Original article

Silibinin, up-regulates chemokine receptor expression in MDA-MB-231 breast cancer cell line

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Abstract:

Introduction: Silibinin, a polyphenolic flavonoid isolated from the milk thistle plant (Silybummarianum), has various applications in cancer therapy. This investigation aimed to examine the effects of silibinin on proliferation and chemokine receptor expression on MDA-MB-231 cells, a highly metastatic human breast cancer cell line. ***Methods:*** The cytotoxic effect of silibinin on MDA-MB-231 cells was determined by micro-culture tetrazolium test (MTT) assay. In addition, the expression of chemokine receptors CXCR3, CCR5 and CCR7 genes in response to silibinin was evaluated by Real-Time PCR. ***Results:*** Data analysis from MTT assay showed that silibinin had dose-dependent and time-dependent inhibitory effects on MDA-MB-231 cell line. Moreover, Real-Time PCR analysis showed that silibinin not only had no inhibitory effects on CXCR3, CCR5 and CCR7 gene expressions, but also could increase significantly the expression of these genes in a dose and time-dependent manner. ***Conclusion:*** These results revealed for the first time the increased possibility of CXCR3, CCR5 and CCR7 genes expression in response to silibinin in human breast cancer cells.

Keywords: Silibinin; MDA-MB-231; CXCR3; CCR5; CCR7

Introduction

Chemokines are a family of chemoattractantproteinwith a molecular mass approximately 8 to 17 kDawhich belong to a superfamily that is divided into 4 groups (CXCL, CX3CL, CCL, and XCL) based on the motif displayed by the first two cysteine residues located near their N-terminal end[1]. Their biological activities have been most clearly defined in leukocytes, where chemokines coordinate development, differentiation, anatomic distribution, trafficking, and their effector functions and thereby regulate innate and adaptive immune responses[2],in particular, during infection,cancerand inflammation. In inflammation and cancer, chemokines contribute to the rolling, tethering, and invasion of leukocytes from blood vessels, through the endothelial cell basement membrane, and into the parenchyma indiseased tissues[3-5].

The cells respond to chemokines through a family of seven transmembrane G protein–coupled receptors (GPCR) called chemokine receptors that are also divided into families: CXCR1-5 bind CXC chemokines, CCR1-10 bind the CC chemokines, and CX3CR1 that only be connected to the CX3CL1 chemokine, and XCR1 that binds the two the both XCL1 and XCL2 chemokines. Each receptor isdistinct whit overlapping ligand specificity[6,7].

A growing body of evidence indicates that many types of tumor cells can express chemokinesand chemokine receptors that contribute to a number of tumor-related processes and regulating various forms of the transformed phenotype, such as angiogenesis/angiostasis,tumor cell growth, local invasion, and mediating organ-specific metastases of cancer[8,9],that share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors[10].

In breast cancer, specific chemokine receptors have important roles in disease progression. In addition,

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it is reported that the chemokine receptorsCXCR3, CCR5 and CCR7 are highly expressed in human breast cancer cells, malignant breasttumors and metastases. In breast cancer cells, signaling through CXCR3, CCR5 or CCR7 mediates actin polymerization and pseudopodia formation, and subsequently induces chemotactic and invasive responses[10-12].

Recently, the pharmacological analysis of chemokine receptors and GPCR drugs has beenreported at early stages of development[2].Furthermore, recent results could be useful in understanding silibinineffect in breast tumor[13]. Silibinin, a flavonoid antioxidant extracted from the medicinal plant *Silybummarianum* (milk thistle)have traditionally been used for the treatment of liver diseases. In the last decades silibininhas attracted attention for chemoprevention and chemotherapy of tumor cells. Silibinincanalso exert the significant anti-neoplastic effects in a variety of in vitro and in vivo epithelial cancer models, including breast, lung, skin, prostate, bladder, colon and kidney carcinomas[14,15].

In the present study, the effect of silibinin on expression of chemokine receptors CXCR3, CCR5 and CCR7 in MDA-MB-231 cells, a highly metastatic human breast cancer cell line, was investigated by Real-Time PCR. Furthermore,MTTwas performed to evaluate the silibinin effects on proliferation of MDA-MB-231 cells.

