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Influence of PAC, a Curcumin Analog Inhibitor of *NF-κB*, on the Radiosensitivity of Breast Tumor Cells

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Abstract: The search for NF-κB blockers to optimize antioxidants and counteract inflammation and carcinogenesis has identified several promising natural compounds, encompassing curcumin. Nonetheless, despite the pleiotropic health attributes of curcumin and established safety, its in vivo efficacy is limited by its poor pharmacokinetic properties, primarily due to its low bioavailability because of its low free serum concentrations and short half-life. To overcome these restrictions, we investigated the efficacy of the synthetic curcumin analog PAC in breast cancer cells. Additionally, MCF-7 and MDA-MB-231 cell lines were employed to explore the anticancer effects of PAC by assessing cell viability, apoptosis, clonogenic survival, and the expression of NF-κB, p53, Bcl-2, and Bax. Our results validate that PAC effectively inhibits cell proliferation and induces apoptosis in a dose-dependent manner. PAC also significantly enhances the radiosensitivity of breast cancer cells, indicating a potential synergistic effect with radiation therapy. Mechanistically, PAC exerts anticancer effects by inhibiting NF-κB signaling and modulating apoptotic genes like p53, Bax, and Bcl-2. These findings highlight its potential as a breast cancer therapeutic, enhancing existing treatments.

Keywords: PAC; antioxidant; antitumor; apoptosis; breast cancer; radiosensitive; curcumin analog

1. Introduction

Natural remedies have been extensively utilized as medicine since ancient times to treat diverse illnesses affecting human health [1]. Turmeric, the primary source of curcumin, is one of the most extensively explored natural compounds and has a long history of use in Chinese and Indian medicine for various therapeutic uses [2,3]. Curcumin has demonstrated numerous pharmacological effects, exhibiting potential as a therapeutic agent for several chronic diseases and as a chemopreventive compound [4].

Curcumin has been intensively investigated as a chemopreventative agent in several cancer approaches and has been utilized in clinical oncology for its anti-inflammatory, antioxidant, and antitumoral properties. These effects primarily stem from the ability of curcumin to inhibit the proinflammatory and prosurvival transcription factor NF-κB [5]. Abnormal activation of NF-κB contributes to several human diseases, encompassing cancer and inflammatory disorders [6]. NF-κB activation induces diverse pro-proliferative and anti-apoptotic genes, and NF-κB signaling crosstalk affects several signaling pathways, such as STAT3, AP1, HIF1, interferon regulatory factors, NRF2, Notch, WNT–β-catenin, and p53. Virtually, all known hallmarks of cancer involve NF-κB activation.

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Furthermore, NF-κB plays a critical role in reducing cell death in response to radiation treatment by promoting the expression of anti-apoptotic proteins, encompassing members of the Bcl-2 family (Bcl-2, Bcl-xL, A1, FLIP, and Bfl-1) and the IAP family member c-IAP2), while also activating the cellular antioxidant defense system [6].

Most anticancer therapies ultimately induce cell death through various mechanisms, particularly programmed cell death or apoptosis in cancer cells [7]. The recognition and removal of apoptotic cells by professional phagocytes, comprising dendritic cells and macrophages, help maintain immune self-tolerance and prevent chronic inflammation and autoimmune pathologies [8]. In this context, the NF-κB transcription factor plays an essential role [9,10]. Among the multiple signaling molecules and pathways influenced directly or indirectly by NF-κB, TP53—a transcription factor typically mutated or inactivated in malignancies—plays a pivotal role in regulating key cellular activities, encompassing cell cycle, senescence, and apoptosis [11,12]. Additionally, TP53 strongly suppresses inflammation and carcinogenesis and affects the response to chemotherapy and radiation treatment [13–17].

Curcumin offers a plethora of therapeutic benefits mostly owing to its anti-inflammatory, anti-proliferative, and antioxidant properties due to its ability to interact with various molecular targets [18,19]. Nonetheless, its low bioavailability caused by poor water solubility, poor oral absorbability, low serum levels, limited tissue distribution, brief half-life, and rapid metabolism limits its employment in clinical settings [2,20]. Curcumin tends to accumulate in the intestine, colon, and liver, which may explain its greater potential for treating gastrointestinal diseases compared with other organs [21].

Due to its poor bioavailability, which restricts the therapeutic potential of curcumin, numerous strategies have been developed to enhance its absorption [22]. One approach for increasing curcumin bioavailability is the employment of adjuvants, such as piperine, which can modify its metabolic pathways [23]. Additionally, new formulations, including nanoparticles, liposomes, micelles, and phospholipid complexes, offer extended circulation, enhanced permeability, and resistance to metabolic processes [24]. The use of synthetic analogs is another way to optimize the efficacy of naturally occurring dietary compounds and overcome their limitations, such as low water solubility, poor in vivo bioavailability, and unfavorable pharmacokinetics [25]. Several curcumin analogs have been created with the goal of elevating the efficacy while maintaining the same safety profile [26].

Considering the prevalence of NF-κB activation in cancer-related inflammation, it presents an attractive therapeutic target because of its potential to subvert adaptive immunity and impair responses to hormones, immunotherapy, radiotherapy, and chemotherapeutic agents. Nevertheless, NF-κB inhibition alone has thus far exhibited limited success in human cancer treatment [27]. We postulate that integrating radiotherapy with the inhibition of NF-kB pathways could be an alternative mechanism to sensitize tumor cells to ionizing radiation. This approach holds significant potential to enhance radiotherapy outcomes and overcome resistance and cancer recurrence.

