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Review

Anticancer Potential of Silymarin: From Bench to Bed Side

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Abstract. *Silymarin consists of a family of flavonoids (silybin, isosilybin, silychristin, silydianin and taxifoline) commonly found in the dried fruit of the milk thistle plant Silybum marianum. Although silymarin's role as an antioxidant and hepatoprotective agent is well known, its role as an anticancer agent has begun to emerge. Extensive research within the last decade has shown that silymarin can suppress the proliferation of a variety of tumor cells (e.g., prostate, breast, ovary, colon, lung, bladder); this is accomplished through cell cycle arrest at the G1/S-phase, induction of cyclin-dependent kinase inhibitors (such as p15, p21 and p27), down-regulation of anti-apoptotic gene products (e.g., Bcl-2 and Bcl-xL), inhibition of cell-survival kinases (AKT, PKC and MAPK) and inhibition of inflammatory transcription factors (e.g., NF-κB). Silymarin can also down-regulate gene products involved in the proliferation of tumor cells (cyclin D1, EGFR, COX-2, TGF-β, IGF-IR), invasion (MMP-9), angiogenesis (VEGF) and metastasis (adhesion molecules). The anti-inflammatory effects of silymarin are mediated through suppression of NF-κB-regulated gene products, including COX-2, LOX, inducible iNOS, TNF and IL-1. Numerous studies have indicated that silymarin is a chemopreventive agent in vivo against a variety of carcinogens/tumor promoters, including UV light, 7,12-dimethylbenz(a)anthracene (DMBA), phorbol 12-myristate 13-acetate (PMA) and others. Silymarin has also been shown to sensitize tumors to chemotherapeutic agents through down-regulation of the MDR protein and other mechanisms. It binds to both estrogen and androgen receptors, and down-regulates PSA. In addition to its chemopreventive effects, silymarin exhibits antitumor activity against human tumors (e.g., prostate and ovary) in rodents. Various clinical trials have indicated that silymarin is bioavailable and pharmacologically safe. Studies are now in progress to demonstrate the clinical efficacy of silymarin against various cancers.*

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Abbreviations: AAP, alanine-aminopeptidase; AAF, acrylamino-fluorene; ACTH, adrenocorticotropic hormone; ADP, adenosine diphosphate; Aica-P, 4,5-amino-imidazole-carboxamide-phosphate; AP, alkaline phosphatase; sASG, ascorbigen; AR, androgen receptor; B(α)P, benzo(α)pyrene; BPO, benzoyl peroxide; CAH, chronic active hepatitis; CAPD, continuous ambulatory peritoneal dialysis; CCl_4, carbon tetrachloride, CDK, cyclin-dependent kinase; CDDP, cisplatin; COX, cyclooxygenase; CP, cyclophosphamide; CYP, cytochrome P450; CV, cyclic voltammetry; DHT, dihydrotestosterone; DMBA, 7,12-dimethylbenz(α)anthracene; DNM, daunomycin; DPH, 16-diphenyl-1,3,5-hexan; DR, TRAIL death receptor; DTD, DT-diaphorase=NAD(P)H-quinone reductase; DOX, doxorubicin; EBO, extrahepatic biliary obstruction; EDC, ethanol-derived calories; EROD, ethoxyresorufin O-deethylase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GGTP, γ glutamyl transpeptidase; GSH, glutathione; GSHPx, glutathione peroxidase; GST, glutathione S-transferase; HaCaT, human epithelial cell; HD, high-dose; HbA1c, glycosylated hemoglobin; HFK, human foreskin keratinocyte; HMBC, heteronuclear multiple bond correlation; HMC, human mesangial cell; HOCl, hypochlorous acid; HUVEC, human umbilical vein endothelial cells; IAP, inhibitor-of-apoptosis protein; IBI/S, silybin-β-cyclodextrin; IGF-IR, insulin; IκB, inhibitory subunit of NF-κB; IKK, IκBα kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; HPLC, high performance liquid chromatography; OC, osteocalcin; PCA, prostate cancer; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; JNK, c-Jun NH_2-terminal kinase; LC/MS, liquid chromatography/mass spectrometry; LCK, log10 cell kill; LDH, lactate dehydrogenase; LOX, lipooxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase: MDA, malondialdehyde; MDR, multidrug resistance; MMP, matrix

metalloproteinase; MRP, multidrug resistance-associated protein; MTDQ, 6,6-methylene-bis(2,2,4-trimethyl)-1,2-dihydroquinoline; NAG, non-steroidal anti-inflammatory drug-activated gene-1; NBT, nitro blue tetrazolium; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; NQO, 4-nitroquinoline 1-oxide; NSAIDS, non-steroidal anti-inflammatory drugs; PARP, poly (ADP ribose) polymerase; P-gp, P-glycoprotein; PG, prostaglandin; Akt, protein kinase B; PIIINP, propeptide of procollagen; PMLNs, polymorphonuclear leukocytes; PMS, phenazine methoxysulfate; PSA, prostate-specific antigen; OA, okadaic acid; ODC, ornithine decarboxylase; 3-OH-BP, 3-OH-benzo(α)pyrene; QR, quinone reductase; Rb, retinoblastoma; ROS, reactive oxygen species; SAR, structure activity relationship; SDH, silibin dihemisussinate; SOD, superoxide dismutase; STAT, signal transducer and activator of transcription; TBARS, thiobarbituric acid reactive substance; T2DM, non-insulin-dependent diabetes mellitus; TGF, transforming growth factor; TNBF, trinitrobenzenesulphonic acid; TPA, 7,12-tetradecanoyl phorbol-13-acetate; TRAP, tartrate resistant acid phosphatase; UDPGA, UDP-glucuronic acid; UDP-GDH, UDP-glucose dehydrogenase; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor; VBL, vinblastin. Silibinin is also called silybin.

Contents

1. Introduction

It has been estimated that approximately 50% of all drugs approved by the U.S. Food and Drug Administration (FDA) are withdrawn within 5 years of approval, either because of lack of safety (*e.g.*, Vioxx) or lack of efficacy, despite the claim that, on average, it costs one billion dollars to develop a new drug. In addition to safety, the cost of a drug is a major consideration. For instance, a 10-month course of the colon cancer drug Avastin (anti-VEGF antibody) costs about $49,000; a 4-month course of another colon cancer drug, Erbitux (EGFR inhibitor), costs about $38,000; eight doses of etanercept (Enbrel; soluble TNF receptor) at 25 μg subcutaneously costs approximately $1,600; and eight doses of infliximab (Remicade; anti-TNF antibody) costs approximately $9,000. Most of these modern targeted drug therapies, especially those for cancer, have only a marginal effect on the overall survival of the patient.

Table I. *Comparison of cancers incidence between western (USA) and eastern (India and Japan) countries.*

Cancer (rates per 100,000)		United States	India	Japan
Prostate	(Male)	61.8	6.9	3.3
Breast	(Female)	89.2	24.6	5.8
Colon/rectum	(Male)	46.3	6.4	7.8
	(Female)	33.2	5.1	5.9
Lung	(Male)	64.3	14.0	27.7
	(Female)	29.9	3.0	7.7
Stomach	(Male)	8.0	7.3	40.8
	(Female)	3.5	4.3	19.0
Esophagus	(Male)	4.0	11.4	6.9
	(Female)	1.3	8.4	1.1

Source: Food, Nutrition and the Prevention of Cancer: a global perspective by the American Institute for Cancer Research, 1997.

Table II. *War on cancer.*

Cancer	Total deaths due to cancer in the USA	
	1971	2001
Male		
Lung	53,000	90,000
Colorectal	22,000	28,000
Prostate	17,000	32,000
Pancreas	10,000	14,000
CML	----	4,500
Female		
Lung	11,000	67,000
Colorectal	24,000	29,000
Breast	31,000	----

Table III. *Comparison of cancer incidence.*

	USA		India		Japan	
Cancer	Cases	Deaths	Cases	Deaths	Cases	Deaths
Oral cavity	50	11	102	60	29	12
Nasopharynx	4	2	4	3	3	2
Other pharynx	19	9	57	42	10	7
Esophagus	31	31	63	59	58	43
Stomach	56	34	43	39	489	225
Colon/rectum	356	139	40	26	342	143
Liver	30	31	17	16	186	146
Pancreas	72	68	11	11	76	71
Larynx	33	11	35	22	17	5
Lung	463	402	55	51	262	214
Melanoma of skin	113	21	3	1	3	2
Breast	914	212	191	99	314	77
Cervix uteri	78	33	307	174	111	30
Corpus uteri	155	20	17	5	45	13
Ovary *etc.*	106	62	49	29	66	37
Prostate	1,043	179	46	28	111	55
Testis	40	2	6	3	13	2
Bladder	144	28	20	16	56	17
Kidney *etc.*	86	31	8	6	42	19
Brain, nervous system	54	37	21	16	24	9
Thyroid	46	3	14	4	31	5
Non-Hodgkin's lymphoma	135	59	24	19	58	30
Hodgkin's diseases	22	4	8	4	3	1
Multiple myeloma	35	26	8	6	16	12
Leukemia	80	54	26	20	48	34
All sites but skin	3,223	1,391	1,017	688	2,230	1,213

Cases shown are after standardization with the world standard population, called world standardized incidence or mortality rate. It is also expressed per million. Ferlay J *et al.,* GLOBOCON 2000.

This history of drug development is also in contrast to cancer statistics from the World Health Organization (WHO) and other sources, indicating a higher incidence of cancer and more cancer-linked deaths than almost 30 years ago, when the "war on cancer" was initiated with the establishment of the National Cancer Institute (NCI) (Table I, II and III). These numbers convincingly emphasize that a change in the approach for preventing and treating cancer is required to combat this deadly disease. NCI has begun to realize that, in most instances, cancer is a preventable disease. Evidence is increasingly clear that consumption of red meat (1-3), tobacco and alcohol increase cancer incidence, whereas consumption of fruits and vegetables decreases the incidence. Accordingly, the NCI has revised its recommendations about the consumption of fruits and vegetables, from the initial five servings per day to eight servings. Which fruits and vegetables and how often to consume them are still unclear. Even less clear is how these fruits and vegetables might prevent or even treat cancer. The beneficial substances in these plant products and their molecular targets are also not well understood (4). Interestingly, however, reviews of the literature reveal that the active components of some foods, such as turmeric (curcumin) and red grapes (resveratrol), modulate the same multiple molecular targets, such as cyclooxygenase (COX)-2, vascular endothelial growth factor (VEGF), AKT, epidermal growth factor receptor (EGFR), signal transducers and activators of transcription (STAT) 3, tumor necrosis factor (TNF) and nuclear factor (NF)-κB, that pharmaceutical companies typically target with rational drug design (5, 6).

In this review, we describe silymarin, a compound from milk thistle plant (also called wild artichoke) that has an impressive potential against cancer (Figure 1). The focus of this review is: what silymarin is; how it inhibits different cell signaling pathways; what are the various molecular targets; how the compound inhibits proliferation, invasion and angiogenesis; how it can be used to prevent and treat cancer; and its pharmacokinetics, pharmacodynamics, tissue distribution, bioavailability and safety in humans. Silybin also has a potent antibacterial activity against Gram-positive bacteria (7), but such applications are beyond the scope of this review.

2. Extraction of silymarin

Silybin, isosilybin, silychristin, silydianin and taxifolin are the main active flavonoids, generally found in the dried fruits of *Silybum marianum*, also called milk thistle. The seeds contain flavonolignan and dihydroflavonol compounds that have interesting and important therapeutic activities. It is worth noting that silybin is mentioned in the literature under different names such as silybinin, silibinin and silibin. Wallace *et al.* have examined the extraction of silymarin from milk thistle (8). The maximum yields of taxifolin, silychristin, silydianin, silybinin A and silybinin B in ethanol were 0.6, 4.0, 0.4, 4.0 and 7.0 mg/g of defatted seed, respectively. They suggested that, if silybinin A is the diastereoisomer of choice, methanol would be the preferred extraction solvent because it yields the highest silybinin A to silybinin B ratio. Interestingly, lipid removal is an important extraction step, because defatted material yields twice the silymarin concentration. Barreto *et al.* used a hot water extraction procedure (9). After 210 minutes of extraction at 100˚C, the yield of taxifolin was 1.2 mg/g of seed, an about 6-fold increase over the results obtained in a Soxhlet extraction with ethanol on pretreated (defatted) seeds. Similarly, the yield of silychristin was 5.0 mg/g of seed, an about 4-fold increase. The yields of silybinin A and silybinin B were 1.8 and 3.3 mg/g of seed, respectively, roughly accounting for 30% of the Soxhlet yield. The ratios of the extracted compounds, and particularly the ratios at long extraction times, showed that the more polar compounds (taxifolin and silychristin) were preferentially extracted at 85˚C, whereas the less polar silybinin was favored at 100˚C.

Concentrations of these compounds, except taxifolin, are usually expressed together as silymarin content. Campodonico *et al.* have described a simple dissolution test developed to estimate silymarin in pharmaceutical formulations (10). Five commercial products were tested with this new method (including tablets, sugar tablets and capsules): two from Argentina, one from Brazil, one from Spain and one from Italy. The results demonstrated that, provided the dosage form disintegrate, the amounts dissolved ranged from 50 to 90% of the labeled value. The products were analyzed by high-performance liquid chromatography (HPLC) and UV spectrophotometry.

Moreover, hot water has been attracting attention as an extraction solvent for the recovery of compounds from plant material as the search for milder and "greener" solvents intensifies. The use of hot water as an extraction solvent for milk thistle at temperatures above 100˚C was explored by Duan *et al.* They observed that the maximum extraction yield of each of the silymarin compounds and taxifolin did not increase with rising temperatures, most probably because of the degradation of the compounds. However, the time required for the yields of the compounds to reach their maximum was reduced from 200 to 55 minutes when the extraction temperature was increased from 100 to 140˚C. Severe degradation of silymarin compounds was observed, with first-order degradation kinetics at 140˚C (11).

3. Structure and formation of silymarin

Abrol's group incorporated and evaluated silymarin in lipid microstructured systems (12). Various constituents of lipid microspheres – namely, the internal oily core, surfactants such as soybean lecithin and cosurfactants such as span 20, tween 20, tween 80 and propylene glycol – were tried in different concentrations to optimize the final formulation characteristics, such as globule size range, structural integrity, sustainability and drug-holding capacity percent. The final formulation was characterized with respect to size and morphology using transmission electron microscopy and a laser diffraction technique. The enhanced mean release percent of 57% was observed in 36 hours with silymarin-loaded lipid microspheres, as compared to 19% with silymarin solution. These findings suggested that a stable delivery system with increased hepatoprotective effects of silymarin and soybean lecithin could be produced for passive targeting of the liver.

Lee and Liu examined the molecular structure and stereochemistry of silybin A, silybin B, isosilybin A and isosilybin B (18) (Table IV; Figure 2). Two pairs of diastereoisomeric flavonolignans, silybin A and silybin B, isosilybin A and isosilybin B, were separated from *Silybum marianum* by sequential silica gel column chromatography, preparative reversed-phase HPLC and recrystallization. The complete stereochemical assignments at C-2, C-3, C-7' and C-8' of these flavonolignans have been determined. The stereochemistry of these diastereoisomers was determined as: silybin A, 2R, 3R, 7'R, 8'R; silybin B, 2R, 3R, 7'S, 8'S; isosilybin A, 2R, 3R, 7'R, 8'R; and isosilybin B, 2R, 3R, 7'S, 8'S, by X-ray crystallographic analysis and optical rotation data, coupled with comprehensive ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data interpretation, including COSY, HMQC and HMBC. The content and composition of the main silymarin components (silybin, isosilybin, silydianin and silychristin) in various pharmaceuticals have also been analyzed using HPLC and the newly-developed capillary zone electrophoresis method (19).

Reversed-phase liquid chromatography (RP-LC) and liquid chromatography/mass spectrometry (LC/MS) have been used to separate and quantitatively determine silymarin components, as well as two diastereoisomers of silybin and isosilybin (15). Using mobile phase: methanol and solvent mixture (water:dioxane = 9:1) by gradient; flow rate: 1.5 ml/min; column temperature: 40˚C; detector wavelength: 288 nm, the recovery of 99.66% for silychristin, 99.48% for silydianin, 100.0% for silybin and 98.72% for isosilybin have been reported (15).

Analogs of silymarin. Silymarin, isolated from *Silybum marianum*, is a mixture of three isomers: silybin, silydianin and silychristin. Silybin is the most active antihepatotoxic agent and contains a 1,4-dioxane ring in addition to a flavonoid moiety. Based on the skeleton of silybin, Ahmed's group prepared some flavones and coumarins containing the 1,4-dioxane ring system and evaluated them for antihepatotoxic activity against carbon tetrachloride-induced hepatotoxicity in albino rats (13). The compounds 3',4'(1'',4''-dioxino) flavone and 3',4'(2-hydroxy methyl, 1'',4''-dioxino) flavone exhibited an activity comparable to that of the standard drug silymarin (silybon-70). Other compounds also exhibited good activity. In structure activity relationship (SAR) studies, the flavonoid analogs containing a hydroxy methyl group at position-2" in the dioxane ring exhibited superior antihepatotoxic activity in comparison to the coumarin derivatives. Hypolipidemic silybin analog 3',4'-ethylenedioxyflavonoids (14) and various types of silybin analogs have been synthesized for pharmacological investigation. Many of the benzodioxane analogs of chalcones, chalcone epoxides, isoflavones, flavanones and aurones have been found to possess hypolipidemic activity. Silybin 11-O-phosphate 3 was synthesized by selective phosphorylation of silybin with $POCl_3$ (15). The pharmacological activity was evaluated in the rat by the praseodymium poisoning test. The compound showed antihepatotoxic activity, however the potency was lower compared to the reference drug silybin hemisuccinate. By selective oxidation process, Gazak *et al.* prepared carboxylic acid derivatives of silybin and 2,3-dehydrosilybin with improved water solubility (20). Dehydrogenation at C(2)-C(3) in flavonolignans (silybin *vs.* 2,3-dehydrosilybin; silybinic acid *vs.* 2,3-dehydrosilybinic acid) strongly improved the antioxidative properties (analogously to the flavonoids taxifolin *vs.* quercetin). The antioxidative property of dehydrosilybin was superior to silybin by one order, but its water solubility was too low for application in aqueous milieu. On the other hand, 2,3-dehydrosilybinic acid was a relatively soluble derivative with antilipoperoxidation and antiradical activities superior to those of silybin.

Recently, Davis-Searles *et al.* identified seven distinct flavonolignan compounds and a flavonoid from commercial silymarin extracts (21). Most notably, two pairs of diastereomers, silybin A and silybin B and isosilybin A and isosilybin B, were among those compounds. Silybin is composed only of a 1:1 mixture of silybin A and silybin B. Among these, relative to either the pure constituents or the commercial extracts, isosilybin B was the most potent suppressor of cell growth of human prostate cancer cells. Isosilybin A and isosilybin B also were the most effective suppressors of prostate-specific antigen (PSA) secretion by androgen-dependent LNCaP cells. Silymarin and silybin were shown to suppress the activity of the DNA topoisomerase II alpha gene promoter in DU145 cells, and among the pure compounds, isosilybin B was again the most effective. Isosilybin B accounts for no more than 5% of silymarin and is absent from silybin. It was suggested that preparations enriched with isosilybin B, or isosilybin B alone, might possess relatively improved biological activity.

4. Antioxidant effects of silymarin

4A. Silymarin mediates antioxidant effects in vitro. Like curcumin, resveratrol, and other phytochemicals, silymarin exhibits potent antioxidant activity (Table V). Silybin has been reported to exert antioxidant and free-radical scavenging activities through a variety of mechanisms and in a variety of systems. Work from our laboratory has shown that silymarin suppresses the TNF-induced production of reactive oxygen intermediates and lipid peroxidation (22). Basaga *et al.* examined the free-radical scavenging and antioxidative properties of silybin complexes on microsomal lipid peroxidation (23). They evaluated the antioxidant properties of silybin complexes, the water-soluble form silybin dihemisuccinate (SDH), the lipid-soluble form and the silybin-phosphatidylcholine complex (IdB 1016), according to their ability to react with the superoxide radical anion (O_2^-) and the hydroxyl radical (OH•). The superoxide radicals were generated by the PMS-NADH system and measured for their ability to reduce nitro blue tetrazolium (NBT). The IC_{50} concentrations sufficient to inhibit the NBT reduction by SDH and IdB 1016 were 25 μM and 316 μM, respectively. Both silybin complexes inhibited xanthine oxidase activity. SDH reacted rapidly with OH• radicals and chelated Fe^{2+} in solution. In hepatic microsomes, SDH showed relatively better inhibition of Fe^{2+}-induced lipid peroxidation and xanthine oxidase activity, whereas IdB 1016 exerted a better protective effect when lipid peroxidation was induced by CuOOH. In both pulmonary and hepatic microsomal systems, lipid peroxidation proceeded through a thiol-depletion mechanism that could be restored in the presence of silybin complexes. This study suggested that silybin complexes, with increased bioavailability, have better free-radical scavenging and antioxidative properties.

