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

Aiyat Hasan Yahya Al-khafaji*, Inaam Sameh Arif*.
Department of Pharmacology and Toxicology, Colloge of Pharmacy, Mustansirya University/Iraq.
ahy_pharmacy89@yahoo.com

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 anathracycline chemotherapy drug (doxo-rubicin) 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 respect-tively. 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 doxoru-bicin 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.

تقييم الفعاليه المضاده لكل من سيمفاستاتين واتورفاستاتين على خط خلايا MCF7 ومقارنتها مع دكسوروبسين باستخدام فحص MTT

ايات حسن يحيى الخفاجي*
انعام سامح عارف*
* فرع الادوية والسموم كلية الصيدلة / الجامعة المستنصرية / العراق

الخلاصة:

الستاتين وهي عقاقير تستعمل لخفض مستوى الكولسترول والحمايه ضد امراض القلب والاوعيه الدمويه . وقد اظهرت الدراسات السريريه وقبل السريريه ان الستاتينات لها تاثير عديد المظاهر ويمكن استخدامها لعلاج العديد من انواع الامراض مثل الاضطرابات العصبيه ، السكته الدماغيه ، والاتهابات البكتريه والسرطان.

تمنع الستاتينات تكاثر الخلايا عن طريق قمع مسار الميفالونات مما يؤدي الى التاثير على تنظيم نقل الاشارات الخلويه لبعض البروتينات وهو امر مهم لنسخ الجين.

وقد اجريت هذه الدراسه لتقييم النشاط المضاد للتكاثر لكل من السيمفاستاتين والاتورفاستاتين على خط خلايا الثدي السرطانيه (MCF7) وان التركيز القابل لمنع نمو خلايا الثدي السرطانيه بنسبه خمسين بالمائه هو 10.10μM و 12.3μ على التوالي. عند دمج السمفاستاتين والاستورفاستاتين مع الدوكسوروبسين سوف يظهر تاثير سام افضل وان التركيز القابل لمنع نمو خلايا الثدي السرطانيه بنسبه خمسين بالمائه هو 0.28μM و 0.5μM على التوالي مقارنه مع دواء الدوكسوروبسين لوحده 1.1μM.

الملخص هو وجود تاثير سام لسيمفاستاتين واتورفاستاتين على خط خلايا الثدي السرطانيه (MCF7) وعند الدمج مع الدكسوروبسين يظهر تاثير اكثر فعاليه مقارنه بالدكسوروبسين لوحده على نفس نوع الخلايا الذي سيساعد في الحد من التركيز المطلوب للدوكسوروبسين مع تقليل الاثار الجانبيه للدواء

الكلمات المفتاحية : دوكسوروبسين ، سيمفاستاتين ، اتورفاستاتين ، النشاط المضاد لمنع نمو الخلايا ، خط خلايا الثدي السرطانية .

Introduction:

Breast cancer is the most common malignancy in women worldwide, account 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., geranylpyrophosphate (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)-2.5diphenyltetra-zolium bromide] (Promega , USA)

Methods:

MCF7 cell line at 1 x 104/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 (15 μl dye solution) for 4 hours in each well and incubated in a CO_2 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
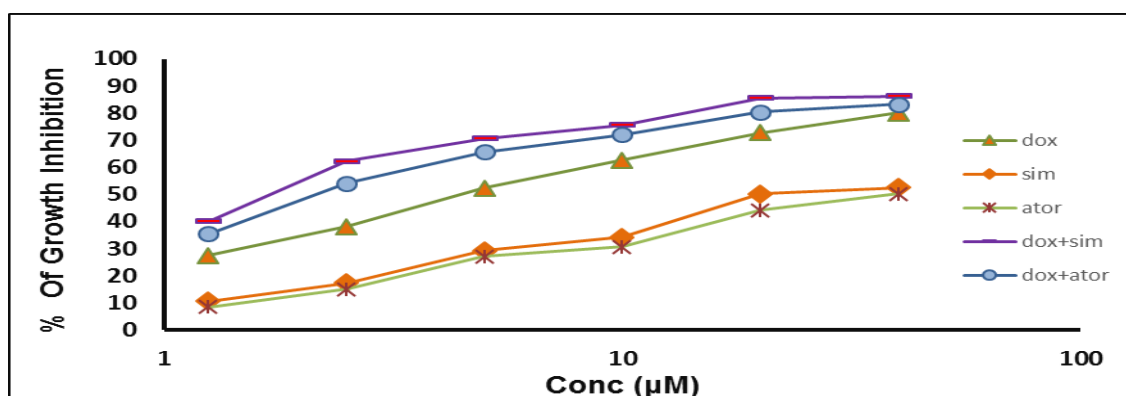

