


Synergistic Cytotoxic Effect of Imiquimod and Cisplatin Combination Therapy on A549 Lung Cancer Cell

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Abstract

Background: Recently, combination therapy has become a promising approach to overcoming chemotherapy problems. In the present study, we describe a combinational treatment regime using cisplatin (Cis) and imiquimod (IMQ) to increase the antitumor response of the therapy in A549 lung cancer cells.

Methods: A549 cells were either treated with increasing concentrations of Cis or IMQ or with Cis-IMQ combinations for 24h. Cell growth inhibition, cell cycle analysis, and inductive apoptosis were evaluated using MTT assay and annexin V assay using flow cytometry, respectively. Kruskal-Wallis was used to analyze differences in cell groups' means.

Results: A549 cell viability was affected by single therapy of Cis and IMQ in a dose and time-dependent manner ($P < 0.001$). The combination index (CI) analysis revealed that the combined effect of Cis-IMQ exerted a wide range of synergy in lung cancer cells as well as 0.58 to 0.84 for IC10 to IC90. More interestingly, the combination of Cis and IMQ reduced the dose of Cis by 1.86-fold. In terms of cell apoptosis induction, Cis (IC20)-IMQ (IC90) displayed a synergistic effect on A549 cells, compared to the single drug ($P < 0.0001$). Co-treatment of A549 cells with Cis and IMQ significantly caused SubG1 arrest compared to Single therapy and control group.

Conclusion: These results indicated that an IMQ-based combination using Cis has synergistic effects on cell proliferation and apoptosis induction in A549 cells and deserves further preclinical and clinical studies.

Introduction

For a long time, chemotherapy along with surgery and radiotherapy was the only approach to cancer therapy. Despite the effective clinical response of chemo agents, acquired resistance and cytotoxicity have become major obstacles to successful cancer treatment.^{1,2} Several strategies such as encapsulation and combination therapy have been introduced to conquer chemotherapy problems. Increasing evidence has demonstrated that synergistic combination therapy compared with monotherapy can overcome therapy resistance and allows for dosage reduction of chemo drugs that improve the clinical outcome of cancer patients.^{3,4} It has been reported that a combination of chemo drugs with nontoxic anticancer agents may result in increased sensitivity of cancer cells to chemotherapy, reducing the dose of chemo drug and ultimately ameliorating side toxic effects.⁵

Cisplatin (Cis-diamminedichloroplatinum II; Cis), a platinum-based drug, is one of the effective chemo agents in cancer therapy as monotherapy or in combination with other treatments.⁶ Cis is used as the first-line treatment for various cancer such as lung, ovarian, and cervical in the clinical setting.

As a strong DNA chelating agent, it can induce tumor cell apoptosis and interfere with transcription and cell proliferation, resulting in toxicity.⁷ Resistant therapy and cytotoxicity are the biggest challenges of the clinical use of Cis in cancer therapy.⁸ Considering the cytotoxicity of cis, it is necessary to find the optimal agent and dosage combination treatment with less toxicity and high apoptosis induction. Several preclinical studies provide evidence that the combination of chemo agents with an immunomodulator such as toll-like receptor (TLR) agonists achieve a promising synergistic anticancer effect

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through the induction of cellular immunity as well as apoptosis.⁹⁻¹¹

Imiquimod (IMQ) is a synthetic agonist of TLR7 that acts as an antitumor agent by stimulating cellular immune responses, inducing apoptosis and immunogenic cell death (ICD).¹² IMQ as topical formulation of (5% cream) was approved by the FDA for the treatment of skin cancers.¹³ Numerous studies demonstrated that co-delivery of chemo agents such as OXA and Cis with TLR agonists strengthened the antitumor effect and reduces systemic and immune toxicity compared with chemotherapy alone.^{1,11,14,15} The present study attempted to find out whether combination therapy with IMQ, an immune system modulator, plus Cis as ICD inducer agent can trigger potent tumor cell apoptosis with lower cytotoxicity in the A549 lung cancer cell line.

Materials and Methods

Reagents and cell culture study

The human lung cancer cell line A549 was prepared by the Pasteur Institute (Tehran, Iran). Cis was provided by Sigma Aldrich. IMQ powder was purchased from InvivoGen (San Diego, United States). The A549 cells were cultured in RPMI Medium 1640 containing 10% (v/v) FBS (Fetal Bovine Serum) and 1% (v/v) penicillin–streptomycin at 37°C in an atmosphere of 5% CO₂.

