RESEARCH ARTICLE

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Enhancing the Effective Chemotherapy: The Combined Inhibition of Rhinacanthin-C, 5-Fluorouracil, and Etoposide on Oral Cancer Cells

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Abstract

Objective: To investigate the effects of rhinacanthin-C (Rh-C), 5-FU, and etoposide on growth inhibition, as well as the effects of a combination of these inhibitors on the oral cell lines SCC9 and HSC4. **Methods:** Cancer cell growth inhibition and inhibition combination were determined using the SRB assay. Cell viability and early apoptosis were determined using flow cytometry on cells stained with Annexin 5 and PI. Western blotting was performed to study the molecular mechanism of these inhibitors on oral cancer cells. **Results:** The results showed that etoposide, 5-FU, and Rh-C exhibited more potent anti-proliferative effects on HSC4 cells compared to SCC9 cells in a time- and concentration-dependent manner. The combination of Rh-C and 5-FU was more effective in inhibiting cell growth than the drugs used alone. The combination of 5-FU and Rh-C resulted in a decrease in live HSC4 cells, with the highest percentage of cell death observed at a ratio of 40:6 μM. Furthermore, the combination of 5-FU and Rh-C reduced P-Akt levels leading to a decrease in cell survival. **Conclusions:** HSC4 cells were found to be more sensitive to the inhibitory effect of these drugs compared to SCC9 cells. These findings suggest that the use of Rh-C as a complementary therapy with 5-FU may have the potential for the treatment of oral cancer. the underlying mechanisms responsible for this difference in sensitivity between the two cell lines need to be further investigated.

Keywords: rhinacanthin-C- HSC4- SCC9- anti-proliferation- combination inhibition

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Introduction

Cancer is a global health concern that affects the quality of life and mortality (Sung et al., 2021). Oral cancer has been reported as the 13th most common cancer worldwide, with an estimated 377,713 new cases and 177,757 deaths per year in the year 2020 (Salari et al., 2022). Tobacco use and infection with the human papillomavirus (HPV) are the primary risk factors for oral cancer, causing DNA damage and gene mutations (Johnson et al., 2020). A genomic study of head and neck squamous cell carcinoma showed that a mutation in PIK3CA, AKT, and PTEN genes leads to the activation of growth signaling pathways, contributing to tumor formation (García-Escudero et al., 2018; Starzyńska et al., 2021). Targeting the proteins encoded by mutated genes in oral cancer is a potential therapeutic approach.

In managing oral squamous cell carcinoma (OSCC), clinical trials strive to enhance treatment efficacy, uncover genetic markers for individualized therapy, and potentially transform the field of oral cancer treatment. Among these strategies, chemotherapy was the most frequently utilized (36.8%), while targeted therapy and immunotherapy each constituted 15.2% of the approaches evaluated (Zou et al., 2022). Patients with advanced or recurrent Oral squamous cell carcinoma (OSCC) are typically treated with surgery and chemotherapy. 5-Fluorouracil (5-FU), an anticancer drug, is an essential therapy for gastrointestinal cancers, including OSCC (Ţigu et al., 2020). However, due to its severe side effects and resistance to the 5-FU might develop, combining it with other anticancer medications or adjuvants may allow for a lower dose of 5-FU. Ţigu et al., (2020) demonstrated that the combination of allicin (main active compounds derived from garlic) and 5-FU suppressed the migration and proliferation of colorectal and lung cancer cells. Compared to single-agent treatments of 5-FU and allicin, the co-treatment resulted in a lower rate of cell survival. Additionally, etoposide, derived from podophyllotoxin, is an anticancer medication that inhibits topoisomerase II. It has strong therapeutic efficacy in the treatment of juvenile leukemia, testicular cancer, and small-cell lung cancer (SCLC) (Sen et al., 2016). The combination of etoposide with platinum compounds (cisplatin or carboplatin) is recommended as first-line

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treatment for aggressive lung cancers, with an average 75% response rate (Sen et al., 2016). However, resistance and tolerance to chemotherapeutic agents lead to a major barrier to effective treatment. The frequency of cisplatin resistance and 5-FU resistance was found to be 33% and 40.2%, respectively (Atashi et al., 2021). To enhance the efficacy of chemotherapy and reduce its side effects, natural compounds have been used in combination with chemotherapeutic drugs.

The use of natural products has a crucial role in cancer treatment, as they modulate signaling pathways that induce cell death in cancer cells. Due to their broad therapeutic targets and low toxicity, natural compounds have emerged as potential novel agents for cancer treatment (Castañeda et al., 2022). Plant-based medicine and edible plants are major sources of bioactive compounds that have demonstrated promising results in inhibiting cancer progression and reducing adverse reactions (Subramaniam et al., 2019). In recent years, phytochemicals isolated from these medicinal and edible plants have been found to modulate gene and protein expression, leading to the inhibition of cell proliferation, the arrest of the cell cycle, and the induction of apoptosis (Subramaniam et al., 2019). Our preliminary results indicated that rhinacanthin-C (Rh-C) isolated from R. nasutus inhibited the growth of HSC4 cells in a dose-dependent manner. In addition, Rh-C has been shown to enhance the anti-cancer effects of doxorubicin cytotoxicity in breast cancer Chaisit et al., 2017. Moreover, rhinacanthin-C (Rh-C) has been documented to suppress migration and invasion in cholangiocarcinoma cells (Boueroy et al., 2018). The synthetic derivative of Rh-C has also demonstrated the anti-proliferative effect on human cervical carcinoma cell lines (Gotoh et al., 2004). However, the combined anti-proliferative effect of Rh-C and the known drug (5-FU and etoposide) on oral squamous cell carcinoma remains unknown.

