

Oral Silibinin Inhibits *In vivo* Human Bladder Tumor Xenograft Growth Involving Down-Regulation of Survivin

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Abstract Purpose: Chemoprevention is an upcoming approach to control bladder cancer, which is one of the commonly diagnosed malignancies showing recurrence rate of 70% or even higher. Recently, we observed the *in vitro* efficacy of silibinin, a flavanolignan, in human bladder transitional cell papilloma RT4 cells. Here, we investigated the antitumor efficacy and associated mechanisms of silibinin in RT4 tumor xenograft.

Experimental Design: RT4 tumor xenograft was implanted s.c. in athymic nude mice, and then animals were oral gavaged with silibinin at 100 and 200 mg/kg doses, 5 days/week for 12 weeks. Tumor growth, body weight, and diet consumption were recorded, and tumors were analyzed for proliferation, apoptosis, and angiogenesis biomarkers and molecular alterations by immunohistochemistry, immunoblot analysis, and ELISA. p53 small interfering RNA was used in cell culture to examine the role of p53 in survivin expression.

Results: Silibinin feeding inhibited tumor xenograft growth without any gross signs of toxicity. Silibinin decreased tumor volume by 51% to 58% ($P \leq 0.01$) and tumor weight by 44% to 49% ($P < 0.05$). Silibinin moderately ($P < 0.001$) decreased cell proliferation and microvessel density and strongly ($P < 0.001$) increased apoptosis in tumors. Silibinin robustly decreased survivin protein expression and its nuclear localization, as well as tumor-secreted level in mouse plasma, but increased p53 and cleaved caspase-3 levels in tumors. Silibinin-caused decrease in survivin was independent of p53.

Conclusion: These findings identified *in vivo* antitumor efficacy of silibinin against human bladder tumor cells involving down-regulation of survivin and an increase in p53 expression together with enhanced apoptosis.

Bladder cancer is one of the most common genitourinary malignancies and has a high rate of tumor recurrence (1, 2). It is more common in men than women, as well as in aged people. Smokers are twice to thrice more likely to develop this malignancy than nonsmokers, and the risk increases with intensity of smoking (1, 2). Caucasians develop bladder cancer approximately twice as frequently as African or Hispanic Americans (1). More than 90% of bladder cancers arise from the urothelium, which is a specialized transitional epithelium lining in the urinary bladder. Bladder cancer has two distinct biological phenotypes. The low-grade papillary tumors in clinical staging account for ~70% of urothelial cancers and

have a high frequency of recurrence after cystoscopic removal (3). Papillary tumors represent an indolent form of urothelial cancer and are difficult to be identified for their malignant potential (4). This phenotype may lead to the second phenotype representing the muscle invasive urothelial carcinoma with high malignant potential and poor therapeutic response and survival (3, 4). Many biomarkers are being investigated; however, due to heterogeneity of bladder cancer, their successful applications are limited. Recently, survivin has been identified as a promising marker for the bladder cancer diagnosis and prognosis (5, 6). Usually, this protein is known to express during embryonic development; however, it remains completely absent in normal differentiated adult tissues. Numerous reports have established the expression of survivin in a variety of tumors, including bladder cancers (5, 6). A positive correlation between survivin expression and tumor grading, as well as recurrence, has been observed (5, 7). Therefore, survivin is regarded as one of the best known diagnostic and prognostic markers in monitoring the bladder cancer patients.

Survivin inhibits the proapoptotic enzymes caspase-3 and caspase-7 to prevent programmed cell death (8, 9). A large number of genetic changes have been associated with the genesis and progression of bladder cancer, in particular, the loss of p53 function has been linked to the development of muscle invasive disease (4). Normal urothelium has an extremely low rate of turnover; however, it possesses a high regenerative capacity in response to damage or injury (3, 10). Therefore,

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excreted urinary carcinogens may promote carcinogenesis, not only by direct genotoxicity but also by damaging the epithelium and driving proliferation toward restoration of a urinary barrier. Thus, chemoprevention of bladder cancer could be a practical approach to control this malignancy (11). In this regard, cancer chemopreventive agents, such as silibinin, excreted in urine as well as present in blood circulation, may effectively interfere with bladder tumorigenesis. The cancer chemopreventive activity of silibinin has already been shown in different animal models of carcinogenesis (12–14). Silymarin, the parent mixture of silibinin isomers, is shown to inhibit chemical carcinogen-induced bladder tumorigenesis in mice (15).

Many cell lines have been established from human urothelial cancer representing tumors of different grades and stages. Among all human cell lines derived from the urothelial cell carcinoma, only the RT4 cell line retains a well-differentiated papillary noninvasive tumor phenotype representing the most common form of human bladder cancer (16). In our previous cell culture studies, silibinin decreased survivin expression and enhanced p53 expression, as well as apoptosis involving caspase activation in RT4 cells (17, 18). In the present study, we established ectopic RT4 tumor xenograft and assessed the *in vivo* efficacy and associated mechanisms of oral silibinin in which, consistent with our *in vitro* findings, down-regulation of survivin together with an increase in p53 protein expression were identified as potential targets for silibinin efficacy.

Materials and Methods

Cell line and reagents. Human bladder transitional cell papilloma RT4 cell line was from American Type Culture Collection and cultured in DMEM (Life Technologies Bethesda Research Laboratories) with 10% fetal bovine serum under standard culture conditions (37°C, 95% humidified air and 5% CO₂). Silibinin was from Sigma-Aldrich Chemical Company and analyzed by HPLC as a pure agent (12).

