Evaluation of the cancer chemopreventive efficacy of silibinin in genetic mouse models of prostate and intestinal carcinogenesis: Relationship with silibinin levels

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Grant support: Cancer Research UK Programme grant C325/A6691, UK Medical Research Council Programme grant G0100874.

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Running Title: Chemopreventive efficacy of silibinin

Key Words: Chemoprevention, preclinical models, silibinin, IGF,

Abstract

Silibinin, a flavonolignan from milk thistle seeds, possesses cancer chemopreventive properties in rodent models of prostate and colorectal carcinogenesis. We tested the hypotheses that silibinin or silipide, a formulation of silibinin with phospholipids to improve systemic availability, delay tumor development in TRAMP or *Apc*^{Min} mice, genetic models of prostate or intestinal malignancies, respectively. Mice received unformulated silibinin or silipide with their diet (0.2% silibinin equivalents) from weaning for their life time. Intervention with silipide reduced the size of well differentiated TRAMP adenocarcinomas by 31% and decreased incidence of poorly differentiated carcinomas by 61% compared to mice on control diet. Unformulated silibinin reduced only incidence of poorly differentiated carcinomas. Silipide decreased plasma levels of insulin-like growth factor (IGF)-1 by 36%, and levels of circulating IGF binding protein (IGFBP)-3 in mice on silipide or silibinin were 3.9- or 5.9–fold, respectively, elevated over those in control TRAMP mice. In *Apc*^{Min} mice on unformulated silibinin, adenoma numbers were slightly but not significantly reduced, whilst silipide had no effect. Blood and tissue levels of silibinin were measured by HPLC analysis. Plasma and prostate levels of silibinin were much higher in mice on silipide than in those on silibinin, whilst levels of silibinin in the intestinal mucosa of mice on unformulated silibinin were 2.5-fold higher than those in mice on silipide. The results support the advancement of silipide to the stage of clinical investigation in prostate cancer. Unformulated silibinin may be appropriate for evaluation to inhibit colorectal adenoma recurrence.

Introduction

The polyphenolic phytochemical silibinin, a flavonolignan (for structure see Figure 1), is a major constituent of the seeds of milk thistle (*Silybum marianum L.*). Silibinin and silymarin, a standardized milk thistle extract of which silibinin is a major component, are widely consumed as dietary supplements, especially in the USA. Amongst claims as to health benefits related to milk thistle components is the suggestion that they possess anticarcinogenic properties. This claim is supported by evidence in rodents according to which silibinin and silymarin can interfere with experimental malignancies of the prostate, intestinal tract, skin and bladder (1-8). The clinical evaluation of flavonoids such as silibinin has been hampered by their poor systemic availability associated with, at least in part, their propensity to undergo avid conjugative metabolism. In order to improve the bioavailability of silibinin, it has been formulated with phosphatidylcholine ("silipide", Indena SpA, Milan, Italy). Clinical evaluation of this formulation at single or repeated doses in healthy volunteers and cancer patients demonstrated its safety (9, 10) and the superior bioavailability of silibinin released from silipide when compared to silymarin (11).

Genetically induced rodent models of carcinogenesis can be exploited to generate information on the efficacy, pharmacodynamics and pharmacokinetics of novel putative cancer chemoprevention agents, and such information is extremely valuable in the planning of clinical intervention studies. The "Transgenic Adenocarcinoma of the Mouse Prostate" (TRAMP) is a model of prostate cancer which mimics progressive forms of the human disease. Expression of the SV40 early genes (T and t antigen) in TRAMP mice is driven by the prostate-specific promoter probasin, leading to cell transformation within the prostate (12). All male TRAMP mice develop prostate cancer from approximately 18 weeks of age, and the disease

progresses from prostatic intraepithelial neoplasia to histologic cancer and finally to carcinoma, which can metastasize to lymph nodes, lungs, liver and bone (13). The *Apc*^{Min} mouse is a model of intestinal carcinogenesis genetically driven by a truncating *Apc* gene mutation (12). It resembles the human heritable condition familial adenomatous polyposis coli (FAP). Tea polyphenols, genistein and curcumin are polyphenolic phytochemicals currently at varying stages of clinical evaluation, which have been found to impede carcinogenesis in these two models. The first two interventions interfered with carcinogenesis in TRAMP mice (15, 16), and curcumin slowed adenoma development in *Apc*^{Min} mice (17, 18).

Several mechanisms have been proposed to explain how silibinin may interfere with carcinogenesis. Among these mechanisms are impairment of receptor tyrosine kinase and erbB1 signalling, upregulation of cyclin-dependent kinase inhibitors causing attenuation of cancer cell growth and perturbation of cell cycle progression (19, 20), induction of cancer cell differentiation (21) and anti-angiogenesis (22). Especially noteworthy is the finding that silibinin is able to modulate the insulin-like growth factor (IGF) system. IGFs are mediators of cell survival in that they can inhibit apoptosis and influence differentiation of many normal and malignant cell types (23-25). The IGF system is regulated by IGF binding proteins (IGFBPs), especially IGFBP-3, which bind IGFs in the extra-cellular milieu with high affinity and specificity, thus reducing the bioavailibility of IGFs. Epidemiological studies have linked increased serum concentrations of IGF-1, decreased concentrations of IGFBP-3, or both, with an increased risk of advanced prostate cancer (26), although the validity of such an association has recently been questioned in a large case-control study (27). Silibinin increased levels of IGFBP-3 in prostate cancer cells *in vitro* (28) and in prostate tumor-bearing rodents *in vivo* (1).

These results intimate that circulating IGF-1/IGFBP-3 levels may be suitable candidates for pharmacodynamic markers of efficacy in prostate cancer intervention studies with silibinin.

Two recent pilot studies of phytosomal silibinin formulations in patients with colorectal (29) or prostate cancer (10) define doses and schedules suitable for further clinical studies. Nevertheless many issues pertinent to the pharmacodynamics and pharmacokinetics of silibinin require optimization prior to the further clinical evaluation of its potential value in cancer chemoprevention. In order to accrue preclinical information which might help define how best to evaluate silibinin or silipide in the clinic, we compared aspects of their pharmacology in TRAMP and *Apc*^{Min} mice. The work was specifically designed to test the hypotheses that silibinin and silipide delay prostate carcinogenesis and affect circulating levels of IGF-1/IGFBP-3 in TRAMP mice, and that they interfere with intestinal carcinogenesis in *Apc*^{Min} mice. Furthermore, we explored whether any differences in efficacy between silipide and silibinin may be explained in terms of plasma and tissue levels of silibinin.

