Natural Products and Biotechnology

Vol. 1 No. 2
pp. 64-74
(2021)

Docetaxel Regulates the Interaction of p53 with MDM2 and Sin3A to Suppress MCF-7 Breast Cancer Cells

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Article History

Received : *Aug 19, 2021*
Revised : *Oct 22, 2021*
Accepted : *Dec 08, 2021*

Keywords

Apoptosis,
Cancer,
Chemotherapy,
Docetaxel,
MDM2

Abstract

Docetaxel is one of the most actively used chemotherapeutic agent in breast cancer which is the most frequent tumor in women. Recent studies propose that blocking the p53-MDM2 interaction may be effective in cancer treatment while the Sin3A mutation enhances cell proliferation in estrogen receptor (ER)-positive breast cancers. We aimed to investigate the effects of docetaxel on gene expression interactions and apoptosis in ER-positive breast cancer cell lines (MCF-7). MCF-7 cells were incubated for 24h with the treatment of escalating molar concentrations of docetaxel. The p53, MDM2 and Sin3A gene expression levels were measured by Real-Time PCR. The MTT assay was used to determine cellular viability. Apoptotic cells were detected by TUNEL. The mRNA expressions of p53, MDM2, and Sin3A increased in the same dose-dependent manner suggesting the highest effective level is 100nM docetaxel concentration ($p<0.001$). The p53 expression levels were strongly correlated with MDM2 ($r=0.9379$; $p<10^{-7}$) and Sin3A ($r=0.9965$; $p<10^{-13}$) in untreated, 10nM, 100nM and 1µM docetaxel concentrations. Cell viability of MCF-7 cells decreased dramatically in the 10µM and 100µM docetaxel treatments ($p<0.001$) and the IC_{50} value was 10µM. Apoptotic cell density was enhanced with the treatments of 10nM, 100nM, and 1µM docetaxel ($p<0.001$) in response to the gene expression levels. Our findings suggest that docetaxel directs the MCF-7 breast cancer cells to apoptosis in a dose-dependent manner and may thus further regulate the interaction of tumor suppressor p53 expression, protecting it from MDM2-mediated degradation and inhibiting Sin3A-mediated cell proliferation in compliance with the apoptotic cell density.

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Cite this article as:
Kurt, N., Bakan, N., Kara, A., Ozkanlar, S., Balkan, E., & Ozgeris, F. B. (2021). Docetaxel regulates the interaction of p53 with MDM2 and Sin3A to suppress MCF-7 Breast Cancer Cells. *Natural Products and Biotechnology*, *1*(2), 64-74.

1. INTRODUCTION

Docetaxel, used as an effective chemotherapeutic agent in breast cancer, binds to β-tubulins and inhibits the depolymerization of microtubulins, causing cell cycle arrest and apoptosis (McGrogan *et al.*, 2008). It causes apoptosis in different ways, either directly or indirectly. The main therapeutic effect of docetaxel is the suppression of microtubule dynamics (assembly and separation), cell cycle disruption, and phosphorylation of Bcl-2 (Dumontet and Jordan, 2010). In a recent study, docetaxel at a dose of 100 mg/m^2 has been found to be effective in patients with advanced or recurrent breast cancer with supportive therapies (Hirata *et al.*, 2021). The tumor suppressor gene TP53, encoding the p53 protein, is one of the most common mutated gene in breast cancer. The tumor suppressor p53 is a pivotal regulator in controlling cell proliferation and death in response to potentially oncogenic conditions, and therefore, the

interactors of p53-family proteins unveil regulatory elements that could be targeted more efficient use of drugs for cancer treatment (Collavin *et al.,* 2010).