In another study, the free radical scavenging and anti-lipoperoxidation properties of silybin glycosides (silybin galactoside, glucoside, lactoside and maltoside) were investigated (24). These glycosides were found to be weaker electron donors than silybin, but were more potent scavengers of the 1,1-diphenyl-2-picrylhydrazyl and the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)-derived radicals. These glycosides were more efficient than silybin in preventing tert-butylhydroperoxide-induced lipoperoxidation of rat liver mitochondrial membranes and damage of rat erythrocytes and primary hepatocytes in culture. All together, these results suggested that silybin glycosides are soluble derivatives of silybin, and might have therapeutic potential

Figure 1. *Silymarin extracted from Silybum marianum.*

against oxidative damage. Silymarin's effects on oxygen radical-generated DNA damage in human lymphocytes have been investigated with the COMET assay (25). Hydrogen peroxide (H_2O_2) produced clear dose-related responses, and silymarin was protective against its effects.

Lang *et al.* examined the effect of silymarin on superoxide dismutase (SOD) activity and expression *in vitro* in erythrocytes and lymphocytes from patients with chronic alcoholic liver disease (26). *In vitro* incubation with the agent in a concentration corresponding to the usual therapeutic dosage markedly increased both SOD expression and SOD activities in lymphocytes. Muzes *et al.* also investigated the effect of silymarin on *in vitro* activity and expression of the SOD enzyme in erythrocytes and lymphocytes from patients suffering from chronic alcoholic liver disease and from healthy controls (27). An *in vivo* achievable concentration

Table IV. *Silymarin and its analogs.*

Natural
Silybin A,B (13)
Isosilybin A,B (13)
Silychristin (13)
Isosilychristin (13)
Silydianin (13)
Isosilydianin (13)
Taxifoline (13)
Synthetic
3,4'-ethylene-3-dioxyflavonoids (14)
Silybin-C-2,3-dihydrogensuccinate disodium salt 5,7,4''-trimethylsilybin (15)
Peracetylsilybin (15)
Peracetyl-5-,7,4''-trimethylsilybin (15)
Silybin dihemisuccinate (15)
*Silipid (15)
IBI/S (silybin-β-cyclodextrin) (16)
Silybin-β-galactoside (14)
Silybin-β-glucoside (14)
Silybin-β-maltoside (14)
Silybin-β-lactoside (14)
IdB1016 (silybin-phosphatidylcholine complex) (17)

*Silipid is a silybin-phosphatidylcholine complex.

(10 μg/ml) of silymarin significantly increased the SOD activity of both the erythrocytes and lymphocytes of patients with liver disease, whereas the SOD expression of the lymphocytes was enhanced considerably. These studies indicate the free radical scavenger role of silymarin in its antioxidant protection of cells.

Wenzel *et al.* showed that high glucose-induced alterations of matrix protein fibronectin (FN) turnover in human mesangial cell (HMC) cultures are suppressed by silymarin (28). The high glucose concentration resulted in an extracellular accumulation of FN *via* an expansion of the matrix-associated pericellular FN and an increase of the soluble molecule in the culture medium. The high glucose-induced FN alterations were not due to osmotic effects, but mediated by oxygen-free radicals as it was prevented by either a water-soluble derivative of silybin, silybin-C-2,3-dihydrogensuccinate disodium salt or a radical scavenger cocktail. Incubation of HMC with high glucose resulted in an increased malondialdehyde formation, which was completely blocked by either silybin or the radical scavenger cocktail. This study suggests that high glucose concentrations induce oxidative stress and, therefore, silybin could play a role in the amelioration of glucose cytotoxicity in renal cells.

Varga *et al.* showed inhibition of superoxide anion release and hydrogen peroxide formation in PMA-stimulated PMNLs by flavonolignans mixture (Legalon: *silybin*, isosilybin, silydianin and silychristin) and derivatives of silybin, as well as vitamin E (50). They reported the inhibitory effect of flavonolignans on O_2^- release in the order of 5,7,4''- trimethylsilybin vitamin E > Legalon ≥ peracetylsilybin > silybin > peracetyl-5,7,4''-trimethylsilybin. A similar order of the inhibitory effect of flavonolignans was also observed on H_2O_2 formation. Here, it should be noted that the trimethyl derivative of silybin has a greater inhibitory effect than silybin itself, suggesting that the antioxidant efficacy could be dependent on the lipophilicity of the molecules. However, this is underscored by the fact that paracetylation of all the hydroxyl groups in silybin halted the molecule's antioxidant activity.

Iron overload is known for inducing oxidative stress. In this regard, Borsari *et al.* showed that silybin is an iron-chelating agent (53). The formation of the complex silybin-Ga (III) in anhydrous dimethyl sulfoxide (DMSO)-d6 has been studied by ^1H nuclear magnetic resonance (NMR) spectroscopy. The formation of a silybin-Fe(III) complex has also been confirmed by mass spectrometry and infrared spectroscopy. Silybin was found to bind Fe(III) even at acidic pH. Different ternary complexes were observed at increasing methoxide ion concentrations. Therefore, a possible role for silybin in the chelation therapy of chronic iron overload, as occurs in the treatment of Cooley's anemia, is expected.

Valenzuela *et al.* examined a differential effect of SDH on rat liver microsomal oxygen consumption and on lipid peroxidation induced by NADPH-Fe^{2+}-ADP and *t*-butyl hydroperoxide (54). These results were ascribed to the antioxidant properties of the flavonoid. The differences observed in the effects of the catalysts may be a consequence of the different capacity of silybin to act as a scavenger of free radicals formed by NADPH-Fe^{2+}-ADP or *t*-butyl hydroperoxide. Koch *et al.* showed an inhibitory effect of silymarin on lipid peroxidation in human platelets through the thiobarbituric acid method (37). Psotova *et al.* reported that silymarin and its flavonolignans inhibited doxorubicin-iron-induced lipid peroxidation in rat heart microsomes and mitochondria (47). These studies suggested that silymarin could prevent doxorubicin and iron-mediated damage of membrane primarily through a free-radical scavenging mechanism.

Dehmlow *et al.* examined the scavenging of reactive oxygen species (ROS) and inhibition of arachidonic acid metabolism by silybin in human platelets, white blood cells and endothelial cells (52). Silybin proved to be a strong scavenger of hypochlorous acid (HOCI) (IC_{50} 7 μM), but not of O_2^- (IC_{50} >200 μM) produced by human granulocytes. The formation of leukotrienes *via* the 5-lipooxygenase pathway was also strongly inhibited. Unlike these findings, a 3- to 4-fold silybin concentration was needed to half maximally inhibit the cyclooxygenase pathway. For prostaglandin E_2 (PGE_2) formation by human monocytes, an

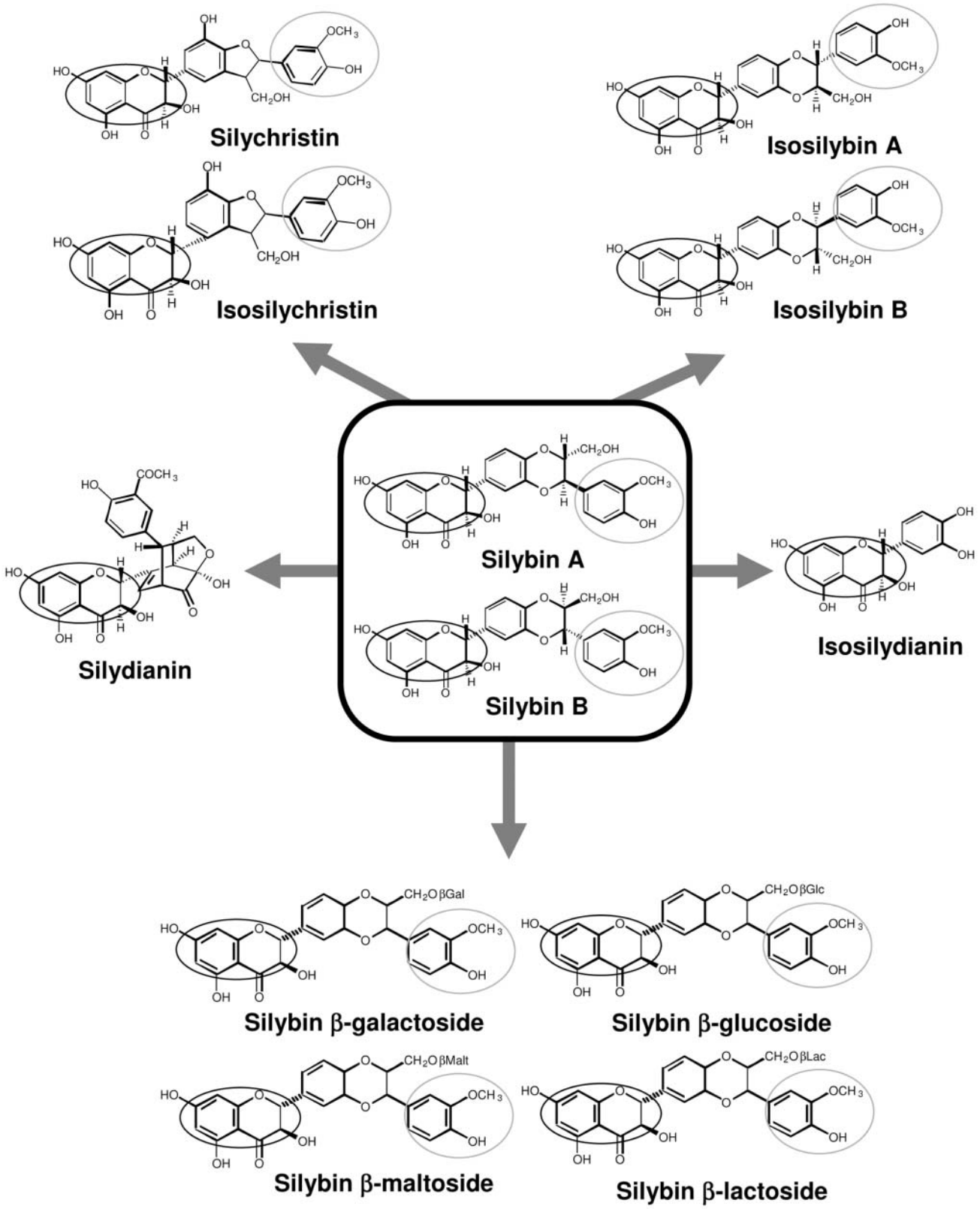

Figure 2. *Structure of silybin and its analogs.*

Table V. *Antioxidative effect of silymarin.*

Patients with alcoholic cirrhosis (29) ↑GSH
Hepatic oxidative stress in rats with biliary obstruction (30) ↑GSH transferase
Acetoaminophen-induced liver damage in rats (31) ↑GSH-PX
Paracetamol-induced liver damage in rats (32) ↑GSH-PX
Doxorubicin induced-oxidative stress (33) ↑GSH-PX
Paracetamol induced-oxidative stress (33) ↑GSH-PX
Benzoyl peroxide-induced oxidative stess in SENCAR mouse skin (34) ↑GSH-PX
Radiation-induced hepatotoxicity (35) ↑GSH-PX
Alloxan-induced diabetes in rat pancreas (36) ↑GSH-PX
Hepatic oxidative stress in rats with biliary obstruction (30) ↑GSH-PX
Acetoaminophen-induced liver damage in rats (31) ↑GR
N-ethyl maleimide-induced lipid peroxidation in human platelets (37) ↑GR
Acetoaminophen-induced liver damage in rats (31) ↓Lipid peroxidation
CCl_4-induced lipid peroxidation in mice (38) ↓Lipid peroxidation
CCl_4-induced membrane lipid alternations (39) ↓Lipid peroxidation
CCl_4-induced lipid peroxidation in rats (40) ↓Lipid peroxidation
Paracetamol-induced liver damage in rats (32) ↓Lipid peroxidation
ADP/Fe^{2+} and NADPH generated damage in rat liver microsomes and freshly isolated hepatocytes (31) ↓Lipid peroxidation
Cyclosporin-induced liver damage in rats (41) ↓Lipid peroxidation
Alloxan-induced diabetes mellitus in rats (42) ↓Lipid peroxidation
Patients with alcoholic cirrhosis (43) ↓Lipid peroxidation
Hepatic oxidative stress in rats with biliary obstruction (30) ↓Lipid peroxidation
Alloxan-induced diabetes in rat pancreas (36) ↓Lipid peroxidation
Alcohol-induced hepatic fibrosis in baboons (44) ↓Lipid peroxidation
High-sucrose diet-induced oxidative damage (45) ↓Lipid peroxidation
Iron-induced accumulation of malondialdehyde in mitochondria (46) ↓Lipid peroxidation
Fe^{3+}/ascorbate-induced rat liver microsome lipid peroxidation (43) ↓Lipid peroxidation
Fe^{2+}-induced hepatic microsomal lipid peroxidation (23) ↓Lipid peroxidation
CuOOH-induced hepatic microsomal lipid peroxidation (31) ↓Lipid peroxidation
Tert-butylhydroperoxidase-induced lipoperoxidation of rat liver mitochondrial membranes (24) ↓Lipid peroxidation
Doxorubicin-iron-induced lipid peroxidation in rat heart microsomes (47)↓Lipid peroxidation
Ischemia-reperfusion stress in rat kidney (48) ↓Lipid peroxidation
LPS-stimulated macrophages (49) ↓Lipid peroxidation
LPS-stimulated macrophages (49) ↓NO
LPS-stimulated RAW 264.7 cells (49) ↓iNOS
Oxidative burst of PMA-stimulated human PMNLs (50) ↓H_2O_2
Lymphocytes from patients with chronic liver disease (26) ↓H_2O_2
Erythrocytes from patients with alcoholic cirrhosis (51) ↑SOD
Erythrocytes and lymphocytes of patients with chronic alcoholic liver diseases (27) ↑SOD
Doxorubicin-induced oxidative stress (33) ↑SOD
Paracetamol-induced oxidative stress (33) ↑SOD
Benzoyl peroxide-induced oxidative stess in SENCAR mouse skin (34) ↑SOD
Alloxan-induced diabetes in rat pancreas (42) ↑Catalase
Alloxan-induced diabetes in rat pancreas (42) ↓HOCl
Human granulocyte-produced HOCl (52) ↓DNA damage
H_2O_2-generated DNA damage in human lymphocytes in the COMET assay (25) ↓Xanthine dehydrogenase / xanthine oxidase ratio
Oxidative burst of PMA-stimulated human PMNLs (50) ↓Xanthine dehydrogenase / xanthine oxidase ratio
Oxidative burst of PMA-stimulated human PMNLs (50) ↓O_2^-
PMS-NADH system in hepatic microsome (32) ↓O_2^-

Silymarin consists of silybin, isosilybin, silychristin and silydianin. ADP, adenosine diphosphate; SOD, superoxide dismutase; GSH, glutathione; GSH-PX, glutathione peroxidase; iNOS, inducible NO synthase; CCl_4, carbon tetrachloride, O_2^-, superoxide anion radical; H_2O_2, hydrogen peroxide; PMA, 12-myristate 13-acetate; PMLNs, polymorphonuclear leukocytes; PMS, phenazine methoxysulfate; HOCl, hypochlorous acid; GR, glutathione reductase; LPS, lipopolysaccharide. References in brackets.

IC_{50} value for 45 μM silybin was found. IC_{50} values of 69 μM and 52 μM silybin were determined for the inhibition of thromboxane (TXB)2 formation by human thrombocytes and of 6-K-prostaglandin F (PGF)1α formation by human omentum endothelial cells, respectively. This study suggested that clinically relevant doses of silybin can protect against the deleterious effects of HOCI, which can lead to cell death, and those of leukotrienes, which are especially important in

inflammatory reactions. Furthermore, these effects may contribute to the cytoprotective activities of silymarin in many organs and tissues, including liver.

The antioxidant effects of silymarin are beneficial in most situations, but they could be harmful under some circumstances. For instance, acetaminophen hepatotoxicity is characterized by glutathione depletion and the formation of the reactive electrophilic metabolite N-acetyl-*p*-benzoquinone imine. The induction of oxidative stress, expressed as lipid peroxidation, is controversial in acute acetaminophen intoxication. Garrido *et al.* showed that silymarin potentiates the acetaminophen-induced toxicity in isolated rat hepatocytes (55). One proposed explanation is that when hepatocytes are incubated with a high concentration of acetaminophen, the drug may accumulate in the cells because of saturation and/or inhibition of detoxification pathways (as in the case of silybin). Under these conditions, hepatocyte oxidative stress may be inhibited because of the antioxidant behavior of acetaminophen.

Acetaminophen, a safe analgesic when dosed properly but hepatotoxic at overdoses, has been reported to induce DNA strand-breaks, but it is unclear whether this event precedes hepatocyte toxicity or is only obvious in cases of overt cytotoxicity. Moreover, it is not known whether ROS formation is involved in the formation of the DNA strand-breaks. Lewerenz *et al.* showed that silymarin protect primary rat hepatocyte cultures from acetaminophen-induced DNA strand-breaks, but not from acetaminophen-induced cytotoxicity. A non-cytotoxic concentration of silybin (25 μM) was unable to inhibit toxicity at any acetaminophen concentration, but completely prevented DNA strand-breaks. Altorjay *et al.* examined the effect of Legalon (silybin) on Adriblastina (doxorubicin) and paracetamol-induced toxicity in human erythrocytes (33). They found that Adriblastina enhances the lipid peroxidation of the membrane of red blood cells, while paracetamol causes significant depletion of the intracellular glutathione level, decreasing the free-radical-eliminating capacity of the glutathione peroxidase system. Legalon, on the other hand, increases the activity of both superoxide dismutase and glutathione peroxidase, suggesting the protective effect of the drug against free radicals and the stabilizing effect on the red blood cell membrane, as shown by the longer increase in time for full hemolysis.

Mira *et al.* investigated the scavenging of ROS by SDH by studying the drug's ability to react with relevant biological oxidants, such as $O2^{-}$ H_2O_2, HO• and HOCl (56). SDH was not a good scavenger of $O2^{-}$ and no reaction with H_2O_2 was detected (56). However, SDH reacts rapidly with HO• radicals in free solution and appears to be a weak iron ion chelator. SDH, at concentrations in the micromolar range, protected against HOCl-induced inactivation of alpha 1-antiproteinase, indicating its potent scavenger activity for this oxidizing species. Luminol-dependent chemiluminescence induced by HOCl was also inhibited by SDH. Further, SDH inhibited lipid peroxidation induced by Fe^{3+}/ascorbate in rat liver microsome.

Human adrenocortical cells from different adrenal disorders exhibit pathologically-altered corticosteroid synthesis, and free-radical mechanisms may induce pathological changes in the activities of corticosteroid biosynthetic enzymes (cytochrome P-450). Racz *et al.* examined silybin's effect on basal and adrenocorticotropic hormone (ACTH)-stimulated secretion of several corticosteroids in isolated adrenal cells from aldosterone-producing, adenoma-atrophied adrenal tissues surrounding the adenoma and hyperplastic adrenals of patients with Cushing's syndrome (57). A high concentration (100 μM) of silybin inhibited the secretion of basal aldosterone, corticosterone, cortisol, 18-OH-corticosterone and 11-deoxycorticosterone. In contrast, a low concentration (0.01 μM) of silybin, which failed to produce a clear effect on basal corticosteroid secretion, resulted in the potentiation of ACTH-stimulated secretion of several corticosteroids in the adenomatous and hyperplastic adrenocortical cells. This study suggests that the dose-dependent dual effect of silybin on corticosteroid secretion could be due to the changes in the activities of cytochrome P-450 enzymes. They also suggest that stimulation of ACTH-induced corticosteroidogenesis by silybin could be due to the antioxidant property of the drug.

Mercury is a highly toxic heavy metal, and exposure to mercury in humans and animals causes damage to several organs or systems, including the immune system. Kim *et al.* examined silymarin's effect on the cytotoxicity of inorganic mercury in murine T lymphoma (EL4) and B-lymphoma (A20) cell lines (58). Mercury decreased the cell viability, membrane integrity and proliferation in a dose-dependent manner in both EL4 and A20 cells, which were accompanied by an increased ROS production. Silymarin decreased mercury-induced ROS generation and lactate dehydrogenase (LDH) release, and also inhibited mercury-induced cytotoxicity involving both apoptosis and necrosis. The cellular thiol level is an important reserve in maintaing the redox state of the cell. In this regard, silymarin was shown to restore the cellular thiol status of peritoneal macrophages from continuous ambulatory peritoneal dialysis (CAPD) patients (59).

4B. Silymarin mediates antioxidant effects in vivo. Numerous studies suggested that silymarin is a potent antioxidant, not only *in vitro,* but also *in vivo* (Table V). Halim *et al.* found that silymarin protects rats from liver injury (40). The researchers used rats chronically intoxicated with carbon tetrachloride (CCl_4) as a model of liver injury, terminating with fibrosis or cirrhosis. Oral silymarin (30 mg/kg) ameliorated the necrotic and fibrotic changes caused by CCl_4. Soto *et al.* showed that silymarin increased antioxidant

enzymes in alloxan-induced diabetes mellitus in rat pancreas (36). It has been suggested that silymarin's protection against pancreatic damage induced by alloxan is attributable to increased activity of antioxidant enzymes that, in addition to the glutathione system, constitute the more important defense mechanisms against free-radical damage.

