Synergistic Anti-proliferative Effects of Metformin and Silibinin Combination on T47D Breast Cancer Cells via hTERT and Cyclin D1 Inhibition

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ABSTRACT
Background There is a growing body of data that chemotherapeutic combination strategies would be more effective in reducing drug toxicity, inhibiting tumor progression in comparison to either drug alone.
Objective To explore a chemopreventive strategy for improving breast cancer treatment efficacy, the anticancer effects of a combination of Metformin (MET) and Silibinin (SIL) were investigated in T47D breast cancer cells.
Materials and Methods Cytotoxicity of the drugs individually and in combination was evaluated using MTT assay. The precise nature of the interaction between MET and SIL was further analyzed through the median-effect method. In addition, qRT-PCR was applied to determine the expression levels of hTERT and cyclin D1 genes after 48 h drug exposure.
Results MTT assays showed that MET and SIL individually inhibited the cell viability in a dose and time-dependent manner, and the obtained combination indices (CIs) were < 1 for all the combination treatments, indicating that the anticancer agents synergistically induced growth inhibition in the breast cancer cells. qPCR findings revealed that the drug combination also synergistically down-regulated the expression levels of hTERT and cyclin D1 at all used concentrations compared with the drugs used alone after 48 h treatment (P ≤ 0.05).
Conclusion The results provide evidence that synergistic antiproliferative effects of MET and SIL, linking to the down-regulation of Cyclin D1 and hTERT genes, and propose that MET + SIL may have therapeutic value in breast cancer therapy.

Introduction
About 12% U.S. women will develop invasive breast cancer over the during her lifetime. In 2017, an estimated 252,710 new cases of invasive breast cancer are predictable to be identified in U.S. women, along with 63,410 new cases of non-invasive breast cancer [1]. Breast cancer is considered as a heterogeneous disease with several clinical features and risk factors. Breast lumps, shape change in breast, skin dimpling, nipple discharge, a scaly or red skin patch, swollen lymph nodes, bone pain and breath shortness are the common signs of breast cancer [2]. Established risk factors for develop-
ing breast cancer include variable life style factors including being female, obesity, lack of physical exercise, hormone replacement therapy and alcohol consumption, and; reproductive factors such as low parity and breast-feeding, late menopause and early menarche.; and inherent genetic factors [3].

Although several chemotherapeutics such as doxorubicin, etoposide, and paclitaxel have been used to treat this type of cancer, issues remain such as low survival rates, high reoccurrence and severe side effects after conventional chemotherapy. Thus, novel chemotherapeutics and approaches should be developed [4, 5].

Nowadays, numerous natural bioactive compounds or phytochemicals have been isolated, described, and their potential anticancer effects have been evaluated [6–9]. Although, efficiency of interactions between various dietary compounds needs further investigation.

Silibinin (SIL), a flavonolignan extracted from Silybum marianum, has been widely investigated for its hepatoprotective, anti-inflammatory, antioxidant and anticancer properties (structures shown in ▶ Fig. 1a) [10, 11]. Furthermore, it has demonstrated considerable efficiency in inhibiting or delaying both tumor initiation-and promotion-related incidences in various pre-clinical cancer models such as prostate, colorectal, lung and breast cancer [12, 13]. Also, recent studies show that combination of SIL with some substances can enhance the therapeutic properties [4, 14].

Also, several recent preclinical and clinical data all suggest that metformin (MET) as a biguanide originated from the French lilac or goat’s rue (Galega officinalis) (structures shown in ▶ Fig. 1b) with known pharmacokinetics, high safety profiles, and relatively inexpensive might be effective against various types of cancer including breast cancer [5, 15, 16].

More recent cancer therapies are established on an effective combination of various chemotherapeutics with different mechanisms. A combination approach may increase the anti-cancer effects or even results in a synergic inhibitory effect on a causal and contributing factor in carcinogenesis. This strategy, specifically in the removal of hindrances such as low drug efficiency and tumor resistance, can be valuable to improve the total therapeutic effectiveness of the single drug [17, 18]. Currently, CompuSyn has become a popular tool to calculate a combination index (CI) through Chou-Talalay method. This method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the link between single entity and multiple entities, and first order and higher order dynamics. This common equation includes the Michaelis-Menten, Scatchard, Henderson-Hasselbalch and Hill equations in biophysics and biochemistry [19].

