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SHORT COMMUNICATION

Consumption of silibinin, a flavonolignan from milk thistle, and mammary cancer development in the C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma (TAg) mouse

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Abstract Silibinin is a flavonolignan extracted from milk thistle with cancer chemopreventive activity in preclinical models of prostate and colorectal cancer. A milk thistle extract, of which silibin is a major component, has recently been shown to exacerbate mammary carcinogenesis in two rodent models. We tested the hypothesis that consumption of silibinin or silipide, a silibinin formulation with pharmaceutical properties superior to the unformulated agent, affect breast cancer development in the C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma mouse model. Mice received silibinin or silipide (0.2% silibinin equivalents) with their diet from weaning, and tumour development was monitored by weekly palpation and the number and weight of neoplasms at the end of the experiment. Intervention neither promoted, nor interfered with, tumour development. The result suggests that promotion of carcinogenesis is not a feature of silibinin consistent across rodent models of mammary carcinogenesis.

Keywords Chemoprevention · Preclinical models · Silibinin · Breast cancer

Introduction

The seeds of milk thistle (*Silybum marianum L.*) contain polyphenolic flavonolignans exemplified by silibinin (for structure see Fig. 1). Milk thistle extracts have been used for centuries in traditional medicine, and they are widely consumed herbal remedies with several putative beneficial effects on health, prominent among them are hepatoprotective properties. In rodents silibinin and silymarin, a crude milk thistle extract containing four flavonolignans with silibinin as the major component, interfered with experimental malignancies of the prostate, intestinal tract, skin and bladder [1–8]. The clinical development of silibinin has been hampered by its poor systemic availability, and in order to improve bioavailability, it has been formulated with phosphatidylcholine ("silipide", Indena SpA, Milan, Italy). This formulation has been found to be safe when administered at single or repeated doses in healthy volunteers and cancer patients [9, 10], and the bioavailability of silibinin after consumption of silipide was superior to that after silymarin [11]. Phytosomal preparations of silibinin such as silipide are currently in early clinical evaluation [10, 12]. Silymarin was recently found to modestly, but significantly, increase tumour incidence and severity in two rodent models of mammary carcinogenesis, one a carcinogen (1-methyl-1-nitrosourea, MNU) induced rat model and the other a transgenic model, the MMTV-neu/HER2 mouse [13]. This observation gives cause for concern, as milk thistle constituents are perceived to be harmless and of potential benefit in cancer prevention. In order to substantiate this disquieting finding, we investigated the effect of silibinin and silipide on mammary carcinogenesis in another genetic murine model, the C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma (TAg) mouse. In this model expression of the SV40 transforming sequences is targeted to the mammary epithelium by a fragment of the rat prostatic steroid binding protein promoter C3(1) [14]. The T-antigen is thought to bind to, and functionally inactivate, the *p53* and *Rb* tumor

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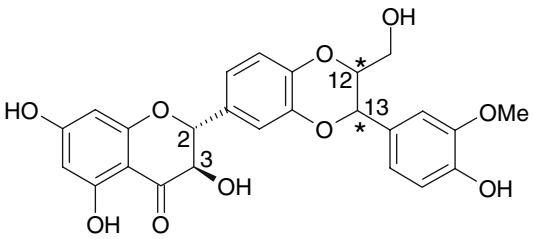

Fig. 1 Structure of silibinin, a diastereoisomeric mixture (1:1) of the two forms 2R, 3R, 12 S, 13S and 2R, 3R, 12R and 13R. *Asterisks* indicate optically active centres

suppressor genes [15, 16]. The consequent perturbation of cell homeostasis is probably responsible for mammary carcinogenesis, and all female TAg mice develop palpable tumours from approximately 12 weeks of age [14].

Materials and methods

Animals and agents

Breeding colonies were established in the Leicester University Biomedical Services facility with TAg mice on an FVB background obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Ear tissue from mice at approximately 10–14 days of age served to assess the presence of the transgene using PCR, as described previously [14]. Silibinin was purchased from Sigma–Aldrich Comp Ltd (Gillingham, UK). "Silipide" (IdB 1016), a phytosome product marketed for use as a hepatoprotectant (see http://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 approximates to 40% silibinin and 60% phosphatidylcholine.

