Original Article

Effects of EF-24, RAD001, and paclitaxel on the expression profiles of apoptotic and anti-apoptotic genes

ABSTRACT

Context: Cancer cells exert differential responses to chemotherapeutics and inhibitors. To the best of our knowledge, a few or no research has been performed until now to determine the effect of EF-24 and RAD001 on MDA-MB-231 breast cancer cells with regard to mRNA expression of apoptotic and anti-apoptotic genes.

Aims: In this study, we aimed to investigate the mRNA expression levels of apoptotic (caspase 2 [*CASP2*], *CASP8*, and *CASP9*) and anti-apoptotic (B-cell lymphoma 2 [*BCL2*] and *BCL2*-like protein 1 [*BCL2L1*]) genes after exposure to paclitaxel, EF-24, and RAD001 in MDA-MB-231 cells.

Materials and Methods: After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure cell viability. mRNA expressions were analyzed using quantitative real-time polymerase chain reaction.

Results: Decrease in cell viability ratios was seen in a dose-dependent manner for all chemicals. MDA-MB-231 cells responded slightly different to paclitaxel, EF-24, and RAD001 at the transcriptional level of apoptotic and anti-apoptotic genes.

Conclusions: Our results showed that response of these cells to paclitaxel, EF-24, and RAD001 was found different at the transcriptional level of apoptotic and antiapoptotic genes. Therefore, understanding transcriptional changes after these drug exposure may give us a change to figure out more realistic results of the apoptotic pathway inhibition.

KEY WORDS: Apoptosis, breast cancer, EF-24, paclitaxel, RAD001

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INTRODUCTION

Breast cancer is one of the most common cancers with poor prognosis and the second leading cause of cancer-related deaths in women.[1] Advances in the diagnosis and treatment notwithstanding, breast cancer treatment remains a major challenge for physicians due to considerable side effect of current therapies.[2] Hence, new drugs are developing to diminish the adverse effect of classical chemotherapeutic agents.[3]

While numerous factors contribute to the growth of cancer, one hallmark is the loss of balance between cell proliferation and cell death.[4] Apoptosis or programed cell death is regulated by complex interactions between anti-apoptotic and pro-apoptotic proteins.[5] Overexpression of anti-apoptotic B-cell lymphoma 2 (*BCL2*) or *BCL2*-like protein 1 (*BCL2L1*) (known as *BCL-xL*) likely appears in more than half of all cancers.[6] Caspases (*CASPs*) are another group of proteins which is responsible for the regulation of apoptosis. Dysregulation of *CASPs* has been induced a number of diseases including cancers.[7]

Profiling transcriptional changes after drug exposure is a powerful approach for investigating cellular responses to drugs. This approach has paved the way for greater understanding of pathway inhibition and off-target drug effects.[8] In addition, functional dysregulation in apoptotic signal transduction may translate into drug resistance of cancer as well.[6] It is known that transcript levels are known to exhibit dose-responsive behavior against pharmacological agents.[9-11]

Paclitaxel and its derivatives are broadly used in the treatment of breast cancer[12] and several

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other solid tumors, such as nonsmall cell lung cancer[13] and ovarian cancer.[14] In addition to stabilization of microtubules, it induces apoptosis by not only *CASP*-dependent but also *CASP*-independent pathways and inhibits the anti-apoptotic protein *BCL2*. Despite its successful use as an anti-cancer drug, raising the resistance of cancer cells to paclitaxel has created a drawback. On the other hand, the major limitation is extreme toxicity of paclitaxel. To overcome the problem, studies have been focused on identifying natural compounds that could increase the therapeutic index.[15] Curcumin, obtained from the rhizome of the perennial herb *Curcuma longa*, has been shown significant therapeutic potential against carcinogenesis in several cancer cell types *in vitro* because it suppresses cancer cell proliferation, induces cell cycle arrest and apoptosis by means of the *CASP* cascade.[16,17] Unfortunately, bioavailability of curcumin is low due to very low absorptive capacity, therefore its therapeutic benefit is limited.[18] In light of these findings, design and synthesis of novel structural analogs have been investigated for cancer therapy.[19] One novel analog is EF-24 which exhibited approximately 10- and 20-fold enhanced cytotoxic activity against various cancer cell lines relative to curcumin.[17]

RAD001 (everolimus), one of the mechanistic targets of rapamycin (mTOR) inhibitor, is recently accepted for the treatment of renal cell carcinoma and breast cancer.[20] RAD001 has shown broad anti-tumor activities in preclinical models.[21,22] Dysregulation of the mTOR signaling pathway is closely associated with tumorigenesis because the mTOR plays a central role in cell growth and many biological activities including gene transcription, protein translation initiation, ribosome biosynthesis, and cellular apoptosis. As a result, mTOR represents a valuable target for anti-tumor therapy.[23]

In this study, we aimed to investigate the mRNA expression levels of apoptotic (*CASP2*, *CASP8*, and *CASP9*) and anti-apoptotic (*BCL2* and *BCL2L1*) genes after exposure to paclitaxel, EF-24, and RAD001 in breast cancer cell line (MDA-MB-231).