Tumor xenograft study. To establish RT4 tumors in mice, RT4 cells were grown in culture, then detached by trypsinization, washed, and resuspended in serum-free DMEM. Six-week-old athymic nu/nu male mice (National Cancer Institute) were s.c. injected with 5 × 10⁶ RT4 cells mixed with matrigel (1:1) in the right flank of each mouse to initiate tumor growth. Mice were randomly divided into three groups, each having 10 mice. After 24 h, mice in control (first) group were fed with 0.2 mL saline/day by oral gavage, and second and third groups with 100 and 200 mg/kg/day doses of silibinin in 0.2 mL of saline 5 days/week, respectively, for 12 weeks. Body weight and diet consumption were recorded twice weekly throughout the study. After xenografts started growing, their sizes were measured twice weekly. The tumor volume was calculated by the formula 0.5236L₁(L₂)², wherein L₁ is the long axis and L₂ is the short axis of the tumor (13). At the end of experiment, tumors were excised and weighed, and one part is fixed in buffered formalin and the remaining part is stored at -80°C until further analysis. Blood was collected in heparinized tubes by cardiac puncture for harvesting plasma samples.

Immunohistochemical studies for proliferating cell nuclear antigen, CD31, survivin, and cleaved caspase-3. Tumor samples were fixed in 10% buffered formalin for 12 h and processed as detailed recently (12, 13). The paraffin-embedded tumor sections (5-μm thick) were processed as described recently (12, 13) and incubated with specific primary antibodies, including mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; 1:400 dilutions; Dako), goat polyclonal anti-CD31 (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-

cleaved caspase-3 (Asp¹⁷⁵; 1:100 dilutions; Cell Signaling Technology, Inc.), and rabbit polyclonal anti-survivin (Novus Biologicals) for 1 h at 37°C followed by overnight at 4°C in humidity chamber. Negative controls were incubated only with universal negative control antibodies

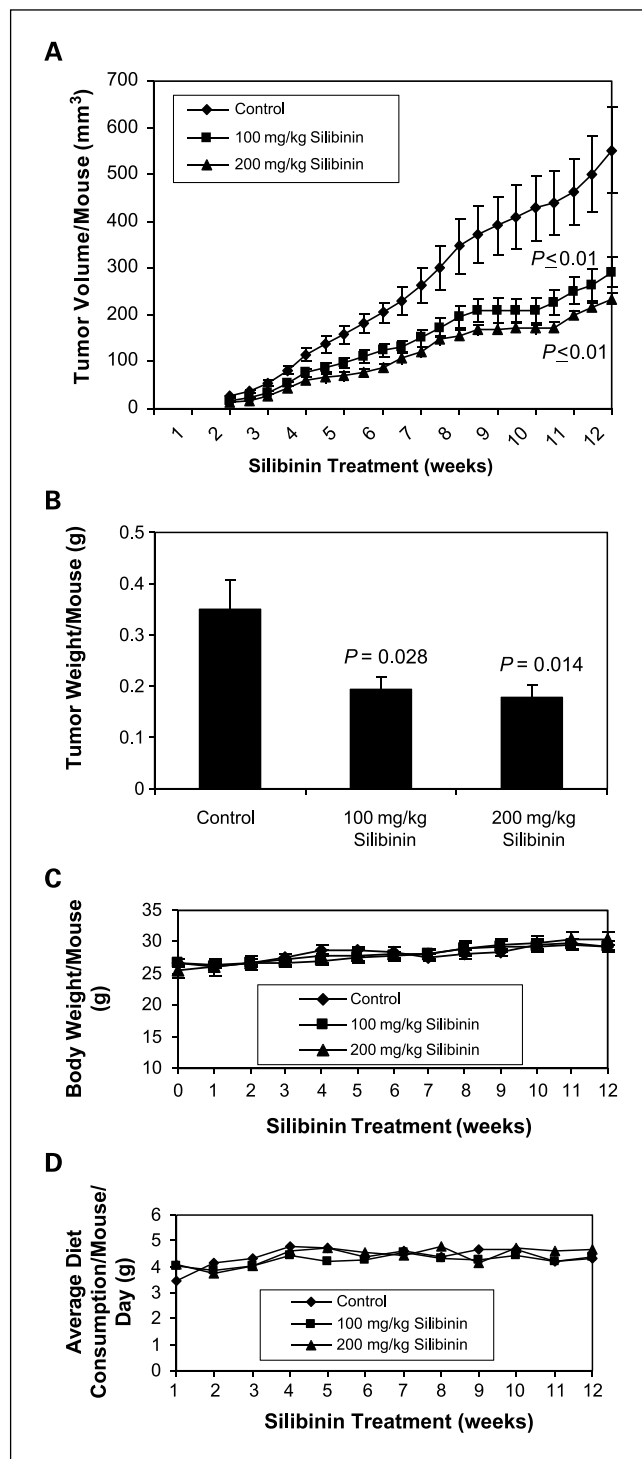


Fig. 1. Effect of oral silibinin on human bladder transitional cell papilloma RT4 tumor xenograft growth in athymic nude mice. Mice were ectopically implanted with RT4 cells and, after 24 h, gavaged with saline (control) or 100 and 200 mg/kg doses of silibinin in saline for 5 d/wk for 12 wk as detailed in Materials and Methods. Effects of silibinin on tumor volume (A), tumor weight (B), body weight (C), and diet consumption (D) were recorded as detailed in Materials and Methods. Data shown in A-C are mean of 10 mice in each group; bars, SE. Diet consumption per mouse per day is shown as an average from 10 mice in each group.

under identical conditions. The sections were then incubated with appropriate biotinylated secondary antibody (1:200-400 dilutions) for 45 to 60 min at room temperature. Thereafter, sections were incubated with conjugated horseradish peroxidase streptavidin (Dako) for 45 to

60 min, followed with 3,3'-diaminobenzidine (Sigma Chemical Co.) working solution, and counterstained with hematoxylin (12, 13).