In this study, we aimed to investigate the anti-proliferative effect of combined Rh-C (a natural product extracted from R. nasutus and known chemotherapeutic drugs (5-FU and etoposide) on HSC4 and SCC9 cell lines. Moreover, the apoptotic cell population was determined. The combination of Rh-C and 5-FU with a ratio of 40:6 μM showed the most synergistic effect on HSC.

Materials and Methods

Reagents and chemicals

Etoposide (lot number 3574604) and 5-Fluorouracil (lot number MKCD1558) were obtained from Merck - Sigma-Aldrich (St Louis, MO). Rh-C was obtained according to the previous report by Pouyfung et al., (2014). The Iscove's Modified Dulbecco's media and M199 media and Fetal bovine serum used for cell cultures were purchased from Gibco BRL (Massachusetts, USA). penicillin, streptomycin, and fungizone were sourced from Sigma (St Louis, Missouri). Annexin V-FITC Kit (tot number 5220708668) was purchased from Thermo Fisher Scientific (Massachusetts, United States).

Cell lines and cultures

The HSC4 and SCC9 oral tongue SCC cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank and ATCC CRL-1929, respectively. The cells were maintained in a mixture of Dulbecco's modified Eagle medium and Ham's F12 (DMEM) medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μL streptomycin, and 1% fungizone. The cells were cultured in a humidified incubator at 37°C with 5% carbon dioxide.

In vitro anti-cell growth assay

Growth inhibitory effect of Rh-C, 5-FU, and etoposide on HSC4 and SCC9 was determined using the sulforhodamine B (SRB) assay as described previously (Pouyfung et al., 2019). Briefly, HSC4 and SCC9 cells were seeded at a density of 7,500 cells per well in a 96-well plate and incubated overnight. Cells were treated with Rh-C, 5-FU, and etoposide ranging from 0-100 μM. After 24, 48, and 73 hours of incubation at 37°C in a 5% carbon dioxide incubator, the growth of cells was determined using an SRB assay based on the measurement of cellular protein content. The optical density of the wells was measured at a wavelength of 595 nm. Fifty percent of the growth inhibition (GI_{50}) for each inhibitor was calculated from a graph between the percent cell viability and log concentrations of each test inhibitor using GraphPad Prism 5.

Combination inhibition of test inhibitors on HSC4 cells

The effects of test inhibitor combinations (Rh-C + 5-FU; Rh-C + etoposide; and etoposide + 5-FU) on HSC4 cell growth were investigated. In brief, 7,500 HSC4 cells were seeded in a 96-well microplate and incubated for 24 hours at 37°C in 5% carbon dioxide. The cells were then treated with different combinations of the drugs, including one-fourth GI_{50} of inhibitor 1 and three-fourth GI_{50} of inhibitor 2, one-half GI_{50} of each inhibitor, and one-fourth GI_{50} of inhibitor 1 and three-fourth GI_{50} of inhibitor 2. After 24 hours of further incubation at 37°C in a 5% carbon dioxide incubator, the number of cells was determined using an SRB assay.

Effect of combined 5-FU and Rh-C on HSC4 cell apoptosis

100,000 HSC4 cells were seeded in a 6-well plate and incubated for 24 hours at 37°C in 5% carbon dioxide. HSC4 cells were treated with 5-FU and Rh-C in the ratio of 120: 2 μM, 80: 4 μM, 40: 6 μM. After 24 h, cells were harvested and stained with Annexin V-FITC for 15 minutes at room temperature in the dark, followed by the addition of propidium iodide (PI) for 5 minutes in the dark. The cell suspension is then loaded into the flow cytometer. The cell population was gated to identify live cells (FITC-negative, PI-negative), early apoptotic cells (FITC-positive, PI-negative), and dead cells (FITC-positive, PI-positive).

SDS-PAGE and Western blotting

Approximately $1x10^6$ HSC4 cells (in a 6-well plate) were treated with 8 μM Rh-C, 160 μM 5-FU, 50 μM Etoposide, and combinations of 4 μM Rh-C + 80 μM 5-FU, 6 μM Rh-C + 40 μM 5-FU, 4 μM Rh-C + 25

μM Etoposide, and 25 μM Etoposide + 80 μM 5-FU. Incubation with DMSO was used as a control experiment. After a 24-hour incubation at 37°C, 5% CO_2, cells were lysed using a mammalian lysis buffer (GE Healthcare, Piscataway, NJ, USA) supplemented with a protease and phosphatase inhibitor (Roche, Indianapolis, IN, USA). The proteins were then subjected to SDS-PAGE and Western blotting, following established methodologies (Pouyfung et al., 2019). The primary antibodies used were specifically targeted against BcL-2 (Santa Cruz Biotechnology, Dallas, TX, USA), Bax (Santa Cruz Biotechnology), Phospho-Akt (Cell Signaling Technology Inc., Danvers, MA, USA), and Akt (Cell Signaling). Either anti-rabbit IgG or anti-mouse IgG (Bio-Rad, Hercules, CA, USA) conjugated with horseradish peroxidase was used as the secondary antibody. To achieve normalized protein loading, immunoblotting was performed using the polyclonal human β-actin antibody (Sigma-Aldrich).