Materials and Methods

Animals

TRAMP mice on a C57BL/6J background and C57BL/6J Min/+ (*Apc*^{Min}) mice were bred in the Leicester University Biomedical Services facility using animals originally obtained from either the NCI Mouse Repository (NCI Frederick, TRAMP) or the Jackson Laboratory (Bar Harbor, ME, *Apc*^{Min}). Ear tissue was obtained from mice at approximately 10–14 days of age in order to assess the presence of the

transgene using PCR as described previously (18, The Jackson Laboratory website: www.jax.org).

Interventions

Silibinin (>98 % pure as checked by HPLC analysis) was purchased from Sigma-Aldrich Comp Ltd (Gillingham, UK). “Silipide” (IdB 1016), a phytosome product marketed for use as a hepatoprotectant (see www.indena.it/pdf/prodottiweb.pdf), was provided by Indena SpA (Milan, Italy). Silipide contains silibinin and soy phosphatidylcholine at a molar ratio of 1:1, in terms of percentage weight this equals approximately 40 % silibinin and 60 % phosphatidylcholine.

Animal Experiments and Dosing

Experiments were carried out under animal project license PPL 40/2496, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UK Co-ordinating Committee on Cancer Research guidelines (30). At four weeks of age mice received standard AIN 93G diet (Dyets Inc, Bethlehem, PA) or AIN diet supplemented with silibinin or silipide (Indena SpA, Milan, Italy) (0.2 % in terms of silibinin) to the end of the animals’ life. The dietary dose of silibinin used (0.2%, approximately 300 mg/kg per day) in mice equates to approximately 1.8 g per human per day, assuming a body surface area of 1.8 square meters accompanying a body weight of 70 Kg, when extrapolated on the basis of body surface area (31). This dose is similar to high doses employed in clinical trials (9, 10). Appearance and weight of the mice were checked

on a weekly basis. Mice showing signs of distress, weight loss or very large tumors were killed as stipulated in the licence. In the analytical chemical experiments using C57BL/6J wild-type mice, mice received silibinin or silipide (0.2% in terms of silibinin) with their diet for 21 days, after which they were killed. Murine blood was obtained by cardiac exsanguination (halothane anesthesia). Prostate and liver tissues were excised, plasma was obtained, and tissue and plasma samples were frozen (-80^0 C) until analysis.

Assessment of Tumor Development

From 11 weeks of age TRAMP mice were palpated once or twice weekly for presence of tumor. Animals were killed in week 28 (TRAMP) or 18 (*Apc*^{Min}). Murine tissues were excised, weighed and placed in buffered formalin for histopathology (see below). In the TRAMP mice, tumor tissue constituted 90% or more of total genitourinary (GU) tract mass (prostate, prostate tumor, seminal vesicles, empty bladder). Separation of prostate tumor from seminal vesicles was often difficult. Therefore in the results, GU tract weight values are given to reflect tumor development. The presence in some TRAMP mice of large poorly differentiated (pd) carcinomas confounded comparison of tumor size between groups. Therefore consequence of intervention in this model was assessed in two ways: by GU tract weight in the case of well differentiated (wd) adenocarcinomas, and by incidence in the case of pd tumors. *Apc*^{Min} mice were killed and their intestinal tract was removed and flushed with phosphate-buffered saline. Intestinal tissue was cut open longitudinally and examined under a magnifying lens. Multiplicity, location and size of adenomas (18), and packed red cell volume (hematocrit) (32) were measured as described previously. In some TRAMP and *Apc*^{Min} mice blood was obtained by

cardiac puncture under terminal halothane anesthesia into heparinized tubes. PCR analysis of TRAMP tumor tissue obtained from mice on control diet, silibinin or silipide suggests that neither intervention interfered with the expression of the SV40 transgene (result not shown).

Histopathology

Maxillary gland, lungs, liver, kidneys, seminal vesicles, prostate and dorsal abdominal connective tissue of TRAMP or C57BL/6J wild-type mice and the intestinal tract from *Apc*^{Min} mice were fixed in formalin for a minimum of 2 weeks. All tissues were embedded in paraffin wax, and standard sections (5 µm thick) were cut and stained with hematoxylin and eosin before microscopic examination.

Measurement of IGF-1 and IGFBP-3 Concentrations

TRAMP mice received standard diet or diet fortified with silibinin or silipide (0.2% silibinin equivalents) for 30 weeks post weaning, after which they were exsanguinated under terminal halothane anesthesia. Levels of IGF-1 and IGFBP-3 were determined in the plasma using the enzyme-linked immunosorbent assay (ELISA) kits “Quantikine Mouse IGF-1” (catalogue number MG100) and “Mouse IGFBP-3” (catalogue number DY775, both from R+D Systems, Abingdon, UK). The IGF-1 kit procedure contained an acid-ethanol extraction step to separate IGFs from their binding sites. The assays were validated and performed according to the vendor’s instructions. Information on circulating IGFBP-3 levels in mice is sparse, and published values vary considerably between studies (16, 33). Therefore in a preparatory experiment we analyzed IGFBP-3 levels in plasma from several mouse strains to determine typical levels and variability. Mean values varied between 1120

and 1450 ng/mL depending on strain, with intra-strain coefficients of variation of 25-35% (n=6-10). The IGFBP-3 level observed in C57BL6J mice in the experiment described here (920±310 ng/mL) is compatible with these values. The molar ratio of IGF-1 to IGFBP-3 was calculated as (0.13 x IGF-1 concentration [ng/mL]) / (0.036 x IGFBP-3 concentration [ng/mL]) (34).

