RESEARCH ARTICLE

Editorial Process: Submission:07/31/2017 Acceptance:07/14/2018

Derivative of Stevioside; CPUK02; Restores ESR1 Gene Methylation in MDA-MB 231

Saeed Khazayel[1], Pooneh Mokarram[1,2]*, Zeinab Mohammadi[1], Fatemeh Ramezani[1], Zhang Dayong[3]*

Abstract

Background: CPUK02 (15-Oxosteviol benzyl ester) is a new ent-kaurenoid derivative of stevioside and exhibits strong anti-cancer activity. Nowadays, the pattern of epigenetic in cancer has been topic of many studies and DNA methylation targeting represents a relevant strategy for cancer treatment. Since, no study conducted to this mechanism, we attempt to evaluate whether CPUK02 induce its anti-cancer effects via alteration the level of mRNA DNMT3B, DNMT3A expression and ESR1 methylation pattern in breast cancer cells line. **Methods:** MCF-7 (ER +) and MDA-MB231 (ER-) cell lines were treated for 24, 48 hours with 1 μM CPUK02 and 5-AZA-CdR (DNA methyltransferase inhibitor). Quantitative expression of DNMT3B and DNMT3A genes and ESR1 promoter methylation was assessed by Real-Time PCR and MS-PCR, respectively. **Results:** CPUK02 restored ESR1 promoter unmethylated allele in MDA-MB 231 cells. Also treatment with CPUK02 decreased the expression of both DNMT3A and DNMT3B genes like 5-AZA. The expression of DNMT genes were diminished by half compared with control cells. **Conclusions:** These results showed that CPUK02 has an anticancer effect on MDA-MB 231 cells which this effect can be done through several pathways.

Keywords: CPUK02- DNMT- Gene expression- Methylation pattern- Estrogen receptor- stevioside

Asian Pac J Cancer Prev, **19 (8)**, 2117-2123

Introduction

Breast cancer is one of the common malignancies amid women and a major public health issue around the world. The incidence rate of breast cancer in industrialized countries were higher than developing countries (Becker, 2010; Ferlay et al., 2010). In the United States, according to data published in 2018, new cases of invasive breast cancer incidence and mortality rate of breast cancer were More than 200,000, and 40,000, respectively (O'Sullivan et al., 2018). Breast cancer can be caused by various factors) multifactorial (which genetic and epigenetic alterations contribute to cancer initiation and progression (Sismondi et al., 2015). Approximately, 5-10 % of all breast cancer cases have a genetic or hereditary background (Silva and Zurrida, 2005).

Currently, estrogen receptors (ESRs) is one of the most important markers for prognosis and response to endocrine therapy in clinical tests (Katzenellenbogen et al., 2018). The connection between estrogen and its ligands leads to proliferation and differentiation in normal breast tissues. However, unsuitable activation of estrogen signaling is associated with limitless cell proliferation that occurs in most breast cancers (Li et al., 2013). In women, ESR1 amplification is a primary occurrence of cancer. ESR1 amplification was demonstrated in 15.5 % of patient with invasive breast cancer and also can be detectable in their mastopathic tissues (Soysal et al., 2015). Initially, a large number of breast cancers are estrogen-dependent (Haldosén et al., 2014). For breast cancer therapy, tamoxifen has been used in pre and postmenopausal women (Olsson et al., 2015). However, ER-alpha-negative breast cancer cells are unresponsive to tamoxifen treatment (Musgrove and Sutherland, 2009).

There are several mechanisms that have been explained the loss of ESR1 expression in ER-negative breast cancer. One of those mechanisms is epigenetic alterations which can justify the suppression of ESR1 expression in breast cancer via the ESR1 promoter methylation (Nass et al., 2000). In addition, it has been demonstrated that promoter of ESR1 gene is methylated in patients with ER-negative breast cancers (Mirza et al., 2007), On the other hand, ESR1 promoter methylation can lead to losing of ESR1 mRNA expression (Yoshida et al., 2000).

CpG island methylation of cytosine catalyzed by DNA methyltransferase (DNMT) enzymes (Kamei et al., 2010). Furthermore, overexpression of DNMTs are also detected in various malignancies (Subramaniam et al., 2014). It has

*[1]Department of Biochemistry, School of Medicine, [2]Gasteroenterohepatology Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran, [3]Vice Director of Lianyungang Eco. & Tech. Development Zone Management Committee and Drug Research Institute, China Pharmaceutical University, China. *For Correspondence: mokaram2@gmail.com, cpuzdy@163.com*

been shown that the mRNA level of DNMT1, 3a, and 3b had positively correlated with cancer, but DNMT3B expression levels, increased further in comparison with other DNMTs in 30% of patients with Sporadic Breast Carcinomas (Girault et al., 2003). Also it has been found that p53-mediated transcription of tumor suppressor genes are suppressed by DNMT3A when it interacts with (c-terminal domain of) p53 (Wang et al., 2005).

