RESEARCH ARTICLE

Synergistic Anticancer Effects of Silibinin and Chrysin in T47D Breast Cancer Cells

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Abstract

Objective: Breast cancer is one of the most significant causes of female cancer death worldwide. Although several chemotherapeutics have been developed to treat this type of cancer, issues remain such as low survival rates and high reoccurrence after chemotherapy and radiotherapy. To explore a chemopreventive approach to enhancing breast cancer treatment efficacy, the antiproliferative effects of a combination of chrysin and silibinin, two herbal substances, in T47D breast cancer cells were assessed. **Materials and Methods:** Cytotoxicity of the agents singly and in combination was evaluated by MTT assay. Also, qRT-PCR was used to measure the expression levels of hTERT and cyclin D1 genes after 48 h treatment. **Results:** Cell viability assays revealed that chrysin or silibinin alone inhibited proliferation in a dose and time-dependent manner, and combining the drugs synergistically induced growth inhibition in the breast cancer cell line. The precise nature of this interaction was further analyzed by the median-effect method, where the combination indices (CI) were <1 for combination treatments, indicating synergism regarding T47D cell proliferation. qPCR results showed that the drug combination also synergistically down-regulated the mRNA levels of hTERT and cyclin D1 at all used concentrations compared with the drugs used alone after 48 h treatment ($P \leq 0.05$). **Conclusion:** The data provide evidence that synergistic antiproliferative effects of Chrysin and Silibinin are linked to the down-regulation of cyclin D1 and hTERT genes, and suggest that their combination may have therapeutic value in treatment of breast cancer.

Keywords: Breast cancer- synergistic effect- Chrysin- Silibinin

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Introduction

Currently, breast cancer is the most common cancer among women (DeSantis et al., 2014). Although several chemotherapeutics such as doxorubicin, etoposide, and paclitaxel have been developed to treat this type of cancer, issues remain such as low survival rates and high reoccurrence after conventional chemotherapy and radiotherapy. Thus, novel targets and treatment should be developed (Akhtar Siddiqui et al., 2015).

Various therapeutic approach has been suggested for cancer therapy. But, some of these treatments have no considerable improvement on survival rate and metastasized cancer and they may have even some severe and unwanted side effects such as chemotherapy, which causes, nausea, anemia, neutropenia, thrombocytopenia, and hair loss (Akhtar Siddiqui et al., 2015; Heather Greenlee et al., 2016).

Epidemiological reports have consistently revealed that consumption of a healthful diet including vegetables, fruits, and whole grains is powerfully related to decreased risk of cancer and other diseases (González-Vallinas et al., 2013). Nowadays, numerous natural bioactive compounds or phytochemicals have been isolated, characterized, and their potential anticarcinogenic attributes have been assessed (Miller and Snyder, 2012; Alibakhshi et al., 2016; Heather Greenlee et al., 2016). Although, effectiveness of interactions between different dietary components requires further analysis.

There is a growing body of data that chemotherapeutic combination approaches would be more effectual in decreasing drug toxicity, preventing tumor progression in relative to with either drug alone (Kapadia et al., 2013; Mohan et al., 2013; Montgomery et al., 2015). When a combination of two or more drugs display a more strong therapeutic effect than that of individual drugs at equal concentrations, the effect is defined to be a synergistic one (Liu, 2004; Chandra et al., 2012).

In this study, we tested the hypothesis that the bioactive herbal compounds, Silibinin from *Silybum marianum (known as milk thistle)* and Chrysin extracted from many plants, honey and propolis, will work in synergism and

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inhibit the growth of T47D cells. Therefore, Silibinin, Chrysin, and their combination were exploited to assess cytotoxicity and expression levels of hTERT and Cyclin D1 in T47D cell line.

