Evaluation of anti-proliferative activity of simvastatin and atorvastatin on MCF7 cell line compared with doxorubicin using MTT test

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Abstract:
Statins are effective to lower the cholesterol level and protect against cardiovascular disease. Recent studies showed that statins have pleiotropic effect and can be used to treat many types of diseases like neurodegenerative disorders, stroke, and cancer. Statins inhibit cell proliferation by suppression of mevalonate pathway leading to desregulation of cell signal transduction of some membrane receptor protein which is important for gene transcription. This study was done to evaluate the anti-proliferative activity of simvastatin and atorvastatin on MCF7 cell line alone and in combination with an anthracycline chemotherapy drug (doxorubicin) using MTT assay. The results showed that simvastatin and atorvastatin had significant anti-proliferative effect on MCF7 cell line in dose-dependent manner with an IC50 10.10 μM and 12.3 μM respectively. Moreover, the combination of atorvastatin and simvastatin with (1 μM) doxorubicin had higher cytotoxic effect with an IC50 = 0.07 μM and 0.05μM respectively than doxorubicin alone IC50 = 1.9 μM. In conclusion, simvastatin and atorvastatin had anti-proliferative effect on MCF7 cell line and displayed significant synergism with doxorubicin which will help in enhancing efficacy of doxorubicin and decrease the adverse effect.

Key words: doxorubicin, simvastatin, atorvastatin, anti-proliferative activity, MCF7 breast cancer cell lines.
Introduction:
Breast cancer is the most common malignancy in women worldwide, accounting for 25% of all cancers. [1] Breast cancers started from different parts of the breast, mainly from milk ducts (ductal carcinoma), or glands that make milk (lobular carcinoma). Other types of breast cancer are less common [2].

Doxorubicin (DOX) is an anthracycline antibiotic and extracted from Streptomyces peucetius in 1960 is the most common drug used to treat different types of cancer but limited because of adverse effects including cardiotoxicity which is dose-dependent. Features of cardiotoxicity can be manifested as arrhythmia, cardiomyopathy, or congestive heart failure (CHF) [3,4].

Statins are used to lower cholesterol level, leading to protect against coronary events and death from CHD (congestive heart disease) [5]. After taking orally, 30-80% of drug absorbed by intestine, then undergo first pass metabolism resulting in more than 70% of statins elimination from the body. [6] Statins have pleiotropic effect and can be used in autoimmune/chronic inflammatory diseases, neurodegenerative disorders, stroke, bacterial and viral infections and cancer [7]. According to their solubility, statins can be divided into hydrophilic for example (lovastatin) which are largely limited to the liver, and lipophilic compounds (for example simvastatin (SIM), atorvastatin (ATOR)) that invade other tissues. Hydrophilic compounds inhibit cholesterol synthesis in the liver and can cause increase in cholesterol synthesis of extra hepatic tissues. While, lipophilic compounds exert more pleiotropic effects by acting on hepatic and extra-hepatic tissues, in addition to their ability to penetrate the cell membrane and affect cell proliferation, survival, and motility [8,9]. For this reason, several studies have shown that lipophilic compounds have more efficacy as anticancer agents compared to hydrophilic statins [8]. The main mechanism of action was inhibition of mevalonate pathway precursor of cell cycle regulating compounds (e.g., geranylpimorphosphate (GPP), farnesyl pyrophosphate (FPP) and dolichol) which are related to DNA synthesis and numerous tumor proteins production such as Ras and Rho, which contribute in regulation of cellular signal transduction of some membrane receptor proteins which are important for gene transcription involved in cellular proliferation, differentiation, and apoptosis. The preclinical studies exposed that statins cause inhibition of GPP, FPP and dolichol production with capture of tumor cell proliferation [6].

