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RESEARCH PAPER

Glucuronidation and Methylation of Procyanidin Dimers B2 and 3,3″-Di-O-Galloyl-B2 and Corresponding Monomers Epicatechin and 3-O-Galloyl-Epicatechin in Mouse Liver

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ABSTRACT

Purpose The 3,3″-di-O-galloyl ester of procyanidin B2 (B2G2) is a component of grape seed extract that inhibits growth of human prostate carcinoma cell lines. In preparation for studies in mice, its hepatic metabolism was examined in vitro and compared to B2 and the corresponding monomers, epicatechin (EC) and 3-O-galloyl-epicatechin (ECG).

Methods Compounds were incubated with liver microsomes or cytosol containing cofactors for glucuronidation, sulfation or methylation, and products analyzed by liquid chromatography-mass spectrometry (LC-MS). B2G2 was administered orally to mice and plasma analyzed by LC-MS for unmodified procyanidin and metabolites.

Results Glucuronides and methyl ethers of B2 and B2G2 were formed in small amounts. In contrast, EC and ECG were largely or completely converted to glucuronides, sulfates and methyl ethers under the same incubation conditions. B2G2 given orally to mice was partially absorbed intact; no significant metabolites were detected in plasma.

Conclusions Glucuronidation and methylation of procyanidins B2 and B2G2 occurred but were minor processes *in vitro*. B2G2 was partially absorbed intact in mice after oral dosing and did not undergo significant metabolism. Unlike the flavanol monomers EC and ECG, therefore, B2G2 bioavailability should not be limited by metabolism. These results paved the way for ongoing pharmacokinetic and efficacy studies.

KEY WORDS bioavailability · glucuronidation · flavanol · methylation · procyanidin

ABBREVIATIONS

B2	EC-(4β→8)-EC
B2G2	3,3″-di-O-galloyl-B2
CAD	collisionally-activated dissociation
COMT	catechol-O-methyltransferase
EC	(−)-epicatechin
ECG	3-O-galloyl-EC
EIC	extracted ion chromatogram
GSE	grape seed extract
LC-MS	liquid chromatography-mass spectrometry
MS/MS	tandem mass spectrometry
PAPS	adenosine 3′-phosphate 5′-phosphosulfate
SAM	S-(5′-adenosyl)-L-methionine
SRM	selected reaction monitoring
TIC	total ion current
UDPGA	uridine 5′-diphosphoglucuronic acid

INTRODUCTION

Use of various chemopreventive agents, especially those of natural origin, has gained increased attention in recent

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years as these agents are usually part of normal diet or taken as dietary supplements and thus have low associated systemic toxicity (1). Grape seed extract (GSE) is a commonly consumed dietary supplement with several health benefits (2) that are attributed to its high procyanidin content and associated antioxidant capacity (3–5). A number of studies in the last 12 years have shown both anti-cancer and chemopreventive efficacy of GSE against various epithelial cancers including those of skin, breast, colon and lung (6). Our studies of DU145 cells, a prostate cancer cell line, revealed that GSE inhibits growth via inhibition of EGF-induced and constitutively active mitogenic signaling, which have been implicated in transition from androgen dependence to androgen independence in prostate cancer (7) and induces apoptotic cell death cascade in DU145 cells (8). The protective effects of GSE were also observed under *in vivo* conditions; its feeding strongly inhibited growth of hormone refractory advanced prostate tumor xenograft in athymic nude mice via its antiproliferative, proapoptotic and antiangiogenic activities (9). More recently, we also reported chemopreventive efficacy of GSE against prostate cancer in the transgenic adenocarcinoma of mouse prostate (TRAMP) model (10).

GSE is a complex mixture of polyphenols that includes gallic acid and the flavan-3-ols catechin and epicatechin (EC). Other major components present in GSE include procyanidins (proanthocyanidins), i.e. oligomers of catechin and/or EC consisting mainly of dimers such as procyanidin B2 (Fig. 1), trimers and small quantities of higher oligomers (11–13). These compounds are also present as gallate esters at the C-3 hydroxyl group of one or more flavanol unit. Our recent studies employing chromatographic isolation and *in vitro* biological testing of the major flavanols and procyanidins in human DU145 prostate cancer cells have demonstrated that procyanidin B2 esterified at the C3 position of both EC units (B2G2), shown in Fig. 1, is considerably more active than GSE or any other compound tested (14,15). Prior to initiating studies into the effectiveness of B2G2 against prostate cancer in mice, the present work was undertaken to characterize its metabolism *in vitro* and assess the likelihood that hepatic biotransformation may influence its bioavailability.

Numerous studies have demonstrated that catechins and gallocatechins are metabolized extensively by glucuronidation, sulfation and methylation of phenolic hydroxyl groups (16,17); however, little is known about the metabolism of procyanidins. Procyanidin B2 is partially absorbed intact from the gastrointestinal tract of rats (18,19) but no conjugates of B2 have been reported and, to our knowledge, no *in vitro* studies characterizing the hepatic metabolism of B2 or its gallate esters have been published. In view of the potential importance of B2G2 in cancer chemoprevention, and the beneficial properties associated with procyanidins in general (2–5), we examined glucuronidation, sulfation and methylation of B2 and B2G2 in mouse liver and compared results to parallel experiments with the corresponding monomers EC and 3-O-galloyl-EC (ECG). Formation of glucuronides and methyl ethers was confirmed by liquid chromatography-mass spectrometry (LC-MS). Procyanidins were metabolized to a much lesser degree than the flavanols. Analyses of plasma from mice given an oral dose of B2G2 confirmed that this compound is partially absorbed intact