Materials and methods

Cell culture

MDA-MB-231 human breast cancer cell line was purchased from the Pasteur Institute of Iran. MDA-MB-231 cell line was cultured in RPMI 1640 medium (Gibco, invitrogen) supplemented with 10% FBS1640, L-glutamine and 100 U/ml penicillin/streptomycin (P/S). Cells were grown at 37°C and 5% CO2 atmosphere. For *in vitro* studies, silibinin(Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) at a 100mM stock solution and stored at 4°C until the time of use.

In vitro proliferation assay and LC$_{50}$ determination

Cell viability was determined using a colorimetric microculture assay with the MTT (Sigma, USA) end-point,which was performed to determine the cytotoxic effect of the silibinin at various concentrations. In this assay, the ratio of reduced MTTto formazanwas calculated based on the number of viable cells. MDA-MB-231

cell line, were seeded at 4×10^3 cells per well on a 96-well plate. Silibinin was diluted in DMSO as described earlier and added at various concentrations (0, 50, 100, 200 and 400 µM), 4 h after cell attachment. Cells were incubated for 24, 48 and 72h at 37°C and then the medium was removed. The number of viable cells was determined by the tetrazolium dye MTT colorimetric assay: with adding 200 µl of MTT (0.5 mg/ml in tissue culture medium without phenol red) and incubating the plates for 2–4 h, removing medium and adding 200 µl of DMSO to each well to solubilize the formazan dye and then reading the plates by ELISA reader. The 50 % lethal concentration (LC50) value of silibinin on MDA-MB-231 cells at 24, 48 and 72 h was calculated, respectively. LC50 was determined by probit analysis using the Pharm. PCS (Springer-Verlag, USA) statistical package.

RNA extraction and c-DNA synthesis

Treated and un-treated MDA-MB-231 cells were rinsed with cold PBS. Thereafter, 800 µL of RNX-Plus solution (Sinaclon, Iran) was added and vortexed, then incubated at room temperature for 5 min. Afterward, 150 µl of chloroform was added and the mixture incubated at room temperature for 10 min. The mixture was centrifuged at 12000 g (4 °C) for 15 min. The aqueous phase was transferred to a fresh Eppendorf tube and 250 µl of isopropanol added to the mixture. After overnight incubation on -20 °C, the samples were centrifuged at 12000 g (4 °C) for 15 min. Here, RNA pellet will be visible, after this step isopropanol was removed, and ethanol (75 %, 1ml) was added to the RNA pellets, and mixture wasthen centrifuged for 8 min. at 7500 g (4 °C) and supernatant discardedand let the pellet to dry at room temperature for few minutes. The RNA pellets were dissolved in diethyl pyro carbonate (DEPC)-treated water. Finally, the purity and concentration of the RNA preparation was assessed by agarose gel electrophoresis and spectrophotometric measurement of optical density at 260 and 280 nm. The Complementary DNA (cDNA) synthase was performed by cDNA synthase kit (Qiagen, Hilden, Germany) according to manufacturer's protocol; also

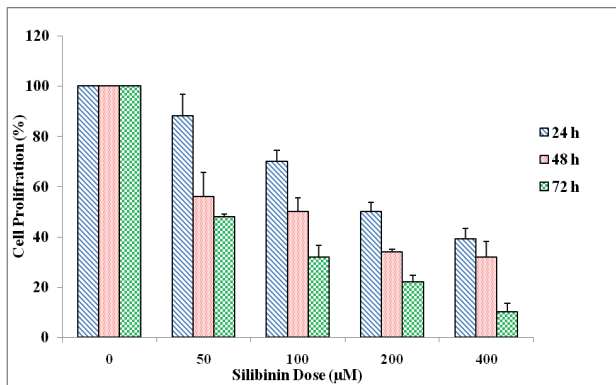| Table 1. Real-Time PCR primers used in this study.		
Gene	**Primer**	**Catalog-No (Qiagen)**
GAPDH	Hs_GAPDH_2_SG QuantiTect Primer Assay	QT01192646
CXCR3	Hs_CXCR3_1_SG QuantiTect Primer Assay	QT00213493
CCR5	Hs_LOC727797_1_SG QuantiTectPrimer Assay	QT01336601
CCR7	Hs_CCR7_2_SG QuantiTectPrimer Assay	QT01666686