Hepatic iron toxicity may be mediated by free-radical species and lipid peroxidation of biological membranes. Pietrangelo *et al.* demonstrated the antioxidant activity of silybin *in vivo* during long-term iron overload in rats (46). The rats were fed a 2.5% carbonyl-iron diet and silybin at 100 mg/kg of body weight per day. The iron overload caused a dramatic accumulation of malondialdehyde-protein adducts in iron-filled periportal hepatocytes, which was appreciably decreased by silybin treatment. Silybin also decreased the iron-induced accumulation of malondialdehyde in mitochondria. The mitochondrial energy wasting and tissue adenosine triphosphate depletion induced by iron overload were successfully counteracted by silybin. The *in vivo* protective effect of silybin against iron-induced hepatic toxicity was related to its prominent antioxidant activity. Feher's *et al.* examined the effect of silymarin on the expression and activity of SOD enzyme in erythrocytes from patients with alcoholic cirrhosis (51). *In vivo* treatment with silymarin increased the SOD expression of lymphocytes, as well as erythrocyte and SOD activity in lymphocytes.

Silipide is a new 1:1 complex of silybin with phosphatidylcholine. Comoglio *et al.* examined the scavenging effect of silipide on ethanol-induced free radicals (60). They found that silipide decreased the spin trapping of hydroxyethyl radicals in microsomes from chronically alcohol-fed rats. Further *in vivo* experiments have shown that silipide administration also decreased detectable hydroxyethyl radical signals detectable in the bile of rats acutely treated with ethanol. Silipide's ability to scavenge ethanol-derived radicals, along with its antioxidant activity, suggests its usefulness in counteracting free-radical-mediated injuries involved in alcohol-related liver damage. Soto *et al.* studied silymarin's effects on rat pancreas and its effects on pancreatic, hepatic, and blood glutathione (GSH) and pancreatic malondialdehyde concentrations in response to alloxan (36). Silymarin itself increased the pancreatic and blood GSH without having any effect on the hepatic GSH or blood glucose level. Silymarin prevented the alloxan-induced increase in lipid peroxidation and plasma glucose level. The investigators also suggested that silymarin had a protective effect against pancreatic damage in experimental diabetes mellitus. This may be related to its antioxidative properties and to increased concentrations of plasma and pancreatic glutathione. Gonzalez-Correa *et al.* investigated the effects of silymarin MZ-80 on hepatic oxidative stress in rats with biliary obstruction (30). Their studies were designed to evaluate the effects of three pharmaceutical forms of silymarin (silymarin MZ-80, silybinin-β-cyclodextrin and silybinin) on the oxidative status of the liver *in vitro* and after oral administration to rats with extrahepatic biliary obstruction (EBO). All three compounds inhibited the production of thiobarbituric acid reactive substance (TBARS) *in vitro,* as well as in EBO rats. Silymarin MZ-80 was most effective in increasing GSH peroxidase and GSH transferase activities and the GSH level. This study suggested the antilipoperoxidation activity of these three silymarin derivatives, from which silymarin MZ-80 also enhanced the GSH antioxidant system.

4C. Silymarin inhibits lipid peroxidation. Silymarin and its derivatives have been shown to inhibit lipid peroxidation in many systems, as mentioned above in different sections. Bosisio *et al.* examined the effect of several *flavonolignans* present in silymarin (silychristin, silydianin, silybin and isosilybin) on lipid peroxidation in rat liver microsomes and freshly isolated hepatocytes (61). In microsomes, lipid peroxidation was generated by ADP/Fe^{2+} and NADPH, which was inhibited by all these *flavonolignans* in a concentration-dependent manner. In hepatocytes, lipid peroxidation was induced by the ADP/Fe^{3+} complex, and cell damage was evaluated as LDH activity released in the medium. *Flavonolignan's* inhibition of the peroxidative process was also evident in this model, even though the potency order differed from that found in microsomes. In contrast, the effect on LDH release was significant only for silybin and isosilybin regarding this parameter. Rastogi *et al.* also showed that silymarin can inhibit aflatoxin B(1)-induced lipid peroxidation in rat liver and kidney (62).

4D. Silymarin lowers serum low-density lipoprotein cholesterol (LDL). Skottova *et al.* showed that dietary silymarin improves LDL levels in the perfused rat liver when administered to rats given a high-cholesterol diet (63). Silymarin normalized the removal of LDL from the medium during recirculating perfusion of the rat livers. The investigators suggested that silymarin's improvement of LDL removal by the liver contributes to its cholesterol-lowering effects.

5. Anti-inflammatory effects of silymarin

5A. Silymarin inhibits proinflammatory pathways. Numerous lines of evidence indicate that silymarin is a potent anti-inflammatory agent (Figure 3). Evidence of silymarin's anti-inflammatory effects includes its antioxidant activity, ability to suppress inflammatory cytokines, suppression of COX-2 and 5-lipooxygenase (LOX), and its ability to suppress NF-κB activation. De La Puerta *et al.* examined the effect of silymarin on different acute inflammation models and on leukocyte migration (64). In carrageenan-induced paw edema in rats, oral silymarin reduced the food-pad abscesses (ED_{50} = 62.42

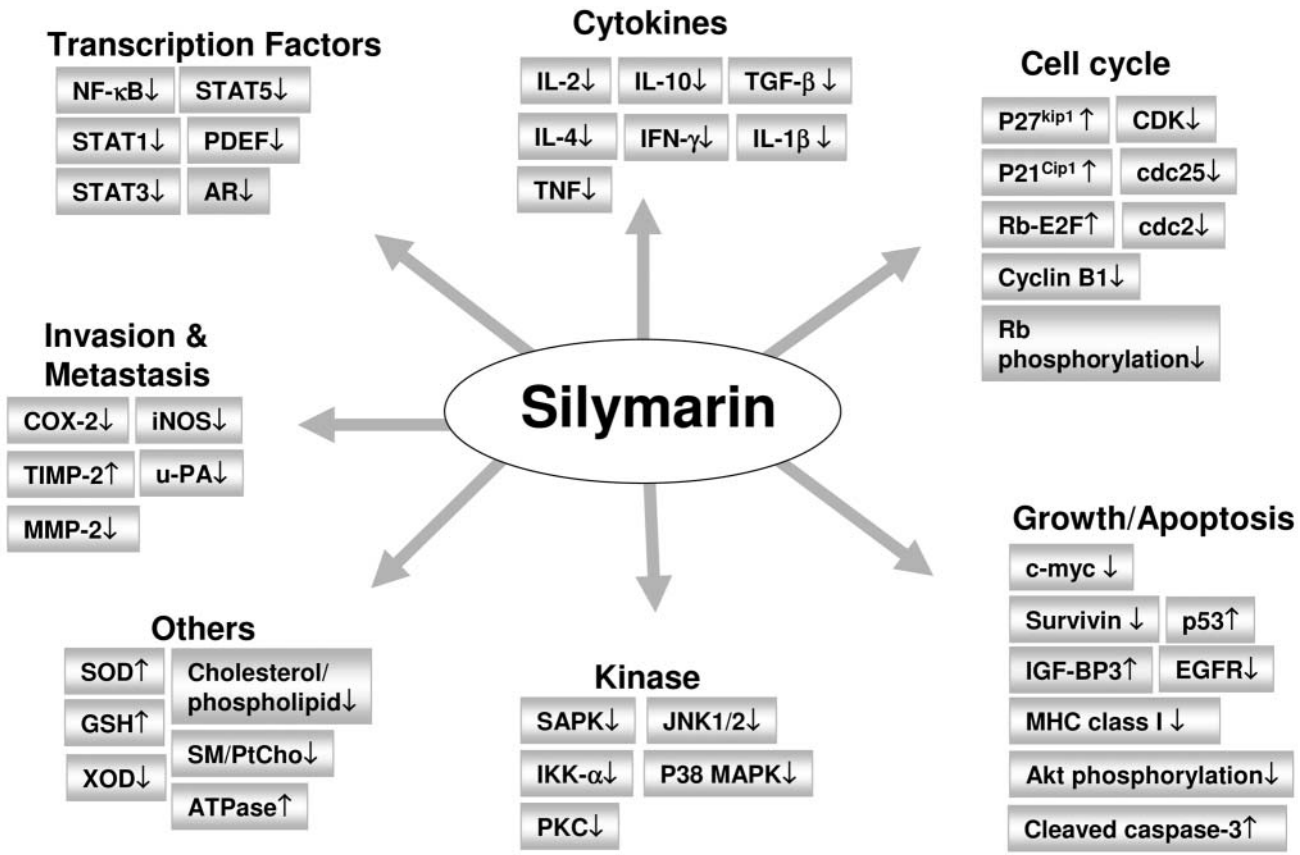

Figure 3. *Molecular targets of silymarin.*

mg/kg). In xylene-induced ear inflammation in mice, silymarin applied topically was more effective than silymarin administered intraperitoneally, with effects comparable to those of indomethacin. Silymarin also inhibited carrageenan-induced leukocyte accumulation in inflammatory exudates in mice, and significantly reduced the number of neutrophils. An inflammatory response in the central nervous system mediated by activation of microglia is a key event in the early stages of the development of neurodegenerative diseases. Wang *et al.* found that silymarin protected dopaminergic neurons against lipopolysaccharide (LPS)-induced neurotoxicity by inhibiting microglia activation (65). They first investigated the neuroprotective effect of silymarin against LPS-induced neurotoxicity in mesencephalic mixed neuron-glia cultures. Their results showed that silymarin significantly inhibited the LPS-induced activation of microglia and the production of inflammatory mediators, such as TNF-α and NO, and reduced the damage to dopaminergic neurons. Silymarin also reduced LPS-induced nitrite, iNOS mRNA, and protein levels, superoxide generation and NF-κB activation, suggesting that silymarin's inhibitory effect on microglia activation could be mediated through inhibition of NF-κB activation.

Cruz *et al.* examined the intestinal anti-inflammatory activity of several doses of silymarin on the acute stage of the trinitrobenzenesulphonic acid (TNBS) model of rat colitis (66). They found that oral silymarin (50 mg/kg) significantly attenuated macroscopic colonic damage and reduced colonic myeloperoxidase activity. The improvements in the oxidative status of the colon, which had been altered during colonic inflammation through the reduced glutathione depletion and malonyldialdehyde production, were also observed. Therefore, it could be suggested that the well-known antioxidant properties of silymarin involved in intestinal anti-inflammatory activity, which was accompanied by the preservation of the colon's absorptive function.

5B. Silymarin modulates COX-2 expression. COX-2 is overexpressed in various tumors and is required for tumor cell proliferation (67). Numerous studies have shown that silymarin suppresses COX-2 expression (68). Our study showed that silymarin inhibited PMA-induced COX-2 expression in mouse epidermis (68). A single topical application of silymarin, at a 3-9 mg dose onto SENCAR

mouse skin, resulted in a 76-95% inhibition of tetradecanoyl phorbol-13-acetate (TPA)-induced skin edema. Silymarin also showed protection against TPA-induced depletion of epidermal SOD, catalase and GSH-PX activity. Pre-treatment with silymarin significantly inhibited TPA-induced epidermal lipid peroxidation (47-66% inhibition) and myeloperoxidase activity (56-100% inhibition). The effect of silymarin on arachidonic acid metabolism pathways involving lipooxygenase and COX was also assessed, and silymarin significantly inhibited TPA-induced epidermal lipooxygenase (49-77% inhibition) and COX activity (35-64% inhibition). The observed effect of silymarin on COX activity was due to inhibition of TPA-inducible COX-2, without any effect on constitutive COX-1 protein levels. Silymarin also showed dose-dependent inhibition of TPA-related induction of epidermal interleukin 1 alpha (IL-1α) protein and mRNA expression. This study provided the biochemical and molecular mechanisms for the silymarin's inhibitory effect on tumor promotion primarily at stage I. Kang *et al.* also showed that silymarin suppresses the LPS-induced production of PGE_2 and completely block mRNA expression of COX-2 in isolated mouse peritoneal macrophages and RAW 264.7 cells (69). Fiebrich and Koch showed that silymarin is a potent inhibitor of prostaglandin synthetase (70). Silybin, silydianin and silychristin inhibited the formation of prostaglandins *in vitro*. The inhibition was log-linearly-dependent on the concentration of the compounds. Overall, these studies suggested COX-2 to be a potential target for silymarin.

5C. Silymarin inhibits LOX expression. Like COX-2, 5-LOX is a pro-inflammatory enzyme that mediates the production of eicosanoids. 5-LOX has been implicated in proliferation of tumor cells (71, 72). Gupta *et al.* showed that the anti-inflammatory and anti-arthritic activities of silymarin were mediated through inhibition of 5-LOX (73). Fiebrich *et al.* also showed that silymarin was an inhibitor of LOX (74). The constituents of silymarin, silybin, silydianin and silychristin inhibited LOX from soybeans *in vitro*.

5D. Silymarin inhibits inflammatory cytokine expression. TNF and IL-1 have been shown to be growth factors for various tumor cells (75-79). Several studies have suggested that silymarin suppressed TNF expression (80-84) and IL-1β expression (69). Kang *et al.* found that, silymarin suppressed the LPS-induced production of IL-1β in isolated mouse peritoneal macrophages and RAW 264.7 cells. Consistent with this effect, silymarin completely blocked the mRNA expression of IL-1β in LPS-stimulated RAW 264.7 cells. He *et al.* found that, in pig kidney epithelial cells (LLC-PK1), fumonisin-B1-induced TNF expression and that pre-treatment with silymarin decreased the fumonisin-B1-induced TNF expression (76).

5E. Silymarin inhibits both TNF signaling and TNF expression. TNF is known to kill tumor cells and act as a tumor promoter; moreover, it is the cause of chemoresistance in many tumor cells (5). Silymarin was reported to inhibit TNF signaling in myeloid leukemia, Jurkat, HeLa and human prostate carcinoma cells (82). Many studies also indicated that silymarin or silybin inhibited toxin/tumor promoter-induced expression of TNF-α in the liver, skin and spleen of the mouse or rat (80, 81, 83, 84). Silymarin inhibited LPS and mycotoxin-induced TNF-α expression in the liver, suggesting a potential mechanism for its hepatoprotective effect (80, 81), and was also found to decrease TPA- and okadaic acid-induced mRNA and protein expression of TNF-α in mouse skin (83). Further, the silybin was reported to inhibit the TNF-α level in the spleen of rats following LPS exposure and partial hepatectomy (84). Overall, these studies indicated that silymarin and silybin are potent inhibitors of TNF expression, as well as of TNF-induced cell signaling, in tumors or in the cells exposed to tumor promoters.

5F. Silymarin inhibits activation of inflammatory NF-κB activation. NF-κB is required for the proliferation and metastasis of tumor cells. NF-κB also plays a critical role in inflammation, and is activated in response to a variety of agents. Our studies indicated that silymarin is a potent inhibitor of NF-κB activation in response to TNF (22). This effect was mediated through the inhibition of phosphorylation and degradation of IκBα, an inhibitor of NF-κB (22). Silymarin blocked the translocation of p65 to the nucleus without affecting its ability to bind to the DNA. NF-κB-dependent reporter gene transcription was also suppressed by silymarin. Furthermore, silymarin blocked NF-κB activation induced by phorbol ester, LPS, okadaic acid and ceramide, whereas H_2O_2-induced NF-κB activation was not significantly affected. Kang *et al.* showed that silymarin suppressed LPS-induced IL-1α and COX-2 expression through suppression of NF-κB (69). It was also shown that silymarin potently suppressed both NF-κB DNA binding activity and its dependent gene expression induced by okadaic acid in the hepatoma cell line HepG2. They found that TNF-induced NF-κB activation was not affected by silymarin, thus, demonstrating a pathway-dependent inhibition by silymarin. Since many genes encoding the proteins of the hepatic acute phase response are under the control of NF-κB, the inhibitory effect of silymarin on NF-κB activation could play a role in its hepatoprotective property. Saliou *et al.* investigated the effects of silymarin on the activation of NF-κB and activator protein-1 (AP-1) in HaCaT keratinocytes after exposure to a solar ultraviolet (UV) simulator (85). UV-induced activation of NF-κB and AP-1 was detected 2 hours after UV exposure and sustained for up to 8 hours. Three hours after exposure, silymarin was found to significantly inhibit NF-κB activation

without any considerable affect on AP-1 activation. Schumann *et al.* found that silybin inhibited concanavalin A-induced intrahepatic activation of NF-κB (86).

Even the most potent agents (*e.g.*, TNF-α) are ineffective in killing human PCA cells, possibly because of a constitutive activation of NF-κB that subsequently activates a large number of anti-apoptotic genes. In such a scenario, the apoptotic agent TNF-α further induces NF-κB activation rather than inducing apoptosis. We have shown that silybin inhibited constitutive and TNF-α-induced activation of NF-κB and sensitized human prostate carcinoma DU145 cells to TNF-α-induced apoptosis (82). The investigators examined whether silybin was effective in inhibiting constitutive NF-κB activation in human PCA cells, which would help in overcoming TNF-α-insensitivity. Consistent with the inhibitory effect on NF-κB DNA binding activity, silybin also reduced the nuclear levels of the p65 and p50 subunits of NF-κB, and increased the level of IκBα with a concomitant decrease in phospho-IκBα, which was accompanied by a decrease in IKK-α kinase activity. Silybin was observed to directly inhibit IKK-α kinase activity. This study also showed that silybin inhibited the TNF-α-induced activation of NF-κB *via* IκBα pathway and sensitized DU145 cells to TNF-α-induced apoptosis.

6. Modulation of cell signaling pathways

6A. Silymarin suppresses proliferation of tumor cells. Silymarin was shown to suppress the proliferation of a variety of tumor cells, including prostate (87, 88), ovarian (89), breast (90, 91), lung (92), skin (91, 93) and bladder (94, 95) (Table VI). Scambia *et al.* investigated the antiproliferative effect of silybin on gynecological malignancies, including human ovarian and breast cancer cell lines (89). Silybin, in concentrations ranging from 0.1 to 20 μM, exerted a dose-dependent growth inhibitory effect on OVCA 433, A2780 parental, and drug-resistant ovarian cancer cells, and MCF-7 doxorubicin (DOX)-resistant breast cancer cells (IC_{50} = 4.8-24 μM). Both the L and D diastereoisomers of silybin were effective in inhibiting A2780 WT cell growth (IC_{50} = 14 and 20 μM, respectively). Silybin induced G0/G1-phase arrest with a concomitant decrease in the percentage of cells in the S- and G2/M-phases of the cell cycle.

Sharma *et al.* also showed that silybin induced growth inhibition and apoptotic cell death in human lung carcinoma cells (92). They used SHP-77 small cell lung carcinoma cells (SCLC) and A-549 non-small cell lung carcinoma cells (NSCLC). Silybin caused growth inhibition and cell death in both cell lines. Cell cycle studies showed a small increase in the G0/G1 population in the SHP-77 cells. In the A-549 cells, a slight increase in the G0/G1- but an increase in S-phase were observed at shorter treatment times, while a great increase in the G0/G1 population was seen at longer treatment times. Silybin also caused apoptotic cell death, with the SHP-77 cells showing more apoptotic effect than A-549 cells. Tyagi *et al.* studied the effect of silybin on established rat PCA cell lines from primary PCA in rats induced by a methylnitrosourea (MNU)-testosterone protocol (88). They showed that silybin induced growth inhibition and reduction in cell viability in these cell lines. Beyond tumor cells, Onat *et al.* found that SDH negatively regulated the proliferation of normal human skin fibroblasts (91). The mechanism by which SDH inhibited this proliferation was not investigated.

Table VI. *Antiproliferative effect of silymarin against cancer cell lines.*

- Human epidermoid carcinoma (A431) (96)
- Human keratinocyte (HaCaT) (29)
- Human ovarian cancer (OVCA433) (89)
- Human breast cancer (MDA-MB 468) (90)
- Human skin fibroblasts (91)
- Human lung cancer (SHP-77, A-549) (92)
- Human prostate cancer (DU145) (97)

References in brackets.

Because silymarin is composed mainly of silybin and small amounts of other stereoisomers of silybin, Bhatia *et al.* examined whether its cancer preventive and anticarcinogenic effects are due to its major component, silybin (98). The treatment of different human prostate, breast and cervical carcinoma cells with silybin resulted in a highly significant inhibition of both cell growth and DNA synthesis, with a large loss of cell viability only in the cervical carcinoma cells. Silybin's effects were consistent and comparable to those of silymarin in terms of cell growth inhibition and DNA synthesis and loss of cell viability. Therefore, it is probable that the cancer chemopreventive and anticarcinogenic effects of silymarin can be attributed to the main constituent silybin.

6B. Silymarin modulates protein kinase C. The activation of protein kinase C (PKC) plays a critical role in tumorigenesis (99). Varga *et al.* found that silybin inhibited the calcium-, phosphatidylserine- and diacylglycerol-dependent PKC translocation activities in PMA-stimulated neutrophils (100) (Tables VII and VIII). Silybin derivatives with different lipid solubility, affected all the studied parameters. The lipid solubility of silybin was enhanced by methylation (5',7',4''trimethylsilybin), whereas a decrease in lipid solubility by acetylation of compound (5',7,'4''-trimethylsilybin-acetate) or by all the hydroxyl groups of silybin (peracetyl-silybin) attenuated silybin's ability to inhibit PKC translocation. Therefore, silybin's increased lipid solubility facilitates its penetration through the cell membrane to enhance its inhibitory effects. This structural modification of silybin could have pharmacological implications.

