Combination of FL118 and NCB-0846 to Inhibit Epithelial-Mesenchymal Transition in Colorectal Cancer Cells

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Directed by Professor Sang Joon Shin

The Master's Thesis submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Chin Woo Kim

December 2022

This certifies that the Master's Thesis of
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The Graduate School
Yonsei University

December 2022

ACKNOWLEDGEMENTS

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마지막으로, 혹시나 여기에 언급하지 못했지만 도와주신 분들께 감사의 말씀을 드리고 싶습니다. 이 과정을 절대 혼자로 못하기에, 다들 도와주신 덕분에 졸업할 수 있게 되었습니다. 감사합니다.

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Colorectal cancer continues to be one of the leading incidences of cancer. Despite new regimens of treatment, colorectal cancer that gains invasive and malignant properties leads to metastasis and a low rate of survival. However, treatments that target this metastatic aspect is largely limited. In this study, we explore how FL118, a camptothecin analogue, in combination with NCB-0846, a TNIK inhibitor, can significantly inhibit both cell viability as well as Epithelial-Mesenchymal Transition (EMT). We found that while FL118 was able to effectively inhibit colorectal cancer cells it also caused an overexpression of Snail. To overcome this, we simultaneously treated cells with NCB-0846 and explored its effects. We explored the underlying mechanisms of cell death and found that combination treatment resulted in G2/M phase cell cycle arrest, apoptosis, and DNA damage. We also determined that the WNT/β-Catenin pathway was inhibited along with other EMT markers which was reflected in the greatly reduced rate of migration. We discovered that Snail expression was critical for both cell viability and migration through siSnail transfection which indicated the importance of Snail inhibition in the treatment of CRC cells. These results clearly demonstrated the ability of FL118 and NCB-0846 to inhibit Snail expression and inhibit colorectal cancer cell viability as well as migration. Thus, the combination of these two compounds could prove to be an effective method of treatment in a clinical setting.

Key words : FL118, NCB-0846, colorectal cancer, epithelial-mesenchymal transistion, WNT/β-catenin

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I. INTRODUCTION

Despite the recent advances in the treatment of Colorectal Cancer (CRC), it continues to remain as one of the leading causes of death in the world; being especially prevalent in Asia[1,2]. CRC has been extensively studied and its carcinogenesis can be attributed to both genetic and environmental factors such as mutations in the APC, K-Ras, and p53 genes[3]. Due to its prevalence, extensive studies and methods of treatment have been carried out on CRC[4]. However, metastatic CRC, which is responsible for the highest cause of death, continues to be a hurdle in established methods of treatment for many patients[5]. Currently, conventional first-line CRC treatment involves the use of 5-Fluorouracil (5-FU) with targetted agents such as bevacizumab, folinic acid, oxaliplatin (FOLFOX), and irinotecan (FOLFIRI)[6]. These methods of treatment, unfortunately, often result in patients forming resistance and thus the need for novel regiments[7].

Epithelial-to-Mesenchymal Transition (EMT) is a phenomenon in which an epithelial cell undergoes a series of fundamental changes that alters it to possess mesenchymal phenotypes[8]. The mechanisms of EMT are classified into three distinct groups which all undergo changes during this process: the effector, core regulator, and inducers[9]. The effector characterizes the epithelial or mesenchymal nature of a cell. These include cell-to-cell junction and cadherin proteins such as N and E cadherin, with the latter being

downregulated during EMT. The core regulators include transcription factors that further drive EMT such as the Snail, Zeb1, and Twist families[10]. Lastly, inducers are signaling pathways in the cell that additionally control the process of EMT. These pathways, such as WNT/β-Catenin, lead to a substantial upregulation in its downstream products such as survivin and cyclin D1 which enhance cell proliferation and EMT[11]. Of these proteins, Snail has also been proven to interact with the WNT/β-Catenin pathway and assist in their stabilization and activation. Thus, tackling these multifaceted EMT processes can curtail cancer metastasis and enhance patient prognosis.

FL118 has been shown to be an effective method of treatment for multiple origins of cancer, including CRC[12,13,14]. As a camptothecin analogue FL118 is structurally similar to irinotecan (SN-38) and topotecan but does not show high binding affinity to Top1 but rather with DDX5 (p68)[13,15].Therefore, FL118 has been reported to overcome irinotecan and topotecan-resistant cells as well as circumvent the ABCG2 efflux pump with great efficiency[12,16]. Thus, making FL118 an attractive alternative form of therapy for those who have resistance to irinotecan or topotecan. FL118 can inhibit anti-apoptotic proteins (Survivin, XIAP, Mcl-1) as well as upregulate anti-proliferative genes such as p53[13,17]. FL118 can further inhibit the cell viability of K-Ras mutants which is often present in CRC[18]. EMT progression of metastatic breast cancer cells have also shown to decrease when treated with FL118 through the inhibition of the WNT/β-Catenin pathway[19]. However, the effect on FL118 on EMT in colorectal cancers has yet to be fully elucidated. As the metastatic properties of CRC is the main cause of death, tackling the migration and invasion phenotypes will be key in treating CRC.

NCB-0846, a TNIK inhibitor, is able to abrogate cancer stemness and inhibit the WNT/β-Catenin pathway[20,21]. As the APC gene is highly mutated in CRC, inhibiting TNIK and its downstream pathways is crucial in lowering cancer cell viability and proliferation[22]. NCB-0846 inhibits cancer stemness markers (CD44, CD133, Oct4, Nanog) as well as EMT markers (Slug, Snail, Vimentin) which, in turn, lowers CRC cell viability by a significant amount[20]. Thus, NCB-0846 was proven to be a viable candidate in barring invasion,

metastasis, and proliferation of CRC cells.

In this study, we compared the effectiveness of FL118 and SN38 in inhibiting colorectal cancer cells as well as protein expression. We then assessed how the use of both FL118 and NCB-0846 was able to affect colorectal cancer cells. We also measured the extent of migration after the treatment of both compounds. We delved deeper into the mechanisms of cell death and explored the underlying reasons for migration. Ultimately, we hypothesized using the conjunction of FL118 and NCB-0846 could lead to a greater synergistic effect in the treatment of CRC.

II. MATERIALS AND METHODS

Reagents and antibodies

FL118 was obtained from Dr. Do Young Jung. NCB-0846 was obtained from the Korean Research Institute of Chemical Technology (KRICT). SN-38 was obtained from the Korea Research Institute of Chemical Technology and all compounds were solubilized in dimethyl sulfoxide (DMSO). Primary antibodies against N-Cadherin (ab18203) were purchased from Abcam, Inc (Cambridge, MA, United Kingdom). β-Catenin (9561), Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (8814), Cleaved Caspase 3 (9664), Cyclin D1 (2978), E-Cadherin (3195), GSK-3β (9315), LC3B (2775), Rad51 (8875), Snail (3879), Survivin (2808), XIAP (2042), and γH2Ax (9718) were purchased from Cell Signaling (Massachusetts, Danvers, USA). Bcl-2 (sc-7382), GAPDH (sc-32233), p21 (sc-6246), p53 (sc-126), Slug (sc-166476), TCF4 (sc-81417), and Vimentin (sc-32322) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Cell line and cell culture

Human colorectal cancer cells SW620, HCT 116, DLD-1, Colo-205, and LoVo was purchased from the Korean Cell Line Bank (Seoul, South Korea). SW620 and HCT 116 was cultured in DMEM medium (HyClone Laboratories Inc, Utah, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a 5% CO2 atmosphere. DLD-1, Colo-205, and LoVo was cultured in RPMI-1640 medium (HyClone Laboratories Inc, Utah, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a 5% CO2 atmosphere.

Cell viability assay (CCK-8)

Cell viability was assessed with a CCK-8 assay. Cells were seeded and cultured in 96-well tissue culture plates at a density of 1 × 104 cells/well 24h before drug treatment. SN-38,

FL118, and NCB-0846 was then added to individual wells and incubated for 48h at 37℃. After 48h, CCK-8 (Dojindo, Kumamoto, Japan) kit was utilized according to the manufacturer's instructions. 10μl was added per well and incubated for 3-4h at 37℃. Absorbance was measured at a wavelength of 450nm with a microplate reader (Molecular Devices, San Jose, CA, USA). Statistical analyses and IC50 values were calculated using Graph Pad Software (San Diego, CA, USA).

Spheroid assay

Spheroid cell viability was assessed through the spheroid assay. 96 U-bottom plates (Greiner Bio-One, Kremsmünster, Austria) were pre-coated with Poly 2-hydroxyethyl methacrylate (Sigma-Aldrich, Missouri, USA) dissolved in 95% Ethanol at a final concentration of 20 mg/ml. 60μl of this solution was added per well and dried for 2-3h in a sterile environment. Plates were then dried overnight at 65℃ overnight. HCT116, SW620, and LoVo cells were seeded at a density of 1×10^3 cells/well. The plate was then centrifuged at 216g for 10 minutes and incubated at 37℃ for 4 days. After 4 days, FL118 and SN38 was treated sequentially at 4, 6, and 8 days after initial seeding. After 11 days the spheroids were stained with HOECHST33342, Calcein AM, and EthD-1 (Thermo Fisher Scientific Inc, Massachusetts, USA) at a final concentration of 33μM, 2μM, and 3μM respectively. After treatment, plates are incubated for 3h at 37℃. Fluoresence imaging was measured with Operetta CLS (PerkinElmer Inc, Massachusetts, USA).

Western Blot Assay

Protein expression was measured through the Western Blot assay. Cells were harvested in ice-cold PBS through scrapping. In order to extract the proteins, we lysed total cell extract using radioimmunoprecipitation assay buffer (Elpis-Biotech, Daejeon, South Korea) with phosphatase and protease inhibitors. Total protein concentrations were calculated through the Bradford assay using bovine serum albumin as a standard. Equal amounts of protein were separated using 8 and 12% SDS-PAGE gel electrophoresis then transferred on a

polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked with 5% skim milk. Primary antibodies were incubated overnight at 4℃. The membranes were then incubated with 1% skim milk containing either horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibodies (Thermo Fisher Scientific Inc, Massachusetts, USA). After each step, the membrane was washed with PBS-T 3 times for 5 minutes each. The membranes were developed after addition of enchanced chemiluminescence (ECL) solution (Thermo Fisher Scientific Inc, Massachusetts, USA).

Immunofluorescence

Cells were seeded in 96-well culture tissue plates (Corning Inc, New York, USA) at a density of 2×10^4 cells/well. They were incubated at 37℃ for 24 hours before trated with or without 10nM FL118 and/or 1μM NCB-0846 for 48h. The wells were then washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. They were then permeabilized with 0.5% Triton X-100 for 10 min and incubated in 3% BSA solution for 30 min at room temperature. Primary antibody against Vimentin (Santa Cruz, CA, USA) was added and incubated at 4℃ overnight. The samples were then washed with PBS and incubated with fluorochrome-conjugated secondary antibody for 1h at room temperature. Lastly, the samples were washed again and incubated with DAPI (Sigma-Aldrich, Missouri, USA) at a concentration of 1μg/ml for nuclear staining. The cells were then observed using Operetta CLS (PerkinElmer Inc, Massachusetts, USA).

Wound Healing Assay

Cells were seeded into 6-well culture tissue plates at a density of 2×10^6 cells/well. They were incubated at 37℃ for 24h until confluent. The wound was created by gently scratching the monolayer using SPLScar kit (SPL Life Sciences Co., Ltd, Gyeonggi-do, South Korea). The wells were washed with PBS to remove the detached cells and fresh media was added along with or without 10nM FL118 and/or 500nM NCB-0846. The cells were photographed using Operetta CLS at 0 and 48h using the same configurations. The wound distance was

calculated using ImageJ (NIH, USA) software. The assay was carried out in triplicates.

Transwell Assay

Cells were seeded into 8μm sized porous chamber at a density of 2×10^6 cells/chamber. They were seeded with serum-free media while the bottom chamber was filled with 600μl of 10% FBS supplemented media. Different concentrations of FL118 and/or NCB-0846 was added to the bottom chamber. Cells were incubated at 37℃ for 48h for migration. After incubation, cells were fixed with 4% paraformaldehyde and stained with crystal violet. The top of the chamber was gently removed with a cotton swab. The chamber images were then captured with Operetta CLS (PerkinElmer Inc, Massachusetts, USA) and counted.