The locally synthesized 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone (PAC) is a novel bioactive curcumin analog that demonstrates greater stability than curcumin in phosphate-buffered saline (PBS) and circulating blood [28]. PAC has been reported to exhibit anticancer properties across several tumor approaches [28–31]. Nonetheless, its combined effect with radiotherapy has not been previously explored. Here, we hypothesize–that the integration of radiotherapy with PAC curcumin analog inhibitor of NF-kB could enhance cell killing and overcome tumor radioresistance. This would occur through the inhibition of the axis of NF-κB/anti-apoptotic-Bcl-2 pathway while inducing the axis of p53/pro-apoptotic-Bax, resulting in increased radiosensitivity and enhanced responses to anticancer treatment. This investigation examined the cytotoxic and radiosensitizing potential of PAC in breast cancer cell lines. The current research explores the effects of PAC on apoptosis induction, cell survival, and gene expression in combination with X-ray radiation.

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Materials and Methods

Reagents and Chemicals

The curcumin analog 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone (PAC) was synthesized and purified by HPLC following the approach described previously [32]. It was stored as a powder at ≤−20°C, protected from light exposure, until needed. PAC was dissolved in DMSO just before use in the experiments.

Cell Culture

The breast cancer cell strains MCF-7 and MDA-MB-231 were obtained from the Biomedical Physics Laboratory at the King Faisal Specialist Hospital and Research Center, Riyadh. These cell strains were derived from cancer patients and have been widely employed in research. They were originally obtained from the American Type Culture Collection (Manassas, VA, USA), making them ethically exempt from signed consent. The characteristics of these breast cancer cell lines are as follows: MCF-7 cells possess wild-type p53 and are positive for estrogen and progesterone receptors (ER and PR). Conversely, MDA-MB-231 cells harbor a p53 mutation in codon 280 and are triple-negative (ER, PR, and HER2), with high PD-L1 expression. The cells were cultured in a culture medium composed of Minimum Essential Medium (Sigma-Aldrich, Co., UK), supplemented with 1% penicillin/streptomycin and 15% fetal bovine serum (Thermo Fisher Scientific Inc, USA). They were maintained as a monolayer in T-25 and T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2.

Real-Time Cell Proliferation Assay

A real-time cell analyzer (RTCA, ACEA Biosciences Inc., San Diego, CA, USA) was utilized to assess cell viability and proliferation, as described previously [33]. Briefly, the well background was measured by employing 100 µl of culture medium to calibrate the plates using the RTCA Software Package 1.2. Subsequently, 10,000 cells were seeded per well in E-Plate 16 to a final volume of 180 µl. The cells were then treated with PAC at a final volume of 200 µL, with concentrations ranging from 0 (control) to 100 µM.

Cell Apoptosis Analysis

Apoptosis analysis was conducted utilizing double labeling (Annexin V and propidium iodide (PI)) with the FITC Alexa Fluor™ 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific Inc, USA) following the manufacturer's protocol. MCF-7 and MDA-MB-231 cells were cultured in T-25 flasks for 24 h before being treated with 1 µM and 5 µM of PAC for 72 h, while control wells remained untreated. The harvested trypsinized cells were washed twice with cold PBS before being resuspended in a binding buffer. Annexin V-FITC and PI were then added to the binding buffer and incubated for 15 min at 37°C in the dark. Analyses were performed using a BD LSR Fortessa flow cytometer [34].

Colony Formation Assay

The effects of PAC on the ability of MCF-7 and MDA-MB-231 cells to form colonies were determined utilizing the clonogenic cell survival assay. The cells were seeded at varying concentrations in six-well plates. After 24 h, the cells were treated with 1 µM of PAC. DMSO served as the control solvent for PAC. After 2–3 weeks of incubation, the cells were fixed, washed, and stained with crystal violet for 30 min. Subsequently, the cells were washed to eliminate residual crystal violet and left to dry at room temperature. The ability of the cells to divide and form colonies containing at least 50 cells was assessed using a BioNuclear stereo zoom microscope (Nikon Instruments Inc., Melville, NY, USA) and recorded as a measure of cell survival. Plating efficiency was calculated by dividing the number of counted colonies by the number of seeded cells.

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Clonogenic Survival Curves

Cells were evaluated through a clonogenic assay as outlined above and described previously [35]. Briefly, breast cancer cells were plated and treated with an IC25 (0.01 μM) concentration of PAC for 24 h before irradiation with doses of 0, 1, 2, 4, or 6 Gy by utilizing an X-RAD 320 Biological Irradiator (Precision X-ray, Madison, CT, USA) at a maximum energy of 320 KVp, 2 mm Al filter, and 1 Gy/min of dose rate. The cells were incubated for 2–3 weeks to allow visible colonies to form. Subsequently, the cells were fixed and stained with crystal violet for 30 min. Colonies comprising at least 50 cells were counted as surviving. At least three independent experiments were performed for each cell strain. Survival curves were fitted and examined by employing the linear-quadratic model. Radiation sensitivity is expressed as the surviving fraction at 2 Gy (SF2) [36]. To evaluate the efficacy of drug treatment, the magnitude of radiosensitization is expressed as a dose-modifying factor, defined as the ratio of radiation doses required to attain the same level of effect (arbitrarily 10% effect survival) with radiation alone compared with combined treatment with PAC.

