Cancer Chemother Pharmacol (2011) 67:255–263
DOI 10.1007/s00280-010-1313-1

ORIGINAL ARTICLE

Pharmacokinetics in mice and metabolism in murine and human liver fractions of the putative cancer chemopreventive agents 3′,4′,5′,5,7-pentamethoxyflavone and tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone)

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Received: 26 January 2010 / Accepted: 16 March 2010 / Published online: 4 April 2010

Abstract
Purpose The flavones tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) and 3′,4′,5′,5,7-pentamethoxyflavone (PMF) are under development as potential colorectal cancer chemopreventive agents as they reduced adenoma development in the Apc^{Min} mouse model of intestinal carcinogenesis. Here, the pharmacokinetic properties and metabolism of these flavones after oral administration were compared in mice.
Methods C57BL/6 J mice received an oral bolus of PMF or tricin (807 μmol/kg). Parent flavone and metabolites were analyzed by HPLC/UV in plasma, liver and gastrointestinal tissues. Flavones were incubated with mouse or human hepatic microsomes or 9000xg supernatant (S9), both fortified with a NADPH-generating system and either uridine 5′-diphosphoglucuronic acid (UDPGA, microsomes) or 3′-phosphoadenosine-5′-phosphosulfate (PAPS, S9). Disappearance of substrate was assessed by HPLC/UV, metabolites were characterized by HPLC/MS/MS.
Results Plasma concentrations and area under the plasma concentration versus time curve for PMF were higher than those for tricin. A mono-*O*-desmethyl PMF and several isomeric mono-*O*-desmethyl PMF glucuronides and sulfonates were major PMF metabolites in murine plasma, liver and intestinal tissue. In murine and human liver fractions, in vitro metabolic removal of tricin was faster than that of PMF. On kinetic analysis of metabolite generation in these incubations, apparent maximal velocity (V_{max}) values for the generation of tricin *O*-glucuronide or *O*-sulfonate were consistently several fold higher than those characterizing the production of mono-*O*-desmethyl PMF glucuronides or sulfonates via the intermediacy of *O*-desmethyl PMF.
Conclusions The results suggest that inclusion of methoxy moieties confers metabolic stability onto the flavone scaffold.

Keywords Chemoprevention · Flavones · Metabolism · Pharmacokinetics

Abbreviation
PMF 3′,4′,5′,5,7-Pentamethoxyflavone

Introduction

Flavonoids are ubiquitous plant polyphenols, many occurring in the diet, which are suspected to exert various beneficial health effects in humans [1]. Their potential application in the treatment or prevention of diseases is confounded by poor systemic availability, a corollary of rapid removal from the organism via metabolic conjugation [2]. Recently published findings suggest that long-term consumption with the diet of two flavones, tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) [3] or 3′,4′,5′,5,7-pentamethoxyflavone (PMF, for structures see Fig. 1a) [4], reduces adenoma development in the Apc^{Min} mouse, a model of human gastrointestinal malignancies associated with mutations in the *Apc* gene [5]. Tricin is a constituent of rice and other grass species, and PMF occurs in the leaves of *Murraya paniculata*, a constituent of traditional

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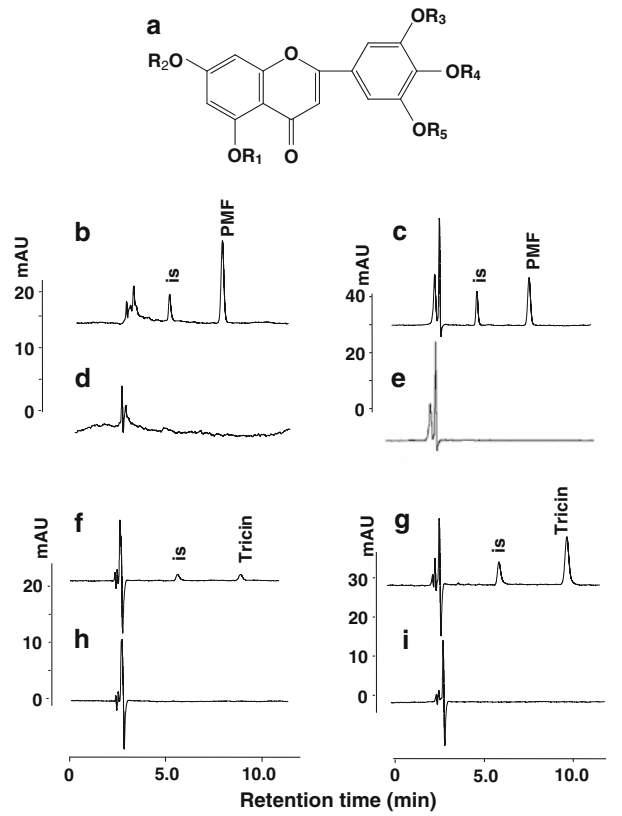