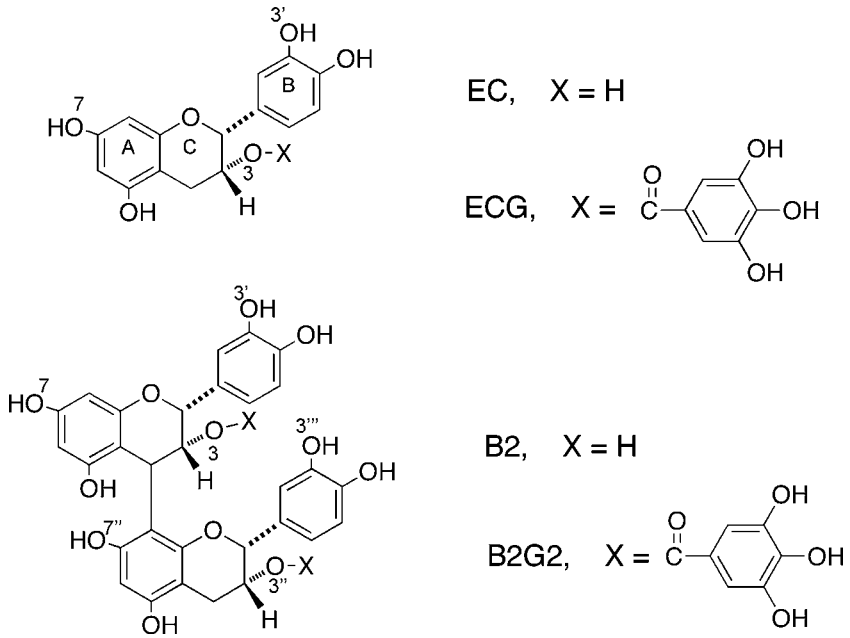

Fig. 1 Structures of flavan-3-ols (−)-epicatechin (EC) and 3-O-galloyl-EC (ECG) and procyanidins EC-(4β→8)-EC (B2) and 3,3″-di-O-galloyl-B2 (B2G2). Ring lettering and atom numbering are shown for structural components mentioned in the text.

and not subsequently conjugated, methylated or hydrolyzed to a significant extent. These results encourage further detailed pharmacokinetic and efficacy studies of this agent with potential activity against prostate cancer.

MATERIALS AND METHODS

Chemicals

EC, ECG, procyanidin B2, uridine 5′-diphosphoglucuronic acid (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate (PAPS), S-(5′-adenosyl)-L-methionine (SAM), ascorbic acid and alamethicin were obtained from Sigma-Aldrich (St. Louis, MO). B2G2 was isolated from GSE and the purity was confirmed by LC-MS as described (14). Solvents and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Incubations and Sample Preparation

For analysis of glucuronidation, pooled liver microsomes from male CD-1 mice were purchased from BD Biosciences (Woburn, MA). Substrates were dissolved in 30 μL of methanol, diluted with water and added to incubates to achieve a final concentration of 0.4 mM substrate in 1% methanol. Incubations contained 2.5 mg of microsomal protein, 200 μg alamethicin, 2.0 mM UDPGA (omitted from controls), 0.15 mM ascorbic acid, 5 mM $MgCl_2$ and 5 mM KCl in 0.4 mM Tris-HCl buffer (pH 7.4) in a total volume of 1.0 mL. Microsomes were pre-incubated in the buffer for 5 min at 37°C, substrate and UDPGA were added, and the reaction mixtures incubated for 60 min and placed on ice for 10 min. Incubates were then loaded onto Waters C18 Sep-Pak Plus solid-phase extraction cartridges (Milford, MA) pre-conditioned with 1.0 mL of methanol followed by 2.0 mL of water. After loading, cartridges were washed with 0.4 mL of water and compounds of interest eluted with 6.0 mL of methanol followed by 1.0 mL of acetonitrile (development of the extraction is shown in Supplementary Material Fig. S1). Combined eluates were concentrated to 0.3 mL under a stream of nitrogen at 25°C. For analysis of sulfation and methylation, cytosol was prepared from the livers of male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) as described (20) and incubated with 0.4 mM substrate at 37°C for 60 min. Reactions contained 5.0 mg/mL of cytosolic protein and 2.0 mM PAPS (omitted from controls) for sulfation studies or 2.5 mg/mL of protein and 5.0 mM SAM for methylation studies. Reactions were terminated by cooling on ice for 10 min and extracted as described above.

LC-MS Analysis

Concentrated extracts were analyzed with a Waters Micromass Quattro mass spectrometer equipped with a Z-spray electrospray interface and an Agilent 1100 HPLC. A 150× 2.0 mm Prodigy 5 μ ODS-2 column (Phenomenex, Torrance, CA) was employed with solvents consisting of water (solvent A) and acetonitrile (solvent B) both containing 10 mM ammonium acetate at a flow rate of 0.20 mL/min. Several different solvent gradients were utilized: (i) for analysis of EC and B2 glucuronidation and sulfation, solvent B was increased from 5% A in B to 25% over 0 to 7 min, to 35% from 7 to 9 min and to 95% from 9 to 12 min; (ii) for EC, ECG and B2 methylation, solvent B was increased from 10 to 40% from 0 to 20 min; (iii) for ECG glucuronidation, solvent B was increased from 5 to 30% from 0 to 9 min; (iv) for ECG sulfation, solvent B was increased from 15 to 23% from 0 to 7 min and to 95% from 7 to 12 min; (v) for B2G2 glucuronidation and sulfation, solvent B was increased from 15 to 60% from 0 to 20 min; and (vi) for B2G2 methylation, solvent B was increased from 15 to 50% from 0 to 20 min. The mass spectrometer was operated in the negative ion mode with scan range generally 100–1000 Da but in some cases the range was reduced to maximize sensitivity. Instrument parameters were as follows: capillary voltage 2.5 kV, cone voltage 30 V (40 V for B2G2), extractor 3 V, source and desolvation gas temperatures 120 and 300°C, respectively. Nitrogen was utilized as both nebulizer (50 L/h) and desolvation gas (600 L/h) and scan time was 1 s. For collisionally-activated dissociation (CAD), argon was the collision gas and collision energies were optimized for each compound. Instrument control, data acquisition and data analyses were performed with Micromass MassLynx software v4.0.

