Pilot Study of Oral Silibinin, a Putative Chemopreventive Agent, in Colorectal Cancer Patients: Silibinin Levels in Plasma, Colorectum, and Liver and Their Pharmacodynamic Consequences

Carmen Hoh,[1] David Boocock,[1] Tim Marczylo,[1] Rajinder Singh,[1] David P. Berry,[2] Ashley R. Dennison,[2] David Hemingway,[3] Andrew Miller,[3] Kevin West,[4] Stephanie Euden,[1] Giuseppe Garcea,[2] Peter B. Farmer,[1] William P. Steward,[1] and Andreas J. Gescher[1]

Abstract Silibinin, a flavonolignan from milk thistle, has intestinal cancer chemopreventive efficacy in rodents. It is a strong antioxidant and modulates the insulin-like growth factor (IGF) system by increasing circulating levels of IGF-binding protein 3 (IGFBP-3) and decreasing levels of IGF-I. Here, the hypothesis was tested that administration of oral silibinin generates agent levels in human blood and colorectal and hepatic tissues consistent with pharmacologic activity. Patients with confirmed colorectal adenocarcinoma received silibinin formulated with phosphatidylcholine (silipide) at dosages of 360, 720, or 1,440 mg silibinin daily for 7 days. Blood and biopsy samples of normal and malignant colorectum or liver were obtained before dosing, and blood and colorectal or hepatic tissues were collected at resection surgery after the final silipide dose. Levels of silibinin were quantified by high-pressure liquid chromatography-UV, and plasma metabolites were identified by liquid chromatography-mass spectrometry. Blood levels of IGFBP-3, IGF-I, and the oxidative DNA damage pyrimidopurinone adduct of deoxyguanosine (M_1dG) were determined. Repeated administration of silipide was safe and achieved levels of silibinin of 0.3 to 4 μmol/L in the plasma, 0.3 to 2.5 nmol/g tissue in the liver, and 20 to 141 nmol/g tissue in colorectal tissue. Silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate were identified in the plasma. Intervention with silipide did not affect circulating levels of IGFBP-3, IGF-I, or M_1dG. The high silibinin levels achieved in the human colorectal mucosa after consumption of safe silibinin doses support its further exploration as a potential human colorectal cancer chemopreventive agent.

Silibinin, a flavonolignan (for structure, see Fig. 1), is a major constituent of the seeds of milk thistle (*Silybum marianum* L.). Extracts of milk thistle are widely consumed as a dietary supplement especially in the United States. Silymarin, a standardized milk thistle extract, of which silibinin is the main component, has been evaluated clinically in the treatment of hepatitis and liver damage inflicted by alcohol and long-term treatment with psychotropic drugs (1–4). Recent evidence in rodents suggests that silymarin and silibinin may be useful in the chemoprevention of malignancies at a variety of sites, including the intestinal tract (5–10). Dietary silymarin delayed the development of intestinal adenocarcinomas in rats induced by dimethylhydrazine (8) or azoxymethane (9). It also suppressed aberrant crypt foci in rats, which had been exposed to azoxymethane, but this suppression was dose independent and did not involve induction of apoptosis (10). In our laboratory, silibinin interfered moderately with small intestinal carcinogenesis in the Apc^{Min} mouse model.[5] Silibinin has been formulated with phosphatidylcholine (silipide, Indena SpA, Milan, Italy) to improve its systemic availability, and clinical evaluation of this formulation at single or repeated doses reflects both its safety (summarized in ref. 11) and its improved bioavailability with respect to silymarin (12). Several mechanisms have been proposed to explain how silibinin may interfere with carcinogenesis. Among these mechanisms are impairment of receptor tyrosine kinase and erbB1 signaling and up-regulation of cyclin-dependent kinase inhibitors causing attenuation of cancer cell growth and perturbation of cell cycle progression (13, 14), induction of cancer cell differentiation (15), and antiangiogenesis (16). Silibinin has also been suggested to modulate the insulin-like growth factor (IGF) system. IGFs are mediators of cell survival in that they can inhibit apoptosis

Authors' Affiliations: [1]Cancer Biomarkers and Prevention Group, Departments of Cancer Studies and Biochemistry, University of Leicester and Departments of [2]Hepatobiliary Surgery, [3]Coloproctology, and [4]Histopathology, University Hospitals of Leicester, Leicester, United Kingdom

Received 12/14/05; revised 1/13/06; accepted 3/1/06.

Grant support: United Kingdom Medical Research Council Programme grant G0100874.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Andreas Gescher, Department of Cancer Studies, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, University of Leicester, Leicester LE2 7LX, United Kingdom. Phone: 44-116-223-1856; Fax: 44-116-223-1855; E-mail: ag15@le.ac.uk.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-2724

[5] Verschoyle, Ho, Steward, Gescher, unpublished data.