Materials and Methods

Materials

RPMI1640 and Fetal Bovine Serum (FBS) were purchased from Gibco, Invitrogen, UK. Trizol-reagent were Purchased from Invitrogen (Germany). Syber Green Real Time PCR Master Mix kit was Purchased from Roche (Germany). Penicillin G, Streptomycin, (3- (4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), was purchased from Sigma-Aldrich. Silibinin and Chrysin were purchased from Sigma-Aldrich. T47D breast cancer cell line was prepared from Pasteur Institute cell bank of Iran.

Cell culture and treatment

T47D human breast cancer was grown in DMEM with 10% FBS and 1% penicillin/streptomycin, and maintained at 37˚C in a humidified incubator with a 5% CO_2 atmosphere. Exponentially growing T47D cells were seeded into desired plates and allowed to attach for 24 h before treatment. Cells were treated with various doses of Silibinin and Chrysin dissolved in DMSO. The identical volume of DMSO was used as a control.

Cell proliferation assay

The effects of Silibinin and Chrysin on cell proliferation were detected by MTT assay. Briefly, T47D cells were seeded into 96-well plates (10^4 cells/well) and allowed to attach for 24 h before treatment. The cells were exposed to various doses of Silibinin (10-150μM) and/or Chrysin (10-150) for 48 h, and cell viability was evaluated every 24 h by MTT assay according to the manufacturer's instructions. The cell survival rate was measured as follows:

Cell survival rate (%) = experimental OD value/control OD value x 100.

qRT PCR analysis

qRT-PCR method was applied to analyze expression levels of relative mRNA. T47D cells were treated with different concentrations of Silibinin, and Chrysin and Silibinin-Chrysin 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 cDNA. Then, the cDNA was amplified by quantitative real time RT-PCR using specific primers (Takapou Zist Co., Iran) (Table 1) and the SYBR Green-I dye (Roche, Germany) by the Rotor-GeneTM 6,000 system (Corbett research, Australia). 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 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. 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 = $2^{-\Delta\Delta Ct}$).

Table 1. Forward (F) and Reverse (R) Primer Sequences of hTERT and Cyclin D1 and β-actin used in Real-Time PCR

Genes	Sequences	PCR product size (pb)
hTERT	F: 5′-CCCATTTCATCAGCAAGTTTGG-3′ R: 5′-CTTGGCTTTCAGGATGGAGTAG-3′	94
Cyclin D1	F: 5′-TGCCCTCTGTGCCACAGATG-3′ R: 5′-TCTGGAGAGGAAGCGTGTGA-3′	148
β-actin	F: 5′- GGTGAAGGTGACAGCAGT-3′ R: 5′- TGGGGTGGCTTTTAGGAT -3′	154

Statistical analysis

GraphPad Prism 6 was applied for statistical analysis. The findings of each series of experiments (carry out in triplicates) are expressed as the mean values ± standard deviation of the 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 $P \leq 0.05$ were considered significant.

Results

Chrysin and Silibinin enhances growth inhibition of T47D cells

In this study to evaluate the cytotoxic effect of Chrysin or Silibinin alone on T47D breast cancer cell line was treated with different concentration mentioned above. The obtained IC50 of Chrysin for 24 and 48 was 72.2 and 43.4 μM and Silibinin for 24 and 48 was 67.7, 35.4 μM respectively. Our data analysis of the cytotoxicity assay showed that growth of cell line was inhibited by Chrysin and Silibinin alone in a dose-and time-dependent manner (Figure 1).

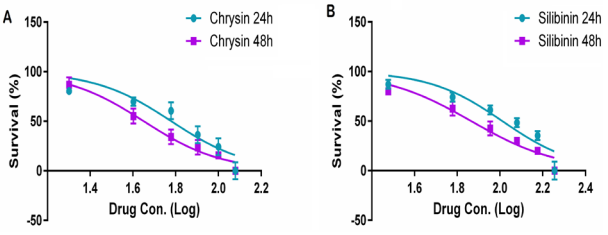

Figure 1. Effects of Chrysin and Silibinin on T47D cell proliferation. T47D cells were treated with different concentrations of (A) Chrysin or (B) Silibinin for 24 or 48 h, and cell viability was evaluated by MTT assay. Data shown are representative of three independent experiments.

