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ORIGINAL ARTICLE

Pharmacokinetics and metabolism of the putative cancer chemopreventive agent cyanidin-3-glucoside in mice

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Abstract
Purpose Cyanidin-3-glucoside (C3G), an anthocyanin component of fruits and berries, possesses cancer chemopreventive properties in mouse models of carcinogenesis. Its pharmacokinetics and metabolism in mice have hitherto not been studied.
Methods C57BL6J mice received C3G by either gavage at 500 mg/kg or tail vein injection at 1 mg/kg. Blood, urine, bile and heart, lung, kidney, liver, prostate, brain and gastrointestinal (gi) mucosal tissues were obtained up to 2 h after administration. Levels of C3G and its anthocyanin metabolites were determined by HPLC with visible detection. Metabolites were identified by LC/MS/MS.
Results After oral administration peak concentrations of anthocyanins occurred within 30 min after administration. Levels were highest in the urine and gi mucosa. In the gi mucosa and liver the predominant flavonoid species after oral administration was C3G, whilst after iv dosing the majority of anthocyanins was C3G metabolites. After oral or iv administration, C3G half-lives in the different biofluids and tissues ranged from 0.7 to 1.8 h and 0.3 to 0.7 h, respectively. Systemic bioavailabilities for parent C3G and total anthocyanins were 1.7 and 3.3%, respectively. The major metabolites of C3G were products of methylation and glucuronidation. Cyanidin was a minor metabolite in the gut.
Conclusion C3G and its metabolites were recovered from murine tissues which may be targets for cancer chemopreventive intervention. Anthocyanin levels achieved in the gi mucosa, prostate and the kidneys were of an order of magnitude consistent with pharmacological activity.

Keywords Anthocyanins · Cancer chemoprevention · Drug development

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Introduction

Anthocyanins, exemplified by cyanidin-3-glucoside (C3G, Fig. 1), are flavonoid pigments occurring ubiquitously in fruits and berries. They are the glycosides of anthocyanidins, cyanidin being the aglycon of C3G. It has been proposed that anthocyanins contribute to the cancer chemopreventive properties of fruits and berries, and evidence accrued using rodent models of carcinogenesis supports the notion that anthocyanins may prevent or delay the development of malignancies [1, 2]. Most studies of health effects of anthocyanins have been conducted using berry preparations or extracts which contain mixtures of often 10 or more anthocyanins. Recently C3G was shown to possess a range of intriguing anticarcinogenic properties: It scavenged ultraviolet B-induced hydroxyl and oxygen radicals, inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced transactivation of the transcription factors NFκB and AP-1 in murine JB6 cells, interfered with the cellular expression of cyclooxygenase-2 and tumour necrosis factor-α, and blocked TPA-induced neoplastic transformation of JB6 cells [3]. Furthermore, C3G when administered in vivo at 9.5 mg/kg ip three times a week reduced the growth of A549 lung tumour xenografts in nude mice [3]. C3G added to the diet delayed adenomagenesis in the *Apc*^{Min} mouse, a model of human gastrointestinal (gi) malignancies characterised by an APC mutation [4]. All these results hint

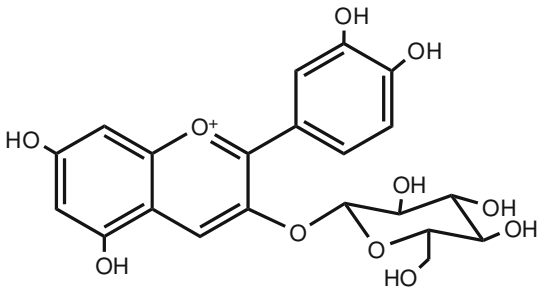

Fig. 1 Structure of C3G

at the possibility that C3G may warrant further evaluation to explore its potential as a cancer chemotherapeutic and/or chemopreventive agent in humans. The pharmacokinetics and metabolism of isolated C3G have hitherto only been studied in rats [5, 6]. In the light of the anticarcinogenic properties of C3G in nude [3] and Apc^{Min} mice [4] we wished to explore its pharmacokinetics and metabolism in this species. To that end levels of C3G and products of C3G metabolism were detected and quantitated in plasma, urine, bile and tissues of mice after oral administration. Metabolites of C3G were identified by LC/MS/MS. Anthocyanins are among the very few naturally occurring flavonoids which are reasonably water soluble. We therefore also studied the murine pharmacokinetics of C3G after iv injection, which when compared with the results from the gavage study allowed computation of anthocyanin bioavailability. Overall the study was designed to aid the development of C3G as a putative cancer chemopreventive agent.

