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RESEARCH ARTICLE

# COMBINED INHIBITION OF THE FAK-Rho-ROCK SIGNALING CASCADE, ONE OF THE IMPORTANT PLAYERS IN MECHANOTRANSDUCTION, IN COLORECTAL CANCERS

**Serap Turkmen[1], Caner Karaca[1], Hasan Kurter[1], Nurcin Kayacik[1], Gizem Calibasi-Kocal[2], Yasemin Basbinar[2]**

[1]Dokuz Eylul University, Institute of Health Sciences, Department of Oncology, Izmir, Turkey
[2]Dokuz Eylul University, Institute of Oncology, Department of Translational Oncology, Izmir, Turkey

ORCID: S.T. 0009-0008-3118-6326; C.K. 0000-0002-9016-3908; H.K. 0000-0001-6851-5279; N.K. 0000-0001-9872-7272; G.C.K. 0000-0002-3201-4752; Y.B. 0000-0001-9439-2217.

**Corresponding author:** Yasemin Basbinar, **E-mail:** yasemin.baskin@deu.edu.tr
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## ABSTRACT

**Purpose:** Colorectal cancer (CRC) is one of the most common cancer types globally, with a high mortality rate. The FAK-Rho-ROCK successive signaling cascade promotes growth, migration and invasion of cancer cells. Focal adhesions are major sites of interactions between extracellular mechanical environments and intracellular biochemical signaling molecules/cytoskeleton and therefore focal adhesion proteins have been proposed to play important roles in mechanotransduction. This study aims to evaluate the effects of combination treatments with Focal Adhesion Kinase (FAK), Rho-ROCK, and YAP/TAZ inhibitors on the proliferative and epithelial-mesenchymal transition (EMT)-related metastatic characteristics of colorectal cancer cells.
**Material and Methods:** In vitro experiments were performed using the HCT-116 colon cancer cell line. The effects of Y-15 (FAK inhibitor), ROCK inhibitor-2, and YAP/TAZ inhibitor-2, either applied alone or in combination, on cell proliferation were analyzed using the WST-1 cell viability assay. Epithelial-mesenchymal transition (EMT) markers, E-cadherin and N-cadherin, were evaluated via immunofluorescence staining, and fluorescent intensity was analyzed using ImageJ software.
**Results:** Y-15, when applied alone or in combination with other inhibitors, significantly reduced cell proliferation ($p \leq 0.005$). Moreover, the combination of Y-15 and ROCK inhibitor-2 increased E-cadherin levels while decreasing N-cadherin levels ($p \leq 0.0159$, $p \leq 0.0286$). While the effect of YAP/TAZ inhibitor-2 alone was limited, specific effects were observed in combination treatments.
**Conclusion:** This study demonstrates the potential of FAK-Rho-ROCK pathway inhibitors in the treatment of colorectal cancer. The ability of Y-15, in particular, to inhibit cell viability/proliferation and metastatic processes suggests that combination strategies targeting these pathways could contribute to the development of new therapeutic approaches for CRC.

**Keywords:** Colorectal cancer, Focal Adhesion Kinase, Rho-ROCK, YAP/TAZ

## INTRODUCTION

Colorectal cancer (CRC) ranks third among cancer types worldwide and stands out as a significant cause of mortality (1,2,3). Due to its impact on health and the burden it places on healthcare costs, new treatment approaches for CRC are constantly being

explored. In recent years, mechanobiology has played a crucial role in the progression of CRC, and the knowledge gained in this field has paved the way for new therapeutic targets (4).

The main components of the cytoskeleton, including microtubules, microfilaments, and intermediate filaments, play critical roles in regulating the viscoelastic properties of cells. These structures are associated with actin/myosin complexes, which are fundamental in determining cell stiffness. Cell stiffness influences key processes such as cancer cell proliferation, differentiation, motility, and adhesion. The Rho-ROCK signaling pathway also contributes significantly to these processes by regulating actomyosin contractility (5). Focal Adhesion Kinase (FAK) emerges as a protein that regulates various biological processes related to cancer cell growth and metastasis. FAK also interacts with Rho family proteins to regulate the actin cytoskeleton, managing cancer cell survival, proliferation, migration, and invasion (6). Focal adhesions play a critical role in linking extracellular mechanical signals with intracellular biochemical signals, with FAK proteins playing a key role in mechanotransduction. The impact of mechanotransduction on cancer progression has garnered attention in recent years, and the inhibition of these processes has become a key target in developing new therapeutic strategies (7,8).

