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Synergistic Effect of Caffeine in B16f10 Cells in Combination with Doxorubicin and Oxaliplatin

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Abstract

Caffeine is a commonly consumed psychoactive substance that has been shown to have various effects on cellular processes, including cell growth and survival. In this study, we investigated the potential of Caffeine to enhance the cytotoxic effects of two commonly used chemotherapeutic agents, doxorubicin and oxaliplatin, on B16F10 cells. We evaluated the cytotoxicity, calculated the IC50 and combination index of the medications, estimated the cell cycle of the cells, and evaluated the apoptotic and anti-apoptotic genes through gene expression analysis. Our results demonstrated that Caffeine significantly potentiated the cytotoxicity of both doxorubicin and oxaliplatin, resulting in a more significant reduction in cell viability compared to treatment with the chemotherapeutic agents alone. Additionally, Caffeine also enhanced the pro-apoptotic effects of the chemotherapeutic agents, leading to increased levels of apoptosis in the B16F10 cells. The combination of doxorubicin and oxaliplatin with Caffeine was found to be synergistic. These findings suggest that caffeine may have the potential to improve the efficacy of current chemotherapeutic regimens and may offer a novel approach to enhance cancer treatment.

Keywords: Anticancer Therapy, Chemotherapy, Melanoma, Xanthine Alkaloids

1. Introduction

Melanoma is a type of skin cancer that develops in the melanocytes, which are the cells that produce the pigment melanin. The B16F10 melanoma cell line is a widely used preclinical model for studying the development and treatment of melanoma[1]. The current standard of care for melanoma includes surgery, radiation therapy, and chemotherapy. However, chemotherapy often has limited efficacy and can cause toxic side effects.[2] Doxorubicin (DOX) and Oxaliplatin (OXP) are widely used chemotherapy drugs that treat various cancers, including melanoma. However, their effectiveness can be limited by the development of drug resistance and toxic side effects on healthy cells[3,4]. Researchers are therefore investigating new strategies to improve the effectiveness of chemotherapy while minimizing its side effects. One such strategy is the combination of chemotherapy with other drugs or natural compounds that can enhance its therapeutic effects.

CAF is a natural stimulant commonly found in coffee, tea, and energy drinks. It has been shown to have various health benefits, including reducing the risk of Parkinson's disease, Alzheimer's disease, and certain types of cancer, including melanoma[5]. Recent studies have demonstrated that coffee consumption is linked to decreased overall cancer incidence and has a negative correlation with certain types of cancers, such as melanoma[6–8]. These effects

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are believed to be facilitated by 1, 3, 7-trimethylxanthine (CAF), a naturally occurring alkaloid methylxanthine that is a primary constituent of coffee and has exhibited anti-tumour properties. Animal experiments have suggested that CAF can hinder cancer cell invasiveness and metastasis and induce cell apoptosis[9–11]. The ability of CAF to inhibit cancer is dependent on high concentrations of CAF (≥1 mM), which may exceed biologically or physiologically relevant levels[12]. High concentrations of CAF could potentially result in a range of negative health consequences, such as central nervous system stimulation, teratogenicity, and mutagenicity[13]. To avoid toxicity, a plasma CAF concentration of 80 µg/mL (412 µM) is recommended[14]. Nevertheless, recent research indicates that even low concentrations of CAF can impede cancer progression. For example, a recent study demonstrated that a low concentration of CAF inhibited the development of Hepatocellular Carcinoma (HCC)[15]. Similarly, another study reported that a low concentration of CAF reduced the viability, migration, and invasion of B16F10 melanoma cells while promoting their apoptosis[16]. However, no information is available on the effect of CAF in a combination of chemotherapeutic drugs such as DOX and OXP in treating melanoma.

Hence the current study evaluated the role of CAF in combination with DOX and OXP in treating B16F10 melanoma cells in vitro, assessing the inhibitory concentration 50 (IC_{50}), Combination Index (CI), cell cycle, and apoptosis *in vitro*. This study is novel in investigating the potential synergistic effects of CAF in combination with chemotherapy drugs for the treatment of melanoma, which may provide a new approach to improving the therapeutic effectiveness of current treatments for melanoma.

