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# Effects of Mebendazole on the Caspase–mediated Apoptosis mechanism in Cancer cell culture

## Efectos del Mebendazol Sobre el Mecanismo de Apoptosis Mediado por Caspasa en Cultivos de Células Cancerosas

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**ABSTRACT**

This study was conducted to compare Mebendazole in terms of its apoptosis–inducing and tubulin–inhibitory effects when combined with vincristine and paclitaxel, both of which are used in cancer treatment. Lung fibroblast cells (MRC–5) and small cell lung carcinoma (NCI–H209) cell lines were used in the study. Concentrations of Mebendazole, vincristine, and paclitaxel at 0.5 µM, 1 µM, 1.5 µM, and 2 µM were separately applied to these cell lines, as well as in combinations. After the cells were kept in the culture medium for 24 hours following drug administration, cell proliferation, apoptotic DNA levels, caspase 3, 8, and 9 levels, and *in vitro* wound healing experiments were performed. It was determined that Mebendazole suppressed cell proliferation and cell healing, increased caspase–3, caspase–8, caspase–9 levels and apoptotic DNA formation in NCI–H209 cancer lung cells. Compared to the groups given Mebendazole and vincristine alone, it was observed that cell proliferation was more suppressed and, the level of apoptosis increased in cancerous cells in the groups given the combination of the two drugs. According to the findings obtained from the present study, it was believe that Mebendazole may possess therapeutic activity against cancerous lung cells (NCI–H209) due to its apoptosis–inducing and cell proliferation–suppressive effects.

**Key words:** Apoptosis; anti–cancer, Mebendazole, Paclitaxel, Vincristine

**RESUMEN**

Este estudio se realizó para comparar el mebendazol en términos de sus efectos inductores de apoptosis e inhibidores de tubulina cuando se combina con vincristina y paclitaxel, ambos utilizados en el tratamiento del cáncer. En el estudio se utilizaron líneas celulares de fibroblastos de pulmón (MRC–5) y de carcinoma de pulmón de células pequeñas (NCI–H209). Se aplicaron concentraciones de mebendazol, vincristina y paclitaxel a 0,5 µM, 1 µM, 1,5 µM y 2 µM por separado a estas líneas celulares, así como en combinaciones. Después de que las células se mantuvieron en el medio de cultivo durante 24 horas después de la administración del fármaco, se realizaron experimentos de proliferación celular, niveles de ADN apoptótico, niveles de caspasa 3, 8 y 9 y experimentos de curación de heridas *in vitro*. Se determinó que el mebendazol suprimía la proliferación celular y la curación celular, aumentaba los niveles de caspasa–3, caspasa–8, caspasa–9 y la formación de ADN apoptótico en células cancerosas de pulmón NCI–H209. En comparación con los grupos que recibieron mebendazol y vincristina solos, se observó que la proliferación celular estaba más suprimida y que el nivel de apoptosis aumentó en las células cancerosas en los grupos que recibieron la combinación de los dos fármacos. Según los hallazgos obtenidos en el presente estudio, debemos creer que el mebendazol puede poseer actividad terapéutica contra las células cancerosas del pulmón (NCI–H209) debido a sus efectos inductores de apoptosis y supresores de la proliferación celular.

**Palabras clave:** Apoptosis; anticáncer, mebendazol, paclitaxel, vincristina

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## INTRODUCTION

The fact that drugs used in the treatment of cancer in humans and animals have serious side effects, and the desired treatment success is still not achieved in many types of cancer, compels scientists to search for new strategies and treatment methods in the battle against cancer. One of these emerging strategies is the investigation of non-cancer drugs that may exhibit anti-cancer activity when used in cancer treatments [1, 2].

In numerous studies examining its anti-cancer activity, Mebendazole has been compared with conventional antineoplastic drugs. The literature reports that the apoptosis-inducing effects of Mebendazole, which contribute to its anti-cancer effects, primarily stem from the destabilization of the tubulin protein an important component of the cytoskeleton [3, 4, 5].

