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β-Sitosterol Synergizes Cisplatin's Anti-Lung Cancer Activity: A Cell Line Study

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ABSTRACT

Background: Cisplatin (Cis) is the primary chemotherapeutic agent for treating non-small cell lung cancer (NSCLC). β-sitosterol (Bs), a plant-derived polyphenol, inhibits tumor proliferation in several malignancies.
Objectives: To evaluate whether Bs in combination with Cis (BCcomb) enhance Cis' anticancer activity against NSCLC cells. Additionally, it investigated the possibility of lowering the Cis dosage when combined with Bs in the treatment of NSCLC.
Materials and methods: The study, conducted in 2024, utilized the A549 cell line as an NSCLC model, with control and three treatment groups (Bs, Cis, and BCcomb). Cell viability was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue exclusion assays. Bs-Cis interaction was analyzed using a combination index, isobologram, and Bliss independence model. The dose reduction index was also used to predict a dose reduction for Bs and Cis in the combination. Expression of apoptotic-related genes (p53, BAX, BCL-2, MCL-1) and the activities of both caspase 3/7 and reactive oxygen species (ROS) were quantified.
Results: This study is the first to demonstrate that Bs, in combination with Cis, significantly synergizes to suppress NSCLC cells, enabling lower doses of Bs or Cis in the BCcomb without reducing efficacy. The combination of Bs with Cis markedly enhances their cytotoxic efficacy, as evidenced by the activation of apoptotic markers and elevated intracellular ROS generation, demonstrating the superiority of the BCcomb compared to Bs or Cis monotherapy.
Conclusion: This study provides evidence that BCcomb may hold promise as a prospective therapeutic strategy for clinically improving NSCLC treatment outcomes.
Keywords: Lung cancer; Cisplatin; β-sitosterol; Apoptosis; Synergism.

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INTRODUCTION

Lung cancer is the most frequently diagnosed cancer and is the primary cause of cancer-related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) represents the predominant subtype, comprising 85% of all lung cancer cases [2]. Despite evolving treatment protocols for NSCLC, cisplatin (Cis), a platinum-based compound, serves as the first-line chemotherapeutic drug for the treatment of NSCLC [3]. However, the aggressive characteristics and unique mechanisms identified in lung cancer cells contribute to Cis resistance, resulting in a poor survival rate [4]. Cis resistance in NSCLC involves multiple mechanisms, particularly the upregulation of anti-apoptotic proteins like Bcl-xL, which enables cancer cells to circumvent apoptosis [5]. In addition, resistant NSCLC cells often exhibit the abrogation of Cis-induced G2/M cell cycle arrest, thereby enabling ongoing proliferation in the presence of Cis [6]. Furthermore, NSCLC cells can develop resistance to Cis through the upregulation of DNA repair-associated proteins, like excision repair cross-complementation group 1 [7]. The interplay of these processes enables NSCLC cells to evade Cis therapy, thereby

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posing significant challenges to effective treatment strategies.

A number of studies have investigated the interaction between plant-derived compounds and Cis. The combination of curcumin or tannic acid with Cis improves therapeutic efficacy in the treatment of NSCLC [8, 9]. β-sitosterol (Bs), which is a phytosterol that exerts its anticancer effects by inhibiting the cell cycle, inducing apoptosis, modulating oxidative stress, disrupting mitochondrial function, and suppressing invasion and metastasis [10]. Bs exhibits anticancer properties against NSCLC cells and has been shown to reduce drug resistance in lung cancer cells to anlotinib, a chemotherapeutic agent for treatment of NSCLC, suggesting its potential role as an adjuvant therapy in overcoming drug resistance in cancer treatment [11].

To the best of our knowledge, no previous studies have evaluated the anticancer effect of Bs in combination with Cis in the treatment of NSCLC; thus, it is currently unclear whether Bs may enhance the antitumor efficacy of Cis in the treatment of lung cancer. Hence, we hypothesized that the combination of Bs with Cis may synergistically improve the chemosensitivity of NSCLC cells to Cis. This may concurrently reduce the necessary dosage of Cis and enhance its therapeutic efficacy against NSCLC cells. This study is the first to evaluate the combined anticancer effects of Bs and Cis on the NSCLC cell line, focusing on their ability to induce apoptosis through the analysis of key apoptotic indicators and intracellular reactive oxygen species (ROS) production.

