

Research Article

Potential Synergistic Interaction Between Curcumin and Sorafenib Enhances Cytotoxicity in NCI-H522 Lung Cancer Cells

Lunawati Lo Bennett *

College of Pharmacy, Union University, 1050 Union University Dr, Jackson, TN 38305, USA; E-Mail: llbennett@uu.edu

* **Correspondence:** Lunawati Lo Bennett; E-Mail: llbennett@uu.edu

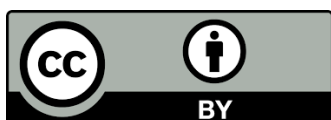
Academic Editor: Ying S. Zou*OBM Genetics*

2025, volume 9, issue 4

doi:10.21926/obm.genet.2504313

Received: August 25, 2025**Accepted:** October 10, 2025**Published:** October 15, 2025**Abstract**

Non-small cell lung cancer (NSCLC), accounting for approximately 85% of lung cancer cases, remains a leading cause of cancer-related mortality. Genetic alteration such as EGFR mutations, tobacco exposure, and resistance to apoptosis, slows its progression and limits therapeutic success. Although targeted therapies and immunotherapy have improved outcomes for select patients, resistance remains a major challenge, highlighting the need for more effective treatments. This study investigated the potential synergistic interaction between curcumin (CUR) and sorafenib (SF) in the p53-null NCI-H522 NSCLC cell line. Cell viability (MTT assay), apoptosis (Hoechst 33342, Rhodamine 123, and Nuclear-ID red/green staining) and protein expression (Western blot) were evaluated following CUR, SF, or combined treatment. The combination markedly increased apoptosis via activation of caspases-3, -7, -8, and -9, elevated Bax/Bcl-2 ratio, and increased ROS production, while suppressing MEK1–4, mTOR, Akt, EGFR, ERK/p-ERK, and HER2. Tumor suppressors p21 and PTEN were upregulated, and cell cycle regulators were downregulated. These findings indicate that CUR potentiates the anticancer effects of SF through multi-pathway inhibition and tumor suppressor activation, supporting its potential as an adjunct therapy for NSCLC, offering a promising strategy to improve NSCLC treatment outcomes.



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Keywords

Non-small cell lung cancer; curcumin; natural anti-neoplastic; caspases; NCI-H522 cells; sorafenib; EGFR

1. Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 85% of cases. Adenocarcinoma has become the most common NSCLC subtype, surpassing squamous cell carcinoma in both men and women, and continues to pose a major public health burden globally [1-4]. In 2024, despite declining smoking rates, an estimated 2.1 million new cases of lung cancer and 1.8 million deaths occurred globally, with the highest incidence in North America, Europe, and parts of Asia [1, 2].

The pathogenesis of NSCLC involves diverse genetic and molecular alterations, including EGFR mutations and tobacco-related mutational burdens, leading to uncontrolled cell growth, survival signaling, and therapy resistance [5-7]. Prognosis depends on stage, histology, and molecular profile. While early-stage disease may achieve five-year survival rates of 60–80% with surgery, advanced NSCLC remains challenging, with substantially lower survival outcomes [8]. Current treatment strategies include surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. Platinum-based chemotherapy remains standard for advanced disease without targetable mutations, whereas targeted agents (e.g., EGFR inhibitors such as Osimertinib) and immunotherapies have expanded therapeutic options [9-12].

Sorafenib (SF), an oral multitargeted tyrosine kinase inhibitor, has shown activity in NSCLC through inhibition of RAF/RAS/MEK/ERK, VEGFR, and PDGFR signaling, suppressing tumor angiogenesis and proliferation [13-17]. Curcumin (CUR), a natural polyphenol from *Curcuma longa*, exhibits antioxidant and antineoplastic activity. Preclinical studies show that CUR can inhibit NSCLC growth by modulating NF- κ B, PI3K/AKT, and MAPK pathways and by enhancing chemotherapeutic efficacy [14, 18-21]. Its dual redox properties—antioxidant under physiological conditions and pro-oxidant in cancer cells—enable selective induction of oxidative stress and apoptosis in malignant cells [22-24].

Recent evidence suggests that CUR and SF act synergistically in several cancers. CUR may downregulate NF- κ B and STAT3 signaling, sensitize cells to SF's pro-apoptotic and anti-angiogenic effects, and reduce drug resistance via inhibition of ABC transporters [25-27]. CUR has also been reported to enhance the efficacy of therapies in different cancer types, when combined with cisplatin, paclitaxel, and gefitinib, highlighting its broad potential as a chemosensitizer [28-31].

In this study, the focus was on the NCI-H522 lung adenocarcinoma cell line, which harbors KRAS mutations and exhibits intrinsic chemoresistance, as a clinically relevant model to investigate whether CUR can enhance SF efficacy and overcome drug resistance [32]. Due to resource limitations, additional NSCLC cell lines could not be included. Future studies should evaluate the combination of CUR and SF in animal studies and across other genotypes of NSCLC to confirm broader applicability.

2. Materials and Methods

2.1 Chemicals and Others

Sorafenib and other anti-neoplastic were obtained from LC Laboratories (Woburn, MA, USA). RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 0.1% sodium dodecyl sulfate), protease and phosphatase inhibitor cocktail, Rhodamine 123, EMEM, MTT reagent, curcumin, were purchased from Sigma (Saint Louis, MO, USA). DPBS, NucBlue™ live cell stain (Hoechst 33342 special formulation) and Nuclear-Live/Dead™ viable/cytotoxicity kit were purchased from Life Technologies (Carlsbad, CA, USA), H2DCFDA from Invitrogen (Eugene, OR, USA). FBS and streptomycin/penicillin bought from ATCC (Manassas, VA, USA). Bradford Reagent was purchased from Fischer Scientific (Waltham, MA, USA). Enhanced Chemiluminescence (ECL) detection kit was supplied by Bio-Rad (Hercules, CA, USA). DMSO was purchased from Ameresco (Solon, OH, USA). Primary antibodies purchased from Cell Signaling Technology include Bax #2772, Bcl2 #15071, Cytochrome C #11940, β -actin #4970, Cas 3 #9665, Cas 7 #12827, Clvd. 7 #8438, Cas 8 #4790, Cas 9 #9502, AIF #5318, Cyclin B1 #4138, Cyclin D1 #2978, p21 #2947, PTEN #9188, HER2 #4290, ERK #4695, p-ERK #5683, CDK2 #28439, mTOR #2972, EGFR #4267, p53 #9282, p13k #4292, and Akt #4691. Other primary antibodies were bought from Santa Cruz Biotechnology (MEK-1 #6250, MEK-2 #398091, MEK-3 #136260, MEK-4 #166197, CDK7 #365075, MDM2 #965) and dry milk. Secondary antibodies from Cell Signalling. Tissue culture dishes and microplates were bought from Greiner-Bio One (Monroe, NC, USA).

2.2 Cell Culture and Media

NCI-H522 human non-small-cell lung carcinoma cells (CRL-5810) was obtained from the American Type Culture Collection (ATCC), derived from a 58-year-old male with adenocarcinoma were cultured in RPMI-1640 medium (ATCC) supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS (ATCC), and 1% penicillin/streptomycin (Sigma Aldrich). These cells were cultured following ATCC's recommended protocols and were obtained directly from ATCC. Cells were routinely tested for mycoplasma contamination. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

2.3 Cell Viability Assay

MTT assays were first used to determine IC₅₀ values for various anticancer agents to identify the compound with the highest cytotoxic effect at the lowest concentration in the NCI-H522 NSCLC cell line using afatinib, irinotecan, doxorubicin, paclitaxel, sorafenib, etoposide, gemcitabine, capecitabine, vincristine, docetaxel, and SF. Among these, SF demonstrated the greatest potency in *vitro* screening.