Paclitaxel acts by stabilizing the microtubulin structure formed through the combination of tubulin dimers, while vincristine functions as a microtubulin destabilizer by depolymerizing the microtubulin protein formed through tubulin dimer polymerization [6, 7]. Both drugs induce cell death by disrupting the mitotic division in cells undergoing mitosis [8]. It has been documented in the literature that Mebendazole exhibits anti-cancer activity by reducing the levels of depolymerized tubulin [9, 10, 11]. However, there is a need for further information and studies on the anti-cancer efficacy of mebendazole when combined with a known antineoplastic drug. Thus, this study aims to evaluate the anti-cancer activity of Mebendazole by comparing its effects in combination with vincristine and paclitaxel.

The present study aims to assess the *in vitro* effects of mebendazole on cell proliferation, cell healing, apoptosis induction, and caspase 3, 8, and 9 levels in healthy and cancerous lung cells (MRC-5 and NCI-H209)

## MATERIALS AND METHODS

### Cell culture and reagents

Human lung fibroblast cells (MRC-5) and human small cell lung carcinoma cells (NCI-H209) were obtained from the Cell Culture Laboratory at HÜKÜK, Alum Institute, Turkey. Dulbecco's Minimum Essential Medium (Thermo Fisher Scientific, DMEM, USA) was used for cell culture. The media were supplemented with 10% FBS (Sigma-Aldrich) and penicillin-streptomycin (100 $U \cdot mL^{-1}$ Invitrogen). All cells were maintained in a standard incubator (Nüve, EC160, Turkey) at 37°C with 5% $CO_2$. Mebendazole (Mbz), Paclitaxel (Pac), and Vincristine (Vin) were purchased from Abcam in analytical purity (Cambridge, UK).

### Cell viability and proliferation assays

Cell viability and proliferation were assessed using the MTT Cell Proliferation assay (Biovision, Massachusetts, USA). The cells were counted on a Thoma slide ($2\times10^4$ cells/well), and then treated with Mbz, Vin, Pac, Vin + Mbz combination, and Pac + Mbz combinations at doses ranging from 0.5 to 250 µM in 96-well plates. To determine the number of viable and proliferating cells, absorbance values were measured using an Epoch Elisa reader (BioTek, Vermont, USA) at a wavelength of 590 nm. The experiments were repeated three times.

### Caspase-3, 8 and 9 experiments

Colorimetric caspase assay kits (Catalog no: K106, K113, K119, Massachusetts, USA) were utilized to determine the levels of caspases. The cells were counted ($1\times10^6$ cells/well), and then treated with Mbz, Vin, Pac, Vin + Mbz combination, and Pac + Mbz combinations in 96-well plates at a dose range of 0.5-2 µM. After 24 hours of drug administration, the experiments were conducted following the experimental protocol. The absorbance values were measured at a wavelength of 400 nm using an EPOCH ELISA reader (BioTek, Vermont, USA). The experiment was repeated at least three times.

### Apoptotic Deoxyribonucleic acid (DNA) laddern level

Apoptotic DNA levels were determined using an assay kit from Biovision (Massachusetts, USA). The drug groups were applied to cells in 96-well plates at a density of $1\times10^6$ cells/well, and the cells were incubated for 24 hours. The experiments were conducted following the protocol provided with the assay kit. At the end of the experiment, DNA fragments were separated on a 1.2% agarose gel containing 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide, and the gel was analyzed using a UV transilluminator device (Maestrogene, Hsinchu, Taiwan). The density of DNA bands was determined using the image analysis program (Image J 1.48s processing software, National Institutes of Health, MD, USA). The experiment was repeated at least twice.

### *In vitro* wound healing experiments

To evaluate cell proliferation and cell migration under in-vitro conditions, a cellular wound healing experiment was conducted [12, 13]. After the cells were counted ($1\times10^6$ cells/well), they were seeded into 6-well plates. Once the cells reached full confluence, a standardized cellular wound was created in each plate by scratching the monolayer cell layer. Following the application of control and drug treatments at a dose range of 0.5-2 µM, the cells were incubated (Thermo Fisher Scientific, DMEM, USA) in serum-free cell media for 24 hours. After the incubation period, the cellular wound areas were visualized using a light microscope, and the resulting images were analyzed using the image analysis program (Image J 1.48s processing software, National Institutes of Health, MD, USA). In this experiment, only MRC-5 cells were used since NCI-H209 cells were not suitable for this particular assay due to their suspension nature

