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ORIGINAL ARTICLE

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Preliminary safety evaluation of the putative cancer chemopreventive agent tricin, a naturally occurring flavone

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Abstract *Purpose*: Naturally occurring flavonoids such as quercetin and genistein possess cancer chemopreventive properties in experimental models. However, adverse effects such as their mutagenicity confound their potential clinical usefulness. Furthermore in leukaemia cells some flavonoids cleave the breakpoint cluster region of the *mixed lineage leukaemia* (*MLL*) gene as a consequence of inhibition of topoisomerase II. The choice of flavonoids to be developed as cancer chemopreventive agents depends crucially on their safety. Here, we explored safety aspects of the novel flavone tricin, a constituent of rice bran and other grass species, which has recently been found to interfere with murine gastrointestinal carcinogenesis. *Methods*: Evidence of pathological or morphological changes in liver, lung, heart, spleen, kidney, adrenal gland, pancreas or thymus tissues was studied in mice which received tricin, genistein or quercetin 1,000 mg/kg daily by the oral route on five consecutive days. The ability of tricin (50 μM) to cleave the *MLL* gene was studied in human leukaemia cells by Southern blotting, and its effect on human topoisomerase II activity was investigated in incubations with supercoiled DNA. The mutagenicity of tricin was assessed in the *Salmonella*/*Escherichia coli* assay, and its clastogenicity was adjudged by chromosomal aberrations in Chinese hamster ovary cells and occurrence of micronuclei in bone marrow erythrocytes in Swiss-Webster mice. *Results*: Neither tricin, quercetin, or genistein caused pathological or morphological changes in any of the murine tissues studied. Tricin (50 μM) failed to cause *MLL* gene breakage, and it inhibited topoisomerase II only at 500 μM, but not at 10, 50 or 100 μM. Tricin lacked genotoxic properties in the systems studied here. *Conclusion*: The results tentatively suggest that tricin may be considered safe enough for clinical development as a cancer chemopreventive agent.

Keywords Flavonoids · Chemoprevention · Genotoxicity · Toxicity

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Introduction

The consumption of naturally occurring flavonoids has long been suspected to exert beneficial health effects, among them the prevention of malignancies of the breast and colorectal tract [1]. Flavonoids occur in plants and diets predominantly in glycosidic form, and it is thought that their health effects are mainly mediated by the aglycones generated from glycosidic precursors on hydrolysis during mastication, or in the stomach. Prominent examples of potentially cancer chemopreventive flavonoid aglycones, which have undergone extensive experimental scrutiny, are quercetin, a flavonol

contained in onions, apples and wine, and genistein, an isoflavone found in soya. Both these agents have been documented to prevent carcinogen-induced carcinogenesis in rodents (e.g. [2–4] for prevention of mammary carcinoma), and together with agents such as epigallocatechin gallate from tea and apigenin from leafy vegetables they can be considered ''benchmark'' flavonoids in the chemoprevention of experimental cancer. There are more than 4,000 naturally occurring flavonoids [5], of which only a handful, including genistein and quercetin, have thus far been examined extensively for cancer chemopreventive efficacy in cells in vitro and rodents in vivo. In addition to efficacy, another pivotal issue, which governs the choice of a molecule for potential development as a chemopreventive agent, is safety. Flavonoids can exert detrimental health effects, as exemplified by the mutagenicity of quercetin [6, 7] and the tumour-promoting ability of genistein in the azoxymethane-induced colorectal carcinogenesis rat model [8]. Most notably, both quercetin and genistein have been shown to induce DNA cleavage in the breakpoint cluster region of the human *mixed lineage leukaemia* (*MLL*) gene as a consequence of DNA topoisomerase II inhibition, which has been causally linked to leukaemia induction in infants [9]. It seems intuitively plausible to surmise that among the large number of naturally occurring flavonoids there are some with cancer chemopreventive properties equal, or even superior, to those of quercetin and genistein and with more favourable safety profiles. Thus characterisation of novel flavonoids in terms of both cancer chemopreventive efficacy and safety is paramount for the optimal exploitation of the plant kingdom for the purpose of improved management of human cancer. Tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone, for structure see Fig. 1) occurs as glycoside in rice bran and other grass species such as wheat, barley and maize. It interfered potently with the growth of human-derived mammary and colonic cancer cells in vitro [10]. Recently, we found that consumption of tricin decreased the burden of intestinal adenomas in Apc^{Min} mice [11]. These mice harbour an inactivatory *Apc* gene