MATERIALS AND METHODS

Cell line and cell culture

MDA-MB-231 breast cancer cell line was purchased from the Foot-and-Mouth Disease Institute of Ministry of Agriculture and Rural Affairs of Turkey. Cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 mg/ml streptomycin (all obtained from HyClone, Thermo, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability assay

The growth inhibitory effect of paclitaxel (Sigma-Aldrich, St. Louis, Missouri, USA), EF-24 (Sigma-Aldrich, St. Louis, Missouri, USA), and RAD001 (Fluka, Hamburg, Germany) were evaluated on MDA-MB-231 cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were suspended in 96-well tissue culture plates (containing 10^4 cells, per well) and seeded in culture medium at 37°C for overnight. After overnight culture, the cells were incubated with paclitaxel (ranges 25–500 nM), EF-24 (ranges 0.125–8 μM), and RAD001 (ranges between 25 and 500 nM) for 24 h and then MTT assay was used to measure cell viability. After the incubation period, 10 μl MTT solution (5 mg/ml) was added to the medium and kept at 37°C for 4 h. To dissolve formazan crystals, dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Missouri, USA) was used and the absorbance values were measured with a SpectraMax M3 microplate reader (Molecular Devices, California, USA) at 570 nM. The experiments were performed in quadruplicate. As a result of MTT assay data, 100 nM paclitaxel, 2 μM EF-24, and 50 nM RAD001 concentrations (IC_{20}) were selected for mRNA analysis.

RNA isolation and cDNA synthesis

Total cellular RNA was isolated after drug treatment of cells using High Pure™ RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Then, quantitation of RNA purity and concentration of each sample was measured using NanoDrop spectrophotometer (NanoDrop ND-1000, Montchanin, DE, USA). The isolated RNA samples were stored at −80°C until required for further experiments. cDNA was synthesized from 1 μg of total RNA via random hegzamer primers using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Eppendorf Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany) was used for cDNA synthesis reaction. The samples were kept at −20°C after reaction.

Quantitative real-time polymerase chain reaction analysis

CASP2, *CASP8*, *CASP9*, *BCL2*, and *BCL2L1* mRNA expression levels were quantified using Light Cycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany). Suitable probes and gene-specific primers spanning exon–exon boundaries were designed at the Universal Probe Library (UPL) Assay Design Center (https://www.universalprobelibrary.com). Primer pairs and UPL probe numbers were indicated in Table 1. Each target gene expression level was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase. Polymerase chain reaction (PCR) efficiency of each gene was determined by serial dilutions. The real-time PCR conditions were as follows: 10 min preincubation at 95°C, followed by 50 cycles of 10 s at 95°C, 20 s at 60°C, and finally a cooling step to 40°C. Each sample was measured in triplicate and mean value was used for further calculation.

Statistical analysis

Cell viability experiments were repeated 4 times, and data were expressed as the mean ± standard deviation from

a representative experiment. Differences of viability were assessed by one-way ANOVA followed by *post hoc* Tukey's test using SigmaStat version 3.5 software (Systat Software, Inc, Point Richmond, CA, USA). Statistical significance levels of differences in mRNA expressions were analyzed by REST 2009 version 2.0.13 (Qiagen, Hilden,Germany).[24] $P < 0.05$ was considered as significant.

RESULTS

Decrease in cell viability ratios was seen in a dose-dependent manner for all chemicals as determined by MTT assay [Figure 1]. Nearly, similar cell viability responses were found at the highest concentration of EF-24 (8 μM) and paclitaxel (500 nM) and about 40% cells death were found at these concentrations after 24 h. On the other hand, RAD001 caused less cell death at the highest concentration as compared with other chemicals (EF-24 and paclitaxel) and 30% cells death were obtained at 500 nM concentrations for 24 h. At 100 nM paclitaxel, 2 μM EF-24, and 50 nM RAD001 concentrations, agents caused 20% cell death (IC_{20}) after 24 h incubation.