### Statistical analysis

The experiments were repeated three times, and standard deviations were calculated. Analysis of variance was performed using a one-way ANOVA test, and within-group comparisons were conducted using Tukey's test. A $P$-value of $<0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

### Effect on cell viability and proliferation

Upon examining the obtained results, it was observed that cell proliferation in NCI-H209 cancer cells decreased in all groups administered with the drugs, depending on the dosage (FIG. 1). Similar decreases in cell proliferation were also observed in healthy MRC-5 cells across all treatment groups (FIG. 1). In the groups where mebendazole was administered solely to healthy lung fibroblast cells, cell viability was noted to be higher compared to other groups. Additionally, the cell viability ratio in healthy lung cells treated with mebendazole and vincristine was observed to surpass the cell viability ratios obtained within the corresponding groups in cancer cells. This implies that the cytotoxic effect of mebendazole, whether administered alone or in combination with vincristine, is lower on

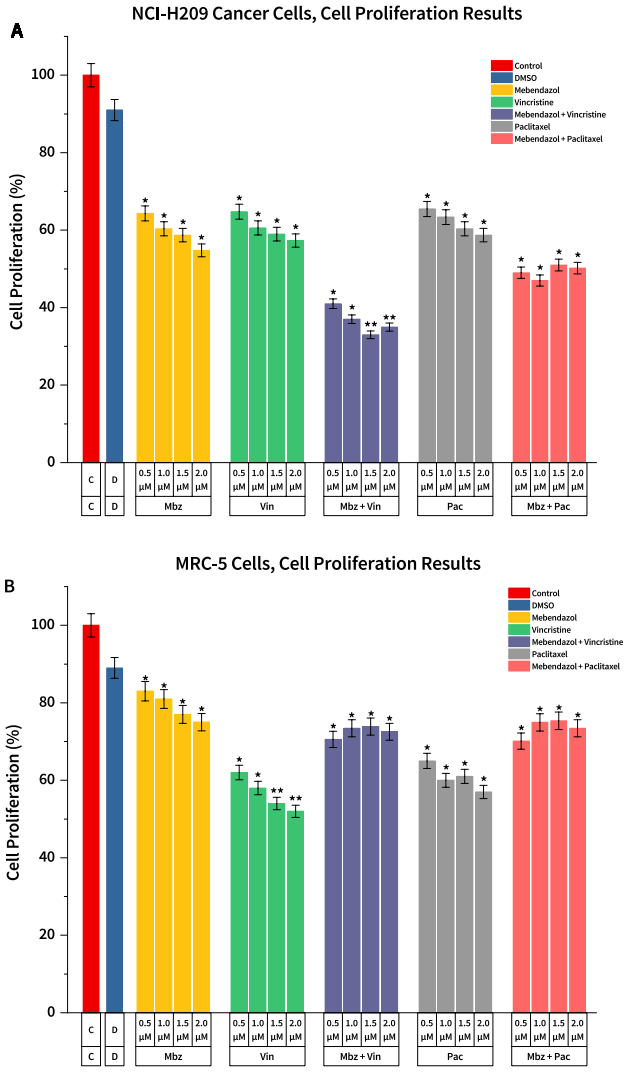


**FIGURE 1. Cell proliferation levels of Mebendazol, Paclitaxel, Vincristine and their combinations applied at increasing concentrations in cancer cells (A) and healthy cells (B) by MTT assay. The drugs were applied to the cells at a dose range of 0.5–250 µM for 24 h. The standard deviations of the obtained data were calculated. **$P$<0.01 compared to the control group; *$P$<0.05**

healthy cells. As the inhibition of cell proliferation was approximately 50% within the dose range of 0.5–2 µM, the study was continued within this dosage range.

## Apoptotic DNA laddern findings

Apoptosis levels of Mbz, Pac, Vin, and their combinations on cancerous NCI–H209 and healthy MRC–5 lung cells were determined using the apoptotic DNA assay. It was observed that apoptotic DNA levels significantly increased in both cancer cells (FIG. 2) and healthy cells (FIG. 2) within a dose–dependent range of 0.5–2 µM compared to the control group (**$P$<0.01; *$P$<0.05). However, when the increases in apoptotic DNA levels in healthy and cancer cells are compared, it is observed that the increase in healthy cells in groups where mebendazole is administered alone is less than that obtained in cancer cells. Additionally, the increase in the rate of apoptosis in cancer cells in groups where mebendazole and vincristine are administered in combination is noteworthy.