Results

The antiproliferative effects of Rh-C (a natural product), etoposide, (a topoisomerase inhibitor), and 5-Fluorouracil (5-FU) (inhibits cellular thymidylate synthase), were preliminary determined against HSC4 and SCC9 oral cancer cell lines. HSC4 and SCC9 cells

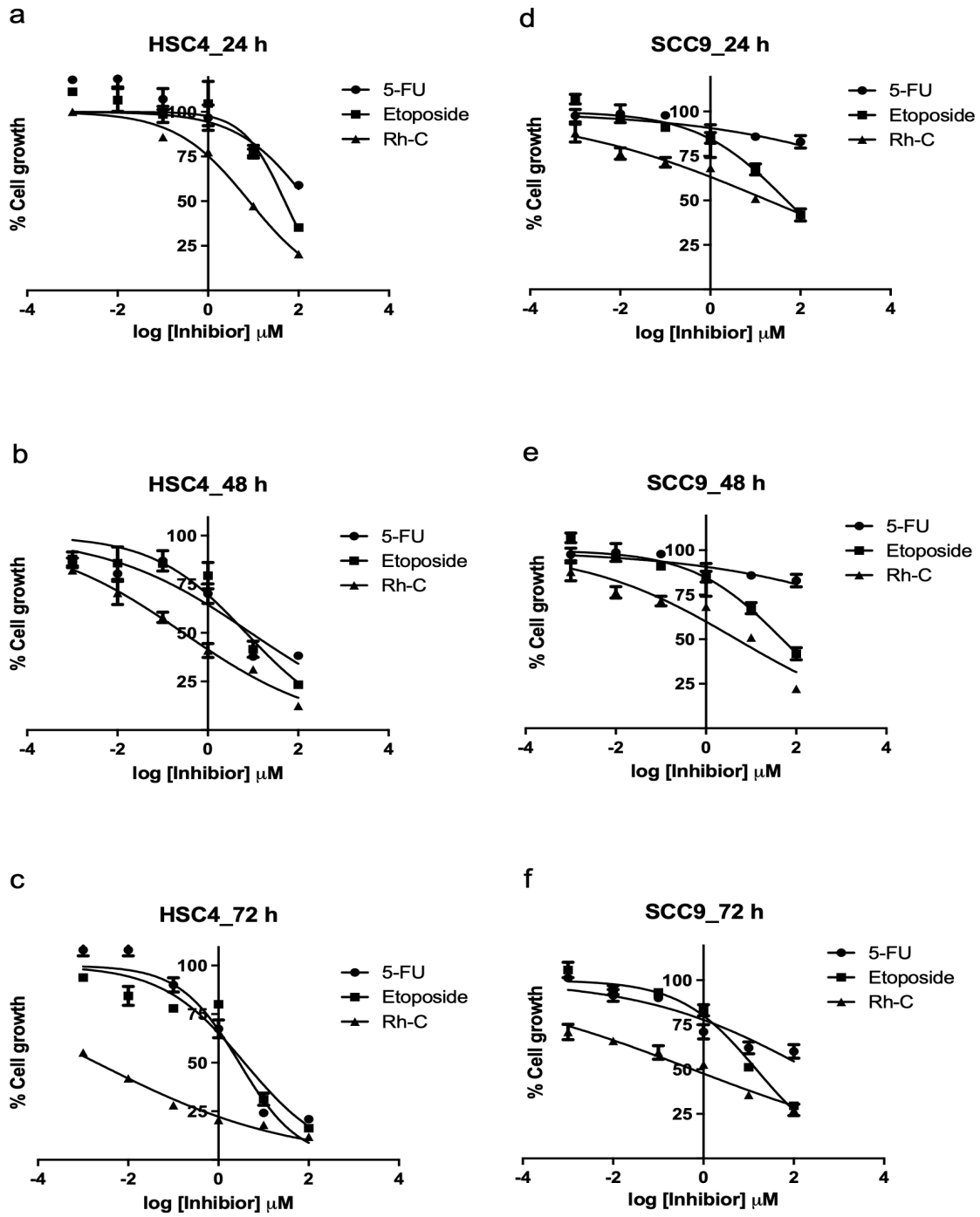

Figure 1. Effect of Rhinacanthin-C, Etoposide, and 5-FU on HSC4 and SCC9 Cell Growth for 24, 48, and 72 h. The cell viability was determined using the SRB assay. Data are r mean ± SEM (n = 3)

