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Tissue distribution in mice and metabolism in murine and human liver of apigenin and tricin, flavones with putative cancer chemopreventive properties

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Running Title: Bioavailability and metabolism of tricin and apigenin

Keywords: Flavonoids, chemoprevention, metabolism

Abstract *Purpose*: The flavones apigenin and tricin, which occur in leafy vegetables and rice bran, respectively, possess cancer chemopreventive properties in preclinical rodent models. Their pharmacology is only poorly understood. We compared their tissue levels in mice *in vivo* and their metabolism in liver fractions *in vitro*. *Methods:* Mice received apigenin or tricin (0.2%) with their diet for 5-7 days, and flavone levels were compared in the plasma, liver and gastrointestinal mucosa using HPLC-UV. Flavone metabolism was investigated in murine and human liver microsomes or cytosol *in vitro* co-incubated with uridine 5'-diphosphoglucuronic acid or 3'-phosphoadenosine-5' phosphosulfate. Flavone metabolites were characterized by on-line HPLC-mass spectrometry. *Results:* After dietary administration of flavones for 7 days, levels of tricin in plasma, liver and mucosa exceeded those of apigenin by 350, 33 and 100 %, respectively. Apigenin was more rapidly glucuronidated than tricin in liver microsomes, whilst tricin underwent swifter sulfonation than apigenin in liver cytosol. For either flavone the rate of glucuronidation was much faster than that of sulfonation. Flavone monoglucuronides and monosulfates were identified as metabolites in microsomal and cytosolic incubations, respectively. *Conclusions:* When consumed with the diet in mice tricin seems to be more available than apigenin in blood and tissues. Differences in their glucuronidation may account for their differential availability. Thus tricin may have a pharmacokinetic advantage over apigenin. This type of information may help decide which flavonoids to select for clinical development.

Introduction

Epidemiological studies suggest that the consumption of fruits and vegetables is associated with a decreased risk of colorectal cancer [1]. Several naturally occurring flavonoids contained in fruits and vegetables share cancer chemopreventive properties and mechanisms with non-steroidal antiinflammatory drugs, but may lack their unfavorable safety profiles [2]. Therefore flavonoids are promising targets of chemoprevention agent discovery activities. This is particularly apposite

in the light of the fact that there are over 4000 flavonoids in the plant kingdom, of which only a handful have hitherto been thoroughly investigated for cancer chemopreventive properties, notably quercetin contained in onions and genistein from soya. In plants flavonoids occur almost exclusively as glycosides, which generate their respective aglycones on hydrolysis caused either at preparation of the foodstuffs for consumption or by intestinal enzmes and/or the intestinal microflora. Tricin (4',5,7-trihydroxy-3',5'-dimethoxyflavone, for structure see Fig. 1), which occurs in rice bran and other grass species, and its closely related cogener apigenin (4',5,7-trihydroxyflavone, see Fig. 1) from leafy vegetables were recently shown to interfere effectively with prostaglandin generation in human colorectal cells [3]. This paper suggests that the two flavones exert an effect on prostaglandin production *via* differential mechanisms, tricin by inhibition of cyclooxygenase (COX) enzyme activity, and apigenin by downregulation of COX-2 expression. Tricin interfered with carcinogenesis in Apc^{Min} mice [4] and C3(1)/SV40 T/t-antigen transgenic ("TAg") mice (Cai, Steward and Gescher, unpublished), genetic models of human carcinogenesis, the former of gastrointestinal origin characterized by an *Apc* gene mutation, the latter of mammary origin associated with dysfunctional Rb and p53 tumor suppressors. Apigenin remains to be tested in these models. In general, flavonoids are characterized by poor systemic availability. The rational selection of flavonoid analogues for advancement into clinical development requires robust knowledge of not only their preclinical efficacy and biochemical mechanisms, but also bioavailability and metabolism. In order to explore the potential suitability of tricin and/or apigenin for clinical development, we compared their availability in blood, liver and gut mucosa in mice after dietary ingestion, and their metabolism in murine and human hepatic and gut fractions *in vitro*. Mice received either flavone with their diet, and flavone levels were measured by HPLC with UV detection. Their metabolism was compared *in vitro* in murine and human liver and gut preparations fortified with suitable cofactors and analyzed by HPLC with UV or mass spectrometric detection.

