Inhibitory Effect of Silibinin against Azoxymethane-Induced Colon Tumorigenesis in A/J Mice

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Abstract

Purpose: Colorectal cancer is the second leading cause of cancer-associated deaths, which suggests that more effort is needed to prevent/control this disease. Herein, for the first time, we investigate in vivo the efficacy of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice.

Experimental Design: Five-week-old male mice were gavaged with vehicle or silibinin (250 and 750 mg/kg) for 25 weeks starting 2 weeks before initiation with azoxymethane (pretreatment regime) or for 16 weeks starting 2 weeks after the last azoxymethane injection (posttreatment regime). The mice were then sacrificed, and colon tissues were examined for tumor multiplicity and size, and molecular markers for proliferation, apoptosis, inflammation, and angiogenesis.

Results: Silibinin feeding showed a dose-dependent decrease in azoxymethane-induced colon tumorigenesis with stronger efficacy in pretreatment versus posttreatment regime. Mechanistic studies in tissue samples showed that silibinin inhibits cell proliferation as evident by a decrease (P < 0.001) in proliferating cell nuclear antigen and cyclin D1, and increased Cip1/p21 levels. Silibinin also decreased (P < 0.001) the levels of inducible nitric oxide synthase, cyclooxygenase-2, and vascular endothelial growth factor, suggesting its anti-inflammatory and antiangiogenic potential in this model. Further, silibinin increased cleaved caspase-3 and poly(ADP-ribose) polymerase levels, indicating its apoptotic effect. In other studies, colonic mucosa and tumors expressed high levels of β-catenin, insulin-like growth factor-1 receptor, phospho Glycogen synthase kinase-3β, and phospho protein kinase B/Akt proteins in azoxymethane-treated mice, which were strongly lowered (P < 0.001) by silibinin treatment. Moreover, azoxymethane reduced insulin-like growth factor binding protein-3 protein level, which was enhanced by silibinin.

Conclusions: Silibinin targets β-catenin and IGF-1Rβ pathways for its chemopreventive efficacy against azoxymethane-induced colon carcinogenesis in A/J mice. Overall, these results support the translational potential of silibinin in colorectal cancer chemoprevention.

Colorectal cancer is the second most common cause of cancer-related deaths and the third most common cancer, with 146,970 new cases and 49,920 deaths predicted in the United States for 2009 (1). Despite the availability of chemotherapy, surgery, and radiation, which is limited for advanced stages of colorectal cancer, there is a high recurrence rate with an overall 5-year survival of about 55% (2). Colon carcinogenesis offers a huge window period of 10 to 15 years, which could be coupled with various screening procedures for the identification of preneoplastic lesions, making this malignancy suitable for the implementation of chemoprevention strategies (3). Azoxymethane-induced colon carcinogenesis is the most frequently employed animal model for an agent efficacy study against colon cancer (4). After metabolic activation, azoxymethane is converted to ultimate carcinogen methylazoxymethanol, which binds to DNA, causing mutations that include K-ras and CTNNB1, which codes for β-catenin (5, 6). The advantages of using the azoxymethane model for chemoprevention studies include the distinction of promotional and protective effects of experimental diets on tumor growth, similarity to humans in progression of aberrant crypt foci to polyps and ultimately to carcinomas, and K-ras mutation in human colorectal cancer, which is present in nearly 60% of azoxymethane-induced colon tumors (6). Most solid tumors, including colorectal tumors, are characterized by increased cell proliferation, ablation of apoptosis, enhanced inflammation, and tumor angiogenesis, which lead to poor prognosis (7–9). The adenosomatous polyposis coli and β-catenin genes are considered critical in colorectal cancer development as alterations in these genes aid in the process of carcinogenesis in both humans and preclinical models (10). Colonic adenomas and adenocarcinomas harbor high expression of β-catenin in the cytoplasm and...
from recent years is silibinin, a flavanolignan isolated 

through molecular changes. This study reports significant inhibition of azoxymethane-caused colonic cell proliferation (proliferating cell nuclear antigen, cyclin D1), inflammation (inducible nitric oxide synthase, cyclooxygenase-2), angiogenesis (vascular endothelial growth factor), and cell survival, accompanied with reduced number and size of colonic tumors by silibinin, suggesting its strong chemopreventive efficacy against colon tumorigenesis. Silibinin also modulated β-catenin, insulin like growth factor-1 receptor, phospho Glycogen synthase kinase-3β, phospho protein kinase B/pAkt, and insulin-like growth factor binding protein-3 protein levels towards inhibition of colon tumorigenesis. Together, these findings suggest the multitargeting effects of silibinin with mechanistic insight and support its translational potential in colorectal cancer chemoprevention.

