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The anticancer potential of silibinin is associated with alterations in gene expression levels of major epigenetic enzymes in prostate carcinoma

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Abstract: Silibinin, a diastereoisomeric mixture extracted from *Silybum marianum L*, with established anti-prostate cancer activity, has been associated with considerable anti-neoplastic ability, in a variety of human cancer types, through interference with the epigenetic machinery. In prostate carcinoma (PCa), high expression of polycomb repressive complex 1 (PRC1) and 2 (PRC2) members, that belong to polycomb group (PcG) proteins, is associated with transcriptional silencing of tumor suppressor genes through histone modifications and chromatin condensation. Our previous results revealed that silibinin reduced the expression levels of PRC2 complex members (EZH2, EED, SUZ12), an ability accompanied by increased H3K27me3 marks. In the current report, treatment of DU145 and PC3 prostate cancer cells with clinically-achievable concentrations (25-75μg/mL) of silibinin, resulted in reduced protein expression levels of PRC1 complex members (RING1a, RING1b and BMI1), in a dose-dependent manner, as obtained from western blot analysis. Next, human epigenetic chromatin modification enzymes-focused DNA microarray and real-time quantitative reverse transcription-PCR (qRT-PCR) analyses, revealed that silibinin modulated differentially the gene expression levels of important enzymes, related with the pathophysiology of the disease, that function at the epigenetic level. Specifically, significant alterations were observed in the expression profile of enzymes associated with gene expression regulation through modification of chromatin configuration, including family members of: i) histone methyltransferases, ii) histone acetyl-transferases, iii) histone demethylases and iv) histone deacetylases along with enzymes inducing gene silencing (*via* DNA methylation) and regulation of cell cycle progression. Our results suggest that the anticancer activity of silibinin could be partially mediated by the disruption of central processes in chromatin configuration-remodeling and alteration of enzymes of the epigenomic landscape that regulate prostate cancer progression.

Keywords: silibinin; prostate cancer; epigenetics, polycomb repressive complex 1 (PRC1), histone methyltransferases, histone acetyltransferases, histone deacetylases

1. Introduction

Prostate cancer (PCa) is one of the most common male malignancies worldwide, especially in Western developed countries. In addition, beyond genetic predisposition onset and progression of PCa is highly related to a number of aberrant epigenetic alterations including: (i) histone modifications-chromatin remodeling, (ii) DNA methylation and (iii) microRNAs expression patterns, resulting in deregulated gene expression [1]. Previously published data indicated that, silibinin, the main constituent of silymarin, a multi-component mixture that is extracted from the plant *Silybum marianum* (L) Gaertn (milk thistle) of the *Asteraceae* family [2], acts as a potent epigenetic regulator in an in vitro model of PCa, through negative regulation of Polycomb Repressive Complex 2 (PRC2) members EZH2, EED and SUZ12 [3]. In addition evidence exists that PRC1 complex (composed by BMI1, RING1A and RING1B) and several protein families including CBX, HPH and PCGF cooperates with PRC2 complex in gene silencing at epigenetic level [4]. Both PRC1 and PRC2 components, are members of the polycomb repressive group (PcG) of proteins that were firstly discovered in Drosophila, as epigenetic regulators of embryonic development. Although PcG proteins and specifically PRC1 and PRC2 complexes play important roles in cellular differentiation and lineage, there is strong evidence that deregulated/abnormal expression and function of PRC members is related to incidence and progression of different types of cancer, including PCa [5]

The purpose of the present study was to investigate the potential ability of silibinin to regulate the protein expression levels of PRC1 complex members (RING1a, RING1b, MBI1), while it further analyzed, in a wide scale, the potential ability of silibinin to modulate gene expression of major components of the epigenetic machinery, in PCa. According to the results silibinin was found to negatively regulate expresssion levels of PRC1 complex members, while altered differentially a variety of major deregulated epigenetic enzymes, implicated in the pathogenesis of PCa.

