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Selenium in the treatment of colon cancer: Investigation of effect on epithelial mesenchymal transition

Kolon kanseri tedavisinde selenyum: Epitel mezenkimal geçiş üzerindeki etkisinin araştırılması

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Abstract

Aim: This study aimed to investigate the therapeutic effect of seleno-L-methionine (SLM), a selenium (Se) compound, on colorectal cancer (CRC) cells through epithelial-mesenchymal transition (EMT)-related transcription factors (ZEB1 and ZEB2).

Materials and Methods: The human colon carcinoma cell line (Caco-2) was treated with different concentrations of SLM (15.6–250 nmol/L) for 24 hours. Cell viability was assessed using the MTT assay, live/dead rate and morphology were evaluated by Calcein-AM/Propidium Iodide staining, and ZEB1 and ZEB2 gene expressions were analyzed by qRT-PCR.

Results: SLM decreased cell viability in a dose-dependent manner (IC_{50}: 31.25 nmol/L). Live/dead analysis revealed a significant increase in non-viable cells in the SLM-treated group compared to controls, although clear apoptotic morphology was not consistently observed. qRT-PCR analysis revealed a significant downregulation of ZEB2 and a slight reduction in ZEB1 expression.

Conclusion: SLM exhibited cytotoxic effects and modulated EMT-related gene expression, suggesting potential therapeutic implications in CRC.

Keywords: Colon cancer; Selenium; Epithelial-to-mesenchymal transition; ZEB 1; ZEB 2; Cell viability.

Öz

Amaç: Bu çalışma, bir selenyum bileşiği olan seleno-L-metiyoninin (SLM), kolorektal kanser (KRK) hücrelerinde epitel-mezenkimal geçiş (EMT) ile ilişkili transkripsiyon faktörleri ZEB1 ve ZEB2 üzerindeki terapötik potansiyelini araştırmayı amaçlamıştır.

Gereç ve Yöntem: İnsan kolon karsinomu hücre hattı (Caco-2) 24 saat boyunca farklı konsantrasyonlarda SLM (15,6-250 nmol/L) ile tedavi edildi. Hücre canlılığı MTT testi ile değerlendirildi, canlı/ölü oranı ve morfoloji kalsein-AM/propidyum iyodür boyama ile değerlendirildi ve ZEB1 ve ZEB2 gen ekspresyonları qRT-PCR ile analiz edildi.

Bulgular: SLM, hücre canlılığını doza bağımlı olarak azalttı (IC_{50}: 31,25 nmol/L). Canlı/ölü boyama sonuçları, SLM uygulanan gruplarda anlamlı düzeyde artmış ölü hücre oranı gösterdi; ancak tipik apoptotik morfolojik özellikler tutarlı şekilde gözlemlenmedi. qRT-PCR analizi, ZEB2'nin önemli bir şekilde azaldığını ve ZEB1 ekspresyonunda hafif bir azalma olduğunu ortaya koydu.

Sonuç: SLM, Caco-2 hücrelerinde sitotoksik etkiler göstermiş ve EMT ile ilişkili gen ekspresyonunu modüle etmiştir. Bu bulgular, SLM'nin CRC'de terapötik potansiyele sahip olabileceğini düşündürmektedir.

Anahtar Kelimeler: Kolon kanseri; Selenyum; Epitelyal mezenkimal geçiş; ZEB 1; ZEB 2; Hücre canlılığı.

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Bu makale araştırma ve yayın etiğine uygun hazırlanmıştır.
intihal incelemesinden geçirilmiştir.

Introduction

Colorectal cancer is a significant global health problem, ranking third in cancer incidence and second in cancer-related mortalities worldwide.[1] Even with advancements in treatment options, the prognosis for advanced colorectal cancer remains poor, emphasizing the need for innovative therapeutic approaches.[2-4]

In colorectal cancer, epithelial-mesenchymal transition (EMT)is associated with tumor progression, metastasis, and resistance to therapy.[4] The transcription factors ZEB1 and ZEB2 are critical regulators of EMT. These factors suppress epithelial genes and activate mesenchymal genes promoting EMT.[5,6] Elevated levels of ZEB1 and ZEB2 expression are linked to poor prognosis and increased metastatic potential in CRC patients.[7,8] ZEB1 reduces E-cadherin expression and enhances stemness in CRC cells. At the same time ZEB2 boosts invasiveness and metastatic capability.[9,10]