The p53, a member of the tumor suppressor gene family, is the most widely studied gene in cancer research. Cellular pathways of p53 mediate tumor suppression resulting in either cellular death or the maintenance of cellular homeostasis. Recent knowledge indicates that cells can activate p53 signaling through positive and negative regulators of p53 having a critical function in cell cycle, apoptosis, senescence, stem cell differentiation, metabolism, DNA repair, ROS, and mitochondria in response to specific stress or a combination of stresses (Levine, 2020). The MDM2 protein encoded by the MDM2 gene can form a complex with the p53 tumor suppressor protein and prevents the apoptosis effect of p53 by keeping p53 under control (Freedman *et al.,* 1999). It is an oncogene-functional intracellular protein that is thought to be a significant target for cancer survival. It stimulates cell survival and growth while arresting the coordinated cell cycle and suppressing apoptosis (Vazquez *et al.,* 2008). The oncogene MDM2 might be an independent negative prognostic marker in breast carcinomas (Turbin *et al.,* 2006). Furthermore, current studies proposed that inhibitors designed to block the MDM2–p53 interaction may be effective in the treatment of human cancer by reactivating the tumor suppressor gene p53 (Wang *et al.,* 2017; Konopleva *et al.,* 2020).

Transcriptional regulator switch-independent 3 family member A (Sin3A) is essential for embryogenesis and T-Cell development and is required for the development and homeostasis of cells in the lymphoid lineage (Cowley *et al.,* 2005). Sin3A may also play an important role in regulating homeostasis and development, including cell death and mitochondrial biogenesis (Pile *et al.,* 2003). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3A (Murphy *et al.,* 1999). The Sin3A gene has been studied for more than two decades in cancer research and is known to be activated in several tumors mediated by interactions with p53, HDAC1/2, Mad, MeCP2, LSD1, REST, NRSF, CTCF, STAT3, and ERα (Murphy *et al.,* 1999; Zilfou *et al.,* 2001; Ellison-Zelski and Alarid, 2010; Watanabe *et al.,* 2018; Yang *et al.,* 2018; Gambi *et al.,* 2019; Zhao *et al.,* 2019; Jayaprakash *et al.,* 2021). Sin3A is a pro-survival protein that promotes growth of estrogen receptor (ER) positive breast cancer cells by preventing apoptosis through the repression of key proapoptotic genes. The Sin3A may therefore be an effective chemotherapeutic target in controlling the survival and growth of ERα-positive tumors (Ellison-Zelski and Alarid, 2010). The Sin3A is critical in the cellular function of cellular proliferation, differentiation, apoptosis, and cell cycle regulation (Kadamb *et al.,* 2013). The role of Sin3A in oncogenic potential is proposed to be associated with the transcriptional repression of tumor suppressor genes (Gambi *et al.,* 2019).

Sin3A mutation enhances MCF-7 cell proliferation through ERα expression and loses its transcriptional repression function due to its cytoplasmic localization. The reduction in Sin3A may also cause the recurrence of ERα-positive breast cancers (Watanabe *et al.,* 2018). Furthermore, the p53-mediated apoptosis in MCF-7 cells is regulated by very complex and diverse mechanisms and negatively regulated by HDAC3–ERα in a caspase-7-dependent manner (Park *et al.,* 2020). It is obvious that breast cancer is controlled by the expressions of a number of cancer-related genes including p53, MDM2, and Sin3A. Therefore, the function and the interaction of suppressive and oncogenic genes provide potential targets for further evaluation of chemotherapy in breast cancer cells. In this research, we aimed to investigate the effects of docetaxel as an actively used chemotherapeutic agent on p53, MDM2, and Sin3A and their interactions in MCF-7 estrogen receptor-positive breast cancer cells. The cell viability measurement and apoptotic cell assay facilitated to evaluation of the function of gene expressions in MCF-7 cells incubated for 24 h and treated with different molar concentrations of docetaxel in vitro.