**Translational Relevance**

Colon tumorigenesis offers a large window of 10 to 15 years and thus makes it suitable for chemopreventive intervention. Azoxymethane-induced colon carcinogenesis in mice has similarity to humans in progression of aberrant crypt foci to polyps, adenoma, and carcinomas, and underlying molecular changes. Various epidemiologic studies and animal experiments have indicated that polyphenolic compounds present in vegetables, fruits, and their constituents have profound beneficial effects in controlling colorectal cancer (13, 14). One of the chemopreventive agents extensively investigated in recent years is silibinin, a flavanolignan isolated from *Silybum marianum*. Silibinin has shown potent anti-cancer activity against various epithelial cancers both in vivo and in vitro (15–19). The pharmacologic significance of silibinin includes its nontoxicity even at higher doses and human acceptance with known hepatoprotective activity (20). Previous studies do suggest an anticancer potential of silibinin (21, 22); however, the in vivo colon cancer chemopreventive efficacy and associated molecular alterations by long-term silibinin administration in the azoxymethane-A/J mouse model are not known. Accordingly, here we examined in vivo the efficacy of long-term feeding of silibinin against azoxymethane-induced colon tumorigenesis in an A/J mouse model and associated molecular changes. Our results clearly show the chemopreventive efficacy of silibinin against colon tumorigenesis and its effect on proliferation [proliferating cell nuclear antigen (PCNA), cyclin D1, and Cip1/p21], apoptosis [cleaved caspase-3 and poly(ADP-ribose) polymerase], inflammation [inducible nitric oxide synthase(iNOS) and cyclooxygenase-2 (COX-2)], angiogenesis [vascular endothelial growth factor (VEGF)], and IGF-1Rβ, IGFBP-3, β-catenin, pGSK-3β, and phospho protein kinase B/pAkt (pAkt).

**Materials and Methods**

**Animals and treatments**

Male A/J mice were purchased from Jackson Laboratory, and experiments were done with an approved protocol by Institutional Animal Care and Use Committee. Silibinin, carboxymethyl cellulose (CMC), and azoxymethane were from Sigma. Silibinin was suspended in 0.5% (w/v) CMC (vehicle) and was gavaged orally, and azoxymethane was dissolved in saline. The animals, maintained under standard conditions with free access to water and food (AIN-76 diet), were divided into seven groups, and as shown in Fig. 1A, were treated as: (a) control group (*n* = 15), 0.5% (w/v) CMC (vehicle); (b) positive group (*n* = 25), injected with 5 mg/kg dose of azoxymethane i.p. once a week for 6 weeks; (c) SB-750 (*n* = 25), 250 mg/kg/day dose of silibinin in CMC initiated 2 weeks prior to azoxymethane and continued for 25 weeks; (d) SB-750 (*n* = 25), 750 mg/kg/day dose of silibinin in CMC initiated 2 weeks prior to azoxymethane and continued for 25 weeks; (e) SB-250 (*n* = 25), 250 mg/kg/day dose of silibinin in CMC initiated 2 weeks after last azoxymethane and continued till 30 weeks of age; (f) SB-750 (*n* = 25), 750 mg/kg/day dose of silibinin in CMC initiated 2 weeks after last azoxymethane and continued till 30 weeks of age; (g) SB-750 (*n* = 15), 750 mg/kg/day dose of silibinin in CMC till 30 weeks of age. All silibinin treatments were 5 days/week throughout the experiment. Body weight and diet consumption were recorded weekly. At 30 weeks of age, the mice were sacrificed. The entire colon was excised starting from ileocecal junction to anal verge, cut open longitudinally along main axis and gently flushed with ice-cold PBS, and divided into three equal sections (proximal, middle, and distal). The tumors were counted, tumor diameters were measured with digital calipers under dissecting microscope, and the tissues were fixed flat in formalin and embedded in paraffin for immunohistochemical studies or frozen in liquid nitrogen.