5-Azacytidine and its deoxy analog, 5-aza-2-deoxycytidine, are two well known complementary nucleotides DNA methylation inhibitors.There are some side effects in patients who consume Azacytidine such as; anemia, neutropenia, thrombocytopenia, gastrointestinal toxicity, hepatotoxicity, and nephrotoxicity (Martens, 2010). While, herbal medicine is a most common and popular form of complementary medicine/traditional medicine, that is widely used as an alternative medicine.

Recently, several studies were conducted on the natural compounds that revealed that herbal compounds such as; epigallocatechin gallate (EGCG), genistein, withaferin A, and resveratrol wereable to reduce transcription of all DNMTs (Mokarram et al., 2015). Stevia Rebaduna Bertoni and its glycoside compounds is a plant that has recently attracted the attention. Stevia mixture, stevioside and rebaudioside A by human intestinal micro flora hydrolyzed to steviol. some studies showed that isosteviol can inhibit human cancer cell growth by inhibition of mammalian DNA polymerases and human DNA topoisomerase (Goyal and Goyal, 2010). In 2012, researchers showed that stevioside had a strong anticancer property via induction of apoptotic cell death and inhibition of DNA synthesis (Paul et al., 2012). Chinese researchers have recently synthesized a new semi-synthetic compound from stevioside that is known as CPUK02 (15-Oxosteviol benzyl ester) (Yang et al., 2012). Nature tetracyclic diterpenoids products especially ent-kaurane diterpenoids which have an enone system in ring D, showed an interesting cytotoxic and anticancer properties. They evaluated the effect of CPUK02 and found that this compound can strongly inhibit cell proliferation in many human cancer cell lines and it also induces apoptosis via p53 (Nagashima et al., 2005; Li et al., 2011). Therefore, epigenetic manipulation could control the fate of cancer cells. Nowadays, epigenetic alteration targeting has gained increasing attention (K-Li et al., 2013). In our previous study, we examined the role of CPUK02 in colorectal cancer cell line and its association with epigenetic alteration (Mokarram et al., 2017). Due to the association between CPUK02 with apoptosis and epigenetic and lack of study which demonstrate this effect in breast cancer, this study was designed to evaluate the effects of CPUK02 on methylation of Estrogen Receptor α promoter and expression of DNMT3B & DNMT3A in MDA-MB231 breast cancer cell line.

Materials and Methods

We have got MDA-MB231, and MCF-7 as human breast cancer cell lines, from the National cell bank of Iran (Pasteur Institute, Iran). Cell culture medium, penicillin, streptomycin medium supplement, glutamine and fetal bovine serum were achieved from Gibco Life Technologies (UK). Biozol Isolation Reagent from Bioflux (China), cDNA Synthesis Kit from Fermentas, EU and SYBR green DNA PCR Master Mix from the Applied Biosystem (ABI) Company, (Foster City, CA USA) were purchased. CPUK02 was kindly provided as a gift from Drug Research Institute, China Pharmaceutical University.

Cell toxicity study

The Alamar blue oxidation-reduction dye as a common indicator, can be used to assess cell growth or viability via fluorometery or spectrophotometery. It is blue, nonfluorescent, oxidized form which converts to pink and fluorescent upon reduction (Ahmed et al., 1994). Breast cancer cells were cultured in RPMI 1640 medium comprising 10% fetal bovine serum 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Cells with 70% confluence were used in this assay. Initially, Alamar Blue® method was used to optimize cell density. So, 2.5, 5, 10, 15, 20, 30, 50, and 100×10^3 MCF-7 and MDA-MB231 cells per well with the 150 ml culture medium were added. The absorbance was determined at 570 nm, by a microplate reader (POLARstar® Omega).

Methylation Specific-PCR analysis

MDA-MB231 and MCF-7 cells were treated with 1µM CPUK02 for 24h and 48h. At the end of treatment, DNA extraction was done from cells as described previously (Sambrook and Russell, 2006). As has already been done, the ESR1 promoter methylation was assessed by chemical treatment with sodium bisulfite following methylation Specific-PCR (MSP) (Mokarram et al., 2015). MSP discriminate unmethylated cytosine from methylated cytosine and amplify specifically either methylated or unmethylated DNA using specific primers. The sequence of primers were used for MSP listed in Table 1. MSP amplification was carried out with an initial denaturation step at 95°C for 5 min and following 35 cycles (95˚C for 30 sec, 55˚C for 45 sec and 72°C for 45 sec with a final extension 72°C for10 min). The PCR products were applied for electrophoresis on 2% agarose gel.