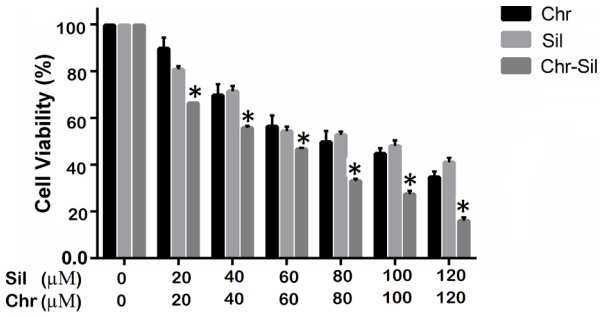

Figure 2. Effects of Combination of Silibinin and Chrysin on T47D Cell Proliferation. T47D cells were treated with Silibinin or Chrysin or both in a fixed ratio (1:1) for 48 h. Cell viability was measured by MTT assay. *p < 0.05 is the statistical difference between the combination form and individual drugs.

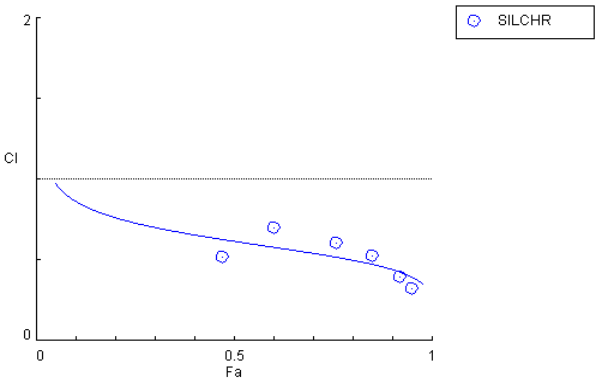

Figure 3. Combined Effects of Chrysin and Silibinin on T47D Cell Proliferation. CI was calculated by isobologram analysis using the Chou-Talalay method. CI =1, additive effect; CI <1, synergistic effect; CI, >1, antagonistic effect. Data represented are from three independent experiments.

Combination of Chrysin and Silibinin synergistically inhibits cell growth

Based on IC50 values for Chrysin or Silibinin alone applied to T47D cell line, Silibinin was combined with Chrysin at equipotent doses (1:1) to investigate potential synergism against the breast cancer cell line. As shown in Figure 2, the combination treatment of Chrysin and Silibinin was more effective in inhibiting the proliferation of T47D cells with IC50 of 24.4 μM when compared with the treatment of either agent alone (P<0.05), which indicating an interaction between the two drugs.

The precise nature of this interaction was further analyzed by the median-effect method, where the combination indices (CI) of less than, equal to, and more than 1 indicate synergistic, additive and antagonistic effects, respectively (Chou, 2013). As shown in Fig 3. the CIs value were <1 for the combination treatments, indicating a synergistic effect between Silbinin and Chrysin on T47D cell proliferation.

Chrysin/Silibinin treatments altered expression of hTERT and Cyclin D1 genes

To further investigate the mechanisms involved in Chrysin and Silibinin combination-mediated inhibition in

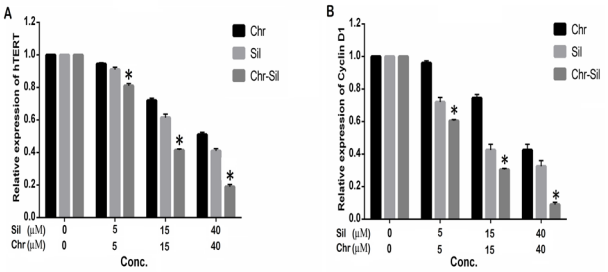

Figure 4. Effects of Chrysin-Silibinin Mixture on Inhibition of (A) hTERT and (B) Cyclin D1 Expression in Comparison with Chrysin or Silibinin Alone. *p < 0.05 is the statistical difference between the combination form and individual drugs

T47D breast cancer cells, qRT-PCR was used to analyze the expression of hTERT and cyclin D1 genes. Thus, the expression of the genes was determined after 48 h drug treatment of breast cancer cells. As shown in Figure 4, mRNA levels hTERT and cyclin D1 decreased largely in the combined treatment groups in all used concentration compared with the groups for which drugs were used alone, the difference was statistically significant ($P \leq 0.05$).