B2G2 Absorption *In Vivo*

Three male C57BL/6 mice were utilized. Food was removed 15 h prior to administering oral doses of 250 mg/kg of B2G2 dissolved in ethanol and diluted with water (16:84% v/v). Total volumes administered were approximately 200 μL. After 2.5 h, mice were euthanized by inhalation of carbon dioxide followed by cervical dislocation. Blood was collected intracardiacally, pooled, placed into heparinized tubes and plasma prepared by centrifugation at 900 g for 20 min. Samples of plasma (1 mL) were treated with 2.5 mL of acetonitrile, vortexed briefly at 30 s intervals over 3 min and centrifuged at 3000 g for 10 min at 4°C. The resulting pellets were separated from the supernatant, 1.5 mL of 1:1 methanol/phosphate buffer (pH 7.0) added and the mixture shaken for 5 min and centrifuged at 2000 g for 30 min. The

supernatants were combined, the organic phase evaporated under a stream of nitrogen at 25°C and compounds of interest extracted with Sep-Pak Plus solid-phase extraction cartridges as described for *in vitro* work. Samples were analyzed by LC-MS with a Sciex 4000 instrument (Applied Biosystems, Foster City, CA) equipped with a Shimadzu HPLC (Columbia, MD) and an electrospray interface. Chromatography was performed on a Zorbax (Agilent Technologies) extended C18 250×4.6 mm column at 40°C and flow rate of 0.4 mL/min. The mobile phase consisted of: solvent A (10 mM ammonium acetate with 0.1% formic acid in water) and solvent B (1:1 acetonitrile/methanol). The solvent program was 5% B in A for 1 min and increased to 95% B from 2 to 7 min and held for an additional 9 min. Ten µL samples were injected and the MS was operated in the positive ion mode under the following conditions: ion spray voltage 5000 V, temperature 450°C, curtain gas at a setting of 10. CAD was conducted with nitrogen gas at an instrument setting of 25 and mass analyzers at unit mass resolution.

RESULTS

Glucuronidation

After incubating B2 with mouse liver microsomes and UDPGA, samples contained mostly unmodified substrate in addition to several small product peaks shown in Fig. 2. The formation of four cofactor-dependent peaks were visible in the extracted ion chromatogram (EIC) for the molecular ion of B2-glucuronide at m/z 753 Da. Selected reaction monitoring (SRM) for the expected fragmentations 753→577 and 753→289 (i.e., formation of B2 and EC product ions, respectively) produced responses for the same four peaks thereby confirming the presence of glucuronides. LC-MS analysis of B2G2 incubations conducted under the same conditions demonstrated that conjugation was also a minor process for the digallate as mostly unchanged substrate remained in the total ion chromatogram (TIC) (Fig. 3). As shown in the figure, one minor product peak was observed in the EIC for B2G2-glucuronide at m/z 1057. MS/MS analysis of this conjugate at relatively low