Materials and methods

Materials

C3G was purified from blackberries using a counter current chromatographic method as described previously [7], its purity was >99% by HPLC. Reagents used in the chemical analysis were purchased from Fisher Scientific (Loughborough, UK).

Study design

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 UKCCCR guidelines [8]. C57BL6J mice were purchased from Harlan (Oxon, UK), and fasted overnight prior to dosing. C3G was dissolved in water immediately prior to administration. In the po study 21 mice (3 per time point) received C3G 500 mg/kg by gavage (injection volume 200 µl), in the iv study 40 mice (5 per time point) received C3G at 1 mg/kg (injection volume 10 µl) via tail vein injection. The 500 mg/kg oral dose is comparable to the daily dietary dose of C3G (0.3%) which was found to interfere with adenoma development in Apc^{Min} mice [4]. The choice of the iv dose (1 mg/kg) was determined by the limit of C3G water solubility. Animals were killed by exsanguination under halothane- or isofluorane-induced terminal anaesthesia 5, 10, 20, 30, 60, 90 or 120 min after administration, in the case of the iv study there was also a 15 min time point. Control mice received vehicle only. Blood, collected by cardiac puncture using a heparinised syringe, was transferred to a heparinised tube and centrifuged (16,060×*g*, 10 min) Plasma was removed and stored (−80°C) until analysis. Urine was collected directly from the bladder. Liver, kidney, heart, lung, brain, prostate, gall bladder and the intestinal tract were excised. The intestine was flushed with phosphate buffered saline, and mucosa was obtained by scraping using a metal spatula. Tissues were flash-frozen in liquid nitrogen, prior to storage at −80°C.

Preparation of biomatrices for analysis

Tissue samples were homogenised (blade X-1020 homogeniser, Ystral GmbH, Ballrechten-Dottingen, Germany) in KCl solution (1.15% w/v, 1:1). In the case of bile, lung (after iv administration only) and prostate, tissues were pooled. In the case of plasma, urine, bile or homogenate of intestinal mucosa and brain, the analyte was extracted using solid-phase columns. Aliquots of plasma, tissue homogenate (200 µl), bile or urine (50–100 µl) were centrifuged (16,060×*g*, 5 min), the supernatant was diluted to 1 ml (water) prior to loading onto Oasis HLB solid-phase extraction cartridges (1 ml; Waters, Elstree, UK), which had previously been conditioned with acetone: formic acid (9:1, 1 ml) and of water: formic acid (9:1, 1 ml). Anthocyanins were eluted (flow rate 1 ml/min) sequentially with 0.2, then 0.1 ml acetone: formic acid (9:1). In the case of prostate, heart, liver, lung and kidney tissues extraction was by organic solvent. To that end homogenates were diluted with 0.6 ml ice-cold acetone and vortexed (20 min), kept at −20°C (20 min), centrifuged (16,060×*g*, 10 min). Eluates of solid-phase extraction or supernatants of acetone extraction were evaporated to dryness (stream of nitrogen). Residues were reconstituted in water: formic acid (9:1, 75 µl) and centrifuged (16,060×*g*, 10 min) prior to HPLC analysis. Efficiency of C3G extraction from human plasma was 71%, with intra- and inter-day variability of 9.0 and 9.1%, respectively. Extraction efficiencies for individual anthocyanins from plasma spiked with an anthocyanin mixture varied by less than 15%.