Discussion

Recent in vitro and in vivo reports exhibit promising findings regarding the chemopreventive properties of phytochemicals in various cancer models (Gatouillat et al., 2015; Shi et al., 2015). The present study supports this notion, Chrysin and Silibinin in combination form synergistically inhibited cell proliferation in T47D breast cancer cells.

Silibinin (3,5,7-trihydroxy-2-[3-(S) -(4-hydroxy-3-methoxyphenyl)-2-(S) -(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl] chroman-4-one), a flavonolignan extracted from milk thistle seeds, is the main active constituent of silymarin and one of the popular dietary additions that has been widely investigated for its hepatoprotective, antioxidant and anticancer properties (Tyagi et al., 2004; Yamamura et al., 2016). Also, it has revealed significant effectiveness in inhibiting or delaying both tumor initiation- and promotion-associated occurrences in different pre-clinical cancer models including that of prostate, colorectal, lung and breast cancer.

Among the different cancer models, the effects of silibinin have been also 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 (Nejati-Koshki et al., 2012; Nasiri et al., 2013; Ting et al., 2013; Kumar et al., 2015).

Chrysin, 5,7-dihydroxy-2-phenyl-4Hchromen-4-one, is a naturally occurring flavone extracted from the honeycomb and blue passion flower. (Mohammadinejad et al., 2014; Mohammadian et al., 2016b). Recently, Chrysin has shown to be a potent inhibitor of aromatase and of human immunodeficiency virus activation in models of latent infection. It has also demonstrated anti-inflammatory and anti-oxidant effects, and has

shown cancer chemopreventive activity through caspase activation and inactivation of the Akt signaling in extensive range of human and rat cell types and inhibition of hTERT has been also observed in some cancer cells treated with chrysin. However, studies of the effects of chrysin on human cancers remain rare (Mohammadian et al., 2016b; Eatemadi et al., 2016)

According to expected results, we found that Chrysin and Silibinin alone inhibits proliferation of breast cancer cells in a dose and time-dependent manner (Figure 1). Also, the combination of Chrysin and Silibinin had significantly synergistic effect in the cells, and the IC50 of Chrysin and Silibinin were significantly reduced (Figure 2 and 3).

As one of novel molecular effect of the combination treatment, it has been revealed that the combined treatment of Chrysin and Silibinin exhibited more inhibitory effect on hTERT and Cyclin D1 genes than either agent alone (Figure 4).

Cyclin D1 overexpression is found in more than 50% of human breast cancers and leads to mammary cancer in transgenic mice (Peurala et al., 2013). The capability of this cyclin to activate the cyclin-dependent kinases (CDKs) CDK4 and CDK6 is the most widely documented mechanism for its oncogenic actions and provides an desirable therapeutic target (Casimiro et al., 2015). It has been reported that Silibinin and Chrysin could partly suppress the expression level of cyclin D1. However, the combination treatment of these phytochemicals promoted a significant decrease in cellular cyclin D1 level compared with the groups for which drugs were used alone.

The drug combination also had significantly synergistic effects on expression levels of hTERT gene. It has been shown that telomerase is activated in the vast majority of breast cancers (over 90% of breast carcinomas) but not in normal adjacent tissues (Mocellin et al., 2013). Telomerase, a cellular reverse transcriptase that maintains the ends of chromosomes (telomeres) and consists of two essential subunits: the functional RNA subunit (in humans called hTR or hTERC), which acts as a template for telomeric DNA synthesis and the other is a catalytic subunit (hTERT) with reverse transcriptase activity (Kazemi-Lomedasht et al., 2013).

hTERT is highly expressed in all tissues irrespective of telomerase activity, but in cancer cells usually have fivefold-higher expression (Buseman et al., 2012; Mocellin et al., 2013). Thus, telomerase could be an attractive target for both diagnosis and therapy because of its distinct pattern of expression.