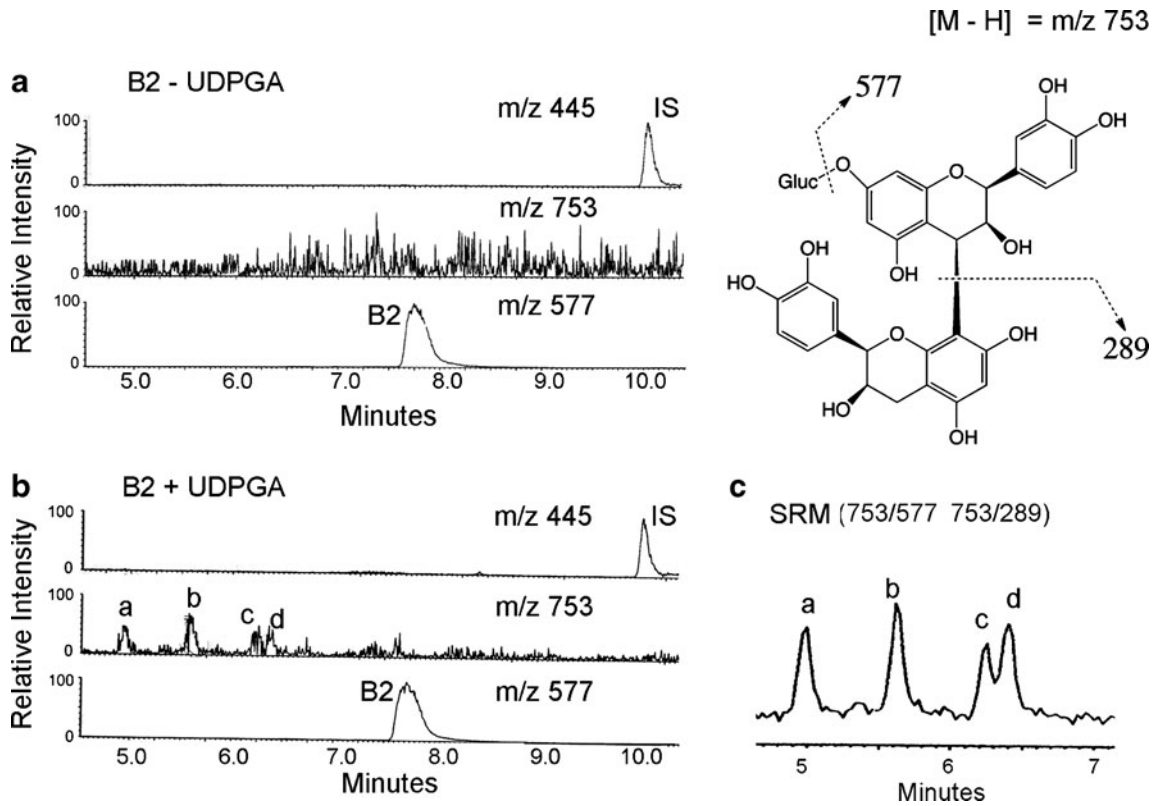

Fig. 2 Glucuronidation of procyanidin B2. After incubation with microsomes, samples were extracted and analyzed by negative ion LC-MS as described in Materials and Methods. (**a**) Control incubation without cofactor with extracted ion chromatograms (EICs) of deprotonated molecular ions for B2 at m/z 577, B2-glucuronide at m/z 753 and the internal standard (baicalin) at m/z 445. (**b**) EICs of an incubation with cofactor present. Peaks labeled a-d contain detectable ion current corresponding to the molecular ion of B2-glucuronide. Mass spectra of peaks a-d are included in Supplementary Material Fig. S2. (**c**) Selected reaction monitoring (SRM) for transitions 753→577 and 753→289, shown for B2-7-O-glucuronide, confirm the presence of glucuronides in peaks a-d.

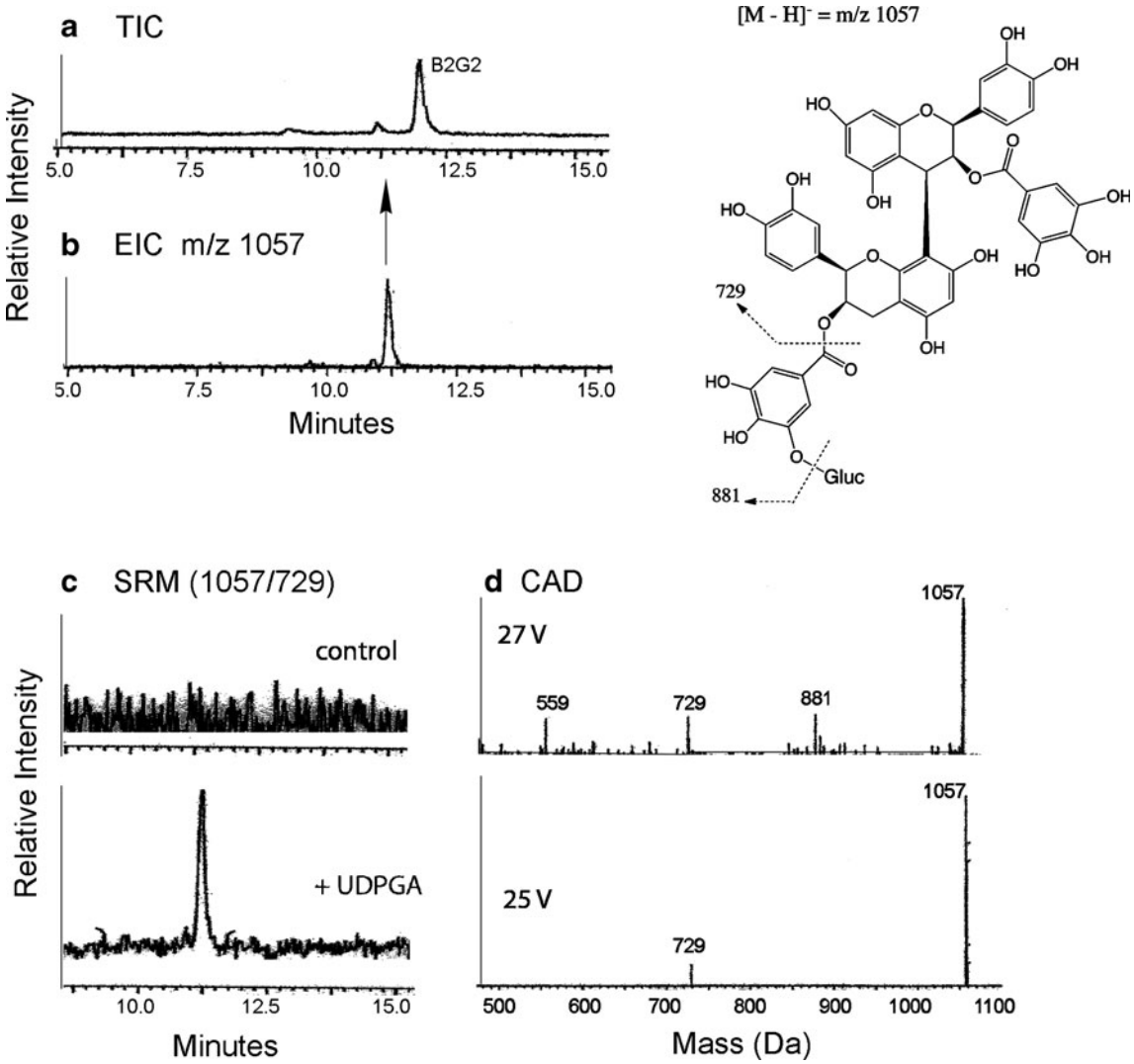

Fig. 3 Glucuronidation of B2G2. (**a**) TIC from a microsomal incubation containing UDPGA. A mass spectrum of the peak at 11.2 min is presented in Supplementary Material Fig. S2. (**b**) The EIC for m/z 1057 corresponding to the molecular ion of B2G2-glucuronide. (**c**) Analysis of incubations with and without UDPGA by SRM of the transition 1057→729. (**d**) Product ion spectra from collision-induced dissociation (CAD) of the m/z 1057 ion at two collision energies. The structure depicts glucuronidation on a galloyl group and proposed fragmentation pathways discussed in the text.