In the current literature, studies have shown that Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitors are used separately in different cancer types, but there is no study investigating their combined use in colorectal cancer. The results of studies in cancer types such as pancreatic and breast cancers suggest that Y-15 is effective both alone and in combination with other inhibitors (9,10). Additionally, research has shown that ROCK inhibitors play a role in preventing metastasis (11,12).

In this study, the effects of the combination of FAK, Rho-ROCK, and YAP/TAZ inhibitors on cell viability/proliferation and epithelial-mesenchymal transition (EMT)-related metastatic characteristics were evaluated in colorectal cancer cells. Cell proliferation was analyzed using the WST-1 assay, and the EMT process was evaluated by immunofluorescence staining of E-cadherin and N-cadherin biomarkers. The results reveal the potential effects of pathway-specific inhibitor combinations on the proliferative and metastatic characteristics of cells associated with cell stiffness.

## MATERIAL AND METHODS

### Cell Culture and Inhibitor Application

In this study, the HCT-116 colon cancer cell line (ATCC® CCL-247) was used to model colorectal cancer under in vitro conditions. The HCT-116 cell line was isolated from the primary colon cancer of an adult male patient at Dukes D stage and carries a mutation in codon 13 of the RAS proto-oncogene. The cell line was cultured in media recommended by the suppliers, supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin, and maintained in an atmosphere of 5% $CO_2$ at 37ºC. For cell preservation, a freezing medium containing 90% FBS and 10% DMSO was used, and the cells were stored at -80ºC.

Y-15 (FAK Inhibitor 14) (MedChemExpress, Cat no: HY-12444), ROCK inhibitor-2 (MedChemExpress, Cat no: HY-119937), and YAP/TAZ inhibitor-2 (MedChemExpress, Cat no: HY-147322) were prepared in solutions according to their stock concentrations. Y-15 was dissolved in PBS and stored at -80°C, while ROCK inhibitor-2 and YAP/TAZ inhibitor-2 were dissolved in DMSO and stored under appropriate conditions.

HCT-116 cells were seeded into 96-well plates at a volume of 100 µl. The cells were incubated at 37ºC in 5% $CO_2$ to allow for proper adherence and morphology. Subsequently, different concentrations of Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitor-2 were applied to the cells.

*This study, conducted as part of a master's thesis, received ethical approval from the Dokuz Eylül University Non-Interventional Research Ethics Committee (Date: 20.07.2022, Decision no: 2022/23-09).

### Cell Viability Assay (WST-1)

The WST-1 viability assay was used to evaluate the effects of the drugs on cell viability. HCT-116 cells were seeded into 96-well plates at a density of $10^4$ cells/100 µl, and drug doses were applied after 24 hours of incubation. After 48 hours of drug treatment, the WST-1 reagent was added, and absorbance values were measured spectrophotometrically at 440 nm.

### Immunofluorescence Staining

To evaluate the effects of the Y-15 and ROCK inhibitor-2 combinations on epithelial-mesenchymal transition (EMT), the immunofluorescence staining

