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EXPERIMENTAL STUDY

Effects of Mdm2 Inhibitors on Cellular Viability of Breast Cancer Cell Lines HP100, MCF7

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ABSTRACT

BACKGROUND: Breast cancer is one of the main Cancers affecting patients all over the world; however, till now, there has been no successful treatment without any side effects on patient health, but there are groups of medications similar to MDM2 inhibitors that have promising effects.

The aim of this study was to find out whether the use of mdm2 inhibitors can help treat breast cancer using three cell lines. The use of treatments belonging to the MDM2 inhibitor alone or with the use of doxorubicin together with a combination of Nutlin-3, Miladometan, Yh239-EE, and doxorubicin in breast cancer cell lines HP100, MCF7.

MATERIALS AND METHODS: Cell lines were treated with different concentrations of MDM2 inhibitors 1,5,10, and 20 micromolar alone or in combinations with doxorubicin. After that, cell viability was estimated by the MTT assay method. Then, we have assessed the expression of caspase as an indicator of cell apoptosis after treatment, and the expression of P53 and MDM2 after and before treatment.

RESULTS: The IC50 of Doxorubicin in the MCf7 cell line was about 1.12 μM. The best IC50% in MDM2 inhibitors was for Nutlin, about 5.9 μM, then for Yh239-EE about 8.45 μM, and Milademetan about 11.07 μM the high IC50% values in normal epithelial cell HBl100. Also, the MDM2 inhibitor increased P53 levels, as did doxorubicin.

CONCLUSION: Our research concluded that Nutlin 3 has a superior effect over Yh239-EE and Miladometan in treating Breast cancer; moreover, the combination group has shown to be more effective than treatment with Doxorubicin or MDM2 inhibitors alone. Interesting information is that Doxorubicin also causes an increase in P53 levels. This result provided us with a promising therapeutic strategy for the treatment of breast cancer. However, more research is required to be conducted on more types of cell lines and in human or animal models *(Tab. 4, Fig. 8, Ref. 33)*. Text in PDF www.elis.sk

KEY WORDS: breast cancer, Miladometan, cell viability, proliferation, therapeutic strategy.

Introduction

Breast cancer is a complex disease that requires a multimodal approach to treatment. There are several medications, chemotherapy, and targeted therapy used to treat cancer. However, there is still a need for more effective and targeted treatments to improve patient outcomes without or with low side effects (1, 2). Therefore, current research focuses on targeted therapy to treat cancer; these pathways include MDM2 inhibitors and P53 activators (3). TP53 is a protein encoded in the P53 gene. Its rule is to protect the cell from mutagenic transformation by trying to fix the mutated DNA inside the cell. However, if this protein cannot resolve the cellular issue, it forces cells to the apoptosis pathway and cell death of cancer cells (4). P53 does this effect by several associated proteins such as P21 and Caspase. However, Mouse double minute 2 (MDM2) is a protein ligase responsible for the suppression of P53 level, so it may be linked to the occurrence of cancer; several studies show that more than 50% of cancers are due to MDM2 increased expression, thus suppressing P53 level (5, 6).

One of the promising types of treatment is MDM2 inhibitors, which will increase the P53 level, and this will help treat cancer and decrease its resistance to chemotherapeutic medication (7). This type of medication (mdm2 inhibitors) includes several drugs such as Yh239-EE, Nutlin-3, and Milademetan; these medications restore p53-mediated tumor suppression and enhance the effectiveness of chemotherapy (7) (Fig. 1).

Doxorubicin is one of the essential chemotherapeutic drugs used for the treatment of several types of cancer, usually as a reference drug; combining MDM2 inhibitors with doxorubicin may improve the effectiveness of Doxorubicin or decrease drug resistance by increasing P53 levels (6, 8).

Materials and methods

Cell lines

The study utilized two human breast cancer cell lines: HP100 and MCF7. The cells were obtained from the Iraqi Center for Genetics and Cancer Research. HP100 is epithelial breast cells, whereas MCF7 cell lines are estrogen receptor (ER) positive and progesterone receptor (PR) positive (9).