**Immunohistochemical analyses**

Paraffin-embedded sections (5 μm) were subjected to antigen retrieval and blocking of endogenous peroxidase activity (21). Sections were incubated with mice monoclonal anti-PCNA (1:250 dilutions; Dako), rabbit polyclonal anti-cyclin D1 (1:100 dilutions; Neomarkers), rabbit polyclonal anti-Cip1/p21 (1:100 dilutions; Santa Cruz), rabbit polyclonal anti-VEGF (1:100 dilutions; Neomarkers), rabbit polyclonal anti-iNOS (1:100 dilutions; Abcam), goat polyclonal anti-COX-2 (1:50 dilutions; Santa Cruz), rabbit polyclonal anti-IGF-1Rβ (1:100 dilutions; Dako), goat polyclonal anti-β-catenin, rabbit monoclonal anti-pGSK-3β, rabbit monoclonal anti-pAkt, and goat monoclonal anti-IGF-1Rβ. The secondary antibodies used were rabbit polyclonal biotinylated anti-goat and horseradish peroxidase (HRP)-conjugated anti-rabbit. The bound antibodies were visualized using the Vector Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine as chromogen.
Cell Signaling), rabbit polyclonal anti-IGFBP-3 (1:100 dilutions; Santa Cruz), rabbit polyclonal anti-p-GSK-3β (1:100 dilutions; Cell Signaling), rabbit polyclonal anti-β-catenin (1:100 dilutions; Santa Cruz), rabbit polyclonal anti-phospho-Akt (1:100 dilutions; Cell Signaling), or rabbit polyclonal anti-cleaved caspase-3 (1:75 dilutions; Cell Signaling) antibody. In all immunohistochemical analyses, negative staining controls were used where sections were incubated with N-Universal Negative Control mouse or rabbit antibody (DAKO) under identical conditions. Sections were then incubated with biotinylated appropriate secondary antibody for 1 hour at room temperature followed by 30-minute incubation with conjugated horseradish peroxidase–streptavidin (Invitrogen) and then incubated with 3,3′-diaminobenzidine (Vector Laboratories, Inc.) working solution at room temperature followed by counterstaining with diluted Harris hematoxylin (Sigma) and mounted. Microscopic immunohistochemical analyses were done using Zeiss Axioscope 2 microscope (Carl Zeiss); photomicrographs were captured by Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. Positive cells for various immunohistochemically stained molecules were quantified by counting brown-stained cells among total number of cells at ×400 magnification. In other cases, immunoreactivity (represented by intensity of brown staining) was scored as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong). Representative images of immunohistochemical staining (∗×400) of colonic adenoma region are shown in each case, and quantification data shown include both colonic mucosa and tumor immunohistochemical staining.

**Western blot analysis**

Tissue lysates of colonic mucosa (scrapped with tumors) from mice treated with azoxymethane alone and with silibinin plus azoxymethane in pretreatment regime were analyzed by immunoblotting as previously described (23, 24). Anti-IGF-1Rβ, anti-pIGF-1Rβ (Tyr1316), anti-Akt, anti-GSK-3β, and anti-cleaved-PARP antibodies were from Cell Signaling; anti-β-actin was from Sigma, and other antibodies were those used in immunohistochemical studies as mentioned above. Densitometry analyses of bands were adjusted with β-actin as loading control.

**Statistical analysis**

Data were analyzed using Sigma Stat software version 2.03 (Jandel Scientific) for statistical significance of difference between different groups. Significance was determined by one-way ANOVA followed by Tukey test for multiple comparisons. \( P < 0.05 \) was considered statistically significant.

**Results**

**Silibinin prevents azoxymethane-induced colon tumorigenesis in mice**

A well-established long-term protocol was used to determine silibinin efficacy in inhibiting azoxymethane-induced
colon tumor formation. Mice in all groups were regularly monitored for any silibinin-associated adverse effects. In terms of body weight gain profiles over the course of study, silibinin did not show any considerable changes among the various groups (data not shown). During necropsy, no pathologic alterations were found in any organs including liver, lung, and kidney, by gross observation in A/J mice in various groups. In terms of colon tumorigenesis, the azoxymethane-treated group showed 11 ± 1.1 colonic tumors/mouse, which was reduced to 6 ± 0.8 and 5 ± 0.7 tumors by 250 and 750 mg/kg doses of silibinin started before azoxymethane (Fig. 1B), accounting for 46% (P < 0.01) and 55% (P < 0.001) decreases in tumor multiplicity, respectively. Lower and higher doses of silibinin treatments in the post-azoxymethane protocol showed a similar trend in decreasing the number of colonic tumors, which were 8 ± 0.9 (P < 0.05) and 7 ± 1 (P < 0.01) per mouse, respectively (Fig. 1B). Moreover, in terms of tumor size distribution, silibinin pretreatment showed much stronger efficacy, accounting for 42% to 50%, 21% to 42%, and 62% to 66% decreases in number of colon tumors sized <1, 1 to 2, and >2 mm, respectively, compared with azoxymethane-alone controls (Fig. 1C). Mice in control or the silibinin only-treated group did not show any colonic tumors. Together, these results show the preventive effect of silibinin on azoxymethane-induced colonic tumor multiplicity, with strongest efficacy in reducing the number of biggest-size tumors (>2 mm) in A/J mice without any apparent toxicity, and suggest that silibinin efficacy is associated with its treatment protocols as well as the duration of treatment with respect to azoxymethane treatment.