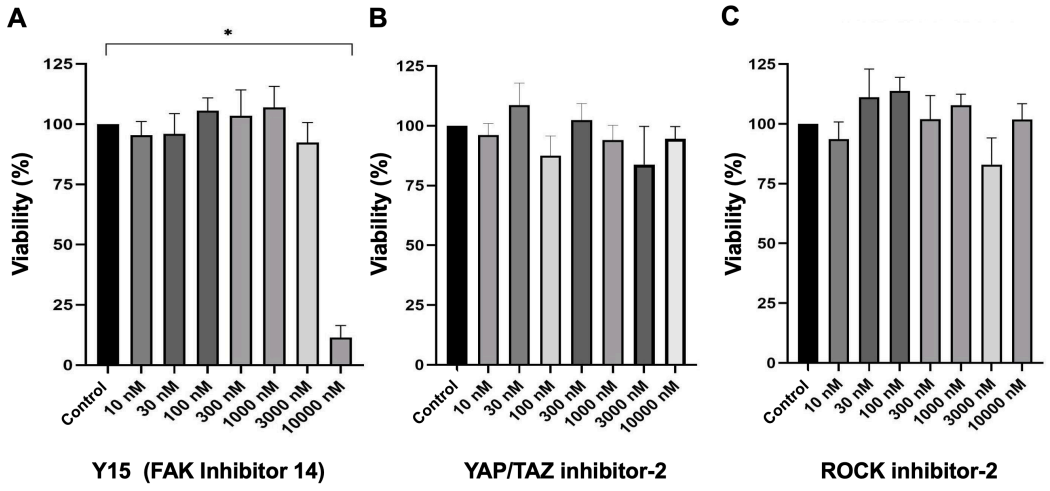


**Figure 1.** A) The effect of Y-15, B) YAP/TAZ inhibitor-2, and C) ROCK inhibitor-2 on cell viability. (* indicates p≤0.05)

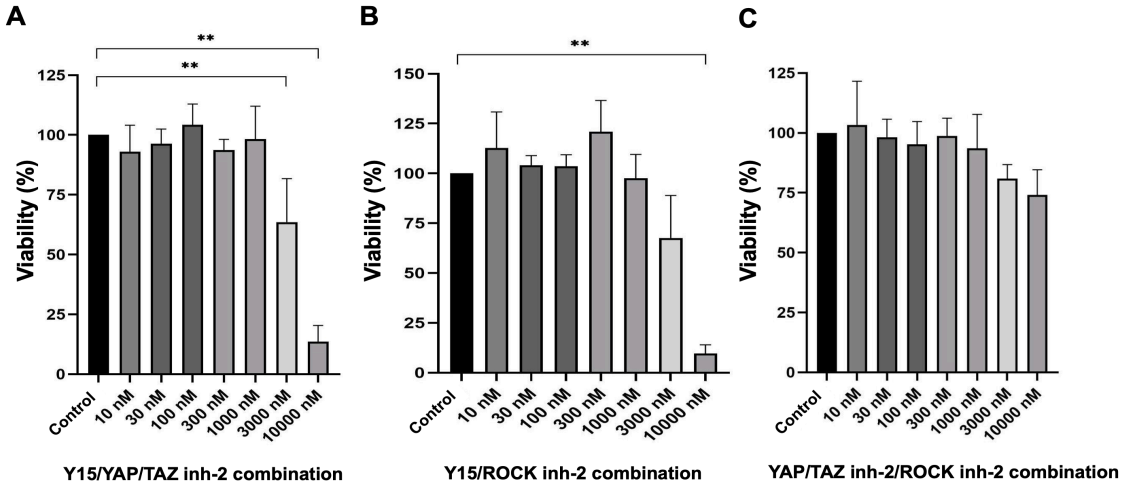


**Figure 2.** The effect of dual combinations on cell viability. A) Combination of Y-15 and YAP/TAZ inhibitor-2. B) Combination of Y-15 and ROCK inhibitor-2. C) Combination of YAP/TAZ inhibitor-2 and ROCK inhibitor-2 (**p≤ 0.002)

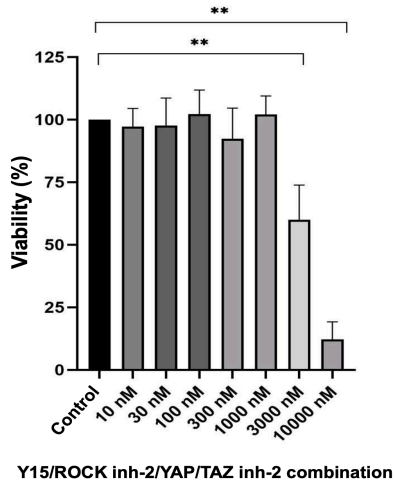


**Figure 3.** The effect of Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitor-2 drug combinations on cell viability (**p≤0.002)

method was used. E-cadherin and N-cadherin biomarkers were stained with Alexa Fluor secondary antibodies, and cells were counterstained with Hoechst 33342 for nuclear staining. The cells were then visualized using a confocal microscope. Fluorescence intensity changes in EMT biomarkers were analyzed using ImageJ software.