Fig. 1 **a** Structure of flavones used in this study: R_1–R_5 = CH_3: PMF; R_1, R_2, R_4 = H, R_3, R_5 = CH_3: tricin. **b–i** HPLC chromatograms of extracts of plasma (**b**, **d**, **f**, **h**) or intestinal mucosa (**c**, **e**, **g**, **i**) obtained from mice 60 min post administration (**b**, **c**, **f**, **g**) by gavage of 807 μmole/kg PMF (**b**, **c**) or tricin (**f**, **g**) and from mice that received vehicle only (**d**, **e**, **h**, **i**). *AU* absorbance units, *is* internal standard (tricin in **b** and **c**, quercetin in **f** and **g**). For experimental conditions, see "Materials and methods"

Indonesian herbal medicines [6], and fruits of the Brazilian *Neoraputia magnifica* [7], both plants being members of the Rutaceae plant family. The intestinal adenoma-reducing activity in the Apc^{Min} mouse model renders tricin and PMF putative cancer chemopreventive agents worthy of further preclinical evaluation. Investigation into the two flavones was originally prompted by the suggestion that inclusion of *O*-methyl moieties in the flavone molecular scaffold in addition to, or in place of, hydroxy functionalities improves cancer chemopreventive efficacy [8]. Consistent with this suggestion, apigenin (4′,5,7-trihydroxyflavone), a hydroxy cogener of tricin and PMF contained in leafy vegetables, failed to affect Apc^{Min} adenoma number [4]. One of the mechanistic determinants that have been proffered to explain the greater chemopreventive efficacy of methoxy-containing flavones vis-à-vis their hydroxy counterparts is differential rate of removal from the biophase via metabolism, with methoxy groups promoting metabolic stability [9].

The pharmacokinetics of tricin and PMF and the metabolism of PMF are not known. As part of a programme dedicated to the preclinical development of the two flavones, we studied their pharmacokinetics after oral dosing in mice in vivo and compared their metabolism in murine and human liver preparations in vitro. The investigation had two specific aims, to characterize the nature of PMF metabolites and to compare PMF and tricin in terms of both metabolic stability and levels achievable in the murine biophase.

Materials and methods

Chemicals

3′,4′,5′,5,7-Pentamethoxyflavone was purchased from Apin Chemicals Ltd. (Abingdon, UK) or synthesised by us as described before [4]; tricin was provided by Dr. I Kapetanovich (NCI Division of Cancer Prevention, Chemoprevention Agent Development Research Group, Bethesda, MD). Both flavones were >99% pure as determined by HPLC analysis. NADPH-generating system, uridine glucuronyl transferase (UGT) reaction mix, pooled human liver microsomes and 9000xg supernatant fraction (S9) were obtained from BD Biosciences (Woburn MA, USA). 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) 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). Water was purified in a laboratory Nano-Pure water purification system (Barnstead, UK).

Animals and treatments

C57BL/6 J mice (aged 9 weeks), the background strain of the Apc^{Min} mouse, were purchased from Charles River (Margate, UK). Mice were housed under sterile conditions in a room maintained at 22°C with a 12-h light/dark cycle. Experiments were carried out under animal project license PPL 80/2167, granted to the University of Leicester 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 [10]. Flavones were mixed with 0.5% aqueous carboxymethylcellulose (CMC) sodium salt solution and sonicated to furnish visually homogenous solutions (60 and 53.2 mg/ml, for PMF and tricin, respectively) for gavage. Female mice (3 per group) received a single oral dose of 807 μmol/kg flavone (300 mg/kg PMF or 266 mg/kg tricin) by gavage. The choice of dose was based on previous

experiments in which daily dietary doses of 0.2% flavones, translating to approximately 300 mg/kg, prevented intestinal adenoma formation in Apc^{Min} mice [3, 4]. Mice were killed 5, 15 or 30 min, or 1, 2, 6 or 24 h after flavone administration by cardiac exsanguination under terminal anesthesia (halothane), and blood was collected into heparinized tubes. Plasma was obtained by centrifugation (13,000×*g*, 20 min, 4°C). The intestinal tract was flushed with phosphate-buffered saline (10 ml) and cut open longitudinally. Tissue epithelial scrapings were collected by gently brushing the epithelial layer with a metal spatula. Liver and intestinal tract tissues were snap-frozen in liquid nitrogen. Biomatrices were kept, no longer than 4 weeks, at −80°C until analysis. Under these conditions, flavones are completely stable in plasma [11].

Tissue sample preparation

Plasma and tissues were thawed to room temperature. For quantitation of PMF in the plasma, a solid-phase extraction method reported previously [11] was used. Briefly, plasma was spiked with internal standard (tricin) and acidified with formic acid (0.5%), then the mixture was loaded onto a an Oasis HLB solid-phase extraction column (1 cc, Waters Corporation, Milford, Ma, USA) pre-conditioned with methanol/water. The column was washed and eluted (formic acid 0.2% in methanol/acetone, v/v, 1:1), then the eluate was evaporated under nitrogen (40°C), and the residue was reconstituted with HPLC mobile phase (100 µl). Quantitation of tricin in the plasma was essentially as described before [12]. Briefly, plasma was spiked with internal standard (quercetin) and mixed with two volumes of acetone containing 0.1 M acetic acid. The mixture was centrifuged, water was added to the supernatant (1:2), and the resultant sample (40 µl) was injected onto the HPLC system for analysis. Intestinal mucosa or liver tissue was mixed with two volumes of isotonic potassium chloride solution and homogenized on ice (Ystral × 10/20 homogeniser, Ballrechten-Dottingen, Germany). The homogenate was spiked with the appropriate internal standard, and an aliquot (100 µl) was added to two volumes of methanol/acetone (v/v, 1:1) containing acetic acid (0.1 M). The mixture was vortexed (10 min) and centrifuged (13,000×*g*, 20 min). The supernatant was mixed with two volumes of mobile phase. Tissue extracts were re-centrifuged (13,000×*g*, 5–10 min), and an aliquot (20–40 µl) of the resultant supernatant was injected onto the HPLC column.