Materials and methods

Chemicals

Apigenin was purchased from Apin Chemicals Ltd. (Abingdon, UK). Tricin was custom-synthesized for the US NCI Division of Cancer Prevention by Syncom (Groningen, the Netherlands). Both flavones were >99% pure as determined by HPLC analysis. Uridine 5'-diphosphoglucuronic acid (UDPGA), 3'-phosphoadenosine-5' phosphosulfate (PAPS), nicotinamide adenine dinucleotide 2'-phosphate (NADPH), quercetin and reagents for HPLC analysis (all of Analytic Reagent grade) were obtained from Sigma Chemical Comp (Poole, UK). HPLC-grade methanol was purchased from Fisher Chemicals (Loughborough, UK), and water was purified in a laboratory Nano-Pure water purification system (Barnstead, UK).

Animals and Treatments

C57BL/6J mice (aged 6-8 weeks), the background strain of the *Apc*^{Min} mouse, were purchased from Harlan (Oxon, UK). Mice were housed under sterile conditions in a room maintained at 22^{0}C with a 12h light/dark cycle. 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 United Kingdom Coordinating Committee on Cancer Research [5]. Mice (4-10 per group) received AIN 93G diet or AIN diet containing 0.2 % flavone (dose: 300 mg/Kg = 0.9 mmol/Kg tricin or 1.1 mmol/Kg apigenin). Dietary administration is customarily used in rodent intervention studies. The choice of dose was based on the fact that tricin at this dose reduced adenoma multiplicity in the *Apc*^{Min} mouse [4]. After 5, 6 or 7 days, mice were killed by cardiac exsanguinations under terminal anaesthesia (halothane), and blood was collected. In animals exposed to flavones for 7 days liver and gastrointestinal tract were also obtained. Plasma

was obtained after centrifugation at 13000 x g (20 min, 4ºC). The intestinal tract was flushed with phosphate-buffered saline (10 mL) to remove contents. Then it was cut open and tissue epithelial scrapings were collected by gently brushing the epithelial layer with a metal spatula. Liver and intestinal mucosa tissues were snap-frozen in liquid nitrogen. Tissues and plasma were kept at -80°C until analysis, for not longer than 4 weeks, conditions under which we had established that flavones in plasma are completely stable.

Tissue sample preparation

Plasma and tissues were thawed at room temperature. Tissues were mixed with an equal volume of isotonic potassium chloride solution and homogenized (Ystral X 10/20 homogeniser, Ballrechten-Dottingen, Germany). An aliquot of plasma or tissue homogenate was added to two volumes of 0.1 M acetic acid in acetone. The mix was vortexed (2×1 min) and centrifuged (13000 x g, 20 min). The supernatant was evaporated under nitrogen and the residue taken up in mobile phase (see below, 50 μl). After further centrifugation (13000xg, 5 min) an aliquot (between 2 and 20 μl) of the resultant supernatant was injected onto the HPLC column for flavone analysis.

Preparation of liver and gut cytosol and microsomes

Healthy liver and colonic tissue resected from three Caucasian traffic accident victims was obtained from the UK Human Tissue Bank (De Montford University, Leicester, UK). Liver and intestinal tissues were excised from six C57BL/6J mice (3 female, 3 male). The mouse intestine was scraped to obtain mucosal tissue. An aliquot (3 g) of tissue was suspended in 27 mL buffered sucrose (sucrose 0.25 M, Tris 10 mM, EDTA 1 mM, pH 7.4), and homogenized under cooling with ice. Human liver homogenates were pooled. Subcellular fractions of human and mouse tissues were isolated, and cytosolic and microsomal protein was quantified, in the usual way [6]. Tissue preparations were kept at -80ºC until analysis.