MATERIALS AND METHODS

This experimental study was carried out from January to September 2024 at the Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Chemical reagents: Cis with a purity of 98%, dimethylsulfoxide (DMSO), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit, and trypan blue were all purchased from Sigma-Aldrich Chemical (MO, USA). β-sitosterol (Bs) with a purity of 98% was purchased from Yuanye Biotechnology (Shanghai, China). Bs and Cis were dissolved in DMSO, ensuring that the final DMSO concentration was less than 0.1%. TRIzol™ reagent and PowerUp™ SYBRTM Green Master Mix were purchased from Thermo Fisher Scientific (IL, USA). qScript cDNA SuperMix was purchased from Quantabio (MA, USA). Caspase-Glo® 3/7 was purchased from Promega (WI, USA). OxiSelect™ Intracellular ROS was purchased from Cell Biolabs (CA, USA).

Cell culture: This study used the A549 lung cancer cell line (ATCC® CCL-185) as a model for NSCLC. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 units/mL). The culture media and supplementary materials have been purchased from Gibco, Thermo Fisher Scientific (MA, USA). The cultures were incubated at 37°C in 5% CO_2 following seeding at a density of 5.0×10^3 cells per 100 μl to allow attachment to the culture plate. A549 cells were collected for drug combination studies via trypsinization with 0.05% trypsin and 1 mM EDTA, re-suspended in media, and then collected by centrifugation at 150 ×g for 3 minutes. The pellet was then resuspended into the culture medium.

Analysis of cell viability and calculation of IC50

Experimental groups: A549 cells were divided into four groups and treated separately with 1 μL of dimethyl sulfoxide (DMSO) at a final concentration of 0.5%. The treatment groups were treated with various serial concentration dilutions of either Bs, Cis, or Bs- Cis in combination (BCcomb) at a constant molar ratio of 4:1. Cells in the control group received treatment solely with phosphate-buffered saline (PBS) containing 0.5% DMSO. The concentrations of Bs or Cis utilized ranged from 1.25 to 40 μM and 0.032 to 4 μM, respectively. The concentrations of the BCcomb varied from 0.032 to 4 μM.

MTT assay: Following the treatment, the plates were incubated for an additional 72 hours, after which the MTT assay was performed as previously published to assess cell viability [12]. Each drug's IC50 was calculated alone or in combination. The experiment's results were obtained by graphing log doses against the percentage of inhibition, which were converted to fraction affected (Fa) (from 0 to 1).

Determination of the combination index, isobologram, and dose reduction index

The Combination index (CI): It was determined using CompuSyn software (version 1.0) to analyze MTT assay results. It was calculated as: **CI = (Bs1/BisX) + (Cis1/CisX)**.

Bs1 and Cis1 represent the concentrations of Bs and Cis alone, respectively, required to achieve a specific effect. BsX and CisX represent the concentrations of Bs and Cis, respectively, in the combination exhibiting equivalent effects.

CI values were plotted against Fa. A CI of 1 indicates an additive effect, <1 denotes synergy, and >1 signifies antagonism [13].

The isobologram analysis: In this graph, the doses on the x-axis indicate the Bs doses that resulted in a specific percentage of inhibition of A549 cell viability, while the doses on the y-axis reflect the Cis doses that can achieve the same effect on cell viability. An isobole was created, representing a straight line that links the dose pairs experimentally identified for each drug that elicit a specific response, such as 50%, 75%, and 90% inhibition of cell viability. The type of the interaction will be additive if the combination data point is located on the isobole, synergistic if the point is situated below the isobole, and antagonistic if the point is positioned above the isobole [14].

The dose reduction index (DRI): This approach was employed to assess the possibility of decreasing the dose of Bs or Cis in the BCcomb while preserving the same level of efficacy utilizing the following equations:

DRI(Bs) = IC(x) of Bs alone/IC(x) of Bs in the combination.

DRI (Cis) = IC(x) of Cis alone/IC(x) of Cis in the combination.

x: The produced % of an effect (such as IC50 for 50% inhibition).

A log DRI value exceeding zero could indicate the potential of reducing Bs and Cis concentrations to a certain extent when they are used in combination while still retaining their effectiveness [15].