Murine and human liver S9 and microsomes

Mouse livers were excised from six healthy C57BL/6J mice (3 female, 3 male, 8–10 weeks of age). An aliquot (3 g) of pooled tissue was suspended in buffered sucrose (27 ml, sucrose 0.25 M, Tris 10 mM, EDTA 1 mM, pH 7.4) and homogenized on ice. Microsomes and 9,000×*g* supernatant (S9) were prepared in the usual way by differential centrifugation, and liver fraction protein content was quantified [13]. Human liver fractions were purchased from BD Biosciences Discovery Labware (Woburn, MA). Human microsomes were pooled from 30 individuals (15 men, 15 women) aged 33–78, human S9 was pooled from 24 donors (16 men, 8 women) aged 21–63. Murine and human liver preparations were stored at −80°C until required.

Flavone metabolism in vitro

For the assessment of substrate disappearance, incubation mixtures contained murine or human liver microsomal or S9 fractions (1 mg/ml protein) and PMF or tricin (10 µM) plus cofactors in a final volume of 200 µl. An aliquot of flavone stock solution in DMSO was added to the incubation mixture, such that the DMSO concentration in the final incubate did not exceed 0.5%. The NADPH-generating system contained in the incubations consisted of nicotinamide adenine dinucleotide phosphate ($NADP^+$, 1.3 mM), glucose-6-phosphate (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 U/ml), sodium citrate buffer (120 µM) and magnesium chloride (33 mM). For the preparation of glucuronides, incubations contained flavones, microsomes, uridine 5′-diphospho-glucuronic acid (UDPGA, 2 mM), Tris–HCl (50 mM), $MgCl_2$ (8 mM) and alamethicin (25 µg/ml) in addition to the NADPH-generating system. For the preparation of sulfonates, incubation mixtures contained S9 fraction, 3-phosphoadenosine-5′-phosphosulfate (PAPS, 200 µM) plus the NADPH-generating system. Each type of incubation included three negative controls: (i) all reaction components except substrate, (ii) substrate and cofactors without microsomes or S9 and (iii) complete incubation mixture with heat-inactivated instead of metabolically competent liver fractions. Incubations were conducted in triplicate at 37°C in a shaking water bath, and the reaction mixtures were pre-incubated for 5 min before the reaction was initiated by addition of cofactors. After 5, 15, 30, 60 or 120 min, the reaction was quenched as described below. In orientation experiments, the conditions of optimal substrate concentration and reaction time were optimized for analysis of enzyme kinetics of metabolite formation. Incubation conditions were essentially as described above, except that substrate concentration was 2.5, 5, 10, 20, 40 and 80 µM for PMF and 0.5, 2.5, 5, 10, 20, 40 and 80 µM for tricin, and incubation times were 30 or 60 min for PMF in mouse or human liver fractions, respectively, and 10 or 15 min for tricin in incubations with microsomes or S9 fraction, respectively. In both types of in vitro experiments, reactions were quenched with an aliquot (160 µl) of ice-cold

methanol/acetic acid (0.1 M) and centrifuged (13,000×*g*, 4°C, 10 min). An aliquot (40 µl) of the supernatant was injected onto the HPLC column for analysis.

HPLC/UV analysis of flavones

For the quantitation of parent flavones in extracts of plasma, tissues or incubation mixtures, previously described reversed phase HPLC methods were used [11, 12]. Briefly, analysis was conducted using a Varian HPLC system consisting of a ProStar 230 pump, a ProStar 410 autosampler and a ProStar 325 UV–visible detector (Varian Inc, Oxford, UK) with UV detection at 324 or 352 nm, the maximum absorbance wavelengths of PMF or tricin, respectively. In some cases, a ProStar 320 photodiode array detector was used to record the UV–visible spectra of flavone species at 200–400 nm. Separation was achieved on a Hypersil BDS C_{18} column (250 × 4.6 mm, particle size 5 µm, Thermo Fisher Scientific, Runcorn, UK). The isocratic mobile phase system used consisted of ammonium acetate buffer (0.1 M, pH 5.1) with EDTA (0.27 mM) and methanol either at 65% for PMF or 55% for tricin. The flow rate was 1 ml/min. For the detection and quantitation of flavone metabolites, a binary mobile phase system was used (A: aqueous ammonium acetate 5 mM; B: ammonium acetate in methanol 5 mM), with gradient elution over 25 min either from 65% A: 35% B to 35% A: 65% B for PMF metabolites or from 75% A: 25% B to 25% A: 75% B for tricin metabolites.