Flavone metabolism *in vitro*

For the study of conjugation reactions, incubation mixtures contained microsomes or cytosol (1mg/mL of protein) from murine or human liver or gut and tricin or apigenin at 50 μM. An appropriate volume of flavone stock solution (12.5 mM) in DMSO was added to the incubation mixture. The DMSO concentration in the final incubate did not exceed 0.4%, which in an orientation experiment did not affect metabolic flavone removal. For analysis of reactions catalysed by glucuronyl transferases, incubates (final volume 0.5 mL) comprised microsomes, flavone and magnesium chloride (10 mM) in Tris-HCl buffer (50 mM, pH 7.4), which were pre-incubated (5 min at 37 °C), after which the metabolic reaction was initiated by addition of UDPGA (1 mM). The mixture was incubated with shaking at 37°C for between 5 to 20 min (mouse microsomes) or to 60 min (human microsomes). For analysis of sulfate conjugation reactions the final volume was 0.2 mL. Incubates comprised cytosol, flavone, magnesium chloride (5 mM) and mercaptoethanol (3 mM) in Tris-HCl buffer (40 mM, pH 7.4), and the metabolic reaction was initiated by addition of PAPS (0.2 mM). The mixture was incubated with shaking at 37 °C for 30 and 60 min. In the experiments, in which the effect of the flavones on activity of glucuronyltransferases and sulfotransferases was studied, liver microsomes or cytosol from mice, which had received flavones in their diet, were incubated for 10 or 30 min, respectively, with tricin, which served as substrate for metabolic conjugation. For investigation of phase I metabolism reactions, incubation mixtures contained mouse liver microsomes (0.5 mg/mL of protein) magnesium chloride (1 mM) and NADPH (1 mM) in Tris-HCl buffer (80 mM, pH 7.4) in a final volume of 0.5 mL. The metabolic reaction was initiated by addition of flavone substrate (final concentration 100 μM), and the mixture was incubated with shaking at 37°C for 30 min open to air. All reactions were terminated by the addition of two volumes of 0.1 M acetic acid in acetone. Mixtures were vortex-mixed (2×1 min) and centrifuged (13000 x g, 10 min), and an

aliquot (20 µl) of the supernatant was injected onto the HPLC column for analysis. Samples of the incubation mixture, which were to be analyzed by on-line HPLC-mass spectrometry, were dried under nitrogen, then reconstituted in methanol:water (50:50, v/v, 0.1 mL). After centrifugation, an aliquot (20 µL) was injected onto the HPLC column.

Flavone analysis by HPLC

Analysis of tricin and apigenin in plasma, tissues and in incubation mixtures with liver or gut fractions was performed using a reversed phase HPLC method described previously [7-9]. Two systems were used: i) a Varian ProStar 230 pump, a ProStar 410 autosampler and a ProStar 325 UV-visible or ProStar 320 photodiode array detector (Varian Inc, Oxford, UK), or ii) an Acquity UPLC system (Waters Corp. Milford, USA) with TUV detector and sample and binary solvent manager. UV detection was at 355 or 336 nm, the maximum absorbance wavelengths of tricin and apigenin, respectively. Separation was achieved on either a Hypersil BDS C_{18} column (250 × 4.6 mm, particle size 5 µm, Thermo Electron Corp., Runcorn, UK) in the case of the Varian system, or an Acquity UPLC BEH C_{18} column (50 x 2.1 mm, particle size 1.7 µm) for the Acquity system. For quantitation of flavone in the biological matrices a mobile phase (methanol 55%: ammonium acetate buffer 0.1 M, pH 5.1, with EDTA 0.27 mM) was used in isocratic mode. The flow rate was 1 mL/min (Varian) or 0.4 mL/min (Acquity). Quercetin was used as internal standard. The UV detector used here afforded a limit of quantitation (LOQ) for tricin and apigenin in plasma of 50 and 25 ng/mL, respectively. The LOQ for tricin constitutes a tenth of the value reported by us previously [8], the precision was <15%. In the case of some HPLC-UV analyses designed to help characterize metabolites (including those which yielded the chromatograms shown in figures 3 and 6) and of HPLC-mass spectrometric analysis, a binary mobile phase system was used (phase A: aqueous ammonium acetate 5 mM; phase B: 5 mM ammonium acetate in methanol), with gradient

elution from 75% A/ 25% B to 10% A/ 90% B over 45 min (Varian) or 70% A/ 30% B to 30% A/ 70% B over 20 min (Acquity).