In addition, antioxidants such as N-acetyl cysteine, alanine, resveratrol, glutathione, and CUR were also tested. CUR was identified to be the most effective. CUR was dissolved in DMSO and added to culture medium to achieve a final working concentration of 12.5 μ M. The final DMSO in all treatment groups was kept below 0.1% to avoid cytotoxic effects associated with the solvent. Although CUR was solubilized in DMSO for in vitro use, its known poor bioavailability and instability in vivo remain major limitations that warrant further formulation-based optimization for clinical

translation. While several antioxidants and anti-cancer were initially screened, only CUR and SF were pursued in detail in the presented study due to their synergistic effect.

Cells were seeded at 1×10^5 cells/ml in 96-well plates and treated with CUR, SF, or their combination. After 24 hr. incubation at 37°C with 5% CO₂, MTT solution (2 mg/ml) was added for 4 h, followed by DMSO addition to dissolve formazan crystals. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices).

2.4 Determination of Synergism

Since the Chou-Talalay software was not available, the combination index (CI) was manually calculated based on the effects of SF, CUR, and their combination at various concentrations. CI values were plotted against the fraction of affected cells (Fa), where $CI < 1$ indicates synergism, $CI = 1$ denotes an additive effect, and $CI > 1$ suggests antagonism.

2.5 Morphology Analysis

The initial morphological changes NCI-H522 cells treated with SF, CUR and their combo for 0 and 24 h were documented using an inverted microscope with 40× capacity (Motic AE31, Hongkong). To obtain more detailed insights, fluorescence microscopy to assess functional and structural cellular alterations, cells were stained with Rhodamine 123 to evaluate mitochondrial membrane potential, H₂DCF-DA to detect ROS generation, and a Live/Dead cell staining kit to assess membrane integrity.

2.6 Migration Analysis (Wound-Healing Assay)

To assess the effect of CUR and SF on cell migration, NCI-H522 cells (1×10^5) were seeded in 12-well plates for 24 hr., followed by scratch-wound creation with a sterile pipette tip. Migration was imaged and quantified at 0 and 24 hr. using a 40× inverted microscope.

2.7 Apoptosis Assay

Nuclear chromatin changes in NCI-H522 cells after CUR and SF treatment were assessed using the NucBlue™ Live Cell Hoechst 33342 assay, following the company's established protocols. Apoptotic and non-apoptotic cells were quantified via Floid Cell Imaging Station, and fluorescence intensity was analyzed with ImageJ (Fiji, NIH). A histogram was generated to compare apoptotic cell percentages across treatment groups.

2.8 Mitochondrial Membrane Potential (Ψ_m) Assay

To identify if there were changes in mitochondrial membrane potential, cells were stained with Rhodamine 123 fluorescence probe following the company's established protocol. The relative intensities of green fluorescence were captured using FLOID cell imaging with a scale bar of 100 μ m. A histogram was prepared to compare the quantities of fluorescence using Image J software.

2.9 Intracellular ROS Assay

Intracellular ROS levels in NCI-H522 cells after CUR, SF, or combination treatment were measured using H₂DCF-DA staining, following manufacturer protocol. Fluorescence was imaged with the Floid

Cell Imaging Station with scale bar of 100 μm , and intensity was quantified using ImageJ to generate a comparative histogram.

2.10 Live and Death Cells Assay

DNA alterations in NCI-H522 cells after CUR, SF, or combination treatment were evaluated using the Nuclear-ID Red/Green Cell Viability Assay per manufacturer's instructions. Live and dead cells were visualized via Floid Cell Imaging Station with scale bar of 100 μm and quantified using ImageJ to generate a comparative histogram.

2.11 Western Blot Analysis

Western blot analysis was performed to assess protein expression in NCI-H522 cells treated with SF, CUR, or their combination. Proteins were extracted using RIPA buffer with protease and phosphatase inhibitors and quantified via Bradford assay. Equal amounts (50 μg) of proteins were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and blocked with 5% milk in TBST for 2 hr. Membranes were incubated overnight at 4°C with primary antibodies under gentle agitation. The next day, after TBST washes, membranes were incubated with secondary antibodies for 2 hr., and bands were visualized using ECL reagents and imaged with the Bio-Rad ChemiDoc XRS+ system.

2.12 Statistical Analysis

All experiments were performed in three independent biological replicates; each conducted on separate days with freshly prepared samples, and the results were expressed as a mean $\pm$ SD. Differences between groups were analyzed using Newman-Keuls one-way ANOVA with ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant.

3. Results

3.1 Cell Viability Assay

Initially, NCI-H522 cells were treated with a range of CUR (0.1–100 μM) and SF (0.1–100 μM) concentrations for 24 hours to determine their respective IC_{50} values. Both agents exhibited dose-dependent cytotoxicity, with IC_{50} values of approximately 25 μM for CUR and 50 μM for SF (Figure 1A, B). Sub-cytotoxic doses 6.3 μM SF and 12.5 μM CUR—resulted in ~80% cell viability and were selected for combination studies to evaluate potential synergistic effects without inducing excessive non-specific cell death (Figure 1C). Combination treatments were then assessed for synergism using manually calculated combination index (CI) values. The combination of SF 6.3 μM + CUR 6.3 μM showed strong synergism (CI = 0.43, Fa = 0.36), while SF 12.5 μM + CUR 12.5 μM also exhibited synergistic effects (CI = 0.73, Fa = 0.42). Based on these results, the combination of SF 6.3 μM and CUR 12.5 μM was chosen for subsequent experiments due to consistent synergistic activity while maintaining cell viability, minimizing non-specific cytotoxicity, as it fell within the observed synergistic range.