HPLC/MS

Flavones and their metabolites were characterized by HPLC–mass spectrometry using a TurboIon Spray (TIS) source in positive and negative ionization mode for PMF and tricin, respectively. Analyses were performed using an API-2000 mass spectrometer (Applied Biosystems, Warringon, UK) attached to an Agilent 1100 series HPLC system. Separation was achieved with the binary mobile phase system described above on a Hypersil BDS C_{18} column (150 × 2.1 mm, particle size 3 µm, Thermo Fisher Scientific) with gradient elution from 60% A: 40% B to 25% A: 75% B over 25 min, at a flow rate of 0.2 ml/min. For identification of flavones and their metabolites mass spectrometry, conditions were as follows: for PMF (positive ion mode) ion source voltage 4,500 V, declustering potential 71 V, focusing potential 270 V, entrance potential 12 V, collision energy 55 V, collision exit potential 40 V and temperature at 450°C; for tricin (negative ion mode) ion source voltage −4,500 V, declustering potential −81 V, focusing potential −230 V, entrance potential −12 V, collision energy −38 V, collision exit potential −46 V, temperature 500°C, as described for tricin previously [14]. Mass to charge ratios (*m/z*) described under Results are either $[M + H]^+$ for PMF and its metabolites or $[M - H]^-$ ions for tricin and its metabolites.

Pharmacokinetic and enzyme kinetic analyses

Pharmacokinetic analysis of flavones was performed by non-compartmental analysis provided with WinNonlin (version 5.2, Pharsight, USA) applying extravascular administration (model 200). Analysis-generated values for areas under the concentration versus time curve (AUC) computed using the linear trapezoid method and terminal phase half-lives ($t_{1/2,\ \beta}$, where $t_{1/2,\ \beta} = 0.639/K_{el}$). Maximal plasma or tissue concentrations (C_{max}) and corresponding times (T_{max}) were determined by visual inspection of the plasma or tissue versus time concentration profiles. Apparent enzyme kinetic parameters, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}), were estimated from curve fitting using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA) for non-linear regression analysis. Apparent V_{max} values were normalized to incubation time and protein concentration and expressed as peak area units. Apparent K_m values were determined from hyperbola plots. Statistical comparison of flavone levels in biomatrices and incubation mixtures was by Student's *t* test, where $P < 0.05$ was considered significant.

Results

Pharmacokinetics of flavones in mice

C57BL/6 J mice received a single dose of 807 µmol/kg of PMF or tricin by gavage. Flavones were measured by HPLC/UV analysis in the plasma and gastrointestinal mucosa (Fig. 1), and analyte concentration was plotted against time *post* administration (Fig. 2). Pharmacokinetic parameters derived from these plots are summarized in Table 1. While there was no substantial difference between the two flavones in terms of $t_{1/2}$ and T_{max}, the C_{max} and AUC values derived from plasma concentration versus time curves of PMF were about twice those seen for tricin. The C_{max} and AUC values describing intestinal mucosa concentrations of PMF exceeded those observed for tricin by a factor of approximately 3.

Identification of flavone metabolites in murine biomatrices

Plasma, liver and gastrointestinal mucosa of mice that had received flavones were analyzed for presence of agent-derived species. On HPLC/UV analysis, species that eluted

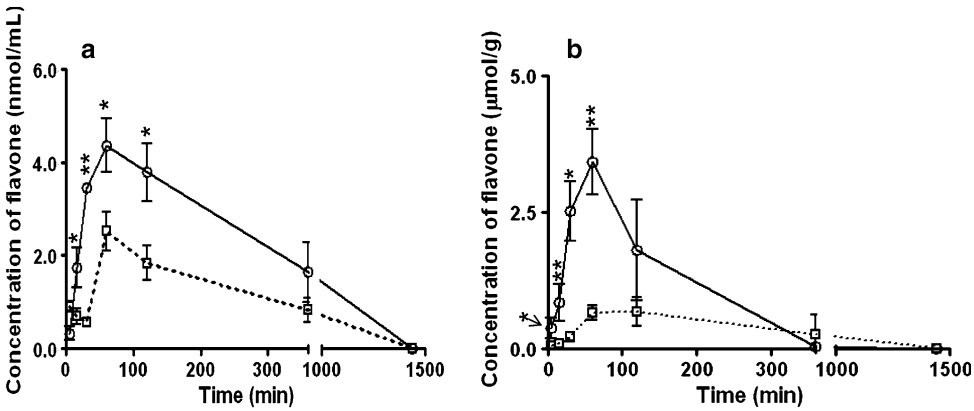

Fig. 2 Concentration *versus* time profiles for PMF (*circles*, *solid lines*) or tricin (*squares*, *broken lines*) in plasma (**a**) or gastrointestinal mucosa (**b**) of mice that received flavones (807 μmole/kg = 300 mg/kg PMF or 266 mg/kg tricin) by gavage. Values are the mean ± SD of 3 mice. *Asterisks* indicate that PMF and tricin levels are significantly different from each other, $*P < 0.05$, $**P < 0.01$

