RESEARCH ARTICLE

Mebendazole Suppresses Tumor Growth and Hinders the Invasion of Triple-Negative Breast Cancer in Model Systems by Disrupting RAN-GTP Regulation

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ABSTRACT

Due to the lack of targeted therapies, triple-negative breast cancer (TNBC), one of the most aggressive types of cancer, has a poor prognosis and a high death rate. TNBC frequently metastasizes to the brain, bones, and lungs. Studies have demonstrated a correlation between the metastatic behavior of A594 lung cancer cells and MDA-MB-231 breast cancer cells and the overexpression of the RAN GTP (RAN) gene. Using a variety of biological assays, this study sought to investigate mebendazole's potential as an anticancer agent by specifically targeting the RAN gene. Both MDA-MB-231 breast cancer cells (IC_{50} 7.5 µM) and A549 lung cancer cells (IC_{50} 48.5 µM) were shown to be resistant to mebendazole's ability to promote cell growth. The cytotoxic effect of mebendazole via the apoptotic pathway was confirmed by Annexin V assays on both cell lines. Furthermore, mebendazole demonstrated enhanced efficacy against TNBC by halting the cell cycle, preventing colony formation, invasion, and migration, and reducing RAN GTPase expression in both cell lines.

Keywords: Apoptosis, Metastasis, Migration, Proliferation, Ran-GTP, TNBC.

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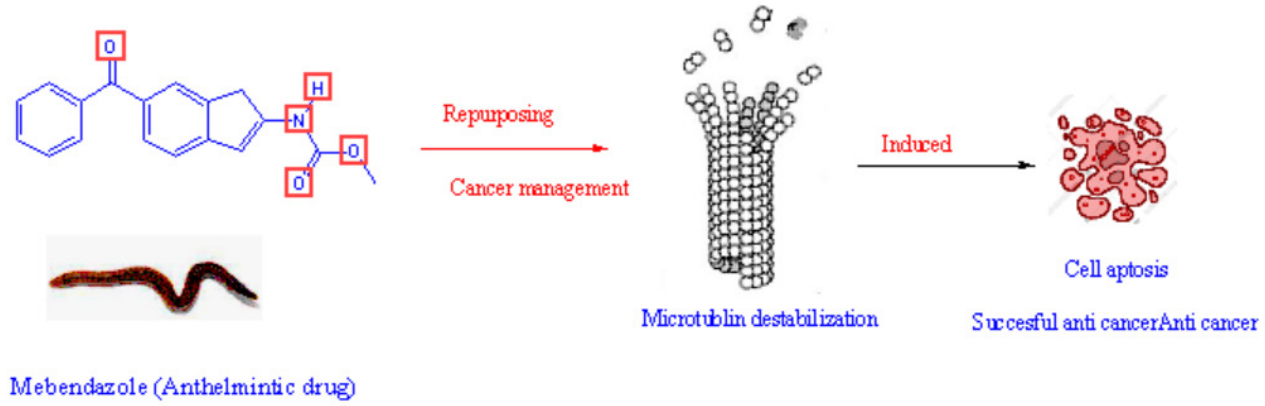

Graphical Abstract

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INTRODUCTION

Genetic abnormalities in cancer result in unregulated cell division and invasion of local tissue. This process, known as metastasis, can cause the disease to spread to distant tissues.[1] In 2018, more than 2.1 million new cases of breast cancer were diagnosed worldwide by medical professionals.[2] Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, constituting 10 to 20% of all breast cancer cases. It is characterized by the low expression of progesterone, estrogen, and human epidermal growth factor receptor 2 (HER2).[3] TNBC has a poor prognosis and cannot be treated with endocrine therapy, also known as targeted therapy. As a result, TNBC requires unique therapeutic strategies because simple medicines that target these receptors are ineffective for the condition.[4] TNBC usually occurs in younger women (<50 years old), especially in women harboring mutations in the BRCA1 gene and women with specific extraction or economic status (more common in African-American and Hispanic origins). Although there is evidence that tumor cross-talk in TNBC causes secondary non-small cell lung carcinoma (NSCLC) to develop, NSCLC metastasis has also been connected to hepatocellular carcinoma, bone cancer, and the brain.[5,6]