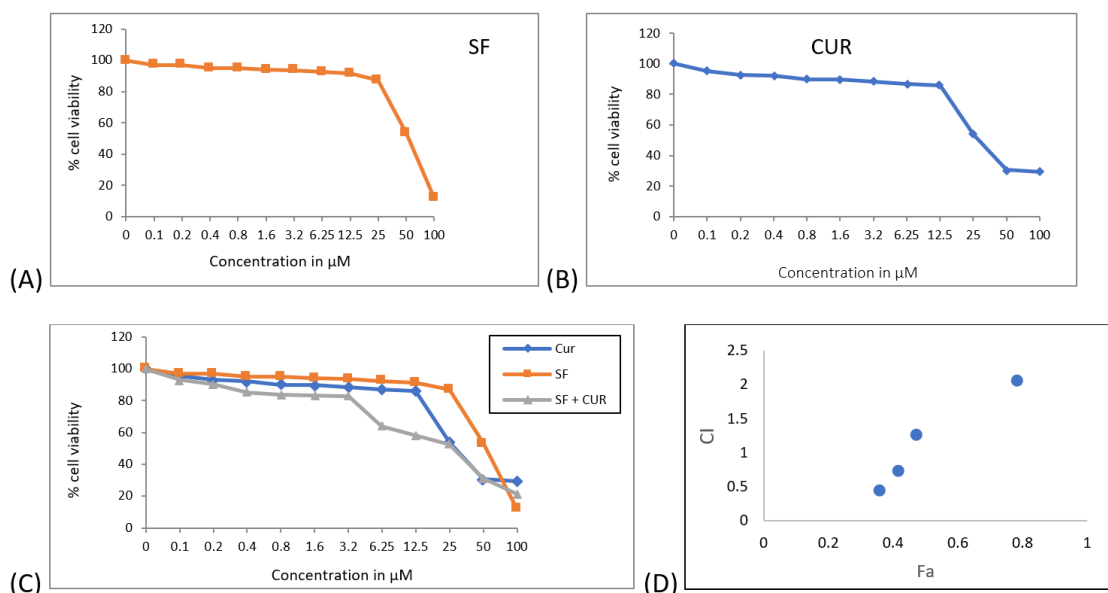


Figure 1 (A) Dose-response of NCI-H522 cells treated with SF (0–100 μM) for 24 h. (B) Dose-response of cells treated with CUR (0–100 μM) for 24 h. (C) SF at 6.3 μM combined with serial concentrations of CUR to determine the optimal synergistic dose; 12.5 μM CUR was selected. (D) Synergistic interaction between SF and CUR evaluated by combination index (CI); SF 6.3 μM + CUR 6.3 μM showed strong synergism (CI = 0.43, Fa = 0.36), and SF 12.5 μM + CUR 12.5 μM also showed synergy (CI = 0.73, Fa = 0.42). Data represent mean $\pm$ SD of three independent experiments.

3.2 Changes in Cell Morphology

All treatment groups exhibited noticeable morphological changes compared to control cells. As shown in Figure 2, control NCI-H522 cells displayed typical epithelial-like growth and increased confluence over 24 hours. SF treatment led to reduced cell density, whereas CUR caused a loss of epithelial characteristics. The combination of SF (6.3 μM) and CUR (12.5 μM) induced extensive cell death, with most cells detaching and floating after 24 hours. These observations indicate that the combination treatment exerts a more pronounced cytotoxic effect than either agent alone.

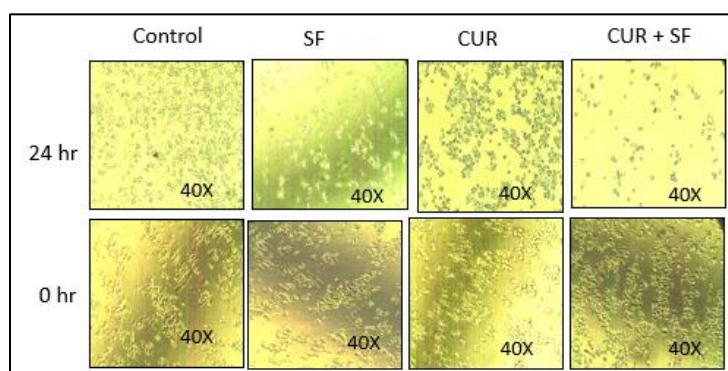


Figure 2 Inverted microscope images of NCI-H522 cells following treatment with SF (6.3 μM), CUR (12.5 μM), or their combination. Control shows typical epithelial-like morphology and high confluency. Data represent mean $\pm$ SD of three independent experiments.

3.3 Cell Migration (Wound-Healing Assay)

The effects of SF, CUR, and their combination on NCI-H522 cell migration were assessed using a wound healing assay. Treatment with SF or CUR individually reduced wound closure, indicating impaired migration. The combination of SF (6.3 μ M) and CUR (12.5 μ M) produced the greatest inhibition, with minimal wound closure observed as shown in Figure 3. It should be noted that the assay reflects both migration and proliferation, and the enhanced cytotoxicity of the combination treatment may partially contribute to reduced wound closure. Nevertheless, these findings suggest that SF and CUR not only decrease viability but also impair migratory potential, with the combination exhibiting synergistic inhibition of cell motility.

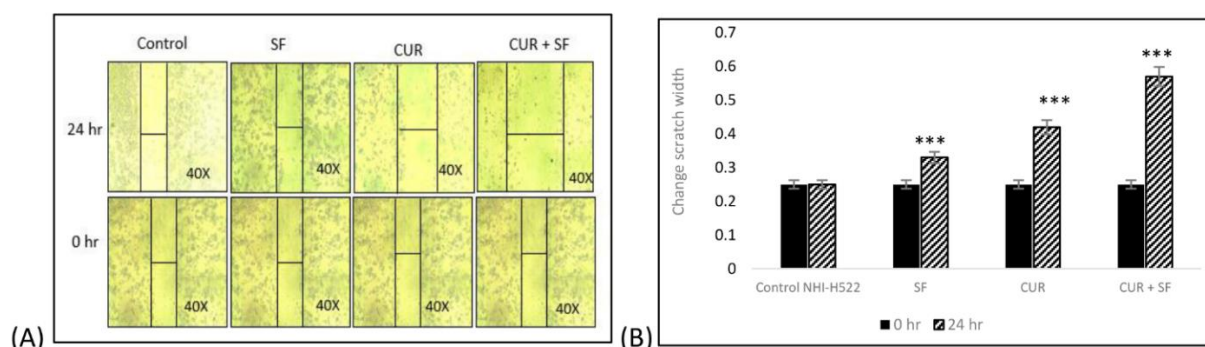


Figure 3 (A) Images show wound closure in control cells and in cells treated with SF (6.3 μ M), CUR (12.5 μ M), or their combination. SF or CUR alone partially inhibited closure. (B) Histogram quantification with statistical analysis via post hoc Newman-Keuls test (***) $p < 0.001$). Data represent mean $\pm$ SD of three independent experiments.

3.4 Induction of Apoptosis

SF and CUR treatments induced apoptosis in NCI-H522 cells as evidenced by Hoechst 33342 staining and quantitative analysis (Figure 4A–B). Western blot analysis (Figure 4C) revealed increased Bax and decreased Bcl-2 expression following treatment, consistent with ROS-mediated apoptosis. Co-treatment with SF (6.3 μ M) and CUR (12.5 μ M) produced a greater increase in Bax and a more pronounced decrease in Bcl-2 compared to either agent alone, supporting a potential synergistic mechanism.