Table VII. *Cell signaling pathways suppressed by silymarin.*

Growth/Apoptosis	Transcription factors	Cytokine	Others
↓EGFR (103, 104)	↓NF-κB (67, 80, 84, 85, 107, 108)	↓IL-1β (69)*	↑SOD (112)*
↑IGFBP-3 (87)	↓STAT1, STAT3, STAT5 (109)	↓IL-2 (86)*	↑GSH (112, 115) *
↑p53 (16)*	↓PDEF (110)	↓IL-4 (86)*	↓Cholesterol/phospholipids (39) *
↓c-myc (105) *	↓AR (111)	↓IL-10 (16)*	↓Sphingomyelin/phosphatidylcholine (39) *
↑Cleaved caspase-3 (16)*		↓TGF-α (103)*	↑ATPase (105) *
↓Survivin (16)*		↓IFN-γ (86)*	↑GR (31)
		↓TNF (22, 82, 86)*	↓XOD (50)
			↓MHC class I (116)

Cell cycle	Kinase	Invasion/Metastasis
↑RB-E2F (106)	↓IKK-α (82)	↓iNOS (86, 109, 112)
↓CDK75 (106)	↓JNK (109)	↓NO (109)
↑p21/Cip1 (90)	↓PKC α,β (100)	↓MMP-2 (110)
↑p27/Kip1 (90)	↓AKT phosphorylation (16)*	↓u-PA (110)
↓RB phosphorylation (106)	↓SAPK/JNK1/2q (16)*	↑TIMP-2 (110)
↓cdc25 (97)*	↓p38 MAPK (16)*	↓VEGF (113)
↓cdc2 (97)*		↓Flt-1 (VEGFR1) (114)
↓cyclin B1 (97)*		↓KDR (VEGFR2) (114)
		↓COX-2 (68)

Silymarin consists of a mixture of silybin (also called silibinin), isosilybin, silychristin and silidianin. SOD, superoxide dismutase; GSH, glutathione; MHC, human major histocompatibility complex; iNOS, inducible NO synthase; TNF, tumor necrosis factor; NF-κB, nuclear factor kappa B; IFN-γ, interferon-γ; RB, retinoblastoma; CDK, cyclin-dependent kinase; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; TIMP, tissue inhibitor of metalloproteinase; PDEF, prostate epithelium-derived Ets transcription factor; u-PA, urokinase-type plasminogen activator; AR, androgen receptor; MMP, matrix-metalloproteinase; STAT, signal transducers and activators of transcription; IGFBP, insulin-like growth factor-binding protein; IL-2, interleukin-2; IL-4, interleulkin-4; IL-10, interleukin-10; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; GR, glutathione reductase; XOD, xanthine oxidase. References in brackets. *indicates *in vivo* study.

In contrast to this study, Kang *et al.* found that silybin induces differentiation of human promyelocytic leukemia HL-60 cells into monocytes by activating PKC. Silybin enhanced PKC activity and increased the protein levels of both PKCα and PKCβ in 1,25-(OH)(2)D(3)-treated HL-60 cells (101).

6C. Silymarin suppresses cellular proliferation by modulating protein kinases. Protein kinases are known to play a major role in the proliferation of tumor cells. Singh *et al.* found that silybin treatment of human epidermoid carcinoma A431 cells in culture resulted in growth inhibition and apoptotic death (102). Silybin also caused a significant decrease in phospho-extracellular signal-regulated kinase (ERK)1/2 levels but an up-regulation of c-Jun N-terminal kinase (JNK)1/2 and p38 mitogen-activated protein kinase (MAPK) activation. The use of the mitogen-activated protein kinase (MEK)1 inhibitor PD98059 showed that inhibition of ERK1/2 signaling, in part, contributed to the cell growth inhibition caused by silybin. This study suggests that silybin's inhibition of ERK1/2 activation and increased activation of JNK1/2 and p38 could be involved in inhibiting proliferation and inducing apoptotic death of A431 cells. Manna *et al.* showed that silymarin also inhibited the TNF-induced activation of MAPK and JNK (22). Silymarin was also shown to inhibit ERK1/2 activation only at lower doses, and induce JNK activation at higher doses in A431 cells (98). These differing effects of silymarin were associated with growth inhibition and apoptotic cell death, respectively. Silymarin-caused growth inhibition was *via* both G2/M and G1 arrests with a decrease in the kinase activity and protein levels of cyclin dependent kinases (CDKs) and cyclins. Together, these results identified distinct signaling pathways for the antiproliferative and apoptotic effects of silymarin/silybin. These studies provided a basis for developing strategies targeted to the ERK and JNK pathways for the prevention of and intervention in malignancies, including skin cancer, by silymarin.

In another study, Bhatia *et al.* found that treatment of A431 cells with silymarin inhibited the activation of the EGFR and the downstream adapter protein SHC (96). Downstream, silybin also inhibited ERK1/2 activation and induced apoptosis at high doses. It is more likely that the lack of an inhibitory effect on ERK1/2 activation could possibly "turn on" an apoptotic cell death response associated with the cancer-preventive and anticarcinogenic effects of the test agent.

Table VIII. *Hepatoprotective effects of silymarin.*

Alcoholic
- ↓Galactosamine-induced hepatitis (117, 118)
- ↓Alcohol-induced hepatic fibrosis (17, 44, 119)
- ↓Cirrhotic liver (120, 121)
- ↓Erythrocytes and lymphocytes in patients with alcoholic liver cirrhoses (122, 209)

Ischemic
- ↓Ischemia-induced liver damage (122)
- ↓Post-ischemic mitochondrial damage (138)

Drug-induced
- ↓Erythromycin estolate, amitriptyline, nortriptyline and tert-butylhydroperoxide-induced liver damage (125)
- ↓Praseodymium-induced hypoglycemia (124)
- ↓Halothane hepatotoxicity (139)
- ↓Acetaminophen-induced liver damage (134)
- ↓α-amanitine-induced liver damage (128)
- ↓ADP/Fe^{2+}-induced hepatocyte lipid peroxidation (61)
- ↓Phenylhydrazine-induced toxicity (129)
- ↓CCl_4-induced liver cirrhosis (38, 39, 130-132)
- ↓Radiation-induced hepatotoxicity (35)
- ↓Microcystin-LR-induced hepatotoxicity (130)
- ↓Paracetamol-induced hepatotoxicity (134)

Others
- ↓Thallium-induced hepatotoxicity (133)
- ↓Aflatoxin B1-induced hepatotoxicity (62)
- ↓Sawfly (*Arge pullata*)-induced hepatotoxicosis (136)
- ↓Methotrexate-induced cytotoxicity in human hepatocyte latent liver damage (125, 210)
- ↓Fumonisin B1-induced liver damage (81, 105)
- ↓Prolonged biliary obstruction-induced liver damage (123)
- ↓Hepatic cell membranes after damage by polycyclic aromatic hydrocarbons (126)
- ↓T-cell-dependent liver damage (86)
- ↓Hepatitis B (135)
- ↓Hepatic damage in *Mastomys natalensis* infected with *Plasmodium berghei* (140)

Silymarin consists of a mixture of silibinin (silybin), isosilybin, silychristin and silydianin. CCl_4, carbon tetrachloride; ADP, adenosine 5'-diphosphate. References in brackets.

6D. Silymarin induces cell cycle arrest. Numerous reports have suggested that silymarin inhibits the proliferation of cells at the G1-phase of the cell cycle (103). Because the EGFR (erbB1) and other members of the erbB family have been shown to play important roles in human PCA progression, efforts should be directed at identifying inhibitors of this pathway for PCA intervention. Zi *et al.* assessed whether silymarin inhibits erbB1 activation and associated downstream events and modulates cell cycle regulatory proteins and progression, leading to growth inhibition of human prostate carcinoma DU145 cells (103). The silymarin treatment of serum-starved cells resulted in a significant inhibition of the TGF-α-mediated activation of ErbB1, but no change in its protein levels. Silymarin also decreased tyrosine phosphorylation of the adaptor protein SHC, as well as its binding to erbB1. In cell cycle studies, silymarin caused an induction of the CDK inhibitors Cip1/p21 and Kip1/p27, and a decrease in CDK4 expression; it also showed an increased binding of CDK inhibitors with CDKs, along with a marked decrease in the kinase activity of CDKs and their associated cyclins. Anti-epidermal growth factor receptor monoclonal antibody clone 225, as well as silymarin, inhibited the constitutive tyrosine phosphorylation of both erbB1 and SHC. Interestingly, the monoclonal antibody C225 showed an increase only in Kip1/p27 and not in Cip1/p21. This study suggested that silymarin-caused inhibition of erbB1 activation could have mediated the induction of Kip1/p27. However, additional pathways independent of erbB1 may be responsible for the increase in Cip1/p21 caused by silymarin. These effects of silymarin led to the induction of G1 arrest in the cell cycle progression and strong inhibition of both the anchorage-dependent and anchorage-independent growth of human prostate carcinoma DU145 cells.

Tyagi *et al.* assessed the effect of silybin on human bladder TCC cell growth, cell cycle modulation, apoptosis induction and the associated molecular alterations, employing two different cell lines representing high-grade invasive tumor (TCC-SUP) and high-grade TCC (T-24) human bladder cancer cells. Silybin caused growth inhibition, together with G1 arrest, only at lower doses in the TCC-SUP cells, but at both lower and higher doses in the T-24 cells. A higher silybin dose resulted in a G2/M arrest in TCC-SUP cells. Silybin treatment induced the expression of Cip1/p21 and Kip1/p27, but decreased G1-phase CDKs and cyclins; it also increased interaction between CDK inhibitors and CDKs, and reduced CDK kinase activity. The G2/M arrest by silybin in TCC-SUP cells was associated with a decrease in pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15), Cdc2 and cyclin B1 protein levels. Further, G2/M arrest was associated with apoptotic death involving caspases in TCC-SUP cells. This study suggested that silybin modulates the CDK inhibitor-CDK-cyclin cascade and activates caspase pathway, for growth inhibition and apoptotic death of human bladder cancer cells (141).

6E. Silymarin induces cell cycle cyclin-dependent kinase inhibitor. Numerous cyclin-dependent kinase inhibitors, that regulate the proliferation of tumor cells, have been identified. These include p15, p21 (also called Cip1) and p27 (also called Kip1). Silymarin/silybin were shown to induce Cip1/p21 and Kip1/p27 in prostate and bladder cancer cells, as discussed above (100, 125) Zi *et al.* showed that, in human breast cancer cells MDA-MB 468, silymarin induced G1 arrest through an increase in Cip1/p21 and a decrease in the kinase activity of CDK and associated cyclins (90). Silymarin treatment resulted in a significantly high to complete inhibition of both anchorage-dependent and anchorage-

independent cell growth. Silymarin's inhibitory effects on cell growth and proliferation were associated with G1 arrest in cell cycle progression concomitant with an induction of up to 19-fold in the protein expression of the CDK inhibitor Cip1/p21. Following silymarin treatment of cells, an incremental binding of the Cip1/p21 with CDK2 and CDK6 paralleled a significant decrease in CDK2-, CDK6-, cyclin D1- and cyclin E-associated kinase activities, along with a decrease in the G1 cyclins D1 and E.

We also showed that silybin up-regulated the expression of cyclin-dependent kinase inhibitors and caused cell cycle arrest in human colon carcinoma HT-29 cells. In this study, silybin resulted in moderate to very strong growth inhibition, which was largely due to a G0/G1 arrest; a higher dose and longer treatment time also caused a G2/M arrest. Silybin treatment resulted in an up-regulation of Kip1/p27 and Cip1/p21 proteins, as well as the mRNA levels. Silybin also decreased CDK2, CDK4, cyclin E and cyclin D1 protein levels, together with inhibition of CDK2 and CDK4 kinase activities. The G2/M arrest was associated with a decrease in Cdc25c, Cdc2/p34, and cyclin B1 protein levels, as well as Cdc2/p34 kinase activity (142).

6F. Silymarin inhibits the EGFR pathway. Enhanced tyrosine kinase activity, due to the aberrant expression or overexpression of receptor and/or non-receptor tyrosine kinases, has been implicated in a variety of human malignancies, including skin cancer. EGFR-mediated tyrosine phosphorylation may be a primary indicator of signal transduction regulating cell growth and proliferation. Silymarin was shown to modulate both EGFR expression and its kinase activity. Ahmad *et al.* showed that silymarin could impair receptor tyrosine kinase signaling, perturbating the cell cycle progression of cancer cells (143). Skin tumor promoters, such as phorbol ester and UVB radiation activate EGFR in mouse skin, as well as in cell culture. Similarly, oxidative stress, which is implicated in skin tumor promotion, also activates EGFR-mediated cell signaling. Employing human epidermoid carcinoma cells A431 that overexpress EGFR, whether the anti-skin tumor-promoting effects of silymarin were due to its inhibitory effect on EGFR activation and the downstream signaling pathway were assessed. Silymarin inhibited the ligand-induced activation of EGFR and decreased the tyrosine phosphorylation of SHC. Silymarin's inhibition of EGFR activation was associated with almost complete inhibition of EGFR intrinsic kinase activity. The observation that silymarin can impair EGFR signaling was accompanied by cell cycle arrest and inhibition of cell proliferation. Further, Qi *et al.* found that rat glioma cells that express EGFR are killed by silybin (104). These investigators generated a cell line with stable expression of human EGFR in rat glioma cell line, 9L, laking endogenous EGFR message or protein. Silybin inhibited EGFR activation by EGF in EGFR expressing cells. EGFR expression was found both necessary and sufficient to confer silybin cytotoxicity in these cells. Therefore, it appears that silybin's toxicity to cancer cells involves the EGFR signaling pathway.

Silymarin has been shown to suppress erbB1-SHC activation in PCA DU145 cells. Sharma *et al.* showed the inhibitory effect of silybin on ligand binding to erbB1 and the associated mitogenic signaling, growth and DNA synthesis (144). Treatment of cells with silybin followed by (125)I-EGF, showed strong inhibition in ligand binding and its internalization in human prostate carcinoma LNCaP and DU145 cells. This was accompanied by strong inhibitory effects on membrane and cytoplasmic signaling molecules including ERK1/2 activation, as well as on cell growth and DNA synthesis. This study also showed the molecular modeling of silybin revealing its highly lipophilic regions, suggesting its interaction with lipid-rich plasma membranes, including binding with erbB1, and thereby competing with the EGF-erbB1 interaction.

6G. Silybin up-regulates insulin-like growth factor-binding protein 3 expression. Zi *et al.* showed that, at pharmacologically achievable concentrations (0.02-20 μM), silybin increased insulin-like growth factor binding protein 3 (IGFBP-3) accumulation in PC-3 cell conditioned medium and showed a 9-fold increase in the IGFBP-3 mRNA level at a 20 μM dose of silybin (87). An IGFBP-3 antisense oligodeoxynucleotide, that attenuated silybin-induced IGFBP-3 gene expression and protein accumulation, also reduced the antiproliferative action of silybin. The investigators observed that silybin reduced insulin receptor substrate 1 tyrosine phosphorylation, indicating an inhibitory effect on the insulin-like growth factor I receptor-mediated signaling pathway. This study suggested the inhibitory effect of silybin on IGF receptor signaling *via* up-regulation of IGFBP-3 for its antiproliferative mechanism in human PCA cells. Silybin has also been found to induce IGFBP-3 in a human PCA tumor xenograft study.

6H. Silymarin down-regulates cell survival proteins. The overexpression of cell survival proteins has been implicated in the proliferation of tumors and their resistance to chemotherapeutic agents and gamma radiation. Survivin, a member of the inhibitor of apoptosis protein (IAP) gene family, is highly up-regulated in a wide range of malignancies, including carcinoma of the bladder urothelium. Recent research has identified survivin as a novel intervention target to induce apoptosis in cancer cells through phytochemicals or synthetic agents. Tyagi *et al.* showed that silymarin down-regulates survivin in human bladder transitional cell papilloma RT4 cells (95). Treatment of the cells for 24 hours with 100 μM silybin showed an almost 50% decrease in the survivin protein level; whereas, 200 μM for 24 and 48 hours

showed a complete loss of survivin protein. Silybin caused a strong to complete decrease in survivin mRNA levels. Overall, the effect of silybin on survivin was associated with prominent caspase-9 and -3, as well as poly(ADP-ribose) polymerase (PARP) cleavage, and apoptotic cell death in RT4 cells.

6I. Silymarin suppresses the expression of the iNOS gene. Inducible nitric oxide (iNOS) is a gene that is regulated by inflammatory cytokines through the activation of many transcription factors including NF-κB. Kang *et al.* investigated the effect of silymarin on nitric oxide (NO) production and iNOS gene expression in macrophages (49). *In vivo* administration of silymarin attenuated NO production by peritoneal macrophages in LPS-treated mice. Silymarin also suppressed the LPS-induced production of NO in isolated mouse peritoneal macrophages and in RAW 264.7, a murine macrophage-like cell line. Moreover, iNOS mRNA and its protein expression were completely abrogated by silymarin in LPS-stimulated RAW 264.7 cells. In the mechanistic study, they observed that silymarin's inhibition of iNOS gene expression was mediated *via* the inhibition of LPS-induced NF-κB activation, involving the inhibition of the degradation of inhibitory factor-κB. Silymarin also inhibited TNF-α-induced NF-κB activation, whereas okadaic acid-induced NF-κB activation was not affected. NF-κB dependent reporter gene expression was also suppressed by silymarin in LPS-stimulated RAW 264.7 cells. This study suggested that silymarin inhibits NO production and iNOS gene expression by inhibiting NF-κB activation, possibly through its radical-scavenging activity.

Besides being hepatoprotective, silymarin also appeared to protect pancreatic beta cells. Matsuda *et al.* showed that silymarin protected pancreatic β-cells against cytokine-mediated toxicity through the suppression of cytokine-induced NO production (109). These cytoprotective effects also appeared to be mediated through the suppression of JNK and the Janus kinase/signal transducer and activator of transcription pathways.

6J. Silymarin inhibits telomerase activity. The androgen-sensitive PCA cell line LNCaP is strongly positive for dihydrotestosterone (DHT)-dependent telomerase activity, which is an important factor in cellular immortality and carcinogenesis. Thelen *et al.* determined silybin's potential to down-regulate telomerase activity (145). LNCaP cells were treated with various concentrations of silybin in the presence or absence of 5α-DHT. They found that silybin decreased the constitutive, as well as DHT-induced telomerase catalytic subunit mRNA level and activity.

6K. Silymarin inhibits Rb phosphorylation and increases Rb-E2F complex formation. The phosphorylation status of retinoblastoma (Rb) and related proteins is important in driving cell cycle progression. In a hyperphosphorylated state, they are growth stimulatory, but their hypophosphorylation is growth inhibitory. Tyagi *et al.* assessed the effect of silybin on total Rb levels and its phosphorylation status, the levels of E2F family members and Rb-E2F binding in LNCaP cells (106). Silybin increased the total Rb levels *via* an increase in unphosphorylated Rb. This effect was mainly attributable to a large decrease (70-97%) in the amount of Rb phosphorylation at specific serine sites. Silybin showed a moderate effect on E2F1, but a strong decrease in E2F2 and E2F3 protein levels. Silybin also increased Rb binding to E2F1, E2F2 and E2F3. CDKs phosphorylate Rb, making transcription factor E2Fs free from Rb-E2F complexes and resulting in cell growth and proliferation. Upstream, silybin induced CDK inhibitors and decreased CDK activity. Consistent with silybin's modulation of the Rb levels and its phosphorylation status, it also induced G1 arrest and growth inhibition, suggesting Rb as a potential molecular target for the antitumor activity of silybin.

In another study, Tyagi *et al.* showed that silybin caused hypophosphorylation of the Rb-related proteins, Rb/p107 and Rb2/p130 *via* modulation of cell cycle regulators. The investigators assessed whether silybin causes hypophosphorylation of Rb-related proteins as its growth inhibitory response in human PCA DU145 cells. Silybin showed a strong increase in the levels of hypophosphorylated Rb/p107 and Rb2/p130, but a strong decrease in protein levels of the transcription factors E2F3, E2F4 and E2F5. This caused an increase in Cip1/p21 and Kip1/p27 levels, and a decrease in the CDK4 and CDK2 levels. Consistent with these molecular alterations, silybin showed a strong G1 arrest, with almost complete growth inhibition and morphological changes suggestive of differentiation.

6L. Silymarin binds to estrogen receptors. Because flavonoids are believed to act through Type II estrogen binding sites (Type II EBS), Scambia *et al.* examined silybin binding to Type II EBS (89). Silybin was able to compete with [^{3}H]E2 for nuclear, but not cytosolic, Type II EBS. Its affinity paralleled its efficacy in inhibiting cell proliferation.

Silymarin also binds to cytosolic estrogen receptors. Seidlova-Wuttke *et al.* demonstrated that this binding is exclusive to the estrogen receptor β (ERβ). Silymarin or estradiol (E2) may allow differentiation of the biological effects mediated by the ERαs or ERβ in ovariectomized (ovx) rats (146). Both E2 and silymarin inhibited the expression of the uterine ERβ gene. In the pituitary, liver and uterus, E2 acts primarily *via* the ERα. Exclusive estrogenic effects of silymarin were observed in the metaphysis of the femur (MF), on osteoblast parameters, such as the gene expressions of IGF1, TGFβ1, osteoprotegerin, collagen-1α1 and osteocalcin, and on the osteoclast activity marker of tartrate-resistant acid

phosphatase (TRAP) gene expression. The ERβ gene was expressed in all organs including developing bones, but not in the MF of adult ovx rats. These investigators concluded that the effects of silymarin in this part of the bone cannot be exerted *via* ERα because it does not bind to this receptor subtype. Despite the failure to detect ERβ mRNA in the MF, they speculated that ERβ protein may be present and mediate the effects of silymarin.