Breast cancer metastasis, angiogenesis, cell motility, and proliferation can all be aided by the receptor for an epidermal growth factor (EGFR). Research has shown that 45% of TNBC patients and 50 to 75% of breast cancer cells express EGFR, which results in a more aggressive tumor compared to cells that do not express this receptor.[7] Therefore, the overexpression of EGFR in TNBC commonly increases resistance to conventional therapies.[8] However, the hepatic growth factor receptor (MET) protein has been shown to play a critical role in the development of breast cancer.[9] Studies have demonstrated that suppressing MET chemically or biologically reduces breast cancer metastasis in the most aggressive subtype of the disease.[10]

Ran GTPase plays a crucial part in cell invasion and division.[11] Generally speaking, in various lung and cancerous breast cell lines, inhibition of Ran GTPase increases the low expression of Met- receptor, which inhibits cell adhesion, migration, and invasion.[12] Additionally, RAN GTPase has the ability to control EGFR expression and the activation of the ERK/AKT signaling pathways, both of which may be linked to cancer at various stages, from the onset of the disease to metastasis.[13,14]

Repurposing drugs, often referred to as therapeutic switching or drug proposition," involves using an established therapeutic

medicine now in use to treat a different or entirely new ailment,[15] this approach is quite helpful as a possible tactical procedure that can aid in lowering the price and development time of novel anticancer medications.[16] Mebendazole, an antiparasitic drug, is effective against mature Schistosoma Mansoni worms.[17] Recent research has revealed that mebendazole can stimulate apoptosis in various malignancy types, including stomach, lung, breast, oral, prostate, bladder, hepatocellular, pancreatic, and ovarian cancers.[18] Mebendazole triggers programmed cell death in certain cells that lack death-associated proteins by enhancing oxidant stress. This is one of several theories proposed to explain its mechanism of action as an anticancer drug.[19]

According to recent research by Choi and his team in 2022,[20] mebendazole combined with radiotherapy has been shown to exert a stronger anticancer effect on TNBC by impeding the cell cycle development, inducing breaks in the double strands of DNA, and enhancing cytotoxicity managed by natural killer cells, thereby overcoming radiation resistance.[20]

A primary goal of this research was to demonstrate that mebendazole inhibited the migration of breast and lung cancerous cells. We selected the formulations the fact that most effectively reduced cell viability to evaluate their effects on migration and invasion, thus streamlining the experimental design.

RESULTS

In-vitro Cytotoxicity Assay

In comparison to DMSO, a cell viability assay was conducted using the MTT protocol to evaluate the cytotoxic effects of mebendazole on MDA/MB/231 and A549 cells. Following 24-hour incubation of the drug treatments, the IC_{50} values were determined, as illustrated in Figure 1A for MDA/MB/231 cells and Figure 1B for A549 cells. The results showed that after 24 hours, the IC_{50} of mebendazole was 10.6 µM for MDA/MB/231 cells and 25.3 µM for A549 cells. In contrast, DMSO, used as a control, had no significant effect.[21]

Colonies Assay

A colony formation technique is a widely utilized *in-vitro* approach to evaluate malignant cell proliferation. This method allows researchers to assess the long-term efficacy of anticancer agents.[22] As shown by the scratch assay, the drugs were expected to exhibit the highest cytotoxicity against MDA/MB/231 and A549 cell lines, with a significant reduction of growth observed from the first day up to 9 days at 1/2 IC_{50}, and almost no colonies at IC_{50} and 2 IC_{50} treatments compared to the media and DMSO controls. Notably, colonies number growing after treatment with mebendazole, as illustrated in Figures 2 and 3, underscores the drug's ability to provide a long-lasting and persistent inhibition of the MDA/MB/231 cancer cell line's growth and division Elayapillai *et al.*[16]

