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In Vitro Evaluation of the Combined Effects of Rosmarinic Acid and 5-Fluorouracil in Prostate Cancer Cells

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Abstract

Introduction: Prostate cancer is the most common malignancy in men. Despite therapeutic advances, drug resistance remains a major challenge, and many patients progress to castration-resistant prostate cancer (CRPC). This study aims to investigate the combined effects of rosmarinic acid (RA), a polyphenolic compound, and 5-FU, a conventional chemotherapeutic agent, on DU145 prostate cancer cells.

Materials and methods: Cell viability was assessed at various doses for RA and 5-FU to determine the IC50, IC30, and IC10 values. For combination studies, IC30 dose was selected, and the effects of the treatments were evaluated using MTT assays, colony formation assays, scratch healing assays and acridine orange/ethidium bromide staining assays.

Results: Monotherapy with RA and 5-FU demonstrated a dose-dependent inhibitory effect on cell viability. The combination of both agents at their IC30 concentrations produced a statistically significant and combined inhibition of colony formation and cell motility compared to the single-agent and control groups. Furthermore, combined treatment induced more pronounced changes in characteristic cell morphology associated with apoptosis and necrosis than monotherapy groups.

Conclusion: This study presents preliminary findings on the combined effects of rosmarinic acid with 5-fluorouracil. The combination significantly suppressed cell viability, colony formation capacity, and motility in CRPC cells in vitro and increased apoptosis- and necrosis-associated cell death compared to single treatments. In conclusion, these in vitro results suggest that combining the chemotherapeutic agent 5-FU with the natural compound RA may enhance treatment efficacy and represent a promising strategy for managing castration-resistant prostate cancer.

Key words: 5-Fluorouracil; drug combinations; prostate cancer; rosmarinic acid.

Introduction

Prostate cancer (PC) is the second most common cancer and the fifth leading cause of cancer-related death among men worldwide (1). Despite technological advances, resistance to therapy and tumor recurrence are the most significant challenges in the treatment of PC. Over time, PC cells may acquire more aggressive characteristics such as increased migration, invasion, and metastasis, which are strongly associated with poor patient survival (2). One of the standard treatments for prostate cancer is androgen-ablation therapy, as the cancer cells are androgen-dependent. Androgen-independent disease often develops over time, which is resistant to treatment and called castration-resistant prostate cancer (CRPC). Despite the utilization of chemotherapeutic agents such as docetaxel and taxane in the treatment of CRPC, the development of resistance remains an unavoidable challenge (3). 5-fluorouracil (5-FU) as a conventional chemotherapeutic agent, is widely used in the treatment of several solid tumors. 5-FU is an uracil analog that enters the cell and is converted into active metabolites. It exerts its cytotoxic effects either by interfering with DNA synthesis by blocking the thymidylate synthase (TS) activity or by incorporation of its metabolites into RNA and DNA (4). Although 5-FU is not a standard first-or second-line agent for CRPC, fluoropyrimidine-based regimens have demonstrated clinical activity. Notably, combination therapy with UFT (tegafur and uracil) and a second-line MAB (maximum androgen blockade) has shown improved progression-free survival compared with MAB alone in CRPC patients, supporting a therapeutic role for fluoropyrimidines in selected clinical settings (6). Furthermore, the clinical use of 5-FU is often limited by the development of drug resistance and systemic toxicity, highlighting the

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need for novel combination treatment strategies to improve the efficacy and safety of 5-FU-based therapies (5). In recent years, natural compounds have been used in cancer treatment studies as promising agents to improve the efficacy of chemotherapeutics and mitigate their toxicity. Rosmarinic acid (RA) is a polyphenolic chemical found in several aromatic herbs, particularly Rosmarinus officinalis. RA has diverse biological characteristics, including antibacterial, antiviral, antioxidant, anti-aging, anti-inflammatory, and anti-cancer activities (7). Rosmarinic acid has been shown to exert anti-cancer activities through inhibition of tumor cell proliferation, apoptosis, and modulation of oncogenic pathways in several cancer types including prostate cancer (8-10). Importantly, RA can exhibit synergistic effects by increasing the efficacy of chemotherapeutic drugs through the reduction of oxidative stress, activation of apoptotic mechanisms, and modulation of the cell cycle (11, 12). Thus, combining the RA and 5-FU may provide a novel approach to overcome chemoresistance and improve efficacy in prostate cancer. The aim of this study is to investigate the combination of RA and 5-FU in DU145, a CRPC model (13), to enhance their potential for anti-cancer efficacy.