### Statistical Analysis

Data analysis was performed using GraphPad Prism software. Variables were expressed as mean ± standard deviation and percentages. All experiments were performed with at least three replicates. Statistical significance was considered at *p≤0.005 and **p≤0.002.

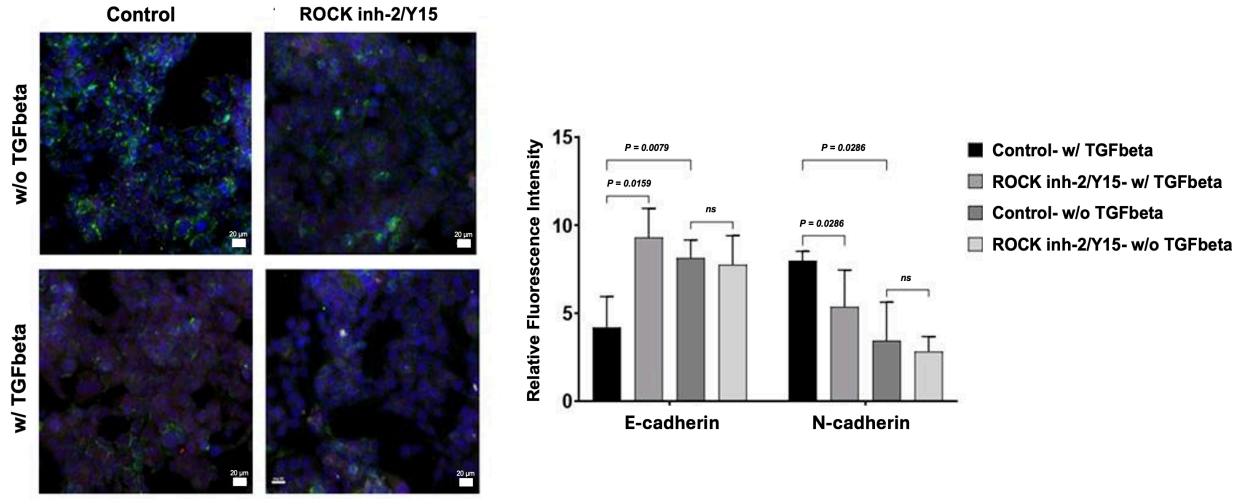


**Figure 4.** The effect of ROCK inhibitor-2/Y-15 inhibitor on epithelial-mesenchymal transition in TGF-β induced and non-induced colorectal cancer HCT-116 cells (Scale bar = 20 µm)

## RESULTS

### Effects of Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitor-2 on Cell Viability

Y-15 inhibitor was applied to HCT-116 cells at concentrations ranging from 10 to 10000 nM. According to the cell viability assay, cell viability was significantly reduced at the 10000 nM dose ($p \leq 0.005$). However, YAP/TAZ inhibitor-2 and ROCK inhibitor-2 did not have a statistically significant effect on cell viability (Figure 1).

### Effects of Dual Drug Combinations on Cell Viability

The combination of Y-15 and YAP/TAZ inhibitor-2 significantly reduced cell viability at 3000 and 10000 nM doses in HCT-116 cells ($p \leq 0.005$). Similarly, a significant reduction in cell viability was observed with the Y-15 and ROCK inhibitor-2 combination at the 10000 nM dose ($p \leq 0.002$) (Figure 2). However, the combination of YAP/TAZ inhibitor-2 and ROCK inhibitor-2 did not show a statistically significant effect on cell viability (Figure 2).