Gene Expression Analysis

The gene expressions of NF-kB, p53, Bcl-2, and Bax were quantified by reverse transcription polymerase chain reaction (RT-PCR), with an endogenous Human RPLP0 (large ribosomal protein) as a control. Total RNA was extracted from MCF-7 and MDA-MB-231 cells utilizing TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Additionally, complementary DNA was synthesized by employing M-MLV Reverse Transcriptase (Sigma-Aldrich, St. Louis, MO, USA). The expression levels were measured utilizing a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and TaqMan Master Mix (Applied Biosystems, USA). The reaction components were mixed in 96-well plates. The thermal cycling conditions included an initial step of 20 s at 50°C, followed by 10 min at 95°C, and 40 cycles of 15-s denaturation at 95°C and 1 min annealing at 60°C. After the reactions, the amplification plots were examined. The baseline and threshold values were adjusted to determine the threshold cycle (CT) of the amplification curves. The results were analyzed by employing the 2-Delta Delta CT relative expression method [37,38].

Statistical Analysis

The data are presented as the mean and standard deviation (SD) from at least three independent experiments. The cell index (CI) for the real-time dynamic cytotoxicity assessment was calculated automatically utilizing the RTCA Software Package 1.2. The normalized CI%) was determined by dividing the cell indices at each time point after compound addition by the CI of the untreated control (0 μM). Comparison between different treatments was conducted by employing one-way repeated measures analysis of variance. The pairwise statistical significance of differences between the experimental groups was calculated using an unpaired, two-tailed, Student's t-test. A P-value of <0.5 was considered statistically significant.

Result

Cell Proliferation Utilizing a RTCA

The cytotoxic effects of PAC on breast cell lines were assessed utilizing the RTCA cell proliferation assay. To evaluate the impact of PAC on cell proliferation, the RTCA data, following PAC addition, were normalized by dividing the CI of the treated wells by that of the untreated control wells (0 μM). The results, presented in Figure 1, demonstrate that increasing concentrations of PAC resulted in a proportional decrease in CI in both cell lines. The inhibitory effect of PAC peaked at 24 h and persisted up to 72 h. At the highest PAC concentrations (100 μM), there was a significant decrease in CI in both cell lines. Specifically, the CI (%) declined to 38.4% (SE = 6.7) and 33.4% (SE = 6.9) at 72 and 96 h in MCF-7, respectively, and to 20.8% (SE = 2.6) and 18.4% (SE = 1.2) at 72 and 96 h in MDA-MB-231, respectively.

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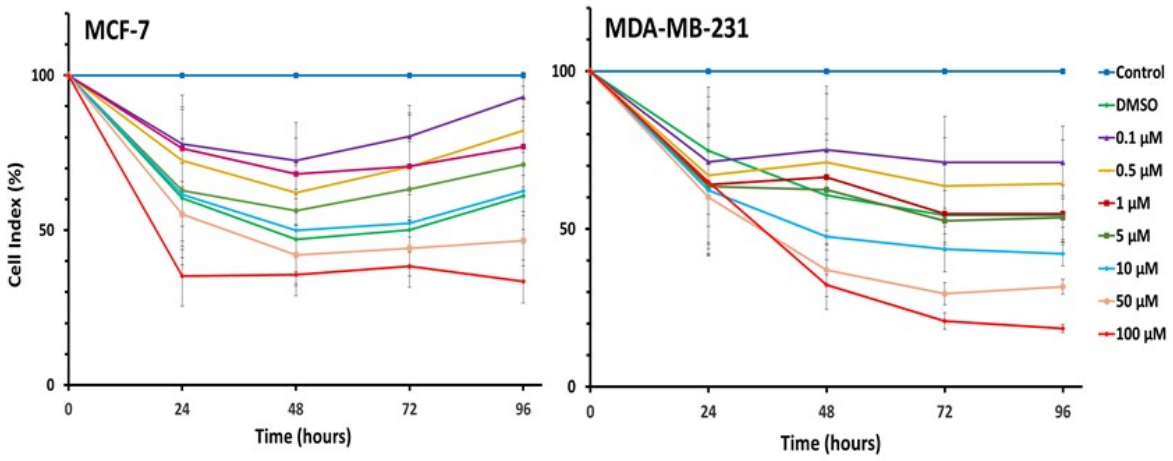

Figure 1. Normalized cell index (CI%) effect of PAC on BC cell lines. Data points represent the average CI ± SD.

PAC Effect on the Clonogenic Assay

The analog PAC inhibited the proliferation of MCF-7 and MDA-MB-231 cells at concentrations ranging from 0.1 to 100 µmol/L (Figure 2). The IC50 values of the curcumin analog PAC in these cancer cell lines were determined to be 5.8 µmol/L for MCF-7 cells and 1.11 µmol/L for MDA-MB-231 cells.

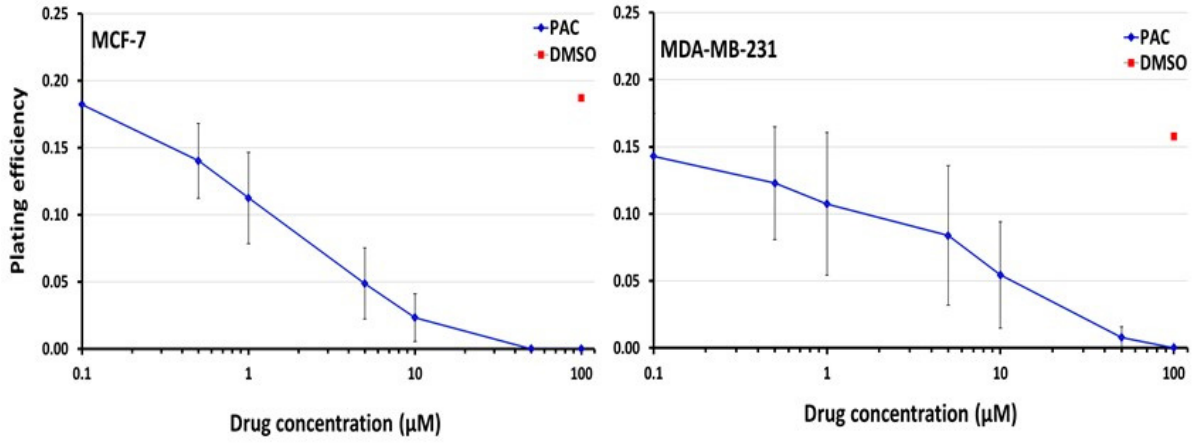

Figure 2. Effect of PAC on BC cell line proliferation. Data represent the average of three independent experiments. Error bars represent the SE of the mean.