HPLC-mass spectrometry

Flavones and their metabolites were characterized by HPLC-mass spectrometry using a TurboIon Spray (TIS) source in negative ionization mode. Analyses were performed using an API-2000 mass spectrometer (Applied Biosystems, Warringon, UK) equipped with an Agilent 1100 series sample delivery system. LC separation of flavones and their metabolites was achieved with the binary mobile phase system, column and flow rate described above (Varian system). Mass spectrometry conditions were as follows: ion source voltage -4000/-4500 V, declustering potential –81/-61 V, focusing potential –160/-230 V, electrode potential –12/-9.5 V, cell entrance potential –14/12 V, cell exit potential –20/-20 V, temperature 500°C. Identification of tricin and apigenin metabolites was by Q1 scan. All mass to charge ratios (*m/z*) described under Results are $[M\text{-}H^{+}]^{-}$ ions.

n-Octanol-water partition coefficient of tricin

Because of the poor water solubility of tricin the *n*-octanol-water partition coefficient had to be determined indirectly by calculating the equilibrium solubility ratio of tricin in *n*-octanol to water, in a manner published for apigenin [10]. An excess amount of tricin was suspended in either solvent (10 mL) in screw-capped tubes (in triplicate). Tubes were rotated (room temperature) for 4 days (Rugged Rotator, Glas-Col, Terre Haute, USA), then centrifuged (3000 x g, 20 min). The supernatant was filtered (0.2 μm syringe filter, Life Science, Ann Arbor, USA). One volume of filtrate was mixed with two volumes each of methanol and HPLC mobile phase (see above). The saturation concentration of tricin was quantitated by HPLC as described above.

Statistical evaluation

Flavone levels in the biomatrices and incubation mixtures were statistically compared with each other by one-way ANOVA followed by Tukeys' pairwise comparison.

Results

Flavone levels in mice after dietary intake

Initially the hypothesis was tested that plasma levels of flavones reach steady state when mice ingest apigenin or tricin for more than 5 days with the diet at 0.2 % (dose ~300 mg/Kg). To that end plasma levels of flavones were determined in animals which had received flavones for either 5, 6 or 7 days. Plasma levels of tricin were 0.33-0.45 nmol/mL (with standard deviation between 19 and 24% of the mean), and those of apigenin 0.08-0.11 nmol/mL (with standard deviation between 120 and 182% of the mean). There was no significant difference in levels between the three time points, suggesting that flavone steady state concentrations were indeed achieved by 5 days. Next flavone levels achieved after consumption of either tricin or apigenin for 7 days in the plasma, liver or intestinal mucosa were compared. Figure 2 shows representative HPLC chromatograms of plasma preparations. Plasma of control mice (unexposed to flavones) did not contain substances which co-eluted with apigenin or tricin (results not shown). In mice exposed to flavones plasma levels of tricin were about 4.5-fold higher than those of apigenin (Table 1). In four out of ten mice on apigenin, plasma concentrations of the flavone were below the limit of quantitation (25 ng/mL, [9]), whilst tricin was measurable in all animals, which had received tricin. Consistent with the results observed in the plasma, hepatic and small intestinal levels of tricin exceeded those of apigenin by 33 and 100 %, respectively (Table 1). The higher tissue recovery of tricin as compared to that of apigenin is noteworthy in the light of the fact that in

molar terms the dose of tricin (~0.9 mmol/Kg) was 20 % smaller than that of apigenin (~1.1 mmol/Kg).

Metabolism of apigenin and tricin in liver microsomes

We reasoned that the difference between levels of tricin and apigenin observed in mouse plasma and liver was related to differential susceptibilities of the two flavones towards hepatic metabolism. To explore this notion flavones (50 μM) were incubated with murine or human liver microsomes in the presence of UDPGA, and incubates were analyzed for presence of parent flavone and metabolites. Chromatograms were similar for both compounds, comprising a major peak (retention times ~10 min) and two minor peaks (retention times 3-5 min) in each case (Fig. 3 A-D). The major peaks, which eluted at the retention times of authentic flavones, afforded the UV spectra of tricin and apigenin characterized by two absorbance maxima each at 352 nm/266.6 nm and 336.3 nm/ 265.4 nm, respectively. Formation of the metabolite peaks depended on the presence of UDPGA. UV spectroscopic analysis of these species allows tentative inferences to be made as to the site of metabolic glucuronidation in the flavone molecule [11]. The spectrum of the tricin conjugate with the retention time of ~3 min was characterized by absorbance maxima at 329.6 nm and 268.6 nm. The hypsochromic shift of the higher absorbance maximum in comparison to the parent tricin suggests glucuronidation in the B-ring, i.e. at the hydroxy in position 4' (see Fig. 1). The tricin conjugate with retention time ~5 min demonstrated absorbance maxima at 352 nm and at 247 nm, and the hypsochromic shift of the lower maximum when compared to tricin intimates glucuronidation of one of the two hydroxy moieties on ring A. In contrast, the UV spectra of the two apigenin metabolites, which both displayed absorbance maxima at 336 nm and 265.1 nm, were too similar to that of parent apigenin to permit inferences to be made as to the site of conjugate attachment. On-line HPLC-mass spectrometric analysis confirmed the identity of the major peaks as apigenin and tricin with molecular ions of *m/z* 269