In-vitro Wound Healing Assay

The scratch technique is a widely used method to assess cancer cell migration by monitoring the closure of a created scratch. In this study, MDA/MB/231 and A549 cells were dealt with half, one and two IC_{50} concentrations of mebendazole alongside media and DMSO controls. Images of the scratch were taken after 48 hours of incubation. The wound closure percentages for MDA/MB/231 and A549 cells at these mebendazole concentrations were compared to the controls, as depicted in Figures 4A-D. For MDA/MB/231 cells, the wound closure percentages were 9.92, 13.17, 0.99, and 31.50%, respectively,

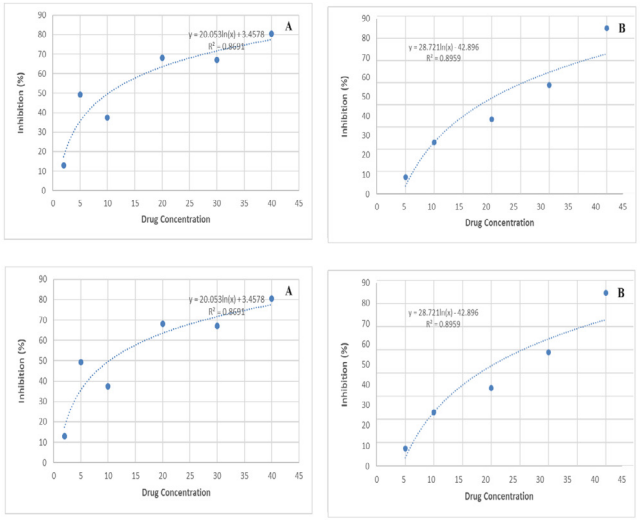

Figure 1: A) %Inhibition of mebendazole on MDA 231 cell line and B) %Inhibition of mebendazole on A549 cell line

For all treatment concentrations, mebendazole treatment resulted in a decrease in relative scratch closure. These results show that mebendazole stops both cell lines (MDA/MB/231 and A549) from migrating, which is very important for stopping breast and lung cancer cells from spreading.[21]

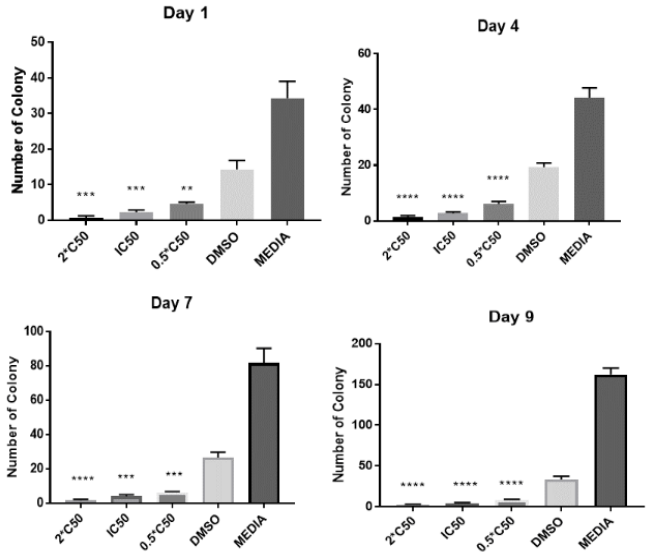

Figure 2: Modulatory impact of mebendazole on the number of colonies of MDA/MB/231 cell lines. The result represents the mean of the number of colonies under different concentrations of mebendazole during 9 days. The final result is stated as averages ± SD (n = 3-4 independent replicates). *: p <0.05, **: p <0.01, *** : p <0.001, ****: p <0.0001 compared to respective DMSO treatment (negative control). The results were analyzed using one-way ANOVA.

whereas the media control showed 99.8% and the DMSO control showed 99.5% closure. For the A549 cell line, the closure percentages were 0.92, 1.0, and 0.99%, compared to the controls: media at 99.7% and DMSO at 99.4%, as demonstrated in Figure 4A and B.