HPLC analysis

Anthocyanin preparations or extracts of biomatrices were analysed for anthocyanin content by HPLC with detection by either ultraviolet–visible (UV–VIS) spectrophotometry or tandem mass spectrometry (LC/MS/MS). The HPLC system (Varian Analytical Instruments, Oxford, UK) comprised a Varian 230 pump, Varian 410 auto-sampler and Varian 325 UV–VIS detector. Separation of anthocyanins was achieved using a Xterra Phenyl column (Waters; 4.6 × 250 mm, 5 μm) with guard column (Waters, 4.6 × 10 mm, 5 μm) at 22°C. Conditions of analysis were as described previously [4] with the following modifications: flow rate 1 ml/min, gradient elution (solvent A: 10% formic acid, solvent B: acetonitrile, the following gradients are presented as % of B): 1–3% up to 12 min, 3–10% 12–18 min, 10% 18–26 min, 10–60% 26–28 min, 60% 28–30 min. Spectrophotometric detection was at 520 nm. Validation of the method for C3G determination using spiked human plasma (0.2 ml) furnished limits of detection and quantitation of 2.5 and 7.5 ng/ml plasma (5 and 15 nM), respectively. Detection was linear over the range 1–1,000 ng/ml ($r^2 = 0.99$); accuracy was 100 ± 9%. Amounts of anthocyanins other than C3G observed in bio-matrices as described under "Results" should be interpreted as semi-quantitative, because they were calculated using standard curves established for C3G, assuming that the absorption coefficients of these anthocyanins are similar to that of C3G. HPLC–electrospray ionisation (ESI) tandem mass spectrometric (MS/MS) analysis was performed using an API2000 mass spectrometer (Applied Biosystems, Warrington, UK) with sample delivery via an 1100 series HPLC instrument (Agilent Technologies UK Ltd., South Queensferry, UK). Separation was achieved with a Xterra phenyl column (2.1 × 150 mm, 3.5 μm) with guard (2.1 × 20 mm, 3.5 μm). Conditions of analysis were as described previously [4]. Mass spectrometric analyses were performed in positive ion mode under the following conditions: declustering potential 55 V, focusing potential 380 V, entrance potential 10 V, collision energy 50 V, collision energy exit potential 16 V, ion-spray voltage 5,000 V, temperature 450°C. C3G and its metabolites were identified in positive ion mode by multiple reaction monitoring (MRM) using transitions characteristic for the loss of the sugar moiety, or in the case of C3G conjugate metabolites the loss of glucuronic acid or sulphate.

Pharmacokinetic analysis

Concentrations of C3G and total anthocyanins in biomatrices after oral or iv dosing were used to analyse the following pharmacokinetic parameters: peak concentration (C_{max}), time of peak concentration (T_{max}), rate of elimination constant (k_e), half-life ($t_{1/2}$) and area under the curve predicted to infinity (AUC_{inf}) using WinNonlin (v2.1, Pharsight Software). Pharmacokinetic parameters were considered to be non-compartmental. Data from the oral dose study were analysed using the model 200 distribution profile for bolus extra vascular input. Data from the iv study were analysed using the model 201 distribution profile for bolus iv input. AUC was calculated utilising the linear trapezoidal rule; AUC_{inf} was extrapolated from the last observed concentration and the last predicted concentration, based on the terminal elimination rate. Bioavailability was calculated using the formula $AUC_{oral} \times dose_{iv} / AUC_{iv} \times dose_{oral} \times 100$.

Results

Plasma and tissue concentrations of C3G

Mice received C3G either at 500 mg/kg administered by gavage or at 1 mg/kg by tail vein injection, and levels of parent agent and total anthocyanins, i.e. all molecular species detectable at 520 nm, were measured. The differential between values for C3G and total anthocyanins reflects the extent of C3G metabolism. Figure 2 shows anthocyanin concentration versus time curves for plasma, urine, bile and six tissues, among them gi mucosa, kidneys, lungs and prostate, potential targets for prevention of malignancies. Levels were also measured in brain (not shown). After oral administration peak concentrations occurred consistently within 30 min after administration, except in bile, in which the peak was delayed. Peak concentrations were highest in the urine and gi mucosa. Anthocyanin levels in the brain after oral C3G were below 1 nmol/g, indicating low propensity to cross the blood–brain barrier. In the gi mucosa and liver the predominant flavonoid species after oral administration was C3G, whilst after iv dosing the majority of anthocyanins in these tissues were C3G metabolites.