Chemical Analysis of Silibinin

Liver and prostate tissues were thawed, weighed and homogenized in an equal part of KCl solution (0.15 M). Samples of plasma or tissue homogenate were mixed with 3 parts of ice-cold methanol. The mixture was centrifuged, 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, employing a gradient system with a two-component mobile phase. The details, characterization and validation (for silibinin) of the method, which separates parent compound from its many conjugate metabolites, have been described before (35). Silibinin is a mixture of two diastereoisomers, and their limits of quantitation were 3 and 5 ng/mL (6 and 11 pmol/mL). The results give values for the sum of both diastereoisomers. Silibinin was quantitated in the plasma, prostate and gastro-intestinal mucosa tissue from wild-type C57BL/6J, TRAMP and *Apc*^{Min} mice using this method.

Statistical evaluation

Evaluation of significance of differences to the appropriate controls was performed by either non-parametric Mann-Whitney *U* test (to assess TRAMP

adenocarcinoma weight) or Student's t test for independent samples (to assess *Apc*^{Min} adenoma number and IGF-1/IGFBP-3 values).

Results

Effect of Silibinin and Silipide on Mouse Body Weight

TRAMP or *Apc*^{Min} mice received unformulated silibinin or silipide at 0.2% (silibinin equivalents) in the diet for their lifetime. Intervention did not affect murine bodyweight (Fig. 2).

Effect of Silibinin and Silipide on Prostate Carcinogenesis

Neoplastic development was assessed in week 28, when tumors were small. Histopathological investigation revealed that prostate tumor had replaced normal prostate tissue in all TRAMP mice (control and treated). Consistent with the original description of the TRAMP model (13), two distinct histological types of malignant tumors were observed: wd adenocarcinomas in the prostatic epithelium and pd carcinomas. The wd tumors showed a cribriform glandular pattern and spread locally into adjoining seminal vesicles. Advanced wd tumors were accompanied by small lung metastases. The pd tumor were composed of large cells showing little or no glandular differentiation, and their size and weight were mostly an order of magnitude higher than those of the wd adenocarcinomas. They infiltrated local tissue including bladder as well as retroperitoneal lymph nodes, and rarely the pancreas. Intervention with silipide reduced GU tract weight in mice with wd adenocarcinomas by 31% (Fig. 3A), whilst consumption of silibinin did not affect GU tract weight. The proportion of mice which presented with large pd carcinomas in either intervention group was 39% of that seen in the control group (Fig. 3B). Neither intervention interfered with the

expression of the SV40 transgene (result not shown). Both interventions decreased metastatic deposits in lymph nodes, but failed to reduce other pathological manifestations associated with TRAMP malignancy (Table 1). These manifestations were metastases in the lungs, atypical hyperplasia (prostatic intraepithelial neoplasia) associated with, or preceding, wd adenocarcinoma, and glandular and stromal hyperplasia in the epithelia of seminal vesicles, which comprised fronds of cells overlaying loose connective tissue stroma. None of the C56BL/6J wild-type mice presented with manifestations of metastasis or hyperplasia.

Effect of Silibinin and Silipide on Circulating Levels IGF-1 and IGFBP-3

IGF-1 and IGFBP-3 levels were determined in the plasma from TRAMP mice which received silibinin or silipide for 30 weeks post weaning and, for comparison, also in the plasma of control C57BL/6J mice, the TRAMP background strain. Circulating IGF-1 levels in plasma from control TRAMP mice were 41% above those in their C57BL/6J wild-type counterparts. IGFBP-3 levels in TRAMP mice were reduced by 84% compared to their background strain, and the molar ratio of IGF-1/IGFBP-3 in TRAMP mice was 4.9-fold above that in C57BL/6J wild-type mice (Fig. 4). Both interventions returned circulating levels of IGF-1 (Fig. 4A) and of IGFBP-3 (Fig. 4B) and their ratio (Fig. 4C), at least partially, towards values observed in the background strain. Levels of IGF-1 in mice on silibinin or silipide were reduced by 11 and 36%, respectively, compared to control TRAMP mice, the latter difference being significant (Fig. 4A). Consumption of silibinin or silipide increased circulating IGFBP-3 levels significantly by factors of 5.9 and 3.9, respectively, in comparison with TRAMP mice on control diet (Fig. 4B). The IGF-1/IGFBP-3 ratio was decreased significantly by silibinin and silipide to 24% and 47%, respectively, of the

corresponding value in TRAMP mice on standard diet (Fig. 4C). In a control experiment to examine any effect of intervention on IGFBP-3 in C57BL/6J wild-type mice, animals received a diet containing 0.2 % silibinin or silipide for 21 days, and plasma IGFBP-3 levels were compared. In contrast to the scenario in TRAMP mice, neither intervention raised IGFBP-3 levels over those in mice on standard diet (results not shown), demonstrating that modulation of this protein by silibinin or silipide can be observed only in mice with the malignant genotype.

Effect of Silibinin and Silipide on Intestinal Carcinogenesis

Intervention with silibinin or silipide reduced adenoma numbers in the small intestine by 18 or 30%, respectively (Fig. 5A), albeit the difference failed to reach levels of significance. Neither intervention affected adenoma numbers in the colon (Fig. 5B). At the late stage of adenoma development *Apc*^{Min} mice suffer from intestinal bleeding, which causes a dramatic fall in hematocrit, and changes in hematocrit in this model reflect adenoma development. Intervention with silibinin raised the hematocrit, measured at the end of the experiment, by 21% (not significant), whilst the hematocrit in mice on silipide was unchanged compared to controls (Fig. 5C).

Histopathological analysis of the small intestine of *Apc*^{Min} mice showed focal proliferative lesions ranging from hyperplastic glands to larger areas of glandular hyperplasia and polypoid adenomas, without any significant differences in morphology between mice on control diet or on silipide or silibinin.

Levels of Silbinin in Blood and Tissues

Silibinin was measured by HPLC analysis in plasma, prostate, liver and gastrointestinal mucosa from C57BL/6J wild-type mice, the background strain of the TRAMP and *Apc*^{Min} mice, which had received either silibinin or silipide (0.2% in terms of silibinin equivalents) in their diet for 21 days prior to analysis. Fig. 6 shows that levels of silibinin recovered from plasma, prostate or liver tissue of mice on unformulated silibinin were close to or below the limit of detection (6 and 11 pmol/mL or g, for both diasteroisomers). In contrast, silibinin levels in plasma, prostate or liver of mice on silipide were 0.5 µM, 24 and 7 nmol/g, respectively, thus easily within the measurable range. Silibinin levels in the intestinal mucosa of mice on either intervention were two orders of magnitude higher than those in prostate or liver. In mice on unformulated silibinin these levels were 1.9 µmol/g tissue, almost 2.5 times higher than those in mice on silipide. Silibinin levels were also quantitated in plasma, prostate and liver tissue from three TRAMP and in plasma and intestinal mucosa from three *Apc*^{Min} mice which had received silibinin/silipide for their lifetime, to ensure that the silibinin levels measured in the wild-type C57BL/6J mice had reached steady state after 21 days consumption of silibinin/silipide, and that they were representative of levels in tumor-bearing mice. Results obtained in this experiment were very similar to those described in Fig. 6.