Materials and Methods

Reagents: RA and 5-FU were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMSO was used to dissolve RA and 5-FU stock solutions, which were then stored at –20°C prior to use. To achieve the ultimate doses, the drug solutions were diluted in the culture media. The final DMSO concentration in all RA-treated wells did not exceed 0.3% (v/v), which is considered non-toxic to cancer cell lines.

Cell culture: DU145, human prostate cancer cells, were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco- Thermo Scientific, Waltham, MA, U.S) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco- Thermo Scientific, Waltham, MA, U.S) and 1% (v/v) penicillin-streptomycin (Pen/Strep, Gibco-Thermo Scientific, Waltham, MA, U.S). Cells were incubated at 37°C in a humidified incubator with 5% CO_2. Cells were subcultured once culture flasks achieved a confluence of about 80%.

Cell viability assay: Cells were seeded at a density of 5 x 10^3 cells per well and maintained overnight under standard cell culture conditions (37°C, 5% CO_2, humidified atmosphere). Following incubation, cells were treated with varying concentrations of RA (50-1000 μM) and 5-FU (50-350 μM). Upon completion of the incubation period, MTT dye was added, and the cells were subjected to a further incubation of three hours. Spectrophotometric analysis was conducted at 570 nm using a microplate reader (Varioskan Lux, Thermo Fisher Scientific).

Colony formation assay: Cells cultured in 6-well plates were exposed to IC10, IC30, and combined IC30 doses of RA and 5-FU for 48 hours. Following trypsinization, cells were collected and seeded into 6-well plates at a density of $8x10^2$ cells/well, and then incubated at 37°C with 5% CO_2 for a duration of 10 days. After the incubation period, cells were washed with PBS and subsequently fixed using methanol, acetic acid, and water solution (1:1:8 v/v). Once the cells had been stained for 20 minutes using crystal violet, any residual dye was removed with distilled water. Clusters comprising more than 50 cells were classified as colonies and subjected to analysis. The colony formation rate was calculated using the formula: (number of colonies)/(number of seeded cells) × 100%.

In vitro scratch assay (Wound Healing): To evaluate wound healing, 1 × 10^5 cells/well were seeded into 24-well plates and incubated overnight at 37°C in a 5% CO_2 incubator. Subsequent to incubation, cells were deprived of serum in fresh medium supplemented with 0.5% FBS for a period of 19 hours. Under 0.5% FBS, proliferation is markedly suppressed but not fully eliminated; therefore, reduced wound closure predominantly reflects impaired motility rather than proliferation-dependent effects. Monolayers of cells were delicately scratched the following day using sterile 200 μl pipette tips. The cells were incubated for 48 hours in serum-free medium, with or without the addition of RA, 5-FU, or a combination of them, at IC30 doses. Microscopic images (using Labscope software, Primovert, Zeiss) were used to assess how the cells were spread out in the scratch area. The MRI Wound Healing Tool (RRID:SCR_025260) within ImageJ allows for the quantitative measurement of wound closure.

Acridine orange/ethidium bromide staining assay: Acridine Orange/Ethidium Bromide (AO/EB) staining was conducted according to a 96-well-based staining method with a minor modification (14). Briefly, cells were seeded at a density of 1 × 10^4 cells per well into a 96-well plate. Following overnight incubation, cells were treated with IC30 doses of RA, 5-FU, and their combinations. 100 μg/ml of AO and EtBr was prepared in PBS, and 16 μl of AO/EB dye mix was added to each well. Images were acquired using a Zeiss LSM 900 microscope and Zen Blue software (10x objective).

Ethical Approval: Ethical approval is not required in this study.

Statistical Analysis: All statistical analyses were performed using GraphPad Prism 10. Data distributions were assessed for normality using the Shapiro–Wilk test. For experiments involving more than two groups (Control, RA, 5-FU, and RA+5-FU), data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple pairwise comparisons. For pairwise comparisons between two independent groups, a two-tailed Student's t-test with Welch's correction was applied. For all experiments, n refers to the number of biologically independent replicates, with a minimum of three replicates per condition. Data are presented as mean ± SEM.