MTT assay

The 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine *in vitro* cytotoxicity of drugs. A549 cells were seeded in 96-well plates (10000 cells per well) and incubated for 24 hours. The medium was replaced by fresh medium containing a range of increasing concentrations of IMQ or Cis. After the desired time of the incubation period (24, 48, and 72 hours), the MTT solvent (0.5 mg/mL) was added to each well and then incubated for 4 hours. Dimethyl sulfoxide (DMSO) solution was applied for dissolving the formazan precipitated. The relative number of viable cells was evaluated by measuring the optical density (OD) value at a wavelength of 560 nm using a microplate spectrophotometer. The percentage of surviving cells was calculated using the following formula: percentage (%) of cell survival = [(average absorbance in treated wells) / (average absorbance in untreated wells)] × 100. All assays were performed in triplicate. The graph of viable cell population was plotted against the concentration of IMQ and Cis. The resulting graph was used to calculate the IC₁₀, IC₂₀, IC₃₀, IC₄₀, and IC₅₀ values of Cis and IC₅₀, IC₇₀, IC₈₀, and IC₉₀ for IMQ.

Dose-response analysis and combination index for detecting synergism additivity or antagonism

Chou and Talalay's method based on the median-effect equation was used to calculate drug combinations effect. The drug-drug interaction is evaluated by the "combination index" (CI), which is based on the

concentration-effect relationship. The CI and dose-reduction index (DRI) for calculating synergism and antagonism between IMQ and Cis combination against A549 cells were calculated as described by CompuSyn software (*version*: 1.0.1.). CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism respectively. For calculating CI, we performed a series of MTT assays using five different IC values of IMQ (IC₁₀, 20, 30, 40, 50) and Cis (IC₅₀, 70, 80, 90) with concentrations in the range of 7–25 μM and 3.5–32 μM for Cis and IMQ respectively.

Annexin V-PI staining to A549 cell apoptosis assay

Apoptosis analysis was conducted with Annexin V/ Povidone-iodine (PI) apoptosis kit according to the manufacturer's instructions. A549 cells were seeded in a 6-wells plate (2 × 10⁵ cell per well) and incubated for 24 hours at 37°C under a humidified atmosphere containing 5% CO₂. Treatment with Cis (IC₂₀, 30, and 40), IMQ (70, 80, and 90), and Cis-IMQ combination numbered 2, 3, and 4 were performed on the cells that were approximately 70% confluent. Based on CI results combination 2, 3, and 4 were selected to apoptosis analysis. Following 24 hours incubation dead and live cells were collected with diluted trypsin. The washed cells with phosphate-buffered saline (PBS) underwent staining with annexin-v (BD Bioscience California, USA) for 30 minutes at room temperature. Then the cells were double stained with Annexin V-FITC (fluorescein isothiocyanate) and PI, and the population of apoptotic cells was measured with the flow cytometer.

Cell cycle analysis

Cell cycle progression analysis was performed based on cellular DNA content to determine the percentage of cell population in four phases of the cycle, Sub-G1 (Sub-gap 1), G1 (Gap1), S (Synthesis) and G2-M (Gap 2-Mitotic), to detect the rate of apoptosis. The cells were cultured into a 6-well plate and incubated at 37°C for 24 hours. The fresh medium containing different concentrations of the Cis, IMQ, and Cis (IC₂₀) -IMQ (IC₉₀) combination replaced the medium of each well. Following 24 h incubation, all of the dead and live cells were harvested and resuspended in 100 μL of cold PBS. After cell incubated with 450 μL of cold ethanol for 24 hours, the cells were incubated with 5 μL of RNase (20 μg/mL final concentration) for 30 minutes. At the end of the incubation period, the cells stained with 50 μg/mL 4',6-diamidino-2-phenylindole (DAPI) on ice for 1h in the dark. The distribution of cells was assessed by flow cytometry using a FACScan system (Becton–Dickinson and Company).

Statistical analysis

For means comparison, one-way variance analysis (ANOVA) was performed among multiple groups. All analyses were made using GraphPad Prism (*version* 9.0.0). Data are presented as means ± standard deviation (SD). *P < 0.05 was considered statistically significant.