Discussion

The results described above show for the first time that silipide, a phospholipid-containing formulation of silibinin, can retard prostate carcinogenesis in the TRAMP mouse. Efficacy was demonstrated by reduction in size of wd adenocarcinoma and in incidence of pd carcinomas. Silipide affected the murine IGF-

1/IGFBP-3 system consistent with anticarcinogenesis. Intervention with silipide decreased plasma levels of IGF-1, increased levels of IGFBP-3 and reduced the IGF-1/IGFBP-3 ratio to levels similar to those observed in wild-type mice. Intervention with unformulated silibinin, which furnished much lower agent concentrations in prostate tissue than silipide, reduced incidence of early stage pd carcinoma but failed to affect wd adenocarcinoma size. The apparently higher potency of silipide as compared to its unformulated counterpart can be explained by its pharmaceutical properties. The lipophilic silibinin-phospholipid complex, which constitutes silipide, is thought to improve silibinin absorption in the gastro-intestinal tract *via* formation of a phospholipid monolayer on the mucosal surface, supporting the transition of silibinin from the hydrophilic gut content across lipophilic membranes into cells (11). In spite of the poor systemic availability of unformulated silibinin in mice, its consumption affected the murine IGF system, in that it restored IGFBP-3 levels and the IGF-1/IGFBP-3 ratio to baseline wild-type values, albeit reducing IGF-1 levels only non-significantly. The prostate cancer-delaying efficacy and pharmacodynamic consequences observed for silibinin, especially as its phospholipid formulation, in TRAMP mice are consistent with the inhibition of prostate tumor development reported for silibinin in nude mice bearing the DU145 human tumor xenograft (1) and for silymarin in rats in which prostate adenocarcinoma were induced by exposure to 3,2'-dimethyl-4-aminobiphenyl (2). Furthermore, silibinin has been previously suggested to ameliorate prostate tumor development in the TRAMP mouse (36), albeit experimental details have hitherto not been published.

One way in which silibinin is thought to lower free IGF-1 levels is by up-regulating IGFBP-3 (1). Enhanced IGF-1 levels can contribute to initiation and progression of prostate cancer by a variety of mechanisms, including induction of

vascular endothelial growth factor (VEGF) and engagement of the angiogenic switch leading to prostatic neovascularisation (37). Type I IGF receptors, *via* which IGFs mediate physiological effects, regulate matrix metalloproteinase (MMP)-2 synthesis (38), and increased IGF signalling has been shown to enhance the expression of VEGF, urokinase plasminogen activator and MMPs, which in turn correlate with tumor angiogenesis and metastasis (37-41). *In vitro* data suggests that IGFBP-3 can affect cell proliferation and apoptosis independently of IGF-1, in addition to its effects mediated *via* IGF-1 reduction. For example, IGFBP-3 compromised proliferation in breast cancer cells unresponsive to IGF-1 (42) and in mouse fibroblasts lacking IGF-1 receptors (43). All of these results are consistent with the notion that a decrease in IGF-1 and/or elevation of IGFBP-3 may have contributed, at least in part, to the anticarcinogenic effects of silipide/silibinin in TRAMP mice.

There was a hint of efficacy of unformulated silibinin as a chemopreventive agent against gastrointestinal carcinogenesis in the Apc^{Min} mouse model, whilst silipide failed altogether to affect intestinal adenoma development. The tentative evidence of efficacy of silibinin complements results of two preclinical studies of silymarin in carcinogen-induced rodent colorectal cancer models (6, 7), implying that milk thistle flavonolignans may delay gastrointestinal carcinogenesis.

The results of the quantitative chemical analysis of silibinin in blood and tissues shows that, when administered with the diet in mice, silipide is superior to unformulated silibinin in terms of systemic silibinin delivery. Differences in activity between the two interventions in the TRAMP and Apc^{Min} mice can be interpreted in the light of the observed discrepancies in silibinin levels. Blood and prostate levels were much higher after consumption of silipide than after unformulated silibinin, compatible with the relative enhanced ability of silipide as compared to unformulated

silibinin to interfere with TRAMP wd adenocarcinoma development. The concentration of silibinin (24 nmol/g) recovered in this study from the prostate of mice which received silipide, is within the concentration range of silibinin which reduced the growth of human–derived DU145 prostate cells in culture (1). This fact intimates the possibility that mechanisms responsible for anticarcinogenesis in prostate cells in culture may also be engaged by silibinin in the TRAMP mouse prostate *in vivo*. Levels of silibinin in the gastrointestinal tract, where exposure does not depend on systemic delivery of agent, were higher after consumption of unformulated silibinin than after silipide, consistent with the tentative potency difference between them in the Apc^{Min} mouse.

How may the findings presented above, together with those published previously, be exploited in the planning of the potential development of milk thistle preparations in humans? Obviously extrapolation to humans of the results obtained here in two genetic carcinogenesis models needs to be made with utmost caution. First and foremost, both interventions were equally well tolerated as reflected by lack of adverse effects on murine body weight, reinforcing the good preclinical safety record of silibinin and silipide. The results in TRAMP mice advocate the advancement of silipide to the stage of clinical evaluation as a prostate cancer delaying agent, eg in patients under active surveillance. Circulating levels of IGF-1 and/or IGFBP-3 were shown here to respond sensitively to silipide in TRAMP mice. Further research needs to establish whether IGF-1/IGFBP-3 might be useful as potential pharmacodynamic markers of clinical efficacy of silibinin. A recent phase I trial in hormone–refractory prostate cancer patients of a phytosomal silibinin formulation similar, but not identical, to silipide, suggests that repeated administration of a daily dose equivalent to 4.3 g silibinin, given in three divided doses, is well tolerated and constitutes a

suitable starting dose for future phase II trials (29). Extrapolation of the results obtained in the *Apc*^{Min} mouse hint at the possibility that silibinin may be useful in the prevention of adenoma recurrence. In the light of the difference between unformulated silibinin and silipide in terms of intestinal levels achieved and putative efficacy, unformulated silibinin may well constitute the preferable pharmaceutical option as compared to silipide, to be employed in such intervention trials.