2. Materials and Methods

2.1 Cell Culture

B16F10 melanoma cells derived from mice were acquired from the National Centre for Cell Science (NCCS; Pune). The B16F10 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) containing 10% Fetal Bovine Serum (FBS) (Gibco, Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St, Louis, MO, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies), and 10 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (HEPES) buffer (Sigma-Aldrich). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and the culture medium was refreshed every three days.

2.2 Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of DOX, OXP, and CAF *in vitro*. B16F10 cells (3×10^5) were seeded into 96-well plates and allowed to grow overnight before being treated with individual compounds or combinations of two compounds at varying concentrations. Control cells were left untreated or treated with Dimethyl Sulfoxide (DMSO). After a 48-hour incubation period, MTT solution was added to the cells, which were then incubated for an additional 4 hours at 37°C. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc. USA) after removing the medium and adding DMSO. Graph pad Prism software was used to calculate the dose-response curve and IC_{50} value (the concentration of drug that results in half-maximal inhibition). CompuSyn software was used to determine the combination index (CI) values for each dose, which were then used to determine the drug combination's synergistic, additive, or antagonistic effect according to the Chou-Talalay method. A CI > 1.1 indicated an antagonistic effect, CI = 0.9-1.1 indicated an additive effect, and CI < 0.9 indicated a synergistic effect[16].

2.3 Apoptosis Assay

B16F10 cells were seeded into 60 mm dishes at a density of 1×10^5 and allowed to incubate overnight. To assess the induction of apoptosis, the cells were treated with DOX and OXP either alone or in combination with CAF for 48 hours. Following treatment, both the supernatants and trypsinized cells were collected and centrifuged at 80 × g for 5 minutes to obtain cell pellets. The harvested cell pellets were then stained with a Propidium Iodide (PI) apoptosis kit following the manufacturer's instructions. The stained samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lake, NJ, and USA).

2.4 Gene Expression Analysis

Twenty-four hours after treatment, total RNA was extracted from PCNs using a TRIzol reagent. cDNA was prepared using a cDNA synthesis kit (Thermo Scientific,

USA) from two µg of total RNA. RT-PCR was carried out using SYBR green master mix (Bio-Rad, USA), a Bio-Rad CFX96, and specific gene primers following the recommended procedure. The Threshold Cycle (CT) values for the target genes calculated from the above runs against a housekeeping gene, GAPDH were normalised, and relative gene expression was calculated. Table 1 represents the gene sequences for the primers used.

Table 1. Primer gene sequence

Gene	Sequence
Bcl2	*5'- ACCGTCGTGACTTCGCAGAG – 3'*
	3' – GGTGTGCAGATGCCGGTTC – 5'
Bax	*5' – CGGCGAATTGGAGATGAACTG – 3'*
	3' - GCAAAGTAGAAGAGG GCAACC – 5'
p53	*5' – GTATTTCACCCTCAAGATCC – 3'*
	3' – TGGGCATCCTTTAACTCTA – 5'
GAPDH	*5' – GAGAAACCTGCCAAGTATG – 3'*
	3' – GGAGTTGCTGTTGAAGTC – 5'

3. Results

3.1 Inhibitory Effect of DOX, OXP, and CAF on B16F10 Cells

The inhibitory effects of CAF (CAF) and two chemotherapeutic drugs, Doxorubicin (DOX) and Oxaliplatin (OXP), on B16F10 murine cells, were evaluated using different concentrations of each substance (0, 1, 5, 10, 15, 30, and 50 µM). Cell viability was determined using the MTT assay after 24 hours of incubation, and IC50 values