2. MATERIAL and METHODS

2.1. Cell Culture, Passaging of Cells, and Cell Count

The MCF-7 cell line (11. passage) was commercially available from the FMD Institute (BioSample: SAMN02054478). MCF-7 cell lines were cultured in EMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) solution. Cultures were incubated at 37 °C with 5% carbon dioxide (CO_2) and 95% humidity. Cell culture studies were performed in the Bio Safety Cabinet (Class II). The medium of the cells that reached certain confluency (80%) was removed and washed with 4-5 mL PBS buffer. Subsequently, 3 mL trypsin/EDTA (Ethylene Diamine Tetraacetic Acid) was added. They were incubated in a CO_2 incubator during the period. 8 mL of fresh medium was then added and centrifuged for 5 min at 800 x g. The supernatant part was removed from the centrifuged cell suspension and 10 mL of fresh medium was added to the pellet. These passaged cell cultures were left to incubation at 37 ^{0}C in a 5% CO_2 incubator containing 95% humidity. 1µL of the cell suspension to be counted was transferred to a 0.5 mL eppendorf tube and 90 µL trypan blue dye was added to it and 10 µL was transferred to both chambers of the hemocytometer and cell count was performed under an inverted microscope. Cell concentration was determined using the formula (1) below.

$$\text{Number of cell /mL} = \text{Average number of cells in chambers} \times DF \times 10^4 \quad (1)$$

DF: Dilution factor.

10^4: Factor arising from chamber sizes on the slide (1 mm^3).

Thus, $5x10^6$ cells were transferred to each 100 mm^2 culture petri dish and 5000 cells to each well of the 96-well culture plate.

2.2. Drug Treatment

Docetaxel drug (Taxotere, Rhone-Poulenc Rorer Pharmaceutical) was used to treat the duplicate of each sample in wells at various concentrations. Docetaxel was diluted by the growth media, and then MCF-7 cells were exposed to the growth medium supplemented with concentrations of 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM and 200µL treated to each well with equal numbers of cells, correspondingly. They were incubated for 24 h at 37°C, 5% CO_2.

2.3. Cell Viability Measurement

The cytotoxic activities of the samples on existing cell lines were measured using the 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit (Cell Proliferation MTT Kit, Roche, Cat No: 11465007001) according to the manufacturer's instructions. The water-insoluble MTT tetrazolium salt is reduced to the water-soluble orange-colored formazan compound by the mitochondrial enzyme activity in metabolically active cells, and the product was measured in a microplate reader at 450 nm. The percent cell cytotoxicity/viability curve was drawn against the applied dose, and the 50% suppressive concentration (IC_{50}) value was calculated with the logarithmic slope graph in Microsoft Excel Program.

2.4. Histochemical Staining and TUNEL Assay

Inoculations were carried out in equal amounts (approximately 5000 cells suspended in 100 µL medium) on the upper surfaces of the lam in each petri dish. Docetaxel treatments were applied after the cells adhered to the lam. Following the end of the 24 h incubation period, PBS was added and cell washing was performed after removing the medium in the petri dish. The cells adhered to the lam were fixed with methanol at -20 ^{0}C for 10 min. The lams were washed with PBS and dried.

Terminal deoxynucleotidyl transferase Mediated Bio-dUTP Nick end Labeling (TUNEL) was used for histochemical detection of apoptosis. The TUNEL kit (Roche, In Situ Cell Death

Detection Kit, TMR red, Cat No: 12156792910) was used for the staining process. Adherent cell staining was performed according to the experimental protocol included in the kit. The "Stereological Optic Fractionator Frame" method was used to compare the results of TUNEL staining between the groups. This assay indicates late-stage apoptosis. The assay was performed under the stereology workstation system (BioPrecision MAC 5000 controller system, Ludl Electronic Products, Hawthorne, NY) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Rochester, VT) under a light microscope with attachment (Leica, Cambridge, UK). For the determination of apoptotic cells on MCF-7 cell preparations, the method of "Unident Counting Frame and Fractionator" was used and the TUNEL positive cell density in each preparation belonging to all groups was calculated according to the following formula (2):

$$PCD = PCN / (FA \times FN) \quad (2)$$

PCD: TUNEL positive cell density per μm^2 area

PCN: TUNEL positive cell count

FA: Frame area (μm^2)

FN: The number of frames

The data obtained are based on duplicate measurements for each group, three preparations from each group were stained.

2.5. RNA Isolation, cDNA Synthesis, mRNA Expression, and Primer Design

The RNA from the samples was isolated by using the MagNA Pure Compact RNA isolation kit (Product No: 04802993001, Roche) in the MagNA Pure Compact automated DNA-RNA isolation instrument (Roche Applied Science, Indianapolis, IN, USA). RNA concentration and purity were measured using the NanoDrop spectrophotometer (ThermoFisher Scientific, USA).