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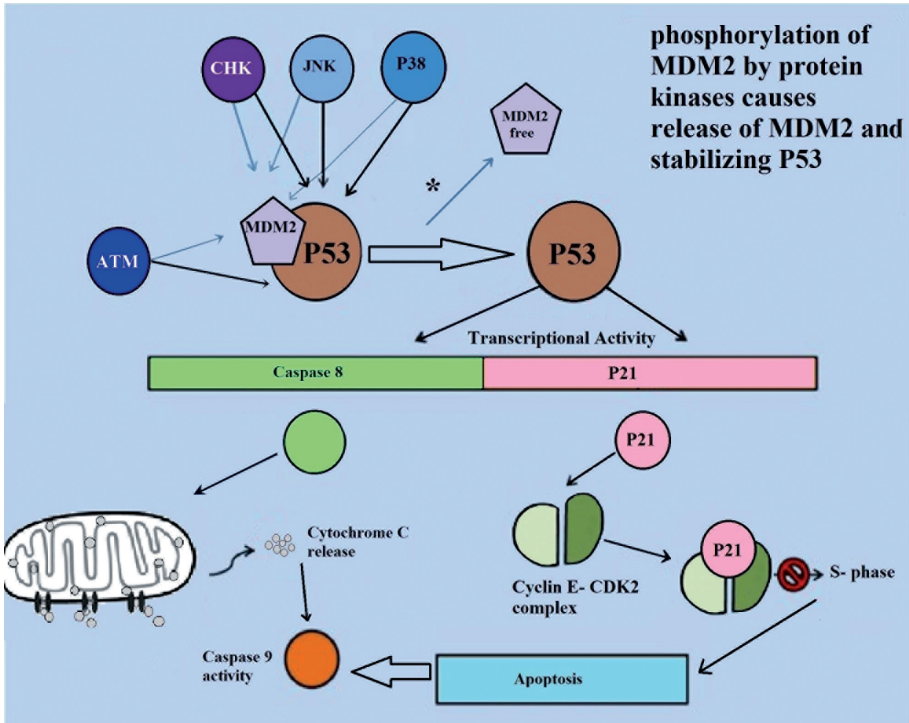

Fig. 1. The author's illustration of P53 and MDM2's roles in apoptosis Using the Paint software.

Reagents

Yh239 -EE, Nutlin-3, and Miladometan was obtained from Hangzhou Hyper Chemicals Limited (LOFT49 Hangzhou China), and doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37°C in a humidified atmosphere of 5% CO_2. For cell counting, MCF7 and HBL100 cells were seeded in 6-well plates at a density of 1 × 10^5 cells per well in complete media. After 24 hours, the cells were treated with Nutlin-3, Miladometan, YH239-EE, Doxorubicin, or combinations of these drugs for 48 hours. After treatment, the Medium will be removed, and the cells will be incubated with 10µL MTT solution (5 mg/ml) for four hours at 37°C in a dark place. Then 150 µL DMSO will be added to each well to dissolve the formazan crystals. The cells were then trypsinized and counted using a hemocytometer under a microscope. A microplate reader will be used to read the absorbance of each well at 570 nm with the background subtraction at 630 nm (14). The survival rate will be calculated according to the following formula (9, 10):

Percentage of cell viability = ((absorbance of treatment)/ (absorbance of control)) × 100.

Annexin V apoptosis test

The cell suspension was to be examined in FITC Annexin V Apoptosis Detection Kit I" (BD Pharmingen™). Still, at least 2–3 x 10^5 cells were transferred to a 15 ml Falcon after 72 hours of incubation with the test substance and centrifuged (5 minutes, 550 Rpm, 20°C, start-up and Braking stage 9). After removing the supernatant, the cell pellet was resuspended for washing in 5 ml of 4°C cold DPBS, centrifuged again, and this washing step was repeated. The supernatant was removed entirely with the help of a pipette, and the cell pellet was transferred to a 5 ml Falcon after resuspension in 100 µL 1X Binding Buffer. The final concentration should be 1 x 10^6 cells/ml. To stain the cells, 5 µl each of FITC Annexin V and PI were added, and the samples were incubated at room temperature for 15 minutes, protected from light. After adding 400 µL 1X binding buffer, the samples were ready for analysis using FACS. The total cell population was evaluated (Fig. 2A), as well as the percentages

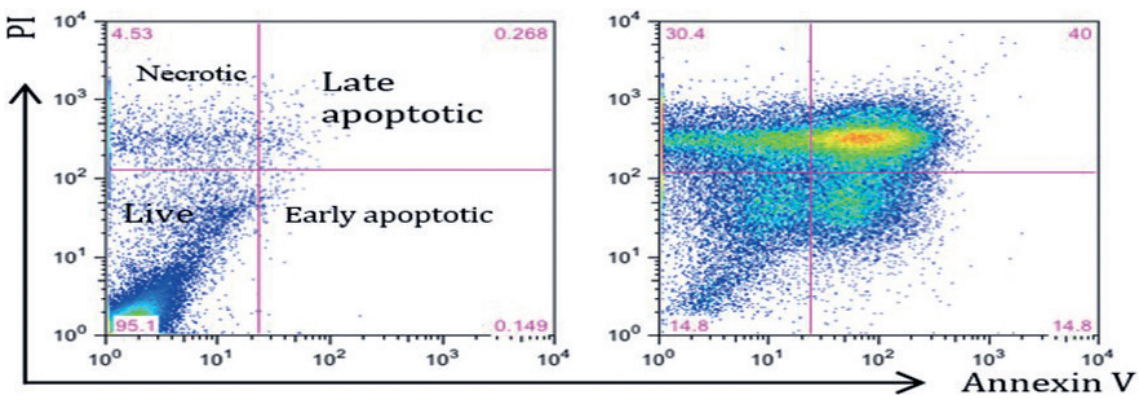

Fig. 2. The apoptosis and necrosis by annexin V test.

of RUQ, ROQ, or LOQ, based on the total cell population (Fig. 2B) to determine the increase or decrease of apoptotic or apoptotic necrotic cells within the sample material (11).

