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Review Article

Silibinin and colorectal cancer chemoprevention: a comprehensive review on mechanisms and efficacy

Komal Raina[1,2], Sushil Kumar[1], Deepanshi Dhar[1], Rajesh Agarwal[1,2,✉]

[1]*Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences;*
[2]*University of Colorado Cancer Center, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA.*

Abstract

Globally, the risk of colorectal cancer (CRC) as well as the incidence of mortality associated with CRC is increasing. Thus, it is imperative that we look at alternative approaches involving intake of non-toxic natural dietary/non-dietary agents, for the prevention of CRC. The ultimate goal of this approach is to reduce the incidence of pre-neoplastic adenomatous polyps and prevent their progression to more advanced forms of CRC, and use these natural agents as a safe intervention strategy during the clinical course of this deadly malignancy. Over the years, pre-clinical studies have shown that silibinin (a flavonolignan isolated from the seeds of milk thistle, *Silybum marianum*) has strong preventive and therapeutic efficacy against various epithelial cancers, including CRC. The focus of the present review is to provide a comprehensive tabular summary, categorically for an easy accessibility and referencing, pertaining to the efficacy and associated mechanisms of silibinin against CRC growth and progression.

Keywords: colorectal cancer, silibinin, cancer chemoprevention, milk thistle

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States men and women combined; statistical estimates for 2015 indicate ~132,700 new CRC cases and 49,700 associated deaths[1]. Though several initiatives involving colon screening and therapeutic interventions have impacted the overall CRC management in the developed countries of the world[2], clearly more efforts are still needed to overcome CRC risk, to arrest the disease progression, and to reduce the mortality numbers associated with this malignancy. One such effort that has gained an appreciable momentum over the last three decades, involves the intake of non-toxic natural dietary/non-dietary agents which can be used globally, for the prevention of CRC. In most cases, these agents, derived from fruits and vegetables as well as herbs and other supplements, have been shown to reduce the incidence of pre-neoplastic adenomatous polyps and/or their progression to more advanced forms of CRC (***Fig. 1***)[3-4]. In the present review, an attempt has been made to tabulate and summarize the beneficial effects of one such natural chemopreventive agent i.e., silibinin, against CRC. Silibinin is a flavonolignan which is isolated from the seeds of milk thistle, *Silybum marianum* (***Fig. 2***); it has a long history of human use for liver ailments[5], widely available as a nutraceutical supplement and now clinically used to manage hepatotoxicity in various countries including the United States.

✉ Corresponding author: Rajesh Agarwal, Ph.D, Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd, C238, Room V20-2117, Aurora, CO 80045, USA. Tel/fax: (303) 24-4055/(303) 724-7266, E-mail: rajesh.agarwal@ucdenver.edu.

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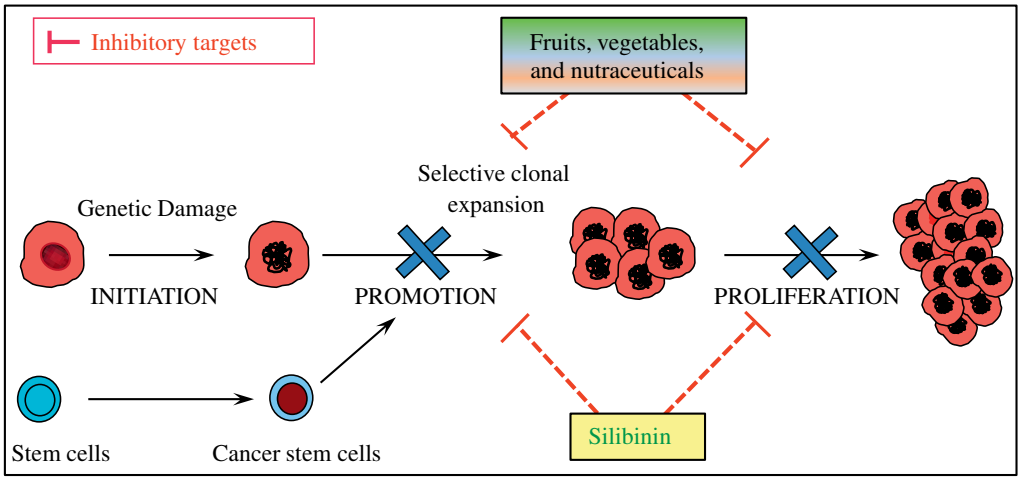

Fig. 1 **Chemoprevention by natural dietary or non-toxic nutraceutical agents.** Natural dietary agents, such as silibinin, can reduce the incidence of pre-neoplastic lesions by targeting cancer stem cells and proliferating bulk tumor cells to prevent the disease progression to more advanced forms of the malignancy. Silibinin inhibits tumorigenesis, inflammatory responses, and angiogenesis involved in CRC growth and progression. CRC: colorectal cancer.

Importantly, silibinin is considered as a very promising cancer chemopreventive agent based on completed studies showing its strong efficacy against various epithelial cancers (***Fig. 3***), including CRC[3-4,6-9]. As elaborated in detail in this review, silibinin has shown significant *in vitro* and *in vivo* anti-CRC efficacy in various pre-clinical models of CRC. Inferences from completed in depth studies investigating the cellular and biological mechanisms associated with anti-CRC effects of silibinin are summarized in ***Tables 1–4***, which detail the effect of silibinin on various molecules regulating cell cycle, cell survival, autophagy, apoptosis, angiogenesis, and inflammation in its efficacy against colon tumorigenesis.