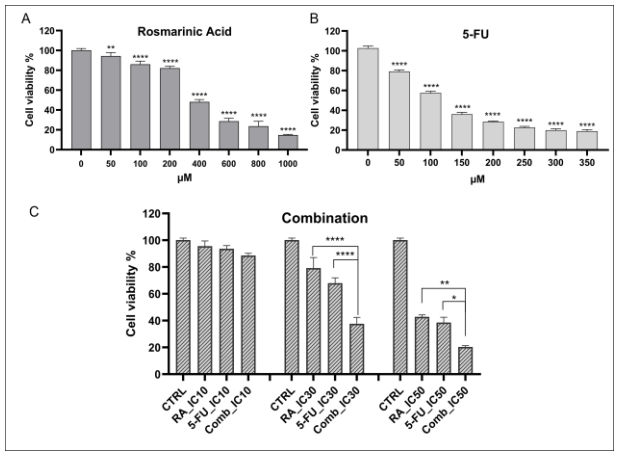

Figure 1: Cytotoxic effects of Rosmarinic Acid (RA), 5-Fluorouracil (5-FU), and their combination on DU145 prostate cancer cells as determined by MTT assay a) Dose-dependent decrease in cell viability following RA treatment (0–1000 μM) (n=4), Statistical comparisons were performed between each RA dose and the untreated control group (0 μM) b) Dose-dependent cytotoxicity of 5-FU (0–350 μM) (n=4), Statistical comparisons were conducted between each 5-FU dose and the untreated control group (0 μM). c) Effects of IC10, IC30, and IC50 doses of RA, 5-FU, and RA+5-FU combination treatments on cell viability (n=3). Within each IC level, statistical comparisons were performed between the combination group and the two corresponding monotherapy groups (RA and 5-FU). For each condition, cells were treated for 48 hours, and viability was measured as the percentage relative to untreated control. Statistical analysis was performed using Student's t-test with Welch's correction for pairwise comparisons and one-way ANOVA followed by Tukey's post-hoc test for multiple pairwise comparisons. Data are presented as mean ± SEM. p-values are provided in Supplementary Table S1 and S2. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001. RA, Rosmarinic Acid; 5-FU, 5-Fluorouracil; CTRL, control; Comb, combination

Results

Rosmarinic acid and 5-fluorouracil decreased cell viability in DU145 cells: The cytotoxic effects of RA and 5-FU on DU145 cells were evaluated using the MTT assay. A dose-dependent reduction in cell viability was observed in both RA-treated and 5-FU-treated groups (Figure 1, A-B). Based on cell viability data, the inhibitory concentrations at 50%, 30%, and 10% (IC50, IC30, and IC10) were calculated. For RA, the IC50, IC30, and IC10 values were determined to be 490 μM, 268 μM, and 58 μM, respectively. In comparison, 5-FU exhibited IC50, IC30, and IC10 values of 135 μM, 70 μM, and 24 μM, respectively. The anticipated enhancement in cytotoxicity was evaluated by combining the IC10, IC30, and IC50 doses. Specifically, combinations of RA and 5-FU at IC10, IC30, and IC50 levels were examined independently. Compared to monotherapies, the combined treatments at IC10, IC30, and IC50 concentrations demonstrated increased cytotoxic effects (Figure 1, C). While the combined IC10 doses exhibited modest efficacy similar to that of individual IC10 treatments, the combined IC30 and IC50 doses significantly reduced cell viability relative to their single-agent counterparts. Considering the extreme toxicity of the IC50 doses, subsequent experiments were conducted using the IC30 dose combinations.

Combination therapy led to a reduction in colony formation: A colony formation assay was conducted to investigate the combined effects of RA and 5-FU on long-term proliferation and colony formation. When evaluated independently, RA exhibited a significant suppression of colony formation at its IC30 concentration; however, 5-FU showed a markedly greater inhibitory effect at its respective IC30 concentration. Notably, cell populations treated with the combination of these agents demonstrated a significantly greater reduction in colony formation compared to those treated with either agent alone (Figure 2).