and 329 (all *m/z* values: $[M-H^+]^-$ ions), respectively. The small peaks, which did not separate satisfactorily on HPLC-mass spectrometric analysis, afforded molecular ions of *m/z* 505 in the case of incubations with tricin and *m/z* 445 for apigenin (Fig. 4 A, B), with prominent loss of 176 atomic mass units (glucuronyl), suggestive of mono-glucuronides of either flavone. Figures 4 A and B show the mass spectra of the metabolites observed in human liver microsomes. The results in mouse microsomes were identical to those shown in the figure. For this HPLC/MS analysis the Varian chromatographic system was used, which did not separate the two isomeric glucuronides as satisfactorily as the Acquity system shown in Fig.3. Thus the mass spectra in Fig. 4 A,B are of mixtures of the two isomeric glucuronides. When liver microsomes were replaced with murine or human gut microsomes, extracts of incubates yielded chromatograms very similar to those shown in Figure 3 for liver (data not shown).

Samples were removed from hepatic microsomal incubates at different time intervals up to 20 (mouse microsomes) or 60 min (human microsomes), and residual flavones were quantitated. At the end of the incubation period, less than 20% of initial amount of flavone remained in the incubation medium (Fig. 5 A, B). Amounts of apigenin left unmetabolized were consistently below those for tricin at each time point in microsomes from either species, suggesting that the rate of metabolic glucuronidation of apigenin was faster than that of tricin. The difference was more marked in human than in murine microsomes.

Apigenin has been suggested to undergo NADPH-dependent phase I drug metabolism in rat liver microsomes *in vitro* to a mono-hydroxylated derivative, luteolin (3',4',5,7-tetrahydroxyflavone) [12]. In order to explore whether tricin also undergoes phase I biotransformation, tricin and apigenin were incubated side-by-side with mouse liver microsomes in the presence of NADPH. Figure 6 shows that analysis of extracts of incubates with apigenin furnished a metabolite peak, whilst analysis of extracts of tricin incubates did not reveal significant peaks in addition to that of parent agent. On UV spectroscopic analysis the apigenin

metabolite showed a high absorbance maximum of 347.0 nm, above that of the equivalent aborbance maximum of apigenin, consistent with this metabolite being luteolin [12]. Unlike apigenin, tricin does not seem to undergo phase I drug metabolism at a measurable rate in mouse liver micosomes.

Next the hypothesis was tested that apigenin or tricin can induce their own metabolic glucuronidation. Liver microsomes from mice, which had ingested dietary flavone (0.2%) for a week, were incubated with tricin in the presence of UDPGA, and removal of substrate was quantitated. The rate of substrate removal by microsomes from mice, which had received tricin, was 9.6±1.3% faster than substrate removal in control microsomes ($p<0.00.1$, $n=4$). Similarly, the rate of substrate glucuronidation by microsomes from mice on apigenin exceeded control metabolism by 12.1±3.3% ($p<0.002$, $n=4$). This result suggest that both flavones weakly induce murine glucuronyl transferase as reflected by tricin glucuronidation.

Metabolism of apigenin and tricin in liver cytosol

Flavones (50 μM) were incubated with murine or human liver cytosol in the presence of PAPS, and incubates were analyzed for presence of parent agent and metabolites. Analysis of the cytosol of either species afforded parent flavone and metabolite peaks with retention times close to 6 min. Incubates with tricin containing cytosol from humans or mice afforded one or two metabolites, respectively (Fig. 3 E, G). Analysis of incubates with apigenin afforded one metabolite (Fig. 3 F, H). Formation of the metabolite peaks depended on the presence of PAPS. The metabolite species derived from tricin with retention time of ~5.8min was characterized by absorbance maxima of 324.4 nm and 268.3 nm, suggesting sulfonation of the hydroxy moiety at position 4'. The tricin metabolite peak with retention time ~6.1 min gave 349.4 nm and 244.9 nm, consistent with sulfate attachment at one of the two hydroxy moieties on ring A. The absorbance maxima of the sulfate metabolite of apigenin were very similar to those of parent apigenin confounding structural