Flow Cytometry

Cells were seeded in 60mm cell culture dish at a density of 8 × 105 and were incubated at 37℃ for 24h. They were then treated with FL118 and/or NCB-0846 for 48h. Cells were harvested through trypsization and centrifugation. Cells were washed with PBS and was resuspended in PBS and fixed in 70% ice-cold ethanol for 2 hours at -20℃. The fixed cells were washed with PBS twice and mixed with propidium iodide (PI) and RNaseA staining buffer (Becton, Dickinson and Company, New Jersey, USA) for 15min at room temperature in the dark. Stained cells were then analyzed using BD FACS LSR II SORP system (Becton Dickinson, Franklin Lakes, NJ, USA). For apoptosis analysis, we stained cells with the PE Annexin V Apoptosis Detection Kit (Thermo Scientific, USA) according the manufacturer's protocol. After the same harvest procedure, cells were resuspended in 1X binding buffer. They were then stainined with 5μL PE Annexin V and 5μL 7-AAD, gently vortexed and incubated for 15 min at room temperature. After incubation, 400μL of 1X binding buffer was added. Measurement was carried out using the BD FACS LSR II SORP system. Further analysis was performed in Flowing software (Turku Bioscience, Turku, Finland).

siRNA Transfection

Cells were seeded into 6-well culture tissue plates at a density of 1×10^6 cells/well. When cells reached 80% confluency, they were transfected with 20nmol/L of control siRNA or siRNA targeting Snail (Bioneer Inc, Daejeon, South Korea). The following sequences were used for forward: GUG AGU AAU GGC UGU CAC U and reverse: AGU GAC AGC CAU UAC UCA C. Transfection was performed using RNAiMAX transfection kit (Thermo Scientific, USA) according to the manufacturer's protocol. In the case of FL118 treatment, FL118 was treated 48h after intial transfection. Cells were seeded in 96-well culture tissue plate at a density of 2×10^4 cells/well for CCK-8 assays.

RNA Extraction

Cells were seeded into 6 well culture plates at a density of 0.6×10^6 cells/well. They were transfected with siRNA targeting Snail after reaching 80% confluency. They were also treated with 10nM of FL118 and DMSO. Cells were harvested after 48 hours. DNA was extracted from the cells using the Ribospin kit (GeneAll, Seoul, South Korea) according to the manufacturer's instructions. The quality of RNA was examined with a Nano-Drop 1000 Spectrophotometer (Thermo Scientific, USA).

Quantitative Real-time Polymerase Chain Reaction

Previously extracted RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA) according to the manufacturer's instructions. The following primers were used in this experiment. β-actin forward: TTG CCG ACA GGA TGC AGA AG, reverse: AGG TGG ACA GCG AGG CCA GG. Snail forward: CTG CAG GAC TCT AAT CCA G, reverse: CAA GGA AGA GAC TGA AGT AG. p53 forward: GGG GAG CAG GGC TCA CTC CAG CCA C, reverse: GTC TGA GTC AGG CCC TTC TGT CTT G. Survivin forward: CAC CGC ATC TCT ACA TTC, reverse: GGT TTC CTT TGC ATG G. Standardization was done with β-actin and target gene expressions were calculated using $2^{-\Delta\Delta CT}$ method.

Organoid

Human colorectal cancer organoids were gifted by the laboratory of Dr. Tae Il Kim. Organoids were maintained in DMEM/F12 (HyClone Laboratories Inc, Utah, USA) with 1% Penicillin-Streptomycin, 1X Glutamax (Gibco, Carksbad, CA, USA), 1X N2 supplement (Gibco), 1X B27 supplement (Gibco), 1mM N-Acetylcysteine (Sigma, St Louis, MO, USA) , 2mM L-Glutamine (Sigma), 10mM Nicotinamide (Sigma), 100ng/mL Noggin (Sigma), 50ng/mL EGF (Peprotech, East Windsor, NJ, USA), 10μM Y-27632 (Sigma), 500nM A-83-01 (Sigma), 10μM SB202190 (Sigma), and 10nM Gastrin I (Sigma). They were seeded in 24 well culture tissue plates with 40μL of 3D matrigel and 700μL of media. The media was changed every 4 days and subcultured when confluency exceeded 80%. For organoid viability assay, organoids were seeded into 96-well culture tissue plates with 10μL of 3D matrigel per well. After 30 min of incubation at 37℃, 100μL of media was added and left to incubate for an additional 24h. Afterwards, 100μL of fresh media including FL118 and/or NCB-0846 was added into each well and incubated for 5 days at 37℃. On the fifth day, Cell-Titer Glo solution (Promega, Madison, WA, USA) was added according to the manufacturer's protocol and measured using a luminescence reader.

Statistical Analysis

All statistical analysis was performed in GraphPad v5.01 software. All statistical figures were repeated independently at least three times. The data is presented as the mean ± SEM. Statistical comparisons were conducted using either an unpaired Student's t-test or One-way analysis of variance (ANOVA). Significance was as follows: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

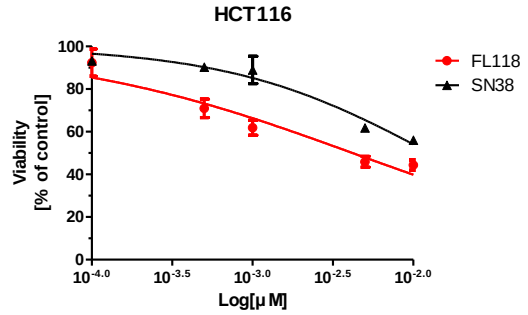
III. RESULTS

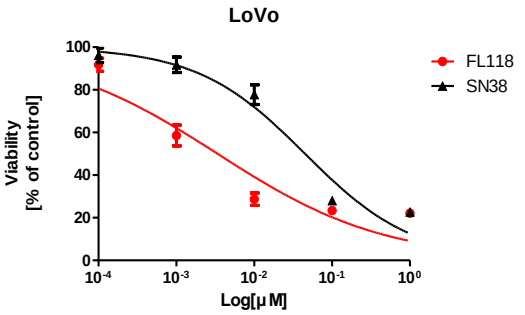
FL118 is a Promising Therapeutic Agent that is More Effective than SN38

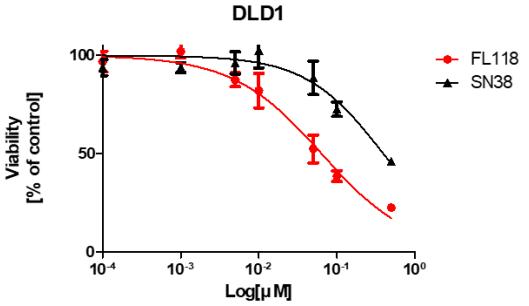
FL118 belongs to the same Camptothecin family as SN38 and thus we first examined the ability of both FL118 and SN38 to inhibit the cell viability and proliferation of various Colorectal Cancer (CRC) cells (HCT116, LoVo, DLD1). Despite SN38 being an established drug currently utilized in clinical settings[23], FL118 demonstrated a higher efficacy in inhibiting CRC cells at lower concentrations (Fig. 1A). Additionally, FL118 was able to effectively inhibit anti-apoptotic proteins (Survivin, XIAP, Bcl-2) after dose-dependent treatment with either FL118 or SN38, further exhibiting its ability in inhibiting CRC cells by promoting apoptosis (Fig. 1B). This is also reflected in the higher protein expression of cleaved caspase 3. Other common methods of cell death further affirmed FL118's effectiveness over SN38. p53, a hallmark tumor suppressor gene, was upregulated to a higher degree when treated with FL118, indicating its anti-tumor capabilities. DNA damage and autophagy were also increased as expressed by the increase in expression of γH2AX and the LC3B I/II ratio respectively. As FL118 treatment resulted in apoptosis, autophagy, and DNA damage at the same or lower relative concentration than SN38, we can determine that FL118 is a more effective therapeutic agent than SN38 in CRC cells.

Through the spheroid assay, we identified the differences in spheroid viability after treatment of either FL118 or SN38 (Fig. 1C). While the size of the spheroid itself was quite similar between both treatments, the degree of dead cells, expressed through Ethidium Homodimer (EthD) staining, was higher when the spheroids were treated with FL118 than SN38. This indicated a higher inhibition of cell viability. Thus, FL118 was clearly more effective in inhibiting CRC cells than SN38.

(A)

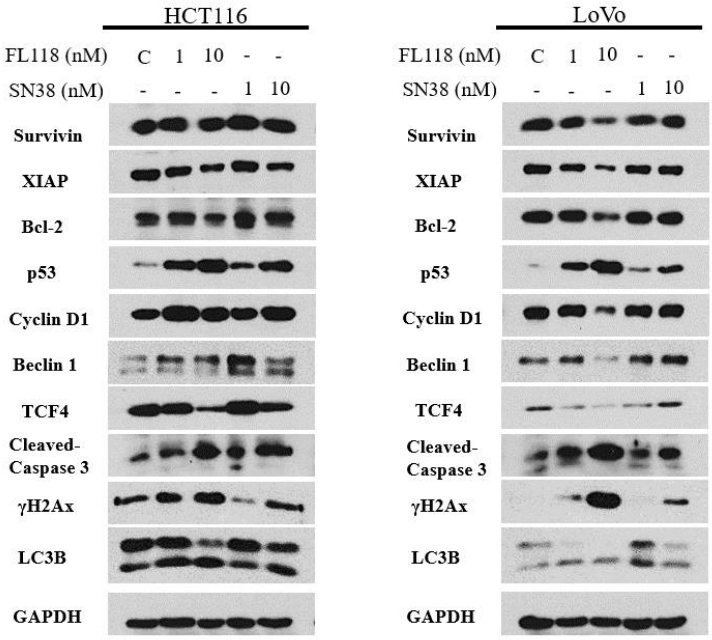






Cell line	FL118 IC_{50} (nM)	SN38 IC_{50} (nM)
HCT116	4.2	12.7
LoVo	3.38	45
DLD1	63.8	394

(B)



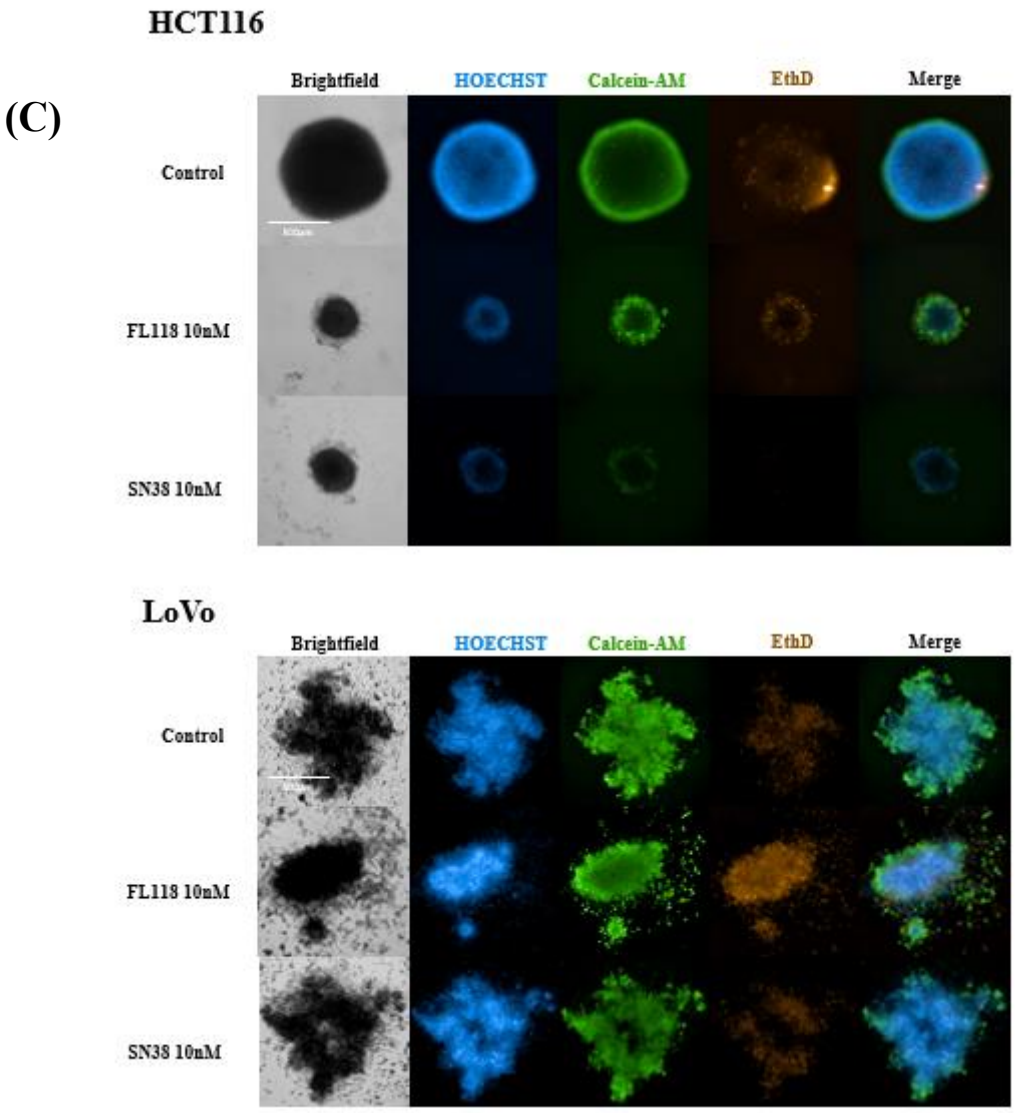
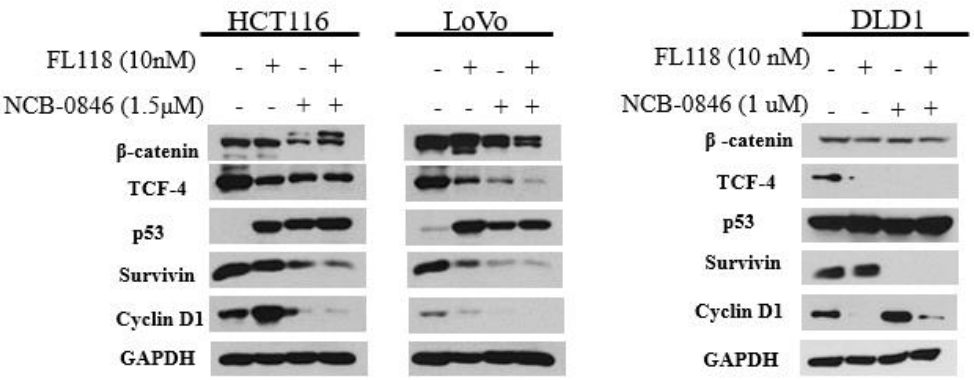