6M. Silymarin inhibits function of the androgen receptor. Agents with novel mechanisms to block the androgen receptor (AR) function may be useful for PCA prevention and therapy. Zhu *et al.* found that silymarin inhibit the function of the AR by reducing nuclear localization of the receptor in the human prostate cancer cell line LNCaP (111). The researchers observed that silymarin and silybin inhibited androgen-stimulated cell proliferation, as well as androgen-stimulated secretion of both PSA and human glandular kallikrein (hK2). Additionally, for the first time, they showed that an immunophilin, FKBP51, is androgen-regulated and that this up-regulation is suppressed by silymarin and silybin. They further demonstrated that the transactivation activity of the AR was diminished by silymarin and silybin using gene transfer of PSA promoter and hK2 androgen-responsive element constructs. However, the expression and steroid-binding ability of the total AR level were not affected by silymarin. Intriguingly, they found that the nuclear AR levels were reduced by silymarin and silybin in the presence of androgens.

6N. Silymarin down-regulates P-glycoprotein. P-glycoprotein (P-gp) is a 170-kDa phosphorylated glycoprotein encoded by the human multidrug-resistant (MDR)1 gene. It is responsible for the systemic disposition of numerous structurally and pharmacologically unrelated lipophilic and amphipathic drugs, carcinogens, toxins and other xenobiotics in many organs, including the intestine, liver, kidney and brain. Like cytochrome P450s (CYP3A4), P-gp is vulnerable to inhibition, activation, or induction by various agents. Flavonoids have been reported to modulate P-gp by directly interacting with the vicinal ATP-binding site, the steroid-binding site, or the substrate-binding site. Inhibition of P-gp may provide a novel approach to reversing multidrug resistance in tumor cells. Modulation of P-gp activity and expression may result in altered absorption and bioavailability of drugs that are P-gp substrates. This is exemplified by the increased oral bioavailability of phenytoin and rifampin with piperine. Thus, the modulation of intestinal P-gp and CYP3A4 represents an important mechanism for many clinically important herb-drug interactions. Maitrejean *et al.* found that the silybin and its hemi-synthetic derivatives modulated P-gp (147). The flavanolignan silybin was first oxidized to dehydrosilybin and then C-alkylated with either prenyl or geranyl bromide. The resulting isoprenoid dehydrosilybins were found to display high *in vitro* affinities for direct binding to P-gp, which ranks them among the best flavonoids ever tested.

Perez-Victoria *et al.* showed high-affinity binding of silybin derivatives to the nucleotide-binding domain of a *Leishmania tropica* P-gp-like transporter and chemosensitization of a MDR parasite to daunomycin (DNM) (148). They studied the effects of derivatives of the flavonolignan silybin and related compounds lacking the monolignol unit on the affinity of binding to a recombinant C-terminal nucleotide-binding domain of the *L. tropica* P-gp-like transporter and the sensitization to DNM on promastigote forms of a MDR *L. tropica* line overexpressing the transporter. Oxidation of silybin to the corresponding flavonol dehydrosilybin, the presence of the monolignol unit, and the addition of a hydrophobic substituent such as dimethylallyl, especially at position 8 of ring A, increased the binding affinity. The *in vitro* binding affinity of these compounds for the recombinant cytosolic domain correlated with their modulation of the drug resistant phenotype. In particular, 8-(3,3-dimethylallyl)-dehydrosilybin effectively sensitized MDR *Leishmania spp*. to DNM. This study suggested that the cytosolic domains could be attractive targets for the rational design of inhibitors against P-gp-like transporters.

Zhang *et al.* examined the effect of silymarin on the P-gp-mediated transport of digoxin and vinblastine in human intestinal Caco-2 cells (149). The accumulation of digoxin and vinblastine in Caco-2 cells was significantly increased by 50 μM silymarin. Therefore, silymarin can inhibit P-gp-mediated efflux in Caco-2 cells, and could potentially increase the absorption/bioavailability of coadministered drugs that are P-gp substrates. Zhang *et al.* also characterized the effect of flavonoids on P-gp-mediated cellular efflux and determined the molecular mechanism(s) of the flavonoid-drug interaction (150). Studies were conducted on the sensitive and MDR human breast cancer cell lines MCF-7 and MDA435/LCC6, and they examined the effects of silymarin on DNM accumulation and doxorubicin cytotoxicity, P-gp ATPase activity, [(3)H]azidopine photoaffinity labeling of P-gp, and cellular P-gp levels. Flavonoids increased [^{3}H]DNM accumulation in P-gp-positive cells, but not P-gp-negative cells, in both flavonoid concentration and P-gp expression dependent manner. Silymarin potentiated the cytoxicity of DOX in P-gp-positive cells and inhibited P-gp ATPase activity, confirming its interaction with P-gp. Silymarin inhibited [^{3}H]azidopine photoaffinity labeling of P-gp, suggesting a direct interaction with P-gp substrate binding. Withdrawal of silymarin did not alter the cellular P-gp level in P-gp-positive cells. This study indicated flavonoid-drug interactions with P-gp substrates involving the inhibition of P-gp-mediated cellular efflux.

Nguyen *et al.* examined the effect of silymarin on multidrug resistance-associated protein1 (MRP)1-mediated transport of DNM and vinblastine (VBL) in human pancreatic adenocarcinoma cell line Panc-1 (151). Silymarin, at 100 μM, increased the accumulation of both DNM and VBL in Panc-1 cells. Cellular GSH concentrations were significantly decreased following a 2-hour incubation with silymarin. These observations indicated that silymarin can inhibit MRP1-mediated drug transport that may involve binding interactions with MRP1, as well as modulation of GSH concentrations.

Cooray *et al.* showed that silymarin interacted with P-gp and modulated the activity of the recently discovered ABC transporter breast cancer resistance protein (BCRP/ABCG2) (152). In two separate BCRP-overexpressing cell lines, silymarin increased the accumulation of the established BCRP substrates mitoxantrone and bodipy-FL-prazosin. Silymarin stimulated the vanadate-inhibitable ATPase activity in membranes prepared from bacteria (*Lactococcus lactis*) expressing BCRP. Therefore, given the high dietary intake of polyphenols, such interactions with BCRP, particularly in the intestines, may play an important role *in vivo* for the distribution of silymarin, as well as other BCRP substrates.

6O. Silymarin is a chemosensitizer. Significant emphasis is being placed on combination chemotherapy using cytotoxic agents and naturally occurring chemopreventive agents, which have different mechanisms of action and no overlapping toxicity. Because chemoresistance is one of the major problems in cancer therapy, agents that can overcome the problem are much needed. Silybin's effects on cisplatin (CDDP) and DOX, the two most commonly used drugs for gynecological tumors have been investigated. Silybin (0.1-1 μM) potentiated the growth inhibitory effect of CDDP (0.1-1 μg/ml) on A2780 WT and CDDP-resistant cells (89). Similar results were obtained on MCF-7 DOX-resistant cells when silybin (0.1 μM) was administered with DOX (0.1-10 μg/ml). The effects of silybin-CDDP and silybin-DOX combinations were synergistic. Further, using the 'stem cell assay', it was found that silybin exerted a dose-dependent inhibition in the clonogenic efficiency of cells derived from three ovarian tumors.

Dhanalakshmi *et al.* showed that silybin sensitized human PCA DU145 cells to CDDP- and carboplatin-induced growth inhibition and apoptotic death (153). CDDP alone at a dose of 2 μ/ml produced 48% cell growth inhibition, whereas a combination with 50-100 μM silybin resulted in 63-80% growth inhibition. Similarly, compared to 68% growth inhibition at 20 μg/ml carboplatin, the addition of 50 to 100 μM silybin caused 80-90% inhibition. A combination of CDDP or carboplatin with silybin resulted in a stronger G2/M arrest, compared to these agents alone. The G2/M arrest caused by these combinations was accompanied by a substantial decrease in the levels of Cdc2, cyclin B1, and Cdc25c. Silybin/platinum combinations also induced apoptosis by almost 2-fold. Apoptosis induction involved cytochrome *c* release from the mitochondria and activation of caspases-9, -3 and -7.

Tyagi *et al.* also assessed whether silybin with DOX showed a synergistic effect against PCA, the effectiveness of DOX being limited because of high systemic toxicity (97). Silybin synergistically increased the growth-inhibitory effect of DOX in human PCA DU145 and LNCaP cells. This combination showed a strong (88%) G2/M arrest in cell cycle progression, compared to silybin (19%) or DOX (41%) alone in DU145 cells. The underlying mechanism of G2/M arrest showed that the combination decreased Cdc25c, Cdc2/p34, and cyclin B1 protein expressions and Cdc2/p34 kinase activity. Silybin-DOX combination also induced apoptosis in DU145 cells.

Giacomelli *et al.* investigated whether the flavonoid silybin and its bioavailable derivative IdB 1016 (silipide) could enhance the antitumor activity of CDDP, the most commonly used drug in the treatment of gynecological malignancies (154). Silybin alone up to 10 μM did not produce relevant *in vitro* growth inhibition of A2780 cells, whereas CDDP was effective, with an IC_{50} value of 0.5 μM. When silybin was combined with CDDP, a dose-dependent increase in the CDDP activity was noted, yielding IC_{50} values of 0.35 and 0.26 μM at silybin concentrations of 1 and 10 μM, respectively. The same trend was observed in *in vivo* experiments. IdB 1016 alone (1350 mg/kg) did not significantly affect tumor growth, whereas CDDP at the maximum tolerated dose (12 mg/kg) reduced the tumor weight by 80% and gave log10 cell kill (LCK) of 0.7. Administration of both drugs resulted in potentiation of the antitumor activity showing a tumor weight decrease 90% and LCK of 1. Interestingly, mice receiving the combination recovered earlier in terms of body weight loss as compared to CDDP-treated mice. This study suggested the sensitizing effect of silybin on the antitumor activity of CDDP.

In another study, we observed that silybin synergized the therapeutic potential of DOX, CDDP, or carboplatin in both estrogen-dependent and -independent human breast carcinoma cell lines, MCF-7 and MDA-MB468, respectively. In both cell lines, the combination of silybin with DOX resulted in much stronger apoptotic death compared to each agent alone in both cell lines. A silybin-cisplatin combination did not show any additional apoptotic effect; however, a silybin-CDDP combination showed a stronger apoptotic effect only in MCF-7 cells. Overall, these studies suggested a possible synergism between silybin and conventional cytotoxic agents for cancer treatment and therefore, pre-clinical and clinical trials with such combinations are warranted (94).

6P. Silymarin has antiangiogenic effects. Angiogenesis is critical for the growth and metastasis of tumors. The anti-angiogenic potential of silymarin has been investigated using human umbilical vein endothelial cells (HUVECs) (113). Silymarin induced apoptotic cell death in HUVECs at doses of >50 μg/ml. Silymarin treatment of HUVECs resulted in a concentration-dependent decrease in the secretion and cellular content of (matrix metalloproteinase) MMP-2/gelatinase A, which was accompanied by the inhibition of HUVEC tube formation on a Matrigel. In other studies, exposure of prostate and breast cancer cells to silymarin decreased the secreted VEGF level in conditioned media. The inhibitory effect of silymarin on VEGF secretion occurred as early as 1 hour. This study indicated that silymarin possesses an anti-angiogenic potential against endothelial cells and can inhibit the secretion of angiogenic cytokine by cancer epithelial cells.

Recently, the anti-angiogenic effect of silymarin was also demonstrated on the colon cancer LoVo cell line (114). A modified *in vitro* system using a coculture of endothelial (EA.hy 926) and LoVo cell lines was adopted in this study, in which silymarin exerted a strong anti-angiogenic effect. Yoo *et al.* examined the effects of silybin on human endothelial ECV304 cells. Silybin was found to suppress the growth of and induce apoptosis in the ECV304 cells (107). Silybin could effectively inhibit constitutive NF-κB activation and NF-κB-dependent luciferase reporter activity. Silybin changed the ratio of Bax/Bcl-2 to favor apoptosis involving the caspase pathway. It was also shown that silybin inhibited angiogenesis *via* fetal liver kinase-1 (Flt-1), but not kinase insert domain-containing receptor (KDR), receptor up-regulation. They designed this study to evaluate the anti-angiogenic effects of silymarin/silybin *in vivo* using a modified chicken chorioallantoic membrane assay (CAM) and on VEGFR gene expression in EA.hy 926 endothelial cells. In CAM, silymarin/silybin showed a dose-dependent decrease in the vascular density index induced by LoVo cells, which was reversed by escalating dosages of VEGF. RT-PCR revealed that silybin up-regulated Flt-1 mRNA expression, but the effect was not statistically significant in case of silymarin. Neither drug affected KDR mRNA expression. Yang *et al.* conclude that the anti-angiogenic effects of silymarin/silybin are associated with the up-regulation of VEGFR-1 (Flt-1) gene expression.

Singh *et al.* investigated the effect of silybin feeding (0.05% and 0.1% (w/w) in the diet for 60 days) on angiogenesis in the prostate tumor xenograft model (155). Immunohistochemical analysis of the tumors for CD31 staining for tumor vasculature showed a 21-38% decrease in tumor microvessel density in tumors from silybin-fed animals as compared to those from control groups of tumors. Silybin also decreased the cytoplasmic immunostaining for VEGF in silybin-fed groups. This study suggested the *in vivo* anti-angiogenic effect of silybin in the inhibition of advanced human prostate tumor xenograft growth in athymic nude mice; however, it was also associated with its *in vivo* antiproliferative and pro-apoptotic effects (32).

6Q. Silymarin alters the expression of TGF-β. He *et al.* investigated the expression of cytokines in mouse liver following treatment with 0, 10, 50 and 250 mg/kg of silymarin once daily for 5 days (105). Silymarin treatment caused significant increases in the expressions of TGF-β and c-myc in the liver. No significant difference in the expression of HGF, IFN-γ, TNF-α and class II major histocompatibility complex was detected among these treatments. This study suggested that alterations of TGF-β and c-myc expressions in the liver could be involved in the hepatoprotective effects of silymarin, as observed in other studies.

6R. Silymarin inhibits the secretion of prostate-specific antigen (PSA). A reduction in the serum PSA levels has been proposed as an end-point biomarker for intervention in hormone-refractory human PCA. In this regard, silybin has been shown to decrease PSA levels in human PCA LNCaP cells (156). Silybin was found to decrease both intracellular and secreted forms of PSA, concomitant with a highly significant to complete inhibition of cell growth *via* a G1 arrest in cell cycle progression. Silybin inhibited 5-α-DHT androgen-stimulated PSA expression and cell growth. Thelen *et al.* also determined silybin's potential to down-regulate PSA together with the coactivator of the AR prostate epithelium specific Ets transcription factor in PCA LNCaP cells (157). Silybin, in the presence or absence of 5-α-DHT, down-regulated PSA mRNA expression and PSA secretion in conditioned medium. Simultaneous stimulation with silybin and 10^{-8} M DHT also resulted in PSA down-regulation.

6S. Silymarin inhibits the expression of cell surface adhesion molecules. The expression of adhesion molecules plays a major role in the metastasis of tumor cells. In this regard, silymarin was shown to inhibit TNF-α-induced mRNA and protein expression of adhesion molecules including VCAM-1, ICAM-1 and E-selectin in HUVECs. In this study, silymarin suppressed the TNF-α-induced DNA binding of NF-κB/Rel in HUVECs, which mediates the expression of adhesion molecules.

7. Silymarin modulates immune system

Several reports have suggested that silymarin exhibits immunomodulatory effects. Johnson *et al.* found that silymarin inhibited T-lymphocyte function at low doses but stimulated inflammatory processes at high doses (158). Silymarin's modulatory effect on the inflammatory immune response was assessed in male BALB/c mice. Intraperitoneal

10-250 mg/kg doses of silymarin once daily for 5 days did not cause any signs of toxicity. Splenic lymphocyte populations showed that the absolute number of CD3+ T-lymphocytes was reduced in the 10- and 50-mg/kg groups, but was significant only at the lowest dose. Concomitant decreases in CD4+ and CD8+ T-cell populations were also observed, but the effect for the CD4+ population was significant only at the lowest dose. Silymarin also enhanced PHA-induced T-lymphocyte proliferation and LPS-induced B-lymphocyte blastogenesis. Similarly, the expressions of TNF, iNOS, IL-1β and IL-6 mRNA were increased dose-dependently. The expressions of IL-2 and IL-4 were reduced in mice treated with 10 and 50 mg/kg silymarin. Overall, this study indicated that *in vivo* parenteral exposure to silymarin results in the suppression of T-lymphocyte function at low doses and stimulation of inflammatory processes at higher doses.

Johnson *et al.* also examined the effect of silymarin on differentiation and cell selection in the thymus *via* alterations in gene expression (159). They found that alterations in thymic differentiation correlated with changes in c-myc gene expression. Using male BALB/c mice and intraperitoneal administration of silymarin, they observed that silymarin increased the absolute numbers of CD4+ and CD8+ T-lymphocytes. C-myc proto-oncogene expression is important in controlling the differentiation and function of thymocytes, which were also increased by silymarin in the thymus. However, the expressions of IL-2 and IL-4 were decreased, whereas MHC II expression did not change. This study indicated the *in vivo* effect of silymarin on the phenotypic selection processes in the thymus at doses that may be encountered in normal medicinal use. Schumann *et al.* found that silybin protected mice from T-cell-dependent liver injury (86). They characterized the hepatoprotective potential of silybin as an immune response modifier in the mouse model of concanavalin A (ConA)-induced, T-cell-dependent hepatitis. Silybin inhibited ConA-induced liver injury as assessed by plasma transaminase activities and intrahepatic DNA fragmentation. It also inhibited the intrahepatic expressions of TNF, IFN-γ, interleukin (IL)-4, IL-2 and iNOS, and the activity of NF-κB, and increased the synthesis of IL-10.

Sakai *et al.* found that silymarin induced major histocompatibility complex class I molecules on human neuroblastoma cell lines (116). Silymarin enhanced the transcriptional activity of the reporter construct containing the MHC class I promoter truncated within -428 bp of transcription initiation, but not the construct containing the promoter truncated within -284 bp. Because an E-box element is located between -428 and -285 bp of the transcription initiation, this study indicated that silymarin induced the transcriptional factors to enhance the MHC class I promoter through the class I E-box element.

8. Silymarin exhibits chemopreventive activity

The chemopreventive effect of silymarin on the free-radical-generating, skin tumor-promoting agent benzoyl peroxide (BPO) has been investigated in SENCAR mouse skin (34) (Table IX). Topical application of silymarin prior to the application of BPO resulted in strong protection against BPO-induced tumor promotion in DMBA-initiated SENCAR mouse skin. The preventive effect of silymarin was evident in a 70% reduction in tumor incidence, a 67% reduction in tumor multiplicity and a 44% decrease in tumor volume/tumor. Silymarin also inhibited the BPO-induced lipid peroxidation and depletion of SOD, catalase, and GPX activities in mouse skin. In inflammatory response studies, silymarin inhibited BPO-induced skin edema, myeloperoxidase activity and the IL-1α protein level in skin. This study suggested that silymarin could be useful in preventing a wide range of carcinogen- and tumor promoter-induced cancers.

Mehta *et al.* examined whether the effective chemopreventive agents including silymarin are active against the initiation or the promotion phases of lesion development by DMBA (160). They exposed mouse mammary glands to 2 mg/ml DMBA followed by a 5-day exposure to TPA, leading to the initiation and promotion phases of lesion development. The chemopreventive agents that were effective when present prior to the carcinogen exposure were considered to be anti-initiators, whereas those that were effective when given after the DMBA-TPA treatments were considered to be antipromoters. Using this model, it was shown that silymarin has antitumor promoter activity.