The proliferation index (per 400× microscopic field) was determined as number of PCNA-positive (brown) cells × 100 per total number of

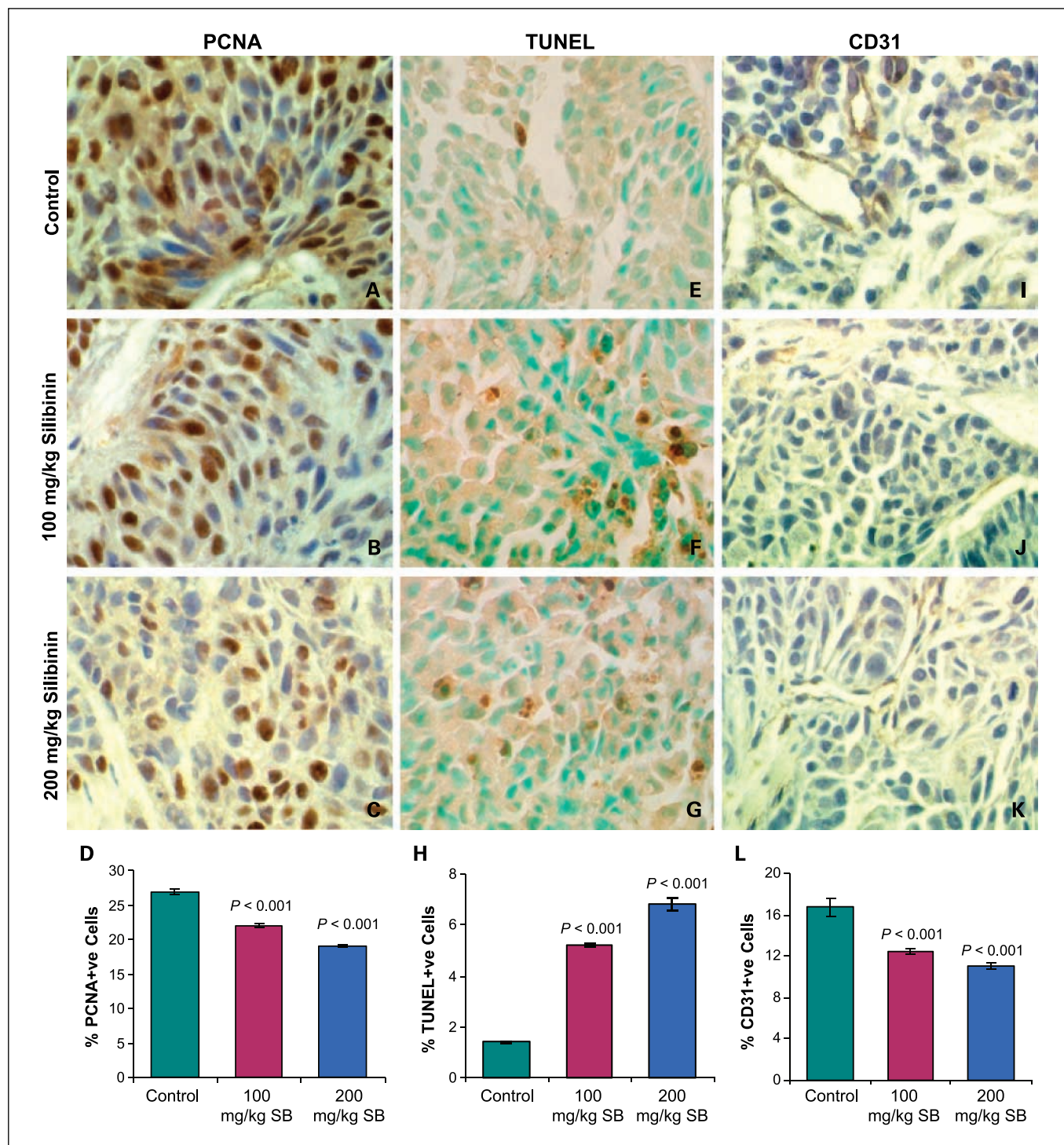


Fig. 2. Effects of silibinin on cell proliferation, apoptosis, and tumor angiogenesis in RT4 tumor xenograft. At the end of the xenograft study detailed in Fig. 1, tumors were excised and processed for immunohistochemical staining for PCNA (A-C), TUNEL (E-G), and CD31 (I-K), an endothelial cell specific marker. A representative picture has been shown for each treatment group in each case. Immunohistochemical analysis was based on diaminobenzidine staining as detailed in Materials and Methods. PCNA-positive cells (D), TUNEL-positive (H), and CD31-positive cells (L) were calculated by number of positive (brown) cells × 100/total number of cells counted under 400× magnification in 10 randomly selected areas in each tumor sample. The pictures are at 400× magnification. Columns, mean of 8 to 10 samples from individual mouse in each group; bars, SE. SB, silibinin.