Determination of Caspase-Glo 3/7 assay method as a parameter of cell death

To obtain information about the signaling pathways leading to apoptosis, the activities of effector caspases 3 and 7, components of the extrinsic pathway, were determined using the Caspase-Glo 3/7 assay system. MCF7 and HBL100 cells were incubated with 5, 10, and 20 µM Nutlin-3a, YH239-EE, or Milademetan for 1, 6, and 24 h. Subsequently, the activity of the caspases was determined luminometrically indirectly via amino luciferin.

Immunocytochemistry study

After about 10,000 cells were seeded and fixated by permeabilization, selective antibody anti-MDM2 and P53 were added and incubated with primary antibody overnight, then followed by washing step to reduce background staining, then washing, after that secondary antibody (fluorescently labeled antibody) then incubation for one hour, then washing, to be ready for visualization step. The images were analyzed by Image J software (12).

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used to analyze the data. The mean ± standard error of the mean (SEM) was used to express the results. Tukey's multiple comparisons test was used after a one-way analysis of variance (ANOVA) to establish the statistical significance. P values were considered statistically significant if they were less than 0.05 (13).

Result

IC50 result

The following was revealed after using different groups of treatments on MCF 7 cell lines. The lowest concentration, which could kill 50% of the cells, is the doxorubicin-treated group, which destroyed 50% of the cells at a concentration of 1.12 µM. As for the highest concentration of drug on MCF 7 cells, it was Milademetan, about 11.07 µM. In 100-HP cells, the lowest concentration was for Doxorubicin, but the highest was for Nutlin, as shown in Table 1 (Fig. 3).

Annexin V test on MDM2 inhibitors (Nutlin 3a) induces apoptosis and necrosis in cell lines MCf7 and HB100

The aim of the test was to determine the proportion of apoptotic and necrotic cells after prolonged exposure to Nutlin-3a, YH239-EE, Milademetan, and doxorubicin. The two cell lines were cultured for 72 h in the presence of 20 µM of the drug, then stained with annexin V and PI and analyzed by FACS (14–16). In MCF7 cells, incubation with 20 µM Nutlin-3a resulted in 85.91% apoptosis and necrosis. In contrast, incubation with 20 µM YH239-EE resulted in 84.31%. Milademetan showed a percentage of apoptosis and necrosis of about 73.16%. The highest value of apoptosis and necrosis was for the doxorubicin 20 µM treated group, which is about 99.87%; the combination groups showed an excellent percentage of apoptosis and necrosis, about 67.73% in 10 µM nutlin+and one µM doxorubicin group and about 71.16 in YH239-EE combination groups and 65.39 in the Milademetan + doxorubicin group regarding none cancer (in epithelial cell line the value of apoptosis

Tab. 1. The inhibitory concentration 50 (IC50) effect of medication on the three cell lines.

IC50	Mcf7	HBL 100
Nutlin 3 in µM	5.9	28.44
Doxorubicin in µM	1.12	0.59
Milademetan in µM	11.07	15.34
Yh239-EE in µM	8.45	21.47