deductions to be made. When liver cytosol was replaced by intestinal cytosol in the incubations, the chromatograms recorded were very similar to those shown in Figure 3 for liver cytosol (data not shown). Characterization of the metabolite peaks by HPLC-mass spectrometry afforded *m/z* 409 for extracts of incubates with tricin and *m/z* 349 for extracts of mixtures with apigenin (Fig. 4 C, D), with prominent loss of 80 atomic mass units (sulfate), consistent with mono-sulfate conjugates. Figure 4 C, D shows the mass spectra of the metabolites observed in human liver cytosol, the results in mouse cytosol were identical to those shown in Figure 4.

Samples were removed form incubates with liver cytosol at different time intervals up to 60 min, and residual flavone was quantitated. Overall, the rate of flavone removal by metabolic sulfonation (Fig. 5 C,D) was much slower than that of glucuronidation (Fig 5 A,B). In murine liver cytosol tricin was metabolically removed more swiftly than apigenin, the difference between tricin and apigenin in terms of amount of flavone left un-metabolized being significant for both time points (Fig 5 C). In contrast, there was no difference in rate of metabolic removal between the two flavones in human cytosol (Fig. 5 D). The results suggest that in mice the rate of metabolic sulfonation of tricin was faster than that of apigenin.

Mouse liver cytosol from mice, which had ingested dietary flavone (0.2%) for a week, was incubated with tricin in the presence of PAPS, and removal of substrate was quantitated. The rate of tricin removal by sulfonation in cytosol from mice which had ingested flavones was indistinguishable from the metabolism seen with cytosol from control mice. This result suggests that the two flavones did not induce sulfotransferase enzymes.

Lipophilicity of tricin

Measurement of the n-octanol-water partition coefficient calculated from the saturation solubility of tricin in n-octanol and water afforded log P=3.24.

Discussion

Flavones are much less abundant in the plant kingdom than the other types of flavonoids. It is therefore not surprising that little is known about the bioavailability and metabolism of flavones as compared to other flavonoids [13]. The work described here explored these areas of flavone pharmacology for tricin and apigenin. The results show that the steady state level achieved in the plasma and liver of mice after dietary consumption of either flavone was higher for tricin than for apigenin, suggesting that tricin is systemically more available than apigenin. For the interpretation of the data presented here it seems useful to note that the dietary dose investigated (~300 mg/kg per day) far exceeds the amount of flavonoids consumed by humans with the diet, which has been estimated to be between 23 mg and 1.1g per day [14]. One of the processes, which determine the systemic availability of flavonoids after oral consumption, is hepatic metabolism. The results of the metabolism experiments in liver preparations *in vitro* described here suggest that the difference in systemic availability may be, at least in part, the corollary of a difference between tricin and apigenin in phase II metabolism. In murine and human hepatic microsomes *in vitro* apigenin underwent more rapid glucuronidation than tricin. In contrast, tricin was more swiftly conjugated with activated sulfate than apigenin in liver from mice, albeit not in liver from humans. Importantly, the rate of flavone glucuronidation *in vitro* was considerably faster than that of sulfonation. Extrapolating these findings to the *in vivo* scenario, it is likely that glucuronide formation contributed more prominently than sulfonation to the overall phase II metabolic clearance of either flavone from the murine organism. Therefore differences in glucuronidation rates between tricin and apigenin may have been, at least in part, responsible for the differential systemic availability of the flavones in mice. The difference in glucuronidation rate between the two flavones was also observed in liver microsomes from humans, hinting at the possibility that the corollary of this difference for the systemic availability of the two flavones in mice may also hold for humans. This notion needs of course clinical pharmacological verification. Tricin has not