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

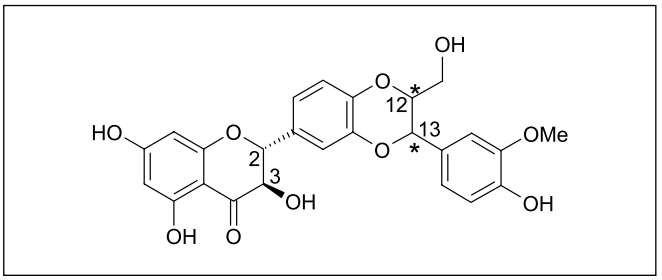

Fig. 1. Structure of naturally occurring silibinin, which is a diastereomeric mixture (1:1) of the two forms 2R, 3R, 12S, and 13S and 2R, 3R, 12R, and 13R. Asterisks, optically active centers.

and influence differentiation of many normal and malignant cell types (17–19). The IGF system is regulated by IGF-binding proteins (IGFBP), especially IGFBP-3, which bind IGFs in the extracellular milieu with high affinity and specificity, thus reducing the bioavailibility of IGFs. Epidemiologic studies have linked increased serum concentrations of IGF-I, decreased concentrations of IGFBP-3, or both with an increased risk of cancer at several sites, including the colorectum (20, 21). Silibinin increased levels of IGFBP-3 in prostate cells *in vitro* (22) and in tumor-bearing rodents *in vivo* (23). Silymarin and silibinin are also powerful antioxidants as a consequence of their polyphenolic structure (11, 24). Elevated oxidative stress has been implicated as an important carcinogenic stimulus, and endogenous or exogenous oxidative events, such as those associated with lipid peroxidation, generate malondialdehyde, a mutagen (25). Malondialdehyde can react with DNA to furnish the pyrimidopurinone adduct of deoxyguanosine M_1dG (26). M_1dG levels may be a suitable marker of chemopreventive efficacy of strong antioxidants, such as silibinin.

Silibinin undergoes extensive metabolism, especially phase II conjugation reactions, both in human liver preparations *in vitro* (27) and in volunteers *in vivo* after consumption of silibinin (28). There is no information available on the pharmacologic properties of metabolic conjugates of silibinin, and it is conceivable that at least some of them share pharmacologic activity with the parent molecule. Taken together, all of these results intimate both the propitiousness and the feasibility of developing silibinin as a colorectal cancer chemopreventive agent. To obtain pharmacokinetic and pharmacodynamic information to aid the design of future colorectal cancer intervention studies with silibinin, we carried out a pilot "presurgery model" study in colorectal cancer patients. Patients consumed silibinin daily for a week before colectomy, and blood and colorectal or hepatic tissue was obtained by biopsy and resection before and after the administration. Blood and tissues were analyzed to explore whether metabolites of silibinin can be identified in human blood and whether silibinin levels achievable in blood, colorectal, or hepatic tissue are comparable with pharmacologically active levels reported in cells *in vitro*. Furthermore we wished to find out if consumption of silibinin affects circulating levels of IGFBP-3, IGF-I, and the oxidative DNA damage adduct M_1dG. Overall, the study was designed to provide data, which helps optimize the design of long-term intervention studies of silibinin as a colorectal cancer chemopreventive agent.

Materials and Methods

Patients and tissues. Twelve patients (1 female and 11 male, ages 55-78 years, mean age 65 ± 7) with confirmed colorectal carcinoma of stages Dukes A (2 patients), B (5), or C (5), who were to undergo colorectal resection, and 12 patients (7 female and 5 male, ages 49-78 years, mean age 62 ± 9, all Dukes D) with hepatic metastatic disease originating from primary colorectal carcinoma, who were to undergo hepatic surgery, were recruited into the trial following approval by the University Hospitals of Leicester (Leicester, United Kingdom) ethics committee. All patients gave written informed consent. Hematologic profiles, plasma levels of urea and electrolytes, and hepatic function were within the reference range defined by the laboratories of the University Hospitals of Leicester. One patient who underwent colectomy had preoperative radiotherapy and none preoperative chemotherapy. All, except two patients who underwent hepatic surgery, had received 5-fluorouracil with folinic acid, oxaliplatin, and/or irinotecan before recruitment. Drug histories included antihypertensives, diuretics, antidepressants, and analgesics. Colorectal tumors in patients who underwent colorectal resection were located in the ascending/transverse colon (one patient), sigmoid colon (four patients), or rectum (seven patients). Tissue biopsy specimens taken at diagnosis weighed 3 to 60 mg (tumor biopsy) and 4 to 60 mg (normal tissue biopsy). Colectomy or hepatic resection was done 3 to 6 hours after the last dose of silipide. The weight of the surgical colorectal/liver tissue samples for chemical analysis was 1.1 to 1.5 g. Samples of peripheral blood were collected in heparinized tubes before intervention and 1 to 4 hours after the last silipide dose. Portal blood was taken at the point of hepatic surgery. Blood samples were centrifuged to generate leukocytes (for M_1dG measurement), serum (for IGF-I/IGFBP-3 measurement), and plasma [for high-pressure liquid chromatography (HPLC) analysis]. Blood components were kept on ice until storage at −80°C. Tissue samples were flash frozen in liquid nitrogen. Biomatrices were kept at −80°C for up to 6 months until analysis. Preliminary HPLC analysis had established that silibinin is stable under these conditions in tissues and plasma.

