ABSTRACT

We have recently reported the anticancer effect of flavonoid antioxidant silybinin, the major component of milk thistle extract, against advanced human prostate carcinoma DU145 cells (X. Zi et al., Cancer Res., 58: 1920–1929, 1998) and later identified that silybinin is the major active component in silymarin responsible for its effect in cell culture studies. On the basis of these observations, here we assessed in vivo growth inhibitory potential of silybinin against advanced human prostate cancer (PCA). Dietary feeding of silybinin at 0.05 and 0.1% doses (w/w) for 60 days, 24 h after s.c. DU145 tumor xenograft implantation in athymic male nude mice, significantly inhibited tumor volume by 35 and 58% (P < 0.05), and wet weight of tumor by 29 and 40% (P < 0.05), respectively. In a second experiment where mice were fed with these test diets for 3 weeks before tumor xenograft implantation and continued on these diets for a total of 63 days, tumor volume and wet weight of tumor were reduced by 53–64% (P < 0.001–0.05) and 31–52% (P < 0.05), respectively. In both studies, animals did not show weight loss or reduced food consumption. These in vivo anticancer effects of silybinin were associated with an increased accumulation (up to 5.8 fold; P < 0.05) of human insulin-like growth factor-binding protein-3 in mouse plasma. In additional studies assessing biological availability of silybinin in nude mice and its antiproliferative activity at such doses in DU145 cells in culture, silybinin levels in plasma and prostate were found to be in the range of 7–13 μg/ml and 3.7–4.6 μg/g, respectively. At these biologically achievable silybinin concentrations, increased IGFBP-3 level in DU145 cell culture medium and a strong DU145 cell growth inhibition were observed that were irreversible in the absence of silybinin in culture medium. These findings extend and translate our observations on in vitro anticancer effect of silybinin/silymarin to an in vivo preclinical PCA model, which may form the basis for a Phase I clinical trial in PCA patients.

INTRODUCTION

Silybinin, a naturally occurring polyphenolic flavonoid, constitutes a major biologically active portion of the plant extract, milk thistle (Silybum marianum) that is widely consumed as a dietary supplement in the United States and Europe, accounting for ~8 million dollar sale in United States market in 1999 (1, 2). Silybinin and its crude form silymarin are clinically used to treat certain liver complications, and are devoid of any toxic effects even at acute or chronic administrations of the drug in both animal and human studies (2). Our cell culture as well as in vivo studies have established the antineoplastic effects of both silymarin and silybinin against different human carcinoma cells including PCA (3–8). However, both efficacy and associated molecular mechanism of silybinin remain to be established in in vivo preclinical PCA models. In the present study, we investigated the in vivo growth inhibitory efficacy of silybinin against advanced human prostate carcinoma DU145 tumor xenografts in nude mice.

Growth factors and associated receptors have been implicated as causative epigenetic events that stimulate proliferation and enhance the possibility of malignant transformation of epithelial cells (Ref. 9 and references therein). For example, several studies indicate that mitogenic/cell survival activity of IGFs are tightly controlled by the presence of IGFBPs and influenced by the balance of these factors in cellular microenvironment (10). IGFBP-3, a high affinity major IGF-binding protein in plasma, exists in ternary complex with IGF-I or IGF-II and an acid-labile unit. In case of inhibition of mitogenic activity of IGFs, soluble IGFBP-3 has been shown to sequester IGFs and prevent their interaction with cell surface-associated IGFBPs and/or IGF receptors (11). Prostate-specific antigen has shown to decrease IGFBP-3 affinity for IGF and potentiate IGF action that significantly contributes to normal as well as malignant prostate growth (12). Although IGFBP-3 is a potent inhibitor of IGF activity, it also has IGF-independent antiproliferative effects on cell growth (13). The tumor suppressor p53-mediated IGFBP-3 expression has also been reported in apoptosis induction in response to cellular stress (13). It has been suggested that IGFBP-3 may serve to protect against potentially carcinogenic effects of growth hormones and IGFs (13, 14). Furthermore, it is important to emphasize here that in recent studies, IGFs/IGFBP-3 plasma levels are being monitored as a potential end point surrogate biomarker for PCA risk (14), and that recently, we showed that silybinin up-regulates IGFBP-3 expression and inhibit its proliferation of human prostate carcinoma PC-3 cells (15).

Taken together, based on above studies, we reasoned that serum level of IGFBP-3 might be one of the useful surrogate end point biomarkers to be evaluated as a potential mechanism of inhibitory efficacy of silybinin against advanced human PCA growth in nude mice. Overall, present study addressed the questions whether: (a) silybinin prevents or inhibits the growth of advance prostate carcinoma in vivo; (b) its in vivo anticancer effect is correlated with up-regulation of IGFBP-3; (c) it is toxic in long-term animal studies; and (d) pharmacologically achievable concentrations have any biological significance.