PAC Effect on Apoptosis

The effect of PAC on apoptosis induction in breast cancer cells was evaluated through flow cytometry utilizing Annexin V/PI staining. Figure 3A illustrates that our results identified four distinct subpopulations: normal (live) cells, early apoptotic cells (Anv), late apoptotic cells (PI + Anv),) and necrotic cells (PI). Most cells (>80%) survived the diverse treatments, but the results revealed a relative increase in the proportion of dead cells (early, late apoptotic, and necrotic) in both MCF-7 and MDA-MB-231 cell lines treated with PAC and/or irradiation compared with the control group. Moreover, Figure 3B depicts the consolidated percentage of all dead cells (Anv, PI + Anv and PI) across the diverse treatments in both cell lines. Notably, PAC and irradiation progressively increased the percentage of dead cells in both cell strains in a dose-dependent manner. Statistical analysis utilizing a t-test confirmed the significance of these distinctions ($p < 0.05$). These results validate that PAC induces apoptosis in breast cancer cells, as evidenced by the elevated number of cells with phosphatidylserine externalization and loss of plasma membrane integrity. DMSO, at the

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concentration employed to dissolve PAC, did not induce apoptosis in either cell line, exhibiting its nontoxic nature. As a positive control, the highest radiation dose (10 Gy) substantially elevated apoptosis in MDA-MB-231 and MCF-7 cells, inducing 17.5% (SE = 2) and 14.1% (SE = 2) apoptotic cells, respectively. The combination treatment of PAC and radiation indicated a synergistic effect, further enhancing apoptosis. In MDA-MB-231 cells, integrating 1-μM PAC with 4-Gy radiation-induced 8.86% apoptotic cells, while 5-μM PAC with 4-Gy radiation-induced 12.02%. Similarly, in MCF-7 cells, the combination treatments resulted in 7.88% and 9.48% apoptotic cells, respectively.

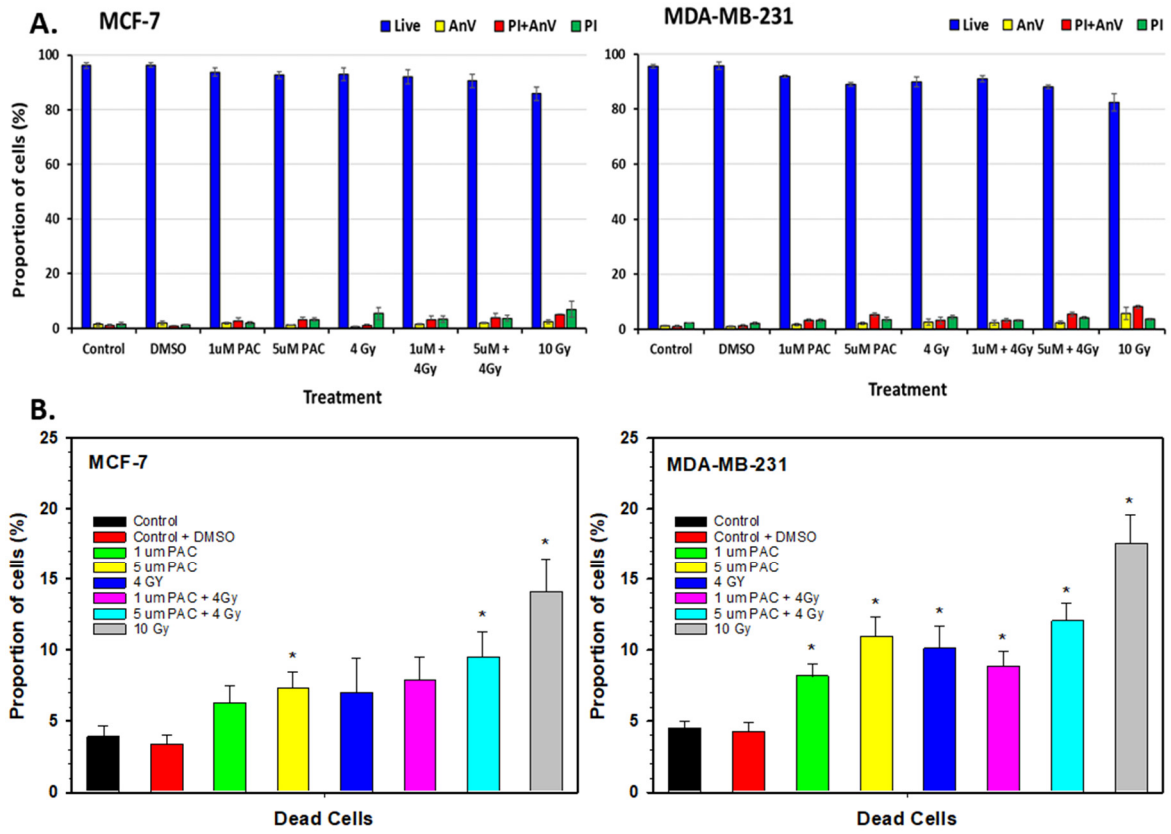

Figure 3. Flow cytometric analysis of apoptosis in MCF-7 and MDA-MB-231 cells. **A.** Percentages of cells at diverse treatments. **B.** Combined percentages of dead cells at multiple treatments. Data represent the mean ± SE of three independent experiments. Statistical significance was determined utilizing a t-test. Stars signify significant distinctions (*$p < 0.05$) between groups.