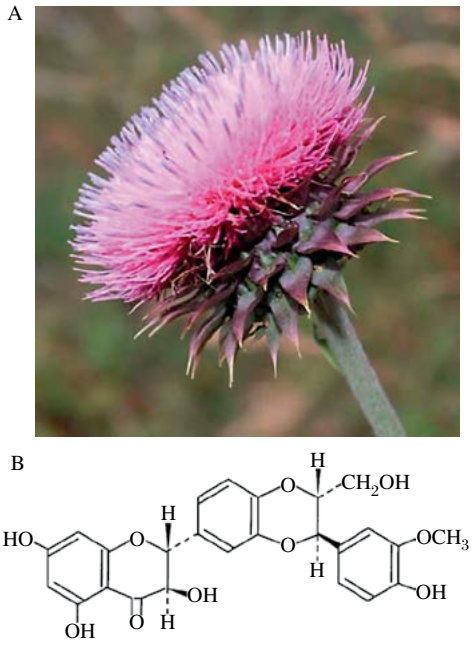

Fig. 2 **Milk Thistle.** A: *Silybum marianum* (Milk Thistle) plant, Family: *Asteraceae.* B: Chemical structure of silibinin - the principal bioactive constituent of milk thistle extract isolated from the dried seeds of milk thistle.

Silibinin efficacy against CRC cell lines in cell culture

Under *in vitro* cell culture conditions, silibinin has been shown to inhibit the growth and proliferation of a wide range of CRC cells (***Table 1***). Mechanistic studies have attributed these inhibitory effects to cell cycle arrest as well as both caspase-dependent and -independent apoptotic pathways (***Table 1***). An increase in cyclin-dependent kinase (CDK) inhibitors Kip1/p27 and Cip1/p21, together with a decrease in the kinase activity of CDK2 and CDK4 in G0/G1 arrested CRC cells; while an increase in the kinase activity of CDC2/p34 molecule along with a decrease in protein expression of cell cycle regulators cdc25C, cdc2/p34 and cyclin B1 by silibinin in G2M arrested CRC cells have been reported (***Table 1***). Furthermore, a decrease in the hyperphosphorylation of Retinoblastoma (Rb) by silibinin has also been reported as one of the mechanisms involved in the early cell cycle arrest induced by silibinin in CRC cells; though total Rb levels are unaffected, a decrease in the protein expression levels of cyclin -D1, -D3, -A and -B1 and CDK-1, -2, -4, and -6 by silibinin has been reported. The *in vitro* effects of silibinin alone or in combination with other molecules are summarized in ***Table 1***[10-17].

Other in depth studies have shown that at physiologically relevant dose (100 μmol/L), silibinin causes

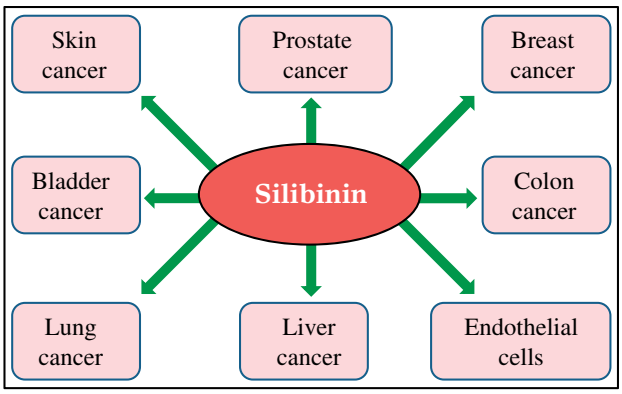

Fig. 3 **The targets of silibinin.** Silibinin inhibits various signaling and regulatory pathways in its chemopreventive and therapeutic efficacy against various epithelial cancers.

oxidative stress in CRC cells, which triggers an apoptotic response, and is followed by a chain of events involving strong inhibition of the PI3K-Akt-mTOR pathway, activation of the ERK1/2 pathway, rough endoplasmic reticulum stress (ER), and suppression of protein translation, which results in autophagy-mediated programmed cell death type II. High-resolution nuclear magnetic resonance spectroscopy (^{1}H, ^{13}C,^{31}P-NMR) studies have further confirmed that silibinin interferes with essential mitochondrial metabolic pathways, and the events associated with both phospholipid as well as protein synthesis[13]. Furthermore, silibinin has also been found to interfere with the glucose uptake in these cells; overall, these events result in energy restriction in CRC cells and their severe and irreparable injury by silibinin[13]. Studies have also shown that significant apoptotic death of CRC cells occurs when high silibinin doses are used[13,18], which suggests that silibinin harbors the potential to induce both apoptotic and autophagy associated programmed cell death in CRC cells.

With regards to silibinin efficacy against CRC stem cells (CSC), silibinin has also been shown to interfere with the kinetics of CSC pool generation, which in turn, decreases the self-renewal and rectifies the aberrant differentiation of the CSC[19-20]. These results are detailed in ***Table 2***; the potential of silibinin to target CSC is being extensively investigated by our group (***Table 2***)[21-22]. Our *in vitro* studies showed that silibinin strongly decreases the percentage of colonosphere formation (a stem cell characteristic) of CRC cells and that this effect on CSC is mediated *via* blocking of interleukin (IL)-4/6 signaling in CRC cell lines. Silibinin also caused a strong decrease in IL-4/6 induced activation of STAT-3 and NF-kB transcriptional activity, which was associated with decreased mRNA/protein levels of various CSC regulatory molecules, and CSC-associated markers and transcription factors (***Table 2***). We also found that silibinin significantly reduces the booster signals of macrophages towards CSC, resulting in decreased colonosphere numbers under both normoxic and hypoxic conditions (Agarwal & colleagues 2015, unpublished data, ***Table 2***). These results are highly significant, given the fact that silibinin has shown efficacy against both CSC and bulk cancer cells, and that CSC are now primarily recognized as the essential factors contributing to initiation/progression as well as the relapse of CRC[23-26].