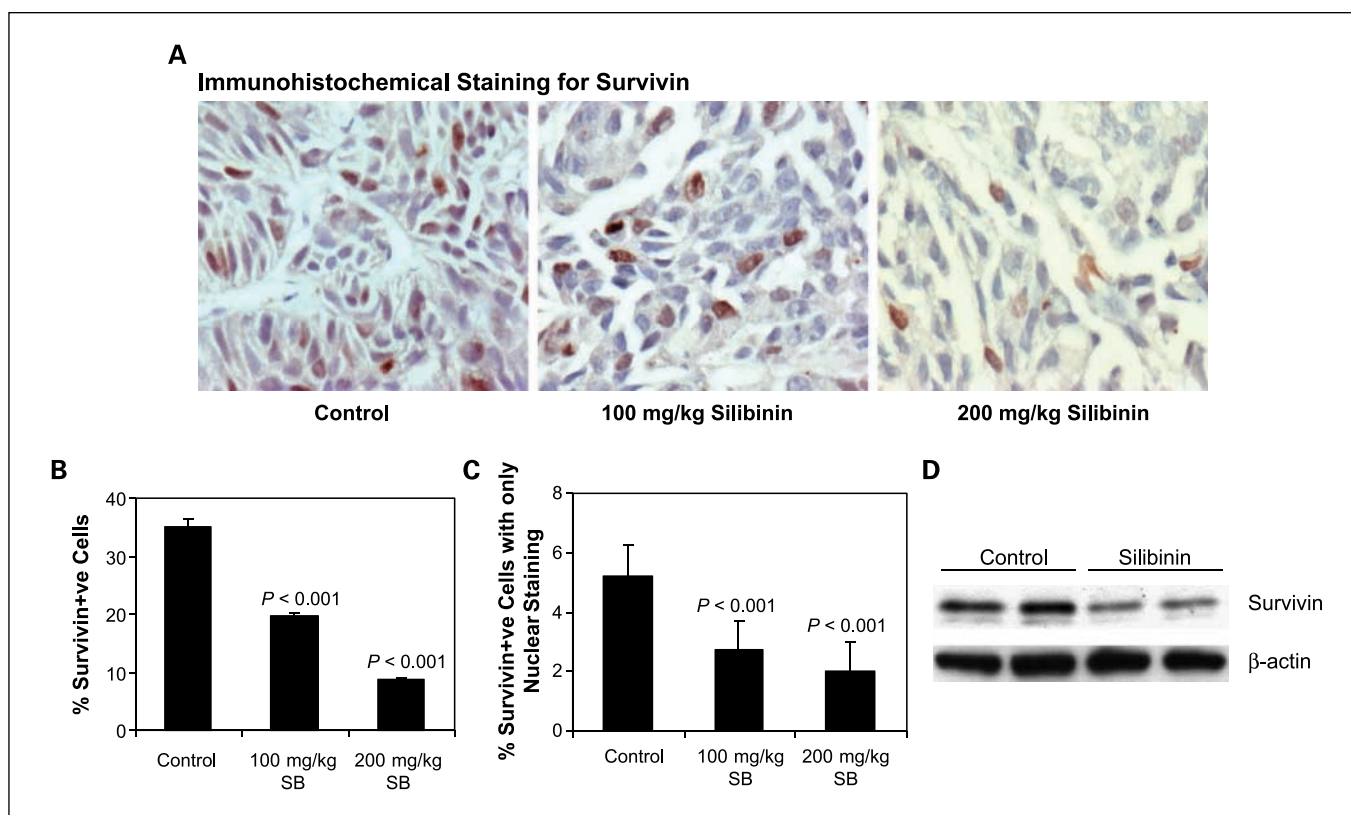


Fig. 3. Effect of silibinin on cellular and nuclear levels of survivin in RT4 tumor xenograft. At the end of the xenograft study detailed in Fig. 1, tumors were processed for diaminobenzidine-based immunohistochemical staining for survivin. *A*, representative picture at 400× magnification from control, 100 mg/kg silibinin, and 200 mg/kg silibinin treatment groups depicting survivin immunoreactivity (brown) in tumors. *B*, quantitative data for percentage of survivin-positive cells in tumors. *C*, percentage of cells showing survivin immunoreactivity strictly limited to nucleus. *B* and *C*, columns, mean from 10 samples in each control and 100 mg/kg silibinin treatment group and seven samples in 200 mg/kg silibinin treatment group; bars, SE. *D*, Western blot analysis was done for survivin expression together with β-actin as loading control from two randomly selected tumors in each control and 100 mg/kg silibinin treatment group as detailed in Materials and Methods. In each case, the Western blot bands shown are from same gel.

cells, and tumor microvessel density was quantified by counting the CD31-positive cells and the total number of cells at 10 randomly selected fields at 400× (12, 13). Survivin and cleaved caspase-3-stained (brown) cells were also quantified as number of positive cells × 100 per total number of cells in 10 random microscopic (400×) fields in each tumor. For survivin, a conspicuous nuclear immunoreactivity was observed in most cells; therefore, only nuclear survivin was also quantified in the similar manner.

Terminal uridine deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. The formalin-fixed and paraffin-embedded 5-μm-thick sections of all tumor samples (those used for PCNA and other staining) were used to identify apoptotic cells by terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using tumor TACS *in situ* apoptosis detection kit (R & D Systems, Inc.), as detailed recently (13). The apoptotic index (per 400× microscopic field) was calculated as number of apoptotic cells × 100 per total number of cells.

Tumor homogenate and Western blot analysis. Tumor samples were homogenized in lysis buffer using Polytron homogenizer and then centrifuged at 14,000 rpm for 30 min as reported recently (12, 14). Supernatants collected were recentrifuged, and protein was estimated in clear supernatant. Total cell lysates (80 μg protein per sample) were denatured twice with sample buffer and resolved on Tris-glycine gels by SDS-PAGE. Separated proteins were transferred onto nitrocellulose membrane by Western blotting, and the membrane was blocked for 1 h with 5% nonfat dry milk. Membranes were then incubated with primary antibodies for survivin, p53 (Novocastra Laboratories Ltd.),

and cleaved caspase-3 followed by peroxidase-conjugated appropriate secondary antibody. Finally, proteins were visualized by enhanced chemiluminescence detection and exposure to X-ray film. To confirm equal protein loading per lane, the membranes were stripped and reprobed with mouse monoclonal anti-β-actin primary antibody (Sigma).

Assay for tumor-secreted survivin level in mouse plasma. At the end of the experiment, the harvested plasma samples were used for the quantification of survivin level secreted by the RT4 tumors using TiterZyme enzyme immunometric assay kit for human total survivin from Assay Designs, Inc. following manufacturer's protocol. The principle of the assay is based on quantitative sandwich enzyme immunoassay using precoated monoclonal antibody specific for human survivin onto a microplate for solid phase ELISA. Briefly, 100 μL plasma was used in the assay, and finally, the absorbance of the developed color was determined using a microplate reader set to 450 nm with correction wavelength at 590 nm. Survivin concentration was extrapolated from the standard curve generated using recombinant human survivin in the assay. The recovery of the human survivin in mouse plasma was 82%, which was included in the calculation of the total survivin concentration in plasma.

Cell culture study with p53 small interfering RNA. Transient transfection using oligofectamine (Invitrogen) with p53 specific (Santa Cruz Biotechnology) and a control nonspecific small interfering RNA duplexes was done to specifically knockdown p53 expression by RNA interference. RT4 cells were cultured in DMEM containing 10% fetal bovine serum and, at ~40% confluency, transfected with 200 nmol/L

small interfering RNA (p53 specific or control nonspecific) according to manufacturer's instructions. After 24 h, p53 small interfering RNA-transfected, as well as parallel growing control, cells were treated with DMSO (0.1% v/v, vehicle for silibinin) or silibinin (150 $\mu\text{mol/L}$) for 16 h. At the end of the treatment, cells were harvested, whole cell lysate was prepared (18), and immunoblot analysis for p53, survivin, cleaved caspase-3, and β -actin was done as described earlier for the tumor lysate samples. The experiment was repeated once.