collision energy caused a small amount of fragmentation but clearly demonstrated only one product ion higher than m/z 500 Da; this ion at 729 Da corresponds to the loss of a glucuronidated galloyl group with charge retention on the resulting B2-monogallate fragment indicating that conjugation occurred on a gallate hydroxyl. With a slightly higher collision energy, additional ions characteristic of this conjugate were produced. Utilizing the 1057→729 transition, selected reaction monitoring clearly confirmed the presence of a UDPGA-dependent metabolite consistent with glucuronidation on one of the galloyl groups.

In contrast to the results summarized above, flavanol monomers EC and ECG were completely degraded in microsomes under the same reaction conditions utilized for the dimers. TICs of incubations with and without the cofactor (Fig. 4) demonstrated the formation of one predominant metabolite in each case. Mass spectra confirmed that these peaks contained only the molecular ion corresponding to EC-glucuronide at 465 Da or ECG-glucuronide at 617 Da. MS/MS analysis yielded product ions at 289 Da for EC-glucuronide or 441 Da for ECG-glucuronide due to the expected losses of glucuronic acid from the molecular species. For the latter conjugate, additional product ions were consistent with glucuronidation on the galloyl group as shown in Fig. 4b.

Sulfation and Methylation

Neither B2 nor B2G2 were metabolized in PAPS-fortified cytosol; no responses were observed when molecular ions for the respective sulfate conjugates were monitored at m/z 657 or 961 Da. In contrast, both EC and ECG were readily