Silibinin inhibits cell proliferation and induces apoptosis

In vivo antiproliferative and apoptosis-inducing effects of silibinin were examined on azoxymethane-induced colonic mucosa and tumors by analyzing the levels of molecular markers, including PCNA, cyclin D1, and Cip1/p21, to relate with tumor cell proliferation and cleaved caspase-3 and PARP for apoptosis by immunohistochemistry and/or immunoblotting. Quantification of PCNA-positive cells showed that 250 and 750 mg/kg/day doses of silibinin started before azoxymethane resulted in 56% and 69% (P < 0.001) decreases in proliferation index, respectively, compared with azoxymethane alone; a similar trend in silibinin inhibitory effect on cell proliferation was also observed in posttreatment regimes (Fig. 2A). Cyclin D1 overexpression is correlated with enhanced cell proliferation (21) and therefore was also studied by us. Similar to PCNA, silibinin pretreatments at 250 and 750 mg/kg/day doses reduced cyclin D1-positive cells by 46% and 63% (P < 0.001), respectively, and also showed a similar but comparatively lesser effect in posttreatment regimen (Fig. 2B). Cip1/p21 plays a significant role in cell cycle arrest by binding with and inhibiting PCNA activity (25). Quantification of Cip1/p21-positive cells showed 5 ± 0.3% cells in the azoxymethane group versus 35 ± 0.7% cells (7-fold increase, P < 0.001) in the group treated with 750 mg/kg/day dose of silibinin; a similar trend was seen in Cip1/p21 induction by silibinin in other treatment protocols (Fig. 2C). The immunohistochemical results shown in Fig. 2A to C were further supported by immunoblot analysis of three randomly selected samples from three individual mice in the azoxymethane and the pre-silibinin plus azoxymethane groups, which showed similar silibinin efficacy patterns. Immunohistochemical analysis for apoptotic cells showed an increase in the number of cleaved caspase-3–positive cells in silibinin-treated groups as compared with the azoxymethane group. Quantification of cleaved caspase-3–positive cells showed an increase in apoptotic index by up to ∼4.6-fold (P < 0.001) at higher dose of silibinin (Fig. 2D). In vivo apoptotic activity of silibinin was also supported by strong levels of cleaved caspase 3 and PARP in silibinin-fed versus azoxymethane-alone samples in immunoblot analyses (Fig. 2D). Together, these results indicate both antiproliferative and proapoptotic effects and associated molecular alterations by silibinin, and their possible involvement in overall preventive efficacy of silibinin against colon tumorigenesis.

Silibinin decreases the levels of inflammatory and angiogenic molecules

The roles of COX-2 and iNOS as inflammatory molecules are well documented in both colon inflammation and carcinogenesis; both molecules are modulated by chemopreventive and anti-inflammatory agents (26, 27). Accordingly, silibinin effect on COX-2 expression was next examined by immunohistochemistry where azoxymethane-group samples showed marked expression of COX-2 that decreased substantially by silibinin treatments (Fig. 3A). Quantification of COX-2 staining based on intensity of immunoreactivity (0-4 scale) showed a 3.1 ± 0.04 positivity score in the azoxymethane group versus 1.8 ± 0.04 and 1.3 ± 0.03 in groups pretreated with low and high doses silibinin, accounting for 42% and 58% (P < 0.001) decreases compared with the azoxymethane group, respectively (Fig. 3A). Silibinin posttreatments also showed a significant decrease (23-39%, P < 0.001) in COX 2 positivity (Fig. 3A). We likewise observed strong immunoreactivity for iNOS in azoxymethane-induced mice, which decreased strongly in silibinin-treated groups (Fig. 3B). Quantification of iNOS immunostaining showed a positivity score of 3.5 ± 0.05 in the azoxymethane-alone group versus 1.8 ± 0.05 and 1.3 ± 0.04 in groups pretreated with low and high doses silibinin, accounting for 48% and 63% (P < 0.001) decrease, respectively (Fig. 3B). Silibinin posttreatment showed a similar decrease in iNOS positivity scores but to a lesser extent.