Figure 1. FL118 is more effective than SN38. (A) HCT116, LoVo, and DLD1 cells were exposed to various concentrations of FL118 and SN38 then subjected to CCK-8 assay. **(B)** HCT116 and LoVo cells were treated with 1 or 10nM of FL118 or SN38. Western Blotting was performed with the following lysates with GAPDH acting as positive control. **(C)** HCT116 and LoVo cells were grown as a spheroid and treated with 10nM of FL118 or SN38. They were measured with confocal microscopy after staining with HOECHST, Calcein-AM, and EthD.

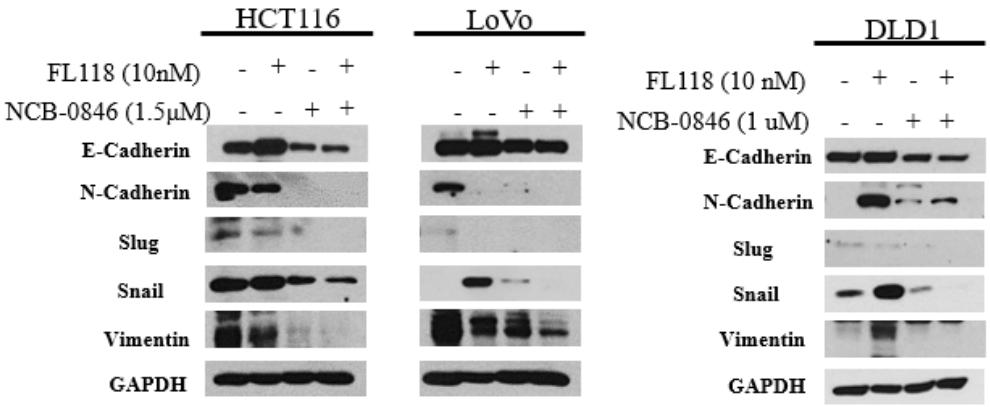
Low Concentrations of FL118 Induced an Increase in EMT Markers which is Attenuated by NCB-0846

Previous studies have demonstrated FL118's ability to inhibit anti-apoptotic proteins as well as Epithelial-to-Mesenchymal (EMT) markers (Snail, Vimentin, N-Cadherin) in multiple lineages of cancers. However, we discovered that lower concentrations (10nM) of FL118 lead to varying degree of increases in the expression of Snail as exhibited by HCT116, LoVo, and DLD1. By treating these CRC cell lines with both FL118 and NCB-0846 in combination, we found a synergistic effect in reducing the WNT/β-Catenin pathway (β-Catenin, TCF4) and its downstream products (Survivin, Cyclin D1) responsible for cancer cell proliferation and anti-apoptosis. We also found that the tumor suppressor, p53, remained highly upregulated after combination treatment (Fig. 2A). HCT116 displayed a slight decrease in E-Cadherin expression, an epithelial marker, when treated in combination. However, N-Cadherin expression, a mesenchymal marker, was downregulated to a comparatively much higher degree. All cell lines exhibited the ability to inhibit EMT-related markers (Vimentin, Slug, Snail) after combination treatment despite the increase in Snail expression after FL118 treatment alone. These changes in EMT markers were further elucidated through the immunofluorescence (IF) of HCT116 cells (Fig. 2B). Vimentin expression was clearly downregulated after combination treatment of both FL118 and NCB-0846 (Fig. 2C). Additionally, there was a significant inhibition in cell viability when treated with both FL118 and NCB-0846 than when treated individually (Fig. 2D). We, therefore, demonstrated how FL118 and NCB-0846 could be treated together in attenuating EMT markers and cell viability.

(A)



(B)



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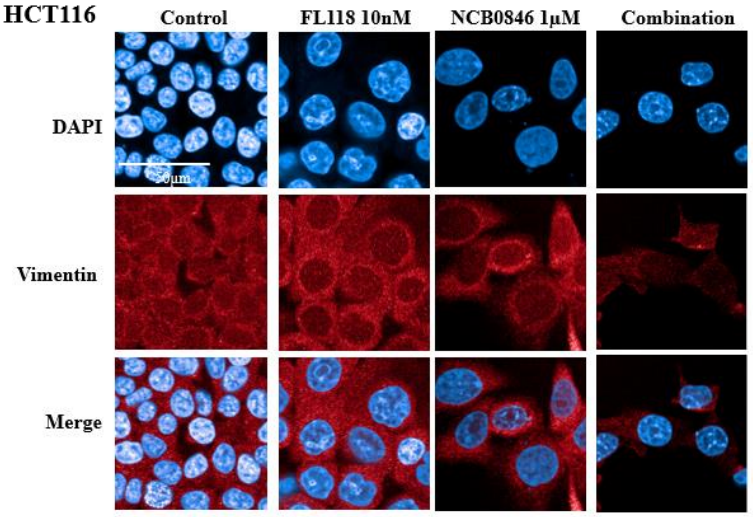

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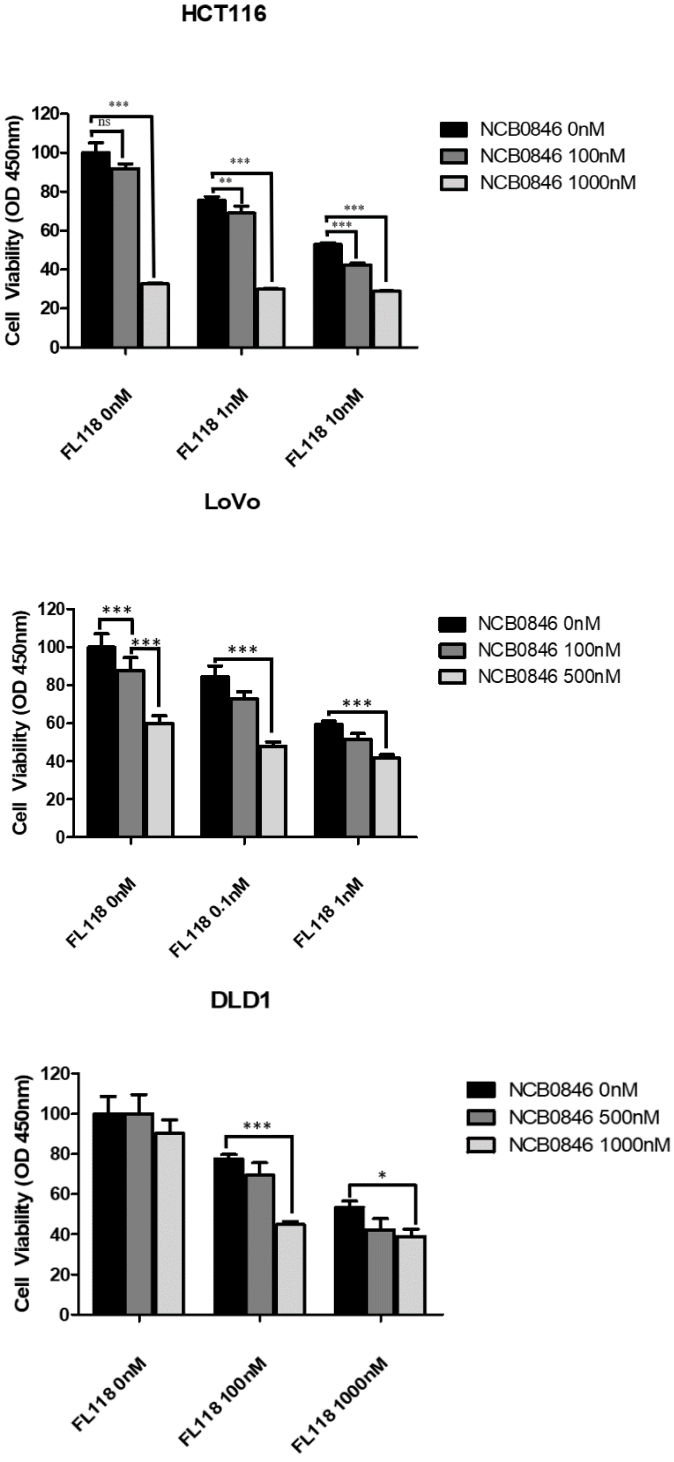