Results

Cis and IMQ induce dose- and time-dependent growth inhibition effects on of A549 lung cancer cells

To explore the inhibitory effects of Cis and IMQ on the A549 cell line, the viability of cells was evaluated by MTT assay using increasing concentrations of Cis and IMQ carried out over a time period of 24 to 72 hours. As shown in Figure 1, time- and dose-dependent cell growth inhibition was observed upon treating the cells with Cis and IMQ. These results were used to determine the IC₅₀ value of the A549 cells, which were 25 μ M and 3.6 μ M for Cis and IMQ at 24 hours respectively.

Synergistic effects of combinational treatment of Cis and IMQ on A549 cells

Over the past decades, efforts have been made to quantify the dose-response relationships of each drug on its own or the combinations in order to detect whether or not given drugs in combination would gain a synergistic effect. In order to accurately assess the effects (additive, synergistic, or antagonistic) of combination treatment on the tumor

cells, the CI was evaluated mathematically using the Chou and Talalay analysis.¹⁶ For this reason, the A549 lung cancer cells were exposed to drugs alone and various combinations of Cis and IMQ at a non-constant ratio in a sequential manner. Combination therapy compared with the drugs-alone treatment, exhibited to have more than an additive effect.

Fraction-affected values (Fa), which represent the fraction of cells inhibited after drug exposure, were evaluated following the treatment of A549 cells at different concentrations of drugs. As shown in Table 1, in three different combination treatments, CI values were below 1, indicating synergistic effects between drugs at low doses. In some concentrations, combination therapy increased CI to more than 1 which in turn suggesting an antagonistic effect.

The median effect plot was applied for determining pharmacological median doses for lethality (LD₅₀), toxicity (TD₅₀), the effect of agonist drugs (ED₅₀) (Figure 2a). To demonstrate the effects at different Fa values, CI values were calculated for each Fa and plotted

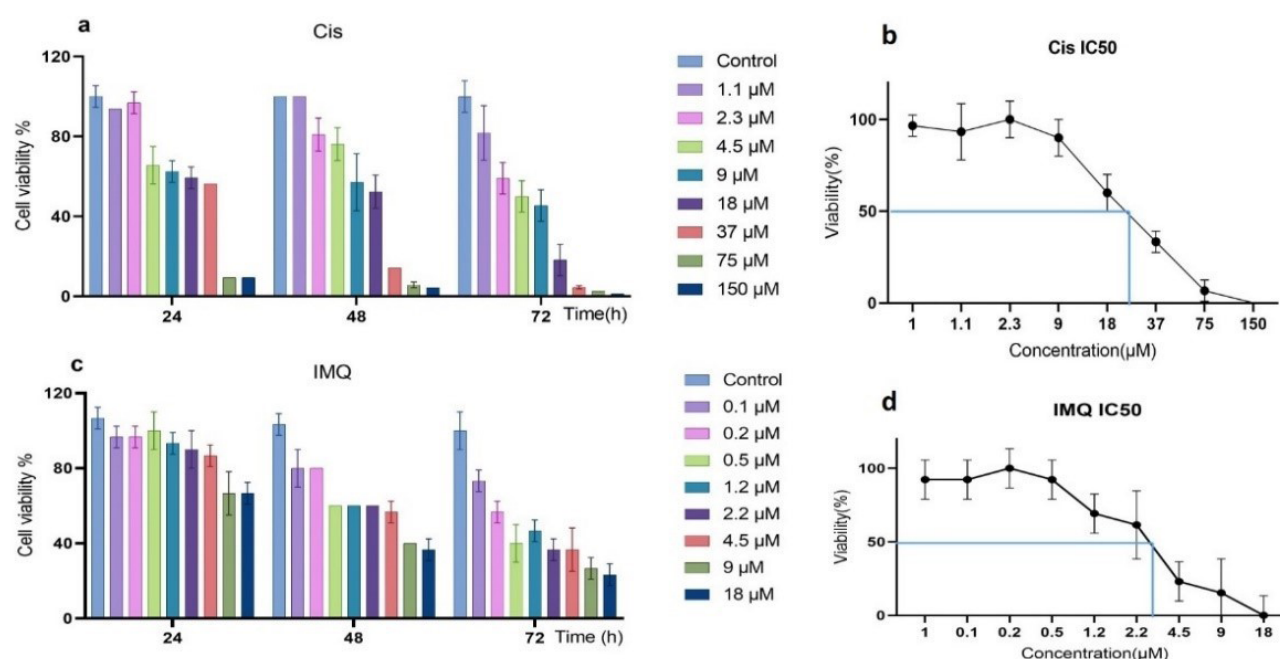


Figure 1. Effects of IMQ and Cis treatment on A549 cell viability. The A549 cells were exposed to increasing concentrations of Cis (a and b) and IMQ (c and d) for indicated hours (24–72 h). The percentages of cell viability were assessed by MTT assay. The error bar of the column represent the SD derived from the results of the three experiments