The Bliss independence model for prediction of the combined effect

A549 cells were seeded in 6-well plates with 2 mL growth media and incubated overnight to estimate viability. Cells were divided into control (PBS-treated) and three treatment groups: 3.2 μM Bs, 0.8 μM Cis, or their combination (3.2 μM

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Bs + 0.8 μM Cis) for 72 hours. Subsequent experiments in our study followed the same group categorization and treatment. After treatment, cells were harvested via trypsinization, re-suspended in 1 mL medium, and mixed with 0.4% trypan blue. Viable (clear cytoplasm) and non-viable (blue cytoplasm) cells were counted using a Neubauer hemocytometer. Viability was calculated as follows:
The percentage of viable cells = Number of viable cells per ml of aliquot/Total number of cells per ml of aliquot × 100 [16].

The Bliss independence criterion was utilized to calculate the additive effect of drug combinations [17], expressed as E (Cis, Bs) = E(Cis) + E(Bs)- E(Cis)×E(Bs). In this context, E (Cis, Bs) denotes the expected combination effect, whereas E(Cis) and E(Bs) indicate the effects of Cis and Bs when used separately.

qRT-PCR analysis for gene expression related to apoptosis

The expression level of four apoptotic-related genes, including p53, BAX, BCL2, and MCL-1, was compared by performing the quantitative reverse transcription polymerase chain reaction (qRT-PCR) following cell treatment as previously described. Total ribonucleic acid (RNA) was extracted using TRIzol™ and reverse-transcribed into cDNA using qScript cDNA SuperMix following preset instructions. The transcripts were amplified with the primers specified in Table 1. GAPDH was selected as a housekeeping gene to normalize target genes expression. The PowerUp™ SYBRTM Green Master Mix was used for qRT-PCR amplification. The relative fold expression was determined using the $2^{-\Delta\Delta CT}$ method [18].

Apoptosis detection via caspase 3/7 activity assay: The treatments were run as detailed above. At 72 hours post-treatment, 100 μL of Caspase-Glo® 3/7 reagent was added to each well at a 1:1 volume ratio. After incubating the samples for 90 minutes at 25°C, we assessed luminescence using a GloMax® microplate reader to quantify caspase 3/7 activity per the manufacturer's directions.

Estimation of intracellular ROS in A549 cells: The OxiSelect™ intracellular ROS assay was conducted according to the manufacturer's directions. We used a fluorescence plate reader with an excitation wavelength of 480 nm and an emission wavelength of 530 nm to quantify the intensity of the formed fluorescence. Fluorescence readings were normalized to control cells, and ROS activities were compared among different treatment groups.

Ethical consideration: The research ethical approval committee of the College of Veterinary Medicine at the University of Al-Qadisiyah approved this study on November 26, 2023, with reference number document 4419. The study did not include any animals or humans; therefore, consent was not required.

Statistical analysis: All the experiments were independently repeated four times, and the resulting data, which were normally distributed, were analyzed using GraphPad Prism 10.4.1. To identify statistically significant differences, a one-way analysis of variance was implemented, followed by Tukey's Honest Significant Difference. A P-value of < 0.05 determined statistical significance.

RESULTS

The effect of BCcomb on A549 cell viability: As expected, both Bs and Cis reduced A549 cell viability in a dose-dependent manner when given individually (Figure 1). BCcomb improves the potency and efficacy of cytotoxicity against A549 cells compared to using Cis or Bs alone (Figure 1). Interestingly, the IC50 was significantly lower (P-value < 0.05) when A549 cells were treated with BCcomb (3.90 ± 0.61) than when Cis (6.04 ± 1.54) or Bis (24.22 ± 3.37) was used alone. This implies that the combination of Bs and Cis increases the sensitivity of lung cancer cells to Cis. Table 2 presents the IC50 values for Bs, Cis, and BCcomb.

Analysis of Bs and Cis interactions in the combination against A549 cells

A- The analysis of CI: To test the hypothesis that Bs exhibited a synergistic inhibitory effect with Cis based on the effect-oriented strategy, we calculated the CIs for each dose combination. Our study showed that when Bs and Cis were combined, they had synergistic effects, with CI values less than 1 for all fractions that were affected (Figure 2a).