TAg mouse experiments

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 [17]. At 4 weeks of age, mice received standard AIN 93G diet (Dyets Inc, Bethlehem, PA, USA) or AIN diet supplemented with silibinin or silipide (0.2% in terms of silibinin) to the end of the experiment. When extrapolated on the basis of body surface area, the dietary dose of silibinin used in mice (0.2%, approximately 300 mg/kg per day) equates to approximately 1.8 g per human per day, assuming a human body surface area of 1.8 m^2 accompanying a body weight of 70 kg [18]. This dose is similar to high doses previously employed in clinical pilot studies [9, 10]. Appearance and weight of the mice were checked on a weekly basis, and mice showing signs of distress, weight loss or very large tumours were humanely killed. From 11 weeks of age mice were palpated once weekly for presence of tumour. Tumour size was measured using callipers, and tumour volume was calculated using the equation:

$$V = 0.5236 \times D \times d^2,$$

with D and d representing the long and the short diameters, respectively. Animals were killed when the combined size of their tumours exceeded 17 mm in diameter, which occurred between 16.3 weeks (114 days) and 25.3 weeks (177 days) of age.

Results and discussion

TAg mice received unformulated silibinin or silipide at 0.2% (silibinin equivalents) in the diet for their lifetime. Intervention did not adversely affect murine bodyweight (Fig. 2), consistent with the reported safety of milk thistle flavonolignans in animals and humans [9–11]. Tumour development was assessed by weekly palpation and by number and weight of neoplasms at the end of the

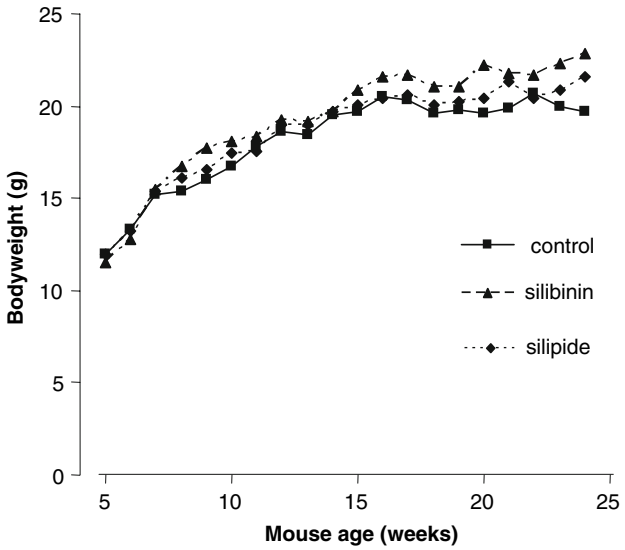

Fig. 2 Lack of effect of unformulated silibinin or silipide on body weight of TAg mice, which received control diet (*squares*, *solid line*) or diet fortified with silipide (*rhombi*, *stippled line*) or silibinin (*triangles*, *broken line*) at 0.2% (silibinin equivalents). Results are the mean of between 13 and 16 mice, SDs were 8–20% of mean values. For details of animal experimentation see "Materials and methods"