Fig. 1: Cytotoxicity and anti-proliferative effects of silibinin on cell culture.a
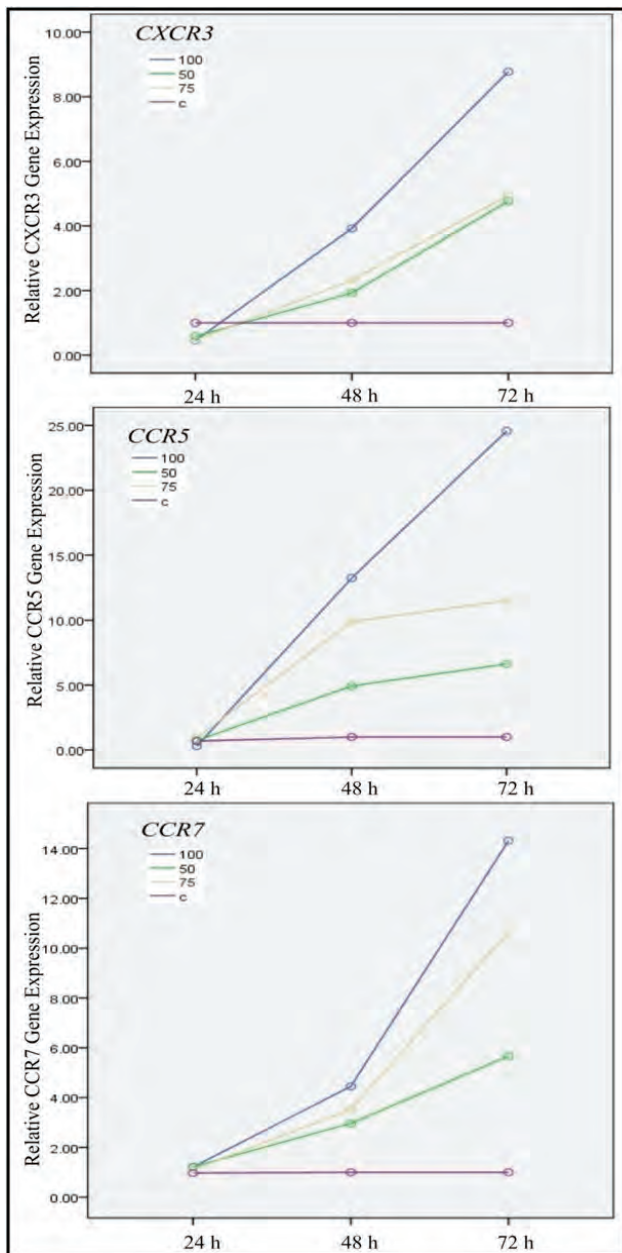

Fig. 2: Real-Time PCR Analysis of CXCR3, CCR5, and CCR7 mRNA expression.

GAPDH was used for housekeeping amplification. The various mRNA, cDNAs were amplified using the following primers as described in the Table 1.

Real-Time PCR

To characterize the silibinin effects on the CXCR3, CCR5 and CCR7 genes expression, Real-Time PCR was performed using the QuantiFast SYBR Green PCR Detection System (Qiagene) on a Rotor-Gene 6000, Thermal Cycler (Corbett Research, Australia) during 35–40 cycles. PCR amplification was performed in 10 µL reaction mixture containing 5 µl ready-to-use SYBR Green RT-PCR master mix (2x), primer A (1 µM), primer B (1 µM), DNA template (≤100 ng/reaction), 1 µLQuantiFast RT Mix and 3 µl RNase-free water. The PCR vessels were place in the real-time cycler and run the cycling program. The following conditions of real-time cycler were: PCR initial activation step (5 min, 95ºC) as first denaturation,HotStarTaq*Plus* DNA polymerase activation, denaturation (10 s, 95ºC), and combined annealing/extension (30 s, 60ºC). All analyses were done in triplicate, and the mean was used for further calculations. Ethical approval was taken prior the study.

Statistical analysis

Statistical analysis was performed with SPSS version 16.0 software. Two-way analyses of variance along with LSC post hoc analysis were carried out to determine the differences betweenmean values. Data are represented Mean ± SD. A *p-value*<0.05 was considered statistically significant.

Results

Effects of silibinin on cell proliferation

To examine the cytotoxicity and anti-proliferative effects of silibinin, we used MTT assay, with incubation times 24, 48 and 72 h of MDA-MB-231 cells suspension with medium (RPMI1640 and 10%FCS) in presence of different silibinin concentrations (0, 50, 100, 200 and 400 µM) along with 5% CO_2, and 37°C.As illustrated in Fig.1,silibinininhibites cell proliferation in a dose and time-dependent manner. Moreover,the LC_{50}valuewas calculated by probit analysis. $LC_{50}$for silibininin 24, 48and 72h were 170.3µg/ml, 90.2µg/ml,and 59.1 µg/ml, respectively. All experiments were repeated at least three times.