Numerous such reports have indicated that silymarin exhibits chemopreventive effects against a variety of carcinogens (Table IX). Vinh *et al.* examined the chemopreventive effects of dietary silymarin on N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN)-induced urinary bladder carcinogenesis in male ICR mice at the end of 32 weeks of the experiment (161). The frequency of bladder lesions, cell proliferation and cell cycle progression activity and cyclin D1 expression were compared. Administration of silymarin in the initiation or post-initiation phase significantly decreased the incidence of bladder neoplasms and preneoplastic lesions. Dietary exposure to this agent significantly reduced proliferation and cyclin D1-positive cells in bladder lesions. This study suggested the chemopreventive effect of silymarin against OH-BBN-induced bladder carcinogenesis in mice. In another study, Lahiri-Chatterjee *et al.* assessed the protective effect of silymarin on DMBA-TPA-induced tumor promotion in the SENCAR mouse skin tumorigenesis model (162). Silymarin reduced tumor incidence, tumor multiplicity and tumor volume. Silymarin's stage specificity against tumor promotion has also been studied. The application of silymarin prior to TPA in stage I

Table IX. *Anticancer effect of silymarin in vivo.*

DMBA-TPA induced
- DMBA-TPA-induced tumor in mammary glands (160)
- DMBA-TPA-induced skin tumor in SENCAR mice (102)
- DMBA-induced tumor promotion in SENCAR mouse skin (163)
- TPA-induced tumor promotion in SENCAR mice (162)

UVB-induced
- UVB-induced non-melanoma skin cancer in female SKH-1 hairless mice (166)
- UVB-induced cell proliferation and apoptosis in SKH-1 hairless mice (93)

Others
- OH-BBN-induced urinary bladder carcinogenesis in male ICR mice (161)
- Azoxymethane-induced colon carcinogenesis in male F344 rats (164)
- NQO-induced tongue carcinogenesis in male F344 rats (165)
- BPO-induced tumor promotion in SENCAR mouse skin (34)

NQO, 4-nitroquinone 1-oxide; BPO, benzoyl peroxide; DMBA, dimethylbenz(α)anthracene; TPA, 7,12-tetradecanoyl phorbol-13-acetate. References in brackets.

or before mezerein in stage II tumor promotion in DMBA-initiated SENCAR mouse skin showed a strong protective effect during stage I: 74% protection against tumor incidence, 92% protection against tumor multiplicity and 96% protection against tumor volume. With regard to stage II tumor promotion, silymarin showed 26, 63 and 54% protection in the above responses, respectively. The application of silymarin during both stage I and stage II protocols showed effects similar to that of the stage I study. Silymarin also inhibited TPA-induced skin edema, epidermal hyperplasia and proliferating cell nuclear antigen-positive cells, DNA synthesis, and epidermal lipid peroxidation, which are regarded as the early markers of TPA-caused changes associated with tumor promotion. Overall, this study suggested the strong protective effects of silymarin against tumor promotion, showing more efficacy against stage I, as well as illustrating the involved mechanisms.

Ornithine decarboxylase (ODC) is a well-known biochemical marker of tumor promotion. In this regard, skin application of silymarin has been shown to inhibit TPA-induced epidermal ODC activity and ODC mRNA levels in SENCAR mice (163). Silymarin also showed inhibition of epidermal ODC activity induced by several other tumor promoters, including free-radical-generating agents. Kohno *et al.* showed that silymarin inhibits azoxymethane (AOM)-induced colon carcinogenesis in male F344 rats (164). Four weeks dietary feeding of silimarin (100-1000 ppm) inhibited the development of AOM-induced colonic aberrant crypt foci (ACF), the putative precursor lesions for colonic adenocarcinoma, and induced the activity of the detoxifying enzymes, glutathione-S-transferase (GST) and quinone reductase in liver and colonic mucosa. In a long-term experiment, dietary feeding of silymarin (100 and 500 ppm) during the initiation or post-initiation phases inhibited the AOM-induced tumor incidence and multiplicity of colonic adenocarcinoma, and lowered the proliferation index but increased the apoptotic index in adenocarcinomas. The β-glucuronidase activity, PGE_2 level and polyamine content were decreased in colonic mucosa. This study clearly indicated that dietary silymarin is chemopreventive against chemically-induced colon tumorigenesis.

Yanaida *et al.* found that dietary silymarin suppressed 4-nitroquinoline 1-oxide (NQO)-induced tongue carcinogenesis in male F344 rats (165). In a short-term study, silymarin reduced tongue dysplasia and increased the activity of phase II detoxifying enzyme in the liver and tongue. Consitent with these observations, in a long-term study, dietary silymarin inhibited 4-NQO-induced tongue carcinogenesis. Silymarin given during the promotion phase lowered the incidence of tongue carcinoma, which was accompanied by a decrease in the proliferating index and an increase in the apoptotic index of tongue carcinoma. Silymarin also decreased the polyamine content and PGE_2 level in the tongue mucosa. This study indicated the chemopreventive activity of silymarin during the promotion phase of 4-NQO-induced rat squamous cell carcinoma of the tongue.

Non-melanoma skin cancer, caused by solar UV radiation, is the most common human cancer. Therefore, it is important to identify agents that can offer protection against this cancer. Katiyar *et al.* investigated the protective effects of silymarin against photocarcinogenesis at different stages of carcinogenesis in female SKH-1 hairless mice (166). The mice were subjected to either UVB-initiated tumor initiation followed by PMA-mediated tumor promotion; or DMBA-induced tumor initiation followed by UVB-mediated tumor promotion; or UVB-induced complete carcinogenesis. Topical application of silymarin in these protocols strongly suppressed tumor incidence, tumor multiplicity and tumor volume. However, silymarin showed much profound effect in the UVB-induced complete carcinogenesis study. In short-term experiments, silymarin inhibited UVB-caused sunburn and apoptotic cell formation, skin edema, depletion of catalase activity and induction of COX and ODC activities and ODC mRNA expression. Thus, silymarin can provide substantial protection against different stages of UVB-induced carcinogenesis, possibly *via* its strong antioxidant properties.

Another study indicated that the effects of silybin on UVB-induced apoptosis are complex. Depending on the dose of UVB, it could inhibit or enhance UVB-induced apoptosis. In this regard, a study with silybin on UVB-induced apoptosis in HaCaT cells showed that silybin can prevent apoptosis induced by the lower doses (15 and 30 mJ/cm^2) of UVB, as

observed by the reversal in UVB-initiated apoptosis, PARP cleavage and caspase-9 activation (29). Silybin also restored survivin, a protein inhibitor of apoptosis with an activation of NF-κB, without any noticeable effect on UVB-initiated AP-1 activation. Silybin up-regulated UVB-induced ERK1/2 phosphorylation and modulated Bcl-2 family proteins as possible survival mechanisms in its protective effects. Silybin also induced S-phase arrest, possibly providing a prolonged time for efficient DNA repair. Surprisingly, at a higher dose of UVB (120 mJ/cm^2), silybin further enhanced UVB-initiated apoptosis together with a strong decrease in AP-1 activation. This study, for the first time, demonstrated the mechanism-based dual efficacy of silybin in protecting or enhancing UVB-initiated apoptosis in the same cellular system. Dhanalakshmi *et al.* also assessed the effect of silybin on UV-induced DNA damage and p53-Cip1/p21 accumulation and their roles in UV-induced cell proliferation and apoptosis in SKH-1 hairless mouse epidermis (93). Topical application of silybin prior to, or immediately after, UV irradiation offered strong protection against UV-induced thymine dimer-positive cells, and increased p53 and Cip1/p21 protein levels. Silybin also inhibited UV-induced epidermal cell proliferation and apoptotic/sunburn cell formation. This study suggested the *in vivo* protective effect of silybin against UV-induced epidermal DNA damage possibly *via* an up-regulation of p53-Cip1/p21, leading to a decrease in both cell proliferation and apoptosis. Also, comparable effects of silybin were observed in both the pre- or post-UV application, excluding a possibility of sunscreen effect for its efficacy.

UVB-induced MAPKs and AKT play critical roles in photocarcinogenesis. Mallikarjuna *et al.* examined silybin efficacy on acute and chronic UVB-caused MAPKs and AKT activation and on the associated biological responses in SKH-1 hairless mouse skin (163). A single UVB exposure at 180 mJ/cm^2 resulted in varying degrees of ERK1/2, JNK1/2, MAPK/p38 and AKT phosphorylation which were inhibited by the topical application or dietary feeding of silybin prior to or immediately after UVB exposure. Silybin also inhibited UVB-initiated phosphorylation of MAPKs and AKT, and induced p53 in a chronic UVB (180 mJ/cm^2/day for 5 days) exposure protocol. This study suggested that silybin inhibits UVB-induced MAPK and AKT signaling and increases p53 in mouse skin, which are possibly responsible for its overall efficacy against photocarcinogenesis (16).

Kohno *et al.* investigated the modifying effects of dietary silymarin on DMAB-induced prostatic carcinogenesis in male F344 rats (164). The rats were given *s.c.* injections of DMAB (25 mg/kg body weight) every other week for 20 weeks, and also received the experimental silymarin diet (100 or 500 ppm) for 40 weeks, starting 1 week after the last dosing of DMAB. Silymarin inhibited the incidence of PCA together with a decrease in proliferative cell nuclear antigen- and cyclin D1-positive indices in adenocarcinomas, prostatic intraepithelial neoplasm and non-lesional glands. Also, silymarin increased the apoptotic index in PCA. This study indicated the chemopreventive effect of silymarin on chemically-induced PCA through apoptosis induction and inhibition of cell proliferation.

9. Silymarin exhibits antitumor effects *in vivo*

Many studies discussed above in the chemoprevention section showed the *in vivo* antitumor effects of silymarin/silybin in different animal models. Furthermore, Singh *et al.* examined the *in vivo* therapeutic efficacy of silymarin against skin tumors with a mechanistic rationale (102) (Table IX). Administration of 0.5% silymarin in an AIN-93M-purified diet (w/w) for 5 weeks to DMBA-TPA-induced established skin papilloma-bearing SENCAR mice showed inhibition (74%) of tumor growth and also caused regression (43%) of the established tumors. These effects were accompanied by the inhibition ERK1/2 phosphorylation and cell proliferation and an induction of apoptosis in tumors.

Singh *et al.* assessed the *in vivo* growth inhibitory potential of silybin against an advanced human PCA ectopic tumor xenograft in male athymic nude mice. Dietary feeding of silybin at 0.05-0.1% doses (w/w) for 60 days significantly inhibited the tumor volume and the wet weight of the tumor without any apparent signs of toxicity (147). Mice fed with silybin for 3 weeks before tumor xenograft implantation showed additional inhibition of tumor growth. These *in vivo* anticancer effects of silybin were associated with an increased accumulation (up to 5.8-fold) of human IGF-BP3 in mouse plasma. In this study, pharmacologically achievable doses were effective in inhibiting growth and increasing IGF-BP3 secretion in a DU145 cell culture study. It was also shown that silybin strongly decreased cell proliferation and angiogenesis, and enhanced apoptosis in the prostate tumor xenografts. Silybin also increased activated caspase-3-positive cells and IGF-BP-3 expression, and decreased VEGF expression in tumors. Overall, this study suggested the inhibition of advanced human prostate tumor xenograft growth in athymic nude mice by dietary silybin, which was associated with its *in vivo* antiproliferative, pro-apoptotic and anti-angiogenic efficacy.

In another study it was observed that oral silybin inhibited A549 lung tumor growth in athymic nude mice and formed a novel chemocombination with DOX and to targeted NF-κB-mediated inducible chemoresistance. It also exerted a strong preventive effect against DOX-caused adverse health effects in mice. Silybin and DOX decreased the proliferation index and tumor vasculature and increased apoptosis; these effects were further enhanced in combination treatment. The pharmacological dose of silybin achieved in the animal study (60 μmol/L) was biologically effective and showed enhanced efficacy in combination with DOX (25 nmol/L) in cell culture.

Table X. *Double blind clinical studies with silymarin in human subjects.*

Trials	Patients	Dose
Liver diseases (195)	97	D.N.A. 4 weeks
Liver cirrhosis (121)	170	140 mg/day x 2 years
Alcoholic liver cirrhosis (196)	60	D.N.A. 1 month
CAH (197)	10 *	240 mg/day
Cirrhotic diabetes (198)	30	600 mg/day x 12 months
Alcoholic liver cirrhosis (199)	200	450 mg/day
Alzheimer's (200)	222	420 mg/day x 1 week + tacrine
Chronic renal diseases (47)	49	858 mg/day x 2 months
Alcoholic liver cirrhosis (43)	60	150 mg; x3/day x 6 months
Chronic alcohol liver and concomitant T2DM (194)	60	135 mg/day x 6 months
Metabolism and disposition of metronidazole (201)	12	140 mg/day x 9 days from 7 days, 140 mg/day + Metronidazole (400 mg/day)
Chronic hepatitis C (202)	82	450 mg/day x 24 weeks

T2DM, non-insulin-dependent diabetes mellitus; CAH, chronic active hepatitis; D.N.A., data not shown.
* 4 males/ 6 females, mean age 50. References in brackets.

Silybin also inhibited DOX-induced NF-κB activation as a possible mechanism for chemoresistance. Furthermore, silybin inhibited COX-2, an NF-κB target, in combination with DOX. This study suggested silybin efficacy against *in vivo* lung tumor growth and its potentiating effect on DOX efficacy, as well as its protective effect against DOX-induced systemic toxicity and chemoresistance involving NF-κB signaling (167).

Cancer metastasis, involving multiple processes and various cytophysiological changes, may complicate the clinical management of cancer and is a primary cause of cancer death. Tumor invasion is one of the major problems in cancer therapy. Chu *et al.* showed that silybin exerted an inhibitory effect on the invasion and motility of highly metastatic A549 cells but had little effect on the adhesion. They showed that silymarin mediated these effects by decreasing the expression of MMP-2 and u-PA and enhancing the expression of tissue inhibitor of MMP (TIMP)-2. Silybin regulated the expression of MMP-2 and urokinase-type plasminogen activator (u-PA) on the transcriptional level, but of TIMP-2 on the translational or post-translational level (110).

10. Protective effects of silymarin

10A. Silymarin protects against alcohol-induced damage. Silymarin/silybin has been shown to protect from the toxicity caused by many agents. La Grange *et al.* investigated whether silymarin-phytosome (PHYTO) could protect the fetus from maternally ingested ethanol in female rats (17). Rats were assigned to pair-fed control, chow-fed control, ethanol and four groups receiving ethanol and PHYTO in varying dosages. The treatment protocol began on day 1 of pregnancy and the experiment was terminated on day 21. Silymarin alone had no significant effect on γ glutamyl transpeptidase (GGTP) activity; however, it inhibited ethanol-induced GGTP activity in the liver and brain tissue from both the fetuses and the dams. Moreland *et al.* also found that silymarin ameliorated some of the negative consequences of *in utero* exposure to ethanol in a rat model (168). They provided pregnant Fisher/344 rats with liquid diets containing 35% ethanol-derived calories throughout the gestational period. A silymarin/phospholipid compound containing 29.8% silybin was co-administered with ethanol. The researchers tested the offspring for laterality preference at 12 weeks of age and sacrificed the rats and perfused their brains for corpus callosum extraction. In the ethanol-only group, the development of the splenium was incomplete in the offsprings. Callosal development was complete in all other treatment groups. Rats from the ethanol-only group displayed a left paw preference, whereas control rats were evenly divided between right and left paw preference. Silymarin groups largely preferred the right paw. Overall, the addition of silymarin to the ethanol diet did confer some ameliorative effects upon the developing fetal rat brains.

10B. Silymarin protects against chemotherapy-induced nephrotoxicity. Cisplatin is one of the most active cytotoxic agents in the treatment of testicular cancer, but its clinical use is associated with side-effects, such as ototoxicity, neurotoxicity and nephrotoxicity. Long-term kidney damage from CDDP particularly affects the proximal tubular apparatus and can be detected by increased urinary excretion of brush-border enzymes, such as L-alanine-aminopeptidase (AAP) and magnesium. Bokemeyer *et al.* used silybin as a nephroprotectant against CDDP-induced nephropathy in a rat model. Infusion of silybin before CDDP significantly decreased glomerular toxicity as indicated by creatinine clearance and serum urea level and tubular kidney toxicity in

terms of excretion of brush-border enzymes and magnesium (169). Silybin given alone had no effect on renal function. In order to rule out the possibility that silybin inhibits the anti-tumor activity of CDDP and 4-hydroperoxy-ifosfamide, the researchers conducted *in vitro* studies in three established human testicular cancer cell lines. Dose-response survival curves for CDDP (3-30,000 nmol) combined with non-toxic doses of silybin (7.25-72.5 μmol) did not deviate significantly from those of CDDP alone during a 5-day assay using the sulphorhodamine-B staining. Silybin also did not influence the cytotoxic activity of 4-hydroperoxy-ifosfamide (30-10,000 nmol) *in vitro*. These observations rule out any significant inhibition of the antitumor activity of the major nephrotoxic components, CDDP and 4-hydroperoxy-ifosfamide by co-administration of silybin in a human germ cell tumor cell line. These results, together with silybin's, demonstrated cytoprotective effects in rats, which could form the basis for a randomized clinical trial of silybin for the protection of cisplatin-associated nephrotoxicity in patients with testicular cancer.

Gaedeke *et al.* evaluated whether silybin could ameliorate alterations in renal glomerular and tubular function and tubular morphology induced by CDDP (170). In a rat model, renal damage was induced by a single injection of CDDP (5 mg/kg) and silybin (200 mg/kg, *i.v.*) was administered 1 hour prior to the administration of CDDP. Kidney function was monitored by analyzing urinary markers of glomerular and tubular function over a period of 11 days. Rats were also sacrificed 4 days after drug application to evaluate tubular morphology. CDDP administration caused a decline in kidney function within one day of treatment. Toxicity was manifested in creatinine clearance and increases in proteinuria, in the urinary activity of the proximal tubular enzymes alanine aminopeptidase and N-acetyl-β-D-glucosaminidase and in renal magnesium wasting. The silybin pre-treatment prevented all these adverse effects of CDDP and alone did not have any effect on kidney function. Treatment with silybin distinctly diminished the morphological alterations observed in the S3-segment of the proximal tubule, 4 days after CDDP administration. Therefore, CDDP's effects on glomerular and proximal tubular function, as well as on proximal tubular morphology may be totally or partly ameliorated by silybin administration. In another study, Vogel *et al.* showed that alpha-amanitin produced pathological changes in the kidneys and that these lesions could be almost completely prevented by pre-treating rats with silymarin, as demonstrated by biochemical and histological evidence (171).

10C. Silymarin protects against chemotherapy-induced cardiotoxicity. Cardiotoxicity is a major concern that limits the success of cancer chemotherapy. Chlopcikova *et al.* tested silymarin and its constituents, namely silybin, dehydrosilybin, silychristin and silydianin for protective effects on rat cardiomyocytes exposed to DOX. Silymarin and individual flavonolignans treatments for 9 hours, did not exert cytotoxicity in the range of 25-100 μM and increased the cellular ATP level. Silymarin and flavonolignans displayed a dose-dependent cytoprotection against DOX. The protective effects of silymarin, silybin, dehydrosilybin and silychristin were comparable to those of dexrasoxane, whereas silydianin exerted the best protective effect. The ability of silymarin and its components to protect cardiomyocytes from DOX-induced oxidative stress was due mainly to their cell membrane stabilization effect, free-radical scavenging and iron chelating potency. In another study, Vereckei *et al.* showed that when silymarin, which does not have a direct antiarrhythmic action, was administered with amiodarone, it potentiated amiodarone's antiarrhythmic actions and prevented sustained atrial flutter by reducing and/or eliminating the excitable gap (172). Further, Chen *et al.* showed that oral administration of silybin (300 mg/kg daily) for 8-12 days reduced mortality in spontaneously hypertensive rats (173). Silybin reduced blood pressure and the incidence of post-occlusion arrhythmias in spontaneously hypertensive rats to the same extent as that of another drug tetrandrine. Both silybin and tetrandrine decreased the severity of ventricular hypertrophy. The results of this study implied that silybin may be beneficial in hypertensive patients who experience acute myocardial infarction.

10D. Silymarin is radioprotective. Systemic toxicity caused by radiotherapy has been known to deteriorate health during cancer treatment. It was observed that silymarin (70 mg/kg *p.o.*) administration twice a day for 7 or 14 days after total body γ-irradiation at a dose of 6 Gy, showed protection against nucleic acid changes (174). At the end of therapy, quantitative changes in nucleic acids were evaluated in the liver, spleen and bone marrow. The nucleic acid changes in irradiated rats were alleviated by the post-radiation application of silymarin in the liver, as well as in the spleen and bone marrow. Therefore, silymarin's protective effect against radiation-induced changes in nucleic acids could be attributable to the activation of cellular metabolism, including the metabolism of nucleic acids. Further, Hakova *et al.* examined the effect of silymarin on radiation injury in rats continuously irradiated with gamma rays (^{60}Co) for 14 days at doses of 0.2 and 0.6 Gy/day (175). In the course of irradiation the animals were treated with silymarin twice daily (70 mg/kg *p.o.*). Silymarin's effects were evaluated on the basis of quantitative changes in nucleic acids in the regenerating liver (after a 70% hepatectomy), spleen, bone marrow and blood. In this study also, silymarin had beneficial effects on the radiation-induced changes of DNA and RNA, especially in the bone marrow. In another study,

Hakova *et al.* showed that silymarin caused a modest increase in the concentration and total content of RNA and DNA in liver and bone marrow at days 7 and 14, and in the total DNA content in spleen at 30 hours. Silymarin administered 1 hour before irradiation moderated radiation-induced changes in nucleic acids in liver, spleen and bone marrow. Therefore, it can be suggested that silymarin's beneficial effects on radiation injury could be mediated *via* its antioxidative properties and its ability to act as a free-radical scavenger (176).