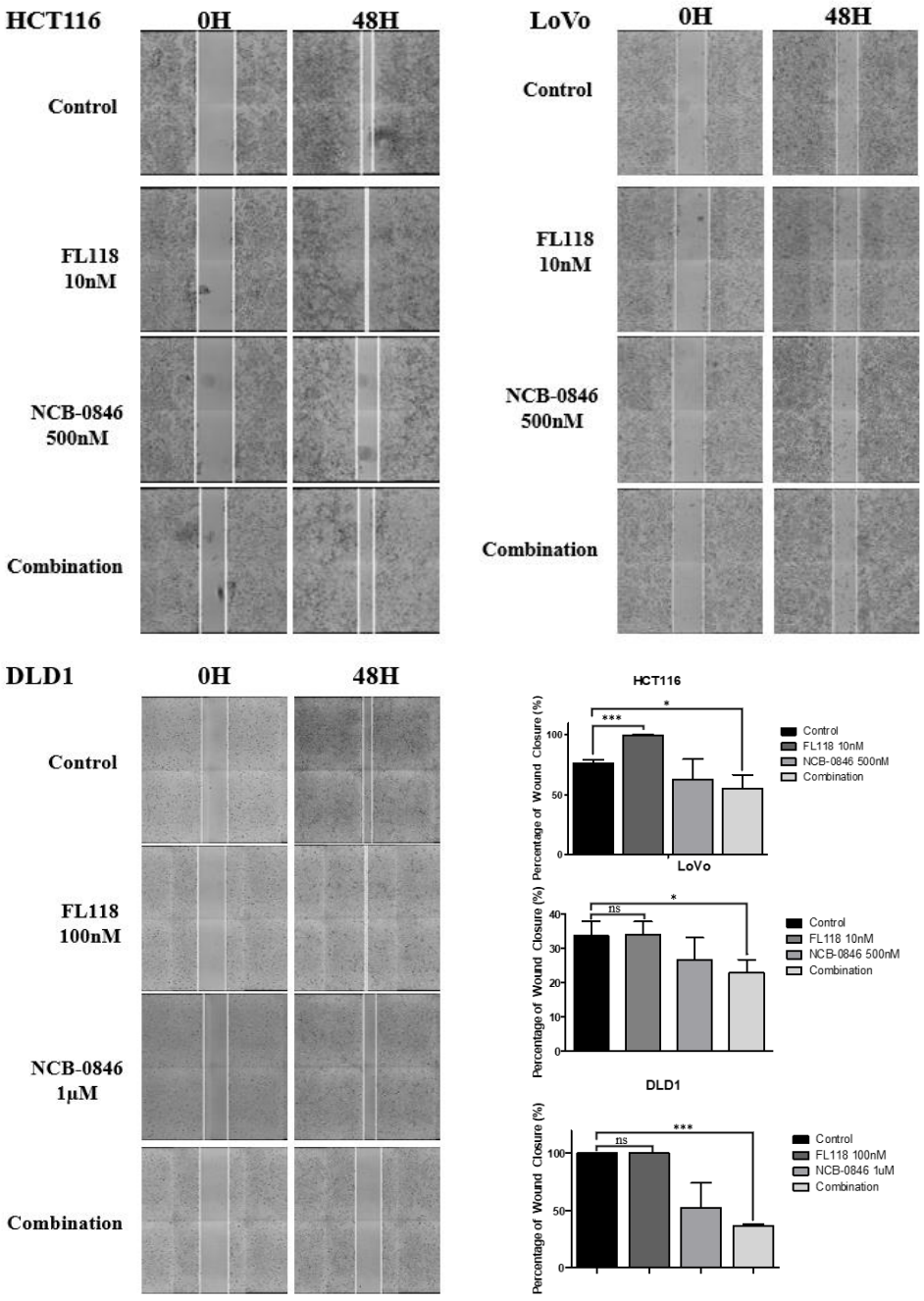
Figure 2. Combination treatment significantly inhibited cell viability and migration. **(A)** HCT 116, LoVo, and DLD1 cells were treated with FL118 and/or NCB-0846. The expression of WNT/β-Catenin pathway and **(B)** EMT pathway genes were analyzed with Western Blotting with GAPDH acting as positive control. **(C)** Immunofluorescence of Vimentin after FL118 and NCB-0846 treatment in HCT116 cells. **(D)** HCT116, LoVo, and DLD1 cells were treated with a combination of a series of FL118 and NCB-0846 then subjected to CCK-8 assay.

Cell Migration is Decreased when Treated in Combination

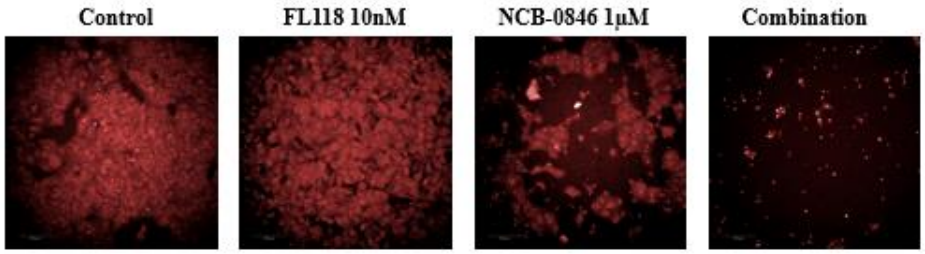
Due to EMT markers being upregulated in low concentrations of FL118, we sought to observe its effects on cell migration and invasion. The degree of cell migration was measured with a Wound Healing Assay. Unsurprisingly, when CRC cells were treated with FL118 alone we saw either an increase or no significant differences in cell migration compared to that of control (Fig. 3A). We hypothesized that despite FL118's ability to inhibit cell viability at lower concentrations, its EMT capabilities are unaffected or conversely increased and thus result in more cells migrating. However, when CRC is treated in combination with NCB-0846, we noticed a marked decrease in the amount of migration in all cell lines.

We further examined the extent of migration after compound treatment through a transwell assay. We found that both LoVo and DLD1 had insignificant changes when treated with FL118 than when compared to control while HCT116 was slightly decreased (Fig. 3B). NCB-0846 was able to further lower the extent of migration than when treated with FL118 alone. It was also demonstrated that combination treatment was able to inhibit migration most effectively in all cell lines.

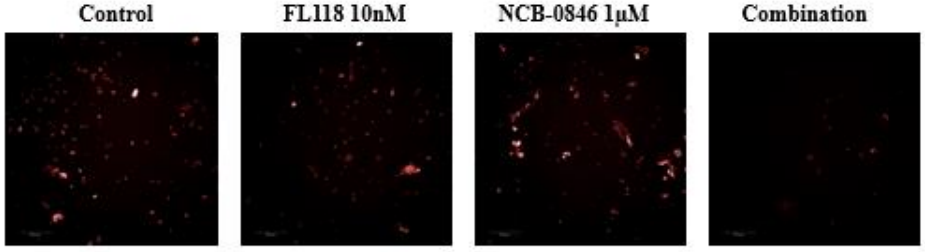
(A)



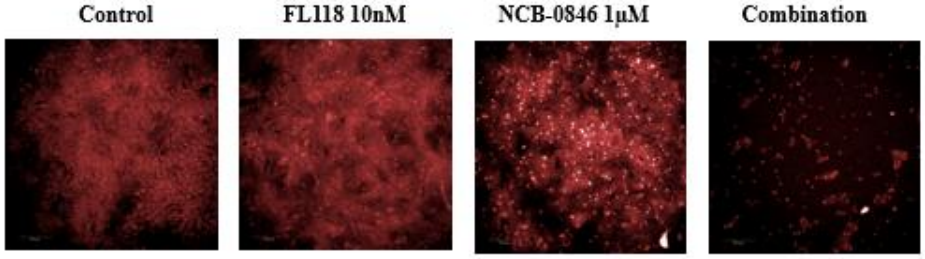
(B) **HCT116**



LoVo



DLD1



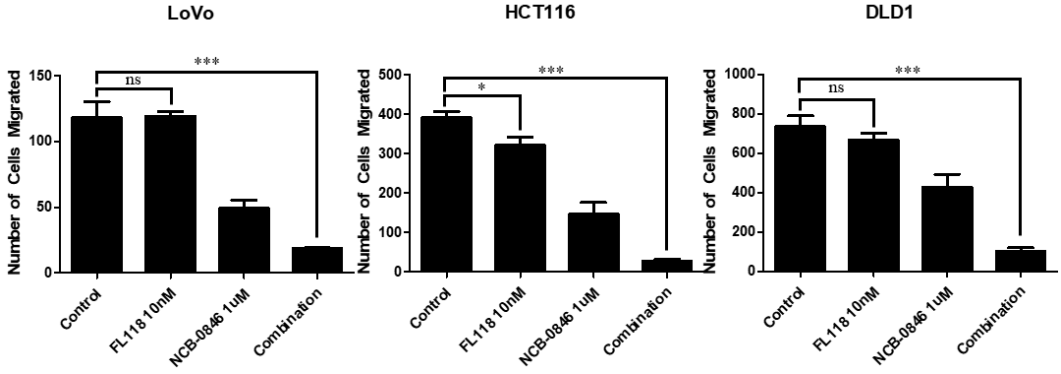


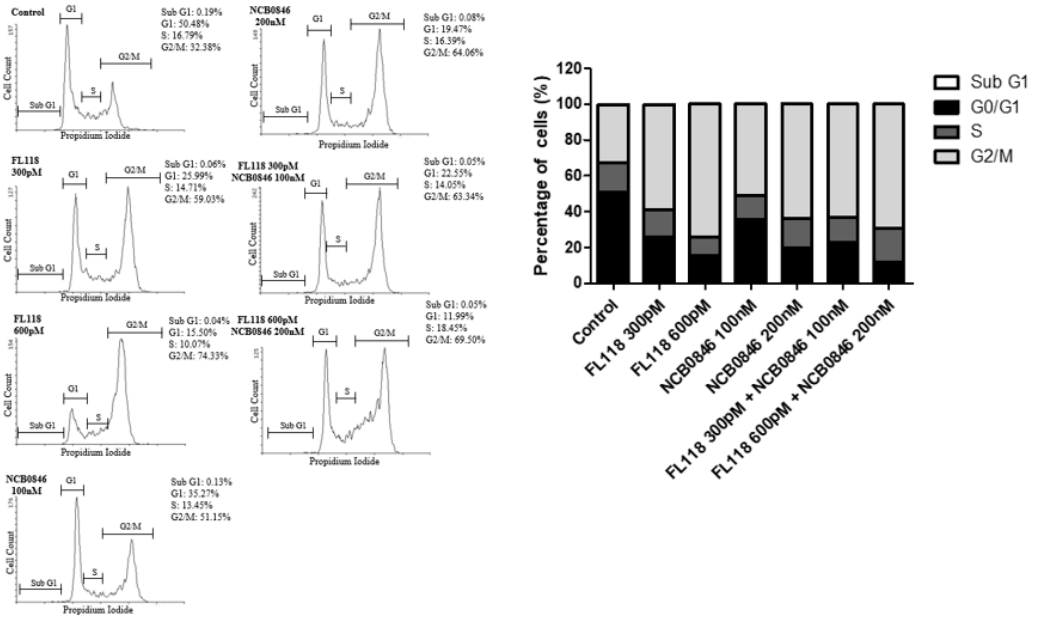
Figure 3. Cell migration is inhibited with combination treatment. (A) Cell migration was analyzed with wound healing assay. HCT116, LoVo, and DLD1 cells were treated with FL118 and/or NCB-0846 after the wound was made. The images were taken on 0 and 48h with the width being measured each time. **(B)** Effect of FL118 and/or NCB-0846 on HCT116, LoVo, and DLD1 cells in a transwell assay to measure cell migration. P value was calculated with student's t-test. ns not significant, *p < 0.05, **p <0.01, and ***p<0.005 vs control.

Combination Treatment Leads to Apoptosis, Cell Cycle Arrest, and DNA Damage

FL118, in a previous publication, has demonstrated its ability to inhibit cell viability through downregulating anti-apoptosis proteins and thus, in turn, enhancing apoptosis[12,13]. However, we did not fully understand how combination treatment resulted in cell death. We, thus, aimed to elucidate methods of cell death with combination treatment. Through cell cycle analysis, we found that both FL118 and NCB-0846 resulted in cells arresting in the G2/M phase of the cell cycle (Fig. 4A). We also demonstrated that CRC cells are arrested in the G2/M phase at lower concentrations than when treated with either compound alone indicating the benefits of combination treatment.

Apoptosis and DNA damage markers were highly upregulated when CRC cells were treated with the combination of FL118 and NCB-0846 (Fig. 4B). We verified previous claims that FL118 treatment could attenuate anti-apoptosis proteins. Combination treatment, however, could further downregulate these proteins leading to an increase in cell death. Accordingly, cleaved caspase 3 expression was greatly increased after combination treatment in HCT116 and LoVo cells. Furthermore, not only was there a higher expression of γH2AX, indicating DNA damage, but also a significant reduction in the expression of DNA repair protein, Rad51. These damage responses were not as evident in DLD1 cells as DLD1 had a much higher IC50 value for FL118 than either HCT116 or LoVo. This could have led to the drugs having a lesser impact and thus had lower expressions of these cell death markers. Nonetheless, the expression of HCT116 and LoVo cells illustrates the ability of combination treatment to inhibit CRC cells in multiple forms of cell death than when treated with either agent alone.