The role of VEGF in angiogenesis is well documented in the literature (28). Based on our results showing that silibinin treatment reduces the number of biggest-size tumors most strongly, we next assessed whether VEGF...
Fig. 2. Silibinin inhibits cell proliferation and induces apoptosis in its chemopreventive efficacy against azoxymethane-induced colon tumorigenesis in A/J mice. At study end, colon samples were collected and subjected to immunohistochemical analysis and quantification as described in Materials and Methods. Representative images of immunohistochemical staining (×400) of colonic adenoma region are shown in each case, and quantification data shown include both colonic mucosa and tumor immunohistochemical staining for PCNA (A), cyclin D1 (B), Cip1/p21 (C), and cleaved caspase-3 (CC3; D) positive cells. Bars, mean ± SE. Colonic mucosa with tumor/s from indicated groups were also analyzed by immunoblotting for PCNA (A), cyclin D1 (B), Cip1/p21 (C), and CC3 and cleaved-PARP (D) levels. Densitometry values shown for each group are mean ± SEM (n = 3) after adjustment with β-actin as loading control. Sb, silibinin.
levels are decreased in silibinin-treated groups compared with azoxymethane alone to support its antiangiogenic activity. Immunostaining for VEGF showed lower immunoreactivity in silibinin-treated groups of mice (Fig. 3C), and quantification of VEGF positivity scores showed ~33% ($P < 0.001$) decrease in the high-dose silibinin-pretreated group compared with azoxymethane alone (Fig. 3C). A comparable silibinin effect was observed in all other treatments in decreasing VEGF positivity scores compared with azoxymethane alone (Fig. 3C). Immunohistochemical results shown in Fig. 3A to C for COX-2, iNOS, and VEGF were further supported by immunoblot analysis of three randomly selected samples from three individual mice in the azoxymethane and the pre-silibinin plus azoxymethane groups, which showed similar silibinin efficacy patterns. Together, these results show the effect of silibinin on two key events of colon carcinogenesis, namely, inflammation and angiogenesis, and their possible role in the overall chemopreventive efficacy of silibinin against colon tumorigenesis.
Silibinin decreases the levels of both nuclear and cytoplasmic β-catenin

Alterations in the β-catenin pathway due to loss of APC function are implicated in colorectal cancer initiation and progression (10, 11). Thus, we next assessed silibinin effect on β-catenin levels by both immunohistochemistry and immunoblotting. The azoxymethane-alone group exhibited significant expression of β-catenin in both nucleus and cytoplasm; silibinin treatments, however, caused a decrease in β-catenin-positive cells (Fig. 4A). Quantification of staining showed that silibinin decreases nuclear β-catenin positive cells by 34% to 49% (P < 0.001) and by 21% to 26% (P < 0.001) in pre- and post-azoxymethane protocols, respectively, compared with azoxymethane alone (Fig. 4B).

Cytoplasmic β-catenin immunoreactivity was scored as an overall intensity of brown staining, and its quantification showed a positivity score of 2.2 ± 0.03 in azoxymethane alone versus 1.0 ± 0.01 in higher silibinin-pretreated group, accounting for a 55% reduction (P < 0.001); other silibinin treatments also showed a decrease ranging from 18% to 30% (P < 0.001; Fig. 4C). These immunohistochemical results were further supported by immunoblot analysis of three randomly selected samples from three individual mice in the azoxymethane group and both groups with doses of pre-silibinin plus azoxymethane, which showed that silibinin treatment decreases total β-catenin levels by 50% to 60% (Fig. 4D). Together, these results indicate that β-catenin could be a potential molecular target for silibinin in chemoprevention of colorectal cancer.

Fig. 4. Silibinin decreases the levels of both nuclear and cytoplasmic β-catenin in its chemopreventive efficacy against azoxymethane-induced colon tumorigenesis in A/J mice. At study end, colon samples were collected and subjected to immunohistochemical analysis and quantification as described in Materials and Methods. A, representative images of immunohistochemical staining (×400) of colonic adenoma region are shown in each case, and quantification data shown include both colonic mucosa and tumor immunohistochemical staining for nuclear β-catenin positive cells (B) and cytoplasmic β-catenin positivity scores (C). Bars, mean ± SE. D, colonic mucosa with tumor/s from indicated groups were also analyzed by immunoblotting for total β-catenin levels. Densitometry values shown for each group are mean ± SE (n = 3) after adjustment with β-actin as loading control.
target for the chemopreventive effects of silibinin against colon tumorigenesis.