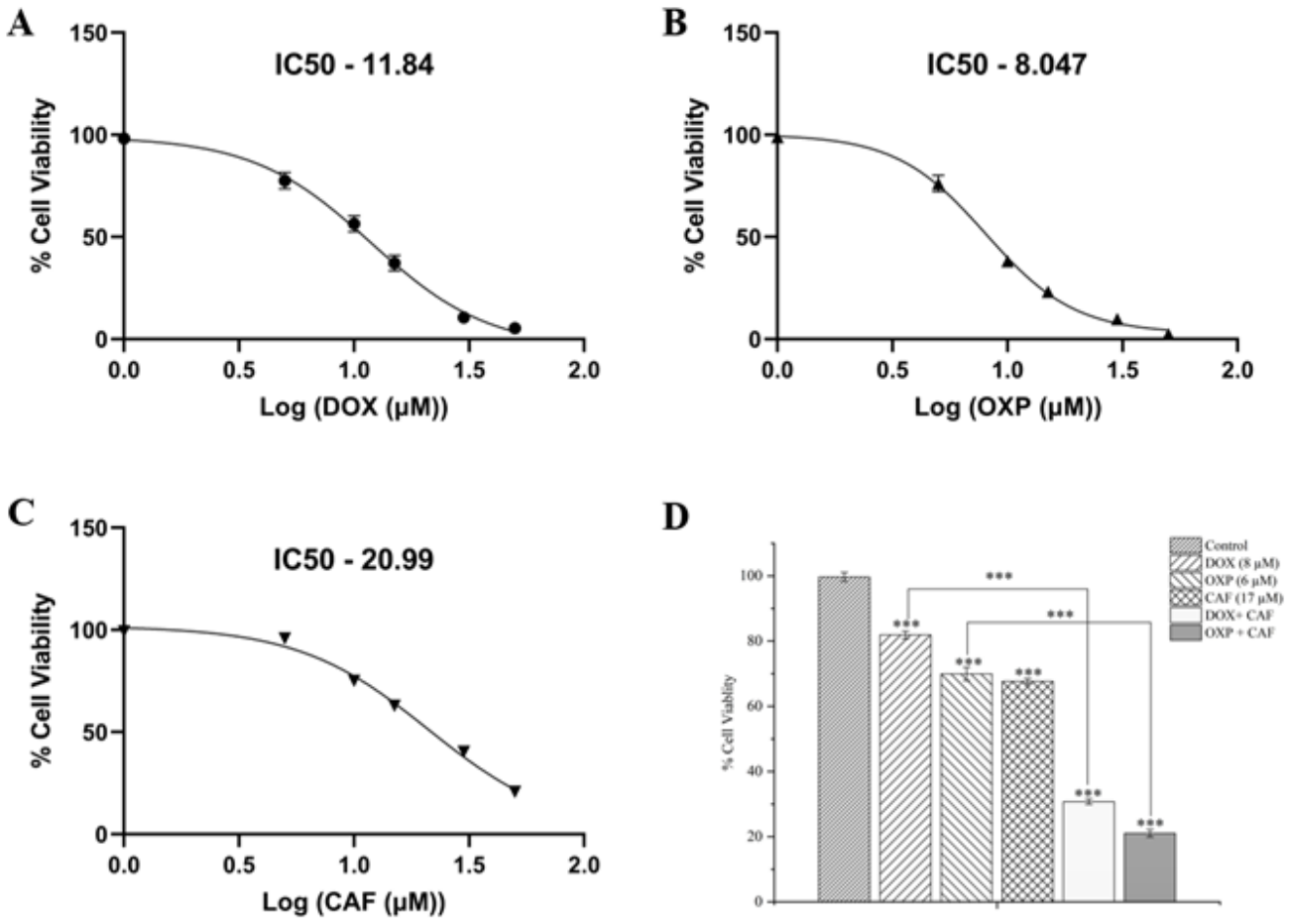


Figure 1. Dose-response curves of **(A)** DOX, **(B)** OXP, **(C)** CAF, and **(D)** % Cell viability of DOX, OXP, CAF, DOX + CAF, and, OXP + CAF in B16F10 cells. Dose-response curves were generated using a graph pad prism to determine IC_{50} value of the drugs. The results are presented as mean ± SEM (n = 3). ONE WAY ANOVA test was done using Tukey's post hoc test and considered P<0.05 significant.

were calculated from the resulting dose-response curves. The IC50 values for the growth inhibitory effects of CAF, DOX, and OXP in B16F10 cells were 20.99 µM, 11.84 µM, and 8.047 µM, respectively, as shown in Figure 1.