Materials:
SIM, ATOR (Tabuk pharmaceutical MFG), DOX powder (Saba/Turkey), DMEM media (Giboco/ USA), fetal calf serum(Biowest/France), DMSO(CDH/India), MCF7 breast cancer cell line (obtained from the Biotechnology center/ Al-Nahrain University), phosphate buffer saline (Sigma Aldrich/USA) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Promega, USA)

Methods:
MCF7 cell line at 1 x 10^4/mL were seeded in 96 well microplate with 100µL DMEM contain 10% FCS in each well and incubated for 24, 48, 72 hours (different plates) in starved media at 37°C. 1-SIM, ATOR and DOX solution in concentration 40; 20; 10; 5; 2.5 and 1.25 µM were added in triplicate 2- SIM and ATOR (40; 20; 10; 5; 2.5 and 1.25 µM) were tested in the presence of 1 µM DOX. Incubation for 24, 48, 72 hours in different plates, then add MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), reagent solution (15 µL dye solution) for 4 hours in each well and incubated in a CO2 incubator at 37°C then add 100 µl solubilization /stop solution for 1 hour. The absorbance of each well was recorded using enzyme linked immunosorbant assay (ELISA) reader at 570nm. The concentration inhibiting 50% (IC50) of the
cell lines was determined by logarithmic scale equation. (10).

Cell viability% = Mean optical density / control optical density × 100%
Inhibition % = 1 - cell viability%

**Statistical analysis**

Throughout the work, the experiments were performed three times independently and results were expressed as the mean ± the standard deviation using SPSS 16 and compared analysis of variance (One Way ANOVA) followed by post hoc LSD. A *p value < 0.05 was considered as statistically significant. Analysis of MTT test between 24, 48, 72 hours was performed using paired sample t test. A *p value < 0.05 was considered as statistically significant.

**Results/Cytotoxic activity assay:**

This work was done in the tissue culture laboratory of the department of Pharmacology and Toxicology in College of Pharmacy / Mustansiriyah University. Data represent the average values from three independent experiments, the cytotoxicity was assessed in 24 h, 48h and 72h (Fig 1, 2, 3) respectively.

![Figure (1): Cytotoxic effect of DOX, SIM and ATOR (40μM, 20μM, 10μM, 5μM, 2.5μM, 1.25μM) alone, the same concentration of SIM and ATOR in combination with (1μM) DOX on MCF7 cell line in 24 hour, each value represent in 3 independent experiment ± SD.](image)

![Figure (2): Cytotoxic effect of DOX, SIM and ATOR (40μM, 20μM, 10μM, 5μM, 2.5μM, 1.25μM) alone, the same concentration of SIM and ATOR in combination with (1μM) DOX on MCF7 cell line in 48 hours, each value represents in 3 independent experiment ± SD.](image)
Six serial dilutions of DOX, SIM and ATOR was prepared (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25µM) and added to MCF7 cell line to determine the anti-proliferative effect. The results showed a significant inhibition of MCF7 cell line in dose dependent manner after 72 hours while compared to the negative control (cells without treatment) (*P<0.05) rather than other incubation periods (24, 48 hour) dose response as shown in (Fig 3). The IC\textsubscript{50} of DOX, SIM, and ATOR was found to be 1.9µM for DOX, 10.10 µM for SIM and 12.3 µM for ATOR.

The results of combination showed higher synergism effect on MCF7 cell line in dose-dependent manner while compared to the negative control (untreated cell) (*P<0.05) after 72 hours rather than other incubation periods as shown in (Fig 3). The IC\textsubscript{50} of ATOR and SIM in combination with DOX was found to be 0.07 µM for (DOX + ATOR) and 0.05 µM for (DOX + SIM).

**Discussion:**

Breast cancer is the major common cause of death in women world-wide attributable to several factors like: early metastasis, aggressive invasion and resistance to chemotherapy drugs \[11\]. Anthracycline (DOX), used alone or in combination with other chemotherapy to enhance the efficacy and reduce the side effect. At cellular level, DOX had several molecular mechanisms such as generation of free radicals, inhibitors of topoisomerase II trigger apoptosis and interference with DNA unwinding \[12\]. DOX is limited because of cardiotoxicity (major adverse effect) which is dose-dependent. Cardiotoxicity caused by formation of reactive oxygen species result from convert the quinine moiety to semi-quinine radical catalyzed by nicotinamide adenine dinucleotide phosphate (NADH) dehydrogenase or deactivate Top2β function causes strand DNA breaks lead to many events like activation of p53, mitochondrial dysfunction and increased apoptosis \[13\]. Other mechanisms involve increase intracellular calcium, form DOX-iron complexes leading to ROS generation and apoptosis, increase ET-1 level and increase production of MMP-2 and MMP-9 cause ECM destruction and cardiomyopathy \[14\].