Table 1. Data of The Chou and Talalay analysis from Cis and IMQ combination therapy

Cell	Combination number	Dose combination		CI value	Fa	DRI values Cis	DRI values IMQ
		Cis μ M (IC value)	IMQ μ M (IC value)				
A549	No. 1	7 (10)	32 (90)	1.44	0.34	1.56	1.24
	No. 2	11 (20)	32 (90)	0.58	0.37	1.86	18.59
	No. 3	15 (30)	14 (80)	0.74	0.37	1.38	44.39
	No. 4	20 (40)	8.5 (70)	0.84	0.35	1.19	161.35
	No. 5	25(50)	3.5 (50)	1.08	0.35	0.9	328.16

The value of CI (combination index), Fa (Fraction-affected), and DRI (dose reduction index) of A549 cell lines treated with five different IC value combinations of IMQ and Cis after 24 h incubation. CI < 1: synergistic effect, CI = 1 additive effect, CI > 1: antagonistic effect.

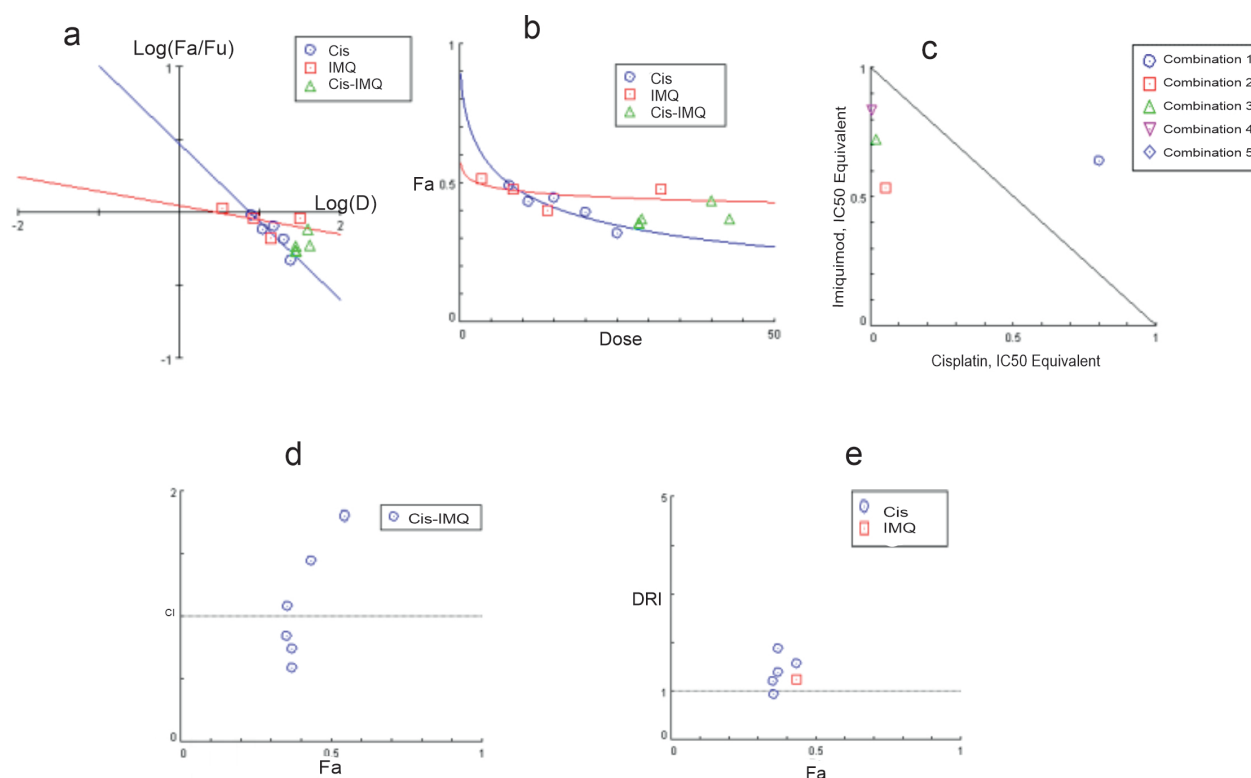


Figure 2. Combination analysis of Cis and IMQ combination therapy on A549 cancer cells. (a) dose-effect curve (b) the median-effect plot for a single and combination therapy (c) Dose-normalized isobologram (d) combination index plot (e) DRI plot for five combinations of Cis and IMQ on A549 cells

in the FA-CI plot, as shown in Figure 2b. The results of the isobologram were concordance with those of the CI values and discovered the existence of synergistic effects in combinations 2, 3, and 4 (Figure 2c). As shown in Figure 2d, the CI in combinations 2, 3, and 4 were below 1 and two combinations (1 and 5) were above 1. The DRI is a measure of how much concentration of each drug in a synergistic combination may be reduced at a given effect level compared to single drug doses. DRI = 1 represent no dose reduction, whereas DRI > 1 and < 1 indicate favorable and negative dose reduction, respectively. In our study, at $F_a = 0.372$, DRI was 1.86 and 18.59 for Cis (IC₂₀) and IMQ (IC₉₀) respectively indicating combination therapy of A549 cells could reduce the Cis dose up to 1.86 times while decreasing the IMQ dose up to 18.59 times. Similarly, at $F_a = 0.371$ and 0.35, a combination regimen could reduce the concentration of both drugs which need to kill tumor cells (Figure 2e and Table 1). These results indicated that combination 2 has strong synergistic effects among combinations tested in this study.