Clonogenic Cell Survival Assay

The ability of PAC to radiosensitize BC cells, irradiated after 24-h incubation at a 1-μM concentration, was evaluated utilizing clonogenic cell survival curves. Figure 4 illustrates that both cell lines exhibited typical cell survival curves, with survival decreasing exponentially as radiation doses increased. The SF2 was 0.48 [95% confidence interval (95% CI): 0.44–0.53] for MDA-MB-231 cells compared with 0.26 (95% CI: 0.24–0.8) for MCF-7 cells, indicating approximately a twofold difference in radiosensitivity. Incubation with PAC enhanced the sensitivity of the cell strains to radiation-induced cytotoxicity, with SF2 values of 0.38 (95% CI: 0.34–0.43) for MDA-MB-231 and 0.19 (95% CI: 0.17–0.21) for MCF-7. The D50 (irradiation dose required to mitigate the survival rate to 50%) was 1 Gy for MCF-7 cells and 1.9 Gy for MDA-MB-231 cells. The survival fractions of the breast cancer cell lines MDA-MB-231 and MCF-7 were evaluated following exposure to 1-Gy radiation, both with and without the administration of the drug. For the MDA-MB-231 cell line, the survival fraction without the drug was 0.78, while the survival fraction with the drug decreased to 0.61, indicating a 17% reduction in survival due to the drug treatment. In the MCF-7 cell line, the survival fraction

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without the drug was 0.51, which declined to 0.39 with the drug, representing a 23.5% reduction in survival attributable to the drug.

The impact of radiation doses on breast cancer cells, both alone and in combination with PAC, was evaluated utilizing a two-sample t-test assuming equal variances. In MCF-7 cells, the p-values for single radiation doses were as follows: 1 Gy ($p = 0.04$), 2 Gy ($p = 0.006$), 4 Gy ($p = 0.09$), and 6 Gy ($p = 0.05$). For the MDA-MB-231 cell line, the p-values were as follows: 1 Gy ($p = 0.01$), 2 Gy ($p = 0.14$), 4 Gy ($p = 0.3$), and 6 Gy ($p = 0.09$). These results validate that PAC can optimize radiosensitivity in both breast cancer cell lines when integrated with low doses of radiation.

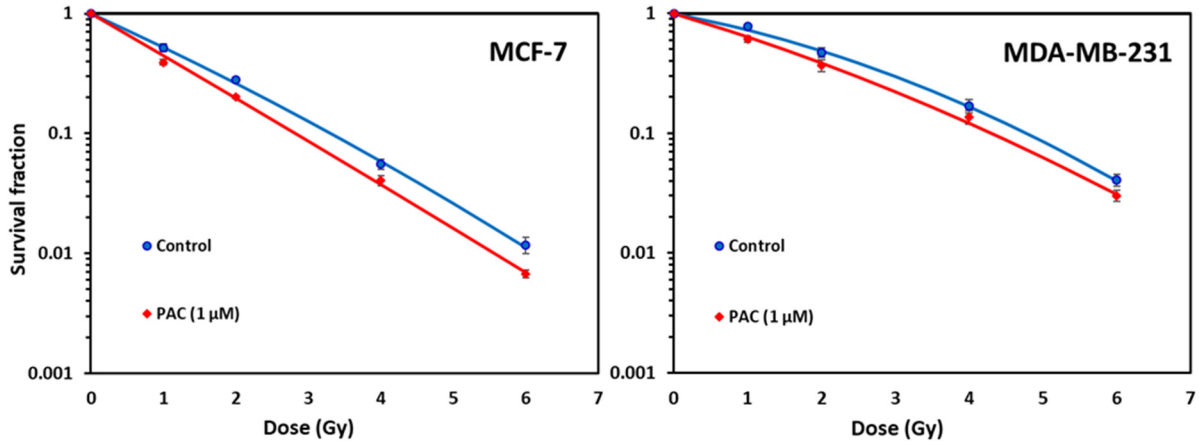

Figure 4. Effect of PAC on the radiosensitivity of BC cell lines. Clonogenic survival curves of BC cells following X-ray irradiation with and without PAC. Data represent the average of three independent experiments. Error bars represent the SE of the mean. Statistical analysis for the entire curve exhibited a substantial difference in PAC + radiation treatment compared with radiation alone ($p = 0.004$ and 0.002 for MCF-7 and MDA-MB-231, respectively).

PAC Effect on the Gene Expression of Molecules Involved in the Cycle, Apoptosis, Migration, and Invasion of Breast Cancer Cells

To explore the underlying mechanism by which PAC affects breast cancer cells, we performed real-time PCR to detect the expression of relevant molecules. Cells were cultured for 24 h in medium containing 5 µmol/L of PAC. Four hours later, the cells were irradiated and then collected 3 h after radiation for detection. In MCF-7 cells, we observed that the expression levels of the gene NF-κB, which promotes cell survival, were downregulated, while the expression levels of the gene Bcl-2, which inhibits apoptosis, were decreased in cells treated with PAC as a single agent. Additionally, the expression levels of the gene p53, which promotes apoptosis, were upregulated in combination with radiation, as were the levels of the Bax gene, which also elevates apoptosis. As expected, MDA-MB-231 cells were more sensitive to irradiation than MCF-7 cells, as exhibited by the expression of all genes. Nonetheless, the expression levels of the genes NF-κB and Bcl-2 were decreased, while the expression levels of the genes p53 and Bax were increased following treatment of cells with PAC as a single agent (Figures 5 and 6). Statistical analysis utilizing a t-test verified the significance of these changes ($p < 0.05$). These results verify that PAC induces apoptosis in breast cancer cells by modulating the expression of key genes involved in apoptosis and inflammation. Moreover, the treatments had a more pronounced effect on gene expression in the MDA-MB-231 cell line than in the MCF-7 cell line.