Figure (1): Cytotoxic effect of DOX, SIM and ATOR (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25uM) alone, the same concentration of SIM and ATOR in combination with (1µM) DOX on MCF7 cell line in 24 hour, each value represent in 3 independent experiment ± SD.
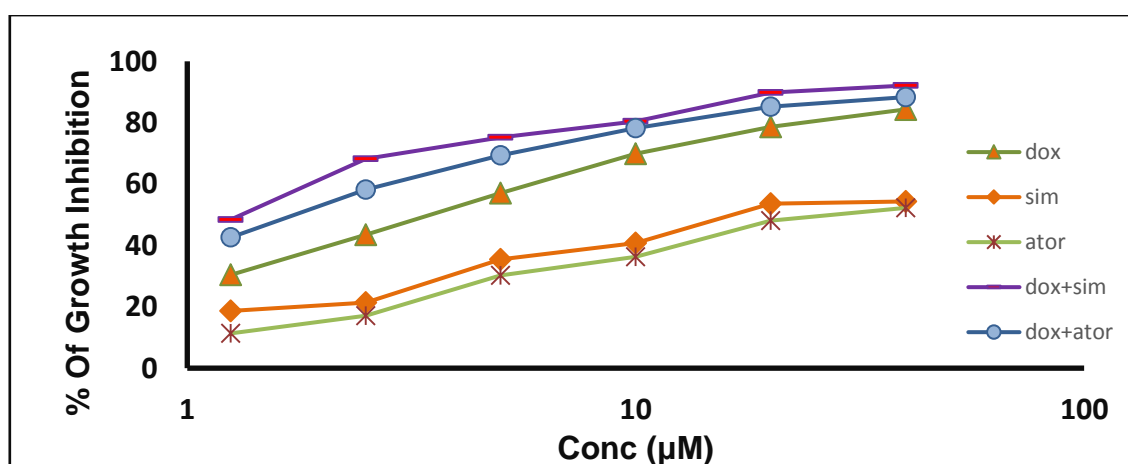

Figure (2) Cytotoxic effect of DOX, SIM and ATOR (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25uM) alone, the same concentration of SIM and ATOR in combination with (1µM) DOX on MCF7 cell line in 48 hours, each value represents in 3 independent experiment ± SD.
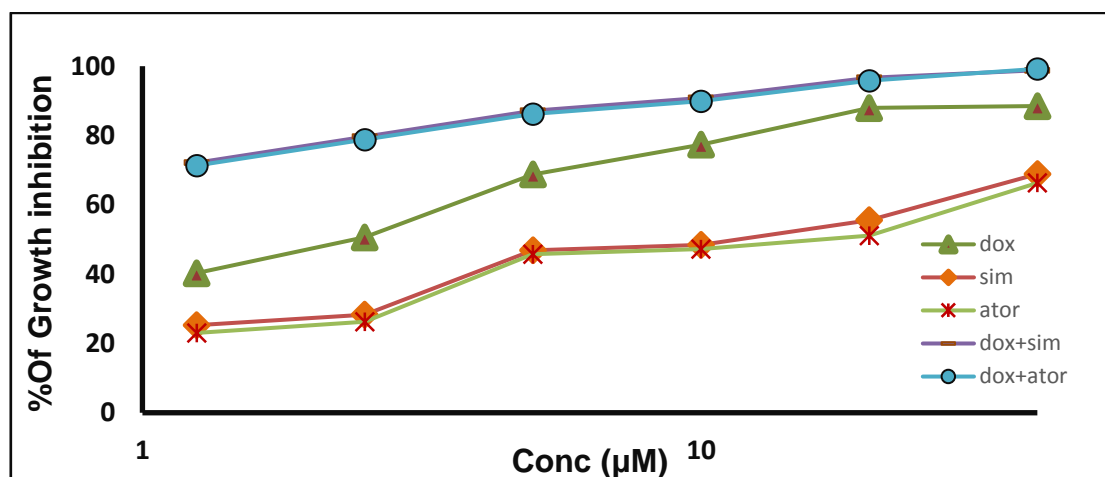
Figure (3) Cytotoxic effect of DOX, SIM and ATOR (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25uM) alone, the same concentration of SIM and ATOR in combination with (1µM) DOX on MCF7 cell line in 72 hours, each value represents in 3 independent experiment ± SD.

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_{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_{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]. Anathrcycline (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. Cardioto-xicity caused by formation of reactive oxygen species result from convert the quinine moiety to semi-quinine radical catalyzed by nicotinamide adenine dinucle-otide phosphate (NADH) dehydrogenase or deactivate Top2β function causes strand DNA breaks lead to many events like activation of p53, mitochondrial dysfun-ction 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 produ-ction 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 hyperchole-sterolemia 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. Sławińska-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 prenlyation [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 (1): The reduction of IC$_{50}$ after combination therapy

Drugs	IC$_{50}$ (µM)	% Reduction of IC$_{50}$ of DOX
DOX	1.9	
DOX+ATOR	0.07	96
DOX+SIM	0.05	97

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 [19].