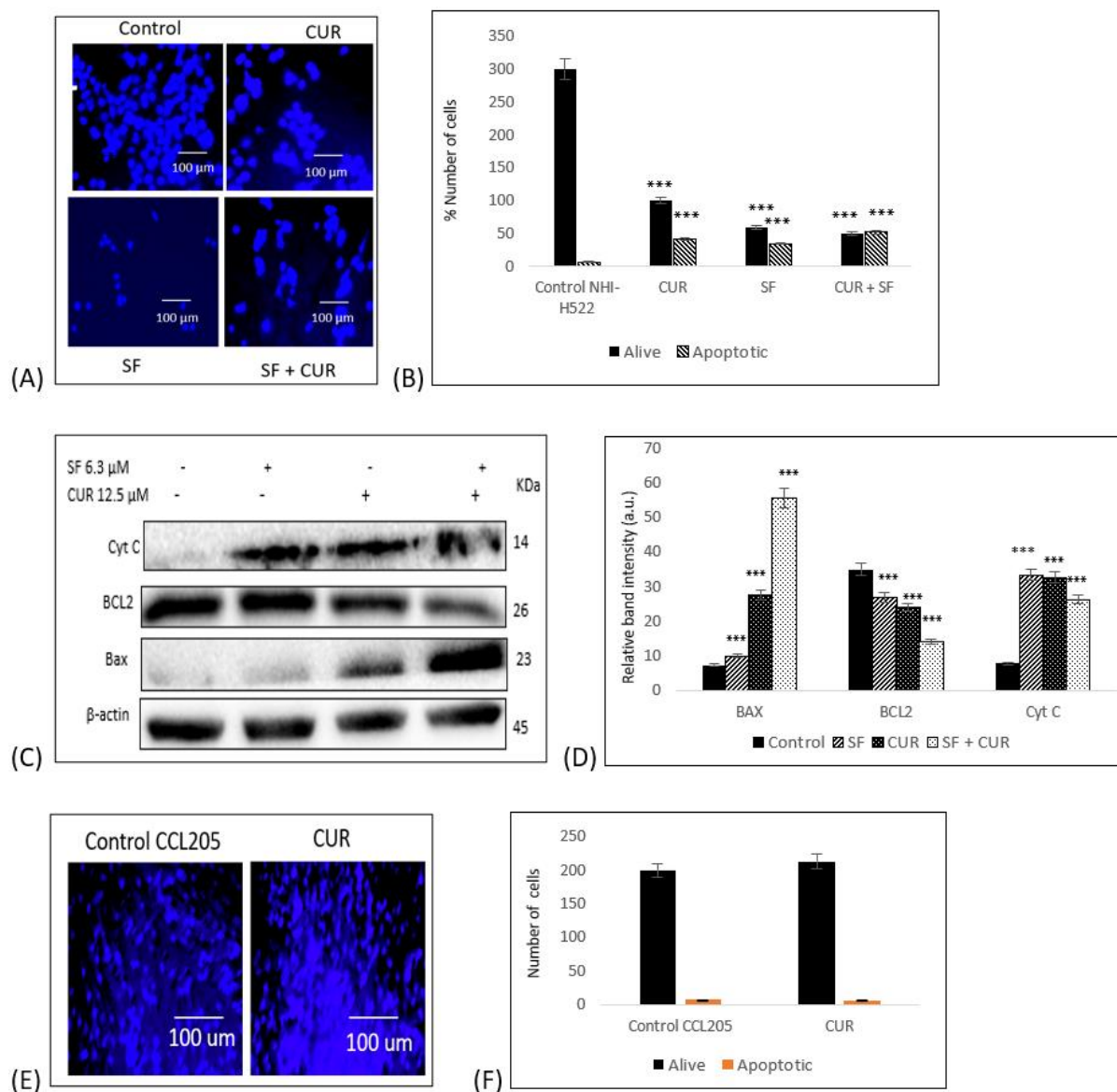


Figure 4 (A–B) Hoechst 33342 staining of NCI-H522 cells; quantification shows increased apoptotic nuclei with CUR and SF treatment, with the combination producing the most pronounced effect. (C) Western blot analysis of Bax and Bcl-2 expression in NCI-H522 cells following treatment. (D) Histogram analysis of proteins expressions. (E–F) Hoechst 33342 staining in normal CCL-205 fibroblasts; CUR (12.5 μ M) does not induce significant nuclear condensation, indicating minimal cytotoxicity. Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

Total cytochrome c levels did not increase in the combination group relative to single treatments (Figure 4D), suggesting that mitochondrial pathway activation may not be the primary driver of apoptosis under these conditions. As the assay measured total cytochrome c rather than its cytosolic translocation, further studies are needed to confirm mitochondrial outer membrane permeabilization and functional release.

Hoechst 33342 staining was also performed in normal lung fibroblasts (CCL-205). Control cells displayed uniform nuclei. Treatment with CUR (12.5 μ M) did not induce significant nuclear

condensation or fragmentation (Figure 4E–F), indicating minimal cytotoxicity in normal fibroblasts at concentrations that induce apoptosis in NCI-H522 cells. These findings support the selective action of the treatment.

3.5 Depletion of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) in NCI-H522 cells was assessed using Rhodamine 123 staining. All treatments SF, CUR, and their combination significantly reduced fluorescence, with the greatest depletion observed in the combination group, indicating enhanced mitochondrial dysfunction (Figure 5A–B). These findings align with activation of intrinsic apoptosis, corroborated by Western blot analysis showing upregulation of apoptosis-inducing factor 1 (AIF-1) and caspases-3, -7, -8, and -9 (Figure 5C–D). Total and cleaved caspase-7 were markedly increased, particularly in the combination group, confirming active caspase-mediated apoptosis.

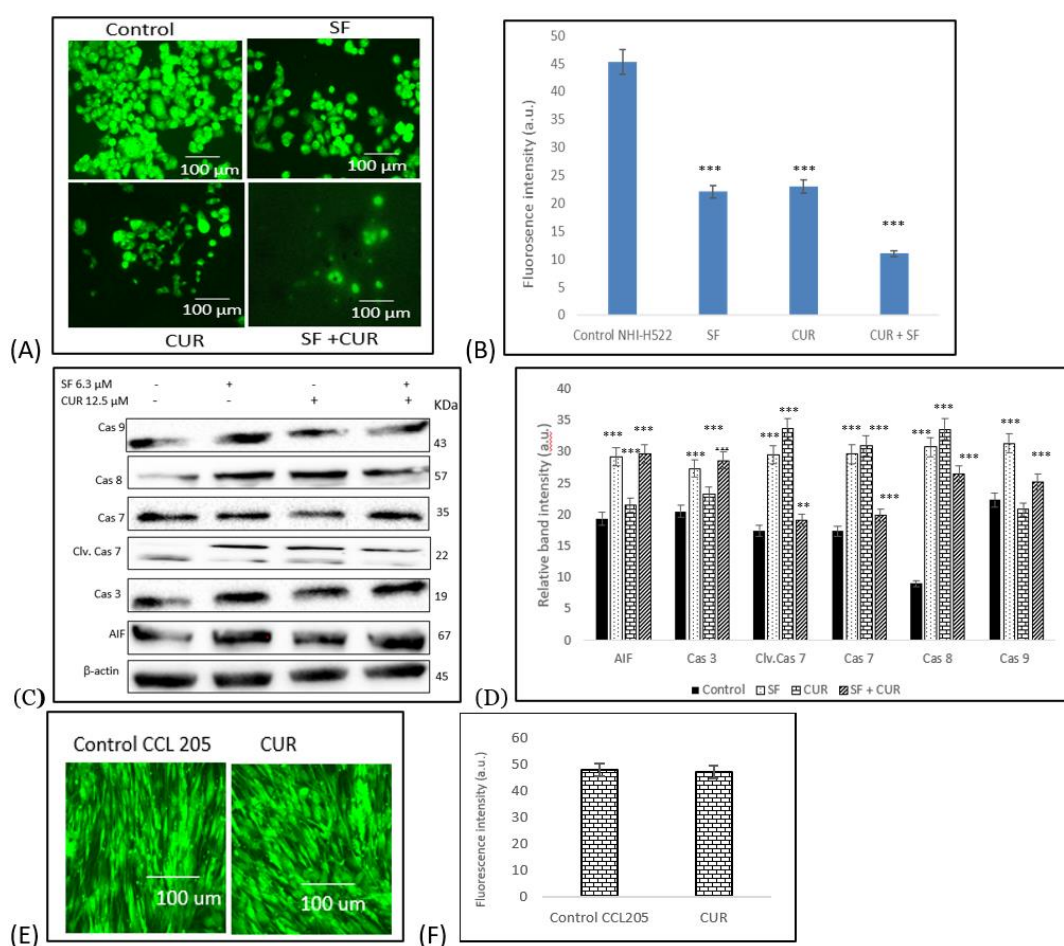


Figure 5 (A–B) Rhodamine 123 staining in NCI-H522 cells with the histogram, showing $\Delta\Psi_m$ loss after treatment; the combination exhibits the greatest depletion. (C–D) Western blot analysis of AIF-1 and caspases-3, -7, -8, and -9 in NCI-H522 cells. Total and cleaved caspase-7 are markedly increased in the combination group. (E–F) Rhodamine 123 staining in CCL-205 fibroblasts; CUR does not induce $\Delta\Psi_m$ loss, demonstrating selective action in cancer cells only. Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

CUR treatment did not alter $\Delta\Psi_m$ in non-cancerous lung fibroblasts (CCL-205) (Figure 5E–F), indicating selective induction of mitochondrial dysfunction in cancer cells while sparing normal cells.