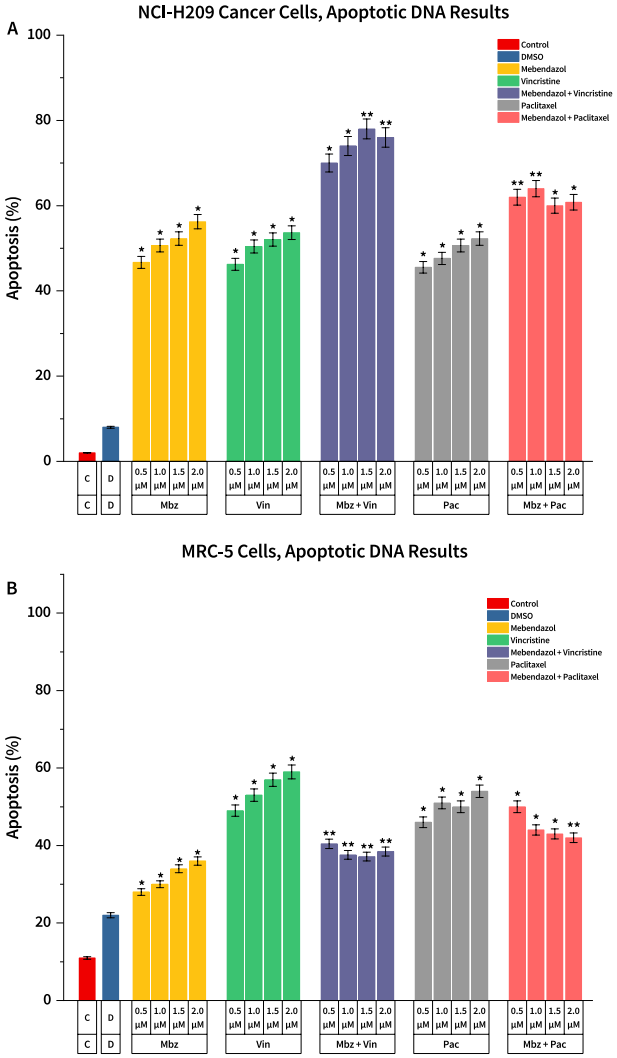


**FIGURE 2. Apoptotic DNA levels (%) found in apoptotic DNA ladder experiments in cancer cells (A) and healthy cells (B) in drug administered groups. When compared with the control group, significant increases in apoptotic DNA levels were observed in all groups. **$P$<0.01; *$P$<0.05**

## Caspase–3 level findings

Caspase–3 levels showed an increase in all groups receiving the drugs, following a dose–dependent pattern compared to the control group. Particularly, the groups treated with Mebendazole in combination with vincristine and paclitaxel exhibited more significant increases in caspase–3 levels in cancerous NCI–H209 cells (FIG. 3). Similarly, elevated caspase–3 levels were observed in healthy MRC–5 cells (FIG. 3). (**$P$<0.01; *$P$<0.05). The increase in groups where mebendazole and vincristine are administered to cancer cells is more pronounced compared to other groups. When comparing the groups where mebendazole is applied alone, it is also observed that the increase in healthy cells is less than the elevation observed in cancer cells.