**Silibinin decreases the levels of key molecules on IGF1 axis**

The IGF1-IGF-1R axis plays a critical role in colorectal cancer development, including its role in proliferation, cell survival, and angiogenesis, as well as in the regulation of β-catenin levels (12, 29). Based on our above discussed findings on silibinin efficacy in preventing azoxymethane-induced colon tumorigenesis together with the in vivo decrease in proliferation, inflammation, and angiogenesis, but increased apoptosis and alteration in associated molecular regulators, including decreased β-catenin levels in tumor tissues, we also analyzed silibinin effect on pAkt, pGSK-3β, IGFBP-3, and IGF-1Rβ levels. In immunohistochemical analysis, azoxymethane-group samples showed very high levels of both pAkt and pGSK-3β, which decreased strongly by silibinin treatments (Fig. 5A and B). Quantification of pAkt in the azoxymethane group showed 27 ± 0.25% pAkt-positive cells versus 10 ± 0.28 and 6 ± 0.17% in groups pretreated with low and high doses silibinin, accounting for 63% and 78% (P < 0.001) decreases, respectively, compared with the azoxymethane group (Fig. 5A). Silibinin posttreatments also showed a significant decrease (42–49%, P < 0.001) in pAkt-positive cells (Fig. 5A). We likewise observed strong immunoreactivity for pGSK-3β in the azoxymethane-alone group, which decreased strongly in silibinin-treated samples (Fig. 5B). Quantification of pGSK-3β-positive cells showed 57% and 74% (P < 0.001) decreases in groups pretreated with low and high doses of silibinin, respectively, compared with azoxymethane alone (Fig. 5B); posttreatment of silibinin showed a similar decrease in pGSK-3β-positive cells but to a lesser extent (Fig. 5B). Immunohistochemistry results shown in Fig. 5A and B for pAkt and pGSK-3β were further supported by immunoblot analysis of three randomly selected samples from three individual mice in the azoxymethane and pre-silibinin plus azoxymethane groups, which showed similar silibinin efficacy patterns, without any effect on total Akt and GSK-3β levels. In other studies, immunohistochemical staining for IGFBP-3 positivity score showed lower levels in the azoxymethane-alone group, which increased strongly (~2.5- to 3.2-fold, P < 0.001), at least in silibinin-pretreated groups (Fig. 5C). Conversely, IGF-1Rβ-positivity score showed high levels in the azoxymethane-alone group, which decreased dose-dependently by 37% to 52% and 22% to 38% (P < 0.001) in the pre- and post-silibinin treatment groups, respectively (Fig. 5D). This was further confirmed by the immunoblot analysis, in which the azoxymethane-alone group showed high levels of both phospho- and total IGF-1Rβ. These azoxymethane-induced levels of phospho- and total IGF-1Rβ were strongly decreased by silibinin treatments (Fig. 5D). Together, these results show strong inhibitory effects of silibinin on the IGF1 axis in azoxymethane-induced mouse colon tumorigenesis, and its possible role in overall chemopreventive efficacy of silibinin.

**Discussion**

The present study provides, for the first time, scientific evidence for the preventive effects of long-term silibinin treatment on azoxymethane-induced colon tumorigenesis in A/J mice. This is an excellent and well-studied preclinical model for colorectal cancer chemoprevention efficacy studies and is also known in the literature for its relevance with respect to the clinical, histopathologic, and molecular features of human colorectal cancer (30, 31). Administration of silibinin significantly prevented colon tumorigenesis by decreasing total tumor numbers in colon in both pre- and post-azoxymethane regimes, with pretreatment groups showing a much prominent effect, which included longer duration of silibinin treatment. Most strikingly, silibinin pretreatments exerted the strongest effect in reducing the number of biggest-size tumors (>2 mm in diameter), possibly by the antiangiogenic activity of silibinin. Silibinin treatments did not show any considerable effects on body weight gain and food consumption profiles throughout the experiment, which is consistent with previous studies where administration of silibinin to various laboratory animals did not show any adverse effects (32, 33). The higher dose of silibinin (750 mg/kg/day) used in the present study was based upon and corresponds to 1% (w/w) dietary-silibinin used in other recent animal studies, without any apparent toxicity (34).

The pathogenesis of colorectal cancer involves a stepwise progression from inflamed and hyperplastic cryptal cells through flat dysplasia to finally adenocarcinoma (35). Colorectal cancer growth and progression to advanced stages involve aberrant cell proliferation, evasion of apoptosis, and initiation of angiogenesis, and are mediated by aberrant Wnt signaling, β-catenin accumulation, and alterations in IGF signaling (8). Several studies in recent years have convincingly argued that targeting a single event alone is not sufficient to halt cancer growth, and accordingly, the agents that could target multiple tumorigenic events and associated mechanisms could be more effective in suppressing colorectal cancer growth and progression (36). Consistent with this concept, in our studies, silibinin showed strong chemopreventive efficacy against azoxymethane-induced colon tumorigenesis by targeting various molecular pathways associated with proliferation, apoptosis, inflammation, and angiogenesis.