Table 1. Effect of Rhinacanthin C, Etoposide, and 5-FU on HSC4 and SCC9 Cell Growth

Test inhibitors	Growth inhibition (GI50) μM					
	SCC9 cell line			HSC4 cell line		
	24 h	48 h	72 h	24 h	48 h	72 h
Rh-C	19.26±4.28	4.79±1.11	0.56±0.06	8.00±1.72	0.28±0.03	0.002±0.00
5-FU	> 100	>100	215.00±21.06	163.30±17.04	8.90±3.24	2.88±1.27
Etoposide	49.12 ±5.00	49.02±5.12	14.75±2.40	49.60±6.25	7.02±2.44	3.76±1.70

were incubated with various concentrations of Rh-C, etoposide, and 5-FU ranging from 0 to 100 μM. After 24, 48, and 72 hours, the growth of cells was assessed using an SRB assay. The results showed that all tested inhibitors exhibited more potent anti-proliferation towards HSC4 cells compared to SCC9 cells in a time- and concentration-dependent manner, as indicated in Table 1 and Figure 1. Rh-C demonstrated a 2.4-fold, 17.1-fold, and 280-fold higher antiproliferative effect on HSC4 cells compared to SCC9 cells (Figure 1). In contrast, 5-FU showed weak inhibition against both cell lines with a GI_{50} value greater than 100 μM against SCC9 cells and GI_{50} values of 163.3 μM (24 hours), 8.9 μM (48 hours), and 2.88 μM (72 hours) against HSC4 cells. Etoposide showed a modulated effect on both HSC4 and SCC9 cells, with GI_{50} values for SCC9 cells being 6.9-fold (48 hours) and 3.9-fold (72 hours) higher than those for HSC4 cells. Moreover, Rh-C, 5-FU, and etoposide exhibited less effect on HGF cell growth, with GI_{50} values greater than 40 μM, 200 μM, and 200 μM, respectively, (Figure 2).

All tested compounds, including Rh-C, etoposide, and 5-FU, exhibited greater anti-cancer properties against HSC4 cells compared to SCC9 cells. To investigate the enhancement of anti-proliferative activity against HSC4 cell growth through the combination of two different chemicals, including Rh-C and etoposide, Rh-C and 5-FU, and etoposide and 5-FU, HSC4 cells were inculcated with combined test inhibitors. The results demonstrated that the combination of 5-FU and Rh-C was more effective in inhibiting cell growth than the drugs used alone (Figure 3). Furthermore, a correlation was observed between an increase in the concentration of Rh-C and a decrease in the viability of HSC4 cells, suggesting that Rh-C enhances the anti-cancer effect of 5-FU. However, an enhanced anti-cancer effect was not observed in normal HGF cells (Figure 3).

The phytochemical compounds derived from a medicinal plant have been found to possess anti-cancer activity through the induction of cell apoptosis. HSC4 cells were treated with Rh-C (at a GI_{50} concentration of 8 μM), 5-FU (at a GI_{50} concentration of 160 μM), and a combination of 5-FU and Rh-C (at ratios of 120:2 μM, 80:4 μM, and 40:6 μM). After 24 hours, the cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) dye. Cell population was analyzed using flow cytometry. The results indicated that the treatment with Rh-C and 5-FU at their GI_{50} concentrations resulted in approximately 60% live cells. In contrast, the