RNA Extraction

To evaluate whether treatment with CPUK02 caused changes on the level of DNMT3A and DNMT3B mRNA, RNA was extracted from cultured cells by means of Biozol isolation reagent (Bioflux, China). The amount of RNA was assessed by optical density (A260/A280 ratio) with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA).

cDNAs synthesis

The cDNAs synthesis from extracted mRNA was done by using the reverse transcription kit (Fermentas, Lithuania), according to the manufacturer´s instructions.

Quantitative Real-Time PCR

Real-time PCR was performed using the ABI real time PCR 7500 system (USA) with PCR reac¬tion mixture

containing 1μL of cDNA, 0.5μL of each forward and reverse primers (10 pM) and 10μL of SYBR green DNA PCR Master Mix in a total volume of 20μL. Steps were as follows: an initial holding step at 95°C for 10 min and 40 cycles (95°C for 15 sec and 60°C for 60 sec) subsequently. After analyzing data using the 7500 Software v2.0.1, the Relative expression level (fold changes) of DNMT3A and DNMT3B gene was computed by the 2 - ΔΔCT formula. Expression levels of target gene were normalized using the GAPDH as housekeeping control gene. Specific primers for DNMT3A, DNMT3B and GAPDH are listed in Table 1.

Statistical analysis

Expression mean differences between groups treated with CPUK02 and 5-AZA were evaluated by Kruskal-Wallis test. Statistical analysis, was carried out by using SPSSsoftware version 21 (SPSS Inc., Chicago, IL). A p-value <0.05 was considered as statistical significance value.

Results

Anti-proliferative effect of CPUK02 against breast cancer cell lines

For a bioassay to be practical, it must provide reproducible and reliable quantitation of the event. Therefore, as a first step in the evaluation of the Alamar Blue assay, the correlation between breast cancer cell numbers and Alamar Blue fluorescence was studied. Breast cancer cell lines were cultured at concentrations ranging from 2,500 to 100,000 cells/well. At each time point, relative fluorescence augmented with increasing cell number. However, in both cell lines, the optimum cell density was chosen 8,000 and 12,000 for 24 and 48h incubation time respectively (data not shown). CPUK02 showed cytotoxicity against the MCF-7 cell lines with IC_{50} of 1.51μM. In MDA-MB231 cell lines, it was observed comparable cytotoxic profiles with

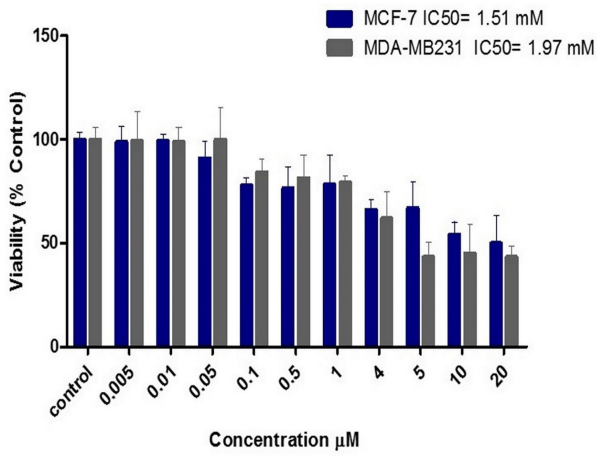

Figure 1. Effect of CPUK02 on Breast Cancer Cell Growth. Breast cancer cells at 70% to 80% confluence were treated with CPUK02 (0.0001-20 μmol/L), in cell culture medium for 24 hours. Control cells were treated with media only. Data are expressed as mean ± SEM of three dependent experiments (n = 3)