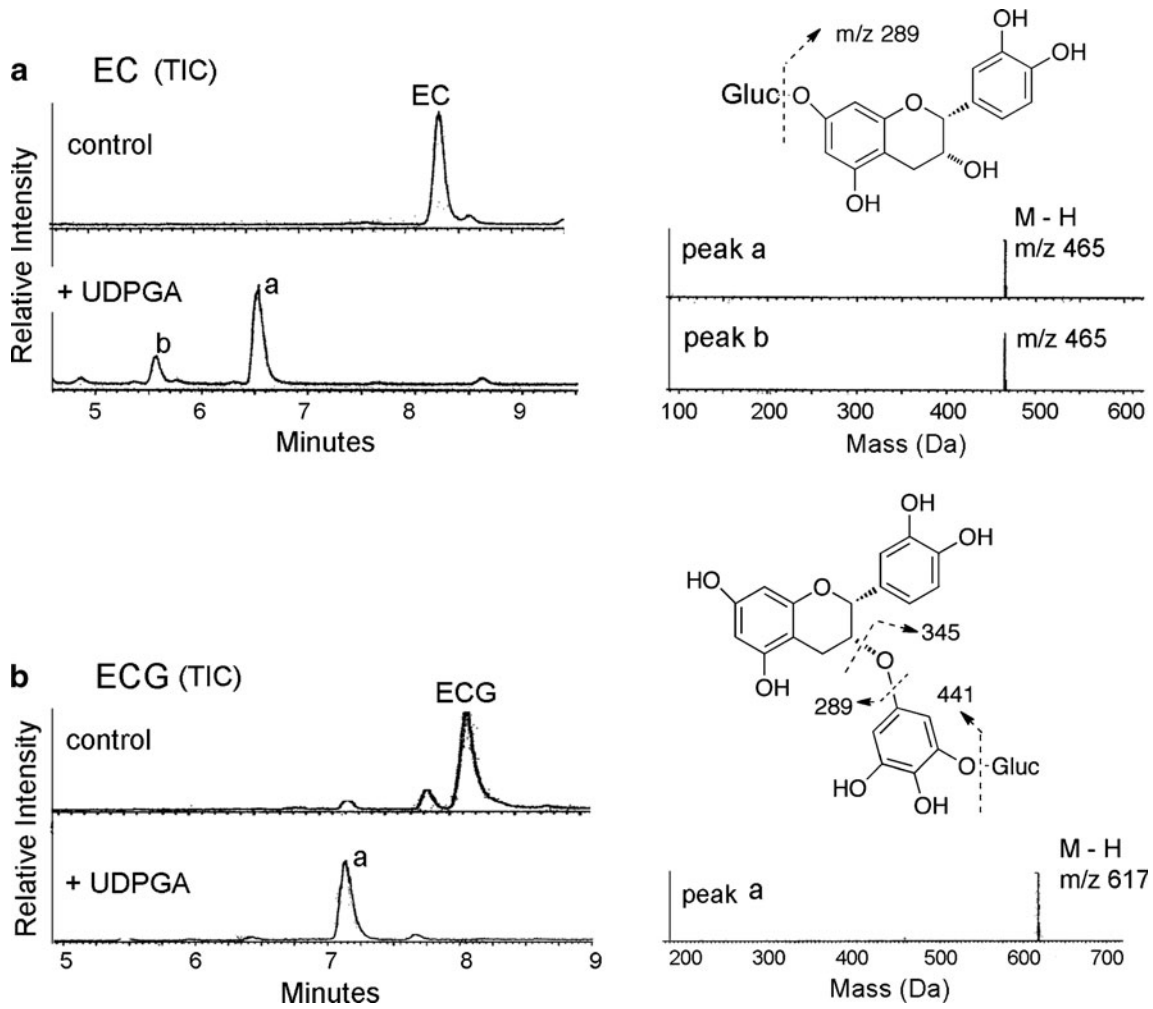

Fig. 4 Glucuronidation of EC and ECG by liver microsomes. (**a**) TIC from LC-MS analyses of EC incubations conducted with and without UDPGA (left panel) and mass spectra of peaks a and b (*right panel*). (**b**) TIC of ECG incubations with and without UDPGA (*left panel*) and mass spectra of the product (*right panel*). In both cases the most likely conjugate structures as described in the text are shown (Gluc=glucuronide) together with fragmentations seen in CAD spectra.

sulfated forming two main products in the former case and one for the latter compound. Mass spectra containing the parent ions of EC-sulfate (369 Da) and ECG-sulfate (521 Da) are shown in Fig. 5, and these conjugates were further analyzed by MS/MS (not shown) with fragmentation involving the losses of sulfate and formation of product ions at m/z 289 for EC or 441 for ECG.

Both B2 and B2G2 were methylated in SAM-fortified cytosol. The TIC for a B2 incubate shown in Fig. 6 contained mostly unmetabolized substrate, but the accompanying EIC demonstrates that the peak at 21.4 min contained methylated B2 with a molecular ion at 591 Da. The TIC from analysis of a B2G2 incubation also contained mostly unmodified substrate together with minor product peaks at 17.4 and 17.8 min in the approximate ratio of 1:2. The EIC for methylated B2G2 at 895 Da clearly demonstrated the presence of two metabolites (Fig. 6). As was the case for glucuronidation and sulfation, the monomers were methylated much more extensively than the dimers. The TIC for EC incubations contained a small peak for unchanged substrate at 12.0 min and three SAM-dependent products eluting between 12.5 and 13.5 min (Fig. 7). The EIC clearly showed one major and two minor products with molecular ions corresponding to methyl-EC at 303 Da. ECG was also partially methylated in SAM-fortified cytosol; the EIC for the molecular ion at 455 Da indicated formation of a single ECG-methyl ether.

Oral Administration of B2G2

In order to determine whether B2G2 undergoes *in vivo* absorption intact, the pure compound was administered orally to mice and blood samples taken after 2.5 h. This time point was selected as previous studies involving administration of oral flavanols and procyandins to rats indicated that maximum plasma levels occur in the

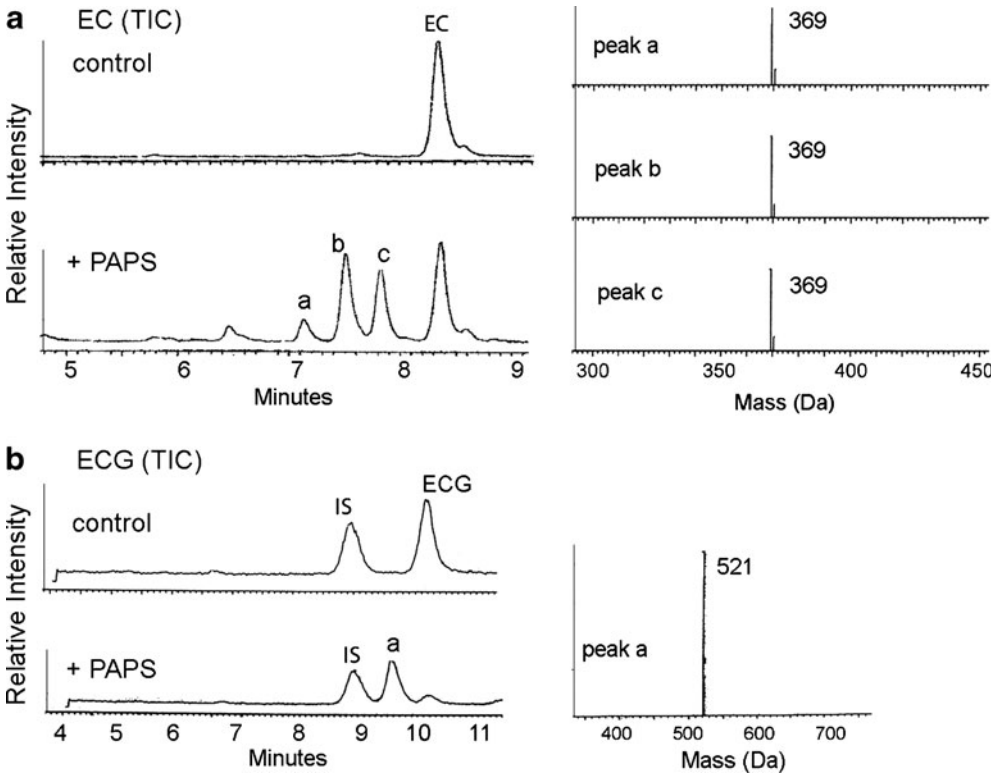

Fig. 5 LC-MS analysis of EC and ECG sulfation by liver cytosol. (**a**) TIC of EC incubations with and without PAPS (*left panel*) and LC-MS spectra of the products labeled a-c (*right panel*). (**b**) TIC of ECG incubations with and without PAPS (*left panel*) and a mass spectrum of the product labeled a (*right panel*). This ECG incubation also contained the internal standard (IS) 4-nitrophenyl sulfate.

approximate range of 1–3 h after dosing (18). Higher LC-MS sensitivity was required than for analyses of *in vitro* samples; optimal conditions with the instrument utilized for plasma samples involved a mobile phase containing formic acid and monitoring positive rather than negative ions. Protonated molecular ions for B2G2 (m/z 883), B2G2-glucuronide (m/z 1059), methyl-B2G2 (m/z 897) and the hydrolysis products B2-monogallate (mz 731) and B2 (m/z 589) were monitored. Except for very small peaks (not shown) that may be due to hydrolysis products, B2G2 was the only compound detected in these extracts (Fig. 8). An MS/MS product ion formed by interflavan cleavage confirmed the presence of this procyanidin in plasma.