Notably, several *in vitro* mechanistic studies have used high doses of silibinin (300 µmol/L); the clinical relevance of such high doses warrants caution; this is founded on the results of several preclinical as well as clinical trial studies that underline limiting the silibinin concentration to ~100 µmol/L dose in *in vitro* studies based on silibinin concentrations achievable in mouse plasma[27] and colon tissues of CRC patients[28-29]. Specifically, the results of the clinical trial data showed that silibinin feeding in the form of 720 mg/day (formulated with phosphatidylcholine) as 'silipide capsules' for 7 days to CRC patients leads to high bioavailability of silibinin in colonic tissue (121 nmol/g of silibinin traced in colonic tissue which is equivalent to 121 µmol/L silibinin concentration).

Efficacy of silibinin against CRC growth and progression in pre-clinical rodent models

Using azoxymethane (AOM) and 1,2-dimethylhydrazine (DMH) as potential carcinogens to induce sporadic CRC in rodent models, various research groups have demonstrated the potential of silibinin to decrease the number of carcinogen-induced aberrant crypt foci (ACF), crypt multiplicity as well as colonic tumors in these colon tumorigenesis animal models (***Table 3***). To determine the stage specific efficacy of silibinin feeding, different silibinin feeding protocols based on whether silibinin feeding started before the carcinogen exposure (pre-initiation phase), during the carcinogen exposure (initiation phase), after the carcinogen exposure (post-initiation phase), or through the entire period of the study (pre-/-post initiation phase) were carried out; though all silibinin feeding protocols showed efficacy against CRC growth and progression, the percentage of CRC inhibition was stronger in the animals on the pre-/-post-initiation protocol[30-37]. Silibinin has also shown significant efficacy against spontaneous intestinal tumorigenesis in *APC*$^{min/+}$ mice model (***Table 3***). Both short and long term intervention studies with silibinin have confirmed that total intestinal polyps are significantly reduced in number and size by silibinin feeding[16,38-40]. While a decrease in

Table 1 **Biological effects of silibinin against human colorectal cancer (CRC) cell lines under *in vitro* cell culture conditions.**

CRC cell lines	*In vitro* effects of silibinin	Model and methods	Mechanism of action	Research group
HT-29	• growth inhibition: dose (50-100 µg/mL) and time (24-72h) dependent. • G0/G1 cell cycle arrest (lower/higher doses). • G2/M cell cycle arrest (higher dose: 100 µg/mL). • no apoptosis at lower doses. • apoptotic death (~ 15%) after 48 hours with 100 µg/mL dose. • no induction of cellular differentiation.	• FACS based cell cycle distribution analysis. • annexin V staining for apoptosis, caspase activity assay, and cytochrome c localization analysis. • immunoprecipitation based CDK2-and cdc2/p34-associated H1 histone kinase assays. • Northern blot hybridization with 32P labeled Kip/p27 and Cip/p21.	• mRNA and protein levels of Kip/p27 and Cip/ p21 ↑ • protein levels of cdc25c, cdc2/p34, and cyclin B1 ↓ • kinase activity of cdc2/p34 ↓ • caspase independent apoptosis.	• Agarwal *et al*. 2003[10]
	• inhibitory effects of silibinin (100 µmol/L dose) on β-catenin mediated signaling.	• TCF-luciferase reporter plasmids based assays.	• β-catenin-dependent TCF-4 transcriptional activity ↓	• Rajamanickam *et al*. 2010[39]
	• inhibitory effects of silibinin (1-100 µmol/L dose) on CDK4 signaling pathway.	• MTT cell viability assays. • FACS based Ki67 labeling analysis. • immunoblotting for cell cycle regulatory molecules.	• protein levels of CDK-4, and cyclin D1 ↓ • hyper phosphorylation of retinoblastoma ↓	• Karim *et al*. 2013[16]
	• dose and time dependent growth inhibition.	• cell count assays.	• Not explored	• Akhtar *et al*. 2014[15]
	• apoptosis of HT29 cells via EGR-l-mediated NSAID-activated gene-1 (NAG-1) up-regulation (silibinin: 50-100 µmol/L dose). • inhibitor of p38 MAPK (SB203580) attenuated silibinin-induced NAG-1 expression.	• p53 wild-type and p53-null cancer cell lines. • siRNA and MAPK inhibitors based confirmatory • assays.	• NAG-1 up-regulation in p53-independent manner. • up-regulation of EGR-1 expression. • ectopic expression of EGR-1 significantly upregulates NAG-1 promoter activity and NAG-1 protein expression in a dose-dependent manner.	• Woo *et al*. 2014[17]
Fet, Geo, and HCT116	• G2/M cell cycle arrest in Fet and Geo cell lines. G1 arrest in HCT116 cells. • IC50 in Fet and Geo lines is 75 µg/mL and 40 µg/mL for HCT116 cells at 72 hours. • growth inhibitory effects more due to inhibition of cell cycle regulatory molecules than due to apoptosis.	• MTT cell viability assays. • FACS based cell cycle distribution and apoptosis analysis. • immunoblotting for cell cycle regulatory molecules.	• protein levels of Kip/p27 and Cip/p21 ↑ • protein levels of Cyclin Bl/Dl and CDK-2 ↓ • no effect on Cox-2 levels.	• Hogan *et al*. 2007[14]
	• dose (50-200 µmol/L) and time (24-72 hours) dependent growth inhibition. • G1 cell cycle arrest (lower/higher doses) as well as G2M arrest with 200 µmol/L. • significant apoptotic death at 100-200 µmol/L.	• FACS based cell cycle distribution analysis. • annexin V staining for apoptosis. • immunoblotting for cell cycle regulatory molecules.	• protein levels of cleaved caspase -3 and -9, and cleaved PARP ↑ • protein levels of Kip/p27 and Cip/p21 ↑ • protein levels of Cyclin- D1/-D3/-A/-B1 and CDK-1/-2/-4/-6 ↓ • hyper phosphorylation of Retinoblastoma ↓	• Kaur *et al*. 2009[12]