In recent years, selenium (Se), an essential trace element found in foods like nuts, fish, and whole grains, has gained attention for its potential anticancer properties in various malignancies, including colorectal cancer.[9-12] This micronutrient has been reported to exhibit chemopreventive and therapeutic effects in cancer, playing crucial roles in key physiological processes, including antioxidant defense, immune response, and thyroid hormone metabolism.[13] Epidemiological studies have suggested an inverse relationship between Se intake and CRC risk, indicating that higher Se levels may be associated with a reduced likelihood of developing colorectal tumors. However, the molecular mechanisms underlying the anticancer effects of Se in CRC remain incompletely understood, necessitating further investigation to elucidate its potential as a therapeutic agent.[9-11,13,14]

This study aims to elucidate the effect of the Se compound SLM on ZEB1 and ZEB2 expression and on cell viability at the single-cell level using live/dead quantification in colorectal cancer cells using the Caco-2 cell line as an experimental model. Accordingly, we treated Caco-2 cells with varying concentrations of SLM-containing compounds and evaluated their effects on cell viability, apoptosis and expression of markers associated with EMT.

Materials and Methods

Chemicals

Seleno-L-methionine [(L(+) SeMet] (SLM) was obtained from Sigma Chemical (St. Louis, MO, USA). Live/dead cell viability assay kits were obtained from AAT Bioquest®, Inc. (formerly ABD Bioquest, Inc.).

Cells and culture conditions

The human colon adenocarcinoma cell line Caco-2 (ATCC HTB-37, passages 6–16) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Invitrogen) supplemented with 10 % fetal bovine serum (Atlanta Biologicals, GA, USA), 1 % non-essential amino acids (NEAA), 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine. Cells were cultured as adherent monolayers in an incubator (Memmert GmbH, Schwabach, Germany) set to 37 °C, 5 % CO_2, 5 % O_2, and ≥ 90 % relative humidity. Subculturing was performed at approximately 80 % confluence using 0.05 % trypsin-EDTA (Sigma-Aldrich), and cells were seeded at a 1:8 split ratio onto fresh plates at a density of 1×10^5 cells/cm². Routine morphological assessments, including daily phase-contrast imaging and contamination checks, were conducted throughout using an inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). All experiments were performed in triplicate.

Quantitative assessment of SLM cytotoxicity in Caco-2 cells via IC_{50} determination using MTT assay

An MTT viability assay was used to investigate SLM's dose- and time-dependent effects on cell viability and to determine the IC50 value. Cells were seeded in 96-well plates at $1.5x10^4$ cells/well and incubated for 24 h. Subsequently, varying concentrations of SLM (250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 nmol/L) were administered for a 24h. Following incubation, the medium was removed. A solution comprising 10 µL MTT from a 5 mg/mL stock solution and 90 µL of

medium was added to each well, followed by a 2-hour incubation under dark conditions at 37°C with 5% CO_2. Subsequently, the medium was removed, and the resultant purple formazan crystals were solubilized in 100 µL/well DMSO by agitation on a plate shaker for 5 min. Optical density (OD) readings were obtained at 570 nm using a microplate enzyme-linked immunosorbent assay (ELISA) reader (BioTek, ELx808IU, USA).

Morphological analysis using phase-contrast inverted microscopy

Daily imaging was performed in all experiments during routine cell culture maintenance using an inverted phase-contrast microscope (Zeiss Axiovert). Cells were regularly observed under this microscope to monitor morphological parameters such as cell shape, confluence, contamination, and detachment. Representative images were captured at 0, 12, and 24 h time points to document dynamic changes consistently across all experimental runs.