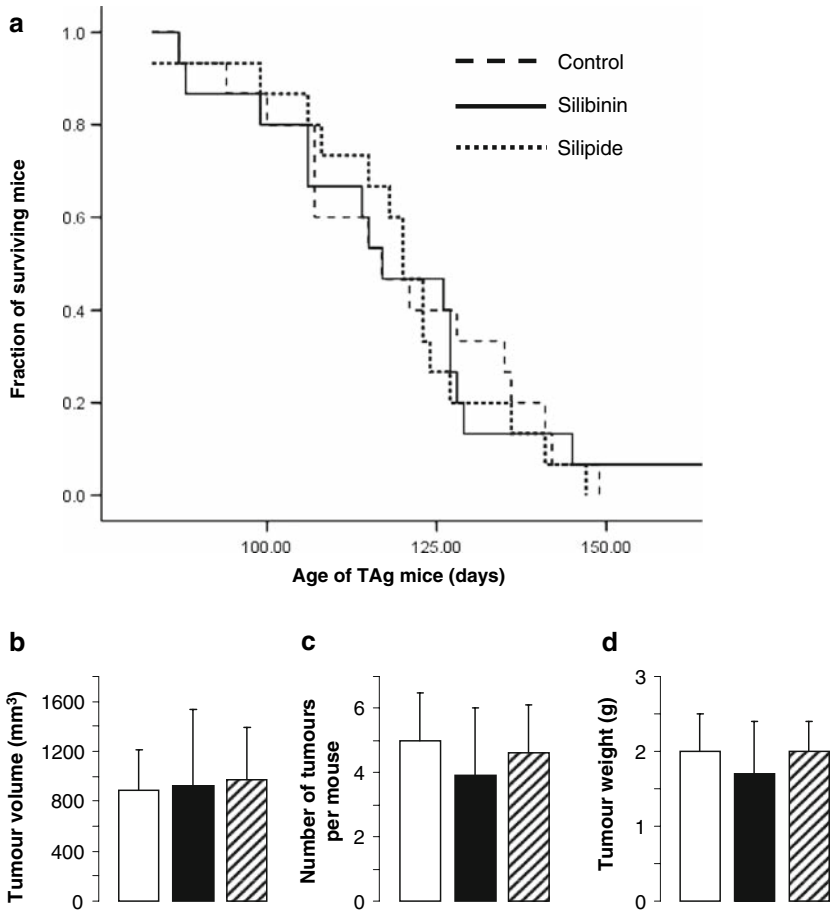

Fig. 3 Mammary tumour development in TAg mice which received standard diet (*broken line* in **a**, *open bars* in **b**–**d**) or diet fortified with unformulated silibinin (*solid line* in **a**, *filled bars* in **b**–**d**) or silipide (*dotted line* in **a**, *striped bars* in **b**–**d**) at 0.2% (silibinin equivalents) for their lifetime. Tumour development is reflected by survival (**a**), cumulative tumour volume (**b**), number of tumours per mouse (**c**) and tumour weight at the end of the experiment (**d**). Initial group size was 13–16. Animals were killed when total tumour size exceeded 17 mm, values in **b**–**d** are the mean ± SD (n = 13–16). For details of animal experiments and assessment of mammary tumour development see "Materials and methods"

experiment. Consumption of silibinin or silipide failed to significantly impede or exacerbate mammary carcinogenesis, as reflected by survival of animals (Fig. 3a), tumour volume (Fig. 3b), number of tumours per mouse (Fig. 3c) and tumour weight at the end of the experiment (Fig. 3d). So in the TAg mouse silibinin or silipide neither interfered with, nor exacerbated, mammary carcinogenesis. Silipide differs from unformulated silibinin in terms of the systemic availability of silibinin. We have found previously that dietary consumption of silipide by mice at the dose used here generated plasma concentrations of silibinin of 0.5 μM of silibinin, whereas dietary unformulated silibinin afforded concentrations below the limit of quantitation, which was near 10 nM (Verschoyle et al., submitted). 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 contrast to the result described here in TAg mice, the difference in the systemic availability of silibinin between silipide and unformulated silibinin translated into differential potency in the TRAMP mouse model of prostate carcinogenesis (Verschoyle et al., submitted). The TRAMP mouse resembles the TAg model employed here in that malignancy in both is initiated by SV40 transforming sequences. Among agents shown to delay mammary carcinogenesis in the TAg mouse are retinoids, di-fluoromethylornithine, dehydroepiandrosterone, nonsteroidal anti-inflammatory drugs and green and black tea extracts [19, 20]. The finding that silibinin does not affect breast tumour development in TAg mice is at variance with results in two mammary carcinogenesis models, rats exposed to MNU and mice bearing the MMTV-neu/HER2 transgene. In both of these models silymarin at 0.03–1.0% in the diet exacerbated tumour development [13]. Silymarin consists to 70–80% of silibinin, so the dietary dose used by us here (0.2%) is within the dose range reported to have caused the detrimental effect. The weak oestrogenic properties of milk thistle flavonolignans have been speculatively implicated in promotion of tumour development [13]. It is conceivable that events, which drive mammary carcinogenesis in the MNU-induced rat and MMTV-neu/HER2 mouse models, are more responsive to hormonal stimuli than those in TAg mice, thus accounting for the observed discrepancy between the models. It needs to be stressed that silymarin is a mixture, and the study described here was conducted on

silibinin, one silymarin constituent. Therefore the possibility cannot be ruled out that the promotion of mammary tumour development by silymarin was due to one or more silymarin constituents other than silibinin.

The results described here suggest that promotion of carcinogenesis is not a feature of silibinin consistent across all rodent models of mammary carcinogenesis. Nevertheless, development of phytosomal silibinin formulations for breast cancer prevention in humans does not seem worthy of consideration. In contrast, robust evidence accrued in preclinical models of prostate carcinogenesis [1, 2] supports the clinical development of phytosomal silibinin in human prostate cancer prevention (Verschoyle et al., submitted).

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