MATERIALS AND METHODS

Cell Line and Reagents. DU145 human prostate carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 95% air and 5% CO2 atmosphere. DU145 cells grown as mono-
layer were harvested by brief incubation with 0.25% trypsin–EDTA solution (Life Technologies, Inc.) and used for xenograft implantation in nude mice.

**Animals and Diet.** Athymic (nu/nu) male nude mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in our nude mice animal care facility at standard laboratory conditions (in laminar airflow cabinets under pathogen-free conditions with a 12 h light/12 h dark schedule) and fed autoclaved Harlan Teklad Sterilizable rodent diet (W) and water *ad libitum.*

The principle of the assay was based on quantitative phospho-IGFBP-3 ELISA. At 60% confluency, cells were treated either with DMSO alone (0.05%, v/v) or 15, 30, and 50 μM silibinin each day for 4 days and then treatment medium was replaced with fresh medium without silibinin at the end of the 4 days of treatment, and cells were harvested at days 5, 6, 7, and 8, and counted. In another subset of similar experiment, cells were treated with the same doses of silibinin each day for 4 days and then treatment medium was replaced with fresh medium without silibinin at the end of the 4 days of treatment, and cells were harvested at days 5, 6, 7, and 8, and counted. Each treatment and time point had two independent plates, and each sample was counted in duplicate. The experiment was repeated with similar results.

**Statistical Analysis.** The data were analyzed using the Jandel Scientific SigmaStat 2.0 software. For all of the measurements, one-way ANOVA followed by Turkey test was used to assess the statistical significance of difference between control and silibinin-treated groups in animal and cell culture studies. A statistically significant difference was considered to be present at $P < 0.05$. **RESULTS**

Silibinin Inhibits Human Prostate Carcinoma DU145 Tumor Xenograft Growth in Nude Mice. Consistent with our recent cell culture studies showing strong growth inhibition of DU145 cells by silibinin and silymarin, in the present study, dietary feeding of silibinin at 0.05% or 0.1% (w/w) dose to nude mice showed a dose-dependent inhibition of tumor xenograft growth in terms of tumor volume as well as wet weight of tumors. In the first set of the experiment, where dietary feeding of silibinin (0.05% or 0.1%) started 24 h after s.c. injection of DU145 cells, tumor volume was inhibited by 35 and 58% ($P < 0.05$; Fig. 1A), and wet weight of tumor was...
around 6 weeks of feeding, which remained so up to the end of the study (Fig. 1A).

In the second set of the experiment we studied whether silibinin feeding before tumor implantation in mice has any additional inhibitory effect on the growth of advanced PCA tumor xenograft. In this study, where mice were first fed with the 0.05 or 0.1% silibinin-containing diet for 3 weeks before the implantation of DU145 cells and continued with same diet for a total 63 days, tumor volume was reduced by 53 and 64% (P < 0.05; Fig. 1B) and wet weight of tumor was decreased by 31 and 52% (P < 0.05; Fig. 1C), respectively, at the end of experiment. These results are indicative that silibinin has an additional growth inhibitory effect against PCA when mice were pretreated with the test compound before the implantation of tumor xenograft.

Dietary Consumption of Silibinin Does Not Show Any Apparent Sign of Toxicity in Nude Mice. In achieving chemopreventive prophylaxis or therapeutic effect of a compound under test, the toxicity and/or side effects are also important determinants to be assessed before any clinical trial in humans and subsequent use. Accordingly, in our nude mice tumor studies, we also assessed any toxicity and/or untoward effects of dietary silibinin feeding throughout the study. In this regard, dietary feeding of silibinin did not show any adverse effect to the mice as monitored by diet consumption, body weight gain, and prostate weight. As shown in Fig. 2, dietary feeding of silibinin at 0.05 or 0.1% (w/w) dose for 60–63 days did not show any reduction in food consumption (Fig. 2, A and B). When these food consumption levels were extrapolated with silibinin intake, on an average throughout the study, its consumption was found to be 1.78–1.79 and 3.29–3.84 mg/mouse/day (approximately equivalent to 65 and 130 mg/kg body weight) at 0.05 and 0.1% dose levels, respectively (Fig. 2C).

Body weight gain profile is considered as one of the parameters extensively used in cancer chemoprevention/therapy studies, which is closely associated with the adverse side effects of the drug. Consistent with the favorable effect of silibinin on food consumption in nude mice, silibinin did not show any decrease in body weight, and its dietary feeding did not affect the body weight gain profile when compared with control group of mice throughout the study (Fig. 3, A and B). Because our aim was to establish anticancer effect of silibinin against PCA, we also estimated the prostate weight at the termination of experiments to evaluate the adverse effect of the compound, if any, such as hypertrophy or hyperplasia. Our results did not show any increase in the prostate weight of the mice fed with dietary silibinin as compared with their control groups (Fig. 3C). In fact, silibinin feeding caused a slight decrease in prostate weight, although it was not significant in all of the treatment groups except at lower dose in first set of experiment (Fig. 3C).