In this work we used β-actin, as a reference gene, for qRT-PCR studies in breast cancer cells.

qRT-PCR is a strong method for investigating gene expression changes during tumorigenesis. Target gene expression is generally normalized by a stably expressed endogenous reference gene; however, reference gene expression may vary amongst tissues under various conditions.

β-actin was Frequently used as a reference gene for breast cancer studies and recently in a study β-actin introduced as an optimal and reliable reference gene for human breast cancer research (Liu et al., 2015)

In this study, we surveyed the alteration in expression levels of Cyclin D1 and hTERT genes to describe the synergistic anticancer effects of chrysin and silibinin 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 chrysin and silibinin. 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, nanoformulation of the drugs may enhance bioavailability of these natural therapeutic agents and result in strong synergistic anticancer effects on breast cancer cells

In conclusion, our study demonstrated that the combination treatment of Silibinin and Chrysin causes synergistic anti-proliferative effect in T47D breast cells. It is possible that the synergistic outcome is caused, at least in part, by down-regulation of cyclin D1 and hTERT. Their possible roles in the demonstrated synergism between Silibinin and Chrysin must be validated by further in vitro or in vivo studies. Based on this finding, we suggest that the combination of Silibinin and Chrysin may emerge as an attractive strategy based on herbal substances for the treatment breast cancer.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

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References

Akhtar Siddiqui J, Singh A, Chagtoo M, et al (2015). Phytochemicals for breast cancer therapy: current status and future implications.*Curr Cancer Drug Targets*, **15**, 116-35.

Alibakhshi A, Ranjbari J, Pilehvar-Soltanahmadi Y, et al (2016). An update on phytochemicals substances in molecular target therapy of cancer: Potential inhibitory effect on telomerase activity. *Curr Med Chem*, **23**, 2380-93.

Buseman C, Wright W, Shay J (2012). Is telomerase a viable target in cancer?. *Mutat Res Fund Mol Mech Mut*, **730**, 90-7.

Casimiro MC, Arnold A, Pestell RG (2015). Kinase independent oncogenic cyclin D1. *Aging (Albany NY)*, **7**, 455.

Chandra S, Sah K, Bagewadi A, et al (2012). Additive and synergistic effect of phytochemicals in prevention of oral cancer. *European J Gen Dent*, **1**, 142.

Chou T-C (2013). Computerized quantification of synergism or antagonism in anticancer drug combination with different mechanisms and/or modes of actions. *Cancer Res*, **73**, 3276.

DeSantis C, Ma J, Bryan L, et al (2014). Breast cancer statistics, 2013. *CA Cancer J Clin*, **64**, 52-62.

Eatemadi A, Daraee H, Aiyelabegan HT, et al (2016). Synthesis and characterization of chrysin-loaded PCL-PEG-PCL nanoparticle and its effect on breast cancer cell line. *Biomed Pharmacother*, **84**, 1915-22.

Gatouillat G, Magid AA, Bertin E, et al (2015). Medicarpin and millepurpan, two flavonoids isolated from Medicago sativa, induce apoptosis and overcome multidrug resistance in