Transcriptor First Strand cDNA synthesis kit (Product No: 04896866001, Roche Diagnostics) was used for complementary DNA (cDNA) synthesis. The cDNA synthesis process was carried out according to the experimental protocol included in the kit. The specific primers used in these tests included p53 (F:5′-TCTCCCCAGCAAAAGAAAAA-3′ R:3′-CTTCGGGTAGCTGGAGTGAG-5′), MDM2 (F:5′-CGAGCTTGGCTGCTTCTGGG-3′, R:3′-GCTGGAATCTGTGAGGTGGT-5′) Sin3A (F:5'-TTGTCTCCAATGCTGTTCGC-3', R:3'-GGTTGGCGAATCCTGCGCTC-5') and β actin (ACT) (F:5’-TCCCTGGAGAAGAGCTACG-3’, R:3'-GTAGTTTCGTGGATGCCACA-5'). p53, MDM2, and Sin3A gene expressions were analyzed using a LightCycler 480 Real-Time Polymerase Chain Reaction (PCR) detection system (Roche Diagnostics). Reference gene ACT was used in the analysis. The composition of PCR reactions for p53, MDM2, and Sin3A consisted of 5µL cDNA, 8 µL ddH_2O, 5 µL Probe Master mix, 2 µL primer. The program below was selected.

Denaturation : 10 min 95 ^{0}C

Amplification : 10 s 95 ^{0}C, 30 s 60 ^{0}C, 60 s 72 ^{0}C (45 spins)

Cooling : 30 s 40 ^{0}C

The relative amount of mRNA to ACT in each sample was calculated using a relative quantification standard curve.

2.6. Statistical Analysis

Statistical analysis was performed by One-Way ANOVA with Duncan’s post hoc test after determining a homogeneous distribution using SPSS software version 20.0 (SPSS Inc. Chicago, IL, USA) and a p value less than 0.05 considered as significant difference between the groups. Linear regression analysis was used to determine the correlation between p53,

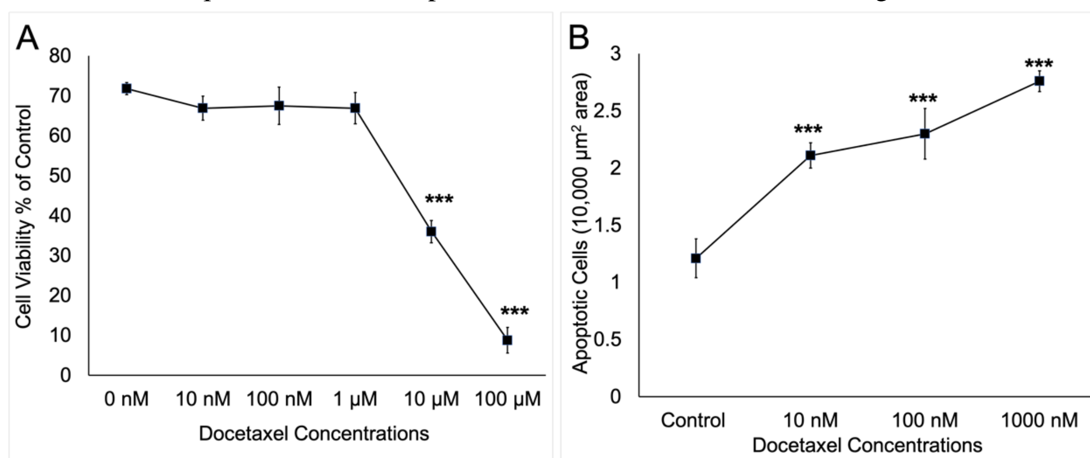
MDM2, and Sin3A variables. Significance between the correlations of dependent and independent variables was determined by means of Fisher's exact test.

3. RESULTS and DISCUSSION

The MCF-7 cell viability in control without any treatment was the highest and the treatments with docetaxel dramatically reduced the cell viability in the 10µM and 100µM concentrations ($p<0.001$). The lowest cell viability rate was in the 100µM docetaxel group and the IC_{50} value for MCF-7 cells was in 10 µM concentration (Figure 1A).