Table 1 Pharmacokinetic parameters of PMF or tricin in plasma or intestinal mucosa of mice that received PMF or tricin at 806 μmol/kg by gavage

	C_{max} (nmol/ml or g)	T_{max} (min)	AUC (nmol/ml or g min)	$T_{½}$ (min, β)
Mice on PMF				
Plasma	4.4	60	1,924	201
Mucosa	3,425	60	520×10^3	140
Mice on tricin				
Plasma	2.5	60	969	195
Mucosa	676	120	196×10^3	252

C_{max}, maximum concentration; T_{max}, maximum time; AUC, area under concentration curve; $T_{½}$, terminal phase half-life

before parent flavones reflecting their higher polarity were suspected to be metabolites. When subjected to photodiode array UV/VIS detection, peaks of putative PMF metabolites displayed band I and band II absorbances at 320–340 and 262–266 nm, respectively, diagnostic of flavones [15] (results not shown). Analysis of PMF metabolites in liver and gastrointestinal tissues by HPLC/MS detected molecular ions with *m/z* values of 359, 535 or 439. LC/MS/MS analysis using MRM transitions identified three types of isomeric PMF metabolites, mono-*O*-desmethyl PMF (*m/z* 359 > 343, corresponding to loss of oxygen), mono-*O*-desmethyl PMF glucuronide (*m/z* 535 > 359, loss of glucuronate) and mono-*O*-desmethyl PMF sulfonate (*m/z* 439 > 359, loss of sulfate). PMF harbors five methoxy groups, each potentially susceptible to metabolic *O*-demethylation, giving rise to species prone to undergo conjugation reactions, which in turn can yield several positionally isomeric conjugate metabolites. A mono-*O*-desmethyl PMF was the only PMF metabolite recovered from the plasma (Table 2). Metabolites detected in the

Table 2 Identification of flavones and their metabolites by LC/MS/MS using MRM transitions in the plasma (p), liver (l) or gastrointestinal mucosa (m) of mice that received PMF or tricin at 806 μmol/kg by gavage

	MRM (*m/z*)	Retention time (min)
Mice on PMF		
PMF	373 > 283	23.6 (p, l, m)
Monodesmethyl PMF	359 > 343	18.3 (p, l, m), 20.1 (l, m)
Monodesmethyl PMF glucuronide	535 > 359	8.8 (l, m), 11.5 (l, m), 16.0 (m)
Monodesmethyl PMF sulfonate	439 > 359	10.1 (l, m), 10.2 (m)
Mice on tricin		
Tricin[a]	329 > 279	20.7 (p, l, m)
Tricin monoglucuronide	505 > 329	7.3, 12.4 (both p, m)
Tricin monosulfonate	409 > 329	14.8, 15.6 (m)

[a] Chromatographic conditions for tricin and its metabolites were different from those used for PMF and its metabolites

biomatrices of mice that had received tricin exhibited molecular ions of *m/z* 505 and *m/z* 409 when analyzed by HPLC/MS, while tandem LC/MS/MS analyses afforded MRM transitions consistent with tricin *O*-monoglucuronide (*m/z* 505 > 329) and tricin *O*-monosulfonate (*m/z* 409 > 329), which occurred as multiple peaks indicative of the presence of positional isomers. Two tricin *O*-monoglucuronides and two tricin *O*-monosulfonates were recovered from the gastrointestinal mucosa, also two *O*-monoglucuronides from the plasma and one from the liver (Table 2).

Comparison of metabolism of PMF and tricin in murine and human liver fractions

We reasoned that the differences in AUC and C_{max} between PMF and tricin may be related, at least in part, to differences in the rate of metabolic removal in vivo. To test this hypothesis, flavones (10 μM) were incubated with murine or human liver fractions suitably fortified with cofactors, and metabolic removal of substrate was measured. Metabolites generated by the liver fractions were identified and quantitated, and their formation was subjected to kinetic analyses. Flavones were incubated in the presence of a NADPH-generating system with either microsomes plus UDPGA or S9 fraction plus PAPS. The rate of removal of PMF from the metabolic incubations was markedly slower than that of tricin (Fig. 3 a, b), so that 60% or more of the initially available PMF was recovered from the mixture at the end of the incubation period, while only 20% or less of the initial amount of tricin remained unchanged. This result is consistent with the notion that the rates of metabolic oxidation plus glucuronidation or sulfonation of PMF were significantly slower than the rates of glucuronidation or sulfonation of tricin.