B- The analysis of isobolograms: To further examine our hypothesis, the isobologram analysis, which is a dose-oriented approach, was employed. We plotted the isobologram for the BCcomb at Fa values of 0.5, 0.75, and 0.90. The doses of Bs and Cis in the combination doses falls below the additive line, suggesting that the drugs synergize and require a lower dose of each to produce the cytotoxic effect on A549 cells (Figure 2b).

C- The analysis of DRI: As it explained before, the IC50 requires 6.04 ± 1.54 μM of Cis and 24.22 ± 3.37 μM of Bs as a combined treatment. The DRI analysis showed that Bs in combination with Cis could lower the amount of Cis needed to reach an IC50 by as much 7.73-fold less plus 7.74-fold less Bs to achieve the same 50% inhibition in A549 cells (i.e., 3.129 μM Bs+ 0.781 μM Cis, as 4:1 molar ratio in the combination). The BCcomb can produce the desired cytotoxic effect against A549 cells at lower concentrations of both Cis and Bs (Figure 2c).

Analysis of the combined effect by Bliss independent model: The observed combination's effect resulted from combining the test concentrations of Bs and Cis (43 ± 6.1) significantly (P-value < 0.05) exceeded the expected effect of an additive interaction (28 ± 2.5). This result is consistent with the synergy indicated by CI and isobologram analyses, thereby confirming that Bs synergizes with Cis in A549 cells (Figure 3).

qRT-PCR analysis of gene expression related to apoptosis: The A549 cells treated with BCcomb exhibited a notable increase in p53 and BAX expression by 6.2- and 5.8-fold, respectively, when compared to the control group. Meanwhile, the expression levels of BCL2 and MCL-1 significantly decreased (P-value < 0.05) to 0.27 and 0.11-fold, respectively, in relation to the control group. The increase in BAX and p53 mRNA expression levels, coupled with the decrease in MCL-1 and BCL-2 mRNA expression levels, was significantly (P-value < 0.05) greater in cells treated with the BCcomb compared to those treated with either Bs or Cis alone (Figure 4).

BCcomb activates caspase 3/7 activity in A549 cells: The activity of caspase-3/7 in the control group was set as 1, and all data in the treated groups are indicated relative to this value. The activity of caspase-3/7 was significantly (P-value < 0.05) higher in the groups that were treated with Cis (2.9 ± 0.7) or Bs alone (2.1 ± 0.7) compared to the control cells. It's important to note that cells treated with BCcomb

Table 1. Primer sequence used for qRT-PCR [19, 20].

Gene	Sense primer	Antisense primer
P53	5'-CCTCAGCATCTTATCCGAGTGG -3'	5' -TGGATGGTGGTACAGTCAGAGC -3'
BAX	5'- GGACGAACTGGACAGTAACATGG-3'	5'-GCAAAGTAGAAAAGGGCGACAAC-3'
BCL2	5' - ATCGCCCTGTGGATGACTGAG-3'	5'- CAGCCAGGAGAAATCAAACAGAGG -3'
MCL-1	5' -TCGTAAGGACAAAACGGGAC-3'	5' -CATTCCTGATGCCACCTTCT-3'
GAPDH	5' - GAGTCAACGGATTTGGTCGT-3'	5' -GACAAGCTTCCCGTTCTCAG-3'

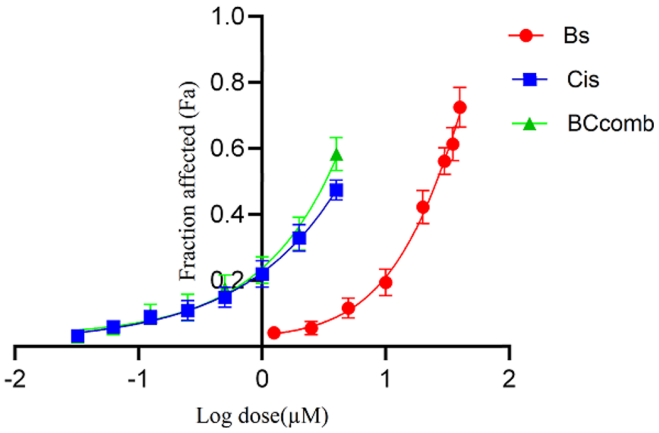

Figure 1. Log dose-effect Curve. The dose values were transformed into the logarithmic values, and the percentages of growth inhibition for Bs, Cis, and BCcomb in the A549 cell line were also converted to the fractions affected (fa) (from 0 to 1). Data presented as mean ± SD (μM); n = 4.