DOX = doxorubicin

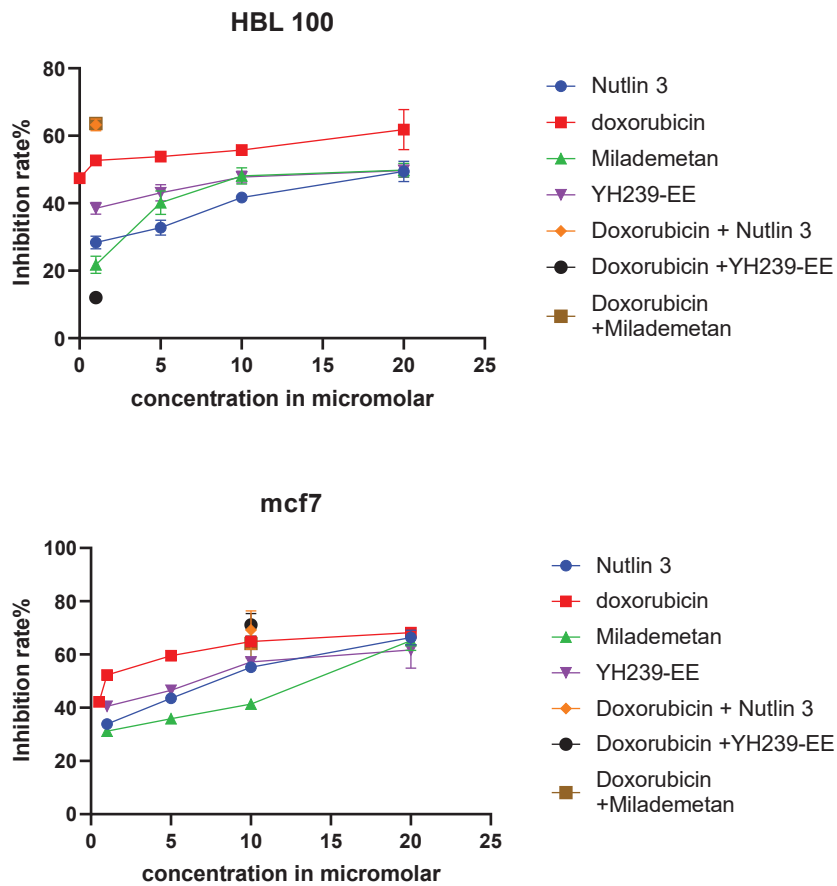

Fig. 3. The cytotoxic effect of medication on the MCF7 and HBL100 cell lines.

Tab. 2. Proportion of apoptotic and necrotic cells after treatment of 2 cell lines with MDM2 antagonists alone and in combination.

Cell line	Nutlin-3a	Dox + Nutlin 3	YH239-EE	Dox+YH239-EE	Milademetan	Milademetan + Dox	Dox	Untreated
MCF7 (%)	85.91	67.73±0.4	84.31	71.36±1.9	73.16	65.39±2.5	99.87	8.38
HB100 (%)	49.44	70.49±0.4	49.71	15.38±0.6	49.81	69.34±2.5	61.86	10.4