Pharmacokinetics and bioavailability of C3G

Elimination rate constants, half-lives and AUC values for C3G in eight biomatrices are shown in Table 1. After oral administration, elimination rate constants and half-lives ranged from 0.39 (mucosa) to 1.08 h^{-1} (lung), and from 0.7 (lung) to 1.8 h (mucosa), respectively. C3G half-lives in tissues mirrored that in plasma except in the case of the lungs. After iv administration, elimination rate constants were between 0.97 (gi mucosa) and 2.28 h^{-1} (heart). C3G half-life values after iv dosing fluctuated from 0.3 (heart) to 0.7 h (mucosa), thus they were lower than those observed after gavage, possibly a consequence of the difference in dose between the two administration modes. Pharmacokinetic data could not be derived for urine, bile, brain and prostate after iv administration, as lack of a declining phase

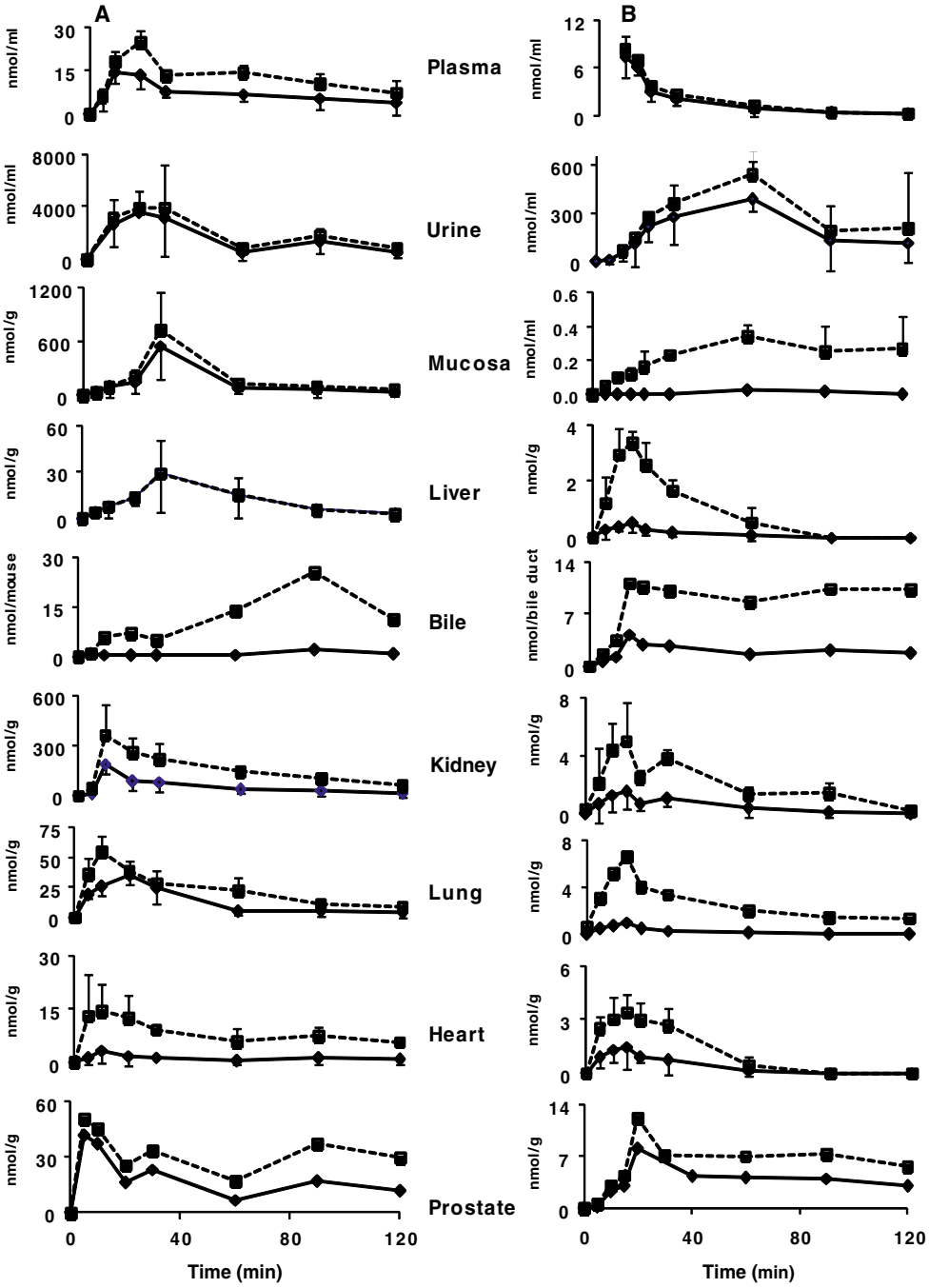

Fig. 2 Concentrations of C3G (*diamond*, *solid line*) and total anthocyanins (*square*, *broken line*) in biofluids and tissues from mice, which received C3G by gavage (500 mg/kg) (**a**) or by tail vein injection (1 mg/kg) (**b**). Values, which are the mean ± SD of 3 (**a**) or 5 mice (**b**), presented as nmol per g tissue or ml biofluid. Single values for bile, lung (after iv administration) and prostate are from pooled biomatrices. Values for the bile are nmol per total bile recovered from a mouse

in the biomatrix concentration versus time curves prohibited evaluation by WinNonlin curve fitting.