Cyclin D1 overexpression is found in more than 50 % of human breast cancers and leads to mammary cancer in transgenic mice. In addition to inducing progression of cell cycle via cyclin-dependent kinases (CDKs) activity regulation, cyclin D1 promotes other regulatory molecules by CDK-independent mechanisms [19]. Transcriptional activity of signal transducer and activator of transcription 3 (STAT3) was suppressed by cyclin D1. Losing antiapoptotic activity of STAT3 and causing apoptosis induction. Cyclin D1 plays role in as an intermediary metabolite in other cell cycle control pathways including, Rac1, 5’ adenosine monophosphate-activated protein kinase (AMPK) and nuclear factor- B (NFκB) signaling pathways. reduction in Rac1 levels causes NFκB signaling inhibition and prompts decrease of cyclin D1. Active AMPK results in loss of cyclin D1 in mRNA and protein levels [20].

Telomerase has been known as an attractive therapeutic target for treatment of different cancers, as it preserves tumor cells division and survival and decreases apoptosis induction. It has been shown that telomerase is active in 90 % of breast carcinomas and 85 % of human cancers, while in normal cells it is not active or detectable [21, 22]. It was reported that telomerase reactivation was considerably related with advanced breast cancer stage, nodal metastasis and histopathological grade, and no significant association between telomerase activity and menopausal status, or tumor size was found. Besides, it was revealed that no significant association between tumor hTERT (human telomerase reverse transcriptase) expression and patient’s age, tumor size, grade, nodal metastasis, estrogen receptor (ER) positivity and lymphovascular (LVI) [23, 24]. Thus, Inhibition of telomerase activity specially its catalytic subunit, hTERT, in breast cancer cells may reactivate telomere shortening and might be a hopeful target in breast cancer treatment [15, 25].

Regarding to the demonstrated anti-cancer effects of MET and SIL as safe anticancer agents and lack of studies showing combined inhibitory effects of the drugs, The aim of this study was to evaluate the hypothesis that Silibinin and Metformin will work in synergism and inhibit the growth of T47D cells as a model breast cancer cell line. Thus, Silibinin, Metformin and their combination in constant ratios with regard to their IC50s were used to determine the cytotoxicity and expression levels of hTERT and Cyclin D1 in T47D breast cancer cells.

▶ Fig. 1 Chemical structures of a MET and b SIL.
Materials and Methods

Chemicals and reagents
Silibinin, Metformin and MTT powder were obtained from Sigma (Deisenhofen, Germany); T47D breast cancer cell line purchased from the Pasteur Institute of Iran, Tehran, Iran; fetal bovine serum (FBS) and phenol-red free RPMI 1640 with L glutamine were purchased from Gibco BRL (Life Technologies, Grand Island, NY, USA); Sodium bicarbonate and Streptomycin were purchased from Merck (Darmstadt, Germany); Penicillin G was purchased from SERVA (Heidelberg, Germany); TRIZOL reagent was purchased from Invitrogen (Eugene, OR, USA); First-Strand cDNA Synthesis kit was purchased from Fermentas (Hanover, MD, USA); and Syber Green-I reagent was purchased from Takara Bio (Otsu, Japan).

In vitro cytotoxicity assay
RPMI 1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin were used to culture T47D breast cancer cells. The cells were incubated in a humified atmosphere containing 5 % CO2 at 37 °C.

Cytotoxic activity was studied using MTT cell viability assay after 24 and 48 h treatment of MET, SIL and MET + SIL to the cells. Metabolically active cells decrease the tetrazolium constituent of MTT in to purple colored formazan crystals. Briefly, 2 x 10^4 cells/well were cultivated in 96-well plates for 24 h and then, treated with different concentrations of MET (0, 1, 5, 10, 15, 20 and 25 mM), SIL (0, 5, 25, 50, 75, 100 and 125 μM) and MET + SIL (1000:5, 5000:25, 10000:50, 15000:75, 20000:100, 25000:125 μM). After 24 and 48 h exposure time, medium was replaced with fresh medium and 2 mg/ml of MTT was added to each well and plates were covered with aluminum foil and incubated for 4 h at 37 °C. Thereafter, the content of the wells was removed and 200 μl pure DMSO and 25 μl Sorensen’s glycine buffer were added. In the next step, absorbance of each well was read at 570 nm using ELISA plate reader (Bio-Tek Instruments) with reference wavelength of 630 nm [26].

IC50s of MET, SIL and MET + SIL against T47D were measured using GraphPad Prism version 6.7 (GraphPad Software, Inc., San Diego, CA, USA). Also, Compusyn software version 1.0 (CompuSyn, Inc.) was used to determine the nature of interaction between MET and SIL in combination form

RNA extraction, cDNA synthesis and Real-time PCR
qRT-PCR assay was used to analyze expression levels of relative mRNA of hTERT and Cyclin D1 genes. T47D cells were treated with different concentrations of MET, SIL and MET + SIL for 48 h. After drug exposure time, total RNA was isolated using the Trizol reagent according manufactures protocol. Then, the quantity and quality of total RNA was assessed based on OD260/280 ratio measurements and electrophoresis on 1.5 % agarose, respectively.