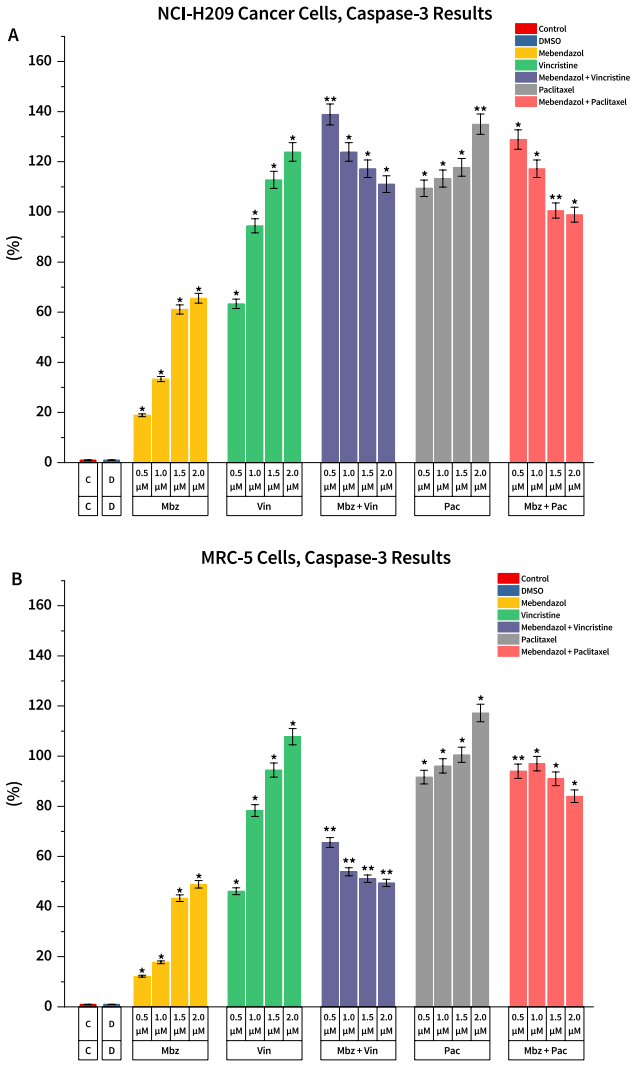


**FIGURE 3. Caspase–3 levels measured in NCI–H209 cancer cells (A) and MRC–5 healthy lung cells (B) treated with increasing concentrations of the drug compared to the control group. Caspase–3 levels increased in all groups compared to the control group (%). **$P$<0.01; *$P$<0.05**

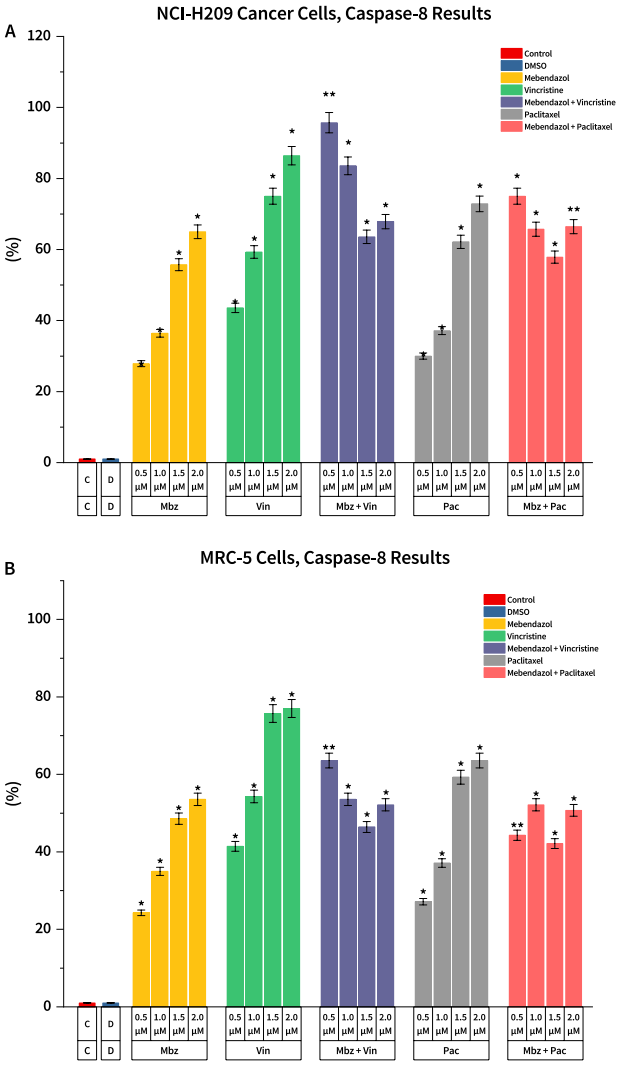


**FIGURE 4. Caspase–8 levels in NCI–H209 cancer cells (A) and MRC–5 healthy lung cells (B) compared to the control group. Caspase–8 levels increased in all groups compared to the control group (%). **$P$<0.01; *$P$<0.05**

## Caspase–8 level findings

A dose–dependent increase in caspase–8 levels was observed in both cancerous NCI–H209 cells and healthy MRC–5 cells (FIG. 4) compared to the control group. The increase in caspase–8 levels was consistent with the increase in caspase–3 levels (FIG. 3). (**$P$<0.01; *$P$<0.05).