### Effects of Triple Drug Combinations on Cell Viability

The triple combination of Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitor-2 significantly reduced cell viability at 3000 and 10000 nM doses in HCT-116 cells ($p \leq 0.002$). The effect of this combination is attributed to the strong inhibitory properties of Y-15 (Figure 3).

### Effects of the Y-15 and ROCK inhibitor-2 Combination on EMT

In the process of Epithelial-Mesenchymal Transition (EMT), HCT-116 cells induced by TGF-β showed a decrease in E-cadherin levels and an increase in N-cadherin levels. In the induced cells treated with the Y-15 and ROCK inhibitor-2 combination, a significant increase in E-cadherin levels ($p=0.0159$) and a significant decrease in N-cadherin levels ($p=0.0286$) were observed. These findings suggest that the treatment combination is particularly effective on cells undergoing the metastatic process (Figure 4).

## DISCUSSION

Research on tumor biology related to cell mechanics has shown that FAK-targeted agents are typically used alone or in dual combinations. In our study, the effects of Y-15, ROCK inhibitor-2, and YAP/TAZ inhibitors on colorectal cancer cell lines were examined. Studies on the role of FAK in processes such as extracellular matrix (ECM) remodeling, immune cell filtration, and epithelial-mesenchymal transition (EMT) have demonstrated the critical role of the FAK pathway in cancer progression (13,14). These findings are consistent with our results, which showed that the Y-15 inhibitor significantly reduced cell viability when used alone.

In addition to FAK inhibition, the effects of the ROCK pathway on cancer cell proliferation and metastasis are also significant. In our study, ROCK inhibitor-2, when used either alone or in combination, did not show a significant effect on cell viability. However, the

literature suggests that ROCK inhibitors, when combined with chemotherapy and immunotherapies, enhance efficacy in cancer treatment (15). Furthermore, studies by Nam et al. have shown that ROCK inhibition can increase the efficacy of doxorubicin, reducing tumor burden (16). In contrast, in our study, ROCK inhibition alone did not have a significant effect on colorectal cancer cells.

The YAP/TAZ pathway is associated with the mechanics and responses of cancer cells to their microenvironment. In our study, YAP/TAZ inhibitor-2 alone did not significantly affect cell viability, but a limited reduction in cell proliferation was observed when combined with other inhibitors. The literature indicates that YAP/TAZ inhibitors, when used in combination with SWI/SNF complex agents, can affect mechanotransduction processes in cancer cells (12).

In our study, it was found that combining Y-15 with other inhibitors further reduced cell stiffness and proliferative properties. Specifically, Y-15 stands out as a promising agent in colorectal cancer treatment by inhibiting cancer cell survival and metastasis through FAK inhibition (17, 18). Studies by Golubovskaya et al. have also shown that Y-15 blocks tumor growth through FAK inhibition in various cancer types, including colon, pancreatic, and breast cancers (18).

## CONCLUSION

In conclusion, this study demonstrated that the inhibition of the FAK-Rho-ROCK signaling cascade suppresses proliferation and metastasis in colorectal cancer cells. Given the effects of cell stiffness and mechanics on cancer progression, combination therapies using inhibitors of this pathway provide opportunities for developing new therapeutic strategies. As highlighted in the literature, considering the contribution of matrix stiffness to cancer progression and its relationship with resistance to anti-VEGF therapy, agents such as FAK and YAP inhibitors may be more effective in combination treatments (19).

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**Author contribution:** Analysis: ST, HK, NK, GCK. Critical review: GCK, YB. Conception: CK, YB. Data collection: ST, HK, NK. Design: ST, CK, YB. Funding: YB. Literature review: ST, CK, GCK. Materials: YB. Supervision: CK, HK, NK, GCK Writing: ST, GCK, YB.

**Conflict of interests:** The authors declare no conflict of interest.

**Ethical approval:** This study was approved by the Non-Interventional Research Ethics Committee of Dokuz Eylul University (Date: 20.07.2022, Number: 2022/23-09).

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