Silibinin formulation and dose. Silibinin was formulated in capsules as silipide (IdB 1016), a phytosome product marketed by Indena SpA for use as a hepatoprotectant (see www.indena.it/pdf/prodottiweb.pdf). Each silipide capsule contained 120 mg silibinin (validated by HPLC-UV analysis, see below) and soy phosphatidylcholine at a molar ratio of 1:1, which in terms of percentage weight constitutes ~40% silibinin and 60% phosphatidylcholine. Patients received silipide at dosages of either 360, 720, or 1,440 mg silibinin daily for 7 days before surgery; each daily dose was divided in three equal portions taken in the morning, at noon, and in the evening. There were eight individuals per dose level (four patients who underwent colectomy and four who had liver resection). The first and second portions of the first dose were taken at noon and in the evening, respectively, of day 1; the last dose portion was ingested in the morning of day 8 before surgery so that, in total, the seven daily doses were distributed >8 days. The doses used in this pilot study were based approximately on the dose that raised plasma IGFBP-3 levels in mice (23). In that study, mice received silibinin at 0.05% or 0.1% in the diet, which equates to ~75 and 150 mg/kg daily, respectively. These doses extrapolated to humans based on body surface area (420 mg/m^2 in mice; ref. 29) would amount to ~450 or ~900 mg silibinin/person daily, assuming a body surface area of 1.8 m^2 accompanying a body weight of 70 kg. Although such extrapolation has to be interpreted with caution, the doses proposed for the pilot study here cover the dose shown to be efficacious in mice.

Analysis of silibinin and silibinin conjugates. Biomatrices were thawed, weighed, and homogenized in an equal part of KCl solution (0.15 mol/L). Samples of plasma or tissue homogenate were mixed with 3 parts ice-cold methanol. The mixture was centrifuged, and the supernatant was decanted and dried under nitrogen, reconstituted in aqueous methanol (70%, containing 5% acetic acid), and analyzed for silibinin by HPLC with UV detection using a gradient system with a

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

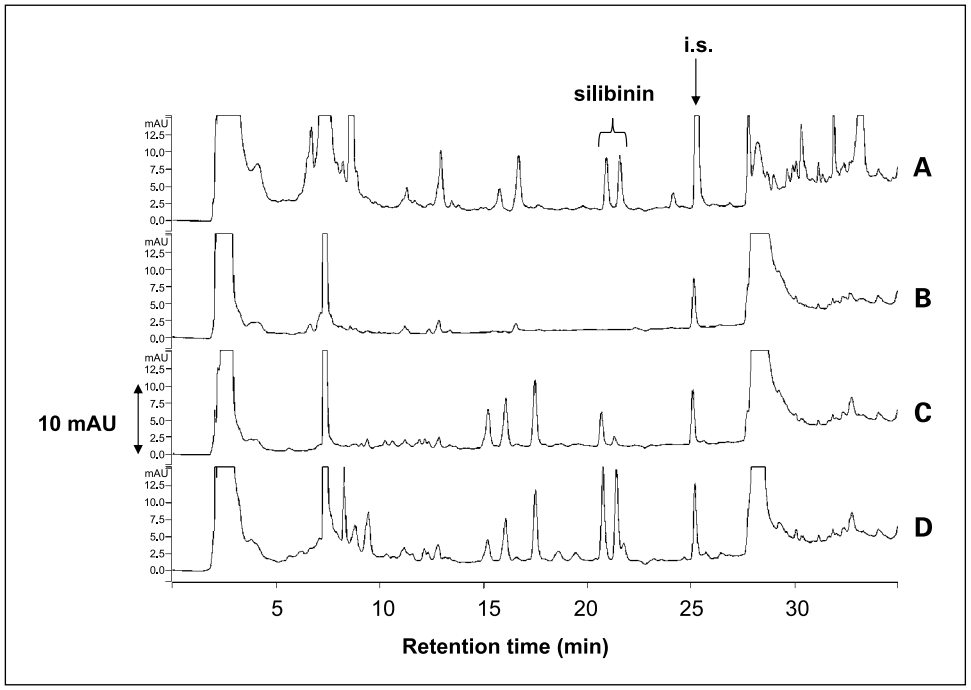

Fig. 2. HPLC-UV chromatograms of extracts of pooled plasma added to silibinin (400 ng/mL) (*A*), plasma obtained from a patient before the first dose of silipide (*B*) or 3 hours after the last of seven daily doses of silipide (1,440 mg silibinin, administered in three divided portions; (*C*) and plasma sample *C* incubated for 1 hour with sulfate- and glucuronide-deconjugating enzymes from *H. pomatia* (*D*). i.s., internal standard. For details of dosing, sample preparation, and HPLC analysis, see Materials and Methods.

two-component mobile phase. The details, characterization, and validation of the method are described in a separate article.[6] The limit of quantitation for the two silibinin diastereoisomers was 5 and 3 ng/mL. Some plasma samples were incubated [1 hour, 37°C, 0.1 mol/L ammonium acetate buffer (pH 6.0)] with β-glucuronidase from *Helix pomatia* (type H-2, containing sulfatase; Sigma-Aldrich, Poole, United Kingdom) before extraction.