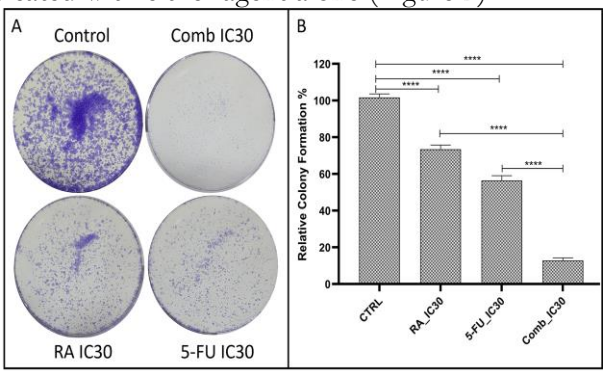

Figure 2: Effects of IC30 doses of Rosmarinic Acid (RA), 5-Fluorouracil (5-FU), and their combination on long-term clonogenic survival of DU145 cells. a) Representative crystal violet-stained colony formation images for control, IC30 doses of RA, 5-FU, and combination groups. b) Quantification of relative colony formation (%), normalized to the untreated control. Statistical analyses were performed between each treatment group and the control, and additionally between the combination group and monotherapies using one-way ANOVA followed by Tukey's post-hoc test (n=3). Data are presented as mean ± SEM. Adjusted p-values are provided in Supplementary Table S3. *p < 0.05, **p<0.01, ***p<0.001). RA, Rosmarinic Acid; 5-FU, 5-Fluorouracil; CTRL, control; Comb, combination.

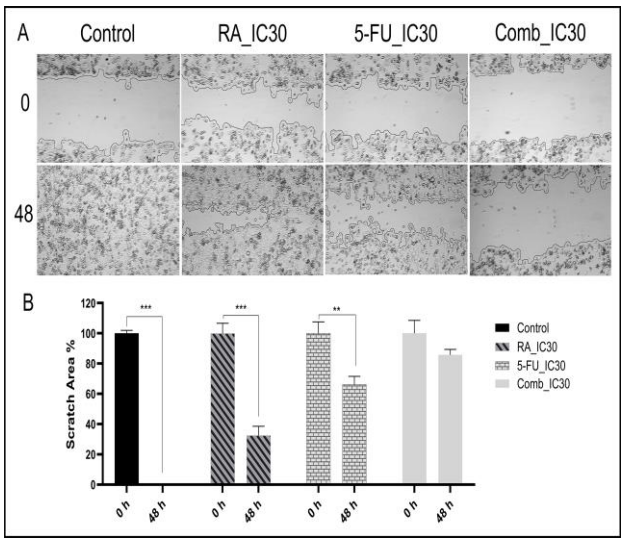

Figure 3: Effects of IC30 doses of Rosmarinic Acid (RA), 5-Fluorouracil (5-FU), and their combination on DU145 cell motility assessed by in vitro scratch assay. a) Representative phase-contrast images of wound closure at 0 h and 48 h for control, IC30 doses of RA, 5-FU, and combination groups. Wound edges are outlined to visualize migration. b) Quantification of scratch area (%) at 0 h and 48 h for each treatment. Statistical comparisons were performed between 0 h and 48 h within each treatment group, allowing assessment of migration inhibition relative to baseline. Statistical analysis was performed using Student's t-test with Welch's correction (n=3). Data are presented as mean ± SEM. Exact p-values are provided in Supplementary Table S4. *p < 0.05, **p<0.01, ***p<0.001. RA, Rosmarinic Acid; 5-FU, 5-Fluorouracil; CTRL, control; Comb, combination.

Combination therapy decreased cell motility: An in vitro scratch assay was employed to determine the effects of a combination of RA and 5-FU on cell motility. The untreated cell group was able to fully cover the scratched area following a 48-hour incubation period. There was a considerable gap in the scratch area with IC30 doses of RA and 5-FU; however, when the two drugs were administered concurrently, the scratch area was observed to be significantly wider than when they were administered individually. The combination therapy resulted in a more pronounced suppression of cell motility in DU145 cells (Figure 3).

Rosmarinic acid, 5-fluorouracil and combination treatment induce apoptotic/necrotic cell morphology: To investigate the morphological alterations of DU145 cells induced by RA and 5-FU, the cells were subjected to AO/EB staining. Viable cells showed a bright green fluorescence throughout, whereas cells undergoing apoptosis or necrosis were characterized by orange staining or the presence of orange nuclear fragments. As shown in Figure 4, untreated control cells demonstrated bright green fluorescence along with normal cellular morphology. In contrast, DU145 cells treated with RA, 5-FU, or their combination at IC30 concentrations exhibited orange nuclear dots, indicative of apoptotic- or necrotic- like morphological features. Quantitative analysis further revealed a significant increase in the percentage of EtBr-positive cells in all treatment groups compared with control group. On the other hand, the combination treatment showed a non-significant trend toward higher cell death.