Effects of combinational treatment of Cis and IMQ on A549 cells apoptosis

In order to compare the effect of a combined and single treatment in inducing cellular apoptosis, flow cytometry analysis was performed. The result of the Annexin-V/PI assay represented that the single therapy of the A549 cells with IMQ (11 μ M) or Cis (32 μ M) induced 9.84 and 23.81% of the cells to enter apoptosis at 24 hours, respectively (Figure 3a). The treatment of A549 cells

with Cis (IC₂₀) and IMQ (IC₉₀) (combination 2) led to an increase in the population of apoptotic cells up to 39% at 24 hours. Although apoptotic cells were markedly increased in the late phase (upper right panel) and early phase (lower right panel), combination therapy of A549 cells resulted in insignificant induction of apoptosis at the early stages more than treatment with each drug. As shown in Figure 3b and 3c, compared to single therapy with Cis and IMQ the rate of early and late apoptotic cells significantly increased after treatment with Cis and IMQ combination ($P < 0.0001$). However, a significant synergistic apoptotic effect on A549 cells was detected using combination 2 compared to each drug and other combination treatments ($P < 0.0001$) (Figures 3d and 3c). Combinations 3 and 4 did not result in any additive apoptotic effect on A549 cells when compared to single or other combinations therapies ($P > 0.3$). Combination 2 was therefore selected for the cell cycle analysis.

Effects of Cis and IMQ combination therapy on cell cycle progression in A549 cells

Cell cycle analysis was carried out to determine the effects of each drug and combination 2 (Cis IC₂₀ + IMQ IC₉₀) on the cell cycle progression of A549 cells.

The result showed that the single-agent treatment of the A549 cells with Cis (32 μ M) or IMQ (11 μ M) caused 3.88% ($P = 0.01$) and 2.69% ($P = 0.002$) of the cells to be observed in subG1 phase respectively. However, the treatment of A549 cells with combination 2 led to a higher level of subG1 arrest (7.75%) compared to single agents and