2. Experiments

2.1 Materials and reagents

Cell culture media, along with other cell culture materials (FBS, antibiotics, trypsin), silibinin, polyvinylidene difluoride (PVDF) membranes, prestained molecular markers, chemiluminescent reagents, seconrady antibodies, proteinase/phosphatase inhibitors and assay for protein estimation were purchased from companies as previously described [3]. Primary antibodies used in the experiments included: anti-Ring1A, anti-Ring1B, anti-Bmi1 and anti-lamin B2 (P8P3U), were obtained from Cell Signaling Technology (Boston, MA, USA). Trizol, DNTPs, and Platinum SYBR green were from Invitrogen (Life Technologies, Carlsbad, CA, USA), while random hexamers and Prime Script Reverse Transcriptase were from Takara (Shiga, Japan). RT² Profiler PCR array for epigenetic enzymes was purchased from Sabioscience (Qiagen, Venlo, Netherlands) (Cat. no. 330231 PAHS-085ZA).

2.2 Cell cultures and treatments

Prostate human cancer cell lines DU-145 and PC-3, were obtained from the American Type Culture Collection (Rockville, MD, USA and were cultured in Dulbecco's modified Eagle medium F-12 (DMEM F-12). Culture media were supplemented with 10% fetal bovine serum, L-glutamine (4 mM), 1.5 g/L sodium bicarbonate, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in an atmosphere of 95% O_2, 5% CO_2 in a humidified incubator, while cell cultures were passaged at 2- to 3-day intervals, depending on cell line type and growth characteristics. In all experimental procedures logarithmically growing cells were used. For immunoblot analysis, and RT² profiler™ PCR array/RT-PCR assays, cells were seeded at a density of $1.0\text{-}1.2\times10^6$ cells per dish (100 mm). Cells were allowed to attach to the surface of culture plates for 24h and when reached 70-80% confluency, they were treated with increasing concentrations of silibinin (25-75 μg/ml) for 48h. Following treatments, the culture medium was aspirated and collection of cells were performed with a gum rubber-scraping device.

2.2 Protein Extraction, preparation of cell lysates and Western blot analysis

Following cell treatments, the culture medium was removed, and the cells were washed twice with PBS. Next, cells were collected with a gum rubber-scrapping device. Samples were centrifuged at 2,500 *rpm* for 2 min, supernatant was discarded while protein extraction, preparation of cell lysates and western blot analysis was performed as previously described [3]. Western blot membranes were hybridized overnight at 4^0 C with the primary antibodies at 1/1000 dilution against anti-Ring 1A, anti-Ring 1B, anti-Bmi1 and 1/2000 dilution against anti-lamin B2. Incubation of membranes with secondary antibodies, development of immunoblot bands, stripping procedure and scanning densitometry was permormed as described elsewhere [3].

2.3 RNA extraction, cDNA synthesis and RT² profiler TM PCR Array for Human epigenetic Chromatin Modification Enzymes

Following treatment, cells were trypsinized, collected and RNA extraction was performed by using Trizol Reagent. For cDNA synthesis, RT² first strand kit was used as follows: total RNA (0.5 μg) was mixed with 2 μl of 5X GE2 buffer, in a final volume of 10 μl, followed by incubation at 42°C for 5 min and on ice for 3 min. Then, samples (20 μl) were prepared by addition of 4μl of BC3 buffer, 1μl P2 control, and RE3 reverse transcription mix, followed by incubation at 42°C for 15 min and at 95°C for 5 min. Next, a volume of 1339 μl of dH2O and 1350 μl of 2X RT² SYBR green master mix were added to the reaction mixture. Each mixture (25 μl) was added to each well of a 96-well PCR array plate (RT² Profiler ™ PCR Array for human epigenetic chromatin modification enzymes/PAHS-085Z). Finally, reactions were performed in a Roche LightCycler 480, by using the following parameters: 10 min at 95°C, 15 sec at 95°C, 1 min at 60°C (45 cycles), 15 sec at 60°C, followed by continuous incubation at 95°C. Cycle thresholds (Cts) were calculated by using absolute quantification/second derivative max selection of the Roche LightCycler. Web-based Sabiosciences RT² Profiler PCR array data analysis (version 3.5) was used to analyze results, while all experimental data were analyzed by the $2^{-\Delta\Delta CT}$ method. Finally, Web-based Sabiosciences RT² Profiler PCR array data analysis (version 3.5) was used for clustergrams generation.