Acknowledgements

The authors thank Dr Paolo Morazzoni, Indena S.p.a., for generous provision of silipide, Mrs J Edwards (MRC Toxicology Unit, University of Leicester) for help with the histology, and the staff in the Leicester University Biomedical Services facility for help with animal husbandry.

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Table 1. Incidence of malignancy-related pathology in TRAMP mice which received dietary silibinin or silipide (0.2% silibinin equivalents).

	Metastasis		Atypical hyperplasia (PIN)	Glandular/stromal hyperplasia in seminal vesicles	
	Lymph	Lungs		Mild/moderate	Severe
Wild type C57BL/6J	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
TRAMP control	3/16 (19%)	1/16 (6%)	5/16 (31%)	14/16 (88%)	3/16 (19%)
TRAMP on silibinin	0/16 (0%)	3/16 (19%)	6/16 (38%)	15/16 (94%)	2/16 (13%)
TRAMP on silipide	1/15 (7%)	1/15 (7%)	3/15 (20%)	14/15 (93%)	2/15 (13%)

Figure legends

Figure 1. Structure of silibinin, a diastereoisomeric mixture (1:1) of the two forms 2R, 3R, 12 S, 13S and 2R, 3R, 12R and 13R. Asterisks indicate optically active centres.

Figure 2. Lack of effect of unformulated silibinin or silipide on body weight of *Apc*^{Min} (**A**) or TRAMP mice (**B**), which received control diet (squares, solid line) or diet fortified with silipide (rhombi, broken line) or silibinin (triangles, broken line) at 0.2% (silibinin equivalents). Results are the mean of between 13 and 19 mice. SDs were 10-21 % (**A**) or 7-20 % (**C**) of the mean values. For details of animal experimentation see Materials and Methods.

Figure 3. Prostate cancer development in TRAMP mice which received standard diet (open bars) or diet fortified with unformulated silibinin (closed bar) or silipide (hatched bar) at 0.2% (silibinin equivalents) for 24 weeks post weaning. Tumor development is reflected by weight of genitourinary tract (prostate, prostate tumor, seminal vesicles and empty bladder) in mice bearing well-differentiated prostate adenocarcinomas (**A**) and by percentage of mice bearing large poorly differentiated prostate carcinomas (**B).** Asterisk indicates that the difference between mice on silipide and those on control diet was significant ($p<0.005$, Mann-Whitney). Values are the mean ±SD. Total group size was between 16 and 19. For comparison, the genitourinary tract weight in wild-type C57BL/6J mice kept on standard diet was

0.5±0.2g (n=6). For details of animal experiments and assessment of tumor development see Materials and Methods.

Figure 4. Plasma levels of IGF-1 (**A**), IGFBP-3 (**B**) and molar ratio of levels of IGF1 over IGFBP-3 (**C**) in TRAMP mice which received standard diet (open bars) or diet fortified with unformulated silibinin (closed bars) or silipide (hatched bars) at 0.2% (silibinin equivalents) for 30 weeks post weaning and, for comparison, in C57BL/6J wild-type mice (stippled bars). Values are the mean ±SD of 13-16 mice. Asterisks indicates that the difference between TRAMP mice on silibinin or silipide and those on control diet was significant, *p<0.02, **p<0.005, ***p<0.001. For details of animal experimentation and IGF-1/IGFBP-3 measurements see Materials and Methods.

Figure 5. Adenoma numbers in the small intestinal tract (**A**) or colon (**B**) and packed cell volume (**C**) in *Apc*^{Min} mice which received standard diet (open bars) or diet fortified with unformulated silibinin (closed bar) or silipide (hatched bar) at 0.2% (silibinin equivalents). The results are the mean ±SD of groups of 15-16; the p value suggests that the difference in small intestinal adenoma numbers between control and intervention with silibinin was outside significance levels. For details of animal experiments and assessment of adenoma development see Materials and Methods.

Figure 6. Levels of silibinin in the plasma (**A**), prostate (**B**), liver (**C**) and gastrointestinal mucosa (**D**) of C57BL/6J mice, the TRAMP and *Apc*^{Min} background strain, which received unformulated silibinin (closed bars) or silipide (crossed bars) at 0.2% (silibinin equivalents) with their diet for 21 days. Levels, which constitute the

sum of both stereoisomers of silibinin, were analyzed by HPLC. There was no silibinin in tissues of mice on control diet. Results are the mean±SD of 8 mice. For details of animal experiments and HPLC analysis see Materials and Methods.