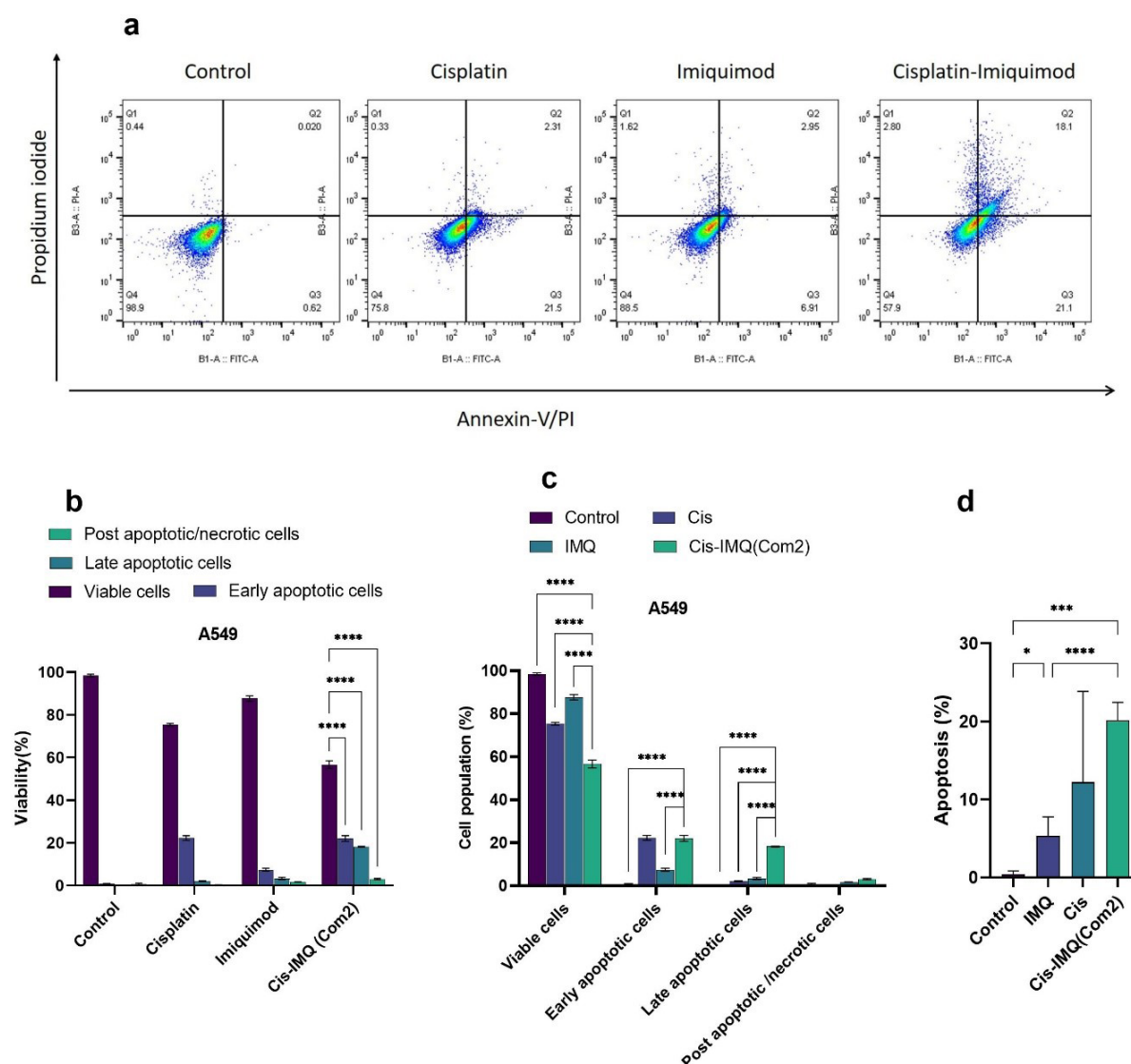


Figure 3. Effects of the combination 2 (IMQ 11µM and Cis 32µM) on A549 cell viability and apoptosis. **(a)** Dot plots from Annexin V-FITC/PI double staining flow cytometry, showing the apoptotic status of A549 cells. **(b)** The overall percentage of viable cells. **(c)** Breakdown of cell populations: percentage of viable, late, and early apoptotic cells. **(d)** Total percentage of apoptotic cells in A549 cells treated with the combination2 for 24h.

untreated cells (Figure 4a). The percentage of subG1 cells significantly increased in cells treated with combination 2 compared to Cis or IMQ treatment ($P < 0.001$) (Figure 4b). Also, the rate of G2M phase arrested cells significantly decreased in cells treated with combination 2 (Cis; 11 µM and IMQ; 32 µM) compared to Cis and control group ($P = 0.0001$). Compared to the control group and single treatment with Cis and IMQ, the rate of arrested cells in S phase significantly decreased in combination 2 ($P < 0.001$) (Figure 4c).