3. Results

3.1 Treatment of DU-145 and PC-3 cells with silibinin resulted in reduced protein expression levels of PRC1 complex members in a dose dependent manner.

Our previous results indicated that silibinin was able to reduce the expression levels of PRC2 complex members EZH2, EED and SUZ12, an effect accompanied by increased marks of H3K27me3 [3]. Next we sought to investigate whether silibinin was able to negatively regulate PRC1 complex members, highlighting any potential disruption of this central epigenetic complex that in cooperation with PRC2 regulates gene expression, favoring PCa progression. Western blot analysis in DU145 **(Figure 1A)** and PC3 **(Figure 1B)** cells, following 48h incubation with clinically achievable concentrations of silibinin (25–75 μg/mL), revealed a significant decrease in protein levels of all PRC1 complex members (Ring 1A, Ring lB and Bmi1), at each concentration tested.

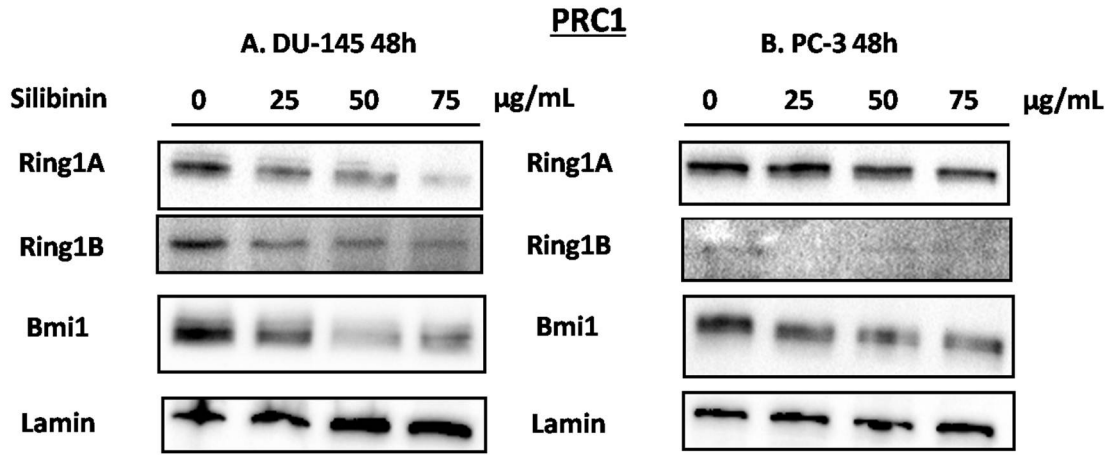

Figure 1. **Effect of silibinin on protein expression levels of PRC1 (Polycomb Repressive Complex 1) members in human prostate carcinoma cells**. DU-145 (A) and PC-3 (B) cells were treated with increasing concentrations (25-75µg/mL) of silibinin for 48h. Following treatment, whole cell extracts were analyzed by immunoblot analysis for Ring1A, Ring1B and Bmi1 levels. Representative blots are shown. Membranes were stripped and re-probed with lamin to verify equal protein loading.

3.2 Silibinin modulates the expression levels of genes involved in epigenetic modifications in human prostate carcinoma

Previously it was shown that silibinin was able to interfere and regulate important epigenetic enzymes that possess a significant role in the regulation of PCa by modulating chromatin structure and configuration. To this end, we next sought to analyze in a wide scale, changes in the expression of genes involved with the modification of chromatin configuration and other epigenetic mechanisms in silibinin-treated (25-75 mg/mL for 48h) and untreated DU-145 cells. For this purpose, we used the RT² profiler real-time PCR array composed of 84 genes encoding enzymes and proteins that participate in cellular epigenetic modifications, including methyltransferases, histone acetylases and deacetylases.