Continued

Table 1 **(continued)**

CRC cell lines	*In vitro* effects of silibinin	Model and methods	Mechanism of action	Research group
LoVo	• anti-angiogenic effect. • inhibits the chemotaxis migration of endothelial cells EA.hy.926 towards CRC cells (IC50: 0.66 μmol/L dose). • inhibits EA.hy.926 capillary formation (IC50: 2.6 μmol/L dose). • ↓ vascular density index in the choriallontoic membrane assay by 20 μmol/L dose.	• transwell migration and matrigel based capillary tube formation assay. • chicken egg based choriallontoic membrane assay. • mRNA levels by RT-PCR analysis.	• mRNA levels of VEGFR-l(Flt-l) ↑ • ↓ VEGF secretion by LoVo cells (IC50: 131.7 μmol/L dose).	• Yang *et al*. 2003/2005[42-43]
	• dose 10^{-6} mol/L. • invasiveness of CRC cells ↓ • IL-6 induced proliferation and invasion of LoVo cells ↓	• [^{3}H] thymidine incorporation assay. • cell invasion assays. • EMSA and MMP-2 promoter activity based luciferase assays. • confocal microscopy based MMP-2 localization analysis.	• ↓ MMP-2 promoter activity via attenuation of AP-1 binding activity. • MMP-2 expression ↓	• Lin *et al*. 2012[44]
SW480	• cell growth inhibition by 50-200 μmol/L dose after 24-72 hours. • no death till 72 hours with doses up to 100 μmol/L. • only 200 μmol/L dose affects viability at early time points. • inhibitory effects on β-catenin mediated signaling.	• viable cell count assays. • TCF-luciferase reporter plasmids based assays. • confocal microscopy based β-catenin localization analysis. • immunoblotting analysis for protein expression.	• nuclear and cytoplasmic β-catenin levels ↓ • expression of β-catenin regulator CDK-8 ↓ • β-catenin-dependent TCF-4 transcriptional activity ↓ • expression of β-catenin transcriptional targets: c-Myc and cyclin D1 ↓	• Kaur *et al*. 2010[11]
HT-29, LoVo, and SW480	• anti-inflammatory effect (50-100 μmol/L) dose. • inhibits TNFα-induced NFκB activation. • effects independent of COX-2 expression.	• immunoblotting analysis for protein expression. • EMSA based gel super shift assays.	• nuclear levels of p65 and p50 ↓ • IκBα protein levels ↑ • phospho- IκBα levels ↓ • NFκB transcriptional activity ↓	• Raina *et al*. 2013[45]
SW480 and SW620	• 300 μmol/L dose synergizes with TRAIL to cause apoptotic death. • assumed that autophagy plays a cytoprotective role.	• DNA fragmentation assays, FACS analysis and caspase inhibitors based confirmatory assays. • mitochondrial membrane potential analysis. • mRNA levels by RT-PCR analysis. • human recombinant DR5/Fc chimera protein based studies.	• mRNA and protein levels of death receptors DR4/-5 ↑ • both intrinsic and extrinsic apoptotic pathways involved. • Mcl-1 and XIAP ↓	• Kuantz *et al*. 2011/2012[18,46]
	• 300 μmol/L dose synergizes with HD AC inhibitors: (SAHA and trichostatin A) to cause cellular death.	• FACS based cell cycle analysis. • HDAC and DNMT activity measurement.	• DNMT inhibition.	• Kuantz *et al*. 2013[47]

Continued

Table 1 **(continued)**

CRC cell lines	*In vitro* effects of silibinin	Model and methods	Mechanism of action	Research group
HT-29, LoVo, and SW480	• inhibits mitogenic/growth promoting signals induced by IGF-1 and EGF.	• IGF-1 and EGF based effects on cell growth and proliferation. • FACS based analysis. • immunoblotting for protein expression levels.	• PI3K-Akt-mTOR pathway ↓ and ERK1/2 pathway ↑ • no inhibitory effect on normal human colon NCM 460 cells.	• Raina *et al*. 2013[13]
SW480	• oxidative stress and early on slight apoptosis. • intense vacuolization of cytoplasm and rough endoplasmic reticulum swelling. events associated with autophagy ↑ • long term exposure causes autophagic cell death (100 μmol/L dose) while higher doses (≥ 200 μmol/L dose) cause apoptosis. • potential to cause both apoptotic and autophagic cell death.	• oxidative stress and mitochondrial membrane potential analysis, and inhibitors based confirmatory assays. • transmission electron microscopy, and dynamics of LC3-I and LC3-II tracking. • metabolomics study utilizing 13C, 1H, 3IP based NMR spectroscopy.	• early on reactive oxygen species generation. • PI3K-Akt-mTOR pathway ↓ and ERK1/2 pathway ↑ • interference in mitochondrial metabolism, phopspholipid and protein synthesis, and glucose uptake. • energy restrictions causing starvation lead to autophagic cell death.	• Raina *et al*. 2013[13]

Abbreviations: CRC, Colorectal cancer; FACS; fluorescence- activated cell sorting; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; RT-PCR, reverse transcription polymerase chain reaction; TCF-4, T-cell factor-4; TRAIL, TNF-related apoptosis-inducing ligand; HDAC, histone deacetylase; DNMT, DNA methyltransferase; SAHA, suberoylanilide hydroxamic acid; IGF-1, insulin-like growth factor-1; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; NMR, nuclear magnetic resonance.