Fig. 1

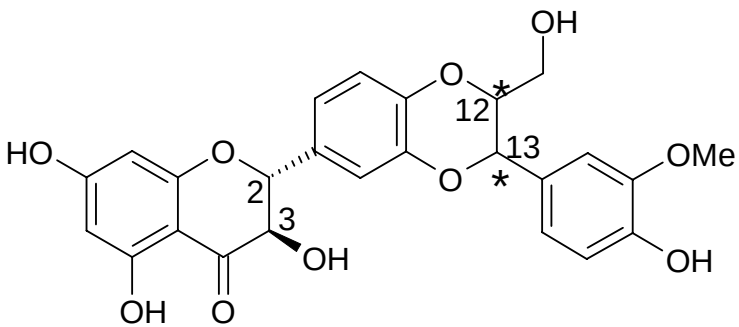


Fig. 2

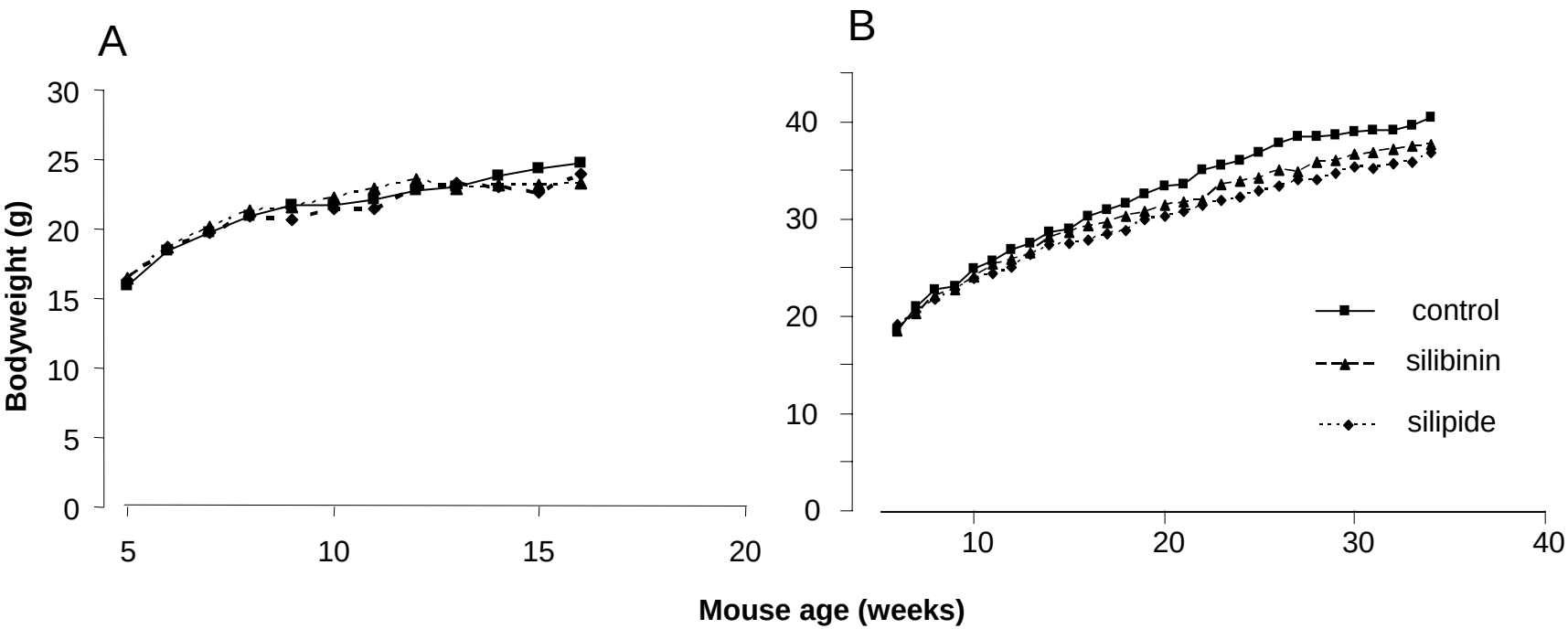

A
B
Bodyweight (g)
Mouse age (weeks)
control
silibinin
silipide
0
5
10
15
20
25
30
40

Fig. 3

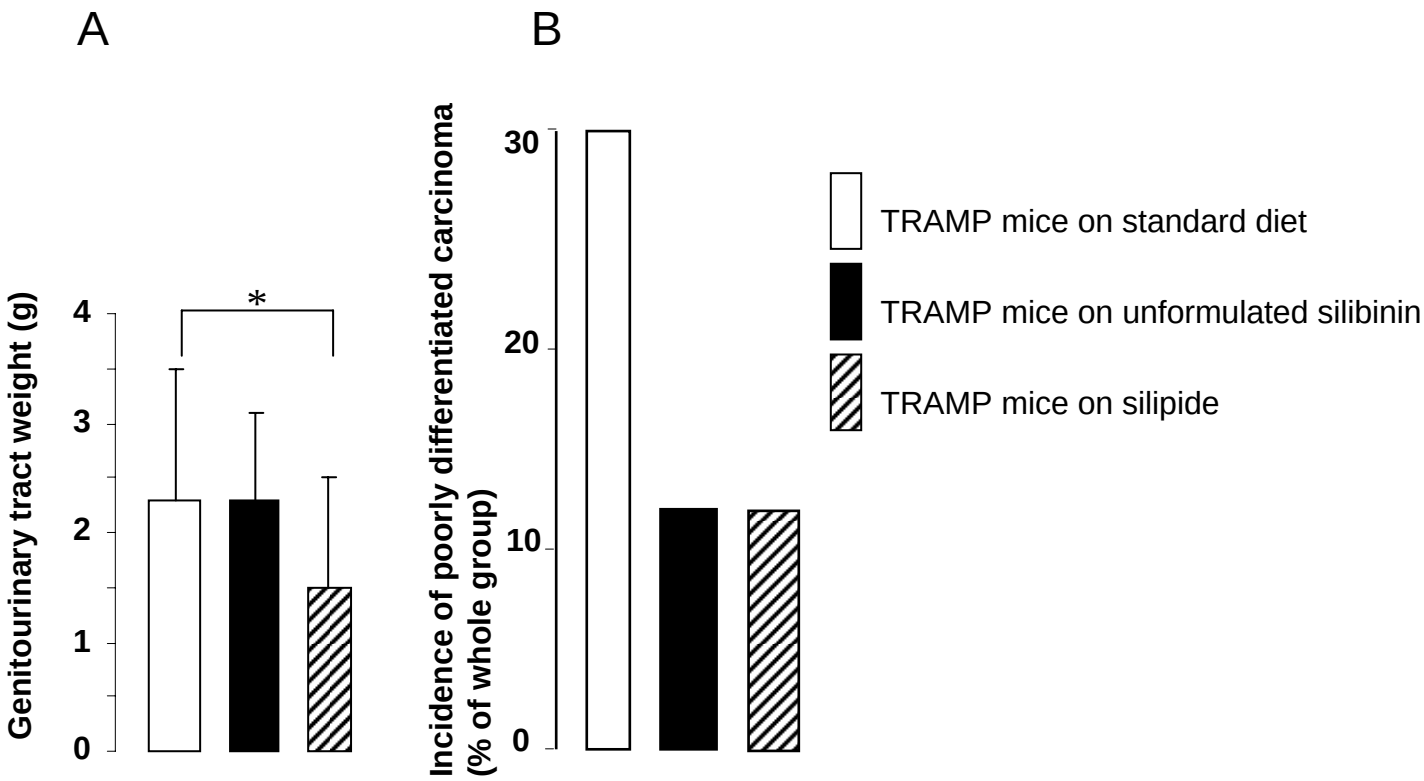

A
B
Genitourinary tract weight (g)
4
3
2
1
0
*
Incidence of poorly differentiated carcinoma (% of whole group)
30
20
10
0
TRAMP mice on standard diet
TRAMP mice on unformulated silibinin
TRAMP mice on silipide