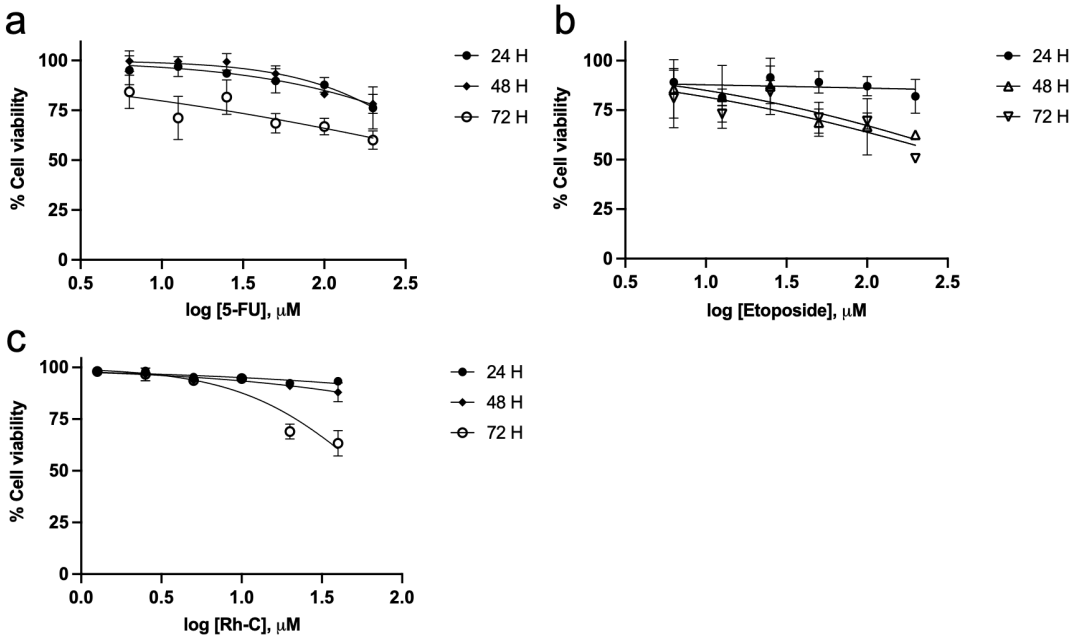

Figure 2. Growth Inhibition of HGF Cells (Normal Gingival Fibroblasts) Treated with 5-FU (A), etoposide (B), and Rh-C (C). For the experiment, 104 HGF cells were exposed to varying concentrations of Rh-C (0-40 μM), 5-FU (0-200 μM), and etoposide (0-200 μM) over three distinct periods: 24 hours, 48 hours, and 72 hours. The cell viability was subsequently evaluated using the SRB assay. The presented data are the mean ± SEM from three independent experiments (n = 3).

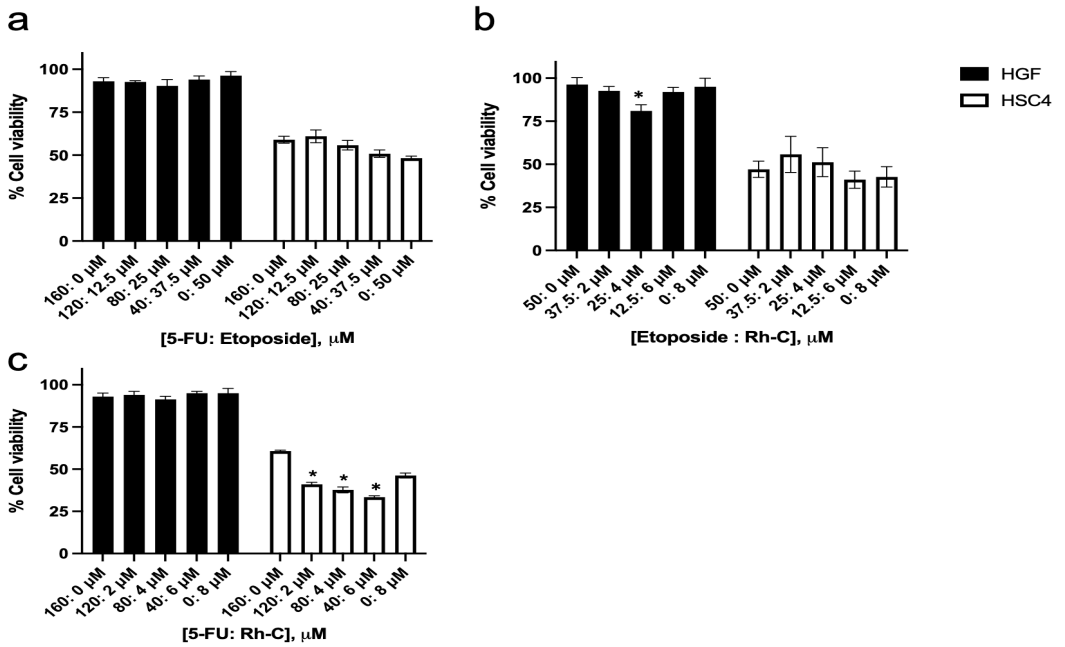

Figure 3. The Combination Inhibition of 5-FU and Etoposide (a), etoposide and rhinacanthin-C (Rh-C) (b), and rhinacanthin-C and 5-FU (c) on the growth inhibition of HSC4 and HGFcells. HSC4 or HGF cells were treated with different combinations of the inhibitors, and cell growth inhibition was determined using the SRB assay. Data are represented as mean ± SEM (n = 3). *p < 0.05, compared to the test inhibitor alone.