## Caspase–9 level findings

There was a dose–dependent increase (%) in caspase–9 levels in the groups treated with drugs compared to the control group. Increased caspase–9 levels were observed in both cancerous NCI–H209 cells and healthy MRC–5 cells (FIG. 5) upon administration of Mebendazole (**$P$<0.01; *$P$<0.05).

## *In vitro* wound healing level findings

In the cellular wound healing experiments with MRC–5 cells, the level of wound healing decreased in all treatment groups compared to the control group, and this decrease was statistically significant (FIG. 6) (**$P$<0.01; *$P$<0.05). The positive progression of the wound healing area, as indicated by the plotted data in the image analysis program, is visually depicted in FIG. 7. In terms of wound healing, the groups receiving mebendazole alone exhibited the highest levels of wound recovery. Additionally, in the groups where mebendazole was administered in combination with vincristine, the wound healing rate was higher compared to the groups where vincristine was administered alone.

In this study, we evaluated the effects of mebendazole on cell proliferation, apoptotic DNA, caspase 3–8–9 levels, and cell healing in cancerous NCI–H209 and healthy lung cells under *in vitro* conditions. The current data showed that mebendazole has a strong antiproliferative effect on cancer cells, leading to increased caspase 3–8–9 levels and apoptosis. Furthermore, the combination of Mebendazole with vincristine demonstrated more effective anti–cancer activity compared to paclitaxel. The obtained findings suggest that

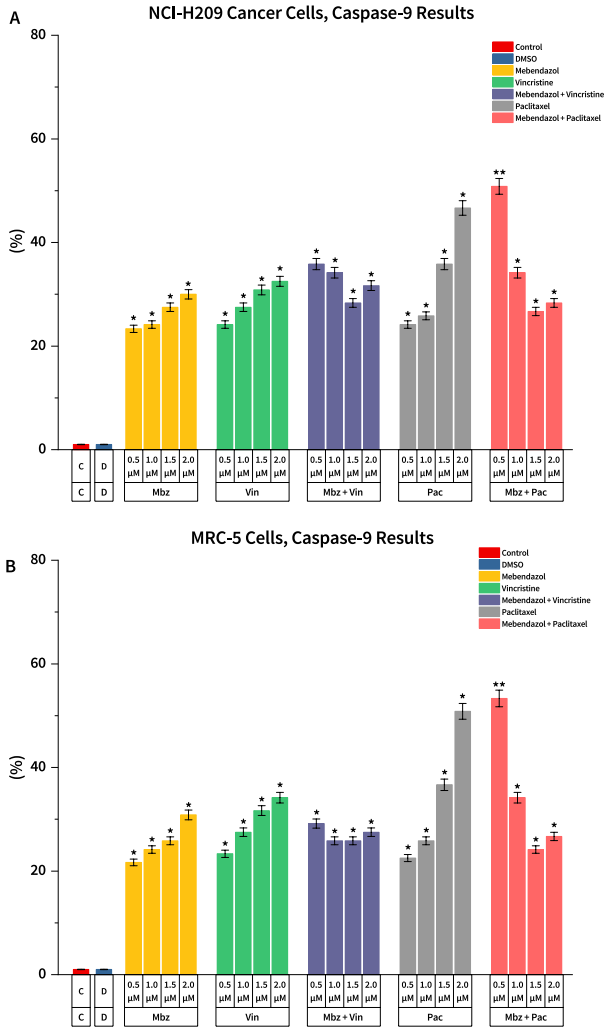


**FIGURE 5. Caspase-9 levels measured in NCI-H209 cancer cells (A) and MRC-5 healthy lung cells (B) treated with increasing concentrations of drugs compared to the control group (%). **$P$<0.01; *$P$<0.05**