Discussion

Despite chemotherapy being the gold standard therapy option for cancer treatment, undesired cytotoxicity and the development of drug resistance limited its clinical application. Increasing studies have demonstrated that synergistic combination therapy can increase the benefit of therapy and overcome drug resistance as well as reduce the toxic effects.⁴ Synergistic effects of drug combination are evaluated to demonstrate additive effects of each

drug, causing dosage reduction and therefore decreasing adverse side effects.² Cis is a well-known chemotherapy agent, which is used in clinical to treat a variety of cancers. However, despite its robust effect and the wide range of activity, its clinical usage is limited due to high toxicity and several adverse side effects.^{6,17} In contrast, IMQ is a non-toxic agent with proven anti-cancer effects against various cancers.¹⁸ Combination treatment of Cis with other agents has been studied in various cancer types.^{7,19,20} In this study, we proposed that the combination of IMQ and Cis leads to synergistic effects and enhances their anticancer effects in A549 lung cancer cells.

Cytotoxic effects of Cis and IMQ have completely different mechanisms for activity. The mechanism of action of Cis and other chemo agents is mediated by binding to nuclear DNA and interference with normal transcription and replication of the DNA resulting in DNA damage and following activation of apoptosis within cancer cells.^{21,22} IMQ (also called Aldara), a synthetic nucleotide-like TLR7 agonist, which is widely investigated