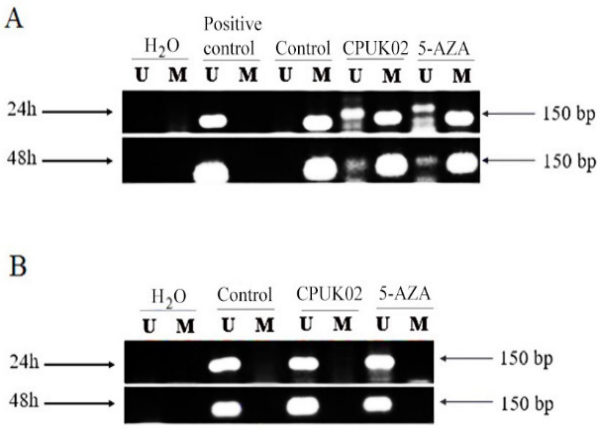

Figure 2. Representative Example of MSP-PCR for Promotor Methylation Analysis of ESR1 in MDA_MB231 Breast Cancer Cell Line, A: MDA-MB231. Positive control (PC): MCF_7 cell line, control: MDA_MB231 cell line, B: MCF-7, control: untreated MCF-7 cell line. Both cell lines were treated under two conditions (24h and 48h).

IC_{50} of 1.97μM. We observed that treatment of cells with CPUK02 showed a concentration-dependent decrease in cell viability. Dose-response curve was exhibited in Figure 1. At concentrations above 4 μM, CPUK02 significantly decreased cell viability of both breast cancer cells (Figure 1). The CPUK02 optimum concentration was determined 1μM for expression and methylation assays.

The effect of CPUK02 treatment on ESR1 gene promoter methylation

In breast cancers without estrogen receptor, methylation of the CpG islands of the ESR1 gene has been correlated with lack of ESR1 gene expression. Thus, methylation of ESR1 promoter site might be utilize as an indicator for breast cancer detection, prognosis, and treatment (Wei et al., 2008).

MS-PCR was done to evaluate whether the treatment of ER-negative breast cancer cell line (MDA-MB231) and ER-positive breast cancer cell line (MCF-7) with CPUK02 could restore unmethylation status of ESR1 promoter. The effects of CPUK02 on restoring of ESR1 gene was compared with 5-aza-2- deoxycytidine, a demethylating agent. Treatment of MDA-MB231cells with CPUK02 appeared an unmethylated band. In addition, unmethylated band intensity in cells treated with CPUK02 was even more than 5-aza-2- deoxycytidine. As expected, in MCF-7 cell lines, treatment with CPUK02 and 5-aza-2- deoxycytidine created only unmethylated band similar to control ones. These results suggesting CPUK02 was even more powerful than 5-aza-2- deoxycytidine in restoring of ESR1 promoter methylation of MDA-MB cell lines (Figure 2).

The effect of CPUK02 treatment on mRNA expression

Since, hypermethylated cell lines like MDA-MB231, overexpress total DNMT activity, i.e.: DNMT3B (Roll et al., 2008), we evaluated whether CPUK02 treatment of cells could decrease of DNMT3A and DNMT3B genes expression. Incubation of MDA-MB231

Table 1. Primer Sequences Utilized for MS-PCR and Real-time PCR

Gene	Accession Number	Annealing	Size (bp)	Sequence	Ref
ESR1 U	NC_000006.12	55	158	F: 5′-ATGAGTTGGAGTTTTTGAATTGTTT-3′	(Lapidus et al., 1998)
				R: 5′-ATAAACCTACACATTAACAACAACCA-3′	
ESR1 M	NC_000006.12	55	151	F: 5′-CGAGTTGGAGTTTTTGAATCGTTC-3′ R: 5′-CTACGCGTTAACGACGACCG-3′	(Lapidus et al., 1998)
DNMT3A	NM_153759.3	60	111	F: 5′-TATTGATGAGCGCACAAGAGAGC-3′	(Wu et al., 2007)
				R: 5′-GGGTGTTCCAGGGTAACATTGAG-3′	
DNMT3B	NM_006892.3	60	113	F: 5′-GGCAAGTTCTCCGAGGTCTCTG-3′	(Wu et al., 2007)
				R: 5′-TGGTACATGGCTTTTCGATAGGA-3′	
GPDH	NM_002046.6	60	232	F: 5′-CGACCACTTTGTCAAGCTCA-3′	(Wu et al., 2007)
				R: 5′-AGGGGTCTACATGGCAACTG-3′	

cells with CPUK02 induced a marked decrease in DNMT3A and DNMT3B expression level in both time of treatment, 24 and 48h (Figure 3). Treatment with CPUK02 lowered expression of both DNMT3A and DNMT3B genes to the same amount as 5-AZA reduced. The expression of DNMT genes were diminished by half compared with control cells following treatment with CPUK02. Incubation of MDA-MB 231 cells with CPU0K (P=0.036) and 5-AZA (P<0.01) significantly reduced the mRNA expression level of DNMT3A by half after 24h. However, it was decreased by 0.73-fold and 0.68-fold following 48h treatment with CPU0K (P=0.028) and 5-AZA (P<0.01), respectively. Similar to DNMT3A, the expression level of DNMT3B was reduced considerably during 24h by 0.53-fold and 0.64-fold while cells were treated with CPU0K (P<0.01) and 5-AZA (P=0.046), respectively. However, after 48h incubation time, this ratio was 0.78- fold and 0.82- fold following CPU0K and 5-AZA therapy respectively and the P value was not significant.