Systemic bioavailabilities were calculated from plasma AUC and dose values after oral and iv administration of C3G. The bioavailability values for parent C3G and total anthocyanins were 1.7 and 3.3%, respectively.

Metabolites of C3G in murine biofluids and tissues

Analysis of plasma, urine, bile, gi mucosa, liver and kidney by HPLC with UV–VIS detection suggested the presence of two clusters of metabolites, with either higher or lower polarity compared to C3G (Fig. 3). Analysis of lung, prostate, brain and heart afforded metabolite patterns similar to those in liver and kidney (results not shown). Confirmatory analysis by LC/MS/MS using MRM transitions allowed identification of eight discreet metabolic species (Table 2). In all biomatrices investigated, the presence of C3G was corroborated by the MRM transition *m/z* 449 > 287 for the peak at retention time 13 min, consistent with authentic C3G. The group of metabolites with polarity superior to C3G consisted of C3G glucuronide, methyl C3G glucuronide,

Table 1 Elimination rate constants, half-lives and AUC values for C3G in plasma, urine and tissues from mice which received C3G either by gavage (500 mg/kg) or tail vein injection (1 mg/kg)

Biomatrix	Elimination rate constant (h^{-1})		Half-life (h)		AUC_{inf} (nmol h/ml)	
	po	iv	po	iv	po	iv
Plasma	0.43[a]	1.84	1.6	0.4	25.1[b]	3.01[c]
Urine	0.99	ND[d]	0.7	ND	3,655	ND
Gi mucosa	0.39	0.97	1.8	0.7	448	0.03
Liver	0.45	1.92	1.6	0.4	35.3	0.08
Kidney	0.60	1.54	1.2	0.5	217	0.97
Lung	1.08	1.79	0.6	0.4	28.3	0.43
Prostate	0.58	ND	1.2	ND	49.6	ND
Heart	0.56	2.28	1.2	0.3	3.6	0.72

[a] Number of mice 3–5

[b] Coefficients of variation (CV, in %) for measured biomatrix concentrations ranged from 9 (at 0.5 h) to 78 (2 h) in plasma, 48 (2 h) to 88 (0.5 h) in urine, 27 (1 h) to 86 (1.5 h) in gi mucosa, 24 (0.3 h) to 86 (1 h) in liver, 5 (0.18 h, kidney) to 103 (0.33 h, prostate) in kidney/lung/heart/prostate

[c] CVs (in %) for biomatrix concentrations ranged from 11 (0.25 h) to 80 (1.5 h) in plasma, 14 (0.25 h) to 112 (1 h) in liver, 20 (0.33 h, lung) to 119 (0.17 h, heart) in kidney/lung/heart; mucosal levels were too low to yield meaningful CVs

[d] Could not be determined

cyanidin glucuronide and cyanidin sulphate. C3G metabolites with lower polarity than the parent comprised methyl cyanidin glucuronide, methyl C3G, cyanidin and methyl cyanidin. The C3G and cyanidin molecules harbour several hydroxyl moieties susceptible to metabolic conjugation, predisposing for the formation of positional isomers. Four metabolites occurred as isomers, with observed transitions suggesting two isomeric species for methyl C3G, four each for methyl cyanidin glucuronide and C3G glucuronide and six for methyl C3G glucuronide (Table 2).

C3G glucuronide, methyl C3G and methyl C3G glucuronide were found in plasma, urine, bile and most tissues investigated, although the latter species was not seen in gi mucosa. Methyl cyanidin glucuronide was detected in plasma, urine, bile and gi mucosa, methyl cyanidin in urine, bile and gi mucosa, cyanidin glucuronide in urine and gi mucosa, and cyanidin only in gi mucosa.