Table 2 **Biological effects of silibinin against human colorectal cancer stem cells (CSC)**

Colorectal cancer stem cells (CSC)	Effects of silibinin	Model and methods	Mechanism of action	Research group
Primary human tumor cells and HT-29	***in vitro:*** ● dose up to 100 µg/mL. ● decreases coionosphere formation.	● CSC from primary CRC isolated from human tumor with Duke C3 CRC stage, and HT-29 cells. ● FACS, immunofluorescence, and ● immunoblotting for protein levels.	● CD 133 expression ↓ ● more differentiated clones ↑ ● PP2A-Akt-mTOR pathway ↓	● Wang *et al* 2012[20]
HT-29, LoVo, and SW480	***in vitro:*** ● 25-100 µmol/L dose used. ● decreases coionosphere formation. ● targets both colon CSC and bulk tumor cells.	● CSC enriched coionosphere assays. ● mitogen and IL4 and IL6 mediated signaling effects on kinetics of CSC spheroids. ● FACS, immunoblotting, EMSA based gel super shift assays. ● RT-PCR and RT^2PCR analysis. ● 3D differentiation and confocal microscopy based z stack and localization assays.	● CSC: $CD44^+$ $EpCAM^{high}$ cells ↓ ● number and size of coionospheres ↓ ● IL4 and IL6 mediated effects on CSC ↓ ● IL4 and IL6 induced effects on NFκB and STAT-3 ↓ ● mRNA and protein levels of LGR5, ASCL2, CD44, CD133, OCT 4, NANOG. MSI-1, BMI-1, and HES-1 ↓ ● symmetric self-renewal of CSC ↓ ● more differentiated clones ↑	● Kumar *et al* 2014[19]
HT-29 and SW480	***in vitro:*** ● 50-100 µM dose used. ● booster signals of macrophages (U937 and THP-1) towards colon CSC ↓ ● resulting in decreased coionosphere numbers under both normoxic and hypoxic conditions.	● CSC enriched coionosphere assays. ● macrophage mediated (hypoxic and normoxic) signaling effects on CSC spheroids. ● FACS, immunoblotting, RT-PCR and RT^2PCR analysis, cytokine arrays. ● confocal microscopy based z stack and localization assays.	● CSC: $CD44^+$ $EpCAM^{high}$ cells ↓ ● macrophage mediated effects on CSC ↓ ● silibinin modifies the cytokine profile of macrophage conditioned media.	● Agarwal & colleagues 2015: ● unpublished
HT-29	***in vivo:*** ● tumorigenic potential of CSC ● ($CD44^+Epcam^{high}$ CRC cells) ↓ ● CSC self-renewal in serial transplantation studies ↓ ● inhibitory effects on both CSC and bulk daughter cells.	● s.c cell injections of sorted CSC ($CD44^+Epcam^{high}$) HT-29 cells mixed with matrigel (1:1) in flank of NOD/SCID male mice. ● after 24 hours of cell injections; oral gavage with silibinin (200 mg/kg) in CMC vehicle every day for ~40 days. ● serial transplantation of unsorted/sorted tumors in NOD/SCID mice. ● diffusion weighted MRI, DCE-MRI, and ^{18}FDG-PET; FACS, confocal microscopy, and paired cell analysis.	● tumor cellularity, vascularity, glycolytic activity ↓ ● tumor growth in serial transplantation studies ↓ ● symmetric self-renewal of CSC ↓ ● asymmetric self-renewal of CSC ↑ ● shifts the cell division to symmetric proliferation resulting in generation of two daughter cells ↑ ● transforms/differentiates $CD44^+$ population into a CD44- phenotype.	● Agarwal & colleagues 2015a: ● -Kumar *et al* 2015[22]

Abbreviations: CRC, Colorectal cancer; CSC, colorectal cancer stem cells; FACS; fluorescence- activated cell sorting; RT-PCR, reverse transcription polymerase chain reaction; RT^2PCR, real -time RT-PCR; EMSA, electrophoretic mobility shift assay; MRI, magnetic resonance imaging; DCE-MRI, dynamic contrast enhanced-MRI; ^{18}FDG-PET, $^{18}Fluorine$-2-Deoxy-Glucose (FDG) positron emission tomography.

Table 3 **Biological effects of silibinin against tumor growth and progression in preclinical animal models of colon tumorigenesis.**

Animal model	*In vivo* effects of silibinin	Treatment modality	Mechanism of action	Research group
		Carcinogen-induced sporadic colon tumorigenesis model		
AOM	• dose dependent decrease in azoxy-methane (AOM)-induced pre-neoplastic aberrant crypt (ACF) formation and crypt multiplicity. • inhibition more pronounced when silibinin given prior to initiation and continued till study end (pre-/-post initiation protocol).	• male Fischer 344 rats. AOM (s.c 15mg/ kg): once a week for 2 weeks. • 0.033% -1% w/w silibinin supplemented AIN-76A diets. • rats sacrificed after 8 weeks of 2nd AOM injection. • silibinin feeding protocols: pre-initiation; post-initiation; and pre-/-post initiation.	• PCNA positive cells ↓ • apoptotic cells ↑ ; cleaved PARP ↑ • cyclin Dl, Cox-2, and iNOS ↓	• Velmurugan *et al*. 2008[30]
AOM	• dose dependent decrease in AOM-induced colon tumorigenesis. • decrease in tumor multiplicity and number of bigger tumors (>2mm). • inhibition more pronounced when silibinin given prior to initiation and continued till study end (pre-/-post initiation protocol).	• male A/J mice. AOM (i.p 5mg/kg): once a week for 6 weeks. • oral gavage with silibinin (250-750 mg/ kg) in CMC vehicle for 5 days a week. • mice sacrificed after 18 weeks of last AOM injection. • silibinin feeding protocols: pre-/-post initiation; post-initiation.	• PCNA positive cells ↓ • apoptotic cells ↑, cleaved (caspase-3 and PARP) and p21 ↑ • cyclin Dl, Cox-2, VEGF, and iNOS ↓ • nuclear and cytoplasmic β-catenin ↓ • key molecules of IGF-1 axis: phospho (IGF-1 Rβ, Akt and GSK-3β) ↓ • IGFRP-3 ↑	• Ravichandran *et al*. 2010[31]
AOM	• reduction in AOM-induced hyper proliferative crypts and ACF formation.	• male Wistar rats. AOM (i.p 15mg/kg): once a week for 2 weeks. • oral gavage with silibinin (300 mg/kg) in CMC vehicle every day. • mice sacrificed 7 weeks after AOM injection. • silibinin feeding protocol: start one week after last AOM (post-initiation).	• apoptotic cells ↑ • protein levels of Bcl-2 ↓ and Bax levels ↑ • mRNA levels of inflammatory markers: MMP-7, ELl-β, and TNFα ↓ • protein levels of MMP-7 ↓	• Kauntz *et al*. 2012[32]
DMH	• inhibits 1, 2-dimethylhydrazine (DMH)-induced colonic pre-neoplastic changes. • ACF formation, dysplastic ACF, and tumor incidence ↓ • restores the levels of GSH-dependent enzymes. • normalizes phase I/II xenobiotic metabolizing enzymes. • Lipid peroxidation in colonic tissues ↓ and enzymic anti-oxidants ↑	• male albino Wistar rats. DMH (s.c 20mg/ kg): once a week for 15 weeks. • oral gavage with silibinin (50 mg/kg) in CMC vehicle every day. • mice sacrificed 13 weeks after last DMH injection. • silibinin feeding protocols: initiation; post-initiation; pre-/-post initiation (entire period).	• fecal biotransforming microbial enzymes ↓ • colonic and fecal β-glucuronidase ↓ • mucosal and fecal activities of β-glucosidase and β-galactosidase ↓ • nitroreductase and sulphatase ↓ • β-catenin, PCNA, agyrophillic nucleolar organizing regions, and cyclin Dl ↓ • prevents suppression of CDX2 mRNA and protein levels.	• Sangeetha *et al*. 2009/ 2010a/2010b/2012/2014[33-37]