Dietary Feeding of Silibinin Induces Human IGFBP-3 Level in Mouse Plasma. On the basis of our cell culture study where silibinin has shown an antiproliferative effect against androgen-independent human prostate carcinoma PC-3 cells, which was associated with up-regulation of IGFBP-3 (15), we also assessed whether the same mechanism is operative in in vivo condition in inhibiting DU145 tumor xenograft growth in nude mice. For this, using quantikine ELISA, we measured the human IGFBP-3 level in mice plasma secreted by DU145 tumor xenograft after silibinin treatment at the end of experiment. A standard curve was made using recombinant human IGFBP-3 (supplied with kit) in the assay to extrapolate the plasma level of IGFBP-3 (Fig. 4A). Dietary feeding of silibinin at 0.05 or 0.1% (w/w) dose for 60 days resulted in 2.33 ± 0.46 and 3.38 ± 0.92 ng IGFBP-3/ml plasma as compared with 0.58 ± 0.14 (ng/ml) in control group, respectively (Fig. 4B), which accounted for 4- and 5.8-fold (P < 0.05) increases as compared with control value, respectively. This observation provides the first evidence for an in vivo
association between IGFBP-3 up-regulation and inhibition of PCA tumor xenograft growth by silibinin.

Inhibition of Tumor Xenograft Growth by Silibinin Is at Pharmacologically Achievable Doses Effective in Cell Culture. After establishing the anticancer effect of silibinin against human PCA tumor xenograft growth in nude mice, we next asked the question what are the pharmacological levels of silibinin in nude mice studies and whether such levels are effective in inhibiting DU145 cell growth in culture. To assess the levels of silibinin in plasma and prostate of the silibinin-fed nude mice, first a standard HPLC profile of silibinin was developed and its retention time was determined (data not shown). On the basis of this HPLC profile of silibinin, a linear detection range of silibinin was next established (data not shown). The silibinin concentrations in plasma and prostate were then calculated under the linear range of detection using area under curve of silibinin peak in HPLC profiles of these samples. As shown in Fig. 5A, compared with the control samples showing undetectable levels of silibinin, 0.05 or 0.1% silibinin feeding in diet (w/w), resulted in $7.1 \pm 0.05$ and $12.8 \pm 0.09 \mu g$ silibinin/ml plasma (corresponding to $15$ and $27 \mu M$) in first, and $6.7 \pm 0.3$ and $10.2 \pm 0.5 \mu g$ silibinin/ml plasma (corresponding to $14$ and $21 \mu M$) in the second set of tumor experiment, respectively (Fig. 5A). Because of this similar trend in dose-related bioavailability of silibinin in both protocols, the silibinin level in prostate was determined only in first set of tumor study. Both dietary doses of silibinin showed similar trend (as in plasma) in in situ

Fig. 2. Effect of silibinin on daily diet and corresponding silibinin consumption in nude mice during DU145 tumor xenograft studies. In the experiments detailed in Fig. 1, daily food intake was recorded throughout the feeding regimen in each group, and corresponding silibinin consumption levels were calculated. A and B, diet consumption/mouse/day (g) is plotted as a function of time (week) for 10 mice in each group from the experiments described in Fig. 1. C, silibinin consumption/mouse/day (mg) is extrapolated from the diet intake during total experimental duration in both sets of the experiment. SB, silibinin; bars, ± SE.

Fig. 3. Effect of dietary feeding of silibinin on body and prostate weights of nude mice during DU145 tumor xenograft study. In the experiments detailed in Fig. 1, body weight of each mouse in different groups was recorded twice a week throughout the experiment, and at the termination of the studies, prostate weight of each mouse in different groups was also recorded. A and B, body weights of mice are represented as mean of 10 mice in each group as a function of time in weeks from the experiments described in Fig. 1. C, prostate weight (mg) is represented as a ratio of body weight (g) of 10 individual mice from each group as mean in both sets of the experiment. SB, silibinin; *, $P < 0.05$; bars, ± SE.
SILIBININ INHIBITS IN VIVO GROWTH OF PROSTATE CANCER

Pharmacologically Achievable Doses of Silibinin Increases IGFBP-3 Level in DU145 Cell Culture Medium. Consistent with inducing effect of silibinin on IGFBP-3 secretion from DU145 tumors in nude mice study, pharmacologically achievable doses of silibinin (15 and 30 μM or a higher dose, 50 μM) resulted in significant increase in IGFBP-3 levels in DU145 cell culture medium. After 24 h of treatment, these silibinin doses increased IGFBP-3 level in medium by 1.1-, 1.3-, (P < 0.05), and 1.4- (P < 0.05) fold as compared with control, respectively (Fig. 5D). Treatment with 15 and 30 μM of silibinin doses for 48 h resulted in 1.3- (P < 0.05) and 1.6- (P < 0.01) fold increase in IGFBP-3 levels secreted from cells in culture medium over that of control value (Fig. 5D).