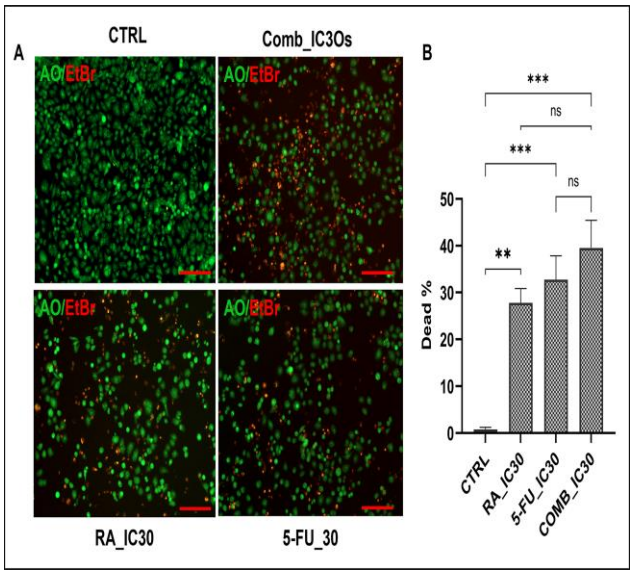

Figure 4: Fluorescence-based assessment of cell death induced by IC30 doses of Rosmarinic Acid (RA), 5-Fluorouracil (5-FU), and their combination in DU145 cells using AO/EtBr staining. a) Representative images show viable cells (AO; green fluorescence) and non-viable/apoptotic cells (EtBr; red fluorescence) in Control, RA_IC30, 5-FU_IC30, and Combination_IC30 groups. b) The percentage of dead cell was quantified from 4 field of view based on AO/EtBr fluorescence staining, where EtBr-positive (red) cells were considered as dead, and AO-positive (green) cells were classified as viable. Statistical comparisons were performed between each treatment group and the control group, as well as between the combination group and corresponding monotherapy groups using one-way ANOVA followed by Tukey's post-hoc test. Data are presented as mean ± SEM. Adjusted p-values are provided in Supplementary Table S5. *p < 0.05, **p<0.01, ***p<0.001. The scale bar = 100 µm. Magnification: 10x. AO, Acridine Orange; EtBr, Ethidium Bromide; RA, Rosmarinic Acid; 5-FU, 5-Fluorouracil; CTRL, control; Comb, combination.

Discussion

Managing prostate cancer, especially in its advanced forms, presents a significant challenge due to the limited effectiveness and notable systemic toxicity linked to current chemotherapeutic protocols (15). Consequently, the development of innovative therapeutic approaches is of critical importance. Current paradigms in oncology are increasingly shifting towards multi-modal combination therapies; a strategy developed to improve therapeutic efficacy