To investigate whether CAF could enhance the anti-cancer effects of DOX and OXP in B16F10 cells, cells were treated with CAF alone or in combination with either DOX or OXP. As shown in Figure. 1f, treatment with a combination of CAF (17 µM) and DOX (8 µM) or CAF (17 µM) and OXP (6 µM) for 24 hours resulted in a significant reduction in cell viability compared to control or individual agent treatment. The combination therapy of CAF and DOX had a Combination Index (CI) value of 0.42, indicating a synergistic effect, while the combination

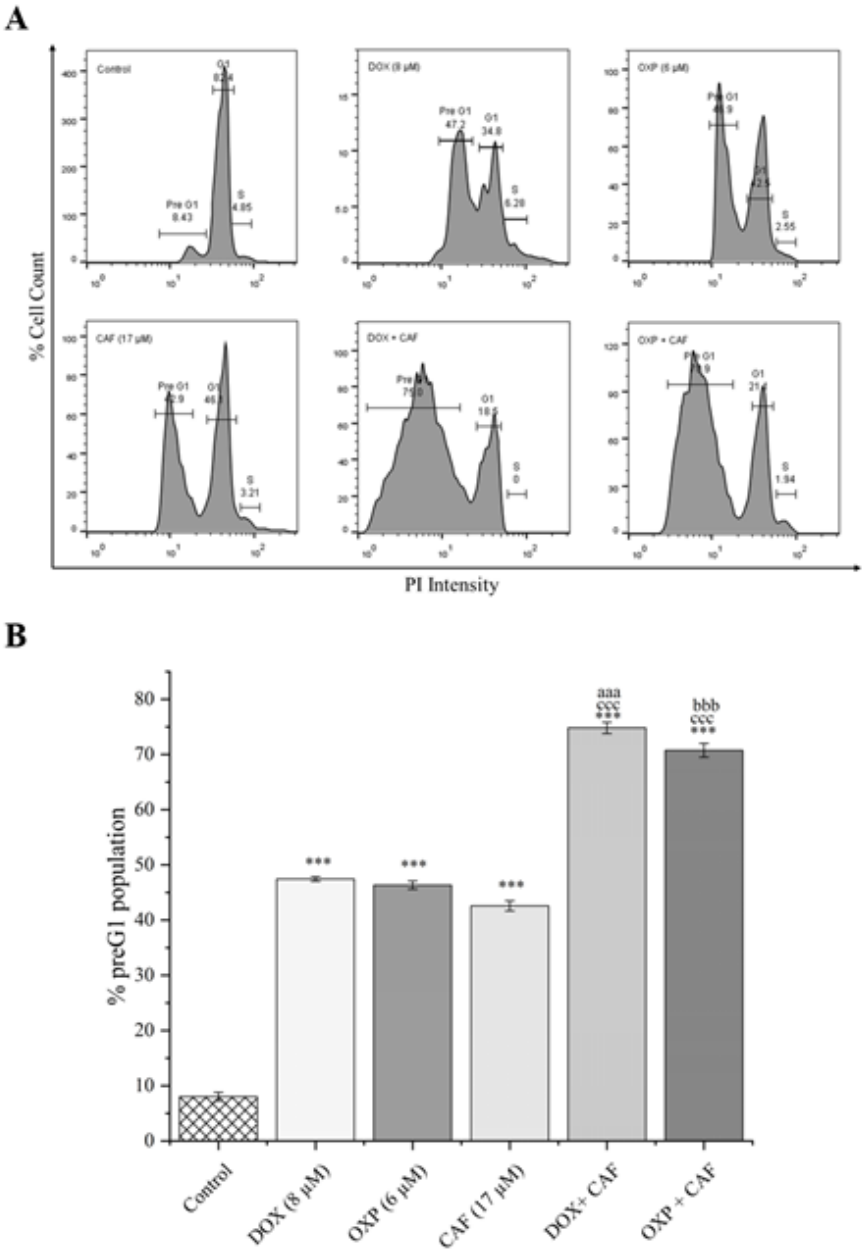


Figure 2. Effect of CAF in combination with DOX and OXP on Apoptosis of B16F10 cells. **(A)** Pre-G1 gated population showing apoptosis in different treatment groups, **(B)** Quantification of the pre-G1 gated population in different treatment groups; The results are presented as mean ± SEM (n = 3). ONE WAY ANOVA test was done with Tukey's post hoc test and considered $P < 0.05$ significant. *** $P < 0.001$ when compare Control; $^{aaa}P < 0.001$when compared to DOX, $^{bbb}P < 0.001$ when compared to OXP, $^{ccc}P < 0.001$ when compared to CAF.