In Figure 1a, MDA-MB-231 cell viability significantly reduced after 24 h paclitaxel incubation at doses higher than 100 nM ($P < 0.05$) and it was found 63.8% at highest concentration (500 nM). Another agent EF-24 at doses of 2, 4, and 8 μM significantly decreased the cell viability after 24 h incubation ($P < 0.05$) [Figure 1b]. After 24 h RAD001 incubation at doses higher than 50 nM, cell viability was significantly reduced ($P < 0.05$); it was found that 79.6% and 70.2% at 50 nM and highest concentration (500 nM), respectively [Figure 1c]. To continue for further analysis, IC_{20} values were selected.

We treated the cells with paclitaxel, EF-24, and RAD001 at selected doses for the mRNA expression analysis of the apoptotic (*CASP2*, *CASP8*, and *CASP9*) and anti-apoptotic (*BCL2* and *BCL2L1*) genes in MDA-MB-231 cell line. The results of quantitative PCR analysis of the *CASP2*, *CASP8*, *CASP9*, *BCL2*, and *BCL2L1* genes were given in Figure 2 for paclitaxel, EF-24, and RAD001.

Although *CASP8* and *CASP2* mRNA expression were not induced, *CASP9* mRNA expression was induced after paclitaxel treatment, but not statistically significant ($P > 0.05$). In addition, paclitaxel was slightly decreased in *BCL2* and *BCL2L1* mRNA expression, but this decline was not statistically significant ($P > 0.05$). Even though EF-24 treatment increased *CASP8*, *BCL2*, *BCL2L1*, and *CASP2* mRNA expression level, this treatment lightly decreased *CASP9* mRNA expression. However, these changes not reached statistically significant

Table 1: mRNA specific primer sequences of studied genes and Universal Probe Library probe numbers

Gene	Forward primer	Reverse primer	UPL probe number
CASP2	5'-CGCCATCTATGGTGTGGAT-3'	5'-CAGTTGGCGTTGTCAAAGAG-3'	78
CASP8	5'-TCCAAATGCAAACTGGATGA-3'	5'-TCCCAGGATGACCCTCTTCT-3'	62
CASP9	5'-CCATATGATCGAGGACATCCA-3'	5'-GACTCCCTCGAGTCTCCAGAT-3'	27
BCL2L1	5'-AGCCTTGGATCCAGGAGAA-3'	5'-GCTGCATTGTTCCCATAGAGT-3'	28
BCL2	5'-AGTACCTGAACCGGCACCT-3	5'-GGCCGTACAGTTCCACAAA-3'	75
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'	60

UPL=Universal Probe Library

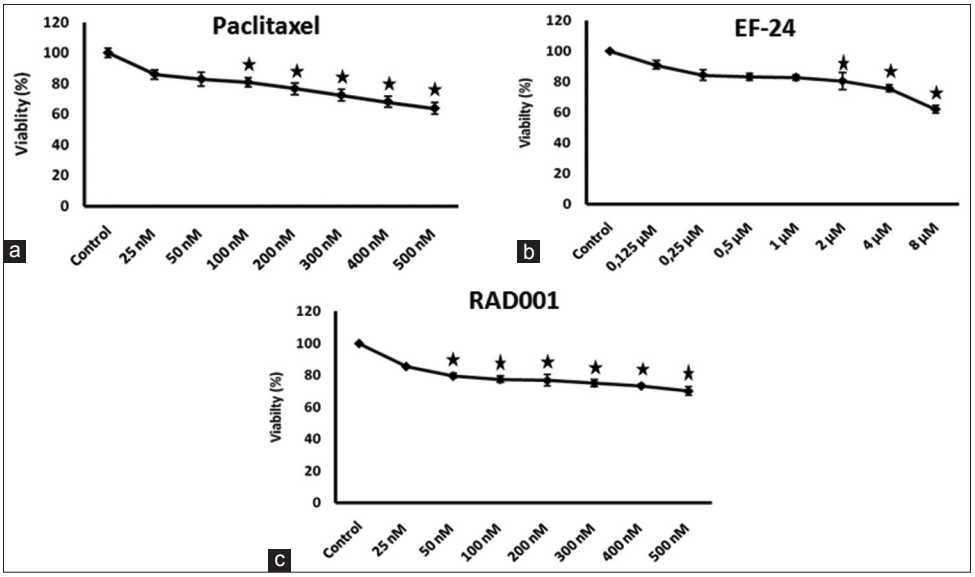

Figure 1: Effects on cell viability of paclitaxel (a) EF-24, (b) and RAD001, (c) on MDA-MB-231 cells measured by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay. The results are expressed as a percent of control. Data shown are the means of four independent experiments. *$P < 0.05$