Ramadan *et al.* investigated the radioprotective effects of silymarin against radiation-induced hepatotoxicity at 3 or 6 Gy doses. Different modes of treatment were tested at 1, 3 and 7 days post-irradiation. Whole-body γ-irradiation led to an increase in serum alkaline phosphatase (AP) activity, as well as liver glutathione reductase (GR) and glutathione peroxidase (GSHPx) activities on the first post-exposure day. However, 3 days after exposure, these parameters (except serum activity) showed a significant decrease below the control level, which persisted until the end of the experiment. Serum AP activity increased again on the 7th post-exposure day at 3 Gy of radiation. A gradual increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and GGTP activities were observed throughout the duration of the experiment. A single dose of silymarin (70 mg/kg) or fractionated (490 mg/kg) oral dose or as an intravenous (*i.v.*) injection (50 mg/kg) provided significant protection, with the later showing the most pronounced protection. Consistent with other studies, silymarin's protective effect was attributed to its antioxidant and free-radical scavenging properties (35).

11. Silymarin modulates global gene expression

Silymarin was shown to alter the expression of many genes. Gallo *et al.* investigated the antitumor activity of IdB 1016 (Silipide), in an *in vivo* experimental model of ovarian cancer (177). They identified the mechanism of the anti-angiogenic action of silybin by assessing VEGF levels and by using microarray technology to evaluate the regulation of a panel of genes involved in angiogenesis. Female nude mice bearing human ovarian cancer xenografts (A2780) received 450 mg/kg/day IdB 1016 daily by oral gavage until the end of the study. At sacrifice, blood and tumor specimens were collected and subsequently processed to determine VEGF levels or a gene expression profile. IdB 1016 was significantly active in inhibiting ovarian tumor growth. The array analysis suggested the down-regulation of the VEGF receptor 3 and the up-regulation of angiopoietin-2 as potential mechanisms for the antiangiogenic activity. This study also suggested IdB 1016 as a good candidate for the management of recurrent ovarian cancer, and formed a basis for an ongoing phase II, non-randomized clinical study in the serological recurrence of ovarian cancer.

12. Pharmacology and metabolism of silymarin

12A. Pharmokinetics of silymarin. Numerous investigators have examined the pharmacokinetics, pharmacodynamics, metabolism and tissue distribution of silymarin in rodents and humans. Valenzuela *et al.* found that silymarin selectively increased the GSH content in different tissues of the rat (115). Silymarin increased the redox state and the total glutathione content of the liver, intestine and stomach, but did not affect the levels of the tripeptides in the kidney, lung and spleen. The selective effect of silymarin on the digestive organs was ascribed to their pharmacokinetics in the digestive tract, where the biliary concentration of silymarin was increased and maintained *via* the enterohepatic circulation. Skottova *et al.* investigated the pharmacokinetics of iodine-labeled silybin (I-SB) in the rat (178). To evaluate the potential for use of radiolabeled silybin, two silybin derivatives, separated by HPLC after iodination, (125)I-SB(1) and (125)I-SB(2), and their complexes 1:1 with phosphatidylcholine, (125)I-SPC(1) and (125)I-SPC(2), were administered concurrently with a single intragastric dose of 5.0 or 50 mg/kg of unlabeled silybin. Pharmacokinetic parameters, as well as organ uptake of (125)I-SB(1)-derived radioactivity, showed a dose-response pattern. The parameters of bioavailability after (125)I-SPC(1) intake were not influenced by unlabeled silybin (complexed with phosphatidylcholine) because maximal levels were achieved with the lower dose of unlabeled compound. The superior bioavailability of (125)I-SPC(1) at the lower dose of unlabeled compound was evidenced by elevated AUC and maximal percentage of administered radioactivity and increased radioactivity in liver, kidney, spleen and heart. An absence of these characteristics with (125)I-SB(2) and (125)I-SPC(2) suggested that (125)I-SB(1) could be used to study modulation of its bioavailability.

Morazzoni *et al.* evaluated the plasma level profile and biliary excretion of silymarin in rats after single equimolar oral doses of 200 mg/kg silybin, in the form of silipide and silymarin (179). Silybin was assayed with a specific HPLC method that allowed the presence of other flavanolignans to be determined in the biological fluids after administration of silymarin. After administration of oral silipide, silybin reached peak plasma levels within 2 hours, with a C_{max} of 9.0 μg/ml for unconjugated drug and 93.4 μg/ml for total drug. Maximum total biliary concentrations of silybin (2989 μg/ml) were observed within 2 hours, and the biliary recovery after 24 hours accounted for about 13% of the administered amount. Compared to silipide, silymarin showed lower levels of unconjugated and total plasma silybin, as well as biliary excretion. In case of silymarin, the recovered dose of silybin after 24 hours was approximately 2% of the administered dose. Oral silymarin also showed the presence of silydianin, silychristin and, to a greater extent, isosilybin which was relatively more than silybin in plasma and

bile. The relative bioavailability of silipide was 10-fold higher than that of silymarin.

Morazzoni *et al.* has also examined the comparative bioavailability of silipide (180). The comparative pharmacokinetics of silipide and silybin were investigated by measuring unconjugated and total plasma silybin levels, as well as total biliary and urinary silybin excretion in rats after administration of a single oral dose (200 mg/kg as silybin). Mean peak levels of unconjugated and total silybin were 8.17 and 74.23 μg/ml, respectively. Approximately, 94% of the plasma silybin was present in a conjugated form. Cumulative biliary (0-24 hours) and urinary (0-72 hours) excretion values were 3.73% and 3.26% of the administered dose, respectively. However, after silybin administration, these values were only 0.001% and 0.032% of the dose, respectively. Again, this study suggested the superior bioavailability of silybin when administered orally as silipide, *via* its increased gastrointestinal absorption.

Schandalik *et al.* examined the pharmacokinetics of silybin following oral administration of silipide in 14 patients with cholestasis secondary to biliary extrahepatic obstruction (181). Each patient received a single oral dose of silipide (120 mg, as silybin) and blood samples collected at frequent intervals for up to 24 hours, were analyzed for free and total (free+conjugated) silybin. Silipide was rapidly absorbed from the gastrointestinal tract and level of free drug peaked within 3 hours in most patients. Thereafter, the decline in plasma free silybin levels was relatively rapid and at 12 hours approached the limit of quantitation (2 ng/ml). Total silybin levels reached a peak at about 3 to 4 hours and persisted at relatively high values (≥400 ng/ml) throughout the entire sampling period and were about more than 40-fold higher than the free silybin. The results of this study suggested that the extrahepatic biliary obstruction was associated with a reduced clearance of conjugated silybin, probably because of impaired excretion of the conjugate in bile.

Schandalik *et al.* also examined the pharmacokinetics of silybin in bile following a single administration of silipide and silymarin (120 mg, as silybin) in cholecystectomy patients with T-tube drainage (182). After intake of silipide, the concentration of silybin in bile peaked within 4 hours and declined thereafter, with a mean time of about 10 hours. After administration of silymarin, biliary silybin concentrations were several fold lower than those observed after intake of silipide. Silymarin intake also showed considerable amounts of isosilybin and very low levels of silydianin and silychristin in bile. Total silybin recovered in bile within 48 hours accounted for 11% of the dose of silipide and for 3% of the dose of silymarin. Silybin levels in three subjects were lower than those in bile after silipide intake; however, it was mostly undetectable after silymarin intake. This study again confirmed the superior bioavalability of silybin from silipide as compared to silymarin.

Weyhenmeyer *et al.* examined the dose-linearity of the pharmacokinetics of silybin diastereomers using a stereospecific assay (183). Single doses of 102, 153, 203 and 254 mg silybin were administered as silymarin capsules (Legalon 140) to six healthy male volunteers. The researchers assayed both diastereomers of silybin in plasma as unconjugated compounds, as well as total isomers after hydrolysis. Approximately 10% of total silybin in plasma was unconjugated. The ratio of the silybin isomers was reversed, when unconjugated and total isomers were compared. For unconjugated silybin, the half-lives was less than 1 hour, but the terminal half-life were probably not observed, because after 4-6 hours, the levels already have fell below the detection limit of 2.5 ng diastereomer/ml. The elimination half-life for total silybin was estimated to be approximately 6 hours. About 5% of the dose is excreted into urine as total silybin, corresponding to a renal clearance of approximately 30 ml/min. No adverse health events were noted, indicating that silymarin, even in doses up to 5 capsules of Legalon 140, was well-tolerated.

Another pharmacokinetic study was performed in six human subjects with 560 mg of silymarin (silybin 240 mg = 8 Legalon dragees) (184). The maximum serum concentrations were low, ranging from 0.18 to 0.62 μg/ml. The same was true of renal excretion, showing only 1-2% of the silybin dose over 24 hours. However, for patients who had undergone cholecystectomy and given 140 mg of silymarin (silybin 60 mg), bile analyses showed maximum silybin concentrations between 11 and 47 μg/ml which were again very high compared to that of serum. In this study, since complete collection of bile from these patients was impossible and it was not possible to collect individual samples in the time interval from 12-24 hours, the conventional pharmacokinetic parameters required for bioavailability studies could not be calculated. However, computation of the value for AUC(0-24) and for 'mean time' provided data that enabled bioavailability comparisons of the absorption of Legalon 35 and Legalon 70 dragees, which could also be used in case of bile.

Optically pure silybin A (1) was glucuronidated *via* bovine liver glucuronyl transferase resulting in three beta-glucuronides of silybin. The substituted phenolic OH groups were at the positions C-20 (2), C-7 (3) and C-5 (4) with a yield of 27%, 62.5% and 2.5%, respectively. Kren *et al.* showed that the main silybin conjugate in humans was (2) and (3) was formed in lower proportions (185). The conjugation rate of silybin diastereomers 10S, 11S and 10R, 11R, as well as their metabolism in humans was somewhat different. The free-radical scavenging activity of (2) was considerably lower than that of its aglycone (1); however, the activity of (3) was higher than silybin. This is the first pharmacological study using optically pure silybin suggesting that at physiological pH, the exclusive target for one-electron

oxidation of the silybin molecule is the o-methoxy-phenolic structure at C-19, C-20. Gunaratna *et al.* showed that when silybin was incubated with human liver microsomes, it produced one major metabolite and at least two minor metabolites. Tandem mass spectrometry studies confirmed that the major metabolite was demethylated silybin and two minor metabolites were mono-hydroxy and di-hydroxy silybin. The K_m value for the demethylation indicated that silybin has a strong affinity for the cytochrome P-450 enzymes. Overall, since the conjugation of silybin, leads to ineffectivity the modification of these conjugating positions could add to an effective silybin drug formulation.

12B. Liposomal silymarin. Maheshwari *et al.* encased a drug into liposomal structures to make them more effective, safe and targeted to liver cells (186). Small unilamellar liposomal vesicles were prepared through the ethanol injection method and formulation and process variables were optimized to improve drug entrapment efficiency. The study included the selection of lipid composition, impact of the charge-imparting agent, nature of the hydration medium, and determination of the stability and size parameters. In mouse studies, liposomal systems were studied for hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity and gastroprotective activity using the pyloric ligation method. Cholesterol showed a significant effect on drug-entrapment and drug-leakage characteristics. The most frequent size distribution of vesicles ranges from 0.266 to 0.466 micron showing the drug loading of approximately 90%. The lipid cholesterol mass ratio of 10:2 showed a maximum entrapment of 87.2%. *In vivo* studies revealed the improved performance of silymarin for hepatoprotection and anti-ulcer activities in liposomes as compared to plain drugs.

12C. Bioavailability of silymarin. Kim *et al.* examined the comparative bioavailability of a Liverman capsule to a Legalon capsule and a silymarin tablet in 24 healthy Korean male volunteers (187), who received a silybin dose of 120 mg in a 3x3 crossover study, with a 1-week washout period among the doses. Plasma concentrations of silybin were monitored over a period of 12 hours after the administration. After an oral administration of Liverman capsule, the pharmacokinetic parameters of silybin, such as AUC (0-12 hours) and AUC inf were significantly greater; C_{max} was significantly higher; and T_{max} was significantly faster than those after the Legalon capsule and silymarin tablet. This study indicated a faster absorption and a greater extent of relative oral bioavailability of silybin from the Liverman capsule as compared to the Legalon capsule or silymarin tablet.

Svager *et al.* assessed the role of plasma lipoproteins in the transport of silybin, using (125)I-labeled silybin administered orally to the rat (188). The plasma (125)I-silybin-derived radioactivity was distributed among plasma lipoproteins according to their lipophilicity, and in the fraction of d >1.215 containing albumin and other proteins, a minimal amount of radioactivity was found. Administration of (125)I-silybin in a complex with phosphatidylcholine resulted in proportionally higher radioactivity in all fractions and tissues. Dietary olive oil had a slight diminishing effect on plasma concentrations of silybin on (125)I-silybin-derived radioactivity in plasma and liver. In the TAG-rich lipoprotein fraction and HDL, olive oil had no effects on the levels of (125)I-silybin-derived radioactivity; however, it was significantly decreased at a 30-min interval in LDL and the heart. This study suggested that silybin was not resorbed by the chylomicron pathway, and the endogenous lipoprotein pathway (VLDL → LDL) may play a role in silybin transport from the liver to the extrahepatic tissues.

To increase silybin's bioavailability, Comoglio *et al.* complexed it in 1:1 ratio with phosphatidylcholine to produce IdB 1016 (60). One hour after intragastric administration of IdB 1016 (1.5 g/kg) to rats, the concentration of silybin in liver microsomes was approximately 2.5 μg/mg protein, corresponding to a final concentration of about 10 μM. It also decreased the lipid peroxidation induced in microsomes by NADPH, CCl_4 and cumene hydroperoxide by about 40%. Spin trapping experiments showed lipid dienyl radical scavenging ability of the complexed form of silybin. In addition, it also interacted with free-radical intermediates produced during the metabolic activation of carbon tetrachloride and methylhydrazine. Overall, IdB 1016 was observed as a potentially protective agent against free-radical-mediated toxic damage.

Savio *et al.* used soft gelatin capsule technology as an enhancer device for the absorption of silymarin in humans (189). To evaluate whether a patented soft gelatin capsule could improve the bioavailability of silybin (CAS 22888-70-6) compared to that of a hard shell capsule, the investigators performed an open, single-dose, two-way, balanced crossover study. The study was conducted on 12 healthy subjects (6 males and 6 females) with 80 mg dose of silybin in a 1:2 complex with phosphatidylcholine. Blood samples were collected at 0, 1, 2, 3, 4, 6 and 8 hours. Plasma analyses demonstrated that both C_{max} and AUC (0-1) were increased by more than 3- and 2-fold, respectively, when the patented soft gelatin capsule formulations were administered. Barzaghi *et al.* also determined plasma silybin levels after administering a single oral dose of IdB 1016 and silymarin (equivalent to 360 mg silybin) to nine healthy volunteers (190). Although absorption was rapid in both preparations, the bioavailability of IdB 1016 was comparatively much greater. Regardless of the preparation used, the terminal half-life was relatively short (generally less than 4 hours). In a subsequent study, nine healthy volunteers received IdB 1016 (equivalent to 120 mg silybin) for 8 consecutive days and showed similar silybin plasma levels from day 1 to day 8. Most of the silybin present

in the systemic circulation was in conjugated form. Less than 3% of the administered total silybin dose was secreted in the urine. The researchers concluded that the oral bioavailability of silybin increased when complexed with phosphatidylcholine in IdB 1016, probably through facilitation of its passage across the gastrointestinal mucosa.

In another study, Gatti *et al.* examined the plasma concentrations of free and conjugated silybin after oral intake of a single dose of silipide (equivalent to 80 mg silybin) in 12 healthy volunteers (191). Free silybin concentrations reached a peak of 141 ng/ml at 2.4 hours and declined thereafter with a half-life of about 2 hours. The peak concentration of conjugated silybin was 255 ng/ml with a half life of about 3.8 hours and showed slower elimination. AUC values for conjugated silybin were about 3-fold greater than those of free drug. This study also indicated that after oral intake of silipide, silybin extensively converted into conjugated derivative(s) that are retained in the circulation at relatively large concentrations.

12D. Tissue distribution of silymarin in mice. Zhao *et al.* investigated the distribution and conjugate formation of systemically administered silybin in liver, lung, stomach, skin, prostate and pancreas (68). SENCAR mice were starved for 24 hours, orally fed with silybin (50 mg/kg dose) and killed after 0.5, 1, 2, 3, 4 and 8 hours. Tissues were collected and homogenized, and then extracted with butanol:methanol for HPLC analysis. Portion of the homogenates was digested with sulfatase and β-glucuronidase for the total extraction of silybin. Silybin levels peaked at 0.5 hour after administration in liver, lung, stomach and pancreas, accounting for 8.8, 4.3, 123 and 5.8 μg silybin/g of tissue, respectively. In the case of skin and prostate, the peak levels of silybin were 1.4 and 2.5, respectively, and were achieved 1 hour after administration. Sulfate and β-glucuronidate conjugates of silybin showed peak levels at 1 hour in all tissues except lung and stomach, in which peak levels were shown at 0.5 hour. Levels of both free and conjugated silybin declined after 0.5 or 1 hour in an exponential fashion with an elimination half-life of 57-127 min for free silybin and 45-94 min for conjugated silybin. Oral feeding of silybin at 100 and 200 mg/kg/day doses also showed a moderate to highly significant increase in both glutathione S-transferase and quinone reductase activities in liver, lung, stomach, skin and small bowel.

13. Silymarin inhibits glucuronidation

Chrungoo *et al.* examined the influence of silymarin on UDP-glucuronic acid (UDPGA) and glucuronidation activity of freshly isolated rat hepatocytes in suspension and in rat liver *in vivo* (192). Silymarin at 0.4 mM depleted UDPGA by more than 60% after 4 hours of incubation. The fall in the nucleotide pool was rapid and concentration dependent. Silybin inhibited the rate of glucuronidation of 3-OH-benzo(α)pyrene (3-OH-BP) three times more effectively than silymarin. Combining silymarin/silybin with D-galactosamine (GalN) further attenuated the glucuronidation functions. Silymarin/silybin also strongly inhibited UDP-glucose dehydrogenase (UDP-GDH) activity in the liver cytosolic fraction, whereas the activity in hepatocytes was not affected even after 4 hours of incubation. Flavonoids completely abolished the GalN-induced strong inhibition of UDP-GDH in isolated hepatocytes. The decrease in UDPGA apparently did not involve the activation of UDPGA-pyrophosphatase activity or the inhibition of UDP-GDH activity in hepatocytes. The flavonoids also inhibited hepatic UDP-glucuronyltransferase activity towards 3-OH-BP (UGT). In contrast, silymarin (70 mg/kg, *i.p.*) administered to rats for 3 hours increased the hepatic UDPGA by 2-fold, whereas GalN (400 mg/kg) reduced the nucleotide content to 50% of control. Co-administration of silymarin and GalN restored the UDPGA content, without having any effect on UDP-GDH or UGT. The results of this study indicated that silymarin had different effects on the rate of glucuronidation and contents of UDPGA in isolated rat hepatocytes and liver. The flavonoid counteracted D-GalN-induced lowering of UDPGA, most likely by relieving UDP-GDH of *in vivo* inhibition caused by the GalN-metabolite.

Han *et al.* also examined the glucuronidation of silymarin. The results showed that silybin is extensively metabolized and the major sites for glucuronidation are the C-20, C-7 at phenolic OH groups. A significant stereoselectivity in the glucuronidation process of silybin was observed. Silybin B was glucuronidated at a more efficient rate than its diastereoisomer, and glucuronidation of silybin B was much preferred at the C-20 position. However, glucuronidation was similar at the C-7 and C-20 in case of silybin A (193).

14. Clinical trials

Although several clinical studies of silymarin have been carried out in humans to investigate its pharmacokinetics and its antioxidant, immunomodulatory, antidiabetic and hepatoprotective potential (Table X), no study for its anticancer potential has been reported in humans. Here, some of the relevent findings of the completed clinical trials are described. Lucena *et al.* examined the effects of silymarin MZ-80 on oxidative stress in patients with alcoholic cirrhosis in a randomized, double-blind, placebo-controlled trial (43). They investigated the clinical outcome, biochemical profile and the antiperoxidative effects of silymarin MZ-80 during 6 months of treatment. Sixty patients were randomized and matched for demographics and baseline clinical and laboratory parameters to receive either silymarin MZ-80 (150 mg *t.i.d.* per day) or placebo for 6 months. GSH content, MDA and serum amino-terminal propeptide of

procollagen Type III (PIIINP) were determined at baseline and at the end of treatment. Forty-nine patients completed the study (24 silymarin and 25 placebo). Silymarin significantly increased total GSH at 6 months (4.5 to 5.8 μmol/g of hemoglobin), whereas in the placebo group, it remained unchanged (4.1 to 4.4 μmol/g of hemoglobin), and platelet-derived non-induced MDA decreased by 33%. A parallel decrease in PIIINP values was seen with silymarin (1.82 to 1.36 U/ml) but not with placebo (1.31 to 1.27 U/ml). Overall, silymarin was well tolerated and produced a small increase in GSH and a decrease in lipid peroxidation in peripheral blood cells in patients with alcoholic liver cirrhosis; however, no changes in routine liver function tests were observed during the course of therapy.

Cyclic voltametry (CV) is a simple, time- and cost-effective and relatively reliable method to assess body antioxidant status. Using this method, Psotova *et al.* determined total antioxidant capacity of silymarin in healthy volunteers and patients with chronic renal diseases (47). The first group consisted of 29 volunteers (males, range 18-21 years of age) who were administered silymarin at a dose of 858 mg/day or a placebo. After two months of silymarin administration, a significant increase in total antioxidant capacity was observed as compared to placebo. No changes were seen in TBARS, SH-groups, creatinine, urea or uric acid concentrations. The second group comprised 49 patients with chronic renal disease who were undergoing dialysis. After dialysis, CV revealed a decrease in total antioxidant capacity in the plasma that was related to a decrease in creatinine, urea and uric acid. CV was performed using a system consisting of a working glassy carbon electrode, an auxiliary platinum electrode, and a reference saturated calomel electrode; a linear change in voltage of 200 mV/s was applied.