Table 2. The IC50 values of Bs, Cis, and BCcomb in A540 cells using MTT assay. IC50 values are expressed as mean ± SD (μM). Different lowercase letters indicate statistically significant differences (P-value < 0.05; n = 4).

Compound	IC50 in A549 cells (μM)
Bs	24.22 ± 3.37[a]
Cis	6.04 ± 1.54[b]
BCcomb	3.90 ± 0.61[c]

had 6.5 ± 1.4 times more caspase 3/7 activity than control cells, which is significantly (P-value < 0.05) higher than cells treated with either Bs or Cis alone (Figure 5).

BCcomb generates ROS in A549 cells: The ROS activity in the control group was set to 1, and all data in the treated groups are presented relative to this value. Cells treated with Bs or Cis separately significantly increased the activity of ROS (1.35 ± 0.18 and 1.5 ± 0.25, respectively) compared to cells in the control group (P-value < 0.05). Cells treated with the BCcomb exhibited a significantly greater ROS activity (2.2 ± 0.33) in comparison to those treated with either Bs or Cis alone (P-value < 0.05). This indicates a significant increase in Cis's ability to produce intracellular ROS in A49 cells when combined with Bs (Figure 6).

DISCUSSION

This is the first report to demonstrate that Bs, when used in combination with Cis, has a significantly synergistic inhibitory effect on NSCLC cell line, allowing reduced Cis dosages while preserving the anticancer efficacy of Cis in the combination. BCcomb markedly activated apoptotic markers and increase the generation of ROS in A549 cells. Our results are consistent with the current theory that natural compounds, such as Bs, can improve the efficacy of conventional chemotherapeutics by specifically targeting multiple cancer survival pathways [10]. Our study showed that Bs and Cis have a synergistic anticancer effect in A549 cells, confirmed by CI analysis and isobologram results. The Bliss independent approach further validated this synergy. We used a 4.0 μM BCcomb concentration as it represents the strongest synergistic effect based on CI values for actual experimentally combined doses. Trypan blue staining was employed as it does not assume a specific cell death mechanism [21]. Our synergy results are consistent with prior research on another cell type, which showed that the combination of Bs and Cis has synergistic anticancer effects in ovarian cancer cells [22]. Additionally, the favorable DRI suggests lower Cis doses can achieve similar effects when used in combination with Bs, supporting combination therapy benefits like reduced side effects and enhance overall therapeutic efficacy [13]. BCcomb outperformed Cis alone, aligning with the principle of "independent similar action," where the cumulative effect of the two drugs equals the superiority of their separate effects [23].

The current study suggests that the Bs in the combination with Cis improves p53-driven apoptosis in A549 cells which is consistent with another study stated that A549 cells are more sensitive to Cis when p53 is overexpressed, which increases apoptosis [24]. In the context of elevated P53 expression, the treatment with BCcomb led to an increase in Bax expression while simultaneously decreasing BCL-2 and MCL-1 expression. This strongly indicates that the combination treatment promotes apoptosis by favoring pro-apoptotic signaling pathways. This is consistent with previous studies have found that a reduction in Bcl-2 protein levels in A549 or the downregulation of Mcl-1 can increase the sensitivity of cancer cells to Cis-induced apoptosis [25, 26]. Interestingly, inhibiting Mcl-1 can activate Bax, thereby promoting apoptosis in cancer cells [27].

The elevated activity of caspase 3/7 in the combination-treated cells indicates that Bs enhances the apoptotic cascade triggered by Cis. Prior researches indicate that both Cis and Bs individually promote caspase-mediated apoptosis in lung cancer cells [28, 29]. Consistent with previous research conducted on A549 cells [29, 30], the present study demonstrated that Bs or Cis act as anti-cancer agents by enhancing intracellular ROS production and initiating apoptosis. Interestingly, Bs demonstrates dual roles in modulating oxidative stress,