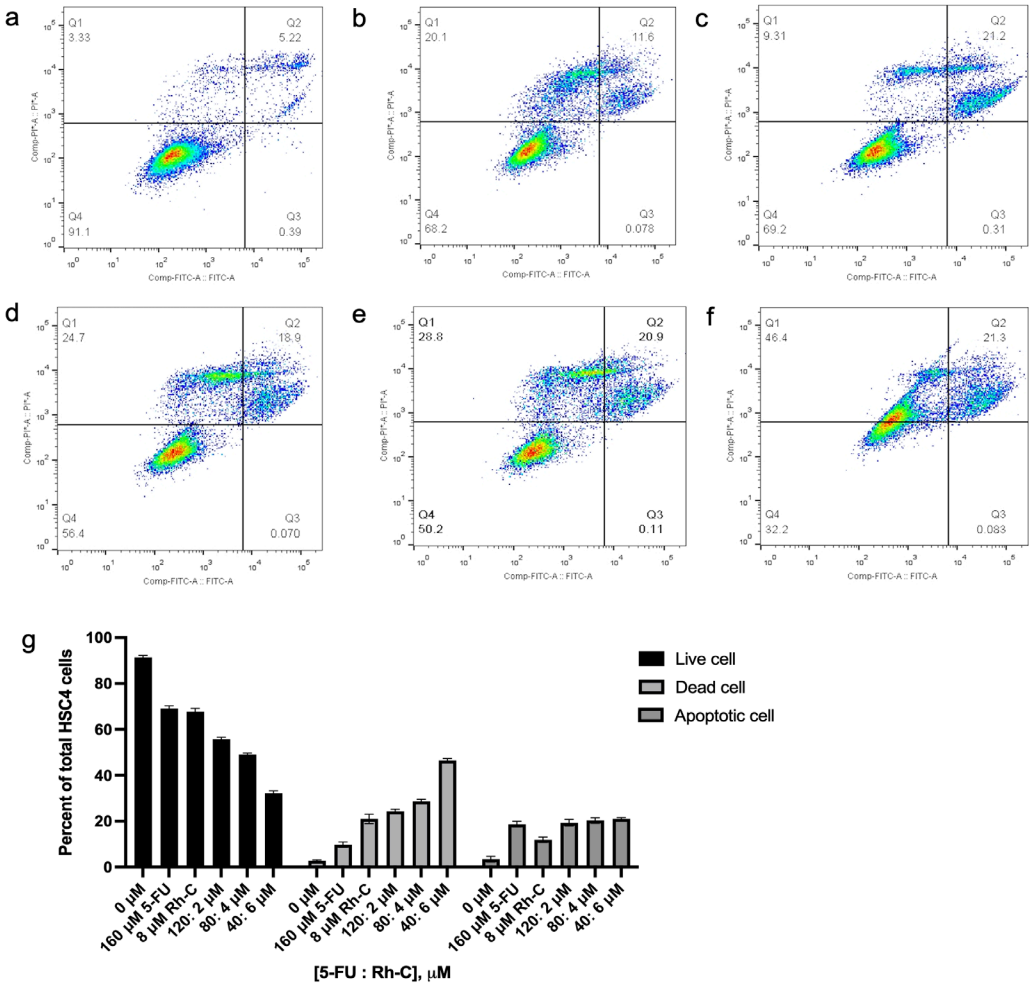

Figure 4. The Combination Inhibition of Rh-C, 5-FU, and Etoposide on HSC4 Cells. HSC4 cells were treated with no inhibitor (a), 8 μM Rh-C (b), 160 μM 5-FU (c), combination of Rh-C and 5-FU at ratios of 2:120 μM (d), 4:80 μM (e), and 6:40 μM (f). The percentage of early apoptotic cells (Anexin 5 positive, PI negative) and death cells (Anexin 5 negative, PI positive) was determined by using flow cytometry. Data are represented as mean ± SEM (n = 3).

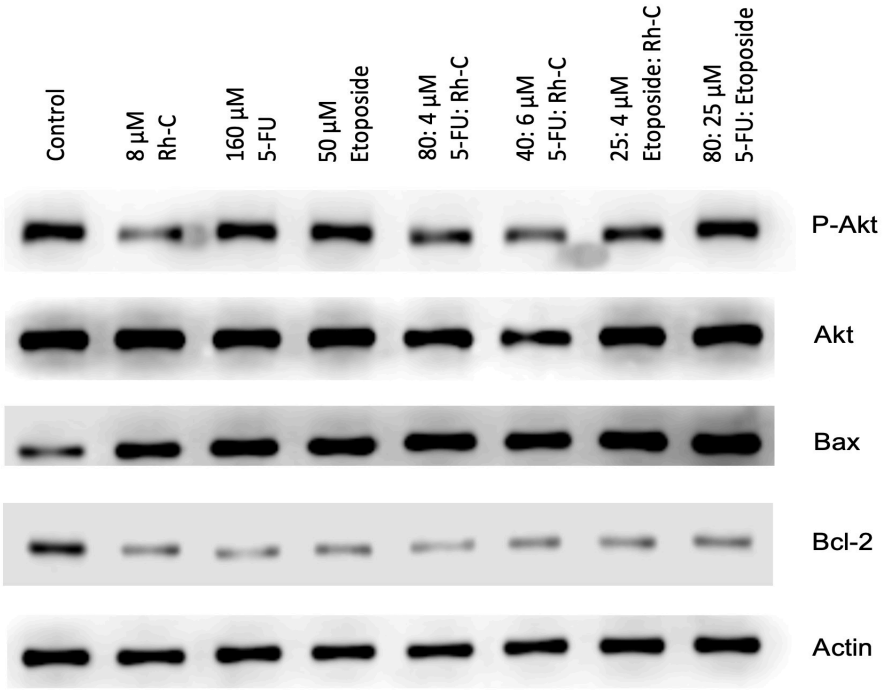

Figure 5. The Effects of Rh-C, 5-FU, Etoposide, as well as the Combination of Rh-C with 5-FU/etoposide, and etoposide with 5-FU, on the protein expression levels of P-Akt, Akt, Bax, and Bcl-2. HSC4 cells were treated with 8 µM Rh-C, 160 µM 5-FU, 50 µM etoposide, and a combination of 4 µM Rh-C + 80 µM 5-FU, 6 µM Rh-C + 40 µM 5-FU, 4 µM Rh-C + 25 µM etoposide, and 25 µM etoposide +80 µM 5-FU for 24 hours. Following protein extraction, 15 µg of crude protein was subjected to SDS-PAGE and Western blotting, employing the primary antibodies for P-Akt, Akt, Bax, and Bcl-2. Actin was used as a loading control for the proteins.

combination of 5-FU and Rh-C resulted in a decrease in live HSC4 cells, with the highest percentage of cell death observed at a ratio of 40:6 µM (Figure 4). The combination therapy showed a synergistic effect in suppressing cancer cell growth and increasing the sensitivity of cancer cells to 5-FU.