Fluorescent live/dead assessment of SLM treated Caco-2 cells using calcein AM/PI dual staining

The effect of SLM on cell viability under in vitro cell culture conditions was assessed using a Calcein-AM/PI live/dead staining kit Calcein-AM, which is non-fluorescent in its original form, is converted into green-fluorescent calcein by intracellular esterases in viable cells following acetoxymethyl ester hydrolysis. Conversely, propidium iodide binds to the DNA of nonviable cells and emits red fluorescence. Cells that appear green are viable, whereas cells that appear red are nonviable. Caco-2 cells were seeded into 6-well plates at a density of 4×10^5 cells per well and incubated for 24 h. Subsequently, the cells were treated with SLM at the previously determined IC_{50} dose (31.25 nmol/L) for an additional 24 h; each condition was tested in triplicate wells and repeated across three independent experiments. Following the incubation period, cells were washed twice with PBS and incubated with 2 µM Calcein AM and 4 µM propidium iodide in PBS in the dark at 37 °C for 20–30 min. Following a second wash with PBS, images were captured immediately using a fluorescence microscope equipped with FITC and Texas Red filters. Post-treatment, cells were washed and immediately imaged using a fluorescence microscope with FITC and Texas Red filters. For the control group, in each of the three independent experiments, live (green) and dead (red) cells were manually counted by three independent observers across four randomly selected, non-overlapping fields per well. Similarly, in each experiment's treatment (SLM) group, counts were conducted by the same three observers across five randomly selected, non-overlapping fields per well. Cell viability was then calculated as the percentage of green-positive cells relative to the total cell count per field, with results averaged across fields, observers, and replicate experiments.

qRT PCR analysis of EMT related gene expression in SLM treated Caco-2 cells

Total RNA was extracted from treated and control Caco-2 cells (n=3 independent experiments) using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, Cat. No. 217684), per manufacturer's instructions. RNA quality and concentration were measured by Nanodrop® ND 1000 (Thermo Fisher Scientific, USA). First-strand cDNA synthesis was performed using the RT² First Strand Kit (Qiagen, Cat. No. 330404) from 1 µg total RNA and stored at –20 °C. qRT PCR was performed using RT² SYBR® Green qPCR Mastermix (Qiagen, Cat. No. 330502) on a Rotor Gene Q 5 plex+HR instrument (Qiagen). Reactions (20 µL) contained 10 µL SYBR Mastermix, 1 µL diluted cDNA (1:10), 0.5 µL forward and reverse primers (250 nM each), and nuclease-free water. Primers were custom designed using NCBI Primer-Blast interface (Table 1). PCR cycling conditions were: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s, with fluorescence acquisition during extension. Technical triplicates and no-template controls were included for all samples. Relative expression of ZEB1 and ZEB2 was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference.

Table 1. Primer sequences and annealing temperatures for target genes.

Gene	Sequences	Size (bp)	Tm (°C)	Amplicon size (bp)
GAPDH	**F:** GCACAAGAGGAAGAGAGAGACC	22	56.70	78
	R: AGGGGAGATTCAGTGTGGTG	20	53.83	
ZEB1	**F:** TGAGTGCAGTCTTCTGAACCA	21	52.40	76
	R: GAGGCTGATCATTGTTCTTGG	21	52.40	
ZEB2	**F:** CAAGAGGCGCAAACAAGCC	19	59.00	128
	R: GGTTGGCAATACCGCATCC	20	59.00	

Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD) based on three independent experiments (n=3). One way ANOVA was conducted to assess differences among groups, assuming normal distribution and homogeneity of variances as checked by Kolmogorov–Smirnov and Levene's tests. For comparisons against the control group, Dunnett's post hoc test was used, while Tukey's HSD test was applied for pairwise group comparisons. Statistical significance was defined as follows: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Results

The dose response relationship between SLM and Caco-2 cell viability was examined via MTT assay (Figure 1). At the two lowest concentrations tested (3.9 and 7.8 nmol/L SLM), cell viability exhibited a modest but statistically significant increase to 102.1 ± 3.5% ($p<0.05$) and 108.5 ± 5.2% ($p<0.05$) of control levels, respectively, suggesting a potential hormetic effect without cytotoxicity. Treatment with 15.6 nmol/L resulted in a significant viability reduction to 87.6 ± 4.8% ($p<0.05$). A sharp dose-dependent cytotoxic effect emerged at 31.25 nmol/L, where cell viability dropped to 50.2 ± 2.9% ($p<0.0001$). At higher concentrations, viability continued to decline steeply: 62.5 nmol/L yielded 22.5 ± 3.1% viability, and 125 nmol/L led to 17.0 ± 2.4% viability both significantly lower than control ($p<0.0001$). The highest dose, 250 nmol/L, resulted in a dramatic decrease to 12.5 ± 1.8% cell viability ($p<0.0001$). The resulting dose response curve generated using SPSS 13.0 and excel yielded an IC_{50} value of 31.25 nmol/L for SLM in Caco-2 cells. This value is considerably lower than reported IC_{50} values for SeMet in other colorectal cancer cell lines indicating a higher potency of SLM at nanomolar levels.