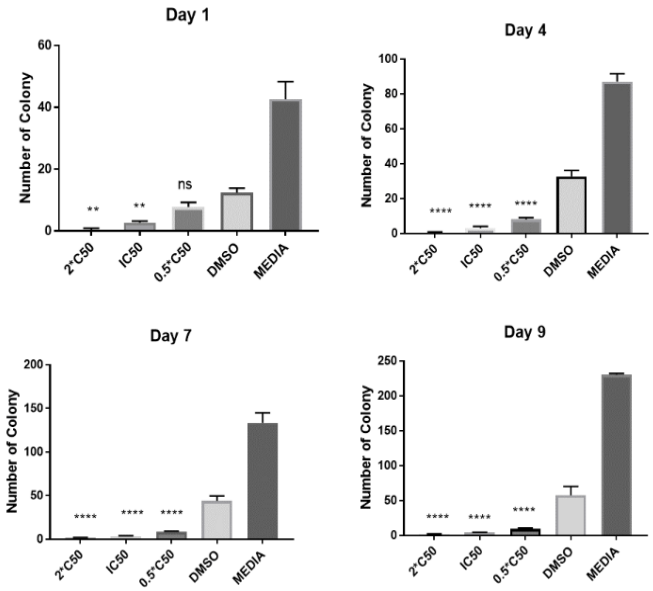

Figure 3: Modulatory effect of mebendazole on the number of colony of A549 cell lines. The result represents the mean of the number of colonies under different concentrations of mebendazole during 9 days. The final result is stated as averages. ± SD (n = 3-4 independent replicates). *: p <0.05, **: p <0.01, ***: p <0.001, ****: p <0.0001 compared to respective DMSO treatment (negative control). The result was analyzed using one-way ANOVA

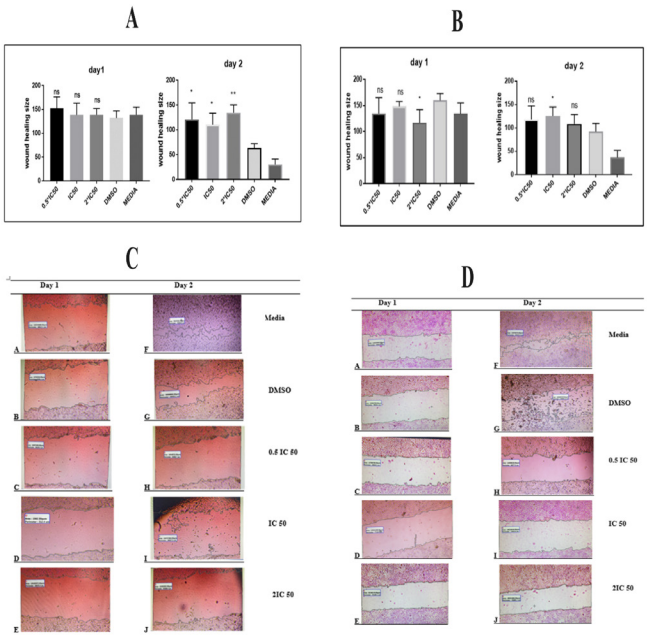

Figure 4: A) Modulatory Effect of Mebendazole on Wound Healing of MDA/MB/231 Cell Lines; B) Analyzed using one-way ANOVA; C) Microscopy images of MDA/MB/231- cell treated lines showing the effect of mebendazole on cell migration, and; D) Microscopy images of A549-treated cell lines displaying the effect of mebendazole on cell migration[23]

Interestingly, the images show that the percentage of the closed area was inversely related to the concentration of treated cells at 1/2 IC_{50}, IC_{50}, and 2 IC_{50} in both the MDA/MB/231 and A549