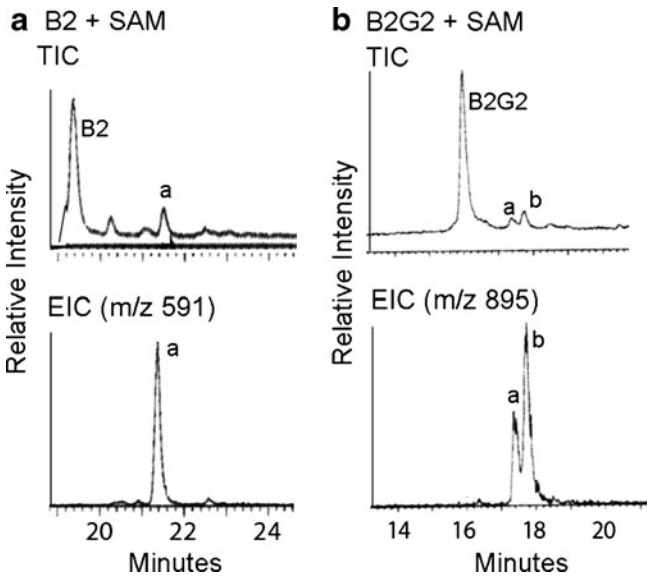

Fig. 6 LC-MS analysis of B2 and B2G2 methylation by liver cytosol. (**a**) TIC of a B2 incubation in the presence of SAM and the corresponding EIC for methyl-B2 at 591 Da corresponding to peak a in the TIC. (**b**) TIC of a B2G2 incubation with SAM and the corresponding EIC for methyl-B2G2 at m/z 895. The mass spectra of methyl ethers are presented in Supplementary Material Fig. S3.

DISCUSSION

Procyanidins and their gallate esters are constituents of several edible plants and are especially abundant in grape seeds (12–14,21–23). Although several potentially beneficial activities have been described for procyanidin B2 and related dimers and timers (21,24), this compound is inactive in cellular models of prostate cancer that are otherwise responsive to GSE (11,14,15). Gallate esters of procyanidin B2, however, are considerably more potent than GSE and this is especially true for B2G2 suggesting its potential value as an anticancer or chemopreventive agent. Other investigators have examined the antioxidant capacity of B2G2 (13) and its ability to inhibit both DNA polymerase alpha (25) and squalene epoxidase (23), but there are no reports on the absorption or metabolism of B2 gallates.

In preparation for a detailed pharmacokinetic study, we explored the conjugation and methylation of B2G2 *in vitro*.

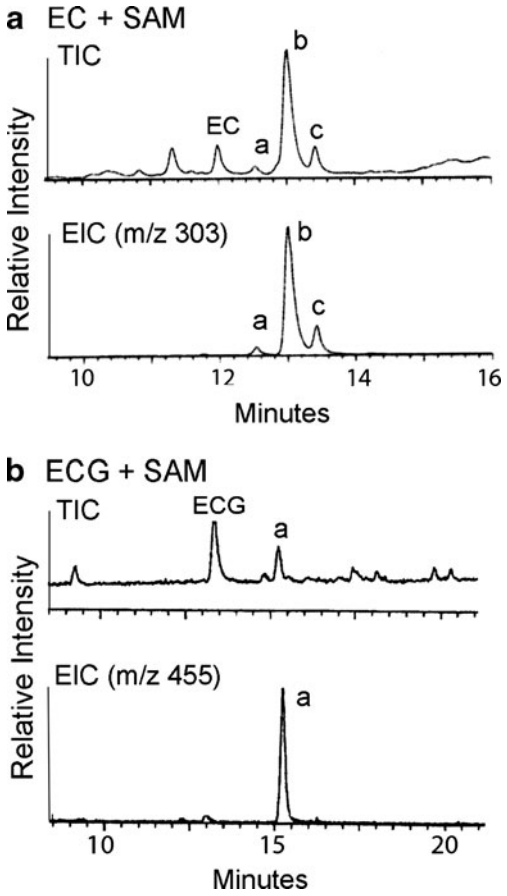

Fig. 7 LC-MS analysis of EC and ECG methylation by liver cytosol. (**a**) TIC of an incubation containing EC and SAM and the corresponding EIC for the molecular ion of methyl-EC at 303 Da. (**b**) TIC of an incubation with ECG and SAM and the corresponding EIC for m/z 455 corresponding to methyl-ECG.