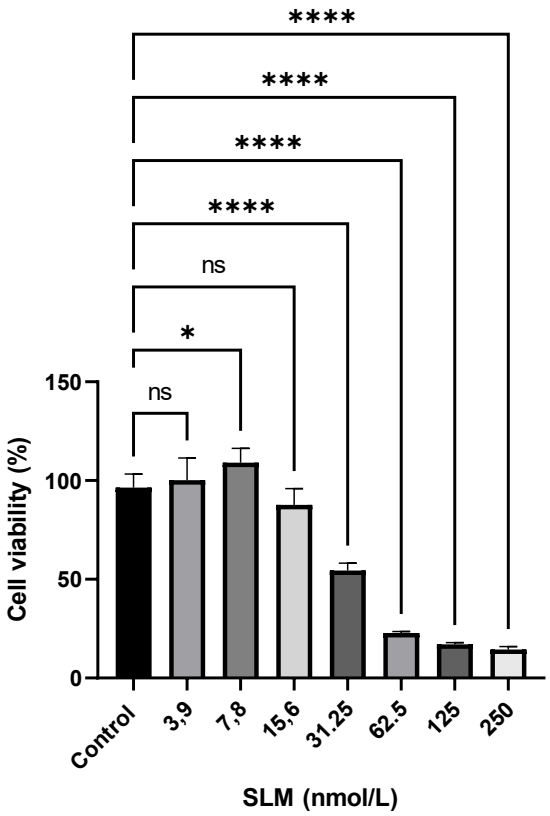


Figure 1. Cytotoxic effects of SLM on Caco-2 cells. Cell viability following SLM treatment was assessed using the MTT assay. Results are expressed as the percentage of viable cells relative to the untreated control group. Data represent the mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

The concentration of 31.25 nmol/L SLM, identified as the IC_{50}, was utilized in all follow-up experiments. This dose ensured a balance between sufficient cytotoxic effect and cell survival adequate for further viability and molecular analyses, including Calcein-AM/PI staining and qRT-PCR assays targeting EMT-related genes.

Observation with an inverted microscope revealed no significant morphological alterations in Caco-2 cells following treatment

with the IC_{50} dose of 31.25 nmol/L SLM. Cell structure, including cytoplasmic and nuclear morphology, remained unchanged, and no detectable differences were observed under phase-contrast at this magnification when compared to untreated controls (Figure 2). It should be noted that no high-resolution structural analyses -such as electron microscopy or cytoskeletal/immunofluorescence staining- were performed in this study to investigate potential submicroscopic alterations.

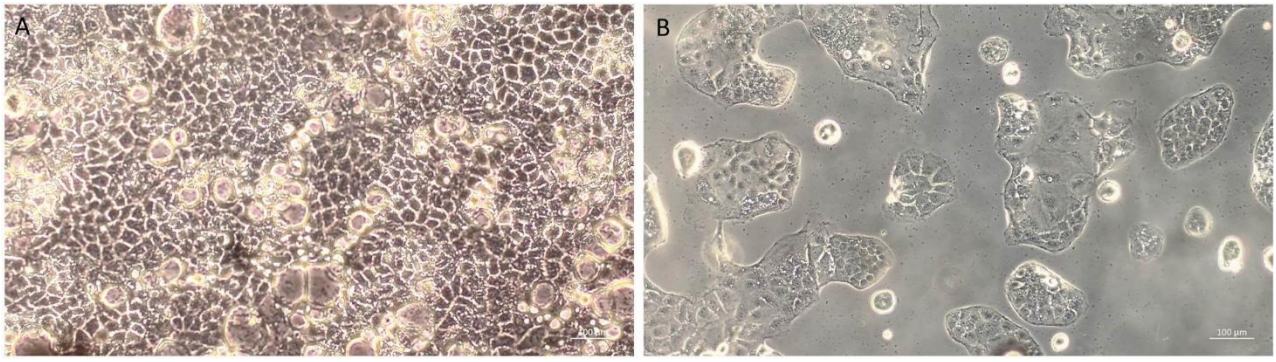


Figure 2. Morphological alterations were assessed using inverted microscopy. A) Untreated Caco-2 cells at passage 6, day 6; B) Caco-2 cells treated with 31.25 nmol/L selenium for 24h at passage 6, day 2. The difference in cell density is attributable to the passage day rather than treatment-related morphological changes.