Fig. 4

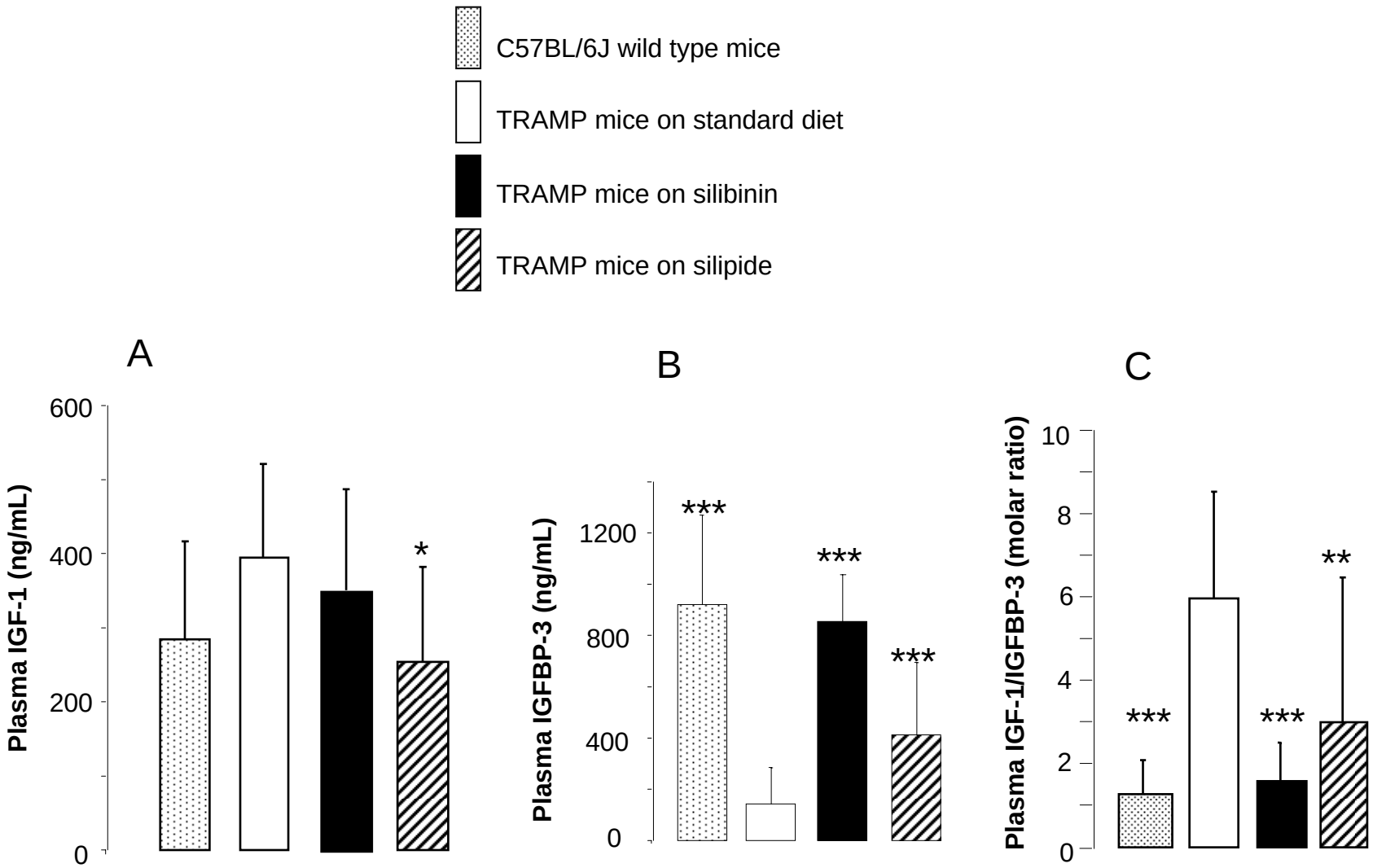

C57BL/6J wild type mice
TRAMP mice on standard diet
TRAMP mice on silibinin
TRAMP mice on silipide
A
Plasma IGF-1 (ng/mL)
600
400
200
0
*
B
Plasma IGFBP-3 (ng/mL)
1200
800
400
0
***
***
***
C
Plasma IGF-1/IGFBP-3 (molar ratio)
10
8
6
4
2
0
***
***
**