Flavone metabolites generated by liver fractions were identified by online HPLC/MS. Metabolism of PMF (Fig. 4) afforded mono-*O*-desmethyl PMF species in incubations containing a NADPH-generating system, while mono-*O*-desmethyl PMF glucuronides and mono-*O*-desmethyl PMF sulfonates were produced in the presence of UDPGA and PAPS, respectively. The formation of these metabolites is consistent with the species identified in tissues of mice that had received PMF. As described previously (Cai et al. 2006), tricin failed to generate detectable amounts of products of oxidative metabolism (i.e., *O*-desmethyl tricin) in microsomes with NADPH but was metabolized by microsomes in the presence of UDPGA to two isomeric *O*-monoglucuronides and by S9 fortified with PAPS to an *O*-monosulfonate (Fig. 4). Conjugate formation was subjected to Michaelis–Menten kinetic analysis evaluating metabolite peak area (Table 3). The apparent K_m values characterizing metabolic conjugate formation for both flavones were approximately similar to each other. In contrast, the apparent V_{max} values for the metabolic generation of the conjugates differed markedly between flavones. The apparent V_{max} values for tricin glucuronides in microsomes were between 8- and 300-fold higher than those characterizing the production of *O*-mono-desmethyl PMF glucuronides via the intermediacy of *O*-desmethyl PMF. Likewise, the apparent V_{max} for generation of tricin

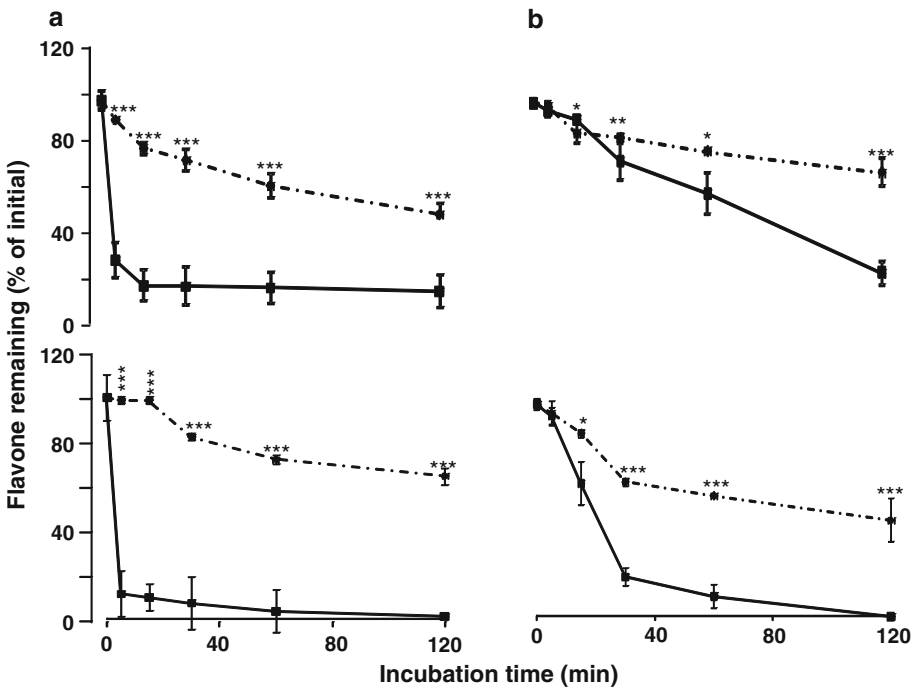

Fig. 3 Metabolic removal of PMF (*broken line*) or tricin (*solid line*) from incubations with either hepatic microsomes (**a**) or S9 fraction (**b**) from humans (*top graphs*) or mice (*bottom graphs*) and a NADPH-generating system with either UDPGA (**a**) or PAPS (**b**). Substrate concentration was 10 μM. Values are expressed as percentage of control incubations with heat-inactivated microsomes or S9, and they are the mean ± SD of 6 separate incubates using pooled liver fractions from 6 mice or 24 (microsomes) or 30 (S9) humans. *Asterisks* 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"