During Calcein-AM/PI live/dead staining, cells in the control group appeared predominantly green (viable), whereas the SLM-treated group showed a marked increase in red fluorescence (non-viable cells). Occasional treated cells displayed features such as nuclear condensation or subtle membrane irregularities; however, these morphological changes were neither widespread nor definitive. Thus, while this imaging approach confirms a significant reduction in cell viability, it does not allow conclusive identification of apoptotic morphology, and interpretations should remain cautious (Figure 3 and 4). Figure 3 presents the quantitative morphometric analysis. In control wells, the percentage of live (green) cells was $84.7 \pm 5.2\%$, while dead (red) cells accounted for $15.3 \pm 5.2\%$. In SLM-treated wells, live cell percentage decreased to $59.3 \pm 7.8\%$ with a corresponding increase in dead cells to $40.7 \pm 7.8\%$. The differences in both live and dead cell percentages between control and SLM-treated groups were statistically significant ($p<0.05$; independent two-sample T-test).

Furthermore, the expression levels of EMT-related transcription factors, ZEB1 and ZEB2, were analyzed in Caco-2 cells by the help of quantitative reverse transcription polymerase chain reaction (qRT-PCR) following treatment with the IC_{50} dose of SLM for 24 hours. The results indicated that SLM treatment modulated the expression of both ZEB1 and ZEB2 genes (Figure 5). Although a slight decrease in ZEB1 expression was observed compared to the control group, this change was insignificant ($p>0.05$). Conversely, a significant downregulation of ZEB2 expression was detected, with its relative mRNA levels reduced to approximately 0.45-fold ($p<0.05$). However, a significant decrease in ZEB2 mRNA level was observed; however, neither protein-level confirmation nor functional EMT indicators (e.g. cadherin switching, cell motility) were analyzed. Therefore, the results should be interpreted as a precursor signal for EMT modulation. These findings suggest that the suppression of Caco-2 cell migration and invasion by SLM may, in part, be mediated through the regulation of EMT-related markers, particularly the marked inhibition of ZEB2.

Discussion

Here, we examined the cytotoxic effects, morphological changes, and the modulation of EMT-related transcriptional markers, ZEB1 and ZEB2, in Caco-2 colorectal cancer cells treated with SLM. Our findings demonstrate a dose-dependent antiproliferative effect of SLM, beginning at 15.6 nmol/L. Interestingly, a slight proliferation-inducing effect was observed at lower doses (3.9 and 7.8 nmol/L), suggesting that SLM might have a biphasic

dose-response relationship, which is a phenomenon previously observed with other compounds in cancer treatment [15,16]. This initial proliferation at lower concentrations may reflect adaptive cellular mechanisms that respond to low levels of stress, which warrants further investigation to determine the underlying signaling pathways involved.