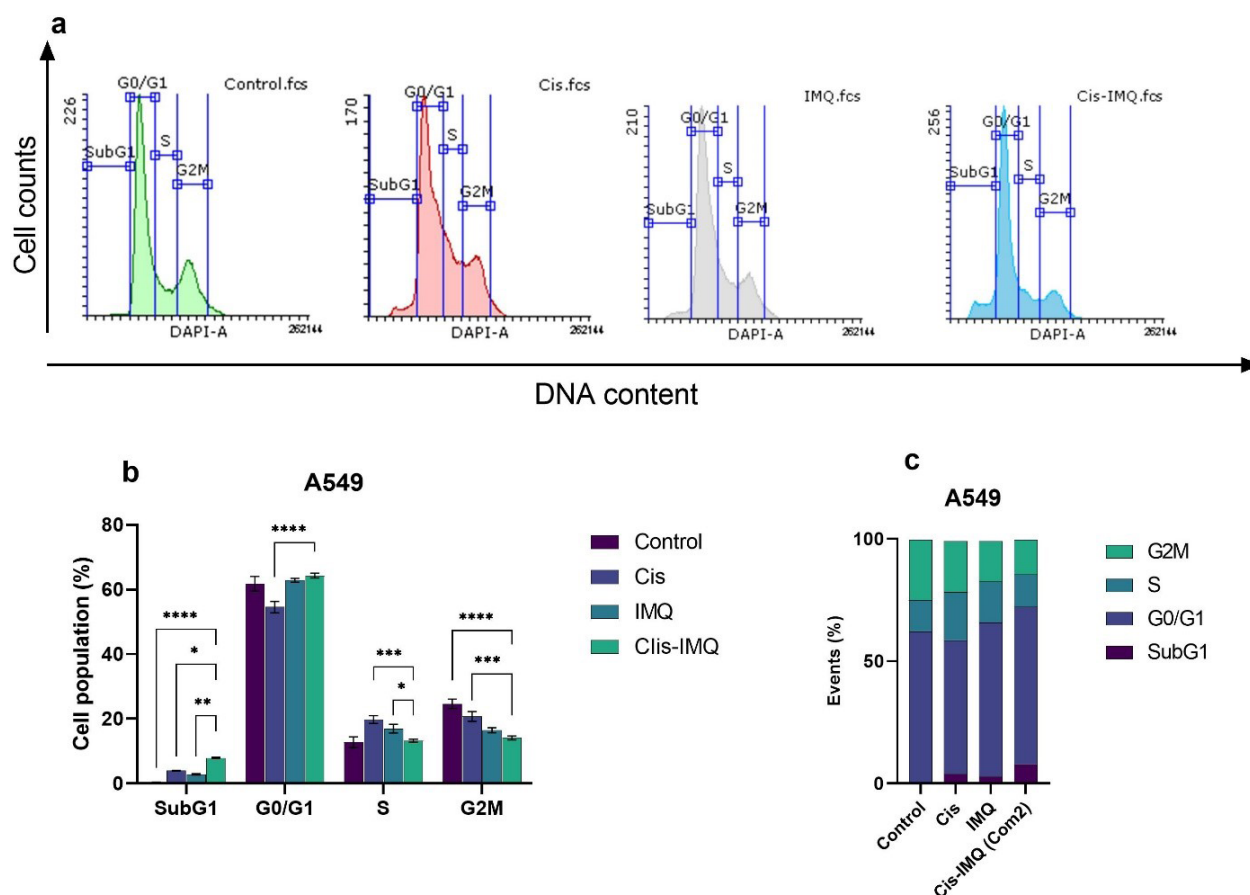


Figure 4. Effects of IMQ and Cis combination treatment on the A549 cells cycle. (a) Analyses of cell cycle progression with DAPI staining flow cytometry illustrating SubG1 increasing in A549 cells upon combination 2. (b and c) Ratio of each phase of the cell cycle in A549 cells treated with the concentration 2 (IMQ 11 μ M and Cis 32 μ M) for 24h

for the treatment of various cancers and has been approved for the treatment of skin carcinoma.²³ IMQ eradicate cancer cells by activating cellular immunity and directly inducing apoptosis in cancer cells.¹² IMQ through TLR7 activates ATM/ATR downstream signaling pathways and induces ROS production to stimulate p53-dependent apoptosis.²⁴ These results suggest that the mechanisms of action of Cis and IMQ are distinctively independent and a combination of these can act as a potential option for cancer cells that induce further apoptosis on cancer cells.

In this study, we investigated the proper concentration ratio of IMQ and Cis to determine the extent of the synergistic effects, by evaluating the cellular response of different dosages and combinations of each drug. The CI values and isobolograms obtained using the MTT assay clearly exhibited that the treatment of the A549 cells with a combination of IMQ and Cis resulted in a synergistic, antagonistic, and dose-dependent increase in growth inhibitory activity.

The binary combination of Cis with IMQ resulted in an antagonistic effect in two combinations (1 and 5) and three combinations (2, 3, and 4) represent synergistic effects, of which combination 2 (IMQ 11 μ M + Cis 32 μ M) had lower CI (0.58). Drug antagonism was detected at dose levels of the IC₅₀ values of each drug. Moderate synergism was observed in concentration ranges close

to IC₈₀₋₇₀ and IC₃₀₋₄₀ for IMQ and Cis respectively. Synergism at Cis dose levels lower than its IC₅₀ value plus IMQ at dose levels 10-fold higher than its IC₅₀, indicating that the significantly low dose of Cis would be sufficient to trigger the synergistic effects of the drug combination.

Annexin V-FITC and cell cycle analyses were carried out on the treated and untreated A549 cells to evaluate whether the growth inhibitory effects of combinations 2, 3, and 4 could be attributed to apoptosis. The results of these assays showed that the synergistic effects of combination 2 (Cis IC₂₀ and IMQ IC₉₀) were caused by an augmented apoptotic response. However, IMQ and Cis alone showed great cell death but when the two drugs were combined the rate of cell death significantly increased. This effect could also be attributed to a different mode of action to those employed by Cis and IMQ. We also demonstrated that the dose of Cis was reduced almost 2-fold in combination 2. The result of cell cycle analysis showed that combination 2 significantly arrest the cells in SubG1, S, and G2M phases compared to the single drug and control group. As reported in animal model studies, IMQ stimulates cellular immune responses and its anti-tumor response increased when combined with a chemo agent.²⁵ Based on our results combination therapy of A549 lung cancer cells with Cis and IMQ can result in further anti-tumor results through induction of immune responses as well as apoptosis.

Conclusion

Our results point out that combination therapy with Cis and IMQ considerably enhances the antitumor efficacy of the drug on A549 lung cancer cells. These findings suggest that Cis and IMQ co-delivery have the potential to work synergistically toward the inhibition of the growth of A549 lung cancer cells by reducing Cis-mediated toxicity. Further in vivo studies are required to elucidate the therapeutic efficacy of the proposed IMQ and Cis combination.

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Authors' Contribution

Conceptualization: Leila Rostamizadeh

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Competing Interests

The authors declare that there are no conflicts of interest.

Data Availability Statement

The data described in this article are available.

Ethical Approval

Ethical approval for this study was obtained from Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.056).

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