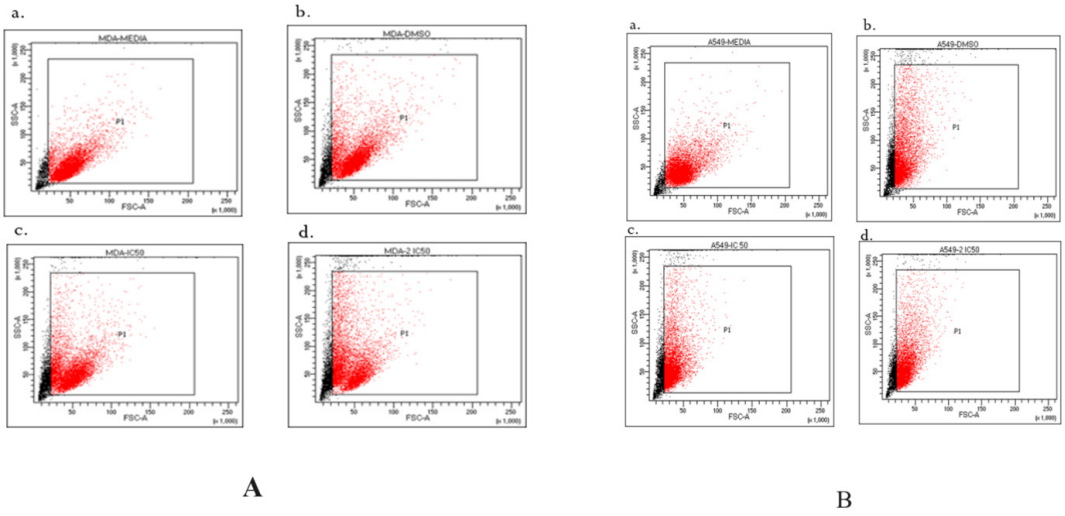

Figure 5: A) Flow cytometric technique in MDA/MB/231 Cell Line of Breast Cancer after Treatment with Mebendazole. a. untreated cells (media), b. control cells (DMSO), c. treated cells (IC50), d. treated cells (2IC50), B) Flow cytometric analysis in A549 lung cancer cell line after treatment with mebendazole. a. untreated cells (media), b. control cells (DMSO), c. treated cells (IC50), d. treated cells (2IC50).

cell lines, as seen in Figure 4C and D. As the concentration of treated cells increased, the percentage of closure decreased, reaching around 1% for both cell lines at IC_{50} and 2 IC_{50} drug concentrations.

The result represents the mean wound healing size under different concentrations of mebendazole.. is the following indicates Statistical significance as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the corresponding DMSO treatment (negative control), corresponding to non-significant, The results were analyzed using one-way ANOVA, B) Modulatory effect of mebendazole on wound healing in A549 cell lines. The result represents the mean wound healing size under different concentrations of mebendazole.. the following indicates Statistical significance: *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the respective DMSO treatment (negative control), ns corresponding to non-significant. The results were

Flow cytometry Results

At the approved IC_{50} concentration, the flow cytometry test displayed that cell viability was 42.3%, which decreased to 13.9% at double the IC_{50} value. We concluded a concentration-dependent elevate in the total percentage of apoptosis. Furthermore, findings suggested an increase in late apoptosis in MDA/MB/231 cells upon expose to twice the treatment's IC_{50} drug concentration (Figure 5A), indicating potential chromosomal fragmentation and nuclear membrane degradation with increasing treatment concentrations. Flow cytometry analysis on A549 cells confirmed that both the IC_{50} concentration and twice the IC_{50} concentration of the treatment initiated the late apoptosis process (Figure 5B). Li *et al.*[24]

qRT-PCR-Gene

The MDA/MB/231 and A549 cell lines were seeded and treated with the IC_{50} concentration of mebendazole for 24 hours, using DMSO as a control. After treatment, cells were collected and

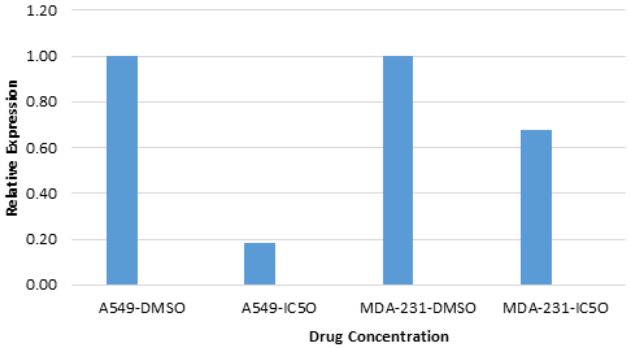

Figure 6: RAN Gene Expression Measured by qRT-PCR After a 24-Hours, where MDA/MB/231 Cell Line Treated with 10.6 µM and A549 Cell Line Treated with 25.3 µM of Mebendazole, Control was DMSO. There was a notable decrease in the expression of RAN in both cell lines when treated with mebendazole as a result

analyzed for Ran mRNA levels. Results showed that Ran expression was reduced by up to 70% in MDA/MB/231 cells and by 30% in A549 cells after 24 hours, while the control showed no significant change as appeared in Figure 6 Chen *et al.*[25]