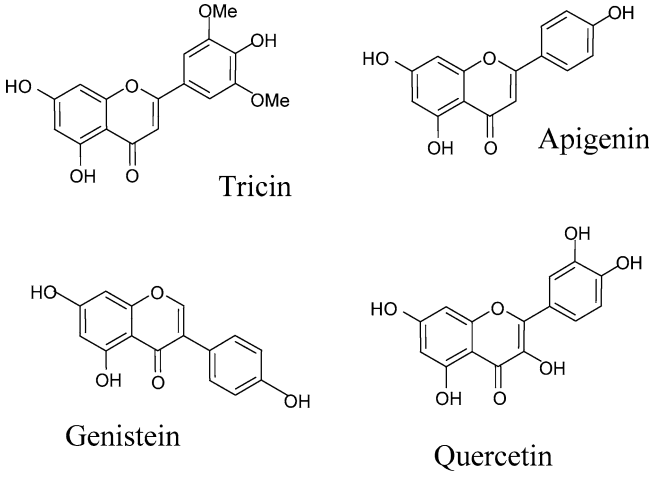

Fig. 1 Structures of flavonoids discussed in this study

mutation, closely resembling the gene abnormality, which underlies the human heritable defect familial adenomatous polyposis coli, a condition predisposing strongly for development of colorectal carcinoma. Furthermore, preliminary data indicates mammary carcinogenesis-delaying activity of tricin in the C3(1)/SV40 T/t-antigen transgenic (TAg) mouse (Cai, Verschoyle, Steward and Gescher, unpublished). In the light of these encouraging results with respect to efficacy, it seems highly germane to further investigate the suitability of tricin as a potential cancer chemopreventive agent in humans. As establishment of its safety is undoubtedly an exceedingly important part of such studies, we conducted experiments aimed at characterising, in a preliminary fashion, potential toxic properties of tricin. In particular, we wished to establish whether repeated oral administration of tricin at a high dose adversely affects murine pathogy, and if, in analogy to quercetin and genistein, it induces *MLL* breaks or inhibits DNA topoisomerase II. In addition, we report results from an NCI-sponsored evaluation of potential genotoxicity or clastogenicity of tricin.

Materials and methods

Agents

Tricin was custom-synthesised by Syncom (Groningen, The Netherlands), for the NCI Chemoprevention Branch. Genistein and quercetin were purchased from Sigma-Aldrich Comp Ltd (Poole, UK).

Assessment of acute toxicity of oral tricin, genistein and quercetin

Experiments were conducted as stipulated by the Animals (Scientific Procedures) Act 1986 UK Home Office Project Licence 40/2496. They complied with the UKCCCR guidelines for the welfare of animals in experimental neoplasia [12] and were approved by the Leicester University Animal Welfare Committee. For investigation of the effect of tricin on body weight when administered via the diet, Apc^{Min} mice received tricin at 0.2% (= approximately 300 mg/kg body weight) in their AIN-93 diet (Dyet Incorp., Bethlehem, PA, USA) from week 4 to 18 of age, and animals were weighed once a week. For the study of the effect of high-dose flavonoid given as an oral bolus, 4-week-old female C57Bl6 mice (the Apc^{Min} background species) were maintained on standard RM3 rodent diet (Special Diet Services, Witham, UK) and water ad libitum. These mice (three in control group, six per intervention group) received AIN-93 and tricin, genistein or quercetin (suspended in aqueous methylcellulose 1%) at 1,000 mg/kg body weight, or vehicle only, daily for 5 days by oral intubation. One day after the final dose animals were killed (halothane anaesthesia) by terminal

exsanguination. The animals were subjected to full autopsy examination, which included inspection for any macroscopic abnormalities. The following tissues were selected for histological examination: liver, lung, heart, spleen, kidney, adrenal gland, pancreas and thymus. Tissues were fixed, processed, sectioned, stained with haematoxylin and eosin and examined microscopically. Concentrations of tricin were measured by HPLC analysis [13] in the plasma, liver and gastrointestinal tract of mice 24 h after they had received the last of five daily doses of tricin.