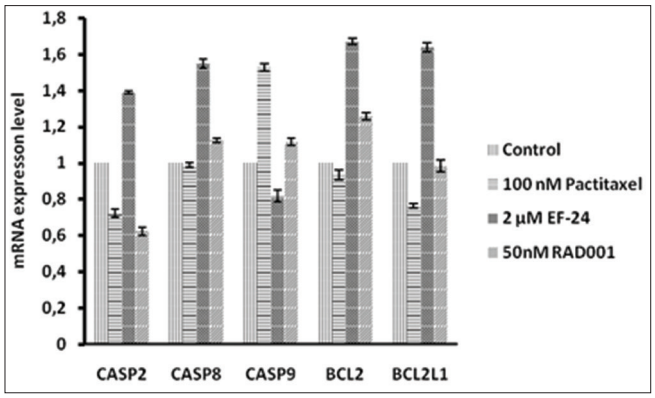

Figure 2: mRNA expression of caspase 8, caspase 9, B-cell lymphoma 2, B-cell lymphoma 2-like protein 1, and caspase 2 genes in MDA-MB-231 cells treated with paclitaxel, EF-24, and everolimus (RAD001) for 24 h. Glyceraldehyde-3-phosphate dehydrogenase used as a housekeeping gene to normalize data

value ($P > 0.05$). After RAD001 treatment, mRNA expression levels of *CASP8*, *CASP9*, and *BCL2* were slightly increased, but *CASP2* and *BCL2L1* mRNA expressions were slightly decreased in MDA-MB-231 cell line. These changes were also not statistically significant ($P > 0.05$).

DISCUSSION

In a recent study by performing microarray analysis, it has been shown that breast cancer cell lines showed quite distinct transcriptional responses to chemotherapeutics.[25] Despite the anti-tumor activity of paclitaxel toward cancer including breast cancer,[15] rising drug resistance to paclitaxel poses a challenge for future cancer treatment.[26] Liu *et al*. showed that very high concentrations of paclitaxel did not further increase growth inhibition and apoptosis.[27] Paclitaxel resistance was also found in breast cancer cell lines including MDA-MB-231.[28] In our study, cell viability was significantly reduced at doses higher than 100 nM paclitaxel.

Imbalance between pro- and anti-apoptotic members of *BCL2* family may contribute to the progression of human cancers and rise drug resistance.[29] Ferlini *et al*. showed that *BCL2* mRNA expression is down-regulated in resistant cell lines with respect to parental paclitaxel sensitive cells in human ovarian A2780 cell line.[30] In our study, despite the down-regulation tendency of *BCL2* and *BCL2L1* mRNA expression, statistically significant value was not found. *CASP8* and *CASP9* mRNA expression did not also significantly change after paclitaxel treatment. As for *CASP2*, to the best of our knowledge, there was no study with respect to *CASP2* expression in MDA-MB-231 cell line after paclitaxel treatment. Jelínek *et al*. indicated that inhibition of *CASP2* expression using siRNA resulted in increased number of surviving MCF-7 and SK-BR-3 cells and decreased cleavage of initiator *CASP8*, *CASP9* in both cells after paclitaxel treatment.[31] In our study, *CASP2* mRNA expression was not significantly altered after paclitaxel treatment. We recommended that further studies are needed to clarify the potential role of paclitaxel on mRNA expression of *CASP2* in MDA-MB-231 cells.

Our previous report has shown that EF-24 significantly decreased MSTO-211H cell viability.[22] In addition, Tan *et al*. indicated that EF-24 significantly suppressed proliferation of ovarian cell lines at ≤2 μM concentration.[32] According to our results, it also significantly inhibited the proliferation of MDA-MB-231 cells at ≥2 μM concentration. With regard to *CASP2*, *CASP8*, *CASP9*, *BCL2L1*, and *BCL2* mRNA expressions, we did not find significant changes after EF-24 treatment in MDA-MB-231. However, we previously showed[22] that *BCL2L1* mRNA was significantly decreased in MSTO-211H cells after 2 μM EF-24 treatment.

The antiproliferative effects of mTOR inhibition have been shown in various cell lines.[22,33-35] We showed that cell viability was significantly reduced after RAD001 incubation at doses higher than 50 nM. However, we did not find significant changes in the expression of *CASP2*, *CASP8*, *CASP9*, *BCL2L1*, and *BCL2* mRNAs after RAD001 treatment and we are unable to compare our results due to lack of similar studies in the literature.

In general, mRNA analysis showed that MDA-MB-231 cells responded slightly different to paclitaxel, EF-24, and RAD001 at the transcriptional level of apoptotic (*CASP2*, *8*, *9*) and anti-apoptotic (*BCL2* and *BCL2L1*) genes. This molecular study might uncover the need for further wide investigation on this pathway and approaches to the development of effective chemotherapy by targeting appropriate signal transducers.

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Conflicts of interest
There are no conflicts of interest.

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