Immunohistochemical and statistical analyses. Zeiss Axioscop 2 microscope (Carl Zeiss, Inc.) was used for microscopic immunohistochemical analyses. Microscopic images were taken by AxioCam MrC5 camera at 400 \times magnification, and processed by AxioVision software documentation system (Carl Zeiss, Inc.). All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific). Quantitative data are presented as mean and SE. Control and silibinin-fed groups were compared for the statistical significance by ANOVA and Tukey test for multiple comparisons. Student's *t* test was used as needed, and $P < 0.05$ was considered significant.

Results

Silibinin feeding inhibits human bladder tumor xenograft growth in athymic nude mice. Oral gavage feeding of silibinin at 100 and 200 mg/kg body weight doses 5 days/week for 12 weeks caused a marked time-dependent inhibition in RT4 tumor xenograft growth. Silibinin decreased tumor volume from 552.4 mm^3 per mouse in control group to 272.7 and 233.4 mm^3 per mouse in 100 and 200 mg/kg silibinin groups, respectively, which accounted for 51% to 58% ($P \leq 0.01$) decrease after 12 weeks of the treatment (Fig. 1A). Correspondingly, tumor weight in silibinin-fed groups was also decreased by 44% to 49% ($P < 0.05$) when compared with the control group of mice gavaged only with saline (Fig. 1B). We did not observe any gross sign of toxicity and/or possible side effects with silibinin feeding as measured by two variables, namely body weight and diet consumption throughout the study protocol, where there was no considerable change in body weight gain (Fig. 1C) and diet intake (Fig. 1D) profiles among control and silibinin-fed groups. These results suggest the *in vivo* antitumor efficacy of oral silibinin against human bladder tumor growth without any toxicity. Next, we analyzed the potential biomarkers of silibinin efficacy in tumor xenograft.

Silibinin inhibits cell proliferation, induces apoptosis, and suppresses tumor angiogenesis in RT4 xenograft. Since we observed tumor growth inhibition by silibinin, first, we analyzed its effect on tumor cell proliferation by immunohistochemical staining of xenograft sections for PCNA. In microscopic observation of tumors, lesser number of PCNA immunoreactivity was observed in silibinin-fed groups (Fig. 2A-C), which accounted for 18% to 29% decrease ($P < 0.001$) in PCNA-positive cells compared with control group (Fig. 2D). TUNEL staining was next done to assess the apoptotic effect of silibinin in tumors, which showed an increased number of TUNEL-positive cells in silibinin-fed groups compared with control group (Fig. 2E-G). The increase in apoptotic cells by silibinin was 4-fold to 5-fold ($P < 0.001$) over that of control group (Fig. 2H). The effect of silibinin on tumor angiogenesis was also analyzed by CD31 staining, which is a specific marker of endothelial cells. The microscopic examination revealed smaller number of CD31-positive cells in silibinin-fed groups of tumors (Fig. 2I-K). The quantification of CD31-positive

tumor microvessel cells showed 26% to 34% decrease ($P < 0.001$) in silibinin-fed groups from that of control group (Fig. 2L). Among these three biomarkers, namely, proliferation, apoptosis, and angiogenesis, we observed that silibinin has profound effect on apoptosis induction in tumors, and therefore, we further focused on molecular alterations which could be involved in mediating apoptotic effect of silibinin.

Silibinin decreases survivin expression in RT4 xenograft. Many studies suggest that survivin plays an important role in bladder tumor cell survival; we anticipated that silibinin might decrease survivin expression as a potential mechanism of apoptosis induction as earlier observed in RT4 cell culture study (17). First, we examined the level of survivin expression in tumor xenograft by immunohistochemical analysis. The microscopic examination of stained tumor sections showed strong immunoreactivity for survivin (brown color) in control group which was decreased in silibinin-fed groups of tumors (Fig. 3A). The quantification of survivin staining showed 35 ± 2 , 20 ± 0.6 , and 9 ± 0.3 survivin-positive cells in control, 100 mg/kg dose of silibinin, and 200 mg/kg dose of silibinin groups, respectively, which accounted for 43% to 74%

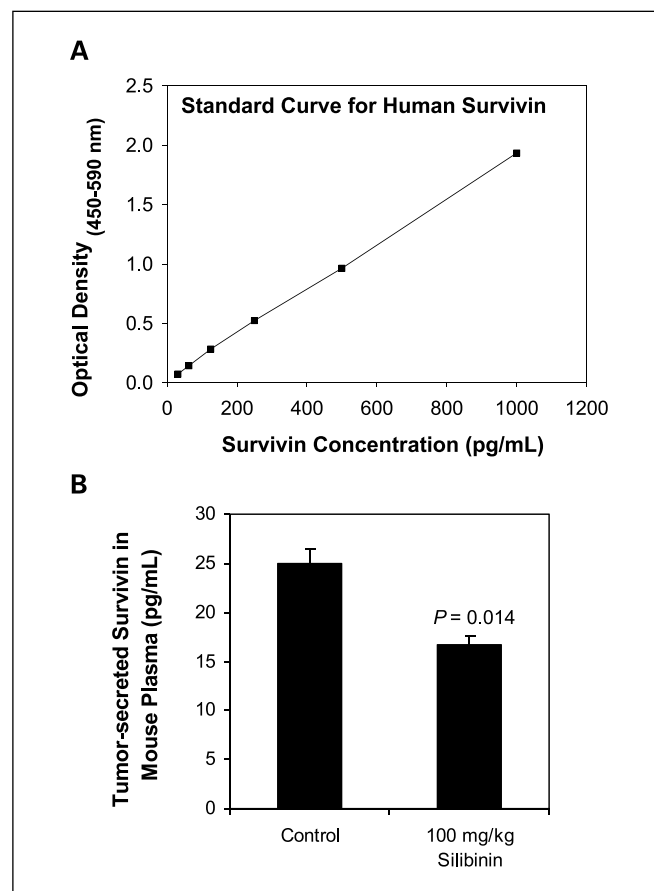


Fig. 4. Effect of silibinin on tumor-secreted level of survivin in RT4 tumor xenograft study. At the end of the xenograft study detailed in Fig. 1, mouse plasma was analyzed for the tumor-secreted level of survivin following manufacturer's protocol as detailed in Materials and Methods. First, a standard curve for survivin was made using different concentrations of recombinant human survivin in the assay (A) and then concentration (pg/mL) of survivin was calculated from the unknown plasma samples for the tumor-secreted level of survivin (B). The data are shown as mean from eight samples in each control and 100 mg/kg silibinin treatment group; bars, SE.