Silibinin and some of its metabolites were characterized by LC-mass spectrometry (MS) using a turbospray source in negative electrospray ionization mode. Analyses were done using an API 2000 LC-MS (Applied Biosystems/MDS Sciex, Warringon, United Kingdom) equipped with an Agilent (South Queensferry, United Kingdom) 1100 series sample delivery system. Separation of silibinin and metabolites was achieved using the method mentioned above. MS conditions were as follows: declustering potential, −26 V; focusing potential, −350 V; electrode potential, −12 V; cell entrance potential, −16 V; cell exit potential, −20 V; and temperature, 500°C. Identification of silibinin-derived species was by selected ion monitoring.

Pharmacodynamic measurements. IGF-I and IGFBP-3 concentrations in patients' serum were determined using ELISA kits 10-5600 Active and 10-6600 Active, respectively, from Diagnostic Systems Laboratories (Oxon, United Kingdom). The IGF-I kit procedure contained an acid-ethanol extraction step to separate IGFs from their binding sites. The assays were validated and done according to the instructions of the manufacturer. The molar ratio of IGF-I to IGFBP-3 was calculated as [0.13 × IGF-I concentration (ng/mL)] / [0.036 × IGFBP-3 concentration (ng/mL)].

DNA was extracted from leukocytes or tissues using the Qiagen (Crawley, United Kingdom) kit, and M_1dG adduct levels were measured in triplicate by immunoslot blot using a murine monoclonal anti-M_1dG antibody provided by Dr. Lawrence Marnett (Vanderbilt University, Nashville, TN) as described previously (30). Goat and anti-mouse horseradish peroxidase–conjugated secondary antibodies were purchased from Dako Cytomation (Ely, United Kingdom). Discrepancies in the amount of DNA per slot were corrected for by staining the nitrocellulose filter with propidium iodide and doing UV light densitometry. The detection limit for M_1dG was ~1 adduct/10^7 nucleotides.

Results

Identification of silibinin and metabolites in blood. Patients with confirmed colorectal cancer received silibinin formulated as silipide at 360, 720, or 1,440 mg daily for a week before colorectal or hepatic surgery (for removal of liver metastases). This intervention was not associated with any adverse effect of silibinin. Silibinin exists as two *trans*-diastereoisomers (Fig. 1), furnishing two peaks on HPLC analysis with retention times near 21 minutes. All three doses afforded measurable peaks in the plasma at the retention times of authentic silibinin. Figure 2 shows the chromatogram of peripheral plasma from a patient who received the highest dose. These peaks were unambiguously identified as silibinin by MS, as they afforded *m/z* 481 $[M\text{-}H^+]^-$ consistent with the mass spectrum of authentic silibinin. Plasma sample extracts were also scanned by MS for metabolic species derived from silibinin. Silibinin monoglucuronide, silibinin monosulfate, and silibinin diglucuronide

Table 1. HPLC-MS analysis of species related to silibinin in plasma from humans who had received silibinin (1,440 mg) daily for 7 days as silipide capsules

m/z $[M\text{-}H^+]^-$	Inference	Retention time (min)
481	Silibinin	20.8; 21.4
657	Silibinin glucuronide	12.6; 15.6; 16.4; 17.8
833	Silibinin diglucuronide	9.2; 10.8
561	Silibinin sulfate	17; 17.8; 18.8

[6] C.S.L. Hoh, et al. Quantitation of silibinin, a putative cancer chemopreventive agent, in human plasma by high performance liquid chromatography, submitted for publication.

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

could be detected (Table 1). Single ion monitoring yielded multiple peaks, which could be assigned to silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate (Fig. 3). There was also some evidence for the presence of silibinin triglucuronide (*m/z* 1,008) and *O*-desmethyl silibinin glucuronide (*m/z* 643; data not shown). Because of the occurrence of multiple diastereoisomers of silibinin derivatives and given the absence of authentic reference materials, it is impossible to assign specific positional isomers to the multiple peaks seen on single ion monitoring. Figure 2 illustrates that incubation with conjugate-hydrolyzing enzymes furnished a marked increase in the height of the parent silibinin peaks, consistent with the abundant presence of silibinin sulfate and/or glucuronide conjugates.

Silibinin levels in blood and tissues. Extracts of samples of plasma or of normal or malignant colorectal or hepatic tissue were subjected to quantitative HPLC-UV analysis. Peripheral plasma levels were between 0.3 and 4 μmol/L, and they were related to silipide dose (Table 2). Silibinin levels in portal plasma were similar to those in peripheral plasma. Levels of silibinin in normal and malignant colorectal tissue showed considerable variation. They were between 20 and 141 nmol/g and not strictly related to silipide dose. Concentrations of silibinin in hepatic tissue were similar to those in plasma (Table 2).

Effect of silibinin on circulating levels of IGFBP-3, IGF-I, and M_1dG. Levels of IGFBP-3, IGF-I, and M_1dG were studied as potential markers of silibinin efficacy in the peripheral blood from 24 patients obtained before the first dose of silibinin and between 1 and 4 hours after the last (i.e., seventh) dose. Figure 4 shows that there was no significant difference in concentration of IGFBP-3 or IGF-I between pretreatment and posttreatment serum at any of the dose levels. Neither did comparison of the molar ratio of IGF-I to IGFBP-3 reveal any difference between pre-intervention and post-intervention serum (data not shown). Statistical analysis of the difference between pre-intervention and post-intervention values for IGF-I in serum from patients on 1,440 mg silibinin (Fig. 4B) afforded $P = 0.07$, tentatively hinting at the possibility that, given a larger cohort of individuals and/or a longer period of intervention, this dose may decrease IGF-I levels. When IGFBP-3/IGF-I levels were compared between patients with different disease stage, there was no obvious difference between Dukes stage on the one side and IGFBP-3/IGF-I levels or susceptibility toward modulation of biomarker levels by silibinin on the other (data not shown).