Assessment of induction of *MLL* gene cleavage and inhibition of topoisomerase II activity

To detect *MLL* gene breakpoints, BV173 and CCEM leukaemia cells (obtained from the German Collection of Microorganisms and Cell Cultures DSMZ; Braunschweig, Germany) were grown in RPMI medium supplemented with 10% FCS. Cells were exposed to tricin (50 μM) for 24 h, after which cells were harvested. High molecular DNA was isolated according to standard procedures. An aliquot of DNA (5 μg) was digested with *Bam*HI (Roche Diagnostics, Mannheim, Germany). The DNA was separated by electrophoresis in a 0.7% agarose gel at 25 V overnight and transferred onto nylon membranes. The blot was probed with an 859 bp *Bam*HI fragment, which spans the *MLL* break cluster region. The probe had been labelled by incorporating digoxigenine 11-dUTP. The filter was hybridised at 41°C for 10 h using the EasyHyb solution (Roche Diagnostics) followed by two washes with 2×SSC/0.1% SDS and 0.2×SSC/0.1% SDS, respectively. Detection was carried out with an anti-digoxigenine antibody (Roche Diagnostics) that was conjugated with alkaline phosphatase.

Inhibition of topoisomerase II activity was assessed using supercoiled DNA (pcDNA3 plasmid, Invitrogen SRL, Milan, Italy) and human topoisomerase II (Amersham Buchler GmbH, Braunschweig, Germany), and analysed on an agarose gel stained with ethidium bromide, as described by Codegoni et al. [14]. For comparison, the known topoisomerase II inhibitors etoposide and 4′-(9-acridinylamino)-methanesulfone-*m*-anisidide (*m*-AMSA) were included in the assay.

Evaluation of mutagenicity and ability to cause chromosomal changes

Evaluation of the ability of tricin to cause mutations or chromosomal changes was commissioned by the Division of Cancer Prevention, Chemoprevention Agent Development Research Group, US National Cancer Institute, and assessed by SRI International (formerly Stanford Research Institute), Menlo Park, CA, USA. The ability of tricin to induce chromosome aberrations was tested in Chinese hamster ovary cells in the presence and absence of rat hepatic S-9 homogenate, providing a metabolic activation system. Cells were analysed for chromosomal aberrations at tricin concentrations of 19–606 μM in the absence, and at 152–606 μM in the presence, of rat hepatic S9 homogenate affording a metabolic activation system. The mutagenic potential of tricin was explored in the *Salmonella typhimurium–Escherichia coli*/microsome preincubation assay, using strains TA1535, TA1537, TA98 and TA100 and *E. coli* strain WP2 (*uvr*A) in the presence of an Aroclor 1254-induced rat liver S9 homogenate. Tricin concentrations were 47–3,030 nmol/plate. The ability of tricin to induce micronuclei in bone marrow erythrocytes was explored in Swiss Webster mice, which received a single dose of 12.5, 25 or 50 mg (= 625, 1,250 or 2,500 mg/kg, gavage). Bone marrow was evaluated for cytotoxicity and micronucleus formation 24 and 48 h later. Results were compiled in the following three SRI International Technical Reports: ''Evaluation of tricin in the Chinese hamster ovary chromosome aberration assay'' (2003), ''Evaluation of tricin in the mouse bone marrow micronucleus assay'' (2003), and ''Evaluation of tricin in the *Salmonella–Escherichia coli*/microsome preincubation assay'' (2004).

Results

Effect of repeated administration of tricin on murine pathology

In chemoprevention studies in rodents, agents are often administered with the diet. Apc^{Min} mice received tricin at 0.2% with their diet (= approximately 6 mg per mouse = ~300 mg/kg per day) from weaning (week 4) to the end of the experiment (week 17). This intervention delayed adenoma development [11]. The bodyweight of mice on tricin was indistinguishable from that of controls (Fig. 2), suggesting that tricin at this dose did not interfere with food intake.

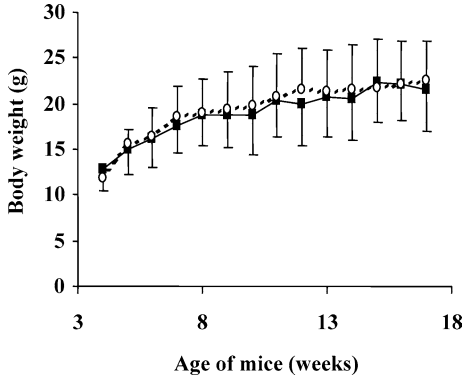

Fig. 2 Weight of Apc^{Min} mice which received control diet (*squares*, *unbroken line*) or tricin (0.2%, *circles*, *broken line*) with their diet from week 4 until the end of the experiment. Results are the mean ± SD of 14 mice

Next we wished to explore the effect of high-dose tricin. Mice received tricin or, for comparison, quercetin or genistein (20 mg = 1,000 mg/kg per day) by gavage once daily for five consecutive days. There were no treatment-related macroscopic or microscopic changes in any organs or tissues in mice on any of the interventions. A small number of lesions were observed in all treatment groups, but these were typical spontaneous lesions expected in mice of this age and type. These lesions were dysplasia in the renal medulla (one of three control mice), minimal focal chronic inflammation in the pancreas (one of six mice on genistein), and minimal chronic inflammation in the lungs (one of six mice on quercetin). Final body weights or kidney weights did not differ between treated and untreated mice. The mean liver weights in mice treated with tricin or genistein were slightly above those of controls (Table 1).