Docetaxel treatments increased apoptotic cell density compared to the control group with an escalating dose manner. The apoptotic cell density was enhanced with the increased concentrations of 10nM, 100nM, and 1µM docetaxel ($p<0.001$) (Figure 1B).

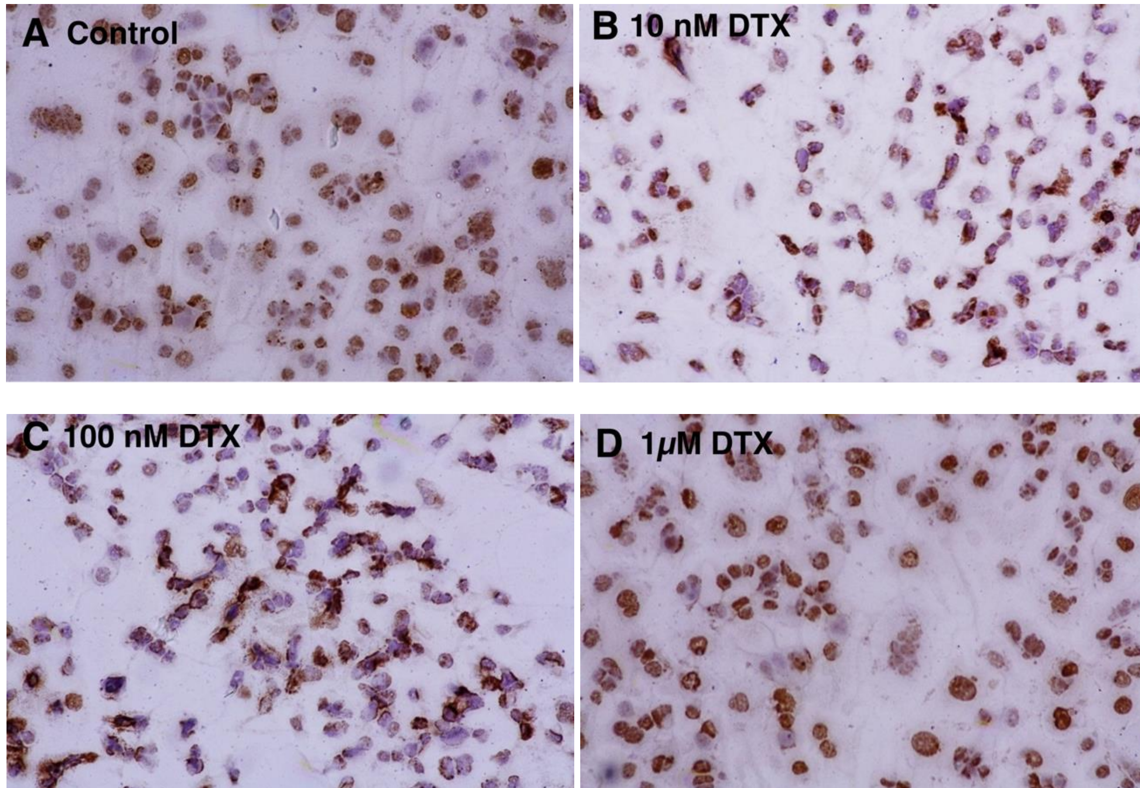
Figure 1. A: Cell viability percentage of MTT measurement in MCF-7 cells treated with docetaxel for 24 h incubation. **B**: Apoptotic cell count of TUNEL assay in MCF-7 cells treated with docetaxel for 24 h incubation. Statistical comparisons were made by One-Way ANOVA with Duncan's post hoc test and a p value less than 0.05 considered as significant difference.



Untreated cells were characterized by a round-shape, and mostly unstained by TUNEL staining. The dark brown cell staining was detected in docetaxel treatment groups indicating apoptosis at an early stage. The percentage of dark brown-stained cells increased in apoptotic MCF-7 cells indicating nuclear DNA fragmentation. The intergroup comparisons of TUNEL staining results of histochemical images are presented in Figure 2.