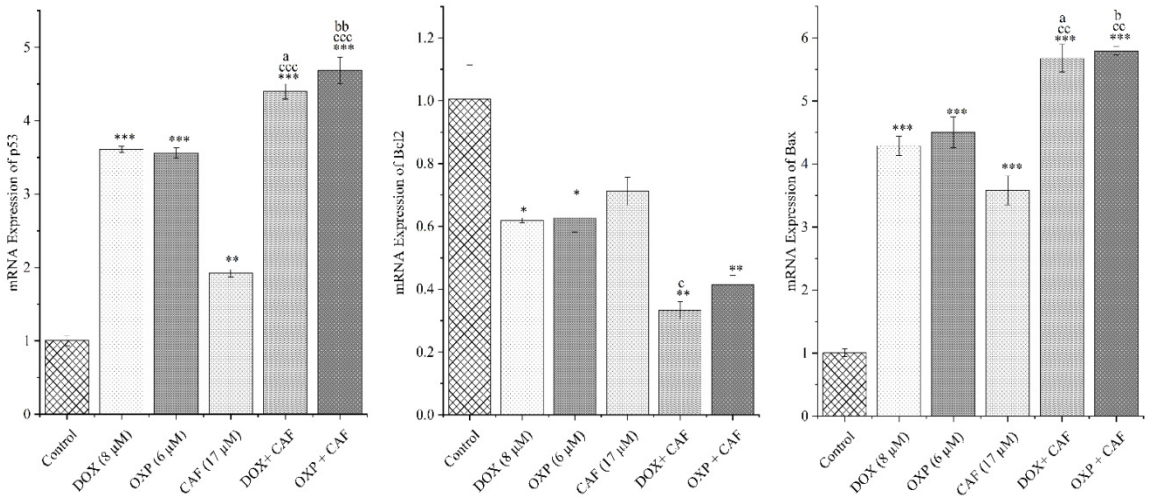


Figure 3. Relative expression of genes in B16F10 cells. **(A)** P53, **(B)** Bcl2, **(C)** Bax. The results are presented as mean ± SEM (n = 3). ONE WAY ANOVA test was done using Tukey's post hoc test and considered $P < 0.05$ significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compare Control; $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$when compared to DOX, $^{b}P < 0.05$, $^{bb}P < 0.01$,$^{bbb}P < 0.001$ when compared to OXP, $^{c}P < 0.05$, $^{cc}P < 0.01$,$^{ccc}P < 0.001$ when compared to CAF.

of CAF and OXP had a CI value of 0.57, also indicating a synergistic effect. According to the Chou-Talalay method, a CI value below 0.9 is defined as a synergistic inhibitory effect.

3.2 Apoptosis

Induction of apoptosis in the murine melanoma cancer cells (B16F10) using the drugs CAF, DOX, and OXP in combination. One of the hallmarks of apoptosis is the fragmentation of DNA, which can be detected by flow cytometry as a sub-G1 peak or a pre-G1 population. This sub-G1 peak corresponds to the apoptotic cells with fragmented DNA, which have a lower DNA content than the normal diploid cells and appear in the DNA histogram before the G1 phase peak[17]. CAF significantly potentiated ($p < 0.001$) the apoptotic activity of DOX and OXP increased by 25% which is shown in Figure 2.

3.3 Gene Expression Analysis

To further validate the pathway of apoptosis, gene expression analysis was carried out on the apoptotic and anti-apoptotic genes such as p53, bax, and bcl2 respectively. CAF significantly increased the apoptotic genes while it decreased anti-apoptotic genes when given in combination with DOX or OXP in comparison to the cells treated with individual drugs. The results were shown in Figure 3.

4. Discussion

Melanoma is a particularly aggressive form of skin cancer that poses significant treatment challenges due to its ability to spread quickly and develop resistance to chemotherapy drugs[18]. While current treatment options like Doxorubicin and oxaliplatin are commonly used, their efficacy is often limited by drug resistance and toxic side effects[19]. However, promising new research has emerged suggesting that CAF, a natural stimulant commonly found in coffee, tea, and other beverages, maybe a key ingredient in improving the effectiveness of melanoma treatment[20]. Recent studies have indicated that CAF may have several beneficial effects on the body, including stimulating the central nervous system, increasing heart rate, and improving cognitive function[21]. Additionally, CAF has been shown to have significant anticancer properties, such as inhibiting cell proliferation, inducing cell death, and preventing the formation of new blood vessels that supply tumours with nutrients[22]. This makes it a potentially powerful ally in the fight against melanoma. One promising approach is the use of combination therapy, in which multiple drugs with complementary mechanisms of action are used together to enhance their efficacy and reduce the risk of drug resistance[23].