M_1dG levels in leukocytes from peripheral blood isolated before and after the intervention from 20 patients were 3.7 ± 3.7 and 2.3 ± 1.8 adducts per 10^7 nucleotides, respectively. In the remaining four individuals, leukocytic pre-intervention M_1dG levels were below the limit of detection. The values are of the same order of magnitude as those reported previously for human blood (31). Statistical comparison between pre-intervention and post-intervention values of the individual dose groups or the combined doses failed to reveal significant differences, suggesting that consumption of silibinin for a week did not markedly alter leukocytic M_1dG. We also compared M_1dG levels in normal and malignant tissues obtained by biopsy and after surgery. The values measured in tissue samples were extremely variable between patients, with a substantial number of them close to or at the limit of detection, confounding meaningful interpretation.

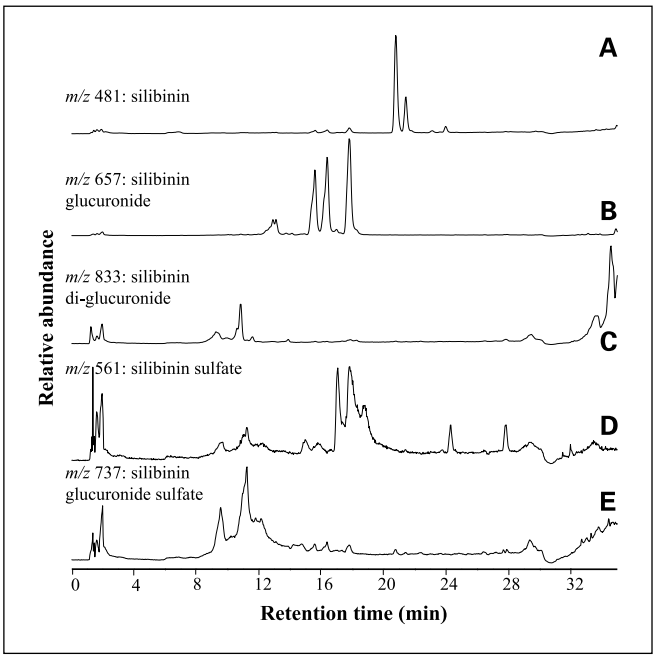

Fig. 3. HPLC-MS selected ion monitoring chromatograms of an extract of a patient's plasma obtained 3 hours after the last of seven daily doses of silipide (1,440 mg silibinin, administered in three divided portions). The following $[M\text{-}H^+]^-$ ions were monitored: 481 (*m/z* silibinin; *A*), 657 (*m/z* silibinin glucuronide; *B*), 833 (*m/z* silibinin diglucuronide; *C*), 561 (*m/z* silibinin sulfate; *D*), and 737 (*m/z* silibinin glucuronide sulfate; *E*). For details of dosing and analysis, see Materials and Methods.

Discussion

The outcome of the pilot study described here supports the notion that the repeated administration of silibinin at daily doses up to 1.44 g for a week is safe. This conclusion is consistent with the result of the original evaluation of silipide in human volunteers (12), and a similar inference was made in a preliminary report of a current phase I study of silibinin in hormone-refractory prostate cancer patients, in which up to 20 g silibinin was administered orally daily for a month (32). We describe here for the first time the identification of silibinin plasma metabolites and measurement of silibinin tissue levels in humans who ingested silibinin. Consistent with results obtained previously using liver preparations incubated with silibinin *in vitro* (27), the results outlined here suggest that silibinin undergoes multiple conjugation reactions in humans. The presence of metabolic conjugates of silibinin in the human biomatrix has hitherto been shown only indirectly (28), in that raised levels of the parent molecule after enzymatic hydrolysis was taken to indicate the presence of conjugates. In contrast, here, the conjugate species silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate were unambiguously identified. The silibinin molecule possesses five hydroxy moieties (Fig. 1), three of which are phenolic in nature, but the analysis described here does not allow the exact position of conjugation on the silibinin molecule to be elucidated. On the assumption that the antioxidant activity of silibinin is a function of its polyphenolic structure, the presence of silibinin monosulfate and monoglucuronide, which bear (at least) two intact phenol moieties,

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

Table 2. Silibinin levels in blood and colorectal or hepatic tissues of colorectal cancer patients who had received silibinin daily for 7 days as silipide capsules

Dose (mg/d)	Peripheral blood levels (μmol/L)	Colorectal tissue levels (nmol/g)	
		Normal	Malignant
360	0.3 ± 0.3*	28 ± 31	37 ± 51
720	0.7 ± 0.6	121 ± 181	20 ± 26
1,440	3 ± 2.3	141 ± 169	68 ± 98

Dose (mg/d)	Blood levels (μmol/L)		Hepatic tissue levels (nmol/g)	
	Peripheral	Portal	Normal	Malignant
360	0.4 ± 0.2	0.5 ± 0.3	1 ± 0.9	0.6 ± 0.5
720	1.4 ± 1	0.5 ± 0.4	1.2 ± 1.1	0.3 ± 0.1
1,440	4 ± 5.3	1.6 ± 0.3	2.5 ± 2.4	0.6 ± 0.7

*Mean ± SD of four patients.

suggests that appreciable amounts of circulating silibinin-derived species may share, at least to some extent, the antioxidant potency of the parent molecule.

