotecan treatment groups in HCT-116 cells. Levels of mRNA determined by RT-qPCR. β-actin was used as an internal control. Data is shown as mean ± SE derived from a minimum of three independent experiments. ****p<0.0001

The expression of TGF-β1 mRNA showed a significant increase with inhibitor treatment, which was further enhanced in the 200 µM group in a dose-dependent manner. While inhibitor treatment increased TGF-β1 expression, the expression levels of SMAD2, and SMAD4 were also elevated compared to the control group, although the increase was less dramatic than that of TGF-β1. And also the inhibitor treatment increased TGF-β1 expression, the expression levels of TGFβRII were decreased slightly compared to the control group.

When the inhibitor was combined with 200 µM irinotecan, the TGF-β1 mRNA expression increased more ($p<0.0001$).

These results indicate that TGF-β1 expression is enhanced by the inhibitor and irinotecan in a dose-dependent manner, and that the expression of TGFβRII, SMAD2, and SMAD4 is similarly correlated with TGF-β1 levels. Overall, these findings demonstrate how TGF-β signaling is modulated in a dose- and inhibitor-dependent manner and how these changes impact other components of the signaling pathway.

Following treatment of HCT-116 cells, the mRNA expression levels of cell cycle-related genes (P53, P21) and apoptosis-related genes (BAX, BCL2) were determined (Figure 3). The expression of the P21 gene showed a significant dose- and inhibitor-dependent increase, with the most dramatic increase observed in the 200µM dose group ($p<0.0001$). The expression levels of BAX and BCL2 proteins also increased, particularly in the 200µM dose group ($p<0.0001$). While P53 expression was higher than in the control group, its increase was less pronounced compared to the other groups ($p<0.0001$).

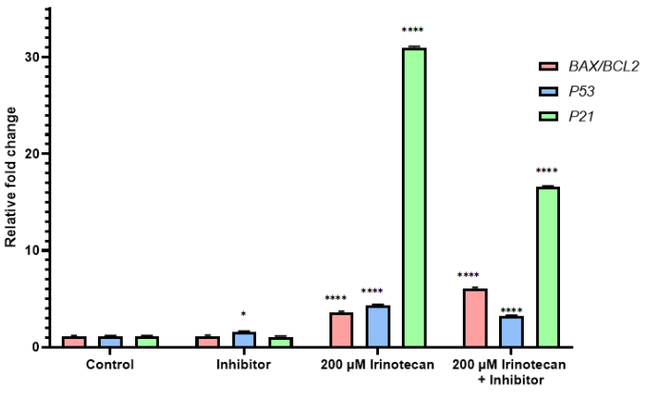

Figure 3. mRNA levels of apoptosis-cell cycle related genes (BAX/BCL2, P21, P53) in the LY2109761 inhibitor and irinotecan treatment groups in HCT-116 cells. Levels of mRNA determined by RT-qPCR. β-actin was used as an internal control. Data is shown as mean ± SE derived from a minimum of three independent experiments. *p < 0.05, and ****p<0.0001

Inhibitor treatments generally elevated the gene expression of these proteins, although the increase was not as significant as that of P21. Overall, p21 gene expression exhibited a marked, dose-dependent increase in response to the inhibitor. The expression levels of BAX and BCL2 also increased in parallel with this increase. P53 gene expression showed an increase as well, but it was less substantial compared to the expression changes of the other proteins.

These findings demonstrate how irinotecan and inhibitor doses primarily affect the gene expression of P21, along with associated increases in BAX and BCL2 expression, in a dose-dependent manner. Additionally, the effects on P53 gene expression, while evident, are less prominent in comparison to the other genes. After treatment applications to HCT-116 cells, gene expression levels of autophagy genes BECLIN1 and ATG7 were shown at the mRNA level (Figure 4). While ATG 7 and BECLIN1 gene expressions increased in an inhibitory and dose-dependent manner, the most significant increase for BECLIN1 was observed in the 200 µM +inhibitor dose group (p<0.001).

Discussion

Understanding the role of TGF-β receptors in the mechanism of action of chemotherapy drugs is critical for developing new strategies that can increase sensitivity to existing treatments or reduce drug resistance (26).

We demonstrated that inhibition of the TGF-β signaling pathway enhances the antiproliferative and cytotoxic effects of irinotecan in CRC cells. Combined treatment with irinotecan and LY2109761 significantly reduced cell viability in HCT116 cells compared to irinotecan alone, indicating enhanced cytotoxic efficacy. These findings suggest that blockade of TGF-β signaling via LY2109761 effectively potentiates irinotecan activity in CRC cells.