Initial results indicated that both B2 and B2G2 were resistant to metabolism. The conversions of EC and ECG, known to be readily glucuronidated, sulfated and methylated (16,17), were also evaluated to provide a direct comparison of these monomers to their corresponding dimers. EC was fully or mostly consumed in incubations with mouse liver fractions and metabolite formation was confirmed by LC-MS techniques. The results were consistent with earlier studies demonstrating extensive conversions to conjugates and methyl ethers in rat liver fractions (26,27) and after oral administration of EC to rats (28). Other investigators concluded that glucuronidation of EC occurs primarily at the 7-hydroxyl group of ring A (Fig. 4) and secondarily at the 3′-hydroxyl group of ring B (29). The EC dimer procyanidin B2 remained mostly unmetabolized in microsomal incubations with a small percentage converted to four minor glucuronide products detected by LC-MS. This finding appears to be the first direct observation of B2 glucuronide formation, although the specific conjugation sites were not determined due to the small amounts of products and facile losses of glucuronic acid from these molecular ions during CAD. The presence of four products, however, was consistent with conjugation at the 3′- and 7-hydroxyl groups of each EC unit of the dimer. In cytosol EC was extensively sulfated as expected from earlier work (26) however no B2 sulfates were formed under identical reaction conditions. Methylation of EC readily occurred in cytosol but was a minor process for the dimer resulting in one main product in both cases. The 3′-hydroxyl group of ring B is believed to be the preferred site of EC methylation (27,30) and the present result demonstrated formation of a single B2 methyl ether, indicating that enzymatic attack took place on only one of the EC units. The relatively large molecular size of B2 relative to EC most likely restricts its access to the active sites of these phase 2 enzymes. The interflavan linkage results in a more bulky structure than other glucuronidation substrates

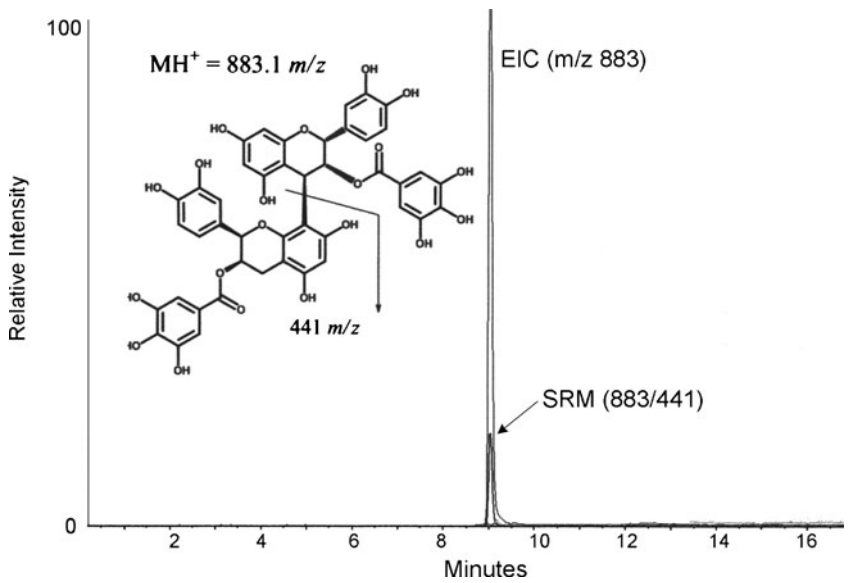

Fig. 8 LC-MS analysis of a plasma extract from mice that had received an oral dose of B2G2. Treatment and sample preparation are described in Materials and Methods. Analysis were carried out in the positive ion mode as discussed in the text with selected ion monitoring for the $(M+H)^+$ ion of B2G2 (m/z 883) and SRM for the transition 883→441.

of similar molecular mass such as bilirubin and digoxin monodigitoxide.

Galloylation had little effect on metabolism of EC or B2. ECG was extensively consumed in liver microsomes and cytosol but the corresponding dimer B2G2 was mostly unchanged. Both gallate derivatives yielded predominantly one glucuronide and MS/MS data strongly indicated that both were conjugated on a galloyl moiety rather than a flavanol ring as shown in Figs. 3 and 4b. There are previous examples of glucuronidation of gallate groups by rats for both ECG and epigallocatechin gallate (31). In PAPS-fortified cytosol, ECG underwent nearly complete conversion to a sulfate conjugate, however no B2G2 sulfate was detected by LC-MS. Under methylation conditions, ECG was converted to one product whereas B2G2 formed smaller amounts of two methyl ethers. Zhu *et al.* (27) reported that ECG is methylated at the 3′-hydroxy group in ring B, so it is likely that methylation occurred on both catechol rings of B2G2.

Earlier studies in which procyanidin B2 was administered orally to rats or humans, either in pure form (18,32) or as a component of GSE (33,34), cocoa (19,35) or apples (36), demonstrated that absorption was no more than 5 to 10% that of EC absorption. Most of this work involved analyses of blood or urine after hydrolysis by β-glucuronidase and sulfatase and, therefore, provided no information on the possibility that B2 is conjugated *in vivo*. Poor absorption of intact procyanidins after oral administration has been linked to their degradation by gut microflora to form low molecular weight phenolic acids and other catabolites (18,19,22,35,36); however, the contributions of such processes depend on several factors including the form and amount of the dose.

There are no published data on the absorption or metabolism of the corresponding gallate esters. Our data demonstrate that in mice B2G2 is at least partially absorbed intact from oral administration of the pure compound. The fact that no conjugates or methyl ethers were detected in plasma is consistent with *in vitro* results demonstrating that B2G2 is fairly stable to hepatic metabolism. Only traces of B2 and B2-monogallate were detected, demonstrating that gallate hydrolysis also does not occur to a significant extent. These data indicate that the bioavailability of B2G2 is not limited by hepatic metabolism. Ongoing quantitative studies involving time courses of absorption and excretion will define the pharmacokinetic profile of this compound in mice in preparation for further efficacy studies for protection against prostate cancer.

ACKNOWLEDGMENTS & DISCLOSURES

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