The current study elucidated the effect of CAF on melanoma cells individually and in combination with conventional chemotherapeutic drugs such as DOX and OXP.

These both are known for the cytotoxicity and apoptosis of cancer cells[24,25]. The results of this study demonstrated that CAF has a potent inhibitory effect on the growth of B16F10 melanoma cells, with an IC50 value of 20.99 µM. This is consistent with previous studies showing that CAF has antiproliferative effects on a wide range of cancer cell types, including melanoma[26]. The study also showed that CAF potentiates the apoptotic response of DOX and OXP in B16F10 cells, resulting in a significant reduction in cell viability. The combination therapy of CAF and DOX had a Combination Index (CI) value of 0.42, indicating a synergistic effect, while the combination of CAF and OXP had a CI value of 0.57, also indicating a synergistic effect. These findings suggest that CAF has the potential to enhance the efficacy of existing chemotherapy drugs in the treatment of melanoma.

Apoptosis is an important mechanism of cell death that is often targeted in cancer therapy. CAF has been shown to induce apoptosis or programmed cell death in various cell types[27]. The study demonstrated that CAF significantly potentiated the apoptotic activity of DOX and OXP in B16F10 cells, as evidenced by the increased sub-G1 peak detected by flow cytometry. CAF potentiated the cell cycle arrest at G0/G1 arrest, which can be evident from the results that it increased pre-G1 population in B16F10 cells which is on par with the previous study in which CAF inhibited cell proliferation by G0/G1 arrest in JB6 cells[28]. This indicates that CAF can induce apoptosis in melanoma cells and that this effect is enhanced when used in combination with DOX or OXP. The researchers found that CAF inhibited cell proliferation by causing cell cycle arrest in the G0/G1 phase, associated with decreased cyclin D1 and CDK4 protein expression. They also observed that CAF inhibited the activation of AP-1 and NF-kB transcription factors, which are known to be involved in skin carcinogenesis[29]. The upregulation of apoptotic genes such as p53 and bax and the down-regulation of anti-apoptotic genes such as bcl2 further support the involvement of the apoptotic pathway in CAF-induced cell death. These findings suggest that CAF has the potential to induce apoptosis in melanoma cells and that this effect can be further enhanced when used in combination with existing chemotherapy drugs.

One of the major challenges in cancer therapy is the development of drug resistance. Combination therapy has been proposed as a strategy to overcome this challenge by targeting multiple pathways and reducing the risk of resistance. The results of this study suggest that 17 µM of CAF has the potential to enhance the efficacy of existing chemotherapy drugs in the treatment of melanoma. The synergistic effect observed with the combination therapy of CAF and DOX or OXP suggests that the two drugs have complementary mechanisms of action and that their combination may be more effective than either drug alone. Furthermore, the involvement of the apoptotic pathway in CAF-induced cell death suggests that this compound may be able to overcome drug resistance by targeting a different pathway than existing chemotherapy drugs. These findings are consistent with previous research[30] and provide further evidence that CAF may be a promising adjunct therapy for melanoma treatment, especially in combination with conventional chemotherapy drugs like DOX and OXP.

The results of this study also have important implications for the development of new melanoma treatments. The fact that CAF has potent inhibitory effects on melanoma cells, and can enhance the efficacy of existing chemotherapy drugs, suggests that it may be a promising candidate for further development as a cancer therapy. Moreover, the fact that CAF is a natural compound with low toxicity and few side effects makes it an attractive candidate for further development as a cancer therapy.

5. Conclusion

In conclusion, the potentiation of doxorubicin and oxaliplatin by CAF in melanoma appears to be a promising area of research. Further studies are needed to determine the optimal dose and timing of CAF administration and the potential for drug interactions and side effects. The results could potentially improve the outcomes for patients with melanoma and other cancers and effective adjunct therapy.

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