According to our results, incubation of DU-145 cells with the lowest concentration of silibinin (25µg/mL) altered the gene expression of a small number of chromatin-modifying enzymes after 48h of treatment. By setting a threshold of fold regulation ≥0.5 and p-value ≥0.05, we were able to select further a set of genes that their expression was modulated following silibinin exposure **(Figures. 2.I and 3A)**. According to the clustergram of the selected genes **(Figure 2I)**, the lowest concentration of silibinin used was capable of down-regulating gene expression levels of three distinct enzymes: *ESCO2*, *AURKA*, and *HDAC1* **(Figure 3A)**.

On the other hand, when DU-145 cells were treated with 50µg/mL of silibinin for 48h, a wide range of genes altered expression with the majority turning down-regulated (Figure 2II and 3B). By setting a threshold of fold-change ≥0.5 and p-value ≥0.05 we were able to select further a set of genes that their expression levels was altered following silibinin exposure. According to the results, the clustergram of the selected genes demonstrated the existence of two distinct clusters, including a set of 13 genes. Cluster A represents the up-regulated genes and cluster B represents the down-regulated genes (Figure 2II) in DU-145 cells after exposure to silibinin compared to control (untreated cells). Specifically, in cluster A, the genes *HDAC9*, *AURKC* and *KAT8* were found to be significantly up-regulated, whereas, in cluster B, the genes *HDAC3*, *SMYD3*, *AURKA*, *AURKB*, *SUV39H1*, *KAT6B*, *HDAC1*, *ESCO2*, *HAT1* and *RPS6KA5* were found to be down-regulated (Figures 2II and 3B).

Finally, we examined the expression profile of chromatin modifying enzymes in DU-145 cells for 48h, using the maximum concentration of silibinin (75µg/mL). As expected, silibinin was shown to alter the expression profile of the tested proteins with the majority of genes being down-regulated

(Figure 2.III). Again, based on the comparative quantification through the ΔΔCt method between the untreated (con) and treated (SB 75μg/mL) DU-145 cells we were able to select a set of 18 genes for further analysis by setting the fold-change of gene expression (≥0.5) and statistical significance (p-value ≥0.05) as above **(Figures 2.III and 3C)**. According to the selected genes, two distinct clusters were identified. In cluster A, the genes *AURKA* and *UBE2B* turned to be positively regulated by silibinin, whereas, in cluster B, the genes *HDAC3, KDM4C, SMYD3, KDM1A, MBD2, AURKA, AURKB, ESCO2, HDAC1, SUV39H1, HAT1, SUV420H1, NCOA1, KAT6B* and *RPS6KA5* were found to be negatively regulated by silibinin **(Figures 2.III and 3C)**.