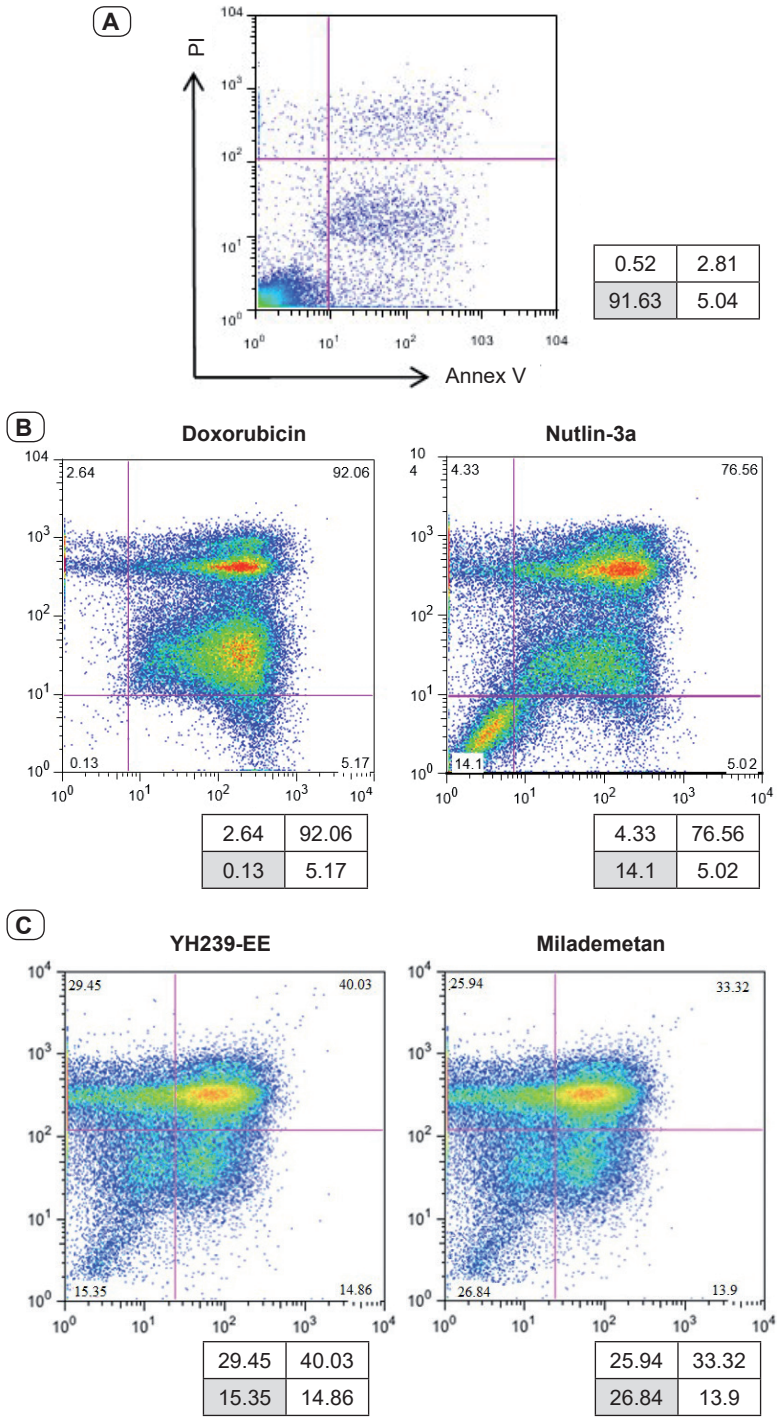

Fig. 4. Determination of apoptotic and necrotic cells by Annexin V/PI staining and FACS. Incubate the MCF7 cell lines with A = Not treated, B = Nutlin-3a, C = Doxorubicin, D = YH239-EE, and E = Miladametan for 72 h. The values show the sum of LUQ, RUQ, and RLQ in %.

and necrosis was decreased significantly in the YH239-EE groups, about 15.38%) all these results summarized in Table 2 and illustrated in Figure 4.

Result of Caspase-Glo 3/7 assay

In the presence of 20 µM Nutlin-3a, caspase activity increased 5.4 times after six h and 7.1 times after 24 h (Fig. 5). Delayed and more moderate caspase activity in cells treated with YH239-EE increased 3.7 folds after 24 h. As expected, incubation with the negative control YH239 did not result in activation. About possible side effects due to high concentrations of MDM2 antagonists, the activation of caspases at lower concentrations was subsequently investigated. While the caspase activity increased significantly 5.2 times after 24 h at five µM Nutlin-3a, 20 µM YH239-EE was required for a 3.7-fold increase in activity. Lower concentrations proved to be ineffective (Fig. 6).

The results showed that there was an increase in MDM2 in the control group with about (47.8±2.9%) fluorescent effect; this value was decreased in the presence of MDM2 inhibitors Nutlin 3 (21.28±1.8), Milademetan (19.1±0.819) and YH239-EE (24.9±7.8). Surprisingly, doxorubicin also decreased MDM2 to a value of (32.100±2.85). In the combination group, the result of MDM2 was also reduced in the doxorubicin+ Nutlin group; the value was (30.76±0.55), (29±1.55), and (35.36±1) in the Doxorubicin +YH239-EE and Doxorubicin +Milademetan group, respectively (Fig. 7).

The expression of MDM2

Expression of the MDM2 in the mcf7 cell line. First, the control group (placebo) showed the highest percent of expression, which is nearly 45%, then doxorubicin and Milademetan, which is about 38%, the lowest volume of expression or the lowest level of expression of MDM2 in mcf7 cell line according to immunofluorescent assay is the Milademetan which is about 20%. However, the strangest thing about this expression is the MDM2 in the doxorubicin-treated group was about 35% (Tab. 3, Fig. 8).

P53 level after this treatment

The lowest value of p53 was recorded in the control group, meaning there was limited or no

Tab. 3. The MDM2 expression level.

Drug name	Fluorescent level	Standard deviation	p
Control	47.83	1.55	
Nutlin 3	21.28	1.88	<0.0001
doxorubicin	32.10	2.86	0.0011
Milademetan	19.10	0.82	<0.0001
YH239-EE	24.90	7.88	0.0078
Doxorubicin + Nutlin 3	30.77	0.55	<0.0001
Doxorubicin +YH239-EE	29.03	1.55	0.0001
Doxorubicin +Milademetan	35.37	1.00	3.00

p values were calculated using the unpaid *t*-test using the Graph Prism program. p value < 0.05 is considered significant, and <0.0001 is highly important.

Tab. 4. The p53 level.

Drug name	Fluorescent level	Standard deviation	p
Control	4.00	2.21	
Nutlin 3	36.43	3.72	0.0002
Doxorubicin	15.33	1.05	0.0013
Milademetan	27.80	3.69	0.0007
YH239-EE	33.45	0.63	<0.0001
Doxorubicin + Nutlin 3	32.83	4.13	0.0004
Doxorubicin +YH239-EE	29.03	1.55	<0.0001
Doxorubicin +Milademetan	35.37	1.00	<0.0001

p values were calculated using the unpaid *t*-test using the Graph Prism program. p value < 0.05 is considered significant, and <0.0001 is highly important.

expression occurring in the control group (untreated group). The percent of P53 level increased to about 37 percent after MDM2 inhibitors (most values are near to each other). It's also interesting to know that the level of P53 expression in the doxorubicin-treated group is about 18 percent (Tab. 4).