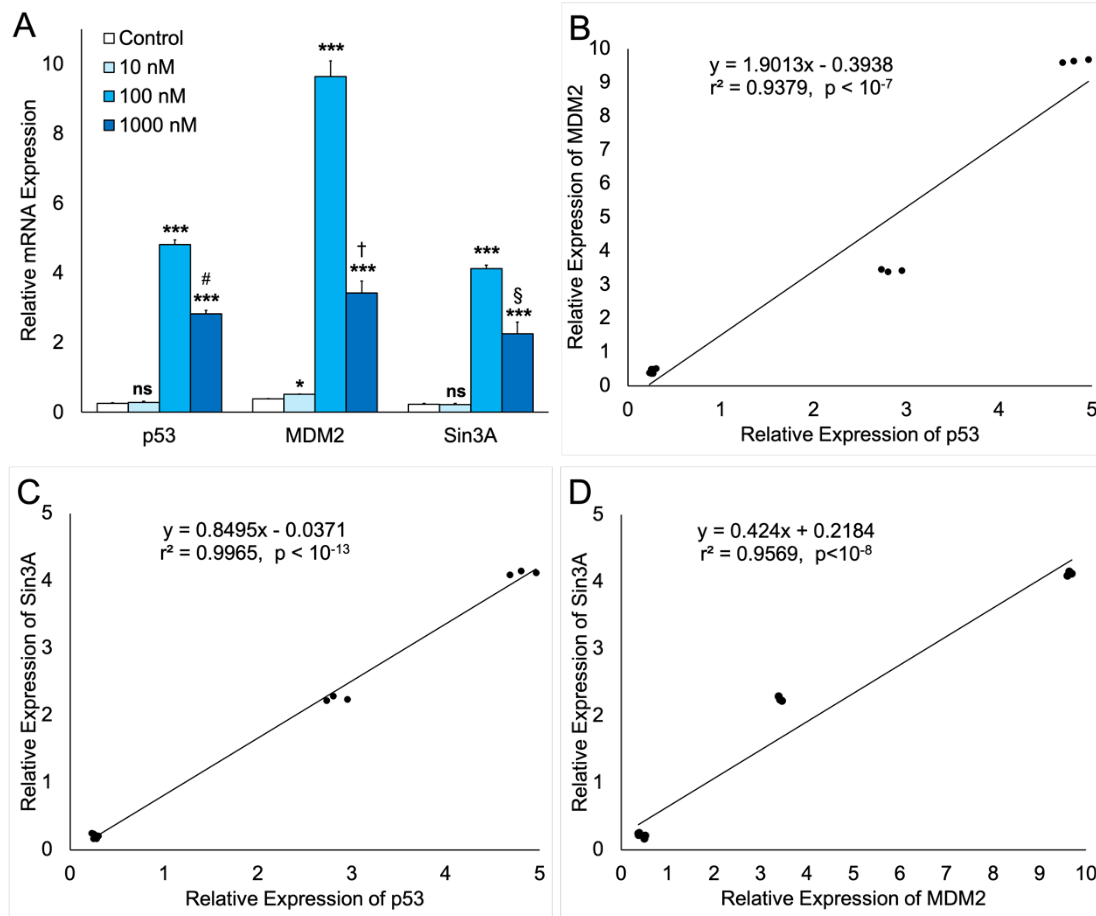
The mRNA expression levels were increased by docetaxel treatment after 24 h incubation. The expressions of p53, MDM2, and Sin3A increased at 100 nM concentration with the same dose-dependent manner compared to control ($p<0.001$) and decreased at 1 µM concentration compared to 100 nM concentration ($p<0.001$). The highest effective level was calculated as 100nM docetaxel concentration ($p<0.001$). Gene expression levels in docetaxel treatment groups are given in Figure 3A. The p53 expression levels were strongly correlated with MDM2 ($r=0.9379$; $p<10^{-7}$) and Sin3A ($r=0.9965$; $p<10^{-13}$) in untreated, 10nM, 100nM and 1µM concentrations. Additionally, the Sin3A expression levels were strongly correlated with MDM2 ($r=0.9569$; $p<10^{-8}$). The p53, MDM2 and Sin3A correlations are given in Figure 3B-D.