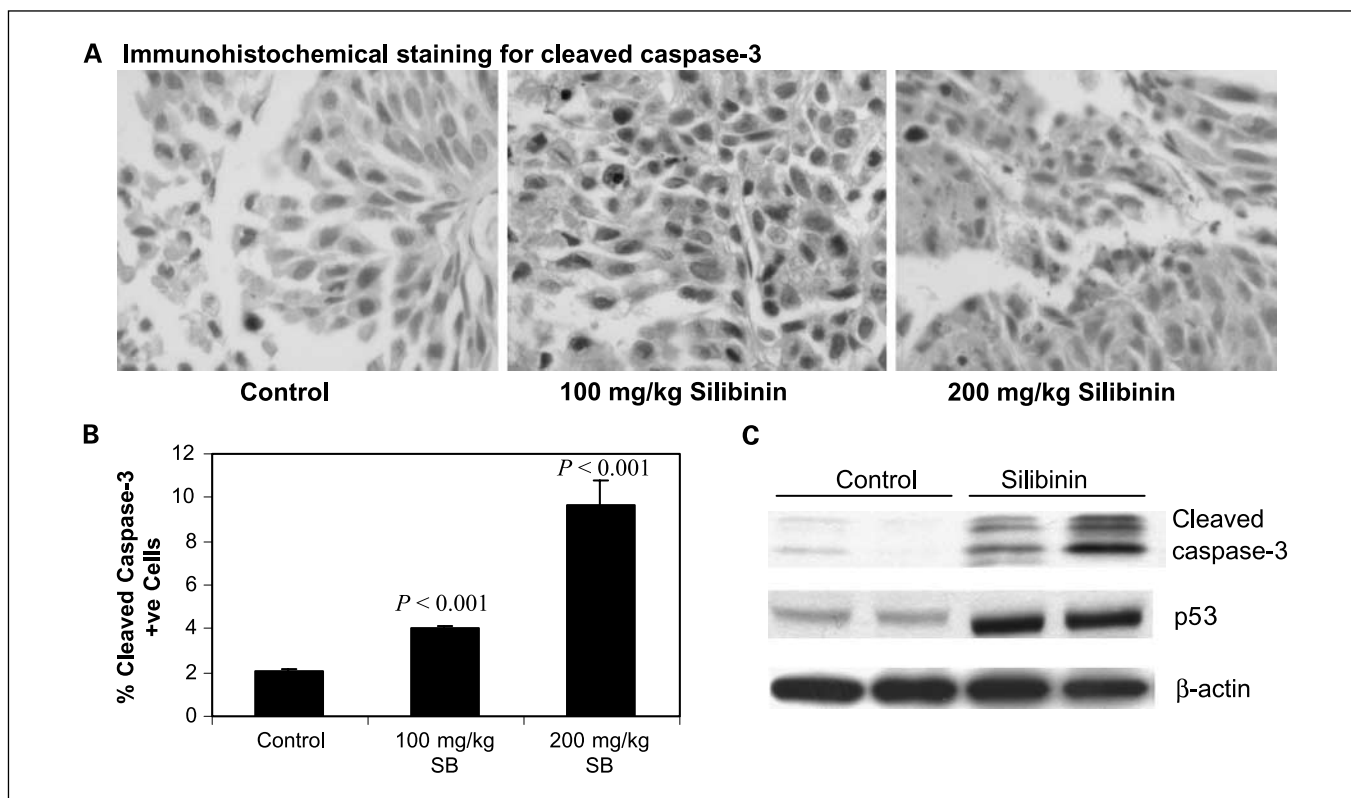


Fig. 5. Effect of silibinin on cleaved caspase-3 and p53 levels in RT4 tumor xenograft study. At the end of the xenograft study detailed in Fig. 1, tumors were processed for diaminobenzidine-based immunohistochemical staining for cleaved caspase-3. *A*, representative picture at 400× magnification from control, 100 mg/kg silibinin, and 200 mg/kg silibinin treatment groups showing cleaved caspase-3 immunoreactivity in tumors. *B*, columns, mean quantitative data for percentage of cleaved caspase-3-positive cells from 10 samples each in control and 100 mg/kg silibinin treatment group and seven samples in 200 mg/kg silibinin treatment group; bars, SE. *C*, Western blot analysis was done for cleaved caspase-3 and p53 expression levels together with β-actin as loading control from two randomly selected tumors in each control and 100 mg/kg silibinin treatment group as detailed in Materials and Methods. In each case, the Western blot bands shown are from same gel.

($P < 0.001$) decrease in silibinin-fed groups from that of control group (Fig. 3B). During microscopic examination of these tumor sections, we also observed that some cells showed survivin immunoreactivity, which was conspicuously localized in the nucleus. Therefore, we specifically quantified survivin-positive nucleus only among survivin-positive cells that showed 5 ± 0.3 cells in control group, which were reduced to 3 ± 0.2 ($P < 0.001$) and 2 ± 0.1 ($P < 0.001$) cells in 100 and 200 mg/kg doses of silibinin groups, respectively (Fig. 3C). Furthermore, we confirmed the effect of silibinin on decrease in survivin expression by Western blot analysis in tumor lysates from control and 100 mg/kg silibinin dose groups which showed a decreased level of survivin protein in silibinin-fed group of tumors. Overall, these results indicated that silibinin could target survivin expression, as well as nuclear localization, for its *in vivo* apoptotic effect in RT4 tumors.