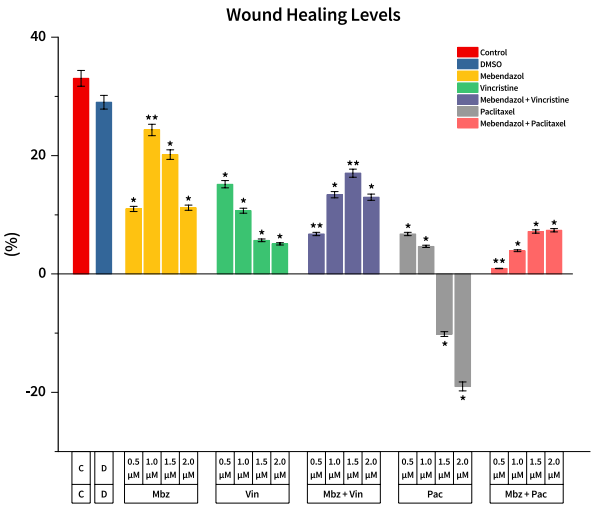


**FIGURE 6. Wound healing levels achieved on healthy lung cells (MRC-5) treated with increasing doses of drugs. While cellular wound healing was positive in other application groups other than Paclitaxel, cellular wound level showed a negative course in the groups treated with 1.5 and 2.0 µM Paclitaxel. (**$P$<0.01; *$P$<0.05)**

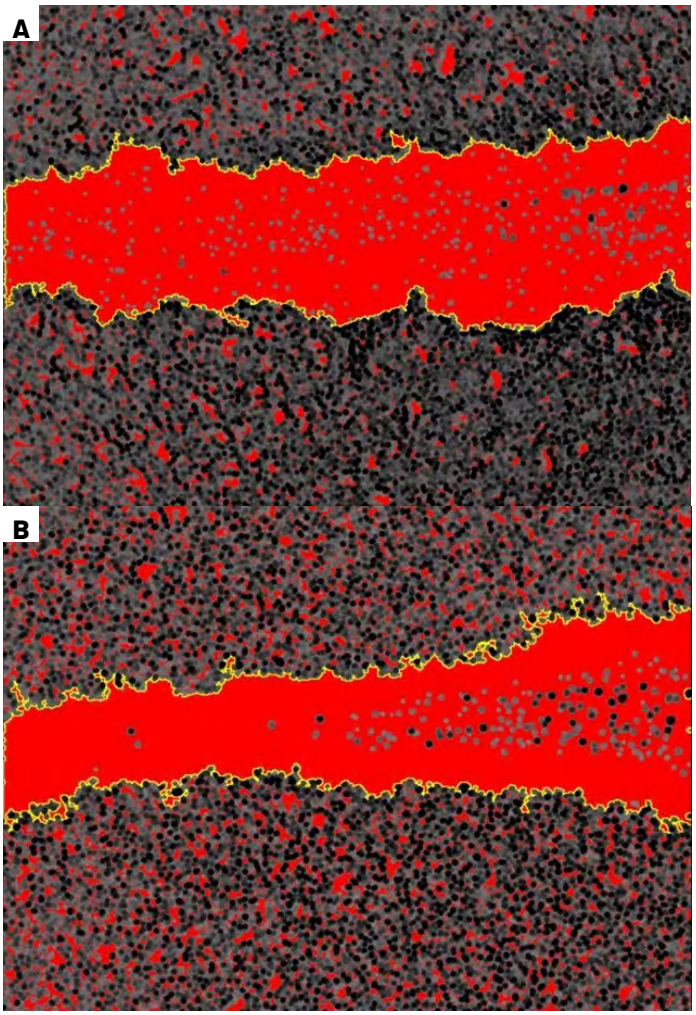


**FIGURE 7. Wound healing image with positive direction. Plotted images of the injured area before drug administration (A) and images taken from the same area after drug trial (B) in the image analyzer. The red areas represent the plotted wound area, the yellow contour lines represent the wound lip formation, and the gray areas represent the monolayer layer**

Mebendazole exerts its suppressive effects on cancer cells through caspase-mediated apoptosis. In healthy MRC-5 cells, an increase in apoptoze and caspase levels has also been observed. However, it is noted that the antiproliferative and apoptotic effects obtained in normal cell groups treated with mebendazole are minimal compared to the findings obtained from cancer cells.