(A)



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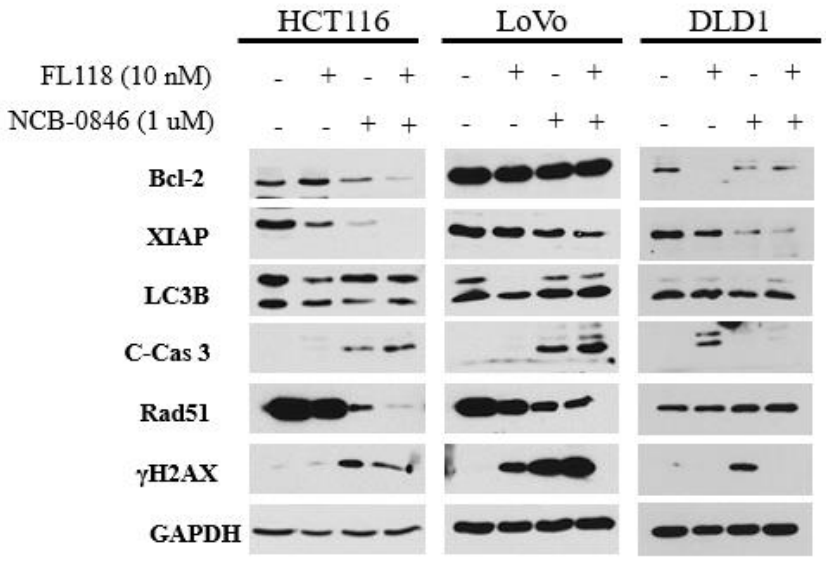


Figure 4. Combination treatment leads to G2/M phase cell cycle arrest as well as apoptosis and DNA damage. (A) Cell cycle analysis of HCT116 after treatment of FL118 and/or NCB-0846 with PI staining and flow cytometry. **(B)** Anti-apoptotic genes and cell death markers were analyzed with Western Blotting after treatment with FL118 and NCB-0846 with GAPDH acting as positive control.

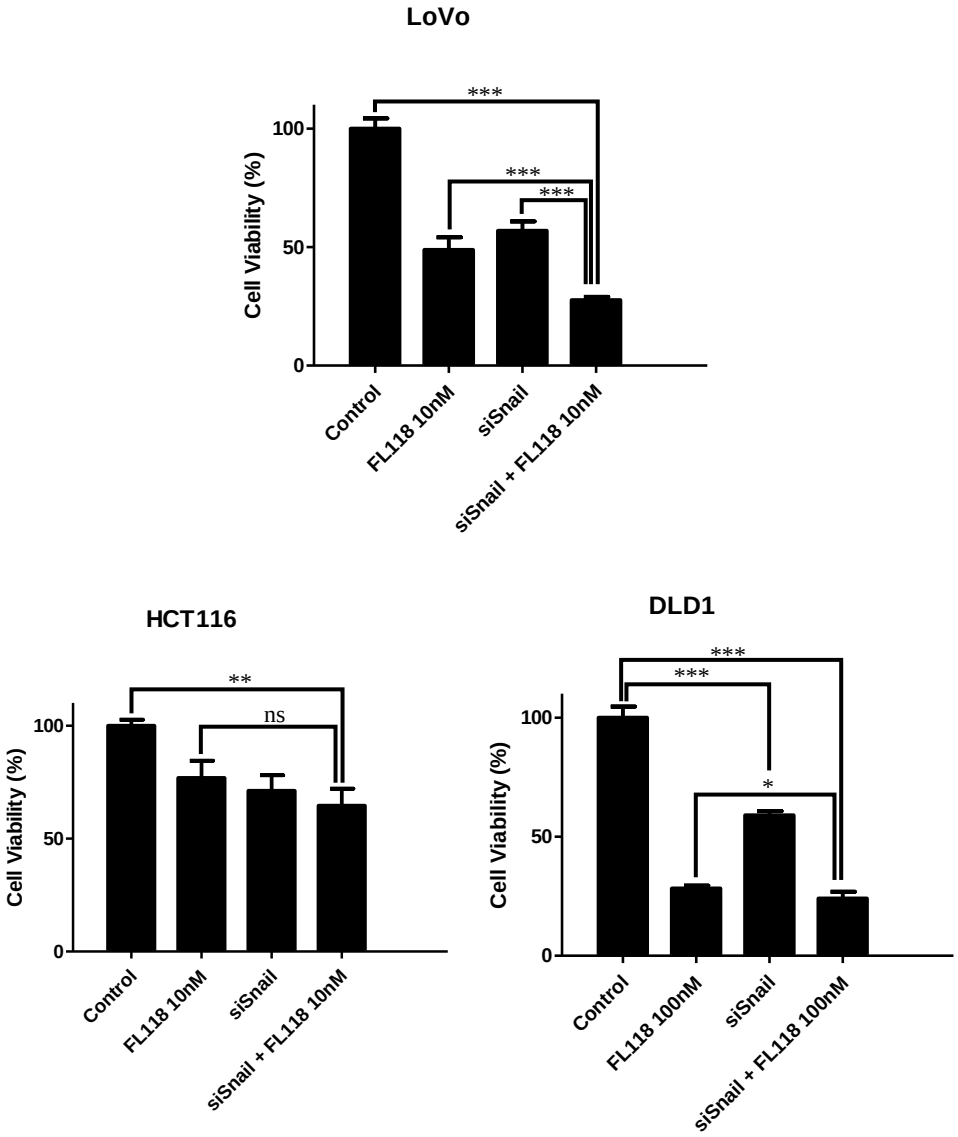
Snail is a Key Regulator of Cell Viability and Migration

High snail expression correlates with increased cell proliferation and processes of EMT such as migration and invasion[24,25]. In colorectal cancer patients, Snail expression is upregulated, and this overexpression correlates with poor prognosis (Fig. S1A). As there was an increase in Snail protein expression when treated with low concentrations of FL118, we explored how this could affect CRC cells in conjunction with FL118. We first analyzed how Snail affected cell viability in CRC cells. We found that cell viability was significantly reduced when Snail was knocked down through siRNA transfection (Fig. 5A). Furthermore, when FL118 was treated together with siSnail, we found an even greater cumulative reduction in cell viability in all CRC cell lines. Similarly, cell migration was also significantly decreased when transfected with siSnail (Fig.5B). However, siSnail was the main factor in the decrease of migration. FL118 treated together with siSnail only resulted in a slight, but non-significant change in cell migration, highlighting the importance of Snail in cell migration.

While all CRC cell lines showed a significant reduction in cell viability and migration when transfected with siSnail and an even greater effect when treated with FL118, we found that there were some differences between protein expression in the CRC cell lines (Fig. 5C). The results indicated the inhibition of Snail after siSnail transfection and slight increases to Snail expression after FL118 treatment as previously mentioned. After Snail knockdown, HCT116 saw a reduction of XIAP as well as γH2AX expression with no significant differences in other cell death markers. Nonetheless, when HCT116 was treated together with FL118, we saw an increase in both p53 and γH2AX while maintaining the inhibition of XIAP. LoVo and DLD1, however, both downregulated survivin expression rather than XIAP while upregulating p53, cleaved caspase 3, and γH2AX when treated with both siSnail and FL118. HCT116 and LoVo had a decrease in TCF4 expression after Snail knockdown which demonstrates Snail's ability to interact with the WNT/β-Catenin pathway leading to cell survival and migration. To further explore how siSnail transfection lead to the differences in protein expression, we examined the changes in gene expression

(Fig. 5D). We found that in both LoVo and DLD1 cells when FL118 was treated there was an increase in Snail gene expression and thus we can ascertain that the increase in Snail protein expression is directly correlated to its genetic expression. We can also deduce that post-translation modifications do not play a major role in the increase of Snail expression after FL118 treatment. Additionally, all cell lines had increased p53 and decreased levels of survivin expression after siSnail transfection and FL118 treatment. This clearly demonstrated siSnail and FL118 to target CRC cells through both activation and repression of genetic expression. Despite some differences in protein expression, all cell lines continued to show lowered cell viability when treated with both siSnail and FL118.

(A)



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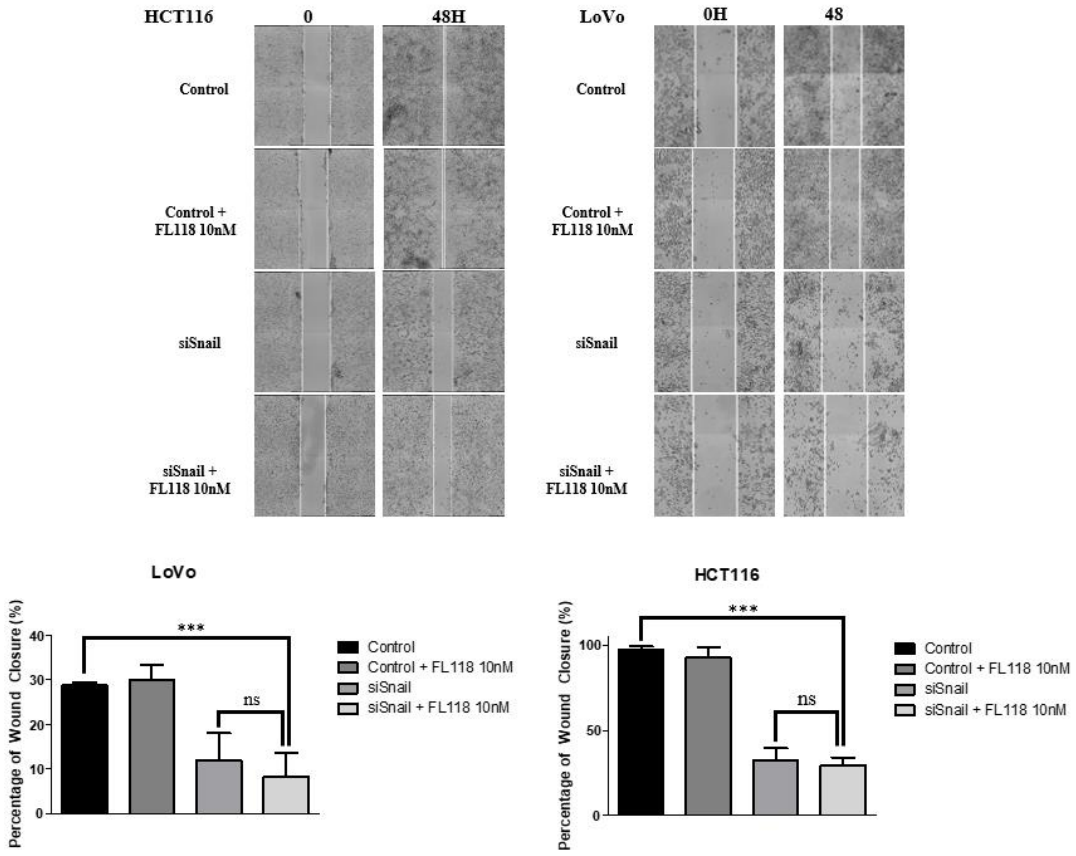

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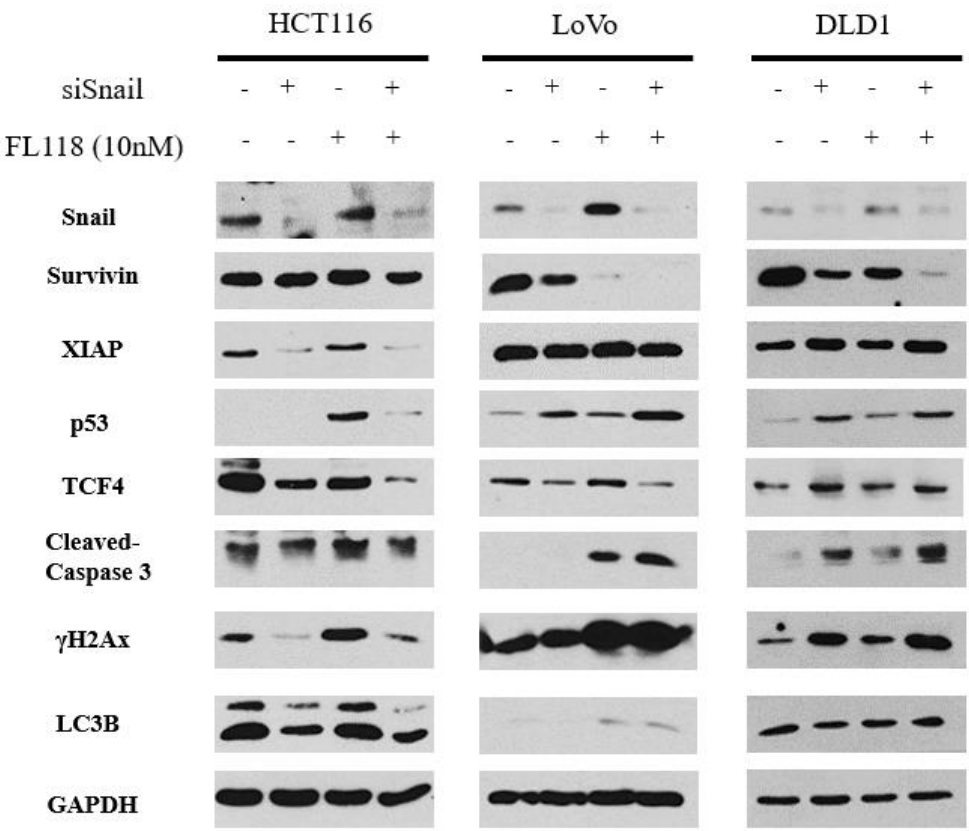