Our results showed that treatment of MDA-MB231 cell line with CPUK02 could restore ESR1 gene unmethylated phenotype by diminishing of DNMT3A and DNMT3B genes expression levels.

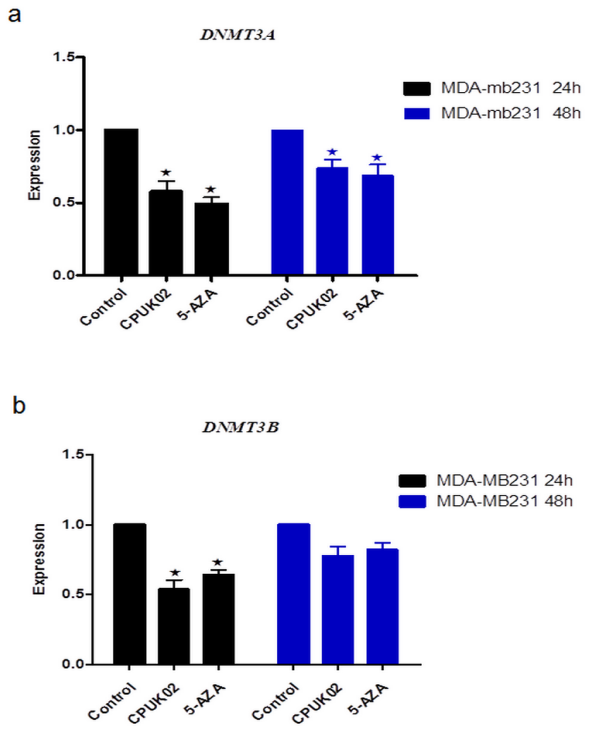

Figure 3. The Effect of CPUK02 on DNMT3A & DNMT3B Expression Level. A: expression level of DNMT3A gene in MDA-MB231. B: expression level of DNMT3B gene in MDA-MB23.CPUK02 and 5-aza were able to decrease mRNA level of DNMT3A and DNMT3B genes compare with the control group in MDA-MB231 cell line.

Discussion

The current study was the first attempt to investigate the CPUK02 effect on epigenetic alteration in ER- breast cancer cell line. According to our results, it was found that CPUK02 as the ent-kauranoeid compound, can mimic the 5-aza-2- deoxycytidine propertyto restore the unmethylated allele. It appeared unmethylated allele in the ESR1 gene promoter in MDA-MB231 breast cancer cell line. Furthermore, our data showed that the level of DNMT3A and DNMT3B mRNA have significantly decreased in MDA-MB231 cells treated with CPUK02 for 24 and 48 hours.

Generally, there are several mechanisms for epigenetic changes in breast cancer, one of the most important of these changes, is promoter hypermethylation in estrogen-response elements. It has been made clear that amplification of DNA methyltransferase activity and gene hypermethylation in CpG islands of ESR1 promoter is associated with ER-negative phenotype. DNMTs inhibitors, such as 5-aza-2- deoxycytidine is widely used for genes re-expression that are silenced by promoter hypermethylation (Luo et al., 2015). Ferguson and colleagues demonstrated that 5-aza-2-deoxycytidine makes demethylated σ gene promoter in breast cancer cells (Ferguson et al., 2000) and they also showed that 5-aza-2- deoxycytidine relatively could be demethylated ESR1 CpG islands in MDA-MB231 cells, and this event leads to increased expression of the ESR1 (Ferguson et al., 1995). On the other hand, Lapidus and his colleagues showed that DNA was highly hypermethylated in ER-negative breast cancer cells, and ESR1 CpG islands were demethylated along with the demethylating agent (5-aza-2- deoxycytidine) (Lapidus et al., 1998) and also in our previous study, we point out that CPUK02 and 5-aza-2- deoxycytidine

were able to restore the unmethylated pattern of SFRP2 gene in HCT116 colorectal cancer cell line (Mokarram et al., 2017). According to the aforementioned results and our study, it seems that CPUK02 are capable of playing a role in ESR1 gene promoter demethylation like 5-aza-2- deoxycytidine. So, our data confirm previous results about 5-aza-2- deoxycytidine and it seems CPUK02 mimic the 5-aza-2- deoxycytidine role.