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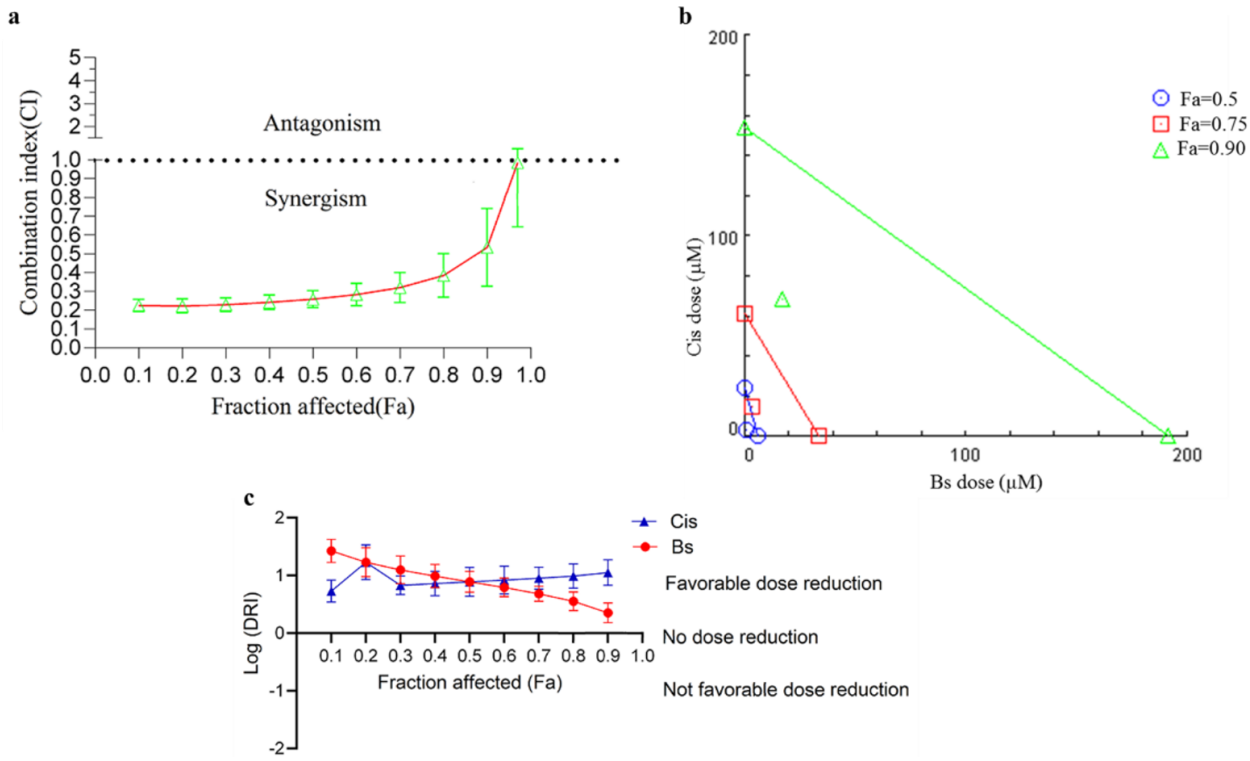

Figure 2. Evaluation of Bs and Cis interaction as combined treatment in A549 cells. (a) CI values of BCcomb plotted against Fa, indicating synergy (CI < 1), additivity (CI = 1), or antagonism (CI > 1). (b) Isobologram illustrating Bs (x-axis) and Cis (y-axis) doses needed for a certain effect when administered in combination; points below/above the additivity line indicate synergy/antagonism. (c) Favorable dose reduction occurs when log (DRI) > 0. Data are presented as the mean ± SD; n = 4.