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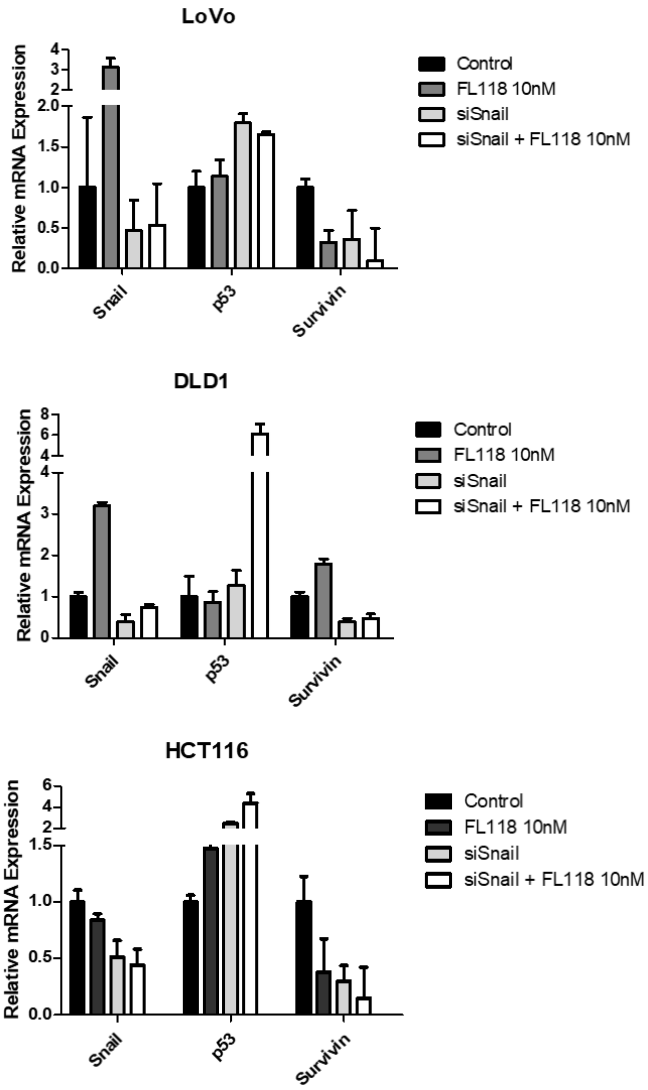
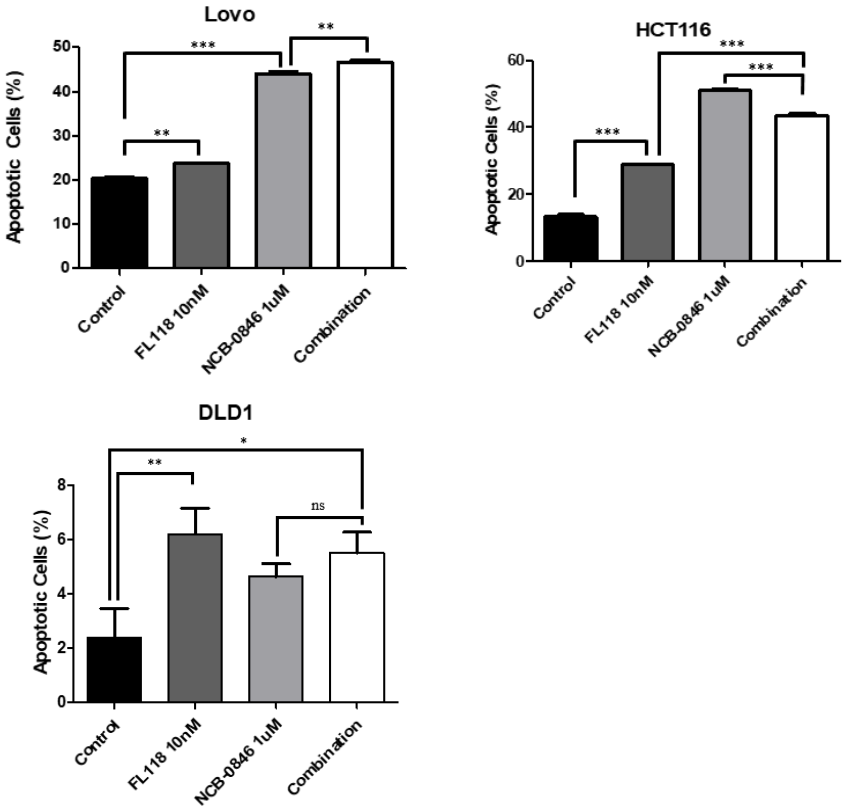


Figure 5. Snail expression is a key factor in cell viability and migration (A) Cell viability of HCT116, LoVo, and DLD1 cells after treatment with 10nM FL118 and/or knockdown with siSnail. **(B)** HCT116 and LoVo cell migration after treatment with 10nM FL118 and/or siSnail knockdown quantified using wound healing assay. **(C)** Protein expression of anti-apoptotic genes and cell death markers after treatment with 10nM FL118 and/or siSnail knockdown. student's t-test. **(D)** Quantitative real-time PCR results for Snail, p53, and Survivin after siSnail transfection and FL118 treatment. **p <0.01 and ***p<0.005 vs control.

Snail Expression has no Effects on Apoptosis in Colorectal Cancer Cell Lines

As we previously discovered that cleaved caspase 3 expression was significantly increased when CRC cells were treated in combination, we sought to see the mechanisms of apoptosis more thoroughly. We found that combination treatment in HCT116 had significant but slightly less apoptosis than NCB-0846 treatment while LoVo maintained the highest degree of apoptosis during combination treatment. As DLD1 had the highest IC50 values for both FL118 and NCB-0846 there was only minimal amounts of apoptosis after FL118 and NCB-0846 treatment. (Fig. 6A). Therefore, we realized that apoptosis is a critical aspect of cell death during combination treatment. We hypothesized that Snail was acting as an anti-apoptotic protein, limiting the ability of cells to go through apoptosis and thus further boost proliferation. However, when Snail expression was knocked down, we observed that all three cell lines did not incur any additional apoptosis compared to control (Fig. 6B). Additional FL118 treatment resulted in an increase of apoptosis further than when treated with siSnail alone. However, this increase in apoptosis was not significantly different than when treated with FL118 alone. This indicates that FL118 treatment alone is responsible for the increase in apoptosis. Thus, we can ascertain that while Snail expression led to increased cell survival and migration, the lack of its expression did not lead to apoptosis.

(A)



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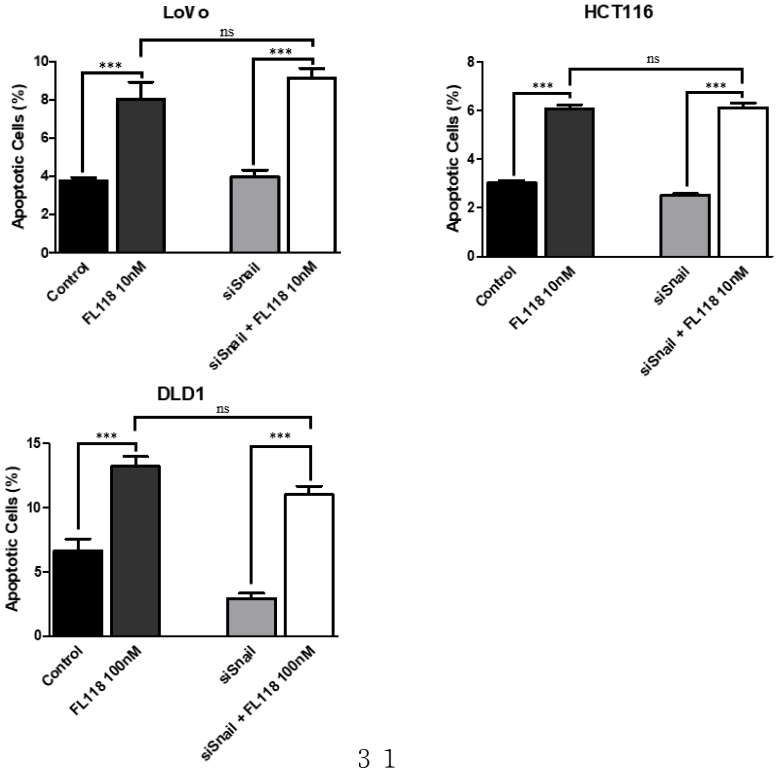

Figure 6. Apoptosis analysis through flow cytometry (A) Examination of apoptosis after treatment of 10nM FL118 and/or 1μM NCB-0846 on HCT116 and LoVo cells. **(B)** Apoptosis after treatment of 10nM or 100nM FL118 and/or siSnail knockdown of HCT116, LoVo, and DLD1 cells. All cells were stained with PE and Annexin V then analyzed through flow cytometry. P value was calculated with student's t-test. ns not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ vs control.

Combination Treatment is Effective in Reducing Organoid Viability

To see if FL118 and NCB-0846 can effectively inhibit cancer cells in an environment like the human body, we tested the viability of patient-derived organoids after drug treatment. After drug treatment, we used the Glo assay to assess organoid viability. We found that FL118 and NCB-0846 both inhibited organoid viability significantly which was even further inhibited when treated in combination. We observed that in organoids 0007 and 0009, NCB-0846 had greater efficacy in inhibiting organoid viability than FL118. In organoid 0013, however, FL118 treatment was more effective than NCB-0846. Despite this, combination treatment was still able to inhibit organoid viability the most in all three organoids. This revealed that, while there were differences in how sensitive each organoid was towards FL118 and NCB-0846, combination treatment remained the most effective in inhibiting all three patient-derived organoids.

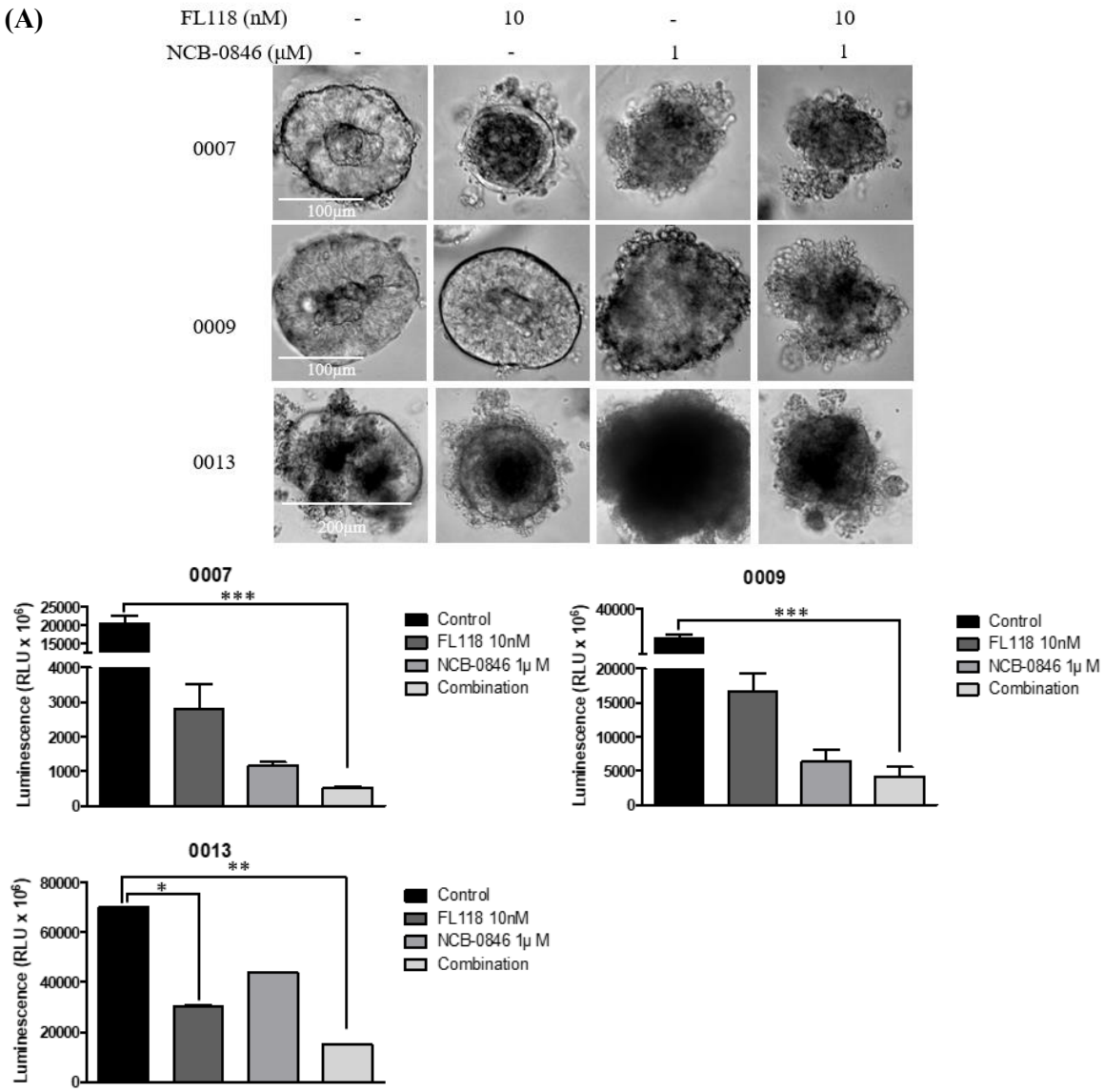

Figure 7. Organoid imaging and viability after combination treatment. (A) Organoid viability after treatment of 10nM FL118 and/or 1μM NCB-0846. Organoids 0007, 0009, and 0013 were incubated with FL118 and NCB-0846 for 5 days, after which Glo solution was added, and luminescence measured. P value was calculated with one-way ANOVA. *p < 0.05, **p <0.01, and ***p<0.005 vs control