When tricin was measured by HPLC in blood and tissues of the mice 24 h after their last dose of tricin, levels in plasma and liver were below the detection limit (1.5 nmol/ml or g) [13], whilst small intestinal mucosa contained 27.4 ± 12.7 nmol tricin/g tissue ($n = 5$). Similar intestinal levels were observed in wild-type (C57Bl6) mice, which received tricin with their diet for a weeks (result not shown). This result suggests that tricin after repeated dosing does not accumulate in the mouse organism.

Effect of tricin on the *MLL* gene

In order to assess the ability of tricin to induce site-specific DNA cleavage in the breakpoint cluster region of the *MLL* gene, human leukaemia cells were exposed to tricin (50 μM) for 24 h, and *MLL* rearrangements were investigated by Southern blotting. At this concentration genistein and quercetin have been reported to potently cleave the breakpoint cluster region in the *MLL* gene [9]. Tricin failed to cause *MLL* gene cleavage (result not shown), in contrast to genistein and quercetin.

Table 1 Weights of whole bodies, livers or kidneys of mice, which received tricin, genistein or quercetin (1,000 mg/kg per day by gavage) daily for five consecutive days

Agent	Weights (g)		
	Body	Liver[a]	Kidney[a,b]
None	18.7 ± 0.7[c]	0.92 ± 0.60	0.23 ± 0.13
Tricin	18.9 ± 1.4	1.06 ± 0.66*	0.24 ± 0.10
Genistein	18.9 ± 1.1	1.06 ± 0.98*	0.23 ± 0.14
Quercetin	18.6 ± 1.0	0.98 ± 0.66	0.24 ± 0.17

Weights were determined on day six
[a]Weight per 20 g mouse
[b]Combined kidneys
[c]Mean ± SD, $n = 3$ for controls, $n = 6$ for intervention groups*$P < 0.05$ compared to control mice

Effect of tricin on topoisomerase II activity

Tricin and, for comparison, the known topoisomerase II inhibitors etoposide and *m*-AMSA were incubated with supercoiled DNA and human topoisomerase II enzyme. Agarose gel analysis suggests that relaxation of DNA by topoisomerase II was blocked by etoposide and *m*-AMSA (either at 50 μM), while tricin inhibited topoisomerase II only at 500 μM, but not at 10, 50 or 100 μM (Fig. 3). This result is consistent with the inability of tricin (50 μM) to cleave the *MLL* gene.

Effect of tricin on genetic and chromosomal integrity

The ability of tricin to induce chromosome aberrations was tested in Chinese hamster ovary cells in the presence and absence of rat hepatic S-9 homogenate, providing a metabolic activation system. Increases in structural chromosomal aberrations or polyploidy were not observed. The mutagenic potential of tricin was studied in the *S. typhimurium–E. coli*/microsome preincubation assay. There was no dose-related increase in the number of microbial revertants. The ability of tricin to induce micronuclei in bone marrow erythrocytes was explored in Swiss Webster mice. Tricin at any of the concentrations tested failed to cause significant suppression of polychromatic erythrocytes among red blood cells, and it did not increase the frequency of micronucleated polychromatic erythrocytes.

Discussion

The preliminary evaluation described above tentatively suggests that tricin possesses a favourable safety profile, which may render it an attractive candidate amongst flavonoids for further exploration as a cancer chemopreventive agent. This suggestion is buttressed by the following three findings: firstly in an acute toxicity study in mice five daily doses of 1,000 mg/kg tricin (by gavage) failed to cause any signs of general toxicity or specific tissue damage. Similar results were obtained for quercetin and genistein, which underline the lack of acute adverse manifestations elicited by flavonoids in general. Secondly, tricin lacked *MLL* gene cleavage-inducing and DNA topoisomerase II-inhibiting properties, unlike quercetin and genistein [9]. Thirdly tricin, unlike quercetin, was deficient of genotoxic properties, as reflected by its inability to induce either chromosomal aberrations in Chinese hamster ovary cells, micronuclei in bone marrow erythrocytes in Swiss-Webster mice, or revertant colonies in the *Salmonella–E. coli* assay. Aspects of the safety profile of tricin discussed here vis-à-vis those of quercetin and genistein are summarised in Table 2. It still needs to be established whether tricin shares with genistein [8] the ability of promoting carcinogen-induced colorectal carcinogenesis. Its intestinal tumour-suppressing efficacy in the Apc^{Min} mouse [11] tentatively