Equal amount of RNA was taken from all the samples and reverse transcribed using RevertAid First strand cDNA synthesis Kit (Fermentas, St Leon-Rot, Germany) to gain complementary DNA (cDNA). The reverse transcription reaction product can be directly used in PCR applications or stored at -20 °C for less than one week. For longer storage, -70 °C is recommended.

hTERT and cyclin D1 gene expression in T47D breast cancer cells after exposure to MET, SIL and MET + SIL for 48 h was assessed by real-time PCR. The synthesized cDNA was diluted 1:5, 1:10 and 1:20. The concentration of 1:20 of cDNA was used as template for real-time PCR. Then, the synthesized cDNA was amplified by quantitative real-time RT-PCR using specific primers (Takapou Zist Co., Iran) and the SYBR Green-I dye (Roche, Germany) by the Rotor-GeneTM 6000 system (Corbett research, Australia). The quality of real-time PCR reactions was controlled by running standard samples as duplicated 5 times serial dilutions of cDNA obtained from the T47D breast cell line served as samples with strong expression of hTERT gene. The program for real-time PCR reaction was as follows; Initial denaturation at 95 °C for 10 min, followed by cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Finally, amplicons were measured by melting curve analysis of 70 °C to 95 °C. The real-time PCR efficiencies were determined for each gene. Relative hTERT and cyclin D1 expression levels was normalized by housekeeping gene (β-actin) and relative expression of the genes calculated by this formula: (normalized relative ratio = 2- Ct).

The program for real-time PCR reaction was as follows; Initial denaturation at 95 °C for 15 min (1 cycle), followed by cycles of denaturation at 95 °C for 15 s (45 cycle), annealing at 59 °C for 30 s and extension at 72 °C for 20 s. Finally, amplicons were assessed by melting curve analysis of 59-95 °C to for 5 s for each degree (1 cycle).

Statistical analysis
All experiments were done in three replicates and values displayed are representative for at least three independent experiments. Graph Pad Prism 6.7 was used for statistical analysis. All the results of the experiments were expressed as the mean ± standard deviation (Mean ± SD). Levels of the statistical significance were measured using the paired Student t test when comparing two groups, or by analysis of variance (ANOVA). P values of P ≤ 0.05 were considered significant.

Results
In vitro cytotoxicity
The effect of free MET, free SIL and MET + SIL on growth inhibition of T47D were examined using colorimetric MTT assay after 24 and 48 h treatment. the cytotoxicity assay findings revealed that both MET and SIL significantly inhibited the proliferation of T47D cells growth in a time and dose-dependent manner (Fig. 2).

Table 1 displays the half maximal inhibitory concentration (IC50) and combination index (CI50) values for the drug formulations against T47D and for 24 and 48 h incubation time. According to the data analysis of the cytotoxicity assay, the combination of MET and SIL showed more growth inhibitory effect with a drastic reduction in IC50s of MET and SIL in the breast cancer cells than the single treatments after 24 and 48 incubation time.

Compusyn software was used to analysis the precise nature of the interaction between MET and SIL in combination through Median-effect method. Based on this method, the obtained combination index plot showed that the CI50 values of MET + SIL for T47D breast cancer cells were measured to be 0.86 and 0.63, respective-
ly, indicating the synergistic growth inhibitory effect of MET and SIL in combination form in a time dependent manner (Fig. 3).

**Gene expression findings**

To further explore the mechanisms involved in MET and SIL combination-mediated inhibition on T47D breast cancer cells, qRT-PCR was applied to measure the expression levels of hTERT and cyclin D1 genes. Therefore, the expression levels of the genes was determined after 48 h drug treatment of breast cancer cells. Real-time PCR results showed that exposure of T47D cells to the various concentrations of MET and SIL lead to a significant reduction in the expression levels of hTERT and cyclin D1 as a dose dependent manner in compared with control group. As shown in Fig. 4, Importantly, hTERT and cyclin D1 mRNA levels decreased highly in the combined treatment groups in all applied concentration in relative to the groups for which drugs were applied alone (P ≤ 0.05).

**Discussion**

In the current work, the cytotoxicity of MET + SIL were assessed against T47D breast cancer cells. The assessment disclosed high capability of the combination to diminish the growth of T47D breast cancer cells through inhibition of hTERT and cyclin D1 expression levels.