Continued

Table 3 (continued)

Animal model	*In vivo* effects of silibinin	Treatment modality	Mechanism of action	Research group
Mouse model of spontaneous intestinal tumorigenesis				
APC$^{min/+}$ mice	• intestinal adenoma numbers ↓ • unformulated silibinin more effective than silibinin-phospholipid complex preparation (*silipide).	• 0.2% w/w silibinin supplemented AIN-93G diets or silipide for 21 days. • (dosing equivalent to silibinin: 300 mg/kg per day).	• Not explored	• Verschoyle *et al*. 2008[40]
APC$^{min/+}$ mice	• decrease in total number of intestinal polyps (34%-55% ↓).	• 6 weeks old APC$^{min/+}$ mice. • oral gavage with silibinin (250-750 mg/kg) in CMC vehicle for 5 days a week. • mice sacrificed after 6 weeks of silibinin feeding.	• PCNA positive cells ↓ • apoptotic cells ↑ • β-catenin, cyclin Dl, c-myc, phospho (Akt and GSK-3β), Cox-2, and iNOS, levels ↓ • nitrotyrosine and nitrite levels ↓	• Rajamanickam *et al*. 2009[38]
APC$^{min/+}$ mice	• decrease in total number of intestinal polyps in proximal (27% ↓), middle (34% ↓), and distal regions (49% ↓). • decrease in colonic polyps (55% ↓). • decrease in polyp numbers in size range > 2-3 mm (92% ↓). • no polyps > 3 mm. • anti-proliferative, pro-apoptotic, anti inflammatory, and anti-angiogenic effects (normal crypt-villus region unaffected).	• 6 weeks old APC$^{min/+}$ mice. • oral gavage with silibinin (750 mg/kg) in CMC vehicle for 5 days a week. • mice sacrificed after 13 weeks of silibinin feeding.	• PCNA positive cells ↓ • apoptotic cells ↑, cleaved (caspase-3 and PARP) ↑ • immunoreactivity of HIF-lα, VEGF, eNOS, and Nestin ↓ • inhibitory effects on β-catenin mediated signaling. • nuclear β-catenin, cyclin Dl, Cox-2, and PGE2 levels ↓ • modulates cytokine profile in intestinal polyps.	• Rajamanickam *et al*. 2010[39]
APC$^{min/+}$ mice	• decrease in small and large intestinal adenomas.	• 4 weeks old APC$^{min/+}$ mice. • 0.2% w/w silibinin supplemented AIN-93G diets. Mice sacrificed after 3 months of silibinin feeding	• Ki67 positive cells ↓ • apoptotic cells ↑	• Karim *et al*. 2013[16]
Inflammation based mouse model of colitis associated colon tumorigenesis				
AOM/DSS	• incidence of high grade dysplasia and intramuscular carcinoma ↓ • mice with prolapsed rectum ↓ • inflammatory cells/markers and associated transcription factors ↓	• male balb/c mice. AOM (i.p 10 mg/kg): once only. 2% DSS in drinking water for 7 days. • oral gavage siliphos (*silibinin-phytosome preparation-600mg/kg in CMC vehicle every day: pre-/-post initiation). • mice sacrificed after 3, 8, 12 and 16 weeks post DSS.	• mast cells, macrophages, NFκB, STAT-3, Cox-2, IL-6, and IL-4 ↓ • colon CSC markers/regulatory factors/ transcription factors ↓ • CD44^{+} cells ↓ and BMI-1+/CD44+ dual stained cells ↓ • nuclear (Sox2, Nanog, and Oct 3/4) ↓	• Agarwal & colleagues 2015b: • -Tyagi *et al*. 2014[21]

Abbreviations: AOM, azoxymethane; ACF, aberrant crypt foci; DMH, 1, 2- dimethylhydrazine; CMC, carboxymethyl cellulose; DSS, dextran sodium sulphate; CSC, colorectal cancer stem cells * Siliphos or silibinin-phytosome (from Indena, Italy) that contains silibinin and phosphatidylcholine in 1:2 ratio. * silipide (from Indena, Italy) that contains silibinin and phosphatidylcholine in 1:1 ratio.