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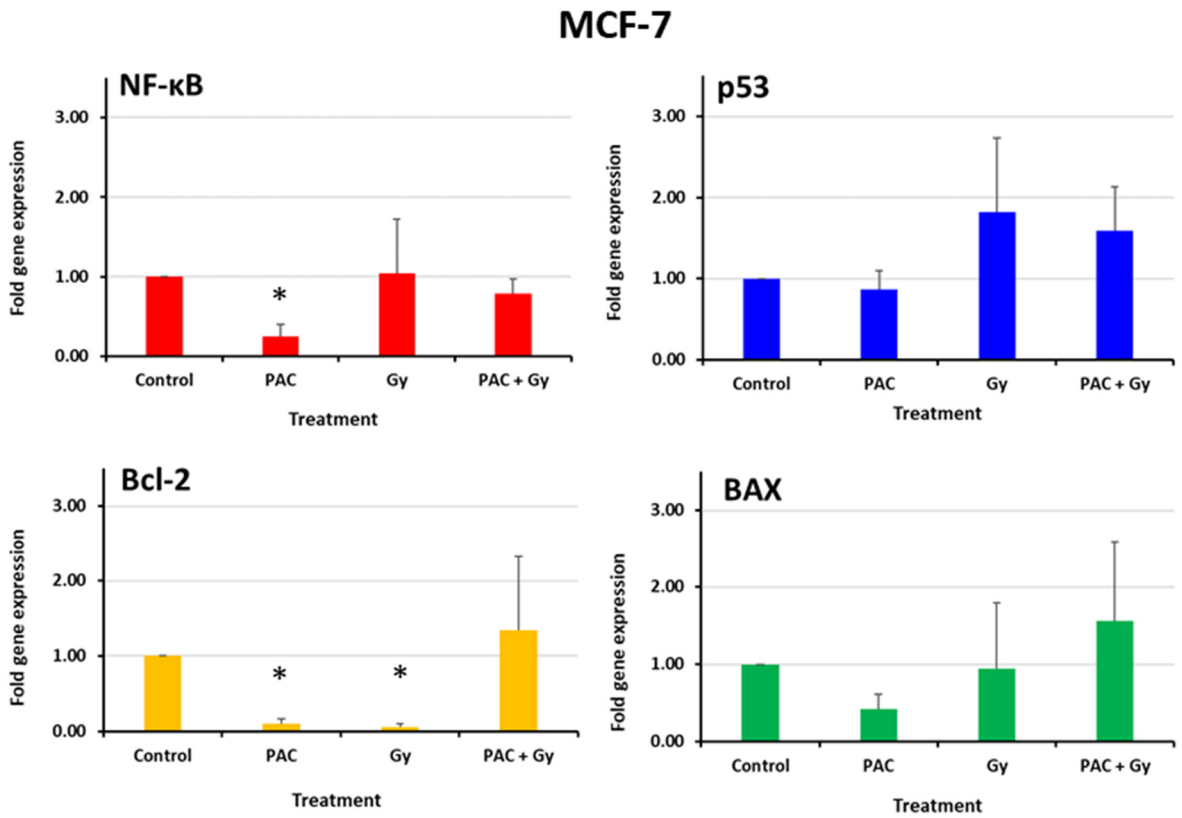

Figure 5. Effect of PAC (5 μM) on gene expression analyzed by RT-PCR on the NF-κB, p53, Bcl-2, and Bax genes in the MCF-7 cell line. Bars represent the mean ± SE of three independent experiments. Statistical significance was determined utilizing a t-test. Stars indicate significant differences (*$p < 0.05$) between groups.