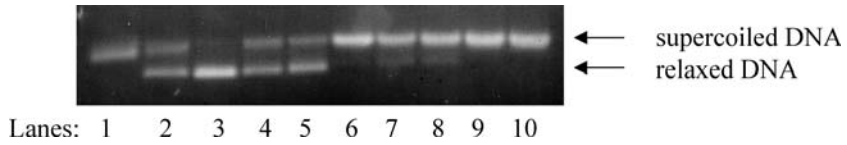

Fig. 3 Effect of tricin, *m*-AMSA or etoposide on ability of human topoisomerase II to relax supercoiled DNA. Allocation of lanes is as follows: *1* supercoiled DNA, *2* supercoiled DNA and toposomerase II, *3* as lane 2 plus tricin 10 μM, *4* as lane 2 plus tricin 50 μM, *5* as lane 2 plus tricin 100 μM, *6* as lane 2 plus tricin 500 μM, *7* as lane 2 plus *m*-AMSA 50 μM, *8* as lane 2 plus *m*-AMSA 100 μM, *9* as lane 2 with etoposide 50 μM, *10* as lane 2 with etoposide 100 μM. The results are representative of two independent experiments

Table 2 Summary of adverse effect spectrum for quercetin, genistein and tricin discussed in this manuscript

	Genotoxicity	Carcinogenicity	Induction of *MLL* cleavage
Quercetin	+	+	+
Genistein	–	+	+
Tricin	–	?	–

militates against this possibility. In contrast to tricin, genistein [15] and quercetin [16] failed to interfere with adenoma development in the Apc^{Min} mouse.

In the original paper on the ability of bioflavonoids, to elicit *MLL* gene rearrangement [9], molecules in which phenolic hydroxy moieties were replaced by methoxy functionalities, lacked *MLL* gene-cleaving ability. For example, in contrast to genistein, which cleaved the *MLL* gene, biochanin A, the 4′-methyl ether of genistein, did not [9]. The finding presented here, according to which tricin is devoid of this side effect, albeit its *bis*-desmethoxy cogener apigenin (see Fig. 1) cleaves the *MLL* gene [9], supports a "protective role" against *MLL* gene damaging properties conferred on flavonoids by the presence of methoxy groups.

We have previously shown that in mice which received tricin with the diet, tricin levels in the plasma were low, e.g. only 0.28 μM after dietary intake of 0.5% (~750 mg/kg per day) for a week [13]. Levels of tricin in the liver were 8 and 2.2 nmol/g tissue, after 0.2 and 0.5% dietary intake, respectively, for a week, while the analogous intestinal mucosa levels were as high as 238 and 462 nmol/g tissue [13]. This data and the indication, presented above, that tricin did not accumulate in mouse tissues after five consecutive daily doses of 1,000 mg/kg, are consistent with low bioavailability. Low systemic availability renders tricin particularly suitable for consideration as a potential candidate for chemopreventive intervention against gastrointestinal malignancies, i.e. in an organ, which is amenable to pharmacological intervention without the necessity for delivery via the bloodstream.

Among naturally occurring flavonoids, quercetin and soya isoflavones such as genistein have arguably progressed furthest in terms of development as potential cancer chemopreventive agents. Quercetin was subjected to an exploratory clinical phase I trial in ovarian cancer patients [17], and soya isoflavones are currently in phase II clinical evaluation in individuals with elevated prostate specific antigen levels (http://cancer.gov/search/clinical_trials/). The work described here, together with the indication of chemopreventive efficacy of tricin in a preclinical model of gastrointestinal carcinogenesis [11], fuels the suspicion that among the ~4,000 naturally occurring flavonoids there may be some molecules, which are more attractive for development as potential chemopreventive agents than either genistein or quercetin. Thus this work supports the propitiousness of both, consideration of tricin for the clinical development as cancer chemopreventive agent, and continuation of the search in the plant kingdom for novel efficacious and safe flavonoids.

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