Discussion

The results presented above constitute the first report of the pharmacokinetics of C3G in mice, in which plasma and tissue levels were explored after both oral and iv administration. The results allow several novel insights to be gained into the biodisposition and metabolism of C3G, and they also corroborate findings made previously in other rodent species. Times of peak plasma levels and half-lives were similar to those reported previously for C3G in the rat [5], although the oral dose administered here was five times the

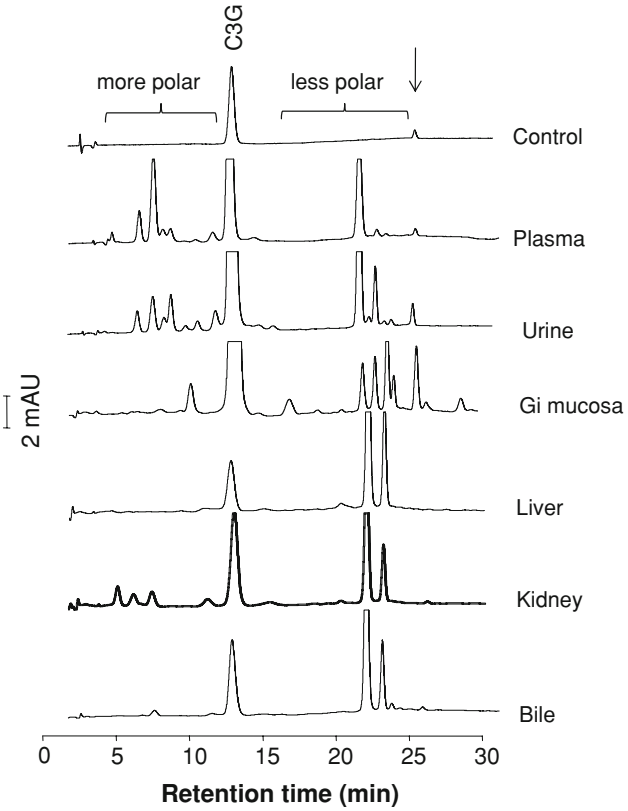

Fig. 3 HPLC/VIS chromatograms of a solution of authentic C3G (*top trace*) and of extracts of plasma, urine, gi mucosa, liver, kidney and bile from mice which had received C3G (500 mg/kg) by gavage. Biomatrices were collected at time points when anthocyanin concentration was maximal, i.e. 20 min post-administration for plasma and urine, 30 min for gi mucosa and liver, 10 min for kidney or 90 min for bile. Authentic C3G eluted at retention time 13 min, and more and less polar metabolites were observed. *Arrow* indicates retention time of unidentified contaminant in the original C3G preparation

Table 2 Metabolites of C3G identified by LC/MS/MS using MRM transitions in biofluids and tissues of mice which received C3G (500 mg/kg) by gavage

Metabolite	Transition (m/z)	Retention time (min)	Number of isomers
C3G glucuronide	625 > 287	4, 7, 8, 11	4
Methyl C3G glucuronide	639 > 301	8, 12, 13, 15	6
Cyanidin glucuronide	463 > 287	9	1
C3G sulphate	529 > 286	15	1
Methyl cyanidin glucuronide	477 > 301	22, 23, 24, 25	4
Methyl C3G	463 > 301	23, 24	2
Cyanidin	287 > 137	24	–
Methyl cyanidin	301 > 201	30	1

dose used in the rat study. C3G peak plasma levels in the rat were 0.19 μM compared to 14.9 μM in the mouse determined here. The disposition of C3G after oral administration in kidney, lung, heart and prostate tissues mimicked its plasma concentration-time profile. After oral administration anthocyanin levels in the gi mucosa were in the 10^{-4}–10^{-3} M range. These concentrations are higher than those seen in any other of the tissues investigated, and they are similar to concentrations which have been shown to elicit pharmacological effects commensurate with cancer chemoprevention in experiments in cells in vitro [1]. This finding, together with the adenoma development-retarding activity of C3G in Apc^{Min} mice [4], supports the development of C3G as a potential agent for colorectal cancer chemopreventive intervention. Anthocyanin peak levels after oral administration of C3G were 25 μM in plasma, and about 50, 60 and 400 nmol/g in prostate, lung and kidney, respectively. These tissue values are in the range of pharmacologically efficacious concentrations, hinting at the suitability of further exploration of C3G in the prevention of malignancies also in the prostate, lung or kidney. It is tempting to speculate on levels of anthocyanins which accompanied the reported chemotherapeutic effect of C3G in the A549 lung tumour in nude mice [3]. The dose in that study was 9.5 mg/kg administered three times a week for approximately 5 weeks. In a simplistic approximation assuming a linear relationship between dose and tissue levels, this dose might have generated peak levels of about a 50th of the values shown in mice here, about 0.5 μM in the plasma and near 1 nmol/g in lung and prostate tissue. This tissue concentration is only a tenth of the concentration which was shown to compromise A549 cell growth in vitro [3].