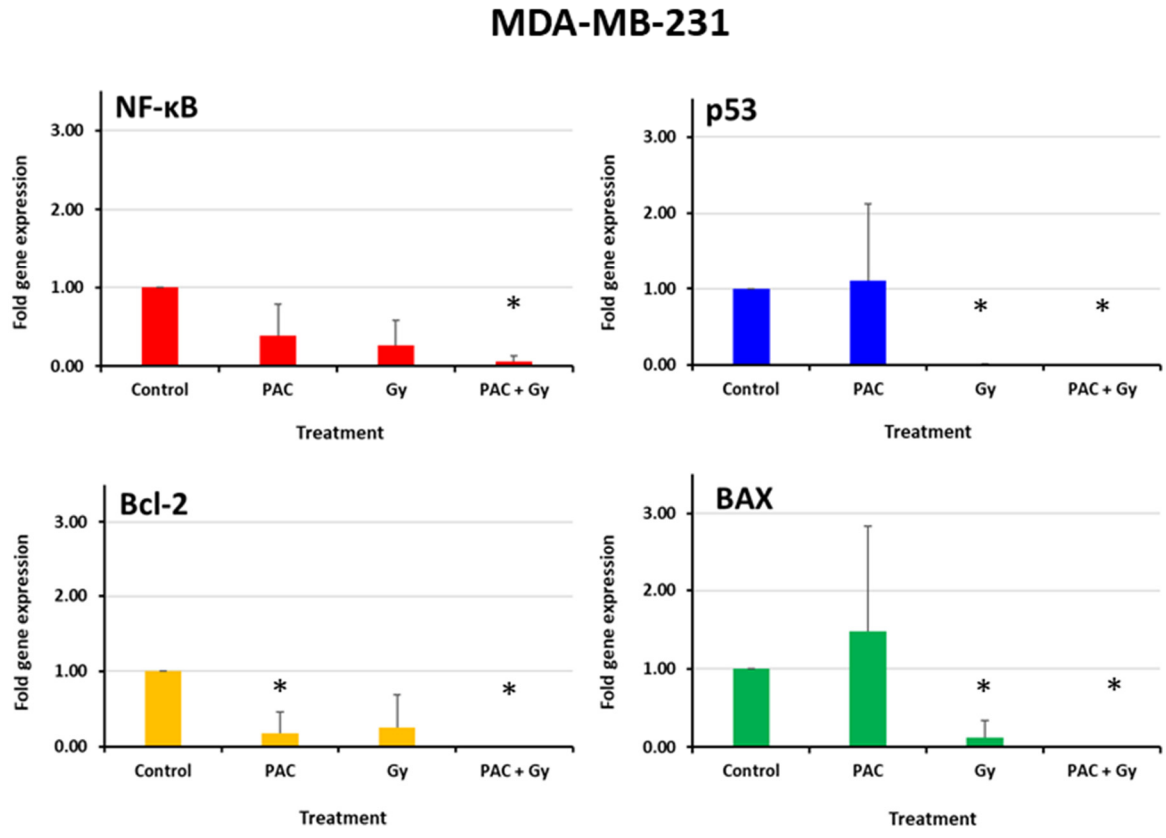

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Figure 6. Effect of PAC (5 μM) on gene expression analyzed by RT-PCR on the NF-κB, p53, Bcl-2, and Bax genes in the MDA-MB-231 cell line. Bars represent the mean ± SE of three independent experiments. Statistical significance was determined utilizing a t-test. Stars indicate significant (*$p < 0.05$) differences between groups.

Discussion

NF-κB activation in cancer-related inflammation fosters tumor progression, therapy resistance, and immunosuppression, making it an attractive therapeutic target. Constitutive NF-κB activation optimizes radiation resistance, while its inhibition, such as by curcumin, has the potential to safeguard normal tissues and sensitize tumors to radiotherapy by modulating diverse signaling pathways and epigenetic mechanisms. In the current research, we demonstrated the efficacy of the curcumin analog (PAC) against breast cancer cells.

The anticancer properties of PAC were established through multiple analytical strategies. Utilizing a real-time cell proliferation assay, we demonstrated that PAC could affect cell proliferation and cell viability in a time-dependent and concentration-dependent manner (Figure 1). High concentrations of (50 and 100 μM) resulted in a drop in CI values to below 50% at 48–96 h for both cell lines compared with 70%–80% for the lowest PAC concentrations (0.1 to 10 μM), which remained relatively constant from 24 to 96 h. Another study demonstrated the proliferation inhibition of another curcumin analog (B14) of 8.84 μM and 8.33 μM in MCF-7 and MDA-MB-231 cells, respectively [39].

To further evaluate the potential of the analog PAC as a chemotherapeutic agent for breast cancer, we assessed the sensitivity of the cell lines to PAC across a range of nontoxic and toxic concentrations utilizing a classical clonogenic assay (Figure 2). The inhibitory concentration values were determined to be 1.11 μM and 5.86 μM for MCF-7 and MDA-MB-231 cells, respectively. In comparison with a previous study by [39], where breast cancer cells were cultured for 24 h in a medium containing 5-μM curcumin or B14, the results validated that B14 significantly inhibited clonal proliferation of both breast cancer cell lines compared with the curcumin and control (DMSO) groups. This suggests the need to consider higher doses or distinct routes of administration to attain greater efficacy.

Our findings align with previous studies demonstrating the potent pro-apoptotic effects of PAC on breast cancer cells. Al-Howil and colleagues [28] affirmed that PAC-induced apoptosis in MCF-7 and MDA-MB-231 cells at micromolar concentrations, similar to our observations. Additionally, other studies have asserted that PAC can target diverse molecular pathways involved in cancer cell proliferation and survival, encompassing the NF-κB signaling pathway, the PI3K/Akt pathway, and the MAPK pathway [40,41] The results of our apoptosis analysis strongly support the hypothesis that PAC has a substantial pro-apoptotic effect on breast cancer cell lines (Figure 3). The observed increase in the percentage of dead cells across multiple treatment regimens, encompassing PAC alone and in combination with radiation, highlights the potential of PAC as a promising therapeutic agent for breast cancer. These results align with those of previous studies, such as Al-Howail et al. [28], which exhibited the potent apoptotic effects of PAC on breast cancer cells. PAC alone conveyed a modest increase in late apoptosis compared with the radiation-only control, but the combined therapy displayed a synergistic effect, substantially boosting cell death. This suggests that PAC may potentiate the effects of radiation therapy, potentially resulting in enhanced clinical outcomes. Future research could explore the role of specific apoptotic proteins, such as caspases and Bcl-2 family members, in PAC-induced cell death to further elucidate the mechanisms underlying the pro-apoptotic effects of PAC. Additionally, it would be valuable to assess the efficacy of PAC in combination with other chemotherapeutic agents or targeted therapies to optimize its therapeutic potential.