and simultaneously lessen the negative side effects observed in high-dose monotherapies (16). A notably promising strategy within this framework comprises the combination of traditional chemotherapeutic drugs with natural compounds. This integrative approach holds the potential to enhance the efficacy of cancer eradication while concurrently decreasing the necessary dosage of cytotoxic chemotherapy (17–19). Advancing this research, the present study examines the cytotoxic and anti-proliferative properties of RA, a naturally occurring polyphenol, in conjunction with 5-FU, a widely utilized chemotherapeutic agent, on the androgen-independent prostate cancer cell line DU145. We evaluated the agents both as monotherapies and in combination to determine their interaction and combined cytotoxic/antiproliferative potential. Cell viability assays revealed that RA and 5-FU treatment resulted in a dose-dependent decrease in DU145 cells. However, the combination of RA and 5-FU, administered at concentrations corresponding to their IC30 values, exhibited markedly enhanced cytotoxic effects relative to the individual treatments. Various studies have reported that the anticancer efficacy of 5-FU is potentiated by several natural compounds (20-23). Consistent with existing literature, our results suggest that the combined treatment exerts an enhanced effect on cell viability. This implies that a therapeutic outcome might be possible with lower doses of 5-FU, thus possibly minimizing the adverse effects. The examination of variable dosages in various tissue types may elucidate more prominent synergistic impacts. In order to evaluate the known efficacy of polyphenols in inhibiting long-term clonogenic effects, we carried out a colony formation assay (24–26). Treatment with RA and 5-FU individually demonstrated their capacity to suppress colony formation, with 5-FU exhibiting greater potency as a single agent. Notably, the combined administration of RA and 5-FU at IC30 concentrations led to a dramatic decrease in colony numbers, significantly surpassing the effects of the individual treatments. This indicates that the combination therapy effectively impairs the long-term proliferation potential of DU145 cells. Cellular motility is one of the key factors in the abnormal migratory abilities exhibited by tumor cells (27,28). Therefore, developing therapeutic strategies focused on inhibiting cellular motility is a crucial objective in oncology. We examined the combined effects of RA and 5-FU in DU145 cell line to assess this therapeutic potential. In vitro scratch assay demonstrated that treatment with individual IC30 concentrations of RA and 5-FU partially impeded cell motility, as evidenced by incomplete wound closure relative to the control group. Remarkably, the combined administration of RA and 5-FU produced a greater gap on the scratch region, significantly exceeding the individual administrations. These results indicate a robust suppression of cellular motility. Collectively, the data suggest that the combination of RA and 5-FU not only reduces cell viability but also directly impairs the migratory behavior of cancer cells, underscoring its promise in disrupting a pivotal step in the metastatic cascade. An extensive investigation assessing the expression levels of cellular components implicated in this cascade and 3D invasion models would yield comprehensive data regarding the effectiveness of the combination therapy. Further research will elucidate the potential of combining 5-FU and rosmarinic acid as an agent to overcome resistance and prevent metastasis in androgen-independent, resistant prostate cancers such as DU145. Moreover, it should be noted that the wound-healing assay represents a composite readout influenced by cell motility, residual proliferation, and viability. While the combination treatment reduced 48-hour viability by ~60%, a substantial proportion of cells remained viable and morphologically intact during the assay. Given that DU145 cells exhibit limited proliferation under 0.5% FBS, the impaired wound closure is unlikely to be explained solely by reduced cell number and instead reflects a genuine suppression of migratory capacity. Acridine orange and ethidium bromide staining is a critical method used to distinguish cell death morphologically (29,30). In this study, we used this method to evaluate the morphological outcomes resulting from the cytotoxic effects of RA and 5-FU alone and in combination. Our findings clearly demonstrated that RA and 5-FU induced cell death in DU145 cells. In contrast to robust green fluorescence in the control group, orange nuclear condensation was observed in the treatment groups, which is a morphological hallmark of apoptosis/early necrosis. Notably, although the combination treatment inhibited the highest mean percentage of dead cells, this increase does not statistically significant when compared with either individual treatment groups. This suggests a trend toward enhanced cell death rather than a definitive combined induction of apoptosis. Given that AO/EtBr staining provides a semi-quantitative insight into membrane integrity rather than a mechanistic assessment of apoptotic pathways, these findings should be interpreted as supportive of the overall cytotoxic phenotype observed with combination treatment rather than as evidence of enhanced apoptosis. In summary, our findings

demonstrate that RA and 5-FU exert complementary cytotoxic and antiproliferative effects in prostate cancer cells, supporting their potential mechanistic interaction.

Study Limitations: This study provides valuable insights however, the mechanistic basis remains to be fully elucidated, and the observed effects require further validation in more physiologically relevant 3D tumor models. Moreover, quantitative confirmation through combination index analysis was beyond the scope of this study. Future work employing the Chou–Talalay method or related approaches would provide a more precise evaluation of the interaction between RA and 5-FU. Future studies should incorporate mechanistic pathway analyses, combination index evaluation, and advanced 3D or animal models to validate and expand upon these preliminary findings.

Conclusion

In conclusion, this preliminary study suggests that rosmarinic acid enhances the cytotoxic, anti-proliferative, and anti-migratory effects of 5-fluorouracil in androgen-independent prostate cancer cells (DU145). The combination of these agents exerts a greater inhibitory effect on cell viability, clonogenic potential, and motility compared to monotherapies, suggesting its potential enhanced effects. These findings show the potential of RA as a chemosensitizing natural compound, potentiating 5-FU efficacy and reducing the dosage of chemotherapy. Future studies incorporating mechanistic pathway analyses, combination index assessment, and validation in 3D and in vivo models are necessary to confirm and expand these observations.

Ethical approval: This study does not involve human participants; therefore, ethical approval was not required.

Conflict of interest: The authors have no conflict of interest regarding this study.

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Author contributions: Concept: C.S., C.S., S.K.A, B.Y., Design: C.S., C.S., S.K.A, B.Y., Data Collection and/or Processing: C.S., C.S., Analysis or Interpretation: C.S., C.S., B.Y., Literature Search: C.S., C.S., S.K.A, B.Y., Writing: C.S., C.S.

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