Anthocyanins are excreted mainly via the kidneys into the urine [9]. The isolation of C3G-derived species from bile demonstrated here suggests that in mice biliary excretion also occurs, albeit to a minor extent. The low bioavailability of both parent C3G (1.7%) and total anthocyanin species (3.3%) after C3G consumption in mice is consistent with values for rats published in the literature. Total anthocyanin bioavailability values in rats after administration of either isolated delphinidin-3-rutinoside [10] or extracts of bilberry [11] or raspberry [12], which contain C3G as major constituent, were 0.49, 0.98 and 1.2%, respectively. Corresponding values in humans after berry consumption are of a similar order of magnitude [9]. Anthocyanins share low systemic availability with almost all flavonoids occurring in the plant kingdom [13].

The predominant routes of C3G biotransformation in the mouse were glucuronidation and *O*-methylation, and Fig. 4 rationalises metabolite generation taking previous metabolite identification in the rat into account [5, 6]. It is not known whether the metabolic species identified here can exert pharmacological effects, and if so, how potent they may be vis-a-vis the parent molecule. The relative abundance of C3G metabolites in comparison to parent species found in the gi mucosa after iv administration is consistent with excretion of C3G via the bile into the gut lumen predominantly as metabolites. Consistent with other studies in rodents, which suggest that anthocyanins can be biotransformed by hydrolysing enzymes in the gut to their respective aglycons [14, 15], cyanidin was recovered here as a C3G metabolite from the murine gi mucosa. Furthermore, identification of the *O*-methyl and glucuronide metabolites of cyanidin in the gi mucosa, urine and bile imply the metabolic intermediacy of cyanidin. These findings have potential pharmacological ramifications, as anthocyanidins have been suggested to exert more potent effects than their glycoside counterparts in cells in vitro [16]. Nevertheless, the amount of cyanidin recovered from the gi mucosa of mice, when estimated grossly by peak area, was low (result not shown), so unlikely to contribute substantially to the overall efficacy of C3G. Anthocyanins and especially their aglycons can undergo spontaneous and enzyme-catalysed decomposition to a phenolic acid and the exquisitely unstable phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde) [17]. As these species are not detected by the analytical methods usually employed to measure anthocyanins, this degradation may contribute to the poor apparent systemic availability observed for anthocyanins. Protocatechuic acid, generated from C3G in the microflora has recently been measured in human plasma at concentrations far in excess of C3G consumed as a constituent of Sicilian blood oranges [18]. However, it is unclear to what extent protocatechuic acid is formed from C3G in species other than humans, as it could not be found in the biophase of rats which had received C3G [19]. A study in cells in vitro, in which protocatechuic and other phenolic acids were compared with their progenitor anthocyanidins or anthocyanins, tentatively concluded that phenolic acid metabolites may contribute to the pharmacological activity of the parent flavonoids, albeit probably only to a limited degree [20]. In the study described here, protocatechuic acid was not measured in mice.

In summary, C3G and its metabolites could be recovered from a variety of murine tissues which may be the target of cancer chemopreventive intervention. Anthocyanin levels achieved in the gi mucosa, prostate and the kidneys were of an order of magnitude consistent with pharmacological activity. As known for other rodent species, the bioavailability of C3G in mice was poor. Glucuronidation and methylation were the major metabolic pathways of C3G in mice. If the preliminary indication of potential chemotherapeutic effect of C3G [3] is supported by further studies in models other than the A549 lung carcinoma, C3G may be considered worthy of clinical evaluation as a chemotherapeutic agent. In this context its water solubility is a pharmaceutical