leukemia P388 cells. *Phytomedicine*, **22**, 1186-94.
González-Vallinas M, González-Castejón M, Rodríguez-Casado A, et al (2013). Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives. *Nutr Rev*, **71**, 585-99.
Heather Greenlee N, Neugut AI, Falci L, et al (2016). Association between complementary and alternative medicine use and breast cancer chemotherapy initiation the breast cancer quality of care (BQUAL) study. *JAMA Oncol*, **2**, 170-6.
Kapadia GJ, Rao GS, Ramachandran C, et al (2013). Synergistic cytotoxicity of red beetroot (Beta vulgaris L.) extract with doxorubicin in human pancreatic, breast and prostate cancer cell lines. *J Complement Integr Med*, **10**, 113-22.
Kazemi-Lomedasht F, Rami A, Zarghami N (2013). Comparison of inhibitory effect of curcumin nanoparticles and free curcumin in human telomerase reverse transcriptase gene expression in breast cancer. *Adv Pharm Bull*, **3**, 127-30.
Kumar S, Kumar D, Raina K, et al (2015). Oral silibinin inhibits tumorigenic potential of colon cancer stem cells. *Cancer Res*, **75**, 2807.
Liu L-L, Zhao H, Ma T-F, et al (2015). Identification of valid reference genes for the normalization of RT-qPCR expression studies in human breast cancer cell lines treated with and without transient transfection. *PloS One*, **10**, e0117058.
Liu RH (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr*, **134**, 3479-85.
Miller PE, Snyder DC (2012). Phytochemicals and cancer risk: a review of the epidemiological evidence. *Nutr Clin Pract*, **27**, 599-612.
Mocellin S, Pooley KA, Nitti D (2013). Telomerase and the search for the end of cancer. *Trends Mol Med*, **19**, 125-33.
Mohammadian F, Pilehvar-Soltanahmadi Y, Mofarrah M, et al (2016b). Down regulation of miR-18a, miR-21 and miR-221 genes in gastric cancer cell line by chrysin-loaded PLGA-PEG nanoparticles. Artificial cells, nanomedicine, and biotechnology, 44, 1972-8.
Mohammadinejad S, Akbarzadeh A, Rahmati-Yamchi M, et al (2014). Preparation and evaluation of chrysin encapsulated in PLGA-PEG nanoparticles in the T47-D breast cancer cell line. *Asian Pac J Cancer Prev*, **16**, 3753-8.
Mohan A, Narayanan S, Sethuraman S, et al (2013). Combinations of plant polyphenols and anti-cancer molecules: a novel treatment strategy for cancer chemotherapy. *Anticancer Agents Med Chem*, **13**, 281-95.
Montgomery A, Daniel N, Ezekiel U (2015). Effect of curcumin and silymarin in combination exerts synergistic inhibition of colon cancer cell proliferation. *FASEB J*, **29**, 721-2.
Nasiri M, Zarghami N, Koshki KN, et al (2013). Curcumin and silibinin inhibit telomerase expression in T47D human breast cancer cells. *Asian Pac J Cancer Prev*, **14**, 3449-53.
Nejati-Koshki K, Zarghami N, Pourhassan-Moghaddam M, et al (2012). Inhibition of leptin gene expression and secretion by silibinin: possible role of estrogen receptors. *Cytotechnology*, **64**, 719-26.
Peurala E, Koivunen P, Haapasaari K-M, et al (2013). The prognostic significance and value of cyclin D1, CDK4 and p16 in human breast cancer. *Breast Cancer Res*, **15**, 1.
Shi Y, Sun L, Chen G, et al (2015). A combination of the telomerase inhibitor, BIBR1532, and paclitaxel synergistically inhibit cell proliferation in breast cancer cell lines. *Target Oncol*, **10**, 565-73.
Ting H, Deep G, Agarwal R (2013). Molecular mechanisms of silibinin-mediated cancer chemoprevention with major emphasis on prostate cancer. *AAPS J*, **15**, 707-16.
Tyagi AK, Agarwal C, Chan DC, et al (2004). Synergistic anti-cancer effects of silibinin with conventional cytotoxic agents doxorubicin, cisplatin and carboplatin against human breast carcinoma MCF-7 and MDA-MB468 cells. *Oncol Rep*, **11**, 493-9.
Yamamura S, Mitsui Y, Majid S, et al (2016). Anticancer effects of silibinin-induced small nucleolar RNA 11B on bladder cancer cells. *Cancer Res*, **76**, 956-.