IV. DISCUSSION

Colorectal cancer remains one the highest occurring cancers in the world[1,26]. Despite advancements in treatment, malignancy and metastasis remain as major hurdles in overcoming CRC-related deaths[27]. Metastasis occurs when cells go through a process of Epithelial-to-Mesenchymal Transition (EMT) in which a cell loses its epithelial phenotypes to become more mesenchymal and invasive[8]. Epithelial markers such as E-Cadherin are downregulated while mesenchymal markers such as N-Cadherin, Vimentin, and Snail are upregulated. To effectively tackle CRC, we must regulate the extent of EMT. Currently, SN38, an activated metabolite of irinotecan, is often used to treat CRC combined with 5-Fluorouracil (5-FU). However, we offer an alternative that could potentially be used in a clinical setting.

Here, we validated FL118, a member of the same Camptothecin family, as a superior alternative to SN38 in the treatment of CRC in-vitro. Past studies have demonstrated FL118's efficacy in inhibiting CRC cells[12,13]. We also validated this and found that FL118 was able to inhibit CRC cells at lower concentrations than SN38. FL118 further demonstrated its ability to inhibit markers pertinent to cancer cell proliferation and migration more effectively than SN38. Despite FL118's qualities over established compounds effectiveness in suppressing cancer cells, we identified, at lower concentrations, that Snail expression remained unaffected or conversely was highly upregulated after treatment with FL118.

The importance of Snail has been reported in multiple different publications over the years[9,10,24,33,34,35,36]. Snail expression has been shown to be linked with cancer cell proliferation, EMT, and metastasis. This was reflected in our study as FL118-treated cells displayed no changes or, rather, increased in migration. This was again validated through the transwell assay with both LoVo and DLD1 cells having no changes to migration after FL118 treatment. HCT116 cells resulted in a minor decrease of cell migration but this decrease could be partly due to Snail expression not increasing after FL118 treatment. Snail

has also been proven to promote WNT target genes and vice versa[33]. Thus, we sought to utilize NCB-0846, a TNIK inhibitor, to attempt to further decrease EMT. NCB-0846 has been shown to be able to not only attenuate colorectal cancer cell viability but also its EMT and stemness properties[20]. Similarly, we found that combination treatment with NCB-0846 was able to significantly downregulate WNT/β-Catenin pathway proteins as well as those related to EMT. Through this combination, we were able to determine that both cell viability and migration were significantly inhibited.

While combination treatment proved to be effective in inhibiting cell viability, we aimed to further explore the underlying mechanisms. We first examined the changes in the cell cycle through PI staining. We found that both FL118 and NCB-0846 exhibited G2/M phase cell cycle arrest which eventually leads to cell death. TP53, a tumor suppressor gene, is known to be crucial in the regulation of the cell cycle such as the G2/M transition[28]. This study suggested that the overexpression of p53 caused an increase in G2 arrest after genotoxic damage. Another study has further revealed that with the increase of DNA damage, as expressed by γH2AX expression, p53 and Rb works to downregulate genes that are required for G2/M causing an arrest[38]. As both compounds significantly increase p53 and γH2AX expression in our study, this DNA damage and subsequent increase of p53 could be a reason for the cause for the G2/M arrest. We also explored different methods of cell death through protein expression. Interestingly the two events of the increase of γH2AX and decrease of Rad51 coupled simultaneously could lead to even greater cell death. Rad51 was shown to co-localize with phosphorylated H2AX after DNA damage to repair the cell[29]. Thus, due to the decrease of Rad51 expression, this interaction could be severely disrupted. Caspase 3 has also been reported to suppress DNA damage-induced necrosis[39]. Therefore, the increase of cleaved caspase 3 after NCB-0846 treatment can further sensitive colorectal cancer cells towards camptothecins such as FL118. As expected, when cells were treated together with both FL118 and NCB-0846, the amount of apoptosis was significantly greater than when treated with just FL118 alone. Together, through the expression of these cell death markers, we found the treatment of both FL118 and NCB-0846 to have the

greatest effect in lowering cell viability and migration.

As mentioned previously, Snail was also highly upregulated when cells were treated with lower concentrations of FL118. Previous publications have also outlined how commonly prescribed medication such as Doxorubicin could increase the migration of tumor cells through the overexpression of oncogenes and its downstream pathways[31]. This was also reflected in our study. We found that while Snail knockdown was able to inhibit cells, adding FL118 further lowered cell viability. Here we demonstrated Snail's ability to suppress cell death and limit FL118's efficacy. Interestingly, when Snail was knocked down cell migration was significantly inhibited but the addition of FL118 did not significantly inhibit migration further. While this did indicate Snail's ability to increase migration, we sought to understand why FL118 did not lead to further inhibition. We reason that this is due to Snail acting as a critical limiting factor in migration where the addition of FL118 provides no additional value in mitigating these properties. FL118 alone was also unable to, other than N-Cadherein in LoVo cells, effectively inhibit other EMT markers. In the case of NCB-0846, Snail expression, other key EMT targets, and WNT/β-catenin pathway genes were inhibited to a greater extent and even greater when treated together with FL118 which can all attribute to the additional inhibition of the cells' migration properties.

LoVo and DLD1 cells had similar p53 and survivin expression after siSnail transfection. However, HCT116 protein expression had a different pattern from that of LoVo and DLD1. CRC cells are stratified according to their varied molecular subtypes and can act as an accurate model to predict a cell's properties based on their genomic profiles[32]. As LoVo and DLD1 cells are grouped in to the consensus molecular subtype (CMS) 1 while HCT116 cells are part of the CMS4 group, this could be a potential reason to explain the discrepancy in protein expression to this genetic homogeneity. This is further validated with gene expression analysis after siSnail transfection and FL118 treatment. All cell lines had the same gene level changes where p53 increased while survivin decreased. Despite this, only LoVo and DLD1 cells reflected these results in their protein expression while it was different for HCT116. This suggests that LoVo and DLD1 cells transcriptionally regulate

their protein expression while HCT116 cells relies more on post-translational controls. LoVo and DLD1 cells also had significantly increased genetic levels of Snail when treated with FL118. As we previously mentioned the importance of Snail in cell survival, this increase of Snail could be a method to ensure cell viability. Therefore, these two cell lines exhibiting greater sensitivity to Snail knockdown suggests a greater reliance on Snail expression for survival.

We observed that FL118 treatment alone was able to increase apoptosis in all three cell lines. However, despite the drastic effects of siSnail transfection, we failed to see any significant increases in apoptosis. FL118 treatment together with siSnail transfection also did not have a significant change compared to that of FL118 treatment alone which indicates that the apoptosis could be attributed to only FL118 treatment. Therefore, Snail-related cell death could be attributed to other forms of cell death such as necrosis, DNA damage, and cell cycle arrest.

Combination treatment was also able to effectively inhibit the viability of patient-derived organoids. As organoids mimic the microenvironment of human physiology much more closely than that of cancer cell lines, we can more accurately examine the effects of both compounds. While both compounds significantly inhibited organoid viability, combination treatment remained superior, demonstrating its effectiveness in multiple different micro-environments

V. CONCLUSION

In conclusion, FL118 and NCB-0846 treatment alone still provides anti-tumor capabilities. However, the combination of these two compounds elevates both their effectiveness to a much greater extent revealing their synergistic nature. Combination treatment can attenuate cell viability by inducing G2/M cell cycle arrest, apoptosis, and DNA damage. It is also able to mitigate EMT through the downregulation of EMT-related markers such as Snail, Vimentin, and N-Cadherin. This was validated by the significant inhibition of migration of CRC cells. We also validated Snail expression as vital for colorectal cancer cells to survive as well as migrate which was solved through combination treatment. Overall, combination treatment could potentially be able to limit cancer growth in patients as well as attenuate possible metastasis, dramatically increasing the chances of survival in a clinical setting.

REFERENCES

1. Xi Y, Xu P. Global colorectal cancer burden in 2020 and projections to 2040. Translational Oncology [Internet]. 2021 Oct 1;14(10):101174. Available from: https://www.sciencedirect.com/science/article/pii/S1936523321001662

2. Nam S, Choi YJ, Kim DW, Park E-C, Kang J-G. Risk Factors for Colorectal Cancer in Korea: A Population-Based Retrospective Cohort Study. Annals of Coloproctology [Internet]. 2019 Dec 1 [cited 2022 Feb 16];35(6):347–56. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6968718/

3. Jung YS, Kim NH, Yang H-J, Park S-K, Park JH, Park DI, et al. Family history of gastric cancer is associated with the risk of colorectal neoplasia in Korean population. Digestive and Liver Disease: Official Journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver [Internet]. 2017 Oct 1 [cited 2022 Feb 16];49(10):1155–61. Available from: https://pubmed.ncbi.nlm.nih.gov/28801179/

4. Xie Y-H, Chen Y-X, Fang J-Y. Comprehensive review of targeted therapy for colorectal cancer. Signal Transduction and Targeted Therapy. 2020 Mar 20;5(1).

5. Biller LH, Schrag D. Diagnosis and Treatment of Metastatic Colorectal Cancer. JAMA. 2021 Feb 16;325(7):669.

6. Colucci G, Gebbia V, Paoletti G, Giuliani F, Caruso M, Gebbia N, et al. Phase III Randomized Trial of FOLFIRI Versus FOLFOX4 in the Treatment of Advanced Colorectal Cancer: A Multicenter Study of the Gruppo Oncologico Dell'Italia Meridionale. Journal of Clinical Oncology. 2005 Aug 1;23(22):4866–75.

7. Yaffee P, Osipov A, Tan C, Tuli R, Hendifar A. Review of systemic therapies for locally advanced and metastatic rectal cancer. Journal of Gastrointestinal Oncology [Internet]. 2015 Apr 1 [cited 2021 Jul 23];6(2):185–200. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4311092/

8. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. Journal of Clinical Investigation [Internet]. 2009 Jun 1;119(6):1420–8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2689101/

9. Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes & Development [Internet]. 2013 Oct 15 [cited 2019 Nov 7];27(20):2192–206. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3814640/

10. Zheng H, Kang Y. Multilayer control of the EMT master regulators. Oncogene. 2013 Apr 22;33(14):1755–63.

11. Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, et al. Variable B-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proceedings of the National Academy of Sciences. 2001 Aug 28;98(18):10356–61.

12. Westover D, Ling X, Lam H, Welch J, Jin C, Gongora C, et al. FL118, a novel camptothecin derivative, is insensitive to ABCG2 expression and shows improved efficacy in comparison with irinotecan in colon and lung cancer models with ABCG2-induced resistance. Molecular Cancer. 2015 Apr 28;14(1).