Although colon carcinogenesis is a multistage process, enhanced cell proliferation and ablation of apoptosis are early events in the progression of this cancer (37). An increase in cell turnover accompanied by epithelial cell damage can increase mitotic aberrations and induce changes both at genetic and epigenetic levels that favor cancer promotion (35). Cell proliferation and apoptosis biomarkers are generally used to test the efficacy of any chemopreventive agent (38). Increased proliferation is well correlated with PCNA expression (34), and in the present study silibinin decreased PCNA levels dose-dependently. Our previous studies had suggested the inhibitory role of silibinin against tumor cell proliferation in azoxymethane-exposed Fisher 344 rats (24). Further, we observed silibinin-caused
Fig. 5. Silibinin decreases the levels of key molecules on the IGF1 axis in its chemopreventive efficacy against azoxymethane-induced colon tumorigenesis in A/J mice. At study end, colon samples were collected and subjected to immunohistochemical analysis and quantification as described in Materials and Methods. Representative images of immunohistochemical staining (×400) of colonic adenoma region are shown in each case, and quantification data shown include both colonic mucosa and tumor immunohistochemical staining for pAkt- (A), pGSK-3β- (B), IGFBP-3- (C), and IGF-1Rβ-positive (D) cells or positivity scores. Bars, mean ± SE. Colonic mucosa with tumor/s from indicated groups were also analyzed by immunoblotting for pAkt and total Akt (A), pGSK-3β and total GSK-3β (B), and pIGF-1Rβ (Tyr1316) and total IGF-1Rβ levels (D). Densitometry values shown for each group are mean ± SE (n = 3) after adjustment with β-actin as loading control.
downregulation of cyclin D1, a marker for proliferation and cell cycle progression as well as an important downstream molecule of the β-catenin pathway, unraveling the role of silibinin in exerting its antiproliferative effect possibly by inhibiting cell cycle progression. The dynamic equilibrium established between proliferating and apoptotic cells in normal tissues seems to be lost in cancerous tissues (39). In cancer, apoptotic cells are decreased and outnumbered by proliferating cells. Expression of Cip1/p21, an inhibitor of cyclin-dependent kinases, is downregulated upon azoxymethane administration in colon tumor development (40). A controversy regarding the role of Cip1/p21 does exist regarding whether it is proapoptotic or antiapoptotic. Nevertheless, it is well cited that in its absence, apoptosis evasion is witnessed in azoxymethane models (25). In our studies, silibinin enhanced Cip1/p21 levels, supporting both anti-proliferative and proapoptotic activities.

During different stages of colon carcinogenesis, proinflammatory mediators like COX-2 and iNOS are elevated (41). iNOS generates mutagenic concentration of nitric oxide, which plays an important role in carcinogenesis, tumor progression, and angiogenesis, and it is overexpressed in colorectal cancer (42). Prostaglandin E2, a product of COX-2, increases pGSK-3β and accumulates β-catenin, and the resulting β-catenin/T-cell factor (Tcf)-dependent transcription aids in the production of cyclin D1 (12). Our results show that silibinin decreases the levels of COX-2 and iNOS significantly, suggesting the possibility that silibinin downregulates β-catenin/Tcf signaling which in turn reduces COX-2 expression, as it is one of the downstream targets of the β-catenin pathway (43). The antiangiogenic potential of silibinin has been shown in HT29 xenograft and in cell culture studies (21). Angiogenesis characterized by proliferation, migration, and capillary formation by endothelial cells is a prerequisite for solid tumor growth and metastasis (44). VEGF is one of the biomarkers for angiogenesis, and our results showed a dose-dependent decrease in VEGF levels by silibinin. β-Catenin, a pleiotropic molecule, is associated with E-cadherin for cell-cell adhesion and Wnt-adenomatous polyposis coli-mediated signal transduction (43). Maintenance of normal levels of β-catenin in membrane, cytoplasm, and nuclear pools is crucial for signaling and cell adhesion functions. In normal colon, β-catenin is localized in cell to cell junction in conjunction with E-cadherin, and its cytoplasmic and nuclear levels are low. Aberrant Wnt signaling or mutant β-catenin, APC, or axin results in the accumulation of β-catenin in cytoplasm which in turn translocates it to the nucleus where it forms a complex with Tcf-4/Lymphoid enhancer-binding factor-1 resulting in the transcription of proliferative genes (29). In the present study, azoxymethane-induced colon tumors exhibited a substantial increase in β-catenin expression, and this increase was accompanied by its redistribution in both cytoplasm and nucleus. In contrast, silibinin caused a significant reduction in the accumulation of β-catenin in both cytoplasm and nucleus.