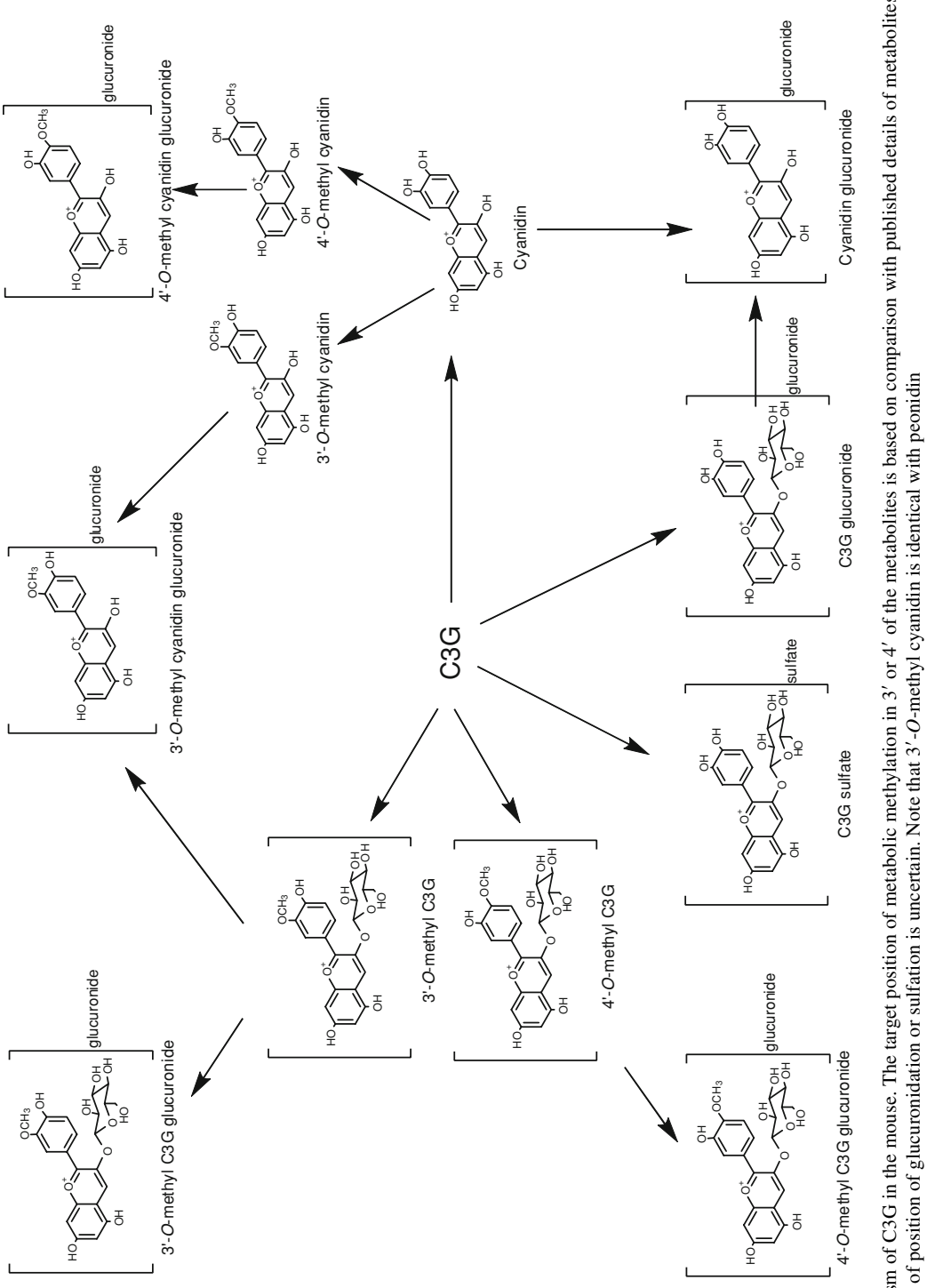

Fig. 4 Metabolism of C3G in the mouse. The target position of metabolic methylation in 3′ or 4′ of the metabolites is based on comparison with published details of metabolites of C3G in rats [5, 6]. Allocation of position of glucuronidation or sulfation is uncertain. Note that 3′-*O*-methyl cyanidin is identical with peonidin

advantage which renders parenteral administration possible, as demonstrated here. In the light of the interesting anticarcinogenic properties of C3G in mice, the information presented here may help optimise its future development as a putative cancer chemopreventive or chemotherapeutic agent.

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