Statins lower the cholesterol level by inhibit mevalonate pathway (precursor of cholesterol) in patients with hypercholesterolemia and cardiovascular disease \[15\]. Many studies show that statins have a pleiotropic effect and used in the treatment of many diseases, especially in cancer \[16\]. Researchers found that statins show
cytotoxic effect and induce apoptosis in many cancers cell line including breast cancer [17]. Lipophilic statins (SIM, ATOR) exert their effects on tumor cells better than hydrophilic statins (pravastatin), because of their ability to invade hepatic and extra hepatic tissues and exert more pleiotropic effect [18]. Although the useful effect of HMG-COA reductase inhibitor to decrease cholesterol is well known, the useful effect in cancer treatment, still clarification. The main adopted mechanism is interference with level of FPP and GGPP (isoprenoid intermediate), which is important in modification of intracellular G-protein RAS, Rho because these proteins are critical for gene expression include cell proliferation, differentiation and apoptosis [19].

In the present study, it was found that SIM and ATOR decrease viability of MCF7 cell line significantly in dose-dependent manner compared with untreated control cell. According to the American National Cancer Institute (NCI), a compound with IC50 lower than 50 µg/mL (119.45 µM) is considered to have cytotoxic effect on cancer cell lines and can be classified in to very active (IC50<5 µg/mL), active (IC50 5-10 µg/mL) and moderate potential (IC50 11-30 µg/mL) (10). According to the results obtained in this study, IC50 of SIM was 10.10µM (4.22 µg/ml) and IC50 of ATOR was 12.3 µM (5.14 µg/ml) which show significant cytotoxic effect against MCF7 cancer cell line (Fig 3) (*p<0.05) after 72 hour rather than other incubation period (24, 48 hours) because the cytotoxic effect is directly proportional with time interval. SIM exerted more cytotoxic effect than ATOR due to the difference in pharmacokinetic properties especially lipophilicity. Slawińska-Brych A et al (2014) reported the IC50 of SIM on MCF7 and MDM-231 ranging 1.26 to 91 µM and different with other cancerous cells [20]. The cytotoxicity of SIM and ATOR and DOX were measured by MTT. MTT is a colorimetric assay depending on the metabolic activity of cells. The assay in reality reflects the number of viable cells which can convert MTT by help of mitochondrial reductase into soluble formazan [21]. The cytotoxicity of SIM and ATOR can be explained by their ability to interfere with expression of tumor suppressor factors, CDK inhibitors, Rho A and desregulation of ROS productions as induction of apoptosis [22]. ATOR and SIM were found to have the ability to downregulate Bcl-2 expression and Rho A prenylation [23]. In present study, the combination of DOX with ATOR (IC50=0.07 µM, 0.02 µg/ml) and DOX with SIM (IC50= 0.05 µM, 0.02 µg/ml) resulted in enhanced cytotoxicity of DOX (Fig 3) than DOX alone IC50= 1.9 µM (0.79 µg/ml), (*p < 0.05) after 72 hour rather than other incubation period (24, 48 hours) because the cytotoxic effect is directly proportional with time interval. The enhancement of the cytotoxicity is manifested by the decrease in IC50 for DOX as shown below:

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 (µM)</th>
<th>% Reduction of IC50 of DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>DOX+ATOR</td>
<td>0.07</td>
<td>96</td>
</tr>
<tr>
<td>DOX+SIM</td>
<td>0.05</td>
<td>97</td>
</tr>
</tbody>
</table>

By using the compusyn software, it was shown that the interaction of DOX with statins (ATOR, SIM) involves synergism. This synergism would be useful, since it will help using lower concentration of DOX to achieve the same required response of cytotoxicity (DOX+ATOR=96% reduction, DOX+SIM = 97%) table (1) Eventually, the
possibility of the predicted side effects will be reduced which means enhanced efficacy and safety. According to the Mechanism by which the combination enhanced cytotoxic effect of DOX would be effect on the cell cycle by upregulating p21 level and downregulated the level of cyclin D1, release of cytochrome C and caspase 3 leading to apoptosis in addition to inhibiting the mevalonate pathway lead to reduce the level of FFP and GGP and cause modification of intracellular G-protein leading to apoptosis \cite{19}.

References:


