
Cytotoxic and toxicogenomic effects of silibinin in bladder cancer cells with different *TP53* status

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Silibinin is a natural phenol found in the seeds of the milk thistle plant. Recent data have shown its effectiveness for preventing/treating bladder tumours. Therefore, in this study we investigated the cytotoxic and toxicogenetic activity of silibinin in bladder cancer cells with different *TP53* statuses. Two bladder urothelial carcinoma cell lines were used: RT4 (wild-type *TP53* gene) and T24 (mutated *TP53* gene). Cell proliferation, clonogenic survival, apoptosis rates, genotoxicity and relative expression profile of *FRAP/mTOR*, *FGFR3*, *AKT2* and *DNMT1* genes and of *miR100* and *miR203* were evaluated. Silibinin promoted decreased proliferation and increased late apoptosis in *TP53* mutated cells. Increased early apoptosis rates, primary DNA damage, and decrease of cell colonies in the clonogenic survival assay were detected in both RT4 and T24 cell lines. Down-regulation of *FRAP/mTOR*, *AKT2*, *FGFR3*, *DNMT1* and *miR100* expression occurred in RT4 cells. Modulation of *miR203* was observed in both cell lines. In conclusion, despite the reduction of clone formation in both cell lines, the toxicogenomic effect of silibinin on *FRAP/mTOR*, *AKT2*, *FGFR3*, *DNMT1* and *miR100* was dependent on the *TP53* status. Taken together, the data confirmed the role of silibinin as an antiproliferative compound, whose mechanism of action was related to the *TP53* status.

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1. Introduction

Among urinary bladder tumours, urothelial cell carcinoma (UCC) is one of the most common malignancies, with high prevalence especially in industrialized countries (Jemal *et al.* 2007). The most frequent gene mutations found in these bladder carcinoma cells occur in *TP53* and are related to the cell transformation, malignancy and high recurrence rates (Cheng *et al.* 2011). Numerous studies have demonstrated

the relationship between *TP53* or p53 mutations and the poor response to antineoplastic therapies (Saint *et al.* 2004; Esuvaranathan *et al.* 2007). At present, only tumour grade and stage determine the treatment's protocols, including surgery, immunotherapy, radiotherapy and chemotherapy, for improving UCC management (Mayr *et al.* 2014; Patel *et al.* 2015). Transurethral resection is the gold standard intervention for non-muscle invasive UCC, followed by adjuvant intravesical therapy with Bacillus Calmette-Guerin

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(van Lingen *et al.* 2013). Topical chemotherapy with mitomycin, thiotepa and epirubicin has also been used as an alternative treatment, but it has no impact on long-term survival, disease progression and recurrence (Retz *et al.* 2013). Additional treatment protocols for UCC include MVAC, a combination of methotrexate, vinblastine, doxorubicin and cisplatin, and the combination of gemcitabine and cisplatin (Raghavan *et al.* 2012).

However, despite their effectiveness, some antineoplastic drugs have limited use because of their adverse side effects, including neurotoxicity, nephrotoxicity, myelosuppression, etc. (Li *et al.* 2015; Sinha *et al.* 2015). Therefore, treatment improvement with use of alternative compounds to reduce these undesirable effects has become imperative. Because of their wide spectrum of chemical and biological properties, some plants have been studied to identify natural compounds with potential anti-tumour activity (Dumont *et al.* 2007; Chawla *et al.* 2010). Recent findings about complementary and alternative medicine for prostate and bladder cancer treatment have shown that curcumin, resveratrol and silibinin have potential anticancer properties through multiple molecular targets (Philippou *et al.* 2013). Silibinin, one of the less investigated compound, is a natural phenol of the flavonolignan family, and is derived from the seeds of the milk thistle (*Silybum marianum*; Asteraceae) plant (Tyagi *et al.* 2007). This substance is effective in preventing the recurrence of superficial bladder tumours in mice (Zeng *et al.* 2011), in addition to its broad-spectrum efficacy against prostate cancer progression both *in vitro* and *in vivo* models without noticeable toxicity (Ting *et al.* 2013). However, little is known about the safety, efficacy, cost-effectiveness and mechanism of action of these phytochemicals (Philippou *et al.* 2013).

Indeed, it is known that some dietary and natural compounds may regulate gene expression by modulating DNA methylation profile (Zwergel *et al.* 2016), mRNAs (Sávio *et al.* 2015) and microRNAs (miRNAs or miR) (Ahmad *et al.* 2012) expressions. DNA methylation is a genetically programmed modification catalysed by DNA methyltransferases (DNMTs). Although DNA methylation is essential for embryonic development or cell differentiation, aberrant expression and/or activities of DNMTs are involved in several diseases, including several cancer types (Zwergel *et al.* 2016). Recently it was demonstrated that both green tea, polyphenol epigallocatechin 3-gallate, and kazinol Q, a natural product from Formosan plants, inhibited *DNMT* expression and suppressed colon HCT 116, breast MCF-7 and prostate LNCaP cancer cell proliferation, respectively (Moseley *et al.* 2013; Weng *et al.* 2014). Additionally, recent reports have shown that *TP53* inactivation may alter the DNA demethylation process through regulation of *TDG* (thymine DNA glycosylase), a base excision repair gene (Maiti and Drohat 2011; da Costa *et al.* 2012). Recently it was demonstrated that silibinin could inhibit *DNMT* activity in colon cancer cell lines (Kauntz *et al.* 2013). Moreover, such a compound alone or in association

with histone deacetylators or DNMT inhibitors can restore the methylated-inhibited E-cadherin gene expression, decreasing the migratory and invasive potential of non-small-cell lung carcinoma cell lines (Mateen *et al.* 2013).

Another gene regulation mechanism now widely studied is mediated by miRNAs. These are small noncoding RNAs that direct gene regulation through translational repression and/or degradation of complementary mRNA (Wu and Belasco 2008). Growing evidence has suggested that miRNAs are implicated as oncogenes and tumour suppressors in a variety of human cancers, and that more than 50% are located in cancer-associated genomic regions or in fragile sites (Bartel 2004; Aravindaram and Yang 2010). Furthermore, the *TP53* gene may facilitate the processing of primary miRNAs to precursor miRNAs through interaction with Drosha processing (Suzuki *et al.* 2009). Interference of *miR100* and *miR203* in tumour cells has been strengthened by the identification of their targets, which play important role in regulation of cell growth, cellular proliferation and senescence (Nagaraja *et al.* 2010). Among those targets are genes related to the PI3K-FRAP/mTOR or PI3K/AKT2 pathways, associated with a majority of sporadic human cancers, such as breast, ovarian and bladder cancers (Gingras *et al.* 2001; Luo *et al.* 2003; Shaw and Cantley 2006; Dobbin and Landen 2013; Gonzalez-Angulo and Blumenschein 2013).

Therefore, given the evidence of urothelial antineoplastic potential of silibinin, and taking into account that its mechanism of action is not completely understood, this study aimed to investigate the molecular events underlying the antiproliferative activity of silibinin in bladder cancer cell lines with wild-type (RT4) and mutated (T24) *TP53* gene.

2. Materials and methods

2.1 Cell lines and tested compound

The established cell lines RT4 and T24 from human bladder UCC were purchased from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. No specific mutation was detected in RT4 cells, which was established from a low-grade papillary bladder tumour. The T24 cells were obtained from an invasive tumour with a *TP53* allele encoding an in-frame deletion of tyrosine 126. The cell lines were maintained as previously described by (da Silva *et al.* 2010). Silibinin (CAS Number 22888-70-6) was purchased from Sigma-Aldrich (USA) and diluted into 2% dimethyl sulphoxide (DMSO), prior to use.

2.2 Cell proliferation

Cytotoxicity and cell proliferation rates were assessed using the Cell Proliferation Kit II (XTT) from ROCHE Diagnostics

(Mannheim, Germany). Briefly, cells were seeded into 12-well culture plates (6×10^4 cells/well) and were treated 24 h later with different concentrations of silibinin (100, 115, 130, 150, 200 and 250 μM) for 48 h (cell proliferation). Untreated cells and cells treated only with DMSO were cultured as negative controls. After treatment, cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4 , 0.04 g Na_2HPO_4 , 0.35 g NaHCO_3 , 1 g glucose and 8 g NaCl in 1 L H_2O) and then a 50 μL aliquot of XTT solution (1 mL XTT labelling solution/20 μL of electron-coupling reagent) was added to each well. The absorbance was measured at 492 and 690 nm after 40 min (absorbance results are proportional to the percentage of viable cells). Treatments were performed in triplicate.

2.3 Clonogenic survival

Clonogenic assay was performed for evaluating the long-term effects of silibinin. To determine clonogenic ability, cells were plated at a density of $1 \times 10^6/25 \text{ cm}^3$ culture flask. After 24 h, silibinin (100, 115, 150 and 200 μM) was added to the culture medium and cells incubated again at 37°C , 5% CO_2 , for 24 h. Afterwards, cultures were rinsed with Hank's solution and trypsinized. Approximately 1000 cells were plated into 25 cm^3 culture flasks and allowed to grow for 15 days to form colonies. Cells were stained with Giemsa, and colonies with 50 or more cells were counted. Silibinin treatments and controls were performed in triplicate.

2.4 Comet assay

The Comet assay was carried out according to the technique described by Singh *et al.* (1988) and Tice *et al.* (1991). The alkaline version used was able to detect DNA strand breaks and alkaline-labile sites (ALS) in DNA. Initially, 6×10^4 cells were seeded into 12-well plates for 24 h. Then, cells were treated with silibinin at concentrations of 100, 115, 130 and 150 μM for 24 h. Treatment with 0.006 M methyl methane-sulfonate (Sigma-Aldrich, Inc.; St. Louis, MO, USA) for 5 min at 37°C was used as positive control. Briefly, 10 μL of cells were added to 100 μL of 0.5% low-melting-point agarose at 37°C . This mixture was layered onto pre-coated slides with 1.5% standard agarose and covered with a coverslip. The agarose was allowed to solidify at 4°C , and then, the coverslip was gently removed. Afterwards, the slides were immersed into lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl at pH 10, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) overnight at 4°C , and then incubated in an alkaline buffer (0.3 mM NaOH and 1 mM EDTA; pH>13) for 20 min to allow DNA unwinding and alkali-labile site expression. Electrophoresis was conducted in the same alkaline buffer at 4°C , for 20 min, at 25 V

(0.86 V cm^{-1}) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5) solution for 15 min, fixed with absolute ethanol and stored at room temperature until analysis. All steps were conducted in the dark to prevent any additional DNA damage. The slides were stained with SYBR Gold (1:10,000; Invitrogen, NY), immediately before analysis. Cell viability was assessed using the trypan blue (0.4% trypan blue; Sigma-Aldrich, Inc., St. Louis, MO) exclusion test, and was never below 90%. A total of 150 randomly selected nucleoids per treatment were analysed under $400\times$ magnification, in a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill, UK). Tail intensity (% DNA in tail) was used to estimate DNA damage. The slides were prepared in duplicate from three independent treatments.

2.5 Apoptosis assay

A quantitative assessment of apoptosis was performed using a Guava Annexin reagent (Merck Millipore). Annexin V was used for detecting the externalization of phosphatidylserine to the cell surface and 7-AAD as an indicator of cell membrane structural integrity. Briefly, 2×10^5 cells were seeded into a 25 cm^3 culture flask. After 24 h, the cells were treated with silibinin at concentrations 100, 150, 200 and 250 μM for 48 h. Untreated cells and cells treated with only DMSO were cultured as controls. Afterwards, cells were washed with Hank's solution, trypsinized, resuspended into 100 μL Guava Nexin reagent for 20 min in the dark, and immediately analysed in the Guava easyCyte flow cytometer (Millipore) using the Guava System software. Data from 5000 cells were collected in each data file. Cellular status was defined as follows: unstained cells were classified as 'alive'; cells stained by only annexin V were classified as 'early apoptotic'; cells stained by both annexin V and 7-AAD were classified as 'late apoptotic'; and cells stained by only 7-AAD were classified as 'dead'.

2.6 FRAP/mTOR, FGFR3, DNMT1 and AKT2 gene expression

Cells were seeded into plates at a density of 1×10^6 cells/plate. Twenty-four hours later, cells were treated with silibinin (135, 150 and 200 μM) for 24 h. Afterwards, cells were washed with Hank's solution and collected for miRNA and RNA extraction. Total RNA was isolated using the RNeasy Mini kit® (Qiagen), according to the manufacturer's instructions. RNA concentrations and purities were determined using a NanoVue spectrophotometer (GE Healthcare). Reverse transcription was performed using 6 μL of random hexamer primers ($10\times$), 6 μL of reaction buffer ($10\times$), 2.5 μL

of dNTPs (25×) and 3 µL of MultiScribe (50 U/mL; High Capacity, Applied Biosystems, Foster City, CA). After incubation (25°C for 10 min, 37°C for 2 h and 4°C undetermined), cDNA was stored at -20°C. *FRAP/mTOR* (Hs_00234508_m1), *FGFR3* (Hs00179829_m1), *DNMT1* (Hs00945875_m1) and *AKT2* (Hs01086102_m1) gene expressions were assayed using the TaqMan system (Applied Biosystems). Each tube contained 2 µL cDNA template, 5 µL Master Mix TaqMan 2X (Applied Biosystems) and 0.5 µL of 20× primers/probe (Assays-on-Demand gene expression products; Applied Biosystems). *GAPDH* was used as a housekeeping gene. The reaction was performed using the following thermal cycler conditions: 94°C for 10 min followed by 40 cycles at 94°C for 30 s and 60°C for 1 min. Fluorescence data were collected during each annealing/extension step. The reactions were performed using the Applied Biosystems 7500 FAST Real-Time PCR System and the SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems; Applied Biosystems). For every PCR sample, a negative (no template) control was processed as a routine control. Relative gene expression data were analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Each silibinin treatment was performed in triplicate. The interaction network was created using String 10 software (http://string-db.org/newstring.cgi/show_network_section.pl). The active prediction method used was the Experiments, with confidence score of 0.400.

2.7 miR100 and miR203 expression

miRNA was isolated using the miRNeasy kit® (Qiagen), according to the manufacturer instructions. Complementary DNA was synthesized using the TaqMan Assays Small RNA kit, according to the manufacturer's protocol (0.15 µL 100 mM dNTPs, 1 µL MultiScribe™ reverse transcriptase 50 U/µL, 1.50 µL reverse transcription buffer 10×, 0.19 µL RNase inhibitor 20 U/µL, 4.16 µL DNase and RNase free water, 3 µL RT primer 5× and 5 µL miRNA sample; Applied Biosystems).

miR100 (2142) and *miR203* (000507) expressions were assayed using the TaqMan system (Applied Biosystems). Quantitative PCR was performed in an automatic thermocycler (ABI Prism 7500 Sequence Detection System; Applied Biosystems), using 1.0 µL TaqMan Assay Small RNA (20×), 1.33 µL reverse transcription product, 10 µL TaqMan Universal PCR Master Mix II (2×) and 7.67 µL DNase and RNase free water, totalling 20 µL per sample. *RNU6B* was used as control. The reaction was performed using the following thermal cycler conditions: 94°C for 10 min followed by 40 cycles at 94°C for 30 s and 60°C for 1 min. For every PCR sample, a negative control was processed. Assays were performed in triplicate. Relative

gene expression data were analysed using the $2^{-\Delta\Delta CT}$ method.

2.8 Statistical analysis

For the cytotoxicity, cell proliferation and clonogenic survival tests, apoptosis, gene expression and comet assay, the data were analysed using the one-way ANOVA, and *post hoc* analysis by Tukey's test. The results were considered statistically significant with $p < 0.05$. Statistical analyses were carried out using the SigmaStat Version 3.5 (Dundas Software LTD, Germany).

3. Results

3.1 Silibinin inhibits human UCC growth

As shown in figure 1, inhibited cell proliferation was observed in the T24 cell line 48 h after treatment with silibinin at concentrations equal to and higher than 150 µM. The data of the clonogenic survival assay showed significant decrease of colonies in both RT4 and T24 cell lines after silibinin treatment with all the tested concentrations, suggesting that silibinin inhibited the formation of cell colonies independent of the *TP53* status (figure 2).

3.2 Silibinin induces DNA damage

Using Comet Assay alkaline version, increased DNA damage was detected after treatment with silibinin at 150 µM, in both cell lines (RT4 and T24). The comparison between the

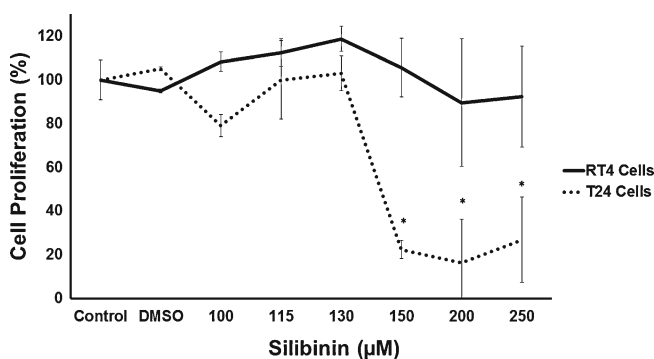


Figure 1. Percentage of cell proliferation (logarithmic scale) in the urinary bladder carcinoma RT4 and T24 lineages 48 h after silibinin treatment. Each point represents the mean value \pm standard deviation obtained from three independent treatments with the same concentration of silibinin. * $p < 0.05$ in relation to the silibinin vehicle control (2% DMSO).

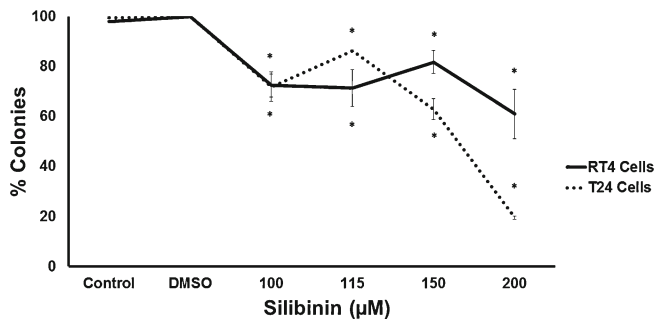


Figure 2. Percentage of colonies (logarithmic scale) in the urinary bladder carcinoma RT4 and T24 lineages 15 days after silibinin treatment. Each point represents the mean value \pm standard deviation obtained from three independent treatments with the same concentration of silibinin. * $p < 0.05$ in relation to the silibinin vehicle control (2% DMSO).

two cell lines was done only for the concentration (150 μM) which caused DNA damage in both. We tried to investigate whether cells presented the same sensitivity to the test compound. However, no statistically difference was detected in the amount of damage between the two lineages (figure 3).

3.3 Silibinin induces low apoptosis rates in human UCC

Increased amount of late (100, 150, 200 and 250 μM) and early (250 μM) apoptosis was observed in T24 cells, 48 h after treatment with silibinin. For RT4 cells, high level of early apoptosis (200 μM) was detected after 48 h (figure 4). These results suggest growth inhibition by silibinin is not related to the high apoptosis rates in UCC cells.

3.4 Silibinin induces FRAP/mTOR, FGFR3, DNMT1, AKT2 and miR100 modulation only in the wild-type TP53 human UCC

Modulated gene *FRAP/mTOR*, *FGFR3*, *AKT2* and *DNMT1* expression was observed after exposure to silibinin. These genes were altered in a majority of human cancers and play important roles in many cellular processes, including the regulation of growth. Moreover, these genes interact with *TP53*, which is the reason why the cell lines with different *TP53* statuses were chosen. The *FRAP/mTOR*, *FGFR3*, *DNMT1* and *AKT2* genes and *miR100* were down-regulated in RT4 cells after treatment with silibinin at 135, 150 and 200 μM . No gene modulation was detected in T24 cells (figure 5). *miR203* was modulated in both cell lines after silibinin treatments (figure 6). Figure 7 depicts the network involving the investigated genes, indicating interaction

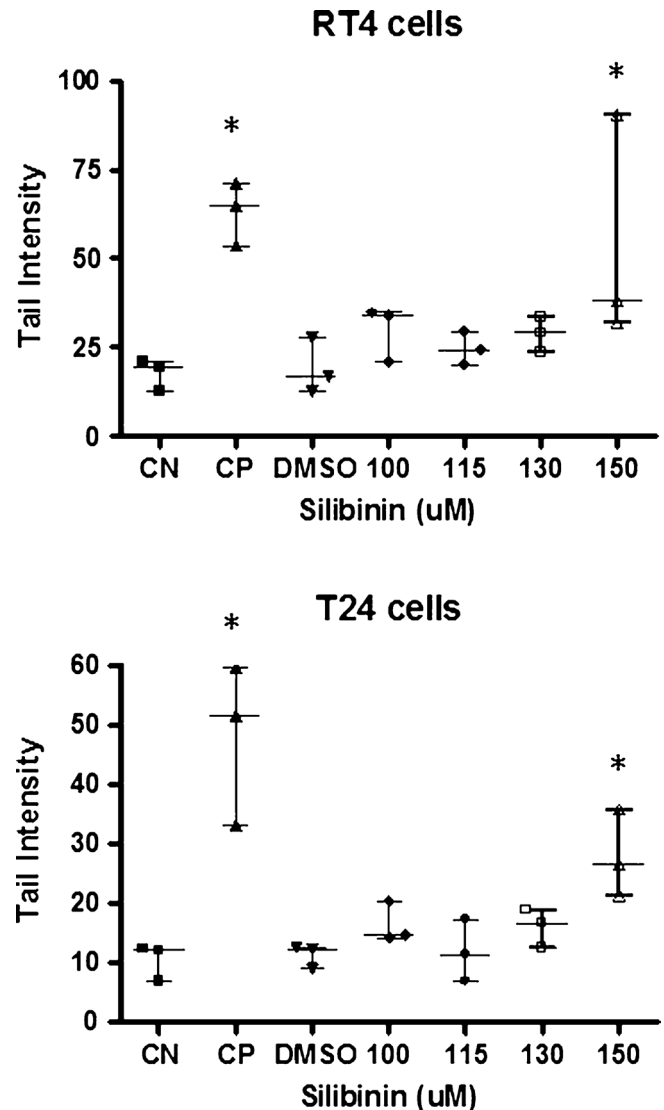


Figure 3. DNA damage (tail intensity) in RT4 and T24 bladder carcinoma cell lines after silibinin treatment. * $p < 0.05$ in relation to the silibinin vehicle control (2% DMSO). CN: negative control; CP: positive control.

among *DNMT1*, *TP53*, *FRAP/MTOR* and *AKT2*. Taken together, these results clearly indicate that the effect of silibinin in terms of molecular mechanism is dependent on the *TP53* status.

4. Discussion

The use of natural products for treating diseases is the oldest and most widespread form of medication (Halberstein 2005). However, limited knowledge about the effectiveness or

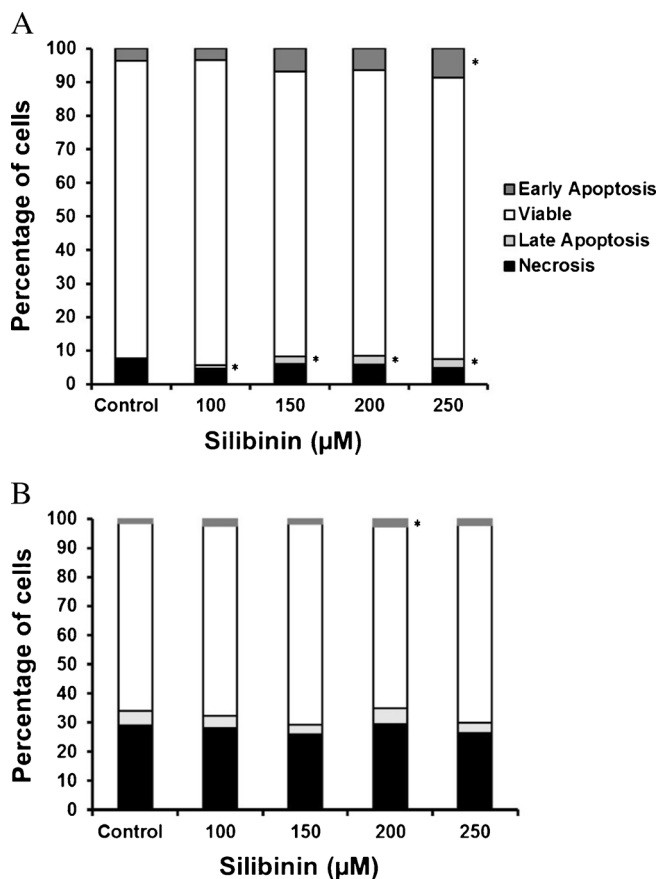


Figure 4. Percentage of cells at early and late apoptosis, viable and death (necrosis) cells in the urinary bladder carcinoma T24 and RT4 cell lines after silibinin treatments. * $p < 0.05$ compared to the silibinin vehicle control (2% DMSO). (A) T24 cells; (B) RT4 cells.

benefits has delayed the incorporation of such products into the clinical practice (Ahmad *et al.* 2012). Regarding silibinin, recent findings have supported its clinical usefulness for colorectal cancer intervention and therapy (Kumar *et al.* 2014). Furthermore, some studies have demonstrated its strong antiproliferative, pro-apoptotic and anti-inflammatory effects, including potential to cause severe and irreparable damage to colon tumour cells (Kaur *et al.* 2009; Raina *et al.* 2013; Rajamanickam *et al.* 2010). In light of these, the present study focused on the molecular mechanisms related to the antiproliferative activity of silibinin in UCCs with wild-type and mutated *TP53* gene.

Recent findings have demonstrated that non-muscle-invasive and muscle-invasive bladder cancers have distinct pathways in carcinogenesis. The pathway with mutation of *FGF receptor 3* gives rise to low-grade non-muscle-invasive papillary tumours that frequently recur but seldom invade. In contrast, deletions or mutations of the *TP53*, and other important suppressor genes (*RBI*, *ERBB2*, or *PTEN*), are

related to the development of muscle-invasive bladder cancer and carcinoma *in situ* (Knowles and Hurst 2014).

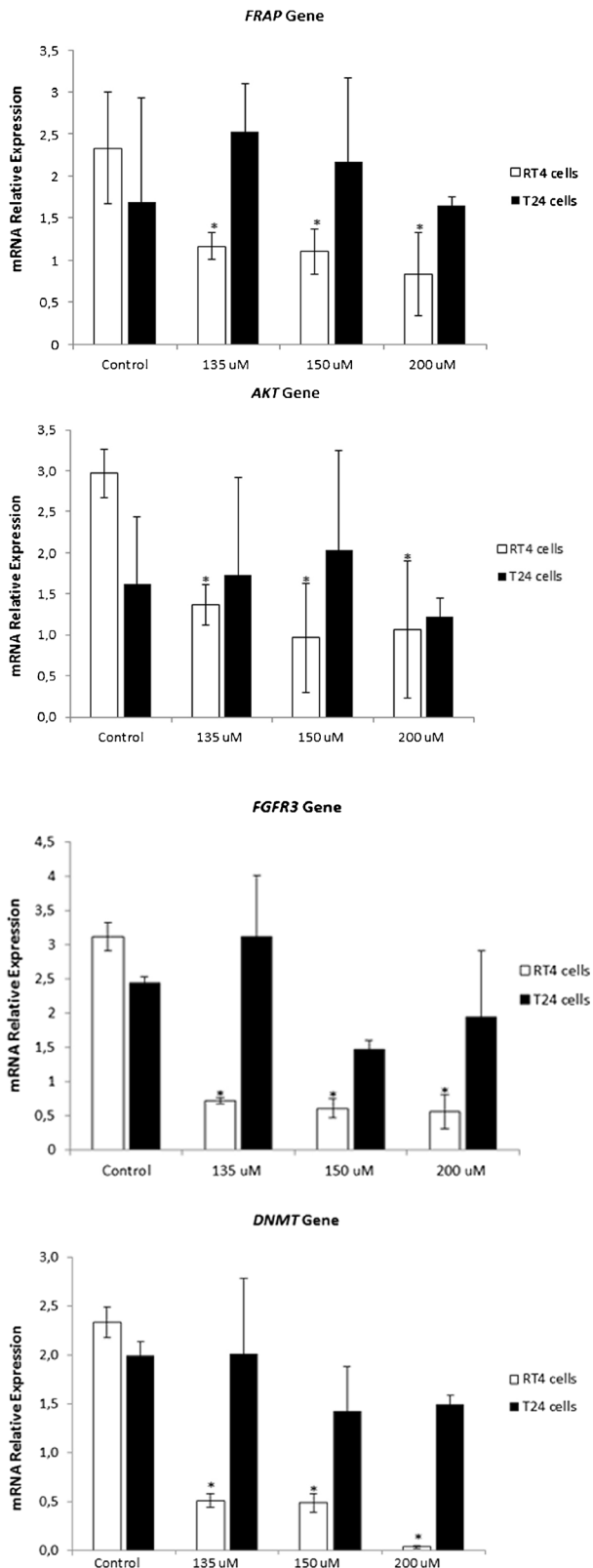
It is known that p53 protein is stabilized and activated in response to various genotoxic stresses to induce cell cycle arrest, DNA repair, and apoptosis. Hence, the induction of DNA damage by chemotherapy and radiotherapy to stabilize p53 is a promising strategy to induce apoptotic cell death in malignant tumours. However, p53 is mutated in half of the human cancers and the mt-p53 loses its function that causes drug resistance (Dashzeveg *et al.* 2016).

Thus, the presence of a functional *TP53* is associated with a good response to chemotherapy (Vinall *et al.* 2012), and impacts the clinical decisions regarding adjuvant therapy (Zhang *et al.* 2013). Indeed, compelling evidence shows that the mutational spectrum of cancer genome can strongly influence the patient's cancer response to treatment (Garnett *et al.* 2012).

We observed that silibinin was able to increase the primary DNA damage at the highest concentration (150 µM) in both wild-type and mutated *TP53* bladder cancer cells. The genotoxic potential of silibinin was previously demonstrated in oral carcinoma (KB), hepatoma (HepG2), and osteosarcoma cell lines through a mechanism that involves mitochondrial stress and ROS generation (Angeli *et al.* 2010; Gohulkumar *et al.* 2014; Leon *et al.* 2014). Moreover, such stress can alter the mitochondrial membrane potential, leading to apoptosis (Gohulkumar *et al.* 2014).

Here, we observed that silibinin decreased cell proliferation only in *TP53* mutated cells, 2 days after treatment with the highest concentrations (150–250 µM). On the other hand, the clonogenic survival assay revealed in both cell lines a significant decrease in cell colonies formation 15 days after silibinin treatment at all tested concentrations. Similarly, it has been reported that silibinin treatment inhibited metabolic activity and clonogenic potential of human neuroblastoma (SK-N-MC) cells in a dose-dependent manner (Yousefi *et al.* 2012).

Taken together, these results provide evidence that silibinin promotes reduction of the number of cell colonies, most likely because of DNA damage induction. However, despite its genotoxic potential, no effect of silibinin was observed on RT4 cell proliferation 48 h after exposure. Interestingly, it was previously demonstrated that silibinin may modulate nucleotide excision repair (NER) process in proficient *TP53* wild-type fibroblast cells through the increase of p53 and GADD45 α expression, both key regulators of NER pathway (Guillermo-Lagae *et al.* 2015). Perhaps in RT4 cells, the acute exposure to silibinin may have a protective effect. It is important to highlight that the clonogenic potential measured by the clonogenic survival assay is a very relevant endpoint to be evaluated, since cells unable to



produce colonies are also unlikely to produce tumours (Tannock *et al.* 2001). The chronic response of both cell lines evaluated 15 days after silibinin treatment (clonogenic assay) was characterized by a significant decrease in colonies formation, suggesting that such compound may alter the cell reproductive potential.

Thus, considering that silibinin was not genotoxic in lower concentrations, these findings may suggest that other mechanisms than DNA damage are primarily responsible for the silibinin effect on cell proliferation (clonogenic potential). Additionally, we noted that acute response to silibinin involves the induction of cell death through p53-independent pathways, since early (250 μ M) and late (100, 150, 200 and 250 μ M) apoptosis were detected in T24, and only early (200 μ M) apoptosis in RT4 cells. It has been suggested (Tyagi *et al.* 2004) that the selectivity and specificity of silibinin-induced apoptosis in two UCC cell lines (TCC-SUP and T24), showing a dose- and time-dependent apoptotic death only in TCC-SUP cells, associated with the cleaved forms of caspase 3 and poly(ADP-ribose) polymerase. Furthermore, while silibinin suppressed tumour growth and induced apoptosis by the *Bax/Bcl-2* pathway through caspases activation in human endothelial cells (ECV304) (Yoo *et al.* 2004), in human hepatocellular (HuH7) and renal carcinoma (Caki-1) cells, this compound promoted apoptosis by down-regulating survivin (Li *et al.* 2008; Cui *et al.* 2009). Thereby, it is possible to believe that silibinin induces apoptosis through cellular-specific mechanisms.

To better understand silibinin mechanisms of action, its acute effects on expression of genes related to cellular proliferation, invasion and migration (PI3K/AKT and PI3K/FRAP-mTOR pathways) and DNA methylation (*DNMT1*) were also investigated. Down-regulation of *FRAP/mTOR*, *FGFR3* and *AKT2* gene expression in *TP53* wild-type cells (RT4) was detected. These findings suggest inhibition of the PI3K/AKT/FRAP-mTOR pathway, decreasing clone formation and increasing cell death, possibly through p53 mechanism. Indeed, simultaneous pharmacological modulation of p53 and AKT/mTOR pathway could provide enhanced anticancer efficacy through cell proliferation and growth inhibition (Budanov and Karin 2008; Levine and Oren 2009). However, silibinin could have also acted through different mechanisms, since reduced clone formation without gene expression modulation was observed in mutated *TP53* cells (T24). Since *TP53* acts as a molecular node, modulation of cell response could also be caused by

◀ **Figure 5.** Relative expression of *FRAP/mTOR* and *AKT2*, *FGFR3* and *DNMT1* genes in RT4 and T24 cells treated with silibinin. Each column represents the mean value \pm standard deviation obtained from three independent treatments with the same concentration of silibinin. Control – treated with 2% DMSO (control vehicle); * $p < 0.05$ compared with control.