Discussion

Cytotoxicity results of MDM2 inhibitors in MCF7 and HBL 100 cells

In the current study, Nutlin showed significant inhibition of cell growth and viability, as shown in Table 1. This effect is also linked to increased expression of P53 and decreased MDM2 effect. The Nutlin IC50 in MCF7 cells is about 5.9 µM. This result is good. Still, if we compare it with another result, it appears more because the IC50 of Nutlin ranges from 90 n M in the same types of blood leukemia in another study may be about 13 n M. However, these IC50 values are still near other Nutlin derivatives, AM-8553, about 6.8 µM, and Nutlin 3B, about 13.6 µM (18–20). Nutlin has a more cytotoxic effect on cancer cell MCF7 (IC505.9 µM) in MCF7 and HBL 100 compared with a less toxic impact on normal cells HBL100 (IC50 28.44). This means that this drug is relatively safe for normal cells in the patient. However, our study agreed with another research (15, 21), which showed that Nutlin could have an anticancer effect against several cell lines, including breast cancer cell line MCF7. The IC50 result of Miladametan was 11.07 in cancer cells, but this value increased to 15.34 µM in normal HBL100 cells. This means that this drug may cause toxicity and side effects; if we compare this result to other published results, the IC50 value was about 0.0034 µM in WaGa cell lines. This value reflects that Miladametan is more effective in treating Merkel cell carcinomas than breast cancer cells. Several types of

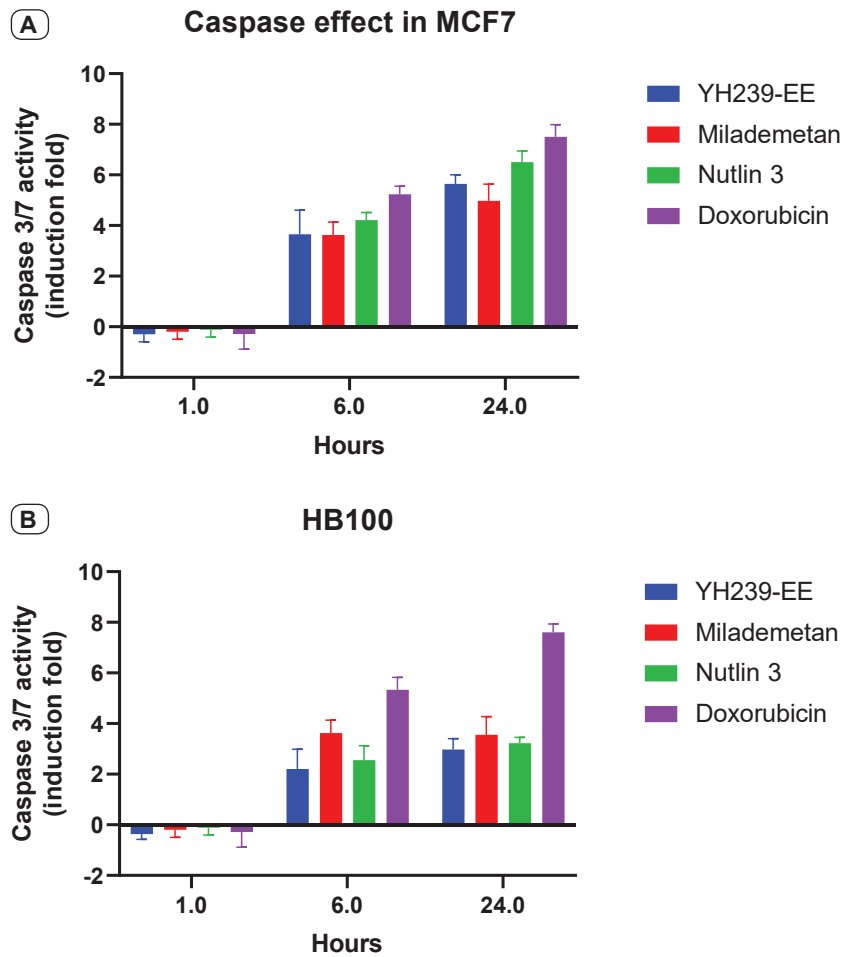

Fig. 5. Activity measurement of caspases 3 and 7. (A) Caspase 3/7 activity in MCF7 cells after 1, 6, and 24 h incubation with 20 µM Nutlin-3a, YH239-EE, or Miladametan. (B): Caspase 3/7 activity in HB100 cells after 1, 6, and 24 h incubation with 20 µM Nutlin-3a, YH239-EE, or Miladametan. All values are shown as n-fold induction compared to untreated cells. The column charts indicate the mean values (n=3), and the error bars represent the standard deviation.