Fig. 5

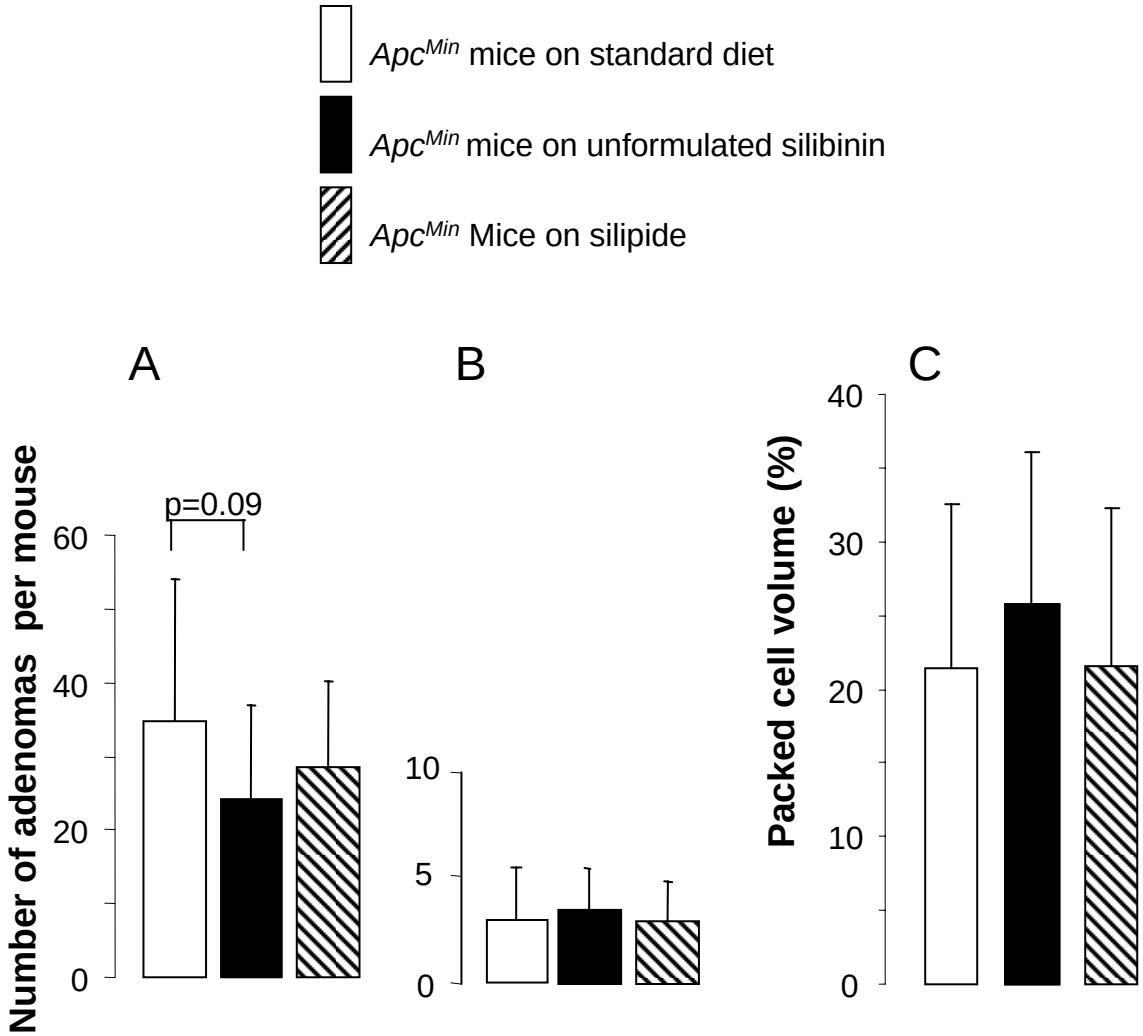
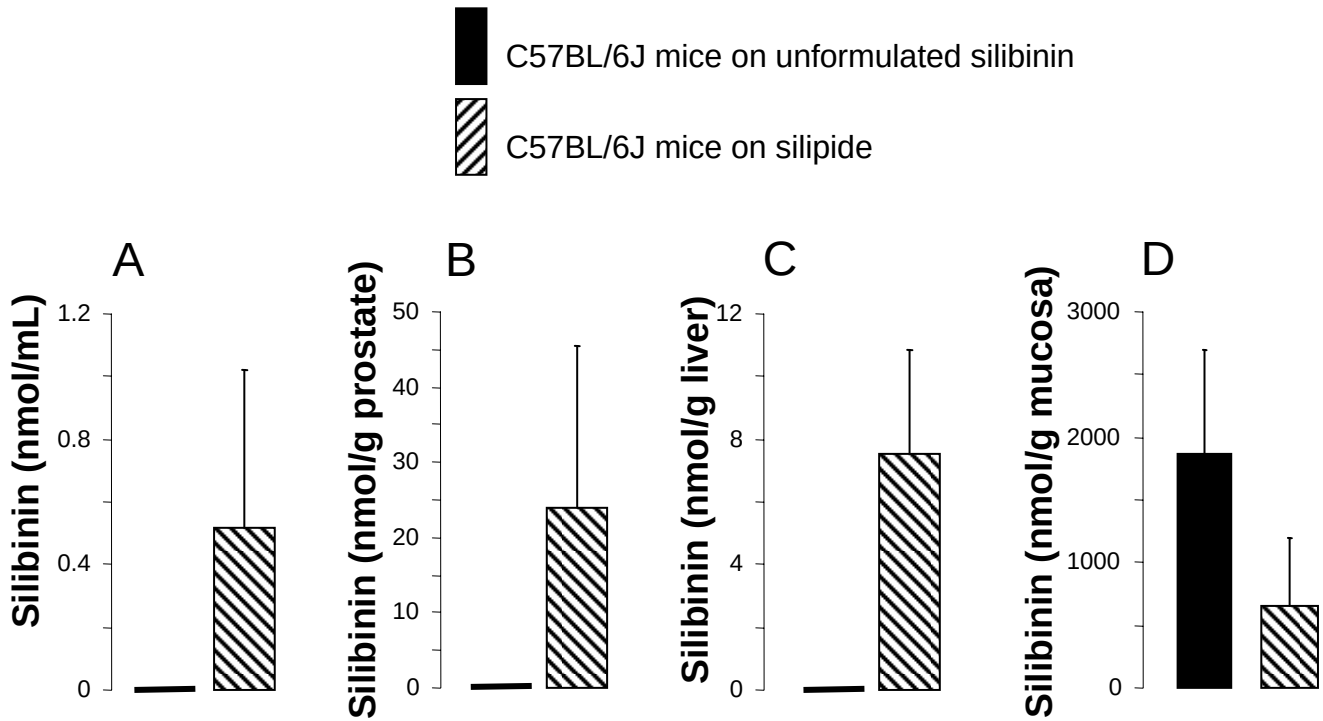

Apc^Min mice on standard diet
Apc^Min mice on unformulated silibinin
Apc^Min Mice on silipide
A
B
C
Number of adenomas per mouse
p=0.09
60
40
20
0
10
5
0
Packed cell volume (%)
40
30
20
10
0

Fig. 6


C57BL/6J mice on unformulated silibinin
C57BL/6J mice on silipide
A
Silibinin (nmol/mL)
1.2
0.8
0.4
0
B
Silibinin (nmol/g prostate)
50
40
30
20
10
0
C
Silibinin (nmol/g liver)
12
8
4
0
D
Silibinin (nmol/g mucosa)
3000
2000
1000
0