As previously mentioned, DNMTs are the enzymes that responsible for DNA methylation. At the second line of our study, we evaluate the effects of CPUK02 on DNMT3B and DNMT3A mRNA expression. In this regard, the newly investigation was carried out about natural compound has shown their role in herbal medicine (Mirza et al., 2013). CPUK02 can induce apoptosis (Yang et al., 2012), and on the other hand, it has been shown that DNMT3A interacts with p53 (Wang et al., 2005). several studies have determined that DNMT3B mRNA expression increased more than DNMT1 and DNMT3A in breast cancer and colorectal cancer (Girault et al., 2003; Roll et al., 2008) which leads to CIMP-high phenotype in colorectal cancer (Nosho et al., 2009) and epigenetic silencing of multiple genes methylation-sensitive such as; CDH1, ESR1, GNA11, CEACAM6,MUC1,CST6 and MYB in breast cancer cells (Roll et al., 2008). Regin Schneider-Stuck and his colleagues showed that 5-aza-2- deoxycytidine reduces the transcription of DNMT1 and DNMT3A genes in HCT-116 colorectal cancer cells (Schneider-Stock et al., 2005) and also in the previous study which performed on HCT116 colorectal cell line, CPUK02 and 5-aza were able to go down mRNA level of DNMT3B gene (Mokarram et al., 2017). On the other hand, in 2012, it has been found that DNMTs were decreased in mice that treated with 5-aza-2- deoxycytidine (Ding et al., 2012). Our study showed that CPUK02, like 5-aza-2- deoxycytidine, is able to significantly reduce DNMT3B and DNMT3A mRNA expression. However, decreasing gene expression mechanism by 5-aza-2- deoxycytidine not well understood yet. This decrease in gene expression via CPUK02 can be explained by post-transcription modification. A study has been shown that the expression of miRNAs including; miR-26, miR-29 and miR-203 was increased in cancer cells that treated with 5-aza-2- deoxycytidine. It seems while miRNAs was increased, expression of DNMT3A and DNMT3B were down-regulated at the mRNA level (Gasque Schoof et al., 2015). It has been found that miR-203 inversely related to DNMT3B in breast cancer cells. Additionally, it has been seen that hypermethylation of DNA caused down-regulation of miR-203 and consequently, DNMT3B is overexpressed (Sandhu et al., 2014). It was also reported that miR-29 family are inversely related to the expression of DNMT3A and DNMT3B (Bian et al., 2015). The miR-29b directly targeting DNMT3A and DNMT3B mRNA, and over expression of the miR-29b leads to DNA hypomethylation and decrease at both protein and mRNA levels in acute myeloid leukemia and it also modulating DNMT3A and DNMT3B expression in female mouse germ cells (Garzon et al., 2009; Takada et al., 2009). So, miRNAs rising is one of the mechanisms that reduced expression of DNMT3A and DNMT3B. Another mechanism for post- transcriptional mRNA regulation is Hu-antigen R which belongs to the family of embryonic-lethal, abnormal vision (ELAV)-like proteins (Grammatikakis et al., 2017). In breast cancer, changes in HuR-bound mRNAs associated with tumorigenic phenotype and tumor progression (Mazan-Mamczarz et al., 2008). In addition to, a study in 2009 was shown that HuR is able to bind to DNMT3B mRNA and stabilize mRNA and increases DNMT3B expression (de Silanes et al., 2009). Another investigation also mentioned that HuR cytoplasmic expression has been declined in breast cancer cells treated with 5-aza-2- deoxycytidine (Pryzbylkowski et al., 2008).

It appears that anticancer effect of CPUK02 is associated with several pathways such as apoptosis, methylation inhibition, and miRNAs expression increasing or cytoplasmic HuR protein decreasing. However, further study is needed to investigate other antitumor aspects of the CPUK02 action.

Funding

This present article was extracted from the thesis written by Saeed Khazayel and financially supported by Shiraz University of Medical Sciences Grant No, 93-6971.

Disclosure

Authors declare they have no financial disclosure.

References

Ahmed SA, Gogal RM, Walsh JE (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. *J Immunol Methods*, **170**, 211-24.

Becker N (2010). Breast cancer epidemiology. In 'Management of Breast Diseases', Eds Springer, pp 121-30.