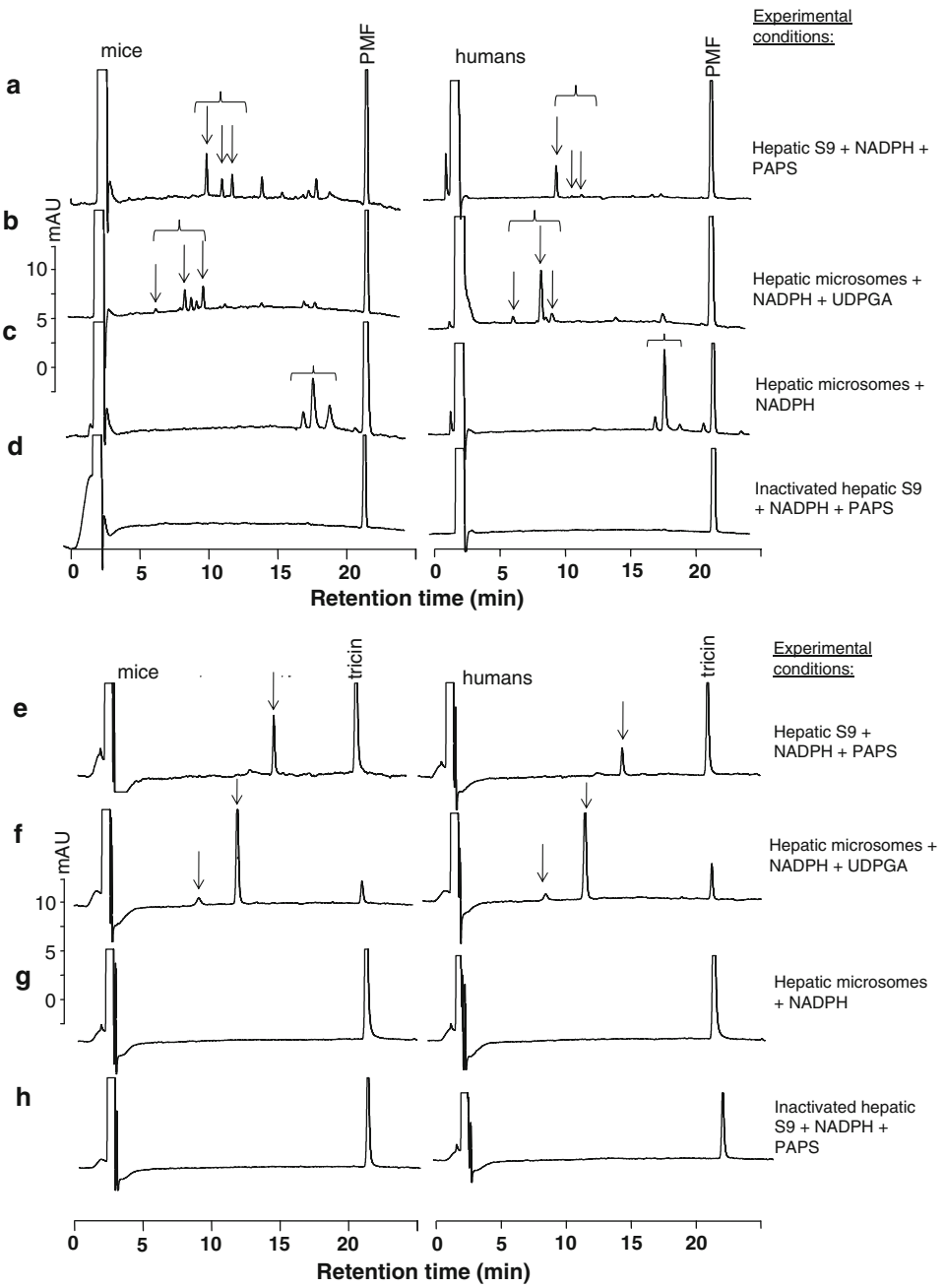

Fig. 4 HPLC–UV chromatograms of extracts of incubation mixtures of PMF (**a**–**d**) or tricin (**e**–**h**) with functionally competent (**a**, **e**) or heat-inactivated (**d**, **h**) hepatic S9 fraction or of hepatic microsomes (**b**, **c**, **f**, **g**) from mice (*left*) or humans (*right*) and a NADPH-generating system without (**c**, **g**) or with either PAPS (**a**, **d**, **e**, **h**) or UDPGA (**b**, **f**). Incubations with heat-inactivated microsomes afforded chromatograms identical to those shown here for heat-inactivated S9 (**d**, **h**) and are therefore not shown. Substrate concentration was 10 μM, incubation times were 10 and 30 min for murine and human liver preparations, respectively, and hepatic fraction protein concentrations were 1 and 0.2 mg/ml for PMF and tricin, respectively. Chromatograms are representative of 3 separate experiments, pooled liver fractions were from 6 mice or 24 (microsomes) or 30 humans (S9). *AU* absorbance units. Brackets mark retention times of putative *O*-desmethyl sulfonate (**a**), *O*-desmethyl glucuronide (**b**) and *O*-desmethyl species (**c**) derived from PMF; *arrows* denote peaks of flavone metabolites, the formation of which was subjected to enzyme kinetic analysis (see Table 3). Note that chromatographic conditions were different from those shown in Fig. 1, therefore retention times differ. For details of incubation and chromatography, see "Materials and methods"

O-sulfonate in S9 was between 3- and 28-fold faster than the equivalent values for production of mono-*O*-desmethyl PMF sulfonates.

Discussion

This study is the first to describe the pharmacokinetic properties in mice of PMF and tricin after oral bolus administration. We show here that after administration of equimolar doses of these flavones, systemic exposure of mice to PMF was higher than that to tricin, as reflected by AUC and C_{max} values. In analogy to these findings, consumption by Apc^{Min} mice of PMF or tricin at 0.2% with the diet for 3 months has previously been shown to generate mean steady state plasma levels of 1.2 and 0.5 μM, respectively, indicative of superior systemic availability of PMF also after dietary ingestion [4]. Similar differences in plasma levels between methoxy flavone and hydroxy congener have previously been demonstrated in rodents for 5,7-dimethoxyflavone vis-à-vis chrysin (5,7-dihydroxyflavone) and for 5,7,4′-trimethoxyflavone vis-à-vis apigenin (5,7,4′-trihydroxyflavone) [8]. All of these results are consistent with the notion that the presence of methoxy

Table 3 Kinetic analysis of metabolic formation of glucuronide and sulfonate metabolites from PMF and tricin in murine and human liver fractions