Rh-C was previously demonstrated to induce apoptosis by upregulating Bax and downregulating both Bcl-2 and phosphorylated-Akt (P-Akt). Therefore, we next assessed the protein expression levels of P-AKt, Akt, Bax, and Bcl-2 in HSC4 cells after treatment with Rh-C, 5-FU, etoposide at their GI_{50} concentrations, and their combinations (Rh-C+5-FU, Rh-C+etoposide, and 5-FU+etoposide). As shown in Figure 5, treatment with Rh-C, 5-FU, etoposide alone, and their combinations resulted in increased expression of Bax, a pro-apoptotic protein, and decreased expression of Bcl-2, an anti-apoptotic protein, leading to cell apoptosis. Interestingly, only Rh-C, but not 5-FU and etoposide were capable of reducing P-Akt. The most potent effect was observed in the combination of 5-FU and Rh-C at a ratio of 40:6 µM, followed by 5-FU and Rh-C at a ratio of 80:4 µM. However, the combinations of Rh-C + etoposide and 5-FU + etoposide demonstrated a lesser effect on suppressing P-Akt protein expression. The observed synergistic effect of 5-FU and Rh-C on the apoptosis of HSC4 cells in this study might be a result from the suppression of the PI3K/Akt signaling pathway.

Discussion

The present study aimed to investigate the effect of etoposide, 5-fluorouracil (5-FU), and Rh-C on HSC4 and SCC9 cell growth. Etoposide and 5-FU are chemotherapy drugs commonly used to treat various cancers, including lung, breast, and colorectal cancer (Sen et al., 2016; Ţigu et al., 2020), while Rh-C is a bioactive compound isolated from R. nasutus that has been reported to have anticancer and anti-inflammatory properties (Chaisit and Jianmongkol, 2021; Chaisit et al., 2017). The results indicated that all three inhibitors exhibited more potent anti-proliferation effects towards HSC4 cells compared to SCC9 cells in a time- and concentration-dependent manner. However, a significant difference was observed in the inhibitory effect of the test inhibitor between the two cell lines. In Figure 1, Rh-C showed the strongest inhibitory effect on HSC4 cell growth with a GI_{50} value of 8 µM at 24 h, which was 6-fold and 20-fold lower than that of etoposide and 5-FU, respectively. Notably, Rhinacanthins had a weaker antiproliferative impact on normal cell lines (HGF), as demonstrated by GI_{50} values higher than 40 µM (Figure 2). The higher sensitivity of HSC4 cells to these drugs may be attributed to differences in the molecular characteristics of the two cell lines. The different oncogene mutation statuses of HSC4 and SCC9 cells may contribute to their different interactions with drugs and other therapies, as well as their difference in cancer cell growth. Further studies are warranted to explore the underlying mechanisms responsible for this

difference in sensitivity between the two cell lines. Our results are consistent with those of a previous study by Songserm et al., (2022) found the treatment of ORL-136 and ORL-48 cell lines with R. nasutus leaf extract for a period of 48 hours resulted in dose-dependent cytotoxicity, with an GI_{50} value of 1.24 mg/mL for ORL-136 and 2.35 mg/mL for ORL-48. Similarly, Boonyaketgoson et al., 2017 found that naphthoquinones isolated from R. nasutus exhibited cytotoxicity against oral human carcinoma (KB cell line) with GI_{50} values ranging from 11.66-41.77 μM (Boonyaketgoson et al., 2018). In our study, we found that 5-FU inhibited HSC4 cells with an GI_{50} value of 8.90 μM at 48 hours. Moreover, effect of 5-FU on HSC4 is consistent with the previous report of Harada et al., 2014 who found that 5-FU inhibited HSC4 cells with an GI_{50} value of 1.4 μg/mL (10.8 μM) at 48 hours (Harada et al., 2014).