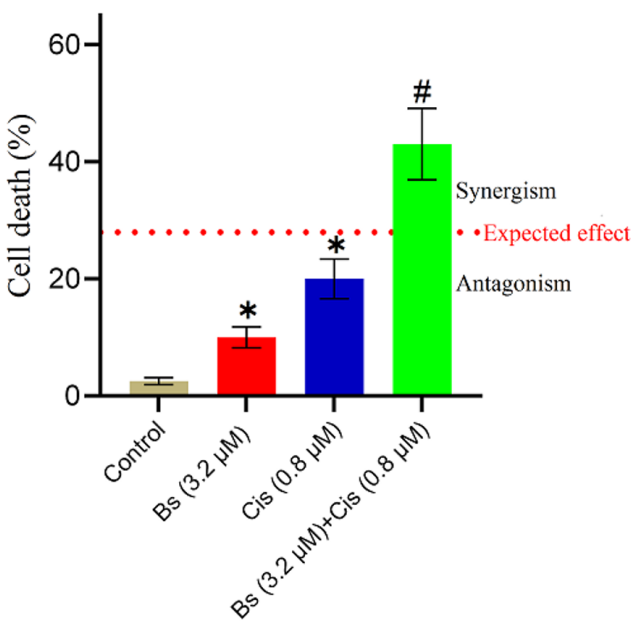

Figure 3. The observed and predicted effects based on the Bliss independence criterion for BCcomb. * Denotes significance vs. control, # vs. Cis or Bs (P-value < 0.05). Data expressed as mean ± SD; n = 4.

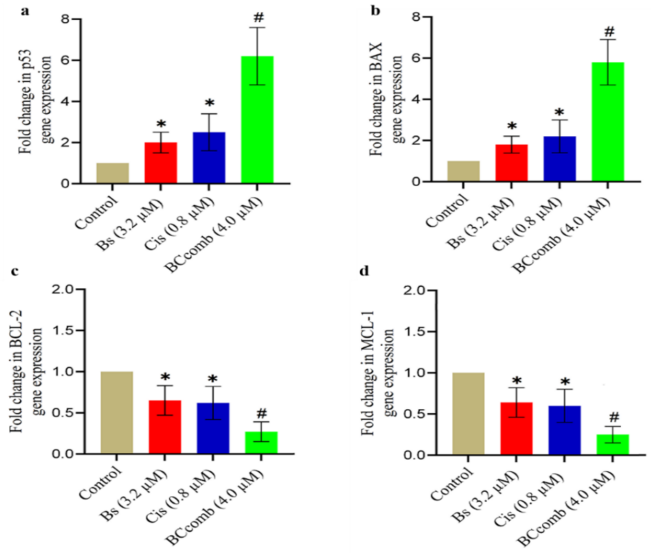

Figure 4. qRT-PCR analysis of apoptotic gene expression in A549 cells treated with BCcomb. mRNA levels of p53 (a), BAX (b), BCL2 (c), and MCL-1 (d) in group treated with the BCcomb were compared to Bs, Cis, and control groups. Data are presented as mean ± SD (n = 4). *P-value < 0.05 vs. control; # P-value < 0.05 vs. Bs or Cis.

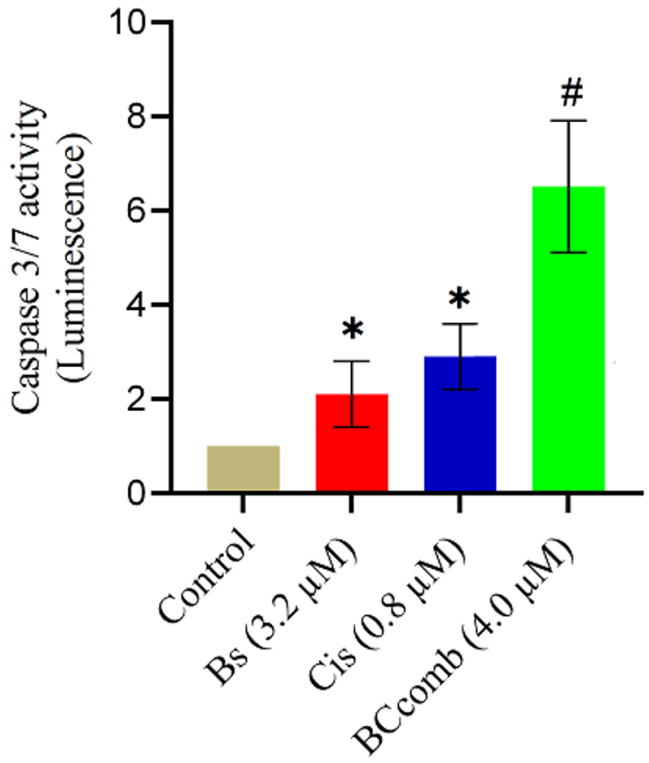

Figure 5. BCcomb increases caspase 3/7 activity: Data are mean ± SD from four biological replicates. *P-value < 0.05 vs. control; # P-value < 0.05 vs. Bs or Cis.