3.6 Induction of Intracellular ROS

Intracellular ROS generation in NCI-H522 cells was assessed using H2DCFDA staining. Control cells displayed low baseline ROS levels, as indicated by darker green fluorescence. Treatment with SF or CUR alone elevated ROS, while the combination produced the strongest effect, characterized by markedly brighter fluorescence (Figure 6A). Quantitative analysis confirmed that ROS levels were significantly higher in the combination group compared to either agent alone (Figure 6B), suggesting that co-treatment enhances oxidative stress and may potentiate downstream apoptotic signaling.

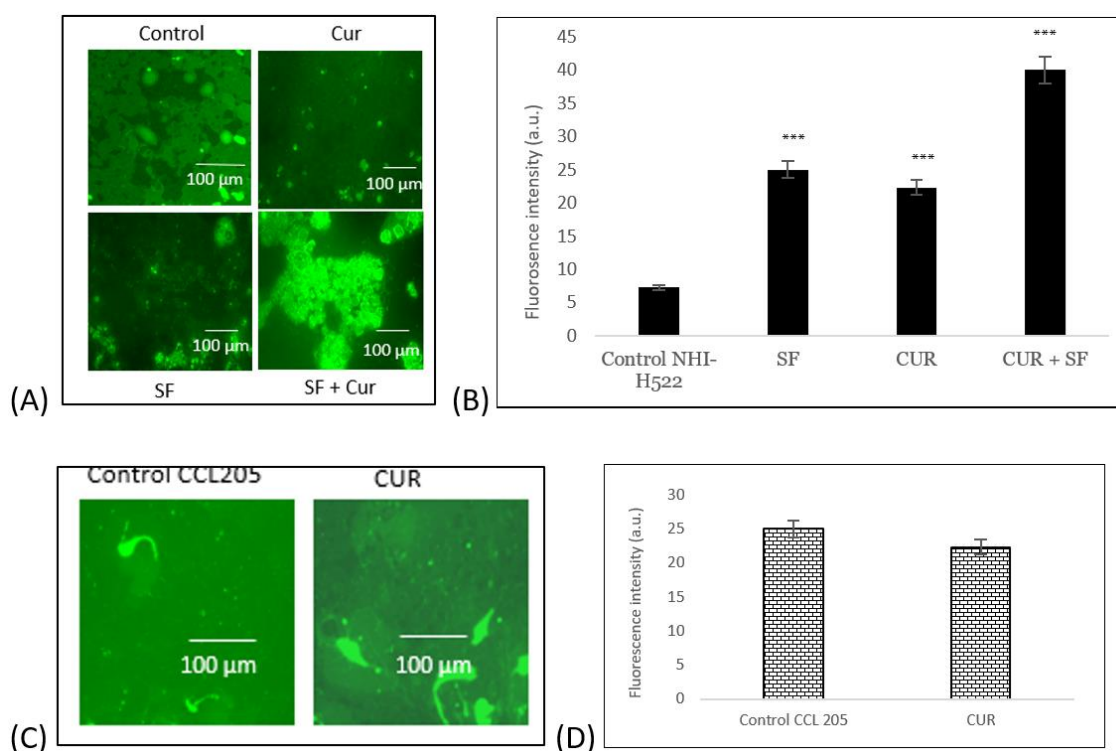


Figure 6 (A) Representative fluorescence images of H2DCFDA staining in NCI-H522 cells showing low basal ROS in controls, elevated ROS with SF or CUR, and markedly increased ROS with the combination. (B) Histogram quantification of ROS intensity in NCI-H522 cells. (C) H2DCFDA staining in CCL-205 fibroblasts showing no significant ROS increase with CUR treatment. (D) Quantification of ROS levels in CCL-205 cells. Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. *** $p < 0.001$ versus control determined by Newman-Keuls post hoc test.

In contrast, CUR at 12.5 μM did not significantly increase ROS in non-cancerous lung fibroblasts (CCL-205), as evidenced by unchanged fluorescence intensity (Figure 6C) and corresponding histogram quantification (Figure 6D). These findings indicate that CUR selectively augments oxidative stress in cancer cells but spares normal fibroblasts, underscoring its potential therapeutic advantage by enhancing tumor-specific cytotoxicity while minimizing off-target effects. Data represent mean $\pm$ SD from three independent biological replicates.

3.7 Induction of Cell Death

Cell viability after treatment was further examined using Nuclear-ID Red/Green staining to distinguish live and dead cells. Figure 7A, control NCI-H522 cells exhibited predominantly green fluorescence, consistent with high viability. Treatment with SF (6.3 μ M) induced the greatest red fluorescence signal, indicating marked cell death, while CUR (12.5 μ M) produced a moderate increase in red staining. Figure 7B, showing the relative proportions of live (green) and dead (red) cells across treatment groups. To evaluate potential cytotoxicity toward non-cancerous cells, CCL-205 fibroblasts were similarly analyzed. As shown in Figure 7C, CUR treatment at 12.5 μ M did not appreciably alter fluorescence patterns compared to untreated controls, and histogram quantification (Figure 7D) confirmed negligible loss of viability. These results demonstrate that CUR selectively enhances cytotoxicity in cancer cells, while sparing normal fibroblasts.