Figure 2. TUNEL staining in adhered MCF-7 cells in untreated/Control (**A**), and docetaxel (DTX) concentrations of 10 nM (**B**), 100 nM (**C**) and 1µM (**D**). Brown nucleus indicates TUNEL-positive cells. Untreated cells were characterized by a round-shape, and mostly unstained. The dark brown cells indicate apoptosis at an early stage of nuclear DNA fragmentation by the docetaxel treatment.



In this study, the cell viability decreased and the apoptotic cell density increased after docetaxel treatments with a dose-dependent manner in MCF-7 cells that clearly indicate the chemotherapeutic effectiveness of the drug. The tumor suppressor p53 expression increased at 100 nM docetaxel concentration compared to control after 24 h incubation. However, the p53 inhibitor MDM2 and oncogenic Sin3A expression levels were also increased similar to the p53 level. Apoptotic effect was detected to start at 10 nM and 100 nM docetaxel concentrations based on the TUNEL assay while 10 µM and higher doses were found to be toxic for MCF-7 cells according to MTT analysis. The apoptotic effect of the drug, therefore, seems to depend on gene expressions suggesting p53-mediated apoptosis. These findings reveal that cytotoxicity and apoptosis are mostly related to genetic regulations of chemotherapy in breast cancer.

Figure 3. Relative gene expressions of p53, MDM2 and Sin3A (**A**). The interactions between p53 and MDM2 (**B**), p53 and Sin3A (**C**), MDM2 and Sin3A (**D**).



Statistical comparisons were made by One-Way ANOVA with Duncan's post hoc test and a p value less than 0.05 considered as significant difference. Correlations were determined by linear regression analysis between the variables. Significance between dependent and independent variables was determined by Fisher's exact test.

In previous studies, there has been a higher increase in p53 gene expression with docetaxel administration in the MCF-7 cell line compared to the doxorubicin and resveratrol administration, while there has been a marginal increase in the administration of docetaxel-resveratrol combination (Al-Abd *et al.,* 2011). Additionally, extreme dilutions of paclitaxel and docetaxel alter p53, p21, COX-2, TUBB2A, and TUBB3 gene expressions with little or no cytotoxic/viability effect on MCF-7 cells incubated 72 h (Seker *et al.,* 2018). Similar to these reports, we found that docetaxel administration caused an increase in p53 expression in MCF-7 cells incubated 24 h. The highest p53 expression has been observed in the group treated with 100 nM docetaxel, then the expression levels started to decrease in the group treated with 1 µM docetaxel after 24 h incubation of docetaxel treatment. The decrease in gene expressions at 1 µM concentration is thought to be related to the high concentration of docetaxel due to cytotoxicity.

Most agents restore the function of mutant p53 and inhibit MDM2, which is often overexpressed in human tumors (Vazquez *et al.,* 2008). MDM2, a negative regulator of p53, is an oncogene-functional intracellular protein that is thought to be a significant target for cancer survival. The oncogene MDM2 is an independent negative prognostic marker in breast carcinomas (Turbin *et al.,* 2006) and prevents the apoptosis effect of p53 protein (Freedman *et al.,* 1999). Therefore, blocking the MDM2–p53 interaction can reactive the tumor suppressor gene p53 (Wang *et al.,* 2017; Konopleva *et al.,* 2020). Since MDM2 negatively regulates p53 through the ubiquitin–proteasome pathway, the increase in p53 may also reactivate MDM2

levels to increase because of the feedback mechanism. Docetaxel decreases MDM2 protein level and does not change p53 mRNA level in LNCaP cells (bearing wild-type p53) for human prostate adenocarcinoma (Gan *et al.,* 2011). In advanced-stage breast cancer, docetaxel decreases MDM2 protein level compared to the combination of methotrexate and 5-Fluorouracil (Sjostrom *et al.,* 2000). In this study, the increase in MDM2 gene expression has been observed in the 10 and 100 nM docetaxel concentrations compared to the control group, and the highest expression has been in the 100 nM docetaxel concentration which is similar to the p53 expression reaction. MDM2 level decreased at 1 μM docetaxel as seen in p53 level. We found that an increase and a decrease in p53 level that is strongly correlated with MDM2 level in MCF-7 cells treated with docetaxel. The decrease in p53 gene expression may be due to the decrease in the MDM2 protein level. Although the p53 level may indicate the chemotherapeutic effectiveness of a drug, cancer variability and drug treatment affect the genetic response of the cells.