13. Ling X, Cao S, Cheng Q, Keefe JT, Rustum YM, Li F. A Novel Small Molecule FL118 That Selectively Inhibits Survivin, Mcl-1, XIAP and cIAP2 in a p53-Independent Manner,

Shows Superior Antitumor Activity. Gartel AL, editor. PLoS ONE. 2012 Sep 19;7(9):e45571.

14. Wang J, Liu Z, Zhang D, Liu R, Lin Q, Liu J, et al. FL118, a novel survivin inhibitor, wins the battle against drug-resistant and metastatic lung cancers through inhibition of cancer stem cell-like properties. American Journal of Translational Research [Internet]. 2017 Aug 15 [cited 2021 Jan 19];9(8):3676–86. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5575181/

15. Ling X, Wu W, Aljahdali IAM, Liao J, Santha S, Fountzilas C, et al. FL118, acting as a "molecular glue degrader", binds to, dephosphorylates and degrades the oncoprotein DDX5 (p68) to control c-Myc, survivin and mutant Kras against colorectal and pancreatic cancer with high efficacy. Clinical and Translational Medicine. 2022 May;12(5).

16. Ling X, Liu X, Zhong K, Smith N, Prey J, Li F. FL118, a novel camptothecin analogue, overcomes irinotecan and topotecan resistance in human tumor xenograft models. American Journal of Translational Research [Internet]. 2015 Oct 15;7(10):1765–81. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4656756/

17. Ling X, Xu C, Fan C, Zhong K, Li F, Wang X. FL118 Induces p53-Dependent Senescence in Colorectal Cancer Cells by Promoting Degradation of MdmX. Cancer research [Internet]. 2014 Dec 15 [cited 2022 Feb 16];74(24):7487–97. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4448973/

18. Thangaiyan R, Aljahdali IA, Lent-Moore KY, Liao J, Ling X, Li F. Kras mutation subtypes distinctly affect colorectal cancer cell sensitivity to FL118, a novel inhibitor of survivin, Mcl-1, XIAP, cIAP2 and MdmX. American Journal of Translational Research [Internet]. 2021 Jul 15 [cited 2022 Oct 15];13(7):7458–74. Available from:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8340187/

19. Yang Z, Ji L, Jiang G, Liu R, Liu Z, Yang Y, et al. FL118, a novel camptothecin analogue, suppressed migration and invasion of human breast cancer cells by inhibiting epithelial-mesenchymal transition via the Wnt/β-catenin signaling pathway. BioScience Trends. 2018;12(1):40–6.

20. Masuda M, Uno Y, Ohbayashi N, Ohata H, Mimata A, Kukimoto-Niino M, et al. TNIK inhibition abrogates colorectal cancer stemness. Nature Communications [Internet]. 2016 Aug 26 [cited 2022 Feb 17];7(1):12586. Available from: https://www.nature.com/articles/ncomms12586

21. Sugano T, Masuda M, Uno Y, Goto N, Seike M, Sawa M, et al. Abstract 2034: Traf2- and Nck-interacting kinase (TNIK) inhibitor, NCB-0846, suppresses TGF-β1-induced epithelial-mesenchymal transition in lung cancer. Cancer Research [Internet]. 2018 Jul 1 [cited 2022 Feb 17];78(13_Supplement):2034–4. Available from: https://aacrjournals.org/cancerres/article/78/13_Supplement/2034/626440/Abstract-2034-Traf2-and-Nck-interacting-kinase

22. Zhang L, Shay JW. Multiple Roles of APC and its Therapeutic Implications in Colorectal Cancer. JNCI: Journal of the National Cancer Institute [Internet]. 2017 Apr 7;109(8). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5963831/

23. Matsumura Y. Preclinical and clinical studies of NK012, an SN-38-incorporating polymeric micelles, which is designed based on EPR effect. Advanced Drug Delivery Reviews. 2011 Mar;63(3):184–92.

24. Wang Y, Shi J, Chai K, Ying X, Zhou BP. The Role of Snail in EMT and Tumorigenesis. Current cancer drug targets [Internet]. 2013 Nov 1 [cited 2022 Oct 15];13(9):963–72.

Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4004763/#

25. Fan F, Samuel S, Evans KW, Lu J, Xia L, Zhou Y, et al. Overexpression of Snail induces epithelial-mesenchymal transition and a cancer stem cell-like phenotype in human colorectal cancer cells. Cancer Medicine [Internet]. 2012 Jun 8 [cited 2020 Jan 7];1(1):5–16. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3544430/

26. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians [Internet]. 2021 Feb 4;71(3):209–49. Available from: https://acsjournals.onlinelibrary.wiley.com/doi/10.3322/caac.21660

27. Feng Y, Jin H, Guo K, Wasan HS, Ruan S, Chen C. Causes of Death After Colorectal Cancer Diagnosis: A Population-Based Study. Frontiers in Oncology. 2021 Mar 30;11.

28. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene [Internet]. 2001 Apr;20(15):1803–15. Available from: https://www.nature.com/articles/1204252

29. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Current Biology. 2000 Aug;10(15):886–95.

30. Fulda S, Debatin K-M. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene [Internet]. 2006 Aug;25(34):4798–811. Available from: https://www.nature.com/articles/1209608

31. Malakouti P, Mohammadi M, Boshagh MA, Amini A, Rezaee MA, Rahmani MR. Combined effects of pioglitazone and doxorubicin on migration and invasion of MDA-

MB-231 breast cancer cells. Journal of the Egyptian National Cancer Institute. 2022 Mar 28;34(1).

32. Berg KCG, Eide PW, Eilertsen IA, Johannessen B, Bruun J, Danielsen SA, et al. Multi-omics of 34 colorectal cancer cell lines - a resource for biomedical studies. Molecular Cancer. 2017 Jul 6;16(1).

33. Stemmer V, de Craene B, Berx G, Behrens J. Snail promotes Wnt target gene expression and interacts with β-catenin. Oncogene. 2008 May 12;27(37):5075–80.

34. Vega S. Snail blocks the cell cycle and confers resistance to cell death. Genes & Development. 2004 May 6;18(10):1131–43.

35. Alaaeldin R, Abuo-Rahma GE-DA, Zhao Q-L, Fathy M. Modulation of Apoptosis and Epithelial-Mesenchymal Transition E-cadherin/TGF-β/Snail/TWIST Pathways by a New Ciprofloxacin Chalcone in Breast Cancer Cells. Anticancer Research [Internet]. 2021 May 1 [cited 2022 Oct 16];41(5):2383–95. Available from: https://pubmed.ncbi.nlm.nih.gov/33952463/

36. Kurrey NK, Jalgaonkar SP, Joglekar AV, Ghanate AD, Chaskar PD, Doiphode RY, et al. Snail and Slug Mediate Radioresistance and Chemoresistance by Antagonizing p53-Mediated Apoptosis and Acquiring a Stem-Like Phenotype in Ovarian Cancer Cells. Stem Cells. 2009 Sep;27(9):2059–68.

37. Sun G, Guzman E, Balasanyan V, Conner CM, Wong K, Zhou HR, et al. A molecular signature for anastasis, recovery from the brink of apoptotic cell death. The Journal of Cell Biology [Internet]. 2017 Oct 2 [cited 2020 Oct 11];216(10):3355–68. Available from: https://pubmed.ncbi.nlm.nih.gov/28768686/

38. Stark GR, Taylor WR. Analyzing the G2/M checkpoint. Methods in Molecular Biology (Clifton, NJ) [Internet]. 2004 [cited 2020 Jul 15];280:51–82. Available from: https://pubmed.ncbi.nlm.nih.gov/15187249/

39. Brown MF, Leibowitz BJ, Chen D, He K, Zou F, Sobol RW, et al. Loss of Caspase-3 sensitizes colon cancer cells to genotoxic stress via RIP1-dependent necrosis. Cell Death & Disease [Internet]. 2015 Apr [cited 2019 Sep 25];6(4):e1729–9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4650537/

APPENDICES

(A) **(B)**

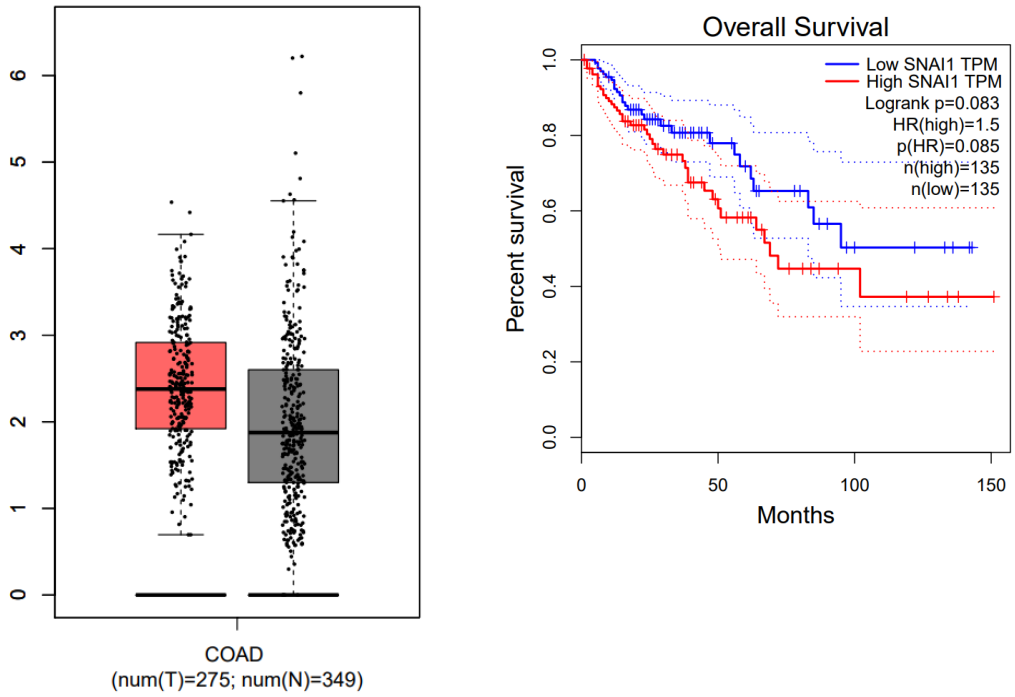

Figure S1. Snail Expression and its Clinical Relevance. (A) The amount of Snail expression in colorectal adenocarcinoma patients with the red bar indicating tumor samples. **(B)** Overall survival graph of colorectal adenocarcinoma patients according to their expression of Snail. The red line indicates high Snail expression. Both graphs were obtained from GEPIA (Tang, Z. et al).

대장암 세포주에서 상피-중간엽 전이를 효과적으로 억제하기 위한 FL118 및 NCB-0846의 복합 치료

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김진우

EMT (Epithelial-Mesenchymal Transition)는 대장암의 진행과정 동안 상피세포가 전이 능력과 침윤 능력을 가지는 세포로 변화하는 과정이다. EMT는 세포 사멸의 억제, 세포주기 진행 및 방사선요법 또는 화학요법에 대한 내성에 관여하며, 높은 사망률과 좋지 않은 예후를 초래한다. 본 연구에서는 대장암 세포에서 EMT 억제에 대한 Campotothecin 아날로그인 FL118과 Wnt/β-catenin 신호전달에 관여하는 TNIK 억제제인 NCB-0846의 병용 투여 효과를 확인하였다.

강력한 세포독성 효과를 나타내는 FL118은 survival mechanism으로 EMT 인자 중 특징적으로 Snail의 과발현을 유도하였으며, 이는 NCB-0846의 병용 처리에 의해 효과적으로 Snail의 발현이 저해되면서 더 높은 항종양 활성을 나타내었다. 이를 통해 Snail이 세포의 증식 및 전이 능력에 중요한 조절 인자이며, 암세포 사멸을 위해 EMT 과정에서 Snail 단백질의 억제에 대한 필요성을 제시하였다. 또한 FL118과 NCB-0846의 병용 처리는 단독 처리와 비교하여 G2/M기의 세포주기 정지를 유도하고, 세포사멸 및 DNA 손상이 증가되는 것을 확인하였다. 이러한 결과는 대장암에서 효과적으로 EMT를 저해할 수 있는 FL118과 NCB-0846의 병용 요법을 개선된 치료 전략으로 제시할 수 있다.

핵심되는 말 :FL118, NCB-0846, 대장암, 상피-중간엽 전이, WNT/β-Catenin