yet been studied in humans, and there is to our knowledge only one report on the metabolism of apigenin in humans, according to which apigenin monoglucurono- and monosulfo-conjugates were recovered from the urine [15]. Our result demonstrating that apigenin underwent NADPH-dependent phase I metabolism to luteolin in mouse liver microsomes at a detectable rate, whilst tricin did not, suggests that this difference, if it applies to flavone biotransformation also *in vivo*, may have played a role in the superior systemic availability of tricin over apigenin. It remains to be investigated whether hydroxylation contributes indeed to the metabolic clearance of apigenin *in vivo*. Experiments in the perfused rat liver model suggest that, unlike in liver microsomes *in vitro*, phase I metabolites of apigenin could not be found in this *ex-vivo* system [12], intimating the possibility that apigenin hydroxylation may be quantitatively insignificant *in vivo*. It needs to be stressed, that the difference in systemic levels between apigenin and tricin after dietary intake may also have been caused by processes unrelated to metabolism, for example by differential absorption. It is conceivable that the two methoxy moieties in positions 3' and 5' of the flavone scaffold increase the lipophilicity of tricin in comparison with apigenin, thus possibly rendering tricin more easily absorbable than apigenin on physicochemical grounds. Consistent with this notion is the difference between the two flavones in *n*-octanol water partition coefficient. The log P value for apigenin has previously been calculated as 2.87 [10], whilst we established 3.24 as the log P for tricin.

On the basis of experiments in hepatoma cells *in vitro* apigenin has been suggested to induce metabolic glucuronidation [16]. Consistent with this observation both flavones were found here to be weak inducers of the glucuronyl transferase-catalyzed metabolism of tricin in mice *in vivo*.

We characterize here for the first time tricin sulfate and tricin glucuronide as tricin metabolites in murine and human liver preparations. Apigenin and tricin possess three hydroxy moieties, which are potentially susceptible to conjugative metabolism. Our analytical method did

not allow localization of the exact position of glucuronyl or sulfate attachment. Previously three monoglucuronides of apigenin have been identified in incubations with rat liver microsomes, again without exact definition of positional attachment of the glucuronyl moiety [12]. One of these metabolites was quantitatively prevalent. The UV spectroscopic analysis of the tricin conjugates described above suggests that tricin is predominantly glucuronidated at one of the two hydroxy moieties in ring A. The fact that apigenin was glucuronidated *in vitro* faster than tricin may be related to the presence of two methoxy moieties in positions 3' and 5' in tricin, which are absent from apigenin. These methoxy groups may have somewhat hindered the interaction of the tricin molecule with glucuronyl transferase. Levels of tricin exceeded those of apigenin also in the small intestinal tract, and it remains to be seen, whether apigenin will accordingly be less efficacious than tricin in the Apc^{Min} mouse model, in which tricin interfered significantly with small intestinal adenoma development [4]. Flavone metabolites could not be detected in the plasma or liver or gastro-intestinal tissues of mice, which had received dietary flavones, whilst preliminary inspection of the urine of mice exposed to flavones suggests the presence of three conjugate metabolites for apigenin and two for tricin (results not shown). The findings presented here need to be interpreded side-by side with some other results, which may allow a tentative comparison of their pharmacological profile. Both apigenin and tricin lack mutagenic properties, as reflected by a variety of tests [17, 18]. Their ability to inhibit cancer cell growth has been determined in human breast cancer-derived MDA MB 468 and in human colon cancer-derived HCA-7 and SW 480 cells. MDA MB 468 cells were more sensitive to the growth-retarding properties of tricin than those of apigenin, with IC_{50} values of 0.6 and 33 μM for tricin and apigenin, respectively [19, 20]. Colon cancer cells were more sensitive to apigenin, as reflected by IC_{50} values of >40 and 18 μM for tricin and apigenin, respectively, in HCA-7 cells [3] and of >40 and 23 μM in SW 480 cells (Al-Fayez, Cai, Steward and Gescher, unpublished). It is conceivable that flavonoid conjugate metabolites contribute to the pharmacological activity ascribed to the

parent flavonoid. A good example is quercetin 3-O-glucuronide, which has been shown to inhibit c-Jun terminal kinase (JNK) activity and binding to DNA of transcription factor activator protein-1 (AP-1) in vascular smooth muscle cells [21].

Overall the results discussed here are germane to the planning of clinical trials, in which the hypothesis is tested that COX-modulatory flavonoids can be used *in lieu* of non-steroidal anti-inflammatory drugs to interfere with adenoma recurrence and/or colorectal cancer development in humans. The type of data presented here contributes to the knowledge base, which will enable prioritization of flavonoid analogs for advancement to the stage of clinic evaluation. The comparison of tricin and apigenin hints at a potential pharmacokinetic advantage, which tricin may offer over apigenin in terms of superior systemic and gastro-intestinal availability.