We found an increase in Sin3A gene expression in the MCF-7 cell line with docetaxel administration. However, there is a limited report investigating the effect of docetaxel on Sin3A in the previous studies. Sin3A and Sin3B differentially regulate breast cancer metastasis (Lewis, 2016). Low levels of Sin3A and Sin3B expressions have been associated with the progression of many cancers. Ellison-Zelski *et al*. (2010) examined the function of Sin3A in the development and gene expression of breast cancer cells. They observed that the absence of Sin3A inhibits the growth of breast cancer cells with an increase in apoptosis. In the evaluation of both ERα-positive and ERα-negative cell lines, the effects of Sin3A on growth are found to be specific to cell type, and Sin3A expression provides maximum growth only in ERα-positive cells suggesting that the Sin3A protein is significantly increased by estrogen. When the Sin3A gene is transferred to MCF-7 cells, Sin3A suppresses the expression of key proapoptotic genes. Sin3A is important in the regulation of development, survival, and gene expression in ERα-positive breast cancer cells that Sin3A could be a new therapeutic target. Furthermore, Yang *et al*. (2018) suggested that LSD1/Sin3A/HDAC complex could be a target for breast cancer therapeutic strategies. Additionally, the interaction between p53 and Sin3A has been studied in MCF-7 and A2780 ovarian cancer cells with a DNA damaging agent. The Sin3A-p53 complex increased approximately four times in MCF-7 cells (Murphy *et al.,* 1999). The mSin3A corepressor binds to both wild-type and mutant p53 accompanied by DNA binding. Zilfou *et al*. (2001) suggested that Sin3A stabilizes p53 and inhibits the degradation of p53 by MDM2 in MCF-7 cells. In this study, Sin3A gene expression levels were high at the 100 nM and 1 μM docetaxel concentrations compared to the control but the level decreased at 1 μM compared to 100 nM concentration. The reduction in Sin3A mRNA has been reported to be correlated with the recurrence of ER-positive breast cancers since the Sin3A mutation has lost its transcriptional repression function due to its cytoplasmic localization (Watanabe *et al.,* 2018). The Sin3A mutation enhances MCF-7 cell proliferation. The increase in p53 expression strongly correlated with the increase in Sin3A at the same doses, herein. ER-positive MCF-7 breast cancer cells are prone to Sin3A elevation and this increase may be related to cell proliferation.

4. CONCLUSION

The efficacy of docetaxel is supported by p53 overexpression according to the enhancement of apoptotic cell density. Sin3A expression may lose its transcriptional repression function related to the drug concentration. The response of MCF-7 cells to docetaxel treatment seems to be in favor of p53 gene overexpression. In conclusion, MCF-7 breast cancer cells respond to docetaxel treatment in a dose-dependent manner and may thus further regulate the interaction of tumor suppressor p53 expression with MDM2 and Sin3A. Furthermore, docetaxel protects p53 from MDM2-mediated degradation and inhibition of Sin3A-mediated cell proliferation according to the enhancement of apoptotic cell density and the reduction of cell

viability. The present study may lead the evaluation of the treatment of breast cancer regarding the genetic and chemotherapeutic factors that have a regulatory role for p53.

Acknowledgements

This research was supported by the Ataturk University Scientific Research Project (SRP: 2012/10).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Nezahat Kurt: Conceptualization, Writing- Original draft preparation, Writing – review, Editing. **Nuri Bakan**: Supervision. **Adem Kara:** Methodology, Data collecting. **Seckin Ozkanlar:** Methodology, Data collecting. **Eda Balkan**: Methodology, Data analysis. **Fatma Betul Ozgeris:** Methodology, Data analysis.

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