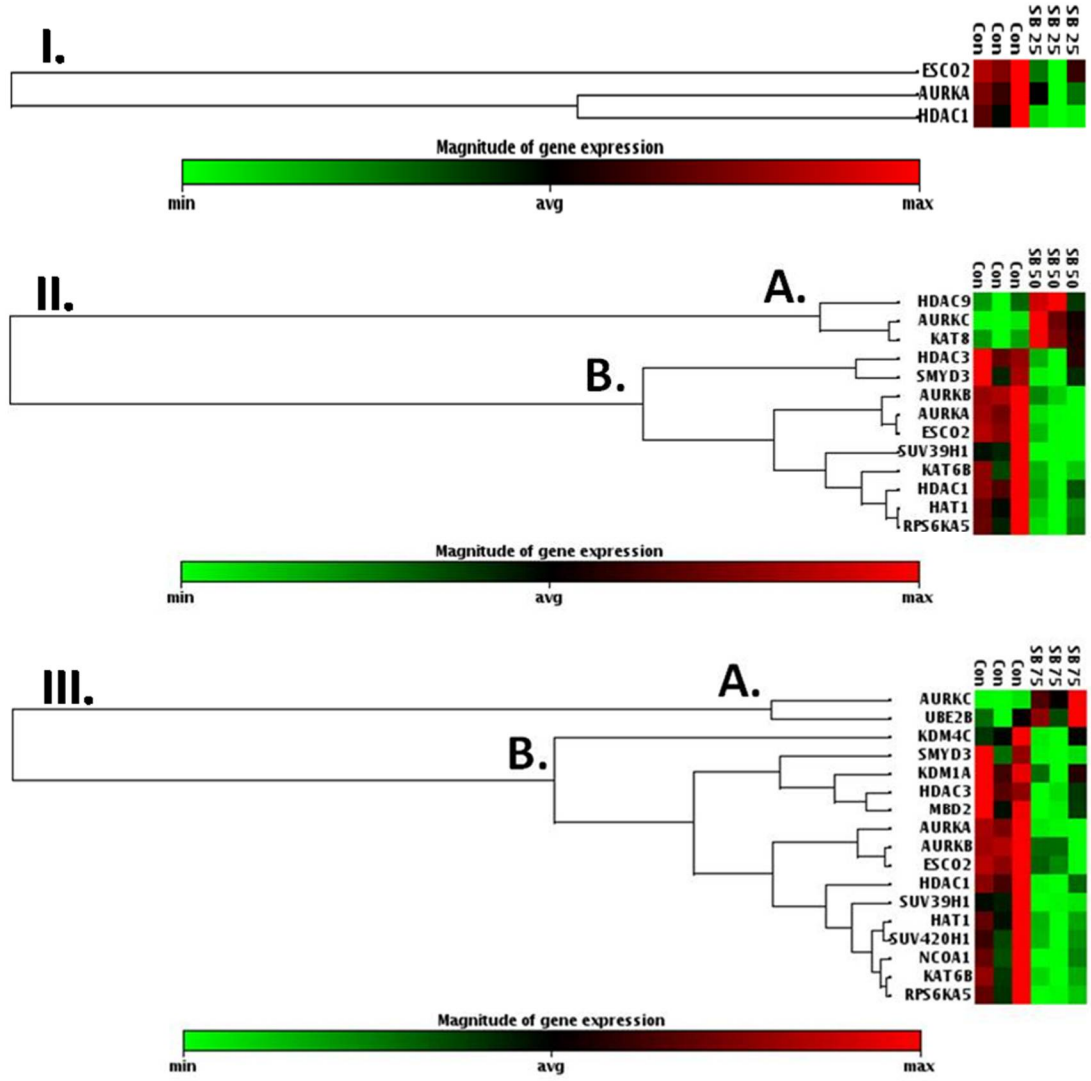

Figure 2. **Clustergram obtained after hierarchical clustering of gene expression data in DU-145 prostate cancer cells following exposure to silibinin (25-75 μg/mL) for 48h**. Clustergram displays a heat map of the magnitude of gene expression obtained from RT² PCR array for human epigenetic chromatin modification enzymes with genes being organized according to their expression pattern in dendrograms. Color saturation indicates the respective magnitude of gene expression. Specifically, green squares indicate lower gene expression, red squares indicate maximum gene expression, while black squares indicate no change in gene expression. The x-axis represents untreated DU-145 cells (Con) and cells treated with **(I)** 25 **(II)** 50 and **(III)** 75 μg/mL of silibinin (SB 25-75) for 48h, while the y-axis represents RT² PCR array genes. Gene expression was normalized with the use of five housekeeping genes (*B2M, HPRT1, RPL13A, GAPDH* and *ACTB*). Gene expression patterns were obtained from three independent experiments.