It was found that single treatments of MET and SIL exhibited proliferative inhibitory effect against breast cancer cells in a dose- and time-dependent manner. The results are in harmony with those from previous studies which displayed the antiproliferative activity of MET + SIL against different types of breast cancer cell lines.

In this study, it was evaluated whether combining MET with SIL has a greater cytotoxicity against T47D breast cancer cells compared to the single treatments of the anticancer agents. The results propose that MET + SIL were strongly effectual in killing the breast cancer cells.

The quiddity of the combination effect was further analyzed by the median-effect method [27]. A series of “dose (D) and effect (fa)” were entered into CompuSyn for each drug alone and their combination, the software automatically simulated the CI values at different fa levels in seconds, based on the CI algorithm. The resulting CI theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI<1), and antagonism (CI>1) in drug combinations. According to our results, The CI values were < 1 for the combination treatments after 24 and 48 h, indicating an inhibitory synergistic effect of MET + SIL against proliferation of T47D breast cancer cells.

The effect of SIL in combination with various anti-cancer agents such as cisplatin, doxorubicin, carboplatin, and curcumin against breast cancer cells has been studied. Combination of SIL with doxorubicin caused a stronger apoptotic death in MCF-7 and MDA-MB468 breast cancer cells compared to either drug alone. Besides, SIL in combination with carboplatin resulted in a much stronger apoptotic effect in MCF-7 cells. On the other hand, SIL plus cisplatin revealed no further apoptotic activity in MCF-7 and MDAMB468 cells [28]. Combination of SIL with curcumin also inhibited the proliferation and telomerase expression of T47D breast cancer cells [12]. Hence, It was suggested that SIL + curcumin might be a hopeful therapeutic approach in breast cancer therapy.

Also, the combination of MET with chemotherapeutic agents such as paclitaxel, carboplatin, epirubicin, doxorubicin, 5-FU, and cyclophosphamide have been extensively reported [29–31]. Recent data showed that MET in combination with trastuzumab killed cancer stem cells and inactivated ErbB2/IGF-1 R interactions in a synergistic manner via inhibiting Src kinase and/or PI3K/Akt interactions, causing overwhelming primary resistance to trastuzumab in HER2 positive breast cancer cells [32, 33]. Ma and colleagues showed that MET reduced migration and invasion of cancer cells in tamoxifen-resistant breast cancer cells and in combination with tamoxifen synergistically inhibited proliferation of ER positive breast cancer via the Bax/Bcl-2 and AMPK/mTOR/p70S6K pathways [34, 35]. It was found that the combination treatment of MET and...
paclitaxel arrested in G2/M phase, increased apoptosis and reduced cell proliferation in MCF-7 breast cancer cells [36].

To explore the mechanisms involved in MET and SIL combination-mediated inhibition on breast cancer cells, the expression levels of hTERT and cyclin D1 genes were assessed and our data suggested that the synergistic cytotoxic activity of the combination involves simultaneous inhibition of hTERT and cyclin D1 expression.

Based on a study by Cantrell et al., telomerase activity can be regulated through MET treatment in various cancer cells. It was shown that MET strongly inhibit proliferation of endometrial cancer cells in a dose dependent manner. Moreover, MET caused hTERT reduction, G1 arrest and apoptosis induction [37].

Among the different cancer models, the effects of silibinin have been strongly evaluated in the prevention and growth control of breast cancer through extensive in vitro and in vivo studies conducted in our laboratory and other researchers. Based on our previous studies, it has been revealed that SIL inhibited proliferation of T47D breast cancer cells through reduction of TERT expression as well as telomerase activity [11, 12]. Also, SIL has been shown to potently inhibit breast cancer through targeting multiple cell signaling pathways, inducing apoptosis, and inhibiting invasion, metastasis, and angiogenesis [38, 39].

In this study, we surveyed the alteration in expression levels of cyclin D1 and hTERT genes to describe the synergistic anticancer effects of MET and SIL in molecular level. However, further studies are needed to provide insight into the mechanisms involved in the elicited anticancer effects of the combination treatment of MET and SIL. Also, studies showed that unfavorable properties of these drugs such as poor solubility in water and low cellular uptake can limit the effectiveness of the drugs. So, using co-nanodelivery systems may enhance bioavailability of these natural therapeutic agents and result in strong synergistic anticancer effects on breast cancer cells.

**Conclusion**

In conclusion, the current study showed that the combination of MET and SIL exerts synergistically growth inhibitory effects against T47D breast cancer cells through down-regulation of hTERT and Cyclin D1 expression levels. Based on this finding, it can be suggested that the combinatorial chemotherapy based on MET and SIL may be a rationale and effective strategy for breast cancer therapy.
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Conflict of Interest

The authors declare that they have no conflict of interest.

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