References:

1- Alwan NAS. Breast Cancer Among Iraqi Women: Preliminary Findings from a Regional Comparative Breast Cancer Research Project. J Glob Oncol. 2016 Oct;2(5):255–8.

2- Kabel AM, Baali FH. Breast Cancer: Insights into Risk Factors, Pathogenesis, Diagnosis and Management. J Cancer Res Treat J Cancer Res Treat. 2015 Sep 15;3(2):28–33.

3- Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. J Mol Cell Cardiol. 2012 Jun;52(6):1213–25.

4- Jungsuwadee P. Doxorubicin-induced cardiomyopathy: an update beyond oxidative stress and myocardial cell death. Cardiovascular Regenerative Medicine. 2016 Jan 12;3.

5- Whalen K, Finkel R, Panavelil TA. Lippincott's illustrated reviews: pharmacology. Philadelphia, Pa.: Wolters Kluwer; 2015.

6- Abdelmaksoud B. Role of Statins in Breast Cancer Management: Current Issues and Future Directions. Cancer Ther Oncol Int J. 2017 Mar 27;3.

7- Davies JT, Delfino SF, Feinberg CE, Johnson MF, Nappi VL, Olinger JT, et al. Current and Emerging Uses of Statins in Clinical Therapeutics: A Review. Lipid Insights. 2016 Nov 14; 9:13–29.

8- Moonindranath S, Shen H. Statins and Breast Cancer: An Overview of the Current Situation. Adv Breast Cancer Res. 2016 Jan 11;05(01):14.

9- Campbell MJ, Esserman LJ, Zhou Y, Shoemaker M, Lobo M, Borman E, et al. Breast cancer growth prevention by statins. Cancer Res. 2006 Sep 1;66(17):8707–14.

10- Putra B, Wahyuningsih M, Sholikhah E. Cytotoxic activity of simvastatin in T47D breast cancer cell lines and its effect on cyclin D1 expression and apoptosis. J Thee Med Sci Berk Ilmu Kedokt. 2017 Apr 11; 49:47–55.

11- Polyak K. Breast cancer: origins and evolution. J Clin Invest. 2007 Nov;117(11):3155–63.

12- Lovitt CJ, Shelper TB, Avery VM. Doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. BMC Cancer. 2018 06;18(1):41.

13- Renu K, V G A, P B TP, Arunachalam S. Molecular mechanism of doxorubicin-induced cardiomyopathy - An update. Eur J Pharmacol. 2018 Jan 5; 818:241–53.

14- McGowan JV, Chung R, Maulik A, Piotrowska I, Walker JM, Yellon DM. Anthracycline Chemotherapy and Cardiotoxicity. Cardiovasc Drugs Ther. 2017;31(1):63–75.

15- Bjarnadottir O, Kimbung S, Johansson I, Veerla S, Jönsson M, Bendahl P-O, et al. Global Transcriptional Changes Following Statin Treatment in Breast Cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2015 Aug 1;21 (15):3402–11.

16- Matusewicz L, Meissner J, Toporkiewicz M, Sikorski AF. The effect of statins on cancer cells-- review. Tumour Biol J Int Soc

Oncodevelopmental Biol Med. 2015 Jul;36(7):4889–904.

17- Fredersdorf S, Burns J, Milne AM, Packham G, Fallis L, Gillett CE, et al. High-level expression of p27(kip1) and cyclin D1 in some human breast cancer cells: inverse correlation between the expression of p27(kip1) and degree of malignancy in human breast and colorectal cancers. Proc Natl Acad Sci U S A. 1997 Jun 10;94(12):6380–5.

18- Bjarnadottir O, Romero Q, Bendahl P-O, Jirström K, Rydén L, Loman N, et al. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial. Breast Cancer Res Treat. 2013 Apr;138(2):499–508.

19- Buranrat B, Suwannaloet W, Naowaboot J. Simvastatin potentiates doxorubicin activity against MCF-7 breast cancer cells. Oncol Lett. 2017 Nov;14(5):6243–50.

20- Sławińska-Brych A, Zdzisińska B, Kandefer-Szerszeń M. Fluvastatin inhibits growth and alters the malignant phenotype of the C6 glioma cell line. Pharmacol Rep. 2014 Feb 1;66(1):121–9.

21- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983 Dec 16;65(1–2):55–63.

22- Feldt M, Bjarnadottir O, Kimbung S, Jirström K, Bendahl P-O, Veerla S, et al. Statin-induced anti-proliferative effects via cyclin D1 and p27 in a window-of-opportunity breast cancer trial. J Transl Med. 2015 Apr 29; 13:133.

23- Wood WG, Igbavboa U, Muller WE, Eckert GP. Statins, Bcl-2, and apoptosis: cell death or cell protection? Mol Neurobiol. 2013 Oct; 48 (2):308–14.