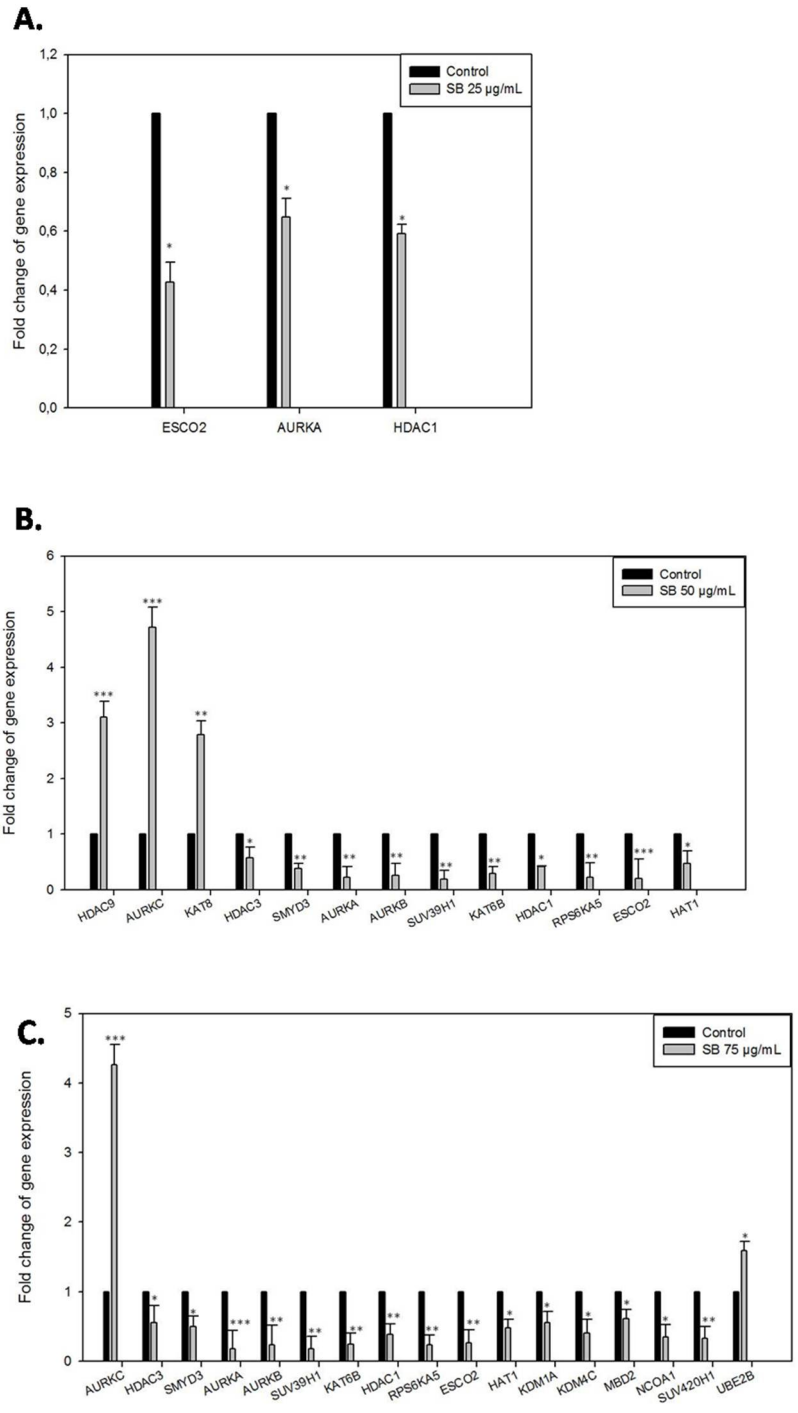

Figure 3. Vertical bar chart of the fold change of expression for the selected genes from the RT² PCR array in DU-145 cells after exposure of silibinin (25-75 µg/mL) for 48h. The fold change of gene expression in the selected group of genes from the PCR array is presented. Five housekeeping genes (*B2M*, *HPRT1*, *RPL13A*, *GAPDH*, and *ACTB*) were used for the normalization of gene expression. **(A)** Genes ESCO2, AURKA and HDAC1, were found to be negatively regulated in DU-145 cells treated with 25 µg/ml of silibinin. **(B)** Genes *HDAC9*, *AURKC* and *KAT8* were found to be positively regulated, while genes *HDAC3*, *SMYD3*, *AURKA*, *AURKB*, *SUV39H1*, *KAT6B*, *HDAC1*, *RPS6KA5*, *ESCO2* and *HAT1* exhibited negative regulation in DU-145 cells treated with 50 µg/mL of silibinin for 48h, compared to untreated cells. **(C)** *AURKC* and *UBE2B* were found to be positively regulated, while genes *HDAC3*, *SMYD3*, *AURKA*, *AURKB*, *SUV39H1*, *KAT6B*, *HDAC1*, *RPS6KA5*, *ESCO2*, *HAT1*, *KDM1A*, *KDM4C*, *MBD2*, *NCOA1* and *SUV420H*1 exhibited negative regulation in DU-145 cells treated with 75 µg/mL of silibinin