The potential of PAC to boost radiosensitivity in breast cancer cells was investigated utilizing a clonogenic cell survival assay. The resulting data revealed characteristic survival curves, highlighting an exponential decrease in cell survival with rising radiation doses (Figure 4). The administration of PAC substantially impacted cell survival in both breast cancer cell lines under radiation exposure. In

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the MDA-MB-231 cell line, the survival fraction declined from 0.78 to 0.61 following treatment with PAC, demonstrating a 17% decrease in cell survival. This result validates that PAC optimizes the radiosensitivity of MDA-MB-231 cells, making them more vulnerable to radiation-induced damage. Similarly, the MCF-7 cell line exhibited a reduction in survival from 0.51 to 0.39 with PAC treatment, specifying a 23.5% reduction in survival. These findings further support the hypothesis that PAC effectively amplifies the efficacy of radiation therapy by mitigating cell viability. Collectively, these findings highlight the crucial role of PAC in elevating the effects of radiation therapy across diverse breast cancer cell lines, emphasizing the potential therapeutic benefits of integrating this drug with radiation treatment and the need for further exploration of its underlying mechanisms and clinical implications.

From a gene expression analysis, considerable research has affirmed that curcumin is a pharmacologically safe compound with therapeutic benefits for cancers and human health, largely due to its anti-inflammatory and antioxidant properties, possibly through the suppression of NF-κB, among other factors [19]. While curcumin can exert inhibitory effects on NF-κB signaling, most chemotherapeutic agents induce the expression of genes correlated with cellular survival, proliferation, invasion, and metastatic processes [42].

Numerous curcumin analogs have been investigated as anti-inflammatory and antiproliferative drugs [43]. Numerous heterocyclic cyclohexanone analogs of curcumin were synthesized by [44] and tested for their capacity to inhibit NF-κB transactivation in nonadherent K562 leukemia cells [45]. PAC has been exhibited to modulate DNA repair pathways by upregulating multiple genes in breast cancer cell lines [46]. Additionally, it inhibits cell proliferation, induces apoptosis and autophagy, and reduces oxidative stress [31]. The effects of PAC encompass multiple signaling pathways, covering NF-κB, MAPK, and Wnt [40]. These results are consistent with previous research on the anticancer properties of curcumin, which have demonstrated its efficacy in inhibiting breast cancer cell proliferation and targeting critical genes involved in angiogenesis, apoptosis, cell cycle, and metastasis [47].

In the current research, we investigated whether PAC could enhance the effects of chemotherapy in breast cancer cell lines. Our findings indicate that PAC decreases NF-κB expression, supporting conclusions from previous reports. The results also validated that PAC integrated with radiation can optimize the effects on (p53 and Bax) gene expression in comparison with the substance effect alone in MCF-7 cells. Conversely, PAC treatment alone decreased Bcl-2 gene expression in MCF-7 cells compared with the control. Regarding NF-κB expression, PAC alone reduced its levels compared with the control, but this effect was more pronounced when PAC was integrated with radiation. In the MDA-MB-231 cell line, PAC integrated with radiation further decreased NF-κB gene expression compared with both the control and PAC alone. Meanwhile, p53 gene expression exhibited a slight increase with PAC treatment in combination with radiation. Additionally, PAC alone reduced Bcl-2 expression while increasing Bax protein expression in MDA-MB-231 cells, further supporting its pro-apoptotic effects.

The inhibition of protein activity has been suggested to enhance the efficacy of chemotherapeutic agents. The observed changes in gene expression, confirmed by statistical analysis ($p < 0.05$), offer compelling evidence that PAC induces apoptosis in breast cancer cells by modulating key apoptotic and inflammatory pathways. Moreover, our findings validate that the treatments had a more pronounced effect on gene expression in the MDA-MB-231 cell line than in the MCF-7 cell line. This highlights that the MDA-MB-231 cell line is more responsive to the applied treatments, underscoring potential variations in biological behavior and treatment responses between these two breast cancer cell lines (Figures 5 and 6). A comparison with a similar study [48] found that gene expression analysis utilizing RT-qPCR indicated that treatment with 15-μM curcumin significantly ($P < 0.05$) decreased p53, Bcl-xL, and NF-κB gene expression while increasing Bax expression in the MCF-7 cell line. In MDA-MB-231 cells, curcumin at 30 μM significantly ($P < 0.05$) increased p53 and Bax gene expression, whereas Bcl-xL was significantly reduced compared with the control. Notably, the concentrations used in that study were much higher than those employed in our research.

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Further studies are essential to elucidate how PAC's anti-inflammatory properties and immunomodulatory effects contribute to its anticancer activity. A deeper understanding of these mechanisms could result in the development of novel therapeutic approaches targeting multiple hallmarks of cancer.

Conclusions

In conclusion, the current research investigated PAC, a curcumin analog, for its anticancer effects in breast cancer. PAC effectively inhibited cell growth and induced apoptosis, both alone and in combination with radiation therapy. Mechanistically, PAC likely targets key pathways, encompassing NF-κB, which is known to regulate inflammation and tumor growth. Notably, PAC significantly optimized the sensitivity of breast cancer cells to radiation. These findings, supported by the observed changes in apoptotic gene expression (p53, Bax, and Bcl-2), strongly suggest the potential of PAC as a promising anticancer therapeutic. Preclinical studies and subsequent clinical trials are warranted to translate these promising findings into effective clinical treatments, including its potential immunomodulatory effects.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: RTCA Cell Proliferation Assay; Figure S2: PAC Effect on Clonogenic Assay; Figure S3: PAC Effect on Apoptosis; Figure S4: Clonogenic Cell Survival Assay; Fihure S5: PAC Effect on Gene Expression using Q-PCR.

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