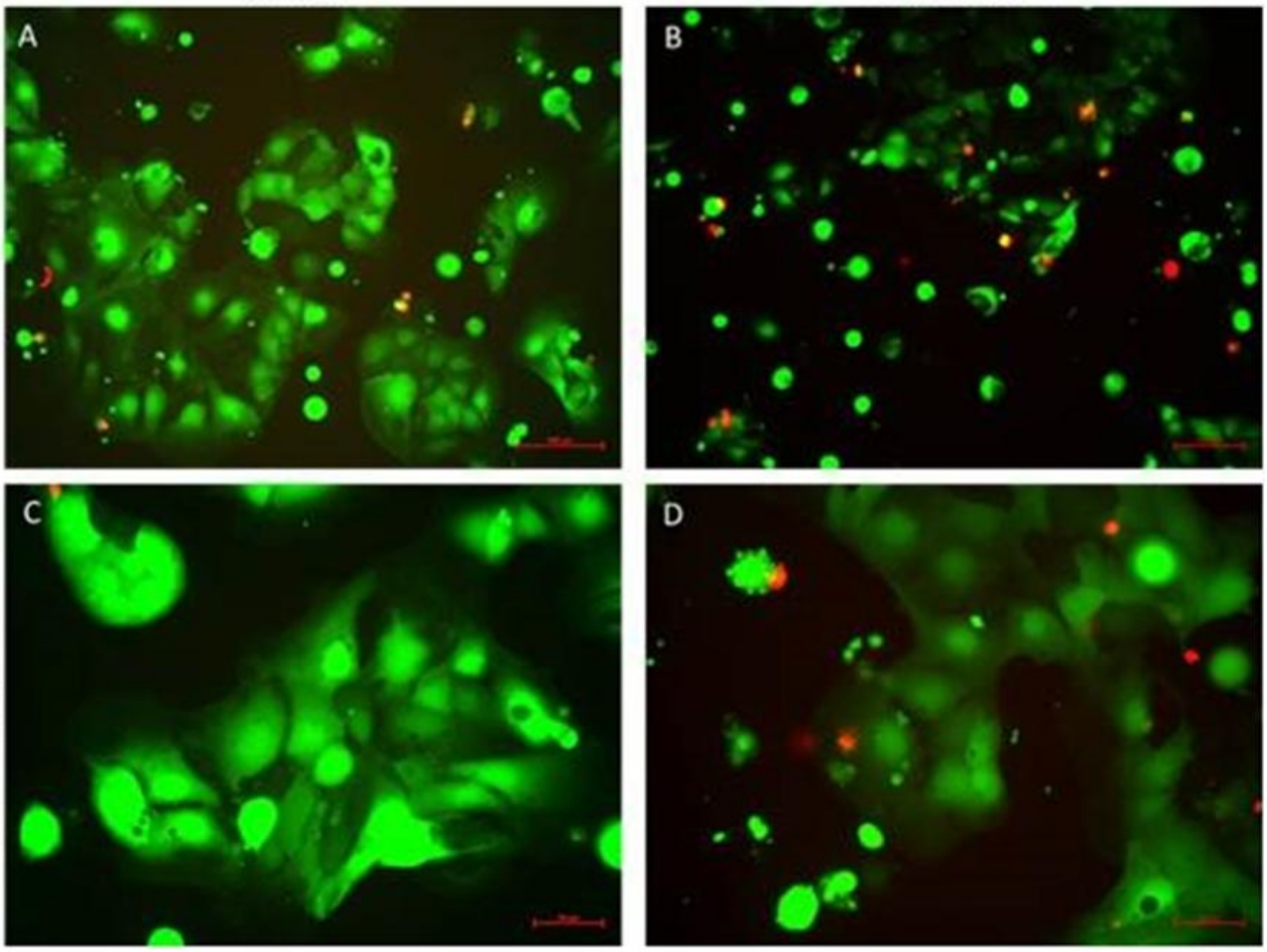


Figure 3. Representative fluorescence ımaging of live/dead staining in Caco-2 cells (control vs SLM treatment). Caco-2 cells were stained with Calcein-AM (green, viable) and Propidium Iodide (red, nonviable) after 24 h treatment. Representative images were randomly selected from four independent fields per well for the control group and five independent fields per well for the SLM-treated group, across three independent experiments. Cell viability was quantified manually by three independent observers. Scale bars: 100 µm (A, B) and 50 µm (C, D).