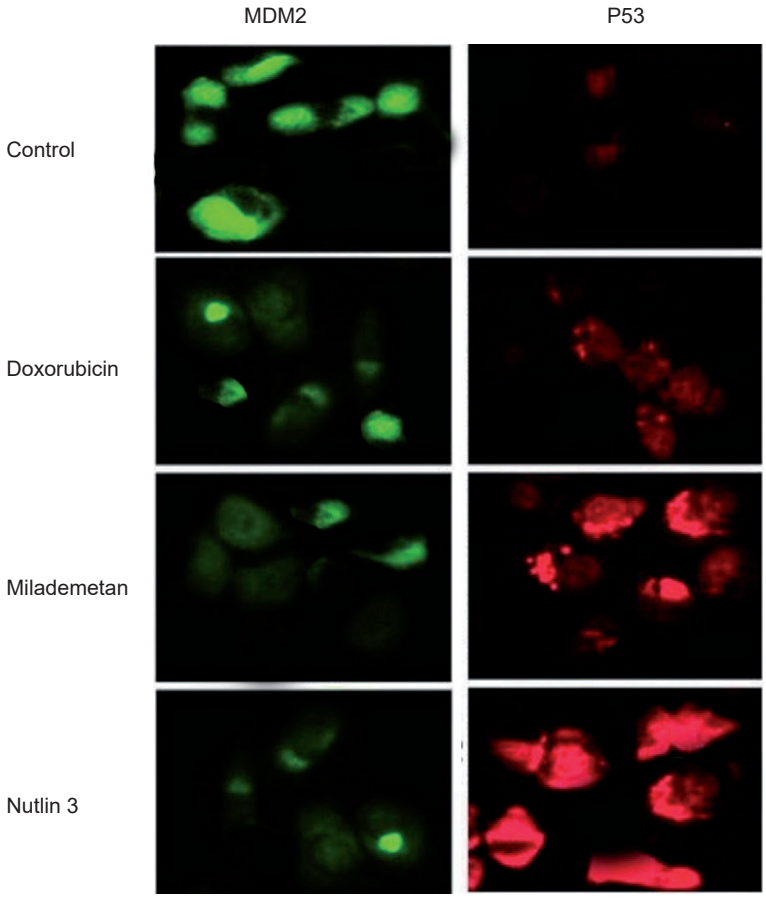

Fig. 6. The fluorescent activity of MDM2 and P53 in treatment groups.

cancer also show the same result as the IC50, about 21.9 nM in SK-N-SH, neuroblastoma cell line (17, 18, 22). The YH239-EE also shows selectivity on MCf7 cells compared to normal epithelial cells 8.45 μM to 21.47 μM, respectively.

Doxorubicin

Applying doxorubicin to the MCF7 significantly inhibits cell growth and proliferation. Doxorubicin shows IC50 about 1.12 μM in cancer cells and about 0.59 μM in normal cells. The IC50 of doxorubicin was about 1.12 μM. This result means this drug is less selective and has high side effects. However, the IC50 values are in line with other studies. In a study by Yao H and his colleagues, the doxorubicin in cell lines MDA-MB-453 was recorded at about 0.69 μM, and in MDA-MB-468 cells at about 0.27 μM and showed effectiveness in treating breast cancer (22–24).

Combinational treatment and its IC50

Nutlin group

The combination of 1 μM doxorubicin with ten μM Nutlin 3 showed a notable increase in the inhibitory effect (69.25% with a standard deviation of 5.9) compared to the impact of 10 μM Milademetan alone (55.23%) and one μM doxorubicin alone (52.25%). This suggests a synergistic interaction between Doxorubicin and Nutlin in targeting MCF7

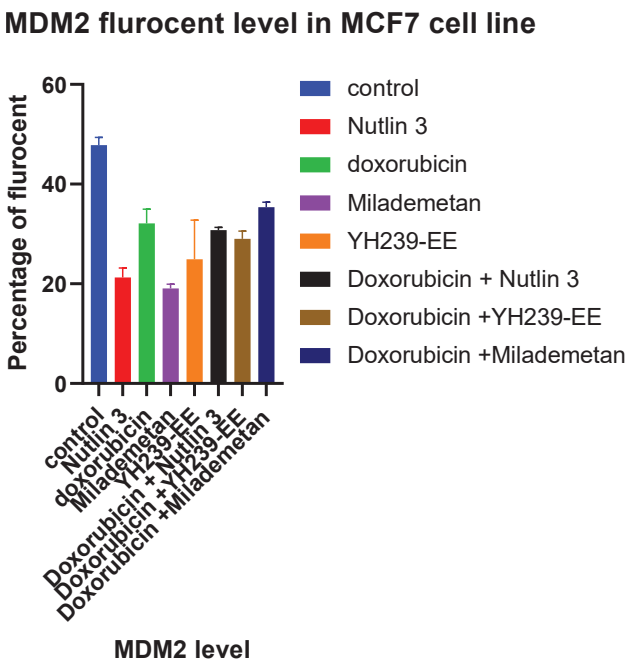

Fig. 7. The expression of MDM2 in the MCF7 cell line after treatment.