DISCUSSION

The study investigates into the detailed analysis and implications of the results obtained from the study on the effectiveness of mebendazole as an anticancer agent, particularly focusing on its effects on MDA/MB/231 and and A549 cell line ofbreast cancer cellsand lung cancer cells respectively.

The study demonstrates the mebendazole drug effectively disrupts the AKT and Ran signaling pathways, leading to reduced levels of Ran-GTPase in cancer cells. This disruption halts the proliferation of triple-negative-breast-cancer (TNBC)

and non-small-cell-lung (NSCL) by causing apoptosis, a type of planned cell death that is necessary to halt the growth and spread of cancer cells.

Subsequent examination uncovers the dose-dependent cytotoxic effects of metronidazole on the cancer cell-lines; MDA/MB/231 cells exhibit the highest level of toxicity, with an IC_{50} of 10.6 μM. This demonstrates the potency and efficiency of mebendazole in preventing the durability and expansion of cells in a concentration-direct relation way.

The scratch assay results indicate a significant prevention of cancer cell migration, a critical factor in cancer metastasis. Mebendazole effectively prevents the migration of both MDA/MB/231 and A549 cells, with almost complete inhibition at IC_{50} and 2IC50 concentrations. This highlights mebendazole's potential as an antimetastatic agent, capable of preventing the spread of cancer cells to other parts of the body.

Colony formation assays further confirm mebendazole's respectively, anticancer efficacy, showing a marked reduction in colony inhibits cancer cell growth and proliferation. The flow cytometric analysis corroborates these findings, revealing increased apoptosis and cell fragmentation upon mebendazole treatment, signifying its role in promoting cell death and reducing tumor viability.

The study also notes the potential therapeutic implications of mebendazole, particularly its ability to overcome drug resistance and its synergistic effects when combined with other cancer therapies. This opens up new avenues for research and treatment strategies, particularly for aggressive cancers like TNBC and NSCL, which are often challenging to treat due to their resistance to conventional therapies.

This study highlights several key aspects. One of the main strengths is the innovative application of mebendazole, traditionally an antiparasitic medication, as an anticancer agent against triple-negative-breas-cancer (TNBC) and lung cancer. This approach not only sheds light on the drug's novel anticancer properties but also suggests a potential pathway for repurposing existing drugs, which could significantly reduce the time and financial resources required for new drug development. The study provides a thorough mechanistic understanding of how mebendazole disrupts cancer cell proliferation and survival, particularly *via* the inhibition of AKT and Ran signaling possible pathways and the induction of apoptosis, employing a variety of biological assays to support these findings.

The interpretation of the study's results aligns well with existing literature, reinforcing the notion that mebendazole could be a viable candidate for cancer therapy, given its ability to inhibit cancer cell migration and invasion, thereby preventing metastasis. This aligns with other published studies that have noted similar mebendazole's anticancer effects, further supporting its repurposing for cancer treatment.

The results provides a comprehensive understanding of the anticancer effects of mebendazole on TNBC and lung cancer cells. The study demonstrates that mebendazole effectively disrupts the AKT and Ran signaling pathways, likely through a reduction in Ran-GTPase levels within cancer cells, leading to the induction of apoptosis as a means of preventing the growth of cancer cells.

This finding aligns with previous research, such as the work by Osada *et al*.,[26] which showed mebendazole's effectiveness against pancreatic cancer cells by inducing rapid phosphorylation of ERK and inhibiting cell populations.