The plasma levels of silibinin described here need to be compared with those reported previously in healthy volunteers who received oral silipide on a repeated dose schedule (12). In that study, silipide at 720 mg (equivalent to 240 mg silibinin) given daily for 7 consecutive days furnished a mean peak plasma level of 0.38 μmol/L (0.18 μg/mL) reached 0.9 hour after administration of the last dose, and the terminal plasma half-life of the last dose was 3.4 hours. This published data allows a tentative prediction of the levels achieved at the time points at which peripheral blood samples were taken for silibinin analysis in the pilot study described here (between 1 and 4 hours after the last silipide dose). Blood was collected at times that coincide approximately with peak levels on the one side and ~0.9 half-life beyond peak levels on the other. The mean plasma levels for the 360-mg daily doses observed here, which were 0.3 to 0.4 μmol/L (0.14-0.19 μg/mL), are broadly consistent with the earlier volunteer study. The results presented here suggest that the concentration of silibinin achieved after consumption of seven daily doses of up to 1.44 g daily is insufficient to affect circulating levels of IGF-I, IGFBP-3, and M_1dG. It is of course conceivable that these putative efficacy biomarkers would be amenable to modulation by these doses of silibinin when given over longer periods of intervention. One week might have been too short to achieve a long-lasting effect on the IGF axis. In athymic mice bearing the DU-145 prostate tumor, administration of silibinin at 0.05% and 0.1% in the diet (equivalent to ~75 and ~150 mg/kg body weight daily, respectively) for the lifetime of animals caused 4- to 6-fold elevation of IGFBP-3 levels over controls, for the two doses, respectively (23). In terms of dose extrapolation based on surface area from mice to humans, these doses are comparable with those used here (see Materials and Methods). Steady-state plasma levels of silibinin, which

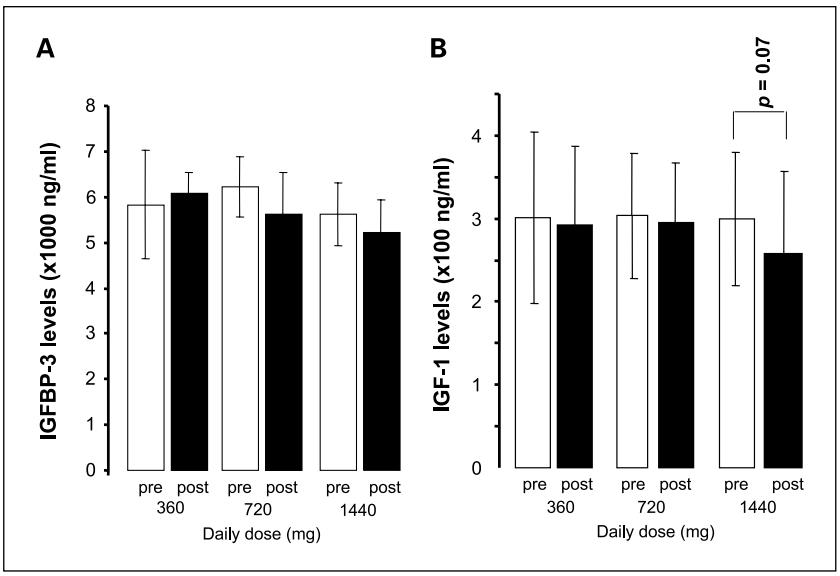

Fig. 4. Concentrations of IGFBP-3 (*A*) and IGF-I (*B*) in serum from patients obtained before dosing with silipide (*pre; white columns*) or 1 to 4 hours after (*post; black columns*) the last of eight daily doses of silipide (360, 720, or 1,440 mg silibinin, administered in three divided portions). Analysis was by ELISA using commercially available kits. Columns, mean of eight patients for the 720-mg and 1,440-mg dose levels and five patients for the 360-mg dose; bars, SD. *P* derived by statistical comparison using paired Student's *t* test. For details of IGFBP-3 and IGF-I analyses, see Materials and Methods.

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

accompanied the IGFBP-3-lowering activity in mice, were between 7 and 21 μmol/L (3.4 and 10.1 μg/mL), thus, ~2- to 7-fold above the plasma levels measured in our human pilot study for the 1.44 g (highest) dose of silibinin. Intervention with 9-*cis*-retinoic acid in former smokers was recently reported to cause a significant decrease in serum IGF-I and an increase in IGFBP-3 (33). It is pertinent to note that the period of intervention in that study was 3 months, substantially longer than the period of intervention with silibinin described here. Recently, preliminary evidence for the chemotherapeutic activity of silipide has been published. Repeated daily administration of silipide by oral gavage (at 450 mg/kg silipide, equivalent to 180 mg/kg silibinin) caused inhibition of ovarian tumor growth in nude mice, and levels of silibinin in the plasma and tumor after termination of the experiment were 15 μmol/L (7.2 μg/mL) and 0.38 nmol/g (0.2 μg/g) tissue, respectively (34). Furthermore, silipide at a dose of 1,800 mg/kg (equivalent to 720 mg/kg silibinin) given concomitantly with chemotherapy enhanced the antitumor activity of *cis*-platinum in a nude mouse model bearing the human A2780 ovarian cancer (35).