Silibinin inhibits tumor-secreted level of survivin in mouse plasma. Studies suggest that bladder tumor secretes survivin, and its elevated level in urine of bladder cancer patients could be a diagnostic, as well as prognostic, biomarker (6, 7). Therefore, in our study, we anticipated that ectopically growing RT4 tumors will secrete survivin, which could be detected in mouse blood circulation. Accordingly, we analyzed plasma samples from control and 100 mg/kg silibinin groups harvested at the end of the study for the tumor-secreted levels of survivin. A standard curve was generated using human recombinant survivin in the

assay to calculate survivin level in plasma (Fig. 4A). Results showed the detectable levels of survivin in plasma, which was 25.04 ± 1.45 pg/mL in control group and reduced to 16.68 ± 0.89 pg/mL in silibinin-fed group that accounted for 33% decrease ($P < 0.014$; Fig. 4B). This result suggests that consistent with the decrease in survivin protein expression, silibinin also decreased tumor-secreted level of survivin.

Silibinin enhances caspase activation and p53 expression in RT4 xenograft. Because survivin is known to directly interact with caspase-3 and subsequently inhibit its activity, next, we analyzed tumor sections for the levels of cleaved caspase-3 by immunohistochemistry. Microscopic examination of cleaved caspase-3 staining showed fewer positive cells in control group compared with silibinin-fed groups (Fig. 5A). Quantification of cleaved caspase-3-positive cells showed $2 \pm 0.1\%$ cells in control group, which were significantly increased to $4 \pm 0.1\%$ ($P < 0.023$) and $10 \pm 1.1\%$ ($P < 0.001$) in 100 and 200 mg/kg doses of silibinin groups, respectively (Fig. 5B). Furthermore, tumor lysate from control and lower dose of silibinin groups were analyzed by Western immunoblotting for cleaved caspase-3 to check the results from immunohistochemical analysis. As expected, enhanced levels of cleaved caspase-3 protein were observed in silibinin-fed group of tumors compared with control group of tumors (Fig. 5C). Additionally, we observed that silibinin feeding also enhanced the level of p53 protein in tumors (Fig. 5C), which may be associated with its apoptotic

response, as we recently observed in an *in vitro* study (18). Because, p53 is known to suppress survivin expression; next, we did RT4 cell culture study using p53 RNA interference to dissect their potential link in response to silibinin treatment.

Role of p53 in survivin expression in RT4 cells. We have observed that inhibition of silibinin-induced p53 expression suppresses caspase-mediated apoptosis in RT4 cells (18); however, it is not known whether this effect of p53 by silibinin is mediated via survivin. Many studies suggest that p53 acts as transcriptional repressor of survivin expression in many cell types (19, 20). To investigate such possibility in our study, we used a p53-specific small interfering RNA to knockdown p53 expression in RT4 cell culture and examined the level of survivin protein expression. Surprisingly, the decrease in p53 protein level by p53 small interfering RNA did not show any considerable decrease in survivin protein level in control cells (Fig. 6). Silibinin (150 $\mu\text{mol/L}$ for 16 h) enhanced p53 level but decreased survivin level as expected; however, knockdown of p53 level did not affect the inhibitory effect of silibinin on survivin expression (Fig. 6). Furthermore, knockdown of p53 level only partially reduced the silibinin-caused increase in cleaved caspase-3 level (Fig. 6). These results suggest that silibinin enhances p53 expression in parallel with the down-regulation of survivin expression, which is independent of p53; nevertheless, both these events could sum up for its apoptotic/antitumor effect in RT4 cells.

Discussion

The central finding of the present study is that oral silibinin inhibits the growth of human bladder transitional cell

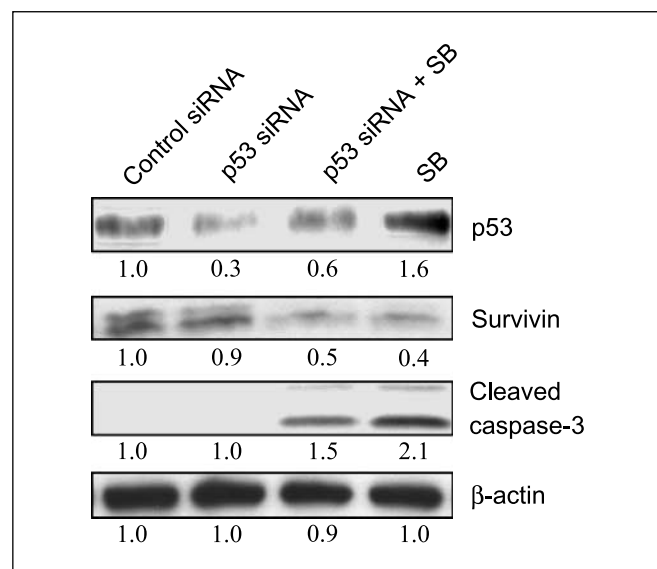


Fig. 6. Role of p53 in silibinin-caused down-regulation of survivin in RT4 cells. RT4 cells were cultured in 10% serum condition till ~40% confluency and then transfected with 200 nmol/L small interfering RNA (siRNA; p53 specific or control nonspecific). After 24 h of transfection, cells were treated with or without 150 $\mu\text{mol/L}$ silibinin for 16 h. At the end of the treatment, cells were harvested and whole cell lysate was analyzed by Western blotting for p53, survivin, cleaved caspase-3, and β -actin (loading control) protein levels as described in Materials and Methods. In each case, the Western blot bands are from same gel. The mean density of each band was analyzed by the Scion Image program (NIH). In each case, the densitometry data presented below the bands are fold change compared with control. SB, 150 $\mu\text{mol/L}$ silibinin.

papilloma RT4 tumor xenograft growth in athymic nude mice without any apparent signs of toxicity. This antitumor efficacy of silibinin was associated with a moderate decrease in tumor cell proliferation and angiogenesis, along with a strong increase in apoptotic cell death. In mechanistic investigation for the proapoptotic effect of silibinin, survivin was identified as a potential *in vivo* molecular target for silibinin efficacy. Secondly, silibinin also induced p53 protein levels in tumors, and therefore, both these molecular alterations could be associated with the apoptotic effect of silibinin as observed by an increase in cleaved caspase-3 level and TUNEL staining. Interestingly, p53 is known to transcriptionally repress survivin expression; however, silibinin-caused down-regulation of survivin was found to be independent of p53 in RT4 cells.