Table 4 **Biological effects of silibinin against human colorectal cancer (CRC) tumor xenografts in animal models.**

CRC cell lines	*In vivo* effects of silibinin	Animal model and treatment modality	Mechanism of action	Research group
HT-29	• inhibits tumor growth, cell proliferation, and angiogenesis. • tumor volume (48% ↓) and tumor weight (42% ↓) decreased by silibinin. • *silibinin-phytosome showed more significant inhibitory effect due to better bioavailability.	• s.c cell injections of 4 × 10^6 HT-29 cells mixed with matrigel (1:1) in right flank of athymic BALB/c nu/nu male mice. • after 5 days of implantation oral gavage with silibinin or * silibinin-phytosome (SP) combination in CMC vehicle, 5 days a week for 32 days. • daily dose: silibinin (200 mg/kg); silibinin-phytosome (300-600 mg/kg)-which is equivalent to 100-200 mg/kg silibinin.	• PCNA positive cells ↓ • apoptotic cells ↑ • cyclin Dl and phospho (ERK1/2 and Akt) ↓ • VEGF and CD31J, • Cox-2, HIF-lα, iNOS, and NOS3 ↓	• Singh *et al*. 2008[27]
LoVo	• inhibits tumor growth and cell proliferation. • tumor volume (34% - 46% ↓) and tumor weight (38% - 49% ↓) decreased by 100 and 200 mg/kg silibinin, respectively.	• s.c cell injections of 5 × 10^6 LoVo cells mixed with matrigel (1:1) in right flank of athymic BALB/c nu/nu male mice. • after 24 h of cell injections; oral gavage with silibinin in CMC vehicle, 5 days a week for 6 weeks. • daily dose: silibinin (100 and 200 mg/kg).	• PCNA positive cells ↓ • apoptotic cells ↑ • Kip/p27 protein levels ↑ • no effect on protein levels of CDK's, cyclins, and Cip/p21. • phosphorylation of Rb at Ser 795, Ser 807/811 sites and total Rb levels ↓	• Kaur *et al*. 2009[12]
SW480	• inhibits tumor growth and cell proliferation but induces apoptosis. • tumor volume (26% - 46% ↓) and tumor weight (29% - 52% ↓) decreased by 100 and 200 mg/kg silibinin, respectively. • inhibitory effects on β-catenin mediated signaling.	• s.c cell injections of 5 × 10^6 SW480 cells mixed with matrigel (1:1) in right flank of athymic BALB/c nu/nu male mice. • after 24 h of cell injections; oral gavage with silibinin in CMC vehicle, 5 days a week for 6 weeks. • daily dose: silibinin (100 and 200 mg/kg).	• PCNA positive cells ↑ • apoptotic cells ↑ • down regulation of β-catenin dependent signaling. • expression of β-catenin signaling associated molecules: c-myc, cyclin Dl, and CDK8 ↓	• Kaur *et al*. 2010[11]
SW480	• Protocol I: even after silibinin withdrawal, a decrease in tumor volume sustains. • Protocol II: inhibitory effect of silibinin observed on established tumors.	• s.c cell injections of 5 × 10^6 SW480 cells mixed with matrigel (1:1) in right flank of athymic BALB/c nu/nu male mice. • Protocol I: Silibinin fed to growing tumors, after 24 h cell injections for 28 days; after 28 days silibinin stopped but tumor study continued for more 21 days. • Protocol II: 25 days past cell injections, silibinin fed to mice with established tumors, and tumor study continued for 16 more days in presence of silibinin. • (silibinin daily dose in both protocols: 200 mg/kg).	• anti-proliferative, pro-apoptotic, and anti-angiogenic effects. • PCNA positive cells ↓ • apoptotic cells ↑ • expression of p-catenin and phospho- GSK-3β, cyclin Dl, c-myc, and survivin ↓ • VEGF, iNOS, and CD31 ↓	• Velmurugan *et al*. 2010[41]

Continued

Table 4

CRC cell lines	*In vivo* effects of silibinin	Animal model and treatment modality	Mechanism of action	Research group
Primary human tumor cells and HT- 29	• Silibinin treated cancer stem like cells showed decreased tumorigenicity in xenograft model.	Xenografts of: • primary tumor cells from Duke C3 CRC stage. • CRC cells from primary tumors in spheroid culture (or HT-29 cells) with and without silibinin treatment (5 μg/mL) for 15 days. • 10^6 to 2×10^6 spheroid culture enriched cells were silibinin treated prior to injections. • s.c cell injections of 5×10^3 cells done and tumor growth monitored for 6 weeks to 6 months.	• in vitro effect on cancer stem cells in spheroid culture inhibits tumorigenicity and tumor growth under *in vivo* conditions.	• Wang *et al*. 2012[20]
LoVo and SW480	• inhibitory effect on tumor growth and progression accompanied with anti-inflammatory effects. • NFκB activation and transcriptional activity ↓ • anti-inflammatory effect independent of COX-2 expression.	• Archived tumor tissues from LoVo and SW480 xenografts used[11-12]	• total p65 immunoreactivity score ↓ • NFκB transcriptional activity ↓ • levels of NFκB regulated molecules: cyclin Dl, Bcl-2, VEGF, MMP-9, and iNOS ↓	• Raina *et al*. 2013[45]
SW480	• induction of starvation induced autophagy.	• Archived tumor tissues from SW480 xenografts used[11].	• immunoreactivity score of SQSTM1 ↓ • SQSTM1 protein expression in tumor lysates ↓. [SQSTM1 is selective substrate of autophagy and its protein levels are decreased during starvation induced autophagy].	• Raina *et al*. 2013[13]

Abbreviations: CRC, Colorectal cancer; CMC, carboxymethyl cellulose; Rb, retinoblastoma. *silibinin-phytosome (from Indena, Italy) that contains silibinin and phosphatidylcholine in 1:2 ratio.