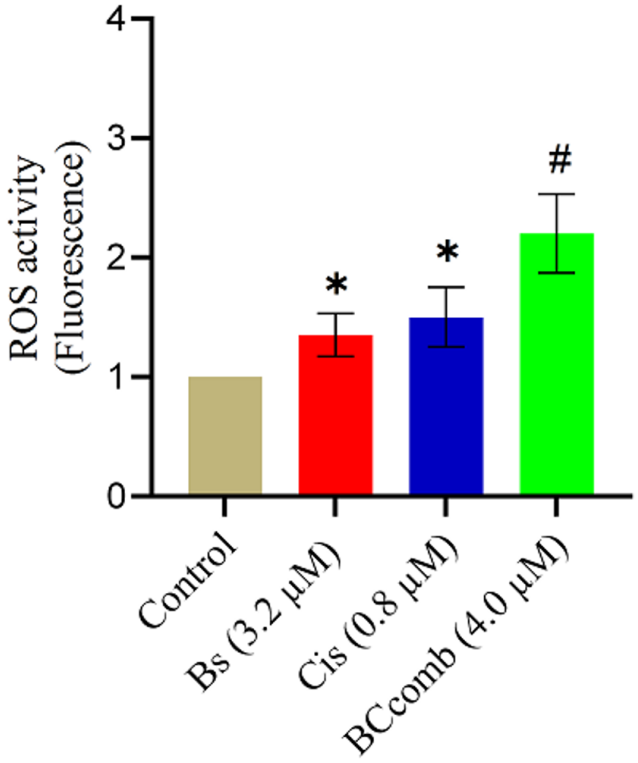

Figure 6. BCcomb increases ROS activity: ROS levels are shown as fold increases over control. P-value < 0.05 vs. control (*); vs. Cis or Bs (#). (n = 4).

functioning as an antioxidant in normal cells while promoting oxidative stress in cancer cells [10]. The mechanism behind Bs-induced excessive ROS accumulation in A549 cells is partly attributed to the significant downregulation of TRX1 and TRXR1 proteins, which are crucial for ROS clearance [29]. Our results confirmed that Bs significantly increases the ROS production triggered by Cis when used in combination, aligning with earlier studies that indicate Bs amplifies the cytotoxic effects of Cis and promotes apoptosis in various ovarian cancer cell lines through increased ROS production [22]. Although these studies do not concentrate on NSCLC cells, they indicate a possible mechanism by which Bs could enhance Cis-induced cytotoxicity via elevated ROS production.

The study is limited by its focus on BCcomb's anticancer effects on lung cancer cells in vitro, without comparison to normal cells. This leaves gaps in understanding its selective toxicity. Further in vivo studies with longer durations are needed to confirm findings and assess off-target effects. Adjustments to the Bs-to-Cis molar ratio may be required for optimal synergy. Future research should also explore molecular pathways, including DNA disruption, mitochondrial function, autophagy, microtubule organization and vascular remodeling, while investigating other therapeutic applications.

CONCLUSION

Combining Bs with Cis enhances its anticancer efficacy against NSCLC synergistically. The combination lowers required doses, potentially reducing the incidence of side effects. Our research indicates that the Bs in combination with Cis considerably increases the pro-apoptotic effects of Cis by increasing the mRNA expression of p53 and BAX while reducing BCL-2 and MCL-1 mRNA expression, increasing caspase 3/7 activity, and increasing intracellular ROS production. This provides evidence that combinatorial therapy of Bs and Cis is a promising strategy for the treatment of NSCLC compared to monotherapy.

ETHICAL DECLARATIONS

Acknowledgments

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Ethics Approval and Consent to Participate

The research ethical approval committee of the College of Veterinary Medicine at the University of Al-Qadisiyah approved this study on November 26, 2023, with reference number document 4419. The study did not include any animals or humans; therefore, consent was not required.

Consent for Publication

Not applicable (no individual personal data included).

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Availability of Data and Material

Data generated during this study are available from the corresponding author upon reasonable request.

Competing Interests

The author declares that there is no conflict of interest.

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Authors' Contributions

All listed authors equally designed the research, wrote the paper, analysed the data, intellectually contributed to the work, and approved the final version of the manuscript for publication.

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