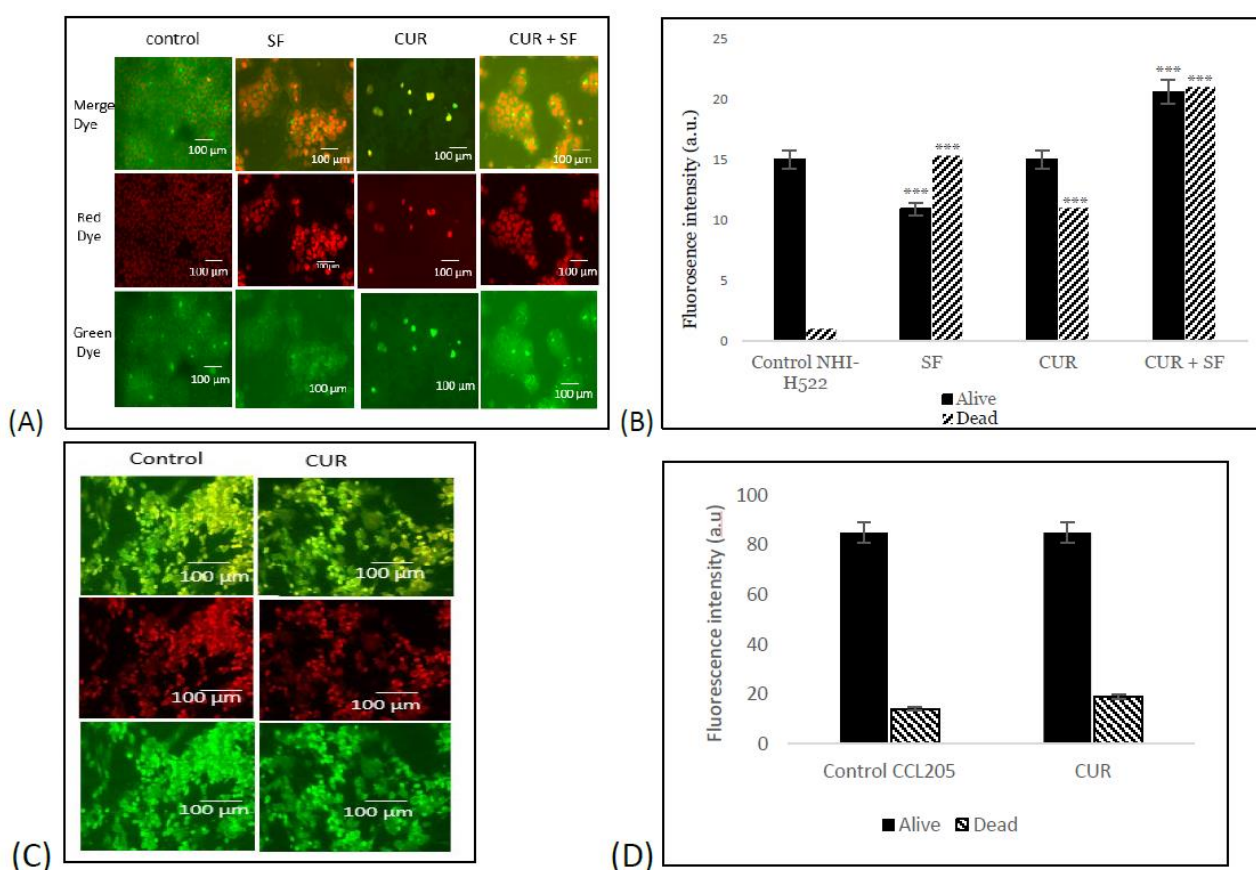


Figure 7 (A) Representative fluorescence images of NCI-H522 cells stained with Nuclear-ID Red/Green following 24 h treatment with SF (6.25 μ M), CUR (12.5 μ M), or SF + CUR. Live cells fluoresce green, while dead cells fluoresce red. (B) Histogram quantification of live/dead ratios. (C) Representative images of CCL-205 fibroblasts treated with control or CUR (12.5 μ M). (D) Corresponding histogram shows negligible cytotoxicity in normal fibroblasts. Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

3.8 Modulation of Cell Cycle Proteins

Inhibition of cell cycle regulators is a key therapeutic approach in NSCLC. Western blot analysis revealed that treatment with SF, CUR, or their combination reduced the expression of critical cell cycle proteins in NCI-H522 cells. Specifically, band intensities for CDK2, CDK7, cyclin B1, and cyclin D1 were all decreased compared to control, with the most pronounced suppression observed in the combination group (Figure 8A). Quantitative densitometry analysis (Figure 8B) confirmed these reductions, supporting the role of CUR and SF, particularly in combination, in disrupting cell cycle progression.

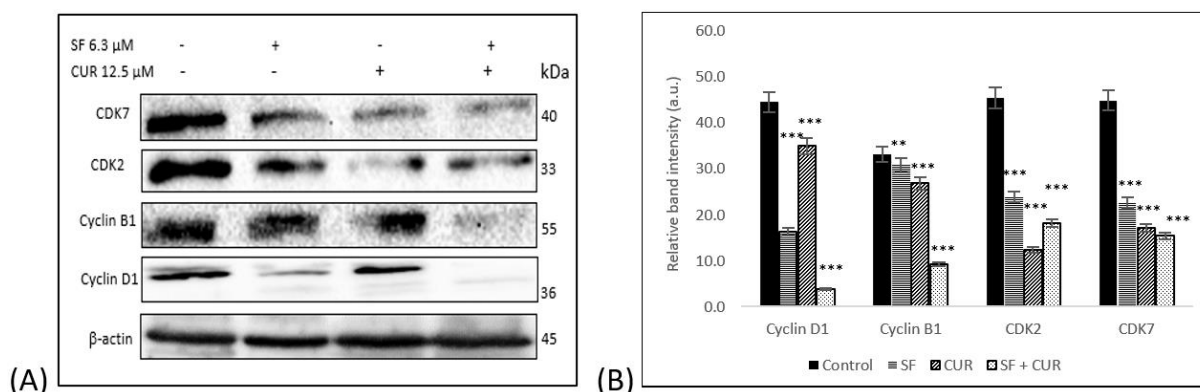


Figure 8 (A) Western blot analysis of CDK2, CDK7, cyclin B1, and cyclin D1 expression in NCI-H522 cells following treatment with SF (6.3 μM), CUR (12.5 μM), or their combination. (B) Band intensities were quantified by densitometry using ImageJ. Data represent mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

3.9 Inhibition of MEK 1-4 Proteins

The MAPK/ERK signaling cascade is a critical driver of NSCLC progression, and its inhibition is an established therapeutic strategy. Western blot analysis demonstrated that treatment with SF, CUR, or their combination suppressed expression of MEK1, MEK2, MEK3, and MEK4 in NCI-H522 cells compared with untreated controls (Figure 9A). The reduction was most pronounced in the combination group, consistent with enhanced pathway inhibition. Figure 9B confirmed significant decreases across all MEK isoforms, suggesting that CUR enhances the ability of SF to disrupt MAPK/ERK pathway activation and thereby contributes to the observed apoptotic and anti-proliferative effects.

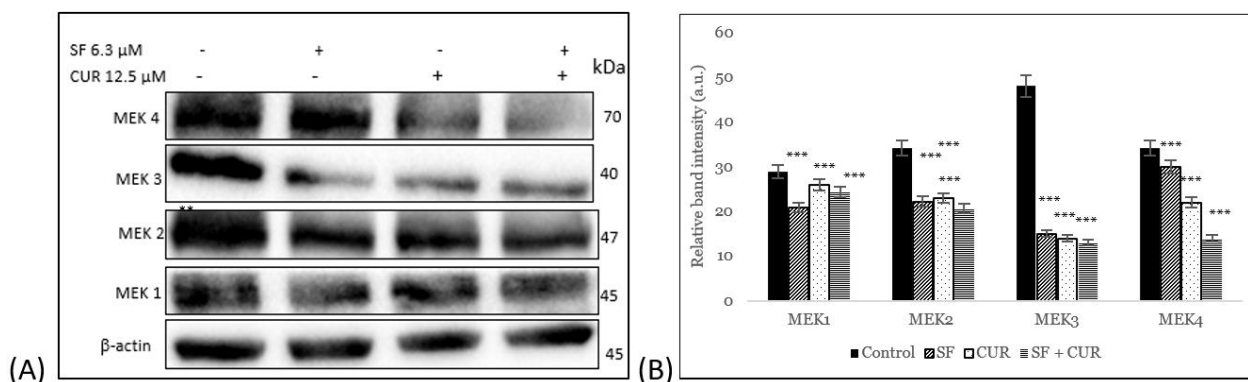


Figure 9 (A) Western blot analysis of MEK1, MEK2, MEK3, and MEK4 expression in NCI-H522 cells following treatment with SF (6.3 μ M), CUR (12.5 μ M), or their combination. (B) Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