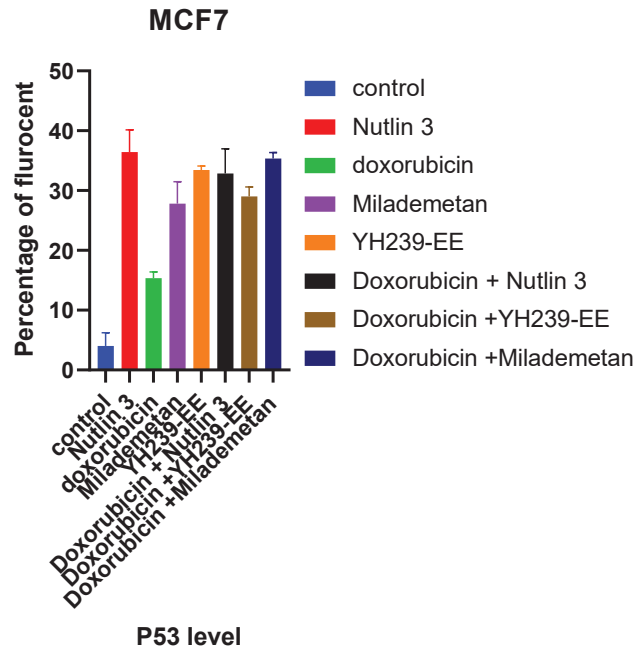

Fig. 8. The expression of P53 in the MCF7 cell line after treatment.

cells. This enhanced efficacy could be attributed to the simultaneous disruption of DNA replication (by doxorubicin) and the inhibition of the p53-MDM2 interaction (by Nutlin), leading to increased apoptosis. (19, 25). Milademetan (10 μM) and doxorubicin (1 μM) demonstrated an inhibitory effect of 64.15%, a significant increase compared to the 41.4% inhibition observed with Milademetan 10 μM alone. This indicates that doxorubicin may potentiate the effects of Milademetan, possibly through a complementary mechanism of action. The specific interaction between these two drugs and its impact on the MCF7 cell line's response needs further exploration (26–28). Combining Doxorubicin with YH239-EE resulted in a higher inhibitory effect of 71.13%, compared to 57.18% with YH239-EE alone. This outcome suggests combining these two compounds is more effective than either agent alone, indicating a potential additive or synergistic effect. The mechanism behind this enhanced cytotoxicity could involve the interaction of Doxorubicin's DNA-damaging effects with the specific apoptotic pathways targeted by YH239-EE. This result comes in line with the article published in 2016 by Oliver et al (29, 30).

The immunocytochemistry part of our research contains insightful information, and there is a high MDM2 level in the cell lines; however, after MdM2 inhibitors, this level decreased, and P53 increased (31, 32). In this immunocytochemistry, we used the Image J software program to analyze MDM2 antibody levels in cell lines.

- The observed decrease in MDM2 levels in cells treated with Nutlin 3 (21.28±1.8), Milademetan (19.1±0.819), and YH239-EE (24.9±7.8), compared to the control group (47.8±2.9%), highlights the efficacy of these inhibitors in reducing MDM2 expression. This aligns with the expected mechanism of action of MDM2 inhibitors, which are designed to antagonize MDM2 function, thereby potentially restoring p53 activity in cancer cells.
- The different degrees of reduction in MDM2 levels among these inhibitors may reflect variations in their potency, affinity, or mechanism of action. Nutlin 3 and Milademetan, showing the most significant decrease, could effectively inhibit MDM2.
- Surprisingly, doxorubicin, a chemotherapeutic agent known for its DNA-damaging effects, also reduced MDM2 levels to 32.100±2.85. This suggests that doxorubicin may have additional mechanisms of action, possibly influencing the MDM2-p53 regulatory pathway. This could represent a novel aspect of doxorubicin's anticancer activity, warranting further investigation.

Conclusion

This research was conducted to determine the effect of doxorubicin and MDM2 inhibitors (Nutlin 3, Milademetan, and YH239-EE). It's concluded that these drugs alone or in combination caused significant changes in P53 and MDM2 levels in breast cancer cell lines; all medical substances (not approved drugs) show substantial decreases in MDM2 levels in cell lines. However, the unexpected changes in doxorubicin cause a mild reduction in MDM2 expression and a significant increase in P53 level. These findings not only support the use of MDM2 in the treatment of Breast cancer but also give us a new possible mechanism of action of doxorubicin in the treatment of cancer, a medication best known for destroying DNA. The MDM2 inhibitors and doxorubicin show an increased cytotoxic effect compared to MDM2 alone or doxorubicin 1 Micro alone. This implies that although combination medicines may have advantages, such as lowering drug resistance or adverse effects, the primary influence of combination therapies on MDM2 levels is probably due to the MDM2 inhibitors alone.

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