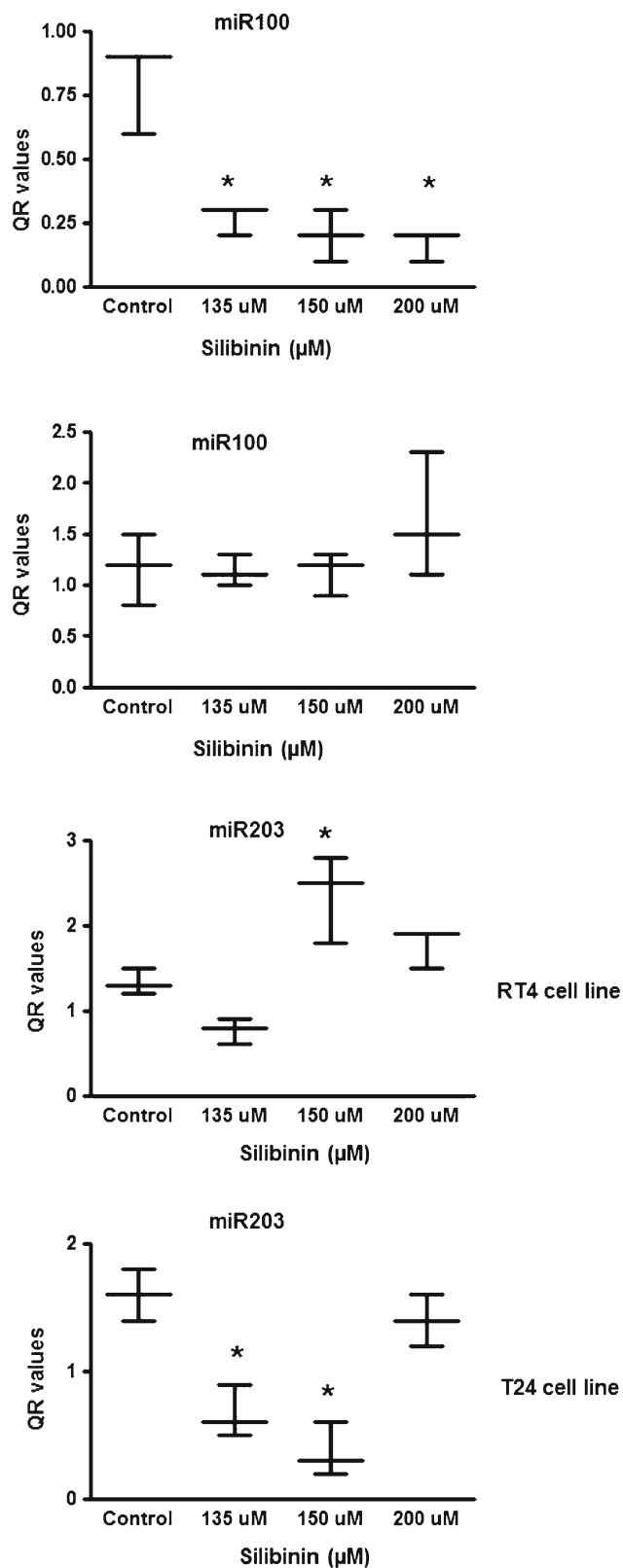


Figure 6. Relative expression of miR100 and miR203 in RT4 and T24 cells treated with silibinin. Control – treated with 2% DMSO (vehicle control). Cell cultures were performed in triplicate.

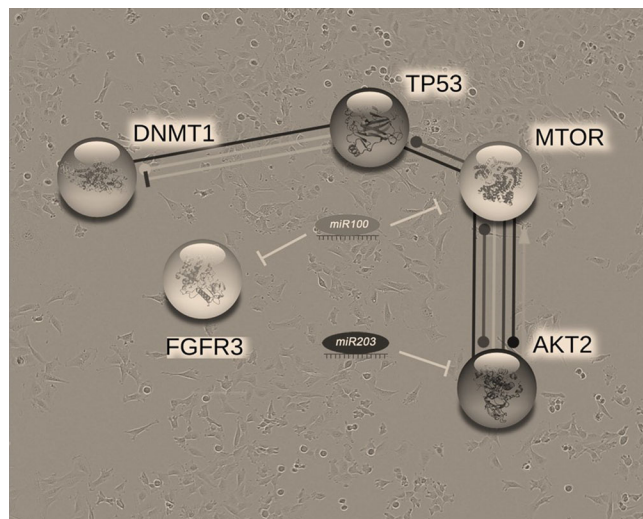


Figure 7. Gene network created using *in silico* data from String 10 software. Continuous lines represent gene interactions. Lines with arrows indicate gene activation and with lines with balls indicate post-transcriptional activation.

other altered target genes. In a previous study we reported that gene networks of three cell lines of UCC (RT4, 5637 and T24), determined by Bayesian interpolation, showed interactions among *TP53* and several other genes, suggesting that other pathways might be associated with the malignant phenotype (da Silva *et al.* 2010).

The down-regulation of *DNMT1* only in the *TP53* wild-type cells (RT4) may indicate a decrease of genome methylation, with consequent activation of genes that should be silenced, such as tumour suppressor genes. Recently it was demonstrated that *DNMTs* inhibition can arrest tumour growth and cells invasiveness and induce differentiation; however, their increased expression was shown in numerous cancer types (Zwergel *et al.* 2016). In fact, Fang *et al.* (2015) have found an up-regulation of *DNMT1* in hepatocellular carcinoma cells. These authors demonstrated that cells with active AKT mechanism show *de novo* DNA methylation and up-regulation of DNA methyltransferase 1 via an AKT/ β -transducin repeat-containing protein (β TrCP)-mediated ubiquitin-proteasome pathway. In our study, both *AKT* and *DNMT1* expression were inhibited in RT4 cells, suggesting that the antiproliferative effects could also be due to simultaneous modulation of these genes.

Another mechanism investigated was the effect of silibinin on relative expression of two microRNAs (*miR203* and *miR100*) involved in the post-transcriptional regulation of *Akt2/mTOR*, *FGFR3* and *FRAP* genes. Dysregulation of *miR100* and *miR203* has been reported to be involved in tumorigenesis and tumour progression of several cancer types. However, its expression patterns in tumours are controversial (Nagaraja *et al.* 2010; Saini *et al.* 2011; Wang

et al. 2013). In our study, since *FGFR3* gene was down-regulated in wild-type *TP53* cells, we were expecting *miR100* to be up-regulated, but the results showed down-regulation of this miRNA. Some authors corroborate our results, suggesting that other mechanisms besides PI3K-FRAP/mTOR must be involved in the toxicogenomic effect of silibinin. Gong *et al.* (2015) showed that silencing *miR100* expression with anti-miRNA-100 oligonucleotide initiated apoptosis of breast cancer SK-BR-3 cells *in vitro* and *in vivo*, suggesting down-regulation of *miR100*-sensitized breast cancer cells to chemotherapy. *miR203* up-regulation was observed in RT4 cells and down-regulation was observed in T24 cells, but not at all concentrations, suggesting that the expression does not depend on *TP53* status. Rihani *et al.* (2015) did not observe direct binding of p53 to miR203 in neuroblastoma cells. Thus, the miR100 and miR203 function in predicting the effect of silibinin still needs to be clarified.

In conclusion, despite silibinin inhibiting clone formation and activating apoptosis in both wild-type and *TP53* mutated bladder cancer cell lines, modulation of *FRAP/mTOR*, *AKT2*, *FGFR3* and *DNMT1* expression occurred in a *TP53*-status-dependent way. However, although the results show promise regarding the important role of silibinin as an antiproliferative compound, further *in vivo* studies that include the complexity of living body need to be performed to better understand the mechanisms by which silibinin exerts its antineoplastic activity.

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