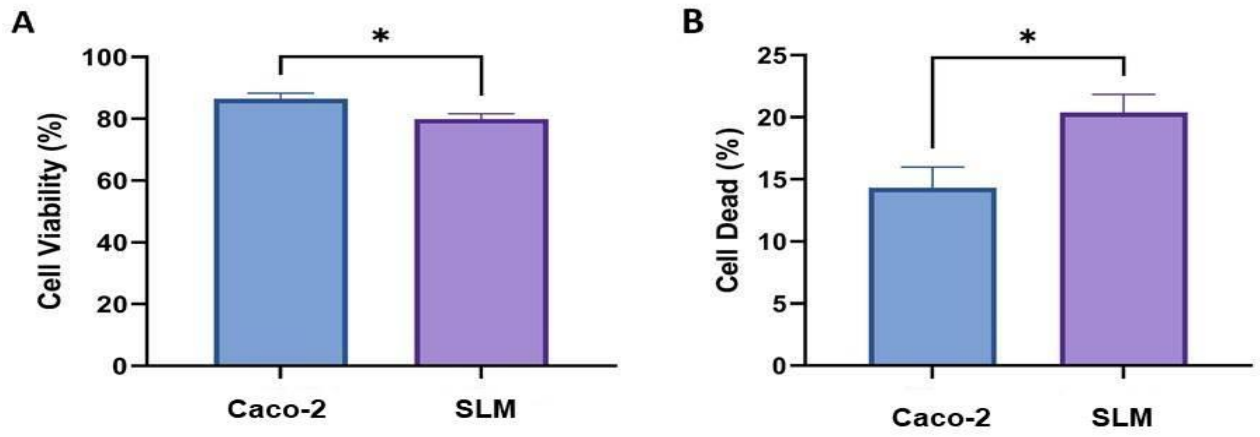


Figure 4. Quantitative comparison of live and dead cell percentages in untreated (control) and SLM-treated Caco-2 cells. Live (green) and dead (red) cells were manually counted from fluorescence micrographs acquired in four randomly selected, non-overlapping fields per well in the Control group and five fields per well in the SLM group, across three independent experiments. Cell percentages were calculated relative to total cell counts per field. Statistical significance was assessed using independent two-sample t-tests. *p<0.05. Control (n = 12 fields); SLM (n = 15 fields).

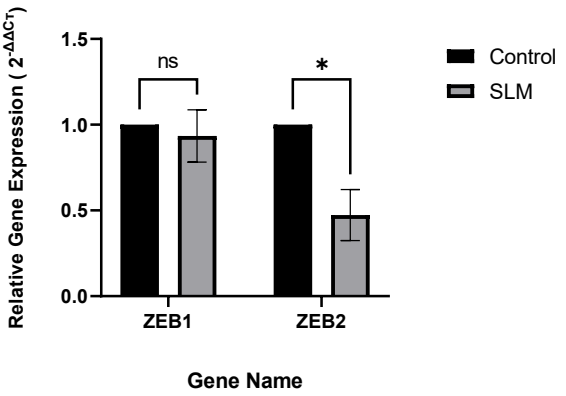


Figure 5. Effect of SLM on EMT-related gene expression in Caco-2 cells. Data represent mean ± SD from at least three independent experiments ($n \geq 3$). Statistical significance is denoted as *p<0.05; ns indicates not significant.

In terms of cytotoxicity, the MTT assay demonstrated a significant decrease in cell viability, confirming the dose-dependent cytotoxic effect of SLM on Caco-2 cells, with an IC_{50} value calculated as 31.25 nmol/L. These results are consistent with previous studies reporting SLM's anticancer properties in various cell lines, including colorectal cancer.[10,17-19] The observed decrease in cell viability suggests that SLM effectively inhibits colorectal cancer cell growth, which may be attributed to its ability to induce cellular stress responses, such as apoptosis.

The morphological analysis using calcein-AM/PI live/dead staining demonstrated a clear reduction in cell viability following SLM treatment, as evidenced by the increased proportion of PI-positive (red) cells. While some treated cells displayed potential apoptotic features—such as nuclear condensation and occasional membrane irregularities—these morphological signs were not consistently or definitively observed across the cell population. The morphological analysis using Calcein AM/PI live/dead staining demonstrated reduced cell viability following SLM treatment, with some cells exhibiting features suggestive of apoptosis. However, we did not perform apoptosis specific assays (e.g., Annexin V FITC/PI staining, caspase activity assays, or TUNEL analysis), and thus cannot conclusively determine whether cell death occurred via apoptosis. Future studies should include these assays to clarify the mechanism of SLM induced cytotoxicity. Nevertheless, previous studies have shown that selenomethionine can induce p53-mediated apoptosis in colon cancer lines, including Caco-2, via Annexin V/PI staining and caspase activation. Similarly, other selenium compounds have demonstrated apoptosis induction in CRC cells.[10,11,13,17,18,22-25] Although the differences observed in Calcein-AM/PI live/dead staining were statistically significant, the limited morphological resolution of this fluorescent-based assay prevents definitive distinction between apoptotic and necrotic cell death. Analyses relying on membrane integrity and esterase activity -such as this- offer only preliminary biological insight and should be supported by complementary techniques (e.g., flow cytometry or cell death–specific immunostaining) before drawing firm conclusions. Nevertheless, these findings can be viewed as a biological counterpart to the significant reduction in ZEB2 mRNA levels, reinforcing the notion that SLM treatment reduces the proportion of viable cells and promotes cell death.