Acknowledgements This work was supported by programme grant G0100874 from the Medical Research Council and a "Rapid Access to Preventive Intervention Development" (RAPID) grant from the US National Cancer Institute. The authors thank Ms Delphine Reynaud for help with the HPLC analysis and Dr Izet M Kapetanovic (NCI Div of Cancer Prevention, Bethesda MD) for advice and efficient organization of tricin synthesis and delivery.

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Table 1 Steady-state level of tricin and apigenin in mice after ingestion of flavones with their diet (0.2%) for a week

Tissue	Flavone		
	Tricin	Apigenin	p value[a]
	(nmol/mL or nmol/g)		
Plasma	0.45±0.12[b]	0.09±0.08[c]	<0.001
Liver	2.0±0.5	1.5±1.0	<0.05
Small intestinal mucosa	174±97	86±47	0.001

[a]By one way ANOVA followed by Tukey's pairwise comparison.

[b]Values are the mean±SD (n=8-10 mice).

[c]Including plasma samples, in which apigenin was undetectable .

Figure legends

Fig. 1 Chemical structure of tricin (A) and apigenin (B)

Fig. 2 HPLC chromatograms of plasma from mice, which had ingested tricin (A) or apigenin (B) at 0.2% for a week in their diet. Each chromatogram is representative of 4 mice. Quercetin served as internal standard. AU=absorbance units. Chromatographic analysis was conducted using the Acquity system in isocratic elution mode, for conditions see Materials and methods. The retention times of tricin and apigenin in spiked samples were 2.84 and 2.83 min, respectively, thus not identical, but very close to each other. On further chromatographic investigation using several conditions allowing increased retention times the peaks just after the solvent front in B did not co-elute with apigenin conjugates.

Fig. 3 HPLC chromatograms of incubation mixtures of liver microsomes (A-D) or cytosol (E-H) from humans (A, B, E, F) or mice (C, D, G, H) with tricin (A, C, E, G) or apigenin (B, D, F, H). Each chromatogram shown is representative of 6 analyses, of pooled livers of 3 humans or 3 mice. Incubations included cofactors UDPGA (A-D) or PAPS (E-H). AU=absorbance units. Arrows denote metabolite peaks. Incubation times were 10 and 30 min for murine and human liver preparations, respectively. Chromatographic analysis was conducted using the Waters Acquity system in gradient elution mode, thus retention times are different from those shown in Fig. 2. For details of incubation and chromatography see Materials and methods.

Fig. 4 On-line HPLC-MS mass spectra of metabolite peaks in extracts of incubates with human liver microsomes (A, B) or cytosol (C, D) including tricin (A, C) or apigenin (B, D).

Each spectrum is representative of 6 samples from pooled preparations from 3 humans. HPLC separation was achieved using the Varian system. The separation of the glucuronide peaks was not as satisfactory as that shown in Fig. 3 using the Acquity system, therefore the mass spectra of the glucuronides (A, B) are of mixtures of two isomeric flavone metabolites. Identical spectra were obtained when human liver fractions were replaced with mouse liver fractions. m/z=mass:charge ratio, amu=atomic mass unit, cps=counts per second. For conditions of incubation and chromatography see Materials and methods

Fig. 5 Metabolic removal of apigenin (closed squares, solid line) or tricin (open squares, broken line, both flavones at 50 μM) from incubations with hepatic microsomes (A, B) or cytosol (C, D) from mice (A, C) or humans (B, D) including UDPGA (A, B) or PAPS (C, D) as cofactors. Values are the mean±SD of 6 samples, from pooled livers of 3 humans or 3 mice. Incubations omitting cofactors furnished values which were close to 100% at all time points. Stars indicate that values are significantly different from each other (*$p<0.05$, **$p<0.01$, ***$p<0.001$). For conditions of incubation and chromatography see Materials and methods.

Fig. 6 HPLC chromatograms of extracts of incubation mixtures of mouse liver microsomes with NADPH and apigenin (A) or tricin (C) (both at 100 μM) or without flavones (B, D). Duration of incubation was 30 min. The chromatograms are representative of two experiments using pooled microsomes from 3 mice. Arrow marks metabolite peak. Control incubations omitting NADPH afforded chromatograms identical to those shown in B and D (in which flavones were omitted). AU=absorbance units. Chromatographic analysis was conducted using the Varian system (gradient elution), thus retention times are different from those shown in Fig 3. For conditions of incubation and chromatography see Materials and methods.