The study's use of a scratch assay to measure migration by evaluating gap closure validates mebendazole's anti-proliferative and anti-invasive properties. The results demonstrate a notable decline in the rate of wound closure for both MDA/MB/231 and A549 of breast cancer cells lung cancer cells compared to controls, highlighting the efficiency of mebendazole in limiting cancer cell migration. This is particularly important as it suggests formation, indicative of its long-term

Mebendazole's potential as a powerful antimetastatic drug, capable of inhibiting tumor growth, inducing apoptosis, and preventing cancer invasion through the dysregulation of Ran-GTPase.

Colony formation assays confirmed mebendazole's effectiveness, showing a marked reduction in colony formation from the first 24 hours up to 9 days of treatment, further supporting its long-term inhibitory effects on cancer cell proliferation. Flow cytometric analysis corroborated these findings, revealing an increase in apoptotic cell fragments in treated cells, indicating enhanced cell death and reduced viability.

The study's findings are compatible with prior research, such as that by Sheng *et al*.,[27] which found that knocking down Ran GTPase slows wound healing, suggesting a vital component of Ran in malignant cell migration and proliferation. Additionally, the study by Yuen *et al*.[28] Ran knockdown drastically reduced colony formation in invasive MDA/MB/231 cells, confirming that Ran contributes significantly to the development of cancer. The study proposes that *in-vivo* tests be used in the future to confirm mebendazole's safety and effectiveness in animal models.

This would be a critical step before moving towards clinical trials. Investigating the potential synergistic effects of mebendazole when combined with other chemotherapy agents could also uncover new therapeutic strategies that enhance its anticancer effects.[29]

In conclusion, the study presents compelling evidence of mebendazole's anticancer effects against TNBC and lung cancer *in-vitro*, mediated through the disruption of critical cellular signaling pathways and the promotion of apoptosis. While the findings are promising, they underscore the need for further in-depth research, particularly through *in-vivo* studies, to fully elucidate the drug's potential as a cancer therapeutic agent and to move closer to clinical application.

In conclusion, the detailed discussion in the article emphasizes mebendazole's promising role as an anticancer agent, with significant implications for cancer treatment, especially in overcoming medication resistance and enhancing

the effective of existing targeting therapies. The study paves the way for further research into mebendazole's potential applications in cancer treatment, underscoring the importance of repurposing existing drugs for new therapeutic uses in oncology.

CONCLUSION

This research marks the inaugural documentation of the anticancer properties of mebendazole, an FDA-approved medication already utilized in clinical practice, particularly exhibiting efficacy against TNBC. since the cellular investigation results demonstrated the effectiveness in inhibiting cell proliferation and induced apoptosis and anti-invasive activity of both MDA/MB/231 and A-549 cell lines compared to DMSO and media control after 24 hours with the superiority effect of IC_{50} and $2IC_{50}$ of mebendazole concentration in wound closure. Thus, mebendazole has potential as a therapeutic agent to prevent cancer cells with the potential to spread by preventing their invasion and migration, but it can also promote cancer growth by interfering with the regulation of Ran GTPase.

Mebendazole's ability to overcome drug resistance may contribute to its synergistic effect with other medications, opening up new possibilities for future research with tyrosine kinase inhibitors. This groundbreaking study demonstrated the anticancer properties of mebendazole, either by itself or in conjunction with other medications, against lung cancer and TNBC. Emphasizing the drug's suitability in the clinic is crucial due to mebendazole's existing commercialization and popularity among patients. In conclusion, we hypothesize that the produced nanoformulation, which serves as a carrier for mebendazole alone or in conjunction with various tyrosine kinase inhibitors, may increase mebendazole's cytotoxic effect on lung cancer cell lines *in-vitro*. This combination could potentially synergize to act as an effective antimetastatic treatment by impeding the activity of these encapsulation-targeting medications against Ran GTPase..

Materials

The MDA-MB-231 human breast cancer cell line and the A549 lung cancer cell line, both obtained from the University of Jordan, were used in cell culture experiments.

Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, penicillin/streptomycin solution, and foetal bovine serum (FBS) were obtained from EuroClone (UK). Dimethyl sulfoxide (DMSO) was purchased from Gainland Chemical Company (UK), and trypsin and phosphate buffered saline (PBS) were provided by EuroClone (UK). Alcohol is 70% from AAU Lab. 3′-(4, 5 dimethyl-thiazol-2-yl)-2, 5-diphenyl yellow tetrazolium bromide (MTT) reagents, Trypan blue staining, stop solution, dimethyl sulfoxide (DMSO), and EDTA were supplied from Promega (USA). TACS® Annexin V-FITC Apoptosis Detection Kit (Cat. #: 4830-01-K) containing TACS Annexin V-FITC, 10X binding buffer, and propidium iodide (PI) staining was brought by Biotechne (UK).

In-vitro Cytotoxicity Assay

MDA-MB-231 breast cancer and A549 lung cancer cell lines were seeded in 96-well plates at a density of 1 x 10^4 cells per well. Subsequently, they were cultured in 100 µl of DMEM medium per well and allowed to adhere overnight. Following this, cells were subjected to varying concentrations of mebendazole (2, 5, 10, 20, 30, 40 µM), while control cells received DMSO (5 µM). Next, up to 100 µl of DMEM was added to each well, and they were incubated for 24 hours at 37°C with 5% CO_2. Following the incubation period, each well received 15 µL of MTT dye solution, which was added from a stock solution containing 5 mg/mL. After an additional 4 hours of incubation at 37°C, 100 µL of stop solution was added to each well, and the plates were left to incubate for an additional 30 minutes at room temperature. Using the Microplate Reader BioTekTM ELx800TM, the optical density (OD) at 590/630 nm was used to measure the growth of the cells. With GraphPad Prism 6.01 (GraphPad Software, San Diego, USA), the dose response curve was constructed and the IC50 for each medication was determined.[30]

Cell Migration Assay (Wound Healing Assay)

Six-well plates were seeded with a suspension of 7.5 × 10^5 MDA/MB/231 and A549 cells in 2 ml of DMEM medium per well. For 24 hours, the cells were incubated at 37°C with 5% CO2. Using a sterile pipette tip, a scratch was made across the cell monolayer once confluence was reached. After removing the medium, 1-mL of PBS was used to wash the wells before they were aspirated. Sub-cytotoxic doses of the drug (IC50, 2IC50, and 1/2IC50) were added to 2 mL of media along with a DMSO control to treat the cells. These doses were based on IC_{50} values. Light microscopy was used to measure scratch closure at the beginning of the treatment and 24 hours later. Using image analysis software (MOTIK), the migration distance was computed by deducting the width of the wounds after 24 hours from the initial wound width. Every experiment was conducted three times.With GraphPad Prism 6.01 (GraphPad Software, San Diego, USA), the dose response curve was constructed and the IC50 for each medication was determined. [30]

Colony Formation Assay

MDA/MB/231 breast cancer cells and A549 lung cancer cells were seeded in 6-well plates with 2 mL of media per well for the colony formation assay. Two days were given to the cells for attachment and colony formation. Following this time frame, the cells were treated with different sub-cytotoxic doses (IC_{50}, 2IC50, and 1/2IC50), while the controls were untreated cells in media and DMSO. Using DMEM, the total volume was changed to 4 mL per well. After that, the plates were incubated with 5% CO_2 at 37°C.[30]

Flow Cytometry Analysis (AnnexinV Assay)

Using propidium iodide (PI) staining, the percentage of cells in the sub-G1 phase was used to assess apoptosis. Following the manufacturer's instructions, Annexin V staining was carried out using the Roche Annexin V-Fluos staining kit. The stained

cells were then examined using a BD LSRII flow cytometer in accordance with the guidelines provided by Yuen *et al*.

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AUTHOR CONTRIBUTION

R.R. conducted all experiments, managed data curation, utilized software, and drafted the original manuscript. M.E.-T., F.M., and M.A. contributed to the conceptualization, methodology, investigation, project administration, data validation, and also assisted in drafting the original manuscript. All authors have reviewed and approved the final published version of the manuscript.

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