Levels of silibinin in human colorectal and liver tissue have not been described previously, although silibinin-containing remedies have long been marketed as liver protectants. In our study, the colorectal mucosal levels of silibinin were highly variable and not related to silipide dose, which may, at least to some extent, be the corollary of the difference between patients in time period (3-6 hours), which elapsed between the consumption of the last dose and surgery. The recommended oral dose of, for example, Legalon (Madaus, Germany), which contains 70 mg silymarin/tablet, is two tablets, taken thrice daily. So the daily dose of silymarin recommended for liver protection is 420 mg. Silymarin contains silydianin and silychristin as well as silibinin. On the assumption that silymarin contains ~80% silibinin, this silymarin dose would translate into ~340 mg silibinin, which is similar to the low dose of silibinin administered in the pilot study described here. Based on this gross calculation, one may tentatively infer that silibinin concentrations in liver tissue of an order of magnitude similar to those measured here after the 360-mg dose (i.e., 0.3-0.5 μg/g or 0.6-1 μmol/L in concentration terms) can afford protection of the human liver against toxic insult. In contrast to the relatively low systemic and hepatic levels of silibinin, levels achieved in colorectal tissue, between 9.6 and 68 μg/g or 20 to 141 μmol/L in concentration terms, are highly likely to elicit pharmacologic effects in the light of the concentrations reported to cause responses in cells in culture. For example, in cultured DU-145 prostate cancer cells, 15 and 30 μmol/L (7.2-14.5 μg/mL) silibinin were sufficient to significantly compromise cell proliferation and increase IGFBP-3 in the cellular supernatant (22).

In summary, repeated administration of silipide achieved levels of silibinin in the colorectal tract similar to those known to exert pharmacologic activity. Several silibinin sulfates and glucuronides have been identified in human blood, some of them retaining the intact phenol structure, a pharmacophoric feature, which may mediate, at least in part, pharmacologic activity. Intervention for periods of a week seemed to be insufficient for orally consumed silibinin to affect the IGF-I/IGFBP-3 system in humans, and circulating silibinin-derived species were not abundant enough to reduce blood levels of M_1dG significantly. Nevertheless, in the light of the colorectal cancer chemopreventive activity of silibinin in rodents (8–10), the high silibinin levels achieved in the human colorectal mucosa after consumption of safe silibinin doses support its further exploration as a human colorectal cancer chemopreventive agent.

Acknowledgments

We thank Dr. Paolo Morazzoni (Indena SpA) for generous provision of silipide capsules, Dr. Lawrence Marnett (Vanderbilt University) for supplying the primary anti-M_1dG antibody, and Sharon Platton for technical assistance.

References

1. Ferenci P, Dragosics B, Dittrich H, et al. Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. J Hepatol 1989;9:105–13.
2. Pares A, Planas R, Torres M, et al. Effects of silymarin in alcoholic patients with cirrhosis of the liver: results of a controlled, double-blind, randomized, and multicenter trial. J Hepatol 1998;28:615–21.
3. Salmi HA, Sarna S. Effect of silymarin on chemical, functional, and morphological alterations of the liver. A double-blind controlled study. Scand J Gastroenterol 1982;17:517–21.
4. Trinchet JC, Coste T, Levy VG, et al. Treatment of alcoholic hepatitis with silymarin. A double blind comparative study in 116 patients. Gastroenterol Clin Biol 1989;3:120–4.
5. Katiyar SK, Korman NJ, Mukhtar H, Agarwal R. Protective effects of silymarin against photocarcinogenesis in a mouse skin model. J Natl Cancer Inst 1997;89:556–66.
6. Lahiri-Chatterjee M, Katiyar SK, Mohan RR, Agarwal R. A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. Cancer Res 1999;59:622–32.
7. Vinh PQ, Sugie S, Tanaka T, et al. Chemopreventive effects of a flavonoid antioxidant silymarin on *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced urinary bladder carcinogenesis in male ICR mice. Jpn J Cancer Res 2002;93:42–9.
8. Gershbein LL. Action of dietary trypsin, pressed coffee, silymarin, and iron salt on 1,2-dimethylhydrazine tumorigenesis by gavage. Anticancer Res 1994;14:1113–6.
9. Kohno H, Tanaka T, Kawabata K, et al. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Int J Cancer 2002;101:461–8.
10. Volate SR, Davenport DM, Muga SJ, Wargovich MJ. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng, and rutin). Carcinogenesis 2005;26:1450–6.
11. Comoglio A, Leonarduzzzi G, Carini R, et al. Studies on the antioxidant and free radical scavenging properties of IdB 1016: a new flavonolignan complex. Free Radic Res Commun 1990;11:109–15.
12. Barzaghi N, Crema F, Gatti G, et al. Pharmacokinetic studies on IdB 1016, a sylibin-phosphatidylcholine complex, in healthy human subjects. Eur J Drug Metab Pharmacokinet 1990;15:333–8.
13. Ahmad N, Gali H, Javed S, Agarwal R. Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation of cell cycle progression. Biochem Biophys Res Commun 1998;248:294–301.
14. Zi X, Grasso AW, Kung HJ, Agarwal R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G_1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res 1998;58:1920–9.
15. Zi X, Agarwal R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G_1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. Proc Natl Acad Sci U S A 1999;96:7490–5.
16. Jiang C, Agarwal R, Lu J. Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells. Biochem Biophys Res Commun 2000;276:371–8.
17. Butt AJ, Firth SM, Baxter RC. The IGF axis and programmed cell death. Immunol Cell Biol 1999;77:256–69.
18. Lopez T, Hanahan D. Elevated levels of IGF-1 receptor convey invasive and metastatic capability in mouse model of pancreatic islet tumorigenesis. Cancer Cell 2002;1:339–53.
19. Samani AA, Chevet E, Fallavollita L, et al. Loss of