Non-insulin-dependent diabetes mellitus (type 2 diabetes mellitus, T2DM) is associated with chronic liver disease, increased plasma levels of glucose, insulin and triglycerides and enhanced lipid peroxidation and depletion of endogenous antioxidant reserves. Therefore, it was hypothesized that the rebalancing of cell redox levels and amelioration of liver function would result in a better glucose and lipid metabolism (194). Investigators assessed the effect of a new oral formulation of silybin-beta-cyclodextrin (IBI/S) in patients with chronic alcoholic liver disease associated with T2DM. Forty-two patients (21 in the IBI/S group, who received 135 mg/day silybin were examined and 21 in the placebo group) completed the 6-month treatment period. In addition to conventional liver function tests, other parameters, including fasting and mean daily plasma glucose levels, glycosylated hemoglobin (HbA1c), basal and stimulated C-peptide and insulin levels and sensitivity, total cholesterol, HDL cholesterol, triglyceride and MDA levels. Fasting blood glucose levels decreased by 15% in the IBI/S group, while remained unchanged in the placebo group. A comparison of the groups at 6 months also showed a significant reduction in glucose levels in the IBI/S group. The same trend was observed in mean daily blood glucose levels, HbA1c and insulin sensitivity, although the differences were not significant. C-peptide values suggested that insulin secretion was virtually unaffected. Plasma triglyceride concentrations dropped from a baseline value of 186 to 111 mg/dl in the IBI/S group, while it increased from 159 to 185 mg/dl in the placebo group. The results from total and HDL cholesterol levels and liver function tests did not show significant changes and no clinically relevant side-effects were observed in either group. MDA decreased significantly only in the group receiving IBI/S. Therefore, it could be suggested that oral administration of IBI/S to patients with T2DM and compensated chronic alcoholic liver disease decrease plasma glucose and triglyceride levels probably *via* the recovery of energy substrates and improved insulin activity.

Several studies have demonstrated that diabetic patients with cirrhosis require insulin treatment because of insulin resistance. As chronic alcoholic liver damage is partly due to the lipoperoxidation of hepatic cell membranes, antioxidants may be useful in treating or preventing free-radical damage. In a 12-month study, Velussi *et al.* found that silymarin (600 mg/day plus standard therapy) was effective in reducing hyperinsulinemia, the need for exogenous insulin and malondialdehyde levels in diabetic patients with cirrhosis (198). A similar result was observed on fasting blood glucose levels, mean daily blood glucose levels, daily glucosuria, HbA1c and insulin requirement as reported in the previous IBI/S study. MDA levels were also decreased. This study also suggested that silymarin reduced the lipoperoxidation of cell membranes and insulin resistance. Lang *et al.* examined the hepatoprotective and immunomodulatory effects of silymarin and amino-imidazole-carboxamide-phosphate in 40 patients with alcoholic cirrhosis of the liver in a 1-month double-blind clinical trial (203). These drugs normalized the elevated levels of aspartate aminotransferase, alanine amino-transferase and serum bilirubin. A marked reduction in the high level of gamma-glutamyl transferase, an increase in lectin-induced lymphoblast transformation, a decrease in the percentage of OKT8+ cells and suppression of lymphocytotoxicity were observed. The hepatoprotective effects of these drugs in alcoholic cirrhosis were partially mediated *via* immunomodulatory activity.

Most of the hepatoprotective drugs belong to a group of free-radical scavengers. The mechanism of action involves membrane stabilization, neutralization of free radicals and immunomodulation. In order to assess the hepato-protective activity and antioxidant properties of a IdB-1016, Buzzelli *et al.* carried out a short-term pilot study on 20 patients with chronic active hepatitis, who were randomly assigned to 240 mg equivalent of silybin *b.i.d.* (197). Silybin significantly reduced the serum

concentrations of aspartate aminotransferase from 88.0 to 65.9 u/l; alanine aminotransferase from 115.9 to 82.5 u/l; γ-glutamyltranspeptidase from 51.4 to 41.3 u/l; and total bilirubin from 0.76 to 0.53 mg/dl. Alkaline phosphatase reduced slightly and no significant changes were observed in serum concentrations of MDA, copper or zinc. This study suggested that IdB-1016 could improve results of liver function tests related to hepatocellular necrosis and/or increases in membrane permeability in patients affected by chronic active hepatitis.

To study the efficacy of silymarin in human liver cirrhosis, Pares *et al.* examined its effect in alcoholics with liver cirrhosis in terms of survival and clinical and laboratory changes in a controlled, double-blind, randomized, multicenter trial. From February 1986 to June 1989, 200 alcoholic patients were enrolled with histologically or laparoscopically proven liver cirrhosis, who were given 150 mg silymarin thrice a day (n=103) or placebo (n=97) (199). Primarily, survival time, and secondarily, progression of liver failure were monitored. A 2-year study period was completed with 125 patients (57, silymarin; 68, placebo). Survival was similar in both groups; 15 patients in the silymarin and 14 in the placebo group died during the trial. Silymarin had no significant effect on the course of the disease, and no relevant side-effects were observed in either group. This study suggested that silymarin had no effect on survival or the clinical course of alcoholic liver cirrhosis. However, in another randomized, controlled trial study with silymarin in 170 patients with cirrhosis, beneficial effect of slymarin was observed (121). Eighty-seven patients received 140 mg of silymarin three times per day, and 83 patients received a placebo. All patients received their designated treatment until the last patient entered had finished 2 years of treatment. The mean observation period was 41 months. No side-effects were observed. In the placebo group, 37 (+2 drop-outs) patients died, and 31 out of these were related to liver disease. In the silymarin group, 24 (+4 drop-outs) patients died, with 18 due to the liver disease. The 4-year survival rate was 58% in the silymarin group and 39% in the placebo group (p=0.036). Further analysis of subgroups indicated that silymarin was significantly effective in patients with alcoholic cirrhosis and those initially rated as Child A.

Lang *et al.* studied the hepatoprotective and immunomodulatory effects of silymarin and amino-imidazol-carboxamid-phosphate in 60 patients with compensated alcoholic cirrhosis of the liver in a 1-month double-blind clinical trial (26). These drugs normalized the elevated levels of aspartate aminotransferase, alanine aminotransferase and serum bilirubin; markedly reduced the high level of gamma-glutamyl transferase; increased lectin-induced lymphoblast-transformation; decreased the percentage of $CD8^+$ cells; and suppressed lymphocytotoxicity, suggesting their immunomodulatory effects in hepatoprotection.

Feherand *et al.* examined the *in vitro* and *in vivo* effects of silybin on the expression and activity of SOD enzyme in lymphocytes from patients with chronic alcoholic liver disease (119). Silybin concentrations corresponding to the usual therapeutic dosage markedly increased the SOD expression of lymphocytes *in vitro*. Silybin treatment *in vivo* restored the originally low SOD activity of the patients' lymphocytes. This study supported the role of antioxidant activity of silybin in hepatoprotection. In another study, *in vitro* and *in vivo* effects of four hepatoprotective agents – silymarin (Legalon®), (+)-cyanidanol-3 (Catergen®), 6,6-methylene-bis (2,2,4-trimethyl-1,2-dihydroquinoline) (MTDQ) and 4,5-amino-imidazole-carboxamide-phosphate (Aica-P) – were examined on the expression and activity of SOD enzyme and on cellular immune reactions in lymphocytes (and erythrocytes) from patients with cirrhosis and from healthy control subjects (204). These drugs inhibited lectin-induced lymphocyte transformation and some decreased antibody-dependent, spontaneous and lectin-induced lymphocytotoxicity *in vitro*. MTDQ, silymarin and Aica-P enhanced the SOD activity of erythrocytes and lymphocytes, and silymarin and Aica-Pand (+)-cyanidanol-3 increased the superoxide expression in lymphocytes. Aica-P restored the originally low lymphocyte transformation values of patient lymphocytes. This study suggested the role of both antioxidant and immunomodulatory activities in the hepatoprotective efficacy of these drugs.

Salmi *et al.* evaluated the effect of silymarin on chemical, functional and morphological alterations of the liver in a double-blind controlled study involving 106 consecutive patients with liver disease and elevated serum transaminase levels (195). Patients in the series had relatively slight acute and subacute liver disease, mostly induced by alcohol abuse. Silymarin showed a greater decrease in S-SGPT (S-ALAT) and S-SGOT (S-ASAT) than the placebo. Serum total and conjugated bilirubin decreased in the treated patients than in the control group, but the differences were not statistically significant. BSP retention returned to normal more often in the treated group. Silymarin treatment also showed more normalization of histological changes than placebo.

Tacrine, the first drug marketed for Alzheimer's disease (AD), induces an elevation of serum liver transaminase, limiting the use of an effective dosage in many patients. Allain *et al.* examined the effect of silymarin in tacrine-treated patients with AD in a 12-week randomized, double-blind, placebo-controlled study at 22 French neurology and geriatric centers (200). Outpatients suffering from mild-to-moderate dementia of the Alzheimer type were randomly assigned to two treatment groups: tacrine plus silymarin (n=110) and tacrine plus placebo (n=112). Silymarin (420 mg/day) was given first for 1 week. Tacrine was added at 40 mg/day for 6 weeks and then increased to 80 mg/day for 6 weeks. Serum ALAT was the main evaluation criteria and

Table XI. *Adverse drug effects of recent FDA-approved drugs.*

Drug	Target	Use	Year approved	Year withdrawn/ warning label	Reason
Voixx (Rofecoxib)	COX-2	RA	1999	2004	Heart attack
Celebrex* (Celecoxib)	COX-2	RA	1998	2004	Heart attack
Bextra *(Valdecoxib)	COX-2	RA	2001	2004	Heart attack
Iressa (Gefinitib)	EGFR tyrosine kinase	NSCLC	2002	2004	No clinical benefits
Remicade (anti-TNF antibody)	TNF	CD RA	1998	2001**	High risk of lymphoma
Prozac (Fluoxetine)	Serotonin	Antidepressant	1986	2004**	Suicidal thoughts and behavior
Stattera (Atomoxetine hydrochloride)	Norepinephrine reuptake inhibitor	Attention deficit Hyperactivity disorder	2002	2004**	Hepatotoxic
Fen-phen (Phentermine/ Fenfluramine)	Serotonin/catecholamines	Anti-obesity	1959/ 1973	1997	Valvular heart disease, PPH
Rezulin (Troglitazone)	PPARγ	Diabetes	1999	2000	Hepatotoxic
Avandia (Rosiglitazone)	PPARγ	Diabetes	1999	2001**	Hepatotoxic
Propulsid (Cisapride)	Potassium channels	Heart burn	1993	2000	Cardiotoxic
PPA	ANS	Cold, cough		2000	Hemorrhagic stroke
Baycol (Cerivastatin)	HMG-CoA reductase	Cholesterol lowering	2000	2003	Rhabdomyolysis
Arava (Leflunomide)	Dihydroorotate dehydrogenase	RA	1997	2000	Peripheral neuropathy
Lontex (Alosetron hydrochloride)	5-HT3 receptor antagonist	Irritable bowel	2000	2004	Ischemic colitis
Serzone (Nefradone hydrochloride)	Serotonin type 2 receptor	Antidepressant			Liver injury

*Although Celebrex has been linked with cardiotoxicity, no black label warning has yet been issued. **Black box warning.
ANS, autonomic nervous system; CD, Crohn's dosease; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; HMG-CoA, 3-hydroxy-3-methylglutamyl coenzyme A; 5-HT3R, serotonin 5-HT3A receptor; TNF, tumor necrosis factor; NSCLC, non-small cell lung carcinoma; PPH, primary pulmonary hypertension; PPAR, peroxisome proliferators-activated receptor; PPA, phenylpropanolamine; RA, rheumatoid arthritis.

serum ASAT, adverse side-effects and cognitive performance were secondary. No statistical difference was observed between the two groups for serum ALAT. Adverse effects, including gastrointestinal disorders were rare in the silymarin group. Cognitive performance remained unchanged in both groups. This study suggested the possible role of silymarin in reducing the tacrine-induced gastrointestinal and cholinergic side-effects, without any impact on cognitive status and, therefore, leading to an improvement of the tolerability of tarcine in the initial phases of AD treatment.

El-Zayadi *et al.* investigated the effect of silymarin for amelioration of necro-inflammation among chronic hepatitis C (CHC) patients with elevated ALT and detectable hepatitis C virus (HCV), who could not afford IFN-based therapy. They were randomly allocated either to non-interferon-based therapy (N-IFN-BT) (group I) or silymarin therapy (group II). Group I (n=87) was administered a daily combination of ribavirin (600-800 mg) plus amantadine (200 mg) and ursodeoxycholic acid (UDCA) (500 mg); Group II (n=83) was administered silymarin 450 mg/day for 24 weeks. A significant normalization of ALT was achieved in 58.5% and 15.3%, whereas end-of-treatment virologic response (ETVR) was achieved in 2.4% and 0% of Groups I and II, respectively. Sustained biochemical response (SBR) was significantly achieved in 28% and 2.8%, while sustained virological response (SVR) was maintained in 2.4% and 0% of the patients in Groups I and II, respectively. Overall, 24-week N-IFN-BT achieved a 4-fold-higher ETVR and a 10-fold-higher SBR compared to silymarin therapy, and showed an improvement in necroinflammatory activity.

15. Drug interaction with silymarin

Piscitelli *et al.* examined the effect of silymarin on the pharmacokinetics and efficacy of indinavir (205). Baseline pharmacokinetics for indinavir were established from the blood samples of 10 healthy volunteers who were given four doses of indinavir 800 mg every 8 hours on an empty stomach. Then volunteers took milk thistle 175 mg, confirmed to contain 153 mg of silymarin, 3 times/day for 3 weeks and exposed to similar dosing of indinavir. After an 11-day washout, Indinavir concentrations were measured. The parameters determined were the highest concentration (C_{max}), 0-hour concentration, 8-hour concentration (C8), time to reach C_{max} and AUC over the 8-hour dosing interval (AUC8). Silymarin did not significantly alter the overall

Table XII. *Sources of silymarin.*

Brand	Web site	Caps or Tbs
Human use		
BioSorb (St. Charles, MI, USA)	http://www.biosorb-inc.com/	250 mg
Country Life (Hauppauge, NY, USA)	http://www.country-life.com/	158 mg
Doctor's Trust (Orlando, FL, USA)	http://www.doctorstrust.com/	250 mg
Douglas Labs Bayh (Pittsburgh, PA, USA)	http://www.douglaslabs.com/	200 mg
Dream Pharm (Glendale, CA, USA)	http://dreampharm.com/	160 mg
Eclectic Institute (Sandy, OR, USA)	http://www.eclecticherb.com/	24 mg
Futurebiotechs (Happauge, NY, USA)	http://www.futurebiotics.com/	140 mg
Gaia herbs (Brevard, NC, USA)	http://www.gaiaherbs.com/	---
Jarrow (Los Angeles, CA, USA)	http://www.jarrow.com/	120 mg
Karuna (Novato, CA, USA)	http://www.karunahealth.com/	136 mg
Life Link (Grover Beach, CA, USA)	http://www.lifelinknet.com/	250 mg
Maxi Health Research (Brooklyn, NY, USA)	http://www.maxihealth.com/	---
Metabolic Maintenance (Sisters, OR, USA)	http://www.metabolicmaintenance.com/	300 mg
Metagenics (Clemente, CA, USA)	http://www.metagenics.com/	56 mg
Natural Factors (Everett, WA, USA)	http://www.naturalfactors.com/	200 mg
Nature's Herbs (American Fork, UT, USA)	http://www.naturesherbs.com/index.htm	160 mg
Nature's Way (Springville, UT, USA)	http://www.naturesway.com/	175 mg
Now Foods (Bloomingdale, IL, USA)	http://www.nowfoods.com/	300 mg*
Nutraceutical Science Institute (NSI)		300 mg
Oregon's Wild Herbes (Sandy, OR, USA)	http://oregonswildharvest.com/	320 mg
Paradise Herbs (Fountain valley, CA, USA)	http://paradiseherbs.com/	200 mg
Planetary Formulas (Soquel, CA, USA)	http://www.planetaryformulas.com/	260mg
Pure Encapsulations (Sudbury, MA, USA)	http://www.purecaps.com/	200 mg
Rx Vitamins (Larchmont, NY, USA)	http://www.rxvitamins.com/	120 mg
Solgar (Leonia, NJ, USA)	http://www.solgar.com/	140 mg
Source Naturals (Scotts Valley, CA, USA)	http://www.sourcenaturals.com/	189 mg
Wonder Laboratories (White House TN, USA)	http://www.wonderlabs.com/	160 mg
YERBA PRIMA (Ashland, OR, USA)	http://www.yerba.com/	26.4 mg
ZAND Herbal formula (Ferndale, WA, USA)	http://www.zand.com/	---
Laboratory use		
Indena USA (Seattle, WA, USA)	http://www.indena.com	N/A
LKT (St. Paul, MN, USA)	Http://www.lktlabs.com/	N/A
Madaus (Germany)	http://www.madaus.com	N/A
Sigma-Aldrich (Milwaukee, WI, USA)	http://www.sigmaaldrich.com/	N/A

*Supplement including 300 mg silymarin and 700 mg turmeric extracts.

exposure of indinavir; however, the C8 level was significantly decreased by 25%. Silymarin did not interfere with indinavir therapy in patients infected with the human immunodeficiency virus.

In another study, Leber *et al.* examined the influence of silymarin on drug metabolizing enzymes in rats and humans (206). Male rats were treated orally with a daily dose of 100 mg silymarin (Legalon)/kg for 4 or 10 days. These treatments increased the activity of the mixed function oxidation system including cytochrome P-450, aminopyrine demethylation and p-nitroanisole demethylation. No alterations were observed in the body weight, liver weight, microsomal protein content, cytochrome b5 content and activities of glucose-6-phosphatase and glucuronidase (4-methylumbelliferone). Oral administration of silymarin 6 hours prior to CCl_4 application could not prevent CCl_4-caused decrease 4 hours later in the activity of mixed function oxidation system and glucose-6-phosphatase. In humans, treatment with daily doses of 3x70 mg of silymarin (Legalon) over 28 days had no influence on the metabolism of aminopyrine and phenylbutazone. This study indicated that silymarin in therapeutic doses (Legalon), despite having effects in animals, has no influence on drug metabolism in humans.

DiCenzo *et al.* also examined whether silymarin has any effect on the pharmacokinetics of indinavir (207). In this study, four doses of indinavir, 800 mg three times/day, were given on days 1 and 2 and silymarin, 160 mg three times/day, was given on days 3-15. On day 16 and for one dose on day 17, both drugs were given at the same dosages. Indinavir's pharmacokinetic parameters were evaluated at steady state

both before and after 14 days of silymarin treatment. Blood samples were collected at 0.25, 0.5, 1, 2, 3, 4 and 5 hours after indinavir dosing. The final pharmacokinetic model had first-order absorption after a lag time and two compartments with first-order elimination from the central compartment. When given alone or combined with silymarin, respectively, the geometric mean steady-state indinavir area under the plasma concentration time-curve was 20.7 hours x mg/L and 19.4 hours x mg/L and the through plasma concentration was 0.340 mg/L and 0.232 mg/L. Therefore, silymarin had no apparent effect on indinavir plasma concentrations. However, Sridar *et al.* reported that silybin inactivated cytochromes P-450 3A4 and 2C9 and inhibited major hepatic glucuronosyltransferases (UGT). Thus, careful administration of silybin with drugs primarily cleared by P-450s 3A4 or 2C9 is advised because possible drug interactions cannot be excluded. However, at present, the clinical significance of *in vitro* UGT1A1 inhibition is not known (208).

Conclusion

The pharmacological activities assigned to silymarin indicate that this phytochemical (or neutraceutical) mimics Celebrex (COX-2 blocker), Avastin (VEGF blocker), Iressa (EGFR receptor blocker) and Remicade/Humira/Enabrel (TNF blockers) drugs combine a market value of over 10 billion dollars. More than 80% of the world's population cannot afford modern medicine. A cancer therapy that costs several thousand dollars for a gain in survival of months (*e.g.*, Herceptin, Avastin, Iressa) can help very few people (Table XI). Thus, novel treatments that are safe, inexpensive and effective are needed. We believe that agents such as curcumin, resveratrol, or silymarin, as described in this review, have the potential to comply with these issues (Table XII). However, further investigation is required. At the molecular level, it is becoming clear that cancer results from the alteration of 300 or more genes, indicating that a tumor cell uses multiple pathways to survive and prosper. Drugs that intervene a single pathway (such as Herceptin, Avastin or Iressa) are unlikely to succeed. In spite of their cost, none of these agents alone was found to be effective. The advantage of plant-derived products, as described here, is that they intervene in multiple pathways. This characteristic further supports that they may have better anticancer potential. However, systematic trials in humans are required with silymarin and other agents in order to fully understand their potential.

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