proliferation index and an increase in apoptotic index were observed in the polyps of silibinin treated mice, the normal crypt-villus regions of the intestine were not affected. Furthermore, our lab has also used a colitis-related AOM/DSS-induced colon tumorigenesis model to assess the role of inflammatory conditions on colon CSC generation and expansion, and their modulation by silibinin[21]. Our results have indicated the protective effect of oral silibinin feeding in this model as evidenced by the absence of large macroadenomas (>2-3 mm) in the colon. This effect was accompanied by minimal colonic inflammation (decrease in recruitment of inflammatory cells); tissue analysis indicated a decrease in the expression of pro-inflammatory cytokines, and associated transcription factors: STAT-3 and NF-kB levels in the colonic tissues (***Table 3***). A decrease in transformed stem cell population (for CSC pool expansion) was also identified (dual staining for BMI-1 and CD44) in the colonic tissues. Silibinin feeding has also shown significant preventive and/or therapeutic efficacy against human CRC xenograft tumors in athymic nude mice (***Table 4***)[11-12,27,41]. These studies indicate that not only has silibinin an inhibitory effect on tumor growth, but this inhibitory effect is sustained even after silibinin withdrawal; furthermore, silibinin has also shown significant efficacy against established xenograft tumors[41].

Conclusion

The summarized studies in four tables provide adequate evidence highlighting the potential of silibinin intake to significantly impact CRC growth and progression. These results also indicate silibinin potential to interfere with CSC pool expansion *via* targeting

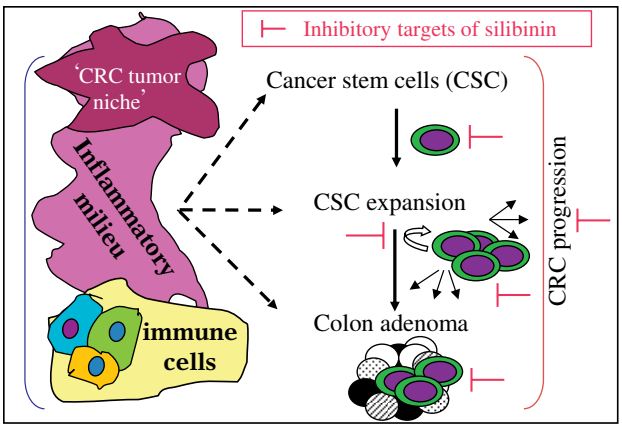

Fig.4 **Targeted proteins of silibinin in CRC.** The inflammatory milieu of the colorectal cancer stem cells (CSC) niche is an important growth regulator for both CSC and progenitor cell population. Silibinin has the potential to target colon CSC self-renewal and aberrant differentiation, and associated inflammatory niche during CRC inhibition.

both CSC, bulk daughter cells and their inflammatory niche, as well as associated signals involved with the survival and multiplication of colon CSC pool (***Fig. 4***); the various mechanisms involved in these inhibitory effects are: a) silibinin causes cell cycle arrest resulting in decreased proliferation, and induces multiple programmed cell death mechanisms; b) silibinin interferes with cellular metabolism to induce energy restriction like conditions; and c) silibinin inhibits various signaling and regulatory pathways involved in tumorigenesis, inflammatory responses, and angiogenesis.

Given that silibinin has the potential to target the CSC or the "tumor initiating cells", it can be beneficial for use in the early stages of CRC development, as well as

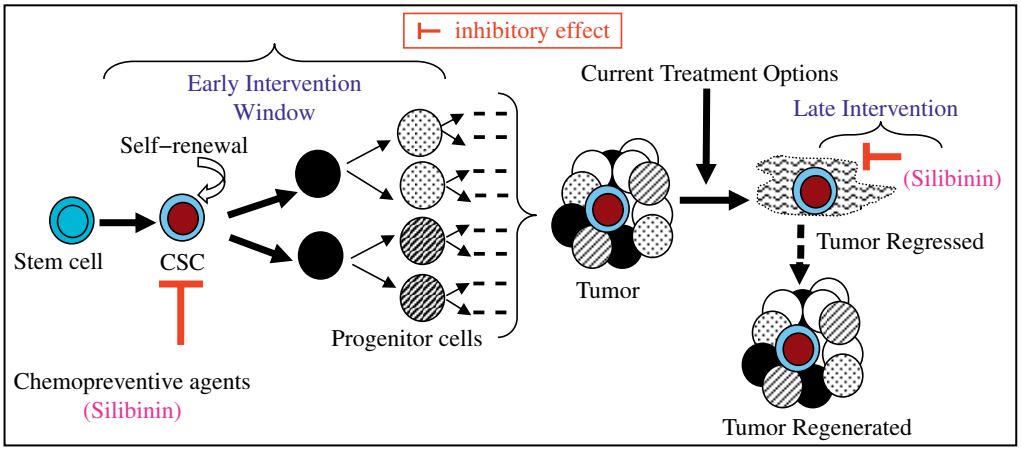

Fig. 5 **Stem cells or their progenitors transformed into colorectal cancer stem cells (CSC) are considered as major factors responsible for colorectal cancer (CRC).** Novel preventive and therapeutic strategies are needed to reduce CSC number, target their self-renewal capacity or rectify their aberrant differentiation, or interfere with the pro-tumorigenic signals arising in the colon 'niche' that affects CSC population. Silibinin acts as a 'double edged sword'-striking both CRC 'initiators' and the 'initiated' cells.

in later stages in cancer treatment to eradicate the CSC pool and bulk tumor cells to prevent cancer relapse (***Fig. 5***). Given the fact that silibinin consumption is exceptionally safe and that it has high bioavailability in colonic tissue of CRC patients, the efficacy evaluation of silibinin in clinical trials is advocated, both as a CRC preventive agent and as an 'adjunct therapy' for its clinical use in CRC cases which are incurable by current therapeutic modalities.

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