for 48h, compared to untreated cells. Results are shown as mean ± SE. For each sample three independent experiments were performed. *p≤0.05, **p≤0.01, ***p≤0.001.

4. Discussion

4.1 Silibinin reduces the expression levels of the PRC2 complex components

The polycomb group (PcG) proteins are important epigenetic regulators acting as transcriptional repressors. Specifically PcG proteins consist of two distinct complexes namely PRC1 and PRC2 that via histone post-translational modifications cooperate to silence downstream target genes, thus contributing to the development and progression of PCa [5]. In support to this, the repressive H3K27 histone mark induced by PRC2 complex, is thought to act as a scaffold for the recruitment of PRC1 complex, to stabilize the condensed chromating configuration, through ubiquitination at lysine 119 residues on histone H2A (H2AK119ub1), leading to gene repression at specific gene loci [6]. On the other hand, deregulated expression levels of different members of PRC2 complex that is composed of Bmi1, Ring1a and Ring1b, are associated with significant clinicopathological features in PCa. For instance high expression levels of Bmi1 in PCa patients samples are directly correlated with unfavorable prognosis, low survival and poor clinical outcome [7], while a similar elevated expression pattern of Ring1B (the catalytic subunit of PRC1 complex), responsible for the mono-ubiquitination of H2A, is also observed in PCa biopsies [8]. According to our results, silibinin was found to negatively regulate expression levels of all PRC1 members in a dose-denpendent way, in DU145 and PC3 cells. High expression levels of Bmi1 and Ring 1B have been associated with increased proliferative rate and PCa promotion through the inhibition of p16 and p14, as well as resistance to docetaxel treatment [9,10,11]. In this context, taking into consideration our previous results that demonstrated that silibinin causes inhibition of the PCR2 members expression, and especially that of EZH2, which is highly expressed in PCa and associated with poor clinical prognosis [3], the silibinin-induced downregulation of PRC1 members could indicate a further potential mechanism at an epigenetic level, through which silibinin exerts its anticancer effects by disrupting PRC complexes' cooperation and ability to regulate gene repression in PCa. Identification in future experiments of how such activity is able to restore expression of downstream target genes of both PRC complexes, in favor of PCa inhibition remains to be elucidated.

4.2 Silibinin modulates the expression levels of genes involved in epigenetic modifications in human prostate carcinoma

According to our previous results, silibinin was observed to interfere with the epigenetic machinery that is deregulated in PCa. Specifically, silibinin was found to modulate the abnormal expression of major epigenetic enzymes including the histone methyltransferase EZH2 (catalytic subunit of the PCR2 complex) along with negative regulation of histone deacetylases 1-2 (HDACs1-2), known to regulate gene expression at an epigenetic level in PCa *via* induced modifications in chromatin configuration. To this end, we sought to analyze the expression levels of crucial epigenetic modifying enzymes in DU-145 cells following incubation with increasing concentrations of silibinin (25-75μg/mL), using the RT^2 profiler PCR assay. According to the results, silibinin was found to modulate the expression of various genes that are important players of the epigenetic landscape in PCa.