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

tumorigenicity and metastatic potential in carcinoma cells expressing the extracellular domain of the type 1 insulin-like growth factor receptor. Cancer Res 2004; 64:3380–5.

20. Kaaks R, Toniolo P, Akhmedkhanov A, et al. Serum C-peptide, insulin-like growth factor (IGF)-1, IGF binding proteins, and colorectal cancer risk in women. J Natl Cancer Inst 2000;92:1592–600.

21. Hassan AB, Macaulay VM. The insulin-like growth factor system as a therapeutic target in colorectal cancer. Ann Oncol 2002;13:349–56.

22. Zi X, Zhang J, Agarwal R, Pollak M. Silibinin up-regulates insulin-like growth factor binding protein-3 expression and inhibits proliferation of androgen-independent prostate cancer cells. Cancer Res 2000; 60:5617–20.

23. Singh RP, Dhanalakshmi S, Tyagi AK, et al. Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. Cancer Res 2002;62:3063–9.

24. Zhao J, Lahiri-Chatterjee M, Sharma Y, Agarwal R. Inhibitory effect of flavonoid antioxidant silymarin on benzoyl peroxide-induced tumor promotion, oxidative stress, and inflammatory responses in SENCAR mouse skin. Carcinogenesis 2000;21:811–6.

25. Marnett LJ. Lipid peroxidation—DNA damage by malondialdehyde. Mutat Res-Fund Mol Mech 1999; 424:83–95.

26. Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis 2000;21:361–70.

27. Gunaratna C, Zhang T. Application of liquid chromatography-electrospray ionization-ion trap mass spectrometry to investigate the metabolism of silibinin in human liver microsomes. J Chromatogr 2003;794: 303–10.

28. Gatti G, Perucca E. Plasma concentrations of free and conjugated silybin after oral intake of a sylibin-phosphatidylcholine complex (silipide) in healthy volunteers. Int J Clin Pharmacol Ther 1994;32:614–7.

29. Freireich EJ, Gehan EA, Rall DP, et al. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 1966;50:219–44.

30. Singh R, Leuratti C, Josyula S, et al. Lobe-specific increases in malondialdehyde DNA adduct formation in the livers of mice following infection with *Helicobacter hepaticus*. Carcinogenesis 2001;22:1281–7.

31. Leuratti C, Singh R, Lagneau C, et al. Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. Carcinogenesis 1998;19:1919–24.

32. Flaig T, Agarwal R, Su LJ, et al. A phase I study of silibinin in hormone-refractory prostate cancer [abstract 4698]. J Clin Oncol 2005;23:16S.

33. Lee HY, Chang YS, Han JY, et al. Effects of 9-*cis*-retinoic acid on the insulin-like growth factor axis in former smokers. J Clin Oncol 2005;23:4439–49.

34. Gallo D, Giacomelli S, Ferlini C, et al. Antitumour activity of the silybin-phosphatidylcholine complex IdB 1016 against human ovarian cancer. Eur J Cancer 2003;39:2403–10.

35. Giacomelli S, Gallo D, Apollonio P, et al. Silybin and its bioavailable phospholipid complex (IdB 1016) potentiate *in vitro* and *in vivo* the activity of cisplatin. Life Sci 2002;70:1447–59.

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.

AACR American Association for Cancer Research

Clinical Cancer Research

Pilot Study of Oral Silibinin, a Putative Chemopreventive Agent, in Colorectal Cancer Patients: Silibinin Levels in Plasma, Colorectum, and Liver and Their Pharmacodynamic Consequences

Carmen Hoh, David Boocock, Tim Marczylo, et al.

Clin Cancer Res 2006;12:2944-2950.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/12/9/2944

Cited articles	This article cites 35 articles, 7 of which you can access for free at: http://clincancerres.aacrjournals.org/content/12/9/2944.full#ref-list-1
Citing articles	This article has been cited by 13 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/12/9/2944.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/12/9/2944. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from clincancerres.aacrjournals.org on June 23, 2021. © 2006 American Association for Cancer Research.