Colonetic mucosa and tumors of azoxymethane-treated animals expressed high levels of IGF-1Rβ, pAkt, and pGSK-3β, and a low level of IGFBP-3. IGF-1R is a membrane-associated receptor tyrosine kinase. The role of IGF-1-IGF-1R in colorectal cancer is well documented; specifically, IGFBP-3 level is decreased, resulting in an increased level of IGF-1, and these molecules are potential targets for colorectal cancer chemoprevention (45). The results of the present study show the reduced levels of activated as well as total IGF-1Rβ by silibinin. On the other hand, silibinin increases the level of IGFBP-3, which can...
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sequester IGF-I and thereby reducing its free level available to interact with IGF-1Rβ. Thus, via both these effects, silibinin could downregulate IGF-1Rβ signaling. Further, the silibinin-induced level of IGFBP-3 could exert its antineoplastic effect through IGF-1-dependent and/or -independent mechanisms, which remains to be investigated. These effects of silibinin are also supported by the similar observations reported in prostate cancer (18). Akt, up-stream of β-catenin, has its importance in cell cycle regulation, and its sustained activity is implicated in growth factor-mediated transition through G1, as well as in apoptosis evasion (46). GSK-3β plays a key role in colorectal cancer development and is phosphorylated by phosphoinositide 3-kinase/Akt via IGF, and its inactivation (phosphorylation) leads to dissociation of APC/axin/β-catenin complex. Several studies have supported the role of GSK-3β as a tumor suppressor that downregulates neoplastic transformation and tumorigenesis (47). One of the prominent substrates for GSK-3β is β-catenin, and it serves as a regulator of the Wnt/β-catenin pathway. Our results show that GSK-3β is phosphorylated at Ser9 (inactive form) and accumulated in the azoxymethane group, and is accompanied by dysregulation of the β-catenin pathway. Silibinin downregulated the inactive (phosphorylated) form of GSK-3β level and therefore could have controlled the β-catenin pathway.

Silibinin inhibited azoxymethane-induced colonic cell proliferation, inflammation, angiogenesis, and cell survival. Because molecular markers studied for these biological events are regulated by IGF-1R as well as β-catenin signaling, it is logical to conclude that silibinin targets the IGF-1R-β-catenin pathway for suppressing azoxymethane-induced colon tumorigenesis (Fig. 6). Based on this assumption, we also speculate that silibinin possibly modulates glucose, insulin, and IGFBP-3 in plasma/serum towards inhibition of IGF-1R signaling. Nevertheless, further experiments involving pharmacologic agents or genetic approaches for inhibition and/or induction of selected targets (such as IGF-1R, Akt, GSK-3β, and β-catenin) are needed to define the phenotypic events as well as their sequence which are regulated by IGF-1R signaling in response to silibinin. At molecular level, it is not known whether silibinin targets the extracellular domain of the receptor, interferes with the ligand-receptor interaction, changes the membrane dynamics of lipid raft, or interferes with intracellular kinase domain of the IGF-1R receptor. Therefore, further studies are also needed to explore these possible mechanisms of IGF-1R inhibition by silibinin. Nevertheless, our data suggest that silibinin increases IGFBP-3 level that could sequester IGFs, and that it decreases both activated and total IGF-1Rβ level, as two potential mechanisms to suppress IGF-1R signaling.

Overall, azoxymethane can enhance the expression and thereby the activity of IGF-1Rβ and reduce IGFBP-3 levels, leading to activation of IGF-1Rβ signaling cascades involving Akt and GSK-3β and ultimately activating the β-catenin pathway. After translocating to nucleus, β-catenin stimulates proliferation, ablation of apoptosis, inflammation, and angiogenesis (Fig. 6). More importantly, these azoxymethane-caused molecular changes are brought closer to normalcy by silibinin treatment causing in vivo antiproliferative, apoptotic, anti-inflammatory, and antiangiogenic effects in its overall chemopreventive efficacy against azoxymethane-induced colon tumorigenesis (Fig. 6).

Taken together, our findings suggest that silibinin could be a potent chemopreventive agent against human colorectal cancer, and therefore warrants further studies for broadening the scope of its application to colorectal cancer patients. Importantly, silibinin is physiologically achievable up to 165 μmol/L in plasma at 2 g/kg oral dose without any toxicity in mice (34). Regarding humans, a recently completed phase I study in prostate cancer patients with silibinin (as silybin-phytosome) has shown up to 100 μmol/L of silibinin in plasma with a half-life of up to 5 hours, and its subsequent conjugation and excretion in urine (48). Another clinical study has also reported the absorption and oral bioavailability of silibinin given in different formulations (49). Moreover, a pilot study with silibinin in human colorectal cancer patients has reported its bioavailability and nontoxicity, and suggested its further exploration (50). Thus, silibinin has promise and potential to be developed as a chemopreventive agent against colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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