Tumor xenograft study represents a well-established preclinical animal model for evaluating *in vivo* anticancer efficacy and associated mechanisms of a test agent. Because we had already observed that silibinin causes cell growth inhibition and apoptotic cell death in human bladder transitional cell papilloma RT4 cells in culture (17), in the present study, our primary goal was to establish *in vivo* antitumor efficacy and associated molecular biomarkers of oral silibinin against RT4 tumor xenograft growth. Both lower and higher doses of silibinin were effective in slowing the xenograft growth; however, we did not observe a significant difference between the two silibinin doses. Importantly, 12 weeks of oral administration of silibinin did not show any observable decrease in body weight or diet consumption. While analyzing for biomarkers of silibinin efficacy, we observed its moderate inhibitory effects on cell proliferation (by PCNA staining) and tumor angiogenesis (by CD31 staining) and a strong enhancing effect on apoptosis (by TUNEL staining) in tumor xenograft. Therefore, among these three biomarkers, the apoptotic effect of silibinin could be a major contributor for its antitumor efficacy.

Survivin is a member of inhibitor of apoptosis protein and shows very exclusive features when compared with other apoptosis regulators (21). It is almost undetectable in normal differentiated tissues but overexpressed in most human cancers, including bladder cancer (8, 22–24). Survivin is capable of suppressing apoptosis and can also induce resistance to cytotoxic drugs; however, it is also involved in cell cycle regulation (8, 9). Many studies suggest that survivin overexpression is associated with bladder tumor growth and progression and an alarming prognostic factor for this malignancy (5, 7, 25, 26). Therefore, targeting of survivin could have a dual favorable advantage, checking cell proliferation, as well as inducing apoptotic cell death, in bladder cancer. Accordingly, in the present study, silibinin decreased survivin protein levels in RT4 tumors, as well as tumor-secreted levels of survivin in mouse plasma. A corresponding decrease was also observed in the nuclear level of survivin by silibinin treatments. The nuclear localization of survivin could have a direct relevance in inhibiting nuclear caspase activity, which is known to degrade critical nuclear proteins, such as poly-ADP ribose polymerase during apoptosis (27). Therefore, down-regulation of survivin by silibinin could be a major mechanism for its *in vivo* antitumor efficacy against bladder tumors, wherein we also observed a decrease in cell proliferation, as well as a marked increase in apoptotic cell death in tumors.

Additionally, silibinin also decreased tumor angiogenesis, which is consistent with its antiangiogenic effect as we have reported in many studies (12, 14, 28, 29).

Survivin is reported to interact with caspase-3 and caspase-7 to block their activation, which can be measured by the level of cleaved caspases that corresponds to their protease activity (9). Based on our finding, we anticipated that survivin may decrease caspase-3 activity in RT4 tumors to enhance its survival and that could be suppressed by silibinin, leading to its apoptotic effect on tumor cells. This anticipation was also based on our completed cell culture study, wherein silibinin has been found to down-regulate survivin and increase the level of cleaved caspase-3 in RT4 cells (17). Accordingly, in the present study, silibinin increased cleaved caspase-3 levels in tumors. Therefore, a decrease in survivin with a concomitant increase in the activated form of caspase-3 by silibinin could be potential *in vivo* mechanism for apoptosis induction in RT4 tumors.

RT4 cells contain wild-type p53, which is also known to inhibit cell proliferation and induce apoptosis (30, 31). Our recently completed study suggests that silibinin induces p53 level in RT4 cells in culture (18); hence, we also assessed the level of p53 in RT4 tumors. A strong increase in p53 level was observed in silibinin-fed group of tumors. Therefore, p53 could be another potential *in vivo* molecular target for silibinin efficacy in RT4 tumors. p53 is known to transcriptionally suppress survivin expression in many studies (19, 20); however, it is not known whether (a) similar mechanism operates in RT4 cells and (b) silibinin-caused increase in p53 protein level down-regulates survivin expression. To explore these mecha-

nisms, we did a cell culture study using transient RNA interference strategy. Surprisingly, as opposed to the reported studies, knock-down of p53 did not show any appreciable change in the level of survivin expression nor it influenced the silibinin-caused decrease in the level of survivin in RT4 cells. Thus, silibinin-induced p53 was not causally linked with decreased expression of survivin, and therefore, both could be parallel mechanisms/molecular targets for antitumor efficacy of silibinin in p53 wild-type bladder tumor cells. This conclusion is supported by the observation that silibinin-caused increase in cleaved caspase-3 level is partially reduced by knocking down of p53 level by RNA interference. On the other hand, it also indicates that silibinin could also be effective in p53-mutated bladder tumor cells. This hypothesis is supported by the fact that, in a recently completed study, silibinin showed apoptotic cell death of human bladder carcinoma T24 and TCC-SUP cell lines (32), which are mutated for p53 in exon 5 and exon 10, respectively (33).

In summary, oral silibinin suppresses *in vivo* growth of human bladder transitional cell papilloma tumors in nude mice, which is accompanied with decreased cell proliferation, as well as angiogenesis, and a marked increase in apoptosis. Survivin was identified as a potential *in vivo* molecular target of silibinin efficacy, in parallel with p53. Because silibinin has already completed phase I trial, and now in phase II trial in prostate cancer patients (34), and physiologically achievable in blood (34, 35), as well as excreted in urine (36), the findings in this preclinical study could be useful for the potential clinical trial of silibinin in human bladder cancer patients.

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