Previous studies have reported the antiproliferative, proapoptotic, antiangiogenic, migration, and metastasis suppressive effects of Mebendazole in different types of cancer by disrupting tubulin protein structure and affecting critical steps in cellular processes [3, 4, 14, 15, 16, 17, 18]. The anti-cancer activity of Mebendazole in human lung cells has been documented [3]. Another study evaluating Mebendazole's potential anti-cancer effect on lung cells reported that it disrupts tubulin protein structure and induces apoptosis in cancer cells with damaged mitotic processes. The study also compared the effectiveness of Mebendazole with paclitaxel and found no side effects associated with Mebendazole administration [4]. The disruption of tubulin polymerization is considered the primary mechanism underlying Mebendazole's anti-cancer activity in various cancer cells [4, 16, 19].

Classical anti-neoplastic agents such as paclitaxel and vincristine exert their anti-cancer effects by targeting tubulin protein [6, 20, 21, 22]. It has been reported in the literature that vincristine, like mebendazole, inhibits tubulin polymerization [23]. Our study's results are consistent with these literature sources. Based on our study's data (FIG. 1), we suggest that mebendazole's antiproliferative effects on the cancerous lung cell line are closely related to its inhibition of tubulin polymerization, and the increased antiproliferative activity in the combination groups with vincristine likely utilizes a similar mechanism of action.

Apoptosis is defined as a programmed cell death mechanism, vital for eliminating damaged cells and regulating regular cell proliferation [24]. Utilizing apoptosis and its mechanisms in cancer treatment is considered an ideal approach [25]. The observation of apoptotic DNA fragments in cells undergoing apoptosis serves as a marker to distinguish necrosis from apoptosis [26]. The increased levels of apoptotic DNA in cancerous NCI-H209 cells and MRC-5 cells in our study (FIG. 2) indicate that the antiproliferative effect of mebendazole in cancer cells is mediated through apoptosis. In the mechanisms of apoptosis formation, the effector caspase-3 is activated through intrinsic and extrinsic pathways, initiating the apoptosis process [27]. The increase in caspase-3 levels observed in both cancerous and healthy cells in our study (FIG. 3) also suggests that the antiproliferative effect of mebendazole in cancer cells occurs through apoptosis. While caspase-8 is crucial for the intrinsic activation of apoptosis, caspase-9 plays a role in the receptor-mediated extrinsic pathway [28]. We determined the levels of caspase-8 (FIG. 4) and caspase-9 (FIG. 5) in the cancerous and healthy cell lines used in our study. Comparing the caspase-8 and caspase-9 levels obtained in our study, the increase in caspase-8 levels shows a parallel trend with the increase observed in the caspase-3 experiments. Similar increases in caspase-3 and caspase-8 levels indicate that the increase in apoptotic DNA levels in our study occurred through the intrinsic pathway. Furthermore, this finding explains that the increased efficacy in the groups where mebendazole is combined with vincristine and paclitaxel is not receptor-mediated.

Cellular wound healing assays are used to assess the migration abilities of cancerous cells under *in vitro* conditions [13, 29, 30]. In the present study, the findings from the wound healing experiment using healthy MRC-5 cells (FIG. 6) demonstrate that Mebendazole and vincristine positively enhance wound healing in a dose-dependent manner. Conversely, high doses of paclitaxel inhibit wound healing and negatively impact the wound. These results obtained in a healthy cell line indicate that the combined use of Mebendazole with vincristine mitigates the undesirable antiproliferative effect of vincristine on healthy cells.

## CONCLUSION

In this study, a dose-dependent decrease in proliferation, an elevation in caspase levels, and an increase in apoptosis were observed in both cancerous and healthy lung cells. However, in the groups treated with mebendazole in healthy cells, these increases were found to be at a minimal level compared to cancer cells. In conclusion, based on the data obtained from the current study, it can be concluded that Mebendazole inhibits cell proliferation in cancerous cells through intrinsic apoptotic pathways, leading to apoptosis. Furthermore, the combination of Mebendazole with vincristine enhances its anti-cancer activity. In healthy cells, however, the antiproliferative and apoptotic effects of mebendazole are minimal compared to cancer cells. These findings suggest that Mebendazole holds therapeutic potential for cancer treatment. However, further *in vitro* and *in vivo* experiments with diverse cancer types are required to validate these results.

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### Institutional review board statement

Not applicable.

### Informed consent statement

Not applicable.

### Data availability statement

The data are available by the corresponding author upon.

### Conflicts of interest

The authors declare no conflict of interest.

### Sample availability

Samples of the compounds are available from the authors.

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