DISCUSSION

Overall, the major findings of the present study are that (a) silibinin significantly inhibits advanced human prostate carcinoma growth in an in vivo preclinical PCA model, (b) which is accompanied by a strong increase in plasma level of human IGFBP-3, (c) without any apparent signs of toxicity in athymic nude mice; and that (d) plasma levels of silibinin in the nude mice study exert strong inhibition of DU145 cell growth in culture. The additional cancer chemopreventive effect of silibinin was also evident by the results in which mice were fed with silibinin for 3 weeks before the implantation of the tumor xenograft.

In recent years, naturally occurring plant products are getting increased attention toward the prevention and/or intervention of the (a) early stages of carcinogenesis and (b) neoplastic progression before invasive malignant disease occurs. On the basis of this idea, certain foods, including many vegetables, fruits, and grains, as well as phytochemicals of diversified pharmacological efficacy have been shown to offer significant protection against various cancers (17). Furthermore, there is an increased focus on providing scientific basis to use these agents in prevention strategy for the people at high risk for cancer. In this regard, our extensive investigations with silymarin/silibinin have shown promising results against different epithelial cancers including PCA (3–8). Furthermore, in the present study, we provide first in vivo evidence for the efficacy of silibinin against advanced prostate carcinoma growth in athymic nude mice. Translating the observed in vivo efficacy of silibinin in inhibiting PCA xenograft growth in nude mice to its practical implications in human PCA, it is important to emphasize here that the observed inhibitory effects of silibinin were not complete in either of the two studies. However, because the major task here is to control its growth even in terms of slowing it down, one could argue that the inhibition observed by silibinin is of significant merit in terms of moving forward with this agent and conduct additional preclinical studies in PCA models followed by clinical trials in human PCA patients.

Previous reports have shown that both silymarin and silibinin are devoid of any toxicity and untoward effects in animal studies, and that they are used clinically as antihepatotoxic agents (2) and consumed extensively as a dietary supplement around the world including the United States (1). Consistent with these reports, in our study, dietary feeding of silibinin (up to 0.1%, w/w) did not show any adverse effect on diet consumption, animal body weight gain, and prostate weight. This finding not only additionally support the notion that silibinin and silymarin, as well as the milk thistle extract (consumed as dietary supplement) that constitute them, are devoid of toxicity, but also suggests that the doses of silibinin higher than those used in the present study should be evaluated for their inhibitory effect on PCA
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PCA xenograft growth inhibition in nude mice. To support this conclusion additional studies are needed to trace the effect of silibinin on IGF-1-IGF-1R-phosphatidylinositol 3′-kinase signaling under in vivo condition. Additionally, IGF-1-independent association between PCA risk and IGFBP-3 level has also been observed where IGFBP-3 has been shown to induce apoptosis independent of its IGF-sequencing action (23). Whether, in vivo inhibitory effect of silibinin on PCA xenograft growth is in part via such a mechanism also remains to be established. However, based on the data of the present study, we suggest that in a silibinin chemoprevention trial in human PCA patients, the circulating level of IGFBP-3 in plasma may serve as a prognostic biomarker as a surrogate measure of success of silibinin treatment. In such studies, the physiological manifestation of IGFBP-3 in a population seems to be an important target in correlating increased/decreased incidence of PCA.

One important component in cancer chemoprevention research that is receiving increased attention is what the pharmacological levels are of the agent being tested and whether such levels have any biological efficacy. Accordingly, one of our other aims in this study was to address this issue. First we determined the levels of silibinin in both plasma and prostate tissue samples from both of the tumor experiments, and then evaluated the biological significance of plasma concentrations of silibinin in DU145 cells in culture. The pharmacologically achievable levels (15–30 μM) of silibinin showed dose- as well as time-dependent inhibition of DU145 cells growth in culture. Furthermore, the wash out experiments, where silibinin was removed from the medium after 4 days of treatment, did not show any change in the rate of cell proliferation as compared with control. This result suggested that the antineoplastic effect of silibinin is static and that after its removal from the medium, it does not exceed the deregulated PCA cell proliferation when compared with control. This observation also indicated that it could be mostly the epigenetic cell signaling events that are being modulated by silibinin in DU145 cells to produce its antiproliferative effect against these cancer cells; a possibility supported by several studies in recent years by us in PCA cell culture (3–8, 15, 22).

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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

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