3.10 Modulation of MDM2, PTEN, and p21 Pathways

To investigate whether CUR, SF, or their combination modulates pathways involved in DNA repair, cell cycle regulation, apoptosis, and angiogenesis, expression of key regulators was analyzed by Western blot. As shown in Figure 10A, the cyclin-dependent kinase inhibitor p21 was upregulated in CUR- and combination-treated cells but remained low in SF-treated cells. Consistent with the p53-null status of NCI-H522 cells, p53 expression was not detected. Importantly, the oncogenic regulator mouse double minute 2 homolog (MDM2) was markedly downregulated in all treatment groups, suggesting reduced negative regulation of tumor suppressors. In contrast, phosphatase and tensin homolog (PTEN) were significantly upregulated by SF, CUR, and especially the combination, indicating strong activation of a tumor-suppressive pathway. Densitometric analysis (Figure 10B) confirmed these trends. Collectively, these results indicate that the CUR–SF combination promotes apoptosis and cell cycle inhibition through a p53-independent mechanism involving MDM2 suppression and p21/PTEN induction.

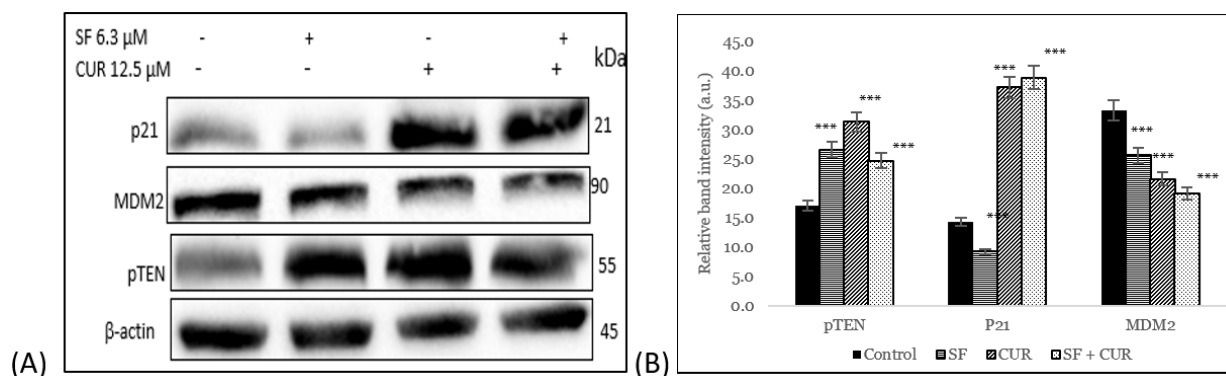


Figure 10 (A) Western blot analysis of MDM2, p21, p53, and PTEN expression in NCI-H522 cells following treatment with SF (6.3 μ M), CUR (12.5 μ M), or their combination. (B) Band intensities were quantified by densitometry using ImageJ. Data represent mean $\pm$ SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

3.11 Inhibition of p13k/Akt/mTOR Signaling Pathways

To assess whether SF and CUR regulate the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, a key signaling cascade driving cancer cell growth and proliferation, Western blot analysis was performed. As shown in Figure 11A, expression of PI3K, Akt, and mTOR was decreased in NCI-H522 cells following treatment with SF, CUR, or the combination compared with control. CUR alone markedly suppressed mTOR, and the combination achieved the strongest inhibition. This pattern suggests that CUR plays a dominant role in targeting mTOR, and that co-treatment with SF enhances this effect in a potentially synergistic manner. Quantitative analysis of protein band intensities (Figure 11B) confirmed these differences across groups.

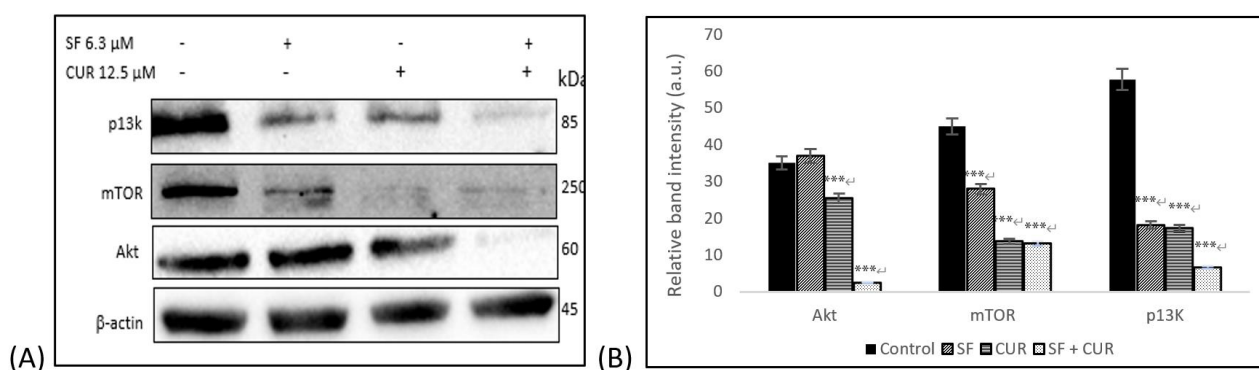


Figure 11 (A) Western blot analysis of PI3K, Akt, and mTOR expression in NCI-H522 cells treated with SF (6.3 μM), CUR (12.5 μM), or their combination. (B) Histogram showing densitometric quantification of protein band intensities normalized to loading controls. Band intensities were quantified by densitometry using ImageJ. Data represent mean ± SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

3.12 Modulation of EGFR/ERK/p-ERK/HER2 Signaling Pathway

To investigate whether SF, CUR, or their combination modulate the EGFR/ERK/HER2 signaling axis, Western blot analysis was performed. As shown in Figure 12A, EGFR and HER2 expression levels were reduced in all treatment groups, with the most pronounced suppression observed in the combination. Total ERK expression remained relatively unchanged; however, phosphorylated ERK (p-ERK) was significantly decreased across all treatments, particularly with CUR and the combination. This pattern indicates that these agents primarily inhibit ERK activation rather than total protein expression. Densitometric quantification (Figure 12B) confirmed these effects, supporting the conclusion that SF and CUR target upstream receptor tyrosine kinases and downstream MAPK signaling, thereby attenuating proliferative and survival signaling in NCI-H522 cells.