The effect of silibinin on cell proliferation using 4×10^3 MDA-MB-231 cells in 96-well plates was assessed by MTT test. The cells were incubated with silibinin for 24, 48and 72 h at various concentrations. silibinin inhibited cell proliferation in MDA-MB-231 cell line in a dose and time-dependent manner. Data are expressed as mean ±SD.

Differential expression of chemokine receptor mRNA in MDA-MB-231 cells

In order to assess the effect of silibinin in various concentrations (0, 50, 75. 100μM) on CXCR3, CCR5 and CCR7 genes expression by breast cancer MDA-MB-231 cell lines following 24, 48 and 72 h treatment, the Real-time PCR was conducted using primers for the mentioned chemokine receptors on RNA extracted from these treated cells.Interestingly, in this experiment,itwas foundedthatsilibinin in various concentrations increased CXCR3, CCR5 and CCR7 genesexpressionin a dose and time-dependent manner.Fig.2 andFig.3 show the results of a representative experiment that was repeated at least 3 times.

Real-Time PCR analysis detected CXCR3, CCR5, and CCR7 mRNAs in MDA-MB-231 cell lines in various concentrations (0, 50, 75 and 100 μM) of
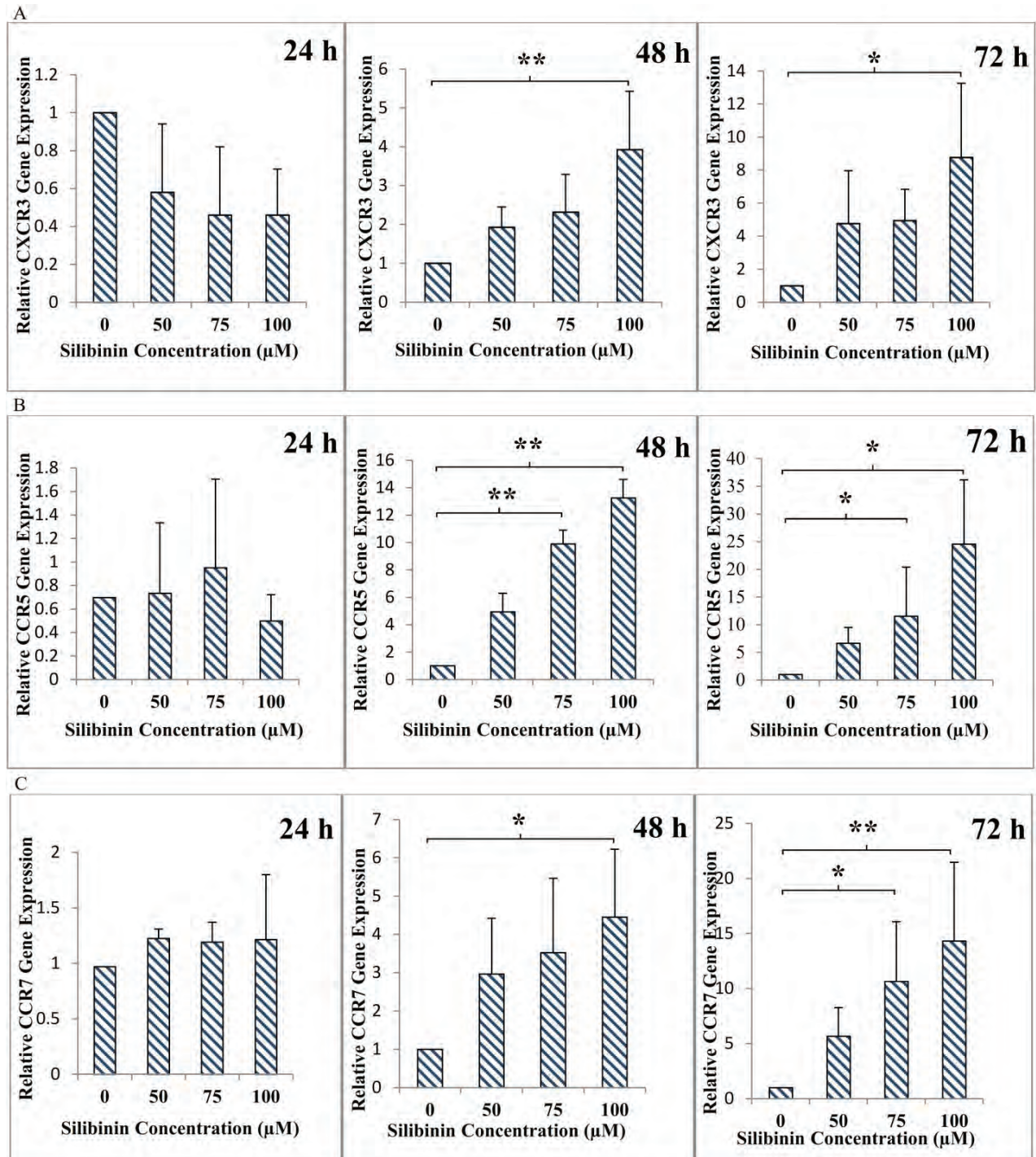