Bian Y, Lei Y, Wang C, et al (2015). Epigenetic regulation of miR-29s affects the lactation activity of dairy cow mammary epithelial cells. *J Cell Physiol*, **230**, 2152-63.

de Silanes IL, Gorospe M, Taniguchi H, et al (2009). The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. N*ucleic Acids Res*, **37**, 2658-71.

Ding Y-B, Long C-L, Liu X-Q, et al (2012). 5-aza-2′-deoxycytidine leads to reduced embryo implantation and reduced expression of DNA methyltransferases and essential endometrial genes. *PLoS One*, **7**, e45364.

Ferguson AT, Evron E, Umbricht CB, et al (2000). High frequency of hypermethylation at the 14-3-3 σ locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci U S A*, **97**, 6049-54.

Ferguson AT, Lapidus RG, Baylin SB, et al (1995). Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res*, **55**, 2279-83.

Ferlay J, Héry C, Autier P, et al (2010). Global burden of breast cancer. In 'Breast Cancer Epidemiology', Eds Springer, pp 1-19.

Garzon R, Liu S, Fabbri M, et al (2009). MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood*, **113**, 6411-8.

Gasque Schoof CR, Izzotti A, Jasiulionis MG, et al (2015). The Roles of miR-26, miR-29, and miR-203 in the silencing of the epigenetic machinery during melanocyte transformation. *Bio Med Res Int*, **2015**, 11.

Girault I, Tozlu S, Lidereau R, et al (2003). Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. *Clin Cancer Res*, **9**, 4415-22.

Goyal S, Goyal R (2010). Stevia (Stevia rebaudiana) a bio-sweetener: a review. *Int J Food Sci Nutr*, **61**, 1-10.

Grammatikakis I, Abdelmohsen K, Gorospe M (2017). Posttranslational control of HuR function. *Wiley Interdiscip Rev RNA*, **8**, e1372.

Haldosén L-A, Zhao C, Dahlman-Wright K (2014). Estrogen receptor beta in breast cancer. *Mol Cell Endocrinol*, **382**, 665-72.

K-Li K, Li F, S Li Q, et al (2013). DNA methylation as a target of epigenetic therapeutics in cancer. *Anticancer Agents Med Chem*, **13**, 242-7.

Kamei Y, Suganami T, Ehara T, et al (2010). Increased expression of DNA methyltransferase 3a in obese adipose tissue: studies with transgenic mice. *Obesity*, **18**, 314-21.

Katzenellenbogen JA, Mayne CG, Katzenellenbogen BS, et al (2018). Structural underpinnings of oestrogen receptor mutations in endocrine therapy resistance. *Nat Rev Cancer*, **1**, 377-88.

Lapidus RG, Nass SJ, Butash KA, et al (1998). Mapping of ER gene CpG island methylation by methylation-specific polymerase chain reaction. *Cancer Res*, **58**, 2515-9.

Li J, Zhang D, Wu X (2011). Synthesis and biological evaluation of novel exo-methylene cyclopentanone tetracyclic diterpenoids as antitumor agents. *Bioorg Med Chem Lett*, **21**, 130-2.

Li Y, Meeran SM, Patel SN, et al (2013). Epigenetic reactivation of estrogen receptor-α (ERα) by genistein enhances hormonal therapy sensitivity in ERα-negative breast cancer. *Mol Cancer*, **12**, 9.

Luo F-Y, Xiao S, Liu Z-H, et al (2015). Kank1 reexpression induced by 5-Aza-2'-deoxycytidine suppresses nasopharyngeal carcinoma cell proliferation and promotes apoptosis. *Int J Clin Exp Pathol*, **8**, 1658.

Martens UM (2010). Small molecules in oncology, Springer, pp 299-324.

Mazan-Mamczarz K, Hagner PR, Corl S, et al (2008). Post-transcriptional gene regulation by HuR promotes a more tumorigenic phenotype. *Oncogene*, **27**, 6151-63.

Mirza S, Sharma G, Parshad R, et al (2013). Expression of DNA methyltransferases in breast cancer patients and to analyze the effect of natural compounds on DNA methyltransferases and associated proteins. *J Breast Cancer*, **16**, 23-31.

Mirza S, Sharma G, Prasad CP, et al (2007). Promoter hypermethylation of TMS1, BRCA1, ERα and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. *Life Sci*, **81**, 280-7.

Mokarram P, Mohammadi Z, Khazayel S, et al (2017). Induction of epigenetic alteration by CPUK02, an ent-kaurenoid derivative of stevioside. *Avicenna J Med Biotechnol*, **9**, 13.