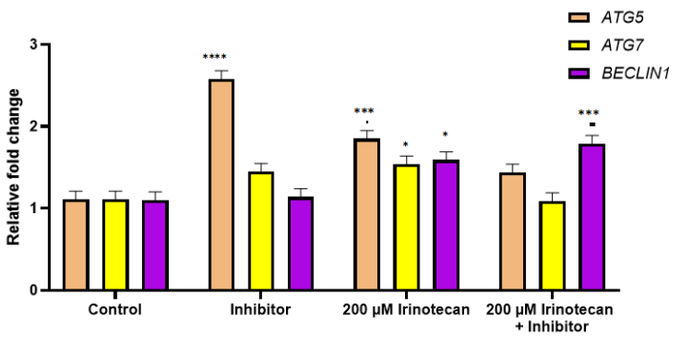

Figure 4. mRNA levels of autophagy-related genes (BECLIN1, ATG5, ATG7) in the LY2109761 inhibitor and irinotecan treatment groups in HCT-116 cells. Levels of mRNA determined by RT-qPCR. β-actin was used as an internal control. Data is shown as mean ± SE derived from a minimum of three independent experiments. *p<0.05, ***p<0.001, and ****p<0.0001

TGF-β triggers diverse biological reactions in cells, which vary based on the cell type and context. Mechanisms such as signal pathway interactions, regulation of cytoplasmic and nuclear antagonist levels, and the composition of transcription factor complexes influence the specificity of these responses. We present direct evidence that inhibition of TGFßR-I/II can influence TGF-β response specificity. Our findings indicate that Smad signaling pathway inactivation can be controlled by inhibiting TGFßR-I/II ,and TGF-β signaling can induce p21 in HCT-116 cells. The inhibition of TGFßR2 leads to varied level of gene expression. We focused on P21 due to its known regulation by TGF-β and its crucial role in inhibiting proliferation and inducing apoptosis. Studying P21, regulated by TGF-β pathways, helps evaluate SMAD signaling's impact on gene regulation. Following TGF-β pathway inhibition, irinotecan treatment exhibited enhanced cytotoxicity, with the drug–inhibitor combination significantly increasing cytotoxic activity compared to single-agent treatments.

Previous studies showing reduced TGFß-R (TGF-β receptor) expression in various cancers and inflammatory states underscore the importance of TGFß-R levels in modulating TGF-β's effects on cells (27,28). In cancers with intact but low-level TGF-β receptors, TGF-β can function both as an oncogene and a tumor suppressor.

This dual role is linked to its activation of SMAD-independent pathways and modulation of TGF-β signaling through interactions with other pathways affected by mutated oncogenes and tumor suppressor genes (29).

TGF-β suppresses G1–S transition in normal cells, but cancer-related mutations abrogate this effect and promote proliferation (30). Tumor proliferation also increases with increased TGF-β signaling (31-33). The mechanism of action of TGF-β receptor kinase inhibitors is to stop TGF-β signaling. Small inhibitor molecules inhibit ATP kinase activity by binding to the ATP-binding domain of TGFβR kinase and block downstream signaling (34-36). In addition, irinotecan, a topoisomerase I inhibitor, affects the S phase of DNA replication (37). In this study, the novel combination of irinotecan and LY2109761 demonstrated a synergistic antitumor effect by arresting the cell cycle at successive phases, resulting in significantly higher cytotoxicity compared to irinotecan alone. These findings provide the first in vitro evidence supporting the enhanced efficacy of this combination. Although the results are promising, further in vitro validation and in vivo studies are required to confirm the therapeutic potential and clinical applicability of this strategy.

Conclusion

This study demonstrates that targeting the TGF-β pathway enhances the cytotoxic efficacy of irinotecan in colorectal cancer, highlighting this novel combination as a promising therapeutic strategy. As the first report to evaluate this approach, our findings warrant further in vitro and in vivo investigation to support irinotecan-based combination therapies.

Ethical Approval: *This study does not involve any clinical or experimental research on humans or animals that would require ethical committee approval.*

Author Contributions:

Concept: E.N.A

Literature Review: E.N.A

Design: E.N.A

Data acquisition: E.N.A, M.S, M.A

Analysis and interpretation: E.N.A, M.S, M.A

Writing manuscript: E.N.A, M.S, M.A

Critical revision of manuscript: E.N.A, M.S, M.A

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