Fig. 2:Real-Time PCR Analysis of CXCR3, CCR5, and CCR7 mRNA expression.

silibinin after 24, 48 and 72 h treatment. GAPDH was used as a control. **A, B, C**: results show an increase in CXCR3, CCR5, and CCR7 genes expression after silibinin treatment in a dose-dependent fashion, respectively.Data are presented as the mean ±SD of triplicate. Error bars, mean ±SD statistics were calculated using t-test and *P values <0.05* were considered significant *(*=P<0.05, **=P<0.01)*.

Silibinin imposed an increase in mRNA level of CXCR3, CCR5, and CCR7 genes in a time-dependent manner (P<0.05).

Conclusion

Silibininis an effective anti-cancer and chemopreventive agent in tumor cells. In the last decades silibinin has attracted attention for chemoprevention and chemotherapy of various epithelial cancer models. It has been reported that silibinininhibits cancer cell growth through mitogenicsignaling pathways. However, the underlying mechanisms are still not well elucidated[16]. In the present study, the inhibitory effect of silibinin on proliferationof MDA-MB-231 cell line,a highly metastatic human breast cancer cell line was investigated by MTT. In addition, the influence of the silibinin on the expression of chemokine receptor mRNAs was assessed by Real-Time PCR.

Momeny et al. demonstrated that silibinin inhibits cell proliferation, in a dose-dependent manner without exerting any cytotoxicity effect in human hepatocellular carcinoma HepG-2 cells[17]. In the recent study Ebrahimnezhad et al. demonstrated that silibinin has a dose dependent cytotoxic effect on T47D breast cancer cell line[18].Moreover, in another study, Dastpeymanet al. in 2012 showed significant dose-dependent inhibitory effect of silibinin on proliferation, migration and adhesion of MDA-MB-231 cells[14]. However, in this study, silibinin showed not only a dose-dependent but alsoa time-dependent inhibitory effect on MDA-MB-231 cell proliferation.

Chemokines and their receptors have effective roles in various stages of the tumor biology including invasion, tumor growth, angiogenesis, and metastasis.Also it is suggested thatsilibininmay exert, at least partly, its anti-breast cancer effect by inhibiting tumor growth, progression, local invasion, metastasis and angiogenesis, probably through the modulation of chemokine receptors expression[9,19,20].

Based on our knowledge, the effect of silibinin on CXCR3, CCR5 and CCR7 genes expression in MDA-MB-231 cell line has never been studied up to now. However, in a study by Ghasemi et al. it is suggested that silibininis able to suppress the transcriptional levels of CCR7 in HepG-2 and PLC/PRF/5 hepatoma cells[21]. Inour study to investigate the effect of various concentrations of silibinin(0, 50, 75 and 100 μM) on CXCR3, CCR5 and CCR7 genes expression on MDA-MB-231 cell lines after 24, 48 and 72 h, Real-Time PCR was used. Interestingly, the result of this study revealed that silibinin not only had noinhibitory effects on CXCR3, CCR5 and CCR7 genes expression, but also could increase significantly the expressionof these genes in a dose and time-dependent manner. Although there is no study in the literature to be compared with this study,but there is a report that thecellular and molecular mechanisms of silibinin function could be cell-cycle arrest and apoptosis, therefore, the only proposed mechanism for inducing the effects of silibinin in chemokine receptor gene expression might beapoptotic cell death which can play an important role in inflammatory processes and reduction inflammatory reactionsrelated tochemokines receptors genes expression[22].

Conflict of interest

The authors declare that they have no conflict of interest.

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