Mokarram P, Zare N, Jafari H, et al (2015). The Methylation Status of MGMT and SFRP2 Genes in Iranian Patients With Polyps. *Thrita*, **4**, e28601.

Musgrove EA, Sutherland RL (2009). Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer*, **9**, 631-43.

Nagashima F, Kondoh M, Fujii M, et al (2005). Novel cytotoxic kaurane-type diterpenoids from the New Zealand liverwort Jungermannia species. *Tetrahedron*, **61**, 4531-44.

Nass SJ, Herman JG, Gabrielson E, et al (2000). Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. *Cancer Res*, **60**, 4346-8.

Nosho K, Shima K, Irahara N, et al (2009). DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. *Clin Cancer Res*, **15**, 3663-71.

O'Sullivan CC, Loprinzi CL, Haddad TC (2018). Updates in the Evaluation and Management of Breast Cancer. *Mayo Clin Proc*, **93**, 794-807.

Olsson H, Einefors R, Broberg P (2015). Abstract P1-12-10: Tamoxifen treated patients have a better survival than patients treated with aromatase inhibitors-A population based registry study in Sweden. *Cancer Res*, **75**, P1-12-0-P1-0.

Paul S, Sengupta S, Bandyopadhyay T, et al (2012). Stevioside induced ROS-mediated apoptosis through mitochondrial pathway in human breast cancer cell line MCF-7. *Nutr Cancer*, **64**, 1087-94.

Pryzbylkowski P, Obajimi O, Keen JC (2008). Trichostatin A and 5 Aza-2′ deoxycytidine decrease estrogen receptor mRNA stability in ER positive MCF7 cells through modulation of HuR. *Breast Cancer Res Treat*, **111**, 15-25.

Roll JD, Rivenbark AG, Jones WD, et al (2008). DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Mol Cancer*, **7**, 1.

Sambrook J, Russell DW (2006). Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harb Protoc*, **2006**, pdb. prot4455.

Sandhu R, Rivenbark AG, Mackler RM, et al (2014). Dysregulation of microRNA expression drives aberrant DNA hypermethylation in basal-like breast cancer. *Int J Oncol*, **44**, 563-72.

Schneider-Stock R, Diab-Assef M, Rohrbeck A, et al (2005). 5-Aza-cytidine is a potent inhibitor of DNA methyltransferase 3a and induces apoptosis in HCT-116 colon cancer cells via Gadd45-and p53-dependent mechanisms. *J Pharmacol Exp Ther*, **312**, 525-36.

Silva OE, Zurrida S (2005). Breast cancer: a practical guide, Elsevier Health Sciences, pp 21-2.

Sismondi P, D'Alonzo M, Bounous VE, et al (2015). Androgen receptor and breast cancer. In 'Frontiers in Gynecological Endocrinology', Eds Springer, pp 179-83.

Soysal SD, Kilic IB, Regenbrecht CR, et al (2015). Status of estrogen receptor 1 (ESR1) gene in mastopathy predicts subsequent development of breast cancer. *Breast Cancer Res Treat*, **151**, 1-7.

Subramaniam D, Thombre R, Dhar A, et al (2014). DNA methyltransferases: a novel target for prevention and therapy. *Front Oncol*, **4**, 80.

Takada S, Berezikov E, Choi YL, et al (2009). Potential role of miR-29b in modulation of Dnmt3a and Dnmt3b expression in primordial germ cells of female mouse embryos. *RNA*, **15**, 1507-14.

Wang YA, Kamarova Y, Shen KC, et al (2005). DNA methyltransferase-3a interacts with p53 and represses p53-mediated gene expression. *Cancer Biol Ther*, **4**, 1138-43.

Wei M, Xu J, Dignam J, et al (2008). Estrogen receptor α, BRCA1, and FANCF promoter methylation occur in distinct subsets of sporadic breast cancers. *Breast Cancer Res Treat*, **111**, 113-20.

Wu Y, Strawn E, Basir Z, et al (2007). Aberrant expression of deoxyribonucleic acid methyltransferases DNMT1, DNMT3A, and DNMT3B in women with endometriosis. *Fertil Steril*, **87**, 24-32.

Yang L, Ke W, Zhang D-Y, et al (2012). A new ent-kaurenoid derivative CPUK02 notably inhibits tumor growth through induction of apoptosis. *Chin J Nat Med*, **10**, 429-35.

Yoshida T, Eguchi H, Nakachi K, et al (2000). Distinct

mechanisms of loss of estrogen receptor α gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. *Carcinogenesis*, **21**, 2193-201.