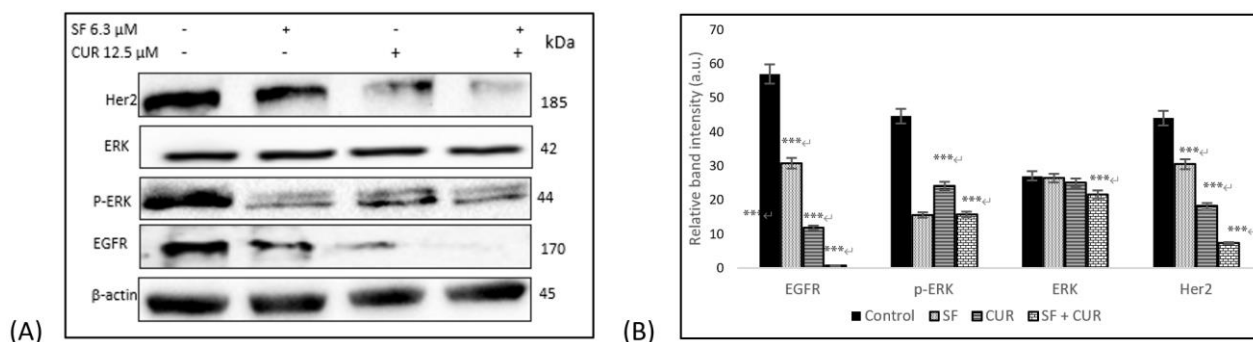


Figure 12 (A) Western blot analysis of EGFR, HER2, ERK, and phosphorylated ERK (p-ERK) expression in NCI-H522 cells treated with SF (6.3 μM), CUR (12.5 μM), or their combination. (B) Band intensities were quantified by densitometry using ImageJ. Data represent mean ± SD of three independent experiments. ***p < 0.001 versus control determined by Newman-Keuls post hoc test.

4. Discussion

This study highlights the anticancer potential of SF, CUR, and their combination (Combo) in p53-null NCI-H522 NSCLC cells, revealing that these agents act through complementary and multi-targeted mechanisms. The data suggests that combination therapy amplifies cytotoxic signaling, disrupts survival pathways, and mitigates resistance, providing insights into strategies for treating aggressive NSCLC subtypes.

The differential modulation of apoptotic pathways by SF and CUR highlights the complexity of their combined action. While SF and CUR individually target extrinsic and intrinsic apoptotic mechanisms, respectively, the Combo surprisingly resulted in a decrease in both caspase-dependent (caspases-3, -7, -8, -9) and caspase-independent (AIF) markers [33]. This suggests that the combined treatment may reduce reliance on conventional apoptosis, potentially engaging alternative forms of cell death. Notably, increased ROS generation appears to act upstream of mitochondrial perturbation, indicating that oxidative stress may contribute to cell death through non-canonical pathways [33-35]. Such a shift could expand the therapeutic impact, especially in p53-null or apoptosis-resistant cancers, by exploiting a broader spectrum of death mechanisms.

Although the apoptosis characterization in this study did not include additional markers such as Annexin V/PI or caspase activation, three independent and complementary approaches—HOECHT staining (nuclear condensation), rhodamine fluorescence (mitochondrial membrane potential loss), and H2DCFDA (ROS generation) were employed—which together capture major hallmarks of apoptosis.

Suppression of multiple cyclin-dependent kinases (CDKs) and cyclins indicate simultaneous disruption of key cell cycle checkpoints, likely contributing to the observed proliferation inhibition [36]. By targeting both G1/S and G2/M transitions, the treatments, particularly the Combo, may prevent tumor cells from compensating via alternative proliferative routes, highlighting the therapeutic relevance of multi-checkpoint intervention in NSCLC. While flow cytometric confirmation was not performed, these molecular changes, together with suppressed proliferation, indicate a strong likelihood of cell cycle arrest.

The wound-healing assay integrates proliferation and inhibition supports the notion that CUR and SF not only induce cytotoxicity but may also impair migratory potential. This suggests a dual

benefit in limiting tumor expansion and dissemination. Future studies employing migration-specific assays (Transwell assay) will clarify these effects.

Downregulation of EGFR, HER2, MEK/ERK, and PI3K/Akt/mTOR pathways underscores the capacity of SF and CUR to target critical drivers of NSCLC survival and therapy resistance. The Combo's enhanced suppression of both upstream receptor tyrosine kinases and downstream survival signals may prevent compensatory signaling—a common mechanism underlying monotherapy failure [37-39]. By simultaneously inhibiting multiple survival pathways, SF–CUR co-treatment not only impairs NSCLC cell proliferation and survival but also enhances sensitivity to other targeted therapies, thereby improving the overall therapeutic effect, particularly in p53-null or therapy-resistant cancers.

Despite the absence of functional p53, upregulation of p21 and PTEN, coupled with MDM2 downregulation, demonstrates activation of alternative tumor-suppressive mechanisms. Stabilization of these proteins may reinforce cell cycle control, metabolic regulation, and apoptotic susceptibility, providing an additional layer of anti-cancer activity that bypasses canonical p53 signaling [40-43].

The integration of apoptotic induction, cell cycle arrest, suppression of oncogenic signaling, and p53-independent tumor suppression highlights a multi-pronged therapeutic strategy. CUR's capacity to modulate compensatory pathways while enhancing SF efficacy positions it as a promising adjuvant. Although systemic bioavailability remains a limitation, short-term exposure *in vitro* supports its mechanistic contribution. These findings support further exploration of this combination as a strategy to improve therapeutic efficacy and mitigate resistance in NSCLC patients.

Key limitations include reliance on a single p53-null cell line, absence of *in vivo* validation, and lack of flow cytometric confirmation of cell cycle arrest. Future work should assess generalizability across diverse NSCLC models, using A549 [KRAS-mutated] or H1975 [EGFR L858R/T790M] to identify if combination of CUR and SF applicable across different cells. Optimizing combinatorial dosing and employing advanced profiling (transcriptomics, phosphoproteomics) to elucidate predictive biomarkers and broader pathway interactions could guide more effective treatment strategies.

5. Conclusions

This study provides the first evidence that SF and CUR, individually and in combination, exert potent anticancer effects in NCI-H522, a p53-null NSCLC model. Combination treatment enhanced efficacy, suggesting that CUR may serve as a promising adjuvant to SF. A limitation of the present study is that, although multiple hallmarks of apoptosis were assessed including HOECHT staining for nuclear condensation, rhodamine for mitochondrial membrane potential loss, and H2DCFDA for ROS generation; however, cytosolic cytochrome c release and caspase inhibition experiments (e.g., with z-VAD-fmk) were not performed to directly confirm caspase dependency. Nevertheless, the consistent changes in mitochondrial potential, ROS levels, and nuclear morphology strongly support intrinsic apoptotic pathway activation. Prior studies indicate that CUR modulates Bax/Bcl-2, promotes cytochrome release, activates caspase-9 and caspase-3, and that these effects are suppressed by pan-caspase inhibitors, suggesting similar mechanisms are likely involved here. Incorporating these assays in future work would strengthen mechanistic validation and clarify the contributions of caspase-dependent versus caspase-independent apoptosis. Additional limitations include the *in vitro* design and use of a single cell line, as well as the lack of direct evaluation of CUR

stability and bioavailability. Future studies should employ multiple NSCLC models, in vivo validation, optimized CUR formulations, and omics analyses to enhance translational relevance.

Author Contributions

L.L.B provides conceptualization, methodology, writing, reviewing, project administration.

Funding

This research was funded by Union University, Jackson, TN, USA.

Competing Interests

The author has declared that no competing interests exist.

Data Availability Statement

The data are available from the corresponding author upon reasonable request.

AI-Assisted Technologies Statement

AI-assisted tools (ChatGPT, OpenAI, San Francisco, CA, USA) were used to improve grammar, refine sentence structure, and enhance the clarity of scientific language in this manuscript. The tools were not used to generate data, perform analyses, or draw conclusions. All scientific content, interpretations, and final decisions remain the sole responsibility of the author.

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