We focused on the expression of EMT-related transcription factors, specifically ZEB1 and ZEB2, which are crucial regulators of epithelial-to-mesenchymal transition (EMT), a process known to contribute to cancer cell migration, invasion, and metastasis.[5-7] Our qRT-PCR analysis revealed that SLM treatment resulted in a significant downregulation of ZEB1 and ZEB2 expression, with a particularly strong reduction in ZEB2 expression. However, EMT cannot be concluded based solely on ZEB2 mRNA reduction, as no protein-level validation or phenotypic assays were performed. This finding should be considered preliminary, warranting further mechanistic confirmation. ZEB2, in particular, has been shown to play a critical role in promoting cancer metastasis by facilitating the transition from an epithelial to a mesenchymal phenotype.[7,8,26,27] The downregulation of ZEB2 observed in our study indicates that SLM may inhibit The downregulation of ZEB2 observed in our study indicates that SLM may inhibit EMT, a crucial step in the metastatic process, thereby potentially reducing the migratory and

invasive abilities of colorectal cancer cells. These findings are consistent with previous studies suggesting that SLM and its derivatives can modulate EMT-associated pathways. The studies have shown that SLM can affect the expression of various EMT-related markers, including E-cadherin, N-cadherin, and vimentin, thereby impacting the metastatic potential of cancer cells.[28,29] By modulating ZEB1 and ZEB2, SLM could be targeting key drivers of EMT and metastasis, which makes it a promising candidate for further investigation as a therapeutic agent in colorectal cancer.

In this study, we investigated the effects of SLM on Caco-2 colorectal cancer cells. Our findings demonstrate that SLM exhibits significant anti-proliferative effects, potentially inducing apoptosis and modulating the expression of key epithelial-to-mesenchymal transition (EMT)-related transcription factors, ZEB1 and ZEB2. These effects suggest that SLM has potential as a therapeutic agent for colorectal cancer, particularly through its capacity to reduce cell viability and target the molecular pathways involved in EMT. Notably, the downregulation of ZEB2 in response to SLM treatment points to its role in modulating cancer metastasis, a critical factor in CRC progression. These results are consistent with previous studies, furher suggesting the notion that SLM compounds can inhibit cancer cell proliferation and migration by affecting EMT pathway. Investigating these pathways could enhance the therapeutic efficacy of SLM and provide insights into its role as an adjunct to conventional cancer treatments. Future studies should include protein-level analyses (e.g., Western blot or immunofluorescence for E-cadherin, N-cadherin, vimentin), as well as functional assays (wound-healing, invasion assays), to establish whether SLM truly modulates EMT.

Conclusion

A limitation of our study is the use of a single CRC cell line (Caco-2). Validation of these findings in additional colorectal cancer lines such as HT-29 and HCT 116 is warranted to confirm the generalizability of the observed effects. In conclusion, our study provides compelling initial evidence supporting the potential of SLM as a therapeutic agent in colorectal cancer. The suggestive induction of apoptosis, based on morphological features, along with the modulation of EMT-related transcription factors (notably ZEB1 and ZEB2), underscores SLM's capacity to reduce cancer cell viability and potentially inhibit metastatic processes. However, the apoptotic mechanism remains inferential, and apoptosis-specific assays are required to confirm this effect. Future research should also explore SLM's efficacy in additional CRC cell lines and in vivo models, as well as investigate synergistic effects with standard chemotherapeutic agents. These steps are essential to fully elucidate the molecular mechanisms and clinical relevance of SLM-based therapies for colorectal cancer.

Ethics Committee Approval

No data were obtained from animal or human experiments for this article. The study does not require ethics committee approval since it does not involve any human or animal subject.

Informed Consent

Consent was obtained from all authors for this article.

Author Contributions

S.G.B.: Conceptualization; investigation; methodology; writing – review, editing and research administration. Ö.T.: Conceptualization; data curation; formal analysis; investigation; visualization; writing– original draft; writing – review and editing. F.B.E: Investigation; methodology. A.E.S: formal analysis; investigation; visualization. O.U: Review and editing.

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Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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Statements

These data have not been presented or published previously.

Peer-review

Externally peer-reviewed.

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