	Glucuronidation[a]				Sulfonation[b]			
	K_M (μM)		V_{max} (peak area/min/μg protein)		K_M (μM)		V_{max} (peak area/min/μg protein)	
	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human
PMF	15.0 ± 4.9[c]	16.7 ± 4.8	50.1 ± 5.8	15.2 ± 1.9	7.9 ± 1.4	11.1 ± 2.8	131 ± 7	46.2 ± 8.7
	9.6 ± 5.6	13.1 ± 5.5	15.0 ± 1.2	103 ± 15	8.3 ± 1.9	14.7 ± 3.3	55.5 ± 3.8	5.0 ± 0.6
	21.4 ± 4.4	9.9 ± 1.9	179 ± 5	17.8 ± 1.3	8.1 ± 1.8	13.5 ± 3.8	68.1 ± 4.4	6.9 ± 0.8
Tricin	7.8 ± 1.3	41.7 ± 4.1	1,488 ± 55	806 ± 31	2.2 ± 0.4	0.5 ± 0.1	1,555 ± 74	134.4 ± 2.3
	18.1 ± 7.2	13.1 ± 2.3	4,668 ± 628	4,773 ± 282				

[a] Microsomes with a NADPH-generating system and UDPGA

[b] S9 with a NADPH-generating system and PAPS

[c] Values are the mean ± SD of three independent experiments

moieties in the flavone scaffold imparts metabolic stability on the molecules, and it is likely that the differences in systemic exposure to the flavones described here reflect discrepancies between them in systemic bioavailability. It needs to be stressed though that in the absence of *iv* pharmacokinetic data, such a conclusion cannot be drawn unequivocally, as a difference between them in ability to permeate membranes may have contributed to the differential levels observed in the biophase.

Mono-*O*-desmethyl PMF, mono-*O*-desmethyl PMF glucuronide and mono-*O*-desmethyl PMF sulfonate have been characterized here for the first time as metabolites of PMF in mice in vivo. The results of the in vitro part of the study described here support the hypothesis that differences in metabolic stability between PMF and tricin may be responsible for or contribute to their dissimilar systemic levels. Under similar incubation conditions, liver fractions were considerably less proficient in generating *O*-desmethyl PMF glucuronide or *O*-desmethyl PMF sulfonate via intermediate formation of *O*-desmethyl PMF than in metabolizing tricin to two *O*-monoglucuronides or an *O*-monosulfonate. These differences in metabolism were observed in liver fractions not only from mice, but also from humans, suggesting that the pharmacokinetic differences between PMF and tricin observed in mice may also apply to humans.

There were marked differences between PMF and tricin in terms of AUC and C_{max} in gastrointestinal mucosa, a tissue directly accessible to orally administered agents avoiding the necessity of transport via the blood stream. Intriguingly, the C_{max} for both PMF and tricin was much higher than the IC_{50} values for growth inhibition in Apc^{Min} mouse adenoma cells in vitro, which were 6 μM for PMF and 12 μM for tricin [4]. This observation is consistent with the finding that both flavones displayed chemopreventive activity in the Apc^{Min} mouse [3, 4]. The Apc^{Min} mouse model is considered a useful preclinical model in the discovery of agents that can prevent adenoma recurrence in humans, as it predicted accurately the clinical efficacy of sulindac [16, 17] and celecoxib [18, 19]. However, there are concerns about the safety of long-term administration of non-steroidal anti-inflammatory drugs [20] and celecoxib [21], which militate against their use as cancer chemopreventive agents. Therefore, the search for novel, safe and efficacious chemopreventive agents is important. The abundance and reasonable safety record of naturally occurring flavonoids, of which there are literally thousands in the plant kingdom, render them a promising potential source of novel chemopreventive agents. The robust activity of PMF and tricin in the Apc^{Min} mouse model tentatively earmarks them for potential clinical development. PMF was a somewhat more potent inhibitor of intestinal adenoma development in Apc^{Min} mice than tricin [4], but discrimination between the two flavones with respect to potential potency is premature, as the potency difference was not significant. Little is known about the mechanisms that may mediate the putative cancer chemopreventive activity of PMF and tricin. Both flavones have been shown to interfere with the metabolic conversion of arachidonate to tumor-promoting prostaglandin E-2 in mice in vivo and to exert antiproliferative activity in human-derived colorectal cancer cells and Apc^{Min} mouse adenoma cells in vitro with IC_{50} values in the 10^{-5} M range [4]. Well-studied flavonoids, such as quercetin, genistein or apigenin, are thought to exert their cancer chemopreventive activity via the parent molecule. This is probably also the case for PMF and tricin, so that their metabolic conjugation described here constitutes probably a pharmacological deactivation. In contrast, it is conceivable that unconjugated *O*-desmethyl PMF contributes to the pharmacological effect of PMF in vivo.

Structural features of flavonoids, which determine pharmacological activity, are only poorly understood. Results of the type described here may help rationalize the

selection of flavonoids for development as agents potentially useful in cancer management. Flavones such as PMF that harbor methoxy moieties supporting metabolic stability seem to be worthy of further investigation for ability to prevent carcinogenesis at sites remote from the gastrointestinal tract and only accessible to orally administered agents via the circulation, such as the prostate, breast, liver or lungs.

Acknowledgments This work was supported by programme grant C325-A6894 from Cancer Research UK. We thank Dr I Kapetanovich (NCI Division of Cancer Prevention) for the provision of tricin.

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