To enhance the efficacy of chemotherapy and reduce its side effects, Rh-C in combination with 5-FU or etoposide was determined. The result found that Rh-C in combination with 5-FU but not Rh-C in combination with etoposide showed stronger cell growth inhibition than the individual test inhibitor alone (Figure 3). Our results showed that treatment with Rh-C and 5-FU at their GI_{50} concentrations resulted in approximately 60% live cells. In contrast, the combination of 5-FU and Rh-C resulted in a significant decrease in live HSC4 cells, with the highest percentage of cell death observed at a ratio of 40:6 μM. Rh-C in combination with 5-FU resulted in an increase in the proportion of the early apoptosis population (Figure 4), suggesting the induction of apoptosis by Rh-C. Moreover, Rh-C has been shown to induce cell cycle arrest and apoptosis through the upregulation of Bax, as well as the downregulation of Bcl-2 and Akt (Chaisit and Jianmongkol, 2021), while 5-FU primarily acts by inhibiting DNA synthesis and repair through its incorporation into RNA and DNA (Noordhuis et al., 2004). Taken together combination inhibition of 5-FU and Rh-C might cause synergistic effect toward HSC4. Further studies are needed to elucidate the molecular mechanisms underlying the observed synergistic effect and to optimize the dosing and timing of the two compounds. Additionally, in vivo studies are needed to determine the safety and efficacy of this combination therapy in preclinical models of oral squamous cell carcinoma. In line with the previous studies of Chaisit., 2017 who found that Rh-C has anti-cancer properties and can enhance the cytotoxicity of doxorubicin in drug-resistant human breast adenocarcinoma cells (MCF-7/DOX cells) (Chaisit et al., 2017). Doxorubicin is a widely used chemotherapeutic drug for breast cancer treatment, but drug resistance often develops, limiting its effectiveness. Rhinacanthin-C has been shown to sensitize drug-resistant human breast adenocarcinoma cells to doxorubicin, making the drug more effective in killing cancer cells. The mechanism behind the enhanced cytotoxicity of doxorubicin by Rh-C is to inhibit P-glycoprotein activity, allowing doxorubicin to enter the cancer cells and cell death. The mechanism of co-inhibition between Rh-C and 5-FU in oral cancer cells is not yet fully understood. In addition, Rh-C has been shown to increase the production of reactive oxygen species (ROS) in cancer cells, leading to apoptosis and cell death (Chaisit and Jianmongkol, 2021). These results provide evidence for the possible use of R. nasutus and Rh-C as anticancer drugs and support the use of 5-FU as an oral squamous cell carcinoma chemotherapy medication. However, additional research is required to determine the optimum dosage and possible adverse effects of these agents in the therapy of oral cancer.

In contrast to Rh-C in combination with 5-FU, Rh-C showed no enhancement of the efficacy of etoposide. It is possible that Rh-C has a similar mechanism of action as etoposide, and they may compete for the same binding site on topoisomerase II, thereby reducing the function of etoposide to cause DNA damage and cell apoptosis. Our findings support earlier studies that reported molecular docking of naphthoquinone derivatives on the ATPase domain of human topoisomerase II. The tested naphthoquinone derivatives could bind to the ATPase domain of human topoisomerase II and inhibit its activity. Inhibition of Rh-C on human topoisomerase II need to be further investigated.

The primary strength of this study lies in the documented synergistic inhibitory effect of Rh-C and 5-FU, a well-known chemotherapeutic agent. This combined effect resulted in a reduced necessary concentration of 5-FU to induce cell apoptosis. Notably, Rh-C exhibited lower toxicity towards HGF, a non-cancerous cell line. Moreover, Rh-C was found to inhibit Phosphorylated-Akt (P-Akt), thereby blocking the PI3K-Akt signaling pathway in HSC4 cells. Given that this signaling pathway—associated with a prevalent PI3K/Akt mutation—is active in over 90% of head and neck squamous cell carcinomas (HNSCC), blocking it may present a promising strategy for targeted therapy by reducing cell survival and inducing apoptosis. With our research objectives in mind, HSC4 emerged as an optimal model for evaluating the effect of Rh-C. Nevertheless, this study has limitations. The cooperative inhibitory mechanism between Rh-C and 5-FU in oral cancer cells is currently only understood in the context of in vitro experiments. To broaden our understanding of the combined inhibitory effects of Rh-C and 5-FU, future exploration in vivo models is warranted.

In conclusion, the current research investigated the growth-inhibition effects of etoposide, 5-FU, and Rh-C on HSC4 and SCC9 cells. The findings revealed that all three inhibitors inhibited cell growth, with HSC4 being more susceptible to inhibition than SCC9. Rh-C in combination with 5-FU inhibited cell growth more effectively than either compound alone, indicating the possibility of a synergistic relationship between these substances. Nonetheless, the addition of Rh-C to etoposide had no effect on the drug's effectiveness. The process underlying the co-inhibition of Rh-C and 5-FU is not fully understood, but it has been hypothesized that Rh-C may increase the cytotoxicity of 5-FU by blockage the cycle progression and stimulation of cell apoptosis. However, additional study is required to explore the underlying processes of the interaction between Rh-C and other chemotherapeutic medicines. The suppression of human topoisomerase II by Rh-C, however, the inhibition of Rh-C on human topoisomerase II requires further investigation.

Author Contribution Statement

Conceptualization, P.P., K.C.; methodology, S.K., P.P., C.K.; formal analysis, S.C.; investigation, S.K. resources, C.K; original draft preparation, S.K. and P.P.; review and editing, P.P., C.K. All authors have read and agreed to the published version of the manuscript..

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Conflict of Interest

The authors have no conflict of interest to declare.

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