Different studies support that aurora kinases (serine/threonine kinases) act as oncogenes in different types of cancers, consisting of three members *AURKA*, *AURKB* and *AURKC* [12,13]. The best-characterized aurora kinases AURKA and AURKB regulate cell cycle progression through mitosis facilitating the G2/M transition in the cell cycle. They are found overexpressed in PCa inducing uncontrolled proliferation and appear to be important targets for repressing cancer progression [12,14,15]. Based on our results, silibinin decreased the expression of *AURKA* and *AURKB*, which could justify the silibinin-induced cell cycle arrest at the G2/M phase in DU-145 cells.

On the other hand, down-regulation of HDAC3 expression by silibinin could further account for the decreased levels of AURKB known to be a positive regulator through deacetylation [16]. Interestingly, *AURKC* was found to be upregulated following silibinin treatment in our study. According to our results, AURKC appears to be expressed in low levels in DU-145 cells. *AURKC* over-expression has been associated with gene amplification and overexpression in highly metastatic breast (MDA-MB-231) and prostate (PC-3) cancer cell line, probably through epigenetic modifications [17]. As differences in *AURKC* expression levels appear to be dependent, at least in part, on the invasive potential of different PCa cell lines, low basal expression levels in DU-145 cells appears to be in consistence with its less invasive capacity compared to PC-3 cells. However, further experiments will clarify the net effect of silibinin on *AURKC* expression patterns in PCa cells, shedding light on its effect in molecular events associated with cell cycle regulation. Deregulated expression of histone methyltransferases (HMTs) along with histone acetyltransferases (HMTs) are implicated in prostate carcinogenesis [18,19]. Histone methyltransferase *SUV39H1* (KMT1A) catalyzes the tri-methylation of lysine 9 residues on histone H3 (H3K9me3) and is associated with transcriptional repression of tumor-suppressor genes in PCa [20]. Among different genes suppressed by HMTs induced histone modification, *GSTP* (detoxification) and *RKIP* (metastasis suppressor) were reported to be down-regulated *via* distinct histone modification marks, such as H3K27me2/3 and H3K9me2/3 [21,22]. Given that silibinin induced an increase in H3K27me3 levels in PCa cells, while it reduced levels of SUV39H1 responsible for elevated H3K9me3 marks, it would be interesting in the future to clarify whether silibinin could restore the expression of these genes by modulating H3K9me3 levels, as they appear to cooperate with H3K27me3 histone marks in suppressing two important genes. Moreover, SUV39H1 has been reported to positively regulate the migratory potential of PCa cells [23]. Therefore, silibinin could mediate the anti-migratory effect against DU-145 cells partially through deregulation of SUV39H1 expression. On the other hand, elevated expression of histone acetyltransferase KAT6B has been associated with the proliferation of DU-145 cells through PI3K-Akt signaling [24], observed to be negatively regulated by silibinin in our experiments. Finally, up-regulation of lysine demethylase KDM1A (LSD1) that catalyzes demethylation of residues of lysine 4 of histone H3 (H3K4me/2m3) has been associated with increased proliferative potential in docetaxel-resistant PCa cells including DU-145 [25], indicating that silibinin could potentially reverse the chemoresistant phenotype. Overall, silibinin appears to negatively modulate major epigenetic enzymes that are known to regulate gene expression in PCa through induction of modification in specific residues of histone H3. However, regulation of gene expression at an epigenetic level is not restricted only to alterations of histones configuration; methylation of the chromatin structure and specifically hypermethylation of gene promoters, that is mediated by the action of methyl CpG binding proteins MBD1/2/3/4 and other factors including MeCP2 and NuRD [26,27], cooperate for gene silencing. To this end, identification of the potential mechanism(s) by which the observed down-regulation of *MBD2* levels by silibinin in DU-145 cells could reverse hypermethylated gene promoters of specific silenced genes observed in PCa appears to be a challenge in future experiments.

5. Conclusions

In conclusion, our study suggests, that the reported anticancer activity of silibinin in PCa could be mediated, at least in part, through the disruption of central processes in chromatin conformation and alteration of key epigenetic enzymes regulating progression of the disease. Further studies are required to clarify in detail the precise molecular mechanism(s) that govern the pleiotropic action of silibinin against PCa.

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